

## In Vitro and Mouse Studies Supporting Therapeutic Utility of Triiodothyroacetic Acid in MCT8 Deficiency

Simone Kersseboom,\* Sigrun Horn,\* W. Edward Visser, Jiesi Chen, Edith C. H. Friesema, Catherine Vours-Barrière, Robin P. Peeters, Heike Heuer,\* and Theo J. Visser\*

Department of Internal Medicine (S.K., W.E.V., E.C.H.F., R.P.P., T.J.V.) and Rotterdam Thyroid Center (S.K., W.E.V., R.P.P., T.J.V.), Erasmus University Medical Center, 3015 CN Rotterdam, The Netherlands; Leibniz Institute for Age Research/Fritz Lipmann Institute (S.H., J.C., H.H.), Jena, Germany; Inserm (C.V.-B.), Unité Mixte de Recherche (UMR) 1103, and Centre National de la Recherche Scientifique (C.V.-B.), UMR6293, F-63001 Clermont-Ferrand, France; Clermont Université (C.V.-B.), Université d'Auvergne, Laboratoire GReD, BP 10448, F-63000 Clermont-Ferrand, France; and Leibniz Research Institute for Environmental Medicine (J.C., H.H.), Düsseldorf, Germany

Monocarboxylate transporter 8 (MCT8) transports thyroid hormone (TH) across the plasma membrane. Mutations in *MCT8* result in the Allan-Herndon-Dudley syndrome, comprising severe psychomotor retardation and elevated serum T<sub>3</sub> levels. Because the neurological symptoms are most likely caused by a lack of TH transport into the central nervous system, the administration of a TH analog that does not require MCT8 for cellular uptake may represent a therapeutic strategy. Here, we investigated the therapeutic potential of the biologically active T<sub>3</sub> metabolite Triac (TA3) by studying TA3 transport, metabolism, and action both in vitro and in vivo. Incubation of SH-SY5Y neuroblastoma cells and MO3.13 oligodendrocytes with labeled substrates showed a time-dependent uptake of T<sub>3</sub> and TA3. In intact SH-SY5Y cells, both T<sub>3</sub> and TA3 were degraded by endogenous type 3 deiodinase, and they influenced gene expression to a similar extent. Fibroblasts from MCT8 patients showed an impaired T<sub>3</sub> uptake compared with controls, whereas TA3 uptake was similar in patient and control fibroblasts. In transfected cells, TA3 did not show significant transport by MCT8. Most importantly, treatment of athyroid *Pax8*-knockout mice and *Mct8/Oatp1c1*-double knockout mice between postnatal days 1 and 12 with TA3 restored T<sub>3</sub>-dependent neural differentiation in the cerebral and cerebellar cortex, indicating that TA3 can replace T<sub>3</sub> in promoting brain development. In conclusion, we demonstrated uptake of TA3 in neuronal cells and in fibroblasts of MCT8 patients and similar gene responses to T<sub>3</sub> and TA3. This indicates that TA3 bypasses MCT8 and may be used to improve the neural status of MCT8 patients. (*Molecular Endocrinology* 28: 1961–1970, 2014)

The widely expressed monocarboxylate transporter 8 (MCT8; SLC16A2) has been characterized as an important thyroid hormone (TH) transporter facilitating cellular uptake and export of T<sub>3</sub> and T<sub>4</sub> (1, 2). The *MCT8* gene is localized on the X-chromosome, and mutations in *MCT8* are associated with the Allan-Herndon-Dudley syndrome (AHDS) (3, 4). This syndrome is characterized

by severe psychomotor retardation in combination with disturbed thyroid function tests, ie, high T<sub>3</sub>, low-normal T<sub>4</sub>, and normal to increased TSH. Clinical features include spastic quadriplegia, axial hypotonia, and severe mental retardation (5–9). These patients also suffer from peripheral thyrotoxicosis, producing tachycardia and muscle wasting. Since the genetic cause of AHDS has been

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\* S.K. and S.H. and H.H. and T.J.V., respectively, contributed equally to this work.

Abbreviations: AHDS, Allan-Herndon-Dudley syndrome; BW, body weight; DITPA, 3,5-diiodothyropropionic acid; dKO, double knockout; FBS, fetal bovine serum; GABA,  $\gamma$ -aminobutyric acid; KO, knockout; MBP, myelin basic protein; MCT8, monocarboxylate transporter 8; P1, postnatal day 1; PC, Purkinje cell; PMA, phorbol 12-myristate 13-acetate; PV, parvalbumin; TA3, 3,3',5-triiodothyroacetic acid; TH, thyroid hormone; TR $\beta$ , TH receptor- $\beta$ ; WT, wild-type.

elucidated, many patients with *MCT8* mutations have been identified worldwide (10). With this increasing prevalence also the need for therapy rises.

Based on the findings of mildly increased serum TSH levels and mildly decreased serum free  $T_4$  levels, several AHDS patients have been treated with  $L-T_4$  (11, 12). This did not improve mental function, whereas it even further increased the already elevated serum  $T_3$  levels and worsened the thyrotoxic state of peripheral tissues. Another therapeutic approach has been the normalization of serum TH levels by block-and-replace therapy using PTU and  $L-T_4$ , which has been applied to older AHDS patients (13, 14). This treatment improved the peripheral thyrotoxicosis, leading to a normalization of the heart rate as well as a gain in body weight (BW). However, it did not improve psychomotor function.

The pathogenic mechanism of *MCT8* mutations is not completely resolved. It is well-known that TH is crucial for brain development (15). Studies in *Mct8*-knockout (KO) mice (16–18) and in vitro studies using patient-derived fibroblast and transfected cells (19–21) have revealed impaired cellular TH transport, in particular into the brain. These findings support the hypothesis that the key factor in the pathogenic mechanism is the lack of  $T_3$  transport via the blood-brain barrier and/or into neurons, causing poor regulation of TH-dependent genes. The lack of transcriptional regulation by  $T_3$  could in theory be bypassed by administration of a TH analog. For the effective treatment of patients with *MCT8* mutations, such an analog should meet several well-defined criteria. It should have 1) high affinity for  $T_3$  receptors, 2) uptake into the brain and into neurons via a transporter other than *MCT8*, 3) a similar pattern of deiodination by  $D3$  as  $T_3$ , and 4)  $T_3$ -like effects on the expression of TH responsive genes in brain cells and should 5) restore neural differentiation in the hypothyroid brain.

The first TH analog that has been studied for possible therapy of AHDS patients is 3,5-diiodothyropropionic acid (DITPA). Its administration to wild-type (WT) and *Mct8*-KO mice resulted in decreased TSH and  $T_4$  levels (22). Although *Mct8*-KO mice do not replicate the neurological phenotype of patients with *MCT8* mutations, they are a good model to evaluate the effects of therapeutic strategies on the hypothalamus-pituitary-thyroid axis and peripheral tissues. Furthermore, DITPA treatment was found to produce TH-like effects in brains of WT and *Mct8*-KO mice, indicating brain uptake in the absence of functional *MCT8*. The clinical efficacy of DITPA was studied in 4 younger *MCT8* patients (23). Although DITPA treatment alleviated the thyrotoxic state in all 4 and resulted in a weight gain in 1 patient, no improvement was seen in psychomotor development.

The natural TH metabolite 3,3',5-triiodothyroacetic acid (Triac, TA3) has some advantages compared with DITPA, such as the extensive clinical experience with its use to suppress TSH in patients with thyroid cancer (24) and in those with TH resistance due to TH receptor- $\beta$  ( $TR\beta$ ) mutations (25, 26). Furthermore, the preserved 3,3',5-iodination pattern makes that TA3 has retained full affinity for the  $TR\alpha1$  receptor, whereas it even has a somewhat higher affinity than  $T_3$  for  $TR\beta$  (criterion 1) (27). The aim of our study was to explore whether TA3 could be a valuable drug for treatment of AHDS patients by studying whether TA3 also fulfills the other 4 criteria required to be effective in the treatment of patients with *MCT8* mutations.

## Materials and Methods

### Materials

Human SH-SY5Y neuroblastoma cells were obtained from ECACC; FuGENE 6 and XtremeGENE 9 transfection reagents, and the High Pure RNA isolation kit from Roche Diagnostics; fetal bovine serum (FBS), penicillin-streptomycin, DMEM/F12+glutamax, RPMI 1640+L-glutamine, and Dulbecco's PBS from Invitrogen; culture dishes from Corning; TaqMan reagents from Applied Biosystems; SYBR Green from Eurogentec; phorbol 12-myristate 13-acetate (PMA), iodothyronine derivatives, BSA,  $Na_2SeO_3$ , D-glucose and dithiothreitol from Sigma-Aldrich; and  $Na^{125}I$  from IDB Holland BV. [ $^{125}I$ ] $T_3$  and [ $^{125}I$ ]TA3 were produced as previously described (28).

### Cell culture and differentiation

The different cell lines were cultured in DMEM/F12+glutamax medium, supplemented with 9% FBS, 100nM  $Na_2SO_4$ , and 1% penicillin/streptomycin in 75-cm<sup>2</sup> flasks at 37°C and 5%  $CO_2$ . Fibroblasts were cultured in RPMI 1640+L-glutamine with the same supplements as described above.

COS1 cells used for deiodinase activity assays were seeded in 6-well culture dishes in culture medium without penicillin/streptomycin and transfected with 1  $\mu$ g hD3-pCneo (2) using FuGENE 6 as previously described. COS1 cells intended for uptake studies were cultured in 24-well culture dishes in culture medium without penicillin/streptomycin and transfected with 200 ng pcDNA3 (empty vector), hMCT8-pcDNA3 (2), or hMCT10-pcDNA3.1 (29) using X-tremeGENE 9 following the manufacturer's protocol.

Fibroblasts from *MCT8* patients and healthy controls were obtained as explained in Supplemental Table 1. Informed consent was obtained from the patients' parents. Patients and controls were age and gender matched. Cells were subcultured at 90% confluence and seeded into 6-well culture dishes for uptake experiments. Medium was refreshed every 3 to 4 days. Experiments were carried out at confluence.

The human SH-SY5Y neuroblastoma cell line was used as a model for neurons. Cells were passaged at 90% confluence and subcultured in 24-well culture dishes for metabolism studies and in 6-well culture dishes for uptake and gene expression experiments. Medium in the culture dishes was changed to DMEM/F12+glutamax without supplements at 80% confluence. To

study gene responses to  $T_3$  and TA3, SH-SY5Y cells were incubated for 6 or 48 hours without or with 100nM  $T_3$  or TA3.

The human MO3.13 oligodendrocyte cell line was used as a model for oligodendrocyte differentiation (30). At 90% confluence, cells were seeded in 6-well culture dishes for uptake experiments. After 24 hours, cells were initiated to differentiate in DMEM/F12+glutamax using 10 ng PMA/mL. Experiments were carried out in proliferating progenitor cells (24 hours after seeding) and in early differentiated cells (7 days in PMA).

### TH transport experiments

TH transport experiments were carried out at confluence for fibroblasts after 2 days of serum deprivation in SH-SY5Y cells and in proliferating and differentiated MO3.13 cells. Cells were rinsed with incubation medium (Dulbecco's PBS, 0.1% D-glucose, and 0.1% BSA) and incubated for 2 to 60 minutes at 37°C and 5%  $CO_2$  in incubation medium containing 1nM ( $10^5$  counts/min) [ $^{125}I$ ] $T_3$  or [ $^{125}I$ ]TA3. After incubation, cells were rinsed with incubation medium and lysed in 0.1M NaOH. Radioactivity in the cell lysates was determined using a  $\gamma$ -counter. Uptake was corrected for cellular protein determined with the Bradford protein assay.

### Deiodination of $T_3$ and TA3 in intact cells and in cell lysates

Intact cell metabolism experiments using endogenous D3 expressing SH-SY5Y cells were carried out after 2 days of serum deprivation (31). Cells were rinsed with incubation medium followed by incubation for 1 to 24 hours at 37°C and 5%  $CO_2$  in incubation medium containing 1nM ( $5 \times 10^5$  cpm) [ $^{125}I$ ] $T_3$  or [ $^{125}I$ ]TA3. After incubation, medium was extracted with ethanol and analyzed by HPLC as described previously (2).

Deiodination of  $T_3$  and TA3 by recombinant human D3 was determined by incubation of different dilutions of transfected COS1 cell lysates for 60 minutes at 37°C with 1nM [ $^{125}I$ ] $T_3$  or [ $^{125}I$ ]TA3 in 0.1M phosphate (pH 7.2), 2mM EDTA and 10mM dithiothreitol. Ethanol extracts of the incubation mixtures were analyzed by HPLC as described previously (2).

### RT-PCR and quantitative PCR

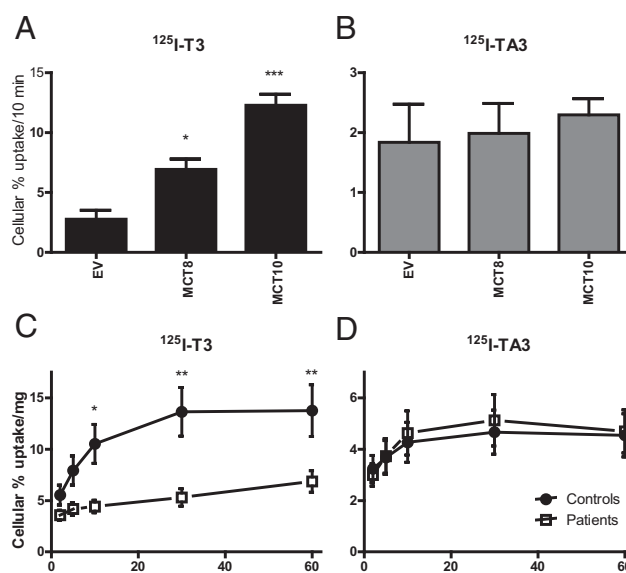
Total RNA was extracted from SH-SY5Y and MO3.13 cells with the High Pure RNA isolation kit using the standard protocol. RNA concentrations were determined by Nanodrop 2000. cDNA was produced from 1  $\mu$ g RNA in a total volume of 50  $\mu$ L using TaqMan reverse transcription reagents following the manufacturer's protocol.

Quantitative PCR was carried out using 1.25  $\mu$ L cDNA in a total volume of 13  $\mu$ L in a 384-well plate. Supplemental Table 2 shows the different primers and probes used for these assays. Expression levels of TH-responsive genes were obtained using 5 pmol/ $\mu$ L primers in combination with SYBR Green following the manufacturer's protocol. Primer-probes and TaqMan Mastermix were used to obtain expression levels of myelin basic protein (*MBP*) and proteolipid protein (*PLP*) and the housekeeping gene cyclophilin A (*PPIA*) following the manufacturer's protocol. TaqMan 7900HT was used for the readout of the plates using SDS version 2.4 software. Data are shown as relative expression to housekeeping gene *PPIA* and as fold change vs untreated cells.

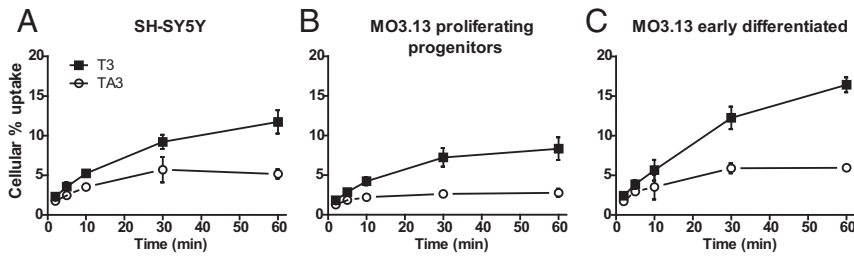
### Mouse studies

The animal studies were approved by the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (TLV Thüringen, Bad Langensalza, Germany). *Pax8*-KO (32) and WT littermates were generated by mating heterozygous *Pax8* mutant mice and genotyped as reported elsewhere (33). Male offspring (4 mice per genotype and treatment) were injected sc once per day between postnatal day 1 (P1) and P12 with either TA3 (200 ng/g BW) or with 0.9% saline as control. The generation and genotyping of *Mct8/Oatp1c1*-double knockout (dKO) mice has been described previously (34). To study TA3 effects in this animal model, male *Mct8/Oatp1c1*-dKO mice were daily injected with either 50 or 400 ng/g BW TA3 between P1 and P12. In addition, WT and *Mct8/Oatp1c1*-dKO mice treated with saline were included as well. At P12 (and 16 hours after the last injection), the animals were deeply anesthetized with isoflurane and subjected to perfusion fixation using 4% paraformaldehyde/PBS. Forebrain and cerebella were dissected and post-fixed overnight in 4% paraformaldehyde/PBS.

To assess the state of Purkinje cell (PC) dendritic development, cerebella were cut sagittally on a vibratome into 50- $\mu$ m-thick sections, and sections of the vermis area were immunostained with rabbit polyclonal antibody against calbindin D28d (Sigma; 1:500) followed by incubation with Alexa Fluor 555-labeled goat antirabbit IgG antibody (Invitrogen; 1:1000). Thickness of the molecular layer at the primary fissure between anterior and posterior lobe was determined by measuring PC dendritic dimension with ImageJ (35) on 3 consecutive section from each animal (n = 4 per genotype).



**FIGURE 1.** A and B, Cellular uptake of [ $^{125}I$ ] $T_3$  (A) and [ $^{125}I$ ]TA3 (B) after 10 minutes in transiently transfected COS1 cells with pcDNA3 (empty vector [EV]) or hMCT8-pcDNA3 or hMCT10-pcDNA3. 1. Results are presented as mean  $\pm$  SEM (n = 3). Significance represents empty vector vs MCT8 or MCT10 using 1-way ANOVA with Bonferroni posttest. C and D, Cellular uptake of [ $^{125}I$ ] $T_3$  (A) and [ $^{125}I$ ]TA3 (B) after 2 to 60 minutes of incubation with fibroblasts from MCT8 patients or age- and gender-matched controls. Results are corrected for protein and presented as mean  $\pm$  SEM (n = 5). Significance represents control fibroblast vs fibroblast of MCT8 patients using 2-way ANOVA with Bonferroni posttest. \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ .



**FIGURE 2.** Cellular uptake of [<sup>125</sup>I]T<sub>3</sub> and [<sup>125</sup>I]TA<sub>3</sub> in SH-SY5Y (A), proliferating progenitor MO3.13 (B), and early differentiated MO3.13 cells (C) at increasing incubation times. Results are corrected for protein and presented as mean ± SEM (n = 2–3).

Frontal forebrain vibratome sections from the same animals were used to visualize a subset of  $\gamma$ -aminobutyric acid (GABA)ergic interneurons that express parvalbumin (PV). For this purpose, sections were incubated with mouse monoclonal anti-PV antibody (Millipore; 1:1000) followed by incubation with Alexa Fluor 555-labeled goat antimouse IgG antibody (Invitrogen; 1:500). PV immunoreactivity was analyzed in the barrel field of the somatosensory cortex as well as in the retrosplenial cortex using 3 consecutive sections between bregma level -0.8 to -1.1 mm. The number of PV-immunopositive neurons was counted and normalized to the respective cortical area using ImageJ.

To assess myelination, frontal forebrain sections were incubated with rat monoclonal anti-MBP antibody (Millipore, 1:200) and Alexa Fluor 555-labeled goat antirat IgG antibody (Invitrogen, 1:1000). The integrated density of MBP in all areas of the cerebral

cortex was determined on 3 consecutive sections located around bregma level 0.1 and normalized to the respective cortical area using ImageJ. For each genotype, 4 animals were used, and results are expressed relative to WT values.

**Statistical analysis**

The results are shown as means and SEM of a minimum of 2 experiments carried out at least in duplicate. GraphPad Prism version 5.01 for Windows (GraphPad Software) was used for statistical

analysis. A 1-way ANOVA with Bonferroni posttest with a cutoff level of  $P < .05$  was used to analyze the difference between the background and the different transporters. Statistical analysis of the effects of T<sub>3</sub> and TA<sub>3</sub> on different genes as well as of TA<sub>3</sub> effects during murine brain development was done using a 2-way ANOVA followed by Bonferroni posttest with a cutoff level of  $P < .05$ .

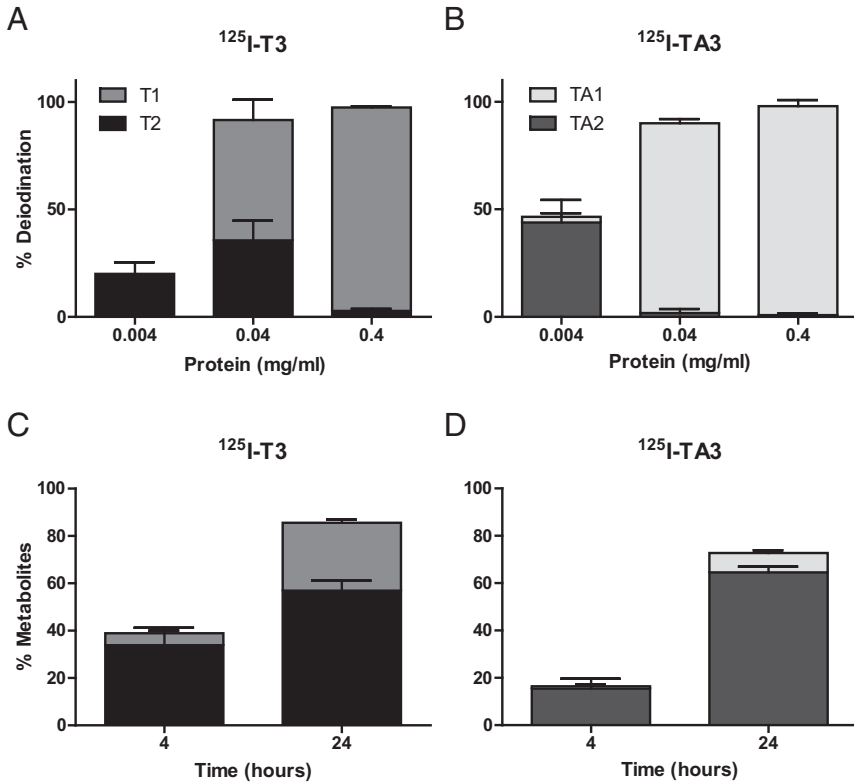
**Results**

**TA3 is taken up by target cells via a transporter other than MCT8**

Transport of [<sup>125</sup>I]T<sub>3</sub> and [<sup>125</sup>I]TA<sub>3</sub> by MCT8 and its homolog MCT10 was studied in transiently transfected

COS1 cells. Cellular T<sub>3</sub> uptake was induced 2.5-fold and 4.4-fold by transfection of cells with MCT8 and MCT10, respectively, vs empty vector control (Figure 1A). In contrast, MCT8 or MCT10 expression did not induce cellular TA<sub>3</sub> uptake (Figure 1B). These findings indicate that TA<sub>3</sub> is not transported by MCT8 or MCT10.

To assure that TA<sub>3</sub> is transported into MCT8-deficient cells, [<sup>125</sup>I]T<sub>3</sub> and [<sup>125</sup>I]TA<sub>3</sub> uptake was studied in fibroblasts from AHDS patients and age and gender-matched controls (Figure 1, C and D). As expected, markedly impaired T<sub>3</sub> uptake was seen throughout the 2- to 60-minute incubation in fibroblasts from AHDS patients compared with control fibroblasts. In contrast, similar TA<sub>3</sub> uptake was observed in fibroblasts from AHDS patients and controls. These findings are in agreement with the results obtained in cells transiently transfected with MCT8, indicating that MCT8 and MCT10 do not facilitate TA<sub>3</sub> transport.



**FIGURE 3.** A and B, Deiodination of T<sub>3</sub> and TA<sub>3</sub> by recombinant human D3. Dilutions of lysates of COS1 cells transfected with human D3 were incubated for 60 minutes at 37°C with [<sup>125</sup>I]T<sub>3</sub> (A) or [<sup>125</sup>I]TA<sub>3</sub> (B). C and D, Conversion of T<sub>3</sub> and TA<sub>3</sub> by endogenous D3 in intact SH-SY5Y cells. Cells were incubated for 4 or 24 hours at 37°C with [<sup>125</sup>I]T<sub>3</sub> (C) or [<sup>125</sup>I]TA<sub>3</sub> (D). Results are presented as mean ± SEM (n = 2–3).

Treatment of AHDS patients with TA3 can be beneficial only if TA3 is transported into neural target cells. As a model for neurons, the SH-SY5Y neuroblastoma cell line was used for studies of T<sub>3</sub> and TA3 transport. Figure 2A shows the time-dependent cellular uptake of [<sup>125</sup>I]T<sub>3</sub> and [<sup>125</sup>I]TA3. Uptake of both substrates increased with time between 2 and 60 minutes of incubation, with TA3 uptake (1.8%–5.2%) roughly amounting to half of T<sub>3</sub> uptake (~2.3%–11.7%). However, it should be realized that these uptake studies were done in the presence of 0.1% BSA in the medium, providing a higher free substrate concentration for T<sub>3</sub> (17.9%) than for TA3 (9.5%) (unpublished results).

Other important TH target cells in brain are oligodendrocytes because the differentiation of these cells is also controlled by TH (15). Therefore, these cells would also be an important target cell for TA3 therapy. The MO3.13 oligodendrocyte cell line was used to study transport of [<sup>125</sup>I]T<sub>3</sub> and [<sup>125</sup>I]TA3 in proliferating cells (Figure 2B) and in early differentiated oligodendrocytes induced by phorbol ester treatment (Figure 2C). Supplemental Figure 1A shows the appearance of immature and mature cells in these cultures. Differentiation was confirmed by the markedly increased expression of mature oligodendrocyte markers (Supplemental Figure 1B). Uptake increased over time for T<sub>3</sub> and TA3 in immature as well as mature MO3.13 cells. Together with the data obtained in MCT8-transfected cells and fibroblasts, the uptake results in SH-SY5Y and MO3.13 cells indicate that TA3 is transported into brain target cells by a transporter other than MCT8 (criterion 2).

### TA3 shows similar metabolism by D3 as T<sub>3</sub>

We compared the deiodination of T<sub>3</sub> and TA3 by recombinant human D3 expressed in transfected COS1 cells. D3 was found to catalyze the inner ring deiodination of T<sub>3</sub> to 3,3'-T2 (Figure 3A) and of TA3 to 3,3'-TA2 (Figure 3B) with equivalent efficacy, although the further inner ring deiodination of 3,3'-TA2 to 3'-TA1 was more rapid than the further deiodination of 3,3'-T2 to 3'-T1.

The metabolism of T<sub>3</sub> and TA3 was also studied in intact SH-SY5Y cells, which express D3 endogenously (31), providing an integral view of T<sub>3</sub> and TA3 transport and metabolism and, consequently, their availability for the T<sub>3</sub> receptor. Initial experiments showed a marked TA3 conversion of 87% after 24 hours. We repeated these experiments to compare TA3 and T<sub>3</sub> using a different batch of FBS and found somewhat lower conversion of TA3 after 24 hours. Between 4 and 24 hours of incubation, we observed a time-dependent increase in [<sup>125</sup>I]T<sub>3</sub> and [<sup>125</sup>I]TA3 metabolism, amounting to 41%–86% for T<sub>3</sub> (Figure 3C) and to 15%–74% for TA3 (Figure 3D).

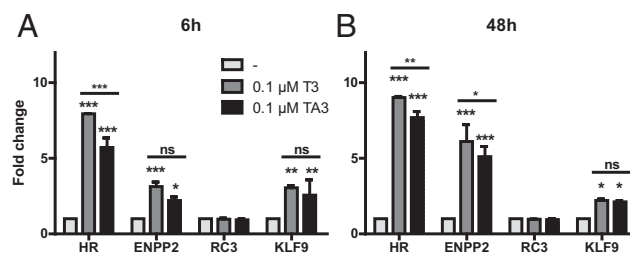
These results support our uptake data, demonstrating that the T<sub>3</sub> and TA3 taken up by SH-SY5Y cells are also available for intracellular deiodination by D3. Therefore, TA3 and T<sub>3</sub> are likewise converted by D3 in neuronal cells, allowing the regulation of intracellular TA3 levels by D3 very much like endogenous T<sub>3</sub> in physiological conditions (criterion 3).

### TA3 has T<sub>3</sub>-like effects on gene expression in neuronal cells

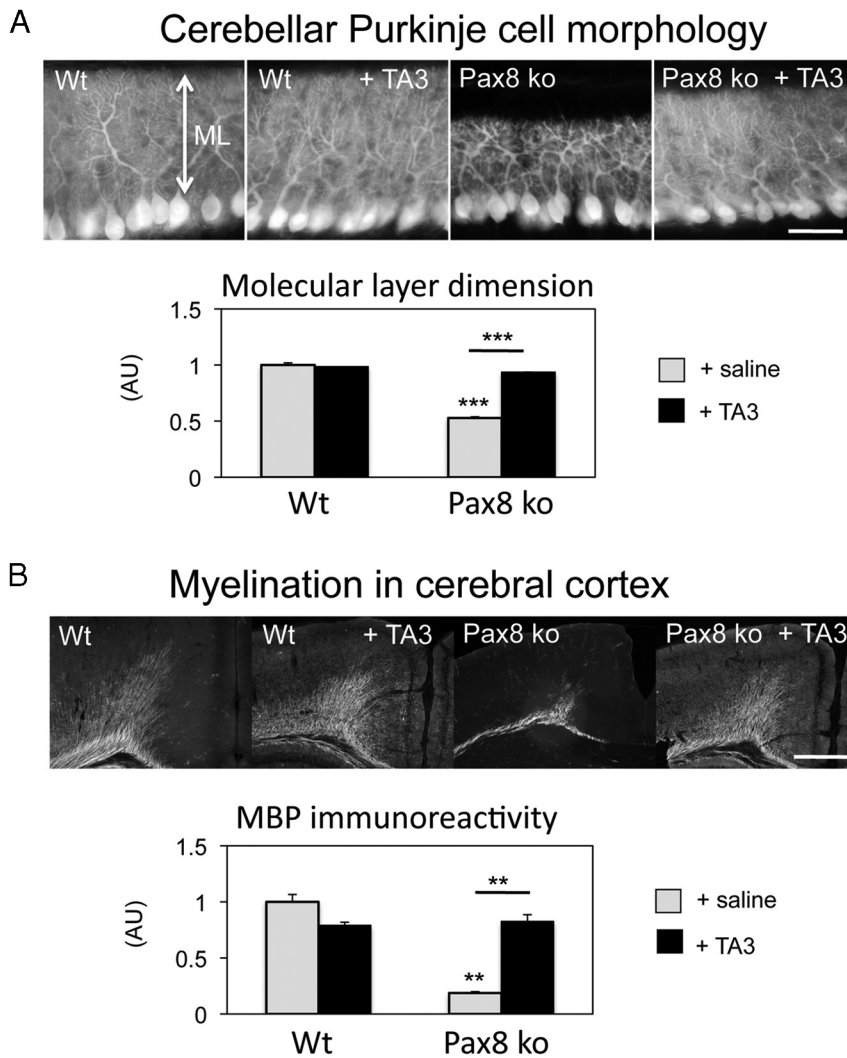
We also used SH-SY5Y cells as a model for central neurons to study the expression of genes in response to incubation for 6 to 48 hours with 100nM T<sub>3</sub> or TA3. No difference in cell appearance was seen after 48 hours of incubation with T<sub>3</sub> or TA3 (Supplemental Figure 2). Expression values were corrected for the housekeeping gene *PPIA*, and the fold change with untreated cells was calculated. T<sub>3</sub> and TA3 significantly induced the expression of the T<sub>3</sub>-responsive genes Hairless (*HR*), ectonucleotide pyrophosphatase/phosphodiesterase 2 (*ENPP2*) and Kruppel-like factor 9 (*KLF9*) after both 6 (Figure 4A) and 48 (Figure 4B) hours. No significant induction of neurogranin (*RC3*) expression was seen by T<sub>3</sub> or TA3 after 6 or 48 hours. This may be related to the brain region-specific regulation of *RC3* expression by T<sub>3</sub> (36, 37). The response to TA3 was slightly lower than to T<sub>3</sub> for *HR* after 6 hours and for *HR* and *ENPP2* after 48 hours. The significant results after 6 hours of incubation with T<sub>3</sub> or TA3 suggest that they were produced by a direct genomic effect. These findings indicate that equal concentrations of T<sub>3</sub> and TA3 have similar effects on the expression of TH-responsive genes (criterion 4). Similar results were obtained using 10nM instead of 100nM T<sub>3</sub> or TA3 (data not shown).

### TA3 restores neural differentiation in the hypothyroid brain

To evaluate the action of TA3 in vivo, we took advantage of athyroid *Pax8*-KO mice that do not produce en-



**FIGURE 4.** Changes in expression of TH-responsive genes after incubation of SH-SY5Y cells for 6 (A) or 48 (B) hours with 0.1 μM T<sub>3</sub> or TA3. Expression values were corrected for the housekeeping gene cyclophilin A, and expressed as fold change vs control incubation without TH. Results are presented as mean ± SEM of 2 experiments performed in triplicate. Significance represents T<sub>3</sub> or TA3 vs no treatment using matched 2-way ANOVA with Bonferroni posttest. \*, *P* < .05; \*\*, *P* < .01; \*\*\*, *P* < .001.



**FIGURE 5.** Thyromimetic actions of TA3 in the developing mouse brain. *Pax8*-KO mice and WT littermates were injected daily with TA3 (200 ng/g BW) starting at P1 and analyzed at P12. Control animals received saline injections. A, PCs in sagittal cerebellar sections through the vermis region were visualized by calbindin immunostaining, and the thickness of the molecular layer (ML) that comprises PC dendrites was determined on 3 consecutive sections of each animal (males;  $n = 4$  per genotype). Whereas in saline-treated *Pax8*-KO mice, PCs showed a strongly reduced dendritic outgrowth and therefore a thinner ML, TA3 treatment of *Pax8*-KO mice restored normal PC outgrowth and consequently ML thickness. B, Coronal forebrain sections of the same animals were immunostained with an antibody against MBP and revealed a significantly reduced immunoreactivity in the cerebral cortex of saline-treated *Pax8*-KO mice. In contrast, TA3 treatment of *Pax8*-KO mice resulted in normal MBP expression. Scale bar, 50  $\mu\text{m}$ . \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ . Abbreviation: AU, arbitrary unit.

ogenous THs and therefore show a severely retarded brain development (32, 38). We treated newborn WT and *Pax8*-KO mice once a day by sc injections of TA3 (200 ng/g BW) or saline and analyzed brain morphology of these animals at P12. Cerebellar development was monitored by visualizing PC morphology with an antibody against calbindin. Compared with WT littermates, saline-treated *Pax8*-KO mice showed a grossly retarded PC dendritic outgrowth consistent with the hypothyroid state of these animals (Figure 5A). TA3 treatment was sufficient

to normalize PC dendrite formation in *Pax8*-KO mice without affecting PC development in WT animals.

Another hallmark of a congenital hypothyroid central nervous system is a reduced myelination because the expression of myelin components such as *MBP* is positively controlled by TH (39). We therefore analyzed *MBP* expression in the cerebral cortex by immunohistochemistry and indeed found a strongly reduced *MBP* immunoreactivity in saline-treated *Pax8*-KO mice compared with WT littermates (Figure 5B). TA3 treatment did not affect *MBP* expression in the cerebral cortex of WT animals but restored normal *MBP* immunoreactivity in *Pax8*-KO mice.

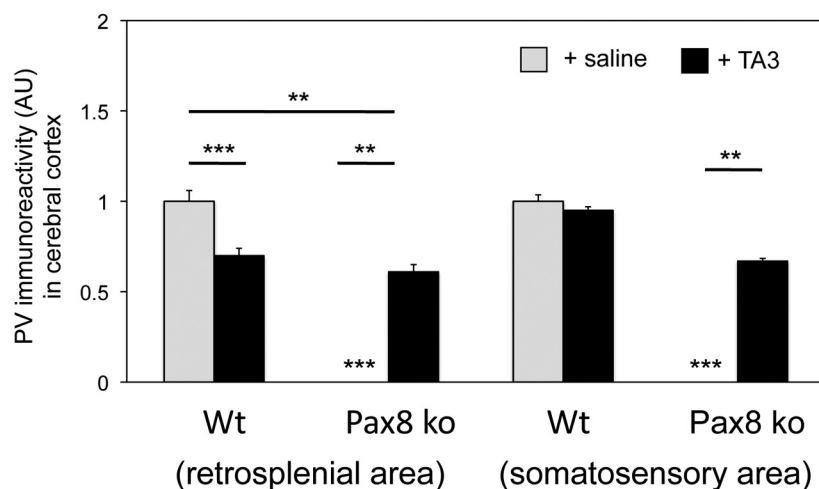
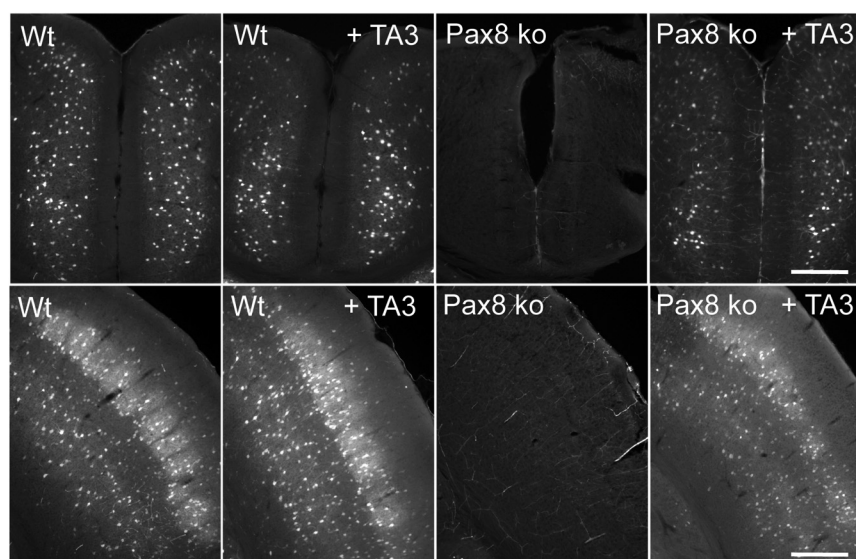
Finally, we determined the number of PV-immunopositive GABA-interneurons in the cerebral cortex where hypothyroidism leads to a strongly reduced PV immunoreactivity (40, 41). As depicted in Figure 6 for the retrosplenial as well as the somatosensory cortex, PV-positive neurons were found in *Pax8*-KO mice only upon TA3 treatment. Altogether, our findings indicate that during early postnatal periods, TA3 can indeed prevent neural abnormalities that are caused by insufficient TH supply (criterion 5).

### TA3 promotes neural development in *Mct8/Oatp1c1*-dKO mice

With the generation of *Mct8/Oatp1c1*-dKO mice, we recently established a mouse model that exhibits

the same abnormal serum TH profile as the patients with AHDS mutations, whereas the brain TH content in these animals is strongly reduced due to a highly compromised transport of TH into the brain (34). To elucidate whether TA3 treatment promotes neural differentiation in these animals as well, we treated *Mct8/Oatp1c1*-dKO mice with either 50 or 400 ng/g BW TA3 daily between P1 and P12 and analyzed cerebellar Purkinje cell morphology and cerebral myelination as described above. In agreement with previous findings (34), saline-injected

## PV- neurons in cerebral cortex



**FIGURE 6.** TA3 stimulates the maturation of PV-immunoreactive neurons in the somatosensory and retrosplenial cortex. Coronal forebrain sections of WT and *Pax8*-KO mice at P12 were immunostained with an antibody against PV that labels a subset of GABAergic interneurons. PV immunoreactivity was quantified on 3 consecutive sections from each animal using 4 animals per genotype and normalized to the respective cortical area. In saline-treated *Pax8*-KO mice, PV immunoreactivity was strongly reduced but could be partially restored upon TA3 treatment. Scale bar, 50  $\mu$ m. \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ . Abbreviation: AU, arbitrary unit.

*Mct8/Oatp1c1*-dKO mice exhibited a strongly reduced thickness of the molecular layer and decreased MBP immunoreactivity compared with saline-treated WT animals (Figure 7). TA3 treatment in turn led to a dose-dependent stimulation of cerebellar Purkinje cell dendritogenesis as well as cortical myelination in *Mct8/Oatp1c1*-dKO mice. Normalization, however, was achieved only by applying the higher TA3 dose (400 g/g BW). These data indicate that TA3 is also able to exert thyromimetic actions in the central nervous system of *Mct8/Oatp1c1*-dKO mice, an animal model for human MCT8 deficiency.

## Discussion

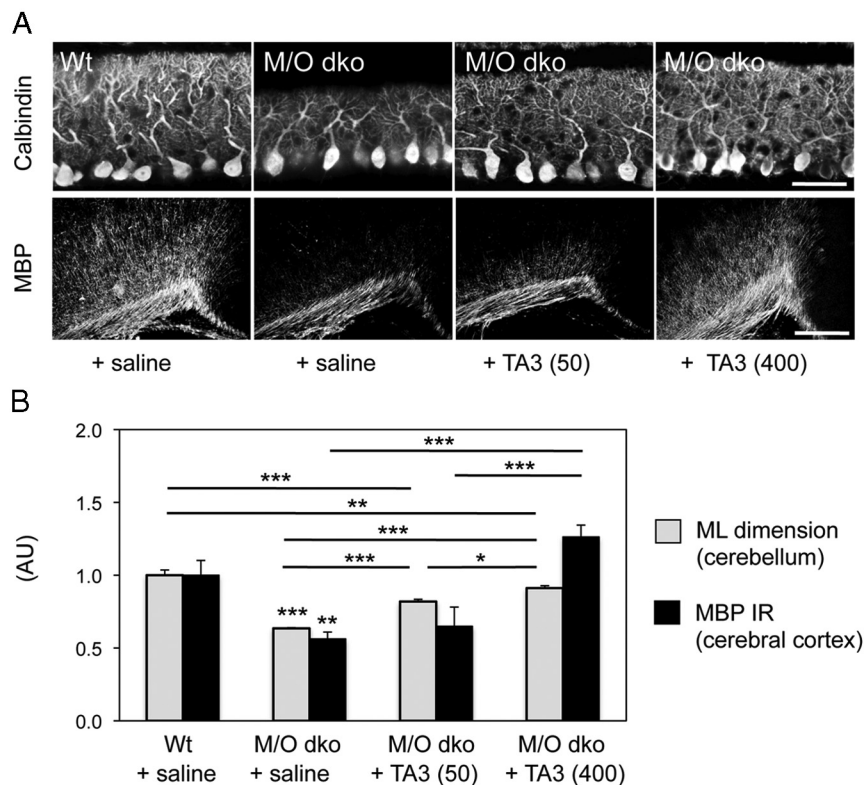
The aim of our study was to obtain evidence that TA3 is a valuable compound for the treatment of patients with MCT8 mutations, in particular when it is administered to young children where TH-dependent brain development is still an ongoing process. TA3 fulfills all 5 criteria required for an effective therapy of these patient: 1) TA3 has a high affinity for the  $T_3$  receptor (27), 2) TA3 is transported into brain cells by a transporter other than MCT8, 3) TA3 is converted by D3 in the same manner as  $T_3$ , 4) TA3 produces similar neuronal gene responses as  $T_3$ , and 5) TA3 restores neural differentiation in the hypothyroid brain.

Cellular transport of TA3 has been studied before in rat anterior pituitary cells (42) and cardiomyocytes (43). Evidence was provided that uptake of TA3 by these cells is mediated by plasma membrane transporters, but the identity of TA3 transporters remains to be determined.

Our *in vitro* studies in neuronal cell lines suggest that TA3 is almost as potent as  $T_3$  in the regulation of gene responses. These data fit well with our previous *in vitro* findings of a robust  $T_3$ -like effect of TA3 on dendrite formation in cerebellar Purkinje cells (44). The  $T_3$ -like effects of TA3 are further supported by our *in vivo* data showing that treatment of athyroid *Pax8*-KO mice as well as of

*Mct8/Oatp1c1*-dKO mice with TA3 results in normal cerebellar PC dendritogenesis, indicating that TA3 can replace  $T_3$  in developing neurons. Moreover, TA3 treatment restores normal MBP expression, suggesting that TA3 can replace  $T_3$  in promoting myelination. Using 3 different test systems, 1) a neuroblastoma cell line, 2) *in vitro* cultured mouse PCs (5), and 3) TH-dependent brain areas, our data demonstrate that TA3 has potent  $T_3$ -like effects in the regulation of neuronal gene expression.

A major advantage of TA3 is the extensive clinical experience with this compound that has been adminis-



**FIGURE 7.** TA3 promotes cerebellar development and cortical myelination in *Mct8/Oatp1c1*-dKO mice. *Mct8/Oatp1c1*-dKO (M/O dKO) mice were daily injected with TA3 at a dose of either 50 ng/g BW (50) or 400 ng/g BW (400) between P1 and P12. As controls, WT and M/O-dKO mice were treated with saline. Visualization of PC dendrites on sagittal cerebellar sections by calbindin immunostaining (A, upper row) revealed a reduced PC arborization in M/O-dKO mice, whereas TA3 treatment visibly stimulated dendritogenesis. Coronal forebrain sections of the same animals were immunostained with an antibody against MBP and revealed a significantly reduced immunoreactivity (IR) in the cerebral cortex of saline-treated M/O-dKO mice, whereas animals receiving TA3 exhibited elevated MBP IR. However, quantification of the thickness of the molecular layer (ML) as well as of the cortical MBP IR revealed that only the application of a high dose of TA3 was sufficient in normalizing both parameters in *Mct8/Oatp1c1*-dKO animals (B). Scale bar in A, 40  $\mu$ m (upper row) and 80  $\mu$ m (lower row). \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ . Abbreviation: AU, arbitrary unit.

tered already for many years to patients with TH resistance caused by TR $\beta$  receptor mutations to reduce thyrotoxicosis (26, 45). It has been administered to thyroid cancer patients to suppress TSH (24). It is known as a safe drug to administer for long periods to suppress TSH in humans. All these features make it an attractive therapeutic option for AHDS patients.

A disadvantage of TA3 is its short half-life of ~6 hours in humans, which makes it necessary to administer it 3 to 4 times a day (46). Therefore, we recently studied also the effects of TA4 as a natural metabolite of T<sub>4</sub> (44). We could demonstrate that TA4 is a good therapeutic option because it is endogenously converted by D1 and D2 to bioactive TA3 and does not have such a short half-life as TA3. The major drawback is the limited experience with TA4 application in humans (47, 48). Consequently, extensive clinical studies would be needed before TA4 may be available for the treatment of AHDS patients. There-

fore, TA3 administration appears more suitable for therapy of AHDS patients despite its short half-life.

What can we expect from administration of TA3 to AHDS patients? Based on the animal studies and the in vitro findings, TA3 administration will reduce serum T<sub>3</sub> levels and, consequently, diminish symptoms of peripheral thyrotoxicosis such as muscle wasting, tachycardia, and weight loss. Of course, this assumes that the effect of the decrease in serum T<sub>3</sub> outweighs the effects of administered TA3 on peripheral tissues. With respect to psychomotor development, we anticipate beneficial effects if TA3 is administered in particular to young children, because TH-dependent brain development still occurs after birth (49). If the treatment is initiated later in life, brain damage is likely to be largely irreversible as is known from other causes of TH insufficiency, such as congenital hypothyroidism (50).

To conclude, our in vitro and in vivo studies indicate that TA3 is a promising compound for the treatment of patients with *MCT8* mutations. It fulfills all criteria required for such a therapeutic approach, ie, 1) high T<sub>3</sub> receptor affinity, 2) MCT8-independent cellular trans-

port, 3) T<sub>3</sub>-like metabolism by D3, 4) T<sub>3</sub>-like effects in neuronal cells, and 5) T<sub>3</sub>-like responses in the hypothyroid brain. These properties combined with the extensive clinical experience with this compound make TA3 an attractive drug to treat the peripheral thyrotoxicosis of *MCT8* patients with the possibility to improve psychomotor development when administered to young children.

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Address all correspondence and requests for reprints to: Theo J. Visser, Erasmus University Medical Center, Wyte-



maweg 80 3015 CN Rotterdam, The Netherlands. E-mail: t.j.visser@erasmusmc.nl.

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