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Bone marrow transplantation increases efficacy of central nervous system-directed enzyme replacement therapy in the murine model of globoid cell leukodystrophy

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Abstract

Globoid cell leukodystrophy (GLD, Krabbe disease), is an autosomal recessive, neurodegenerative disease caused by the deficiency of the lysosomal enzyme galactocerebrosidase (GALC). In the absence of GALC, the toxic metabolite psychosine accumulates in the brain and causes the death of the myelin-producing cells, oligodendrocytes. Currently, the only therapy for GLD is hematopoietic stem cell transplantation using bone marrow (BMT) or umbilical cord blood. However, this is only partially effective. Previous studies have shown that enzyme replacement therapy (ERT) provides some therapeutic benefit in the murine model of GLD, the Twitcher mouse. Experiments have also shown that two disparate therapies can produce synergistic effects when combined. The current study tests the hypothesis that BMT will increase the therapeutic effects of ERT when these two treatments are combined. Twitcher mice were treated with either ERT alone or both ERT and BMT during the first 2–4 days of life. Recombinant enzyme was delivered by intracerebroventricular (ICV) and intrathecal (IT) injections. Twitcher mice receiving ERT had supraphysiological levels of GALC activity in the brain 24 hours after injection. At 36 days of age, ERT-treated Twitcher mice had reduced psychosine levels, reduced neuroinflammation, improved motor function, and increased lifespan. Twitcher mice receiving both ERT and BMT had significantly increased lifespan, improved motor function, reduced psychosine levels, and reduced neuroinflammation in certain areas of the brain compared to untreated or ERT-treated Twitcher mice. Together, these results indicate that BMT enhances the efficacy of ERT in GLD.

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Keywords

globoid cell leukodystrophy; enzyme replacement therapy; bone marrow transplantation; Twitcher mouse; lysosomal storage disease; neurodegenerative disease

1. Introduction

Globoid cell leukodystrophy (GLD, or Krabbe disease), is an autosomal recessive, neurodegenerative disease caused by a deficiency in the lysosomal enzyme galactocerebrosidase (GALC, EC 3.2.1.46) [1,2]. GALC is responsible for the degradation of various galactolipids, including galactosylceramide and psychosine [3]. In the absence of GALC, psychosine accumulates to toxic levels in the central (CNS) and peripheral nervous systems (PNS). Although psychosine is toxic to all cell types, oligodendrocytes are particularly susceptible [4,5]. The preferential loss of oligodendrocytes leads to demyelination and accumulation of Periodic acid-Schiff (PAS)-positive macrophages, or globoid cells, in both the CNS and PNS [5]. The infantile form of GLD leads to a variety of symptoms, including irritability, seizures, and delayed mental and motor development [2,3]. Infantile GLD is generally fatal by the age of two [2,3]. The Twitcher mouse is a naturally occurring murine model of GLD that has a mutation in the GALC gene that completely eliminates enzyme activity [6]. Because it exhibits many of the same clinical and histological features as the human disease [7,8], it has been a valuable tool to study the underlying pathogenesis and develop effective therapies for this invariably fatal inherited pediatric disease.

Currently, the only therapy for GLD is hematopoietic stem cell transplantation (HSCT) using bone marrow [9] or umbilical cord blood [10]. It is hypothesized that HSCT treats the disease by introducing donor-derived, GALC-positive cells of hematopoietic origin into the CNS [11]. The enzyme produced and secreted by the donor cells can then be endocytosed by neighboring host cells via cross-correction [12]. Bone marrow transplantation (BMT) was first studied in young (10-day-old) Twitcher mice using high doses of conditioning radiation (900 rads). The median lifespan increased significantly from ~38 days in untreated Twitcher mice to ~80 days in mice that received BMT [13,14]. Subsequent BMT studies performed in newborn mice (2–3 days old) using lower doses of myeloreductive conditioning radiation (400 rads) resulted in a median lifespan of approximately 44 days [15]. In either case, BMT is only partially effective. This is likely due to the relatively small number of hematopoietic-derived cells that cross the blood-brain barrier and are able to effect therapeutic changes in the brain [16,10]. Although partially effective, BMT has a considerable mortality rate in newborns [17], when therapy for GLD should be most effective [15]. Therefore, alternate therapeutic approaches need to be developed for this disease.

Alternative therapies have been studied in Twitcher mice in order to increase lifespan and efficacy. CNS-directed gene therapy has been shown to increase GALC expression in the brain to several fold greater than normal levels, but only increased the median lifespan to approximately 55 days [18,19]. Systemic enzyme replacement therapy (ERT) via intraperitoneal (IP) injections increased the median lifespan of Twitcher mice to approximately 47 days [20]. CNS-directed ERT via intracerebroventricular (ICV) injections increased lifespan to approximately 50 days [21].

To date, single therapies (BMT, gene therapy, ERT) have resulted in minimal to moderate increases in lifespan. Therefore, we hypothesized that combining disparate therapeutic approaches that target different tissues or different aspects of disease would increase efficacy [22]. Small molecule substrate reduction therapy combined with BMT increased the

lifespan of Twitcher mice to approximately 110 days [23]. CNS-directed gene therapy combined with BMT increased the median lifespan to approximately 105 days [15] or 123 days [24], depending on which regions of the CNS were targeted. When compared to the moderate increases in lifespan from either therapy alone, these studies demonstrate that two disparate therapies can synergize by targeting different aspects of the disease.

The current study examined the effects of combining CNS-directed ERT and BMT in neonatal Twitcher mice. We hypothesized that ERT would supply an immediate and high-level source of enzyme to the CNS, while BMT would provide a persistent, albeit lower, level of enzyme to the brain. Due to the rapidly progressing nature of the disease, an immediate source of enzyme is necessary. Also, although the persistent level of enzyme provided by BMT is low, it is known that low levels of enzyme (5–10% normal) can be beneficial [9]. Therefore, we believed that this combination approach had the potential to be therapeutic.

2. Materials and Methods

2.1. Animal husbandry

Heterozygous Twitcher (GALC +/-) mice on a congenic C57BL/6 background were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained under the supervision of M.S.S. at Washington University School of Medicine under standard housing conditions with free access to food and water. All animal procedures were in accordance with the regulations of the Institutional Animal Care and Use Committee at Washington University School of Medicine. Heterozygous Twitcher mice (GALC +/-) were mated to obtain homozygous Twitcher (GALC -/-) or homozygous wildtype (GALC +/+) mice. Genotypes of newborn pups were identified by PCR for the Twitcher mutation [15]. Only mice surviving until weaning at 28 days were included in this study.

2.2. ERT and BMT treatment

There were six groups of mice in this study: 1) Twitcher mice receiving ERT only (Twi ERT), 2) Twitcher mice receiving both ERT and BMT (Twi ERT+BMT), 3) Twitcher controls receiving enzyme diluent (Twi vehicle), 4) wildtype controls receiving enzyme diluent (WT vehicle), 5) untreated Twitcher mice (Twi), and 6) untreated wildtype mice (WT).

On day 2–3 of life, those mice receiving ERT or vehicle were given one intrathecal (IT) injection [25] and bilateral intracerebroventricular (ICV) injections [21] as previously described. Recombinant GALC enzyme solution and GALC enzyme vehicle were obtained from Shire Human Genetic Therapies. Intrathecal injections were given into the spine at the upper lumbar vertebral column. A total volume of 15µL (13µL GALC enzyme solution at 0.41mg/ml, 2µL Trypan blue dye) was injected. For ICV injections, one injection was given per hemisphere (2 injections total) into the lateral ventricles at 0.5mm posterior from the bregma, 1mm lateral to the midsagittal sinus, and 2mm deep. A total volume of 4µL (2µL GALC enzyme solution at 0.41mg/ml, 2µL Trypan blue) was injected per hemisphere.

Mice receiving the combination treatment of ERT+BMT were given BMT on the day after receiving ERT. BMT was performed as previously described [24]. Briefly, unfractionated, syngeneic bone marrow cells were isolated from the femurs of sex-matched GALC +/+, GFP +/- donors [26]. Mice were exposed to 400 rads of total body radiation from a ¹³⁷Cs source, followed by an IV injection of 1×10⁶ syngeneic bone marrow cells into the superficial temporal vein [27].

2.3. Lifespan and behavioral testing

Ten mice from each group were tested for motor function and clinical condition every five days starting at 25 days of age. They were assessed for: 1) body weight, 2) constant speed rotarod performance, and 3) wire hang performance. Behavioral assays were performed as previously described [18]. In the constant speed rotarod test, each mouse was placed on a constantly rotating rod (3 rpm), and the time to fall off the rod (latency) was measured. In the wire hang test, each mouse was placed on the top of a wire cage lid held 30 cm above a cage bottom with aspen bedding. The cage lid was gently shaken and then inverted, and the time to fall off was measured. For both tests, latency was measured as the average of three trials, with a maximum time of 60 seconds per trial. Mice were given a rest period of at least five minutes between trials. To train the mice to do these tests, two training trials for each test were given at 20 days of age.

The same mice used for behavioral tests were also used for lifespan analysis. Each mouse was allowed to live as long as it could according to humane laboratory animal care ethics. Animals were sacrificed by CO₂ asphyxiation if they had hindlimb paralysis or appeared moribund (25% loss in body weight, lethargy and/or unresponsive to tactile stimulation).

2.4. Bone marrow engraftment

To determine the level of hematopoietic engraftment, three mice treated with ERT+BMT were sacrificed at 36 days of age, and bone marrow was harvested from their femurs. Bone marrow was also harvested from the femurs of GFP +/– and GFP –/– mice for use as controls to establish the appropriate fluorescence-activated cell sorting (FACS) parameters. Red blood cell lysis buffer was added, and the resulting cell suspension was filtered through a 70µm filter (BD Biosciences, Bedford, MA). The bone marrow cells were analyzed via FACS to determine the percentage of GFP (+) cells, which represents the % donor engraftment.

2.5. GALC activity

Three mice from each group were sacrificed at 36 days to determine GALC activity in the brain. These animals were transcardially perfused with PBS. Three Twitcher mice treated with ERT only, two untreated Twitcher controls, and four untreated WT controls were also sacrificed at 24 hours post-injection to determine GALC activity in the brain and spinal cord shortly after injection.

Tissues were flash frozen in liquid nitrogen, subsequently homogenized in water, and the supernatants used for GALC enzyme assays. Brains and spinal cords were analyzed for GALC activity by cleaving ³H-galactosylceramide. Excess uncleaved substrate was extracted using chloroform:methanol saturated with galactose. The amount of radiation remaining, which represents the free ³H-galactose, was measured in a scintillation counter as counts per minute (CPM). The background radiation (CPM measured from a blank) was subtracted from each sample's measurement. Protein assays were performed using the Coomassie dye binding assay (Biorad, Hercules, CA) in order to calculate specific activity (nmole substrate cleaved/mg prot/hr).

2.6. Psychosine quantification

The same brains at 36 days used for GALC assays were also used for psychosine quantification. Psychosine measurements were performed using a column-switching LC-MS/MS method. Prominence HPLC system (Shimadzu, Columbia, MD), equipped with SIL-20A autosamplers and four LC-20AB binary pumps, was used to separate psychosine and internal standard from the other biological matrix. Mobile phases were 0.1% aqueous formic acid (A), 0.1% formic acid in acetonitrile-isopropanol (1:2) (B), 0.1% formic acid in

acetonitrile-water (2:3) (C). The gradient for the trapping column started from 30% of B and held for 0.2 min, gradient increased from 30 to 50% B in 1 min and held for 0.3 min, gradient increased from 50 to 100% B in 0.5 min and held for 4.5 min then ramped back to 30% B in 0.1 min, and finally held at 30% of B for 1.4 min. Chromatographic separation on the analytic column was achieved with isocratic elution using mobile phase C at a flow rate of 0.7 mL/min. The analyte and internal standard eluted from the analytic column with mobile phase C were directed into the electrospray interface of the mass spectrometer. Detection was achieved using an AB SCIEX 4000QTRAP tandem mass spectrometer (Applied Biosystems/MDS Sciex Inc., Ontario, Canada) employing ESI in the positive ion mode along with multiple reaction monitoring (MRM). Analyst software (version 1.5.1, Applied Biosystems/MDS Sciex Inc., Ontario, Canada) was used for the data analysis. Due to endogenous levels of psychosine in the biological samples, methanol-water (1:1) with 0.04 M citric acid was used for the preparation of the calibration curve. The calibration curves (analyte peak area/internal standard peak area for Y-axis and analyte concentration for X-axis) of psychosine were obtained using the least square linear regression fit ($y = ax + b$) and a weighting factor of $1/x^2$. The coefficient of determination (r^2) was set as >0.98 for acceptance criteria of calibration curves.

2.7. Histochemistry

One Twitcher mouse treated with ERT only was sacrificed 24 hours post-injection to determine the distribution of enzyme activity in the brain. One untreated WT and one untreated Twitcher mouse were also sacrificed at this timepoint for comparison. Brains were collected, immediately frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and cryosectioned. Histochemical staining for GALC activity was performed as previously described [28]. Mounted 16 μ m sections were fixed for 15 minutes with chloral-formal-acetone fix, blocked for endogenous β -galactosidase activity using taurodeoxycholic acid and oleic acid, and stained using a solution containing potassium ferricyanide, potassium ferrocyanide, and X-Gal. Sections were counterstained with Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO), dehydrated, and coverslipped.

2.8. Immunohistochemistry

Brains and spinal cords were collected from the mice sacrificed at 36 days. Brain hemispheres were immersion-fixed in 4% paraformaldehyde in PBS. Spinal cords were immersion-fixed and decalcified in Enhanced Decalcification Formulation (StatLab, McKinney, TX). After overnight fixation, both brains and spinal cords were cryoprotected in 30% sucrose for ~48 hours, frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and cryosectioned. Free-floating 20 μ m sections were treated by peroxidase quenching, blocked with normal goat serum (Sigma-Aldrich, St. Louis, MO), and incubated overnight in the appropriate primary antibody at 4°C. Sections were then incubated in the appropriate secondary antibody, followed by incubation in Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA). Stains were developed using a DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA), and sections were mounted, dehydrated, and cover-slipped. For sagittal brain sections, primary antibodies were rabbit anti-mouse glial fibrillary acidic protein (GFAP) (1:500, Immunostar, Hudson, WI), rat anti-mouse CD68 (1:1000, AbD Serotec, Raleigh, NC), and rabbit anti-human myelin basic protein (MBP) (1:500, Sigma-Aldrich, St. Louis, MO). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated biotinylated anti-rabbit (Vector Laboratories, Burlingame, CA) and mouse adsorbed HRP-conjugated biotinylated anti-rat (Vector Laboratories, Burlingame, CA). Spinal cord sections were stained for CD68 only.

Images of the cerebellum (20x), hippocampus and corpus callosum (10x), striatum (20x), and brainstem (20x) regions were taken using an Olympus BX41 microscope and Olympus

DP 20 camera. Whole spinal cord images were taken in the thoracic (4x) and lumbar (4x) regions. Camera settings were kept constant for all images of the same CNS region, and images were taken from the same area of each CNS region.

Sections stained with GFAP and CD68 were quantitatively analyzed. ImageJ was used to quantify the area fraction of GFAP or CD68 staining in each image. Images were converted to 8-bit grayscale, a constant threshold was placed to highlight stained regions, and area fraction was calculated. The threshold was kept constant for all images of the same CNS region and antibody stain. Quantification was performed on three sections of each CNS region per animal.

Sections stained with MBP were analyzed quantitatively using two defined parameters: 1) the thickness of the corpus callosum and 2) the thickness of major axonal bundles in the striatum. All measurements were performed on sections about 1.56mm lateral from the midsagittal sinus [29]. In the corpus callosum, the thickness was measured at approximately 2.75mm posterior to bregma [29]. In the striatum, a major bundle was defined as a bundle with a thickness of ~30–40 μ m, and a minor bundle was defined as having a thickness of 10 μ m. The thickness of major bundles was determined by measuring all of the major bundles in a 1 $\times$ 1mm² field in the center of the striatum. These measurements were performed on two sections of each CNS region per animal.

2.9. Statistical methods

Graphpad Prism (GraphPad Software, Inc., La Jolla, CA) software was used for statistical analyses. For lifespan and behavior, untreated and vehicle-treated WT groups were pooled after determining that there were no significant behavioral differences between these groups. Untreated and vehicle-treated Twitcher mice were also pooled for the same reason. Comparison of lifespan across groups was performed using the Kaplan-Meier method and log-rank test. Comparison of behavioral data across treatment groups at each timepoint was performed using one-way ANOVA and post-hoc Bonferroni comparisons. For analysis of the immunohistochemistry, GALC activity, and psychosine quantification, comparison across groups was performed using one-way ANOVA and post-hoc Bonferroni comparisons.

3. Results

3.1. GALC activity and psychosine levels

The GALC levels in the brains of ERT-treated mice at 24 hours post-injection were significantly ($p<0.001$) greater than in untreated Twitcher mice and approximately 2-fold greater than WT levels (Fig. 1a). The GALC levels in the spinal cords of ERT-treated mice at 24 hours post-injection were significantly ($p<0.01$) greater than in untreated Twitcher mice and approximately 1.7-fold greater than WT levels (Fig. 1b). At 36 days of age, the GALC levels in WT mice were significantly ($p<0.001$) greater than untreated and treated Twitcher groups (Fig. 1c). There was no significant difference between the treated and untreated Twitcher groups at 36 days of age.

Psychosine levels in the brains of untreated Twitcher mice at 36 days were significantly ($p<0.001$) greater than in untreated WT mice (Fig. 1d). Treated Twitcher mice had significantly ($p<0.001$) reduced psychosine levels compared to untreated Twitcher mice, and the ERT+BMT group had a further significant ($p<0.05$) reduction in psychosine compared to the ERT-only group.

3.2. GALC distribution

Twitcher mice receiving ERT had intense GALC staining (blue) in the ependymal lining and choroid plexus at 24 hours post-injection (Fig. 2). The staining was particularly intense in the hippocampus, corpus callosum, periventricular region, and the meninges surrounding the cerebellum and olfactory bulb. There was an apparent decreasing gradient of GALC staining from the periventricular region to the more distal regions of the brain. The brainstem and the remainder of the brain had less intense staining with rare intensely staining cells. WT mice had diffuse staining throughout the entire brain, and untreated Twitcher mice had little to no GALC staining.

3.3. Myelination

Twitcher mice at 36 days of age had an apparent decrease in the intensity of myelin staining compared to WT controls (Fig. 3a). This was most notable in the corpus callosum, brainstem, and cerebellar white matter tracts. The distribution of myelin also appeared less organized in Twitcher mice compared to WT controls. In untreated Twitcher mice, there were focal areas devoid of myelin staining in the cerebellar white matter tracts (arrows), and the striatum appeared less well organized. Although there were still focal areas devoid of myelin staining in the cerebellar white matter tracts in the ERT and ERT+BMT groups (arrows), there appeared to be fewer. The intensity of myelin staining in the corpus callosum and the pattern of staining in the striatum of the ERT+BMT mice appeared more normal when compared to untreated Twitcher mice.

Quantitative measures also showed significant differences in the thickness of the corpus callosum (Fig. 3b) and of the major bundles in the striatum (Fig. 3c). In untreated Twitcher mice, the corpus callosum was significantly ($p<0.05$) thinner than in WT mice. The ERT and ERT+BMT groups had an apparent increase in the thickness of the corpus callosum compared to untreated Twitcher mice; however, this increase was not statistically significant. In the striatum, untreated Twitcher mice had significantly thinner major bundles than WT mice ($p<0.01$) and the ERT-BMT group ($p<0.05$).

3.4. Neuroinflammation

Brain sections from 36-day-old mice were immunostained for GFAP to visualize activated astrocytes (Fig. 4a). Twitcher mice had significantly ($p<0.001$) more GFAP staining compared to WT controls in all brain regions analyzed. Both ERT- and ERT+BMT-treated Twitcher mice showed a significant ($p<0.001$) decrease in GFAP staining compared to untreated Twitcher mice in the cerebellum (Fig. 4b) and brainstem (Fig. 4d). There was a significant ($p<0.01$) decrease in GFAP staining in the ERT-only mice compared to untreated Twitcher mice in the hippocampus and corpus callosum (Fig. 4c), but not in the striatum (Fig. 4e). The ERT+BMT group had significantly less GFAP staining than the ERT-only group in the striatum ($p<0.01$) and brainstem ($p<0.05$).

Brain sections from 36-day-old mice were immunostained for CD68 to visualize activated microglia (Fig. 5a). Twitcher mice showed significantly ($p<0.001$) increased CD68 staining compared to WT controls in all brain regions analyzed (Fig. 5b–e). Both ERT- and ERT+BMT-treated Twitcher groups showed a significant ($p<0.001$) decrease compared to untreated Twitcher controls. The ERT+BMT group showed a further significant ($p<0.01$) decrease from the ERT-only group in the cerebellum (Fig. 5b). Spinal cord sections from 36-day-old mice were immunostained with CD68 (Fig. 6a). Both groups of treated Twitcher mice had a significant ($p<0.001$) decrease in CD68 staining compared to untreated Twitcher controls in the thoracic (Fig. 6b) and lumbar (Fig. 6c) regions.

3.5. Bone marrow engraftment following conditioning radiation

Partial donor hematopoietic chimerism was measured in three ERT+BMT mice at 36 days of age. The level of bone marrow engraftment was measured as the percentage of cells expressing GFP. The mean $\pm$ SEM of the percent donor engraftment was $12.6 \pm 4.3\%$. This level of donor chimerism is consistent with previous studies using the same transplant protocol [15,24].

3.6. Lifespan and behavior

All Twitcher mice (control and treated) had significantly ($p < 0.0001$) shorter lifespans than WT controls (Fig. 7a). Twitcher controls had a median lifespan of 40 days (range 36–45 days). Twitcher mice receiving ERT only had a significant ($p < 0.0001$) increase in median lifespan to 51 days (range 44–59 days). Twitcher mice receiving ERT+BMT had a further significant ($p < 0.01$) increase in median lifespan to 59 days (range 48–69 days). All WT mice were still alive at 100 days of age.

The constant speed rotarod and wire hang tests were used as measures of behavioral and motor function. There was no statistically significant difference in latency on the rotarod across all groups until 35 days of age (Fig. 7b). After 35 days, Twitcher controls showed a precipitous drop in latency. Twitcher mice treated with ERT only and ERT+BMT showed a decrease in latency starting at 40 and 45 days, respectively. The decrease in latency of both treated Twitcher groups showed a roughly parallel decline, and was significantly ($p < 0.001$) different from Twitcher controls starting at 40 days. The ERT+BMT group showed a significant ($p < 0.05$) increase in latency compared to the ERT-only group at 55 days.

In the wire hang test, all Twitcher groups showed a gradual decline starting from day 25 (Fig. 7c). The ERT-only group showed significantly ($p < 0.01$) increased latency compared to Twitcher controls at all timepoints. However, the ERT+BMT group was not significantly different from Twitcher controls at any timepoint. The two treated groups were significantly ($p < 0.05$) different from each other at 25 and 30 days.

Body weight was used as a measure of disease progression (Fig. 7d). WT mice gained weight more quickly than Twitcher mice. Twitcher controls and treated Twitcher mice stopped gaining weight at 25 and 35 days, respectively. The two treated groups were significantly ($p < 0.01$) different from Twitcher controls starting at 35 days of age but were not significantly different from each other.

4. Discussion

This study tested two treatment methods for GLD: 1) delivering recombinant GALC enzyme to the CNS via IT and ICV injections (ERT), and 2) combining ERT and BMT in neonatal Twitcher mice. The results show that this ERT approach led to supraphysiological levels of GALC activity in the brain shortly after injection. Furthermore, both ERT alone and the combination of ERT and BMT are beneficial in increasing the lifespan and correcting the behavioral phenotypes of Twitcher mice.

A previous study demonstrated that a single ICV administration of GALC resulted in widespread distribution of enzyme in the CNS, as well as clinical improvement [21]. In that study, Twitcher mice received an ICV injection of GALC to the right ventricle at 20 days of age, which increased median lifespan in a dose-dependent manner. The median lifespans of treated Twitcher mice were ~45 days after receiving 3 μ g GALC, ~46.5 days for 6 μ g GALC, and ~50 days for 30 μ g GALC. In the current study, we delivered GALC to neonatal mice via bilateral ICV injections, in addition to an IT injection. The IT approach has previously shown efficacy in targeting gene therapy to the CNS of Twitcher mice [24]. Twitcher mice

receiving both ICV and IT injections in this study reached a median lifespan of ~51 days, which is comparable to the ~50-day lifespan of mice receiving 30 μ g GALC in the previously described study [21]. However, in the current study, the total amount of GALC delivered was ~7 μ g (5.33 μ g IT, 0.82 μ g ICV to each ventricle). Although these two studies differ in several important aspects (i.e. time and routes of administration), the data would suggest that a lower total dose of enzyme delivered via ICV and IT injections earlier in life could be more efficacious.

In addition to improvements on the ERT regimen, this study also investigated the effects of combining ERT and BMT in neonatal Twitcher mice. Twitcher mice receiving ERT showed supraphysiological (about 2-fold normal) levels of GALC activity in the brain and spinal cord 24 hours after injection. GALC activity was particularly high in areas associated with the injection sites: the periventricular regions and the meninges. By 36 days of age, GALC was undetectable in the brain in treated Twitcher mice. Psychosine levels were significantly lower in the brains of the ERT+BMT mice compared to the ERT-only mice, which suggests that BMT might have provided a small but undetectable additional source of GALC. The likely presence of very low (below the sensitivity of the assay) GALC activity in the brains of the ERT+BMT mice was probably due to the myeloreductive conditioning regimen (400 rads) and lower engraftment when performing BMT in neonatal animals. This is consistent with previous studies using myeloreductive conditioning and BMT in the Twitcher mouse [15,24].

Previous studies demonstrated that Twitcher mice have increased levels of GFAP and CD68 staining, which represent activated astrocytes and microglia, respectively [15,24]. Those studies also showed that decreased GFAP and CD68 staining correlate with an improved clinical course. In the current study, we observed significantly decreased levels of GFAP and CD68 in the CNS of all treated Twitcher mice compared to untreated Twitcher mice. The ERT+BMT-treated mice had a further significant decrease compared to ERT-only mice in GFAP in the striatum and brainstem, and in CD68 in the cerebellum. This further reduction in neuroinflammation supports that BMT provides an additional therapeutic benefit to ERT. This is consistent with previous reports showing that BMT can reduce neuroinflammation [30,24].

The combination-treated mice also had significantly increased lifespan and rotarod performance compared to mice treated with ERT only. Mice receiving both ERT and BMT reached a median lifespan of ~59 days, compared to ~51 days for ERT only, ~44 days for BMT only (as determined by a previous study in our lab [15]), and ~40 days for untreated Twitcher mice. These results further demonstrate that BMT enhances the efficacy of ERT.

Although these increases in lifespan are significant, they are smaller than those achieved by other combination therapies. In previous studies in our lab, Twitcher mice treated with both CNS-directed gene therapy (GT) and BMT had a median lifespan of ~105 days [15] or ~123 days [24], compared to ~55 days with GT alone [18,19]. The transient nature of ERT may be one of the reasons why the ERT+BMT-treated mice in this study did not perform as well as the GT+BMT-treated mice in previous studies [15,24]. In this study, Twitcher mice were given a one-time bolus of GALC at birth, but did not receive any enzyme during the remainder of their lives. Although the highest levels of GALC activity in the brain in the current ERT study were comparable to previous GT studies (2–5 fold greater than normal), and psychosine accumulation was significantly decreased with a single administration of ERT, the elevated GALC activity in the GT-treated mice was persistent. This is one of the major limitations of ERT. However, this limitation may be overcome using either repeated CNS injections or the implantation of a permanent pump or port [31]. Repeated infusions via an implanted ICV cannula into the mouse model of Niemann-Pick AB resulted in a more

persistent level of the enzyme in the brain, reduced accumulation of the substrate, and improved behavioral function [32]. Similarly, repeated enzyme injections into the cerebrospinal fluid of the mouse model of mucopolysaccharidosis type IIIA also decreased brain substrate levels and improved behavior [33]. These studies have shown that enzyme can be effectively delivered to the CNS over an extended period of time, thereby maintaining a high, consistent level of enzyme activity similar to GT. In future studies, it would be interesting to determine whether combining long-term ERT with BMT would result in lifespan and other clinical improvements more similar to those achieved by GT +BMT.

While the ERT+BMT-treated mice showed equal or improved results compared to the ERT-only mice in most paradigms, this was not the case for the wire hang test. ERT significantly increased wire hang performance, but the addition of BMT appeared to nullify this effect. This may be due to the motor deficits and increased tremor of mice treated with BMT [24,34] and the cerebellar dysplasia associated with radiation conditioning at birth [35]. Increased tremor and cerebellar deficits may decrease the ability of BMT-treated mice to perform the wire hang task, which is thought to involve the PNS and cerebellar-associated motor capabilities. Therefore, adding BMT to ERT is generally beneficial, but the conditioning regimen likely imposes additional deficits on the mice.

Overall, we have shown that delivering recombinant enzyme to the brain and spinal cord of neonatal Twitcher mice supplies an immediate source of the deficient enzyme, decreases CNS inflammation, and improves behavioral performance and lifespan. The addition of BMT to this ERT regimen further increases the treatment efficacy. However, this study also highlights some of the adverse consequences of BMT and the limitations of ERT due to its transient nature. Future studies may improve on this combination treatment by giving ERT throughout life, adding other therapies such as gene therapy or substrate reduction, or increasing the bone marrow engraftment levels.

Supplementary Material

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Abbreviations

GLD	globoid cell leukodystrophy
GALC	galactocerebrosidase
BMT	bone marrow transplantation
ERT	enzyme replacement therapy
ICV	intracerebroventricular
IT	intrathecal

CNS	central nervous system
PNS	peripheral nervous system
PAS	Periodic acid-Schiff
HSCT	hematopoietic stem cell transplantation
IP	intraperitoneal
FACS	fluorescence-activated cell sorting
CPM	counts per minute
GFAP	glial fibrillary acidic protein
MBP	myelin basic protein
HRP	horseradish peroxidase
GT	gene therapy

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Highlights

- BMT enhances the effects of intraventricular + intrathecal ERT in GLD.
- ERT combined with BMT could be an effective treatment for GLD.
- A single dose of GALC decreases psychosine accumulation and neuroinflammation.

Figure 1. GALC activity and psychosine levels

(a) GALC levels in the brains of the ERT-treated mice at 24 hours post-injection were significantly greater than in age-matched WT mice. (b) GALC levels in the spinal cords of the ERT-treated mice at 24 hours post-injection were significantly greater than in untreated Twitcher mice. (c) At 36 days of age, GALC levels in the brains of all Twitcher groups were significantly less than WT. (d) At 36 days of age, psychosine levels were significantly greater in untreated Twitcher mice than in untreated WT mice. Treated Twitcher mice had significantly reduced psychosine levels compared to untreated Twitcher mice, and the ERT +BMT group had a further significant reduction in psychosine compared to the ERT-only group. The horizontal bars represent the means, and the error bars represent one SEM. (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$)

Figure 2. GALC distribution

Twitchee mice treated with ERT had intense GALC staining (blue) in the ependymal lining and choroid plexus at 24 hours post-injection. In addition, there was intense staining in the periventricular region, the hippocampus, corpus callosum, and the meninges surrounding the cerebellum and olfactory bulb. There was an apparent decreasing gradient of GALC staining from the periventricular region to the more distal regions of the brain. WT mice had diffuse, less intense staining throughout the brain, and untreated Twitchee mice had little to no staining. All images are shown at 4x, except for the brainstem, which is at 20x.

Figure 3. Myelin basic protein (MBP) immunohistochemistry

(a) Representative images of MBP staining of the brain at 36 days of age. Twitcher mice had an apparent decrease in myelin staining, especially in the corpus callosum and brainstem, compared to WT controls. Twitcher mice also had regions in the cerebellum devoid of staining (arrows). Treated Twitcher mice appeared to have increased myelin staining compared to untreated Twitcher controls, as well as fewer cerebellar areas devoid of staining (arrows). The ERT+BMT group appeared to have more intense staining in the corpus callosum and more normal organization in the striatum compared to the ERT group. (b) The corpus callosum in untreated Twitcher mice was significantly thinner than in WT controls. The ERT and ERT+BMT groups had a slightly, though not significantly, thicker corpus callosum compared to untreated Twitcher mice. (c) In the striatum, untreated Twitcher mice had significantly thinner major axonal bundles than WT controls and the ERT-BMT group. Although there was an apparent increase in the major bundle thickness of Twitcher mice treated with ERT alone, it was not statistically significant. All images are shown at 20x. (*= $p < 0.05$, **= $p < 0.01$)

Figure 4. GFAP immunohistochemistry

(a) Representative images of GFAP staining of the brain at 36 days of age. In the cerebellum (b), hippocampus/ corpus callosum (c), and brainstem (d), Twitcher mice had significantly increased GFAP immunoreactivity (activated astrocytes) compared to WT controls and treated Twitcher mice. In the striatum (e), Twitcher mice had significantly increased GFAP compared to the WT and ERT+BMT groups, and the ERT+BMT group had a significant decrease from the ERT group. In the brainstem (d), the ERT+BMT group showed a significant decrease compared to the ERT group, and the ERT group had a significant increase from WT. All images are shown at 20x, except for hippocampus/ corpus callosum, which are at 10x. (*=p<0.05, **=p<0.01, ***=p<0.001)

Figure 5. CD68 brain immunohistochemistry

(a) Representative images of CD68 staining of the brain at 36 days of age. In the cerebellum (b), hippocampus/ corpus callosum (c), brainstem (d), and striatum (e), Twitcher mice had significantly increased CD68 immunoreactivity (activated microglia) compared to WT controls and treated Twitcher mice. The ERT+BMT group showed significantly less intense CD68 staining than the ERT group in the cerebellum (b). All images are shown at 20x, except for hippocampus/ corpus callosum, which are at 10x. (**= $p < 0.01$, ***= $p < 0.001$)

Figure 6. CD68 spinal cord immunohistochemistry

(a) Representative images of CD68 staining of the spinal cord at 36 days of age. In the thoracic (b) and lumbar (c) regions, Twitcher mice have significantly increased CD68 immunoreactivity (activated microglia) than WT controls and treated Twitcher mice. All images are shown at 4x. (***)= $p<0.001$)

Figure 7. Lifespan and behavior

(a) The median lifespans for control, ERT-only, and ERT+BMT treated Twitcher mice were 40, 51, and 59 days, respectively. All WT mice were still alive at 100 days of age. All four of these groups were significantly different from each other. (b) Twitcher mice began declining in rotarod performance starting from 35–45 days of age. Both treated Twitcher groups showed a significant ($p < 0.001$) increase in latency compared to untreated and vehicle Twitcher controls starting at 40 days. The ERT+BMT group showed a significant ($p < 0.05$) increase in latency compared to the ERT-only group at 55 days. (c) All Twitcher groups showed a gradual decline in wire hang performance starting at 25 days, while WT mice improved. Both treated Twitcher groups showed a significant ($p < 0.01$) increase in latency compared to the Twitcher controls. The ERT-only group showed a significant ($p < 0.05$) increase in latency compared to the combination-treated (ERT+BMT) group at 25 and 30 days. (d) Untreated and vehicle-treated Twitcher controls stopped gaining weight at 30 days, and treated Twitcher mice stopped gaining weight at 35 days. The two treated Twitcher groups were significantly ($p < 0.005$) different from the untreated and vehicle-treated Twitcher controls starting at 35 days of age.