Neonatal gene therapy achieves sustained disease rescue of maple syrup urine disease in mice

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Maple syrup urine disease (MSUD) is a rare recessively inherited metabolic disorder causing accumulation of branched chain amino acids leading to neonatal death, if untreated. Treatment for MSUD represents an unmet need because the current treatment with life-long low-protein diet is challenging to maintain, and despite treatment the risk of acute decompensations and neuropsychiatric symptoms remains. Here, based on significant liver contribution to the catabolism of the branched chain amino acid leucine, we develop a liver-directed adeno-associated virus (AAV8) gene therapy for MSUD. We establish and characterize the Bckdha (branched chain keto acid dehydrogenase a)−/− mouse that exhibits a lethal neonatal phenotype mimicking human MSUD. Animals were treated at P0 with intravenous human BCKDHA AAV8 vectors under the control of either a ubiquitous or a liver-specific promoter. BCKDHA gene transfer rescued the lethal phenotype. While the use of a ubiquitous promoter fully and sustainably rescued the disease (long-term survival, normal phenotype and correction of biochemical abnormalities), liver-specific expression of BCKDHA led to partial, though sustained rescue. Here we show efficacy of gene therapy for MSUD demonstrating its potential for clinical translation.
Monogenic inborn errors of metabolism are relevant targets for gene therapy as they are autosomal recessive conditions with well-characterized pathophysiology and highly informative biochemical readouts; yet their therapeutic management remains complex and cumbersome. Maple syrup urine disease (MSUD, MIM: 248600) is one of the earliest described metabolic disorders. This autosomal recessive disorder with an incidence of one in 185,000 live births is caused by a defective activity of the branched-chain 2-keto acid dehydrogenase (BCKD) enzyme leading to the accumulation of branched-chain amino acids (BCAA) and other metabolites. MSUD is caused by mutations in BCKDHA, BCKDHB or DBT genes respectively coding for E1_{α}, E1_{β}, and E2 subunits and accounting for 45, 35, and 20% of MSUD patients, respectively. 

Neurotoxicity in MSUD is related to the accumulation of leucine and 2-ketoisocaproic acid (KIC, the ketoacid derived from leucine). In the classical severe form of MSUD (85–95% of cases), with less than 3% residual enzyme activity, this accumulation causes coma and cerebral edema shortly after birth with early death in the absence of aggressive and rapid management. Long-term MSUD management represents an unmet clinical need. Current MSUD treatment is limited to severe and life-long BCAA dietary restriction associated with an oral BCAA-free amino acids mixture. Such treatment is difficult to maintain in the long term and is largely incompatible with a normal quality of life. Further, it does not prevent long-term neurocognitive impairments, the latter affecting roughly 30% of the patients after the age of 20 years.

Orthotopic liver transplantation (OLT), was shown as an effective MSUD therapy allowing removal of dietary restrictions, full protection from acute decompensations during illness, arrest of neurocognitive impairment progression, prevention of liver failure, and metabolic and clinical stability. However, liver transplantation is associated with the potential risk of death and graft failures. As an autosomal recessive monogenic disease, MSUD represents an ideal target for liver-directed gene therapy since clinical OLT data suggest that incomplete restoration of liver BCKD enzyme activity (representing 9–13% of body BCKD activity) is fully therapeutic. This constitutes a strong rationale for testing liver gene transfer as a therapeutic option for MSUD.

Among the available gene-delivery platforms, adeno-associated virus (AAV) vectors are the most suitable for liver gene transfer. AAV liver gene therapy achieved a major milestone with the proof of safety and long-term efficacy in a clinical trial for hemophilia B. Inborn errors of metabolism are good candidates for AAV gene therapy – 17, organic acidemias, and other metabolic conditions with well-characterized pathophysiology and targets for gene therapy as they are autosomal recessive and 20–22. While the majority of patients harbor mutations in BCKDHA and BCKDHB genes, there is no characterized mouse model of MSUD linked to the Bckdha gene, and only one report of a Bckdha knockout mouse with a severe phenotype and lethality within 24 h after birth and elevated BCAA and BCKA in plasma and tissues. Recently, a mouse model with tissue-specific Bckdha knockout in brown adipose tissue was described and showed a reduced tolerance of BCAA loading but no other phenotypic features of MSUD.

We herein extensively characterized a Bckdha−/− mouse line showing that it faithfully recapitulates the classical human MSUD. We further developed a successful AAV gene therapy based on the transfer of human BCKDHA (hBCKDHA) in neonatal Bckdha−/− mice.

Results

Bckdha−/− mouse recapitulates the severe human phenotype of MSUD. We generated Bckdha−/−/+, Bckdha−/−−/− males and females, who did not display any particular phenotype. We found that Bckdha−/− mice show a lethal early-onset phenotype. Half of the mice died before 3 days of age, and the other half around 7 days of age with a maximum lifespan of 12 days (Fig. 1a). Bckdha−/− mice exhibited a major growth delay (Fig. 1b). Animals who survived longer than 1 week showed reduced activity and abnormal hindlimb clamping in response to tail suspension (100% of these short-survivors exhibited a score of 0 compatible with severe neurological impairment). Biochemical analyses showed increased concentrations of plasma BCAA (Fig. 1c and Fig. S1a), along with a significant accumulation of allolucine, a pathognomonic marker of MSUD (Fig. 1c), and increased concentrations of plasma BCKA (Fig. S1b). Conversely, alanine and glutamate plasma concentrations were decreased, leading to an increase in the leucine/alanine ratio (Fig. S1a) as described in MSUD patients. RT-PCR showed a decrease of Bckdha transcripts in the liver, heart, and brain to about 50% in Bckdha−/− mice relative to Bckdha+/+, whereas Bckdha transcripts were barely or not detectable in Bckdha−/− mice in all of these tissues (Fig. 1d). Western blot showed similar results for BCKDHA protein in the liver, heart, and brain, confirming the null nature of the model (Fig. 1e). Furthermore, BCKDHB protein was undetectable in the liver of Bckdha−/− mice as described in BCKDHA defective lymphoblasts from MSUD patients (Fig. S1c). Together, these results indicate the Bckdha−/− mouse model faithfully recapitulates the features of human classical MSUD.

Design and in vitro testing of cassettes for the treatment of MSUD. Transfection of plasmids in Huh7 cells resulted in the expression of BCKDHA for all transgenes with the highest expression detected with the wild-type sequence as assessed by Western blot analysis (Fig. 2b, d). We therefore selected the EF1α (ubiquitous promoter) and hAAT (human alpha 1 antitrypsin, liver-specific promoter) hBCKDHA transgenes with the full-length human BCKDHA cDNA sequence (Fig. 2a, c). Accordingly, codon-optimized constructs were not further used in the study. To ensure that the difference of intron between the EF1α driven construct (containing the EF1α intron) and the hAAT driven construct (containing the SV40 intron) did not affect BCKDHA expression, we designed an additional hAAT driven cassette containing the EF1α intron. Similar levels of BCKDHA protein expression in transfected Huh7 cells between the two constructs (hAAT-EF1α and hAAT iSV40) were obtained (Fig. S2a). As it was initially reported that 80% of BCKD activity was provided by the liver in rodents, we opted for AAV8 vectors given their ability to mainly transduce the liver in mice, along with other tissues such as the heart.

AAV8-EF1α-hBCKDHA gene transfer allows for rescue of MSUD early lethality in Bckdha−/− mice. To determine the minimally effective vector dose that would rescue the MSUD phenotype in Bckdha−/− mice, we performed systemic temporal
Fig. 1 The Bckdha⁻/- mouse recapitulates the MSUD phenotype in humans. 

**a** Kaplan-Meier curve (Bckdha⁻/- n = 15, Bckdha⁺/- n = 44, Bckdha⁺/+ n = 26); log-rank Mantel-Cox test (Bckdha⁻/- vs. Bckdha⁺/- or Bckdha⁺/+; p values = 4.86 × 10⁻¹³ or 2.22 × 10⁻¹⁶, respectively). 

**b** Weight (1 week, Bckdha⁻/- n = 5, Bckdha⁺/- n = 7, Bckdha⁺/+ n = 4). 

**c** Leucine and alloisoleucine plasma concentrations (3 days, Bckdha⁻/- n = 3, Bckdha⁺/- n = 3, Bckdha⁺/+ n = 2). 

**d** Bckdha mRNA and **e** BCKDHA protein levels in the liver, heart, and brain (1 week, Bckdha⁻/- n = 3, Bckdha⁺/- n = 1, Bckdha⁺/+ n = 1). All data are shown as means ± SD. ****P < 0.0001. Source data are provided as a Source data file.
vein injection of the AAV8-EF1α-hBCKDHA vector at either $10^{13}$ vg/kg ($1.5 \times 10^{10}$ vg/mouse, further referred to as low dose) or $10^{14}$ vg/kg ($1.5 \times 10^{11}$ vg/mouse further referred to as high dose) at P0, immediately after birth. All the pups of the litters were injected, without prior knowledge of the genotyping data, which were obtained at P10. Three litters were injected at $10^{13}$ vg/kg and two litters with $10^{14}$ vg/kg of the AAV8-EF1α-hBCKDHA vector. In the litters injected at $10^{13}$ vg/kg, one pup died at P3 (eaten by the dam), one Bckdha$^{-/-}$ pup died at P1, and one at P7 (traumatic urine collection), leaving for the analysis 7 Bckdha$^{-/-}$, 9 Bckdha$^{+/-}$, and 5 Bckdha$^{+/-}$ mice. In the litters injected at $10^{14}$ vg/kg, two Bckdha$^{-/-}$ died at P2 and one heterozygous Bckdha$^{+/-}$ died at P7 in a context of major growth failure, leaving for the analysis 2 Bckdha$^{-/-}$, 9 Bckdha$^{+/-}$, and 4 Bckdha$^{+/-}$ mice. With the injections at $10^{13}$ vg/kg, we observed a partial and transient rescue of the MSUD phenotype in Bckdha$^{-/-}$ mice ($n$ = 7) with important inter-individual variability.
growth without obvious neurological symptoms during the first 3 weeks post vector infusion, but then stopped growing and developed neurological symptoms (ataxia with frequent falls), urging to sacrifice them at age 4 weeks (Fig. 3a). In the remaining 2/7 Bckdha−/− mice growth retardation was obvious very early, evolving into weight loss and finally a moribund status requiring an anticipated sacrifice before age 4 weeks (Fig. 3a). The Bckdha−/− mice injected with the AAV8-EF1α-hBCKDHA vector at 10^{14} vg/kg displayed normal growth without overt neurological symptoms (Fig. 3a) n = 2 Bckdha−/− injected at 10^{14} vg/kg and...
sacrificed at age 4 weeks). We observed a similar dose effect at the biochemical level, with the Bckdha−/− mice injected at 10^{14} vg/kg displaying plasma leucine concentrations slightly higher than the upper limit of the normal range, whereas the Bckdha−/− mice injected at 10^{13} vg/kg and sacrificed at age 4 weeks displayed a marked six-fold increase in the normal in leucine concentrations (Fig. 3b). The two phenotypically most severe Bckdha−/− mice injected at 10^{13} vg/kg and sacrificed before age 4 weeks exhibited extremely high plasma leucine concentrations, consistent with their clinical status, suggesting a loss of treatment efficacy and a relapse of the disease. AAV8 vector genome copies were detectable in the liver and heart and more moderately in the brain with a significant dose effect between injections at 10^{13} vg/kg and 10^{14} vg/kg (Fig. 3c). In the liver and heart, hBCKDHA mRNA transcripts and hBCKDHA protein were detectable with a similar dose effect (Fig. 3d, e). In brain, hBCKDHA mRNA transcripts and hBCKDHA protein were only detectable for injections at 10^{14} vg/kg (Fig. 3d, e). Liver pathology of injected mice did not show significant alteration (Fig. S3). In the brain of Bckdha−/− mice sacrificed before age 4 weeks due to relapse of the disease, we observed an important vacuolization of the striatum (Fig. S3).

These results indicate that rescue or MSUD in mice can be achieved with systemic gene transfer with AAV vectors.

High dose AAV8-EF1α-hBCKDHA gene transfer allows long-term sustained MSUD rescue. To further evaluate the long-term effect of gene therapy in MSUD mice, we next injected the AAV8-EF1α-hBCKDHA vector in nine Bckdha−/− pups from 3 litters at 10^{14} vg/kg. Compared to their wild-type and heterozygous littermates, treated animals exhibited comparable survival and normal growth (Fig. 4a, b). All nine animals were alive at 6 months of age without overt phenotypic abnormalities. The biochemical phenotype was dramatically improved with BCAA concentrations that were reduced from twenty-fold to only two-fold over normal at age 6 months (Figs. 4c and S4) along with a reduction of BCKA concentrations (Fig. S5a). Alloisoleucine was not detectable. Other disturbances of amino acids such as decreased alanine and glutamate concentrations were near completely corrected (Fig. S4). We sacrificed 5 treated Bckdha−/− mice at 6 months of age. In these mice, AAV8 vector genome copies were detectable in the liver and heart and, at lower levels, in the brain (Fig. 4d). hBCKDHA mRNA transcripts were detectable in the liver and heart and, to a lesser extent, in the brain (Fig. 4e). Consistently, hBCKDHA protein was detectable mainly in the liver and heart and present albeit at lower levels in the brain (Fig. 4f). In the liver, hBCKDHA protein was less phosphorylated in Bckdha−/− treated mice than in Bckdha+/+ mice, suggestive of an important active BCKDHA fraction (Fig S5b). BCKDHB protein expression was also rescued in the liver to about 15% of its expression in the liver of wild-type individuals (Fig. S5c). Brain pathology did not show significant alterations as opposed to striatum vacuolization observed in Bckdha−/− mice treated with 10^{13} vg/kg and sacrificed at age 4 weeks (Fig. S3). The remaining four Bckdha−/− mice were still alive without overt phenotypic abnormalities (Fig. S6a, b) at age 12 months with stable, near-normal leucine concentrations in plasma (Fig. S6c). In these 12 months old mice, AAV8 vector genome copies and hBCKDHA protein were detectable in the liver and heart and, at lower levels, in the brain (Fig. S6d, e). Other features such as the phosphorylation state of BCKDHA, partial rescue of BCKDHB protein expression, and correction of other amino acid disturbances were similar between 6- and 12-month-old (treated) Bckdha−/− individuals (Figs. S6f, g and S4). These results indicate that constitutive expression of the hBCKDHA transgene mediates long-term rescue of MSUD in mice.

Lever-restricted gene transfer allows partial but sustained rescue of the MSUD phenotype. We next wanted to evaluate the liver vs. extrahepatic contribution to the total BCKDHA activity responsible for the phenotypic rescue of mice treated with the AAV8-EF1α-hBCKDHA vector. To this aim, we tested the therapeutic efficacy of a vector expressing the hBCKDHA transgene under the control of a liver-specific promoter (AAV8-hAAT-hBCKDHA). Compared with the AAV8-EF1α-hBCKDHA low dose (see above), the 10^{13} vg/kg dose of AAV8-hAAT-hBCKDHA led to the transient rescue of the MSUD phenotype, as the 5/5 Bckdha−/− mice survived for 14 days, either without overt clinical symptoms (3/5) or with delayed though progressive growth (2/5). Thereafter, all mice became rapidly moribund thus requiring sacrifice between P19 and 21 (Fig. S7a). At sacrifice, mice showed a significant increase in plasma leucine, consistent with the severe phenotype, suggesting reduced treatment efficacy (Fig. S7b). We observed a comparable biodistribution of AAV8 in tissues similar to the previous experiments, with the detection of vector genome copies in the liver and heart and, at lower levels, in the brain (Fig. S7c). Results of mRNA and protein analyses were consistent with the hAAT promoter liver specificity (Fig. S7d, e). Therefore, the effects of low dose AAV8-hAAT-hBCKDHA were partly effective yet significantly less than low dose AAV8-EF1α-hBCKDHA.

Subsequently, we tested the intermediate (3 × 10^{13} vg/kg) and high (10^{14} vg/kg) doses of AAV8-hAAT-hBCKDHA along with parallel experiments testing an intermediate dose of AAV8-EF1α-hBCKDHA. The 5 Bckdha−/− mice that survived beyond 48 hours (out of a total of 9 pups) injected with 3 × 10^{13} vg/kg AAV8-hAAT-hBCKDHA vector had 3 weeks of normal growth without overt phenotypic abnormalities. Then they all exhibited abrupt growth arrest, severe neurological impairment with frequent falls, ataxic gait, all with a score of 0 at the hindlimb clasping test (Fig. S8a). This led to sacrifice during the fourth week, 1 week later than the animals treated at 10^{13} vg/kg (Fig. S7a). Conversely, only 1 of the 6 Bckdha−/− mice injected with 3 × 10^{13} vg/kg AAV8-EF1α-hBCKDHA vector that survived beyond 48 h (out of a total of 8 pups, plus 2 dead pups that could not be genotyped) died at 5 weeks post treatment displaying poor growth and ataxic gait. Three of the 5 other pups died or required sacrifice at 9, 10, or 12 weeks of age (Fig. S8c, d), whereas the two remaining...
animals were in good health when they were sacrificed at age 12 weeks at the end of the study. Leucine plasma concentration measured at age 4 weeks in AAV8-hAAT-hBCKDHA treated animals (intermediate dose) was 3460 +/− 750 µmol/L vs. 890 +/− 340 µmol/L for Bckdha−/− mice in the AAV8-EF1α-hBCKDHA intermediate-dose cohort (Fig. S8b, e). These differences agreed with the less severe phenotype observed in AAV8-EF1α-hBCKDHA-treated mice. At age 8 weeks, the mean leucine plasma concentration for the Bckdha−/− mice in the intermediate dose EF1α group was 1070 +/− 230 µmol/L indicating sustained moderate restoration of BCKDHA activity. Tissue analyses confirmed the expression and distribution of BCKDHA.
Fig. 4 Long-term sustained complete rescue of severe MSUD phenotype in Bckdha−/− mice. Mice were injected at 10^{14} vg/kg with AAV8-EF1α-hBCKDHA. a Weight curves for males (Bckdha−/− n = 6, Bckdha+/− n = 3, Bckdha+/+ n = 3) and b females (Bckdha−/− n = 3, Bckdha+/− n = 8, Bckdha+/+ n = 6) for all injected mice. c Plasma leucine concentrations for non-injected Bckdha−/− newborns (n = 3) and at 6 months for injected mice, Bckdha−/−, Bckdha+/−, and Bckdha+/+ (n = 5 each). d Vector genome copy number (VGCN), e human BCKDHA (hBCKDHA) mRNA, and f BCKDHA protein levels in the liver, heart, and brain in mice sacrificed 6 months after neonatal injection (Bckdha−/− injected n = 5, controls: Bckdha+/− n = 1 non-injected and sacrificed at 1 week +1 non-injected Bckdha+/− sacrificed at 6 months for western blots). Analyses were performed in triplicate. The antibody detected both the human BCKDHA and the murine BCKDHA proteins. g Kaplan-Meier curves for all injected Bckdha−/− mice (n = 9) (blue line) during the first 6 months. The red dash line represents the maximum survival of untreated Bckdha−/− mice. All data are shown as mean ± SD. Source data are provided as a Source data file.

mRNA transcripts and protein according to promoter expression profiles as previously described (Fig. S9). Therefore, 3 × 10^{13} vg/kg dose of AAV8-hAAT-hBCKDHA was quite effective until 3 weeks of age, but not beyond, whereas the same dose of AAV8-EF1α-hBCKDHA showed more prolonged efficacy albeit with pup loss between 5 and 12 weeks of age.

Next, we tested the efficacy of 10^{14} vg/kg (high dose) AAV8-hAAT-hBCKDHA gene therapy. We injected six Bckdha−/− mice and their littermates. One Bckdha−/− mouse died on day 28 but the 5 other treated Bckdha−/− animals exhibited long survival (>5 months), similar to mice treated with a high-dose AAV8-EF1α-hBCKDHA vector, albeit with a partial rescue of the remaining MSUD phenotype: lower weight and high plasma leucine concentrations (much higher than in the reciprocal experiment using 10^{14} vg/kg AAV8-EF1α-hBCKDHA) (Figs. 5a–c and S10). BCKD enzyme activity was tested in the liver of Bckdha−/− mice either untreated at age P2-4 or treated and sacrificed at age one and/or six months (Fig. S11). At age P2-4, enzyme activity in untreated Bckdha−/− mice was undetectable, contrasting with enzyme activity in litter-matched controls that was comparable or slightly reduced relative to older mice. Compared to age-matched controls, at age 6 months, Bckdha−/− mice treated with the AAV8-EF1α-hBCKDHA or the AAV8-hAAT-hBCKDHA vectors at 10^{14} vg/kg showed 12 ± 4% and 7 ± 4.5% BCKD activity, respectively. Of note in AAV8-EF1α-hBCKDHA-treated mice at 10^{14} vg/kg, the activity was much greater at age one month than at age 6 months, though with high variance, consistent with progressive vector dilution in the growing liver (Fig. S11). In these two situations, however, the degrees of reconstituted hepatic BCKD activity in the Bckdha−/− mice were markedly lower than the restoration of the hBCKDHA protein to the wild-type levels in the livers (Figs. 3e and 4f). In AAV8-EF1α-hBCKDHA-treated mice at 10^{13} vg/kg that were only transiently and partly rescued, activity was reduced already
markedly at age one month, i.e., 7 ± 5% activity relative to age-matched controls (Fig. S11).

Finally, as the two cassettes (EF1α and hAAT) differed in one intron (iEF1α vs. iSV40, respectively), we wanted to confirm in vitro data showing that in Huh7 cells, there was no overt difference in terms of BCKDHA protein expression between the original hAAT driven construct containing the SV40 intron, iSV40 and an additional hAAT driven construct containing the EF1α intron, iEF1α. We therefore compared outcomes of intermediate dose (3.10^13 vg/kg) injected individuals with these two constructs in a separate experiment. We injected 3 Bckdha^-/- individuals immediately after birth with the AAV8-hAAT-iSV40-hBCKDHA construct, which showed neurological symptoms during the fourth week of life, 2 of them with weight loss, requiring sacrifices before age 4 weeks. Six Bckdha^-/- individuals were injected with the AAV8- hAAT-iEF1α-hBCKDHA construct. Two showed neurological symptoms with weight loss during the fourth week of life requiring sacrifices before age 4 weeks. Three exhibited a major growth arrest during the fourth week of age. All of these mice were sacrificed at age 4 weeks. In keeping with the in vitro data (Fig. S2a), no major differences were found between the two constructs considering growth and survival, plasma leucine concentrations, and BCKDHA protein expression in the liver (Fig. S2). A non-significant trend towards superior rescue by AAV8-hAAT-iEF1α-hBCKDHA cannot be excluded but any such effect falls within interindividual variability.

These data may suggest a significant contribution of non-liver tissues to the whole-body BCKDA transgene activity responsible for the phenotypic rescue of MSUD in neonate mice as they show that at equivalent vector doses, a better phenotypic rescue is achieved with the EF1α-driven AAV8 transgene.

**Discussion**

MSUD represents an unmet medical need with limited therapeutic curative options. Given the beneficial impact of liver transplantation on survival, quality of life, and disease biomarkers in patients with MSUD, we hypothesized that liver-directed gene therapy would be a feasible therapeutic approach. Herein, we showed that a single AAV vector infusion in neonate MSUD mice resulted in the long-term survival of animals and rescue of the disease phenotype.

As mutations in BCKDHA are one of the most frequent causes of MSUD in humans^2, a first step was to establish and characterize a colony of Bckdha^-/- mice. We showed that homozygous Bckdha^-/- mice faithfully recapitulate the human disease features, with affected animals exhibiting extremely high plasma concentrations of leucine and early-onset severe neurological involvement resulting in death before age P12.

Based on the clinical experience, heterozygous human individuals, like Bckdha^-/- mice, exhibit 50% of whole-body BCKD enzyme activity and do not exhibit any phenotypic or biochemical abnormalities. This would suggest that the restoration of 50% of BCKD activity could be fully curative. However, liver transplantation in humans brings approximately 10% of the whole-body BCKD activity^10 and dramatically improves the MSUD phenotype and the metabolic parameters for the great majority of patients^2^28. Thus, based on these considerations, in the context of gene therapy, an increase of BCKDH above 10% of normal would be sufficient to rescue the disease phenotype, while levels around 50% of normal would completely correct MSUD. Emerging data from liver-directed gene therapy trials with AAV vectors suggest that levels of transduction needed to rescue MSUD are indeed achievable, as in some cases supraphysiological levels of transgene expression have been achieved in hemophilia A subjects^29, and whole-body transduction has been documented in infants affected by spinal muscular atrophy^30 and Duchenne muscular dystrophy^31, among others. One of the challenges of AAV vector-mediated liver gene transfer is that, due to the predominantly non-integrating nature of these vectors, transgene expression in rapidly dividing tissues is not stable. This has been shown in several preclinical animal models^32-34 in which AAV vectors have been administered at a time when the liver had not reached the adult size. This is clearly a limitation in the development of neonatal gene therapy for MSUD, as early treatment, ideally shortly after birth, would greatly benefit patients.

Here, by using the ubiquitous promoter EF1α, we were able to achieve full and sustainable phenotypic rescue both clinically and biochemically in neonate Bckdha^-/- mice. BCKDHA transgene mRNA and protein were detectable in the liver, brain, and heart, reflecting the widespread transduction of the vector given at a dose of 10^{14} vg/kg, in line with doses tested in neonatal patients^30. At a lower vector dose of 10^{13} vg/kg, the rescue was partial and transient, indicating subtherapeutic levels of transgene expression. Future studies aiming at optimizing the transgene expression cassette^34, testing of hybrid promoters^35, and possibly testing vectors with broader tropism than AAV8^31, will help lowering the therapeutic vector dose threshold.

By contrast, liver-restricted gene transfer using the hAAT promoter^36 provided partial though sustained rescue of the MSUD phenotype. Indeed, mice injected with vector doses of 10^{14} vg/kg exhibited lower weight growth, plasma leucine concentrations of about 4.5–6 times the normal (vs. 2 times in the ubiquitous approach). This suggests that the lack of extrahepatic expression may reduce therapeutic efficacy, thus consistent with previous findings from our laboratory, showing that multi-tissue expression of alpha-acid glucosidase provides superior rescue of Pompe disease in neonate mice compared to liver-restricted transgene expression^35. An alternative, possibly complementary mechanism suggested by BCKD activity assays is that the hAAT construct may be less active than the EF1α construct in the liver in vivo, though previous reports showed that the hAAT promoter is more potent than the EF1α one^37.

Our data suggesting that in the setting of neonatal gene therapy, long-term disease rescue with the ubiquitous promoter may involve extra-hepatic correction of the molecular defect, are consistent with recent data^38 suggesting that similar to humans, branched-chain amino acid oxidation takes place in most mice tissues including, muscle, brown fat, liver, kidney and heart. In keeping with this, recent findings show disease rescue in a mild mouse model of MSUD (intermediate MSUD [iMSUD] mouse model) by AAV9 vector expressing E2 not solely in the liver but also in muscle at a non-neonatal age^39. This model harbors a mutation in the E2 [DBT subunit of BCKD and has a phenotype that is milder than the classical form of MSUD explaining why it could be rescued by systemic injection at weaning (P21-P28, equivalent to late adolescence in humans). Relative to ubiquitous expression at the highest vector doses tested, liver-specific gene transfer leads to long-term survival but poorer growth and higher concentrations of metabolic parameters. Indeed, in the mouse model used in our study, animals had to be treated immediately at birth in order to rescue the early lethality associated with MSUD. As discussed above, pronounced liver growth during the first weeks of life in mice^32,40 results in the dilution of the AAV vector genomes and loss of transgene expression possibly explaining the partial efficacy of the liver-specific (hAAT) approach. Additionally, the fact that restoration of BCKDHA expression in the liver to supraphysiological levels is not fully correcting the disease can also be due to several other factors. These include the expression of the enzyme in only a subset of hepatocytes. Based on literature data^41 and our own experience.
with AAV vectors, it is safe to assume that at the doses used herein, nearly 100% of mouse hepatocytes would express the enzyme. In large animal models, the efficiency of AAV transduction in the liver is significantly lower as is it also expected to be in humans. The lack of sufficient expression in other tissues, or the production of a non-functional and/or misfolded BCKD enzyme in hepatocytes due to overexpression may also play a role. The recombiantly expressed E1 protein has been shown to depend on molecular chaperones for proper folding and heterotetrameric assembly in cells.

Based on these observations, and given that in humans the liver reaches nearly full size by the age of 10 years, it is reasonable to argue that in the context of MSUD and other similar diseases with early manifestations, strategies to achieve long-term efficacy following AAV gene transfer are highly needed. As an alternative to this approach and a general strategy to achieve the long-lasting rescue of pediatric diseases with primary liver manifestation this approach and a general strategy to achieve the long-lasting rescue of pediatric diseases with primary liver manifestation as an alternative to the production of a non-functional and/or misfolded BCKD enzyme in hepatocytes due to overexpression may also play a role. The recombiantly expressed E1 protein has been shown to depend on molecular chaperones for proper folding and heterotetrameric assembly in cells.

In conclusion, we provide strong evidence of the long-term therapeutic efficacy of AAV8 neonatal gene therapy in a mouse model of MSUD that is lethal shortly after birth. Further research is warranted to precisely evaluate the potential contribution of extrahepatic tissues to disease rescue as opposed to dose-dependent effects within the liver. This study is of interest for a broader subset of genetic conditions characterized by early, postnatal onset and for which both liver and peripheral tissue expression could rescue the disease long term.

Methods

Plasmid design and vector production. The hBCKDHA gene (NCBI gene ID: 593) comprises 9 coding exons, spanning around 27 kb on chromosome 19, resulting in a cDNA of 1.34 kb. The BCKDHA transgene expression cassettes were WT versions of the human BCKDHA coding sequence, encoding the full-length hBCKDHA protein. We designed 2 transgene expression cassettes. The first cassette, termed EFla-hBCKDHA was assembled by cloning the hBCKDHA cDNA into a modified AAV expression cassette that includes the wild-type AAV2 inverted terminal repeats (ITRs), and a ubiquitous human elongation factor 1-alpha promoter (hEF1α) with an EFla intronic sequence to increase the expression of the transgene. To improve mRNA stability, we also included the Woodchuck Hepatitis Virus (WHV) Posttranscriptional Regulatory Element (WPRE), and the bovine growth hormone gene polyadenylation signal (bGH poly(A)) to improve mRNA stability. All DNA sequences (co1 and co2). The AAV vectors were produced using an adenovirus- or hAAT promoters (2 µg/well) was transfected as a control. Seventy-two hours after transfection, cells and conditioned media were harvested and analyzed for hBCKDHA expression by western blot.

Animal study procedures. Mouse studies were performed according to the French and European legislation regarding animal care and experimentation (2010/63/EU) and approved by the local institutional ethical committee (Paris Descartes ethics committee CEA34, APAFIS#22754-2018092017287553 v3). Bckdha<sup>−/−</sup> mice were purchased from The Canadian Mouse Mutant repository (C57BL/6N-Bckdha<sup>−/−</sup>). These mice carried a 266-bp deletion of Ch7 from 25683173 to 25683438 and insAGAGGC at the heterozygous state and Bckdha<sup>−/−</sup> mice have never been characterized. Bckdha<sup>−/−</sup> mice were crossed to generate Bckdha<sup>−/−</sup>/+. To evaluate the therapeutic efficacy of our constructs, we performed single systemic intra-temporal injections of AAV8-EFla1 or hAAT hBCKDHA transgene vectors in neonate mice at P0. The volume of injection was 10 µL. We injected the whole livers and genotyped the pups at P7. Different doses were tested: 10<sup>11</sup> vg/kg, 3 x 10<sup>11</sup> vg/kg, and 10<sup>14</sup> vg/kg. Mice were housed in a temperature (20–22 °C) and humidity (40–50%)-controlled environment with 12 h/12 h light/dark cycle and fed ad libitum with a standard diet.

Blood sampling was performed every 2 weeks and at sacrifice. Plasma was separated by centrifugation at 1000 g for 10 min and stored at −80 °C until analysis. After sacrifice, organs were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis or fixed overnight in 4% paraformaldehyde for pathology studies.

Genotyping. DNA extraction from the tail or ear clip was performed by adding 75 µL of extraction solution (25 mM NaOH, 2 mM EDTA) and by heating at 95 °C for 45 min. Samples were cooled on ice for 5 min. An equal volume of 40 mM Tris HCl (pH 7.5) was then added. 5 µL of DNA was amplified using a Taq DNA Polymerase PCR kit (Pepplab, Germany) according to the manufacturer’s instructions. The first pair of primers, flanking the deleted region in Bckdha (forward: 5′-ctgccacctctttggag-3′; reverse: 5′-cttctggatctggttctg-3′), were used, giving either a 516 bp amplicon for the wild-type allele (without the deletion) or a 256 bp amplicon for the mutated allele (with the deletion). The second set of primers was used with the antisense primer comprised in the deleted region of Bckdha (forward: 5′-ctgcctctctttggag-3′; reverse: 5′-aattgcctgatagaggactct-3′), giving a 339 bp amplicon for the wild-type allele (without deletion), or no amplicon for the mutated allele (with the deletion). Amplification conditions were 95 °C for 10 min then 40 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min.

Vector genome copy number analysis. DNA was extracted from tissues homogenates using DEscreen Blood & Tissue kit (Qiagen, Germany) and quantified. Vector genome copy number was determined by qPCR using 100 ng of DNA (assay with Nanodrop, version 1.6.198). Primers annealed to bGH polyA signal region of the expression cassette sequence: forward: 5′-gcacctcactctcctctc-3′, reverse: 5′-ccacatctgcctgctttg-3′. Data were recorded with QuantStudio (version 5.2).

RNA extraction and expression. Total RNA was extracted from snap-frozen tissue homogenates using the RNeasy mini kit (Qiagen, Germany). RNA was quantified and 0.8 mg (quantified with Nanodrop, version 1.6.198) was retro-transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). RT-reverse reactions were performed as negative controls. For hBCKDHA qPCR or cDNA was performed using SYBR green and primers annealing specifically on the WT hBCKDHA transgene sequence (hBCKDHA forward, 5′-ccacatctgtgagcagcttg-3′; hBCKDHA reverse, 5′-caacatctcaacctcagc-3′) and the mice Bckdha sequence respectively (Bckdha forward, 5′-ggctgctctgcatgctt-3′; Bckdha reverse, 5′-ctgtctgctgctggttctg-3′). Mouse Bckdha gene transcripts were used to normalize BeclinA or hBCKDHA expression (Gapdh forward, 5′-ggctgctgctgctgctgct-3′; Gapdh reverse, 5′-ctgtctgctgctgctgct-3′). Data were recorded with QuantStudio (version 5.2).

Western blot. Snap frozen mouse tissues were weighed, homogenized with RIPA buffer supplemented with protease inhibitors, sonicated using the Bioruptor system (Diagenode, Belgium), and centrifuged 20 min at 20,000 × g at 4°C. Protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific). SDS-PAGE electrophoresis was performed in a 4–12%
polyacrylamide gel. After transfer, the membrane was blocked with a solution of 5% BSA and incubated with an anti-BCKDHA antibody (Rabbit polyclonal; Abcam; cat. no. ab201225; lot GR3244895; WB 1:200) was used and mouse BCKDHA antibody (Rabbit polyclonal; Abcam; cat. no. ab2012125; lot GR3244895; WB 1:200) was used and visualized with the Odyssey imaging system (LI-COR Biosciences) and Image Studio version 5.2.

The polycyclonal anti-BCKDHA antibody recognized both the mouse BCKDHA and human BCKDHA proteins. For quantification, the mBCKDHA and mouse BCKDHA protein expression was normalized to GAPDH or actin (anti-actin, mouse polyclonal; ProteinTech; cat. no. 20358–1-AP; WB 1:1,000). The same protocol was used for the detection and quantification of phosphorylated BCKDHA (Ser293) using the Anti-human phosphorylBCKDHA antibody (Rabbit polyclonal; Abcam; cat. no. ab20057; lot GR394441; WB 1:2,000).

Liver BCKD enzyme activity. We performed an analysis of actual BCKD activity in the liver with a spectrophotometric assay as previously described, based on monitoring of NADH production and using 2-ketoisocaproic acid as substrate. As validated, we did not use a chloroform–methanol extraction buffer and dihydropropiolamide dehydrogenase in the assay buffer. Approximately 25 mg of frozen liver was pulverized with a mortar and homogenized in 200 µL of ice-cold extraction buffer (50 mM HEPES, 3% (w/v) Triton X-100, 2 mM EDTA, 5 mM dithiothretiol (DTT), 0.5 mM thiamine pyrophosphate, 50 mM potassium phosphate, 2% (w/v) bovine serum, 0.1 mM N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), trypsin inhibitor (0.1 mg/mL), leupeptin (0.02 mg/mL); pH 7.4 at 4°C, adjusted with KOH). Centrifugation at 20,000 x g for 5 min at 4°C removed insoluble materials. The supernatant was diluted to 9% PEG, stood on ice for 10 min, and then centrifuged at 12,000 x g for 10 min at 4°C. The pellet was suspended in a mixture of ice, 2.5% (v/v) of 2M HEPES buffer, 0.02% (w/v) Triton X-100, 0.2 mM EDTA, 0.4 mM thioanisamide pyrophosphate, 1 mM DTT, 50 mM KCl, and leupeptin (0.02 mg/mL); pH 7.4 at 37°C, adjusted with KOH). 500 µL of assay buffer (60 mM potassium phosphate dibasic, 4 mM MgCl2, 0.8 mM thiamine pyrophosphate, 0.8 mM CoA, 2 mM NAD + 0.2% (w/v) Triton X-100, 4 mM DTT), adjusted to pH 7.4 at 37°C, and 20 µL of tissue extract was mixed. Enzymatic activity measurement was performed at 30°C after the addition of 20 µL of 50 mM 2-ketoisocaproic acid (final concentration 1 mM; pH of the final assay = 7) by monitoring NADH production at 340 nm (with the software Kinetics version 3.09). Protein concentrations of the tissue extract were assessed with a BCA assay. The activity was normalized to proteins and expressed in arbitrary units.

Pathology studies. Organs were fixed overnight in 4% paraformaldehyde. For conventional bright-field light microscopy, organs were embedded in paraffin. Sections were stained with hematoxylin and Eosin. Numerisation of sections was performed with the software NDP.view2 (version 2.7.41).

Statistical analysis. All the data shown are reported as means ± standard deviation (SD). The statistical analyses were performed using the R software (version 3.4.2; http://www.R-project.org) and GraphPad Prism (version 9.3.11). Non-parametric tests were performed when two groups were compared (two-sided Mann–Whitney test). The survival of Bckdha–/– or Bckdha+/- mice was compared to the survival of Bckdha+/- or Bckdha–/– mice by log-rank Mantel–Cox test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files). Source data are provided with this paper.

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