Delivery of an enzyme-IGFII fusion protein to the mouse brain is therapeutic for mucopolysaccharidosis type IIIB

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Mucopolysaccharidosis type IIIB (MPS IIIB, Sanfilippo syndrome type B) is a lysosomal storage disease characterized by profound intellectual disability, dementia, and a lifespan of about two decades. The cause is mutation in the gene encoding α–*N*-acetylglucosaminidase (NAGLU), deficiency of NAGLU, and accumulation of heparan sulfate. Impediments to enzyme replacement therapy are the absence of mannose 6-phosphate on recombinant human NAGLU and the blood–brain barrier. To overcome the first impediment, a fusion protein of recombinant NAGLU and a fragment of insulin-like growth factor II (IGFII) was prepared for endocytosis by the mannose 6-phosphate/IGFII receptor. To bypass the blood–brain barrier, the fusion protein ("enzyme") in artificial cerebrospinal fluid ("vehicle") was administered intracerebroventricularly to the brain of adult MPS IIIB mice, four times over 2 wk. The brains were analyzed 1–28 d later and compared with brains of MPS IIIB mice that received vehicle alone or control (heterozygous) mice that received vehicle. There was marked uptake of the administered enzyme in many parts of the brain, where it persisted with a half-life of approximately 10 d. Heparan sulfate, and especially disease-specific heparan sulfate, was reduced to control level. A number of secondary accumulations in neurons [β-hexosaminidase, LAMP1(lysosome-associated membrane protein 1), SCMAS (subunit c of mitochondrial ATP synthase), glypican 5, β-amyloid, P-tau] were reduced almost to control level. CD68, a microglial protein, was reduced halfway. A large amount of enzyme also appeared in liver cells, where it reduced heparan sulfate and β-hexosaminidase accumulation to control levels. These results suggest the feasibility of enzyme replacement therapy for MPS IIIB.

Mucopolysaccharidosis type III (MPS III, Sanfilippo syndrome) is a heritable lysosomal disorder of heparan sulfate degradation, divided into four types (A–D), depending on the enzyme deficiency (1, 2). All four MPS III types are characterized by severe neurologic problems and relatively mild somatic ones. Profound intellectual disability that progresses to dementia, behavioral disturbances, and death in the second or third decade bring untold suffering to the MPS III patients and their families. Despite the dire need, treatment for the MPS III disorders has lagged behind other MPS diseases. Hematopoietic stem cell transplantation, an effective procedure for MPS I patients with CNS involvement (3), is not effective for MPS III (4). Enzyme replacement therapy has been available for some years for several MPS with extensive somatic involvement [MPS I (5, 6), II (7), and VI (8)], or is newly approved (MPS IVA), or in clinical trial (MPS VII). However, development of enzyme replacement for MPS III did not seem promising because access to therapeutic enzyme to brain parenchyma would be limited by the blood–brain barrier. With respect to MPS IIIB, a deficiency of α–*N*-acetylglucosaminidase, EC 3.2.1.50) (NAGLU), there is an additional difficulty in that, in contrast to most other soluble lysosomal enzymes, recombinant NAGLU produced in Chinese hamster ovary (CHO) cells contains little or no mannose 6-phosphate (Man6-P) (9–11), the signal for receptor-mediated endocytosis and targeting to lysosomes (12, 13). The reason for the lack of the Man6-P modification is not understood and appears to apply only to the recombinant enzyme, as human urinary NAGLU (14) and endogenous NAGLU made by CHO cells (9) contain Man6-P.

The lack of Man6-P on the enzyme can be overcome by taking advantage of the ability of the cation-independent Man6-P receptor to bind insulin-like growth factor II (IGFII) at a site distinct from the Man6-P binding sites (15–19). Fusion proteins consisting of a fragment of IGFII linked to a lysosomal enzyme

Significance

Mucopolysaccharidosis type IIIB (MPS IIIB) is a devastating and currently untreatable disease affecting mainly the brain. The cause is lack of the lysosomal enzyme, α–*N*-acetylglucosaminidase (NAGLU), and storage of heparan sulfate. Using a mouse model of MPS IIIB, we administered a modified NAGLU by injection into the left ventricle of the brain, bypassing the blood–brain barrier. The modification consisted of a fragment of IGFII, which allows receptor-mediated uptake and delivery to lysosomes. The modified enzyme was taken up avidly by cells in both brain and liver, where it reduced pathological accumulation of heparan sulfate and other metabolites to normal or near-normal levels. The results suggest the possibility of treatment for MPS IIIB.

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with no Man6-P have been made for β-glucuronidase (20), α-glucosidase (21), and NAGLU (11), and shown to have enzymatic activity similar to that of the original lysosomal enzyme and to be taken up by cultured cells in a manner that is dependent on IGFII and independent of Man6-P. In addition, the β-glucuronidase– and α-glucosidase–IGFII fusion proteins, administered i.v. to deficient mice, were found to be taken up by major somatic organs and muscles, respectively, in which they functioned to reduce storage and pathology (20, 21). On the basis of these promising earlier studies, we treated the brain of the MPS IIIB mouse by administering a NAGLU–IGFII fusion protein directly into the left cerebral ventricle, bypassing the blood–brain barrier. The modified enzyme was endocytosed mainly into neurons, where it functioned to reduce the level of stored heparan sulfate and of other accumulated substances to a normal or near-normal level. It also spread to the liver, where it was endocytosed into vascular cells and hepatocytes and eliminated storage of heparan sulfate. The results suggest that the combined use of the IGFII signal for endocytosis with administration directly into the brain may overcome the major obstacles to enzyme replacement therapy for MPS IIIB.

A preliminary account of this work has been presented in abstract form (22, 23).

Results

IGFII-Modified Enzyme Binds to the IGFII Receptor and Is Endocytosed by Cultured Fibroblasts. NAGLU fused at the C terminus to a fragment of IGFII was purified from secretions of a CHO cell line (*Materials and Methods* and *SI Materials and Methods*). The NAGLU–IGFII fusion protein was purified to apparent homogeneity, as demonstrated by a single band on an SDS/PAGE gel, staining intensely with Coomassie blue; it migrated slightly more slowly than the unmodified enzyme and reacted on Western blotting with antibodies against NAGLU and against IGFII (Fig. S1). It had a K_m of 0.2 mM for a fluorogenic synthetic substrate (*Materials and Methods*), similar to the K_m of the unmodified enzyme [previously reported as 0.22 mM (9)]. Its molecular mass was estimated to be ~106 kDa on the denaturing gel and 315 kDa by multiple angle light scattering, indicating a trimeric structure, as was also the case with the unmodified enzyme (~95 kDa and 278 kDa, respectively). Neither NAGLU nor the NAGLU–IGFII fusion protein contained Man6-P (Fig. 1*A*). High mannose chains were also lacking, in agreement with a previous report (11). NAGLU–IGFII, but not unmodified NAGLU, was bound to domains 10–13 of the Man6-P/IGFII receptor, as determined by a competitive binding assay (Fig. 1*B*); its calculated affinity for the receptor (IC_{50} = 0.28 ± 0.05 nM) was over 10-fold better than the affinity for IGFII itself (IC_{50} = 5.4 ± 0.97 nM), presumably due to the multivalency of the trimeric NAGLU–IGFII. NAGLU–IGFII, but not NAGLU, was endocytosed by cultured MPS IIIB fibroblasts with a K_{uptake} (concentration for half-maximal uptake) of 5.4 ± 0.9 nM, expressed as the concentration of trimer (Fig. 1*C*). The uptake was not affected by the presence of 0.5 or 5 mM Man6-P in the medium, but was partially inhibited by 0.1 μM IGFII and almost completely so by 1 μM IGFII (Fig. 1*D*). The catalytic activity of NAGLU–IGFII had a half-life of ~10 d within the MPS IIIB fibroblasts.

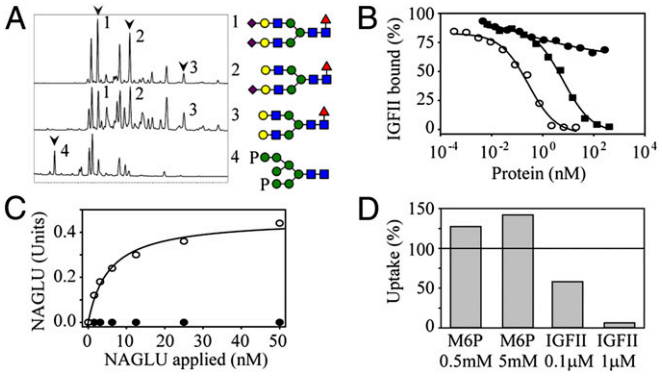

Fig. 1. Characterization of NAGLU–IGFII. (*A*) Glycosylation profiles of NAGLU (*Top* row) and of NAGLU–IGFII (*Middle* row) show that neither contained bisphosphorylated high mannose oligosaccharide (*Bottom* row). The sugars are indicated as follows: blue squares, *N*-acetylglucosamine; green circles, mannose; yellow circles, galactose; purple diamonds, *N*-acetylneuraminic acid; red triangles, L-fucose; P, phosphate. The oligosaccharides and the corresponding peaks on the capillary electrophoresis are numbered. (*B*) NAGLU–IGFII (open circles) was bound to domains 10–13 (21) of the Man6-P/IGFII receptor with much higher affinity than IGFII (squares), whereas unmodified NAGLU was not bound at all (filled circles). (*C*) NAGLU–IGFII (open circles) was endocytosed by MPS IIIB fibroblasts, whereas the unmodified enzyme (filled circles) was not. (*D*) Uptake of NAGLU–IGFII was not inhibited by Man6-P up to 5 mM, but was almost completely inhibited by 1 μM IGFII and partially so by 0.1 μM IGFII; the horizontal bar, set at 100%, represents uptake without inhibitor.

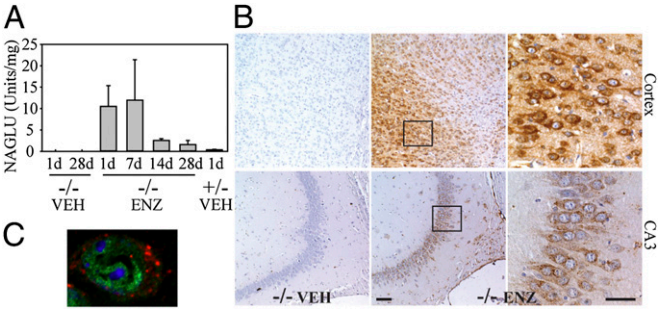

Fig. 2. Uptake of NAGLU–IGFII by MPS IIIB mouse brain after intracerebroventricular administration of NAGLU–IGFII. (*A*) NAGLU taken up, measured as NAGLU catalytic activity in homogenates of slice 1. Measurements are in units per milligram of protein, expressed as mean ± SD. The brains were taken at the times indicated (in days from the last injection) from mice treated with NAGLU–IGFII (ENZ) or vehicle (VEH). The number of mouse brains analyzed at each time point, going from left to right, was 7, 4, 8, 4, 4, 4, and 8. (*B*) Immunohistochemical staining for NAGLU in cortex (slice 2) and in CA3 layer of the hippocampus (slice 4), in the mutant mice treated with vehicle or with enzyme, as indicated. The squares in the *Center* images are enlarged for the images on the *Right*. [Scale bars, 100 μm (*Left* and *Center*) and 50 μm (*Right*).] (*C*) Characteristic image of a neuron stained for NAGLU (red), NeuN (green), and DAPI (blue).

NAGLU–IGFII Administered to the Brain Is Taken Up by Neurons. The design of in vivo experiments is illustrated in Fig. S2*A*. NAGLU–IGFII fusion protein (to be subsequently called "enzyme" in the text and "ENZ" in figures) was administered in artificial cerebrospinal fluid ("vehicle" in the text, "VEH" in figures) to the left lateral cerebral ventricle of 16-wk-old MPS IIIB mice (see Fig. 5 for experiment with 8-wk-old mice) over a 2-wk period; a similar group of MPS IIIB mice and a group of control (heterozygous) mice received vehicle alone. The mice were killed 1, 7, 14, or 28 d after the last injection; the brains were sliced coronally or sagittally, as shown in Fig. S2*B*, and slices were either frozen for subsequent determination of enzyme activity, heparan sulfate and protein concentration, or fixed for subsequent immunohistochemistry and/or immunofluorescence studies. The procedures for administration of enzyme and vehicle and for tissue harvesting are provided in *SI Materials and Methods*.

NAGLU enzyme activity was determined in homogenates of slice 1 (Fig. S2*B*). The activity on the first day postinjection was 30 times higher than in the brain of the heterozygous control and stayed high for 28 d; the half-life of the enzyme in the brain was ~10 d (Fig. 2*A*). The enzyme was located within cells, as shown in Fig. 2*B* by immunohistochemical staining of sample areas (cortex and the CA3 region of the hippocampus). The morphology of

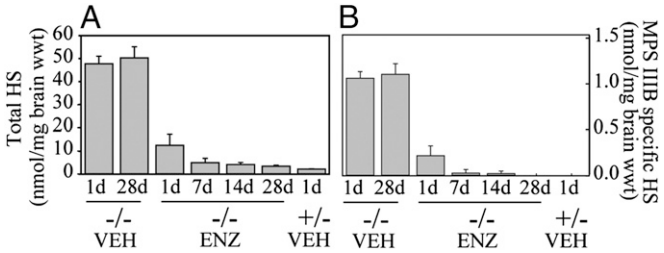

Fig. 3. Reduction of heparan sulfate after intracerebroventricular administration of NAGLU–IGFII. The mouse brains are the same as used for Fig. 2. (*A*) Representation of the total heparan sulfate in slice 3. (*B*) In the same slice as in *A*, the heparan sulfate oligosaccharides with an *N*-acetylglucosamine residue at the nonreducing end and therefore specific to MPS IIIB. The amount of total heparan sulfate (HS) is expressed in nanomoles of disaccharide per milligram wet weight of brain tissue, as mean ± SD; the amount of MPS IIIB-specific HS is expressed as nanomoles of trisaccharide per milligram wet weight of brain tissue, mean ± SD.

the stained cells indicates that most are neurons (Fig. 2*B*); this was confirmed by staining with the neuron-specific marker, NeuN (Fig. 2*C*). It should be noted that the endocytosed NAGLU and NeuN do not colocalize within the neuron; whereas NeuN is nuclear, NAGLU is present in punctate structures in the cytoplasm, as expected of its lysosomal location. The lysosomal location was confirmed by colocalization with LAMP2, a lysosomal membrane protein (Fig. S3*A*).

Heparan Sulfate Storage Is Greatly Reduced. The amount of heparan sulfate, the primary storage material, was very high in brain of MPS IIIB mice treated with vehicle alone, as determined by the Sensi-Pro method (24, 25) (Fig. 3*A*). It decreased rapidly after treatment with enzyme, but even after 28 d, it was not quite as low as in the vehicle-treated heterozygous control. However, the heparan sulfate fragment specific to MPS IIIB (i.e., terminating in nonreducing α–*N*-acetylglucosamine), was reduced to an undetectable level, comparable to the heterozygous control (Fig. 3*B*).

β-Hexosaminidase and LAMP1 Levels Are Reduced. Deficiency of α–*N*-acetylglucosaminidase in brain of MPS IIIB mice is accompanied by increased activity of several other lysosomal enzymes, including β-hexosaminidase (26). Fig. 4*A* shows the activity of β-hexosaminidase in slice 1 of the mutant brain to be more than twice that in the comparable area of the heterozygous control. After treatment with NAGLU–IGFII, the activity of β-hexosaminidase decreased slowly over the period of 28 d, to a level not significantly different from the control brain.

LAMP1, another lysosomal membrane protein, is expected to decrease in amount as lysosomal storage is reduced and the lysosomes become smaller. Fig. 4*B* shows prominent LAMP1 staining in neurons of CA3 and in nonneuronal cells (perhaps astrocytes) of the dentate gyrus; upon treatment with enzyme, LAMP1 staining was much reduced, essentially to the level of the heterozygous control.

Other Pathologic Markers Are Reduced. In addition to the primary storage product, heparan sulfate, neurons in the MPS IIIB mouse brain show elevation of several seemingly unrelated proteins in very specific areas of the brain: subunit c of mitochondrial ATP synthase (SCMAS), glypican 5, and β-amyloid in the medial entorhinal cortex (MEC), and hyperphosphorylated tau (P-thr181) in the dentate gyrus (27, 28). CD68, a marker for activated microglia, is elevated in many areas (29). To determine whether these secondary accumulations would also be reduced by treatment with NAGLU–IGFII, a separate experiment was set up for sagittal cutting of the brain to expose the areas of interest. The experimental design was as described above, except that the mice were 8 wk of age at the start of the experiment and killed 1 d after the last administration of enzyme or vehicle. The accumulation of SCMAS in the MEC of the mutant mice is easily seen by immunohistochemistry (Fig. 5*A*); it disappears after treatment with NAGLU–IGFII, the MEC becoming indistinguishable from the MEC of control brain. The levels of SCMAS, glypican 5, β-amyloid, and CD68 were quantitated by immunofluorescence, and the level of P-tau was quantitated by counting aggregates (28) (Fig. 5*B*). Except for CD68, the accumulation in the enzyme-treated MPS IIIB mice was reduced to almost the level of the heterozygote control, both on the left (ipsilateral) and right (contralateral) sides (reduction in SCMAS, 97% and 97%; glypican 5, 100% and 87%; β-amyloid, 91% and 81%; and P-tau, 92% and 93%). The accumulation of CD68 was reduced partially, by 72% on the left and 44% on the right side.

NAGLU–IGFII Administered to Brain Is also Taken Up by Liver. A considerable amount of NAGLU–IGFII was taken up by the liver, as can be seen by immunohistochemical staining (Fig. 6). NAGLU staining was most intense in cells lining the sinusoids (Fig. 6*A*, *Left*); immunostaining with antibodies to CD31 (a marker for endothelial cells) and Iba1 (a marker for Kupffer cells), showed the former to be rich in NAGLU and the latter to be relatively free of NAGLU (Fig. 6*A*, *Center* and *Right*, respectively). Because of the intense staining of the endothelial cells, it was difficult to see the enzyme in hepatocytes; the presence of NAGLU in hepatocytes was best seen by confocal microscopy, which showed colocalization with LAMP2 (Fig. S3*B*).

The uptake of NAGLU–IGFII into liver could also be determined by measurement of NAGLU enzymatic activity (Fig. 6*B*) and was seen to be five times higher, per milligram of protein, than in homogenates of slice 1 of the brain. β-Hexosaminidase activity was reduced 90% of the way to the normal level, 1 d postfinal injection (Fig. 6*C*). The stored heparan sulfate was decreased to almost zero for up to 28 d after injections were discontinued; the disease-specific heparan sulfate fragment (with terminal α–*N*-acetylglucosamine) was decreased to zero for 14 d postinjection, but a small amount reappeared in 28 d (Fig. 6*D*). These findings indicate that a considerable amount of enzyme that had been injected intracerebroventricularly was taken up by the liver, primarily by endothelial cells and hepatocytes, and that it was functional within lysosomes.

An incidental finding in this experiment was a sex difference in heparan sulfate accumulation in the liver of mutant mice, with males having nearly twice the level of heparan sulfate and MPS

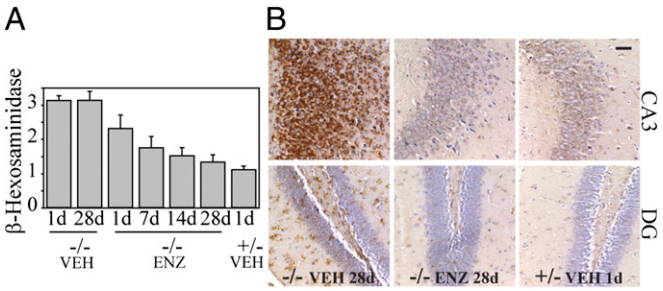

Fig. 4. Reduction of β-hexosaminidase and of LAMP1 after intracerebroventricular administration of NAGLU–IGFII to MPS IIIB mice. (*A*) Decrease in β-hexosaminidase activity (expressed in thousands of units per milligram of protein, mean ± SD) in homogenized brain tissue from slice 1; the mouse brains are the same as were used for Fig. 2. (*B*) Immunohistochemical staining for LAMP1, a marker for lysosomes, in the CA3 and dentate gyrus (DG) areas of the hippocampus (slice 4), in brains removed 28 d after the last injection. (Scale bar, 50 μm.)

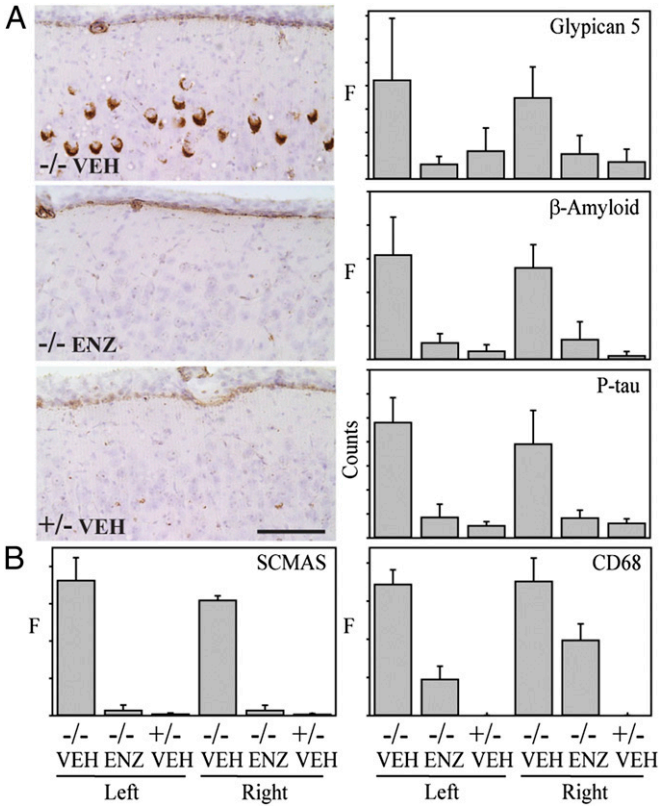

Fig. 5. Reduction of secondary accumulations in MPS IIIB brain upon intracerebroventricular administration of NAGLU–IGFII. (*A*) Immunohistochemistry shows intense staining of SCMAS in the medial entorhinal cortex (MEC) of the *Naglu*$^{-/-}$ untreated brain, which disappeared upon treatment with enzyme, leading to an appearance similar to the MEC of control mice. (Scale bar, 100 μm.) (*B*) Immunofluorescence was used to quantitate all of the biomarkers except for P-tau. F, arbitrary fluorescence units; counts, the number of P-tau inclusions in the dentate gyrus (28). Bar graphs show the mean ± SD for three to four mice in each group. The decrease in the enzyme-treated mice was significant to $P < 0.001$ for all biomarkers examined except for glypican 5, in which the experimental variability decreased the significance to $P < 0.01$.

IIIB-specific heparan sulfate, as females (Fig. 6*D*). No such difference was seen in the brain.

Discussion

This study demonstrates that it is possible to bypass the blood–brain barrier by direct injection of therapeutic enzyme into the cerebral ventricles, using an implanted cannula. The enzyme (a fusion protein of NAGLU and IGFII) was distributed to many areas of the brain, to the contralateral side as well as the ipsilateral. The enzyme was endocytosed by neurons; that was expected, because the Man6-P/IGFII receptor (to which the fusion protein was targeted), is known to be widely distributed in neurons of the central nervous system (30). The enzyme functioned in neuronal lysosomes to degrade heparan sulfate, and in addition caused a decrease of other disease-associated biomarkers, some of which were not lysosomal.

The enzyme administered through the ventricles did not remain confined to the brain, but was also found in substantial amount in the liver. Given that the specific activity of NAGLU in liver homogenates was five times the specific activity found in homogenates of brain slice 1, and that a mouse liver is nearly three times as large as its brain (31), the amount in liver is at least one order of magnitude higher than in brain (more precise calculations would not be meaningful, because the exogenous enzyme is not evenly distributed in either organ). It is likely that the enzyme that was injected into the ventricle mixes with endogenous cerebrospinal fluid and enters into the circulatory pathway of the latter, through blood or lymph (32). In the liver, the enzyme was found concentrated in endothelial cells, but was also present in hepatocytes; very little was found in Kupffer cells. The enzyme functioned to remove the accumulated heparan sulfate. No somatic organs other than liver were examined.

It was somewhat surprising that few cells of the monocyte/macrophage lineage—microglia in brain and Kupffer cells in liver—showed immunostaining of NAGLU. This is consistent with the absence of high mannose chains in the fusion protein, because these cells will efficiently endocytose glycoproteins with glycans containing terminal mannose residues due to the presence of abundant levels of the mannose receptor on the cell surface. However, we must presume that microglia and Kupffer cells do acquire some enzyme, to account for the degradation of all of the accumulated heparan sulfate. This may occur because the requirement for enzyme is below detection by immunostaining (which may have a lower sensitivity than biochemical assays) and/or the cells acquire enzyme by some different mechanism, e.g., by phagocytosis of bits of neurons containing the enzyme, or by recognition of different carbohydrates by macrophage lectins (receptors). Both galactose and L-fucose, which are present on the complex oligosaccharides of the fusion protein (Fig. 1*A*), are recognized by such receptors (33, 34).

The half-life of the fusion protein (or more precisely, of the catalytic activity of its NAGLU moiety) was about 10 d in brain, similar to its half-life in cultured fibroblasts. The amount used in this study was sufficient to prevent reaccumulation of the

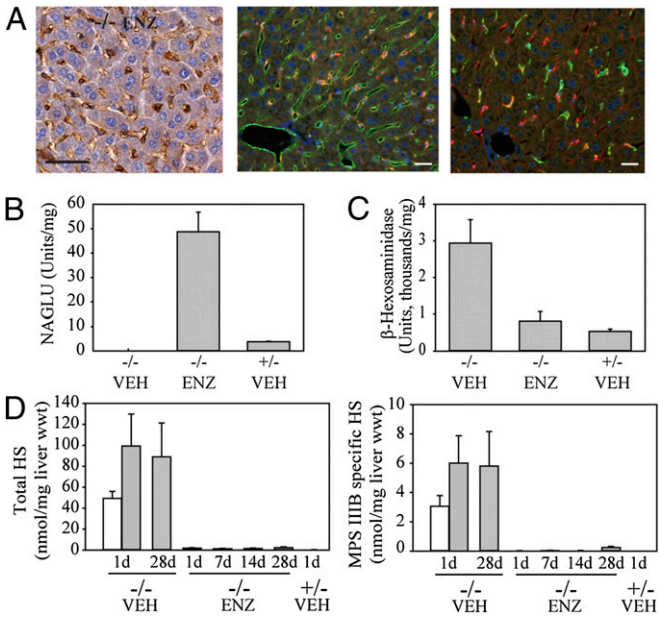

Fig. 6. Uptake of NAGLU–IGFII by liver after intracerebroventricular administration. (*A*) Uptake by *Naglu*$^{-/-}$ mice. (*Left*) Immunostaining of NAGLU. (*Center*) Uptake into endothelial cells (green, CD31; red, NAGLU; blue, DAPI). (*Right*) Uptake into Kupffer cells (green, Iba1; red, NAGLU; blue, DAPI). [Scale bars, 50 μm (*Left*) and 20 μm (*Center* and *Right*).] (*B* and *C*) Enzymatic activity of NAGLU and β-hexosaminidase, respectively, after treating the mice with enzyme or vehicle. (*D*) Total heparan sulfate (*Left*) and MPS IIIB-specific heparan sulfate (*Right*). Gray bars represent male mice (four at each point), and clear bar represents female mice (4). All bars show mean ± SD, and all differences between livers of enzyme-treated and vehicle-treated *Naglu*$^{-/-}$ mice were significant to $P < 0.001$. The difference between male and female mice was significant to $P < 0.05$.

pathogenic substrate for nearly three half-lives. Furthermore, the near normalization of brain biochemistry and pathology was achieved with only 2 wk of treatment of adult mice. These properties bode well for future therapeutic trials of the fusion protein.

Materials and Methods

Because the experiments were performed at three sites, there sometimes were differences in reagents and procedures used. Where these occur, the performance site will be indicated as BioMarin Pharmaceutical, Inc. (BMRN), Los Angeles Biomedical Research Institute at Harbor–UCLA Medical Center (LA BioMed), or University of California, Los Angeles (UCLA).

Enzyme Assays. Catalytic activity of NAGLU and of NAGLU–IGFII was determined by hydrolysis of the fluorogenic substrate, 4-methylumbelliferyl-*N*-acetyl-α-glucosaminide, obtained from CalBiochem (now EMD Millipore Chemicals) or Toronto Research Chemicals, with minor modifications of a published protocol (35), using either 1.8 mM (BMRN) or 1.0 mM (LA BioMed) substrate in the incubation mixture. Catalytic activity of β-hexosaminidase (combined A and B isoforms) was determined by hydrolysis of 4-methylumbelliferyl-*N*-acetyl-β-glucosaminide (EMD Millipore Chemicals) using 1.25 mM substrate in the incubation mixture. For both enzymes, a unit of activity is defined as release of 1 nmol of 4-methylumelliferone (4MU) per hour. Protein concentration was estimated by the Bradford method, using BSA as standard.

Preparation of Human Recombinant NAGLU and NAGLU–IGFII. Human NAGLU and NAGLU–IGFII protein constructs were prepared for production in Chinese hamster ovary cells using the GS (glutamine synthetase) Mammalian Gene Expression System (Lonza Biologics). A human full-length NAGLU cDNA (corresponding to amino acid residues 1–743 with an N-terminal 24-residue signal peptide) was subcloned into a GS vector pEE12.4. A pEE12.4/NAGLU–IGFII expression plasmid was constructed by joining the full-length NAGLU sequence to a 25-residue spacer sequence (amino acids GGGGSGGGGSGGGGSGGGGSGGGPS), followed by a peptide derived from a human IGFII protein (amino acid residues 8–67 with a Arg37Ala mutation; J. LeBowitz and J. Maga, US patent 8,563,691 B2). See *SI Materials and Methods* for a detailed description of expression and purification of the recombinant proteins. The final purified proteins were stored at −80 °C in artificial cerebrospinal fluid (CSF): 1 mM Na_2HPO_4/NaH_2PO_4, 148 mM NaCl, 3 mM KCl, 0.8 mM $MgCl_2$, 1.4 mM $CaCl_2$, pH 7.2.

Analysis of Glycans on NAGLU and NAGLU–IGFII. The glycans were isolated by treatment of NAGLU and NAGLU–IGFII with peptide *N*-glycanase F or endoglycosidase H at 37 °C for 24 h and the oligosaccharides derivatized overnight with 8-aminopyrene-2,3,6-trisulfonic acid in the presence of $NaBH_3CN$ (36). The sample was passed through a column of Sephadex G-10 and subjected to capillary electrophoresis and detected with laser-induced fluorescence. The isolated glycans were further characterized by stepwise digestions with multiple exoglycosidases and alkaline phosphatase. The resulting electrophoretic mobility shifts after each digestion were evaluated relative to the migration of commercially available or known oligosaccharides from a reference protein to determine the oligosaccharide structure.

Endocytosis of Enzyme in Cell Culture. MPS IIIB skin fibroblasts (Coriell Institute; GM02931) were plated onto 24-well plates at 10^5 cells per well and subsequently incubated with NAGLU or NAGLU–IGFII samples in high glucose Dulbecco's modified Eagle medium (Gibco, now Life Technologies) containing 25 mM Hepes buffer pH.7.0, 2 mM glutamine and 0.5 mg/mL BSA. After 4 h, the cells were washed with PBS, lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), and the lysates were assayed for enzyme activity.

IGFII Receptor Binding Assay. A competitive receptor binding assay was used to measure the binding affinity to the IGFII receptor fragment consisting of domains 10–13 (21). The fragment (0.5 μg/100 μL/well) was coated onto a Reacti-Bind plate (Thermo Scientific) overnight at room temperature, followed by a blocking step (SuperBlock T20 Blocking buffer; Thermo Scientific) for 1 h. The plate was incubated with varying concentrations of NAGLU, NAGLU–IGFII, or IGFII (Cell Sciences) in the presence of 3–8 nM biotinylated IGFII (Cell Sciences) for 2 h at room temperature. The amount of biotinylated IGFII bound to the plated receptor fragment was detected with streptavidin conjugated to horseradish peroxidase and a chemiluminescent substrate (SuperSignal ELISA Pico substrate; Thermo Scientific). The ability of NAGLU, NAGLU–IGFII, or IGFII protein to inhibit binding of biotinylated IGFII to the receptor fragment was calculated from inhibition curves and reported as an IC_{50} value.

Experimental Animals. The MPS IIIB mice were from a *Naglu* gene knock-out colony maintained since 1999 (26). The animal protocols were approved by the Animal Care and Use Committee at LA BioMed. Heterozygous females were mated with mutant males to obtain an approximately equal number of mutant ($Naglu^{-/-}$) and heterozygous ($Naglu^{+/-}$) offspring, the latter being used as controls. Experiments were performed on age-matched mice (usually littermates) of mixed sex, unless otherwise specified.

Analysis of Heparan Sulfate. The Sensi-Pro assay was used to quantify both the total heparan sulfate and the heparan sulfate specific to MPS IIIB. Tissue samples were homogenized in water in a bead homogenizer and the glycosaminoglycans were isolated by anion exchange chromatography and depolymerized with heparin lyase (IBEX Technologies). They were tagged by reductive amination with isotopically labeled aniline and quantified by liquid chromatography-mass spectrometry, as previously described (24, 25). The quantity of disaccharides obtained by the lyase digestion is a measure of total heparan sulfate, whereas the quantity of trisaccharide with *N*-acetylglucosamine at the nonreducing end is a measure of heparan sulfate specific to MPS IIIB.

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1. Neufeld EF, Muenzer J (2001) The mucopolysaccharidoses. *The Metabolic and Molecular Bases of Inherited Disease*, eds Scriver CR, Beaudet AL, Sly WS, Valle D (McGraw-Hill, New York), 8th Ed, pp 3421–3452.
2. Valstar MJ, Ruijter GJ, van Diggelen OP, Poorthuis BJ, Wijburg FA (2008) Sanfilippo syndrome: A mini-review. *J Inherit Metab Dis* 31(2):240–252.
3. Souillet G, et al. (2003) Outcome of 27 patients with Hurler's syndrome transplanted from either related or unrelated haematopoietic stem cell sources. *Bone Marrow Transplant* 31(12):1105–1117.
4. Sivakumur P, Wraith JE (1999) Bone marrow transplantation in mucopolysaccharidosis type IIIA: A comparison of an early treated patient with his untreated sibling. *J Inherit Metab Dis* 22(7):849–850.
5. Wraith JE, et al. (2007) Enzyme replacement therapy in patients who have mucopolysaccharidosis I and are younger than 5 years: Results of a multinational study of recombinant human alpha-L-iduronidase (laronidase). *Pediatrics* 120(1):e37–e46.
6. Kakkis ED, et al. (2001) Enzyme-replacement therapy in mucopolysaccharidosis I. *N Engl J Med* 344(3):182–188.
7. Muenzer J, et al. (2006) A phase II/III clinical study of enzyme replacement therapy with idursulfase in mucopolysaccharidosis II (Hunter syndrome). *Genet Med* 8(8):465–473.
8. Harmatz P, et al.; MPS VI Phase 3 Study Group (2006) Enzyme replacement therapy for mucopolysaccharidosis VI: A phase 3, randomized, double-blind, placebo-controlled, multinational study of recombinant human N-acetylgalactosamine 4-sulfatase (recombinant human arylsulfatase B or rhASB) and follow-on, open-label extension study. *J Pediatr* 148(4):533–539.
9. Zhao KW, Neufeld EF (2000) Purification and characterization of recombinant human α-*N*-acetylglucosaminidase secreted by Chinese hamster ovary cells. *Protein Expr Purif* 19(1):202–211.
10. Weber B, Hopwood JJ, Yogalingam G (2001) Expression and characterization of human recombinant and alpha-N-acetylglucosaminidase. *Protein Expr Purif* 21(2):251–259.
11. Kan SH, et al. (2014) Insulin-like growth factor II peptide fusion enables uptake and lysosomal delivery of α-N-acetylglucosaminidase to mucopolysaccharidosis type IIIB fibroblasts. *Biochem J* 458(2):281–289.
12. Kaplan A, Achord DT, Sly WS (1977) Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc Natl Acad Sci USA* 74(5):2026–2030.
13. Kornfeld S (1990) Lysosomal enzyme targeting. *Biochem Soc Trans* 18(3):367–374.
14. von Figura K, Klein U (1979) Isolation and characterization of phosphorylated oligosaccharides from alpha-N-acetylglucosaminidase that are recognized by cell-surface receptors. *Eur J Biochem* 94(2):347–354.
15. Morgan DO, et al. (1987) Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* 329(6137):301–307.
16. Oshima A, Nolan CM, Kyle JW, Grubb JH, Sly WS (1988) The human cation-independent mannose 6-phosphate receptor. Cloning and sequence of the full-length cDNA and expression of functional receptor in COS cells. *J Biol Chem* 263(5):2553–2562.

17. Tong PY, Tollefsen SE, Kornfeld S (1988) The cation-independent mannose 6-phosphate receptor binds insulin-like growth factor II. *J Biol Chem* 263(6):2585–2588.
18. Kiess W, et al. (1988) Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. *J Biol Chem* 263(19):9339–9344.
19. Kornfeld S (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem* 61:307–330.
20. LeBowitz JH, et al. (2004) Glycosylation-independent targeting enhances enzyme delivery to lysosomes and decreases storage in mucopolysaccharidosis type VII mice. *Proc Natl Acad Sci USA* 101(9):3083–3088.
21. Maga JA, et al. (2013) Glycosylation-independent lysosomal targeting of acid α-glucosidase enhances muscle glycogen clearance in pompe mice. *J Biol Chem* 288(3): 1428–1438.
22. Aoyagi-Scharber M, et al. (2014) Engineering of a recombinant NAGLU fusion protein with insulin-like growth factor 2 leads to improved cellular uptake via a glycosuylation-independent lysopsomal targeting pathway. *Mol Genet Metab* 111(2):S20.
23. Kan SH, et al. (2014) Intracerebroventricular enzyme replacement therapy with glycosylation-independent lysosomal targeted NAGLU leads to widespread enzymatic ativity, reduction of lysosomal storage and of secondary defects in brain of mice with Sanfilippo syndrome type B. *Mol Genet Metab* 111(2):S59.
24. Lawrence R, et al. (2012) Disease-specific non-reducing end carbohydrate biomarkers for mucopolysaccharidoses. *Nat Chem Biol* 8(2):197–204.
25. Lawrence R, et al. (2014) Glycan-based biomarkers for mucopolysaccharidoses. *Mol Genet Metab* 111(2):73–83.
26. Li HH, et al. (1999) Mouse model of Sanfilippo syndrome type B produced by targeted disruption of the gene encoding alpha-N-acetylglucosaminidase. *Proc Natl Acad Sci USA* 96(25):14505–14510.
27. Ohmi K, et al. (2009) Sanfilippo syndrome type B, a lysosomal storage disease, is also a tauopathy. *Proc Natl Acad Sci USA* 106(20):8332–8337.
28. Ohmi K, Zhao HZ, Neufeld EF (2011) Defects in the medial entorhinal cortex and dentate gyrus in the mouse model of Sanfilippo syndrome type B. *PLoS ONE* 6(11): e27461.
29. Ohmi K, et al. (2003) Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. *Proc Natl Acad Sci USA* 100(4):1902–1907.
30. Hawkes C, Kar S (2003) Insulin-like growth factor-II/mannose-6-phosphate receptor: Widespread distribution in neurons of the central nervous system including those expressing cholinergic phenotype. *J Comp Neurol* 458(2):113–127.
31. The Jackson Laboratory (2014) Morphometric (organ weight) survey of 11 inbred strains of mice. *MPD:Jaxpheno2*.
32. Brinker T, Stopa E, Morrison J, Klinge P (2014) A new look at cerebrospinal fluid circulation. *Fluids Barriers CNS* 11:10.
33. Stahl PD (1992) The mannose receptor and other macrophage lectins. *Curr Opin Immunol* 4(1):49–52.
34. Fadden AJ, Holt OJ, Drickamer K (2003) Molecular characterization of the rat Kupffer cell glycoprotein receptor. *Glycobiology* 13(7):529–537.
35. Marsh J, Fensom AH (1985) 4-Methylumbelliferyl α-N-acetylglucosaminidase activity for diagnosis of Sanfilippo B disease. *Clin Genet* 27(3):258–262.
36. Szabo Z, Guttman A, Rejtar T, Karger BL (2010) Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. *Electrophoresis* 31(8): 1389–1395.