Biochimica et Biophysica Acta 1832 (2013) 1784–1792

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Chaperone-mediated gene therapy with recombinant AAV-PPCA in a new mouse model of type I sialidosis

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ARTICLE INFO

Article history:
Received 17 April 2013
Received in revised form 28 May 2013
Accepted 3 June 2013
Available online 12 June 2013

Keywords:
AAV
Chaperone-mediated therapy
Gene therapy
Lysosomal storage disease
NEU1
Sialidosis

ABSTRACT

The lysosomal storage disease sialidosis is caused by a primary deficiency of the sialidase N-acetyl-α-neuraminidase-1 (NEU1). Patients with type I sialidosis develop an attenuated, non-neuropathic form of the disease also named cherry red spot myoclonus syndrome, with symptoms arising during juvenile/ adult age. NEU1 requires binding to its chaperone, protective protein/cathepsin A (PPCA), for lysosomal compartmentalization, stability and catalytic activation. We have generated a new mouse model of type I sialidosis that ubiquitously expresses a NEU1 variant carrying a V54M amino acid substitution identified in an adult patient with type I sialidosis. Mutant mice developed signs of lysosomal disease after 1 year of age, predominantly in the kidney, albeit low residual NEU1 activity was detected in most organs and cell types. We demonstrate that the activity of the mutant enzyme could be effectively increased in all systemic tissues by chaperone-mediated gene therapy with a liver-tropic recombinant AAV2/8 vector expressing PPCA. This resulted in clear amelioration of the disease phenotype. These results suggest that at least some of the NEU1 mutations associated with type I sialidosis may respond to PPCA-chaperone-mediated gene therapy.

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1. Introduction

N-acetyl-α-neuraminidase-1 (NEU1) initiates the degradation of sialoglycoconjugates in lysosomes. NEU1 binds its chaperone, protective protein/cathepsin A (PPCA) to route to the lysosome and achieve intralysosomal catalytic activity/stability [1–3]. In the lysosome, NEU1 is active exclusively in a multienzyme complex with PPCA and β-galactosidase. Two lysosomal storage diseases (LSDs) are associated with NEU1 deficiency: sialidosis, which results from structural mutations at the *NEU1* locus on chromosome 6p21 [4–6], and galactosialidosis (GS), which is caused by a primary defect of PPCA leading to a secondary deficiency of NEU1 [7–9]. Because both diseases involve severe NEU1 deficiency, their clinical onset and progression are overlapping. However, certain distinct systemic and neurologic differences exist between the two diseases that likely result from the (near) complete PPCA and partial β-galactosidase deficit in GS patients [10,11].

The numerous clinical manifestations of sialidosis vary in terms of the age of onset and severity of the symptoms, mostly correlating with the levels of residual NEU1 activity [5,12,13]. Patients with type I sialidosis have the attenuated form of the disease, and mostly develop symptoms during adolescence, which include myoclonus, progressive visual loss, and bilateral cherry red spots. All patients diagnosed with this form of the disease have normal intellectual capacity. The extent of disease severity in type I sialidosis largely correlates with the type of NEU1 mutations and the residual NEU1 activity they confer [5]. Some NEU1 variants segregating with type I patients (R294S, 400dupHY, G328S, V54M) showed an increase in their catalytic activity in response to an increase in the levels of PPCA [5]. Congenital type II sialidosis, on the other hand, is a fulminant disease. Patients are stillborn or diagnosed at birth and have severe somatic features, skeletal dysplasia, hepatosplenomegaly, and mental retardation. Infantile or juvenile type II sialidosis patients are relatively healthy at birth, but also develop progressive visceromegaly, dysostosis multiplex, and moderate to severe mental retardation. Also for type II sialidosis there is a clear correlation between the type of NEU1 mutations and the severity of the disease [5].

A disease-threshold model explains the correlation between the residual activity in patients with mild phenotypes [14]. This model predicts that lysosomal hydrolase activity is normally higher than needed for substrate turnover and tissue/organ homeostasis. However, if the enzyme activity falls below the disease threshold, as it is the case in LSD patients, its catalytic activity is not sufficient to metabolize substrates, resulting in disease onset and progression. For many LSDs, the disease threshold may be as low as 1% to 5% of normal

Abbreviations: BMEF, bone marrow extracellular fluid; ERT, enzyme replacement therapy; FACE, fluorophore-assisted carbohydrate electrophoresis; GS, galactosialidosis; IHC, immunohistochemistry; KO, knockout; KO/TG, knockout–transgenic; LSD, lysosomal storage disease; PCT, pharmacologic-chaperone therapy; PPCA, protective protein/cathepsin A; scAAV, self-complementary adeno-associated virus; WT, wild-type

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0925-4439/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbadis.2013.06.002

enzyme levels [14,15]. Therefore, relatively small differences in the level of residual enzyme may explain variability in age of onset, disease severity, and symptoms in patients with sialidosis.

Neu1$^{-/-}$ mice develop a systemic disease that closely mimics the early-onset type II form of sialidosis [16]. In particular, splenomegaly, which is a recurrent feature in patients, coincides with the occurrence of extramedullary hematopoiesis [17]. Previously, we have shown that in *Neu1*$^{-/-}$ mice this and other phenotypes result at least in part from exacerbation of the process of lysosomal exocytosis, which is downstream of the Neu1 deficiency in various cell types, including bone marrow (BM)-derived macrophages and connective tissue fibroblasts [17–19]. In addition, in the fibroblasts of patients with sialidosis we demonstrated an inverse correlation between residual NEU1 activity and the level of lysosomal exocytosis, which may link the exacerbation of this process to multiple disease manifestations [17].

Enzyme replacement therapy (ERT) with recombinant lysosomal hydrolases is an effective treatment for several non-neuropathic patients with LSDs [20]. Unfortunately, due to the unfavorable properties of NEU1 (i.e., it is prone to aggregate, unstable in the absence of PPCA, lack mannose-phosphate recognition marker) no therapy is currently available or foreseeable for patients with sialidosis. Besides ERT, small-molecule, pharmacologic-chaperone therapy (PCT) may be beneficial for LSD patients with non-neuropathic attenuated forms of LSDs [21–23]. PCT is based on the use of specific iminosugars that can bind the active site of a mutant enzyme, thereby promoting better folding, increased lysosomal trafficking and catalytic activity/stability. However, because single therapies are not universally effective, PCT may be more successful in combination with other treatments [24].

Analogously to PCT, we hypothesized that increasing the concentration of NEU1's physiological chaperone, PPCA, could facilitate the lysosomal compartmentalization and stability of mutant forms of NEU1 associated with type I and juvenile, type II sialidosis. To test this hypothesis, we generated a new mouse model of the attenuated, type I form of sialidosis, carrying a V54M-NEU1 amino acid substitution previously identified in a non-neuropathic adult patient [5]. We treated type I sialidosis mice with a good manufacturing practice (GMP)-grade self-complementary adeno-associated virus (scAAV) expressing PPCA under the control of a liver-specific promoter [25]. This vector, expressing human factor IX, has been approved and successfully used in a combined phase 1 and 2 gene-transfer clinical trial in patients with Hemophilia B [26]. Recently we have successfully used the same scAAV2/8 vector expressing PPCA in a preclinical study in GS mice [25], a milestone in the development of a gene therapy clinical trial for GS patients. Here, we present evidence that also some patients with type I sialidosis may benefit from gene therapy treatment with the same PPCA-expressing scAAV2/8 vector.

2. Material and methods

2.1. Cell culture and antibodies

Primary lung fibroblasts were isolated from *Neu1*$^{-/-}$ (KO) mice and maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin). The cells were maintained at 37 °C (5% CO_2). The method for isolating primary macrophages from fresh bone marrow (BM) of KO mice has been described [27]. HEK293T and ecotropic (Phoenix-E) viral-producer cells were grown in DMEM supplemented with 5% FBS. Antibodies against NEU1 and PPCA were produced and affinity purified, as described previously [28]. A monoclonal antibody against the luminal domain of Lamp1 (clone 1D4B) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Retrovirus preparation, transduction, and transient transfection

The MSCV-PPCA-IRES-GFP and MSCV-NEU1-IRES-GFP retroviral constructs have been used previously [29]. The *V54M-NEU1* and *Y370C-NEU1* missense mutations have previously been previously described and transiently expressed in fibroblasts from patients with sialidosis [5]. The mutant *NEU1* cDNAs were cloned into the retroviral vector MSCV-IRES-GFP using standard cloning procedures. Ecotropic retroviruses were generated by transfecting retroviral packaging cell line Phoenix-E with MSCV-NEU1^{WT}-IRES-GFP, MSCV-NEU1^{V54M}-IRES-GFP, or MSCV-NEU1^{Y370C}-IRES-GFP. Mouse KO fibroblasts were either single-transduced with the WT or 1 of 2 mutant MSCV-NEU1-GFP viral constructs, or they were cotransduced with the *NEU1*-expressing viruses and MSCV-PPCA-IRES-GFP. BM-derived KO macrophages were transduced with either the WT or 1 of the 2 mutant MSCV-NEU1-GFP viral constructs. A final concentration of 8 μg/mL polybrene was used to improve transduction efficiency. Two or 3 days after transduction, GFP^{+} cells were sorted by fluorescence-activated cell sorting. Sorted cells were maintained in culture for an extended period of time.

2.3. Animal studies

All experiments were performed on *Neu1*$^{-/-}$ (KO), *Neu1*$^{-/-}$;*NEU1*^{V54M} (KO/TG), *Neu1*$^{+/+}$;*NEU1*^{V54M} (WT/TG), *Ppca*$^{-/-}$ mice in the FVB background [16,17,30]. All experiments and handling procedures described herein were approved by the St. Jude Children's Research Hospital Animal Care and Use Committee.

2.4. Generation of *Neu1*$^{-/-}$;*NEU1*^{V54M} mice

The 1.2-kb human ubiquitin promoter fragment [31] was released from the plasmid pFUGW by the restriction endonucleases PacI and BamHI (New England BioLabs, Ipswich, MA) and treated with Klenow (New England BioLabs) to generate blunt ends. Subsequently, the ubiquitin promoter fragment was cloned upstream of the *NEU1* cDNA into the BamHI-linearized and Klenow-blunted *pSCTOP-NEU1*^{V54M} plasmid DNA vector [5]. A 4.2-kb linear *NEU1*^{V54M} transgene-containing DNA fragment was released by digesting the plasmid DNA with ClaI and SfiI (New England BioLabs). The DNA fragment was injected into the pronuclei of fertilized eggs isolated from FVB/NJ pregnant mice [32]. Transgenic founders were PCR-genotyped using genomic DNA extracted from the tails and 2 human *NEU1* cDNA-specific oligonucleotide primers (sense, 5′-gggcttaagggtgacatctgcgct-3′; antisense, 5′-agcag ttgctccatggtcaccagc-3′) that generate a 288-bp fragment in transgene-positive mice. *NEU1*^{V54M} transgenic FVB mice were bred into the KO FVB background [16] to generate mouse strains that are *Neu1* mRNA-negative, but instead express human mutant NEU1 under the control of the ubiquitin promoter: knockout transgenic (KO/TG) *Neu1*$^{-/-}$;*NEU1*^{V54M} mice.

2.5. Uptake assay of mouse fibroblasts and macrophages in vitro

MSCV-transduced fibroblasts and primary macrophages were seeded in 6-well plates to near confluency. Aliquots of purified BV-PPCA[28] (3, 6, or 9 μg) were added to the culture medium of macrophages and incubated for 48 h, after which the cells were trypsinized, washed in PBS, centrifuged at 500 ×*g*, and frozen at −80 °C.

2.6. Production and titration of the scAAV2/8-LP1-PPCA vector

The production and titration of the scAAV2/8LP1-PPCA construct have been described previously [25]. In brief, the vector contains a liver-specific regulatory element that consists of the core domains from the human apolipoprotein hepatic control region and the human *alpha-1-antitrypsin* gene promoter [33] which drives the hepatocyte-exclusive expression of the 1.44-kb human *PPCA* cDNA

[7]. The scAAV vector particles are made using a GMP process-comparable transient-transfection procedure in the Children's GMP, LLC facility on the St. Jude campus, as previously described for the hemophilia B vector [34].

2.7. Experimental design, injection of AAV, and collection of tissues and urine

A single dose of 2.6×10^{11} vector genomes of scAAV2/8LP1-PPCA was injected into the tail veins of four 16-month-old female KO/TG mice. This dose was identical to the highest and optimal rAAV-dose used previously in GS mice [25]. Four female WT/TG mice were not injected and 4 female KO/TG mice were mock-injected with a sterile saline solution. The mice were monitored for 4 weeks post injection, after which they were euthanized. Urine was collected prior to treatment and again just before euthanasia. Eleven tissues (BM, liver, kidney, spleen, lung, skeletal muscle, heart, salivary gland, brain, and ovary) were collected at the time of euthanasia. Tissues were fixed in 10% neutralized formalin and paraffin-embedded for histologic and IHC analyses or frozen in liquid nitrogen for biochemical analyses.

2.8. Cathepsin A and NEU1 activity assays, Western blot analysis, and sialic acid detection assay

NEU1 and cathepsin A enzyme assays and Western blot analysis were performed on lysates of fibroblasts and macrophages maintained in culture. Briefly, 2 pieces of frozen tissue of all rAAV-treated KO/TG mice, as well as that of untreated WT and KO/TG controls, were homogenized on ice in 3 volumes of water (w/v) using an Omni-Prep Multi-Sample Homogenizer and soft-tissue disposable grinders (Omni International, Kennesaw, GA). Frozen cell pellets were lysed by hypotonic shock in ice-cold water. NEU1 catalytic activity was measured against synthetic 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (Sigma-Aldrich, St. Louis, MO) [35], and cathepsin A activity was measured against the dipeptide Z-Phe-Ala [8]. Tissue homogenate and cell lysate samples (5–30 μg) were resolved on 4% to 12% gradient polyacrylamide gels under denaturing and reducing conditions, transferred onto Hybond-PVDF membranes (Amersham, Piscataway, NJ), and incubated with affinity-purified anti-PPCA and peroxidase-conjugated goat–anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The enzyme was visualized using Super Signal West Femto Chemiluminescence reagent (Thermo Scientific, Rockford, IL) and the Chemi-Doc imaging system (Bio-Rad, Hercules, CA).

Aliquots of bone marrow extracellular fluid (BMEF; 30 μL) isolated from femurs and tibias of KO/TG mice, as described previously [36], were analyzed on Western blots using the Lamp1-specific antibody. The total sialic acid content in mouse kidney homogenates was measured using the Enzychrom Sialic Acid Assay Kit, according to the manufacturer's instructions (BioAssay Systems, Hayward, CA).

2.9. Immunohistochemical analysis of mouse tissue sections

Paraffin-embedded tissue sections (6-μm thick) were subjected to deparaffinization and antigen retrieval using standard histology methods. After blocking with 0.1% bovine serum albumin and 0.5% Tween 20, the sections were incubated overnight at room temperature with anti-PPCA antibody [37]. The sections were washed and incubated with biotinylated secondary goat anti-rabbit antibody (Jackson ImmunoResearch Laboratory) for 1 h. Endogenous peroxidase was removed by incubating the sections with 0.1% hydrogen peroxide for 30 min. Antibody detection was performed using the ABC Kit and diaminobenzidine substrate (Vector Laboratories, Burlingame, CA), and sections were counterstained with hematoxylin according to standard method. For histopathologic examination, paraffin-embedded tissues from all 96 mice were cut into 6-μm thick sections and stained using a standard H&E method.

2.10. Fluorophore assisted carbohydrate electrophoresis (FACE) analysis

Urine samples (10 μL) were tested for the presence of undegraded HMWOs using the FACE urinary carbohydrate analysis kit, according to the manufacturer's protocol (Glyko, Novato, CA).

3. Results

3.1. Low mutant V54M-NEU1 enzyme activity is restored by exogenous PPCA

In previous in vitro studies we showed that mutant and wild-type (WT) NEU1 activity could be substantially augmented by increasing the concentration of its chaperone PPCA [3,5,29]. Based on these initial observations, we decided to design a proof-of-principle in vivo study for the potential use of PPCA, instead of NEU1, to treat non-neuropathic patients with the type I form of sialidosis. We have chosen the V54M-NEU1 as representative mutation of type I sialidosis because this NEU1 variant retains a residual activity that in the patient's fibroblasts was shown to respond well to increased levels of PPCA, and this mutation was identified in an adult patient with a mild form of the disease [5]. To further validate that missense V54M-NEU1 would respond to increased PPCA levels, we transduced mouse *Neu1*$^{-/-}$ (KO) fibroblasts with bicistronic MSCV-NEU1-GFP retroviral constructs expressing either WT-, V54M-, or Y370C-NEU1 with or without coexpression of MSCV-PPCA-GFP. Y370C-NEU1, which was included as a negative control, is a catalytically inactive mutant previously identified in a patient with congenital type II sialidosis [5].

Expression of NEU1 and PPCA in MSCV-transduced cells was first tested on Western blots that showed sustained expression levels of both enzymes (Fig. 1a, lower panel). Coexpression of PPCA and WT-NEU1 resulted in a 3-fold increase in NEU1 activity compared to cells transduced with the WT-NEU1 vector alone (Fig. 1a). V54M-NEU1-expressing fibroblasts showed a similar, albeit overall smaller increase in NEU1 activity in MSCV-PPCA-coexpressing cells (Fig. 1a). In contrast, “catalytically dead” Y370C-NEU1-expressing fibroblasts showed no increase in NEU1 activity when coexpressed with PPCA (Fig. 1a).

We next tested whether instead of de novo synthesized PPCA the endocytic uptake of the enzyme would have a similar stimulatory effect on the NEU1 activity. We have previously demonstrated in the GS mouse model that macrophages internalized baculovirus (BV)-expressed PPCA via the mannose-receptor, thereby efficiently restoring endogenous NEU1 [28]. Therefore, we transduced KO macrophages with the same WT and mutant MSCV-NEU1 vectors, and incubated these cells in the presence or absence of insect cell-produced PPCA precursor (Fig. 1b, lower panel). Analogously to the fibroblast experiment, after uptake of PPCA both WT-NEU1- and V54M-NEU1-expressing macrophages showed a substantial increase in NEU1 activity, whereas Y370C-NEU1-expressing cells did not (Fig. 1b).

3.2. Generation of an accurate mouse model of type I sialidosis

These initial results suggest that PPCA-mediated chaperone therapy could be beneficial for some of the attenuated non-neuropathic forms of sialidosis. To be able to test such therapy in vivo, we engineered a mouse model expressing V54M-NEU1. In mammals NEU1 is ubiquitously, albeit differentially, expressed in various organs and cell types, with highest expression in the kidney [38]. We opted to use the ubiquitin promoter to drive the expression of the V54M-Neu1 transgene instead of the endogenous NEU1 promoter, because it is unknown what size of the endogenous NEU1 promoter should be used to ensure general and sustained expression of the transgene

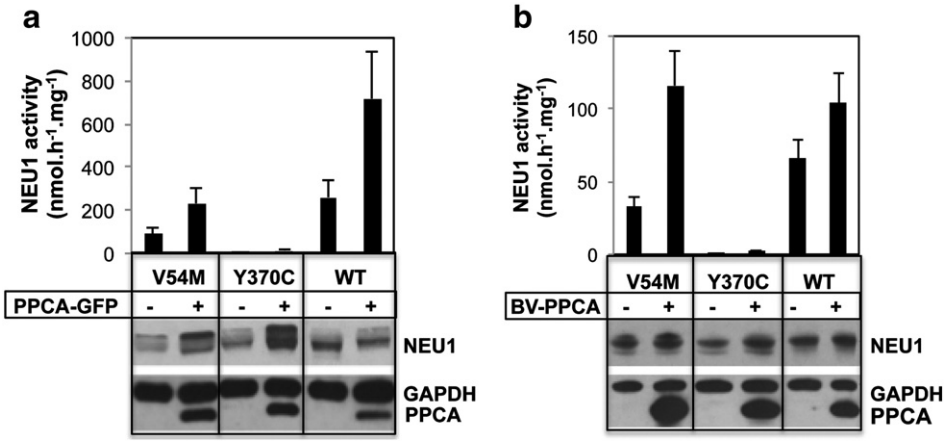

Fig. 1. Rescue of *NEU1* mutations by human PPCA. (a) Murine *Neu1*$^{-/-}$ fibroblasts were transduced with MSCV-based retroviral vectors containing the cDNAs for V54M, Y370C, or WT NEU1 and the GFP marker. GFP-expressing cells were sorted and transduced with an MSCV-PPCA-GFP (PPCA-GFP) retrovirus (+) or incubated with control medium (−). Cells (n = 3) were assayed for NEU1 activity and analyzed on Western blots for NEU1, PPCA, and GAPDH levels. (b) *Neu1*$^{-/-}$ macrophages in culture were transduced with the same MSCV-based NEU1 retroviral vectors and incubated with growth medium (−) or with growth medium containing recombinant BV-PPCA (+) for 48 h. Samples (n = 3) were analyzed as in (a). Error bars indicate standard deviation.

and because to date only limited experimental data are present in the literature that describe the regulatory elements driving NEU1 expression [39]. Instead, the ubiquitin promoter was previously shown to generate robust transgene expression in all major tissues and organs [31]. A *NEU1*^{V54M} transgenic founder was crossed into the *Neu1*$^{-/-}$ (KO) FVB background to generate a new sialidosis knockout transgenic (KO/TG) mouse strain, *Neu1*$^{-/-}$;*NEU1*^{V54M}. These mutant mice were viable and fertile with a gross-normal appearance. They also lacked any obvious neurologic involvement and lived a normal lifespan. Early disease hallmarks of 2- to 6-week-old KO mice (i.e., edema and enlarged kidneys, liver, and spleen) were absent in KO/TG mice as old as 1 year. However, KO/TG mice that were euthanized between 1 and 2 years of age had developed edema and enlarged kidneys similar to what was observed in young KO mice [16,17]. Therefore, the KO/TG mice appear to be a suitable delayed-onset model for non-neuropathic type I sialidosis.

3.3. NEU1 activity is reduced in KO/TG mice

We measured the NEU1 activity in tissue homogenates of adult KO/TG mice and *Neu1*$^{+/+}$;*NEU1*^{V54M} wild-type transgenic (WT/TG) controls (Fig. 2a). The residual NEU1 activity was reduced in all KO/TG organs; however, the extent of reduction varied greatly (Fig. 2a). Low enzyme activity was detected in the BM, liver, salivary glands, and kidney of KO/TG mice (Fig. 2a). In the latter tissue, residual NEU1 activity was only 1% compared to WT/TG control values (Fig. 2a). In the heart, spleen, lung, and brain, the residual NEU1 activity varied between 30% and 80% of WT/TG control values (Fig. 2a). The normal appearance and absence of visceral organ and neurologic abnormalities in adult KO/TG mice younger than 1 year of age indicated that the residual mutant NEU1 activity was sufficient to prevent the onset of the disease in most organs. It is noteworthy that in the KO mice, the complete loss of Neu1 resulted in substantially increased catalytic activity of its chaperone PPCA in virtually all tissues and cell types tested (not shown). In contrast, cathepsin A activity was normal in the organs of KO/TG mice (Fig. 2b), indicating that the partial loss of NEU1 causes biochemical and phenotypical effects that are distinct from those caused by complete loss of the enzyme.

3.4. Adult KO/TG mice show signs of late-onset non-neuropathic sialidosis

The complete deficiency of Neu1 in Neu1-null mice results in exacerbated lysosomal exocytosis in the BM, evident by the aberrant accumulation of active serine proteases and glycosidases in the BM extracellular fluid (BMEF), resulting in the loss of BM-progenitors, and the occurrence of extramedullary hematopoiesis and splenomegaly [17]. A marker of excessive lysosomal exocytosis in KO mice is the presence of large quantities of Lamp1 in the particulate fraction of BMEF (Fig. 3a, lane 1). In contrast, despite the dramatic reduction in Neu1 activity in the BM (Fig. 2a) and the severely reduced amount of Neu1 in BM-derived macrophages (Fig. 3b, lane 3), the BMEF-particulate fraction of KO/TG mice as old as 27 months of age contained low amounts of Lamp1, similar to that in WT/TG mice (Fig. 3). This result indicates that KO/TG BM cells are not excessively exocytic and explains the absence of enlarged spleens in these mice.

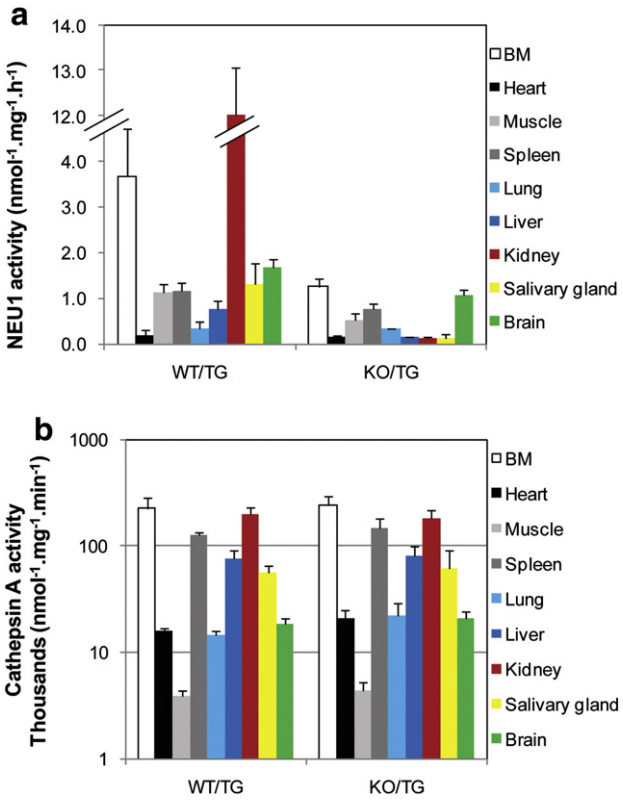

Fig. 2. Measurement of cathepsin A and Neu1 activities in tissues of KO/TG mice. (a) NEU1 and (b) cathepsin A activities assayed in various tissue homogenates from KO/TG (n = 4) or WT/TG (n = 4) mice. Error bars indicate standard deviation.

In *Neu1*-null mice, progressive accumulation of undegraded substrates is evidenced by the extensive lysosomal vacuolization in all visceral organs and the brain, resulting in gross morphologic tissue abnormalities [16,17]. in contrast, hematoxylin and eosin (H&E) staining of KO/TG tissue sections showed normal morphology in all organs in mice as old as 1 year (not shown). However, in older KO/TG mice lysosomal vacuolization was prominent in the glomeruli and Bowman's capsules of the kidney which was comparable to that seen in sections from 3-month-old KO mice (Fig. 4). Vacuolization was not detected in the brains of KO/TG mice (not shown), which correlated with relatively high residual Neu1 activity (Fig. 2a; 65% of WT) and the absence of overt neurological signs [16].

Although H&E staining enabled us to analyze tissue morphology and detect abnormal vacuolization typical of LSDs, it is not quantitative and lacks the sensitivity to assess an attenuated disease phenotype. A more sensitive diagnostic method is the detection of high-molecular weight oligosaccharides (HMWOs) in the urine of patients and mouse models of LSDs [25]. Therefore, we analyzed the urine of 5- to 25-month-old KO/TG mice for the presence of HMWOs by using fluorophore-assisted carbohydrate electrophoresis (FACE). HMWOs were present in the urine of KO/TG mice as young as 5 months of age but were most abundant in urine of a 25-month-old mouse (Fig. 4b). However, the amount of HMWOs in the latter mouse did not reach that observed in a 4-month-old KO mouse (Fig. 4b). Nevertheless, FACE analysis detected the onset of oligosaccharidiuria in KO/TG mice at least 6 months before it could be detected by H&E staining of tissue sections. We conclude that KO/TG mice represent a faithful model of attenuated type I sialidosis, suitable to test PPCA-mediated chaperone therapy.

3.5. Uptake of PPCA by KO/TG macrophages increases NEU1 activity to WT levels

We previously showed that the secondary Neu1 defect in *Ppca*-null mice was rescued upon restoration of PPCA expression [25]. To assess whether KO/TG mice would be receptive to chaperone therapy with PPCA, we first performed in vitro uptake assays with BV-expressed PPCA. BM-derived macrophages isolated from KO/TG, KO, $Ppca^{-/-}$, and WT mice were cultured in the presence or absence of increasing amounts of BV-expressed PPCA precursor [28]. Cells were then analyzed for increased cathepsin A and NEU1 activities (Fig. 5a and c). Efficient uptake of PPCA was confirmed by Western blot analysis of macrophage lysates using a PPCA polyclonal antibody (Fig. 5c). All 4 primary macrophage cultures showed a concentration-dependent increase in cathepsin A activity, which reached a 10 fold increase in KO/TG and KO macrophages (Fig. 5a). Moreover, after uptake of exogenous PPCA NEU1 activity in both $Ppca^{-/-}$ and KO/TG macrophages was comparable to that in untreated WT macrophages (Fig. 5c).

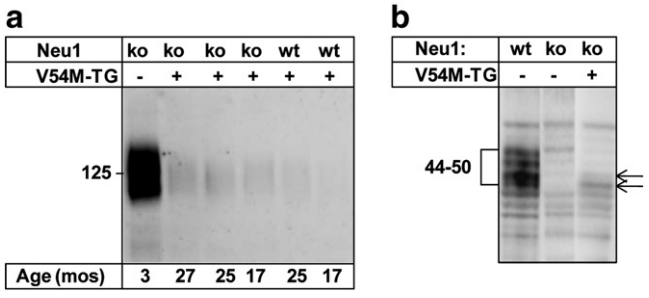

Fig. 3. Western blot analysis of BMEF and BM-derived macrophages. (a) The amount of Lamp1 in BMEF (30 μg) of KO/TG (ko; +) mice (17–27 months old) is comparable to that in 17–25 month-old WT/TG $Neu1^{-/-}$ (wt; +) mice. (b) Western blot analysis of KO/TG macrophages using a Neu1-specific polyclonal antibody shows a dramatic decrease in the expression of Neu1 compared to WT (wt; −) cells. As expected, KO macrophages are devoid of Neu1.

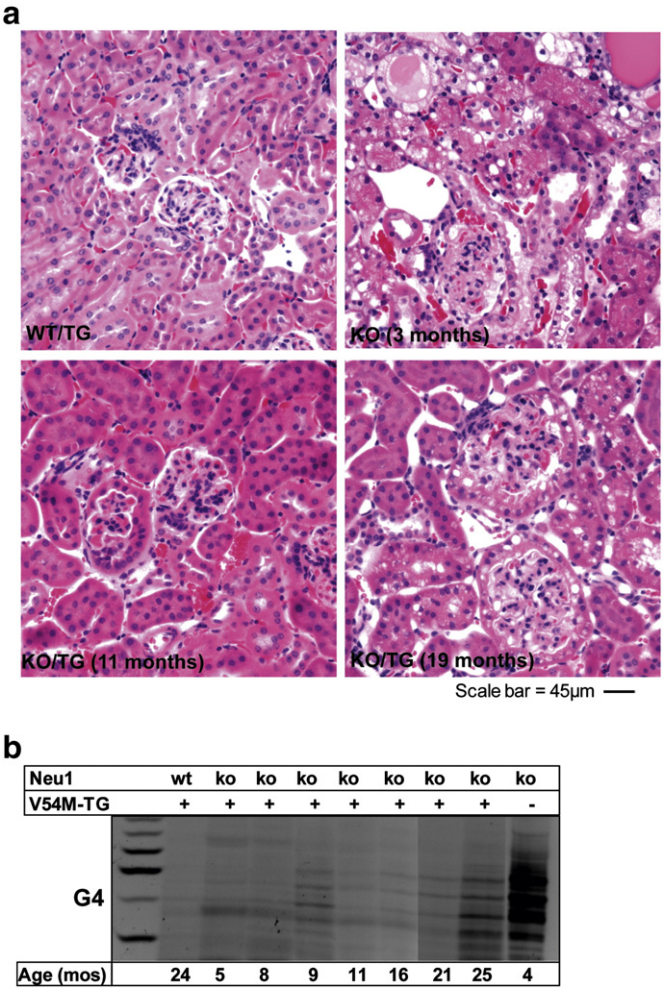

Fig. 4. Late onset of sialidosis in KO/TG mice. (a) H&E staining shows normal kidney morphology in an 11-month-old KO/TG mouse but extensive vacuolization in the glomeruli and Bowman's capsules in a 19-month-old KO/TG mouse, comparable to that seen in a 3-month-old KO mouse with sialidosis. (b) FACE analysis of urine from KO/TG (ko; +) mice (5–25 months old) shows elevated levels of HMWOs, namely glucose-4 (G4). However, the level of HMWOs in urine from a 4-month-old KO (ko; −) mouse with sialidosis is several-fold higher compared to that in a 25-month-old KO/TG mouse. This is consistent with a mild sialidosis phenotype in KO/TG mice.

3.6. ScAAV2/8LP1-PPCA-treated KO/TG mice express high levels of exogenous PPCA

We then assessed whether NEU1 activity could be increased in vivo by treating KO/TG mice with exogenous PPCA. Tissues in KO/TG mice have sufficient endogenous cathepsin A activity to allow for a late onset disease phenotype. Based on our in vitro studies, we predicted that increasing the levels of PPCA in these mice could prevent or ameliorate their disease symptoms. Pilot experiments with tail vein injections of Chinese hamster ovary- or BV-expressed PPCA in young adult KO/TG mice resulted in a moderate (10%–30%) increase in cathepsin A activity in the visceral organs. However, this level of activity did not increase the residual activity of the mutant NEU1 enzyme (not shown), suggesting that this type of approach would not be an effective therapy.

In a recent preclinical gene therapy trial in $Ppca^{-/-}$ mice, we used the liver-specific, scAAV2/8LP1 PPCA-expression vector to correct the disease phenotype [25]. We showed that the liver-produced PPCA zymogen was secreted and efficiently taken up by cells of most systemic organs, restoring both cathepsin A and Neu1 activities. Therefore, we decided to also treat a cohort of KO/TG mice older than 1 year of age with a single dose tail vein injection (2.6×10^{11} vector genomes/

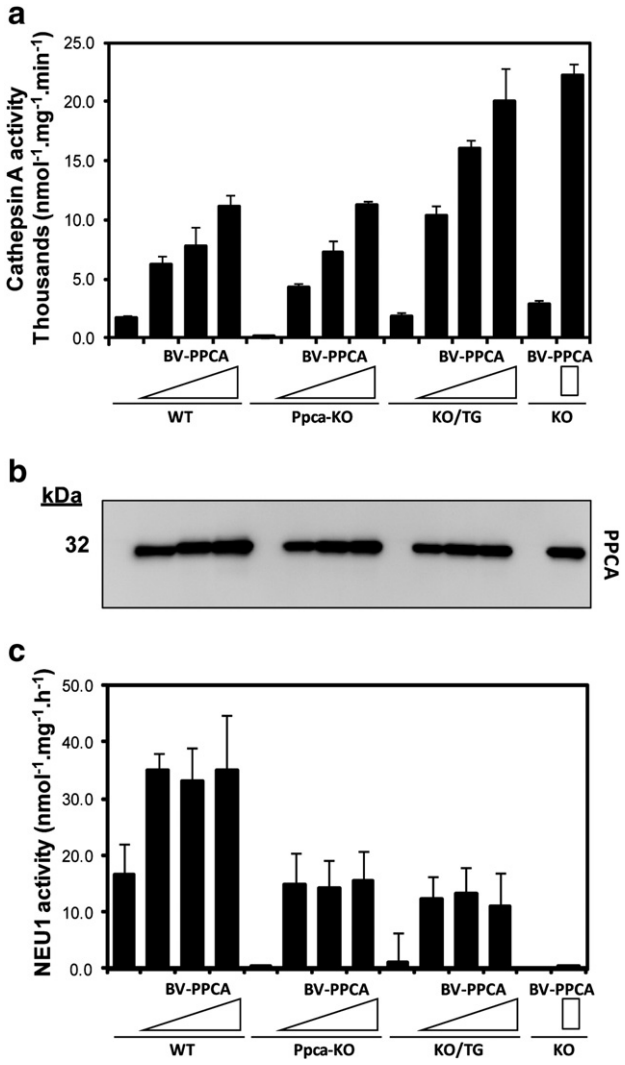

Fig. 5. Uptake of BV-PPCA by KO/TG BM-macrophages rescues mutant NEU1 activity. KO/TG (n = 3), WT (n = 3), and *Ppca*-KO (n = 3) macrophages that were maintained in culture for 2 days in the presence of increasing amounts of BV-PPCA (3, 6, 9 μg) (a) show a PPCA-dose dependent increase in cathepsin A activity. (b) The amount of PPCA detected on a Western blot correlates with cathepsin A activity in the corresponding cells. (c) KO/TG macrophages maintained in culture in the presence of BV-PPCA show a dramatic increase in mutant NEU1 activity that is comparable to that in WT cells and in BV-PPCA-treated *Ppca*-KO macrophages. KO macrophages, which were used as a negative control, show no increase in NEU1 activity because they lack endogenous NEU1. Error bars indicate standard deviation.

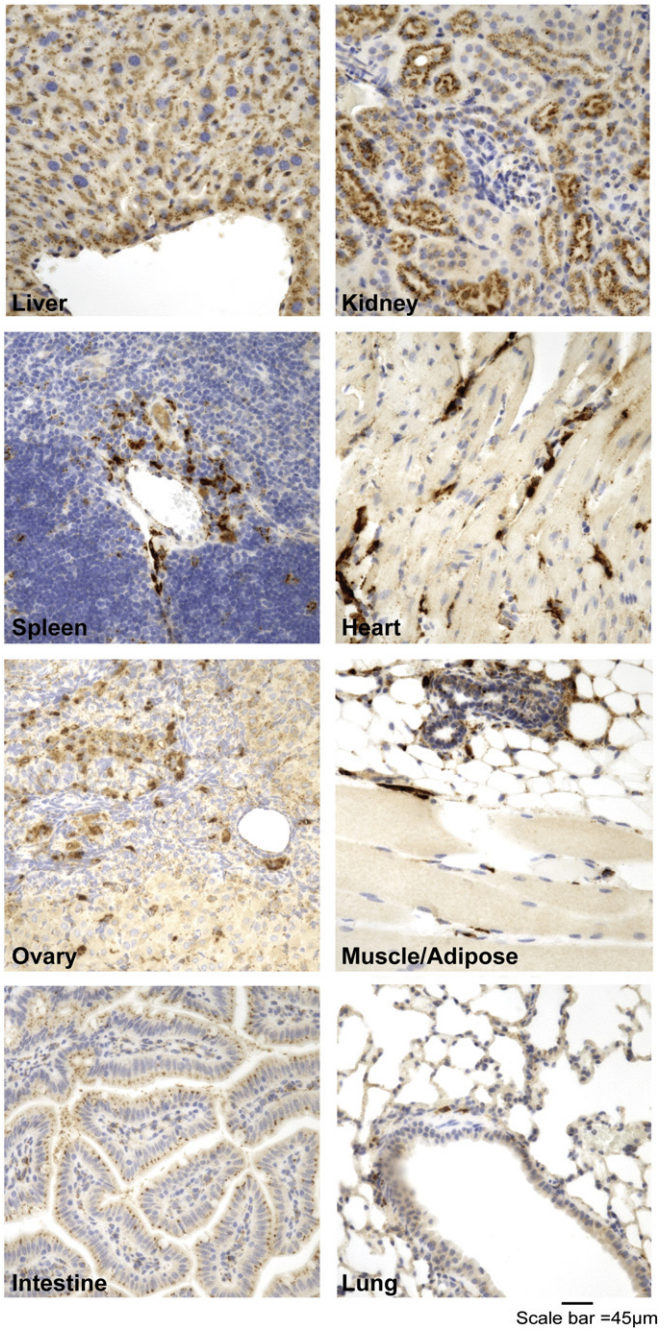

Fig. 6. Immunohistochemical analysis of PPCA in systemic organs of scAAV2/8LP1-PPCA-treated KO/TG mice. A human PPCA-specific antibody readily detects the enzyme (brown punctate staining) in various tissue sections of scAAV2/8LP1-PPCA-treated mice.

mouse) of scAAV2/8LP1-PPCA. We used old mice because they showed clear signs of kidney disease, making it possible to assess whether a short-term gene therapy treatment reversed the phenotype. Treated mice were euthanized 1 month after AAV injection. Immunohistochemical analysis (IHC) using a polyclonal antibody specific for human PPCA showed widespread high levels of PPCA expression in various organs and tissues, including the liver, kidney, spleen, heart, intestine, adipose tissue, and muscle (Fig. 6). However, as anticipated, PPCA was not detected in the brain (not shown). In agreement with the IHC results, cathepsin A activity in the tissue lysates of the AAV-injected mice showed an 86-fold increase in the liver, 9-fold in the BM, 4.4-fold in the spleen, 3.6-fold in the heart, 2.4-fold in the skeletal muscle, 1.9-fold in the kidney, and no increase in the brain (Fig. 7a). Western blot analysis using a human PPCA-specific antibody confirmed the robust expression of exogenous PPCA in the various tissue lysates (Fig. 7b).

3.7. ScAAV2/8LP1-PPCA-treated KO/TG mice express increased levels of mutant NEU1 activity

In concordance with the increase in cathepsin A activity in AAV-PPCA-treated mice, the NEU1 activity was increased in all tested organs, except the brain (Fig. 7c). The average increase in NEU1 activity in tissues of rAAV-PPCA-treated mice was 1.9 fold. Unexpectedly, tissues with the highest increase in cathepsin A activity (liver, BM, and spleen) showed only a moderate 1.3- to 1.6-fold increase in NEU1 activity, whereas tissues with a lower increase in cathepsin A activity (kidney, muscle, heart) showed higher NEU1 activity levels (Fig. 7c). We reasoned that, because the endogenous cathepsin A activity in WT and untreated KO/TG mice was already very high

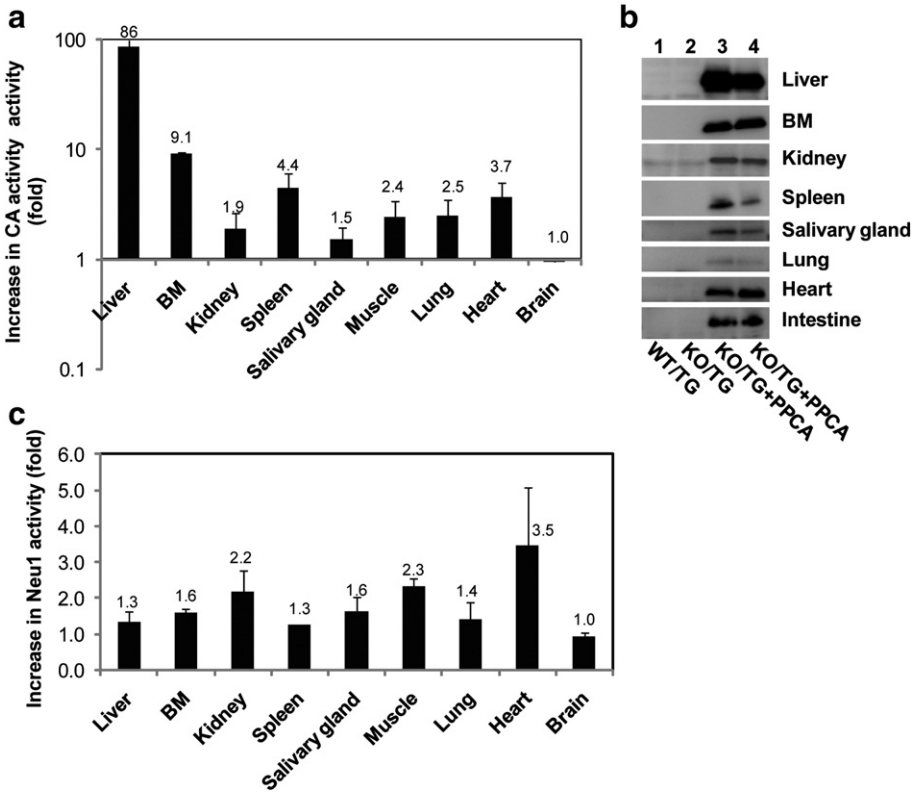

Fig. 7. Cathepsin A and NEU1 activities in scAAV2/8LP1-PPCA-treated KO/TG mice. Cathepsin A and NEU1 activities were measured in tissue homogenates of scAAV2/8LP1-PPCA-treated KO/TG mice (n = 4) and untreated KO/TG (n = 4) control mice. The (a) cathepsin A and (c) NEU1 activities are higher in tissues from rAAV-treated KO/TG mice than in the corresponding untreated KO/TG mice. Error bars indicate standard deviation. (b) Immunoblot analysis of PPCA in tissues homogenates of 2 scAAV2/8LP1-PPCA-treated KO/TG (KO/TG + PPCA) mice and untreated KO/TG and WT/TG controls.

($\geq$100 nmol·min^{-1}·mg^{-1}) in the liver, BM, and spleen (Fig. 2b), the concentration of the enzyme in these tissues was most likely already near the saturation level. This could explain why the rAAV-PPCA-mediated increase in the amount of PPCA above the saturation level resulted only in a modest increase in NEU1 activity (Fig. 7b). In contrast, in the muscle and heart of WT and untreated KO/TG mice, the level of endogenous cathepsin A activity was relatively low ($\leq$15 nmol·min^{-1}·mg^{-1}) and thus less likely to be saturated (Fig. 2b).

3.8. ScAAV2/8LP1-PPCA-treated KO/TG mice have reduced vacuolization in the kidney

Chaperone-mediated gene therapy with scAAV2/8LP1-PPCA increased residual NEU1 activity in non-neurogenic tissues by about 2 fold; thus, we questioned whether this treatment would be sufficient to prevent or reverse vacuolization in the kidney. H&E staining of kidney sections from treated KO/TG mice showed that the glomeruli and Bowman's capsules had reduced vacuolization (Fig. 8a). However, urine from scAAV2/8LP1-PPCA-treated KO/TG mice that was collected post-treatment still contained elevated levels of HMWOs (Fig. 8b). The total (free and bound) sialic acid content in kidney lysates from KO/TG mice was only marginally reduced in scAAV2/8LP1-PPCA-treated KO/TG samples (Fig. 8c). Despite the modest decrease in sialic acid content in the most affected organ, the kidney, the morphology of the tissue appeared considerably improved (Fig. 8a). Because the treated KO/TG mice were old, we think that the overall effects of scAAV-PPCA treatment on the mutant NEU1 activity and improved kidney morphology are very encouraging.

4. Discussion

Here we describe the generation of a new mouse model of the non-neuropathic attenuated type I form of sialidosis, carrying a V54M amino acid substitution identified in a type I sialidosis patient [5]. We used these type I sialidosis mice in an explorative scAAV-based PPCA-mediated chaperone gene therapy study. We first showed that in this mouse model, as it is the case for type I sialidosis patients, disease symptoms and progression occur later in life (>1 year of age) and that the disease is mild and non-neuropathic [12]. Because of the known dependence of NEU1 on its chaperone PPCA for efficient lysosomal routing and intralysosomal activity and stability [1], we hypothesized that raising the concentration of PPCA above endogenous levels, could potentially increase the stability and residual activity of mutant forms of NEU1. As a consequence, the mutant NEU1 activity could be raised near or above the disease threshold level, thereby slowing or reversing the lysosomal accumulation of undegraded substrates.

We recently used the scAAV2/8LP1-PPCA vector in a comprehensive preclinical dose-escalation study in *Ppca*-null mice, a model of GS [25]. In this study we showed that, although the expression of the corrective enzyme was restricted to the liver in the treated mice, the circulating zymogen was efficiently internalized by cells of virtually all visceral organs, thereby restoring endogenous PPCA and NEU1 activity. This resulted in complete reversal of visceral organ histopathology, clearance of HMWOs from urine, and restoration of fertility. This AAV-based dose-escalation study in GS mice set the stage for PPCA-mediated chaperone gene therapy in type I sialidosis mice that, unlike *Neu1*-null mice, have residual mutant NEU1 activity in all organs.

We first proved that in vitro augmentation of PPCA expression in murine type I sialidosis macrophages and fibroblasts expressing V54M-NEU1 raised the mutant NEU1 activity near WT levels. Next, in choosing the deficient mice to be treated with the AAV-PPCA vector, we considered potential age effects on the curative efficacy. For instance, the AAV-treatment of neonatal Fabry mice was shown to be more effective than similar treatment of adult Fabry mice [40]. However, we also had to consider that the most likely candidates

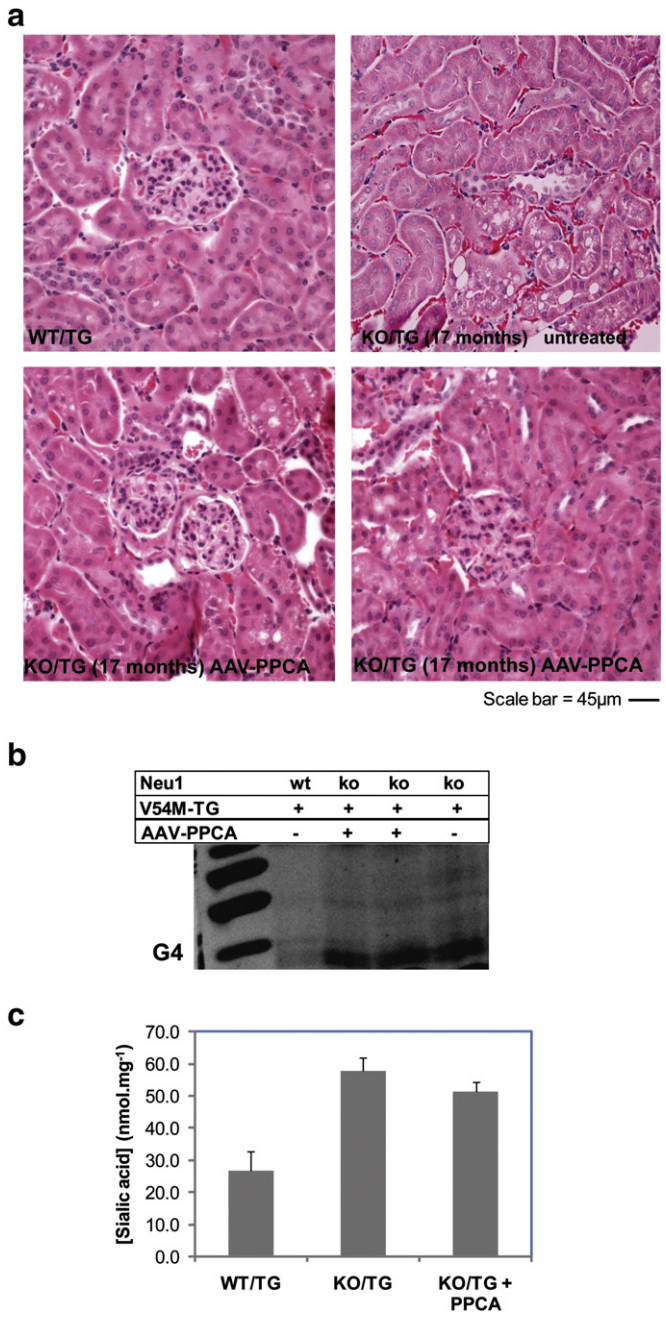

Fig. 8. Kidney morphology in scAAV2/8LP1-PPCA-treated KO/TG mice. (a) H&E staining shows vacuolization in the glomeruli and Bowman's capsules of an untreated KO/TG mouse but little vacuolization in the kidneys of 2 rAAV-PPCA-treated mice. (b) FACE analysis of urine from 2 rAAV-PPCA-treated KO/TG (ko; +; +) mice shows comparable amounts of HMWOs compared to that in untreated KO/TG mice (ko; +; −). (c) Measurement of the total sialic acid content in the kidneys of rAAV-PPCA-treated KO/TG mice (n = 4) shows a 12% decrease in the level of sialic acid compared to that in untreated KO/TG mice (n = 4). Although the decrease in sialic acid content was not statistically significant, the experiment was performed in triplicate and consistently showed a 10% to 12% decrease in sialic acid content in the kidneys of rAAV-PPCA-treated KO/TG mice. Error bars indicate standard deviation.

for PPCA-chaperone therapy would be symptomatic non-neuropathic juvenile and adult patients. Therefore, we chose to treat 16-month-old symptomatic type I sialidosis mice with a single tail vein injection of high-dose scAAV2/8LP1-PPCA vector and assessed the effects of treatment 1 month later. Treated mice showed increased PPCA expression in all organs, except the brain, and consequently, an average of about 2-fold higher residual NEU1 activity than untreated mice. Although this was not enough to completely reverse the lysosomal storage phenotype, it reduced vacuolization and improved tissue morphology in the kidney.

Small synthetic iminosugars are active site-specific compounds that help fold mutated lysosomal glycosidases and allow more enzyme to pass the endoplasmic reticulum-quality control systems and be routed to the lysosomal compartment [21]. However, this strategy will be effective only for mutations that do not involve catalytic site residues. PPCA appears to play a similar physiological role for NEU1, with the added benefit of a 2-fold action: first, PPCA binds to NEU1 early after synthesis to form a heterodimeric complex and as such is routed to the lysosomal compartment. Second, in the lysosomal compartment, complex formation between the 2 enzymes further promotes catalytic activation and stability of NEU1 [1–3]. This approach would not be effective for mutations that involve the catalytic site of NEU1, such as the Y370C mutation identified in a type II sialidosis patient [5], or for those mutations responsible for both visceral organ as well as CNS-pathology in type II sialidosis patients. However, the majority of the NEU1 missense mutations do not involve the catalytic site; therefore, we propose that besides the V54M mutation used in this proof of principle study, also other sialidosis patients with different NEU1 missense mutations could potentially benefit from PPCA-mediated chaperone therapy [5,6]; approximately 50% of patients with sialidosis have the attenuated non-neuropathic type I form of the disease, and they carry at least one of the 11 *NEU1* mutant alleles (400dupHY, E377X, V54M, L111P, V217M, G219A, V275A, R294S, P316S, G328S, T345I) known to confer this phenotype [6]. Because the available amount of PPCA appears to be rate limiting for the level of NEU1 activity, it is unclear whether synthetic pharmacologic chaperones could also be effective in increasing mutant NEU1 levels. Our data indicate that it may be worthwhile to explore strategies aimed at enhancing PPCA expression pharmacologically.

We have also considered using an AAV-based gene therapy approach to treat the type I sialidosis with a scAAV2/8LP1 vector expressing NEU1 instead of PPCA. However, we do not think this would be a better option because, unlike PPCA, NEU1 is poorly mannose-6-phosphorylated, which would result in poor internalization of the liver-produced and secreted enzyme by cells in other systemic organs [1]. In addition, we have previously shown that the NEU1 enzyme is immunogenic, which would raise concerns about the development of adverse immunologic reactions in patients [41]. Therefore, the PPCA-mediated chaperone gene therapy described here could be the best therapeutic option for some patients with type I sialidosis, who could be enrolled in an anticipated scAAV2/8LP-PPCA gene transfer trial designed for non-neuropathic GS patients.

Acknowledgements

We thank Dr. Gerard Grosveld and Mr. John Raucci of the Transgenic/Gene Knockout Facility of St. Jude Children's Research Hospital for generating the mouse transgenic founders used in this study. We thank Drs. John Gray and Jim Allay for constructing the scAAV2/8LP1-PPCA vector and producing the GMP-grade virus. We are grateful to Dr. Angela McArthur for editing this manuscript. A. d'A. holds the Jeweler's Charity Fund Endowed Chair in Genetics & Gene Therapy. This work was supported by grants from the National Institutes of Health (DK52025, GM60905), the Assisi Foundation of Memphis, and the American Lebanese Syrian Associated Charities (ALSAC).

References

[1] A. van der Spoel, E. Bonten, A. d'Azzo, Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A, EMBO J. 17 (1998) 1588–1597.
[2] E.J. Bonten, A. d'Azzo, Lysosomal neuraminidase. Catalytic activation in insect cells is controlled by the protective protein/cathepsin A, J. Biol. Chem. 275 (2000) 37657–37663.

[3] E.J. Bonten, Y. Campos, V. Zaitsev, A. Nourse, B. Waddell, W. Lewis, G. Taylor, A. d'Azzo, Heterodimerization of the sialidase NEU1 with the chaperone protective protein/cathepsin A prevents its premature oligomerization, J. Biol. Chem. 284 (2009) 28430–28441.
[4] E. Bonten, A. van der Spoel, M. Fornerod, G. Grosveld, A. d'Azzo, Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis, Genes Dev. 10 (1996) 3156–3169.
[5] E.J. Bonten, W.F. Arts, M. Beck, A. Covanis, M.A. Donati, R. Parini, E. Zammarchi, A. d'Azzo, Novel mutations in lysosomal neuraminidase identify functional domains and determine clinical severity in sialidosis, Hum. Mol. Genet. 9 (2000) 2715–2725.
[6] V. Seyrantepe, H. Poupetova, R. Froissart, M.T. Zabot, I. Maire, A.V. Pshezhetsky, Molecular pathology of NEU1 gene in sialidosis, Hum. Mutat. 22 (2003) 343–352.
[7] N.J. Galjart, N. Gillemans, A. Harris, G.T. van der Horst, F.W. Verheijen, H. Galjaard, A. d'Azzo, Expression of cDNA encoding the human "protective protein" associated with lysosomal beta-galactosidase and neuraminidase: homology to yeast proteases, Cell 54 (1988) 755–764.
[8] N.J. Galjart, H. Morreau, R. Willemsen, N. Gillemans, E.J. Bonten, A. d'Azzo, Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function, J. Biol. Chem. 266 (1991) 14754–14762.
[9] A. d'Azzo, G. Andria, P. Strisciuglio, H. Galjaard, Galactosialidosis, in: C. Scriver, A. Beaudet, W. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, vol. 3, McGraw-Hill Publishing Co, New York, 2001, pp. 3811–3826.
[10] A. d'Azzo, Protective Protein Cathepsin A (PPCA), Wiley Encyclopedia of Molecular Medicine, John Wiley & Sons, 2002. 2624–2628.
[11] A. d'Azzo, E. Bonten, Molecular mechanisms of pathogenesis in a glycosphingolipid and a glycoprotein storage disease, Biochem. Soc. Trans. 38 (2010) 1453–1457.
[12] G.H. Thomas, Disorders of glycoprotein degradation and structure: a-mannosidosis, b-mannosidosis, fucosidosis, and sialidosis, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, vol. III, McGraw Hill, Inc., New York, 2001, pp. 3507–3534.
[13] A. Caciotti, M. Di Rocco, M. Filocamo, S. Grossi, F. Traverso, A. d'Azzo, C. Cavicchi, A. Messeri, R. Guerrini, E. Zammarchi, M.A. Donati, A. Morrone, Type II sialidosis: review of the clinical spectrum and identification of a new splicing defect with chitotriosidase assessment in two patients, J. Neurol. 256 (2009) 1911–1915.
[14] E. Conzelmann, K. Sandhoff, Partial enzyme deficiencies: residual activities and the development of neurological disorders, Dev. Neurosci. 6 (1983) 58–71.
[15] K. von Figura, Molecular recognition and targeting of lysosomal proteins, Curr. Opin. Cell Biol. 3 (1991) 642–646.
[16] N. de Geest, E. Bonten, L. Mann, J. de Sousa-Hitzler, C. Hahn, A. d'Azzo, Systemic and neurologic abnormalities distinguish the lysosomal disorders sialidosis and galactosialidosis in mice, Hum. Mol. Genet. 11 (2002) 1455–1464.
[17] G. Yogalingam, E.J. Bonten, D. van de Vlekkert, H. Hu, S. Moshiach, S.A. Connell, A. d'Azzo, Neuraminidase 1 is a negative regulator of lysosomal exocytosis, Dev. Cell 15 (2008) 74–86.
[18] E. Zanoteli, D.V. de Vlekkert, E.J. Bonten, H. Hu, L. Mann, E.M. Gomero, A.J. Harris, G. Ghersi, A. d'Azzo, Muscle degeneration in neuraminidase 1-deficient mice results from infiltration of the muscle fibers by expanded connective tissue, Biochim. Biophys. Acta 1802 (2010) 659–672.
[19] X. Wu, K.A. Steigelman, E. Bonten, H. Hu, W. He, T. Ren, J. Zuo, A. d'Azzo, Vacuolization and alterations of lysosomal membrane proteins in cochlear marginal cells contribute to hearing loss in neuraminidase 1-deficient mice, Biochim. Biophys. Acta 1802 (2010) 259–268.
[20] R. Desnick, E.H. Schuchman, Enzyme replacement therapy for lysosomal diseases: lessons from 20 years of experience and remaining challenges, Annu. Rev. Genomics Hum. Genet. 13 (2012) 307–335.
[21] L. Urbanelli, A. Magini, A. Polchi, M. Polidoro, C. Emiliani, Recent developments in therapeutic approaches for lysosomal storage diseases, Recent Pat. CNS Drug Discov. 6 (2011) 1–19.
[22] K.J. Valenzano, R. Khanna, A.C. Powe, R. Boyd, G. Lee, J.J. Flanagan, E.R. Benjamin, Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders, Assay Drug Dev. Technol. (2011) 213–235.
[23] J. Jakóbkiewicz-Banecka, E. Piotrowska, M. Gabig-Cimińska, E. Borysiewicz, M. Słomińska-Wojewódzka, M. Narajczyk, A. Węgrzyn, G. Węgrzyn, Substrate reduction therapies for mucopolysaccharidoses, Curr. Pharm. Biotechnol. 12 (2011) 1860–1865.
[24] J.A. Hawkins-Salsbury, A.S. Reddy, M.S. Sands, Combination therapies for lysosomal storage disease: is the whole greater than the sum of its parts? Hum. Mol. Genet. 20 (2011) R54–R60.
[25] H. Hu, E. Gomero, E. Bonten, J.T. Gray, J. Allay, Y. Wu, J. Wu, C. Calabrese, A. Nienhuis, A. d'Azzo, Preclinical dose-finding study with a liver-tropic, recombinant AAV-2/8 vector in the mouse model of galactosialidosis, Mol. Ther. 20 (2012) 267–274.
[26] A.C. Nathwani, E.G. Tuddenham, S. Rangarajan, C. Rosales, J. McIntosh, D.C. Linch, P. Chowdary, A. Riddell, A.J. Pie, C. Harrington, J. O'Beirne, K. Smith, J. Pasi, B. Glader, P. Rustagi, C.Y. Ng, M.A. Kay, J. Zhou, Y. Spence, C.L. Morton, J. Allay, J. Coleman, S. Sleep, J.M. Cunningham, D. Srivastava, E. Basner-Tschakarjan, F. Mingozzi, K.A. High, J.T. Gray, U.M. Reiss, A.W. Nienhuis, A.M. Davidoff, Adenovirus-associated virus vector-mediated gene transfer in hemophilia B, N. Engl. J. Med. 365 (2011) 2357–2365.
[27] M.D. Sklar, A. Tereba, B.D. Chen, W.S. Walker, Transformation of mouse bone marrow cells by transfection with a human oncogene related to c-myc is associated with the endogenous production of macrophage colony stimulating factor 1, J. Cell. Physiol. 125 (1985) 403–412.
[28] E.J. Bonten, D. Wang, J.N. Toy, L. Mann, A. Mignardot, G. Yogalingam, A. D'Azzo, Targeting macrophages with baculovirus-produced lysosomal enzymes: implications for enzyme replacement therapy of the glycoprotein storage disorder galactosialidosis, FASEB J. 18 (2004) 971–973.
[29] D. Wang, S. Zaitsev, G. Taylor, A. d'Azzo, E. Bonten, Protective protein/cathepsin A rescues N-glycosylation defects in neuraminidase-1, Biochim. Biophys. Acta 1790 (2009) 275–282.
[30] X.Y. Zhou, H. Morreau, R. Rottier, D. Davis, E. Bonten, N. Gillemans, D. Wenger, F.G. Grosveld, P. Doherty, K. Suzuki, G.C. Grosveld, A. d'Azzo, Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells, Genes Dev. 9 (1995) 2623–2634.
[31] C. Lois, E.J. Hong, S. Pease, E.J. Brown, D. Baltimore, Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors, Science 295 (2002) 868–872.
[32] F. Grosveld, G. Blom van Assendelft, D.R. Greaves, G. Kollias, Position-independent, high-level expression of the human b-globin gene in transgenic mice, Cell 51 (1987) 975–985.
[33] A.C. Nathwani, J.T. Gray, C.Y. Ng, J. Zhou, Y. Spence, S.N. Waddington, E.G. Tuddenham, G. Kemball-Cook, J. McIntosh, M. Boon-Spijker, K. Mertens, A.M. Davidoff, Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver, Blood 107 (2006) 2653–2661.
[34] J.A. Allay, S. Sleep, S. Long, D.M. Tillman, R. Clark, G. Carney, P. Fagone, J.H. McIntosh, A.W. Nienhuis, A.M. Davidoff, N. A.C., J.T. Gray, Good manufacturing practice production of self-complementary serotype 8 adeno-associated viral vector for a hemophilia B clinical trial, Hum. Gene Ther. 22 (2011) 595–604.
[35] H. Galjaard, Genetic Metabolic Disease: Diagnosis and Prenatal Analysis, Elsevier Science Publishers B.V, Amsterdam, 1980.
[36] J.P. Levesque, Y. Takamatsu, S.K. Nilsson, D.N. Haylock, P.J. Simmons, Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor, Blood 98 (2001) 1289–1297.
[37] C. Hahn, M. Martin, X. Zhou, L. Mann, A. d'Azzo, Correction of murine galactosialidosis by bone marrow-derived macrophages overexpressing human protective protein/cathepsin A under control of the colony-stimulating factor-1 receptor promoter, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14880–14885.
[38] R. Rottier, E. Bonten, A. d'Azzo, A point mutation in the *neu-1* locus causes the neuraminidase defect in the SM/J mouse, Hum. Mol. Genet. 7 (1998) 313–321.
[39] M.J. Champigny, M. Johnson, S.A. Igdoura, Characterization of the mouse lysosomal sialidase promoter, Gene 319 (2003) 177–187.
[40] K. Ogawa, Y. Hirai, M. Ishizaki, H. Takahashi, H. Hanawa, Y. Fukunaga, T. Shimada, Long-term inhibition of glycosphingolipid accumulation in Fabry model mice by a single systemic injection of AAV1 vector in the neonatal period, Mol. Genet. Metab. 96 (2009) 91–96.
[41] D. Wang, E.J. Bonten, G. Yogalingam, L. Mann, A. d'Azzo, Short-term, high dose enzyme replacement therapy in sialidosis mice, Mol. Genet. Metab. 85 (2005) 181–189.