Tetrac Can Replace Thyroid Hormone During Brain Development in Mouse Mutants Deficient in the Thyroid Hormone Transporter Mct8

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The monocarboxylate transporter 8 (MCT8) plays a critical role in mediating the uptake of thyroid hormones (THs) into the brain. In patients, inactivating mutations in the *MCT8* gene are associated with a severe form of psychomotor retardation and abnormal serum TH levels. Here, we evaluate the therapeutic potential of the TH analog 3,5,3′,5′-tetraiodothyroacetic acid (tetrac) as a replacement for T_4 in brain development. Using COS1 cells transfected with TH transporter and deiodinase constructs, we could show that tetrac, albeit not being transported by MCT8, can be metabolized to the TH receptor active compound 3,3′,5-triiodothyroacetic acid (triac) by type 2 deiodinase and inactivated by type 3 deiodinase. Triac in turn is capable of replacing T_3 in primary murine cerebellar cultures where it potently stimulates Purkinje cell development. In vivo effects of tetrac were assessed in congenital hypothyroid *Pax8*-knockout (KO) and *Mct8*/*Pax8* double-KO mice as well as in *Mct8*-KO and wild-type animals after daily injection of tetrac (400 ng/g body weight) during the first postnatal weeks. This treatment was sufficient to promote TH-dependent neuronal differentiation in the cerebellum, cerebral cortex, and striatum but was ineffective in suppressing hypothalamic TRH expression. In contrast, TSH transcript levels in the pituitary were strongly downregulated in response to tetrac. Based on our findings we propose that tetrac administration offers the opportunity to provide neurons during the postnatal stage with a potent TH receptor agonist, thereby eventually reducing the neurological damage in patients with MCT8 mutations without deteriorating the thyrotoxic situation in peripheral tissues. **(*Endocrinology* 154: 968–979, 2013)**

The physiological importance of the thyroid hormone (TH) transporter monocarboxylate transporter 8 (MCT8) (SLC16A2) for mediating the cellular entry of TH has been highlighted by the identification of patients with inactivating mutations in the X-chromosomal *MCT8* gene (1–3). All patients suffer from a combination of neurological impairments that include severe hypotonia and spastic paraplegia as well as the lack of speech and poor communication skills. In addition to this severe form of psychomotor retardation, all patients exhibit characteristic changes in the TH serum profile with highly elevated serum T_3 levels in the presence of low T_4 and normal TSH values. As a consequence of the increased serum T_3 levels, peripheral tissues such as liver and muscle seem to be in a thyrotoxic state (4–6). The underlying mechanisms causing the severe neurological damage have not been fully elucidated. Based on the well-established role of TH in brain development (7–9), it has been speculated that due to an impaired transport of TH across the blood-brain barrier and/or into neurons in the absence of MCT8, the central nervous system (CNS) is deprived of TH with devastating consequences for neural differentiation and proper brain function.

Currently, the therapeutic options for the patients suffering from this disease are rather limited. Treatment with

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Abbreviations: CNS, central nervous system; D2, type 2 iodothyronine deiodinase; DITPA, 3,5-diiodothyropropionic acid; dKO, double KO; HPT, hypothalamus-pituitary-thyroid; KO, knockout; MCT8, monocarboxylate transporter 8; Oatp, organic anion-transporting polypeptide; PC, Purkinje cell; PTU, propylthiouracil; RC3, neurogranin; TA3, 3,3′,5-triiodothyroacetic acid; TA4, 3,3′,5,5′-tetraiodothyroacetic acid; TH, thyroid hormone; TR, TH receptor; WT, wild-type.

propylthiouracil (PTU) to block endogenous TH production in combination with T_4 supplementation has been shown to ameliorate peripheral symptoms of thyrotoxicosis such as an increased heart rate and body weight loss in one patient but did not result in any improvement of the neurological impairments (10). As another possibility, the treatment of patients with TH analogs that exert thyromimetic actions but do not depend on MCT8 for cellular entry has been considered.

To test the activities of such compounds in vivo, the *Mct8*-KO mouse has been considered as the best available animal model because these mice faithfully replicate the thyroidal abnormalities found in patients (11, 12). Studies in *Mct8*-KO mice have also revealed an impaired uptake of the active hormone T_3 into the murine brain, thereby underscoring a unique function of Mct8 in mediating cellular TH passage (12, 13). However, in contrast to the patients, *Mct8*-KO mice do not show any neurological damage (14). Detailed analysis of the brain of these mice demonstrated only a mild hypothyroid situation with altered inner and outer-ring deiodinase activities (12). This discrepancy between mice and men may be due to the presence of additional TH transporters such as the organic anion-transporting polypeptide (Oatp) 1c1 in the endothelial cells of the mouse CNS that can partially compensate for the absence of Mct8 (15–19). Indeed, the uptake of T_4 into the murine brain was only slightly affected in *Mct8*-KO mice, whereas the human brain may be more dependent on MCT8 for the uptake of both T_4 and T_3 from the circulation.

Despite the lack of neurological symptoms *Mct8*-KO mice have been successfully used to explore the in vivo action of the TH analog 3,5-diiodothyropropionic acid (DITPA) (20). After rendering the animals hypothyroid, DITPA was capable of normalizing changes in transcript levels of TH-regulated gene products in the brain of both wild-type (WT) and *Mct8*-KO mice, indicating that it represents a suitable T_3 substitute in the absence of Mct8. Moreover, application of DITPA to 4 patients with *MCT8* mutations resulted in a normalization of the serum TH parameters without causing any overt side effects (21). As an alternative to DITPA with an eventually even higher therapeutic potential, we considered to determine the in vivo action of tetrac (3,3′,5,5′-tetraiodothyroacetic acid [TA4]) by using the *Mct8*-KO mouse model.

Tetrac and the corresponding T_3 analog triac (3,3′,5-triiodothyroacetic acid [TA3]) represent naturally occurring TH metabolites. They are produced by decarboxylation and oxidative deamination of the alanine side chain in the liver and other peripheral tissues (22–24). Inactivation of these acetic acid derivates occurs similarly to the elimination of TH and includes deiodination by iodothyronine deiodinases and formation of conjugates with glucuronic or sulfuric acid (25). Determination of serum levels in euthyroid humans by RIA revealed a mean concentration of 50 ng/100 ml for TA4 and 2 to 8 ng/100 ml for TA3. Estimation of the daily production rate indicates that the conversion of TH into their acetic acid derivatives constitutes only a minor pathway (22, 23).

Already in the mid 1950s, TH acetic acid derivates were tested in clinical practice. TA4 was evaluated as a potential substitute for T_4 in the treatment of myxedema and was shown to improve the myxedematous appearance without grossly affecting the basal metabolic rate (26–28). TA3 in turn was successfully used in the treatment of patients with resistance to TH syndrome because it potently suppresses pituitary TSH secretion with minimal metabolic side effects (29–31). That TSH expression is efficiently downregulated by TA3 may be explained by the binding properties of this compound to TH receptors (TRs). According to in vitro studies, TA3 binds with an almost 10-fold higher affinity than T_3 to the TRβ1 and TRβ2 receptors that are predominantly involved in the negative feedback regulation at the hypothalamic and pituitary level. With respect to the TRα1 isoform, TA3 displays an equal affinity as T_3 (32). In addition, TA3-induced activation of TR seems to be promoter dependent and to vary between different TH-responsive elements (32). Thus, to consider TA3 as a replacement for T_3, its cell-specific capacity in promoting T_3 effects needs to be carefully monitored.

A major disadvantage for the therapeutic application of TA3 is its short half-life in the circulation. In humans, TA3 has a half-life of only approximately 6 to 8 hours compared with T_3 with a half-life of 23 hours (33), whereas in rats, the half-life of triac at 5.5 hours is even shorter (34). Because TA4 exhibits a half-life of 3 to 4 days and can be converted to TA3 by outer-ring deiodination, it may be beneficial in the treatment of patients with *MCT8* mutations given that the cellular uptake of TA4 and TA3 specifically into the brain is not impeded in the absence of MCT8.

To which extent can TA4 exert TH action during brain development? To address this question, we applied TA4 to newborn mice and monitored TH-dependent neuronal differentiation processes during the first 3 postnatal weeks. To exclude endogenous TH effects, we took advantage of the congenital hypothyroid paired box gene 8 (*Pax8*)-KO mouse that is born without a functional thyroid gland and therefore cannot produce any TH endogenously (35). In addition, we used *Mct8*-KO and *Mct8*/*Pax8*–double-KO (dKO) mice to assess the impact of Mct8 deficiency on central TA4 and TA3 action. Our studies indicate that tetrac and triac are indeed able to promote T_3-dependent gene expression and neuronal differentia-

tion in mice and may be therefore considered as a therapeutic option for patients with inactivating *MCT8* mutations.

Materials and Methods

Animals

Mct8-KO mice were obtained from Deltagen (San Mateo, CA) and genotyped as described previously (12). *Pax8*-KO mice (35) were generated by mating heterozygous *Pax8*-KO mice and genotyped as reported elsewhere (36). *Mct8*/*Pax8*-dKO mice were obtained by mating *Mct8*-KO/*Pax8*$^{+/-}$ animals (37). The generation and phenotype of *Mct8*-KO, *Pax8*-KO, and *Mct8*/*Pax8*-dKO mice have been described previously (37). All animal studies were approved by the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (TLLV Thüringen, Erfurt, Germany). Animals were provided with standard laboratory chow and tap water ad libitum and were kept in accordance with local regulations (TLLV Thüringen) at constant temperature (22°C) and a 12-h light, 12-h dark light cycle.

Offspring of heterozygous *Pax8*$^{+/-}$ and *Mct8*-KO/*Pax8*$^{+/-}$ breeding pairs were sc injected once per day with either tetrac (400 ng/g body weight) or with 0.9% saline as control. For all studies, at least 4 male animals per genotype (*Pax8*$^{+/+}$ [WT], *Pax8*$^{-/-}$ [Pax8-KO], *Mct8*$^{-/y}$/*Pax8*$^{+/+}$ [*Mct8*-KO], *Mct8*$^{-/y}$/*Pax8*$^{-/-}$ [*Mct8*/*Pax8*-dKO]) were used. Animals were killed and tissues were collected 16 hours after the last injection. Detailed information about the procedures of tissue collection and processing are provided in the Supplemental Methods published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Primary cerebellar cultures

Mixed cerebellar cultures from newborn mice were prepared as described previously (38) and cultured in the presence or absence of 1nM T_3 and various concentrations of triac. After 14 days in vitro, cultures were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature and immunostained as described in supplemental data. Dendritic parameters were determined with the help of the ImageJ program as described previously (38). All experiments were performed 3 times.

Measurements of serum THs and brain deiodinase activities

Serum T_4/TA4 and T_3/TA3 concentrations were determined by RIA, and type 2 iodothyronine deiodinase (D2) and D3 activities in the brain were assessed as reported elsewhere (39).

Detailed information about experimental procedures concerning in situ hybridization, quantitative real-time PCR, immunohistochemistry, in vitro transport studies (40), and determination of T_4 and TA4 deiodination by D2 and D3 is provided in Supplemental Methods.

Statistical analysis

The values represent mean ± SEM. Statistical differences were calculated by 2-way ANOVA followed by Bonferroni-Holm post hoc test. $P > .05$ was considered not to be significant.

Results

Triac can promote cerebellar Purkinje cell differentiation in vitro

To elucidate the thyromimetic potential of TA3 in neurons, we took advantage of a well-established cerebellar cell culture system (38). Only in the presence of T_3 did Purkinje cells (PCs), the principal neurons of the cerebellar cortex, develop a highly elaborated dendritic tree that can be easily detected by calbindin-immunofluorescence staining. In previous experiments, we demonstrated that 1nM T_3 added to the serum-free culture medium for 2 weeks is sufficient to exert the most pronounced effects on PC dendritogenesis (38). Here, we performed similar experiments by preparing mixed cerebellar cultures from neonatal WT and *Mct8*-KO mice and incubating them in the presence or absence of 1nM T_3 or 1nM, 10nM, or 100nM TA3. After 14 days in vitro, the cultures were fixed with 4% paraformaldehyde and Purkinje cells were visualized using an antibody against calbindin. The dimensions of PCs obtained from three independent experiments were quantified using ImageJ. As depicted in Figure 1, PCs of both *Mct8*-KO and WT mice did not develop an extensive dendritic tree in the absence of TH but responded robustly to the treatment with 1nM T_3. A similar effect on arborization was also observed in the presence of 10nM or 100nM TA3, indicating that an approximately 10 times higher concentration of triac is needed to fully stimulate PC arborization. These cell culture experiments demonstrate a potent thyromimetic action of TA3 on neuronal differentiation independent of the presence or absence of Mct8.

Deiodination of T_4 and TA4 by D2 and D3

Because TA3 exhibits a very short half-life in humans and rodents, we considered TA4 as an alternative therapeutic option. For patients with *MCT8* mutations, therapeutic intervention using TA4 may be successful only if TA4 is similarly processed as T_4. D2 and D3 play an important role in TH metabolism within the CNS because D2 converts T_4 to T_3 in astrocytes and D3 inactivates both THs by inner-ring deiodination in neurons. Here, we compared the deiodination of T_4 and TA4 by recombinant human D2 and D3 expressed in transfected COS1 cells. The results show that D2 catalyzes the outer-ring deiodination of T_4 to T_3 and of TA4 to TA3 with equal efficacy (Figure 2A). Moreover, D3 catalyzes the inner-ring deiodination of TA4 to rTA3 as efficient as the conversion of T_4 to rT_3.

TA4 is not transported by MCT8 and OATP1C1 in vitro

As a prerequisite for using TA4 as alternative therapeutic option for MCT8 patients, TA4 transport must not

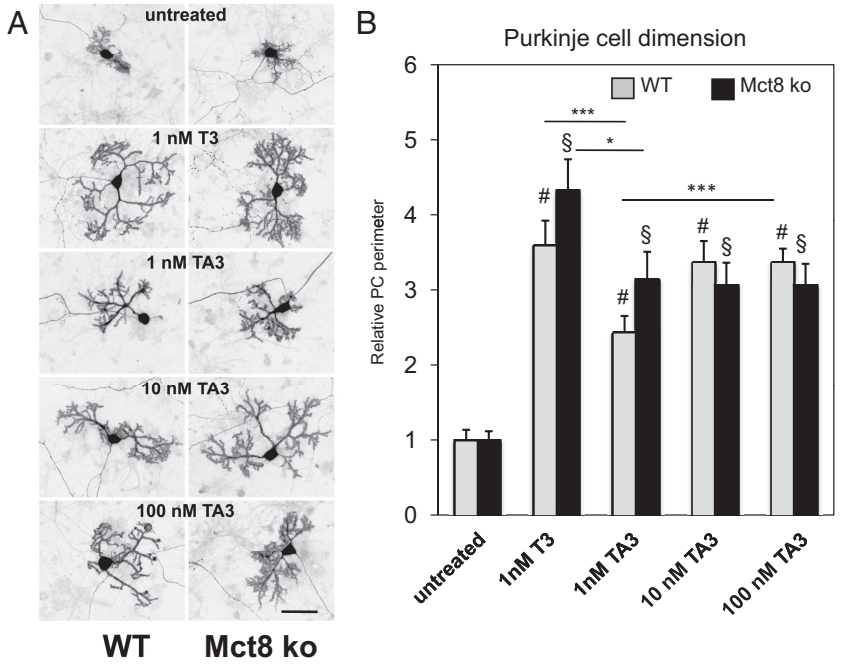

Figure 1. Effects of triac on cerebellar PC dendritogenesis in vitro. Mixed cerebellar cultures from WT and *Mct8*-KO mice were cultured for 14 days in the absence or presence of T_3 or TA3. A, Development of PCs was visualized by calbindin D28k staining. Scale bar, 100 μm. B, For quantification of PC dimension, the perimeter of 25 cells derived from three different cultures was quantified using ImageJ and normalized to the perimeter of PCs in untreated cultures of the respective genotype. Treatment of the cultures with 1nM T_3 resulted in pronounced formation of dendrites as shown by an almost 4-fold increase in PC perimeter. The same stimulating effect was achieved by adding 10nM or 100nM TA3 to the cultures. No differences were observed between cultures derived from WT or *Mct8*-KO mice. $*P < .05$; $***P < .001$; $\#P < .001$ for comparison with untreated WT cells; $\S P < .001$ (for comparison with untreated *Mct8*-KO cells).

be dependent on MCT8. To address this question, we studied TA4 transport by human MCT8 using transiently transfected COS1 cells. We also included human OATP1C1 in our experiments because in mice, this transporter seems to contribute significantly to the uptake of T_4 into the brain (19). Cells transfected with MCT8 or OATP1C1 produced an increase in cellular T_4 uptake during both 30- and 60-minute incubations compared with control cells transfected with empty vector (Figure 2B). At 60 minutes, cellular T_4 content increased from ~4% in control cells to ~7.5% for MCT8 and to ~6% for OATP1C1. Transfection with MCT8 or OATP1C1 did not alter TA4 uptake compared with control cells. The findings indicate that TA4 is not transported by MCT8 or OATP1C1.

T_4 and TA4 metabolism by D3 in intact cells

To study in more detail T_4 and TA4 transport across the plasma membrane by MCT8 and OATP1C1, COS1 cells were cotransfected with D3 and the metabolism of T_4 and TA4 was studied in intact cells. Cells cotransfected with MCT8 or OATP1C1 and D3 produced a significant increase in the conversion of T_4 at 4 hours compared with cells transfected with D3 alone (Figure 2C). This indicates that MCT8 and OATP1C1 enhance the intracellular availability of T_4. In contrast, expression of MCT8 and OATP1C1 did not affect TA4 conversion at either time point (4 or 24 hours) compared with D3 alone. Although our results suggest that TA4 is not as efficiently transported or converted in cells as T_4, incubations carried out without albumin in the medium for only 2 hours resulted in ~84% conversion of TA4 and ~90% conversion of T_4 in the presence of MCT8 (data not shown). These findings indicate that TA4 is transported into cells via transporters other than OATP1C1 and MCT8.

Effects of TA4 on brain development

For evaluating the action of TA4 in vivo, we treated newborn mice by sc injections of 400 ng/g body weight of TA4 once a day. We not only included *Mct8*-KO and WT littermates in our analysis but also took advantage of athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice that do not produce endogenous THs and therefore show severe retardation in brain development. When we analyzed the cerebellar PC development at postnatal day 12, we observed a strongly reduced dendritic outgrowth of PCs in saline-treated *Pax8*-KO and *Mct8*/*Pax8*-dKO mice as assessed by a significant reduction in the thickness of the molecular layer (Figure 3A). In contrast, the molecular layer of saline-injected WT and *Mct8*-KO mice showed a similar expansion of the molecular layer, indicating that PC dendritogenesis is not severely affected by the absence of Mct8. In animals receiving TA4 treatment, we found a similar degree of Purkinje cell dendritogenesis independent of the genotype. These findings suggest that postnatal application of TA4 promotes Purkinje cell dendritogenesis even in the absence of TH.

In the next approach, we studied D2 and D3 activities in cerebellar homogenates of 21-day-old animals (Figure 3, B and C). As expected, athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice showed highly elevated D2 activities in the cerebella of saline-treated animals. This rise in D2 could be partially prevented by TA4 administration. *Mct8*-KO mice also exhibit elevated D2 activities that

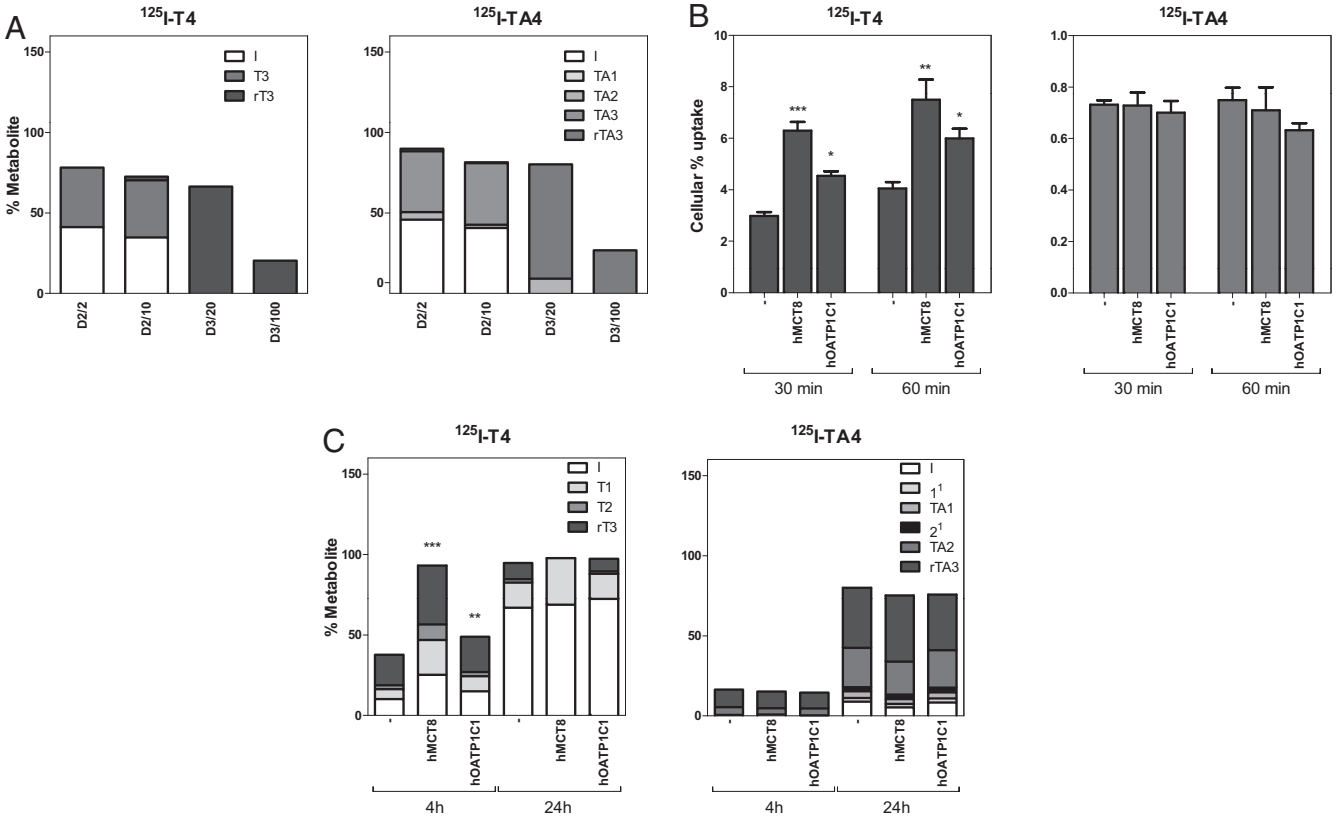

Figure 2. Tetrac serves as a substrate for D2 and D3 and is not transported by MCT8 or OATP1C1. To evaluate the metabolism of TA4 by deiodinases, COS1 cells were transfected with constructs encoding human D2 or human D3. Different dilutions of the cell lysates (1:2 and 1:10 for D2; 1:20 and 1:100 for D3) were incubated for 60 minutes at 37°C with 1nM [^{125}I]T_4 or [^{125}I]TA4, and radiolabeled metabolites were analyzed by HPLC. A, Both deiodinases accept TA4 as substrate equally as well as T_4. To evaluate TA4 transport by MCT8 or OATP1C1, COS1 cells were transiently transfected with constructs encoding human MCT8 (hMCT8) and human OATP1C1 (hOATP1C1) and incubated with [^{125}I]T_4 or [^{125}I]TA4. B, Results of 3 experiments each done in duplicates. In cells expressing either hMCT8 or hOATP1C1, only the uptake of T_4 is stimulated, whereas the transport of TA4 is not enhanced. C, To explore in more detail TA4 transport across the plasma membrane by MCT8 and OATP1C1, cells were cotransfected with human D3, which was shown to deiodinate TA4 as efficiently as T_4. Cells coexpressing OATP1C1 and hMCT8 exhibited increased T_4 metabolism, whereas TA4 metabolism was not altered. $*P < .05$; $**P < .01$; $***P < .001$. 1^1 and 2^1 are unknown metabolites 1 and 2 of TA4.

were reduced by TA4 injections. Surprisingly, in WT mice, TA4 treatment led to increased D2 activities, presumably due to a suppression of endogenous TH production. As a consequence, animals of all four genotypes responded to the TA4 treatment with very similar D2 activities that were twice as high as in euthyroid controls but significantly reduced compared with the activities measured in homogenates of athyroid animals. Compared with WT animals, cerebellar D3 activities were close to the detection limit in athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice but could be stimulated by TA4 substitution. In WT and *Mct8*-KO mice, TA4 treatment resulted in rather reduced D3 levels compared with the saline-injected animals.

We did not restrict our analysis to the cerebellar development but also monitored the thyroidal state in specific neurons of the forebrain. Expression of neurogranin (RC3), a protein involved in calcium/calmodulin and protein kinase C signaling, is strongly altered under hypothyroid conditions particularly in the striatum, an important region for motor control (41). As depicted in Figure 4, RC3-specific in situ hybridization signals were absent in the striatum of athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO, whereas *Mct8*-KO mice were characterized by a slight reduction compared with WT mice. TA4 treatment restored RC3 expression in all four genotypes, indicating that the amount of TA4 was sufficient to exert a thyromimetic action in the striatum.

Other targets of TH action during postnatal brain differentiation are parvalbumin-positive GABA-ergic neurons in distinct areas of the cerebral cortex (42). As illustrated in Figure 5, athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice at P12 displayed a strongly diminished parvalbumin immunoreactivity in the retrosplenial (Figure 5A) and somatosensory (Figure 5B) cortex that was partially normalized by TA4 injections. In *Mct8*-KO mice, TA4 treatment even reduced the number of parvalbumin-positive neurons, whereas WT animals did not show any overt response toward the treatment. We therefore con-

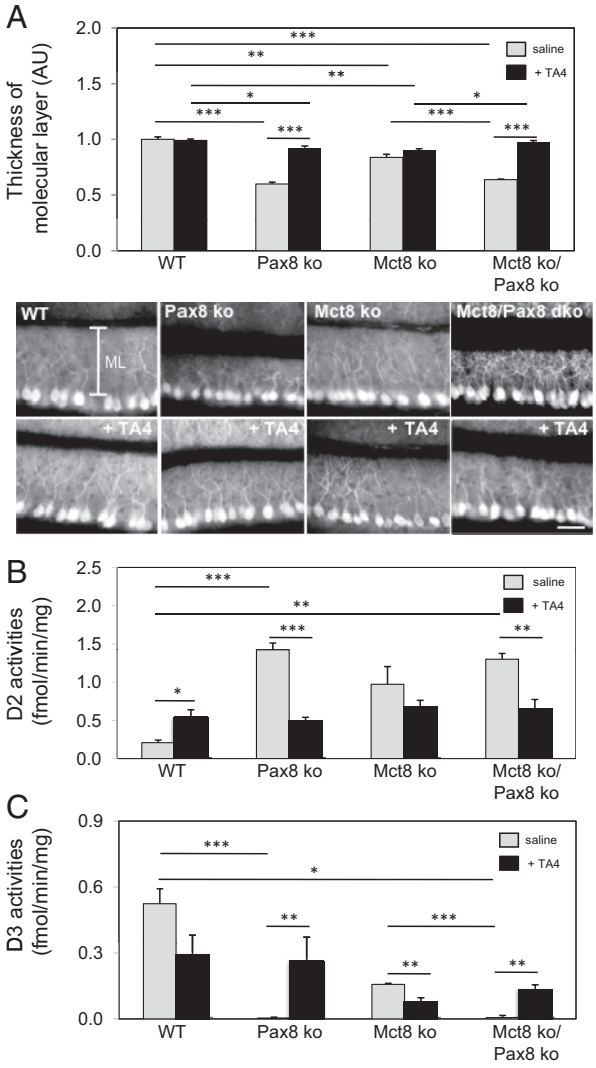

Figure 3. Thyromimetic actions of tetrac in the cerebellum of TH-deficient animals. WT, *Pax8*-KO, *Mct8*-KO, and *Mct8*/*Pax8*-dKO animals were daily injected with tetrac starting at the day of birth (postnatal day 0). Control animals received saline injections. A, Sagittal cerebellar vibratome sections through the vermis regions from animals killed at postnatal day 12 were immunostained with an anti-calbindin D28k antibody to visualize cerebellar PC differentiation. Thickness of the molecular layer that comprises PC dendrites was determined on 3 consecutive sections of each animal (males, n = 4 per genotype) at the primary fissure between anterior and posterior lobe using ImageJ. As a characteristic feature of a hypothyroid cerebellum, PCs show a strongly reduced dendritic arborization in athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO compared with WT and *Mct8*-KO mice, which is also illustrated by a thinner molecular layer. Treatment of the animals with TA4 restored normal thickness of the molecular layer in animals of all genotypes. Scale bar 50 μm. Cerebellar D2 (B) and D3 (C) activities were determined in TA4- and saline-treated animals at postnatal day 21 (males, n = 4 per genotype). Athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice exhibited a 7-fold increase in D2 activities, whereas D3 activities were close to the detection limit. In *Mct8*-KO mice, D2 was 4.5-fold elevated compared with WT levels and D3 activities were reduced to 30% of WT levels. TA4 treatment resulted in similar D2 activities in all four genotypes with a 2- to 2.5-fold increase compared with control animals but a significant reduction of 50%–60% compared with the situation in athyroid animals. Data are expressed as mean ± SEM. ***$P < .001$; **$P < .01$; *$P < .05$. Abbreviation: AU, arbitrary unit.

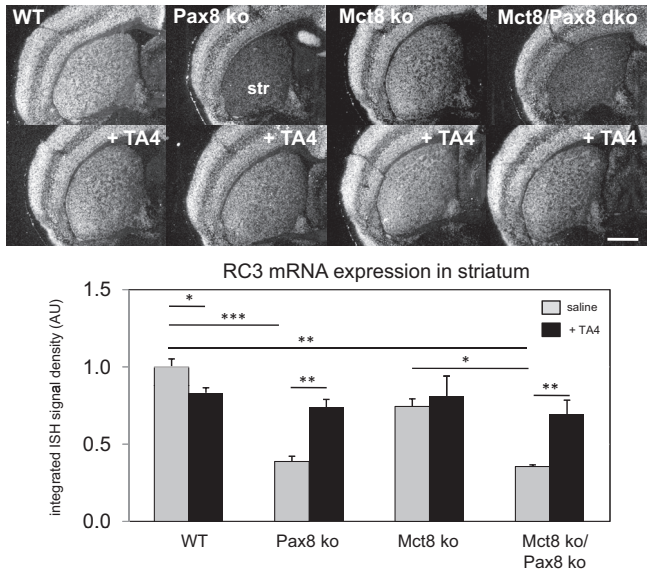

Figure 4. Thyromimetic actions of tetrac in the striatum of TH-deficient animals. Dark-field autoradiograms illustrate TH-dependent alterations in RC3 mRNA expression in striatal areas (str) of 21-day-old animals (males, n = 4 per genotype). Scale bar, 2 mm. In situ hybridization signals in the striatum of 4 animals per experimental group were quantified using imageJ. TA4 treatment was sufficient to induce RC3 expression in athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice similar to the levels of untreated control animals. ***$P < .001$; **$P < .01$; *$P < .05$. Abbreviation: AU, arbitrary unit.

clude that distinct neuronal populations responded differentially to the application of TA4.

Effects of tetrac on the hypothalamus-pituitary-thyroid axis

Another important aspect of our study was to determine the response of the hypothalamus-pituitary-thyroid (HPT) axis to TA4 treatment. Hypothalamic TRH expression was assessed by radioactive in situ hybridization of brain sections derived from 21-day-old mice. As depicted in Figure 6A, athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice demonstrated strongly enhanced signal intensities compared with euthyroid controls. In line with previous findings (12), TRH transcript levels were elevated in *Mct8*-KO mice as well. Intriguingly, TA4 was unable to suppress TRH expression in all four genotypes. Even more surprisingly, TRH expression in WT animals increased upon TA4 treatment, suggesting that these neurons are in a hypothyroid state, presumably again due to a suppression of endogenous TH production.

In contrast to the hypothalamus, TA4 administration resulted in pronounced alterations in the anterior pituitary (Figure 6B). In all 4 groups of TA4-treated animals, TSH mRNA levels were close to the detection limit, indicating a strong suppressive effect of this compound on thyrotrophic cells. To evaluate the consequences of TA4 on endogenous TH production, we determined T_3 and T_4 concentrations in the serum of these animals at postnatal

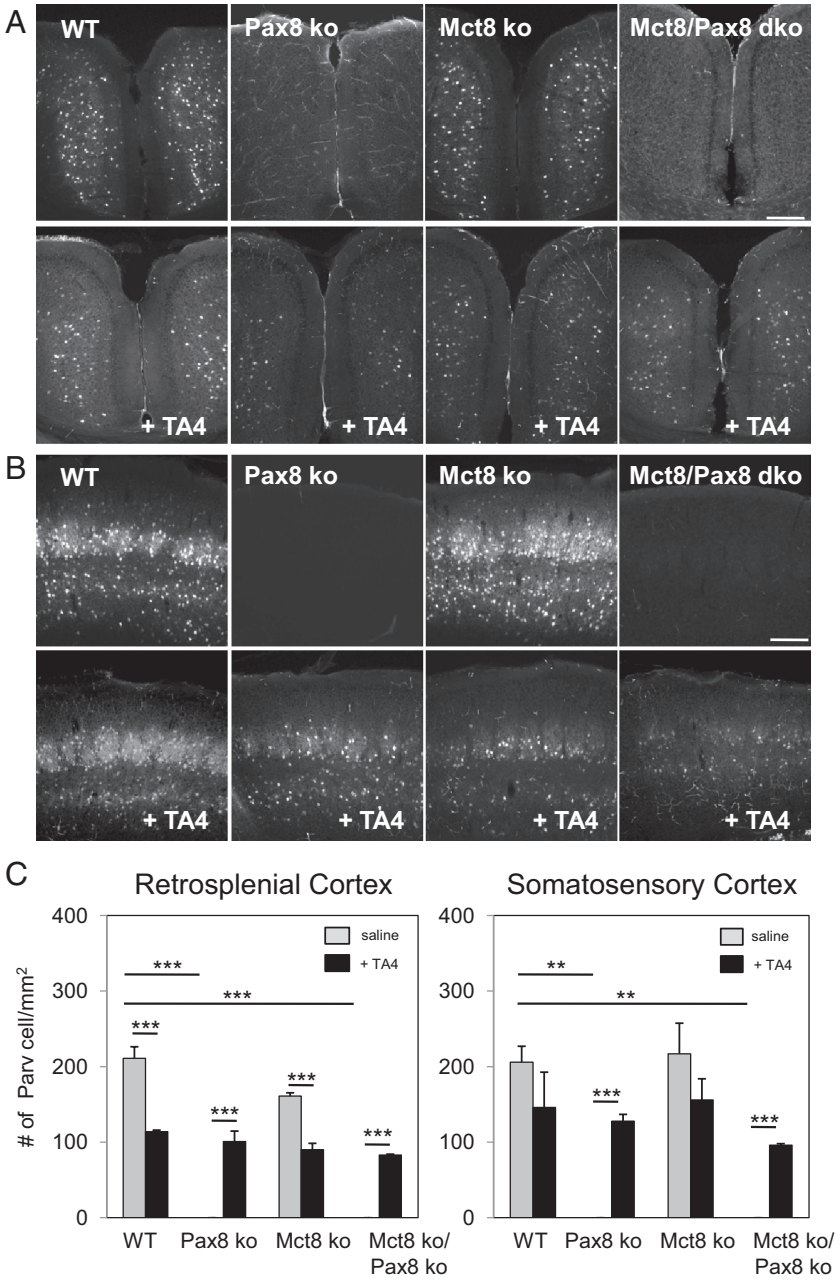

Figure 5. Tetrac stimulates the maturation of parvalbumin-immunoreactive neurons in the somatosensory and restrosplenial cortex. WT, *Pax8*-KO, *Mct8*-KO, and *Mct8*/*Pax8*-dKO animals were daily injected sc with tetrac starting at the day of birth (postnatal day 0). Control animals received saline injections. Animals (males, n = 4 per genotype) were perfusion-fixed at postnatal day 12. Coronal vibratome sections were immunostained with an antibody against parvalbumin that labels a subset of GABAergic interneurons. In athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice, the number of parvalbumin-immunoreactive neurons was strongly diminished in the retrosplenial (A) and somatosensory area (B) of the cerebral cortex. Substitution of the animals with TA4 resulted in a partial recovery. However, in all TA4-treated animals, the number of parvalbumin-immunoreactive neurons was reduced compared with control animals. Scale bars in A and B, 50 μm. C, Numbers of parvalbumin-positive cells were counted on 3 consecutive sections from each animal using 4 animals per genotype. Data are expressed as mean ± SEM. ***$P < .001$; **$P < .01$.

day 21 (Figure 6C). Unfortunately, the antibodies used in the RIA are not able to distinguish between T_4/TA4 and T_3/TA3, respectively. Because *Pax8*-KO and *Mct8*/*Pax8*-dKO do not produce TH endogenously, the values obtained by RIA must solely reflect TA4 and TA3 values and indicate the presence of 1nM TA3 and 120nM TA4 after the substitution of the animals with 400 ng TA4/g body weight. Of note, *Mct8*-KO mice showed rather similar values despite the presence of a functional thyroid gland. Because these animals received the same TA4 dose as the athyroid mutants, endogenous T_3 and T_4 production must substantially be repressed in these animals. This seems not to be the case in WT animals because these mice showed the highest T_4/TA4 and T_3/TA3 values of all TA4-treated animals.

Effects of tetrac on D1 expression in liver and kidneys

Finally, we investigated the impact of the TA4 treatment on D1 transcript levels (Figure 7). D1, mainly present in liver, kidneys, and the thyroid, catalyzes both outer- and inner-ring deiodination and is positively and potently regulated by T_3. Thus, changes in expression levels of this enzyme are a well-suited indicator for alterations in the content of TH in tissues. In TA4-treated WT mice, D1 mRNA levels were 3-fold increased in the liver and 2-fold enhanced in the kidneys compared with animals that received only saline injections. A similar hyperthyroid situation was observed in TA4-treated *Pax8*-KO mice. Intriguingly, the elevation in hepatic D1 expression observed in *Mct8*-KO mice was not further up-regulated by TA4 application. In the kidneys, TA4 treatment even resulted in a mild decrease of the highly increased D1 expression levels. TA4-treated *Mct8*/*Pax8*-dKO mice showed similar hepatic and renal D1 transcript levels as the TA4-treated *Mct8*-KO mice. We therefore conclude that the treatment of Mct8-deficient animals with TA4 is not associated with an aggravation of the thyrotoxic situation in liver and kidneys.

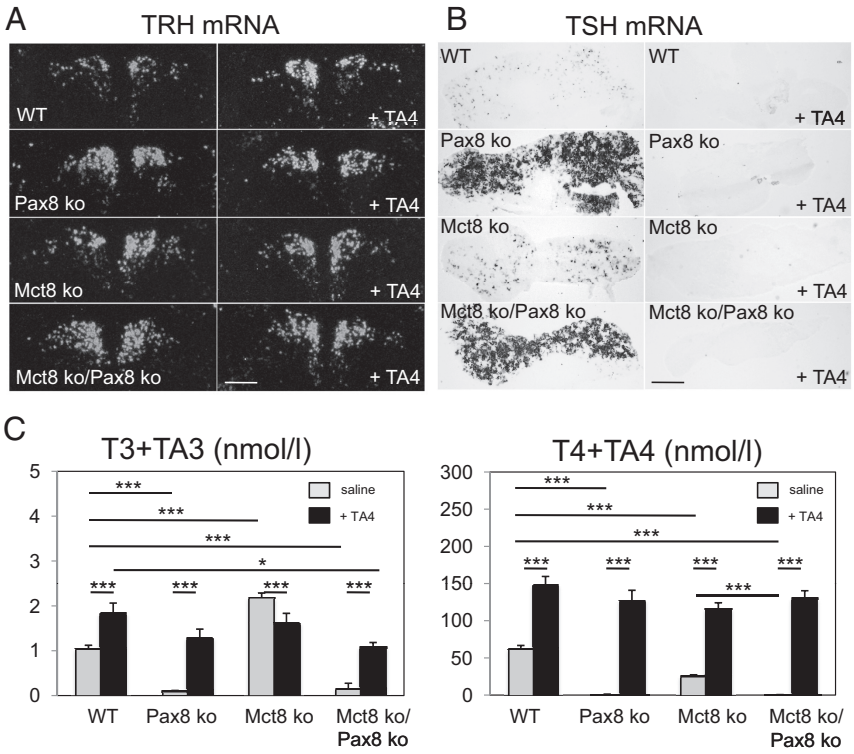

Figure 6. Analysis of tetrac action in the HPT axis. A, Dark-field autoradiograms illustrate TRH mRNA expression in hypothalamic brain sections derived from 21-day-old animals (males, n = 4 per genotype). Compared with WT mice, TRH-specific hybridization signals were visibly increased in the paraventricular hypothalamic nucleus of *Pax8*-KO, *Mct8*-KO, and *Mct8*/*Pax8* double mutant mice (left row). In TA4-treated animals, TRH expression was strongly elevated in all 4 animal groups, indicating that TRH expression was not suppressed by TA4 treatment (right row). Scale bar, 200 μm. B, In contrast, pituitary TSH expression as illustrated by nonradioactive in situ hybridization was strongly suppressed upon TA4 administration in all animals. Scale bar, 400 μm. C, Serum samples from 21-day-old animals were used to determine T_4/TA4 and T_3/TA3 concentrations by RIA. Because *Pax8*-KO and *Mct8*/*Pax8* double-deficient animals do not produce any TH endogenously, the respective values represent the TA4 and TA3 concentrations in the serum. Data are expressed as mean ± SEM. ***$P < .001$; **$P < .01$; *$P < .05$.

Discussion

Because patients with inactivating *MCT8* mutations suffer from severe neurological impairments, it seems of utmost importance to develop a therapeutic strategy that could improve the situation of these patients. Unfortunately, the exact mechanisms that result in the development of the neurological symptoms are still not known. Parameters such as decreased TH concentrations in the cerebrospinal fluid and delayed myelination (43–45) as found in several patients support the hypothesis that a strongly diminished TH supply during critical stages of brain development is substantially linked to the phenotype. However, in contrast to children that suffer from congenital hypothyroidism or neurological cretinism, treatment of patients with TH is not expected to provide any benefit if TH transport into the brain or into neurons is impaired. A more promising approach appears to treat patients with thyromimetic substances that do not depend on MCT8 for cellular uptake and can replace TH in promoting neural differentiation such as proper myelination, dendritogenesis, and synapse formation during postnatal stages. To achieve the most positive effect, treatment should be initiated ideally as soon as possible after birth. However, even a treatment initiated later in life may have some beneficial outcome on the patients' brain functions and should therefore be considered.

The second hallmark of MCT8 deficiency, the abnormal TH serum profile, also represents a target for therapeutic interventions because the high circulating T_3 levels cause a thyrotoxic state of peripheral organs with pronounced alterations in metabolism and energy expenditure. A normalization of serum TH parameters can be achieved only if endogenous TH production is largely turned off. Such an approach was successfully applied to one MCT8 patient who was substituted with T_4 in the presence of high doses of PTU that blocked TH production and probably also inhibited T_4 to T_3 conversion to some extent. Under this treatment, the patient significantly gained weight and tachycardia was abated (10). Continuous PTU treatment in high doses, however, can have severe side effects of which agranulocytosis and liver toxicity are the most severe (46). Thus, alternative procedures to lower serum T_3 levels are of value for the treatment of the patients even if such an achievement is not expected to have any positive effects on the brain.

We put forward the hypothesis that the TH analog triac may exhibit a therapeutic potential for the treatment of patients with *MCT8* mutations based on the following observations. In vitro studies have revealed that triac can activate TRs thereby replacing T_3 (32). Moreover, TA3 has already been used in clinical practice for treating patients who suffer from the syndrome of resistance to TH. Due to mutations in the *TR*β gene, these patients display high TSH and TH levels that could be effectively suppressed by TA3 (29, 30, 47, 48). In general, TA3 treatment was accompanied by the positive trend of various indexes of tissue-specific TH action in these patients (31). Overall, the clinical experience with long-term TA3 treatment in this group of hyperthyroid patients has proven the poten-

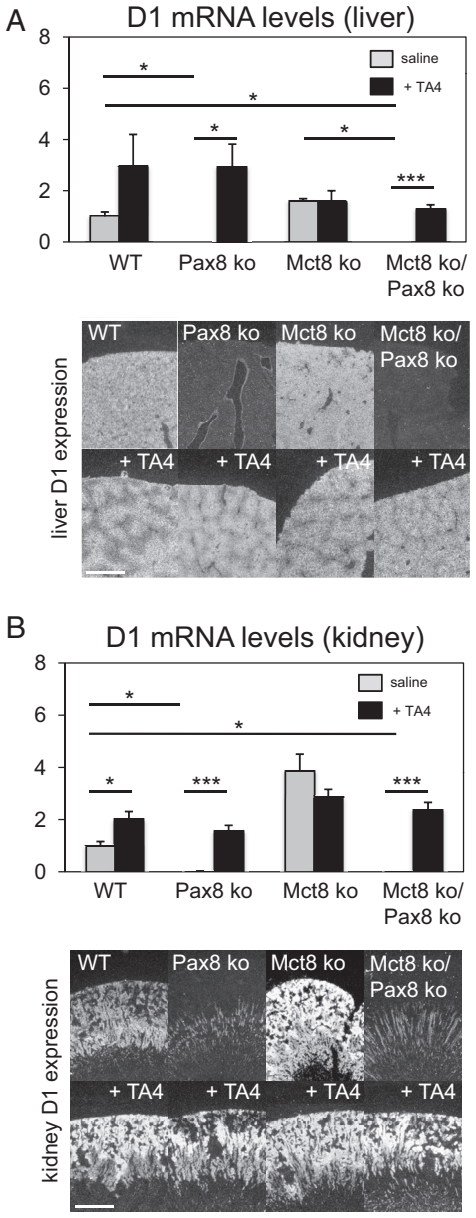

Figure 7. Regulation of renal and hepatic D1 expression by tetrac. As a marker for the tissue-specific thyroidal state, D1 mRNA expression was analyzed by quantitative PCR and radioactive in situ hybridization in liver (A) and kidneys (B) of 21-day-old animals (males, n = 4 per genotype). D1 expression in saline-treated *Pax8*-KO and *Mct8*/*Pax8*-dKO mice was close to the detection limit in both tissues but could be induced by TA4 treatment. In *Mct8*-KO mice that showed highly elevated renal D1 transcript levels, TA4 application resulted in a mild reduction of D1 mRNA levels, whereas hepatic D1 mRNA was not altered by the treatment. Data are expressed as mean ± SEM. $***P < .001$; $**P < .01$; $*P < .05$. Scale bar, 50 μm.

tial efficacy and safety of TA3 even when given during childhood.

TH action is mainly mediated via the nuclear hormone receptor isoforms encoded by the *TR*β and *TR*α genes. The clinical observation of a pronounced repressive action of TA3 on the HPT axis as well as in vitro promoter assays indicate a higher affinity of TA3 toward the TRβ isoforms compared with TRα1. During brain development, however, TRα plays the predominant role because it is rather ubiquitously expressed and already present during prenatal stages, whereas TRβ expression develops later and is restricted to certain neuronal populations (49–51). Consequently, TA3 has to be able to activate TRα1 in neurons to replace TH action in the Mct8-deficient brain. To test the potency of TA3 in promoting neuronal differentiation, we took advantage of the fact that T_3-dependent activation of TRα1 is mandatory for promoting cerebellar PC dendritogenesis in vitro (38). When we cultured primary cerebellar neurons in the presence of either T_3 or TA3, we found a strong, dose-dependent effect of TA3 on PC dendrite formation. These findings indicate that TA3 is indeed able to enter neurons and activate TRα1, although 10 times higher concentrations of triac were needed to achieve the same effect as T_3.

A major disadvantage for the clinical application of TA3 is its short half-life of 6 hours in humans (33). Consequently, TA3 has to be administered at least 3 to 4 times daily or as a continuous-release formulation, which has not been tested in clinical studies. We therefore considered TA4 as an alternative because TA4 does not only have a longer half-life of 3 to 4 days but also can act as a prohormone for TA3 much like T_4 is a prohormone for T_3. To fulfill its anticipated action within the brain, TA4 has to be transported into the brain via the blood-brain barrier and then taken up by astrocytes. That indeed neither MCT8 nor the T_4-specific transporter OATP1C1 is involved in the cellular uptake of TA4 could be demonstrated by transport studies using COS1 cells transfected with the respective transporter cDNA (Figure 2). Still, it remains to be investigated which transporters facilitate the passage of the TH acetic acid derivatives in and out of cells. Once TA4 has entered the brain, it should be subjected to outer-ring deiodination to produce the TR active compound TA3. Using COS1 cells that overexpress either human D2 or D3 we could indeed demonstrate that TA4 is a potent substrate for deiodination by D2 and D3. We therefore assume that similar enzymatic reactions may also take place in vivo.

To test whether TA4 is able to replace T_4 in such a manner, we explored its action in promoting brain development by using *Pax8*-KO mice as a suitable in vivo model system because *Pax8*-KO mice do not produce any endogenous TH that could interfere with our experiments. We also included *Mct8*/*Pax8*-dKO mice in our studies to rule out that Mct8 deficiency indirectly affects TA4/TA3 transport in the CNS. During the first 3 weeks, the animals were daily injected with TA4. Brain development was carefully monitored and compared with untreated *Pax8*-KO and *Mct8*/*Pax8*-dKO mice that exhibit a pro-

nounced hypothyroid brain phenotype as characterized by a delayed cerebellar PC development (Figure 3A) and decelerated maturation of parvalbumin-immunopositive GABAergic interneurons in specific cortical brain areas (Figure 5). Whereas TA4 application restored normal cerebellar development as assessed by the thickness of the molecular layer, only a partial normalization could be achieved with respect to the inhibitory parvalbuminergic system in the cerebral cortex. In the striatum, however, where RC3 expression is under positive control of T_3, TA4 administration was sufficient to induce RC3 expression similar to the levels found in untreated control animals (Figure 4). Overall, these data indicate that TA4 and its receptor-active metabolite TA3 can indeed replace T_4 and T_3 action in various brain areas, although the efficacy may vary between distinct neuronal populations or even between different genes that are controlled in a given cell type by TH in a positive or negative manner.

The enzyme D2 is known to be negatively regulated by T_3 at the transcriptional level and even more so by T_4 at the posttranslational level as T_4 markedly stimulates D2 inactivation (52–54). Accordingly, athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice showed highly elevated D2 activities in the cerebellum that could be partially suppressed by the TA4 treatment. Although these findings clearly indicate that TA4 can act in astrocytes, TA4 seems to be less efficient than T_4 in promoting D2 inactivation, an observation in line with previous reports (55). In contrast to D2, D3 is positively regulated by T_3 (56, 57), and tetrac treatment was indeed able to induce D3 expression in the cerebellum of athyroid *Pax8*-KO and *Mct8*/*Pax8*-dKO mice. Intriguingly, TA4-treated WT animals showed increased D2 and decreased D3 activities compared with saline-treated animals. We speculate that these changes are mainly due to a strongly suppressed endogenous TH production and an insufficient tetrac dose to restore normal D2 and D3 levels. That compared with control animals D2 is still elevated under TA4 treatment may be even considered as an advantage because it may ensure increased local triac production, thereby counteracting the shorter half-life of this compound.

A rather unexpected finding was the lack of responsiveness toward TA4 treatment in TH-sensitive neurons of the hypothalamic PVN. In all genotypes, TRH expression was highly elevated after TA4 administration, indicating a hypothyroid state of these cells. This observation was rather surprising because TRβ isoforms to which TA3 has even a higher affinity than T_3 (32) are predominantly involved in the regulation of TRH expression (58, 59). Indeed, pituitary TSH expression that is also controlled by TRβ isoforms was strongly down-regulated in TA4-treated animals. We therefore cannot exclude that the active metabolite TA3 does not reach the TRH-expressing PVN neurons, eg, due to a lack of suitable transporters in neurons or in tanycytes, and are therefore in a hypothyroid state. However, the increase in TRH expression does not seem to affect endogenous TH production because TSH mRNA levels were strongly suppressed.

To monitor the activity of the HPT axis in response to the treatment, we also aimed to determine TA4, TA3, and TH levels in the circulation and therefore collected serum samples from saline- and TA4-treated animals at postnatal day 21 (Figure 6C). Unfortunately, the antibodies used in the RIA were not able to distinguish between T_3 and TA3 or T_4 and TA4, respectively. However, because *Pax8*-KO and *Mct8*/*Pax8*-dKO mice do not produce any TH endogenously, the values measured in these animals after TA4 treatment must solely reflect the circulating TA3 and TA4 concentrations. Because TA4-treated *Mct8*-KO and WT mice showed similar levels as the TA4-treated athyroid mice, the endogenous TH production in *Mct8*-KO mice must be greatly reduced in response to the administration of TA4. This is a favorable side effect because the peripheral hyperthyroid situation found in *Mct8*-KO mice should ideally be improved by the treatment as well. However, when we analyzed D1 expression in liver and kidneys, we still found highly elevated transcript levels in TA4-treated animals, although the values did not exceed those found in saline-treated *Mct8*-KO mice (Figure 7). Thus, the thyrotoxic situation of the peripheral tissues in the absence of Mct8 was not significantly ameliorated but also not further worsened by the application of TA4. Probably the use of lower TA4 concentrations would be beneficial for normalizing the hyperthyroid state of peripheral tissues, whereas even higher doses may be favorable for promoting brain development.

Overall, our analysis demonstrated the potency of TA4 in replacing TH during brain development even in the absence of Mct8. Thus, TA4 may be considered as a therapeutic option for patients with *MCT8* mutations particularly when these patients are diagnosed very early in life and irreversible brain damage as a consequence of insufficient TH supply can still be prevented. For normalizing serum TH parameters later in life, application of lower TA4 doses may be suited due to the strong suppressive effects of TA4 on endogenous TH production.

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