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Neonatal gene transfer leads to widespread correction of pathology in a murine model of lysosomal storage disease

(adeno-associated virus/mucopolysaccharidosis type VII/animal models/metabolic disease/gene therapy)

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ABSTRACT **For many inborn errors of metabolism, early treatment is critical to prevent long-term developmental sequelae. We have used a gene-therapy approach to demonstrate this concept in a murine model of mucopolysaccharidosis type VII (MPS VII). Newborn MPS VII mice received a single intravenous injection with 5.4×10^6 infectious units of recombinant adeno-associated virus encoding the human β-glucuronidase (GUSB) cDNA. Therapeutic levels of GUSB expression were achieved by 1 week of age in liver, heart, lung, spleen, kidney, brain, and retina. GUSB expression persisted in most organs for the 16-week duration of the study at levels sufficient to either reduce or prevent completely lysosomal storage. Of particular significance, neurons, microglia, and meninges of the central nervous system were virtually cleared of disease. In addition, neonatal treatment of MPS VII mice provided access to the central nervous system via an intravenous route, avoiding a more invasive procedure later in life. These data suggest that gene transfer mediated by adeno-associated virus can achieve therapeutically relevant levels of enzyme very early in life and that the rapid growth and differentiation of tissues does not limit long-term expression.**

Mucopolysaccharidosis type VII (MPS VII) is a lysosomal storage disease caused by the lack of β-glucuronidase (GUSB) activity (1). This defect results in a progressive accumulation of undegraded glycosaminoglycans (GAGs) in lysosomes, leading to lysosomal distention in multiple tissues. The clinical features of MPS VII include skeletal deformities, shortened life span, hearing and vision defects, and mental retardation. Reconstitution of GUSB activity can reverse lysosomal disease both *in vitro* and *in vivo*. This enzyme may be supplied from either an intracellular source (as seen after transduction of affected cells) or taken up from extracellular sources via the mannose-6-phosphate receptor (cross-correction; ref. 2). The murine MPS VII model closely resembles the human disease and has been used to evaluate a number of potential therapies for this condition. Reversal of established lysosomal disease in many tissues can be accomplished in adult animals by either enzyme replacement therapy (3) or bone marrow transplantation (4). It is clear from enzyme replacement therapy and bone marrow transplantation studies that neonatal treatment provides a more complete correction of functional defects, such as skeletal dysplasia (5) and deficiencies in hearing and behavior (6). Unfortunately, the transient nature of enzyme replacement therapy and the lack of suitable donors for bone marrow transplantation limits the clinical application of these therapies. An ideal treatment would be one that can be administered early in life, has low morbidity, and leads to long-term reconstitution of GUSB activity.

Adeno-associated virus (AAV) is a human parvovirus that is being developed as a gene therapy vector (7). Persistent *in vivo* expression from AAV vectors has been shown in many tissues, including retina, muscle, liver, and brain (8–14). This ability of AAV to transduce multiple organs, coupled with the nonpathogenic nature of the wild-type virus, makes AAV a potentially useful vector for the neonatal treatment of lysosomal storage diseases such as MPS VII. Previously, we have shown that, although neonatal intramuscular injection of AAV results in high levels of GUSB expression at the site of injection, the level and kinetics of secretion were insufficient to prevent the accumulation of lysosomal storage in distant tissues (15). However, results from that study suggested that an intravenous route of administration in neonates might provide more widespread delivery of therapeutic amounts of GUSB.

We show here that intravenous AAV-mediated gene therapy in neonatal MPS VII mice results in high-level GUSB expression in multiple tissues at a developmental stage where there is minimal evidence of disease. GUSB activity persisted for the duration of the study and was sufficient to reduce lysosomal distention dramatically in many cell types, including Kupffer cells of the liver, cardiac stromal fibroblasts, retinal pigment epithelial cells, meninges, and central nervous system (CNS) neurons. These data suggest that neonatal AAV-mediated gene transfer may represent an effective and noninvasive method of achieving widespread persistent therapeutic expression of a relevant enzyme for the treatment of lysosomal storage disease.

METHODS

Construction of AAVβGEnh Expression Cassette and Recombinant Virus. The AAV vector AAVβGEnh has been described (15) and consists of the cytomegalovirus enhancer, chicken β-actin promoter, human GUSB cDNA, and rabbit β-globin and simian virus 40 polyadenylation signals. The initial intron of the chicken β-actin gene is included to increase protein expression. Viral stocks were prepared from homogenates of 293 cells after cotransfection of AAVβGEnh and the helper plasmid pIM45 (16) and superinfection with adenovirus type 5 at a multiplicity of infection of 2. Purified virus was isolated from cell lysates over two continuous isopycnic cesium gradients. The final product was concentrated and incubated at 56°C for 45 min to inactivate residual adenovirus. Infectious units were determined by an expression-based assay on a GUSB-deficient cell line. Replication-competent adenovirus

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Abbreviations: AAV, adeno-associated virus; CNS, central nervous system; GAG, glycosaminoglycan; GUSB, β-glucuronidase; MPS VII, mucopolysaccharidosis type VII.

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was absent as determined by cytopathic effect assay on 293 cells. The ratio of wild-type AAV to recombinant AAV was 0.18 as determined by infectious center analysis.

Neonatal Injections. Mutant mice were obtained from heterozygous matings of B6.C-*H-2*^{bm1}/ByBir-*gus*^{mps}/+ mice maintained by M.S.S. at Washington University. Identification of newborn mutants was accomplished by quantitative analysis of GUSB activity in toe clips on the day of birth. Each mouse received a single intravenous injection of 100 μl of viral suspension containing 5.4×10^6 infectious units of recombinant AAV via the superficial temporal vein (5) on day 2 of life, providing a dose of approximately 5×10^9 infectious units per kg.

Quantitative Analysis of Lysosomal Enzyme Activities and Glycosaminoglycan Levels. GUSB activities were measured on tissue homogenates by using fluorometric assays as described (3). For serum samples, reactions were incubated for 48 h at 37°C to increase the sensitivity of the assay. GUSB specific activity (1 unit = 1 nmol of 4-methylumbelliferyl β-D-glucuronide liberated per hour per milligram of total protein) was calculated and compared with GUSB specific activities from age-matched +/+ mice of the same strain and presented as a percentage of normal activity. Glycosaminoglycan levels were measured by the alcian blue method as described (17). Final GAG levels are expressed as micrograms of GAG per milligram of protein.

Histochemical and Histopathological Analyses. Histochemical analysis of GUSB activity was performed on 10-μm thick frozen sections as described by using naphthol-AS-BI β-D-glucuronide (ASBI) as the substrate (3). Sections were counterstained with 1% methyl green. Additional tissue samples were collected, fixed, and prepared for light and electron microscopy as described (4). Tissue samples were immersed in ice-cold 2% glutaraldehyde and 4% paraformaldehyde in PBS and then embedded in Spurr's resin. Sections of tissue 0.5 μm thick were stained with toluidine blue and evaluated for lysosomal storage.

PCR and Southern Analysis of Tissues. The locations of the primers and probes used in this study have been described (15). Primers specific for sequences in exon 6 (5′-CTGTGGCTGTCACCAAGAGC-3′) and exon 7 (5′-GGACACTCATCGATGACCAC-3′) of the human GUSB cDNA were used. These sequences are identical to the human cDNA (18) but have two mismatches within the murine exon 6 sequence (19). Expected products are a 240-bp fragment from the human cDNA and a 454-bp fragment from the endogenous murine gene. Blots were probed with a 527-bp *Cla*I fragment from the human GUSB cDNA. Tissue samples were resuspended in 50 μl of buffer containing 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Triton X-100, 0.45% Tween 20, and 200 μg/ml Proteinase K and incubated at 56°C for 16 h. For each PCR reaction, 10 μl of this reaction was used.

RESULTS

Quantitative GUSB Expression After Neonatal Injection. Newborn MPS VII mice were injected intravenously on day 2 of life with 5.4×10^6 infectious units of the recombinant vector AAVβGEnh. This vector has been described (15) and consists of the human GUSB cDNA driven by the chicken β-actin promoter and cytomegalovirus enhancer element. Significant levels of GUSB activity were detected in multiple organs at time points throughout the first 16 weeks of life (Fig. 1 *A* and *B*). In heart, lung, brain, and kidney, GUSB expression steadily increased for the first 4 weeks of the study, stabilizing at levels ranging from 1% to 2,000% of normal (Fig. 1*A*). These levels have been shown to be sufficient to reduce lysosomal storage in most tissues (3, 20). In contrast, GUSB activity in the liver initially approached normal levels but then decreased sharply over the course of the study, stabilizing at a final level of 1% of normal by week 16 (Fig. 1*B*). Relatively high levels of GUSB activity were observed in the spleen and serum between 1 and 4 weeks of age but then decreased dramatically by week 16 (Fig. 1*B*). Interestingly, the decrease in splenic and serum GUSB levels paralleled the hepatic activity.

Distribution of Expression and Therapeutic Effect After Neonatal AAV Injection. Histochemical and histopathological analyses of organs from treated mice showed widespread therapeutic response to neonatal AAV treatment. An example of this response can be seen in the heart, where GUSB activity was distributed throughout the cardiac muscle at week 16 (Fig. 2*A*). Histopathological lesions in cardiac-valve fibroblasts were decreased in AAV-treated MPS VII mice at week 16 (Fig. 2 *B* and *C*). Persistent GUSB expression was also noted in multiple areas of the CNS in AAV-treated mice. A limited survey at 2 weeks of age showed GUSB staining in the central gray matter,

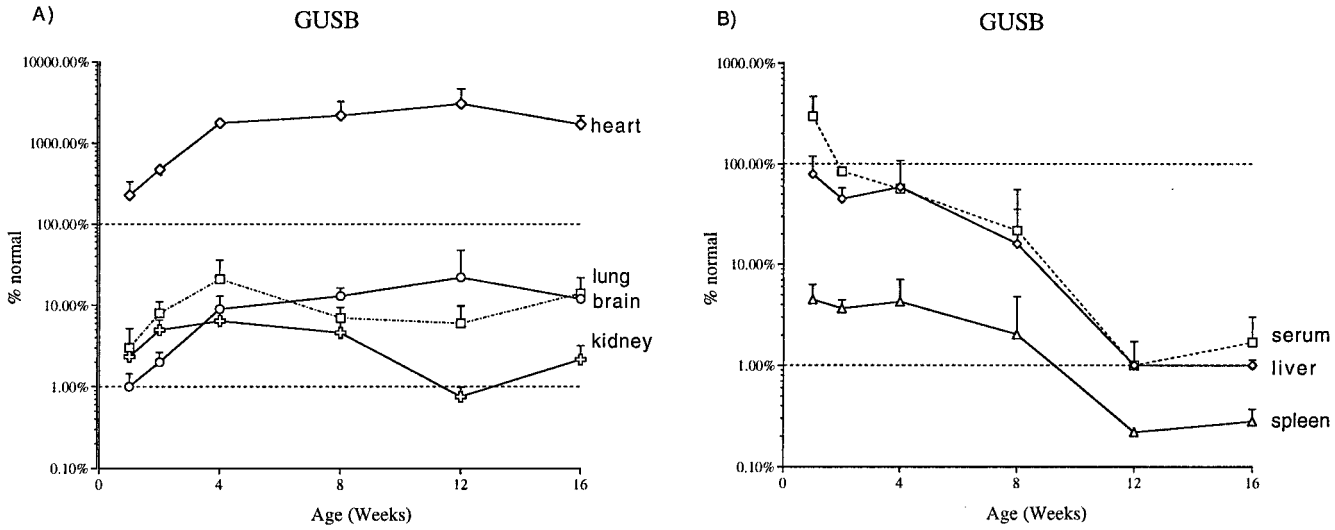

FIG. 1. Mice were killed at time points ranging from 1 to 16 weeks of age, and organs were assayed for GUSB activity with a fluorometric quantitative assay. Each point represents the average and standard deviation from three AAV-treated mice. Results are expressed as a percentage of normal GUSB activity found in the corresponding organs from three age-matched +/+ B6 mice. Dotted lines at 1% and 100% represent the "therapeutic range" of GUSB activity, because previous work with enzyme replacement therapy and bone marrow transplantation has shown that levels of approximately 1% normal are sufficient to reverse storage disease in some organs.

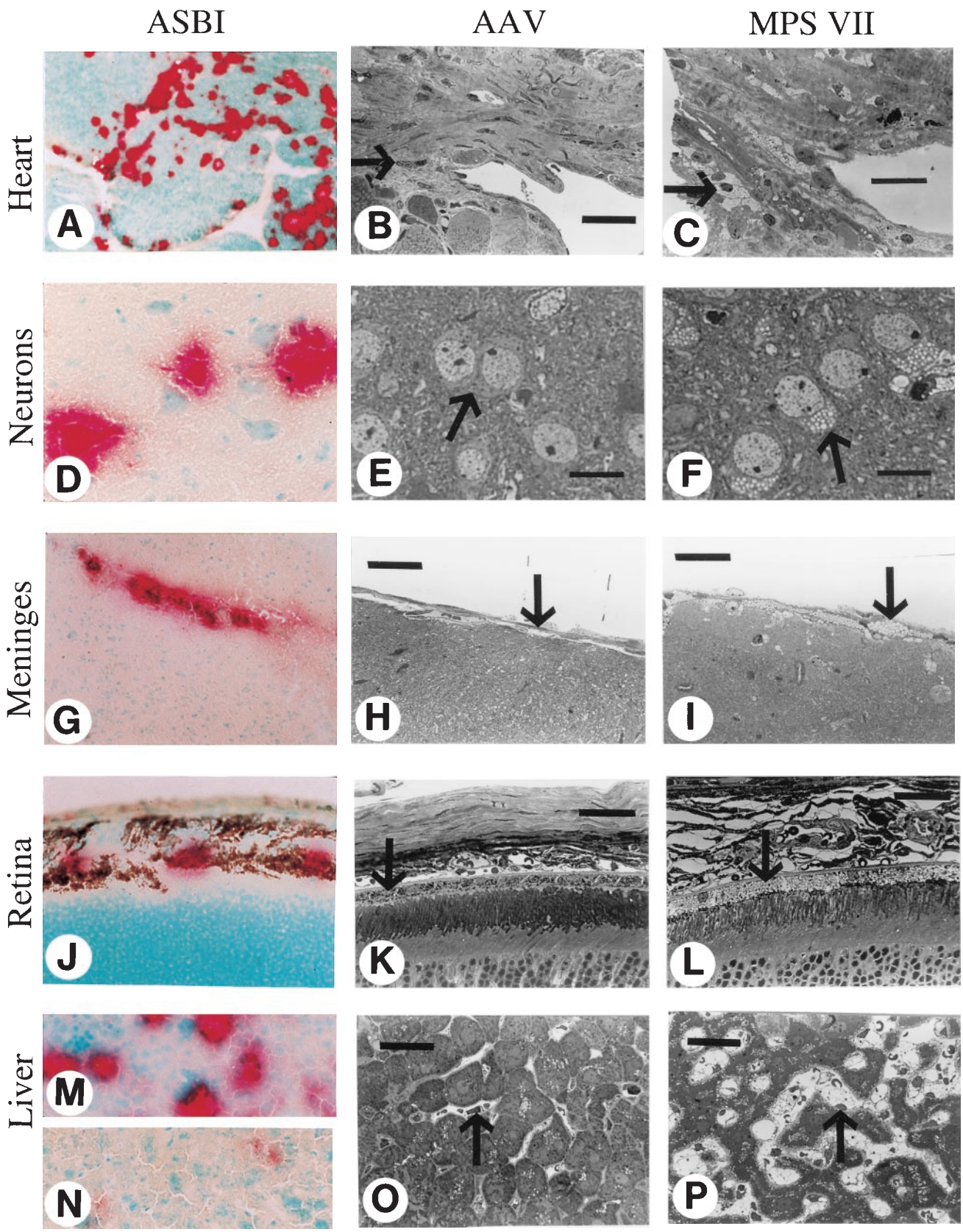

FIG. 2. Tissue sections were examined for GUSB activity and evidence of therapeutic response to AAV treatment. (*Left*) The column labeled ASBI (naphthol-AS-BI β-D-glucuronide, see *Methods*) shows histochemical staining for GUSB activity. Cells that stain red contain GUSB. (*Center* and *Right*) The other columns show histopathological analysis of 16-week-old treated (AAV) and untreated (MPS VII) mutant mice. Widespread GUSB activity is present in cardiac muscle at week 16 (*A*), and a corresponding reduction of disease in cardiac-valve stromal fibroblasts is seen (*B*, arrow) when compared with untreated mutants (*C*, arrow). GUSB activity is also present in sections of polygonal neurons of the basis pons (*D*), meninges (*G*), and retinal pigmented epithelium (*J*) from 16-week-old AAV-treated mice, leading to the nearly complete elimination of lysosomal storage in these organs when compared with the corresponding untreated controls (*Center and Right*). In the liver,

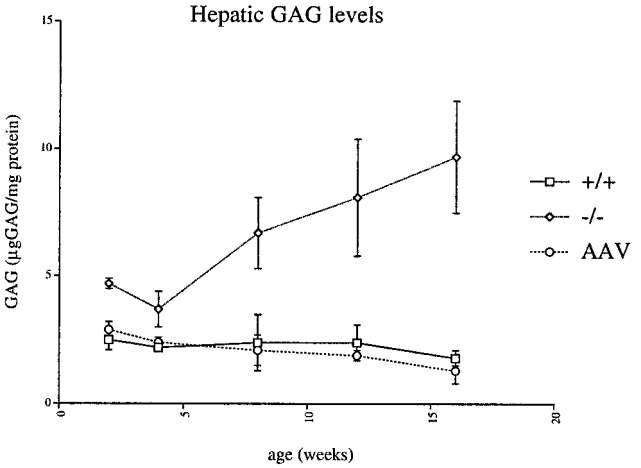

FIG. 3. The total glycosaminoglycan content of hepatic tissue from the normal, mutant, and AAV-treated mice shown in Fig. 2 *M–P* was compared. Each point represents the average GAG content from three mice. Vertical error bars represent the standard deviation.

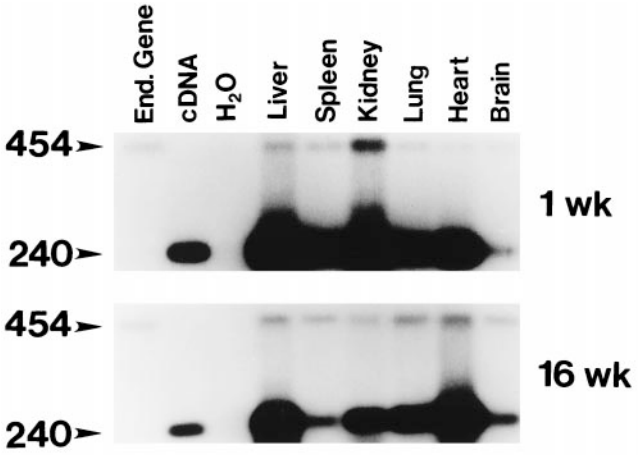

FIG. 4. Tissues were analyzed for the presence of viral cDNA with primers specific for exons 6 and 7 of human GUSB. These primers amplify a 240-bp band from the viral cDNA and a 454-bp band from the endogenous murine GUSB gene. Mismatches between the murine sequence and the human PCR primers, the interruption of the murine coding sequences by an intron, and the use of a human-specific probe cause the human cDNA to be detected more efficiently than the endogenous murine band. At 1 week of age, relatively high levels of viral cDNA are present in all tissues when normalized to the endogenous murine band. At 16 weeks of age, the amount of viral cDNA varies more widely between tissues.

meninges, cortex, and vessels of AAV-treated mice. A more detailed survey at week 16 showed continued expression in central gray matter, meninges (Fig. 2*G*), cortex, and vessels. In addition, GUSB-positive areas were observed in the choroid plexus, pyramidal cell layer of the hippocampus, and molecular layer of the cerebellum. Although specific cell types could not always be determined, in areas such as the polygonal neurons of the basis pons, clear GUSB staining of morphologically distinct neuronal cell bodies was evident (Fig. 2*D*). This observation is in agreement with a recent study that showed preferential infection of neurons in the CNS of rodents by AAV vectors (21). Histopathological analysis showed widespread reduction of lysosomal storage in parenchymal neurons of the cortex (Fig. 2 *E* and *F*) as well as in meninges (Fig. 2 *H* and *I*). Histochemical analysis also showed persistent GUSB activity in the retina, with elimination of the characteristic lysosomal storage in retinal pigment epithelial cells by 16 weeks of age (Fig. 2 *J–L*). Lysosomal storage in the cornea of treated MPS VII mice, however, was reduced only slightly (data not shown).

In the liver, the decline in GUSB activity from 1 to 16 weeks of age seems to be caused by a sharp decrease in the percentage of GUSB-expressing cells (Fig. 2 *M* and *N*). However, the remaining activity at week 16 was still sufficient to reduce lysosomal distention in Kupffer cells of AAV-treated mice when compared with untreated controls (Fig. 2 *O* and *P*). Quantitation of total hepatic GAG content confirmed that the livers of AAV-treated mice had no meaningful accumulation of GAGs over the course of the study, unlike untreated MPS VII mice (Fig. 3). Although the level of GUSB activity in the spleen was less than 1% of normal levels at week 16, a significant reduction of lysosomal storage in the sinus-lining cells was observed (data not shown). There was minimal to no effect on disease in the skeletal system, as evidenced by the development of the characteristic MPS VII phenotype (shortened limbs and facial dysmorphism) in the AAV-treated mice (data not shown).

Presence of Viral cDNA in AAV-Treated Mice. PCR analysis of treated mice showed the presence of viral cDNA in most tissues at 1 week of age (Fig. 4). At week 16, strong viral cDNA signals persisted in heart, lung, brain, kidney, and liver. Levels of viral cDNA in the spleen seemed to decrease somewhat by week 16 of age when normalized to the internal control of the endogenous murine GUSB gene.

DISCUSSION

Our study shows that neonatal animals are suitable targets for intravenous AAV-mediated gene transfer and that the rapid growth and differentiation of tissues after birth does not limit expression from these vectors in the first 16 weeks of life. Several advantages of neonatal treatment are shown in this study. First, therapeutically relevant levels of enzyme can be achieved early in life, which may prevent the development of disease as opposed to reversing established disease. Second, neonatal administration of AAV provides access to the CNS by an intravenous route. This access is especially noteworthy, as it has been difficult to achieve persistent therapeutic levels of GUSB in the CNS by noninvasive means. Although AAV has been shown to infect neurons and retinal cells efficiently after localized injection, widespread infection of these organs was not seen after intravenous injections in adult animals. One possible cause for this improved transduction of the CNS in neonatal mice after intravenous injection could be because of the incompletely formed blood–brain barrier, which is not fully intact until 10–14 days of life in rodents (22). Alternatively, the relatively small size of neonatal mice results in a larger dose of virus per kilogram than can be achieved easily in adult animals. This virus load may reach a threshold for CNS infection that was not achieved in previous studies that used adult mice.

For heart, lung, kidney, and brain, persistent viral cDNA PCR signals correlated with sustained expression of GUSB in these tissues. In contrast, although viral cDNA also persisted in the liver from week 1 to week 16, hepatic GUSB activity sharply decreased during this time. This result suggests that the decline in GUSB expression in the liver occurs at a transcriptional or translational level. This decline may be caused by transcriptional silencing of the cytomegalovirus enhancer el-

10–20% of hepatocytes showed evidence of GUSB activity at week 1 (*M*). Although a much smaller percentage of cells were positive at week 16 (*N*), the residual activity is sufficient to reduce lysosomal distention greatly in treated animals at 16 weeks of age when compared with age-matched untreated controls (*O* and *P*, arrows). (For *A–C* and *G–P*, bars = 34 μm; for *D–F*, bars = 14.5 μm.)

ement (23). In the spleen, however, both viral cDNA and GUSB expression have decreased by week 16, suggesting that this organ is not stably transduced. This distribution of viral cDNA after neonatal injection suggests that the tropism of AAV in neonatal mice is similar to that described in adult mice (24, 25). In addition, the persistence of viral cDNA in multiple organs provides evidence that the primary source of GUSB activity in heart, lung, brain, and kidney is direct infection by AAV and not cross-correction by secreted enzyme. This hypothesis is supported further by the fact that GUSB activity in these organs remains relatively stable, whereas circulating GUSB levels decrease dramatically from 1 to 16 weeks of age.

Although direct infection of tissues by AAV seems to be the primary source of GUSB in most organs studied, some element of cross-correction by circulating enzyme undoubtedly plays a role, especially at early time points when high serum levels are present. Evidence for cross-correction also can be seen in the neurons of the brain. Although only a small percentage of parenchymal cells stained positive for GUSB, lysosomal distention was reduced throughout the cortex. It is unclear whether the corrective enzyme is being supplied from meningeal secretion into the cerebrospinal fluid, axonal transport from neighboring transduced cells, or GUSB circulating early in life before closure of the blood–brain barrier. However, the combination of high circulating levels of GUSB early in life and lower persistent levels that are maintained as the animals age results in the reduction of lysosomal storage in numerous tissues. It is interesting to note that the GUSB activity in the serum and spleen parallels the activity in the liver, suggesting that the liver is the primary source of secreted GUSB in AAV-treated animals. This result has important therapeutic implications. Unlike the muscle, which does not secrete large amounts of GUSB (15), the liver apparently has the capacity to secrete lysosomal enzymes more efficiently. The development of AAV vectors that constitutively express at high levels in the liver may result in more complete correction of disease in tissues that responded only partially in this study, such as the cornea, spleen, and skeletal system. It also will be crucial to determine the short-term and long-term effects of AAV-mediated gene transfer on mental, auditory, and visual functions, as well as on longevity. These kinds of neonatal therapies will be important for the treatment of many childhood genetic diseases, where early treatment is required to prevent long-term developmental damage.

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RESEARCH ARTICLE

Prevention of systemic clinical disease in MPS VII mice following AAV-mediated neonatal gene transfer

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For many inborn errors of metabolism, early treatment is critical to prevent long-term developmental sequelae. We have previously shown that systemic treatment of neonatal mucopolysaccharidosis type VII (MPS VII) mice with recombinant adeno-associated virus (AAV) vectors results in relatively long-term expression of β-glucuronidase (GUSB) in multiple tissues, and a reduction in lysosomal storage. Here, we demonstrate that therapeutic levels of enzyme persist for at least 1 year following a single intravenous injection of virus in neonatal MPS VII mice. The level and distribution of GUSB expression achieved is sufficient to prevent the development of many aspects of clinical disease over the life of the animal. Following treatment, bone lengths, weights and retinal function were maintained at nearly normal levels throughout the life of the animal. In addition, significant improvements in survival and auditory function were seen in AAV-treated MPS VII mice when compared with untreated mutant siblings. These data suggest that AAV-mediated gene transfer in the neonatal period can lead to prevention of many of the clinical symptoms associated with MPS VII in the murine model of this disease. Gene Therapy (2001) **8,** 1291–1298.

Keywords: *adeno-associated virus; lysosomal storage disease; animal models; metabolic disease; gene therapy*

Introduction

Mucopolysaccharidosis type VII (MPS VII) is a lysosomal storage disease caused by the absence of β-glucuronidase (GUSB) activity.[1] Patients with this syndrome have multiorgan disease, including mental and growth retardation, vision and hearing defects, cardiac abnormalities, and premature mortality. Although MPS VII is rare in humans, the disease shares many symptoms with more common lysosomal storage disorders such as Hunter's and Hurler's, and several authentic animal models of MPS VII have been characterized.[2–4] Therefore, MPS VII has been used as a model system for lysosomal disease in general. A murine model of MPS VII has been described that has no detectable GUSB activity and is caused by a single base pair deletion in exon 10 of the murine gene.[3,5] The MPS VII mouse model recapitulates many of the symptoms seen in human patients, and has been widely used in the evaluation of therapeutic strategies for lysosomal storage diseases.

There are a number of characteristics of MPS VII which make it amenable to treatment. The first is that the lysosomal distention characteristic of MPS VII can be reduced upon reconstitution of the enzymatic defect.[6] This allows established disease to be corrected on a histopathological level, even after long periods of accumulation. Secondly, the enzyme used to reconstitute the cell does not need to be produced within the affected cell, but can be taken up from the extracellular milieu by 'cross-correction', a phenomenon where extracellular GUSB can be internalized via binding to mannose-6-phosphate receptors (M6Pr) on the cell surface.[2,7] This ability of cells to internalize GUSB and direct it to the lysosomal compartment forms the biochemical basis of virtually all of the potential therapeutic strategies for MPS VII.

Two methods which take advantage of cross-correction are enzyme replacement therapy (ERT) and bone marrow transplantation (BMT). In ERT, purified GUSB is infused intravenously, and taken up into various tissues based on their vascularity and surface expression of the M6Pr. High levels of GUSB are initially achieved in most tissues following ERT, leading to reversal of lysosomal distention and improvements in higher mental and auditory functions.[8–10] However, this effect is transient, and as the infused GUSB activity decreases lysosomal distention will recur.[11] To provide a more persistent source of GUSB activity, BMT using syngeneic GUSB-positive bone marrow has been examined. Following transplantation, reconstitution of the hematopoietic system with GUSB-positive cells and dissemination of hematopoietic progeny throughout the body leads to GUSB activity in multiple tissues, and results in widespread reduction of lysosomal storage, presumably due to cross-correction.[6]

Similar to most other lysosomal storage disease, MPS VII is progressive in nature, and there is little clinical evidence of disease at birth.[3] Therefore, it is generally believed that early initiation of therapy may be more effective than delayed treatment. A number of studies have examined the benefits of neonatal *versus* adult treat-

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ments in the MPS VII mouse, using BMT, ERT, or a combination of the two.[10–13] These experiments have demonstrated that many of the functional deficits are amenable to therapy early in life, and that irreversible developmental damage does not occur *in utero*. It has been shown that reversal of lysosomal storage can be accomplished in most organs by either neonatal or adult treatments, although the duration of the histopathologic correction varies depending on the tissue and treatment method.[6,9,11,12] A notable exception to this statement is the brain, where the blood–brain barrier blocks ERT-mediated correction in adult life.[9,13,14] Improvements in survival and retinal function can also be achieved by treatment in either the adult or neonatal period.[6,13,15] However, improvements in bone length, auditory function, or higher mental functions have only been documented following neonatal BMT or ERT.[9,10,12,16]

Despite the successes seen in this mouse model, both ERT and BMT have aspects which limit their clinical applicability to humans. As mentioned above, the effects of ERT are transient, requiring repeated administrations of GUSB throughout life to maintain activity *in vivo*. For BMT, the lack of appropriate donors and the morbidity associated with allogeneic BMT limits the uses of BMT in humans. As an alternative to these approaches, we have been examining methods for neonatal gene transfer in the MPS VII mouse. Ideally, neonatal gene transfer would only require a single treatment early in life, which would lead to widespread and persistent *in vivo* expression of GUSB. We have previously shown that a single neonatal injection of an adeno-associated virus (AAV) vector coding for human GUSB leads to relatively long-term (4 months), multi-organ expression of GUSB, which results in a reduction of lysosomal distention into adulthood.[17] In the present study, we have examined the functional effects of this treatment. We show that a single injection of AAV-GUSB at birth results in GUSB expression for at least 1 year, and improves many aspects of the clinical phenotype of MPS VII, including the defects in retinal function, hearing, growth, bone length, survival, dysmorphic phenotype, and reproductive capacity. These data suggest that systemic AAV-mediated gene transfer early in life may represent a non-invasive long-term therapeutic approach for this disease.

Results

Gene transfer and GUSB expression following neonatal injection

AAV-injected mice were killed at ages ranging from 1 week to 1 year of age, and organs were analyzed for GUSB activity using a quantitative assay. Untreated MPS VII mice have no detectable activity at any time-point. MPS VII mice who received neonatal AAV treatment showed GUSB activity as early as 1 week of age in multiple organs (Figure 1), in a distribution similar to that previously described.[17] Activity in most organs achieved therapeutic levels at early time-points, and persisted at detectable levels for the duration of the study. Although not quantified, persistent GUSB activity was also seen in the retinal pigmented epithelium by histochemical staining (data not shown). Levels of GUSB activity greater than 1–5% normal have previously been shown to be sufficient to correct histopathologic disease in many tissues,[6,9,18] and tissue sections from 1-year-old animals in this study showed a marked reduction of lysosomal storage similar to that previously described[17] (data not shown). Persistence of transduced cDNA was documented by PCR in most tissues at 1 year of age (Figure 2), with the notable exception of the spleen, suggesting that the persistence of splenic GUSB activity is primarily due to cross-correction.

AAV treatment results in improvements in growth and phenotype

Treated and untreated MPS VII mice and normal siblings were weighed weekly starting at 4 weeks of age. Untreated MPS VII mice deviate significantly from the normal growth curve at 18 weeks of age, and achieve an average adult weight which is 33% less than normal siblings (Figure 3). In contrast, both male and female AAV-treated MPS VII mice show statistically significant improvements in weight when compared with untreated siblings. In addition, no statistically significant difference was observed between AAV-treated MPS VII males and normal control males throughout the study. The skeletal abnormalities seen in untreated MPS VII mice are also reduced following AAV treatment, with improvements in bone lengths of all extremities (Table 1). This improved bone development results in an improved phenotype, with the characteristic MPS VII phenotype of blunted facies and distorted limbs much reduced in AAV-treated mice when compared with untreated MPS VII controls (Figure 4). Physical activity of AAV-treated MPS VII mice was generally higher than untreated mutant siblings, and reproductive ability of AAV-treated mutants was improved, with treated males successfully impregnating normal females (data not shown).

Retinal function is maintained in AAV-treated mice

In order to evaluate improvements in retinal function (ERG) following AAV treatment, flash electroretinography was performed in groups of treated and untreated mice. We have previously shown that retinal function in MPS VII mice was essentially normal to 8 weeks of age, and then decreased significantly from 12 to 20 weeks of age.[15] Because the retinal dysfunction is progressive, groups were tested at both 12 and 20 weeks of age. While untreated MPS VII mice show decreases in both mesopic and photopic responses at 12 weeks, retinal function in the AAV-treated siblings is maintained at essentially normal levels (Figure 5). This effect is more pronounced at 20 weeks, where ERG amplitudes continued to decrease in the untreated MPS VII group. The AAV-treated mice showed a statistically significant improvement ($P < 0.05$) over untreated mutants in both mesopic and photopic responses, with essentially normal responses maintained into adulthood.

Auditory deficits are partially corrected following AAV treatment

MPS VII mice have a profound and progressive hearing loss, which may be due to a combination of neuronal defects and bony abnormalities of the middle ear.[10,16] This can be seen in untreated MPS VII mice, where the auditory-evoked brainstem response (ABR) threshold increases at all frequencies with age, resulting in profound hearing loss by adulthood (Figure 6). In contrast, mice who have been treated with neonatal AAV injection

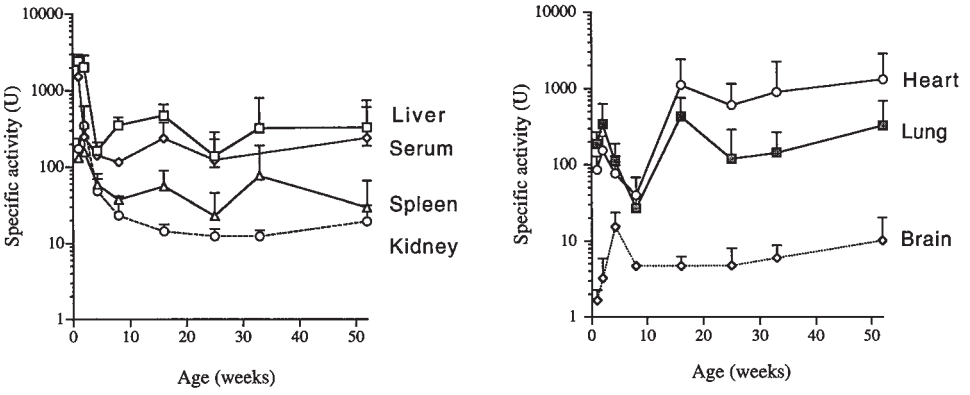

Figure 1 *Persistent therapeutic levels of GUSB are maintained for at least 1 year following neonatal AAV treatment. Mice injected with AAV-GUSB as neonates were killed at time-points ranging from 1 to at least 52 weeks of age, and organs were assayed for GUSB activity using a quantitative flourometric assay. Each point represents the average and standard deviation from three AAV-treated mice. All results except serum are expressed as specific activity (units/mg total protein). Serum levels are expressed as units/ml serum. For comparison, average activities found in normal adult B6 +/+ mice are liver 300 U, brain 40 U, spleen 500 U, heart 10 U, kidney 200 U, lung 160 U, and serum 90 U/ml.*

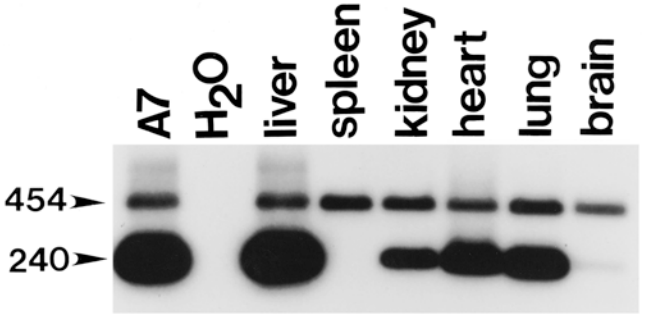

Figure 2 *Transduced viral cDNA persists in most tissues for at least 1 year. Tissues from 1-year-old mice were analyzed for the presence of viral cDNA using primers which amplify a 240 bp band from human cDNA, and a 454 bp band from the endogenous murine GUSB gene. A murine fibroblast line (A7) which has been transduced with a single retroviral copy of the human GUSB cDNA is shown as a positive control. Persistence of AAV-tranduced human GUSB cDNA is seen in most tissues at 1 year. The spleen, however, shows no indication of persistent viral transduction at this late time-point. The Southern blot shows the PCR products from a single mouse and is representative of the pattern observed in three separate mice 1 year after injection.*

have significantly improved responses at 5, 10, and 20 kHz into adulthood when compared with untreated mutant siblings. Although there is also a significant improvement in auditory function at 40 kHz in AAV-treated MPS VII mice, it is less pronounced than at lower frequencies.

AAV-treated mice have prolonged life-span

Twenty-five AAV-treated MPS VII mice, along with sex-matched normal and mutant controls were set aside at birth for longevity studies, and not included in any of the functional testing. While all untreated MPS VII mice were dead by 10 months of age, AAV-treated mutants showed significantly ($P < 0.0001$) improved survival when compared with untreated mutants (Figure 7). At 1 year of age, the AAV-treated mutants had approximately a 65% cumulative probability of survival.

Discussion

MPS VII is a progressive lysosomal storage disease, where accumulation of storage material for the life of the animal results in multi-organ disease and a wide range

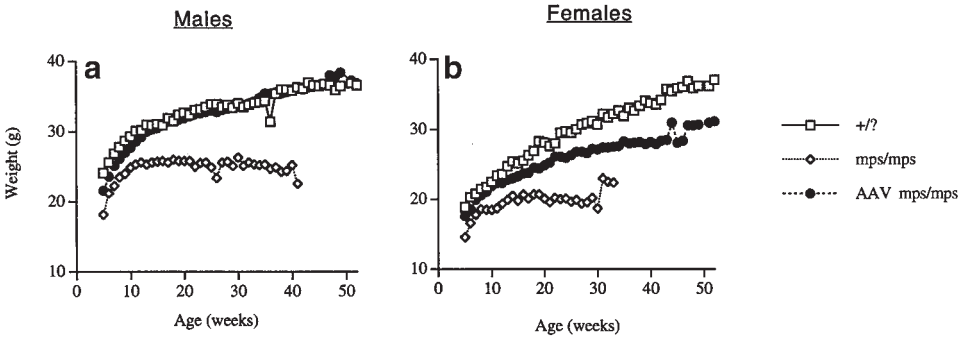

Figure 3 *Body weights of AAV-treated mice are improved. AAV-treated MPS VII mice, along with age-matched normal (+/?) and untreated mutants were weighed weekly starting at 4 weeks of age. Each time-point represents the average of eight to 16 mice, decreasing with time as mice died or were killed for quantitative analysis. Untreated male MPS VII mice show decreased weights as early as 8 weeks of age, and reach a final adult weight which is 7–8 g less than age-matched normal siblings (a). In contrast, AAV-treated MPS VII mice maintain normal weights for the duration of the study. A similar pattern is seen in females (b). All untreated female MPS VII mice were dead at 33 weeks.*

Table 1 Improvements in bone length following neonatal AAV treatment

	Untreated MPS		*AAV-treated MPS*	
	Male (n = 3–5)	*Female (n = 3–4)*	*Male (n = 4)*	*Female (n = 6–8)*
Radius/Ulna	0.87 (0.05)	0.87 (0.02)	0.94 (0.04)*	0.94 (0.03)*
Humerus	0.87 (0.04)	0.83 (0.03)	0.93 (0.02)*	0.93 (0.06)*
Tibia/Fibula	0.82 (0.04)	0.84 (0.02)	0.93 (0.01)*	0.93 (0.05)*
Femur	0.81 (0.04)	0.83 (0.06)	0.87 (0.02)	0.88 (0.04)

MPS VII mice receiving AAV treatment at birth were killed at time-points ranging from 8 weeks to 1 year, and bone lengths were measured radiologically. All lengths are expressed as a percentage of bone length in sex-matched adult B6 mice, with s.d. noted in parenthesis. Data for untreated MPS mice are based on historical controls (Refs 11, 12). AAV-treated mice show improvements in bone growth in most bones (*$P < 0.05$ *versus* untreated sex-matched mutants).

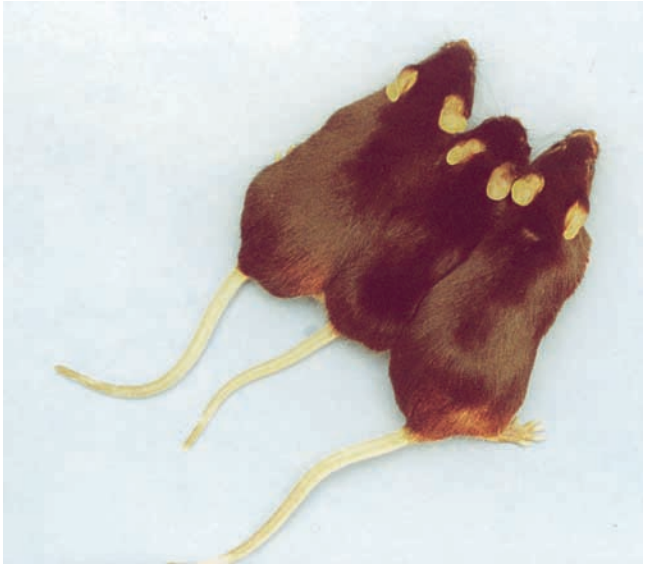

Figure 4 *The phenotype of AAV-treated MPS VII mice is improved compared with untreated mutants. Untreated adult MPS VII mice (middle) show a characteristic phenotype of blunted facies, hunched stature and limb abnormalities when compared with normal siblings (bottom, right). MPS VII mice that received neonatal AAV treatment show a dramatically improved adult phenotype (top, left). Although the mice shown here are 5 months old, the improvement in phenotype persisted throughout the study.*

of functional defects.[1,2] Neonatal treatments such as ERT and BMT have been shown to be more effective than adult treatment in reducing lysosomal storage and improving some functional parameters in the MPS VII mouse.[10,16] This is presumably because the presence of disease can affect critical stages of development, which cannot easily be reversed once tissues have reached their fully differentiated adult state. However, both ERT and BMT have limitations for neonatal treatment. For ERT, the repeated infusions necessary to maintain enzyme levels throughout development would be costly, and require large amounts of clinical grade enzyme. Although BMT offers a more long-lived alternative, the difficulties in locating appropriately matched donors and the potential morbidity of neonatal BMT limits its use as well.

For these reasons, a neonatal gene therapy approach for MPS VII would be appealing. In this study, we have shown that a single intravenous injection of recombinant AAV coding for GUSB given at birth can provide sufficient levels of enzyme to result in lifelong correction of some of the defects in the MPS VII mouse. A number of characteristics of recombinant AAV vectors contribute to this effect. First, the ability of AAV to transduce multiple tissues such as liver, heart and brain *in vivo* results in localized production of GUSB in many organs, which can then locally or distantly cross-correct uninfected cells. Second, the persistence of the recombinant AAV genome as either stable episomes or integrated forms[19,20] provides

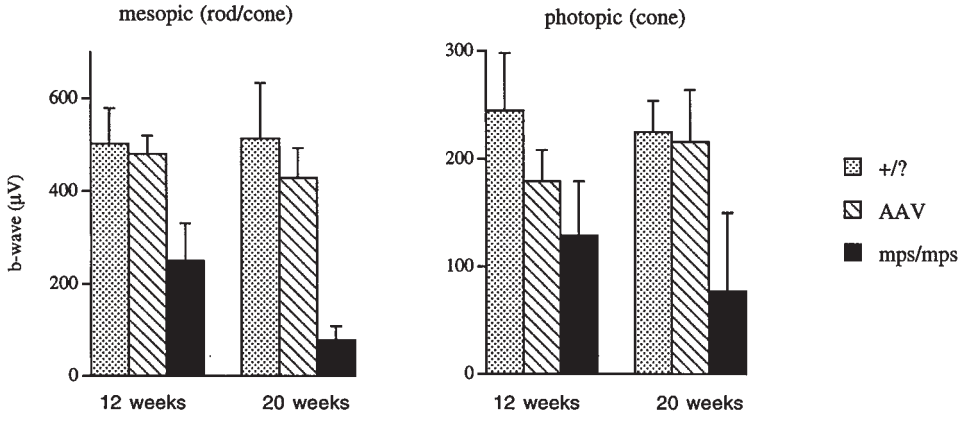

Figure 5 *Retinal dysfunction in MPS VII mice is prevented by AAV-mediated gene transfer. Mice were tested at 12 and 20 weeks by ERG to evaluate the progression of retinal dysfunction. Each group consisted of seven to eight mice. At 12 weeks, untreated MPS VII mice show decreases in both mesopic and photopic responses when compared with normal (+/?) siblings, while responses in AAV-treated mice are preserved. This preservation of function in treated MPS VII mice is more notable at 20 weeks, where retinal dysfuction in untreated MPS VII mice has continued to progress.*

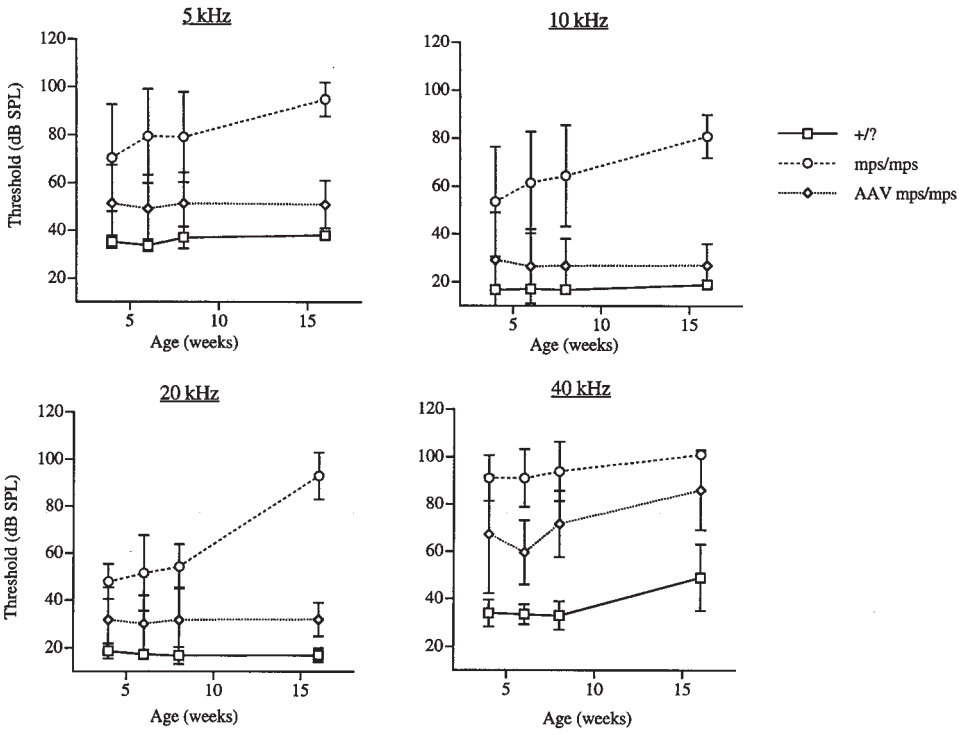

Figure 6 *Auditory function is improved in AAV-treated MPS VII mice. Individual mice were serially tested at 4, 6, 8 and 16 weeks of age. Each group consisted of seven to eight mice. ABRs for each mouse were determined at four frequencies ranging from 5 to 40 kHz. Untreated MPS VII mice show a significantly higher ($P < 0.05$) threshold than normal (+/?) siblings at all frequencies and time-points. AAV-treated mice, however, are significantly improved over untreated mutants at all ages and frequencies ($P < 0.05$).*

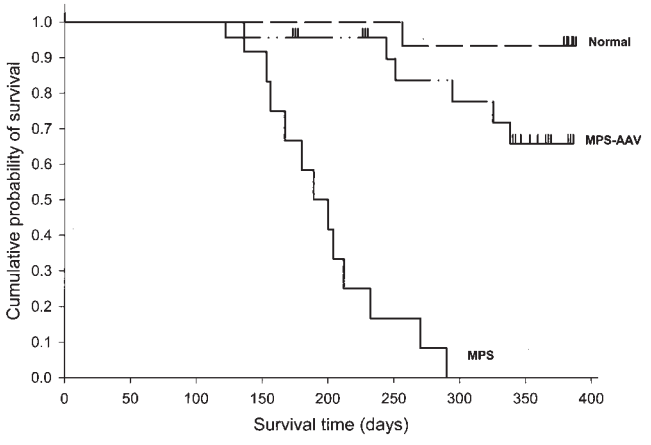

Figure 7 *Neonatal AAV treatment results in prolonged longevity. Newborn MPS VII mice (n = 25) were treated with AAV at birth, and set aside for longevity studies. Matched groups of normal (n = 12) and untreated MPS VII (n = 12) siblings were used as controls. Cumulative probability of survival for each group is shown. Vertical slashes indicate events where mice were removed for analysis, or are still surviving at the end of the study. Untreated MPS VII mice show premature mortality, with 50% dead by 6 months and all mice dead by 10 months of age. MPS VII mice who received neonatal AAV show a significantly ($P < 0.0001$) improved survival compared with untreated MPS VII mice, with nearly a 65% probability of survival at 1 year of age.*

long-term GUSB expression throughout development. Finally, the low immunogenicity of recombinant AAV vectors[21,22] make this virus a potentially acceptable option for neonatal use.

Neonatal gene transfer using AAVβGEnh[17,23] provided high levels of GUSB expression in early life, with persistent expression throughout development. This expression cassette was chosen since it was known to mediate high-level expression in a wide variety of cell types. These characteristics were desirable for this application since overexpression of GUSB does not appear to be toxic,[24] overexpressing cells secrete proportionally higher amounts of enzyme[25] and GUSB is normally expressed in virtually every cell of the body.

Based on previous studies using ERT and BMT, it seems likely that the primary mechanism for improvements in weight, bone length, and dysmorphic features seen in this study are the high levels of GUSB produced during the first month of life.[12,13] As shown previously, animals that received ERT for only the first 5 weeks of life showed essentially the same improvements in bone length and phenotype as animals who received long-term ERT.[11] Persistence of GUSB activity, however, may be more important in organs where progressive dysfunction can continue throughout life, or in tissues where storage material rapidly reaccumulates after therapy is stopped, such as the eye. The electroretinography findings of mesopic (rod/cone) losses before photopic (cone) losses seen in untreated MPS VII mice are similar to those seen in animal models of retinal degeneration, where early loss of the rod layer results in progressive loss of cone cells over time. The prolonged expression of GUSB in RPE-associated cells of AAV-treated mice may help to prevent disruption of the RPE by lysosomal distention, thereby preserving RPE function and maintaining the health of the photoreceptor layer.

The results of ABR testing in AAV-treated mice may

provide an interesting insight into the mechanisms of hearing loss in the MPS VII mouse. Previously, the auditory deficit in MPS VII mice was thought to be caused by a combination of a sensorineural cochlear defect and a conductive loss due to bony abnormalities in the middle ear.[16,26] In this study, although AAV-treated mice show marked improvements over untreated MPS VII mice at most frequencies, some residual defect is seen at 40 kHz, suggesting that the hearing loss has not been completely corrected. Preliminary data indicate that the cochlea of AAV-treated mice are essentially normal, with clearing of the characteristic storage lesions seen in untreated mutants (Ohlemiller, personal communication). This suggests that the residual defects following AAV treatment may be due to inadequate treatment of the bony defects of the middle ear. This is consistent with past observations that skeletal dysplasia is somewhat resistant to both ERT and BMT, and with only partial correction of bone lengths observed in this study. Experiments designed to determine the relative contributions of conductive *versus* sensorineural defects to the hearing losses in MPS VII mice are ongoing.

The general clinical improvement of AAV-treated mice is also seen by the ability of treated males to successfully impregnate normal female mice. It has been demonstrated previously that both male and female MPS VII mice are infertile,[3] and the ability to sire young or become pregnant has been used as a measure of clinical improvement in the MPS VII model.[12,27] The mechanisms of these improvements in the AAV-treated cohort are being evaluated further.

Although not directly measured in this study, we believe that the immune response to this form of therapy is minimal since circulating GUSB levels remain relatively constant and the clinical improvements persist. In previous studies, antibodies against human GUSB were not detected following intravenous or intramuscular injections of AAVβGEnh in neonatal MPS VII mice.[17,23] In addition, anti-GUSB antibodies were not detected following intravenous injections of high doses of recombinant enzyme in neonatal MPS VII mice.[9] The lack of anti-GUSB antibodies is somewhat unexpected since the mutation in the MPS VII mouse results in nearly undetectable amounts of GUSB mRNA and presumably little or no protein.[3] However, there may be several reasons for the lack of immune response in these studies. First, it is possible that the treated animals become tolerized to the transgene product due to the early initiation of therapy (day 2 of life). Second, we recently demonstrated that the MPS VII mouse has a GUSB-dependent immune defect which probably leads to inefficient antigen processing.[28] Finally, it has been demonstrated previously that AAV does not efficiently transduce dendritic cells and elicits only a minimal or no immune response.[22]

We demonstrated previously that a single intravenous injection of AAVβGEnh at birth resulted in persistent GUSB expression in the brains of MPS VII mice.[17] This level of expression was sufficient to prevent the accumulation of lysosomal storage in several regions of the brain. The level of GUSB expression in the brain in the current study is approximately two-fold higher than that measured in the previous study. Although not examined thoroughly, we believe that the extent of lysosomal storage reduction in the current study will be comparable with that reported previously and that the behavioral deficits may be prevented. However, it seems unlikely that an intravenous injection of AAV in a human will result in the same level of transduction and expression in the brain, and may require direct administration of the gene transfer vector to the CNS.

In conclusion, we have demonstrated that a single neonatal injection of recombinant AAV in MPS VII mice can lead to multi-organ correction of disease at a functional level. Although the data in this animal model are promising, there are a number of issues that need to be addressed before these results can be extended to the treatment of human disease. However, the results presented here are a first step toward this end. The unique ability of recombinant AAV to transduce multiple tissues, coupled with the ability to provide persistent activity *in vivo*, provides a promising tool for the treatment of inherited genetic disease.

Materials and methods

Production of recombinant AAV

The AAV vector AAVβGEnh has been previously described,[23] and consists of the CMV enhancer, chicken β-actin promoter, human GUSB cDNA, and rabbit β-globin and SV40 polyadenylation signals. Viral stocks were prepared from homogenates of 293 cells following cotransfection of AAVβGEnh and the helper plasmid pDG, which contains both adenoviral and AAV helper functions.[29] Purified virus was prepared at the University of Florida Department of Molecular Genetics and Microbiology, Gene Therapy Center using iodixanol gradient centrifugation followed by heparin-agarose column chromatography.[30] Genomic titers were determined by Southern blot analysis, while infectious units (IU) were determined by an expression-based assay on a GUSB-deficient cell line.[23]

Neonatal injections and experimental animals

Mutant MPS VII mice were obtained from heterozygous matings of B6.C-*H*-2^{bm1}/ByBir-*gus*^{mps}/+ mice maintained by MSS at Washington University. Homozygous normal (+/+) and heterozygous (+/*mps*) mice were differentiated by PCR.[31] Phenotypically normal control littermates of undetermined genotype (+/+ or +/*mps*) are referred to as +/? animals. Identification of newborn mutants was accomplished by quantitative analysis of GUSB activity in toe clips on the day of birth. Each mouse received a single intravenous injection of 100 μl of viral suspension via the superficial temporal vein[32] on day 2 of life. A successful injection was determined by the lack of extravasation of the injectate into surrounding tissue. Viral titers were 8×10^8 IU/ml (1.5×10^{12} genomes/ml), providing a dose of approximately 8×10^{10} IU/kg. This dose is approximately 15 times that used in our previous study.[17]

Tissue analysis of GUSB expression and lysosomal distention

GUSB activities were measured on tissue homogenates using fluorometric assays previously described.[9] For serum samples, reactions were incubated for 48 h at 37 C to increase the sensitivity of the assay. For histopathologic analysis of tissues, samples were immersed in ice-cold 2% glutaraldehyde, 4% paraformaldehyde in phosphate-buffered saline, and embedded in Spurr's resin.[6]

Sections (0.5 μm thick) of tissue were stained with toluidine blue and evaluated for lysosomal storage.

PCR and Southern analysis of tissues

PCR was performed on proteinase K digests of tissue samples as described previously, using primers specific for exons 6 and 7 of the human GUSB cDNA.[23] Blots were probed using a 510-bp *Cla*I fragment from the murine cDNA. Expected products are a 240-bp fragment from the human cDNA, and a 454-bp fragment from the endogenous murine gene which includes the intervening sequence between exons 6 and 7 of the murine gene. The primers were specifically designed to amplify both human and murine sequences such that the 454-bp murine band would serve as an internal control. Mismatches between the murine sequences and the human-specific PCR primers cause the human cDNA to be amplified more efficiently than the endogenous murine band.

Auditory evoked brainstem response (ABR) testing

ABR testing was done using standard techniques.[33] Mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (80/15 mg/kg), and platinum needle electrodes were placed subdermally at the vertex (reference), below the right ear (active), and on the dorsosacrum (ground). Sinewave auditory stimuli were output to ceramic speakers located 7 cm directly lateral to the right ear, and toneburst stimuli at each frequency were presented 1000 times at 20/s. The minimum sound pressure level required for visual detection of a response was determined at 5, 10, 20 and 40 kHz, using a 5 dB minimum step size. ABR thresholds from 40 to 80 dB indicate a moderate to severe hearing loss, while thresholds above 80 dB indicate a profound deafness.

Electroretinography

Electroretinography was performed as previously described.[15] Briefly, mice were dark-adapted for at least 2 h, then prepared for recording under dim red light. After intraperitoneal anesthesia, pupils were dilated with 2% xylazine, and a recording electrode was placed on the surface of the eye. Each recording included an intensity series obtained under dark adaptation to measure the mesopic (rod-cone) response, followed by 20 min of light adaptation and measurement of the photopic (cone) response. Results for each animal are expressed as the average b-wave amplitude over 5–10 flashes (rod-cone) or 50 flashes (cone). The b-wave amplitude includes elements of both the outer and inner retina, and represents a measure of overall photoreceptor health.

Statistical analysis

Statistical significance of the ABR, ERG and bone length data was determined using Student's *t* test. Longevity data are presented as a Kaplan–Meier plot, and significance determined by log-rank analysis. Comparison of weights between treatment groups and across time was done using a mixed model analysis of variance. The three-way interaction of group, sex and week was significant ($P < 0.02$). A set of 156 pairwise comparisons was performed to determine at which weeks the groups differed. To reduce the chance of declaring too many comparisons significant, a smaller P value was chosen using the Bonferroni adjustment. Comparisons were performed only to 30 weeks of age, since the number of untreated MPS VII animals became too small at that age.

Acknowledgements

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