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Examination of a blood-brain barrier targeting β-galactosidase-monoclonal antibody fusion protein in a murine model of GM1-gangliosidosis

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ABSTRACT

GM1-gangliosidosis is a lysosomal disease resulting from a deficiency in the hydrolase β-galactosidase (β-gal) and subsequent accumulation of gangliosides, primarily in neuronal tissue, leading to progressive neurological deterioration and eventually early death. Lysosomal diseases with neurological involvement have limited non-invasive therapies due to the inability of lysosomal enzymes to cross the blood-brain barrier (BBB). A novel fusion enzyme, labeled mTfR-GLB1, was designed to act as a ferry across the BBB by fusing β-gal to the mouse monoclonal antibody against the mouse transferrin receptor and tested in a murine model of GM1-gangliosidosis (β-gal$^{-/-}$). Twelve hours following a single intravenous dose of mTfR-GLB1 (5.0 mg/kg) into adult β-gal$^{-/-}$ mice showed clearance of enzyme activity in the plasma and an increase in β-gal enzyme activity in the liver and spleen. Long-term efficacy of mTfR-GLB1 was assessed by treating β-gal$^{-/-}$ mice intravenously twice a week with a low (2.5 mg/kg) or high (5.0 mg/kg) dose of mTfR-GLB1 for 17 weeks. Long-term studies showed high dose mice gained weight normally compared to vehicle-treated β-gal$^{-/-}$ mice, which are significantly heavier than heterozygous controls. Behavioral assessment at six months of age using the pole test showed β-gal$^{-/-}$ mice treated with mTfR-GLB1 had improved motor function. Biochemical analysis showed an increase in β-gal enzyme activity in the high dose group from negligible levels to 20% and 11% of heterozygous levels in the liver and spleen, respectively. Together, these data show that mTfR-GLB1 is a catalytically active β-gal fusion enzyme *in vivo* that is readily taken up into tissues.

Despite these indications of bioactivity, behavior tests other than the pole test, including the Barnes maze, inverted screen, and accelerating rotarod, showed limited or no improvement of treated mice compared to β-gal$^{-/-}$ mice receiving vehicle only. Further, administration of mTfR-GLB1 was insufficient to create measurable increases in β-gal enzyme activity in the brain or reduce ganglioside content (biochemically and morphologically).

1. Introduction

Lysosomal diseases are a group of rare, inherited, metabolic disorders that are caused by deficiencies in lysosomal proteins, leading to the accumulation of their cellular substrates within the lysosome. Numerous therapeutics have been developed and tested for treating lysosomal diseases, including enzyme replacement therapy (ERT). However, due to their size, lysosomal enzymes are unable to cross the blood-brain barrier (BBB), limiting their effect on the neurological pathology. For this reason, ERT has not been developed for many lysosomal diseases, including GM1-gangliosidosis, which has widespread neurological involvement.

GM1-gangliosidosis results from a deficiency in the lysosomal hydrolase β-galactosidase (β-gal; E.C. 3.2.1.23), encoded by the GLB1 gene.

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β-gal is responsible for the cleavage of terminal β-galactose residues on GM1 and GA1 gangliosides, which are a major component of the central nervous system (CNS). Patients with the most severe form of the disease, the infantile form, exhibit progressive neurodegeneration, including profound hypotonia and weakness, global developmental delay, and skeletal abnormalities. These patients typically never learn to ambulate by crawling or walking. Most patients succumb to the disease by three years of age [1,2]. Patients with the late-infantile and juvenile phenotypes usually learn to walk, but develop ataxia in early childhood and eventually become non-ambulatory. Death commonly occurs in early to mid-childhood in the late-infantile phenotype and in mid- to late-childhood in the juvenile phenotype [3]. Currently, there are no clinically approved treatments for GM1-gangliosidosis.

ERTs have been developed for many lysosomal diseases (reviewed in [4,5]) and high dose ERTs have shown efficacy in achieving neurological benefits in animal models [6]; however, none are currently approved for treating GM1-gangliosidosis. Preclinical experiments using purified [7] and recombinant [8] feline β-gal have been tested *in vitro* as potential therapies for GM1-gangliosidosis, though neither were utilized in animal models of the disease to test efficacy. More recently, Condori et al. [9] developed and tested a recombinant human β-gal-fusion protein that utilized the non-toxic lectin subunit ribosome-inactivating toxin B (RTB) of ricin from *Ricinus communis* as a protein carrier across the BBB. Previously, fusion of RTB to the deficient protein in the lysosomal disease mucopolysaccharidosis type I (MPS I) has shown the ability of delivering the enzyme, α-L-iduronidase (IDUA), to the brain in a murine model of the disease [10]. *In vitro* experiments with β-gal:RTB showed that the fusion protein was internalized into human fibroblasts, processed into the mature form of β-gal, was catalytically active, and reduced ganglioside content [9]. Recently, Acosta and Cramer [11] described in their review of lectin-mediated delivery of macromolecules into the CNS that intravenous administration of β-gal:RTB into β-gal$^{-/-}$ mice resulted in measurable β-gal enzyme activity in the CNS and mature β-gal protein in the cerebellum and spinal cord of treated mice. However, no data was presented.

An alternative platform for engineering lysosomal enzymes for crossing the BBB has been developed, which utilizes genetic fusion of the lysosomal enzyme to antibodies [12]. For lysosomal diseases, the lysosomal enzyme is fused to a monoclonal antibody against either the human insulin receptor (HIR) or the mouse transferrin receptor (mTfR). For multiple lysosomal diseases, this approach has been utilized in murine experiments, including MPS I (Hurler syndrome) [13], MPS II (Hunter syndrome) [14,15] and MPS IIIA (Sanfilippo syndrome type A) [16]. Additionally, preliminary safety studies in mice have been presented for metachromatic leukodystrophy [17]. In MPS I mice, one hour following intravenous (IV) administration revealed an increase in α-L-iduronidase enzyme activity in the liver, spleen, heart, kidney, serum, and brain of treated mice [13]. Following eight weeks of injections, the level of glycosaminoglycans (GAG) was reduced in the liver, spleen, and heart of treated mice, but not in the kidney or brain, though there was a 73% reduction in the inclusion bodies in the brain. [13]. Following six weeks of intraperitoneal (IP) administration of the fusion protein in MPS IIIA mice, the GAG heparan sulfate was significantly reduced in the brain and liver of treated mice, whereas the GAG dermatan sulfate was only reduced in the liver [16]. Further, motor function assessment utilizing the rotarod showed mice treated with the MPS IIIA fusion protein performed better than sham-treated mice. However, treated animals were not compared to normal mice. Further, studies in rhesus macaques show that these fusion enzymes are capable of penetrating the BBB and are safe for repeated IV injections [18,19]. Results from these studies led to their use in two clinical trials for MPS I and MPS II (Clinical Trial Identifiers MPS I: NCT03071341; MPS II: NCT02262338). For MPS I, the results of the phase 1/2 clinical trial showed that 52 weeks of IV treatment with the enzyme valanafusp alpha was well tolerated, with the primary adverse reactions being infusion reactions and mild hypoglycemia [20]. Further, neurological scores of patients and CSF levels of the GAGs heparan and dermatan sulfate were stable during the study. However, the study did not compare enrolled patients to a control group or natural history data of the MPS I disease, limiting the interpretation of the efficacy of this fusion protein.

In the present study, a novel lysosomal enzyme fusion protein, mTfR-GLB1, was tested as a therapy for GM1-gangliosidosis in β-gal deficient mice (β-gal$^{-/-}$). mTfR-GLB1 is a fusion of the human β-gal enzyme to the carboxyl terminus of each heavy chain of a mouse chimeric monoclonal antibody against the mouse transferrin receptor (mTfR); therefore, each mTfR-GLB1 molecule contains two human β-gal enzymes with a molecular weight of 292 kDa. This study aimed to test the efficacy of long-term intravenous administration of mTfR-GLB1 to determine whether this novel fusion enzyme could be utilized as a therapeutic agent for treating GM1-gangliosidosis and preventing the onset of the neurological symptoms and pathology previously described in the murine model of GM1-gangliosidosis [21].

2. Materials and methods

2.1. Expression and purification of mTfR-GLB1

mTfR-GLB1 was designed and synthesized using established methodologies for monoclonal antibody-lysosomal enzyme fusion proteins [12,13,16–19,22–24]. Briefly, a plasmid encoding the sequence of mTfR-GLB1 was expressed in stable Chinese hamster ovary cells. The supernatant was removed and purified utilizing Protein A purification (HiTrap® MabSelect SuRe™, Cytiva Life Sciences, Marlborough, MA). The final formulation buffer (or vehicle) was adjusted to 25 mM citrate, 150 mM NaCl, pH 6.0.

2.2. Animals and procedures

All animal care and experimental procedures were conducted under the approval of the Institute Animal Care and Use Committee (IACUC) of the University of Minnesota. All animals were housed in specific pathogen-free conditions.

GM1-gangliosidosis mice (β-gal$^{-/-}$) were generated by targeted knockout of 20 bp in exon 8 of the β-gal encoding gene, *Glb1* [21]. All animals were genotyped by PCR. For short-term assessment, two to six-month-old mice received a single intravenous injection of mTfR-GLB1 at a dose of 5 mg of mTfR-GLB1 (molecular weight) per kilogram of body weight (mg/kg) through the lateral tail vein and euthanized after 12 h. For the long-term efficacy study, eight-week-old mice ($n = 12$ per group; $n = 6$ of each sex) were injected intravenously through the lateral tail vein twice per week with a dose of 2.5 or 5.0 mg/kg of mTfR-GLB1 or empty vehicle for 17 weeks. Volume was adjusted to 200 μl for each injection with vehicle buffer. Following injection of mTfR-GLB1, animals were monitored for at least one hour for potential adverse events, including lethargy, respiratory changes, or other abnormal behavior. If mice had an adverse reaction that lasted 30 min or longer, a 10 mg/kg dose of diphenhydramine was administered intraperitoneally (IP). Animals which received diphenhydramine were then given prophylactic diphenhydramine for the duration of the study. Mice were monitored daily for signs of morbidity and mortality, in addition to weekly weights for signs of toxicity to mTfR-GLB1.

For biochemical analysis, animals were sacrificed by CO_2 asphyxiation and perfused *via* transcardial perfusion with 20 ml of ice-cold phosphate-buffered saline. Tissues isolated for biochemical assays were flash frozen in liquid nitrogen and stored at −80 °C until assayed.

2.3. β-Galactosidase (β-gal) enzyme assay

β-gal enzyme activity was determined utilizing a fluorometric assay using 4-methylumbelliferyl-β-D-galactopyranoside (4-MUGal, Millipore-Sigma, St. Louis, MO) as the substrate as previously described [21]. β-gal enzyme activity was expressed in nmol of 4-MU released per

hour, per milligram of protein (nmol/h/mg). Total protein quantification was determined using a Pierce™ 660 nm Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

2.4. Histopathological analysis

Following perfusion and fixation with 10% NBF, tissues were processed into paraffin wax using standard histology techniques, sectioned at a thickness of 4 μm, stained with hematoxylin and eosin (H&E), and evaluated using light microscopy by two A.C.V.P. board-certified pathologists (T.C. and M.G.O'S). Additional evaluation was performed on selected tissues stained with Luxol fast blue (LFB)/H&E combination stain using standard laboratory protocols. All work was done at the Masonic Cancer Center Comparative Pathology Shared Resource laboratory at the University of Minnesota.

2.5. Ganglioside isolation and quantification

GM1 and GA1 ganglioside levels were quantified as previously described [21]. The mouse hippocampus, cerebellum, and cerebral cortex tissues were homogenized in 2% CHAPS solution (40 ml/g for hippocampus and cerebellum tissues; 10 ml/g for cerebral cortex tissue). Protein precipitation with 200 μl of methanol was performed to extract GM1 and GA1 from 50 μl of homogenate in the presence of internal standards (d3-GM1(18:0) for GM1 and d3-GA1(18:0) for GA1). The 10% study sample extracts from each tissue type were pooled to prepare a quality control (QC) sample for that tissue. The QC samples were injected every 10 study samples to monitor the instrument performance.

Sample analysis was performed with a Shimadzu 20 CE HPLC system, coupled to a 6500QTRAP+ mass spectrometer (AB Sciex, Framingham, MA) operated in positive MRM mode. Data processing was conducted with Analyst 1.6.3 (Applied Biosystems). The relative quantification of lipids is provided, and the data were reported as the peak area ratios of the analytes to the corresponding internal standards.

2.6. Mouse behavior analysis

Beginning at approximately 24 weeks of age, corresponding to week 15 of mTfR-GLB1 injections, mice were subjected to a battery of behavior tests to assess neuromotor and neurocognitive function. All behavioral analyses were conducted in the Mouse Behavior Core at the University of Minnesota.

2.6.1. Neuromotor testing

2.6.1.1. Inverted screen. Inverted screen testing [25] was completed utilizing a wire mesh apparatus, approximately 25 cm × 25 cm with 25 mm^2 square openings, placed approximately 30 cm over a cushioned surface. Testing began by placing the mouse on the wire mesh, which was then inverted until the animal was completely upside down. Latency to fall was measured for a max trial duration of 120 s. Mice were subjected to three trials, with a minimum of a 15-min resting period between trials.

2.6.1.2. Pole test. The pole test was conducted as previously described with minor modifications [26]. The test began by placing a mouse head-upward on a vertical pole (2 cm in diameter; 55 cm tall) wrapped in athletic tape to assist with grip. Two measurements were taken: latency to rotate body to face downward was measured, and the total time to complete the turn and descend the pole and place all four paws on the base of the apparatus inside of the cage (*i.e.* total time to complete the test). Maximum test duration was set at 60 s. If a mouse failed to make the initial rotation to face downward, 60 s was recorded for both the turn and total time to complete the task. If the mouse fell from the pole during the testing time, it was placed back at the initial starting point while the time continued until 60 s. Mice were subjected to a single trial.

2.6.1.3. Rotarod. Rotarod analysis was conducted on an accelerating rotarod (Ugo Basile, Comerio, Italy), using an adapted protocol described previously [27]. Briefly, mice were tested for three consecutive days, undergoing four trials per day, with a minimum inter-trial duration of 30 min. Testing was done on a rotarod programmed to accelerate from 5 to 50 rpm over a max trial duration of 300 s. Three to five mice were placed on the divided rod simultaneously, and the apparatus' counter was started. The trial was considered complete when 1) the mouse fell off the rotarod and stopped the counter, 2) the mouse completes two consecutive rotations by holding on to the rod without walking, or 3) when 300 s elapsed.

2.6.2. Neurocognitive testing

2.6.2.1. Barnes maze. The Barnes maze [28] was conducted on an elevated circular platform, 36 in. in diameter, with 20 equally spaced holes around its perimeter. All of the holes on the platform were blocked except for one, which contained an opening to an escape box. The apparatus was placed directly under a bright light source that served as an aversive stimulus for the mouse to escape the platform. Visual cues were placed on each of the walls that act as navigational cues for the mouse. Mice were trained on the Barnes maze for four days, with each mouse subjected to four trials per day, with a max of 3 min per trial, and a minimum of 30 min between trials. Data was collected utilizing the ANY-Maze software (Stoelting Co, Wood Dale, IL).

2.7. Statistical analysis

GraphPad Prism 8 (v. 8.4.2, GraphPad Software, Inc) was used to perform all statistical analyses. For enzyme activity, pole test, and inverted screen behavior tests, a one-way ANOVA, followed by Dunnett's multiple comparisons test was used. For weight and plasma β-gal enzyme activity, a repeated-measures two-way ANOVA, followed by Tukey's multiple comparisons test, was performed. For ganglioside quantification, the accelerating rotarod, and Barnes maze, a two-way ANOVA, followed by Tukey's multiple comparisons test was used. Significance cutoff of $p < 0.05$ was used. All values are presented as mean ± SEM.

3. Results

3.1. Catalytic activity of mTfR-GLB1 in vivo

Preliminary experiments conducted prior to this study comparing purified mTfR-GLB1 to recombinant human β-gal enzyme (hβ-gal) revealed that mTfR-GLB1 had approximately 17% of hβ-gal enzyme activity *in vitro*, when using molecular weight equivalencies (Tanabe Research Laboratories, personal communication). When translated to percent of β-gal enzyme activity per mole of β-gal, mTfR-GLB1 has approximately 34% of hβ-gal enzyme activity. To test the function of mTfR-GLB1 *in vivo,* a short-term experiment was conducted. Two to six-month old β-$gal^{-/-}$ mice were injected with 5.0 mg of mTfR-GLB1 per kilogram body weight (mg/kg) of mTfR-GLB1 ($n = 4$) or vehicle only ($n = 3$) IV through the lateral tail vein. Twelve hours following injection, mice were euthanized and β-gal enzyme activity was measured in the plasma, brain, heart, liver, spleen, and kidney. β-gal enzyme activity was not present in the plasma of treated β-$gal^{-/-}$ mice, suggesting that the enzyme was readily taken up into the peripheral tissue within 12 h (Fig. 1A). In the liver, β-gal enzyme activity was increased approximately 12-fold in mTfR-GLB1 treated β-$gal^{-/-}$ mice (46.02 ± 4.95 nmol/h/mg protein) compared to vehicle only β-$gal^{-/-}$ mice (3.86 ± 0.38), resulting in approximately 38% of heterozygous ($n = 4$) β-gal enzyme activity (Fig. 1B). While not statistically significant ($p = 0.0692$), β-gal

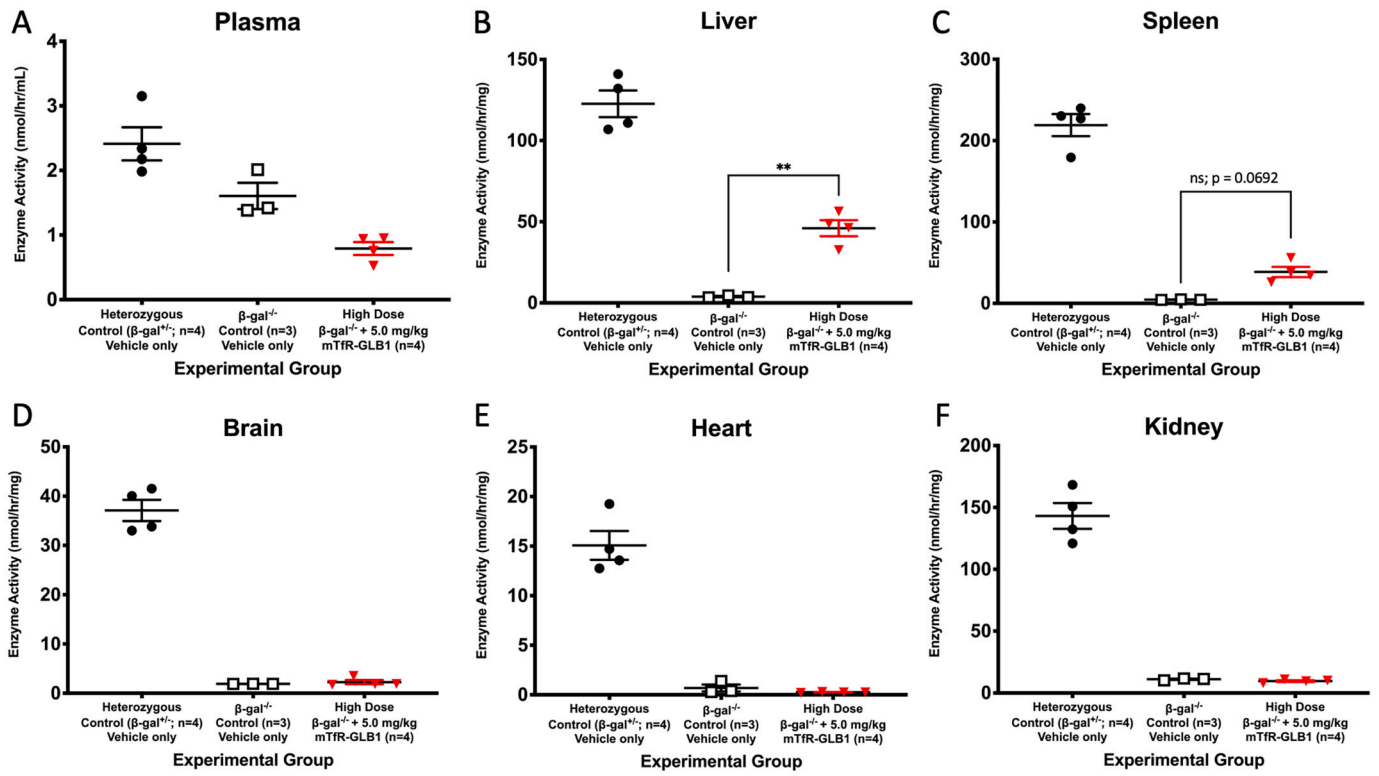

Fig. 1. β-galactosidase enzyme activity 12-h post-injection with mTfR-GLB1.

enzyme activity was increased 8.5-fold in the spleen of mTfR-GLB1 treated mice (38.67 ± 6.3) compared to β-gal$^{-/-}$ mice receiving vehicle only (4.57 ± 0.21) (Fig. 1C). This was approximately 18% of heterozygous β-gal enzyme activity. Surprisingly, β-gal enzyme activity was not increased above β-gal$^{-/-}$ mice receiving vehicle only in the brain, heart, or kidney (Fig. 1D, E, F). Overall, these results showed that mTfR-GLB1 was readily taken up into the tissue and was catalytically active in a murine model of GM1-gangliosidosis.

Two to six-month-old β-gal$^{-/-}$ mice were injected intravenously with 5.0 mg/kg of mTfR-GLB1 ($n = 4$) or empty vehicle ($n = 3$). Control heterozygous β-gal$^{+/-}$ mice (n = 4) were also treated IV with empty vehicle. 12-h following treatment, animals were euthanized and β-gal enzyme activity was measured in the (A) plasma, (B) liver, (C) spleen, (D) brain, (E) heart, and (F) kidney. Mean ± SEM. ***$p < 0.001$ when comparing mTfR-GLB1 treated β-gal$^{-/-}$ mice to vehicle only β-gal$^{-/-}$ mice. One-way ANOVA with adjustments for multiple comparisons.

3.2. Long-term efficacy of mTfR-GLB1 in preventing disease onset in a murine model of GM1-gangliosidosis

To test whether mTfR-GLB1 could prevent the onset of disease, mice were treated for 17 weeks with twice weekly IV injections of mTfR-GLB1, beginning at eight weeks of age. For this experiment, mice were randomly assigned into one of six experimental groups (Table 1). Two groups served as baseline controls for ganglioside quantification, which included six heterozygous mice (β-gal$^{+/-}$) and six β-gal$^{-/-}$ mice, with equal numbers of females and males. These mice were euthanized and analyzed at eight weeks of age. The four remaining groups included two control groups, β-gal$^{+/-}$ and β-gal$^{-/-}$ mice receiving vehicle only, one group of β-gal$^{-/-}$ receiving a low dose of mTfR-GLB1 (2.5 mg/kg) and another group of β-gal$^{-/-}$ mice receiving a high dose of mTfR-GLB1 (5.0 mg/kg). Each group consisted of eight female and eight male mice, resulting in a total of 16 animals in each group. Injections were continued for a total of 17 weeks, or 34 injections. For the duration of the study, weights were recorded weekly and animals were monitored for onset of disease. Additionally, plasma was collected every four weeks to measure β-gal enzyme activity. Beginning at 24 weeks of age, mice were subjected to a battery of behavioral tests to quantify neuromotor and neurocognitive function. Twenty-four hours following the final injection, mice were euthanized, and tissues were collected. Study animals were randomly selected to have the left hemisphere of the brain utilized either for histopathological analysis or for measuring β-gal enzyme activity. Further, β-gal enzyme activity was measured in the liver, spleen, kidney, and heart of all mice. For all animals, the right hemisphere of the brain was microdissected to remove the hippocampus, cerebellum, and cerebral cortex, which were utilized to measure ganglioside levels.

Overall, IV administration with mTfR-GLB1 was tolerated over the duration of the study. At the end of the study, all female β-gal$^{-/-}$ mice receiving mTfR-GLB1, except one mouse receiving the low dose, were receiving prophylactic diphenhydramine. In contrast, only two male β-gal$^{-/-}$ mice receiving the low dose and three receiving the high dose were receiving prophylactic diphenhydramine. In most instances, adverse reactions observed after administration of mTfR-GLB1 included lethargy and respiratory changes. While all male mice in the experiment survived, two female β-gal$^{-/-}$ mice were lost during the study. One β-gal$^{-/-}$ female receiving vehicle only became emaciated nine weeks into the study and required euthanasia. Post-mortem observations revealed severe splenomegaly. The second mouse that was lost during

Table 1
Experimental design.

Group	Group designation	No. of animals		Total mTfR-GLB1 dose (mg/kg)
		Male	Female	
1	β-gal$^{-/-}$, Baseline	3	3	0
2	Heterozygotes, Baseline	3	3	0
3	β-gal$^{-/-}$, Vehicle only	8	8	0
4	Heterozygotes, Vehicle only	8	8	0
5	β-gal$^{-/-}$, Low dose	8	8	2.5
6	β-gal$^{-/-}$, High dose	8	8	5.0

the study was a β-gal$^{-/-}$ mouse that was receiving a high dose of mTfR-GLB1. This mouse became severely lethargic following mTfR-GLB1 administration and did not recover and a decision was made to euthanize the animal.

3.3. Male β-gal$^{-/-}$ mice receiving high dose of mTfR-GLB1 are significantly lighter than vehicle only β-gal$^{-/-}$ mice

For the duration of the experiment, mice were weighed weekly prior to the administration of the first dose of mTfR-GLB1. Previous characterization studies have shown that male β-gal$^{-/-}$ mice become significantly heavier than heterozygous and wildtype littermates [21]. In this study, similar findings were observed, where the weights of female β-gal$^{-/-}$ mice (Fig. 2A) were not significantly different than heterozygotes. Interestingly, throughout the study, the weights of male β-gal$^{-/-}$ mice receiving the high dose of mTfR-GLB1 were lower than vehicle only β-gal$^{-/-}$ mice, even significantly at various points throughout the study (Fig. 2B). Further, the weights of male β-gal$^{-/-}$ mice that received the low dose of mTfR-GLB1 trended lower than β-gal$^{-/-}$ mice receiving vehicle only. Overall, there was an observable dose-weight response for male β-gal$^{-/-}$ mice receiving mTfR-GLB1.

3.4. Presence of β-galactosidase enzyme activity in the liver and spleen of β-gal$^{-/-}$ mice treated with mTfR-GLB1

Plasma was first collected 24 h prior to the first administration of mTfR-GLB1 and β-gal enzyme activity was measured. Plasma was then collected every four weeks, 24 h after the second weekly injection. Both groups receiving mTfR-GLB1 had no measurable increase in β-gal enzyme activity over β-gal$^{-/-}$ mice receiving vehicle only at the time points assessed (Fig. 3A). This further suggests that mTfR-GLB1 is readily taken up into the peripheral tissues following IV administration.

At the conclusion of the study, β-gal enzyme activity was also measured in multiple tissues, 24 h following the final administration of mTfR-GLB1. In the liver, β-gal$^{-/-}$ mice receiving the low dose of mTfR-GLB1 had 4.7-fold higher β-gal enzyme activity (9.76 ± 1.45 nmol/h/mg protein) and mice that received the high dose of mTfR-GLB1 were 7.5-fold higher (15.51 ± 2.69) compared to vehicle-treated β-gal$^{-/-}$ mice (2.06 ± 0.13) (Fig. 3B). In the spleen, enzyme activity was also increased 2.4-fold (14.2 ± 1.66) in mice receiving the low dose and 5.0-fold (29.63 ± 5.98) in mice receiving the high dose of mTfR-GLB1 compared to vehicle only β-gal$^{-/-}$ mice activity (5.93 ± 0.45) (Fig. 3C). However, in both mTfR-GLB1 treatment groups, β-gal enzyme activity in the brain, heart, and kidney did not increase above vehicle only β-gal$^{-/-}$ levels (Figs. 3D, E, F).

3.5. No reduction of ganglioside content in the brain of mTfR-GLB1 treated mice

To determine if long-term treatment of GM1-gangliosidosis mice with mTfR-GLB1 reduced or prevented the accumulation of storage material in the brain, GM1 and GA1 ganglioside levels were measured in the cerebral cortex, cerebellum, and hippocampus (Fig. 4A, B, C). At eight weeks of age, ganglioside levels in baseline β-gal$^{-/-}$ mice were already significantly elevated compared to heterozygous β-gal$^{+/-}$ controls in all tissues assayed. Additionally, as the disease progressed between two months of age when the study was initiated and the end of the study at six months of age, ganglioside content increased 5–54% in the cerebral cortex, 8–45% in the cerebellum, and 26–50% in the hippocampus.

After 17 weeks of treatment, β-gal$^{-/-}$ mice treated with either the low dose or high dose of mTfR-GLB1 showed no reduction in the ganglioside content in the cerebral cortex, cerebellum, or hippocampus. This further supports that mTfR-GLB1 does not cross the BBB as designed or that the relatively low enzyme activity of the fusion protein is not sufficient to reduce the substrate in the brain.

3.6. Partial improvement of motor function in mice treated with mTfR-GLB1

At approximately 24 weeks of age, mice were subjected to a battery of behavioral tests to assess whether treatment with mTfR-GLB1 could improve neuromotor and neurocognitive function. To assess motor function, specifically bradykinesia, the pole test was used. Both the low and high dose groups had a significant improvement ($p < 0.05$ and $p < 0.001$, respectively) in their ability to coordinate their limbs and invert their body on the pole, whereas the β-gal$^{-/-}$ mice receiving vehicle only had extreme difficultly completing this task (Fig. 5A). Moreover, mice receiving the high dose of mTfR-GLB1 had a significant reduction ($p < 0.01$) in the time it took to complete the entire task of turning and descending the pole compared to vehicle-treated β-gal$^{-/-}$ mice (Fig. 5B). Overall, there was an observed dose-response in the ability of the mice to complete the pole test.

Additional testing was conducted to elucidate whether motor or

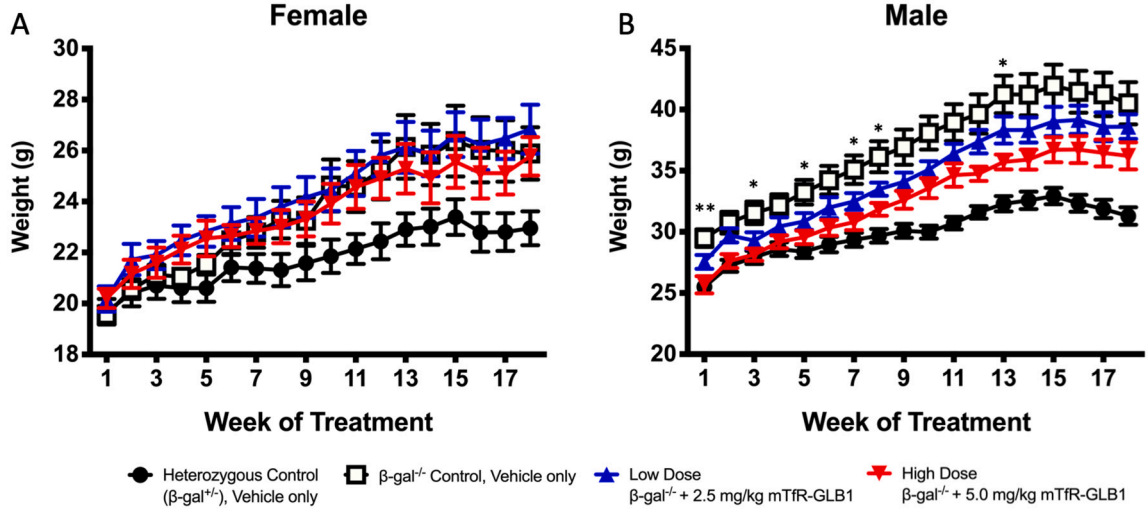

Fig. 2. Effects of mTfR-GLB1 on weight of β-gal$^{-/-}$ mice. For the duration of the study, the weights of (A) female mice and (B) male mice were measured and recorded weekly. Mean ± SEM. All groups $n = 8$, except (A) Female β-gal$^{-/-}$ control and high dose ($n = 7$). *$p < 0.05$ when comparing high dose treated to vehicle-treated β-gal$^{-/-}$ mice; **$p < 0.01$. Repeated-measures two-way ANOVA, followed by Tukey's multiple comparisons test.

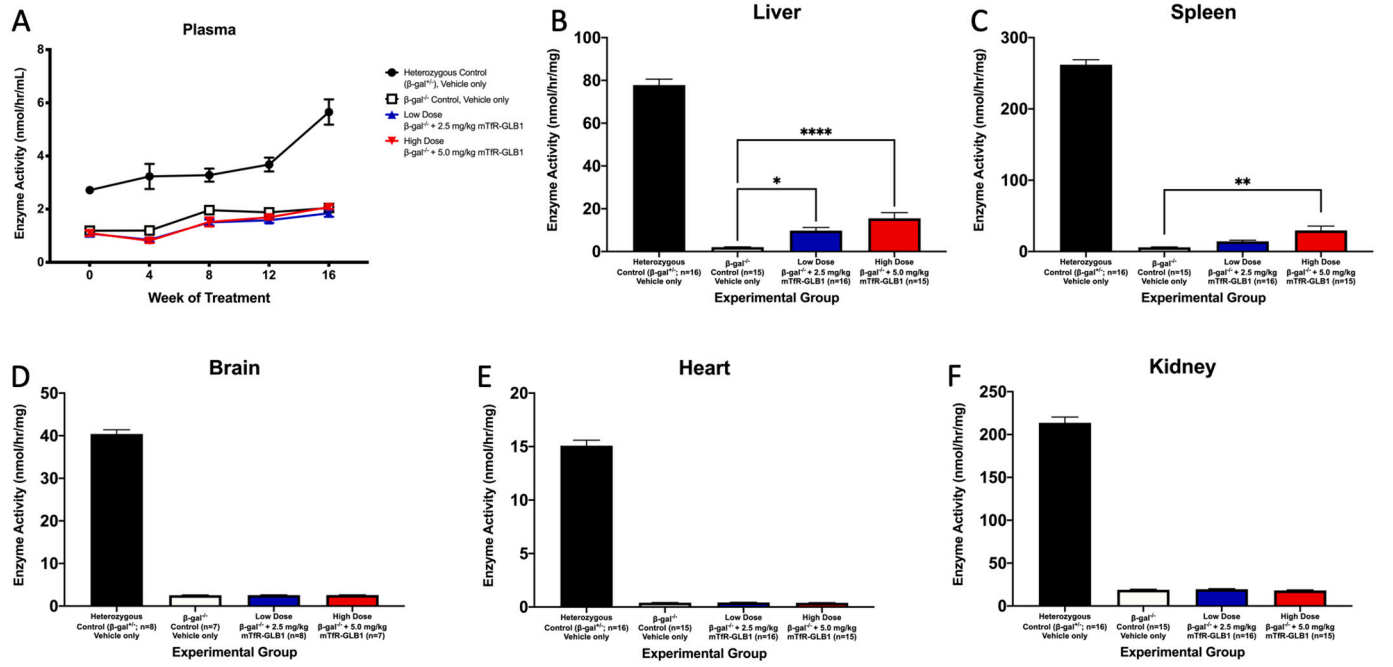

Fig. 3. Plasma and tissue β-gal enzyme activity. (A) Plasma enzyme activity was measured prior to study initiation and 24-h following IV administration of mTfR-GLB1 every four weeks for the duration of the study. For (B) to (F), mice were euthanized 24-h following the final treatment of mTfR-GLB1 (week 17), perfused with phosphate buffered saline and tissues were collected and analyzed for β-gal enzyme activity as described in the materials and methods. Mean ± SEM. $^{*}p < 0.05$ when comparing treated to vehicle only β-gal$^{-/-}$ mice; $^{**}p < 0.01$; $^{****}p < 0.0001$. (A) Repeated-measures two-way ANOVA, followed by Tukey's multiple comparisons test; (B)-(F) One-way ANOVA, followed by Dunnett's multiple comparisons test.

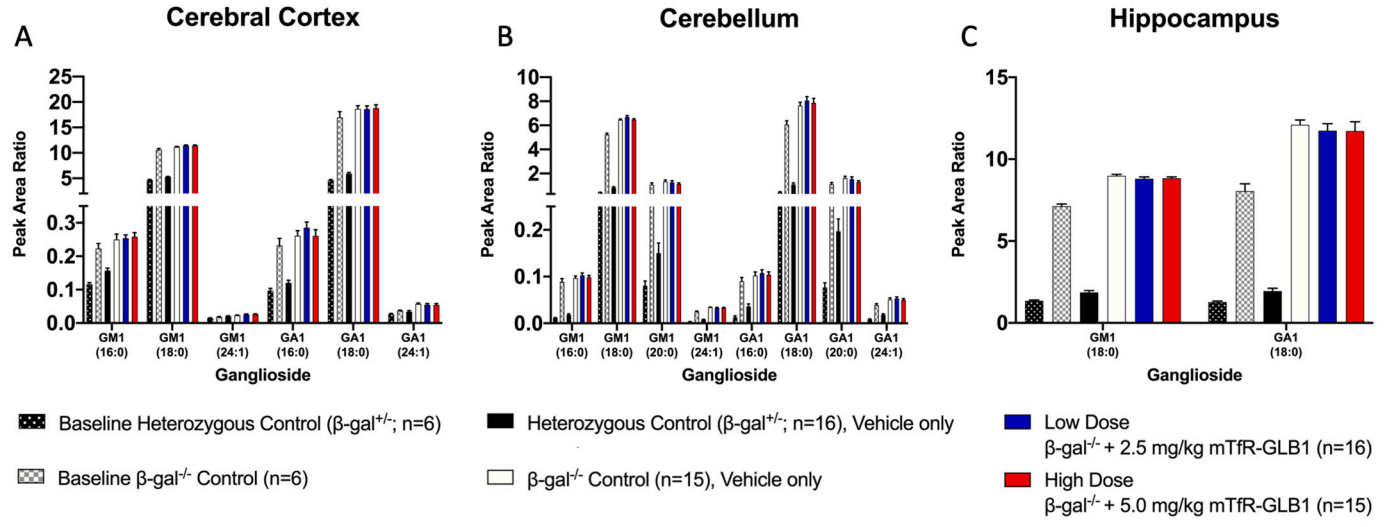

Fig. 4. GM1 and GA1 ganglioside quantification in the brain of mice treated with mTfR-GLB1. Ganglioside levels were measured using high performance liquid chromatography tandem-mass spectrometry in the (A) cerebral cortex, (B) cerebellum, and (C) hippocampus. Mean ± SEM. Two-way ANOVA, followed by Tukey's multiple comparisons test.

neurocognitive function was preserved in β-gal$^{-/-}$ mice treated with mTfR-GLB1. To assess grip strength, the inverted screen was used. In this test, β-gal$^{-/-}$ mice treated with mTfR-GLB1 showed no improvement in their ability to remain on the inverted screen, performing similarly to vehicle-treated β-gal$^{-/-}$ mice, suggesting that grip strength was not maintained (Fig. 6A). Motor function and coordination were further assessed using the accelerating rotarod. Similar to the inverted screen, β-gal$^{-/-}$ mice treated with mTfR-GLB1 performed comparably to vehicle-treated β-gal$^{-/-}$ mice (Fig. 6B).

To assess cognitive function, the Barnes maze was utilized. While heterozygous β-gal$^{+/-}$ mice learned the task and escaped the platform in under 60 s on the final testing day, vehicle-treated β-gal$^{-/-}$ mice failed to learn the task. Similar to β-gal$^{-/-}$ mice receiving vehicle only, mice treated with the low and high dose of mTfR-GLB1 failed to learn and complete the task, exhibiting neurocognitive impairment (Fig. 6C).

3.7. Brain histopathological analysis revealed presence of severe neuronal pathology in mice treated with mTfR-GLB1

When the mice were six months of age, the histology from the left hemisphere of the brains was analyzed to determine whether mTfR-GLB1 reduced or prevented the neuronal vacuolation described

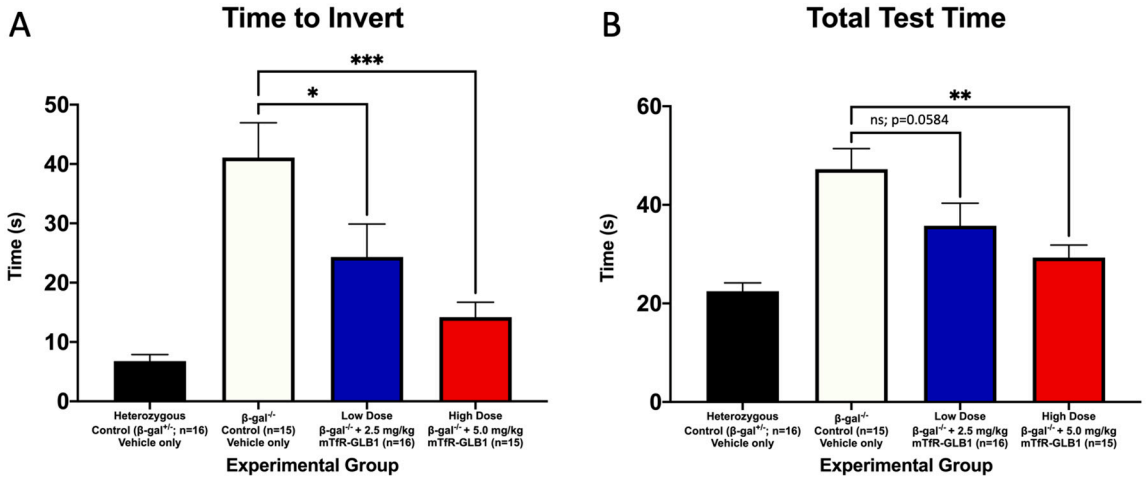

Fig. 5. Assessment of fine motor coordination using the pole test in mice receiving mTfR-GLB1. At six months of age, fine motor skills were tested using the pole test, where animals were placed on a vertical pole facing upwards and the (A) time to invert and face downward was measured. Additionally, the (B) total time to complete the inversion and descend the pole was measured. Mean ± SEM. *p < 0.05 when comparing treated to vehicle-treated β-gal$^{-/-}$ mice; **p < 0.01; ***p < 0.001. One-way ANOVA, followed by Dunnett's multiple comparisons test.

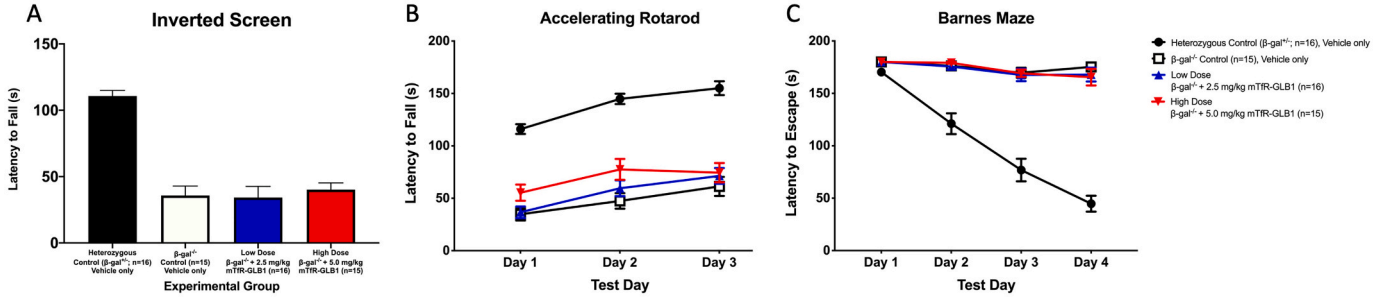

Fig. 6. Further behavioral evaluation of motor and neurocognitive function of β-gal$^{-/-}$ mice treated with mTfR-GLB1. At six months of age, mice were subjected to additional behavioral analyses. (A) Grip strength was assessed by placing mice on an inverted wire screen and timing the latency to fall. (B) Motor coordination was tested using the accelerating rotarod, where the latency to fall is measured. (C) To assess neurocognitive function, mice were subjected to the Barnes maze, which requires mice to learn to locate and escape the brightly-lit platform over the four day duration of the test. Mean ± SEM. (A) One-way ANOVA, followed by Dunnett's multiple comparisons test; (B and C) Repeated-measures two-way ANOVA, followed by Tukey's multiple comparisons test.

previously. The brain tissue of vehicle-treated β-gal$^{-/-}$ mice had neuronal lesions, consistent with the GM1-gangliosidosis phenotype (Fig. 7B). This was characterized by swollen neurons that contained clear to pale-eosinophilic, well-demarcated intracytoplasmic vacuoles, occasional fine-granulation, displacement of the Nissl substance, and occasional nuclear margination. These lesions are moderate to marked and present in all areas of the brain, including the cerebellum, pons, thalamus, hypothalamus, and cerebral cortex. In contrast, all of these observations were absent from the heterozygous β-gal$^{+/-}$ brain tissue. Further, the intracellular storage material in the affected neurons stained positively with Luxol fast blue (Fig. 8B). Analysis of the brain samples from β-gal$^{-/-}$ mice that received low (Fig. 7C) and high (Fig. 7D) doses of mTfR-GLB1 for 17 weeks revealed the presence of neuronal vacuolation that was comparable to that observed in β-gal$^{-/-}$ mice receiving vehicle only. Further, similar to vehicle-treated β-gal$^{-/-}$ mice, this intracellular storage material stained positively with Luxol fast blue (Fig. 8C and D).

4. Discussion

The treatment of lysosomal diseases with ERT using recombinant native enzymes has been beneficial for thousands of patients suffering from these diseases. However, these enzymes continue to lack neurological benefit due to the inability of the lysosomal enzyme to cross the BBB. To solve this problem, fusion enzymes have been developed, which allows for the enzyme to bind to a receptor that is present on the epithelial cells of the BBB and undergo receptor-mediated transcytosis. The present study describes the assessment of such a protein, mTfR-GLB1, as a potential therapy for GM1-gangliosidosis. mTfR-GLB1 fuses the human β-gal enzyme to the carboxyl terminus of each heavy chain of a mouse chimeric monoclonal antibody against the mouse transferrin receptor, meaning there are two human β-gal enzymes present in each molecule. The data presented shows that mTfR-GLB1 is cleared from the bloodstream within 12 h following intravenous administration, as demonstrated by the lack of β-gal enzyme activity in the plasma of treated mice. mTfR-GLB1 has also been shown to be catalytically active and capable of catabolizing the synthetic substrate 4-methylumbelliferyl β-D-galactopyranoside used in β-gal enzyme assays, as shown by liver and spleen enzyme activity. Additionally, weight measurements over the duration of the study showed the weight of male β-gal$^{-/-}$ mice receiving the high dose of mTfR-GLB1 was significantly lower than β-gal$^{-/-}$ mice receiving vehicle only, displaying weights closer to that of the heterozygous control group. Further, behavioral assessment at six months of age using the pole test revealed improved motor function in mice receiving the high dose of mTfR-GLB1.

At eight weeks of age, ganglioside content in β-gal$^{-/-}$ mice was already elevated compared to normal heterozygous β-gal$^{-/-}$ mice. Further, by the end of the study at six months of age, there was a progressive accumulation of gangliosides as the disease advanced, displayed by a significant increase in ganglioside content in six-month-old

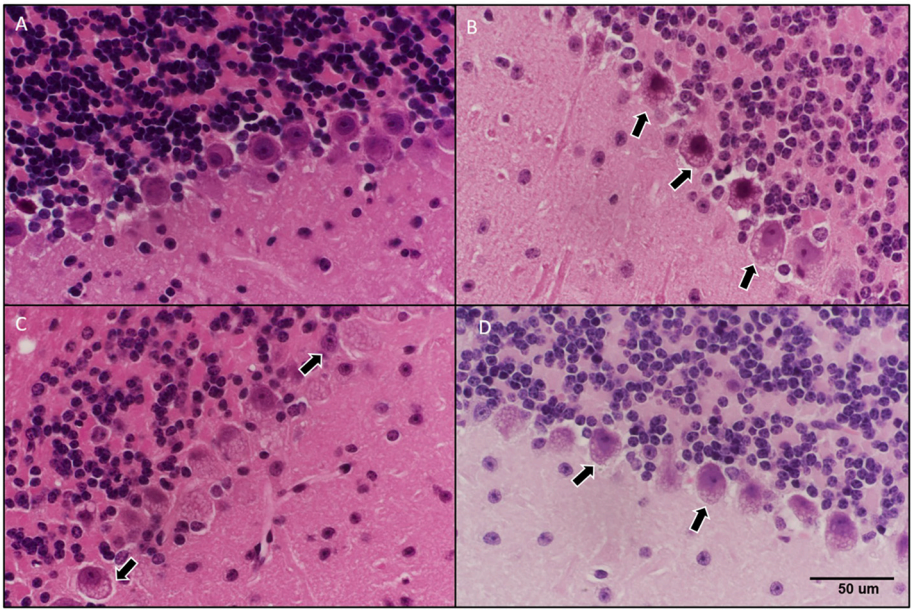

Fig. 7. Neurological pathology in β-gal$^{-/-}$ mice treated with mTfR-GLB1. Hematoxylin and eosin staining of cerebellar tissue from the brain of six-month-old (A) normal β-gal$^{+/-}$ mice, (B) vehicle-treated β-gal$^{-/-}$ mice, (C) β-gal$^{-/-}$ mice treated with 2.5 mg/kg of mTfR-GLB1 and (D) β-gal$^{-/-}$ mice treated with 5.0 mg/kg of mTfR-GLB1. Arrows indicate examples of Purkinje neurons with intracytoplasmic vacuoles. Objective x60, scale bar indicates 50 μm.

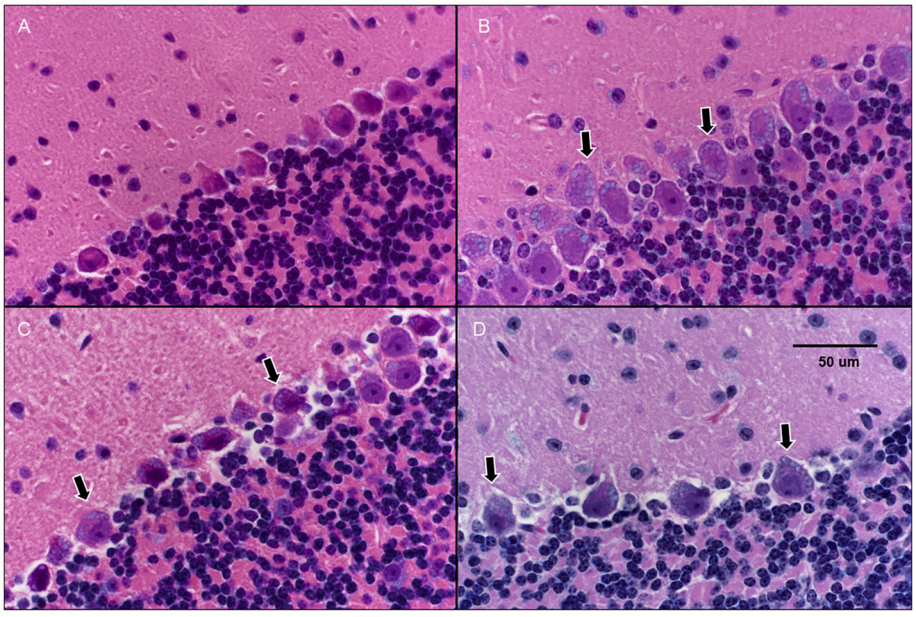

Fig. 8. Luxol fast blue staining of intracellular storage material in mice treated with mTfR-GLB1. Luxol fast blue staining of neurons in the cerebellum of six-month-old (A) heterozygous β-gal$^{+/-}$ mice, (B) vehicle-treated β-gal$^{-/-}$ mice, (C) β-gal$^{-/-}$ mice treated with 2.5 mg/kg of mTfR-GLB1 and (D) β-gal$^{-/-}$ mice treated with 5.0 mg/kg of mTfR-GLB1. Arrows illustrate Purkinje neurons with intracytoplasmic vacuoles that contain intracellular storage material stained positive with Luxol fast blue. Objective x60, scale bar indicates 50 μm.

β-gal$^{-/-}$ mice receiving vehicle only. While this is the first observation of ganglioside accumulation early in adulthood in the β-gal$^{-/-}$ model used in this study, a similar result has been described in another murine model of GM1-gangliosidosis [29–31]. Though treatment with mTfR-GLB1 improved pole test results and mouse weight, full cognitive and motor function was not improved. A possible interpretation of the improved motor function in the pole test is that mTfR-GLB1 provided enough β-gal enzyme activity to increase the physical activity of β-gal$^{-/-}$ mice. This increase in activity then could have contributed to the observed weight reduction, which resulted in less of a burden on the animals during a physical test such as inverting and descending a vertical pole. With significant ganglioside accumulation already present at eight weeks of age, it is possible that even with therapeutic intervention using mTfR-GLB1 or another approach, irreparable neurological damage has been done and earlier treatment is necessary.

In contrast to biodistribution studies testing similar mTfR-lysosomal enzyme fusion proteins, β-gal enzyme activity was not detected in the heart, kidney, or brain of treated β-gal$^{-/-}$ mice at 12- and 24-h following treatment. Prior studies with mTfR fused to IDUA have shown that IDUA enzyme activity was increased in the heart, kidney, brain, liver, spleen, and serum one hour following IV administration in IDUA-deficient mice [13]. Further, brain ganglioside levels were not reduced in mTfR-GLB1 treated β-gal$^{-/-}$ mice compared to vehicle-treated β-gal$^{-/-}$ mice. In another study, Boado et al. [16] showed that one week following the final dose of a six-week treatment with the mTfR-lysosomal enzyme fusion protein that heparan sulfate levels were significantly reduced in the brain of MPS IIIB mice compared to sham-treated MPS IIIB mice. Together, this data suggests mTfR-GLB1 has reduced biodistribution or a

short half-life compared to these other mTfR-fusion enzymes.

Additionally, the absence of a therapeutic benefit in treating the GM1-gangliosidosis pathology in β-gal$^{-/-}$ mice with mTfR-GLB1 can potentially also be attributed to the instability of the human β-gal enzyme. Human β-gal is known to form a dynamic multi-enzyme complex with neuraminidase 1 (NEU1) and protective protein cathepsin A (PPCA) [32], which plays a role in intracellular stability of human β-gal. Recently, Chen et al. [31] demonstrated that recombinant human β-gal formed stable homodimers under acidic conditions at a concentration of 0.1 mg/ml, whereas at a neutral pH, the enzyme became unstable and was predominantly found as a monomer. Further, while human β-gal enzyme can be either in a multi-enzyme complex or as a homodimer, the half-life is extended from a few hours to several days when in complex with NEU1 and PPCA [33]. In cell culture experiments, β-gal-deficient human fibroblasts transduced with retroviruses expressing human β-gal [34] or transfected with plasmids expressing human β-gal [35] revealed no or minimal β-gal enzyme activity in the cell culture media. Further, Lambourne and Potter [35] showed that the catalytic activity of human β-gal was dependent on temperature, showing reduced enzymatic activity at 37 °C, which is the standard human biological temperature. In contrast, the mouse β-gal enzyme provided higher enzyme activity in β-gal-deficient human fibroblasts in cell lysates and the culture media and was very thermostable [34,35].

In addition to the stability of the human β-gal enzyme, the lack of therapeutic efficacy of mTfR-GLB1 could be due to the structure of the mTfR-GLB1 protein. Studies have shown that the presence or location of the receptor-binding component of the engineered enzyme can significantly impact enzyme activity, for example, in β-glucuronidase [23], which is the deficient enzyme in the lysosomal disease MPS VII (Sly syndrome), and β-galactosidase in GM1-gangliosidosis [9]. *In vitro* studies showed that the synthetic mTfR-GLB1 enzyme, which is comprised of two human β-gal enzymes, had 17% of the β-gal enzyme activity as the recombinant human β-gal enzyme (unpublished data, Tanabe Research Laboratories USA, Inc.). In the present murine study, mice receiving the low and high dose of mTfR-GLB1 had approximately 12.5% and 20% of heterozygous levels of β-gal activity in the liver, respectively. It was previously reported that approximately 1% of the injected dose of the lysosomal enzyme fusion protein designed for MPS I crosses the BBB [18]. However, with such a relatively low enzyme activity present in the peripheral tissue, measuring 1% of the injected dose may be outside of the limits of detection of the β-gal enzyme assay. Even so, if β-gal enzyme activity were measured in the brain, the present study showed that long-term IV administration of mTfR-GLB1 at the current doses provided limited benefits for preventing the onset of the GM1-gangliosidosis phenotype and did not reduce ganglioside accumulation in the brain. Recently, Boado et al. [24] re-designed and tested the catalytic activity of a new human β-gal fusion enzyme. This enzyme, termed HIRMAb-HC-LL-GLB1, includes a different linker (LL) sequence between the β-gal enzyme and the monoclonal antibody against the human insulin receptor (HIRMAb), compared to the mTfR-GLB1. This modification resulted in the restoration of β-gal enzyme activity up to 93% of recombinant human β-gal enzyme activity [24]. Whether or not this modification to increase β-gal enzyme activity results in a therapeutic benefit for GM1-gangliosidosis has yet to be elucidated.

Overall, improved motor function in the pole test indicates a response to therapy with mTfR-GLB1, but not sufficient to result in a demonstrative improvement in the Barnes maze, accelerating rotarod, and inverted screen. The association of animal weight with dose of mTfR-GLB1, which may have contributed to improved motor abilities on the pole test, suggests a possible dose-response effect. Administration of mTfR-GLB1 was initiated after affected mice reached adulthood and importantly, a profound pathologic accumulation of ganglioside was shown to be present in the CNS of affected β-gal$^{-/-}$ mice prior to the age of initiation of mTfR-GLB1 administration. Would dosing earlier in disease course, when less neurological damage has occurred, allow for a better therapeutic response? King et al. [3] has described a timeline of clinical changes that occur in the infantile and juvenile gangliosidoses through a clinical perspective natural history study. The gangliosidoses are chronically progressive, but a critical window of time exists in which the patients do not show overt signs of their gangliosidosis condition. Children with the most severe infantile phenotype often appear healthy at birth and have normal Agpar scores. Signs of neurological impairment do not become apparent until four to six months of age, at which time fine and gross motor skills markedly decline, leading parents and caregivers to seek a diagnosis. Cognitive abilities also begin to show decline at this time or shortly thereafter [2]. Children with the classic juvenile phenotype meet their first-year milestones. Signs of disease leading caregivers to seek a diagnosis typically do not occur until after the third to fifth year of life, and most often initially present as changes in ambulation skills and language skills. In an intermediate form, the late-infantile phenotype, patients meet first-year development milestones, followed by appearance of marked changes in ambulation and language skills [3]. Considering the severe and progressive neurological impairment that occurs in the gangliosidoses and concerns about irreversibility of neurological damage, an effective therapy may require therapy initiation to occur early, before disease symptoms are apparent. The encouraging results of the pole test and observed weight normalization in the high dose of mTfR-GLB1 suggest that such variations as a higher dose, genetic modification of the fusion protein to accomplish higher enzyme activity or earlier treatment with mTfR-GLB1 might elicit a clearer therapeutic cognitive and motor skill improvement.

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