Molecular Therapy for Mucopolysaccharidosis Type I

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II. Thesis Abstract

Mucopolysaccharidosis type I (MPS I) is caused by deficiency of the lysosomal hydrolase α-L-iduronidase (IDUA). IDUA is a required component of the step-wise degradative pathway responsible for the catabolism of the glycosaminoglycans (GAGs) heparan sulfate and dermatan sulfate. As a result, these GAGs accumulate within lysosomes causing the development of multisystemic disease. Patients with MPS I present with clinical manifestations of disease within the first two years of life including corneal clouding, hepatoslenomegaly, skeletal dysplasias, cardiopulmonary disease, and obstructive airway disease. Additionally, patients with severe MPS I, also known as Hurler syndrome, develop hydrocephalus and severe neurocognitive decline. The current standard of care for Hurler patients included intravenous administration of recombinant enzyme upon diagnosis followed by hematopoeitic stem cell transplantation (HSCT) once a proper donor cell source is identified. Following HSCT, many patients exhibit a reduced rate of neurological deterioration. However, the potential of HSCT to ameliorate central nervous system manifestations of disease is limited by the inability of IDUA to efficiently cross the blood brain barrier. The results of my experiments demonstrate that GAG storage materials were partially reduced in the brains of MPS I mice following bone marrow transplantation with wild-type donor marrow (Chapter 2). However, pathogenic accumulation of GM3 ganglioside, not normally expressed in the brain, remained present in treated animals. This highlights the necessity to achieve more efficient delivery of IDUA to the central nervous system in order to normalize brain biochemistry. Thus, I propose the application of intracerebroventricular (ICV) infusion of adeno-associated viral

vectors in order to mediate gene transfer and expression of IDUA in the brain. Infusion of an AAV serotype 8 vector into neonatal MPS I mice resulted in widespread long-term expression of high levels of IDUA throughout the brain consistent with normalization of GAG storage material and complete prevention of a neurocognitive deficit in a Morris water maze test (Chapter 3). Infusion of the same vector into adult MPS I animals resulted in low levels of IDUA expression and partial reduction of storage material in the brain consistent with partial improvement in the Morris water maze test (Chapter 4). The results of these experiments support the adoption of ICV infusion of AAV vectors as a supplement to enzyme replacement therapy and HSCT for the treatment of Hurler syndrome.

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Chapter 1

Background and Significance

Overview

The mucopolysaccharidoses (MPSs) are a group of 11 rare, recessively inherited disorders included in the larger family of more than 40 identified lysosomal storage diseases (LSDs) (Table 1). LSDs comprise approximately 14% of all inherited metabolic diseases and affect nearly 1:7,700 births, of which ~30% are MPSs (1, 2). MPSs are caused by genetic mutations in genes that encode lysosomal hydrolases, which are responsible for the catalysis of glycosaminoglycans (GAGs), a component of the exctracellular matrix. Deficiency for any of the enzymes involved in this process results in lysosomal accumulation of GAG storage material and resultant progressive, multisystemic disease (3). MPS diseases are typically manifested by symptoms including growth delay, organomegaly, cardiopulmonary disease, skeletal dysplasias, and obstructive airway disease. Additionally, patients with severe MPS I, II, III, and VII exhibit severe neurocognitive decline. Symptoms develop within the first two years of life and, without treatment, severe MPS patients typically succumb to disease complications within the first decade of life. Current available therapies used to treat the MPSs include allogeneic hematopoietic stem cell transplantation (HSCT) and recombinant enzyme replacement therapy (ERT) for MPS II, VI, and I. Although markedly efficacious in treating manifestations of disease in peripheral organs, the benefits of ERT in the central nervous system (CNS) are limited due to the inefficiency of lysosomal enzymes to cross the blood brain barrier (BBB) (4) Thus, the focus of this thesis is to investigate gene therapy as an alternative form of treatment that could be used as a supplement to HSCT and ERT in order to more effectively deliver lysosomal enzymes to the CNS by conducting relevant experiments in a mouse model of MPS I.

Mucopolysaccharidosis Diseases

MPS I

MPS I has an incidence of nearly 1:100,000 live births and is caused by deficiency of the lysosomal hydrolase α-L-iduronidase (IDUA) (5-7). Deficiency for IDUA leads to progressive lysosomal accumulation of heparan and dermatan sulfate in tissues throughout the body (3). MPS I has a wide spectrum of severity and can present itself in a mild attenuated form, Scheie syndrome (MPS I S), an intermediate form, Hurler-Scheie syndrome (MPS I HS), or the most severe form of the disease, Hurler syndrome (MPS I H) (3, 8). Severity of the disease depends on the amount of residual IDUA expression, determined by the combination of specific recessive mutations present on the two alleles of the *IDUA* gene located at chromosomal position 4p16.3. For instance, combinations of the null mutations W402X, Q70X, and P533R result in Hurler syndrome (9, 10). Patients with attenuated forms of disease present with corneal clouding, joint stiffness, growth delay, aortic valvular disease, and joint stiffness (3). In addition to more severe forms of these symptoms, Hurler patients also present with coarse facial features, obstructive airway disease, hydrocephalus, and severe neurocognitive impairment. Life expectancy for untreated Hurler patients is not more than a decade.

MPS II

MPS II, also known as Hunter syndrome, is caused by deficiency of the enzyme iduronate-2-sulfatase (IDS), resulting in lysosomal accumulation of heparan and dermatan sulfate (3). Unlike other MPSs, MPS II is an x-linked recessive disorder (the *IDS* gene is located on Xq28) and has an incidence of 1:150,000 live births (5, 7). Point

mutations, splice site mutations, and gene deletions occur on the *IDS* gene located at chromosomal position Xq28 (11). However, there does not seem to be a close correlation between genotype and phenotype as is observed in MPS I (12, 13). As is the case with MPS I, most Hunter patients begin to display symptoms including growth delay, loss of hearing, coarse facial features, cardiovascular complications, respiratory disease, and neurocognitive deficiencies within the first 2 years of (14-16). Life expectancy for severe Hunter children is approximately 12 years while less severe individuals can survive into the 5^{th} or 6^{th} decade of life.

MPS III

MPS III or Sanfilippo syndrome consists of a group of 4 related disorders (Sanfilippo syndrome types A, B, C, and D) caused by mutations in one of 4 genes that encode lysosomal enzymes involved in the degradation of heparan sulfate (3). The 4 distinct subtypes of Sanfilippo syndrome, MPS III A, B, C, and D are caused by deficiency of heparan sulfate sulfamidase (SGSH), α-*N*-acetylglucosaminidase (NAGLU), heparan sulfate acetyl-CoA: α-glucosaminide *N*-acetyltransferase (HGSNAT), and *N*-acetylglucosamine 6-sulfatase (GNS) respectively. Although each subtype of the disease is biochemically distinct, the clinical symptoms of each disorder are quite similar. Unlike other MPSs, MPS III is unique, considering that disease symptoms in peripheral organs and tissues are relatively mild while neurological manifestations are quite severe. Notably, patients display hearing loss, behavioral abnormalities, psychiatric disease, and severe mental retardation (17, 18).

MPS VI

MPS VI or Maroteaux-Lamy syndrome is caused by an absence of activity of *N*-acetylgalactosamine 4-sulfatase also known as arylsulfatase B (ARSB) located on chromosome 5 (5q13-5q14) (19, 20). ARSB is responsible for removing sulfate groups from the GAG dermatan sulfate. However, chondroitin 4-sulfate can also act as a substrate for ARSB (although chondroitin 4-sulfate can be hydrolyzed by hyaluronidase and ß-glucuronidase in the absence of ARSB activity) resulting in the formation of sulfated oligosaccharides (19, 21). Therefore, ARSB deficiency leads to lysosomal accumulation of storage material consisting of dermatan sulfate and sulfated oligosaccharides, which results in multisytemic disease. MPS VI makes up between 2 and 18.5% of all MPSs (22). Much like MPS I, MPS VI seems to have a wide spectrum of severity, which is based on the amount of residual ARSB activity present. The severity of disease progression can be somewhat predicted by the level of GAG excreted through the urine with more severe patients excreting higher levels of GAG (> 200 µgGAG/mg creatinine) than more mildly affected patients (23). Severe disease has an onset within 2-3 years of birth with symptoms including growth delay, hepatosplenomegaly, stiffened joints, hearing loss, corneal clouding, cardiopulmonary obstruction, coarsened facial features, and skeletal dysplasias. MPS VI patients generally retain normal intellect although many patients present with CNS manifestations including cervical cord compression, meningeal thickening, and hydrocephalus. Patients with the most progressive MPS VI disease typically die as a result of cardiac malfunction in the 2nd or 3rd decade of life (3).

MPS VII

MPS VII or Sly syndrome is an extremely rare MPS and is characterized by deficiency of ß-glucuronidase (GUSB) activity resulting in progressive lysosomal accumulation of heparan sulfate, dermatan sulfate, and chondroitin sulfate. The *GUSB* gene is located on chromosome 7 and so far 49 different mutations have been discovered and mapped from a group of 56 patients (24). There is a wide variability of clinical symptoms displayed by MPS VII patients. Mild patients present with hepatosplenomegaly and skeletal dysplasias, but retain normal intelligence and survive into the 5th decade of life (3). More severe children demonstrate severe neurological symptoms including progressive neurocognitive decline while the most severe patients display hydrops fetalis at birth and fail to survive more than a few months after birth.

MPS IX

MPS IX, first identified in 1996, is caused by mutations in *HYAL1*, a gene encoding hyaluronidase 1 (25, 26). The deficiency of active hyaluronidase 1 leads to lysosomal accumulation of hyaluronan, a negatively-charged GAG normally found within the extracellular matrix. To date, only one MPS IX patient has been identified. The patient first developed symptoms at 6 years of age including periarticular masses, delayed growth, and coarsened facial features. However, no CNS involvement was identified as the patient retained normal intelligence.

Mechanisms of Neurological Disease

Severe neurological deterioration develops in patients with severe MPS I, II, III, and VII. The pathogenic basis for the development of neurological disease is not well understood and the relationships between the accumulation of GAG storage material and resultant neurological symptoms are not fully characterized. To date, aberrancy of processes including cell signaling (27), apoptosis (28), and oxidative damage have been implicated. Additionally, accumulation of GM2 and GM3 gangliosides has been identified in the brains of MPS patients (29) and animal models (30-33), suggesting that these molecules are involved in pathogenesis. Furthermore, astrogliosis and inflammation have been suggested to contribute to neurological damage.

Fibroblast growth factor 2 (FGF-2) is a cytokine involved in tissue morphonegesis and neurogenesis (34). FGF-2 also has protective properties by promoting neuronal survival and guarding against apoptosis (35). FGF-2 interacts with two types of cellular receptors. High affinity fibroblast growth receptors (FGFR) with tyrosine kinase activity mediate cellular responses while low affinity receptors consisting of heparan sulfate proteoglycans act as extracellular FGF-2 reservoirs and accessory molecules required for the binding of FGF-2 to the high affinity receptor (36). Formation of a complex between FGF-2, heparan sulfate proteoglycans, and FGFRs is necessary for mitogenesis and cellular response (37, 38). GAGs from Hurler tissues consist of both large GAG molecules and small, partially degraded chains or disaccharides (39, 40). Small, abnormally sulfated heparan sulfate chains have been shown to dysregulate stem cell growth and differentiation (41-43). Abnormal heparan sulfate molecules have also been

shown to disturb interactions between FGF-2, FGFRs, and heparan sulfate molecules and to reduce proliferation and survival of multipotent adult progenitor cells (MAPC) from Hurler patients (27). Since MAPCs have been shown to be able to differentiate into neurons and glia when incubated with FGF-2 in culture (44), it is possible that this dysregulation of FGF signaling may contribute to neuropathogenesis.

Besides FGF signaling, analysis of brains from a murine model of MPS IIIB, suggest that apoptosis may play a role in neurological dysfunction. The antiapoptotic genes Bcl2, Birc2, and Tnfr2 were downregulated and proapoptotic genes Apaf1, Tnfr1, and Casp1 1 are upregulated in brain tissue of these mice (28). A TUNEL assay identified apoptotic cells in the brains of these animals suggesting that induction of apoptosis may contribute to development of neurological disease. In addition to apoptosis, the same study also revealed upregulation of genes related to the NADPH oxidase complex, which might implicate an increase in reactive oxygen species-mediated damage to the brain. Finally, downregulation of macrophage migration inhibitor factor (Mif) could indicate an upregulation of cell signaling within inflammatory pathways, which might explain the presence of astrogliosis in the brains of MPS III patients upon autopsy (45, 46), and may further contribute to neuropathogenesis.

In addition to the accumulation of abnormal heparan sulfate molecules, aggregation of additional, seemingly unrelated molecules may also play a role in the development of neurological disease. GM2 and GM3 gangliosides, which are normally not expressed in the adult brain, have been found to be elevated in several types of MPS disease (47, 48).

There is no evidence that gangliosides require lysosomal hydrolases that are deficient in MPS diseases in order to be degraded. However, accumulating GAGs can inhibit the action of other types of lysosomal hydrolases by selectively binding to them (49, 50). Thus, the prevailing explanation for the secondary accumulation of GM2 and GM3 gangliosides is that lysosomal hydrolases responsible for ganglioside degradation are inhibited subsequent to binding excess GAGs. In actuality, a more complex mechanism of ganglioside accumulation may be responsible since GM2 and GM3 gangliosides have been shown to accumulate within separate vesicles in neurons from MPS I, MPS IIIA, and MPS IIIB mice (31). Although it is still unknown exactly how GM2 and GM3 ganglioside accumulation contributes to neurological disease, it is believed to be linked to the reinitiation of dendrite growth and altered synaptic connectivity (51). Additionally, overexpression of GM3 synthase has been shown to result in massive reactive oxygen species production and neuronal cell death (52).

Clearly, the mechanisms governing the emergence of severe neurocognitive disease in MPS I, II, III, and VII are quite complex and not well understood. Multiple cellular processes are disrupted and it is likely a combination of factors that ultimately result in neurological dysfunction. Developing strategies for delivering deficient lysosomal enzymes to the CNS in order to prevent or reverse neurocognitive deterioration has been a major focus of recent literature in the MPS field and is the main focus of this thesis

Current Therapies

Rationale

The biochemical basis for treating MPS diseases was first discovered in the laboratory of Elizabeth Neufeld in 1968. Cultured fibroblasts from an MPS I patient and fibroblasts from an MPS II patient both demonstrated progressive accumulation of GAGs over time. However, when cells from both genotypes were mixed together, they were able to complement each other and degrade GAG in normal fashion (53). Additional experiments revealed that lysosomal enzymes are translated in the rough endoplasmic reticulum (RER) before being transported to the lumen of the organelle through interaction between a hydrophobic amino terminal signal peptide on the nascent protein and an 11-S-ribonucleoprotein signal recognition protein (54, 55). In the lumen of the RER and Golgi, the lysosomal proteins undergo posttranslational modification by the addition of oligosaccharide moeities (mannose and/or mannose 6-phosphate) to Asn residues (56). These modified proteins are then sorted in the Golgi by binding to mannose 6-phosphate receptors (MPRs) (57, 58). The ligand-receptor complexes are then coalesced into coated vesicles, which travel to lysosomes where the ligand-receptor complexes are dissolved by the acidic pH of the lysosome, thereby delivering the lysosomal proteins to their destination (59).

Although most of the lysosomal enzymes are successfully delivered to the lysosome, between 5-20% of the enzymes are released to the extracellular space without being trafficked to the lysosome (60). These extracellular enzymes are able to interact with MPRs on the surface of neighboring cells and become internalized before being shuttled

to the lysosome. This “cross-correction” mechanism by which lysosomal enzymes produced in one cell are able to travel to and degrade GAG storage material in the lysosomes of neighboring cells forms the molecular basis for treating MPSs. Current therapies based on this molecular principle include hematopoietic stem cell transplantation and recombinant enzyme replacement therapy.

Hematopoietic Stem Cell Transplantation (HSCT)

The MPS diseases are amenable to treatment by transplantation of hematopoietic stem cells (HSCs) because donor-derived HSCs are able to engraft into the recipient and differentiate into many cell types including Kupffer cells in the liver (61), tissue macrophages in the spleen and lungs, and microglial cells in the brain (62). These and other donor-derived cells are then able to provide enzyme to deficient cells via metabolic cross-correction, thereby clearing GAG storage material from engrafted tissues. The first bone marrow transplant (BMT) for the treatment of an MPS disorder was performed in 1980 on a child with Hurler syndrome (MPS I H) (63). Since then, there have been approximately 500 children who have received transplants for MPS I, II, III, IV, VI, and VII although most of the patients treated have been afflicted with MPS I H (64). These patients have received cells from a variety of donor sources including either bone marrow or peripheral blood stem cells (PBSCs) from both related and unrelated heterozygous or unaffected individuals as well as PBMCs from unrelated umbilical cord blood (UBC) units. Since it is only possible to identify noncarrier human leukocyte antigen (HLA) matched bone marrow donors for about 1 in every 16 patients, many patients have

recently been treated with unrelated HLA-matched cord blood as an alternate source of donor cells (65).

Prior to infusion of donor cells, patients receive a preconditioning regimen to create space for the engraftment of incoming cells by treatment with cyclophosphamide (Cy) and either busulphan (Bu) or total body irradiation (TBI) (65-67). In MPS I H, successful donor cell engraftment seems to be dependent on cell dose (greater success in patients receiving higher than 3.5 x 10^8 cells/kg). 15-37% of patients failed to engraft and displayed autologous recovery after receiving more than 3.5 x 10^8 cells/kg. However, patients who received less 3.5 x 10^8 cells/kg bone marrow cells failed to engraft at a rate of 72% (67-70). Of patients that did engraft, 45-86% of them fully engrafted to levels of ≥95% donor chimerism. Patients receiving unrelated cord blood transplants have overall fared better, with a graft rejection rate of 8% and with 97% of engrafted patients exhibiting ≥90% donor chimerism (71). In addition to this potential for graft rejection, which decreases the overall rate of long term survival following treatment, patients are also at risk of HSCT-related mortality due to complications including graft-versus-host disease, sepsis, and pneumonia. The rate of graft-versus-host disease has been reported to be between 35% and 50% while the mortality rate due to transplant complications is between 11% and 45% (67, 70). However, the risk for these complications has decreased over time as procedures have been improved and the availability of matched donor cell sources has increased with the use of cord blood (64).

Following successful engraftment of donor HSCs, patients rapidly display phenotypic correction. Within a few months of receiving a transplant, the lungs, spleen, liver, and upper respiratory symptoms show evidence of improvement. Most patients experience resolution of hepatosplenomegaly within a year after transplant. There is a decrease in cardiac hypertrophy, although cardiac valve deformities still persist (71). Growth and development of the musculoskeletal system improves following transplant and many patients report an increase in joint mobility. Corneal clouding typically at least partially resolves and many patients demonstrate improved hearing. Most importantly, overall survival of Hurler patients following transplant is between 50-85% with some of the first transplanted patients living into the 3rd decade of life.

With respect to neurological outcomes following HSCT, the age and severity of disease of the patient at the time of transplant is paramount to the overall benefit achieved (66-68, 72). The earlier the transplant is conducted, the better chance there is of preventing lysosomal accumulation of GAG and associated secondary storage materials GM2 and GM3 ganglioside within the brain (73) There exists a wide variability in overall success of neurological outcomes of patients affected with Hurler syndrome following HSCT. However, it has been reported that hydocephalus improves and neurocognitive decline stabilizes in many patients following transplantation. However, recipients nonetheless continue to exhibit below normal IQ and impaired neurocognitive capability (74, 75). Furthermore, although some improvements in white matter and cribiform lesions have been detected using MRI brain imaging techniques in patients with MPS II and VI after BMT, these improvements are incomplete, variable, and do not occur in all patients (76).

Enzyme Replacement Therapy

As recently as 2003, ERT was approved by the U.S. Food and Drug Administration for the treatment of MPS I. Today, ERT is available for the treatment of MPS I, II, and VI in the form of laronidase, idursulfase, and galsulfase respectively. Laronidase and galsulfase are produced in stably transfected Chinese hamster ovary (CHO) cells while idursulfase is generated in a human cell line. To manufacture each of these enzymes, the recombinant gene encoding the specific lysosomal hydrolase with transcriptional regulatory elements is transfected into the cells in plasmid form along with a gene encoding dihydrofolate reductase (DHFR) (77). Drug selection is applied by growing the cells in the absence of hypoxanthine and thymidine to prevent the growth of non-transformed cells. Surviving cell colonies are expanded and evaluated for expression levels of the recobinant lysosomal hydrolase. The chosen cell lines are then expanded and the recombinant enzymes are harvested from the cells and purified. The resultant drugs are then administered by weekly intravenous infusion and most patients treated by ERT begin treatment immediately upon diagnosis (78, 79). In the case of MPS I, mildly affected patients with Scheie syndrome typically receive ERT while more severely affected Hurler-Scheie and Hurler patients receive ERT followed by HSCT once an appropriate cell donor source has been identified.

Similar to the experience with HSCT, the response to ERT seems related to the severity of disease at the onset of treatment. Generally, following treatment with ERT, patients display a decrease in urine GAG excretion, resolution of hepatoslenomegaly, and

improvement in cardiopulmonary function as measured by a 6 minute walk test within the first 6 months of treatment (80). Although greater than half of the patients treated with ERT develop antibodies to the recombinant proteins, they are mostly IgG antibodies and no connection has been made between development of these antibodies and decrease in efficacy of the drugs (81). Although the results of ERT are promising, intravenous infusion of ERT fails to provide enzyme to the CNS due to inability of the enzymes to efficiently cross the blood-brain barrier (82-87). This limitation has led to recent, ongoing clinical trials whereby these drugs are being infused intrathecally (88).

The goal of intrathecal ERT is to treat spinal cord compression and to provide enzyme to the brain through circulation within the cerebrospinal fluid (CSF). Following intrathecal infusion of laronidase into MPS I dogs, high levels of IDUA activity have been detected in the spinal, cervical, and lumbar meninges, associated with a 57-70% reduction in GAG storage material in these tissues. Furthermore, in MPS I dogs, infusion of laronidase into the cisterna magna resulted in clearance of storage material in glia, perivascular cells, and neurons as well as normalization of quantified GAGs (83, 89). Currently, several clinical trials are underway to assess the safety and efficacy of intrathecal laronidase in MPS I patients with results expected to be published shortly.

Summary

HSCT and ERT have both proved to be efficacious for the treatment of MPSs. However, many challenges remain, including providing enzyme that is able to penetrate the cardiac valves, deep skeletal tissue, and the CNS in sufficient levels to prevent/reverse clinical

outcomes. In the case of HSCT, identifying a proper source of donor HSCs is not trivial and takes precious time during which patients continue to deteriorate. Additionally there is significant morbidity and mortality related to the transplantation procedure itself. With ERT, major limitations include the frequency of intravenous infusions (weekly) as well as the estimated cost of treatment (between $250,000-1,000,000 USD annually per patient) (90). Also, intravenous ERT is not helpful for patients with neurological involvement and questions remain regarding the overall effect of HSCT in preventing or reversing neurocognitive impairment. Intrathecal ERT is a promising approach for delivering enzyme to the CNS, although it isn't clear how often infusions need to be made for optimal effect and the dog studies did not demonstrate a functional neurocognitive benefit as this analysis was not conducted. The focus for the remainder of this thesis will be on the development of more effective strategies to deliver lysosomal enzymes to the CNS by gene transfer.

Gene Delivery Systems

Many preclinical studies have been conducted during the course of the last few decades investigating the use of gene therapy vectors to deliver lysosomal enzymes to the CNS in animal models of MPSs. Both viral and nonviral vectors have been used in these experiments including retroviruses, lentiviruses, adenoviruses, adeno-associated viruses (AAV), and Sleeping Beauty transposons. Different routes of administration have been investigated including *ex vivo* transduction of HSCs and in vivo infusions through intravenous, intrathecal, intracisternal, intraparenchymal, and hydrodynamic injections.

Retroviral and lentiviral vectors

Retroviruses are a group of spherical 80-100nm diameter viruses that utilize two identical strands of RNA as genetic material. The retroviral genome contains three genes, *gag*, *pol*, and *env,* which encode proteins necessary for viral infection and reproduction. These sequences are flanked by long-terminal repeat (LTR) sequences required in cis for reverse transcription and integration. The reverse transcriptase enzyme, encoded by *pol*, produces a double-stranded DNA copy of the RNA viral genome (91) upon entry into a host cell. Once converted into double stranded DNA and transported to the nucleus, the viral DNA is able to integrate into the host genome through interaction between the virally-encoded integrase and the host genome, creating a provirus. Activation of transcription and translation of the integrated viral genes by the host cell machinery then results in production of viral genomes and proteins necessary to produce additional virions and complete the lytic life cycle. Replication incompetent gene therapy gammaretroviral vectors have been engineered whereby the *gag*, *pol* and *env* genes in the viral genome have been replaced by a promoter regulating expression of a gene encoding a therapeutic protein, in this case a specific lysosomal hydrolase deficient in MPS disease. These vectors can be used to mediate gene transfer upon integration of the promoter and therapeutic gene of interest into the host genome. Thus, long-term expression of the desired gene product can be achieved in target cells. However, the use of gammaretroviral vectors as gene transfer vehicles is hindered by their inability to transduce non-dividing cells, deeming them non-ideal for use in transducing brain tissue.

HIV-1-based lentiviral vectors have been engineered to deliver therapeutic genes of interest into target cells (92). The advantage of these lentiviral vectors compared to gammaretroviral vectors is that they are able to transduce non-dividing cells, making them suitable for transducing terminally differentiated cells such as neurons (93). Together, gammaretroviruses and lentiviruses have been used in over 300 clinical trials to treat human disease including the neurological disorders Alzheimer's and Parkinson's disease. However, since retroviruses and lentiviruses integrate semi-randomly into heterochromatic regions of the host genome (94), concerns remain about the risk of insertional mutagenesis when using these types of vectors. During clinical trials for X-linked severe combined immunodeficiency in France and the United Kingdom, several patients developed leukemia following treatment with a gammaretroviral vector due to integration-related transcriptional upregulation of the oncogene *LMO2* (95).

Adeno-associated Viral Vectors (AAV)

AAV is a small (26 nm in diameter) single-stranded DNA parvovirus that causes no known pathogenic disease in infected humans. The wild type AAV genome is 4.7KB in length and contains two open reading frames, *rep* and *cap*, flanked by inverted terminal repeat sequences (ITRs). *Rep* encodes four overlapping genes encoding Rep proteins required for the viral life cycle. *Cap* includes three overlapping sequences encoding the capsid proteins VP1, VP2, and VP3, which assemble in a 1:1:20 ratio to form the icosohedral viral capsid (96-98). AAV is a dependovirus and requires co-infection by adenovirus or herpes simplex virus to complete its viral life cycle (99-101). To generate

therapeuric AAV vectors, the *rep* and *cap* sequences are removed from the viral genome and replaced by an expression cassette including a promoter regulating expression of a therapeutic gene of interest. Three plasmids (one containing the ITRs flanking the therapeutic gene of interest, one containing *cap* and *rep*, and one containing helper proteins) are co-transfected into producer HEK293 cells. The viral genome is packaged into capsid proteins produced within the triply-transfected cells. Recombinant AAV virions can then be purified from cell lysates using density gradients, affinity columns, or ion exchange purification methods (102, 103).

Recombinant AAVs (rAAV) have become effective tools for use as gene therapy vectors for several reasons. rAAVs are non-pathogenic and are capable of transducing a variety of tissues and cell types including brain, liver, heart, and muscle (104-107). Despite persisting in the form of extrachromosomal DNA once inside a host cell, rAAVs are able to maintain stable expression of transgenes for periods greater than 1.5 years in various animal models including mice, dogs, and hamsters. Although wild type AAVs are capable of integrating into the AAVS1 site on chromosome 19 through interaction between the ITRs and the chromosome with the help of Rep proteins, rAAVs integrate into the host genome at very low frequency since the viral Rep gene has been removed (104, 108). The low frequency of viral integration reduces the likelihood of insertional mutagenesis as has been demonstrated with retroviral vectors. In addition to these advantages of using rAAV vectors, several different serotypes have been identified and different capsid mutants have been either isolated from animal tissues or synthetically engineered. AAV serotype 2 (AAV2) was the first serotype discovered and cloned into

bacterial plasmids and used to created rAAV vectors (100, 109). Thus, AAV2 is the most widely studied AAV serotype and has been used to date in 75 clinical trials worldwide. Included in these clinical trials are 14 trials to treat neurological disease including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and epilepsy. Most of the preclinical studies involving the treatment of animal models of MPS disease have used AAV2 vectors, although AAV5 and AAV8 have also been used (see below).

Sleeping Beauty (SB) Transposon System

The SB transposon system is a nonviral, plasmid-based gene transfer system that was constructed by reverse engineering of extinct DNA sequences found in salmonid fish (110-112). The system consists of two components; the transposon, including ~230bp inverted repeat/direct repeat sequences (IR/DRs), which flank a desired genetic cargo (an expression cassette encoding a therapeutic protein of interest under transcriptional regulation of a desired promoter sequence), and the SB transposase enzyme. The gene encoding SB transposase can be included on the same plasmid as the transposon (in cis) or on a separate plasmid (in trans). Following delivery into a target cell, two SB transposase molecules interact with each IR/DR to excise the transposon from the plasmid delivery vehicle. Subsequently, the transposon can randomly integrate into any of the 2×10^8 TA dinucleotide sequences contained in the mammalian genome (113). These integration events allow for stable, long-term expression of genes contained in the genetic cargo of an engineered SB gene therapy vector. The advantages of using the SB transposon system rather than viral vectors for use as gene therapy vehicles include that they are cheaper and easier to purify, are largely non-immunogenic, have no risk of being

contaminated with replication-competent viruses, and allow for large therapeutic cargo sizes (113). However, the SB transposon system does have the potential risk of insertional mutagenesis, although no adverse events have yet to be observed when using the SB system as a gene therapy vector. Additionally, although SB gene therapy vectors have been administered by hydrodynamic delivery to the liver of mice and by high-pressure catheter-mediated infusion to the liver of dogs, *in vivo* gene transfer of therapeutic genes to the CNS has not yet been demonstrated. It should be noted that the SB transposon system is currently being used in clinical trials to treat B-cell lymphoid malignancies.

Administration of Gene Therapy Vectors to Treat Neurological MPS Disease

Gene therapy vectors have been administered in animal models of MPS disease with the intention of achieving gene transfer in the CNS to treat neurological manifestations of disease. Vectors have been infused into these animal models by the intravenous route as well as by direct injection into the CNS. Early studies demonstrated the limitations of the intravenous route to mediate gene transfer in the CNS due to the challenge of crossing the blood-brain barrier. This has led to many studies that have explored different strategies for direct infusion of gene therapy vectors to the brain. The results of these experiments are outlined below.

Systemic Delivery

Intravenous Infusion

Daly *et al.* initially reported intravenous infusion of an AAV2 vector expressing GUSB into MPS VII mice. GUSB expression was detected in the brain, and neurons, microglia, and meninges were cleared of pathological storage material (114). Similarly, intravenous infusion of an AAV2 vector expressing IDUA into 1 day-old MPS I mice resulted in detectable expression of IDUA in the brain, clearance of pathological GAG, and improved neurobehavior in an open field test (115). Intravenous infusion of a retroviral vector derived from Moloney murine leukaemia virus into newborn MPS I animals also prevented accumulation of storage material in the brain, with the effect related to dosage (storage material was cleared when a high dose of 10^9 transducing units was infused while this did not occur when a lower dose of 10^8 transducing units was infused) (116). Additionally, intravenous infusion of a lentiviral vector at birth resulted in transduction of neurons in the brain, consistent with detectable IDUA expression and reduction of GAG storage material in the CNS that was dose dependent (117). MPS I dogs have also been infused at birth with a gamma retroviral vector expressing canine IDUA, demonstrating that this route of administration can be used to deliver IDUA expression to the brain and clear GAG storage material from the CNS in a large animal model (118).

Intravenous infusions into adult mice have resulted in mixed outcomes. Tail vein injection of a lentiviral vector encoding IDUA into 8 to 10-week old MPS I mice failed to mediate detectable IDUA expression or to decrease levels of GAGs in the CNS of treated mice as assessed one month post-treatment (119). The lack of detectable IDUA

expression could be at least partially due to an immune response against human IDUA transgene product, as enzyme-specific antibodies were detected, and a cytotoxic T-lymphocyte response resulting in clearance of IDUA-expressing transduced cells (120). However, immunomodulation prior to infusion with a gamma-retroviral vector resulted in a small amount of detectable IDUA activity (< 3% of normal) in the brains of treated mice with some evidence of reduction in pathological GAG storage (120). The immune response reported in these studies could be avoided by the use of a liver-specific promoter. Intravenous infusion of an AAV8 vector expressing iduronate 2-sulfatase under transcriptional regulation of the liver-specific promoter of the gene encoding thyroxin-binding globin into MPS II mice resulted in detectable expression of IDS in the brain, reduction of storage material, and partial correction of neurobehavior as determined using an open field test, despite a small sample size for neurobehavioral testing (121).

Ex Vivo Transduction of HSCs

Transplantation of unmodified wild-type bone marrow cells into 6 to 8-week old MPS I mice did not result in detectable IDUA levels or reduced storage material in the brain (122). However, ex vivo transduction of the cells with an IDUA-expressing retrovirus prior to transplantation into *IDUA*$^{-/-}$ recipients resulted in expression of IDUA as well as in partial correction of pathology, and detection of normal donor-derived microglia in the brain. In another approach, stromal cells from the bone marrow were harvested and transduced *ex vivo* with a retroviral vector prior to being infused directly into the lateral ventricles of the brains of MPS VII mice (123). Two weeks after infusion, GUSB positive

cells were detected in the olfactory bulb, striatum, and cerebral cortex. GAG levels were reduced to normal, and the animals displayed neurocognitive improvement in a Morris water maze test. However, after 8 weeks, GUSB expression disappeared, likely due to an elicited immune response.

Direct delivery to the CNS

Intracranial Infusion Into the Brain Parenchyma

Lysosomal enzymes have been expressed in the brain, mediated by direct intrastriatial infusion of therapeutic vectors. Following delivery of AAV2 and AAV5 vectors to the striatum by direct injection into the putamen, animals exhibited high levels of NAGLU expression in the brain, especially in the injected hemisphere of adult MPS IIIB mice (124). Expression of NAGLU within neurons contributed to improved lysosomal function, reduced levels of GM2 and GM3 ganglioside, and improved neurobehavior in an open field test, elevated plus maze, and in a home cage analysis. However, only partial neurological improvements were observed in many mice. AAV2 and AAV5 vectors have also been injected intrastriatally into a mouse model of MPS VII to express GUSB. Unilateral infusion of an AAV2 vector into adult MPS VII mice resulted in GUSB levels equivalent to that of unaffected heterozygote animals and cleared lysosomal storage lesions in the injected hemisphere of the brain (125). Bilateral intrastriatal infusions of a similar vector packaged into AAV5 capsids had a more successful outcome as GUSB was expressed in both hemispheres of the brain and lysosomal storage material was also cleared from cells in both hemispheres. These animals displayed improvement in a learning and spatial memory deficit using a repeated acquisition and performance

chamber (RAPC) assay (126). Furthermore, AAV2 and AAV5 vectors have been shown to mediate expression of IDUA throughout the brain of adult MPS I mice following a single unilateral intrastriatal infusion. This treatment prevented accumulation of GM2 and GM3 gangliosides and reversed lysosomal distension with the treatment being more effective in mice that received AAV5 vector compared to mice that received AAV2 vector (127). This same AAV5 vector has subsequently been injected into the striatum of adult MPS I dogs. The vector was infused bilaterally into two sites in each hemisphere: into the putamen and the centrum semiovale. Dogs that were fully immunosuppressed using a combination of mycophenolate mofetil and cyclosporine during the treatment exhibited widespread IDUA expression as well as reduced neuropathology, GAG, and secondary ganglioside accumulation. However, dogs receiving only partial immunosuppression with cyclosporine alone presented with subacute encephalitis, produced antibodies recognizing IDUA, and eliminated genetically modified cells (128).

Besides intrastriatal infusion, other direct intracranial approaches to delivery have been investigated. Multiple injections into both cerebral hemispheres combined with injection into the cerebellum of an HIV-1-based lentiviral vector expressing GUSB were superior to a single unilateral injection into the brain of MPS VII mice. The brains of animals receiving multiple injections had more widespread expression of GUSB and reduction in pathology than animals receiving only a single unilateral intrastriatal infusion (129). Another approach involved injections of an AAV2 serotype vector into the anterior cortex and hippocampus of newborn MPS VII mice. GUSB activity reached wild-type levels in the brain18 weeks post-infusion although much of the GUSB activity was

concentrated near the injection sites. Lysosomal distention was prevented, as was the CNS accumulation of GM2 and GM3 gangliosides. This resulted in improved performance of treated animals in a Morris water maze test of spatial navigation (130).

Intracerebroventricular Infusion

An alternative strategy to intracranial infusion of therapeutics into the parenchyma of the brain is to take advantage of the ventricular system. There are four cerebral ventricles located within the mammalian brain, which are remnants of the neural tube formed during early development. Two paired lateral ventricles are situated within the cerebrum and run parasagittally along much of the dorsal basal ganglia. The lateral ventricles communicate with the third ventricle, located within the center of the diencephalon, by way of the intraventricular foramina. The third ventricle is further connected to the fourth ventricle, located within the hindbrain, by the cerebral aqueduct, which runs through the midbrain. Three foramina further adjoin the fourth ventricle to the subarachnoid space and the central canal allows access between the fourth ventricle and the spinal cord. All of these ventricular spaces are filled with cerebrospinal fluid (CSF), which bathes the brain and spinal cord and protects these structures from injury.

Fraldi *et al*. exploited the ventricular system as a route of access to multiple brain structures using an AAV5 vector co-expressing sulfaminidase (SGSH) and sulfatase modifying factor 1 (SUMF) for treatment of a mouse model of MPS IIIA (131). The investigators infused the vector bilaterally into newborn pups and detected SGSH activity throughout the brain, while demonstrating that co-expression of SUMF and SGSH

resulted in increased SGSH activity in comparisonto animals infused with SGSH vector alone. With a similar AAV5 vector expressing green fluorescent protein (GFP), transduced cells were visualized at high frequency in the olfactory bulb, at an intermediate frequency in the choroid plexus and cerebral cortex, and at a lower frequency in the striatum, hippocampus, thalamus, and cerebellum. Animals treated with the bicistronic vector displayed a reduction in neuropathological storage material and GM2 ganglioside, decreased microglial activation, and prevention of astrogliosis compared to AAV5-GFP treated control animals. The functional results of the analysis demonstrated that the animals displayed improvement in gait as well as in both neurobehavioral and neurocognitive tests as measured using an open-field test and Morris water maze test, respectively.

Intracisternal Infusion

An alternative route of administration to deliver gene therapy vectors into the CSF is direct infusion into the subarachnoid cisterns. The cisterns are openings in the subarachnoid space located between the pia matter and arachnoid layers of the meninges, which surround the brain. The cerebellomedullary cistern (cisterna magna) is the largest of the subarachnoid cisterns and is connected by the flow of CSF from the fourth ventricle by the lateral and median apertures. Following intravenous administration of mannitol, a vasodilator used to increase extracellular space between epithelial cells in the CNS, infusion of an AAV2 vector expressing NAGLU into the cisterna magna resulted in increased longevity of MPS IIIB mice treated at 4-6 weeks of age (132). The overall effectiveness of the infusions was dose dependent. Animals treated with a high dose (5 x

10^{10} vector genomes) of the vector fared better than animals receiving a low dose of vector (1 x 10^{10} vector genomes). Long-term activity of NAGLU was detected in the brains of treated animals, and the highest levels occurred in the hind portion of the brains of animals treated with the high vector dose (~4-fold higher than wild-type). IHC analysis revealed NAGLU expression in Purkinje cells of the cerebellum as well as in neurons of the brainstem and hypothalamus. GAG content in the brain was partially reduced and animals treated with a high dose of the AAV vector displayed improvement in a Morris water maze task, although there was no improvement in a rotarod test of motor function.

Intrathecal Infusion

Yet another strategy for delivering gene therapy vectors into the CSF is intrathecal injection into the spinal canal, in which CSF surrounds the spinal cord. Intrathecal delivery of an AAV2 vector expressing GUSB resulted in restoration of between 3% and 30% of wild type GUSB activity levels in the brains of MPS VII mice treated as neonates and 8% of wild type levels in animals treated as adults (133). This treatment resulted in a reduction of lysosomal storage vacuoles present in the cerebral cortex. Similarly, intrathecal infusion of an AAV2 vector expressing IDUA into adult MPS I mice resulted in enzyme activity levels approaching wild-type, particularly in the hindbrain (134). IDUA-producing neurons were detected in the brains of treated animals by immunostaining of histologic sections, with the highest density of reactive cells detected in the olfactory bulb and cerebellum. In general, IDUA levels correlated with AAV vector dose, and the brains of animals treated with a high dose of vector (4 x 10^{10} particles) appeared to be entirely cleared of storage vacuoles. Although both of these

studies show promise, interpretation of the results is limited by lack of data assessing neurologic function.

Thesis Statement

The studies presented in the above sections outline the current treatments used for the MPS disease. These data demonstrate that intravenous ERT and HSCT are efficacious in clearing GAG storage material and in improving some manifestations of MPS disease in peripheral organs such as the heart, skeletal tissue, and musculature. However, it is clear that there is a need for more effective treatment strategies to efficiently deliver lysosomal enzymes to the CNS. Furthermore, considering the high cost of both ERT and HSCT, as well as the inherent risks involved when conducting transplants, it is clear that there is also room for the development of less expensive and safer therapeutics for the MPSs. As outlined in the preclinical studies in the above sections, gene therapy has the potential to provide safer, cheaper, and more efficient delivery of lysosomal enzymes to the CNS of MPS patients, as demonstrated by current success in animal models. However, to date, no reports have been made to describe results of clinical trials using gene therapy vectors for the treatment of MPS disorders.

The goal of this thesis is to provide preclinical data supporting the adoption of gene therapy for the delivery of IDUA to the CNS of Hurler patients using the MPS I mouse as a model system. Before commencing gene therapy studies, it is first necessary to understand the extent of CNS correction that can be achieved by HSCT in the mouse model. Although HSCT has been conducted in human Hurler patients since 1980 (63),

mouse models of MPS I did not become available until 1997 (33, 135). Thus, Chapter 2 investigates HSCT in a mouse model of MPS I, with an emphasis on determining the extent to which HSCT is able to deliver IDUA to and biochemically correct tissues in both peripheral organs, and more importantly, within the CNS.

After understanding the levels of correction that can be achieved in the brains of MPS I animals following HSCT, the goal was to develop a strategy for delivering high levels of IDUA to the brain using a gene therapy approach. AAV vectors were chosen as the vector of choice due to their ability to efficiently transduce neural tissue, their ability to mediate long-term gene expression, and their lower risk of insertional mutagenesis as compared to lentiviral vectors. The goal is to achieve the most widespread distribution of AAV transduction and associated IDUA expression after a single intracranial injection. We hypothesized that infusion of AAV vector into the lateral ventricles would achieve this goal by dispersion through the CSF. Chapter 3 investigates this route of administration by studying infusion of an AAV8 vector expressing human IDUA (AAV8-IDUA) into newborn murine MPS I pups. This chapter evaluates the distribution patterns and levels of AAV transduction and long-term IDUA expression achieved in the brain. Also, the types of cells transduced by the vector were determined and the level of biochemical correction of storage material accumulation achieved by this treatment was assessed. Finally, the treated animals were evaluated for the prevention of a neurocognitive deficit displayed by untreated MPS I mice in a Morris water maze-based test.

Chapter 3 explores the potential for preventing the emergence of neurological disease by administering an AAV vector to neonatal mice. However, treating newborn mice is not the ideal manner to model correction of human disease, as newborn mice are immunologically naïve (136-140). Also, humans are more likely to be treated as infants rather than as neonates since newborn screening has not been universally established. Thus, a better model for evaluating ICV infusion of AAV vectors is to treat adult MPS I mice and evaluate the extent to which this therapy can prevent or reverse CNS manifestations of disease. Chapter 4 analyzes the results of experiments in which pre-symptomatic young adult and post-symptomatic adult MPS I mice of advanced age were infused ICV with AAV8-IDUA. Together, the following chapters of this thesis explore the efficacy of AAV8-IDUA infused ICV in treating the CNS manifestations of MPS I in a mouse model. These results can be compared to those achieved following BMT with wild-type donor marrow. These results form the basis for further preclinical work in which the efficacy and safety of AAV8-IDUA can be evaluated in a large animal model. The ultimate goal is to develop a protocol for a clinical trial whereby direct administration of AAV to the brain can be tested as an adjuvant to ERT and HSCT in the treatment of Hurler syndrome.

Table 1.1. Summary of the MPS disorders. There are seven classes of MPS disorders with a total of eleven distinct diseases. Each disorder is listed below along with its deficient lysosomal enzyme, type of accumulating storage material, and available therapies.

Disease	Deficient Enzyme	Accumulating GAGs	Available Therapies
MPS I			
Scheie	α-L-iduronidase	Heparan/Dermatan Sulfate	HSCT, ERT (laronidase)
Hurler-Scheie			
Scheie			
MPS II			
Hunter	Iduronate-2-sulfatase	Heparan'Dermatan Sulfate	ERT (Idursulfase)
MPS III			
Sanfilippo A	Heparan-*N*-sulfatase	Heparan Sulfate	
Sanfilippo B	α-*N*-acetylglucosaminidase		
Sanfilippo C	Acetyl-coenzyme A:α-glucosaminide *N*-acetyltransferase		
Sanfilippo D	*N*-Acetylglucosamine 6-sulfatase		
MPS IV			
Morquio A	Galactose 6-sulfatase	Keratan Sulfate	None
Morquio B	β-galactosidase		
MPS VI			
Maroteaux-Lamy	*N*-Acetylgalactosamine 4-sulfatase	Dermatan Sulfate	HSCT, ERT(galsulfase)
MPS VII			
Sly	β-glucuronidase	Heparan/Dermatan/Chondroitin Sulfate	HSCT
MPS IX	Hyaluronidase	Hyaluronan	None

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Chapter 2

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

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 sulfate and dermatan sulfate. Deficiency of the enzyme leads to lysosomal accumulation of these substrates and associated multi-systemic disease, the most severe form known as Hurler syndrome. Since 1981, the treatment of severe MPS I 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. The engrafted animals displayed an increased lifespan, improved cardiac function, partially restored IDUA activity in peripheral organs, and decreased GAG accumulation in both peripheral organs and in the brain. However, elevated levels of GAG and GM3 ganglioside remained in the brain. These results are similar to those observed in Hurler patients and provide a rationale for developing more effective methods for delivering IDUA to the brain as a supplement to HSCT and enzyme replacement therapy.

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 severe Hurler syndrome begin developing symptoms 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 golgi (3) Most of the modified enzyme is shuttled to the lysosomes, but a small percentage of IDUA escapes from the cell and 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. 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 weekly infusions of recombinant enzyme following diagnosis and ultimately HSCT for patients with a matched HLA donor. Allogeneic bone marrow transplantation (BMT) for MPS I was first conducted in 1981 by Hobbs *et al.* (5). 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 (5-12). These and other studies have provided insights into the molecular mechanisms of these treatments and have led to continual improvement in HSCT protocols in order to reduce 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 transplanted recipients (13). 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. Furthermore, neurocognitive deterioration has improved in many patients, although they continue to exhibit subnormal IQ and impaired neurocognitive capability (14, 15).

Despite the long history of HSCT to treat Hurler patients, it wasn't until 1997 that murine models of MPS I were introduced (16-18). 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. In this study, we investigated the effect of BMT in the MPS I mouse to determine the extent to which the outcomes of this treatment recapitulate results observed in human MPS I patients and other animals models. We engrafted *Idua*$^{-/-}$ animals with high levels of syngeneic wild-type marrow. 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 be similarly achieved Hurler patients, particularly with respect to metabolic correction in the brain.

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 C57 BL/6 donors. Thirty-three CD45.2 *Idua*$^{-/-}$ mice between the ages of 4-8 weeks were preconditioned with 750 cGy cesium irradiation. On the following day, whole bone marrow was flushed from the hind limbs of wild-type CD45.1 mice and the preconditioned MPS I mice were infused with 10^7 donor cells through the tail vein. Mean donor cell engraftment as determined by flow cytometry of peripheral blood for the CD45.1 congenic marker and 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 10^7 congenic donor marrow cells. However, donor cell engraftment was undetectable in this cohort of animals.

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 ($P < 0.001$ using log-rank test) (Figure 2.1). 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 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. Activity levels detected in the heart, lungs, spleen, kidneys, and liver were 13%, 38%, 75%, 28%, and 47% of those detected in unaffected heterozygous animals (Figure 2.2a). However, IDUA activity was undetectable in the brains of BMT recipients (Figure 2.2b)

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

As previously reported, we found that *Idua*$^{-/-}$ mice displayed increased levels of GAG storage in peripheral organs and in different sections of the brain in comparison to heterozygous littermates. Additionally, *Idua*$^{-/-}$ animals engrafted with IDUA+ donor

marrow exhibited a statistically significant reduction in GAG storage material in the lungs (**$P < 0.01$), spleen (***$P < 0.0001$), kidneys (***$P < 0.0001$), and liver (*$P < 0.05$) (Figure 2.3). There was also a trend toward reduced GAGs in the hearts 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 (Fig. 2.4). 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, 19).

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 2.5a). 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 2.5b). 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 2.6). Interestingly, the aggregates of GM3 ganglioside in the dentate gyrus appear concentrated within the subgranular zone, which provides a niche for neural stem cells. It remains to be seen whether or not this may result in dysfuction of these stem cells and alter their ability to divide and differentiate although this warrants further investigation. GM3 ganglioside accumulation was absent from the grey matter of unaffected heterozygous mice. Although total brain GAG levels were reduced in the brains of BMT-recipient mice, focal aggregations 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.

Transplanted mice exhibited improved cardiac function.

BMT recipient and control mice were analyzed by high-resolution ultrasound biomicroscopy to assess cardiac function (20). 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 cardiac function, consistent with what has been observed in Hurler patients following successful HSCT (11, 21, 22). 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 (23, 24). 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 explain the relationship between IDUA activity and GAG accumulation in tissues. The relationship fits a hyperbolic equation that validates previous observations whereby very small amounts of IDUA resulted in dramatic reduction in tissue GAGs (2, 19, 24).

Although some BMT studies in MPS I animals indicate that IDUA activity can be detected in the brains of transplanted dogs and mice (25, 26), brain IDUA was undetectable in this study. Despite this result, total GAG accumulation in the brain was significantly reduced as has been reported in cats following BMT (24). This suggests that progenitor cells may have engrafted into the brain and differentiated into microglia (27), providing a small amount of enzyme sufficient 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 in the brain following BMT, there is much room for improvement in the extent of biochemical correction that can be achieved in the brain. Since the relationship between biochemical storage in the nervous system and neurocognitive function has yet to be elucidated, conducting these types of experiments in animal models is an important next step to understanding the degree of intervention that may be required to preserve or restore brain function.

The results from this study validate 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 outcomes achieved following HSCT. Additionally, since many patients now receive enzyme replacement therapy in combination with HSCT (12), it is also important to understand the effects of this combined therapeutic approach on the disease in animal models such as the MPS I mouse. As the major challenge 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 in the MPS I mouse can be compared.

Materials and Methods

Animals and Transplantation Procedure

The *Idua*$^{-/-}$ mouse strain (28) 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, thirty-three (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 C57/BL6 mice with DMEM supplemented by the addition of 10% fetal bovine serum and 10U/mL heparin. Harvested cells were triturated to create a single-cell suspension and viable cells were counted by trypan blue exclusion using a hemacytometer. The donor cells were then infused into the preconditioned *Idua*$^{-/-}$ animals by tail-vein injection of 10^7 donor cells in a 200µl volume.

Flow Cytometry

To determine the level of donor cell engraftment achieved in recipient animals, peripheral blood was collected via the submandibular vein. The blood was heparinized and the red blood cells were lysed using a hypotonic buffer (eBioscience). The remaining nucleated cells were harvested by centrifugation at 200 x g for 3 minutes, incubated with allophycocyanin-conjugated anti-murine CD45.1 (eBioscience) and evaluated by flow cytometry using a FACSCalibur bench top cytometer. The data were analyzed using FlowJo software (Tree Star Inc.) and donor cell engraftment was determined as the percentage CD45.1+ cells present in the gated lymphocyte compartment (29).

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 analysis 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 PBS using a motorized pestle and permeabilized with 0.1% Triton X-100. IDUA activity was determined by fluorometric assay using 4MU-iduronide as the substrate, as previously described (30). 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).

GAG Analysis

The remainder of each tissue homogenate was incubated overnight with proteinase K, DNase1, and RNase (Sigma) as previously described and clarified for 3 min at 12,000 rpm using an Eppendorf tabletop microcentrifuge model Centrifuge 5415D (Eppendorf) (30). GAG concentration was determined using the Blyscan Sulfated Glycosaminoglycan Assay (Accurate Chemical) according to the manufacturer's instructions.

Tissue Staining

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 x 10 minutes in PBS (pH = 7.4) and blocked with 10% normal donkey serum / 0.3% Triton X-100. Primary antibody was added and sections were incubated overnight at 4°C, washed 3 x 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 x 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, Seikagaku). The secondary antibody was a donkey anti-mouse IgM antibody conjugated to Alexa Fluor 488 (1:500, Invitrogen).

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) (31). Doppler imaging was conducted in both the long- and short-axis beneath the aortic valve to determine the presence or absence of 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).

FIGURE 2.1. MPS I mice engrafted with syngeneic marrow displayed an increased lifespan compared to untreated control animals. Following BMT, recipient MPS I animals survived 1.7 times longer than untreated mice (***$P < 0.001$ by log-rank test). BMT recipients lived to a median age of 85 weeks while untreated MPS I animals exhibited a median lifespan of 49 weeks.

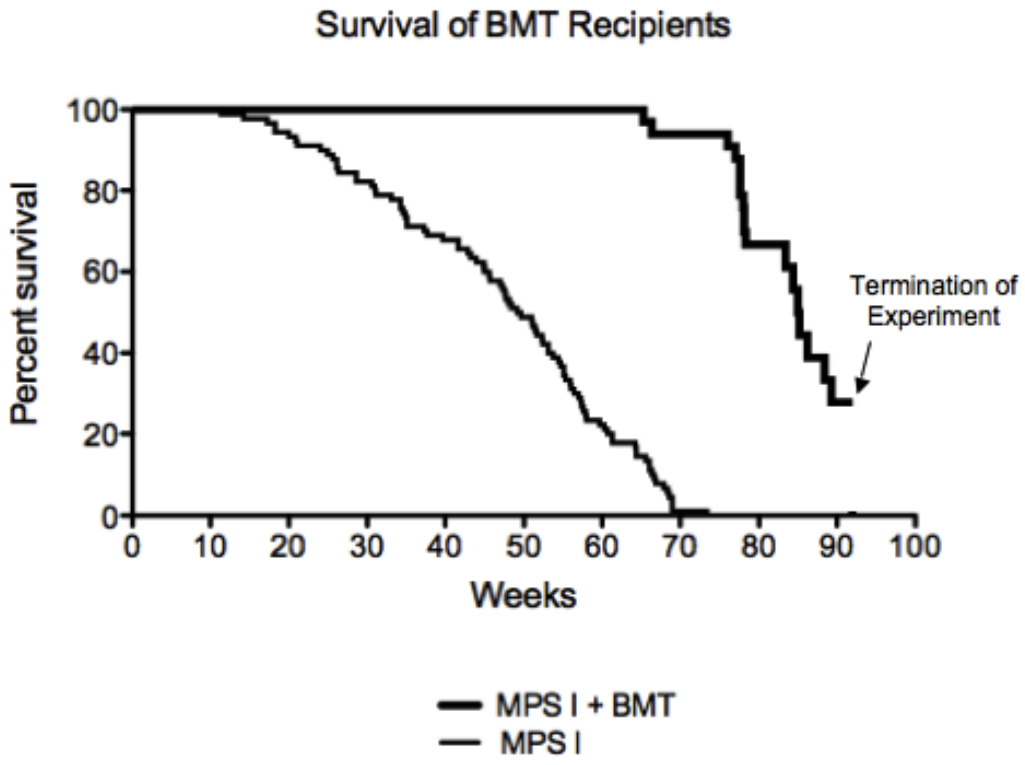


FIGURE 2.2. IDUA activity levels partially restored in peripheral organs, but undetectable in the brain following BMT. (a) IDUA activity was detected in peripheral organs from BMT recipient MPS I animals. The mean percentage of activity detected in the heart, lung, spleen, kidney, and liver of BMT recipients was 13%, 38%, 75%, 28%, and 47% respectively of that detected in unaffected heterozygotes. Activity assays were conducted on peripheral organ samples from untreated MPS I mice, but IDUA activity was undetectable in these samples. (b) IDUA activity was detected in microdissected brain samples from unaffected heterozygote animals. IDUA activity was undetectable in brain samples from both untreated MPS I animals and BMT recipients.

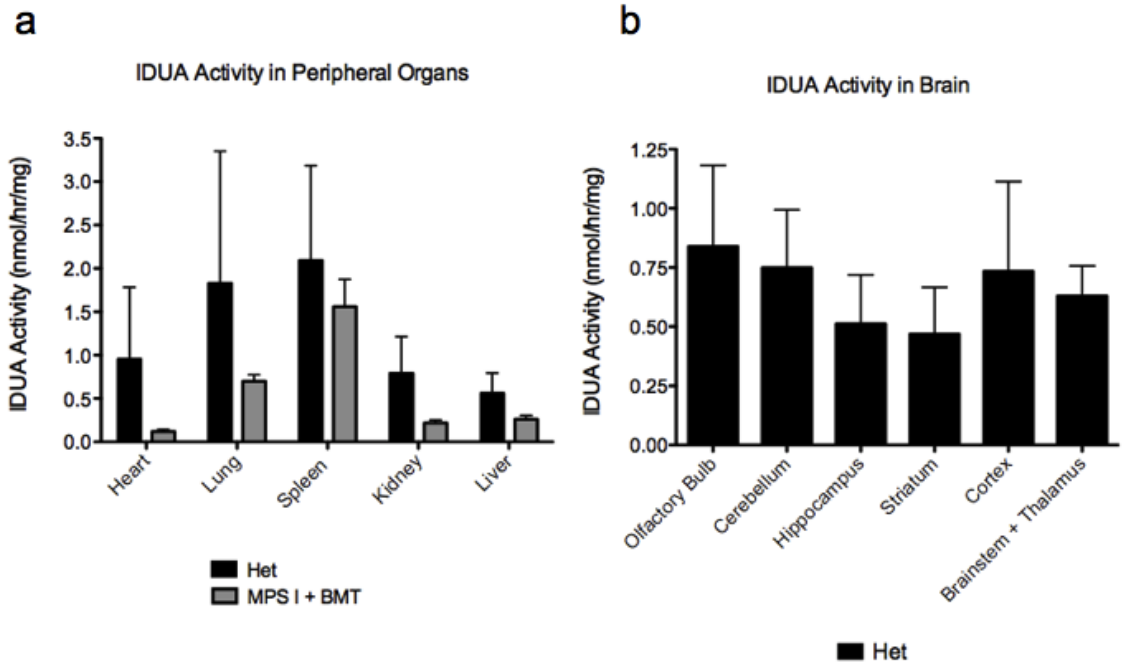


FIGURE 2.3. Reduced GAG storage in peripheral organs following BMT. Levels of GAG storage material were normalized in the liver, spleen, lung, and kidneys of MPS I mice following BMT as compared to unaffected heterozygote animals. GAG levels in the heart were also reduced compared to untreated MPS I mice, although the difference was not statistically significant. (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by one-way ANOVA as compared to untreated MPS I mice; Δ = no statistical significance compared to heterozygous animals by one-way ANOVA)

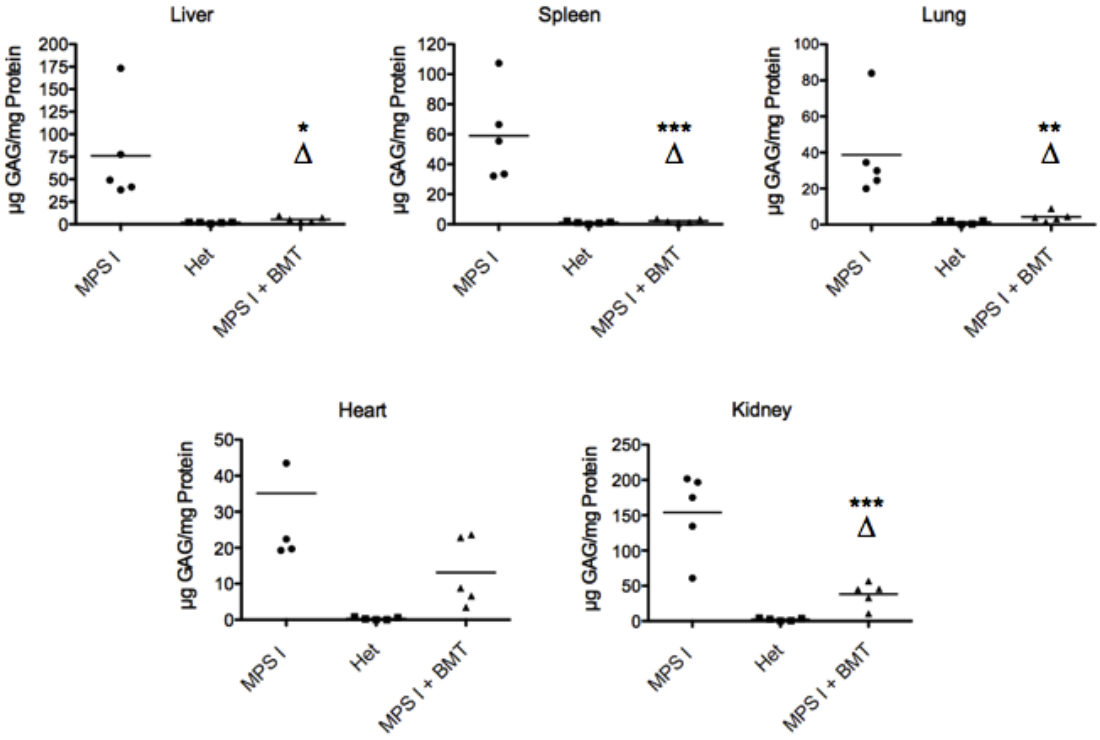


FIGURE 2.4. Correlative relationship between GAG reduction and IDUA activity level in peripheral organs of BMT recipients. The percent reduction of GAG storage material detected in peripheral organs (BMT-treated animals compared to untreated MPS I mice) was plotted vs. the percentage of heterozygote IDUA activity level detected in each sample. The correlative relationship between GAG storage and IDUA activity corresponded to a hyperbolic model with coefficient of determination (R^2) value = 0.95. This model supports previous observations whereby a small amount of IDUA activity is sufficient to provide a drastic reduction in lysosomal GAG storage.

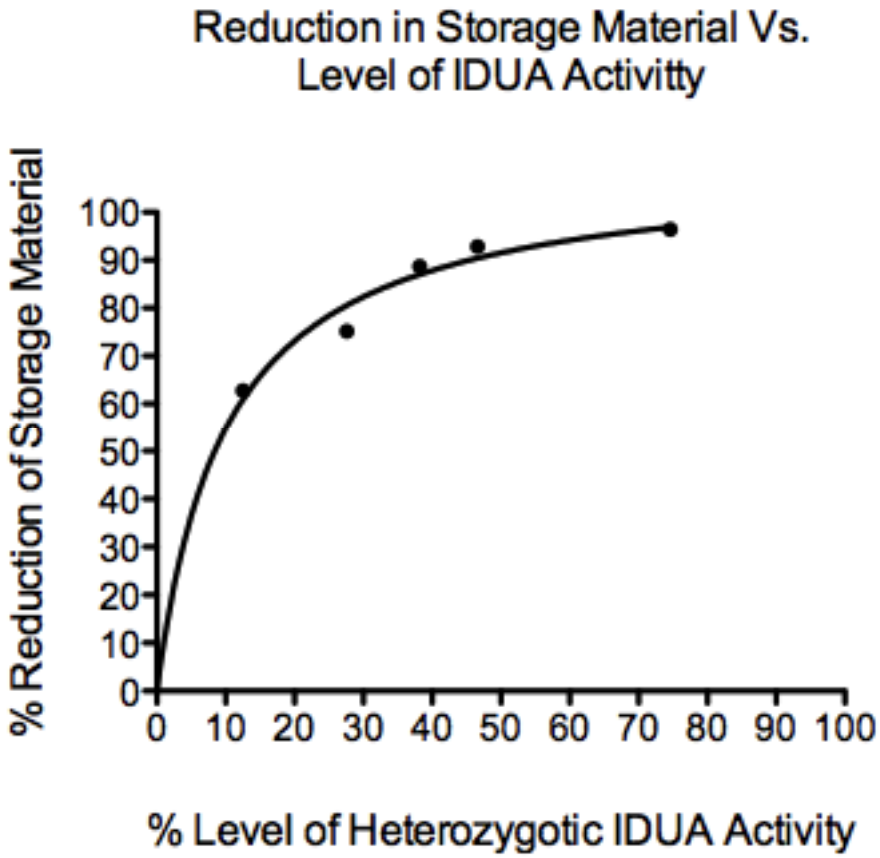


FIGURE 2.5. GAG levels are reduced in the brain. (a) The average levels of GAG detected in the brains of transplanted mice trended lower in every region of the brain than untreated *Idua*$^{-/-}$ mice including in the olfactory bulb, cerebellum, hippocampus, striatum, cortex, and brainstem and thalamus, although not significantly so. However, upon compiling the data from all samples (b), the total GAG content in the brains of BMT-treated mice was significantly lower than that of untreated MPS I animals. (*$P < 0.05$)

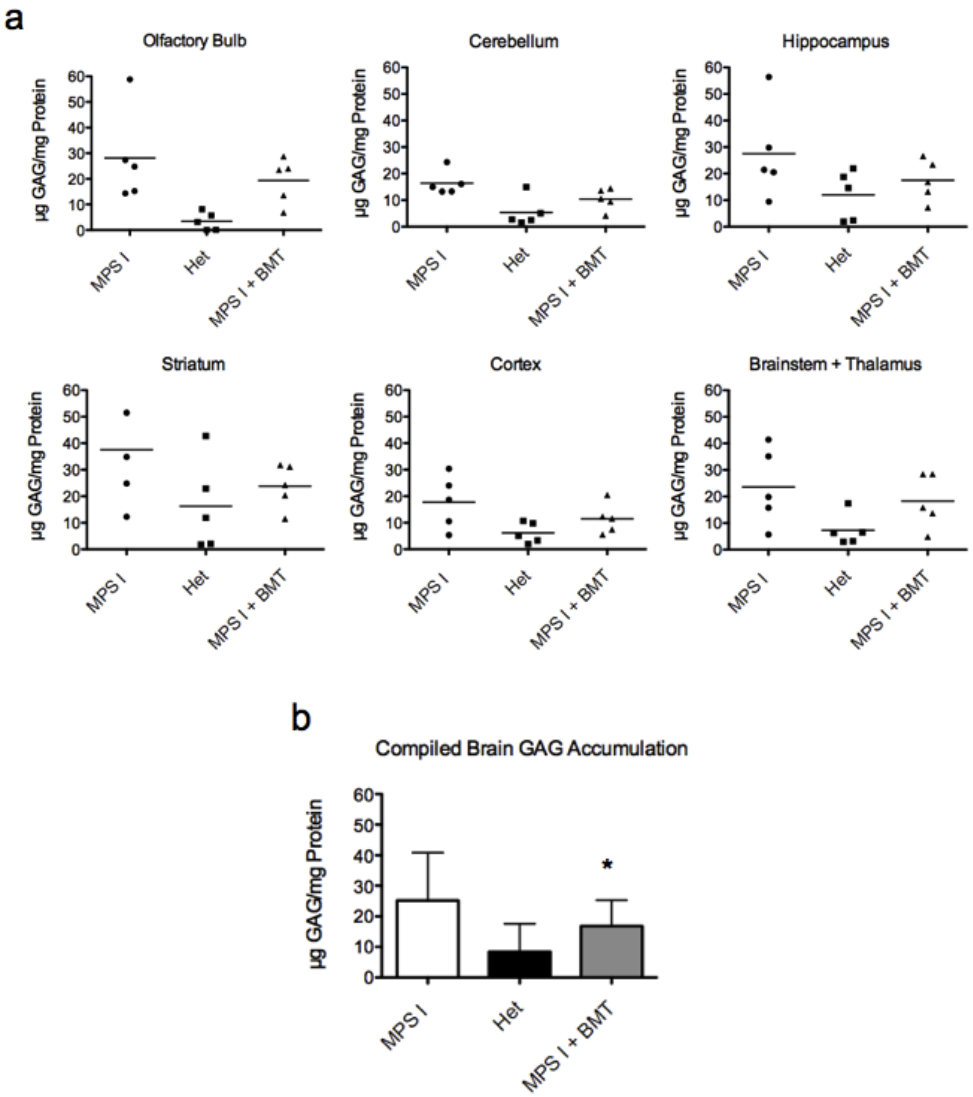
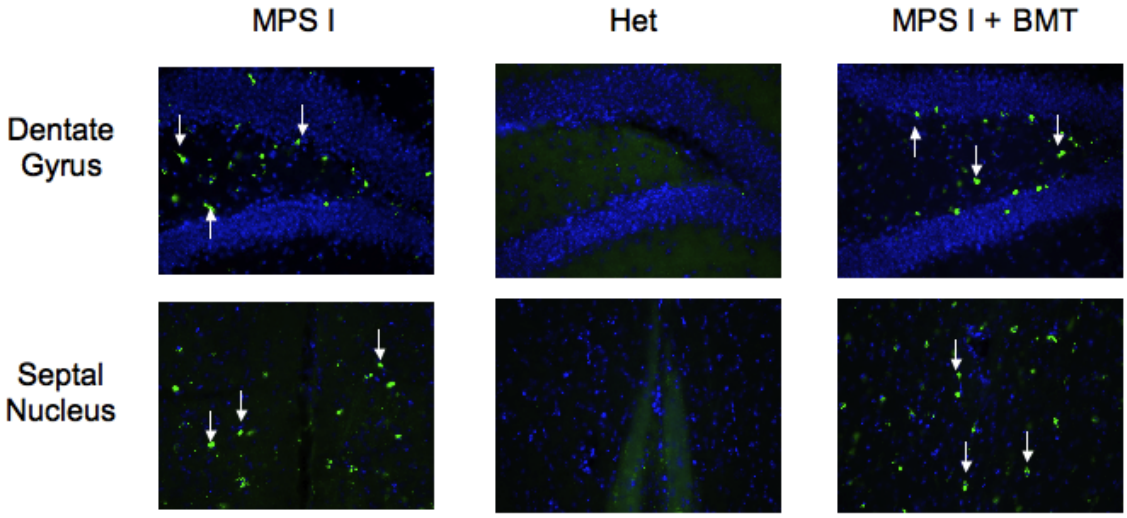


FIGURE 2.6. GM3 ganglioside accumulation in the brain. GM3 ganglioside, a substrate that accumulates in neurons and glia of MPS I animals (32), is visualized by immunofluorescence microscopy as punctate aggregates within grey matter throughout the brains of untreated MPS I mice. Representative images 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 are absent from the grey matter of *Idua*$^{+/-}$ mice, but are present in the brains of BMT recipient mice.



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Chapter 3

Direct Gene Transfer to the CNS Prevents Emergence of Neurologic Disease in a Murine Model of Mucopolysaccharidosis Type I

The mucopolysacharidoses (MPSs) are a group of 11 storage diseases caused by disruptions in glycosaminoglycan (GAG) catabolism, leading to their accumulation in lysosomes (1). Resultant multisystemic disease is manifested by growth delay, hepatosplenomegaly, skeletal dysplasias, cardiopulmonary obstruction, and, in severe MPS I, II, III, and VII, progressive neurocognitive decline. Some MPSs are treated by allogeneic hematopoietic stem cell transplantation (HSCT) and/or recombinant enzyme replacement therapy (ERT), but effectiveness is limited by central nervous system (CNS) access across the blood-brain barrier (2). To provide a high level of gene product to the CNS, we tested neonatal intracerebroventricular (ICV) infusion of an adeno-associated virus (AAV) serotype 8 vector transducing the human α-L-iduronidase gene in MPS I mice. Supranormal levels of iduronidase activity in the brain (including 40x normal levels in the hippocampus) were associated with transduction of neurons in motor and limbic areas identifiable by immunofluorescence staining. The treatment prevented accumulation of GAG and GM3 ganglioside storage materials and emergence of neurocognitive dysfunction in a modified Morris water maze test. The results suggest the potential of improved outcome for MPSs and other neurological diseases when a high level of gene expression can be achieved by direct, early administration of vector to the CNS.

Introduction

The mucopolysaccharidoses (MPSs) are a group of 11 rare, recessive inherited disorders included within the larger family of more than 40 identified lysosomal storage diseases (LSDs). LSDs comprise approximately 14% of all inherited metabolic diseases and affect nearly 1:7,700 births, of which ~30% are MPSs (3, 4). MPSs are caused by mutations in genes that encode any of the lysosomal hydrolases that are responsible for the catabolism of GAGs. Genetic deficiency for any of these specific enzymes is associated with growth delay, organomegaly, cardiopulmonary disease, skeletal dysplasias, and obstructive airway disease (1). Importantly, individuals with the most severe forms of MPS I, II, III, and VII suffer from severe neurological dysfunction and death usually by age 15.

Although HSCT and ERT are currently used to treat MPSs, delivery of enzyme to the central nervous system (CNS) remains a major challenge due to the inability of the deficient lysosomal enzymes to efficiently cross the blood-brain barrier (5, 6). Early intervention with HSCT has been emphasized in order to prevent cognitive decline in the treatment of neuropathic MPSs (7, 8). However, although neurological outcomes for several of the MPSs are improved with donor cell engraftment following HSCT, recipients nonetheless continue to exhibit below normal IQ and impaired neurocognitive capability (9). The aim of this study was to investigate the effects of providing a superior level of enzyme to the brain by direct infusion of an adeno-associated virus (AAV) vector early in life using a murine model of Hurler syndrome, the most severe form of MPS I (also known as MPS IH).

Both AAV2 and AAV5 serotyped vectors have been previously used to express lysosomal enzymes in the CNS and to reduce lysosomal GAG storage material following direct intraparenchymal infusion into mouse models of MPS I (10), MPS IIIB (11), and MPS VII (12-14) as well as a dog model of MPS I (15), in some cases resulting in improved neurobehavior (11, 13, 14). Because AAV8 serotyped vectors have recently been shown to more efficiently transduce neurons within the neonatal mouse brain than AAV2 and AAV1 vectors (16), we generated a vector expressing human α-L-iduronidase (IDUA) packaged into AAV8 virions. This vector was infused into the lateral ventricles of newborn MPS I mice to provide widespread distribution of the vector through the cerebrospinal fluid (CSF) and long-term expression of IDUA throughout the brain. Superabundant levels of IDUA activity in limbic areas of the brain were consistent with normalized GAG and GM3 storage materials. The AAV8-IDUA treated MPS I mice also exhibited memory and spatial navigation character in a modified Morris water maze that was indistinguishable from normal animals. We thus report the first complete prevention of a neurophenotype in a mouse model of MPS I.

Results

We hypothesized that the emergence of neurological disease might be prevented if sufficient levels of the deficient lysosomal enzyme were expressed after vector-mediated gene transfer to the CNS early in life. To investigate this possibility, we generated an AAV8 vector expressing human IDUA under transcriptional regulation by a strong mini-CAGS promoter (17), AAV8-MCI (Figure 3.1a). This vector produced high levels of IDUA activity following transduction of HEK293 cells in culture (Supplementary Figures 3.1a, b). 2 x 10^{10} vector genomes of AAV8-MCI were infused into the right-hemisphere lateral ventricle of neonatal (P4-P6) IDUA-deficient mice, anticipating that this route of administration would provide widespread distribution and vector-mediated IDUA expression throughout the brain by diffusion through the cerebrospinal fluid (CSF).

Intracerebroventricular (ICV) infusion of AAV8-MCI results in supranormal levels of long-term IDUA activity. Animals were sacrificed at 10 months of age, and brain tissue was harvested by microdissection and homogenized in phosphate-buffered saline (PBS). IDUA was undetectable in extracts from IDUA-deficient controls. IDUA enzyme activity levels in extracts from treated animals exceeded levels detected in wild-type animals for all 12 microdissected regions of the brain studied (Figure 3.1b). IDUA activity was the highest in brain structures within close proximity to the ventricles. Most notably, activity levels in the hippocampus were ~40-fold higher than wild-type levels. The striatum, cortex, brainstem, olfactory bulb, and cerebellum exhibited IDUA activity levels that were between 1.5- and 15-fold higher than wild-type levels. Although the AAV8-MCI vector was infused unilaterally into the lateral ventricle of the right-

hemisphere, we did not observe a significant difference in IDUA activity levels between structures in the left and right hemispheres. Furthermore, IDUA activity was not detectable in the liver of treated animals, demonstrating that this method of treatment was not systemic, but was confined largely to the CNS.

Robust IDUA expression is observed within neurons of the limbic system. We further investigated the distribution of IDUA expression at the cellular level by immunofluorescence staining of histologic sections using an antibody to detect human IDUA. In coronal brain sections located at the anterior-posterior coordinates indicated in relation to the bregma (Figure 3.2a), IDUA staining was most intense in, although not limited to, structures within close proximity to the ventricular system (Figures 3.2b,h, and n). Medial to the lateral ventricles, IDUA expression was visualized in the septal nucleus, and in areas CA1, CA2, CA3 and the dentate gyrus of the hippocampus (Figures 3.2f, i, j, l, and m). Dorsal to the lateral ventricles, expression was visualized in the corpus callosum (Supplementary Figure 3.3a) and much of the cerebral cortex including the cingulate cortex (Figure 3.2d) region of the limbic cortex. Additionally, the primary motor cortex (Figure 3.2c), and entorhinal cortex (Figure 3.2k) (regions involved in motor coordination, and learning and memory, respectively) stained positive for IDUA dorsal to the lateral ventricles. Ventral to the lateral ventricles, positive staining was observed in the caudate putamen (Figure 3.2e) and nucleus accumbens (Supplementary Figures 3.3b, c). The indusium griseum (Figure 3.2g) and fasciola cinereum (Supplementary Figure 3.3d) are nuclei lining the 3rd ventricle that stained very intensely with the antibody. Furthermore, Purkinje neurons scattered throughout the cerebellum

also stained positive for IDUA, especially in areas immediately dorsal to the 4th ventricle (Figures 3.2n, o). It is likely that the majority of cells that stained positive for IDUA were transduced cells rather than cross-corrected cells, since animals infused in the same manner with an AAV8 vector expressing green fluorescent protein (GFP) displayed a very similar pattern of GFP expression (Supplementary Figure 3.2). In summary, IDUA expression was observed at the cellular level in many structures of the limbic system, which are involved in the processes of learning and memory as well as in structures involved in motor function. Robust IDUA expression within these structures may be responsible for the resultant neurophenotype displayed by these animals as described below.

In some cases, the type of cell staining positive for IDUA expression was apparent from the morphologic structure revealed by the staining. For example, Purkinje cells in the cerebellum and pyramidal neurons within the hippocampus were easily identifiable by their characteristic morphology. By co-staining for both IDUA and NeuN (a neuronal cell marker), we verified that much of the IDUA-positive staining observed in the brains of these animals was in neurons (Figure 3.2p), as previously reported after infusion of AAV8 vector into the brain (16). However, when co-staining with antibodies to detect both IDUA and GFAP (an astrocytic cell marker), co-localization was not observed (Figure 3.2q), demonstrating that most of the IDUA expression was from neuronal rather than astrocytic cells.

Widespread IDUA activity results in normalization of GAG and GM3 ganglioside storage material. The widespread expression and distribution of IDUA had a profound effect on accumulation of storage materials. Total GAG levels were elevated in untreated MPS I mice, but were normalized throughout the brains of AAV8-MCI-treated mice, including the striatum, hippocampus, olfactory bulb, cerebral cortex, cerebellum, brainstem and thalamus, as they were not significantly different from levels observed in unaffected heterozygous or wild-type animals (Figure 3.3a). Total GAG levels were reduced in the livers of AAV8-MCI-treated mice (perhaps due to enzymatic cross-correction), although they were still significantly higher than those observed in unaffected animals. Secondary accumulation of GM3 ganglioside was apparent in the brains of MPS I animals, as visualized by positive immunofluorescence staining within the white matter as well as in punctate aggregates in cells within the grey matter throughout most of the brain. In contrast, the brains of wild-type and AAV8-MCI treated animals displayed GM3 ganglioside only within white matter (Figure 3.3b).

Treated mice display complete prevention of a neurocognitive deficit in a modified Morris water maze test. Hurler syndrome in children involves severe neurocognitive dysfunction, which is recapitulated in IDUA-deficient mice (18-21). To assess the extent to which the widespread distribution of IDUA expression and associated reduction in GAG and GM3 ganglioside storage material affected neurocognition, we employed a modified Morris water maze (MWM) as a test of spatial navigation and memory (14, 21, 22). Animals were evaluated for the amount of time it took to locate a submerged platform in a tank of opaque water using three-dimensional visual cues positioned on the

sides of the tank (see Methods for detail). At 5 months of age, the animals were subjected to 4 trials of this test per day for a 10-day duration. Unaffected heterozygous littermates exhibited improved performance in this test with repeated trials, requiring an average of 20 seconds to locate the submerged platform at the end of the 10-day testing period. In contrast, untreated MPS I mice displayed a significant deficit in locating the submerged platform (***$P < 0.0001$ by two-way ANOVA) (Figure 3.4a). Remarkably, we observed complete prevention of this neurocognitive impairment in MPS I animals infused with AAV8-MCI at birth, as there was no significant difference observed between these and unaffected heterozygous animals in this test. Wild type animals (not shown) did not perform any better in the water maze than heterozygous animals. On day 11 of the testing period, the submerged platform was moved to the northeast quadrant of the maze and a visual cue was placed directly on the platform. MPS I mice were able to locate the visible platform much faster than the submerged platform and did not perform significantly different than unaffected animals. This demonstrates that the observed phenotype was neurocognitive in nature and not the result of impaired vision or of musculoskeletal or cardiopulmonary dysfunction in the MPS I animals.

Discussion

We observed extraordinarily high levels of long-term IDUA activity throughout the brains of animals infused ICV with AAV8-MCI at birth, consistent with prevention of accumulated GAG and GM3 ganglioside storage materials. The majority of the IDUA expression was in neurons of the hippocampus and other limbic areas as well as in Purkinje cells of the cerebellum. Although a portion of IDUA is able to find its way into the extracellular space (23), most of the IDUA expression observed by immunofluorescence likely came from transduced cells rather than metabolically cross-corrected cells, since the brains of MPS I neonates transduced with AAV8-GFP displayed a similar pattern of immunostaining.

We presume that the complete prevention of a deficit in the modified MWM test is the result of preserved neural circuitry, which is damaged in untreated animals. Importantly, many structures of brain where IDUA expression was visualized by immunofluorescence are intimately involved in the cognitive processes required for learning in the Morris water maze test. For instance, hippocampal place cells are thought to be primarily required for spatial memory and navigation skills in hidden platform learning in the MWM test (24). Rats with quinolinic acid induced lesions in the striatum display a deficit in MWM hidden platform acquisition and spend much of their time swimming along the walls of the maze (thigmotaxis), as observed in our untreated MPS I control group (25). Additionally, rats with lesions of the medial septal nucleus or the cingulate cortex and mice with cerebellar damage all have impaired MWM performance (26-28). IDUA expression was visualized in neurons within all of these areas of the brain, suggesting that

this pattern of expression may be in part responsible for the complete prevention of neurocognitive deficit in the MWM.

While neurological outcomes for several of the neuropathogenic MPSs are improved with engraftment following HSCT, recipients nonetheless continue to exhibit below normal IQ and impaired neurocognitive capability (9). Our results predict that AAV-mediated IDUA gene delivery to the CNS, by way of ICV infusion, will provide a sustained, high level of enzyme expression that vastly exceeds the amount reasonably attainable by ERT or HSCT (29, 30), with improved neurological outcome anticipated. However, there are several limitations in the interpretation of this study when considering the treatment of MPS children. Human IDUA is known to be highly immunogenic, and has been shown to elicit both humoral and cytotoxic T lymphocyte responses in adult MPS I mice, newborn cats, and dogs (31-36). Newborn mice are immunologically naive (37-41), so it is likely that our AAV-treated animals were immunotolerized by the expression of IDUA soon after treatment, before the immune system became fully developed. Also, although we were able to achieve widespread distribution throughout the mouse brain after neonatal ICV infusion of AAV8, a significant challenge will be the application of this strategy to achieve gene transfer in the human brain, which is several thousand-fold larger than the neonatal mouse brain and in which the effectiveness of the procedure will be further limited by diffusion of the vector into deep brain tissues.

The results of this study nonetheless strongly support the adoption of CNS-directed, AAV-mediated gene therapy as a supplement to ERT and HSCT for the treatment of

MPSs (2), thereby providing effective treatment for both peripheral and CNS manifestations in patients with severe lysosomal storage disease. Although there are significant challenges to be faced in scale-up, route of administration, and assessment of procedural safety, the benefits of high-level neonatal AAV-mediated transduction in the CNS are potentially applicable to the treatment of a broad range of neurological disorders.

Methods

AAV8-MCI Vector Construction

The *IDUA* expression cassette (mini-CAGS promoter (17), human *IDUA* cDNA, bovine growth hormone polyadenylation signal) was excised from plasmid pT2/IDUA//Ub-SB11 (42) using *Pme*I and *Eco*RV and ligated into the *Sna*BI site of plasmid pSub201 (43). The resulting plasmid, pTR-MCI, contains the *IDUA* expression cassette flanked by AAV2 inverted terminal repeats, and was packaged into AAV8 virions by the University of Florida Vector Core. Vector titer was 4×10^{12} vector genomes/ml.

Animals and ICV Infusions

The *IDUA*$^{-/-}$ mouse strain was provided by Dr. E. Neufeld (44) and maintained under pathogen-free conditions in AAALAC-accredited facilities. Animal work was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota. *Idua*$^{-/-}$ pups were generated by breeding together homozygous *Idua*$^{-/-}$ animals. All mice were genotyped by PCR as previously described (19). Four to six-day old *Idua*$^{-/-}$ pups were cryoanesthetized and placed on a stereotactic frame. Five microliters (2×10^{10} vector genomes) of AAV8-MCI was infused into the right-side lateral ventricle (stereotactic coordinates AP 0.0, ML 0.5, DV 1.8 mm from bregma) using a Hamilton syringe. The animals were returned to their cages on heating pads for recovery and subsequent study.

IDUA Enzyme Assay

Animals were anesthetized with ketamine/xylazine (100mg ketamine + 10mg xylazine per kg) and transcardially perfused with 70 mL PBS prior to sacrifice. Brains were harvested and microdissected on ice into 12 regions: right and left olfactory bulb, cerebellum, hippocampus, striatum, cortex, and brainstem. The samples were frozen on dry ice and then stored at -80°C. Samples were thawed and homogenized in 1 mL of PBS using a motorized pestle and permeabilized with 0.1% Triton X-100. IDUA activity was determined by fluorometric assay using 4MU-iduronide as the substrate, as previously described (20). Activity is expressed in units (percent substrate converted to product per minute) per mg protein as determined by Bradford assay (BioRad).

Quantitative PCR

Tissue homogenates were supplemented with 300µl of cell lysis buffer (5 Prime) and with 100µg of proteinase K and incubated with gentle rocking overnight at 55°C. DNA was then isolated from the samples by phenol/chloroform extraction. Reaction mixtures of 25µl contained 0.5µg of DNA, 2X IQ^{TM} SYBR Green Supermix (Bio-Rad) and 200 nM each of forward and reverse primer as previously described (16). PCR conditions were: 95° followed by 40 cycles of 95°C for 40 sec, 58°C for 30 sec, and 72°C for 1 min. Mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) sequence served as an internal control for genomic DNA content and was amplified in a separate reaction. Mouse genomic DNA diluted in water was used as a standard curve for *Gapdh*. The standard curve for IDUA consisted of serial dilutions of DNA isolated from a clone of NIH3T3 cells transduced at low multiplicity with LP1CD retroviral vector (26) and

assumed to contain 1 viral integrant per cell containing *IDUA*. This DNA was diluted in wild-type C57BL/6 liver DNA to make serial dilutions. All reactions were carried out in duplicate. GAPDH primers used were forward primer: 5′-TGTCTCCTGCGACTTCAACAGC-3′ and reverse primer: 5′-TGTAGGCCATGAGGTCCACCAC-3′. IDUA primers used were forward primer: 5′-AGGAGATACATCGGTACG -3′ and reverse primer: 5′-TGTCAAAGTCGTGGTGGT -3′.

Glycosaminoglycan Assay

Tissue homogenates were clarified by centrifugation for 3 minutes at 13,000 rpm using an Eppendorf tabletop centrifuge model Centrifuge 5415D (Eppendorf) and incubated overnight with proteinase K, DNase1, and RNase as previously described (20). GAG concentration was determined using the Blyscan Sulfated Glycosaminoglycan Assay (Accurate Chemical) according to the manufacturer's instructions.

Modified Morris Water Maze

A pool measuring 160 cm in diameter was filled 12 cm deep with water (23°C ± 2°C) mixed with 200 mL of non-toxic white acrylic paint. Three-dimensional, multi-colored visual cues were attached to the walls of the pool according to the cardinal directions (North, South, East, and West) and a platform was submerged 1 cm below the surface of the water in the northwest quadrant of the maze. Age-matched, 5-month old mice were released in the maze facing the southern wall of the pool. The mice were given 90 s to explore the maze and the total time spent swimming before locating the submerged

platform was recorded. If a mouse did not reach the platform within 90 s, the animal was guided to the platform and given 30 s to explore it. Animals were given 4 trials each day for 10 days, with 12 minutes between each trial. On day 11, a visual platform test was performed in which the submerged platform was moved to the northeast quadrant of the maze and a visual cue was placed directly on the platform, making it visible. The mice were tested using the same protocol, and the time for each mouse to locate the visual platform was recorded.

Immunofluorescence

Animals were anesthetized with ketamine/xylazine as 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 coronally (30µm thickness) using a freezing microtome. Free-floating sections were washed in PBS and blocked with 10% normal donkey serum / 0.3% Triton X-100. Primary antibodies (see legends to Figs. 2 and 3) were added and sections were incubated overnight at 4°C, washed again with PBS, and incubated for 1 hr at room temperature with fluorescently labeled secondary antibodies diluted in PBS. Sections were then washed in PBS, incubated for 10 minutes with DAPI (Invitrogen), mounted on slides, and visualized using a Zeiss Axioplan 2 upright microscope (Zeiss). Primary antibodies were used to stain human IDUA (1:500, R&D Systems), GFAP (1:400, Sigma), NeuN (1:500, Chemicon), and GM3 ganglioside (1:500, Seikagaku). Alexa-conjugated secondary antibodies and dilution factors were as

follows: donkey anti-mouse IgG Alexa Fluors 555 and 488 (1:500, Invitrogen), and donkey anti-mouse IgM Alexa Fluor 488 (1:500, Invitrogen).

Statistical Analysis

Data are reported as mean ± s.d. One-way ANOVA with Tukey's post test was used to evaluate statistical differences between groups for the IDUA activity assay, GAG assay, and visual platform behavior assay, with $P < 0.05$ considered significant. Two-way ANOVA was used to evaluate behavioral performance in the modified Morris water maze test. Data analysis was performed using Prism 5.0 software (GraphPad Software).

FIGURE 3.1. ICV infusion of AAV8-MCI into neonates results in widespread high-level expression of IDUA. (a) Depiction of the therapeutic AAV construct. AAV serotype 2 inverted terminal repeat (ITR) sequences flank human *IDUA* cDNA regulated by a mini-CAGS (mCAGS) promoter. This construct was packaged using AAV serotype 8 capsid protein to produce AAV8-MCI. (b) IDUA activity levels in brain tissues at 10 months of age. Extracts were prepared from brain tissues microdissected from normal animals (wild-type), animals heterozygous at the *IDUA* locus (heterozygous) and MPS I animals treated at birth by ICV infusion with AAV8-MCI vector and assayed for IDUA activity (mean +/- S.D.). Enzyme activity levels were well above those detected in the brains of wild-type animals in all sections studied. The activity levels follow a gradient pattern with the highest levels of activity present within brain regions in close proximity to the lateral ventricles. IDUA activity was undetectable in extracts prepared from animals homozygous for the $IDUA^{-}$ locus (not shown).

a


ITR
mCAGS
hIDUA
pA
ITR

b

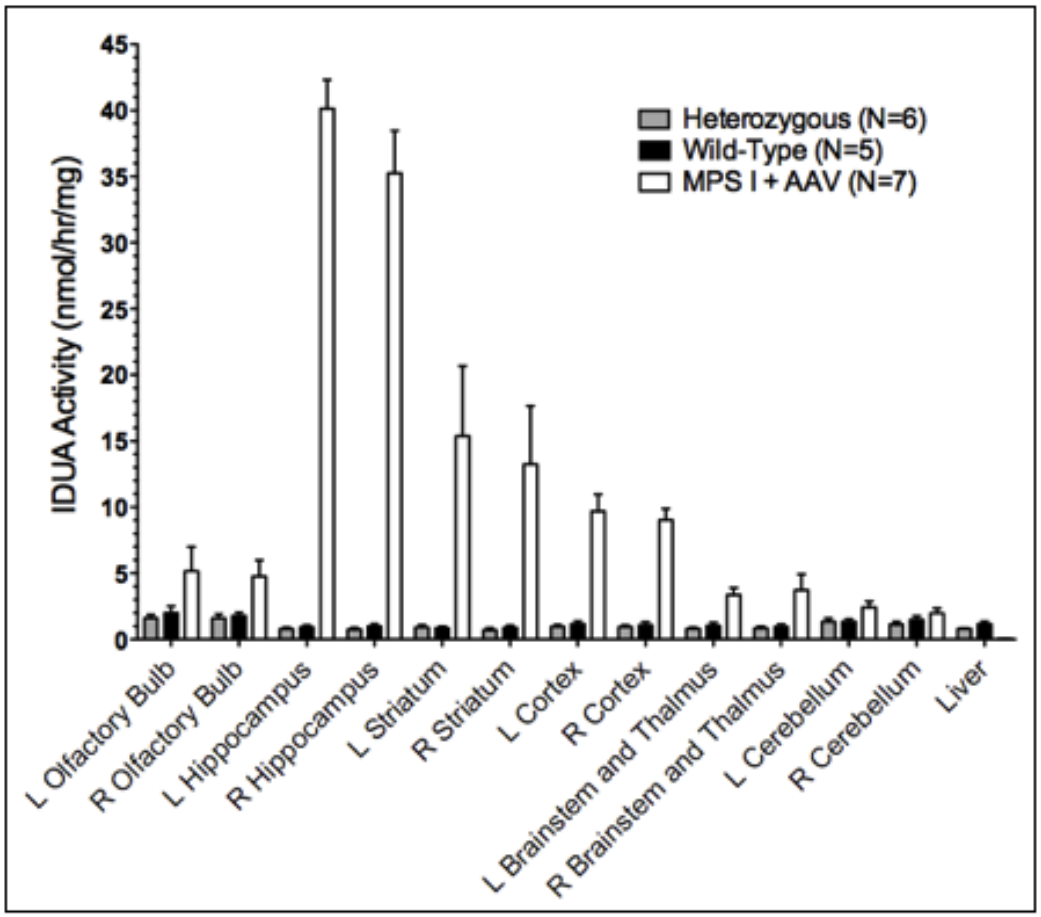

IDUA Activity (nmol/hr/mg)
45
40
35
30
25
20
15
10
5
0
Heterozygous (N=6)
Wild-Type (N=5)
MPS I + AAV (N=7)
L Olfactory Bulb
R Olfactory Bulb
L Hippocampus
R Hippocampus
L Striatum
R Striatum
L Cortex
R Cortex
L Brainstem and Thalmus
R Brainstem and Thalmus
L Cerebellum
R Cerebellum
Liver

FIGURE 3.2. Immunofluorescence staining with an anti-IDUA antibody reveals widespread IDUA expression, mainly from neuronal cells within areas of the limbic system. Coronal brain slices from the displayed Bregma coordinates (a) were stained with an anti-human IDUA antibody. Low magnification images reveal widespread distribution of IDUA expression (red) (b,h,m). Higher-power magnification of specific regions within the section reveals IDUA expression (IDUA (red) and DAPI (blue)) in multiple areas of the limbic system including the cingulate cortex (d), septal nucleus (f), indusium griseum (g), hippocampus (CA1 (I), CA2 (j), CA3 (l), and dentate gyrus (m)), entorhinal cortex (k), and basal ganglia (caudate putamen (e)). Additionally, regions of the brain important for motor function displayed robust IDUA expression, including the primary motor cortex (c) and Purkinje cells lining the 4th ventricle in the cerebellum (o). Cells expressing IDUA displayed a strong co-localization with NeuN (green) resulting in yellow co-staining within pyramidal neurons of area CA3 of the hippocampus (p). However, in the same region, IDUA did not co-localize with the astrocytic marker GFAP (green). Representative white scale bars in panels (c), (o), and (q) are 100μm in length.

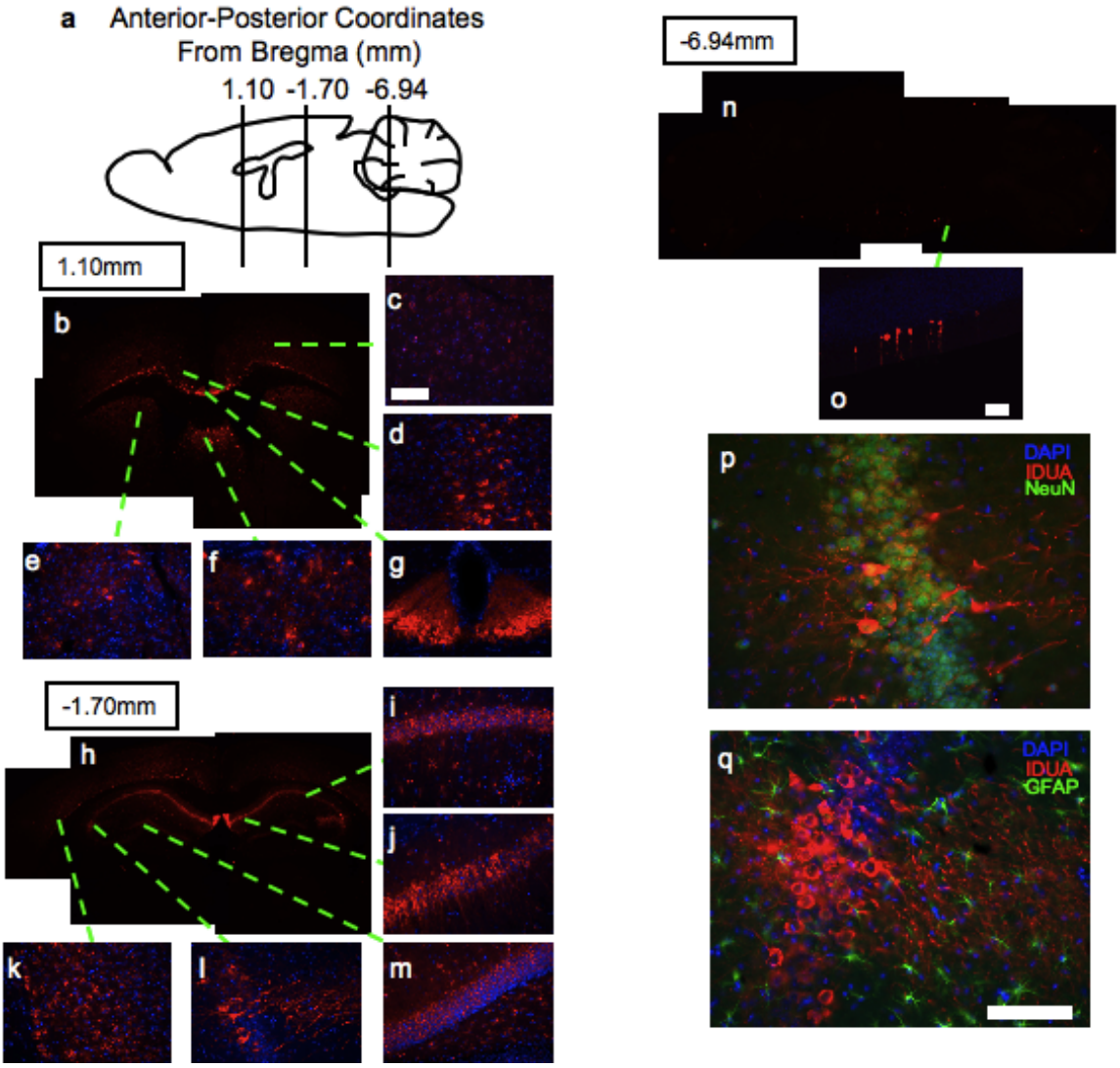

a
Anterior-Posterior Coordinates
From Bregma (mm)
1.10 -1.70 -6.94
1.10mm
b
c
d
e
f
g
-1.70mm
h
i
j
k
l
m
-6.94mm
n
o
p
DAPI
IDUA
NeuN
q
DAPI
IDUA
GFAP

FIGURE 3.3. ICV infusion of AAV8-MCI into neonates normalizes pathogenic storage material 10 months post-infusion to levels of unaffected animals. (a) Total GAG levels in the striatum, olfactory bulb, cerebellum, hippocampus, cortex, striatum, brainstem and thalamus, and livers of wild-type, *IDUA* heterozygous, MPS 1, and AAV8-MCI treated MPS I mice. Data points represent brain samples microdissected from the left (red) and right (blue) hemispheres, respectively. Values are compared to respective values in untreated MPS I animals (horizontal bar: mean; $***P < 0.001$). (b) Punctate GM3 ganglioside accumulation is visualized in grey matter throughout the brains of MPS I animals, including the dentate gyrus and septal nucleus. This pattern of staining is absent in the brains of wild type animals and MPS I animals treated with AAV8-MCI. White scale bar in represents 100μm in length.

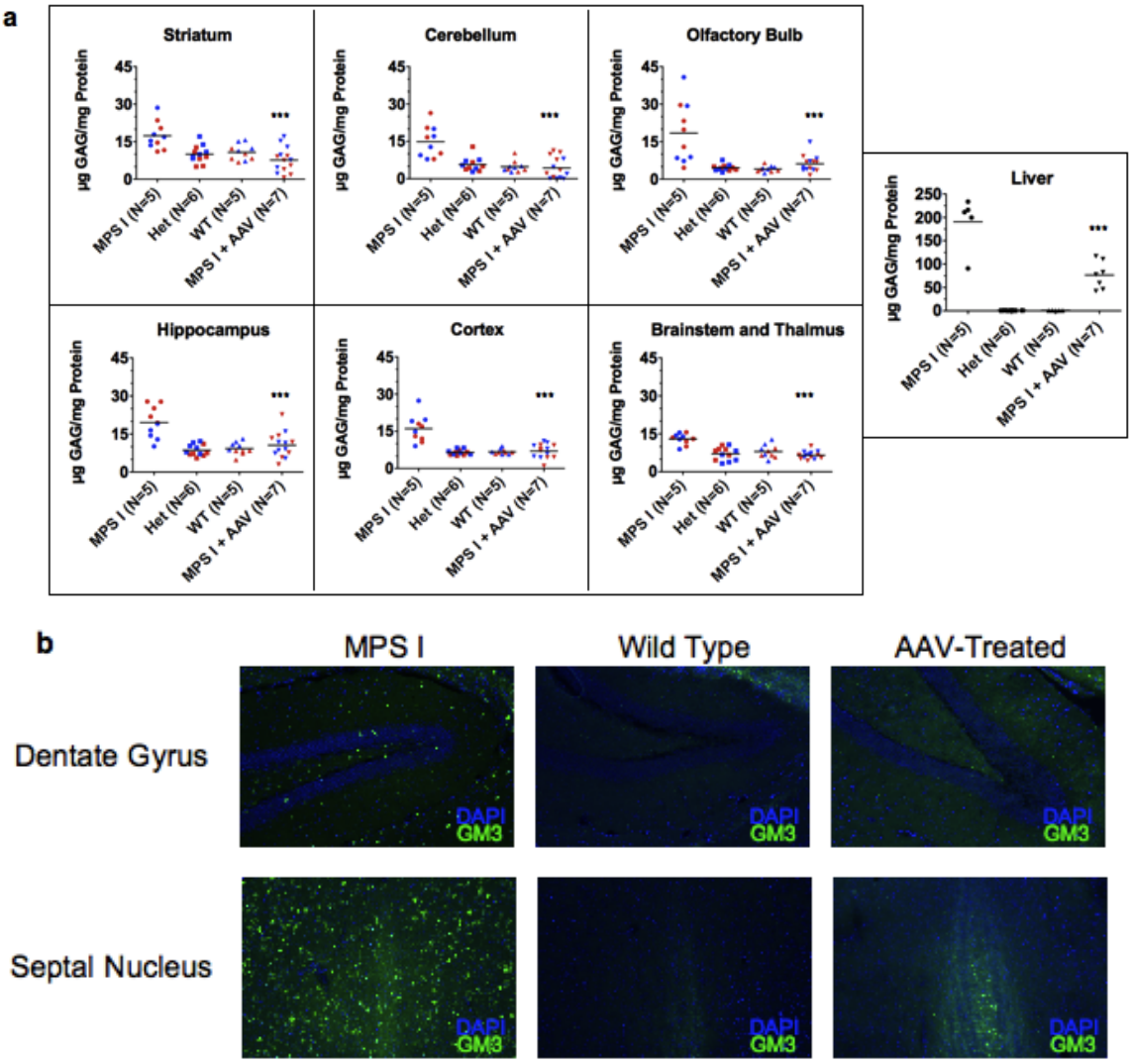
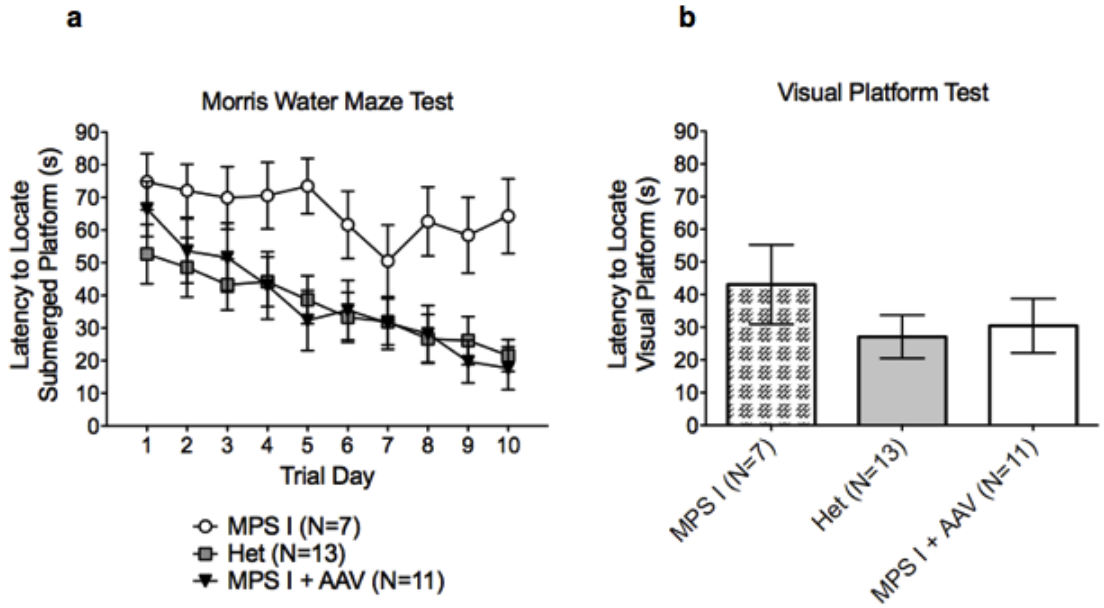
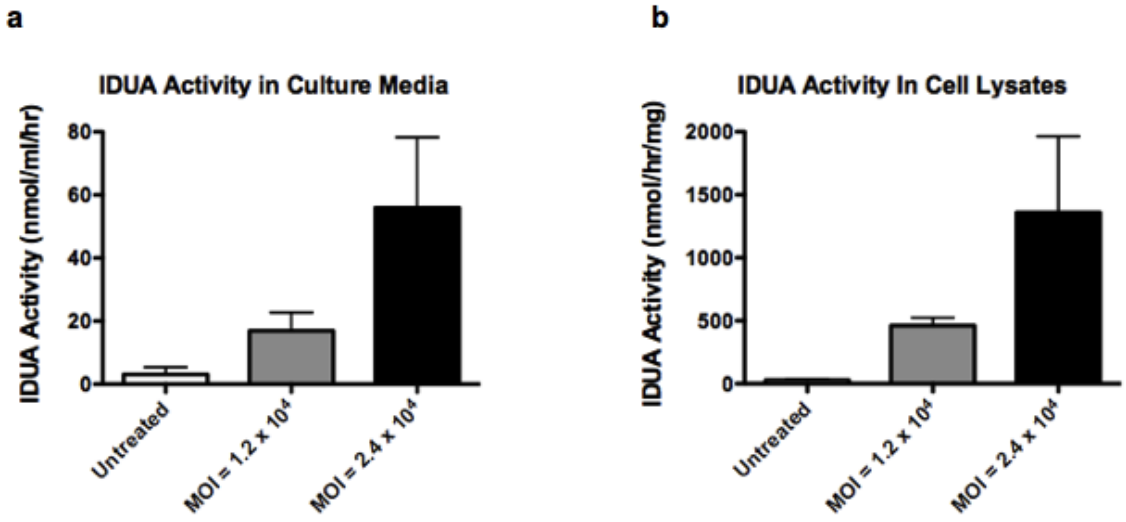

a
Striatum
Cerebellum
Olfactory Bulb
Hippocampus
Cortex
Brainstem and Thalmus
Liver
µg GAG/mg Protein
MPS I (N=5)
Het (N=6)
WT (N=5)
MPS I + AAV (N=7)
***
b
MPS I
Wild Type
AAV-Treated
Dentate Gyrus
Septal Nucleus
DAPI
GM3

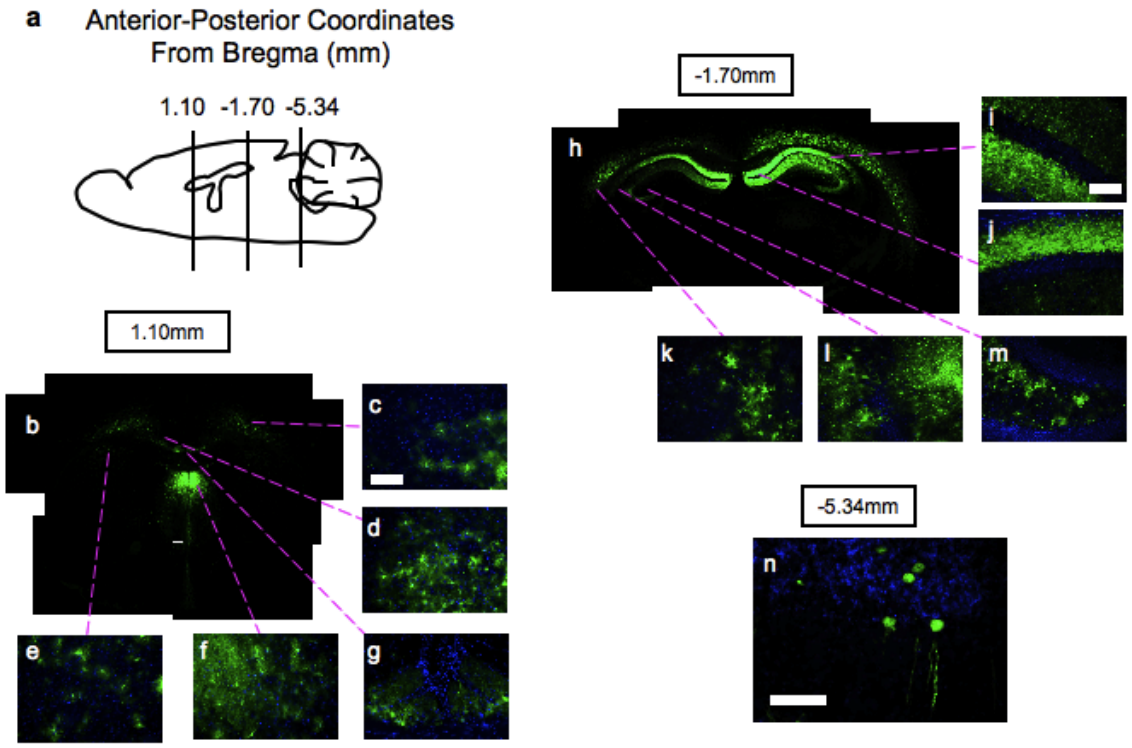
FIGURE 3.4. A spatial learning and memory phenotype displayed by MPS I mice is completely prevented in MPS I mice treated by ICV infusion of AAV8-MCI as neonates. (a) Results of a modified Morris water maze task. MPS I mice displayed a significant deficit in their ability to locate a submerged platform compared to unaffected heterozygous mice ($P < 0.0001$). MPS I mice treated with AAV8-MCI performed equivalent to heterozygous mice ($P < 0.0001$ vs. MPS I mice). (b) To confirm that the deficit displayed by MPS I mice was not due to impaired vision caused by corneal clouding or to musculoskeletal or cardiopulmonary abnormalities, the mice were subjected to a visual platform test. No significant difference was observed in this task between any of the groups of animals.



SUPPLEMENTARY FIGURE 5.1. *In vitro* transduction of HEK293 cells mediates high level expression of IDUA. 10^6 HEK293 cells were plated in 6 cm^2 dishes and transduced with AAV8-MCI at an MOI of either 1.2 x 10^4 or 2.4 x 10^4 vector genomes per target cell. Three days later, IDUA activity was measured in the culture medium (a) or in prepared cell lysates (b) demonstrating robust AAV-mediated IDUA expression.



SUPPLEMENTARY FIGURE 5.2. ICV infusion of an AAV8-GFP vector results in a similar transgene expression pattern as that seen using AAV8-MCI. 2 x 10^{10} vector genomes of AAV8-TRUF11(45) was infused into the right side lateral ventricle of *IDUA*$^{-/-}$ neonates as described in the methods for AAV8-MCI. Coronal brain sections from the displayed anterior-posterior coordinates from bregma (a) were stained with an anti-GFP antibody. Low power magnification images reveal widespread distribution of GFP expression (green) (b,h). Higher-power magnification of specific regions reveals GFP expression (GFP (green) and DAPI (blue)) in multiple areas of the limbic system including the cingulate cortex (d), septal nucleus (f), indusium griseum (g), hippocampus, (CA1 (I), CA2 (j), CA3 (l), and dentate gyrus (m)), entorhinal cortex (k), and basal ganglia (caudate putamen (e)). Additionally, regions of the brain important for motor function displayed robust GFP expression, including the primary motor cortex (c) and Purkinje cells lining the 4th ventricle in the cerebellum (o). This pattern or expression is comparable to that observed after ICV infusion of AAV8-MCI. White scale bars represent 100μm in length.


a
Anterior-Posterior Coordinates
From Bregma (mm)
1.10 -1.70 -5.34
1.10mm
b
c
d
e
f
g
-1.70mm
h
i
j
k
l
m
-5.34mm
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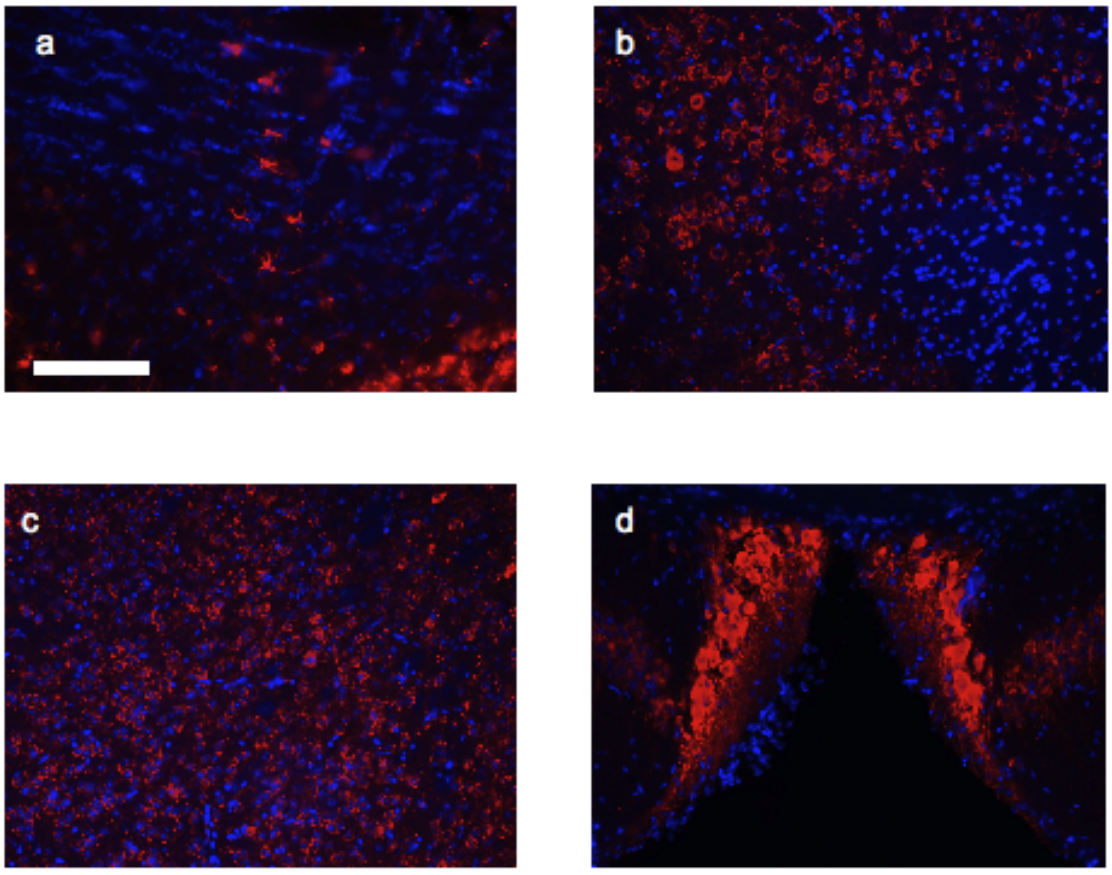
SUPPLEMENTARY FIGURE 3.3. IDUA expression is visualized in multiple brain structures by immunofluorescence staining. Cells staining positive for IDUA are visualized in the corpus callosum (a), nucleus accumbens shell (b), nucleus accumbens core (c), and fasciola cinereum (d). White scale bar represents 100µm in length.



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Chapter 4

Direct Gene Transfer to the Adult CNS in a Murine Model of Mucopolysaccharidosis Type I

Mucopolysaccharidosis type I (MPS I) is progressive lysosomal storage disorder caused by deficiency of α-L iduronidase (IDUA), resulting in multisystemic disease. In addition to cardiopulmonary obstruction, musculoskeletal abnormalities, corneal clouding, and organomegaly, Hurler patients display hydocephalus and severe neurocognitive dysfunction. Treatment this disease by delivery of IDUA to the central nervous system remains a major challenge due to the inability of IDUA to cross the blood-brain barrier. We recently reported widespread distribution and long-term expression of extraordinarily high levels of IDUA throughout the brain as well as normalization of storage material and the complete prevention of neurocognitive deficiency following neonatal intracerebroventricular (ICV) infusion of an adeno-associated viral (AAV) serotype 8 vector expressing IDUA (AAV8-MCI). In the current study we sought to determine the effects of similar treatment in adult MPS I mice. Unilateral ICV infusion of AAV8-MCI into 6-week old and 7-month old animals resulted in effective transduction of neural tissue as determined by quantitative PCR for IDUA vector genomes. However, IDUA expression was detectable only in a small number of treated animals. Glycosaminoglycan storage materials were reduced in specific regions of the brain. Animals treated at 6 weeks of age exhibited partial prevention neurocognitive decline in a Morris water maze acquisition test, while animals treated post-symptomatically at 7-months of age didn't show neurophenotypic improvement. Both groups of treated animals demonstrated an increased lifespan compared to untreated littermates. Results from this study provide insights into the development of treatment strategy to achieve effective AAV-mediated IDUA expression in the brains of adult mice.

Introduction

Mucopolysaccharidosis type I (MPS I) is a rare autosomal recessive inherited disorder with an incidence of nearly 1:100,000 live births (1). This disease is part of a family of 11 related mucopolysaccharidosis disorders, each caused by deficiency of a particular lysosomal enzyme involved in the process of degrading glycosaminoglycans (GAGs). In the case of MPS I, affected patients harbor mutations in the *IDUA* locus leading to deficiency of the lysosomal hydrolase α-L-iduronidase (IDUA), an enzyme that cleaves off the terminal sugar from heparan sulfate (HS) and dermatan sulfate (DS) GAGs. This interrupts the step-wise catalysis of these molecules and results in lysosomal buildup of HS and DS storage materials in multiple tissues as well as accumulation of secondary storage materials such as GM2 and GM3 gangliosides in the brain (2). There are a range of manifestations, including a mild attenuated form, Scheie syndrome (MPS I S), an intermediate form, Hurler-Scheie syndrome (MPS I HS), and the most severe form of the disease, Hurler syndrome (MPS I H) depending on the specific *IDUA* mutations and the resulting level of residual cellular IDUA activity (3). Patients with attenuated forms of the disease exhibit corneal clouding, joint stiffness, growth delay, aortic valvular disease, and joint stiffness (1). Severe Hurler patients additionally exhibit hydrocephalus and severe neurocognitive impairment and rarely survive into the second decade.

Currently, the standard of care for severe Hurler patients involves weekly infusions of recombinant enzyme following diagnosis and ultimately hematopoietic stem cell transplantation (HSCT) for patients with a matched HLA donor (4). HSCT increases survival and improves hepatosplenomegaly as well as musculoskeletal and cardiac

manifestations of the disease (5-7). Despite these successes, challenges remain in delivering therapeutic levels of IDUA to the central nervous system (CNS), due to the inability of IDUA to cross the blood-brain barrier (8, 9). This has led to the investigation of alternative therapeutic approaches, including gene therapy, to more effectively deliver IDUA (and other lysosomal enzymes) to the CNS for the treatment of severe MPS I as well as other neuropathological lysosomal disorders.

Adeno-associated viral vectors (AAV) have become an effective tool for gene transfer into neural tissue. Both AAV2 and AAV5 serotype vectors have been used to express lysosomal enzymes in the CNS and to reduce lysosomal GAG storage material following direct intraparenchymal infusion into mouse models of MPS I (10), MPS IIIB (11), and MPS VII (12-14) as well as in a dog model of MPS I (15), in some cases resulting in improved neurobehavior (11, 13, 14). Adding to this body of literature, we have recently demonstrated that intracebroventricular (ICV) infusion of an AAV serotype 8 vector expressing IDUA under transcriptional regulation by the strong mini-CAGGS promoter (AAV8-MCI) into neonatal MPS I mice resulted in high levels of long-term IDUA expression, completely preventing the emergence of neurologic disease (Chapter 3). Building on these results, the goal of this study was to investigate the extent to which ICV infusion of AAV8-MCI is able to prevent or correct neurologic disease in both young adult and post-symptomatic adult MPS I mice of advanced age, respectively. The results of this study indicate that there are significant hurdles to overcome in order to achieve high-level AAV-mediated IDUA gene transfer in adult mice by ICV infusion.

Results

An AAV8 vector containing a strong mini-CAGS promoter regulating expression of human IDUA (AAV8-MCI) was constructed as previously described (Chapter 3). 2 x 10^{10} vector genomes of AAV8-MCI were stereotactically infused into the right-side lateral ventricle of adult MPS I mice. We treated two distinct groups of animals in this manner. One cohort of mice was treated at 6 weeks of age. The second cohort of mice was treated at 7 months of age after displaying neurocognitive deficit in a Morris water maze test of spatial navigation and memory at 5 months of age (Figure 4.1).

ICV infusion of AAV8-MCI into 6-week old MPS I animals partially prevents neurocognitive decline. At 5 months of age, AAV8-MCI treated and control animals were subjected to a modified Morris water maze test of spatial navigation and learning. The mice were placed in a circular tank filled with opaque water and surrounded by 3-dimensional visual cues. The animals were placed into the maze facing the south wall of the tank and were given 4 trials per day to locate a submerged platform hidden in the northwest quadrant of the maze. Over the course of a 10 day training period, unaffected heterozygous animals were able to rapidly learn the task (Figure 4.1). However, untreated MPS I animals displayed a deficit in their ability to learn this acquisition task, as there was little improvement observed over the 10 day testing period. *Idua*$^{-/-}$ mice treated with AAV-MCI at 6 weeks of age exhibited a significant improvement in their performance of this test in comparison with untreated animals (***$P < 0.0001$ by 2-way ANOVA). ICV infusion of AAV8-MCI at 6 weeks of age thus partially prevented emergence of this neurophenotype in MPS I mice.

9 of the 21 untreated MPS I animals were infused ICV with AAV8-MCI post-symptomatically at 7 months of age. At 9 months of age, the animals treated at six weeks of age were retested in the water maze with the submerged platform remaining in the same location. These animals continued to demonstrate partial improvement in acquisition compared to untreated MPS I animals ($P < 0.01$ by 2-way ANOVA) (Figure 4.2). However, the animals treated post-symptomatically at 7 months of age displayed no statistical improvement in the water maze task compared to untreated littermates. Following the last day of testing, the submerged platform was removed and a visible platform with a visual cue was placed in the northeast quadrant of the maze. The mice were then given 4 trials to locate the visible platform using the same protocol as in the acquisition phase. There was no statistical difference observed between any of the groups of animals in this test, indicating that the results obtained from the acquisition phase of the water maze test were neurocognitive in nature and not due to impaired vision or musculoskeletal deficiencies of the MPS I mice.

Animals treated with AAV8-MCI display an extended lifespan compared to untreated MPS I littermates. Prior to termination of the experiment and sacrifice of experimental animals at 10 months of age, it was observed that both groups of treated MPS I animals exhibited increased longevity compared to untreated littermates ($*P < 0.05$ by log-rank test; Figure 4.3). Nearly half of the untreated animals perished due to disease symptoms prior to termination of the experiment. (It should be noted that two of the animals died suddenly while swimming during water maze testing due to apparent

myocardial infarction). Meanwhile, only 2 treated animals (both treated at 6 weeks of age) died during the course of the experiment.

AAV8-MCI vector genomes detected in the brain of all treated animals. At 10 months of age, experimental animals were perfused with saline and sacrificed. Brains were harvested and microdissected into 12 regions: left and right olfactory bulb, cerebellum, hippocampus, striatum, cerebral cortex, and brainstem and thalamus. The microdissected samples were homogenized and DNA was isolated from a small portion of each homogenate. Quantitative PCR was conducted to detect the vector sequences using primers to amplify a segment of the human *IDUA* cDNA sequence in a SYBR green-based assay (16). AAV8-MCI vector sequence was detected in the brains of all of treated animals, with the highest consistent copy numbers detected in the right striatum, right cortex, and right cerebellum, consistent with access to these structures through diffusion of the AAV vector in the cerebrospinal fluid of the injected lateral ventricle (Tables 4.1 and 4.2). Notably higher copy numbers were detected in the brains of animals treated at 7 months of age (Table 4.2) compared to animals treated at 6 weeks of age (Table 4.1). Vector was also detected in the liver of a few treated animals, suggesting some leakage into the circulation during the procedure that may have contributed to the increased longevity of the treated animals.

Limited IDUA expression in brain tissues of treated animals. IDUA activity was assayed in brain homogenates from several of the AAV8-MCI treated animals. Despite the presence of detectable *IDUA* sequence in the brains of all treated animals, IDUA

enzyme activity was detectable in brain tissue homogenates of only one animal, mouse #5 treated at 7 months of age (Table 4.2). Wild-type animals had IDUA activity levels within the normal range detected in both hemispheres of the cerebellum. Activity levels detected in both hemispheres of the cerebral cortex and right hippocampus of mouse #5 were approximately half of those observed in wild-type animals, while the level of activity detected in the right striatum was approximately 1/3 of that detected in wild-type animals (Chapter 3).

Several of the AAV8-MCI treated animals were perfused with 4% paraformaldehyde prior to sacrifice. The preserved brains were frozen and coronally sliced on a freezing microtome. Sections were then stained using an antibody to detect human IDUA and then visualized by fluorecence microscopy. Of two brains analyzed from each cohort of treated MPS I animals, one brain from each group stained positive for IDUA. IDUA expression was observed in cells with neuronal morphology in and around the CA3 region of the hippocampus in an animal treated at 6 weeks of age (Figure 4.4a). Many of the reactive cells were located within the pyramidal neuronal layer medial to the lateral ventricle. More impressive IDUA staining was detected in the brain of an animal treated at 7 months of age. Dorsomedial to the lateral ventricles, neurons with the indusium griseum and white matter of the corpus callosum demonstrated IDUA expression (Figure 4.4b). Further ventral to this area, the telencephalon cells within the dorsal and medial septal nucleus displayed IDUA expression (Figure 4.4c). Furthermore, cells surrounding the ventral portion of the right-side lateral ventricle were brightly stained, including cells within the ventral portion of the septal nucleus and cells within the caudate putamen

region of the basal ganglia (Figure 4.4d). Rostral to this area, neuronal cells within the septo-hippocampal nucleus stained robustly for IDUA expression (Figure 4.4e).

Treated animals display a reduction in GAG storage material in some areas of the brain. Brain homogenates were assayed for GAG storage material using a colorimetric dye-binding assay. GAG accumulation was not reduced in the cerebellum, hippocampus, or brainstem and thalamus regions of the brain in AAV8-MCI treated animals (Figure 4.5). However, GAG levels were significantly reduced compared to untreated MPS I littermates within the olfactory bulb, cerebral cortex, and striatum regions of the brain. GAG levels were normalized in the striatum of animals treated at 6 weeks of age as there was no statistical difference in GAG levels between these animals and unaffected animals by one-way ANOVA analysis. Additionally, GAG levels in the liver were significantly reduced compared to unaffected animals, consistent with detectable *IDUA* sequence and increased longevity of treated animals.

Despite a reduction in GAG storage material in several regions of the brain, aggregates of GM3 ganglioside were still evident in the brains of treated animals. GM3 ganglioside, normally expressed only within the white matter of the brain, was visualized as punctate aggregates within the grey matter throughout the brains of untreated MPS I animals by immunofluorescence staining (Figure 4.6). Representative images reflect punctate aggregates of GM3 ganglioside within the dentate gyrus region of the hippocampus and within the septal nucleus. Brains from the same animals that stained positive for IDUA expression in Figure 4.4 were stained with an antibody to detect GM3 ganglioside.

Punctate aggregates of GM3 ganglioside remained evident in grey matter areas throughout the brain as depicted by representative images from the dentate gyrus and septal nucleus.

Discussion

We previously observed that unilateral ICV infusion of AAV8-MCI into the lateral ventricles of neonatal MPS I animals resulted in long-term, high-level expression of IDUA throughout the brain, normalization of storage material, and complete prevention of a neurocognitive deficit in the Morris water maze (Chapter 3). In the current study, we found that the same treatment in adult animals yielded results that were not nearly as impressive. The vector sequence was detected in all treated animals, although higher copy numbers were generally detected in the right hemisphere, suggesting that diffusion to the left hemisphere of the brain was not nearly as efficient in the adult brain as in the neonatal brain. Additionally, higher vector copy numbers were detected in the brains of animals treated at 7 months of age compared to animals treated at 6 weeks of age. IDUA activity was detected in the brain of one animal treated at 7 months of age and IDUA expression was visualized by immunofluorescence in half of the brains studied, despite the presence of AAV vector sequences in brain tissues of all treated animals analyzed. One potential explanation for why we only detected low levels of IDUA expression in 3 animals could be the emergence of enzyme-specific antibodies or an adaptive cytotoxic T lymphocyte response to eliminate cells expressing IDUA, as previously observed in gene transfer experiments using the MPS I mouse model (17, 18). Only a small amount of IDUA activity is needed to reduce levels of lysosomal storage material (19). Thus, it is

possible that a small amount of IDUA (undetectable in some cases) was present in tissues of the treated animals and was sufficient to reduce the level of GAG storage material.

The relatively low levels of IDUA expression and activity achieved in the treated animals in this study was enough to reduce levels of GAG storage materials in the olfactory bulb, cerebral cortex, and striatum. This reduction of storage material in specific regions of the brain was enough to partially prevent the emergence of an acquisition deficit in the Morris water test in animals treated at 6 weeks of age. However, the observed reduction in GAG storage had no neurophenotypic effect in animals treated post-symptomatically. This suggests that a small amount of IDUA enzyme may be able to provide a benefit in the prevention of neurological disease, but that a larger amount of enzyme may be required to reverse neurological manifestations after such extensive progression of neuropathology. This is consistent with the results of HSCT studies emphasizing the importance of early intervention in the treatment of severe MPS I (1).

Although we found that ICV infusion of AAV8-MCI was less effective than ICV infusion into neonatal animals, this method of treatment remains a promising strategy for potential use as an adjuvant to HSCT and ERT in the treatment of Hurler syndrome and other neuropathological MPS disorders. There are many potential strategies to improve the outcome of this approach. Although infusing 2×10^{10} vector genomes of AAV8-MCI was sufficient to achieve high levels of IDUA expression in neonates, a higher dose of vector may be needed to achieve effective vector transduction and IDUA expression in adult animals, due to the larger size of the target organ. Additionally, infusion into both

lateral ventricles may provide more widespread distribution of the vector throughout the brain, as was achieved in the animals treated as neonates. Furthermore, administration of a vasodilator such as D-mannitol has been shown to increase distribution of AAV-mediated gene transfer in neural tissue and could be applied to IVC administration of AAV-IDUA vector as well (20-23). Lastly, since expression of human IDUA in adult animals has been shown to illicit both an adaptive T lymphocyte response and an enzyme-specific antibody response, animals could be immunotolerized by administration of ERT prior to AAV treatment or immunosuppressed during and following treatment. Results of this study provide insights for the improvement of protocols involving AAV-mediated IDUA gene transfer in the adult brain following ICV infusion. They also provide further evidence to support the adoption of this method as a supplemental treatment for Hurler syndrome.

Methods

Animals and ICV Infusions

AAV6-MCI was constructed as previously described (Chapter 3) and was packaged into AAV8 virions by the University of Florida Vector Core. Vector titer was 4 x 10^{12} vector genomes/ml as determined by dot blot analysis. The *IDUA*$^{-/-}$ mouse strain was provided by Dr. E. Neufeld (24) and maintained under pathogen-free conditions in AAALAC-accredited facilities. Animal work was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota. *Idua*$^{-/-}$ pups were generated by breeding together homozygous *Idua*$^{-/-}$ animals. All mice were genotyped by PCR as previously described (25). Adult *Idua*$^{-/-}$ mice (either 6 weeks or 7 months of age) were anesthetized using a cocktail of ketamine and xylazine (100mg ketamine + 10mg xylazine per kg) and placed on a stereotactic frame. Five microliters (2 x 10^{10} vector genomes) of AAV8-MCI were infused into the right-side lateral ventricle (stereotactic coordinates AP 0.4, ML 0.8, DV 2.4 mm from bregma) using a Hamilton syringe. The animals were returned to their cages on heating pads for recovery.

Quantitative PCR

Tissue homogenates were supplemented with 300µl of cell lysis buffer (5 Prime) and with 100µg of proteinase K and incubated with gentle rocking overnight at 55°C. DNA was then isolated from the samples by phenol/chloroform extraction. Reaction mixtures of 25µl contained 0.5µg of DNA, 2X IQ^{TM} SYBR Green Supermix (Bio-Rad) and 200 nM each of forward and reverse primer as previously described (16). PCR conditions were: 95° followed by 40 cycles of 95°C for 40 sec, 58°C for 30 sec, and 72°C for 1 min.

Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence served as an internal control for genomic DNA content and was amplified in a separate reaction. Mouse genomic DNA diluted in water was used as a standard curve for GAPDH. The standard curve for IDUA consisted of serial dilutions of DNA isolated from a clone of NIH3T3 cells transduced at low multiplicity with LP1CD retroviral vector (26) and assumed to contain 1 viral integrant per cell containing *IDUA*. This DNA was diluted in wild-type C57BL/6 liver DNA to make serial dilutions. All reactions were carried out in duplicate. GAPDH primers used were forward primer: 5′-TGTCTCCTGCGACTTCAACAGC-3′ and reverse primer: 5′-TGTAGGCCATGAGGTCCACCAC-3′. IDUA primers used were forward primer: 5′-AGGAGATACATCGGTACG -3′ and reverse primer: 5′-TGTCAAAGTCGTGGTGGT -3′.

IDUA Enzyme Assay

Animals were anesthetized using ketamine/xylazine (100mg ketamine + 10mg xylazine per kg) and transcardially perfused with 70 mL PBS prior to sacrifice. Brains were harvested and microdissected on ice into 12 regions: right and left olfactory bulb, cerebellum, hippocampus, striatum, cortex, and brainstem. The samples were frozen on dry ice and then stored at -80°C. Samples were thawed and homogenized in 1 mL of PBS using a motorized pestle and permeabilized with 0.1% Triton X-100. IDUA activity was determined by fluorometric assay using 4MU-iduronide as the substrate, as previously described (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).

Glycosaminoglycan Assay

Tissue homogenates were incubated overnight with proteinase K, DNase1, and RNase as previously described and clarified by centrifugation for 3 min at 12,000 rpm using a tabletop microcentrifuge (27). GAG concentration was determined using the Blyscan Sulfated Glycosaminoglycan Assay (Accurate Chemical) according to the manufacturer's instructions.

Modified Morris Water Maze

A pool measuring 160 cm in diameter was filled 12 cm deep with water (23°C ± 2°C) containing 200 mL of non-toxic white acrylic paint. Three-dimensional, multi-colored visual cues were attached to the walls of the pool according to the cardinal directions (North, South, East, and West) and a platform was submerged 1 cm below the surface of the water in the northwest quadrant of the maze. Age-matched (± 2 weeks), 5-month old mice were released in the maze facing the southern wall of the pool. The mice were given 90 s to explore the maze and the total time spent swimming before locating the submerged platform was recorded. If a mouse did not reach the platform within 90 s, the animal was guided to the platform and given 30 s to explore it. Animals were given 4 trials each day for 10 days, with 12 minutes between each trial. The mice were retested for a duration of 9 days using the same protocol at 9 months of age with the submerged platform located in the same location. Two days following testing, a visual platform test

was performed in which the submerged platform was moved to the northeast quadrant of the maze and a visual cue was placed directly on the platform, making it visible. The mice were tested using the same protocol used for the acquisition phase, and the time for each mouse to locate the visual platform was recorded.

Immunofluorescence Staining

Animals were anesthetized using ketamine/xylazine as 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 coronally (30µm thickness) using a freezing microtome. Free-floating sections were washed in PBS and blocked with 10% normal donkey serum / 0.3% Triton X-100. Primary antibodies (see legends to Figs. 2 and 3) were added and sections were incubated overnight at 4°C, washed again with PBS, and incubated for 1 hr at room temperature with fluorescently labeled secondary antibodies diluted in PBS. Sections were then washed in PBS, incubated for 10 minutes with DAPI (Invitrogen), mounted on slides, and visualized using a Zeiss Axioplan 2 upright microscope (Zeiss). Primary antibodies were used to stain human IDUA (1:500, R&D Systems) and GM3 ganglioside (1:500, Seikagaku). Alexa-conjugated secondary antibodies and dilution factors were as follows: donkey anti-sheep IgG Alexa Fluor 555 (1:500, Invitrogen) and donkey anti-mouse IgM Alexa Fluor 488 (1:500, Invitrogen).

Statistical Analysis

Data are reported as mean ± s.d. One-way ANOVA with Tukey's post test was used to evaluate statistical differences between groups for the IDUA activity assay, GAG assay, and visual platform behavior assay. Two-way ANOVA was used to evaluate behavioral performance in the modified Morris water maze test. Differences in survival were evaluated using the Kaplan-Meier product limit method, by calculating the log rank statistic. In all cases, $P < 0.05$ was considered significant. Data analysis was performed using Prism 5.0 software (GraphPad Software).

TABLE 4.1. Quantitative PCR detects AAV-IDUA vector sequences in the brains of adult animals treated with AAV8-MCI. (a) Vector copy number detected in MPS I animals treated at 6 weeks of age. (b) Vector copy number detected in MPS I animals treated at 7 months of age. (L= left, R= right, OB = olfactory bulb, Hip = hippocampus, Str = striatum, Ctx = cerebral cortex, BS = brainstem + thalamus, Cer = cerebellum, UT= untreated MPS I animal, NA= no amplification occurred in the reaction, NC= not conducted, BD= below the reliable limit of detection of 0.01 copies/cell)

A IDUA Copy Number Per Cell in MPS I Animals Treated at Age = 6 Weeks

Tissue	Mouse1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	UT
L OB	9.8	0.40	0.016	0.022	0.048	0.021	BD
R OB	3.7	0.37	NA	BD	BD	0.018	BD
L Hip	1.2	0.029	NA	BD	BD	BD	BD
R Hip	NC	NC	NC	0.019	BD	BD	BD
L Str	3.3	0.19	BD	BD	0.015	0.018	BD
R Str	0.25	0.019	BD	BD	BD	NA	BD
L Ctx	0.15	BD	BD	BD	BD	BD	BD
R Ctx	0.022	0.021	0.025	0.11	0.054	BD	BD
L BS	0.91	0.11	0.023	0.020	0.013	BD	BD
R BS	0.27	0.13	0.056	0.12	0.014	0.031	BD
L Cer	0.40	NA	NA	BD	BD	BD	BD
R Cer	0.055	NA	NA	BD	BD	BD	BD
Liver	0.027	0.019	BD	BD	BD	BD	BD

B IDUA Copy Number Per Cell in MPS I Animals Treated at Age = 7 Months

Tissue	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	UT
L OB	NA	0.020	0.016	9.77	BD	0.050	0.66	BD
R OB	NA	0.15	BD	BD	BD	37.7	BD	BD
L Hip	BD	0.014	NA	0.034	0.17	NA	0.029	BD
R Hip	NC	NC	2.63	10.5	3.90	NC	32.8	BD
L Str	BD	BD	0.014	BD	BD	0.021	BD	BD
R Str	0.019	0.089	0.99	0.026	0.078	BD	BD	BD
L Ctx	BD	BD	BD	0.015	28.8	BD	BD	BD
R Ctx	BD	0.15	25.0	3.11	168.0	0.38	0.24	BD
L BS	BD	0.035	0.12	0.097	0.15	0.052	0.067	BD
R BS	BD	0.14	0.058	0.13	1.15	0.026	0.018	BD
L Cer	BD	0.021	BD	BD	0.093	BD	BD	BD
R Cer	BD	0.11	BD	BD	5.38	1.41	BD	BD
Liver	NA	BD	BD	BD	BD	0.075	0.34	BD

TABLE 4.2. IDUA activity detected in brain tissues of AAV8-MCI-treated animal #5. IDUA activity was detected in the brain of mouse #5, treated with AAV8-MCI at 7 months of age. IDUA activity was distinguishable from background in the hippocampus, striatum, cortex, and cerebellum. Wild-type levels of IDUA activity are between 1-2 nmol/hr/mg protein, while the background level of the assay is below 0.2 nmol/hr/mg protein. (L= left, R= right, OB = olfactory bulb, Hip = hippocampus, Str = striatum, Ctx = cerebral cortex, BS = brainstem + thalamus, Cer = cerebellum, ND = not detectable)

	L OB	R OB	L Hip	R Hip	L Str	R Str	L Ctx	R Ctx	L BS	R BS	L Cer	R Cer	Liver
IDUA Activity (nmol/hr/mg)	ND	ND	ND	0.50	ND	0.26	0.39	0.76	ND	ND	1.24	2.58	ND

FIGURE 4.1. MPS I mice infused ICV with AAV8-MVI at 6 weeks of age display partial improvement in a Morris water maze test of spatial navigation. Over the course of a 10 day testing period carried out at 5 months of age, unaffected heterozygous animals learn to locate a submerged platform using visual cues. Untreated MPS I mice display an inability to learn this task. MPS I mice treated with AAV8-MCI at 6 weeks of age demonstrate improvement in this learning task compared to untreated MPS I animals (***P < 0.0001 by 2-way ANOVA), although the treated animals also exhibit an impairment compared to unaffected heterozygous animals (***P < 0.0001 by 2-way ANOVA). Following testing, 9 of the untreated MPS I mice were infused ICV with AAV8-MCI at 7 months of age.

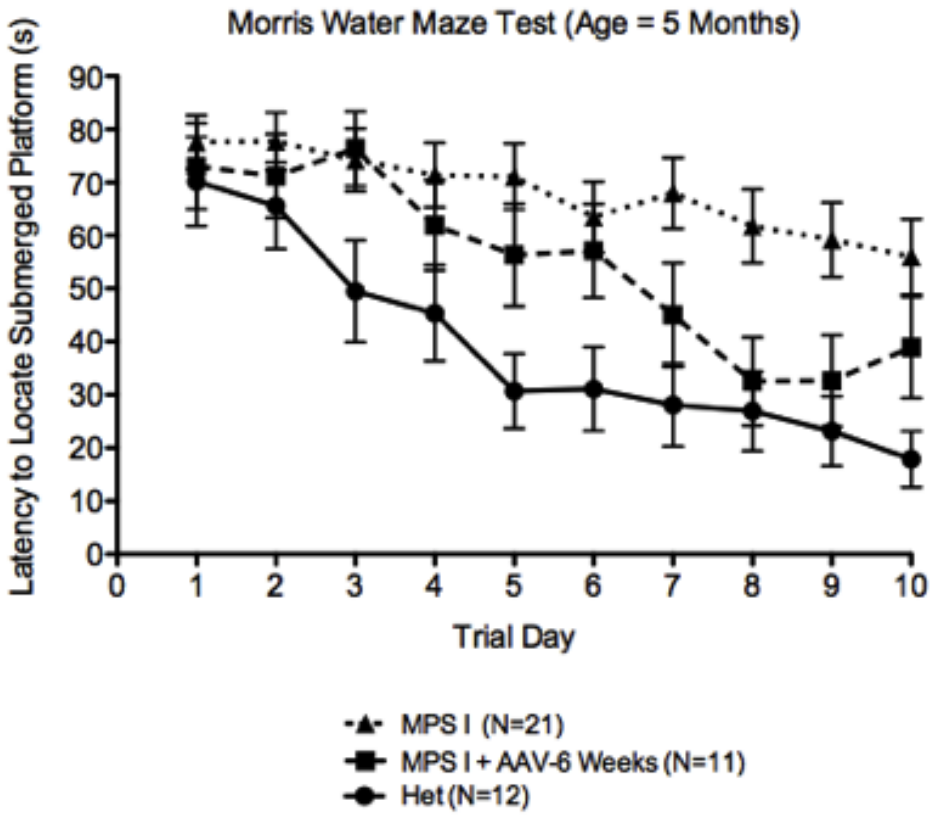


FIGURE 4.2. MPS I mice infused ICV with AAV8-MCI at 6 weeks of age continue to display partial improvement in a Morris water maze test of spatial navigation conducted at 9 months of age. Mice were retested in a Morris water maze test at 9 months of age with the goal of locating a submerged platform placed in the northwest quadrant of the maze. (a) MPS I mice treated with AAV8-MCI continue to demonstrate partial improvement in acquisition compared to untreated MPS I mice ($P < 0.01$ by 2-way ANOVA). However, mice treated at 7 months of age show no significant improvement. (b) Following the completion of acquisition testing, the submerged platform was removed and a visible platform with a visual cue was placed in the northeast quadrant of the maze. There was no statistical difference between any of the groups of animals in ability to locate the visible platform, suggesting that the neurophenotype observed in the water maze is neurocognitive in nature and not due to impaired vision or musculoskeletal deficiencies.

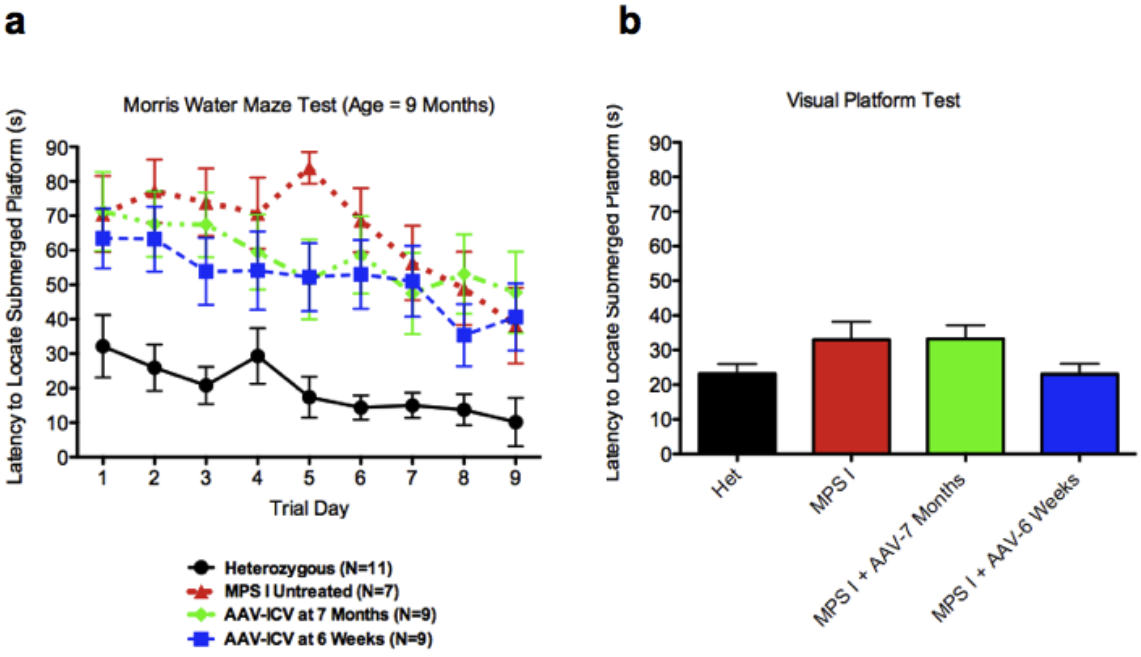


FIGURE 4.3. Adult MPS I animals infused ICV with AAV8-MCI display increased longevity compared to untreated animals. A Kaplan-Meier plot depicts an increase in lifespan of MPS I animals treated with AAV8-MCI. Both groups of treated animals survived significantly longer (*$P < 0.05$ by log-rank test) than untreated MPS I animals. Arrows depict the two time points at which the two groups of treated animals were infused ICV with AAV8-MCI (at 6 weeks and 7 months of age). The experiment was terminated at 43.5 weeks when the animals were sacrificed and tissues harvested for analysis.

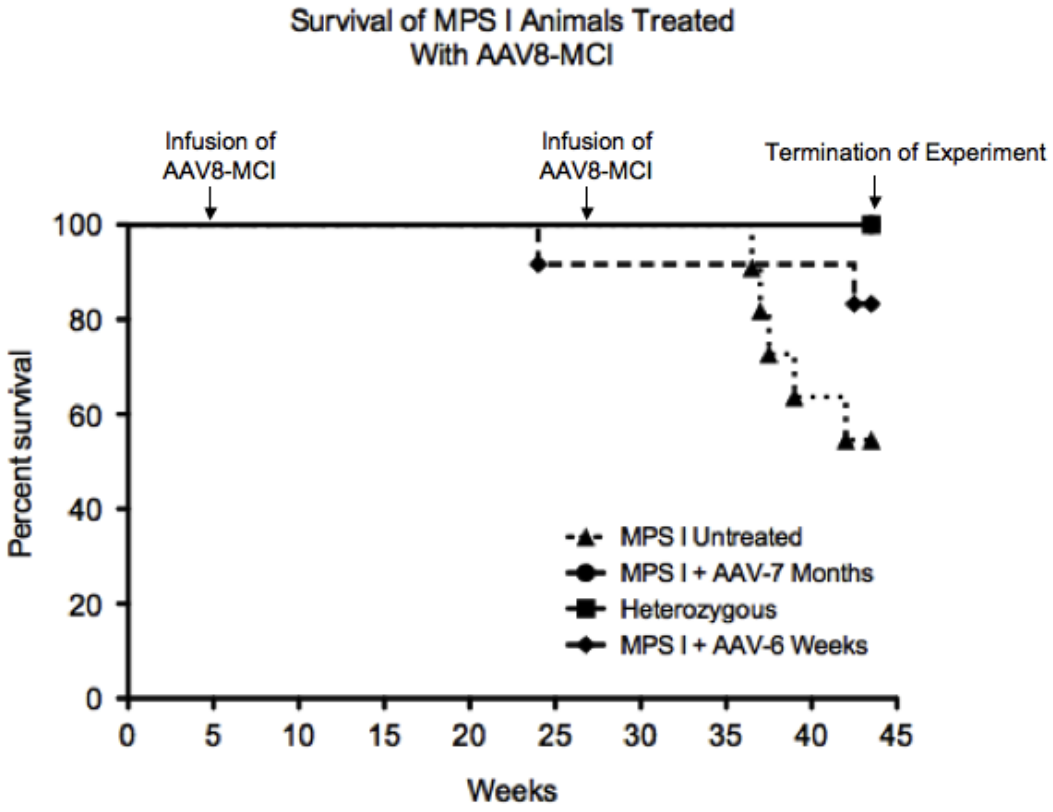


FIGURE 4.4 IDUA visualized by immunofluorescence in structures within the brain. Coronal brain slices from the displayed Bregma coordinates were stained with an anti-human IDUA antibody (red) and counterstained with DAPI to identify cellular nuclei (blue). IDUA expression can be visualized in numerous cells within the CA3 region of the hippocampus, primarily within the pyramidal neuronal cell layer, in an MPS I animal treated with AAV8-MCI at 6 weeks of age (a). In the brain of an MPS I animal treated at 7 months of age, IDUA expression is observed in cells with neuronal morphology within the indusium griseum and corpus callosum (b), septal nucleus (c), surrounding the ventral aspect of the lateral ventricle including a portion of the caudate putamen (d), and in the septohippocampal nucleus.

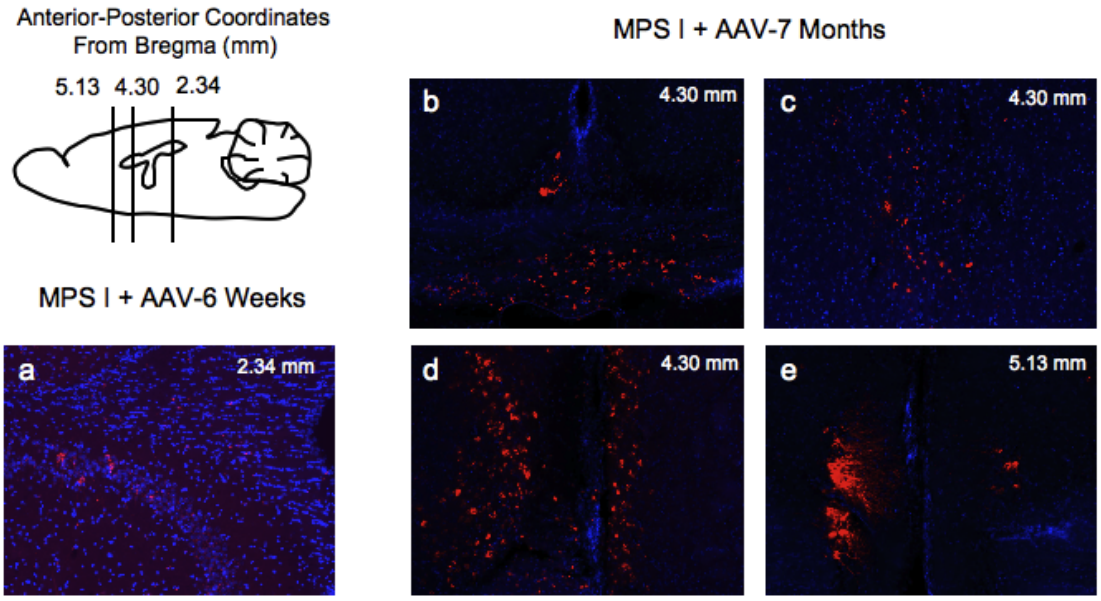


FIGURE 4.5. Levels of GAG storage material were reduced in specific regions of the brain following ICV infusion of AAV8-MCI into adult MPS I mice. The amount of GAG storage material remained unchanged in the hippocampus, brainstem, and cerebellum following ICV infusion of AAV8-MCI into adult MPS I mice. However, GAG levels were significantly reduced in the striatum, cerebral cortex, and olfactory bulb compared to untreated animals. Furthermore, GAG levels were reduced in the liver of animals treated at 7 months of age, but not in animals treated at 6 weeks of age. (horizontal bar: mean; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$) (blue dots = values from the right hemisphere, red dots = values from the left hemisphere)

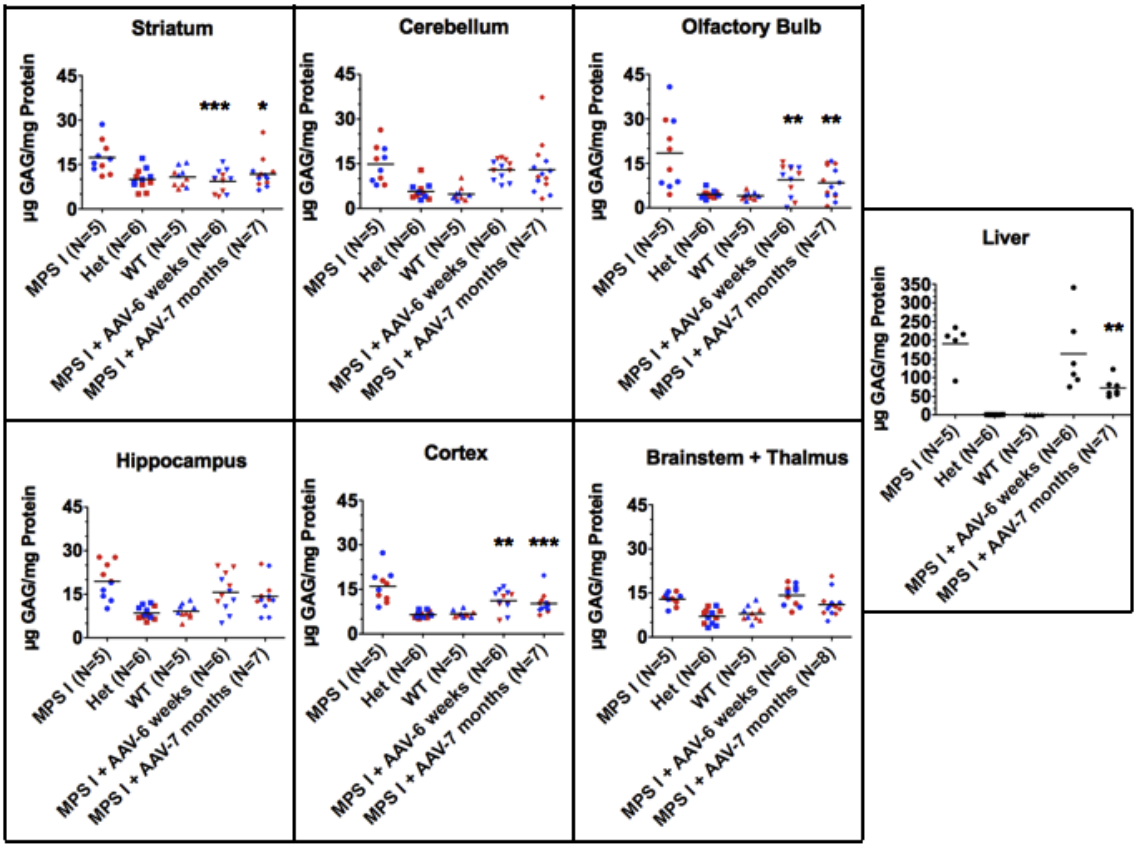
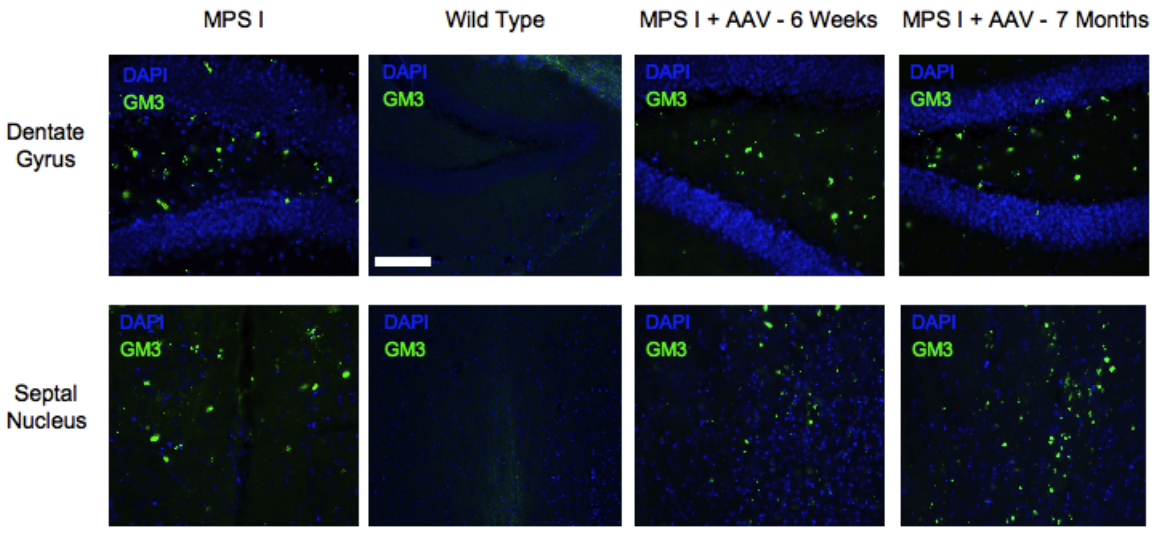


FIGURE 4.6. GM3 ganglioside remains present within the grey matter of brains from adult MPS I animals treated with AAV8-MCI. GM3 ganglioside is normally expressed within white matter of the brain and is not visualized within the grey matter of wild-type animals. However, aggregates of GM3 ganglioside are visible within the grey matter of the brains from untreated MPS I mice in representative areas including the dentate gyrus (hippocampus) and septal nucleus. The aggregates are also present in the brains of animals treated with AAV8-MCI at both 6 weeks and 7 months of age. Representative images are from the same treated animals that displayed IDUA expression in Fig. 4.1.



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Chapter 5

Conclusions

Hematopoeitic Stem Cell Transplantation as a Therapeutic Reference Point

In Chapters 3 and 4 we demonstrated that high levels of IDUA expression can be sustained in the brain following intracerebroventricular infusion of AAV vectors, resulting in clearage of storage material and improvement in neurocognitive behavior. In order to further develop this strategy and other therapeutics toward use in clinical trials for Hurler syndrome, it is first important to understand what can be achieved by current methods. In doing so, the results of experiments investigating new strategies for treatment can be directly compared to results using current therapies. The standard of care for the treatment of Hurler patients involves both hematopoeitic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). ERT, when given through intravenous infusion at standard doses, is not sufficient to provide IDUA to the central nervous system (CNS), due to inability of the lysosomal enzyme to cross the blood-brain barrier. Following HSCT, many patients demonstrate a slowing of neurocognitive decline and better overall function in comparison with untransplanted controls (1). Although transplanted patients show neurocognitive improvement, standard scores assessing neurocognitive skills decline over time and the development of functional skills including communication, socialization, and motor functions occur at a slower rate than in normal children (1, 2). Thus, there is need for the development of more effective strategies for delivering IDUA to the CNS.

To date, the only method of IDUA delivery to the CNS that has reached clinical trials other than HSCT is intrathecal infusion of laronidase (3). While the trials are currently underway and most results have yet to be published, the first report of intrathecal ERT to

a single patient concluded that the protocol was safe and GAG levels were normalized in the cerebrospinal fluid following treatment. Experiments in MPS I dogs have yielded normal levels of enzyme in the brain, spinal cord, and spinal meninges following infusions of laronidase into the cisterna magna, leading to normalization of mean GAG levels and improvement in neuropathology (4, 5). Although this approach seems promising, treatments will likely require continuous administration throughout the lifetime of the patient. The application of gene transfer for delivery of IDUA to the CNS is therefore a viable approach, due to the potential for long-term expression of IDUA that may be achieved in transduced target cells.

Since HSCT has been established as the standard of care for treatment of Hurler patients, it is likely that any gene transfer-based strategy for delivery of IDUA to the CNS would be used as a supplement to HSCT rather than an alternative form of treatment. When considering direct infusion of gene vectors to the CNS, outcome measures should be compared directly to those achieved after HSCT in order to test for biochemical reduction of storage materials and improvement in neurocognitive tasks. Thus, understanding the effects of HSCT on the CNS in animal models of MPS I is and important step to provide benchmarks for evaluating the efficacy of novel therapies.

Studies involving HSCT in animal models of Hurler syndrome have demonstrated that very low levels of IDUA activity can be detected in the brain following transplantation of wild-type donor marrow (6, 7). After treatment, GAG levels are reduced and neuropathology improved compared to unaffected animals (6, 8). In our studies whereby

Idua$^{-/-}$ mice were engrafted with high levels of wild-type donor marrow, IDUA activity was not detectable in the brain, although the levels of total brain GAG were significantly reduced compared to untreated animals. However, total brain GAGs were significantly elevated compared to heterozygous unaffected animals (Chapter 2). The observed reduction in GAG storage material is likely the result of donor-derived cells that have differentiated into microglia in the brain, providing very low levels of IDUA that were below the limit of detection of the enzymatic assay (9). This is consistent with what has been previously observed in the feline model of MPS I (8). The presence of very low, undetectable levels of IDUA activity did not, however, prevent the pathobiological accumulation of focal GM3 ganglioside aggregates within the grey matter of the brain (Chapter 2). These results provide a reference against which the results of preclinical studies testing novel therapeutic strategies for delivery of IDUA to the MPS I mouse brain (and peripheral organs) can be compared. Nevertheless, the most important outcome measure to consider for any particular therapy is the effect on neurocognition and behavior. Thus, further studies are necessary to ascertain functional neurological effects of HSCT in the MPS I mouse and in larger animal models of MPS I.

Prospects for Intraventricular Delivery of AAV

Numerous preclinical studies have been conducted to evaluate the efficacy of direct administration of gene therapy vectors to the CNS by various routes of administration (Chapter 1). We have supplemented this body of literature by exploring intracerebroventricular (ICV) infusion of an adeno-associated viral (AAV) vector as a strategy for delivery of IDUA to the CNS. Following unilateral ICV delivery of an AAV

seroytpe 8 vector expressing IDUA under the transcriptional regulation of a strong miniCAGGs promoter (AAV8-MCI) to neonatal MPS I mice, we observed widespread, long-term expression of IDUA throughout the brain as well as normalization of GAG and GM3 ganglioside storage material (Chapter 3). This resulted in complete prevention of a neurocognitive deficit, as assessed in a modified Morris water maze task of spatial navigation and memory. Neonatal AAV8-MCI infusion thus far surpassed the level of biochemical correction achieved by BMT, providing preclinical support for the adoption of ICV AAV administration as a supplement to HSCT for improved delivery of IDUA to the CNS of Hurler patients.

After the success achieved following ICV infusion of AAV8-MCI into neonatal animals, results from a parallel experiment in adult animals were less dramatic (Chapter 4). IDUA expression was detected in only 3 treated animals, although AAV-MCI vector could be detected in the brains of all animals analyzed. GAG storage material was reduced in specific regions of the brain but remained significantly elevated compared to wild-type animals. Also, there did not appear to be a reduction in punctate GM3 ganglioside aggregates in the grey matter of these animals. *Idua*$^{-/-}$ mice treated at 6 weeks of age displayed significant improvement in the Morris water maze acquisition task, but still displayed a significant deficit compared to unaffected animals. Thus, the overall results of this study did not indicate efficacious delivery of IDUA to the CNS in comparison to BMT (Chapter 2). Potential explanations for the low levels of IDUA expression achieved in the CNS after ICV infusion of AAV8-MCI in adult MPS I animals when compared to neonatal animals include:

1) The vector dose, although sufficient to provide robust IDUA expression in the neonatal mouse brain, was too low to achieve widespread detectable expression of IDUA in the much larger adult brain.
2) Unilateral ICV infusion provides widespread distribution of vector throughout the brain by CSF-mediated access to brain structures in close proximity to the ventricular system in neonatal mice. However, the same may not be true for adult mice, due to their larger brain size and which creates greater diffusional constraint since solute diffusion decreases with the square of the diffusion distance within brain tissue (10). Bilateral infusions may provide more widespread vector distribution in adult animals.
3) Immune responses may have limited IDUA expression through generation of enzyme-specific antibodies and/or a cytotoxic T-cell response, as has been reported for similar experiments in adult MPS I animals (11, 12)
4) AAV8 may have a more favorable tropism for neurons of the neonatal brain than in the adult brain. It is possible that the cellular receptor for AAV8 is more highly expressed in neurons of the neonatal brain than in the adult brain. Studies could be conducted to explore this possibility by analyzing the expression patterns of the proposed AAV8 receptor, the 37/67-kDa laminin receptor, in both the neonatal and adult mouse brain (13).

Before ICV infusion of AAV vectors could be applied as a supplement to BMT and ERT in the treatment of Hurler syndrome in the form of a clinical trial, these issues must be addressed and more promising results must be obtained in large animal models.

Many routes for administering gene therapy vectors directly to the CNS have been explored in preclinical studies, including stereotactic injection into the striatum, cerebral cortex, cerebellum, and cisterns as well as intrathecal infusion into the cisterna magna (Chapter 1). All of these approaches involve risky procedures and/or extensive surgical interventions. An advantage of the ICV route of administration is that there already exists a Hurler patient population in which ICV infusion could potentially be carried out in a manner that minimizes the invasiveness of the procedure. Many Hurler patients develop severe hydrocephalus leading to ventriculomegaly. These patients routinely undergo placement of ventricular shunts that access the lateral ventricles to relieve pressure. The potential exists, therefore, for development of a specialized shunt engineered to contain a port for infusion of a gene therapy vector into the ventricles without the need to carry out additional surgical procedures.

Noninvasive Approaches

As noninvasive approaches for the delivery of IDUA to the CNS are desirable to minimize potential adverse events and costs, we propose here a novel method for noninvasive delivery of IDUA to the CNS by the intranasal route of administration. Proteins such as insulin-like growth factor and erythropoietin, peptides such as hypocretin-1, and neurotrophins such as neutrophil activating protein and activity-dependent neurotrophic factor have been successfully delivered to the CNS by intranasal delivery (14-18). Additionally, viral gene transfer vectors including adenovirus, semliki forest virus, and herpes simplex virus have been shown to reach and transduce cells in the CNS following intranasal administration in rodents (19-22). It is presumed that these

agents reach the brain via the nasal cavity by a combination of pathways involving the olfactory nerves, vasculature, CSF, and lymphatic systems (23). Extracellular rather than intracellular transport is likely, due to the high concentrations of drug that reach the CNS soon after delivery.

We have tested the intranasal route of administration to demonstrate that IDUA activity can be detected throughout the brain within 20 minutes of administering laronidase to the nasal cavity of MPS I mice (Figure 5.1a and b). This delivery method could be used to deliver IDUA (and other lysosomal enzymes) to the CNS as an alternative to intrathecal ERT . In addition to providing a noninvasive method of delivery, another advantage of intranasal administration, in comparison to intrathecal ERT, is the potential to easily administer an unlimited number of consecutive doses. Future studies should be conducted to validate the long-term efficacy of this method and the impact on storage material and neurocognition. Additionally, experiments should be conducted to determine the frequency of administration necessary to achieve biochemical and neurobehavioral correction.

In addition to intranasal delivery of laronidase to the CNS, we have also demonstrated that intranasal administration of AAV8-MCI to *Idua*$^{-/-}$ mice results in detectable IDUA activity in the olfactory bulb (~5-fold higher than wild-type levels) (Figure 5.2). However, IDUA was undetectable in other regions of the brain. To gain more widespread access to the brain, experiments should be conducted to explore the use of agents such as D-mannitol to open the vasculature prior to AAV administration, promoting deeper

penetration of the vector into the CNS. If successful, this approach could be used for widespread AAV delivery to the brain with expression of IDUA as well as other transgenes for treatment of a multitude of other neurological disorders.

Closing Remark

Overall, the results presented in this thesis provide preclinical data supporting the design of a clinical trial to test ICV infusion of AAV vector in Hurler patients in order to achieve high-level IDUA expression in the CNS. Such a strategy would be used as a supplement to HSCT and ERT to create a more comprehensive treatment regimen for MPS I. This strategy could further be applied for the treatment of other lysosomal storage diseases and neurological disorders in which widespread distribution of a gene product is necessary.

Figure 5.1. IDUA activity is detectable in the brain following intranasal administration of laronidase. A series of eight 3μL drops of laronidase that had been concentrated by centrifugation in an Amicon Centriplus YM-10 column to a final concentration of ~1.5mg/ml was administered to the nasal cavity of *Idua*$^{-/-}$ mice. Twenty minutes after the first drop was applied, animals were perfused with saline and sacrificed. IDUA activity assays were performed on homogenized, microdissected brain samples. IDUA activity was detected in all portions of the brain, including the olfactory bulb, cerebellum, hippocampus, striatum, cortex, and brainstem and thalamus (a). Relatively high levels of IDUA activity were also detected in the olfactory epithelium and trigeminal ganglion (b). Pathways supporting the flow of materials through the trigeminal nerves and nasal vasculature have been suggested as routes of transport from the nasal cavity to the brain.

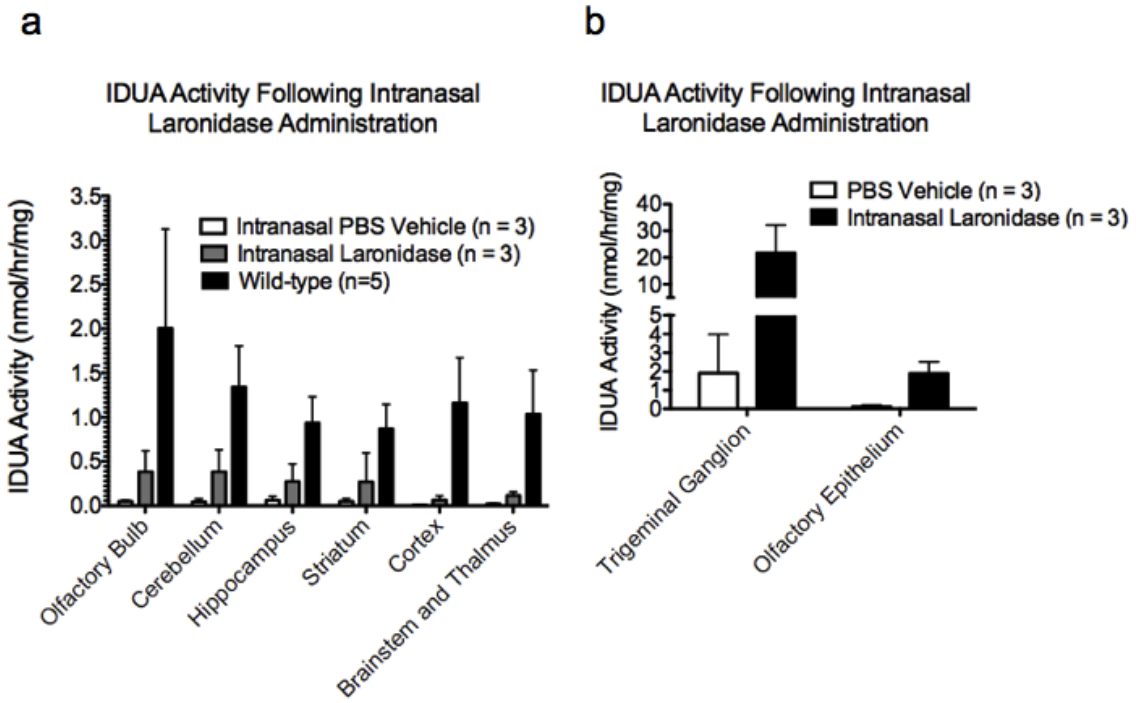
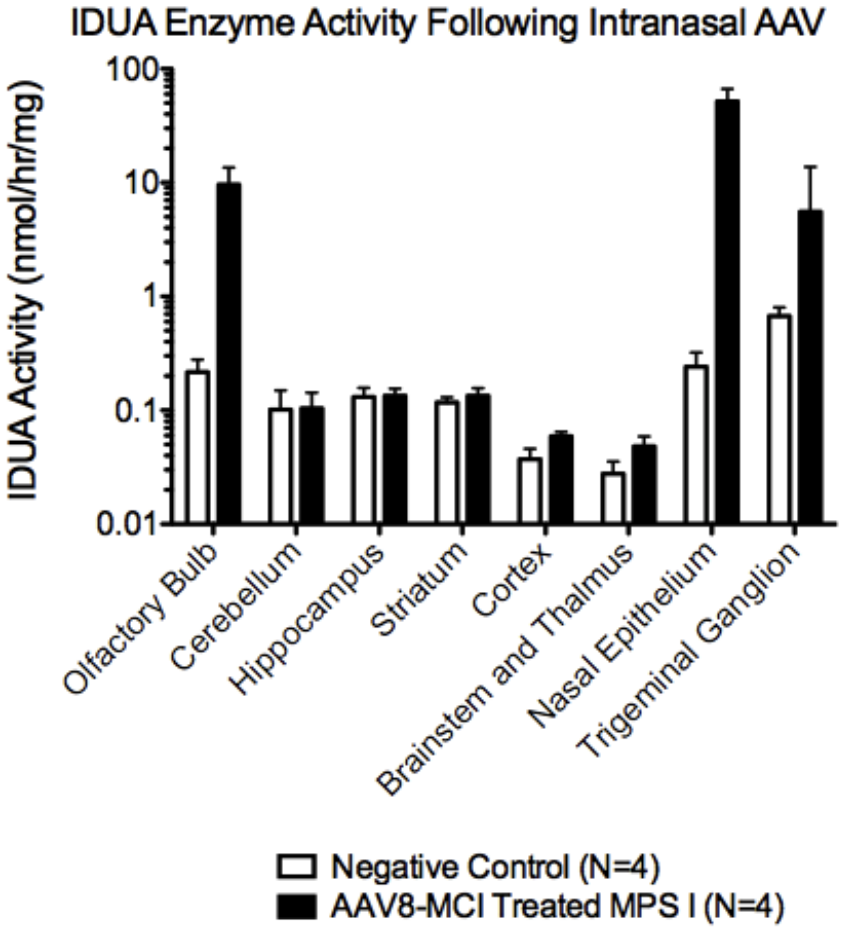


Figure 5.2 IDUA activity is detectable in the olfactory bulb following intranasal delivery of AAV8-MCI. 1.4 x 10^{11} vector genomes of AAV8-MCI were administered to the nasal cavity of NOD/SCID *Idua*$^{-/-}$ mice as described above for laronidase (see legend to Fig. 5.1). Three weeks post-treatment, animals were perfused with saline and sacrificed. IDUA activity assays were performed on homogenized, microdissected brain samples. IDUA activity was detected in the olfactory bulb of the brain with levels reaching ~5-fold higher than wild-type activity. High levels of IDUA activity were also detected in the nasal epithelium and trigeminal ganglion.



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Chapter 6

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Chapter 1

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Chapter 2

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