

mRNA as a Novel Treatment Strategy for Hereditary Spastic Paraplegia Type 5

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Hereditary spastic paraplegia type 5 is a neurodegenerative disease caused by loss-of-function mutations in the *CYP7B1* gene encoding the oxysterol 7- α -hydroxylase involved in bile acid synthesis in the liver. Lack of *CYP7B1* leads to an accumulation of its oxysterol substrates, in particular 25-hydroxycholesterol and 27-hydroxycholesterol that are able to cross the blood-brain barrier and have neurotoxic properties. A potential therapeutic strategy for SPG5 is the replacement of *CYP7B1* by administration of mRNA. Here, we studied the intravenous application of formulated mouse and human *CYP7B1* mRNA in mice lacking the endogenous *Cyp7b1* gene. A single-dose injection of either mouse or human *CYP7B1* mRNA led to a pronounced degradation of oxysterols in liver and serum within 2 days of treatment. Pharmacokinetics indicate a single injection of human *CYP7B1* mRNA to be effective in reducing oxysterols for at least 5 days. Repetitive applications of mRNA were safe for at least 17 days and resulted in a significant reduction of neurotoxic oxysterols not only in liver and serum but also to some extent in the brain. Our study highlights the potential to use mRNA as a novel therapy to treat patients with SPG5 disease.

INTRODUCTION

mRNA-based protein supplementation is a powerful tool to compensate for lack of proteins in monogenetic diseases caused by loss-of-function mutations. It offers a potentially curative treatment option, especially in diseases in which the protein is expressed predominantly in organs that can be reached by intravenous (i.v.) delivery. Here, supplementation of mRNA can restore physiological conditions and may provide a potential novel therapeutic approach.

Hereditary spastic paraplegia (HSP) is a rare monogenetic neurodegenerative disorder characterized by progressive lower-limb spasticity and weakness due to axonal degeneration of the corticospinal tract.¹ Hereditary spastic paraplegia type 5 (SPG5) is caused by autosomal recessive loss-of-function mutations in *CYP7B1*, a gene encoding

for the cytochrome P-450 oxysterol 7- α -hydroxylase, essential for the alternative pathway of bile acid synthesis in liver.^{2,3} Mutations causing SPG5 lead to decreased enzyme activity of *CYP7B1* and consecutively to accumulation of its substrates like 25-hydroxycholesterol (25-HC), 27-hydroxycholesterol (27-HC), and 3 β -hydroxy-5-cholestenoic acid (3 β -HCA) in plasma and cerebrospinal fluid (CSF) of SPG5 patients.^{4,5} The classical as well as the neural pathways of cholesterol metabolism are unaffected by this mutation demonstrated by unaltered levels of total cholesterol as well as 24-hydroxycholesterol (24-HC) in patient's serum and CSF⁶ (Figure 1).

In SPG5 patients, oxysterols accumulate with levels of 25-HC found to be 90-fold increased in serum (178 ng/mL [range, 91–318]; controls, 2 ng/mL [range, 0–11]). The absolute levels of 27-HC in serum are even more elevated, with 878 ng/mL (range, 600–1,270) compared to 154 ng/mL (range, 89–243) in controls (6-fold), and more than 25-fold increased in CSF (12.9 ng/mL [range, 6.5 – 25.2]; controls, 0.5 ng/mL [range, 0.5–0.8]).⁶ An implication of increased oxysterol concentration in disease pathology is demonstrated by impaired metabolic activity and viability of induced pluripotent stem cell (iPSC)-derived cortical neurons treated with various oxysterols and motor neuron cell loss in mice receiving 3 β -HCA intraventricular injections.^{6,7}

To date, no curative treatment for SPG5 is available. Current clinical treatment strategies for SPG5 are based on the reduction of

Received 2 September 2019; accepted 23 October 2019;
<https://doi.org/10.1016/j.omtm.2019.10.011>.

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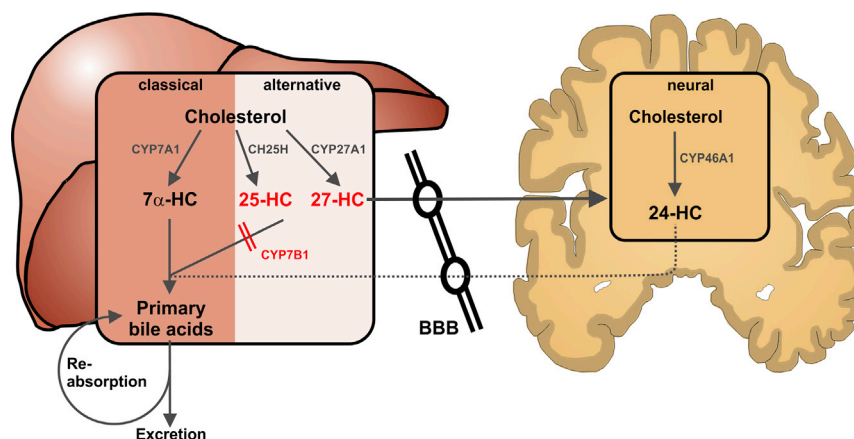


Figure 1. Schematic Overview of Liver (Classical and Alternative Pathway) and Brain (Neural Pathway) Cholesterol Metabolism

In SPG5, mutations in CYP7B1 (red) lead to an accumulation of 25- and 27-hydroxycholesterol (25-HC, 27-HC), which are able to pass the blood-brain barrier (BBB) and have neurotoxic properties. The neural (CYP46A1, 24-HC) as well as the classical cholesterol metabolism (CYP7A1, 7α-HC) are unaffected from this mutation.

cholesterol by applying cholesterol-lowering drugs (statins), which consequently lead to a reduction of oxysterols. A first randomized placebo-controlled double-blind interventional trial shows promising results regarding the reduction of 25-HC (~23%) as well as 27-HC (~31%) in serum of SPG5 patients upon treatment with atorvastatin for 9 weeks.⁶ Although restricted to assessment in serum, the atorvastatin effect has been reproduced with comparable reduction of 27-HC after 2-month treatment.⁸ However, this effect only partly translates into CSF, with a reduction of 27-HC by 8% with no significant difference from the placebo group.⁶ This clearly indicates that a significant lowering of oxysterol levels in the brain by this strategy might be difficult to achieve.

As oxysterols reach the brain most likely by a diffusion gradient across the blood-brain barrier,^{6,9} it is likely that oxysterol levels need to be reduced to almost normal levels as they are found in healthy heterozygous mutation carriers to circumvent the neurotoxic effects driving SPG5. A potential strategy to achieve this goal might be a gene-therapeutic approach using CYP7B1 mRNA. The use of mRNA has emerged as a promising drug platform technology in recent years following initial studies in the early 1990s demonstrating that exogenous mRNA can translate into functional protein expression *in vivo*.^{10,11} Using mRNA as a therapeutic compound has several safety advantages compared to DNA approaches.^{12,13} mRNA consists only of the elements directly required for the expression of the encoded protein. Additionally, mRNA does not localize to the cell nucleus; this helps to exclude potential harmful genomic integration. A third safety advantage is due to the non-replicative nature of mRNA, which serves as a transient carrier of information.

These characteristics make mRNA a promising technological basis to trigger the production of desired proteins for therapeutic purposes. For vaccination against infectious diseases as well as cancer immunotherapies, direct applications of *in vitro* transcribed mRNA are already in clinical trials. Similar approaches for protein replacement are in the preclinical stage.¹⁴ As an example, the functionality and safety of this treatment strategy has been shown with chemically unmodified mRNA coding for erythropoietin; this approach leads to

high systemic protein levels and strong physiological responses *in vivo*.¹⁵ The therapeutic use of mRNA takes advantage of the endogenous cellular translational as well as post-translational machinery to produce therapeutic proteins directly in cells. This may be an advantage over the delivery of therapeutic proteins via conventional protein-based enzyme-replacement therapies.¹⁶ Additionally, the use of lipid nanoparticles (LNPs) to administer mRNA *in vivo* protects the mRNA from degeneration by nucleases during delivery, allows for a safe passage through the body, and directs its efficient entry into the liver.^{15,17–19} The potential of mRNA-based therapies was recently demonstrated in various therapeutic areas, including methylmalonic acidemia/aciduria,²⁰ hemophilia B,^{21,22} alpha 1-antitrypsin deficiency,²³ and glycogen storage disease type Ia.²⁴

In this study, we report the safe and efficient delivery of formulated human CYP7B1 mRNA to mice lacking the endogenous *Cyp7b1* gene (*Cyp7b1*^{−/−} mice). This approach resulted in a significant reduction of neurotoxic oxysterols not only in liver and serum but also in the brain. Our study highlights the use of mRNA as a potential treatment for SPG5, which cannot be medicated via a conventional enzyme-replacement therapy due to the intracellular localization of the affected protein.

RESULTS

***In Vitro* Transfection of CYP7B1 mRNA Led to a Pronounced Protein Expression**

To determine the expression kinetics and localization of the protein *in vitro*, we generated mRNA constructs encoding for a C-terminal HA-tagged murine (MmCyp7b1-HA) or human CYP7B1 (HsCYP7B1-HA). L929 cells were transfected separately with both constructs and harvested at the indicated time points (Figure 2A). Western blot analysis showed a peak expression of the protein between 12 and 24 h post-transfection and a residual expression 72 h post-transfection (Figure 2A). This data indicated a half-life of CYP7B1 protein expression of approximately 10 h *in vitro*. As CYP7B1 is localized in the membrane of the endoplasmic reticulum (ER), we co-stained the transfected L929 cells with the ER-specific marker calreticulin 24 h post-transfection. Immunocytochemical analysis revealed a clear colocalization of the ER-specific marker with CYP7B1 (Figure 2B; Figure S1), indicating that both the mouse and human mRNAs were properly translated, processed, and localized inside the cells.

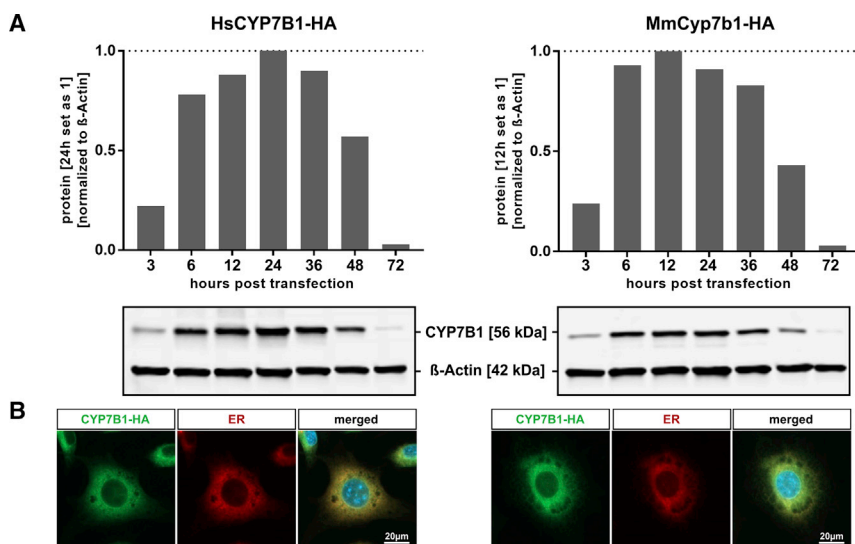


Figure 2. In Vitro Validation of Designed HsCYP7B1-HA and MmCyp7b1-HA mRNAs

(A) Western blot analysis and quantification of CYP7B1 protein expression at various time points (3, 6, 12, 24, 36, 48, 72 h) post-transfection in L929 cells transfected with either HsCYP7B1-HA or MmCyp7b1-HA mRNA. (B) Immunocytochemical co-staining of mRNA-transfected L929 cells with an HA-specific antibody (green) and calreticulin-specific antibody (ER marker; red) 24 h post-transfection (scale bar, 20 μ m).

Establishing an *In Vivo* System for the Administration of CYP7B1 mRNA

To determine the biodistribution of the LNP used in this study and to verify its ability to specifically target the liver, wild-type animals received an i.v. injection of either LNP with mRNA encoding the reporter *Photinus pyralis* luciferase (PpLuc) or PBS. 6 h after injection, animals were sacrificed, and the luciferase expression in different organs was quantified via bioluminescence (Figure 3A). The reporter protein was mainly detected in liver (19.7 ± 4.5 μ g/g) with a 10-fold lower expression in spleen (1.9 ± 0.3 μ g/g). In all other organs, no signal above background (PBS-treated animals) was detected, indicating the high liver specificity of the used LNPs (Figure 3A).

A Cyp7b1 knockout mouse model (*Cyp7b1*^{-/-}) was established in the lab of J.-Å.G. without presenting any obvious phenotypic characteristics compared to wild-type mice.^{25,26} We determined the baseline oxysterol profile by quantification of 25-HC, 27-HC, and 3 β -HCA in the serum, liver, and brain of wild-type (*Cyp7b1*^{+/+}) versus knockout (*Cyp7b1*^{-/-}) mice via mass spectrometry. Comparable to the human situation in SPG5 patients, a drastic increase of these oxysterols has been detected in all three compartments of *Cyp7b1*^{-/-} mice (Figures 3B–3D). In particular, levels of 25-HC were highly elevated with an ~270-fold increase in serum, ~27-fold increase in liver, and ~51-fold increase in brain compared to wild-type animals. However, in contrast to the situation in SPG5 patients, this massive accumulation of oxysterols did not lead to any obvious neurological or general phenotype in the knockout mice. Rotarod motor performance as well as Lab Master analysis did not show any significant differences in moving activity, food and water intake, or coordination between *Cyp7b1*^{-/-} and *Cyp7b1*^{+/+} animals at ~18 months of age (Figure S2).

Single Administration of CYP7B1 mRNA Significantly Reduced 25-HC Levels in Serum and Liver of *Cyp7b1*^{-/-} Mice

To prove the functionality of the CYP7B1 mRNA, LNP carrying either MmCyp7b1 or HsCYP7B1 mRNA were injected i.v. into

Cyp7b1^{-/-} mice (n = 4 per group) with a dosage of 40 μ g of mRNA per injection. LNP loaded with a non-translating mRNA were applied as control (vehicle). Prior to the administration, serum samples were taken to determine basal oxysterol levels. 48 h post-injection, animals were sacrificed, and liver, serum, and brain samples were prepared for oxysterol analysis (schematic illustration, Figure 4A).

Animals treated with mouse or human CYP7B1 mRNA had significantly less oxysterols than mice in the vehicle control group. In liver, 25-HC levels decreased to ~20 ng/mg CHOL (MmCyp7b1, 19.9 ± 4.9 ng/mg CHOL/HsCYP7B1, 20.9 ± 5.1 ng/mg CHOL), which results in a more than 8-fold reduction compared to the vehicle group (174.9 ± 21.4 ng/mg CHOL) (Figure 4B). Changes in levels of 27-HC and 3 β -HCA in liver were insignificant.

In serum, treatment effects were even more impressive, with a > 90% reduction of 25-HC, > 40% reduction of 27-HC, and > 20% reduction in 3 β -HCA following a single CYP7B1 mRNA injection (Figure 4C). The effect of the massive reduction of serum 25-HC levels even translated to the brain, decreasing 25-HC to either 152.2 ± 16.7 ng/mg CHOL (MmCyp7b1) or 158.0 ± 18.5 (HsCYP7B1) compared to 223.4 ± 35.4 ng/mg CHOL in the vehicle group (Figure 4D). Brain values of 27-HC and 3 β -HCA were only slightly reduced by 10%–29% (not significant) upon a single injection.

Measurements of the unrelated neural-specific oxysterol 24-HC as well as total cholesterol in all three compartments did not show any significant differences between the three groups (vehicle, MmCyp7b1, HsCYP7B1), demonstrating the specific targeting of the alternative cholesterol pathway by the mRNA treatment (Figure S3).

Comparing the values of mice receiving MmCyp7b1 mRNA to those receiving HsCYP7B1 mRNA, no significant difference was observed. Thus, both mRNAs and the resulting mouse Cyp7b1 and human CYP7B1 protein were functional in *Cyp7b1*^{-/-} mice. Since human CYP7B1 mRNA was of primary interest as a potential therapeutic tool, further experiments focused on delivery of HsCYP7B1 mRNA.

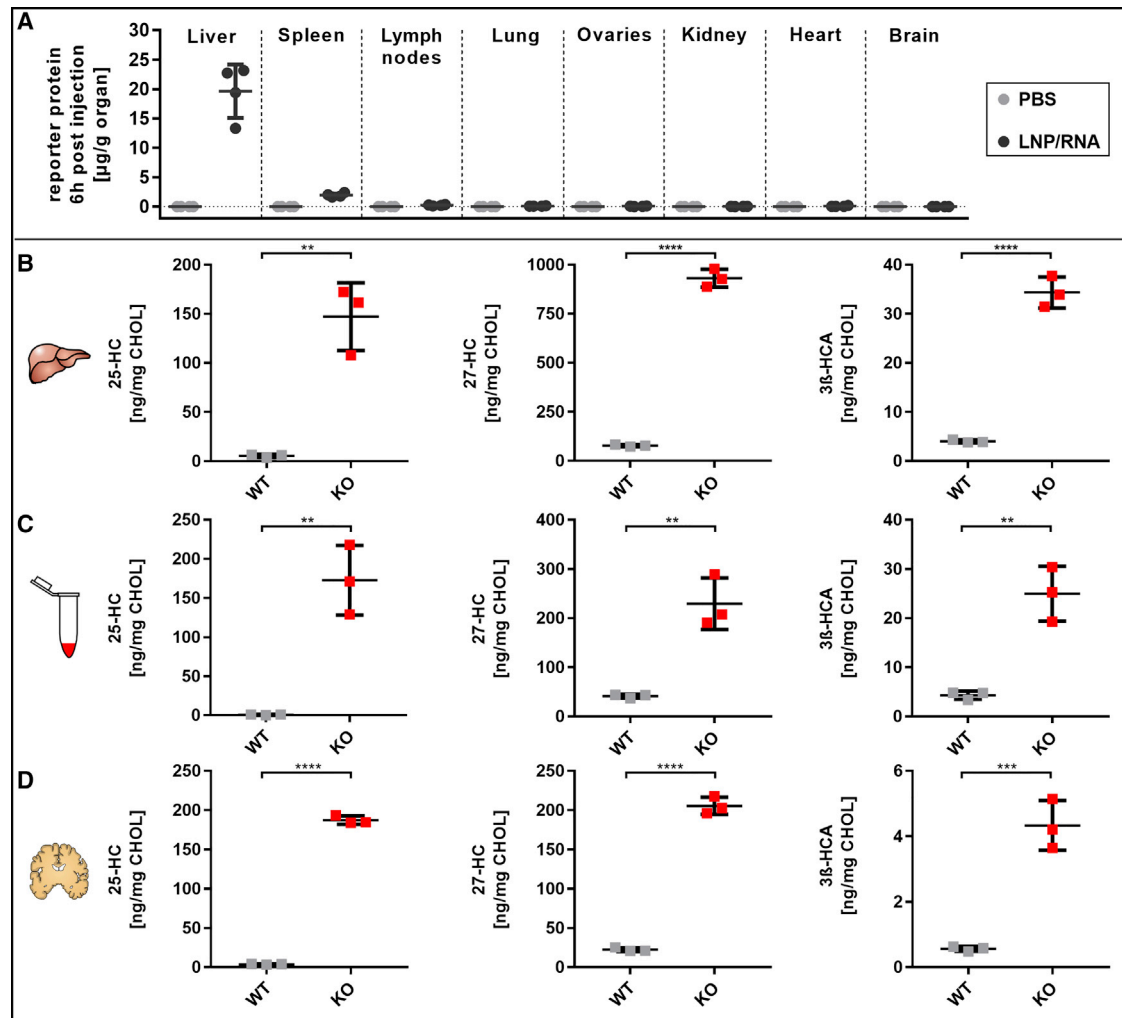


Figure 3. Establishing an *In Vivo* System for mRNA Administration

(A) Wild-type BALB/c mice were dosed with a single i.v. injection of 20 μg of LNP encapsulated with mRNA encoding the reporter PpLuc or PBS as a control. Luciferase expression in various tissues was quantified via bioluminescence 6 h post-injection ($n = 4$ mice per group). (B–D) Mass spectrometric analysis of oxysterols (25-HC, 27-HC, 3β-HCA) in (B) liver, (C) serum, and (D) brain of *Cyp7b1*^{−/−} mice (KO, red) and *Cyp7b1*^{+/+} mice (WT, gray) ($n = 3$ mice per group; unpaired one-tailed t test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, error bars show SD).

Kinetics of Single Administration of CYP7B1 mRNA Revealed a Therapeutic Effect for at Least 5 Days

We speculated that transducing the effect of HsCYP7B1 mRNA treatment to the brain of *Cyp7b1*^{−/−} mice may require repeated i.v. injections for a sustained reduction of serum oxysterols. To determine the optimal administration interval, we studied pharmacokinetics of HsCYP7B1 mRNA *in vivo*. After a single i.v. application of HsCYP7B1 mRNA, animals were sacrificed at days 1, 3, 5, and 7 post-injection ($n = 5$ per group). For baseline assessment of oxysterol levels, blood samples were drawn 3 days prior to mRNA injection administration. LNPs with a non-translating mRNA were used as vehicle control (schematic illustration, Figure 5A).

Again, we observed strong effects of HsCYP7B1 mRNA on 25-HC levels in the liver 24 h post-injection (14.4 ± 6.4 ng/mg CHOL), while

the vehicle group showed a substantial increase of 25-HC (339.0 ± 87.8 ng/mg CHOL) compared to untreated *Cyp7b1*^{−/−} mice (147.3 ± 34.5 ng/mg CHOL) (Figure 5B). Concentrations of 25-HC in the treated group slightly increased over time (72 h, 25.0 ± 8.2 ng/mg CHOL; 120 h, 57.4 ± 13.8 ng/mg CHOL) and reached almost baseline levels 168 h after the single administration (117.5 ± 23.1 ng/mg CHOL).

Once more, only a slight and not significant reduction of 27-HC levels (~10%) was measured in liver samples. Interestingly, the levels of 3β-HCA strongly decreased upon treatment (> 50%) and kept stable over time. However, a time-dependent reduction of 3β-HCA levels was also observed in the vehicle group (from 24 h, 28.1 ± 10.2 ng/mg CHOL to 168 h, 14.5 ± 2.5 ng/mg CHOL).

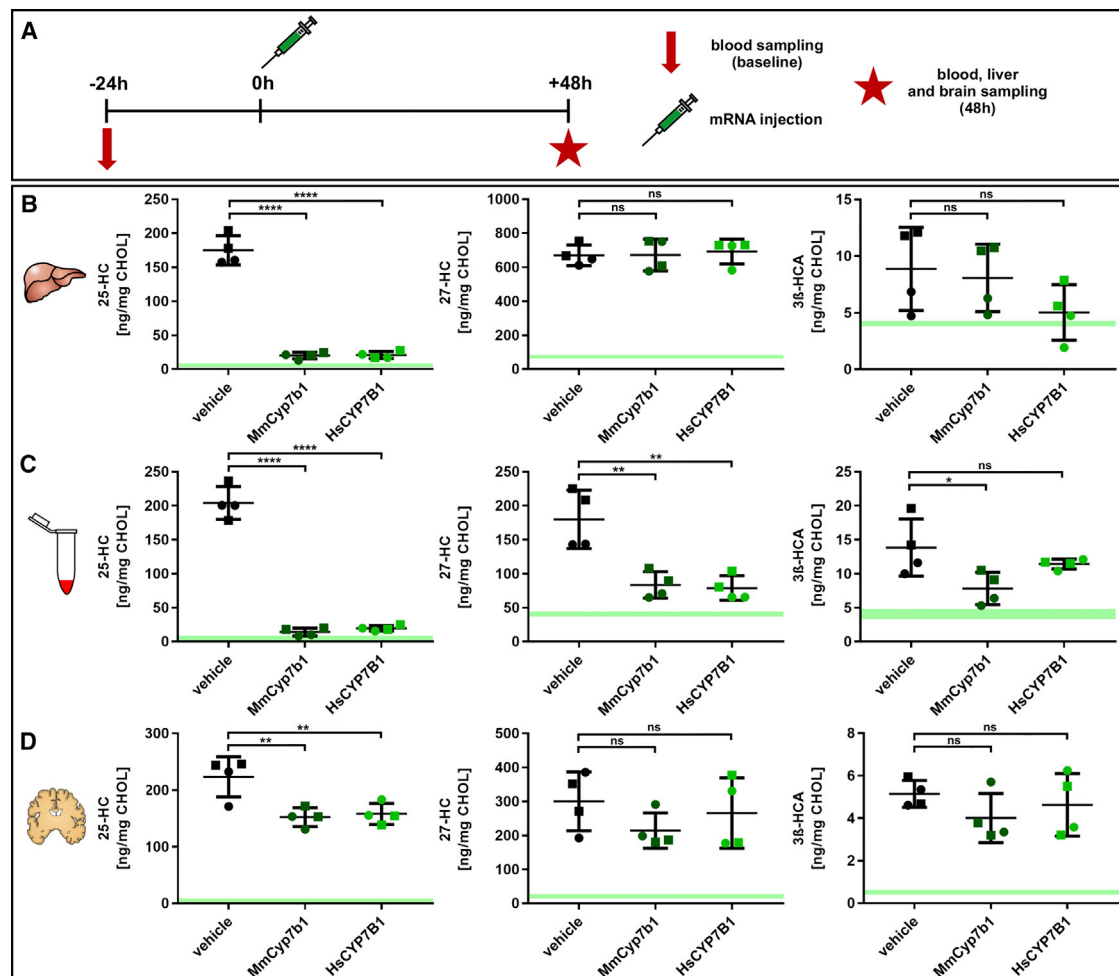


Figure 4. Single Intravenous Administration of HsCYP7B1 or MmCyp7b1 mRNA

(A) *Cyp7b1*^{-/-} mice received a single i.v. injection of 40 μg mRNA LNP (either non-translating mRNA [vehicle, black], MmCyp7b1 [dark green], or HsCYP7B1 mRNA [light green]). 48 h after administration, mice were sacrificed and tissues were processed for further analyses (n = 4 mice per group). (B–D) Oxysterol analysis of (B) liver, (C) serum, and (D) brain samples (square, male; circle, female; green section, wild-type values; ns, not significant; one-way ANOVA, *p < 0.05, **p < 0.01, ****p < 0.0001, error bars show SD).

In serum, 25-HC and 3β-HCA levels decreased to a comparable extent and with a similar time course as in liver (Figure 5C). In contrast to the poor response in liver, 27-HC levels in serum substantially decreased (> 60%) compared to the vehicle group and kept stable for at least 72 h.

In brain samples, a decrease of 25-HC by about 30% was reached over time with HsCYP7B1 mRNA (150.5 ± 40.7 ng/mg CHOL) compared to vehicle-treated animals (222.9 ± 60.7 ng/mg CHOL) (Figure 5D). Again, no significant changes in 27-HC and 3β-HCA concentrations were detected upon treatment.

Western blot analysis of protein levels 24 h after a single application of CYP7B1 mRNA in liver lysates showed expression in all treated mice but with some variability (Figure 5E). By 72 h post-injection, CYP7B1 protein already substantially decreased and was only detectable in

three out of five animals. CYP7B1 protein was not detectable at later time points (120 and 168 h post-injection) (data not shown). Based on this western blot analysis, the *in vivo* half-life of CYP7B1 protein is estimated as 15 h.

Based on the levels of 25-HC, we concluded that a sufficient therapeutic effect was present for at least 5 days (120 h) after a single administration of CYP7B1 mRNA with 61% reduction in liver, 70% reduction in serum, and 30% reduction in brain compared to vehicle.

Systemic Reduction of 25-HC Translated to the Brain after Repeat Intravenous Injection of CYP7B1 mRNA

Based on the pharmacodynamics data, we decided on a dosing interval of 5 days between repeat CYP7B1 mRNA i.v. injections to maintain reduced serum oxysterol levels over time in *Cyp7b1*^{-/-} mice.

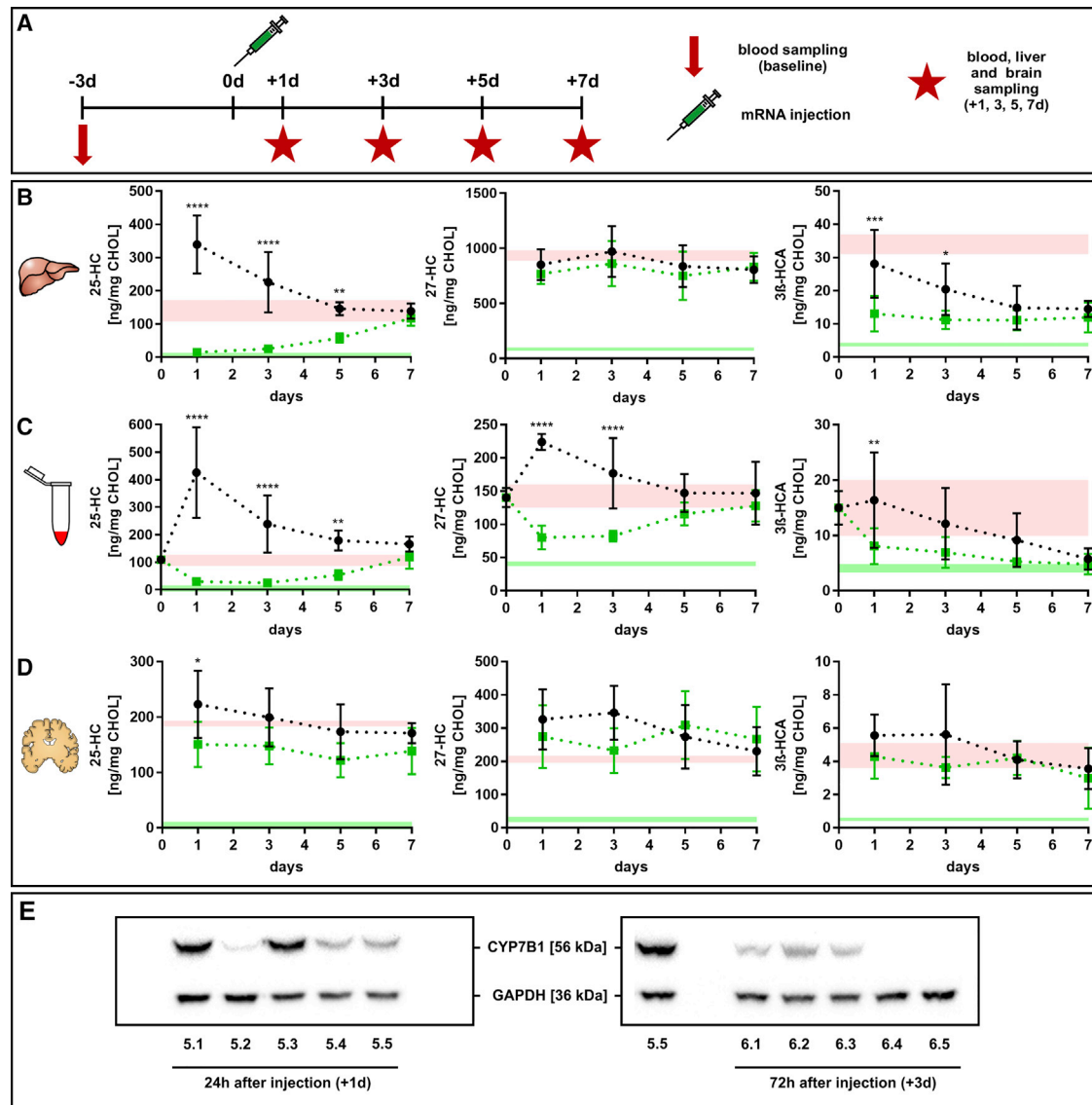


Figure 5. Pharmacodynamics Study of a Single Intravenous Administration of HsCYP7B1 mRNA

(A) *Cyp7b1*^{-/-} mice received a single i.v. injection of 40 μ g mRNA LNP (either non-translating mRNA [vehicle, black] or HsCYP7B1 mRNA [treated, green]) and were sacrificed 1, 3, 5, and 7 days post-injection ($n = 5$ mice per group). (B–D) Mass spectrometric analysis of 25-HC, 27-HC, and 3 β -HCA in (B) liver, (C) serum, and (D) brain samples (green section, range of wild-type values; red section, range of KO values; unpaired one-tailed t test comparing vehicle to treated group at each time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, error bars show SD). (E) Western blot analysis of CYP7B1 in liver extract of mice receiving HsCYP7B1 mRNA 24 h (left image, mice 5.1–5.5) or 72 h (right image, mice 6.1–6.5) after administration.

Safety and efficiency of repetitive applications of mRNA LNPs (40 μ g) were investigated with four consecutive i.v. injections every 5 days (either HsCYP7B1 mRNA [treated] or non-translating mRNA [vehicle]). Two days after the last injection (17 days of treatment), animals ($n = 6$ per group) were sacrificed, and serum, liver, and brain samples were analyzed (schematic illustration, Figure 6A).

Oxysterol analysis of liver samples revealed a more than 8-fold reduction of 25-HC in animals receiving HsCYP7B1 mRNA (Figure 6B) 2 days

after the last mRNA treatment. Additionally, repetitive treatment resulted in a significant decrease of 27-HC (525.8 ± 140.2 in treated animals versus 777.4 ± 82.6 ng/mg CHOL in the vehicle group) and 3 β -HCA (almost 50% reduced) in livers of treated compared to vehicle animals.

These findings were confirmed by oxysterol profiles in serum samples (Figure 6C), with significant reductions of 88% in 25-HC, 53% in 27-HC, and 32% in 3 β -HCA levels of CYP7B1-mRNA-treated animals compared to animals receiving non-translating mRNA.

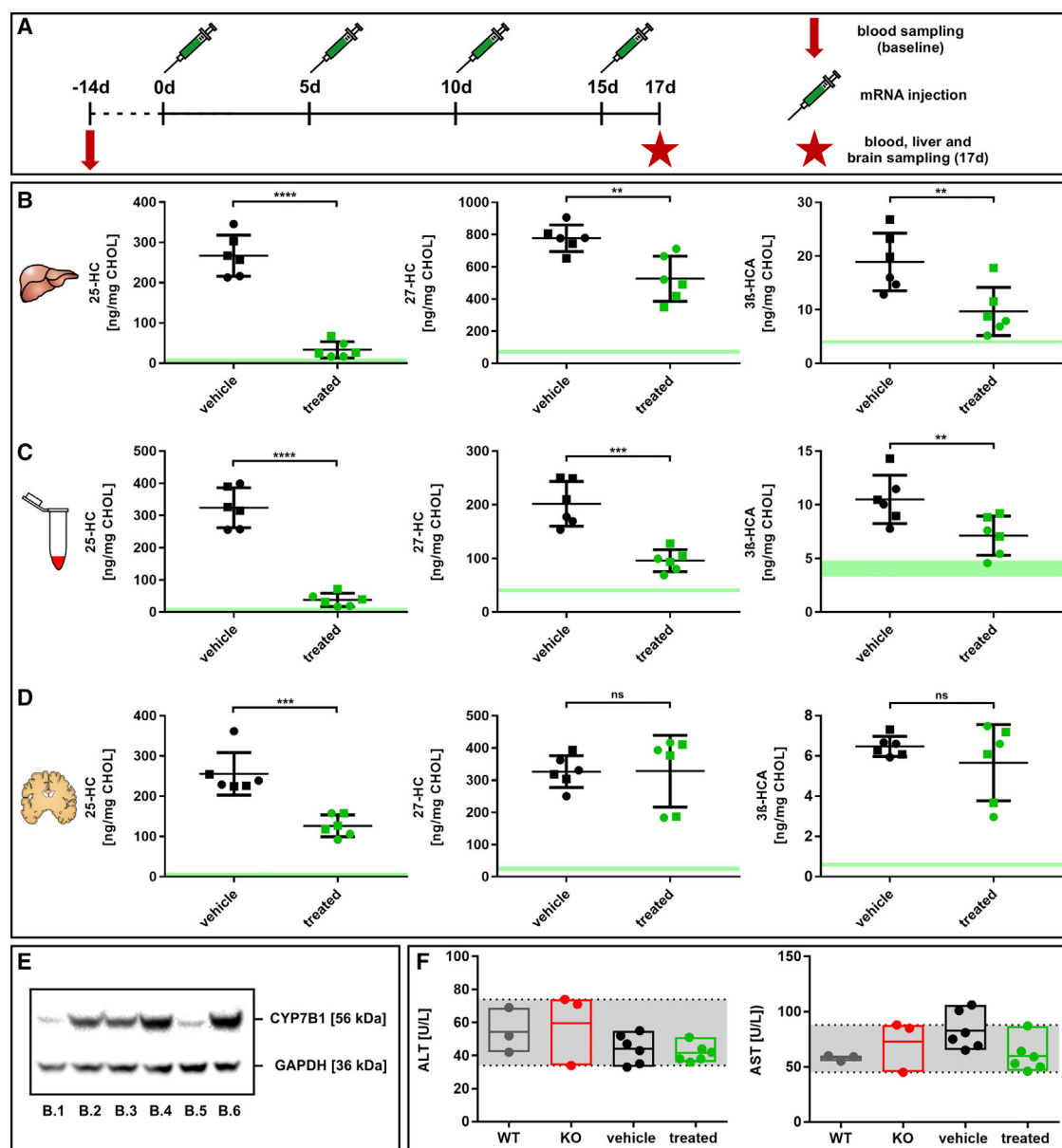


Figure 6. Repeat Intravenous Injection of HsCYP7B1 mRNA

(A) *Cyp7b1*^{-/-} mice received repeat i.v. injections of 40 μ g mRNA LNP (either non-translating mRNA [vehicle, black] or HsCYP7B1 mRNA [treated, green]) with an interval of 5 days for a total of four injections. Mice were sacrificed 2 days after the last injection (in total, 17 days) ($n = 6$ per group). (B–D) Oxysterol analysis (25-HC, 27-HC, 3 β -HCA) in (B) liver, (C) serum, and (D) brain samples (square, male; circle, female; green section; range of wild-type values, unpaired one-tailed t test; ns, not significant; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, error bars show SD). (E) Western blot of CYP7B1 expression in liver of treated animals (B.1–B.6) 2 days after the last injection of HsCYP7B1 mRNA (F) ALT and AST concentrations in serum samples of treated (green) and vehicle (black) animals 2 days after the last injection compared to values in untreated wild-type (WT, gray) and knockout (KO, red) mice (gray section, range of KO values).

Repetitive treatment further reduced brain concentration of 25-HC by more than 50% (126.5 ± 27.0 versus 255.9 ± 53.0 ng/mg CHOL) (Figure 6D). In a grouped analysis, no significant differences in 27-HC and 3 β -HCA values between treated and vehicle animals were identified. Interestingly, two animals (B.2 and B.4) in the treated cohort showed an almost 50% reduc-

tion of 27-HC and 3 β -HCA compared to the mean levels in the vehicle group.

Western blot analysis revealed a stable expression of CYP7B1 protein 2 days after the fourth administration. This clearly demonstrates that repetitive delivery of the LNP/mRNA complex did not adversely

affect the transfection, expression, or translation efficiency *in vivo*. Again, a high variability in protein expression between single animals was observed with two animals showing high CYP7B1 expression (B.4 and B.6), two animals with intermediate expression (B.2 and B.3), and two animals (B.1 and B.5) with low expression (Figure 6E).

By measuring unchanged total cholesterol as well as 24-HC levels in all three compartments as a marker for the classical and neural cholesterol pathway, the specific targeting of the mRNA treatment was confirmed (Figure S4).

Repetitive Intravenous Application of CYP7B1 mRNA Did Not Induce Liver Toxicity Marker

To control for liver toxicity, we assessed alanine aminotransferase (ALT) and aspartate aminotransferase (AST) 2 days after the last injection (Figure 6F). There was no indication of liver toxicity in mice receiving either HsCYP7B1 LNP (ALT, 42 ± 6 U/L; AST, 60 ± 15 U/L) or non-translating RNA LNP (ALT, 44 ± 9 U/L; AST, 83 ± 17 U/L) compared to wild-type (ALT, 54 ± 14 U/L; AST, 58 ± 3 U/L) and untreated *Cyp7b1*^{-/-} mice (ALT, 60 ± 22 U/L; AST, 73 ± 24 U/L).

DISCUSSION

In this study, we demonstrated that i.v. application of human CYP7B1 mRNA is a feasible strategy to restore physiological enzyme activity in liver of CYP7B1-deficient mice, the mouse model of SPG5. As SPG5 is thought to be caused by highly elevated levels of oxysterols, the reduction of these neurotoxic metabolites is a major goal in therapeutic approaches.

In SPG5, loss-of-function mutations in CYP7B1 cause the accumulation of its most important substrates, 25-HC, 27-HC, and 3 β -HCA. All three have been shown to be toxic to different degrees in human neuronal cell cultures as well as in *in vivo* studies.^{6,27,28} 25-HC and especially 27-HC impaired neuronal outgrowth and hampered mitochondrial metabolism in neurons at concentrations close to those observed in serum of SPG5 patients. In contrast, 3 β -HCA exerted a toxic effect only at considerably higher concentrations.⁶

As oxysterol levels largely correlate with cholesterol levels in healthy controls, statin treatment to lower the cholesterol level has been tested in SPG5 patients. Indeed, previous work from our group showed that atorvastatin not only reduced cholesterol in SPG5 patients, but also lowered serum levels of 27-HC by 31% and 25-HC by 23%. This reduction, however, was not sufficient to lower oxysterol levels in CSF over 9 weeks of treatment.⁶

As the brain is exposed to a constant flux of oxysterols from the circulation, especially when serum levels are highly elevated, we aimed for a more efficient way to reduce oxysterol levels using enzyme replacement via i.v. mRNA administration. This strategy provided a reduction of 27-HC levels by 53% and of 25-HC by 88% in serum, bringing levels of both oxysterols close to those of wild-type animals. In addition, 3 β -HCA levels that did not respond to statin treatment were reduced by 32% upon mRNA treatment. Even

more importantly, reduction of oxysterols in serum translated into lowered 25-HC levels in the brain after only 17 days of treatment. It may be speculated that a prolonged treatment could also result in a significant reduction of 27-HC and 3 β -HCA in the brain. Alternatively, a single intrathecal administration of mRNA may help to clear oxysterols that accumulated in the brain before starting a systemic treatment. Recently, the feasibility and effectiveness of an intrathecal mRNA application has been described in Friedreich ataxia.²⁹

Physiologically, it is of interest that 25-HC responds much more directly and extensively to the lack of CYP7B1 as well as its replacement upon mRNA application. This may suggest a substrate preference for 25-HC over 27-HC. In direct comparison, 27-HC appears to be more stable especially in liver. The discrepancy may arise from different cellular compartments of 27-HC storage and CYP7B1 expression (ER). Alternatively, 27-HC may accumulate in compartments (vesicle or membrane bound) or cells that are more difficult to access by restored CYP7B1. This may explain why concentrations of 27-HC in liver are only significantly lowered after a prolonged repetitive treatment with CYP7B1 mRNA over 17 days. However, from a therapeutic perspective, probably more important than 27-HC levels in liver are levels in circulation, as they are likely to drive diffusion into the brain.

Additionally, mRNA treatment may not only reduce neurotoxic substrates of CYP7B1, but also restore its physiological metabolites like 7 α -hydroxylated lipids that may have neuroprotective properties, as reported by Theofilopoulos and colleagues,⁷ who observed a compensatory effect of 3 β ,7 α -diHCA in cultured neuronal cells treated with 3 β -HCA. We found, however, that the serum level of 3 β ,7 α -diHCA in wild-type mice is low and considerably lower than in humans (I.B., unpublished data). This metabolite was therefore barely detectable in *Cyp7b1*^{-/-} mice. A lack of these metabolites has not been shown to be of medical relevance yet and can obviously not be investigated in mice. Nevertheless, an increase of physiological metabolites of CYP7B1 can only be achieved by enzyme replacement therapy and not by strategies aiming to lower oxysterols by reducing its precursors.

Of primary importance, repeat i.v. delivery of CYP7B1 mRNA was found to be safe in this study. Neither the CYP7B1 mRNA nor the restored protein nor the LNP induced liver toxicity. None of the treated animals presented signs of toxic or adverse effects. However, LNP particles encapsulating non-coding mRNA led to a temporary increase oxysterol levels (25-HC and 27-HC) in liver and serum in the vehicle group. Cholesterol is an essential component of LNP since it stabilizes nanoparticles and enhances the activity of cationic lipids,^{30,31} and the 40- μ g mRNA dose used in this therapeutic approach would equate to a cholesterol dose of \sim 300–400 μ g per injection. As cholesterol is the precursor of oxysterols and is shown to correlate closely with oxysterol levels under physiological conditions, we hypothesize that the temporary elevation of 25-HC and 27-HC levels in serum and liver in the vehicle group is likely due to the

LNP injections. Interestingly, this effect was overcompensated in the CYP7B1 mRNA-treated group.

In contrast to other oxysterols, 3 β -HCA was found to decrease upon treatment even in the vehicle group. This may be explained by cross-regulation of cholesterol and 3 β -HCA. When the enzyme CYP27A1 is exposed to high levels of cholesterol, such as following LNP administration, cholesterol rather than 27-HC will be the preferred substrate resulting in a reduced oxidation of 27-HC to 3 β -HCA.³²

There was substantial variability in protein expression levels of CYP7B1 after mRNA treatment between animals that may derive from variable efficacy of cellular uptake of LNP. However, the variation in CYP7B1 expression does not result in strong variation of oxysterol levels, indicating that even low protein levels can compensate for a defect in the cholesterol pathway. This may suggest smaller mRNA doses may have similar therapeutic effects compared to the dose used in this study. In this pilot study, we did not perform dose titration experiments but relied on a most likely supra-maximal dosage to investigate the potential of mRNA treatment in SPG5. To approach a clinical application, a dose-titration study in humans will be required, as the transfer of mouse dosage to humans is not reliable.

This mouse study is limited to a relatively short treatment period of 17 days. As *Cyp7b1*^{-/-} mice do not develop a neurological phenotype, we did not expand the study for a longer period. *Cyp7b1*^{-/-} mice may escape neurological deficits due to lower absolute oxysterol levels compared those of humans affected by SPG5. Additionally, as symptoms in SPG5 patients are mostly restricted to the longest axons in the corticospinal tract innervating the legs but are not present in the shorter axons addressing the arms, metabolic changes may not result in an obvious phenotype in mice due to shorter axons. This hampers the evaluation of clinical effects of the enzyme replacement therapy in the *Cyp7b1*^{-/-} mouse model.

A limitation of the mRNA approach is its relatively short half-life and the resulting brevity of CYP7B1 protein expression, which results in a rather short interval (5 days) between mRNA administrations. This limited time of expression, however, is also an advantage for easy regulation of the protein expression and helps to prevent non-physiological effects of overexpression that might potentially be harmful.

A compelling advantage of mRNA therapy over classical enzyme replacement strategies is its much more physiological expression profile. Despite the success of classical enzyme-replacement therapy in several metabolic disorders, this therapeutic approach is not well suited for treatment of diseases caused by the lack of functional intracellular proteins, especially if the proteins are located in or on intracellular compartments like the endoplasmic reticulum. Additionally, as therapeutic proteins are conventionally manufactured by using human, animal, or even plant cells, it cannot be excluded that pharmacological and biochemical properties of such recombinant proteins may differ from endogenously expressed enzymes. Cellular localiza-

tion, folding, and post-translational modifications can especially be critical for the correct function of a therapeutic protein. Delivery of mRNA can overcome these limitations and is likely to result in expression of a functional protein at a physiological cellular location, as demonstrated in this study.

In summary, we were able to demonstrate that a therapeutic approach with mRNA can restore human CYP7B1 protein that exhibits physiological function and eliminates abnormal cholesterol metabolites *in vivo*. In the next step, these mouse experiments need to be translated into early clinical trials in humans. Data from this *in vivo* study indicate that CYP7B1 mRNA is a promising candidate for causal treatment in SPG5.

MATERIALS AND METHODS

mRNA Production and Formulation

The design and synthesis of mRNA sequences have been described previously.¹⁵ In brief, mRNAs for HsCYP7B1-HA, HsCYP7B1, MmCyp7b1-HA, and MmCyp7b1 as well as non-translating mRNA (used to generate vehicle control) were capped co-transcriptionally to produce Cap0 structure. A template-encoded poly(A) sequence was utilized as described previously.¹⁵ Sequences were codon optimized for human use and did not include chemically modified bases. For *in vivo* studies, mRNAs were further polyadenylated using A-Plus poly(A) polymerase tailing kit (Biozym). Polyadenylation was carried out according to manufacturer's recommendations.

LNP formulation was conducted at Acuitas Therapeutics as described previously.¹⁵ For injections, mRNA/LNP was diluted in PBS (pH 7.4).

In Vitro Transfection

For *in vitro* transfection of L929 cells, mRNAs (MmCyp7b1-HA, HsCYP7B1-HA) were complexed with Lipofectamine2000 (Life Technologies) at 1.5 μ L per μ g of mRNA and transfected into cells according to manufacturer's instructions. For western blot analysis, 3×10^5 L929 cells were seeded 1 day before transfection in 6-well plates and transfected with 2 μ g of complexed mRNA for each construct. For immunocytochemical staining, 5×10^4 L929 cells were seeded on 24-well plates on coverslips (Roth) 1 day before transfection with 1 μ g mRNA as described above. Approximately 2 h after transfection, the transfection mix was replaced with HyClone RPMI 1640 media (GE Healthcare) supplemented with 2 mM L-glutamine (Lonza), 100 U/mL penicillin (Lonza), 100 U/mL streptomycin (Lonza), and 10% fetal calf serum (GE Healthcare).

Western Blotting

Transfected cells were harvested 3 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h post-transfection. Cells were washed once with PBS followed by the addition of 350 μ L RIPA buffer (Sigma-Aldrich) complemented with cOmplete mini protease inhibitor cocktail (Roche) and subsequent incubation for 15 min at 4°C. Cells were collected via scraping and centrifuged at $1,500 \times g$ for 10 min at 4°C, followed by incubation at room temperature (RT) for 30 min with 50–75 U benzonase (Millipore) and at 95°C for 5 min.

Liver protein samples were isolated by addition of 10 μ L RIPA buffer with cOmplete mini protease inhibitor cocktail per mg of tissue and homogenized using the tissue lyser II system (QIAGEN). After lysis, the homogenate was centrifuged (13,500 rpm, 30 min, 4°C) and supernatant was collected.

The total protein content was analyzed by using Pierce BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. For transfected cells, 5 μ g of protein was loaded for western blot analysis. To this end, lysates were prepared by incubation with 1 \times protein sample loading buffer (LI-COR Biosciences) complemented with 2% 2-mercaptoethanol for 15 min at RT followed by heating for 5 min at 95°C. For liver samples, 30 μ g of total protein was loaded.

Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane (Odyssey nitrocellulose membrane 0.22 μ m, LI-COR Biosciences), followed by blocking with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 buffer (TBST) (Sigma-Aldrich) for 2 h. Subsequently, the membranes were incubated for 1 h with anti-HA antibody (Sigma-Aldrich; rabbit, 1:1,000, H6908), anti- β -actin antibody (Abcam; mouse, 1:20,000, ab6276), anti-CYP7B1 antibody (Abcam; rabbit, 1:1,000, ab138497), or anti-GAPDH antibody (Meridian Life Science; mouse, 1:10,000, H86504M) diluted in 0.5% skimmed milk in TBST. Membranes were washed three times with TBST and incubated for 1 h with a secondary antibody against rabbit (IRDye 800CW goat anti-rabbit immunoglobulin G (IgG) heavy and light chain [H + L], 1:20,000 [LI-COR Biosciences] or peroxidase-conjugated AffiniPure goat anti-rabbit, 1:10,000 [Jackson ImmunoResearch]) or mouse (IRDye 680RD goat anti-mouse IgG [H + L], 1:20,000 [LI-COR Biosciences] or peroxidase-conjugated AffiniPure goat anti-mouse, 1:10,000 [Jackson ImmunoResearch]) each diluted in 0.5% skimmed milk in TBST. Membranes were washed three times each for 10 min in TBST and stored in TBS until analysis. Protein detection and image processing was carried out either with the Odyssey CLx imaging system (LI-COR Biosciences) or with the Gel Doc XR+ system (Bio-Rad).

Immunocytochemistry

Transfected cells were harvested 24 h post-transfection. Cells were washed once with PBS and fixed with 500 μ L of 4% paraformaldehyde/2.5% sucrose solution for 10 min at RT followed by three washing steps in PBS. Fixed cells were permeabilized by 10 min incubation in 500 μ L of 0.1% Triton X-100 in PBS and washed two times with PBS. Blocking was performed by incubation in 500 μ L PBS containing 2% BSA (Sigma Aldrich) for 30 min. Afterward, primary antibodies were used at the following dilutions: anti-HA tag (Thermo Fisher Scientific; mouse, 1:100, 26183-D488) and anti-calreticulin antibody (Abcam; rabbit, 1:500, ab92516) diluted in PBS containing 1% BSA. After 60 min incubation and washing three times with PBS, cells were incubated with secondary antibodies using Alexa Fluor 568 (Thermo Fisher Scientific; donkey anti-rabbit, 1:1,000, dA10042) diluted in PBS containing 3% BSA for 60 min in the dark. Nuclei were stained with DAPI by using the mounting medium

Roti-Mount FluorCare DAPI (Roth) on glass slides. Samples were examined using the Axio observer microscope (Zeiss). Images were edited and merged by using the ZEN 2.3 software.

Animal Experiments

All research and animal care procedures were approved by the district government (Tübingen, Germany) and performed according to international guidelines for the use of laboratory animals in the FORS animal facility (Tübingen, Germany).

Mice were housed in standard laboratory cages (type II L) in temperature (22°C \pm 2°C)- and humidity-controlled (55% \pm 10%) rooms. Mice were kept on a 12-h light/dark cycle and had *ad libitum* access to food and water. Male and female *Cyp7b1*^{-/-} mice²⁵ (C57BL/6J background) between 3 and 7 months of age were used in the present studies and evenly distributed in each study group.

For each i.v. application of LNP/mRNA complex, a total of 40 μ g mRNA per injection (injection volume, 100 μ L, c = 0.4 μ g/ μ L, PBS [pH 7.4]) was administered into the tail vein. At desired time points, retrobulbar blood sampling of animals was performed. Serum samples were gained by using serum-gel Z microtubes (Sarstedt) according to manufacturer's guidelines. For liver and brain sampling, mice were perfused with sterile PBS. Both organs were then removed, snap frozen on dry ice, and stored at -80°C till further usage. No adverse event was observed during the animal experiments.

For the biodistribution study, BALB/c mice were i.v. injected with 20 μ g mRNA per injection (injection volume, 100 μ L, c = 0.2 μ g/ μ L, PBS [pH 7.4]) into the tail vein. 6 h post-injection, the animals were sacrificed and the indicated organs were harvested. PBS injection was used as a negative control.

Photinus pyralis Luciferase Assay

Tissue lysates were prepared at Preclinics and used to determine the reporter PpLuc. In brief, lysates were prepared by organ homogenization with 1 \times lysis juice (PJK Biotech) and protease inhibitor (cocktail set I, CalBiochem, EMD Millipore). 30 μ L per mg organ was used and homogenization was performed in a bead disruptor (Disruptor Genie, Scientific Industries). The tissue was disrupted for 3 min at 2,860 rpm, followed by centrifugation at 3,200 \times g for 5 min. For lymph node and ovary homogenization, 50 μ L 1 \times lysis juice and protease inhibitor was used per mg tissue. The homogenates were snap frozen in liquid nitrogen prior to luciferase measurement and stored at -80°C. 20 μ L of the lysate was used to measure PpLuc via the multimode plate reader TriStar2 S LB 942 (Berthold Technologies) by adding 50 μ L Beetle-Juice buffer (PJK Biotech).

Mass Spectrometry Analysis

Oxysterols and 3 β -HCA were assayed by isotope dilution mass spectrometry using deuterium-labeled internal standards as described previously.³³ Values of oxysterols and 3 β -HCA were normalized to total cholesterol content (ng/mg CHOL). Individual values of all analyzed samples can be found in Table S1.

Assessment of Aminotransaminases

Measurement of ALT and AST in mouse sera was conducted by Synovo (Tübingen, Germany).

Accelerating Rotarod Test

To measure the motor coordination abilities and balance of the *Cyp7b1*^{-/-} mice, rotarod analyses were performed. In total, 12 mice at ~18 months of age of each genotype (either knockout [*Cyp7b1*^{-/-}] or wild-type [*Cyp7b1*^{+/+}]) were analyzed. After a 3-day training session with an acceleration from 4 to 12 rpm over 120 s (2 min) (maximum illumination of 100 lux), the final run was performed. Thereby, mice were audited twice on an accelerating rotarod (acceleration from 4 to 40 rpm over 300 s [5 min]; TSE Systems). The latency to fall off the rotating rod was recorded and statistically analyzed.^{34,35}

Home-Cage Activity

To analyze the spontaneous home-cage activity, the Lab Master system (TSE Systems) was used. In total, 12 mice at ~18 months of age of each genotype (either knockout [*Cyp7b1*^{-/-}] or wild-type [*Cyp7b1*^{+/+}]) mice were analyzed. In order to exclude any bias, both transgenic and control mice were analyzed in parallel and were randomly distributed between the test systems. Mouse cages were placed into sensor frames, the number of beam brakes was counted, and the total activity was quantified. Additionally, drinking and feeding behavior was analyzed. The analysis was performed for 70 h (12-h light/dark cycle).

Statistics

Data was statistically analyzed using GraphPad Prism software version 7. Means of treated versus vehicle group were compared by unpaired one-tailed t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). In case of study 1 (Figure 4; Figure S3), means of MmCyp7b1, HsCYP7B1, and vehicle groups were analyzed by one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). All error bars depicted in this publication refer to SD.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtm.2019.10.011>.

AUTHOR CONTRIBUTIONS

Conceptualization, S.H., N.H., and L.S.; Methodology, S.H., M.P., J.H.-S., and I.B.; Formal Analysis, S.H., M.P., and I.B.; Investigation, S.H., M.P., Y.S., P.H., S.S., A.T., R.B., and I.B.; Resources, M.P. and J.-Å.G.; Writing – Original Draft, S.H. and L.S.; Writing – Review & Editing, M.P., P.H., S.S., J.-Å.G., J.H.-S., T.S., F.C.-T., N.H., and I.B.; Visualization, S.H. and M.P.; Supervision, S.H., T.S., F.C.-T., and L.S.; Project Administration, N.H. and L.S.; Funding Acquisition, N.H. and L.S.

CONFLICTS OF INTEREST

M.P., F.C.-T., T.S., N.H., A.T., and R.B. are employees of CureVac AG developing therapeutics based on sequence-engineered mRNA.

ACKNOWLEDGMENTS

LNP formulations used in this study were kindly provided by Acuitas Therapeutics. We gratefully thank P. Baumhof for contributing the biodistribution data of LNPs. We would like to thank N. Casadei for data processing of home-cage activity. We thank Ying Tam for critical reading of the manuscript. Technical assistance by A.L. Sandblom, A. Braunheim, A. Schäfer, F. Polzer, J. Herold, S. Yaycioglu, A. Kulesa, and M. Rauch is gratefully acknowledged. J.-Å.G. is thankful to the Robert A. Welch Foundation for a fellowship. I.B. was supported by grants from the Swedish ALF Projektmedel and the foundation Gamla Tjänarinnor.

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