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

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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 T3 levels in the presence of low T4 and normal TSH values. As a consequence of the increased serum T3 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 T₄ 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 T₃ 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 T₄ 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 T₄ and T₃ 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 T₃ 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 T₃ 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 derivatives 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 derivatives were tested in clinical practice. TA4 was evaluated as a potential substitute for T₄ 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 down-regulated 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 T₃ 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 T₃ (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 T₃, its cell-specific capacity in promoting T₃ 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 T₃ 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 T₃-dependent gene expression and neuronal differen-
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+/− mice and genotyped as reported elsewhere (36). Mct8/Pax8-dKO mice were obtained by mating Mct8−/−/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 breeding pairs were sc injected once per day with either tetrac or with 0.9% saline (0.9% saline). For all experiments 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 T4 in the presence of or absence of Mct8. 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 T4, and D2 and D3 play an important role in TH metabolism within the CNS because D2 converts T4 to T3 in astrocytes and D3 inactivates both THs by inner-ring deiodination in neurons. Here, we compared the deiodination of T4 and TA4 by recombinant human D2 and D3 expressed in transfected COS1 cells. The results show that D2 catalyzes the outer-ring deiodination of T4 to T3 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 T4 to rT3.

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 T3 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 T3 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 T3 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 T3. 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 T4 and TA4 by D2 and D3

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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...
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 T₄ into the brain (19). Cells transfected with MCT8 or OATP1C1 produced an increase in cellular T₄ uptake during both 30- and 60-minute incubations compared with control cells transfected with empty vector (Figure 2B). At 60 minutes, cellular T₄ 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.

T₄ and TA4 metabolism by D3 in intact cells

To study in more detail T₄ and TA4 transport across the plasma membrane by MCT8 and OATP1C1, COS1 cells were cotransfected with D3 and the metabolism of T₄ and TA4 was studied in intact cells. Cells cotransfected with MCT8 or OATP1C1 and D3 produced a significant increase in the conversion of T₄ at 4 hours compared with cells transfected with D3 alone (Figure 2C). This indicates that MCT8 and OATP1C1 enhance the intracellular availability of T₄. 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 T₄, incubations carried out without albumin in the medium for only 2 hours resulted in ~84% conversion of TA4 and ~90% conversion of T₄ 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 [125I]T4 or [125I]TA4, and radiolabeled metabolites were analyzed by HPLC. A, Both deiodinases accept TA4 as substrate equally as well as T4. To evaluate TA4 transport by MCT8 or OATP1C1, COS1 cells were cotransfected with human MCT8 (hMCT8) and human OATP1C1 (hOATP1C1) and incubated with [125I]T4 or [125I]TA4. B, Results of 3 experiments each done in duplicates. In cells expressing either hMCT8 or hOATP1C1, only the uptake of T4 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, transiently transfected with constructs encoding human MCT8 (hMCT8) and human OATP1C1 (hOATP1C1) and incubated with [125I]TA4. B, Results of 3 experiments each done in duplicates. In cells expressing either hMCT8 or hOATP1C1, only the uptake of T4 is stimulated, whereas TA4 metabolism was not altered. *P < .05; **P < .01; ***P < .001. 11 and 21 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 animals. 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). TA4 treatment was sufficient to induce RC3 expression in striatal areas (str) of 21-day-old thyroid-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.

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 thyrotrrophic cells. To evaluate the consequences of TA4 on endogenous TH production, we determined T3 and T4 concentrations in the serum of these animals at postnatal...
Unfortunately, the antibodies used in the RIA are not able to distinguish between T4/TA4 and T3/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 T3 and T4 production must substantially be repressed in these animals. This seems not to be the case in WT animals because these mice showed the highest T4/TA4 and T3/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 T3. 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.
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 T₃ 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 T₄ in the presence of high doses of PTU that blocked TH production and probably also inhibited T₄ to T₃ 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 T₃ 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 T₃ (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 TRβ 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 potential 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.
companied 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 T3-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 T3 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 T3.

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 T4 is a prohormone for T3. 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 T4-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 T4 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-

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 TRβ and TRα 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 T3-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 T3 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 T3.

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To test whether TA4 is able to replace T4 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 T3, 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 T4 and T3 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 T3 at the transcriptional level and even more so by T4 at the posttranslational level as T4 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 T4 in promoting D2 inactivation, an observation in line with previous reports (55). In contrast to D2, D3 is positively regulated by T3 (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 salinetreated 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 T3 (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 T3 and TA3 or T4 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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