Efficacy of adeno-associated virus gene therapy in a MNGIE murine model enhanced by chronic exposure to nucleosides

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ABSTRACT

Background: Preclinical studies have shown that gene therapy is a feasible approach to treat mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). However, the genetic murine model of the disease (Tymp/Upp1 double knockout, dKO) has a limited functional phenotype beyond the metabolic imbalances, and so the studies showing efficacy of gene therapy have relied almost exclusively on demonstrating correction of the biochemical phenotype. Chronic oral administration of thymidine (dTd) and deoxyuridine (dUrd) to dKO mice deteriorates the phenotype of the animals, providing a better model to test therapy approaches.

Methods: dKO mice were treated with both dTThd and dUrd in drinking water from weaning until the end of the study. At 8 - 11 weeks of age, mice were treated with several doses of adeno-associated virus (AAV) serotype 8 vector carrying the human TYMP coding sequence under the control of different liver-specific promoters (TBG, AAT, or HLP). The biochemical profile and functional phenotype were studied over the life of the animals.

Findings: Nucleoside exposure resulted in 30-fold higher plasma nucleoside levels in dKO mice compared with non-exposed wild type mice. AAV-treatment provided elevated TP activity in liver and lowered systemic nucleoside levels in exposed dKO mice. Exposed dKO mice had enlarged brain ventricles (assessed by magnetic resonance imaging) and motor impairment (rotarod test); both were prevented by AAV treatment. Among all promoters tested, AAT showed the best efficacy.

Interpretation: Our results show that AAV-mediated gene therapy restores the biochemical homeostasis in the murine model of MNGIE and, for the first time, demonstrate that this treatment improves the functional phenotype.

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1. Introduction

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is caused by mutations in the nuclear gene TYMP [1], which lead to a deficiency of thymidine phosphorylase (TP) activity and systemic accumulation of its substrates, the nucleosides thymidine (dTd) and deoxyuridine (dUrd) [2,3]. dTThd and dUrd accumulation causes an imbalance in the mitochondrial deoxyribonucleoside triphosphate (dNTP) pool that interferes with mitochondrial DNA (mtDNA) replication, thereby inducing mtDNA depletion, multiple deletions, and point mutations [4-8].

The main clinical features of MNGIE are progressive gastrointestinal dysmotility, cachexia, ptosis, ophthalmoplegia or ophthalmoparesis, demyelinating peripheral neuropathy, symmetrical distal
Research in context

Evidence before this study

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a fatal metabolic disease caused by mutations in the nuclear gene TYMP that encodes thymidine phosphorylase. In patients, thymidine phosphorylase dysfunction causes accumulation of the nucleosides thymidine and deoxyuridine, which interferes with mitochondrial DNA replication. Hence, all therapy approaches for MNGIE are addressed to reach permanent reduction of these toxic metabolites. Over the last years, several preclinical studies have demonstrated that gene therapy is a feasible strategy for this disorder. Transduction of hematopoietic progenitors with the correct version of the gene using lentiviral vectors, or the liver, using an adeno-associated virus (AAV) vector, resulted in effective and sustained restoration of nucleoside homeostasis in the Thymp/Upp1 double knockout murine model of this disorder. However, this animal model does not recapitulate the clinical manifestations of the disease, and all evidence so far collected supporting gene therapy for MNGIE relies almost exclusively on demonstrating correction of the biochemical phenotype in mice.

Added value of this study

Previous studies demonstrated that challenging the MNGIE mouse model with a chronic oral administration of the toxic nucleosides exacerbated the model leading to the manifestation of neurological phenotype and thus providing a better tool to test therapeutic intervention. In this study, we show that AAV-mediated gene therapy prevents the enlargement of brain ventricles and the neuromotor dysfunction observed in the mice. Hence, we prove that liver-targeted gene therapy using an AAV vector prevents the manifestation of a neurological phenotype beyond correcting the biochemical nucleoside imbalance. This is the first time liver-targeted AAV-mediated gene therapy for MNGIE shows efficacy on clinical parameters in the animal model of the disease, which constitutes an important step towards the translation of this therapy to patients.

Implications of all the available evidence

At present, hematopoietic stem cell transplantation and liver transplantation are the only available therapies for MNGIE patients. However, both treatments are constrained by the availability of a suitable donor and involve complex medical interventions associated to life-threatening risks. Gene therapy may constitute a safer alternative, and this study provides evidence that liver targeted AAV-mediated gene therapy is effective on the neurological phenotype in the enhanced animal model of the disease. This will support conducting clinical studies that will bring this potential treatment closer to patients.
[AUC], time to reach plasma concentration in naïve wild type [WT] animals), a single dose was administered by oral gavage (10 μL/g body weight of 16.6 g/L of dThd and dUrd aqueous solution, ie, 166 mg dThd + 166 mg dUrd/kg body weight). Blood samples were collected immediately before nucleoside administration, and at 30 min, 1 h, 1.5 h, 2 h, 3 h, 5 h, and 8 h after administration. In the PK study, mice were deprived of access to drinking water during the first 3 h after gavage.

To detect the influence of possible metabolic adaptation on PK in chronically dThd/dUrd-treated mice (exposed group), acute dThd/dUrd administration was studied under two different conditions: mice without chronic oral dThd/dUrd treatment (naïve group), and mice already receiving chronic dThd/dUrd treatment (exposed group). In this latter case, dThd/dUrd was withdrawn from drinking water 24 h before acute administration for the PK study.

Therapeutic vectors were administered to 8- to 11-week-old dKO mice in a single intravenous injection (tail vein) of AAV-TBG at doses (in vector genomes per kg, vg/kg) of 5 × 10^11, 10^12, 2 × 10^12 and 10^13, and AAV-AAT or AAV-HLP, both at doses of 2 × 10^12 and 10^13.

Blood samples from live animals were collected from the saphenous vein using EDTA capillaries (Microvette 200K3E, Sarstedt). At the end of the study (age 22 months), mice were euthanized by cervical dislocation. Heart blood was immediately collected in EDTA

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2.7. Mitochondrial isolation and dNTP determination

Liver and brain mitochondria were isolated and dNTPs were extracted as previously described [4,19]. For dNTP quantification, dry extracts were thawed and re-dissolved in 40 μL of 40 mM Tris–HCl, pH 7.4. Mitochondrial dNTP content was determined using the previously described polymerase-based method [31] with some modifications [19].

2.8. Mitochondrial DNA copy number analysis

Total DNA was isolated from approximately 25 mg of pulverized frozen tissue with the QiaAamp DNA Mini Kit (Qiagen) and dissolved in 10 mM Tris–HCl (pH 8). MtDNA copy number was quantified by qPCR on the 7900HT Fast Real Time PCR System (Applied Biosystems) using a probe targeted to the mitochondrial gene mt-Rnr2 (encoding the 16S rRNA) and referred to the nuclear gene Ang1, following the protocol detailed elsewhere [32].

2.9. Histochemical staining of small intestine

Longitudinal sections of jejunum were analysed using the adapted “Swiss roll” technique [33]; jejunum sections were fixed in 4% neutral buffered formalin and embedded in paraffin for histological examination. Three-μm-thick slices were stained with Masson’s trichrome. Images were taken using a FSX100 fluorescence microscope and the software FSX-BSW (Olympus, Tokyo, Japan).

2.10. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6 program (GraphPad Software, Inc.). For multiple comparisons (multiple comparisons with one reference group, and multiple comparisons of all groups compared with each other) the Kruskal-Wallis test was used. When only one interest was to compare two groups defined by only one variable (e.g., exposed group vs. non-exposed group), we used the Mann-Whitney U test. In any case, tests used are always indicated in the figure legends. For statistical purposes, undetectable values were considered as zero. Nucleoside PK parameters, including peak plasma concentration, AUC, and time to reach plasma WT concentration, were calculated from the graphical curves obtained by plotting nucleoside plasma concentrations vs time, defined by connecting adjacent measurements with straight lines, and taking as baseline the mean plasma nucleoside concentration at time 0 h in naïve WT mice.

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The disclosed funders had no role in study design, data collection and analysis, interpretation, decision to publish, or preparation of the manuscript.

3. Results

3.1. Pharmacokinetics of dThd and dUrd in the murine model

In agreement with previous reports [19–24], in the absence of oral nucleoside administration (naïve mice), circulating dThd and dUrd concentrations were around 3-fold higher in dKO mice than in WT mice (Fig. 1a and Supplementary Fig. 1). A marked increase in plasma dThd and dUrd concentrations above normal WT levels was observed in the exposed groups. When the time of blood sample collection was not considered, plasma dThd and dUrd concentrations showed wide dispersion in the exposed groups (data not shown). This variability was greatly reduced after changing the protocol and collecting all blood samples between 8 and 9 am (8- to 30-fold increase in exposed WT mice, and 13- to 26-fold increase in exposed dKO mice) (Fig. 1a and Supplementary Fig. 1). As mice were exposed to dThd and dUrd in drinking water ad libitum, it was not possible to determine the time of the last intake before blood collection. This may account for the wide, randomly distributed variation in plasma concentrations; that is, time differences between the last water intake and sample collection.

Thus, we decided to explore a more controlled set of parameters, by studying nucleoside PK in 35 to 40 week-old mice after a single dThd/dUrd administration by oral gavage in naïve WT and dKO mice. We also studied exposed WT and dKO mice to detect whether a possible metabolic adaptation in response to chronic dThd and dUrd exposure influenced PK. After the single oral dose, plasma concentrations rose above naïve dKO levels, reaching a peak value between 1 and 1.5 h after oral gavage (Fig. 1b and Supplementary Fig. 2a). Thereafter, deoxyribonucleoside (dN) levels rapidly declined, even in dKO mice, which lack the required catalytic enzymes, owing to urinary excretion (data not shown). Eight hours after oral administration, dThd and dUrd plasma levels remained above normal plasma levels in all groups. Peak concentrations (Fig. 1c and Supplementary Fig. 2b) and AUCs (Fig. 1d and Supplementary Fig. 2c) showed a clear tendency to be higher in dKO mice than WT mice, in both the naïve and exposed groups, respectively. We also found that AUC values were higher in exposed dKO than in naïve dKO mice (Fig. 1d and Supplementary Fig. 2c).

3.2. AAV-TYMP treatment limits nucleoside stress in chronically dThd/ dUrd-exposed dKO mice

Exposed dKO mice were treated with three different liver-targeted AAV vectors carrying hTYMP (AAV-TBG, AAV-AAT, or AAV-HLP) at different doses. Plasma dThd and dUrd monitoring over 35 weeks showed the following: although AAV treatment failed to reduce circulating levels of these nucleosides at most time points, the frequency of randomly distributed samples in which nucleosides concentrations decreased to levels within the range of naïve dKO mice (Supplementary Fig. 3) was higher than that of untreated exposed dKO mice (Fig. 1a). As the last nucleoside intake (in drinking water) before blood sample collection might have been interfering with detection of the effect of AAV treatment on dN concentration, we analyzed the effect of AAV treatment on PK parameters in gavage-exposed dKO mice (Fig. 1b-e). Before gavage dosing, plasma dThd and dUrd concentrations in all AAV-treated mice but one were in the WT range or below (dThd, 1.9–3.5 μM; dUrd, 2.5–4.2 μM). It should be noted that the group of exposed mice had been deprived of oral dThd and dUrd in drinking water 24 h before PK assessment. AAV treatment reduced peak dThd concentrations to well below the peak concentrations observed in exposed dKO mice, regardless of the vector construct or dose used, although statistical significance was not reached for the two AAV-TBG lowest doses (Fig. 1c). AAV treatment led to even more pronounced reductions in the AUC (Fig. 1d). In contrast to what occurred in AAV-untreated mice, which did not reach WT nucleoside levels after gavage nucleoside administration over 8 h of monitoring (Fig. 1b), all AAV-treated exposed dKO mice reached WT levels in less than 8 h (Fig. 1e). A dose-dependent effect in the time reduction to reach WT dThd concentration was observed with all vectors; a similar, but less marked dose-dependent effect was observed on peak dThd concentration and dThd AUC. Comparison between the different vectors showed that AAV-AAT had the most pronounced effect on dThd PKs.

The greatest effect was achieved with AAV-AAT at 1013 vg/kg, which reduced plasma dThd concentration to ~3.5 μM in a mean of 2.2 h (range 1.8–2.6 h) after oral gavage. Mice treated with 1014 vg/kg of AAV-AAT also showed the lowest mean dThd peak concentration (13.8 μM, range 5.5–16.9) and the lowest mean
dThd AUC (23.8 μmoles dThd x hour/L, range 15.8–29.3). Similar effects of AAV treatment on PK were observed for dUrd (Supplementary Fig. 2).

Nucleoside-exposed mice were treated with dThd/dUrd in drinking water from weaning until the end of the study (at 22 months old). At this point, mice were killed and dThd and dUrd levels were analyzed in plasma and liver (AAV target organ), as well as brain and small intestine (two affected organs in MNGIE patients). Chronic oral nucleoside exposure resulted in increased dThd median levels in all tissues in WT mice (plasma 5-fold, liver 2-fold, brain 4-fold, and small intestine 90-fold, as compared with median levels in naïve WT group), and showed a higher increase in dKO mice (plasma 20-fold, liver 10-fold, brain 7.5-fold, and small intestine 275-fold, as compared with median levels in naïve WT group) (Fig. 2). In addition, AAV-treatment reduced dThd and dUrd levels in nucleoside-exposed mice at the end of the study in liver (reductions compared to exposed dKO AAV-untreated, with levels comparable to those of WT naïve mice) and in brain (reductions compared to untreated exposed dKO AAV, with levels comparable to those of exposed WT mice in all groups and doses except the lowest AAV-TBG dose). In plasma, reductions were especially prominent in mice treated with the AAV-AAT vector (and reached statistical significance for the dose 2 × 10^{12} vg/kg), whereas there was no effect in small intestine with any treatment. The greatest reductions were obtained for the AAV-AAT vector in plasma, liver, and brain. Similar results were observed for the dUrd concentrations (Supplementary Fig. 4).

3.3. AAV-TYMP treatment induces increased TP activity in liver

Chronic nucleoside exposure induced increased TP activity in liver tissue in WT mice (mean value 39 nmoles Thy/h/mg prot in naïve WT, and 112 in exposed WT), and in dKO mice (mean value 1.2 in naïve dKO, and 9.9 in exposed dKO) (Fig. 3a), but not in small intestine, brain, or skeletal muscle (Supplementary Fig. 5). Treatment with AAV vectors provided TP activity to liver of exposed mice in a dose-dependent manner (Fig. 3b). AAV-AAT was the most effective vector...
in providing TP activity to liver, in agreement with its nucleoside reduction. At the highest dose (10^{13} \, \text{vg/kg}), liver TP activity mean values in exposed mice increased 40- to 75-fold compared to WT values: 2091 \pm 140 \, \text{nmoles Thy/h/mg prot} for AAV-TBG (mean \pm SD); 3031 \pm 509 for AAV-AAT and 1535 \pm 456 for AAV-HLP (Fig. 3b).

Vector copy number (VCN) increased in liver in a dose-dependent manner (Fig. 3c). In an attempt to compare the efficacy of the promoters carried by the three vectors, we normalized TP activity per VCN, and the results revealed that TP/VCN ratios were not constant for each vector, but declined as VCN increased (Fig. 3d). This apparent “saturation of efficacy” makes it difficult to compare the efficacies of different promoters, but the fact that the highest TP/VCN ratios were always observed with the AAV-AAT vector for all different VCN values (Fig. 3d), suggests that AAT was the most effective promoter among those tested. The TP vs VCN distribution was adjusted to the nonlinear function: $Y = \text{intercept} + \text{slope} \times \log(X)$, where $Y$ is TP activity and $X$ is VCN. Semilogarithmic transformation of the function showed that the highest slope among the three vectors corresponded to AAV-AAT ($p < 0.05$, extra sum of squares F-test).

Although all the promoters used here are liver-specific, AAV treatment provided TP activity to small intestine in exposed dKO mice, as well as skeletal muscle treated with the highest vector doses, and brain in a few individual mice. AAV-induced TP activities did not reach levels above WT values in non-hepatic tissues except in a single skeletal muscle sample (Supplementary Fig. 5).

### 3.4. Histological analysis of small intestine and mtDNA copy number

Histological analysis of the small intestine (Masson’s trichrome staining) did not reveal any detectable abnormalities induced by nucleoside exposure in this tissue. Also, we could not find that nucleoside exposure induced mtDNA depletion in brain and small intestine. (Supplementary figure 6).

### 3.5. Effect of the AAV-TYMP treatment on neurological phenotype

Motor coordination, balance and exercise tolerance were determined using the rotarod performance test in 25 week-old mice (Fig. 4). The performance in this test can be influenced by impaired function of the skeletal muscles and/or the central nervous system. Multiple comparison analysis of all groups represented in Fig. 4 (Kruskal-Wallis test, followed by the Dunn’s multiple comparisons test) did not detect statistical differences for any group, probably due to the high number of groups. However, when we only analysed the effect of nucleoside exposition, we observed that exposed dKO mice had a reduced time to fall, as compared with WT naïve mice ($p = 0.006$, Mann-Whitney U test) (Fig. 4). In AAV-treated mice, rotarod performance varied widely, but in most AAV-treated mice (69%; 27 out of 39) was within or above the naive WT normal range (19–44 s), whereas only 33% (3 out of 9) of exposed dKO mice showed a lengthier time to fall.
All MNGIE patients have diffuse leukoencephalopathy on brain MRI [9], and chronic oral exposure to dThd and dUrd enhances leukoencephalopathy in the dKO murine model [25]. We performed brain MRI studies in 84-week-old naïve and exposed WT and dKO mice. Larger T2-weighted hyperintense areas (indicative of cerebrospinal fluid) were found in exposed dKO mice than in naïve dKO, naïve WT, or exposed WT mice (Fig. 5a and Supplementary Fig. 7). In contrast to what was reported in the former study of nucleoside protocols [25], Hyperintensity was mainly located in the third and lateral ventricles, suggesting ventricular enlargement. The volume of the third and lateral ventricles was quantified using T2 maps, and it was significantly greater in exposed dKO mice than in naïve WT mice whereas it was unchanged in both naïve dKO and exposed WT mice (Fig. 5b). AAV-AAT and AAV-HLP treatment prevented ventricular enlargement in exposed dKO mice: most animals treated with these vectors had normal ventricular volumes, with the exception of one mouse treated with 10^{11} vg/kg AAV-AAT that developed hydrocephalus. In contrast, AAV-TBG failed to prevent ventricular enlargement in most cases (Fig. 5b).

Ventricular volumes at 84 weeks of age significantly correlated with brain dThd levels at the end of the study (94 weeks) in all groups (Spearman r = 0.324, p = 0.014, n = 57, excluding 2 ventricular volume extreme values; r = 0.358, p = 0.005, n = 59, including all values) and with rotarod test performance (Spearman r = −0.348, p = 0.0068, n = 59, excluding two ventricular volume extreme values). Ventricular volumes did not significantly correlate with plasma or liver dThd levels.

3.6. AAV-TYMP treatment normalized the mitochondrial dNTP balance

In MNGIE, nucleoside overload leads to an unbalanced dNTP mitochondrial pool, resulting in mtDNA instability [4]. We quantified dNTP levels in mitochondrial extracts from liver and brain in 22-month-old mice (Fig 6). dTTP content was significantly increased in brain mitochondria of exposed dKO mice. The same trend was observed in liver mitochondria, but statistical significance was not reached, probably owing to the wide dispersion of the values (liver dTTP range: 5.3–54.7 pmoles/mg protein). The dTTP expansion in liver was largely prevented in exposed dKO mice treated with AAVs, as most values of AAV treated mice returned to dTTP values with the naïve WT range, although only the group treated with AAV-TB at 10^{13} vg/kg reached statistical significance (Fig 6a). AAV treatment also limited dTTP expansion in brain, but the effect was only partial as dNTP in AAV-treated animals did not reach WT levels (and reached statistical significance only in the group treated with AAV-AAT at 2 × 10^{11} vg/kg) (Fig. 6b). The observations regarding mitochondrial...
dCTP levels in liver conversely mirrored those of dTTP; that is, exposed dKO showed dCTP depletion as compared with WT levels, which was largely prevented by AAV treatment, reaching statistical significance for all AAV-AAT treated groups, and for the group treated with AAV-HLP at 2 × 10^12 vg/kg (Fig. 6c). No statistical differences were detected in mitochondrial dCTP levels in brain (Fig. 6d). As to the purine deoxynucleotides, mitochondrial dCTP levels were expanded in liver (an increase that was not prevented by AAV treatment), while dATP showed a tendency to be depleted in the same organ (statistically non-significant), but the effect was prevented in most AAV treated groups. In contrast, mitochondrial dATP and dCTP levels were barely affected or not affected at all in brain (Supplementary Fig. 8).

4. Discussion

Over the last few years, gene therapy has been proposed to reduce the relevant risks associated with the currently available options to treat MNGIE (allogeneic stem cell transplantation and orthotopic liver transplantation) [11-13,15-18,34]. Since the first proof-of-concept preclinical studies demonstrating the feasibility of using lentiviral and AAV vectors [23], several studies have shown that these strategies provide maintained effectiveness at long term in the preclinical model [19-21,24]. The main endpoint used to draw conclusions was the ability of the vectors to achieve genetic correction in target cells of the murine dKO model, thereby providing TP activity and normalization of systemic dThd and dUrd levels.

However, the dKO mouse is a limited model due to the absence of a clinically relevant phenotype, which consists of a lack of TP activity (except for some residual activity in tissues expressing Upp2), moderate dThd and dUrd systemic accumulation, and a modest mitochondrial dNTP imbalance. Any additional traits beyond the biochemical consequences of the absence of TP have only been glimpsed in very old animals and, even so, as elusive and barely detectable signs [5].

The model can be enhanced by providing the animals with dThd and dUrd in drinking water, which exacerbates the phenotype and highlights the importance of dThd and dUrd overload in the pathophysiology of MNGIE [25]. This enhanced model does not recapitulate the main clinical features of the disease (which manifest mainly as severe gastrointestinal dysmotility, myopathy, peripheral neuropathy and brain diffuse leukoencephalopathy). Specifically, the lack of gastrointestinal phenotype is an important limitation of this animal model, because gastrointestinal dysmotility is the most debilitating symptom in MNGIE patients [9]. However, it does show some new functional and radiological alterations (neuromotor dysfunction and ventricular alterations in brain) that are absent or much less prominent in the non-nucleoside exposed murine model. We used this enhanced preclinical model to expand on our previous studies demonstrating the feasibility and efficacy of AAV-based liver-targeted gene therapy for MNGIE [19,21,23].

Systemic dThd and dUrd levels in naïve dKO mice were 2- to 4-fold higher than levels in naïve WT mice, as was originally reported [5] and consistently observed here, which contrasts with the much more pronounced accumulations (more than 50-fold) observed in MNGIE patients [35,36]. In agreement with the study where the stressed model was established [25], we found that oral administration of dThd and dUrd to dKO mice exacerbates systemic accumulation of both dNs. Furthermore, the highest dThd and dUrd accumulations were seen in small intestine, likely because of the presence of ingested dNs, before or during intestinal absorption. In contrast, we found detectable dThd and dUrd levels in all tissues analyzed from naïve WT mice, and exposed dKO mice had dN levels only 20-fold above WT plasma values, and 10-fold above liver and brain WT values. These discrepancies likely result from the use of TP activity inhibitors during sample processing for dN determination, which prevented in vitro dN degradation in WT samples and reduced the differences between naïve WT and exposed dKO concentrations. As was expected, the excess of dThd and dUrd resulted in enhanced mitochondrial dNTP imbalances (dTTP increase and dCTP depletion) in the two tissues analyzed (liver and brain).

We found an unreported effect of dN exposure on mice: an increase in TP activity in liver, but not in skeletal muscle, small intestine, or brain. A similar effect (TYMP upregulation) has been reported in cultured human cells in response to dThd and nucleoside analogues [37]. Exposure to dN doubled liver TP activity in WT mice and, interestingly, also increased activity in dKO animals. Upregulation of Tymp or other genes encoding enzymes with TP activity (such as Upp1 and Upp2) may account for this increase in WT mice. Additionally, dThd excess could be inhibiting the ubiquitin-mediated TP degradation as it has been recently reported in human differentiating reticulocyte cell cultures [38]. In contrast, Tymp and Upp1 genes are molecularly inactivated in the dKO model [5,39]; therefore, dN-induced TP activity in these animals should be mediated by upregulation of another enzyme, likely UP2 (encoded by Upp2). In fact, naïve dKO mice have some detectable TP activity in liver that must be due to UP2.

All MNGIE patients present diffuse leukoencephalopathy on brain MRI, but this abnormality is barely detectable in naïve dKO mice, and is only seen when they are very old [5]. The first study of the nucleoside-exposed dKO model demonstrated that chronic exposure to dThd and dUrd led to increased brain MRI FLAIR signal intensity around the third and lateral ventricles [25]. In our study, we failed to detect MRI differences between naïve dKO and naïve WT mice, showing how elusive this feature is in the unexposed mouse model. Nonetheless, and in agreement with the previous report [25], dKO mice (but not WT mice) did develop the MRI phenotype under dN exposure, although we found enlarged ventricle volumes rather than the diffuse parenchymal leukoencephalopathy that is typically observed in MNGIE patients. Combined analysis of T2 maps and high-resolution T2-weighted images revealed that the periventricular signal hypointensity corresponds to enlarged ventricular volume, with no fluid transfer to the surrounding white matter, in contrast to what is observed in other hydrocephalic models with severe ventricular enlargement [40]. Exposure to dNs also succeeded in the development of a motor function phenotype. At 25 weeks of age, exposed dKO mice showed a shorter time to fall in the rotarod test, which is indicative of motor coordination impairment in this model, in agreement with previous findings [25]. Furthermore, rotarod time to fall at 25 weeks of age inversely correlated with MRI ventricular volume.

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**Fig. 4.** Motor function. Motor function assessed by rotarod test in 25-week-old WT and dKO naïve and exposed mice, and dKO exposed mice treated with different AAVs doses. Horizontal lines show median time to fall in each group. Gray areas indicate the range of values in the naïve WT group. No statistical differences found, Kruskal-Wallis test followed by the Dunn’s multiple comparisons test, all groups compared with dKO exposed).
assessed at 84 weeks of age, which concurs with previous studies associating hydrocephalus with motor function defects [41].

As was discussed above, most effects of dN exposure seen in our study are consistent with previously reported findings [25], but we failed to replicate some other important features. After careful histological analysis (Masson’s trichrome staining), we found no abnormalities in small intestine of exposed mice, and no mtDNA depletion was detected in brain or in small intestine.

In previous preclinical studies, we used the TBG promoter in AAV gene therapy for MNGIE and proved that it is effective for driving hCTMP expression [21,23]. The need to optimize therapeutic gene expression and minimize the vector dose to transfer therapy to MNGIE patients prompted us to explore new vector constructs [19]. The ApoE-AAT enhancer-promoter cassette has been used in several AAV-based preclinical and clinical studies [42-45]. The self-complementary genome configuration resulted in faster and stronger therapeutic gene expression in these studies, and it has also been successfully used in patients [46-48]. We found that use of the AAV-AAT vector to carry hCTMP in the dKO model resulted in greater efficacy as compared to other hepatic promoters or a self-

Fig. 5. Brain MRI analysis. (a) Coronal T2-weighted and T2 map images of 1-mm sections (region corresponding to slice 6 in Supp Figure S7) showing ventricular enlargement in dKO exposed mice (red arrows). (b) Ventricular volume in 80 to 84-weeks old WT (n = 9) and dKO (n = 5) naïve mice, non-treated WT (n = 5) and dKO (n = 12) exposed mice, and dKO exposed mice treated with different AAVs doses (TBG-treated mice with: 5 × 10¹¹ vg/kg n = 2, 10¹² vg/kg n = 3, 2 × 10¹² vg/kg n = 5 and 10¹³ vg/kg n = 5; AAT-treated mice with: 2 × 10¹² vg/kg n = 6 and 10¹³ vg/kg n = 4; HLP-treated mice with: 2 × 10¹² vg/kg n = 6 and 10¹³ vg/kg n = 3). Ventricular volume was quantified in T2 maps as the sum of the ventricular volumes calculated in all sections analyzed in Supp Figure S6 (see materials and methods for calculations). Gray areas indicate the range of values in the naïve WT group. Asterisks indicate statistically significant differences (*p < 0.05; Kruskal-Wallis test followed by the Dunn’s multiple comparisons test, all groups compared with dKO exposed). Ventricular volume values above the mean plus 3 SD of all data were considered outliers and excluded from the analysis. Two outliers were identified in dKO exposed AAV-treated with TBG 10¹² vg/kg, and TBG 2 × 10¹² vg/kg, one in each group.
Now, taking advantage of the enhanced nucleoside-exposed animal model to expand the study, we found that AAV-AAT provides an effect beyond the previously reported biochemical normalization. AAV-AAT performed better than other liver-targeted vectors in restoring biochemical homeostasis (nucleoside reduction, liver TP activity, dNTP normalization), and it also improved the brain MRI abnormalities manifested in the exposed mouse model.

Our attempts to determine the effect of different vectors on dN homeostasis revealed an unexpected limitation of the model. As mice received dNs in their drinking water ad libitum, the variability in dN levels seen over the day was likely determined by the last time the mouse drank water. To circumvent this confounding factor, we decided to determine PK parameters (peak concentration, AUC, and time to reach WT values) after a single gavage administration, instead of single dN values in one blood sample, as more reliable biochemical endpoints to study the effect of the vectors on dN homeostasis in the model. A vector dose-response was observed in most cases, and comparison between the three vectors showed that AAV-AAT was the most efficient to reduce plasma dN levels, which is consistent with their better comparative ability to provide TP activity to liver. As was expected, all vectors led to substantial dose-dependent TP activity in liver, and the analysis of TP activity/vector copy number ratios indicated that the AAT promoter was the most efficient. All these observations confirm and reinforce our previous results on the efficacy of different vectors [19].

Previously, we observed that treatment with AAV-TBG, AAV-HLP and AAV-AAT results in sporadic cases of increased TP activity in non-hepatic tissues [19], and this was confirmed here in an independent set of experiments. In our previous report, we found that this “illegitimate activity” seems not to be caused by ectopic expression, as transgene DNA or mRNA could not be consistently found in samples with TP activity. Hence, this activity might respond to exosomal export from liver [49]. In any case, this extra-hepatic effect was irrelevant in quantitative terms in both the previous and the current study.

dNTP imbalances were ameliorated in liver as a consequence of the systemic dN reduction. The effect in brain, however, was only partial. The fact that liver, but not brain, is the target of the vectors tested here may account for the modest effect in brain. In addition, the anatomic complexity of the brain, constituted by many cell types distributed among different functional areas, may mask local effects, as our method for mitochondrial dNTP determination is performed on total brain mitochondria. Interestingly, dCTP depletion could not be detected in brain mitochondria, which correlates with the absence of mtDNA depletion in this organ, in agreement with previous experimental data showing that the cause of mtDNA depletion in MNGIE is limited availability of dCTP [4].

Of note, this is the first time that AAV-mediated gene therapy has shown efficacy on disease-related features in MNGIE beyond normalization of the biochemical imbalances. Nucleoside-exposed mice treated with AAV-HLP and AAV-AAT vectors recovered normal...
ventricular volumes on MRI, while this effect was only partial (in some mice) and not statistically significant with the AAV-TBG vector. When all mice were taken as a whole, ventricular volumes significantly correlated with dThd levels in brain, reinforcing the notion that normalization of the biochemical imbalances is directly related with improvements in this neurological trait. However, the results did not allow us to draw clear conclusions on the effect of therapy on motor function, as assessed with the rotarod test. Time to fall in the rotarod test was shorter in nucleoside-exposed dKO mice, which can be due to alterations in the central nervous system and/or the skeletal muscle. This reduction seemed to be prevented in most groups of AAV-treated mice. However, if this observation suggests a positive effect of the treatment, it is uncertain why was it not observed at the highest vector doses. The reasons for this apparent paradox remain to be explored.

The results of this study show that the AAV-AAT vector performed the best among those tested, efficiently providing TP activity to the liver and restoring dN and dNTP homeostasis. In addition, functional improvements on the phenotype beyond normalization of biochemical markers were proven for AAV-mediated MNGIE gene therapy in the nucleoside-exposed dKO mouse model. AAV transduction rates are lower in humans than in mice [50;51]; therefore, using more potent promoters, within a reasonable range of potency, will be necessary to make AAV treatment possible in humans at feasible vector doses. This will have an impact on both biosafety and the cost of treatment, and contribute to facilitating implementation of this therapy in patients, which constitutes a significant advance towards the treatment of this devastating disorder with a much less invasive therapy than those currently available.

Contributors

F. Vila-Julià, J. Torres-Torronteras and R. Martí designed the study and wrote the manuscript; F. Vila-Julià, R. Cabrera-Pérez, Y. Cámara, M. Molina-Berenguer, S. Lope-Piedra, and J. Torres-Torronteras performed the experiments and collected and analysed the data; F. Vila-Julià, S. Lope-Piedra, J. Torres-Torronteras and R. Martí participated in data interpretation; M. Hirano generated and provided the mouse model; F. Mingozzi produced and provided the AAV-AAT vector; all the participating authors contributed to the critical revision of the manuscript. All the authors read and approved the final version of the manuscript.

Declaration of Competing Interests

RM, FV and MM report grants from the Instituto de Salud Carlos III during the conduct of the study; JT reports grants from Departament de Salut, Generalitat de Catalunya (PERIS program), during the conduct of the study; RM, MH, JT and YC report grants and non-financial support from Modis Therapeutics, personal fees and other from Modis Therapeutics, outside the submitted work; in addition, RM and MH have a patent “Deoxynucleoside therapy for diseases caused by unbalanced nucleotide pools including mitochondrial DNA depletion syndromes” (PCT/US16/038110) with royalties paid to Modis Therapeutics; MM reports grants and other support from Entrada Therapeutics, grants from Muscular Dystrophy Association and grants from NIH, outside the submitted work; FM is an employee of Spark Therapeutics; SL reports that the Nuclear Magnetic Resonance facility where she works charged the VHIR a fee for the MRI services; and RM, YC, JT and RC have a patent “Treatment of mitochondrial diseases” (PCT/EP2016/062836) with royalties paid to Modis Therapeutics.

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Data sharing statement

The data that support the findings of this study are available from the corresponding authors, RM and JT, upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103133.

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