Gene therapy targeting the blood–brain barrier improves neurological symptoms in a model of genetic MCT8 deficiency

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A genetic deficiency of the solute carrier monocarboxylate transporter 8 (MCT8), termed Allan–Herndon–Dudley syndrome, is an important cause of X-linked intellectual and motor disability. MCT8 transports thyroid hormones across cell membranes. While thyroid hormone analogues improve peripheral changes of MCT8 deficiency, no treatment of the neurological symptoms is available so far. Therefore, we tested a gene replacement therapy in Mct8- and Oatp1c1-deficient mice as a well-established model of the disease. Here, we report that targeting brain endothelial cells for Mct8 expression by intravenously injecting the vector AAV-BR1-Mct8 increased tri-iodothyronine (T3) levels in the brain and ameliorated morphological and functional parameters associated with the disease. Importantly, the therapy resulted in a long-lasting improvement in motor coordination. Thus, the data support the concept that MCT8 mediates the transport of thyroid hormones into the brain and indicate that a readily accessible vascular target can help overcome the consequences of the severe disability associated with MCT8 deficiency.

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Abbreviations: AAV = adeno-associated virus; AHDS = Allan–Herndon–Dudley syndrome; DKO = Mct8 and Oatp1c1 double knockout mice; PBECs = primary brain endothelial cells; T3 = tri-iodothyronine; T4 = thyroxine; TH = thyroid hormone

Introduction

The thyroid hormones (THs) thyroxine (T4) and tri-iodothyronine (T3) are critical for the function of the CNS. Severe hypothyroidism causes cognitive deficits and other neurological symptoms.1,2 Mild TH deficiency has been associated with major depression and dementia.2 In the CNS, nuclear receptors and deiodinases maintain TH homeostasis, but the initial step required for the action of THs is their cellular uptake by solute carriers. Among the latter family of proteins, monocarboxylate transporter 8 (MCT8) and organic anion transporter polypeptide 1c1 (OATP1C1) have been shown to transport THs in vivo.3,5 Mutations of the X-linked MCT8 gene (SLC16A2) cause severe intellectual disability, motor dysfunction and peripheral thyrotoxicosis (Allan–Herndon–Dudley syndrome, AHDS).6,7 It has been estimated that MCT8 mutations are responsible for almost 4% of all X-linked intellectual disabilities.8 Affected boys appear normal at birth but show developmental delay and feeding problems in the first year of life. Some patients cannot fully control their head, speak or walk. Other motor symptoms include muscle weakness, gait ataxia and dystonia. The TH analogues 3,5-diodothyropropionic acid (DITPA) and 3,3',5-triiodothyroacetic acid (TRIAC) improved the peripheral thyroid status,9,10 but treatment of the CNS symptoms that are key to patients’ disabilities is still not available and represents an unmet medical need.

Neuropathological changes underlying symptoms of MCT8 deficiency include an altered cerebellar structure, lack of parvalbumin-positive interneurons, delayed myelination and oligodendrocyte dysfunction,11 indicative of a global TH deficiency in the CNS. Although MCT8 mutations severely affect oligodendrocytes and neurons in humans, these cells either do not contain significant amounts of MCT8 protein or produce it only during development or in subpopulations.12,13 Interestingly, brain barriers, including endothelial cells, express MCT8 into adulthood, pointing to a pivotal role of MCT8 in TH transport through the brain barriers.12,14

Mice

As Oatp1c1−/− and Oatp1c1+/− mice do not differ in most parameters,15 breeding pairs were set up among Mct8−/−;Oatp1c1−/− and Mct8+/−;Oatp1c1−/− mice to obtain Mct8−/−;Oatp1c1−/− or Mct8−/−;Oatp1c1−/− (DKO) and Mct8−/−;Oatp1c1+/− or Mct8+/−;Oatp1c1−/− littermate controls, as reported previously.16 Detailed information is provided in the Supplementary material. Mice were randomized into treatment groups. Experimenters were blinded to group allocation in the phenotype analysis.

Materials and methods

Statistical analysis

Sample sizes were planned based on a power analysis by G*Power. P-values ≤ 0.05 were considered statistically significant. We did not exclude outliers, unless indicated in Supplementary Table 1. Outliers were identified by Grubbs test with an alpha of 0.05. ANOVA and t-test were only applied if assumptions were met, i.e. data sets were examined for Gaussian distribution using the D’Agostino–Pearson or Kolmogrov–Smirnov test, aided by visual inspection of the data, and homogeneity of variances by Brown–Forsythe test. Mostly, Holm–Sidak post hoc analysis was applied to test the significance between groups. For unequal variances, a Welch’s ANOVA test with a Tamhane’s T2 multiple comparisons post hoc test was used.

Study approval

All animal experiments were performed according to German animal welfare regulations and experimental protocols were approved by the local animal ethics committee (No. 11-1/17 and 97-9/19, MELUND, Kiel, Germany).

Data availability

The data that support the findings of this study are available from the corresponding author.
Intravenous administration of AAV-BR1-Mct8 enables the expression of Mct8 in brain endothelial cells and T3 transport into the CNS. (A) Schematic illustration of AAV-BR1-Mct8 vectors to transduce brain endothelial cells. CAG = cytomegalovirus enhancer fused to the chicken beta-actin promoter; WPRE = woodchuck hepatitis posttranscriptional regulatory element. (B) After transduction of primary brain endothelial cells (PBECs) of DKO mice in vitro with AAV-BR1-Mct8, MCT8 was expressed. MCT8 was detected by fluorescence immunostaining and nuclei with DAPI. Arrows indicate MCT8-positive cells. Scale bar = 100 µm. (C) T3 uptake in DKO PBECs was enhanced by AAV-BR1-Mct8 treatment in vitro. Results were obtained from three independent cell culture preparations. **P = 0.0015 (unpaired t-test). (D) Schematic of the experimental design, created by BioRender.com. gp = genomic particles. (E) MCT8 expression (arrow) in CD31-positive endothelial cells of control or DKO mice that received AAV-BR1-Mct8 at P0. In the top right panel, an MCT8-positive cell with the typical astrocytic morphology is visible. Fluorescence immunostaining for MCT8 and CD31 was performed at P33. Scale bars = 100 µm; 10 µm. (F) MCT8 expression in PBECs prepared from P33 mice that received AAV-BR1-Mct8 at P0 (MCT8-positive cells, 8.6 ± 1.1% of CD31-positive PBECs in four independent cell culture preparations, with one mouse per cell culture preparation). Scale bar = 100 µm. (G and H) At P120, Mct8 mRNA (G) and MCT8 protein (H) were detected in control and DKO mice treated with AAV-BR1-Mct8 at P0. Insets: Higher magnification of the boxed areas. Scale bar = 100 µm (G), 5 µm (H), 2 µm (insets). Cx = cortex; Chp = choroid plexus; Hp = hippocampus. (I) T3 and T4 concentrations in brains of control and DKO mice that received AAV-BR1-Mct8 at P0 and were sacrificed at P21. One-way ANOVA: T3, F(3/19) = 104.7, P < 0.0001; T4, F(3/19) = 34.03, P < 0.0001; Holm–Sidak’s post hoc test. Each dot represents one animal. Means ± SEM are shown. **P < 0.01; ***P < 0.001; ****P < 0.0001.
Results

For targeted expression of Mct8 in the blood–brain barrier, we generated a viral Mct8 gene vector (Fig. 1A), packaged in the AAV-BR1 capsid (AAV-BR1-Mct8), which selectively transduces brain endothelial cells in vivo. After incubating primary brain endothelial cells (PBECs) from Mct8;Oatp1c1 DKO mice with AAV-BR1-Mct8, about 10% of the cells immunostained for MCT8, indicating transduction (Fig. 1B). To determine whether virally transduced MCT8 was functional, we measured PBEC uptake of T3. Untreated PBECs from DKO mice did not take up T3 (Fig. 1C). However, transduction of PBECs with AAV-BR1-Mct8 enabled T3 uptake and silychristin, an MCT8 inhibitor, blocked it, confirming that MCT8 is active after viral transduction (Fig. 1C).

For in vivo testing, we injected AAV-BR1-Mct8 intravenously in mice at postnatal Day 0 (P0) and investigated the animals at various time points to monitor the TH-dependent brain development and function (Fig. 1D). At P33, immunohistochemistry demonstrated robust MCT8 expression in brain endothelial cells and in some neurons and astrocytes, too, as previously described for the AAV-BR1 capsid (Fig. 1B). In addition, some scattered epithelial cells in the choroid plexus were transduced with Mct8 but no tanyctyes in the mediobasal hypothalamus (Supplementary Fig. 1A and B). As differentiation of endothelial cells versus pericytes or astrocyte endfeet on immunofluorescence staining can be difficult, we quantified the transduction rate of brain endothelial cells ex vivo by investigating PBECs derived from P33 mice that had received the vector at P0. Of PBECs derived from AAV-BR1-Mct8-treated mice, 8.8 ± 1.1% expressed MCT8 (Fig. 1F). However, the true transduction rate might be higher, as immunofluorescence staining sensitivity for MCT8 was limited and did not detect the downregulated endogenous MCT8 levels of cultured control cells (Fig. 1F).

In the CNS, endothelial cells are non-proliferative and quiescent after mice reach an age of 14–30 days. Thus, loss of the non-integrating AAV vector after P33 is not expected. Accordingly, in situ hybridization and immunofluorescence staining showed that Mct8 was similarly expressed in cerebral vessels of P33 and P120 DKO mice that received AAV-BR1-Mct8 at P0 but not in other tissues, such as pituitary, kidney and liver at P21 and P120 (Fig. 1E and Supplementary Figs 1C and 2). Thus, loss of Mct8 function (Fig. 1F) and lack of MCT8 expression in the choroid plexus were transduced with AAV-BR1-Mct8, however, the molecular layer was thicker than in untreated DKO mice (Fig. 3F) and there were more interneurons expressing the marker genes Gad67 (glutamate decarboxylase 67) and parvalbumin in the somatosensory cortex (Fig. 2A).

AAV-BR1-Mct8 treatment at P0 had a persistent effect. At P33, AAV-BR1-Mct8-treated DKO mice showed significantly thicker layers II–VI containing NeuN-positive neurons in the somatosensory cortex than untreated DKO animals (Fig. 2B). Consistent with the findings at P12, AAV-BR1-Mct8 treatment improved Gad67 fluorescence intensity and the number of parvalbumin-positive cells in the somatosensory cortex of DKO mice at P33 (Fig. 2B).

MRI and autopsy studies showed that myelination is abnormal or delayed in patients with MCT8 deficiency. Reflecting the critical role of THs in postnatal myelination, the celluular level, THs promote differentiation of oligodendrocytes that produce myelin. As expected from the lack of THs in the CNS, DKO mice had significantly fewer Olig2-positive oligodendrocytes in the cortex and corpus callosum than controls at P33 (Fig. 3A and Supplementary Fig. 6). AAV-BR1-Mct8 treatment at P0 significantly attenuated the decrease in Olig2-positive cells in the cortex (Fig. 3A). Under the influence of TH, oligodendrocytes produce myelin basic protein (MBP), a key component of CNS myelin. Like human patients, DKO mice present with low MBP expression in the cortex and corpus callosum (Fig. 3B). However, AAV-BR1-Mct8 treatment at P0 increased MBP levels in the cortex of DKO mice (Fig. 3B). MBP mediates myelin compaction. Accordingly, AAV-BR1-Mct8 treatment increased levels of compact myelin in the corpus callosum as shown by FluoroMyelin staining, indicating better gross myelination (Fig. 3C). The observation that AAV-BR1-Mct8 improved myelination was corroborated by immunoblots of the myelin proteins MBP and proteolipid protein 1 (PLP1) showing significant higher levels in DKO that received the vector at P0 (Fig. 3D and E).

To determine whether sustained Mct8 expression affects neuronal or astrocytic function in the long term, we analysed the expression of established TH-regulated genes by using in situ hybridization and quantitative RT-PCR at P120. The cortical expression of hairless (Hr) that is known to be upregulated by THs was reduced in DKO mice at P120 (Fig. 4A). The cortical expression of hairless (Hr) that is known to be upregulated by THs was reduced in DKO mice at P120 (Fig. 4A). However, the TH-regulated gene trp53rd-related factor 9 (Klf9) encoding a neuronal transcription factor was significantly induced by AAV-BR1-Mct8 treatment of DKO mice (Fig. 4B). After administration of AAV-BR1-Mct8, expression of the TH-regulated neuropeptide N (Nrgn, Rc3) was slightly but non-significantly higher than in untreated DKO animals (Supplementary Fig. 7A). Also, the
Figure 2. AAV-BR1-Mct8 treatment improves neuronal morphology and gene expression. (A) Administration of AAV-BR1-Mct8 at P0 to DKO mice increased thickness of the molecular layer in the cerebellar vermis as well as the relative fluorescence intensity of GAD67- and parvalbumin-positive interneurons of the somatosensory cortex at P12. Administration of AAV-BR1-Gfp at P0 to DKO mice had no effect. One-way ANOVA: molecular layer thickness, $F_{(3/40)} = 50.25, P < 0.0001$; Holm–Sidak’s post hoc test. Welch’s ANOVA: GAD67, $W_{(3/21.43)} = 58.74, P < 0.0001$; parvalbumin-positive interneurons, $W_{(3/15.37)} = 64.72, P < 0.0001$; Tamhane’s T2 post hoc test. Scale bar = 100 µm (top and bottom panel); Scale bar = 200 µm (middle panel). (B) Administration of AAV-BR1-Mct8 at P0 improved the thickness of the somatosensory cortex layers II–VI, the relative fluorescence signal intensity of GAD67 and the number of parvalbumin-positive interneurons in the cortex at P33. One-way ANOVA: cortical thickness, $F_{(2/12)} = 16.4, P = 0.0004$; GAD67, $F_{(2/9)} = 102.0, P < 0.0001$; parvalbumin-positive interneurons, $F_{(2/9)} = 63.01, P < 0.0001$; Holm–Sidak’s post hoc test. Scale bar = 200 µm. Each dot represents one animal. Means ± SEM are shown. Dashed line indicates region of interest. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. 
reduced levels of aldehyde dehydrogenase 1a1 (Aldh1a1), a TH-dependent gene expressed mainly in astrocytes and fibroblasts, were elevated by AAV-BR1-Mct8 (Fig. 4C). In contrast to the four previous genes that are induced by THs, the mRNA of the astrocytic deiodinase 2 (Dio2) that converts T4 into T3 was increased in the cortex of DKO and AAV-BR1-Mct8 had no significant effect on the expression level (Fig. 4D). Thus, the findings demonstrate that AAV-BR1-Mct8 treatment at P0 has a long-lasting effect on the expression of several TH-dependent neuronal and astrocytic genes.

MCT8-deficient patients suffer from coordination deficits. Like AHDS patients, DKO mice exhibited locomotor deficiencies, including impaired motor coordination and learning in comparison to littermate controls when investigated in the rotarod test at P120.
Figure 4 AAV-BR1-Mct8 treatment improves expression of TH-dependent genes and motor function in DKO mice. (A and B) AAV-BR1-Mct8 injected at P0 increased the expression of neuronal Hr and Klf9 in the cortex of DKO mice at P120. Gene expression was visualized by in situ hybridization and quantified by qPCR. In situ hybridization images are representative for several replicates (Supplementary Table 1). Scale bar = 100 µm. One-way ANOVA: Hr, $F(2/20) = 36.92, P < 0.0001; Klf9, F(2/20) = 52.79, P < 0.0001; Holm–Sidak’s post hoc test. (C and D) AAV-BR1-Mct8 injected at P0 increased the expression of astrocytic Aldh1a1 (C) in the cortex of DKO mice at P120. The elevated cortical Dio2 expression (D) in DKO mice was not reduced by AAV-BR1-Mct8.

(Continued)
However, a single intravenous injection of AAV-BR1-Mct8 at P0 significantly improved DKO mouse performance in the rotarod test (Fig. 4E). In the beam walk, another test of coordination and balance, DKO mice treated with AAV-BR1-Mct8 crossed the beam faster than untreated DKO and made fewer errors (Fig. 4F). Moreover, grip strength tended to be greater in AAV-BR1-Mct8-treated DKO mice (Fig. 4G).

If there are no other known cases in the family, AHDS is typically diagnosed with some delay, precluding a therapeutic intervention early after birth. To determine whether AAV-BR1-Mct8 would also improve the phenotype when administered at a juvenile age, we treated DKO mice at P30 with an intravenous injection of AAV-BR1-Mct8 (Fig. 5A). At P51, many capillaries throughout the brain of AAV-BR1-Mct8-treated DKO mice expressed MCT8 (Fig. 5B). In addition, a few neurons and astrocytes were transduced. Intriguingly, AAV-BR1-Mct8 markedly corrected the aberrant expression of the TH-dependent genes Klf9, Hr, Aldh1a1 and Dio2 and even normalized mRNA levels of Klf9, Aldh1a1 and Dio2 (Fig. 5C). Likewise, AAV-BR1-Mct8 corrected the thickness of the cortical layers II–VI and of the molecular layer in the cerebellum (Fig. 5D and G). The gene therapy increased the expression of the interneuron marker GAD67 and the number of parvalbumin-positive interneurons in the somatosensory cortex (Fig. 5E and F). The effect of AAV-BR1-Mct8 on myelin markers was more variable with a normalization of the number of Olig2-positive oligodendrocytes in the corpus callosum and a trend towards more Olig2 protein as determined by immunoblot but no significant effect on MBP levels (Fig. 5H–J). The number of mature CC1-positive oligodendrocytes was also not altered by this treatment regime (Supplementary Fig. 6B). Overall, the data show that AAV-BR1-Mct8, even when administered at a juvenile age, ameliorates deficits in neural gene expression and brain structure, while effects on myelination were more variable and may require more time to fully develop.

**Discussion**

Our study reports a novel gene therapy for MCT8 deficiency, a major cause of X-linked intellectual disability. Previous investigations expressed MCT8 in mice with the help of the AAV9 capsid. After intracerebroventricular injection of the vector, expression in parenchymal cells did not correct low TH concentrations in the brain, presumably because the blood–brain barrier transport of THs was still compromised. In contrast, when the AAV9 vector was intravenously injected at P30, it ameliorated the phenotype of DKO mice. However, the latter study did not investigate the efficacy of an earlier administration or the cellular expression of MCT8. In general, AAV9 is known for its unspecific tropism. It crosses the blood–brain barrier and transduces neural cells and epithelial cells of the choroid plexus, complicating the mechanistic interpretation of the latter study. Moreover, transduction of extraneuronal cells, such as hepatocytes, may cause potentially severe side effects. In the present study, targeting the small endothelial cell population was sufficient to supply T3 to the brain and to improve symptoms of MCT8 deficiency in a mouse model. Thus, the data confirm that endothelial cells are a critical site for postnatal MCT8 function.

Assuming that permeation into the brain is the key problem in MCT8 deficiency, previous studies have attempted to circumvent the blood–brain barrier by administering TH or TH analogues intranasally or intracerebroventricularily, but with limited success. Apparently, endothelial MCT8 is highly efficient in supplying the CNS with TH. After THs enter the CNS through MCT8, uptake in glia or neurons may occur through other transporters, including MCT10, ı-type amino acid transporters (LATs), or OATP1C1. Normal brain development requires TH action already before birth, but how THs cross the prenatal blood–brain barrier is still unclear. Human brain vessels express MCT8 already at gestational Week 14. Lower brain TH concentrations in an MCT8-deficient foetus suggest that MCT8 plays a role in foetal TH transport. On the other hand, patients with MCT8 deficiency are normal at birth and only develop symptoms during the first year of life. Our study now provides functional data that postnatal reconstitution of MCT8 is sufficient to prevent the neurological phenotype of the disease, at least partially. MCT8 expression could be directed to the neonatal or juvenile blood–brain barrier by using an AAV vector with high selectivity for brain endothelial cells. This approach improved transcriptional and morphological parameters of the disease already after 12 days. Importantly, the effect persisted at the behavioural and transcriptional level into adulthood. Thus, brain endothelial cell-specific gene therapy emerges as a potential novel treatment of the disabling neurological signs of AHDS. Before the approach is translated into the clinic, several aspects have to be considered. First, it should be noted that the concept is based on a mouse model, the validity of which is open to challenge. The example of Oatp1c1 shows that there are species differences in the transport of THs. Even on an Oatp1c1-deficient background, the phenotype of Mct8 deficiency seems to be less severe in mice than in humans. It is possible that during human development, transient expression of MCT8 by neurons and neural precursor cells or the low expression in adult neural cells contribute to MCT8 function. Second, it will be important to see whether AAV-BR1 possesses the same tropism for brain endothelial cells in humans as in mice. Recently, another AAV capsid, AAV-BI30, has been reported to target brain endothelial cells in mice after intravenous injection, providing an alternative to implement the gene therapy strategy developed here. Despite the rather low transduction rate of brain endothelial cells in our mouse model, the marked effects are reassuring and suggest that the transduction rate must not be high to achieve a therapeutic benefit in humans. Finally, the therapeutic time window has to be defined because MCT8 deficiency is often diagnosed with some delay. In this respect, it is important that treatment with AAV-BR1-Mct8 was still effective when initiated at a juvenile age (P30). Overall, we believe that brain endothelial targeting could be a valuable strategy to treat AHDS.

**Figure 4** Continued

Scale bar = 100 μm. One-way ANOVA: Aldh1a1, F(2/20) = 440.4, P < 0.0001; Dio2, F(2/19) = 10.01, P = 0.0011; Holm–Sidak’s post hoc test. (E) AAV-BR1-Mct8 treatment at P0 prolonged the time DKO mice were able to balance on the rotarod as a sign of improved coordination and motor learning. Mice were assessed on five consecutive days starting at P120. Repeated-measures ANOVA, F(2/26) = 5.045, P = 0.0141; Holm–Sidak’s post hoc test. (F) AAV-BR1-Mct8 treatment reduced the time DKO mice needed to cross the beam and led to a trend towards fewer errors, indicating better motor coordination. One-way ANOVA: time, F(2/21) = 7.567, P = 0.0034; errors, F(2/21) = 9.274, P = 0.0013; Holm–Sidak’s post hoc test. (G) AAV-BR1-Mct8 treatment tended to increase the grip strength of DKO mice in comparison to control animals. One-way ANOVA, F(2/18) = 8.301, P = 0.0028; Holm–Sidak’s post hoc test. Each dot represents one animal. Means ± SEM are shown. ns = non-significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.
Figure 5 Intravenous administration of AAV-BR1-Mct8 in juvenile mice enables the expression of Mct8 in brain endothelial cells and improves T3-dependent parameters. (A) Schematic of the experimental design, created by BioRender.com. gp = genomic particles. (B) MCT8 expression in CD31-positive endothelial cells of control or DKO mice that received AAV-BR1-Mct8 at P30. Fluorescence immunostaining for MCT8 and CD31 was performed at P51. Scale bar = 100 µm (left), 10 µm (right). (C) AAV-BR1-Mct8 injected at P30 increased the expression of Klf9, Hr and Aldh1a1 and reduced expression of Dio2 in DKO mice at P51. Gene expression was quantified by qPCR. One-way ANOVA: Klf9, F(2/26) = 4.031, P = 0.0299; Hr, F(2/25) = 32.80, P < 0.0001; Aldh1a1, F(2/26) = 8.9, P = 0.0011; Dio2, F(2/26) = 6.076, P = 0.0068; Holm–Sidak’s post hoc test. (D–G) Administration of AAV-BR1-Mct8 at P30 improved the thickness of the somatosensory cortex layers II–VI (D), the relative fluorescence signal intensity of GAD67 (E), the number of parvalbumin-positive interneurons (F) and the thickness of the molecular layer in the cerebellar vermis (G) at P51. One-way ANOVA for cortical thickness, F(2/22) = 5.723, (Continued)
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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain on online.

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