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Increased Longevity and Metabolic Correction Following Syngeneic Bone Marrow Transplantation in a Murine Model of Mucopolysaccharidosis Type I

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Abstract

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive inherited disease caused by deficiency of the glycosidase α-L-iduronidase (IDUA). Deficiency of IDUA leads to lysosomal accumulation of the glycosaminoglycans (GAG) heparan and dermatan sulfate and associated multi-systemic disease, the most severe form known as Hurler syndrome. Since 1981, the treatment of Hurler patients has often included allogeneic bone marrow transplantation (BMT) from a matched donor. However, mouse models of the disease were not developed until 1997. To further characterize the MPS I mouse model and to study the effectiveness of BMT in these animals, we engrafted a cohort (n=33) of 4–8 week-old *Idua*$^{-/-}$ animals with high levels (88.4 ± 10.3%) of wild-type donor marrow. Engrafted animals displayed an increased lifespan, preserved cardiac function, partially restored IDUA activity in peripheral organs, and decreased GAG accumulation in both peripheral organs and in the brain. However, levels of GAG and GM3 ganglioside in the brain remained elevated in comparison to unaffected animals. Since these results are similar to those observed in Hurler patients following BMT, this murine transplantation model can be used to evaluate the effects of novel, more effective methods of delivering IDUA to the brain as an adjunct to BMT.

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Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive inherited disease caused by deficiency of the glycosidase α-L-iduronidase (IDUA). IDUA is required for the degradation of the glycosaminoglycans (GAG) heparan and dermatan sulfate and deficiency of the enzyme leads to lysosomal accumulation of these substrates (1). MPS 1 affects approximately 1 in 125,000 live human births and homozygosity for specific mutations (e.g., W402X, Q70X) leads to the most severe phenotype, Hurler syndrome (2). Patients with Hurler syndrome develop progressively severe manifestations of the disease within the first year of life, including growth delay, hepatosplenomegaly, skeletal deformities, excess urinary GAG, corneal clouding, and severe neurological deficits. Untreated, these patients usually succumb to the disease in the first decade of life due to complications caused by respiratory infection, cardiac failure, and obstructive airway disease.

Early biochemical research led to discoveries that have provided the basis for treatment of Hurler patients by hematopoeitic stem cell transplantation (HSCT). After synthesis in the endoplasmic reticulum, IDUA is post-translationally modified by the addition of mannose-6 phosphate to Asn residues in the rough endoplasmic reticulum and Golgi apparatus (3). Most of the modified enzyme is sorted and translocated to the lysosomes, but a small proportion of IDUA escapes from the cell into the extracellular environment (4). Extracellular IDUA can then interact with mannose-6-phosphate receptors on the surface of neighboring cells, with subsequent endocytosis, and shuttling to the lysosomes (5–7). IDUA-deficient cells can thus be cleared of accumulated lysosomal GAG through the uptake of IDUA released by non-deficient cells. This cross-corrective mechanism constitutes the basis for development of cellular and molecular strategies to treat this disorder.

Currently, the standard of care for severe Hurler patients involves enzyme replacement therapy (ERT) by weekly infusions of recombinant enzyme following diagnosis and ultimately HSCT for patients with an HLA-matched donor. Allogeneic bone marrow transplantation (BMT) for MPS I was first conducted in 1981 by Hobbs *et al.* (8). Since then, a body of literature has accumulated describing studies involving transplantation of hematopoeitic stem cells collected from bone marrow, peripheral blood, or umbilical cord blood of related and unrelated donors (8–15). These and other studies have provided molecular insights into the effectiveness of these treatments and have led to continual improvement in HSCT protocols with reduced transplant complications and morbidity. The evolution of HSCT protocols has resulted in increased access to donor sources and allowed achievement of higher levels of donor chimerism and enzyme activity in transplant recipients (16). Following HSCT, organomegaly, upper respiratory symptoms, corneal clouding, and sleep apnea are generally resolved. Cardiac function is preserved, although valvular deformities may persist. The lifespan of patients significantly improves following successful transplantation, as many patients have now survived into the 3rd decade of life (16). Furthermore, neurologic outcome has improved in many patients, although they continue to exhibit subnormal IQ and impaired neurocognitive capability (17, 18).

Despite the long history of HSCT to treat Hurler patients, it was not until 1997 that murine models of MPS I were introduced (19–21). This advance has provided the ability to characterize the pathobiology of IDUA deficiency in a model that can also be used to develop new approaches for the treatment of MPS I (22). Previous studies have reported some of the biochemical effects of wild-type HSCT into MPS I mice (23, 24). In this study, we further investigated the effects of BMT in the MPS I mouse to determine the extent to which the outcomes of this clinically relevant treatment recapitulate results observed in human MPS I patients and other animal models. We engrafted *Idua*$^{-/-}$ animals with quantifiably high levels of congeneic wild-type marrow and report novel outcomes of HSCT on the lifespan, cardiac function, and GM3 ganglioside accumulation in the brains of treated mice. These results provide further characterization of the MPS I mouse model as well as additional insights into the long-term benefits of HSCT that may similarly be achieved in Hurler patients, particularly with respect to metabolic correction in the brain.

Materials and Methods

Animals and Transplantation Procedure

The *Idua*$^{-/-}$ mouse strain (25) was kindly provided by Dr. Elizabeth Neufeld. The animals were routinely maintained on a C57BL/6 background and provided food and water *ad libitum*. For bone marrow transplantation, 33 (CD45.2) 4–8 week old *Idua*$^{-/-}$ recipient mice were preconditioned by exposure to a sublethal dose of cesium irradiation (750 cGy). The following day, wild-type whole marrow was harvested by flushing the hind limbs of congenic (CD45.1) wild-type C57BL/6 mice into DMEM supplemented with 10% fetal bovine serum and 10U/mL heparin. Harvested cells were triturated into a single-cell suspension and viable cells counted by trypan blue exclusion using a hemacytometer. Ten million donor cells were then infused into the preconditioned *Idua*$^{-/-}$ animals through the lateral tail-vein.

Flow Cytometry

Peripheral blood was collected via the submandibular vein into heparinized tubes and the red cells lysed using a hypotonic buffer (eBioscience, San Diego, CA, USA). The remaining nucleated cells were stained with allophycocyanin-conjugated anti-murine CD45.1 (eBioscience, San Diego, CA, USA) and evaluated by flow cytometry using a FACSCalibur bench top cytometer. The data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA) and donor cell engraftment was determined as the percentage CD45.1+ cells present in the gated lymphocyte compartment (26).

IDUA Activity Assays

Animals were anesthetized with ketamine/xylazine (100mg ketamine + 10mg xylazine per kg) and transcardially perfused with 70 mL PBS prior to sacrifice. Peripheral tissues including the heart, lungs, liver, kidneys, spleen, and brain were harvested. The brain was microdissected on ice into separate regions: right and left olfactory bulb, cerebellum, hippocampus, striatum, cortex, and brainstem and thalamus. For the analyses included in this paper, microdissected brain samples from the left hemisphere were analyzed. The tissue samples were frozen on dry ice and stored at −80°C until use. Samples were thawed and

homogenized in 1mL of phosphate-buffered saline (PBS; Gibco, Invitrogen, Carlsbad, CA, USA) using a motorized pestle and permeabilized by addition of 0.1% Triton X-100. IDUA activity was determined by fluorometric assay using 4MU-iduronide as the substrate, as previously described (22, 27). Activity is expressed as nmol 4-methylumbelliferone released per mg tissue per hour (nmol/mg/h) with the amount of protein in each sample determined by Bradford assay (BioRad, Hercules, CA, USA).

GAG Analysis

The remainder of each tissue homogenate was incubated overnight with proteinase K, DNase1, and RNase (Sigma-Aldrich, St. Louis, MO, USA) as previously described and clarified for 3 min at 12,000 rpm using an Eppendorf tabletop microcentrifuge model Centrifuge 5415D (Eppendorf, Hauppauge, NY, USA) (22, 27). GAG concentration was determined using the Blyscan Sulfated Glycosaminoglycan Assay (Accurate Chemical, Westbury, NY, USA) according to the manufacturer's instructions.

Tissue Staining for GM3 Ganglioside

Animals distinct from those analyzed for IDUA activity and GAG levels were anesthetized with ketamine/xylazine as described above and transcardially perfused with 70mL ice-cold paraformaldehyde (4% w/v in 0.1M PBS) prior to sacrifice. Brains were removed, post-fixed overnight in 4% paraformaldehyde, cryopreserved in sucrose (30% w/v in PBS), frozen on dry ice, and sectioned into coronal slices (30μm thickness) using a freezing microtome. Free-floating sections were washed 3 × 10 minutes in PBS (pH = 7.4) and blocked with 10% normal goat serum / 0.3% Triton X-100. Primary antibody was added and sections were incubated overnight at 4°C, washed 3 × 10 minutes in PBS (pH = 7.4), and incubated for 1 hr at room temperature with a fluorescently labeled secondary antibody diluted in PBS (pH = 7.4). Sections were then washed 3 × 10 minutes in PBS (pH = 7.4), incubated for 10 minutes with DAPI (Invitrogen), mounted on slides, and visualized using a Zeiss Axioplan 2 upright microscope. Primary antibody was used to bind GM3 ganglioside (1:500, Cat. # 370695, Seikagaku Corporation, Tokyo, Japan). The secondary antibody used was a donkey anti-mouse IgM antibody conjugated to Alexa Fluor 488 (1:500, Cat. # A-10680, Molecular Probes, Invitrogen, Carlsbad, CA, USA).

Cardiac Ultrasound

Mice were anesthetized by inhalant isoflurane and ultrasound was conducted using a 30 MHz probe and a Vevo 660 high-resolution ultrasound biomicroscope (VisualSonics, Toronto, Ontario, Canada) (28). Doppler imaging was conducted in both the long- and short-axis beneath the aortic valve to determine the presence or absence of aortic insufficiency (AI).

Statistical Analysis

Data are reported as mean ± s.d. Differences in survival were evaluated using the Kaplan-Meier product limit method, calculating the log rank statistic. One-way ANOVA with Tukey's post-test was used for determining significance between groups for GAG assays. A chi-square test with 95% confidence interval was used to determine significance in cardiac

function manifested by the presence or absence of AI. In all cases, $P < 0.05$ was considered significant and analysis was performed using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

Results

Increased longevity of MPS I mice following BMT with wild-type donor marrow

To evaluate the effect of BMT in MPS I mice, we transplanted young adult *Idua*$^{-/-}$ mice with wild-type marrow from syngeneic C57BL/6 donors. Thirty-three CD45.2 *Idua*$^{-/-}$ mice between the ages of 4–8 weeks were preconditioned with 750 cGy cesium irradiation and on the following day transplanted with 10^7 donor marrow cells harvested from wild-type CD45.1 mice. Mean donor cell engraftment as determined by flow cytometry of peripheral blood for the CD45.1 congenic marker two months post-transplant was 88.4 ± 10.3% (s.d.). Interestingly, we attempted to engraft a second cohort of *Idua*$^{-/-}$ mice at a reduced level by administering 100 cGy cesium irradiation followed by infusion of 1×10^7 congenic donor marrow cells. However, donor cell engraftment was undetectable in this cohort of animals (data not shown).

Upon extended study of these animals, we observed that the recipient *Idua*$^{-/-}$ mice appeared quite healthy compared to untreated MPS I mice as they remained unhunched and active in their cages. The recipient animals displayed increased longevity compared to untreated control animals, with a median lifespan of 85 weeks for transplanted animals compared to a median lifespan of 49 weeks for historical untreated *Idua*$^{-/-}$ animals from the same colony ($P < 0.001$, log-rank test) (Figure 1). The median survival ratio of transplanted mice to untreated MPS I mice was 1.72 (95% CI, 1.09 – 2.36).

Partial restoration of IDUA expression in peripheral organs following BMT

Upon reaching 92 weeks of age, the longest surviving transplanted MPS I mice were trans-cardially perfused with saline and organs were harvested and homogenized. Positive and negative control animals consisted of 13-month old unaffected *Idua*$^{+/-}$ and untreated MPS I mice, respectively. A portion of each tissue homogenate was evaluated for IDUA activity using a fluorometric assay. BMT recipient animals displayed long-term partial restoration of IDUA activity in peripheral tissues 20 months post-transplantation (Figure 2a,b). Activity levels detected in the heart, lungs, spleen, kidneys, and liver were 12%, 37%, 74%, 26%, and 45% of those detected in unaffected heterozygous animals. However, IDUA activity was below the lower limit of detection for this assay in the brains of BMT recipients (Figure 2c).

Reduced GAG storage material in both peripheral organs and the brain following BMT

As previously reported (27), we found that *Idua*$^{-/-}$ mice displayed increased levels of GAG storage in peripheral organs and in different sections of the brain in comparison to unaffected heterozygous animals. Additionally, *Idua*$^{-/-}$ animals engrafted with IDUA-positive donor marrow exhibited a statistically significant reduction in GAG storage material in the lungs (**$P < 0.01$), spleen (***$P < 0.001$), kidneys (***$P < 0.001$), and liver (*$P < 0.05$) (Figure 3). There was also a trend toward reduced GAGs in the heart of BMT

recipients, but the difference was not statistically significant. The mean reduction in GAG storage material in heart, lung, spleen, kidney, and liver of treated animals was 63%, 89%, 96%, 75%, and 93%, respectively, compared to that detected in untreated MPS I animals. The mean percent reduction in storage material was plotted vs. the mean percent of heterozygous IDUA activity detected in the peripheral organs of transplanted animals (Figure 4). A best fit curve was applied to the data set and yielded a hyperbolic relationship between the two variables with a coefficient of determination (R^2) value = 0.95. This relationship between the level of IDUA activity vs. the level of GAG reduction supports previous observations whereby a very small amount of IDUA enzymatic activity is sufficient to provide a substantial reduction in lysosomal GAG accumulation (2, 23).

Although IDUA activity was undetectable in brain samples from transplanted animals, the mean level of GAGs detected was slightly although not significantly lower than that observed in untreated animals in all portions of the brain including the olfactory bulb, cerebellum, hippocampus, striatum, cerebral cortex, and brainstem and thalamus (Figure 5a). When the data from all portions of the brain were compiled, total GAG accumulation in the brain was significantly reduced in the BMT recipients (Figure 5b). These results were likely an underestimation of the actual reduction in GAG storage material since untreated *Idua*$^{-/-}$ mice did not survive to the age of the transplanted animals and thus the BMT-treated animals studied were much older than the control animals.

Focal aggregates of GM3 ganglioside in the brains of BMT recipients

GM3 gangliosides were visualized as focal aggregates in grey matter areas throughout the brains of untreated *Idua*$^{-/-}$ mice by immunofluorescence microscopy. Representative images of GM3 ganglioside accumulation were taken from the dentate gyrus and septal nucleus (Figure 6). Interestingly, the aggregates of GM3 ganglioside in the dentate gyrus appear concentrated within the subgranular zone, which provides a niche for neural stem cells (29). It is not known if this results in dysfunction of these neural stem cells and alters their ability to divide and differentiate: this warrants further investigation. GM3 ganglioside accumulation was absent from the grey matter of unaffected heterozygous mice. Although total brain GAG levels were modestly reduced in the brains of BMT-recipient mice, focal aggregates of GM3 gangliosides were detected in the grey matter of the brains of these animals with the pathology indistinguishable from that observed in untreated MPS I animals.

Preserved valvular heart function in BMT recipients

BMT recipient and control mice were analyzed by high-resolution ultrasound biomicroscopy to assess cardiac valve function (30). Aortic insufficiency (AI) was observed in 9 of 16 (56%) 7.5-month old male untreated MPS I mice while AI was not present in age-matched unaffected male heterozygous animals (n = 6). Six male BMT recipient mice were analyzed and none of the animals displayed AI, demonstrating a significant improvement in aortic function compared to untreated *Idua*$^{-/-}$ animals (**$P < 0.01$ by Chi-square test).

Discussion

Engraftment of wild-type donor marrow into *Idua*$^{-/-}$ mice resulted in a prolonged lifespan and improvement in valvular heart function, consistent with what has been observed in Hurler patients following successful HSCT (14, 31, 32). We also observed partial restoration of enzymatic activity in the heart, liver, kidney, lungs, and spleen of transplanted animals, with a resultant dramatic reduction in GAG storage material in these organs. These results are consistent with data generated from BMT studies in large animals of MPS I disease, including both canine and feline models (33, 34). Upon plotting the mean level of GAG reduction achieved vs. the mean IDUA activity detected in each peripheral organ, we constructed a best-fit curve to depict the relationship between IDUA activity and GAG accumulation in tissues. The relationship validates previous observations whereby very small amounts of IDUA resulted in dramatic reduction in tissue GAGs (2, 23, 34).

Although some BMT studies in MPS I animals have indicated that IDUA activity can be detected in the brains of transplanted dogs and mice (35, 36), brain IDUA was below the lower limit of detection in this study. Nonetheless, total GAG accumulation in the brain was significantly reduced in our study, as reported in cats following BMT (34). This suggests that hematopoietic cells may have engrafted in the brain and differentiated into microglia (37), providing a sufficient amount of enzyme to reduce lysosomal GAG accumulation. However, although this reduction was statistically significant, brain GAG levels were still elevated compared to unaffected heterozygous animals ($^{*}P < 0.05$). Furthermore, punctate aggregates of GM3 ganglioside remained in the treated animals. This demonstrates that although some clearage of storage material occurred following BMT, there is much room for improvement in the extent of biochemical correction that can be achieved in the brain. Because the relationship between the level of biochemical storage in the nervous system and the extent of neurocognitive dysfunction in LSD has yet to be elucidated, experiments in animal models constitute an important step in understanding the degree of intervention that may be required to preserve or restore brain function.

The results from this study corroborate the usefulness of the MPS I mouse as a disease model that recapitulates the human disease. Since the current standard of care for Hurler children involves HSCT from a matched donor, the development and assessment of new therapeutic strategies for improved outcomes should be based on those achieved following HSCT. Additionally, since many patients receive enzyme replacement therapy in combination with HSCT (15), it will be important to understand the effect of this combined therapeutic approach on the disease in animal models such as the MPS I mouse. As one of the major challenges in the treatment of Hurler patients remains efficient delivery of IDUA to the central nervous system, the results of this study provide a benchmark against which novel therapies directed at the CNS in the MPS I mouse can be compared.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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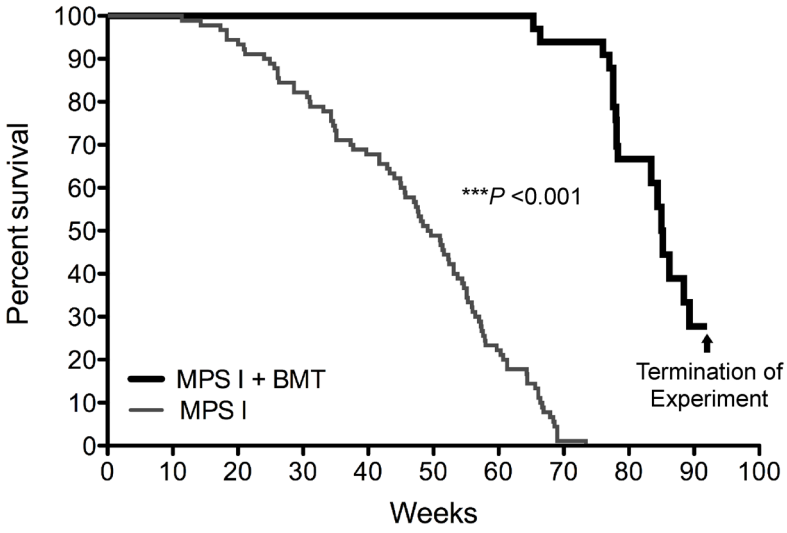

Figure 1. Survival of MPS I mice engrafted with syngeneic marrow compared to untreated MPS I animals

The Kaplan-Meier plot shows the percentage of animals surviving to the indicated age in weeks depicted on the x-axis. Lines on the graph represent untreated *Idua*$^{-/-}$ animals (thin grey) and *Idua*$^{-/-}$ animals treated by BMT at 4–6 weeks of age (thick black).

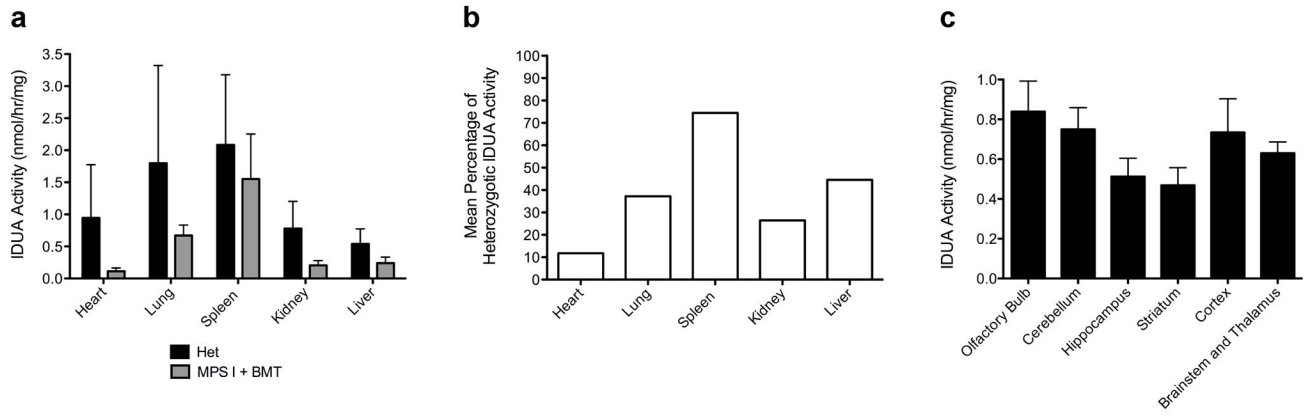

Figure 2. IDUA activity levels in peripheral organs and brain

(a) Mean (± S.D.) IDUA activity detected in peripheral organs of BMT recipient MPS I mice (MPS I + BMT) and heterozygous control animals (Het). (b) Mean percentage of heterozygous IDUA levels detected in the peripheral organs of BMT recipient animals. (c) Mean (± S.D.) IDUA activity in microdissected brain samples from unaffected heterozygous animals. IDUA activity was undetectable in peripheral organ and brain samples from untreated MPS I animals and in brain samples from BMT recipient mice.

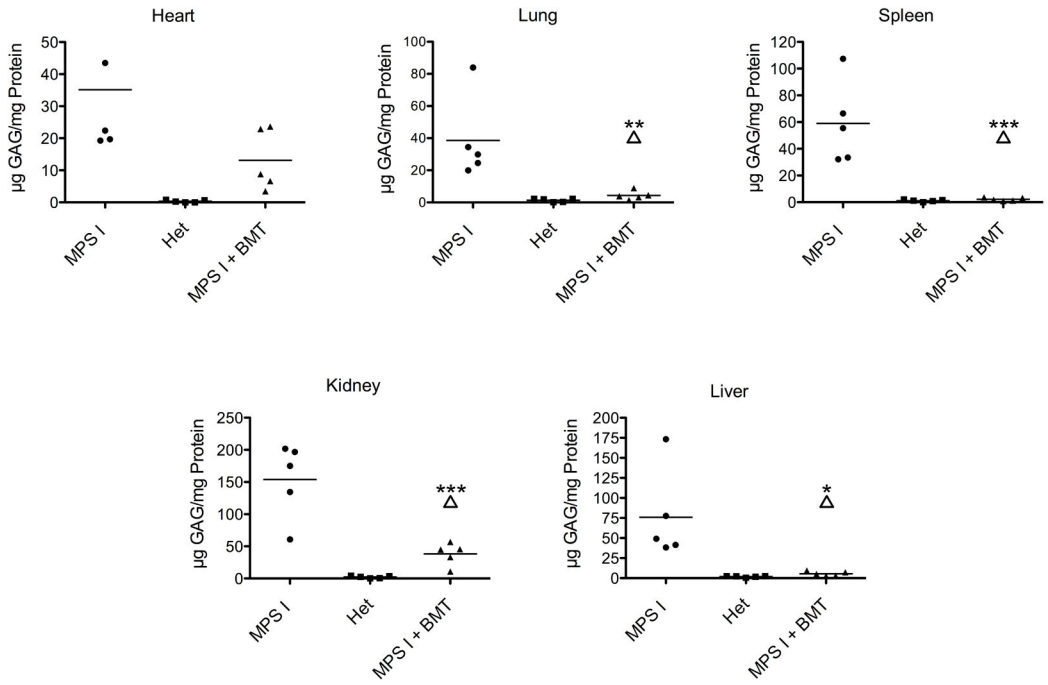

Figure 3. GAG storage in peripheral organs following BMT

Levels of GAG storage material detected in the peripheral organs of MPS I (MPS I, circles), heterozygous (Het, squares) and BMT recipient mice (MPS I + BMT, triangles). The mean is indicated by a solid line. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by one-way ANOVA as compared to untreated MPS I mice; Δ = no statistical significance compared to heterozygous animals by one-way ANOVA.

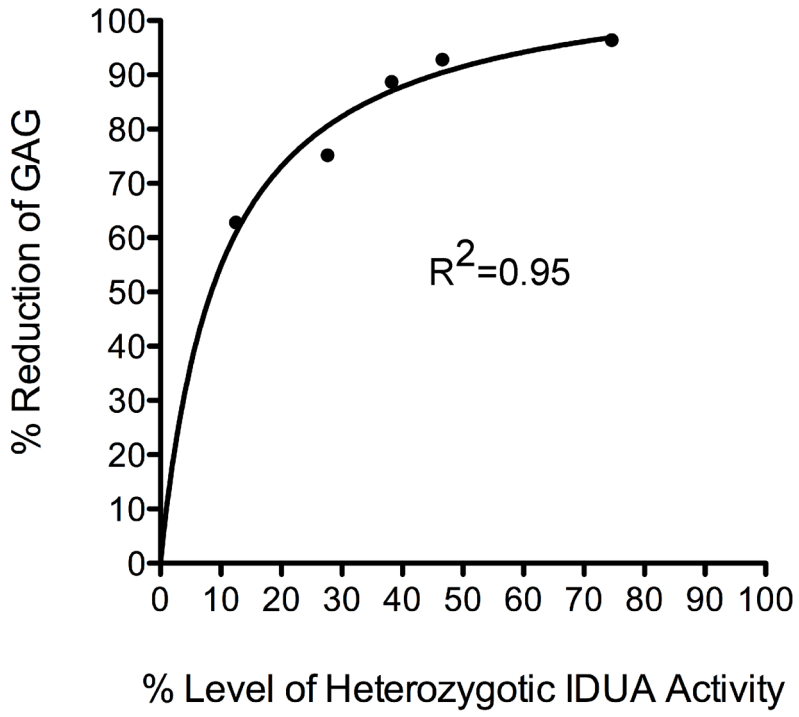

Figure 4. Relationship between GAG reduction and IDUA activity level in peripheral organs of BMT recipients

The mean percent reduction of GAG storage material detected in each peripheral organ (BMT-treated mice compared to untreated MPS I mice) was plotted vs. the mean percentage of heterozygous IDUA activity (BMT-treated mice compared to heterozygous mice) detected in the same organ The correlative relationship between GAG storage and IDUA activity corresponded to a hyperbolic model with coefficient of determination (R^2) value = 0.95.

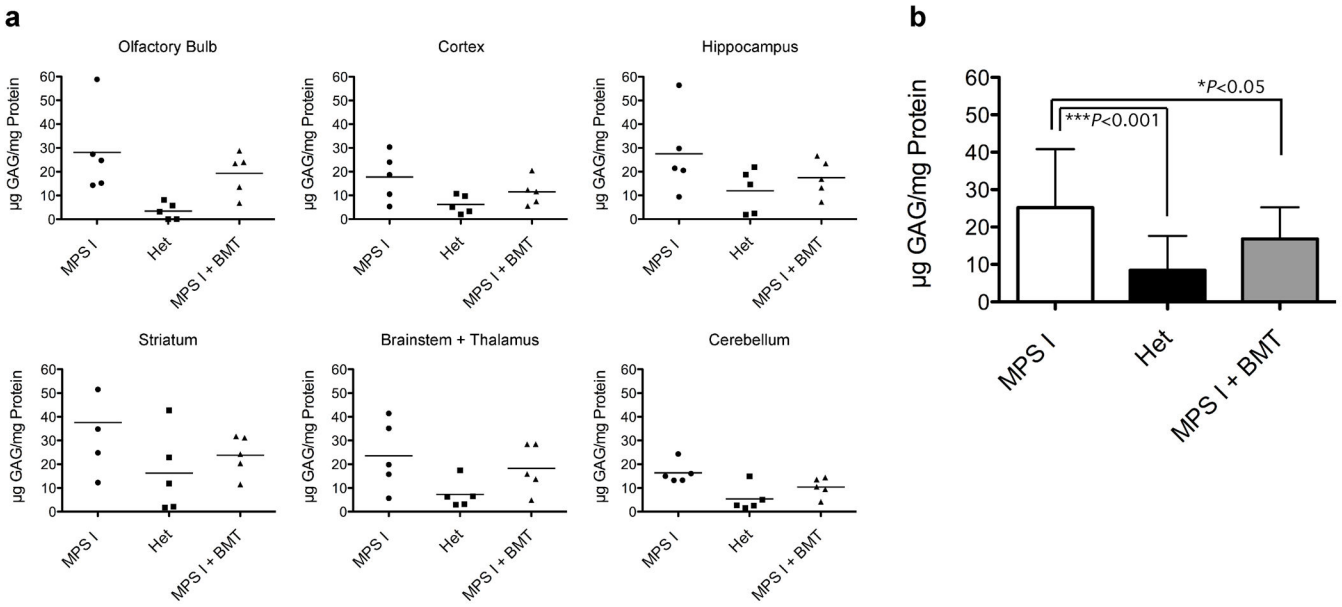

Figure 5. GAG levels in the brain

(a) Levels of GAG storage material detected in microdissected regions of brain tissue collected from MPS I (MPS I, circles), heterozygous (Het, squares) and BMT recipient mice (MPS I + BMT, triangles). The mean is indicated by a solid line. (b) Mean (± S.D.) GAG content detected in the brains of MPS I (MPS I, white bar), heterozygous (Het, black bar) and BMT recipient mice (MPS I + BMT, grey bar) upon compiling all data points in part (a). **P* < 0.05 (MPS I vs. MPS I + BMT), ***P < 0.001 (MPS I vs. Het) by one-way ANOVA.

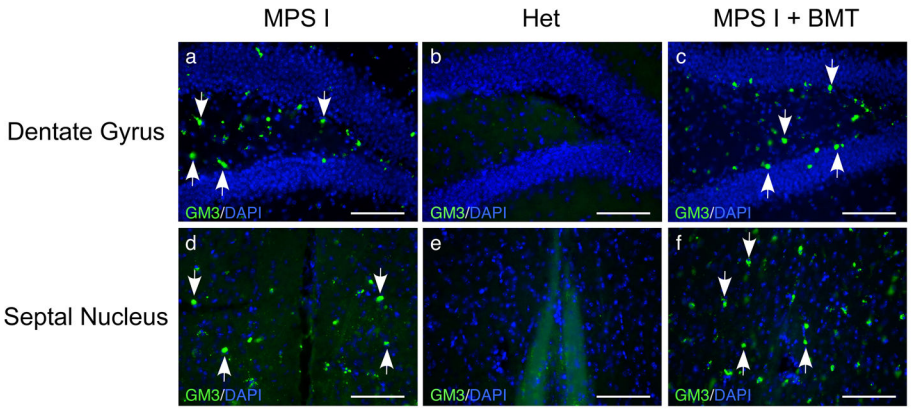

Figure 6. GM3 ganglioside accumulation in the brain

GM3 ganglioside, a substrate that accumulates in neurons and glia of MPS I animals (27, 38), was visualized by immunofluorescence microscopy as punctate aggregates (white arrows) within grey matter throughout the brains of untreated MPS I mice. Representative images demonstrate focal accumulation of GM3 ganglioside in the dentate gyrus region of the hippocampus and within the septal nucleus, both parts of the limbic system. These GM3 ganglioside aggregates were absent from the grey matter of *Idua*$^{+/-}$ mice, but persisted in the brains of BMT recipient mice.