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Neurological Correction of Lysosomal Storage in a Mucopolysaccharidosis IIIB Mouse Model by Adeno-associated Virus-Mediated Gene Delivery

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Mucopolysaccharidosis (MPS) IIIB is characterized by mild somatic features and severe neurological diseases leading to premature death. No definite treatment is available for MPS IIIB patients. We constructed two recombinant adeno-associated virus (rAAV) vectors containing the human α-*N*-acetylglucosaminidase (NaGlu) cDNA driven by either a CMV or a neuron-specific enolase (NSE) promoter. *In vitro*, these rAAV vectors mediated efficient expression of recombinant NaGlu in human MPS IIIB fibroblasts and mouse MPS IIIB somatic and brain primary cell cultures. The secreted rNaGlu was taken up by both human and mouse MPS IIIB cells in culture and degraded the accumulated glycosaminoglycans (GAG). A direct microinjection (10^7 viral particles, 1 μl/10 minutes per injection) of vectors containing the NSE promoter resulted in long-term (6 months, the duration of the experiments) expression of rNaGlu in multiple brain structures/areas of adult MPS IIIB mice. Consistent with previous studies, the main target cells were neurons. However, while vector typically transduced an area of 400–500 μm surrounding the infusion sites, the correction of GAG storage involved neurons of a much broader area (1.5 mm) in a 6-month duration of experiments. These results provide a basis for the development of a treatment for neurological disease in MPS IIIB patients using AAV vectors.

Key words: adeno-associated virus, mucopolysaccharidosis IIIB, lysosomal storage, α-*N*-acetylglucosaminidase

INTRODUCTION

Mucopolysaccharidosis (MPS) IIIB (Sanfilippo syndrome IIIB) is an inherited lysosomal storage disorder caused by deficiency of α-*N*-acetylglucosaminidase (NaGlu). NaGlu deficiency causes intracellular lysosomal accumulation of the glycosaminoglycan (GAG) heparan sulfate. The disease is characterized by multiple organ/system involvement and severe neurological degeneration [1]. Neurological deterioration occurs in most patients by 6 to 10 years of age with rapid and progressive retardation of social and adaptive abilities leading to premature death. No definite treatment is available for patients with MPS IIIB. Therapy is limited to management of complications. Bone marrow transplantation has been shown to be beneficial in MPS I [2,3]. So far, bone marrow transplantation has not been shown to correct the central nervous system (CNS) disease in MPS III patients. The success of long-term enzyme replacement in MPS I offers promise for the treatment of somatic disease in MPS [4]. However, new treatment approaches are needed to restore NaGlu activity in the CNS, and to prevent and control the neurological degeneration in MPS IIIB patients.

The genomic structure of NaGlu has been identified and the cDNA encoding NaGlu has been isolated [5,6]. A mutant mouse model for MPS IIIB has been generated [7], which provides a tool for developing novel therapies and studying the precise process of the disease.

The adeno-associated virus (AAV) is a widespread parvovirus having no known associated pathology in human, although most human tissues are susceptible to the virus [8]. In the recombinant AAV (rAAV) vector system, 96% of the viral genome is removed, leaving only the *cis* terminal repeats that mediate DNA replication and packaging [9,10]. *In vivo* rAAV-mediated gene transfer has resulted in long-term and efficient gene expression in many tissues, including muscle, lung, liver, retina, and CNS [11–19]. Previous studies have shown the expression of β-glucuronidase (GUSB) and the correction of GAG storage in

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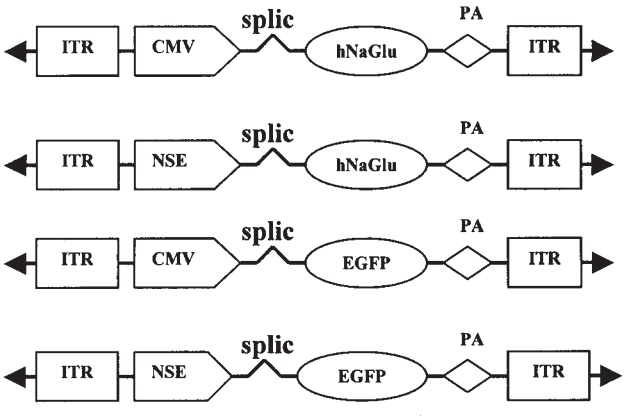

FIG. 1. Schematic diagram of recombinant AAV vectors. ITR, inverted terminal repeat of AAV; CMV, human cytomegalovirus immediate early promoter; NSE, neuron-specific enolase promoter; hNaGlu, human α-*N*-acetylglucosaminidase cDNA; splic, SV_{40} splicing site; PA, SV_{40} polyadenylation site; EGFP, enhanced green fluorescent protein gene.

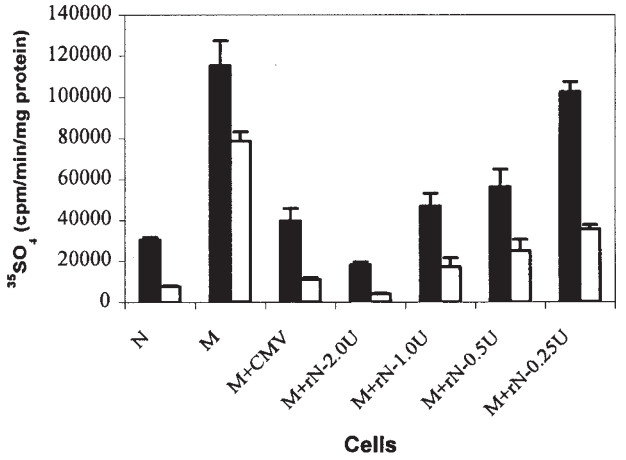

FIG. 2. Uptake of $^{35}SO_4$ in human skin fibroblast cell cultures. N, normal fibroblasts; M, MPSIIIB fibroblasts; CMV, infection with AAV-CMV-hNaGlu; rN, incubation with rNaGlu; 2.0U, 2.0 U/ml; 1.0U, 1.0 U/ml; 0.5U, 0.5 U/ml; 0.25, 0.25 U/ml, in the medium of transduced MPS IIIB cells; filled square, 72 hour labeling; open square, 24 hours after the removal of the labeling medium. The amount of rNaGlu added to the medium is based on NaGlu activity, expressed as unit (U)/ml: 1 U = 1 nmol 4MU released/hour. *n* = 4.

multiple organs/tissues of a mouse model of MPS VII, using rAAV vectors encoding human GUSB [5,20,21].

Here, we constructed AAV vectors containing human NaGlu (hNaGlu) coding sequence cDNA, and driven by a cytomegalovirus (CMV) promoter or a neuron-specific enolase (NSE) promoter (Fig. 1). To investigate the feasibility of AAV-mediated gene therapy for the neurological disease in MPS IIIB using a mutant mouse model, we delivered the viral vectors into different brain structures/areas of adult MPS IIIB mice by direct microinjection. Analysis of vector-treated animals demonstrated AAV-mediated expression of recombinant NaGlu and the correction of GAG accumulation in mouse brain for 6 months (the duration of the experiments).

RESULTS

Expression and Secretion of Functional rNaGlu *in Vitro*

To test AAV-mediated gene therapy for MPS IIIB, we generated AAV-NaGlu viral vectors and infected both human MPS IIIB fibroblasts and/or MPS IIIB mouse brain primary cell cultures (50–100 viral particles/ml in growth medium). NaGlu activity above the level of that in normal human (7.1 U/mg protein in cells, 3.6 U/ml in medium) was detected in the cell lysates (14 U/mg protein) as well as in the medium (6.0 U/ml) of human MPS IIIB fibroblasts transduced with AAV-CMV-hNaGlu (Table 1). MPS IIIB mouse brain primary cell cultures transduced with AAV-CMV-hNaGlu also showed higher NaGlu activity in the cells (3.7 U/mg protein) and in medium (1.6 U/ml; Table 1) than that in the cells (1.2 U/mg protein) and medium (1.2 U/ml) of normal mouse brain cells. The NaGlu activity was also expressed in both the cells (1.2 U/mg protein) and the medium (1.0 U/ml) of MPS IIIB mouse brain primary cell cultures when infected with AAV-NSE-hNaGlu vectors, and was higher than that in normal mouse brain cell cultures (Table 1). The enzyme activity persisted at the same levels for the 5-week duration of the experiments in AAV-transduced MPS IIIB mouse brain primary cell cultures.

TABLE 1: AAV2-mediated *in vitro* NaGlu gene expression in human MPS IIIB fibroblasts and MPS IIIB mouse brain primary cell cultures

Cells	Vector	*n*	[a]NaGlu activity	
			cellular	medium
GM00969D	none	4	7.1 ± 0.18	3.6 ± 0.17
GM02931	none	4	0.0	0.3 ± 0.0
	CMV	4	14.0 ± 0.32	6.0 ± 0.22
$B^{+/+}$	none	3	0.9 ± 0.08	1.2 ± 0.13
$B^{-/-}$	none	3	0.0	0.3 ± 0.08
	CMV	3	3.7 ± 0.16	1.6 ± 0.16
	NSE	3	1.2 ± 0.08	1.0 ± 0.14

[a]The NaGlu activities were means ± SD (standard deviation). NaGlu activity was expressed as U/mg protein for cellular samples or U/ml for medium samples: 1U = 1 nmol of 4MU released/hour or 1 U/ml = 1 nmol of 4MU released/hour.

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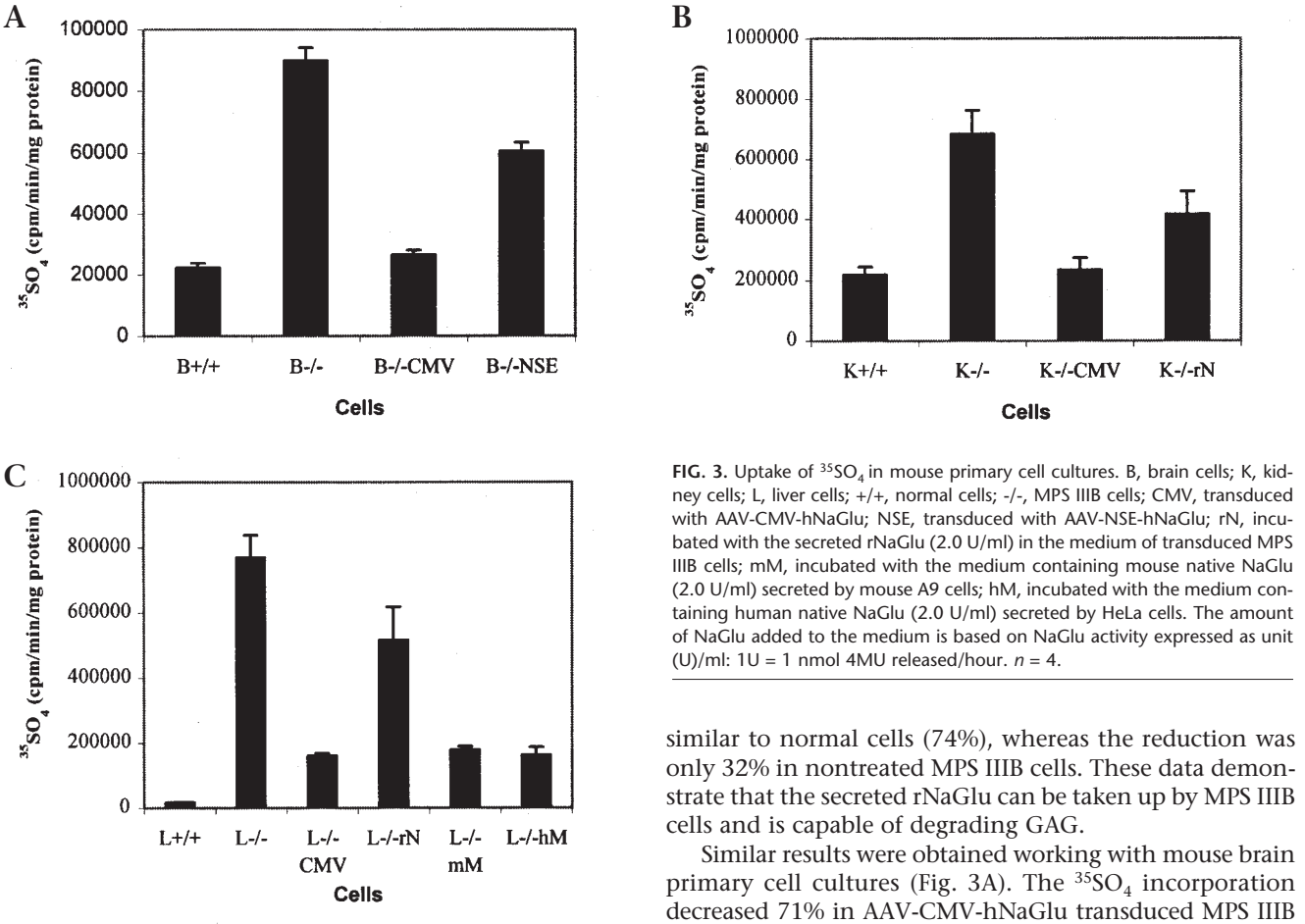

FIG. 3. Uptake of $^{35}SO_4$ in mouse primary cell cultures. B, brain cells; K, kidney cells; L, liver cells; +/+, normal cells; -/-, MPS IIIB cells; CMV, transduced with AAV-CMV-hNaGlu; NSE, transduced with AAV-NSE-hNaGlu; rN, incubated with the secreted rNaGlu (2.0 U/ml) in the medium of transduced MPS IIIB cells; mM, incubated with the medium containing mouse native NaGlu (2.0 U/ml) secreted by mouse A9 cells; hM, incubated with the medium containing human native NaGlu (2.0 U/ml) secreted by HeLa cells. The amount of NaGlu added to the medium is based on NaGlu activity expressed as unit (U)/ml: 1U = 1 nmol 4MU released/hour. *n* = 4.

Intracellular and Extracellular Activity of AAV-Mediated rNaGlu

We carried out $^{35}SO_4$-labeling experiments to study the correction of GAG storage in MPS IIIB cell cultures by AAV-mediated rNaGlu. The accumulation of $^{35}SO_4$-labeled materials in cultured human normal and MPS IIIB fibroblasts, with or without the presence of the AAV-mediated rNaGlu, is summarized in Fig. 2. In corporation of $^{35}SO_4$ in human MPS IIIB fibroblasts decreased 65%, to the level of that in normal cells, when infected with AAV-CMV-hNaGlu, indicating that the recombinant enzyme degraded the sulfated materials in these cells. Human MPS IIIB fibroblasts incorporated 84%, 59%, or 49% less $^{35}SO_4$, respectively, compared with untreated cells, when incubated with medium containing 2.0 U/ml, 1.0 U/ml, or 0.5 U/ml of rNaGlu secreted by AAV-CMV-hNaGlu transduced MPS IIIB cells. When treated with only 0.25 U/ml of rNaGlu, the decrease of $^{35}SO_4$ incorporation in human MPSIIIB fibroblasts was 11% (rNaGlu activity needed to achieve half-maximal correction was 0.6 U/ml in medium). Twenty-four hours after withdrawal of $^{35}SO_4$ from the medium, MPS IIIB cells transduced with AAV vectors or treated with rNaGlu displayed 60–90% reduction in $^{35}SO_4$-labeled materials, similar to normal cells (74%), whereas the reduction was only 32% in nontreated MPS IIIB cells. These data demonstrate that the secreted rNaGlu can be taken up by MPS IIIB cells and is capable of degrading GAG.

Similar results were obtained working with mouse brain primary cell cultures (Fig. 3A). The $^{35}SO_4$ incorporation decreased 71% in AAV-CMV-hNaGlu transduced MPS IIIB mouse brain cells to the level of that seen in normal mouse brain cell cultures. The decrease for AAV-NSE-hNaGlu transduced MPS IIIB mouse brain cells was 33% compared with that in nontransduced MPS IIIB mouse brain cells. In MPS IIIB mouse kidney primary cell cultures, the transduction with AAV-CMV-hNaGlu reduced the $^{35}SO_4$ incorporation by 71% to the normal level, and secreted rNaGlu (1.5 U/ml) decreased $^{35}SO_4$ incorporation by 40% (Fig. 3B) compared with that in nontreated MPS IIIB cells.

We also conducted $^{35}SO_4$ labeling using MPS IIIB primary liver and skin fibroblast cell cultures in the presence of native human NaGlu (from HeLa cells) or native mouse NaGlu (from mouse A9 cells), to determine whether the species difference affected the uptake of the enzyme and the function of NaGlu in the cells. It was shown that both human and mouse NaGlu (2–3 U/ml in the medium) were taken up by the mouse primary liver and skin fibroblasts and reduced the accumulation of $^{35}SO_4$ in the cells to a similar level (Fig. 3C).

In Vivo Expression of Functional rNaGlu

Both AAV-CMV-hNaGlu and AAV-NSE-hNaGlu vectors were microinjected into the inferior colliculus of MPS IIIB mice (0.1 μl/min, 1 μl/site/injection, 10^7 viral particles/μl).

TABLE 2: Expression of rNaGlu in different brain structures of MPS IIIB mutant mice after microinjection of rAAV vectors

Brain sample	*n*	Vectors	Weeks (pi)	[b]NaGlu activity	
				range	mean
Normal	4	none		0.6–1.0	0.9
MPS IIIB	4	none		0.0	0.0
IC	4	CMV	4	3.6–96.7	9.4
IC	1	CMV	12	26.8	
IC	2	NSE	4	93.5–98.6	96.0
IC	4	NSE	12	11.7–56.4	27.2
HIP	7	NSE	4	3.1–51.4	11.6
HIP	9	NSE	12	1.1–49.1	41.5
HYT	5	NSE	4	5.9–149.5	14.6
HYT	7	NSE	12	15.5–82.9	56.6
CTX-1[a]	1	NSE	4	0.7	
-2				2.7	
-3				2.8	
CTX-1[a]	4	NSE	12	0.1–0.3	0.2
-2				0.2–3.6	1.3
-3				0.5–8.0	2.2
THL-1[a]	4	NSE	4	3.2–4.3	3.8
-2				0.2–0.5	0.3

[a]These samples were divided into 2–3 sections due to relatively larger sizes and each section was assayed for NaGlu activity individually.
[b]NaGlu activity was expressed as U/mg protein: 1U = 1 nmol of 4MU released/hour.

AAV-NSE-hNaGlu was also injected into four other brain structures/areas of MPS IIIB mice: hippocampus, hypothalamus, cerebral cortex, and thalamus. These vectors mediated efficient rNaGlu expression in all injected brain structures that persisted for the 12-week duration of the experiments. In total, 48 mice were injected and analyzed (Table 2). The injected cortices and thalamic areas demonstrated relatively lower NaGlu activities, due to the relatively larger size of these structures compared with inferior colliculus, hippocampus, and hypothalamus. They were therefore divided into two or three sections, which were assayed for the NaGlu activity separately. The sections closer to the injection sites demonstrated higher enzyme activities (Table 2).

AAV Tropism for Neurons

We used two vectors expressing enhanced green fluorescent protein (EGFP), AAV-CMV-EGFP and AAV-NSE-EGFP (Fig. 1), to visualize the distribution of AAV-mediated gene expression in mouse brain after microinjection. Results from these experiments help model AAV-hNaGlu gene transfer and expression that we observed in prior experiments (Fig. 4). When 1 μl (10^7 particles) of AAV-NSE-EGFP viral vector was microinjected into different brain sites of the 4-week-old mice, EGFP was detected, 4 weeks after infusion, in the neurons (including large multipolar neurons) of the injected inferior colliculus, hippocampus, hypothalamus, cortex, and thalamus (Figs. 4B–4F). No obvious decrease in EGFP expression was observed after the 3-month duration of the experiments, supporting long-term expression, as previously described with these vectors. EGFP presented throughout neuron cell bodies, in an area of 400–500 μm surrounding the infusion site, and in axons farther away from the injection site, some of which even extended to some areas on the contralateral side of the brain. The AAV-CMV-EGFP vector resulted in similar expression (data not shown) in these brain sites 4 weeks after injection (longer observation with this vector was not done). Consistent with previous observations [12,13,18], no obvious sign of toxicological reaction was observed in the injected mice and no apparent cell damage was found in the areas of the recombinant gene expression following the delivery of AAV vectors by a single microinjection.

Correction of Lysosomal Storage in MPS IIIB Mouse Brain

To study AAV-mediated correction of lysosomal storage in MPS IIIB mouse brain, 1 μl of AAV-NSE-hNaGlu vectors (10^7 viral particles/μl) was injected into the thalamus (Franklin and Paxnos coordinates: Bregma –2.0 mm, lateral right 1.2 mm, down 3.0 mm) over a period of 10 minutes. The animals (four per experimental group) were sacrificed at 3 and 6 months after the injection, and cryostat brain sections were stained with toluidine blue. An obvious decrease in the number and the sizes of swelling lysosomes was observed in the neurons of thalamic nuclei on the injected sides of MPS IIIB mouse brains, compared with that in their contralateral structures (Fig. 5). The decrease of lysosomal storage occurred in the areas approximately 1.5 mm surrounding the injection site and persisted at least 6 months after microinjection. No obvious decrease of lysosomal storage in the neurons in the noninjected contralateral area was observed, compared with that of noninjected MPS IIIB mouse brains.

DISCUSSION

The rAAV therapeutic vectors used in this study contain only minimal regulatory elements required for NaGlu gene expression genes (Fig. 1). We demonstrated that these AAV viral vectors successfully delivered a human NaGlu gene *in vitro* into human MPS IIIB fibroblasts and MPS IIIB mouse brain primary cell cultures, and mediated above-normal levels of rNaGlu expression. The AAV-mediated recombinant NaGlu is functional and secreted. The secreted enzyme can be taken up by both human and

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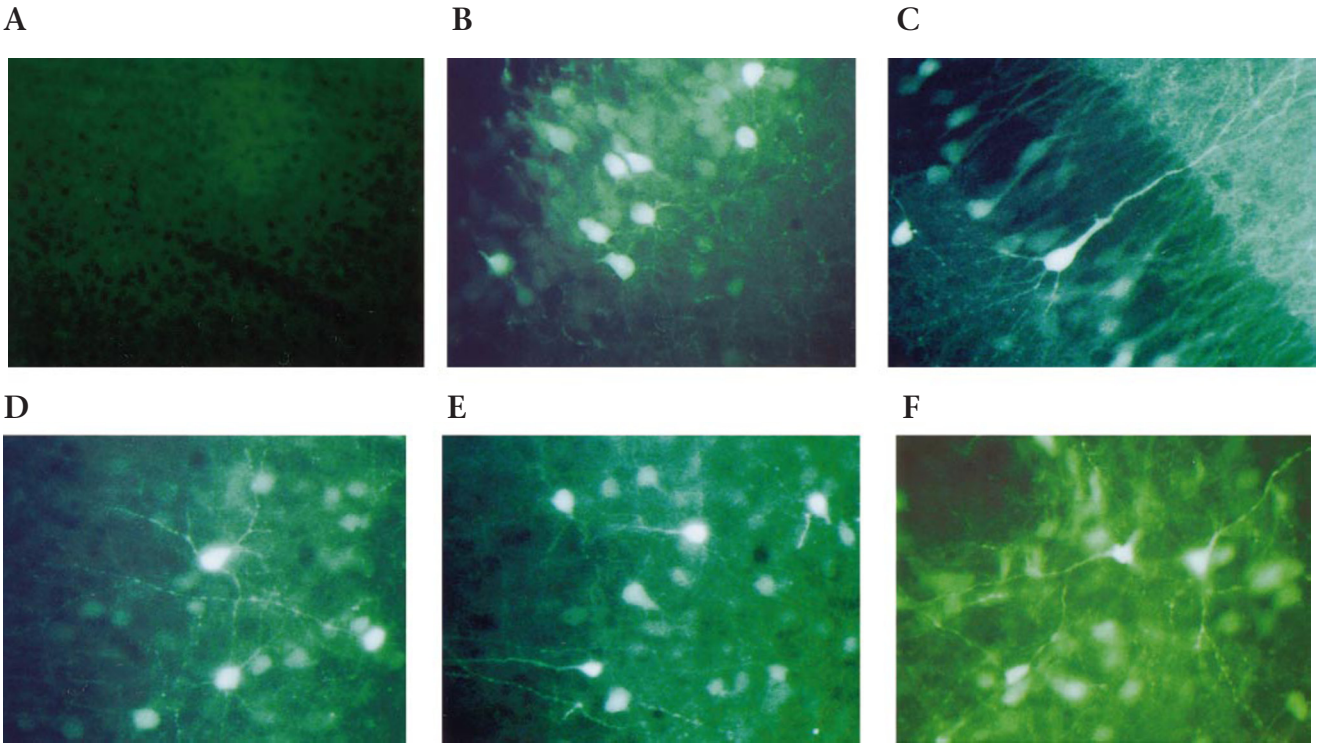

FIG. 4. EGFP expression in different structures/sites of mouse brains after a single injection of AAV-NSE-EGFP. (A) Nontransduced cerebral cortex. (B) Inferior colliculus. (C) Hippocampus. (D) Hypothalamus. (E) Cerebral cortex. (F) Thalamus.

mouse MPS IIIB somatic cells and mouse brain cells *in vitro*, resulting in the correction of GAG accumulation in the cells using only minimal amounts of enzyme (0.5–2.0 U/ml in the medium). It is consistent with previous studies that only very low levels (0.1–10% of normal) of lysosomal enzymes are needed to maintain normal cell function [22–26]. This suggests that expression and secretion of hNaGlu mediated by rAAV may meet therapeutic needs in deficient cells exposed to the enzyme.

The full-length human NaGlu cDNA was also successfully delivered into multiple sites/areas of adult MPS IIIB mouse brain using rAAV viral vectors. These vectors, driven by a NSE promoter, mediated efficient long-term expression of enzymatically active recombinant human NaGlu in all injected mouse brain structures/areas. In mouse brain, the main target cells of AAV vectors seem to be neurons. Using AAV-EGFP reporter vectors, we observed that the transduction after microinjection was limited to the neurons surrounding the injection sites within the injected structures/areas. However, a long-term (6-month duration of the experiments) correction of lysosomal storage in a larger area, a bystander effect, was achieved by a single injection in the MPS IIIB mouse brain. This suggests that *in vivo* correction of GAG storage in MPS IIIB mouse brain does not require transducing all deficient cells. We anticipate that MPS IIIB mouse brain cells transduced by AAV viral vectors can produce and secrete rNaGlu persistently *in vivo*. The secreted rNaGlu may then defuse through brain tissue, be taken up by surrounding neurons, and play the role of degrading accumulated heparan sulfate, not only in transduced cells but also in non-transduced neighboring cells, thus preventing further GAG storage. In addition, we have observed by electron microscopy that enlarged lysosomes are present in axons, while working with MPS II mouse brain samples (H.F., unpublished data). This suggests that lysosomal enzymes may be transported from the neuronal cell body to distal areas of the cell, through axons to the areas remote to the injection sites, even possibly to the contralateral sphere of the brain. In previous studies, complete reversion of lysosomal storage lesions was observed in most neurons in broad brain areas after a single injection of AAV viral vector into the striatum [14] or cerebral cortex [17] of adult MPS VII mice.

Pathological changes of lysosomal storage in the MPS IIIB mouse brain are ubiquitous under the light microscope. However, the morphology of the aberrant storage varies and can be difficult to visualize in the neurons in some brain areas (H.F., unpublished data). The thalamus is a relatively large structure and exhibits the typical microscopic pathology of lysosomal storage, enlarged lysosomes in the cytoplasm of the neurons in most thalamic nuclei of MPS IIIB mouse brain. Therefore it is an ideal model area

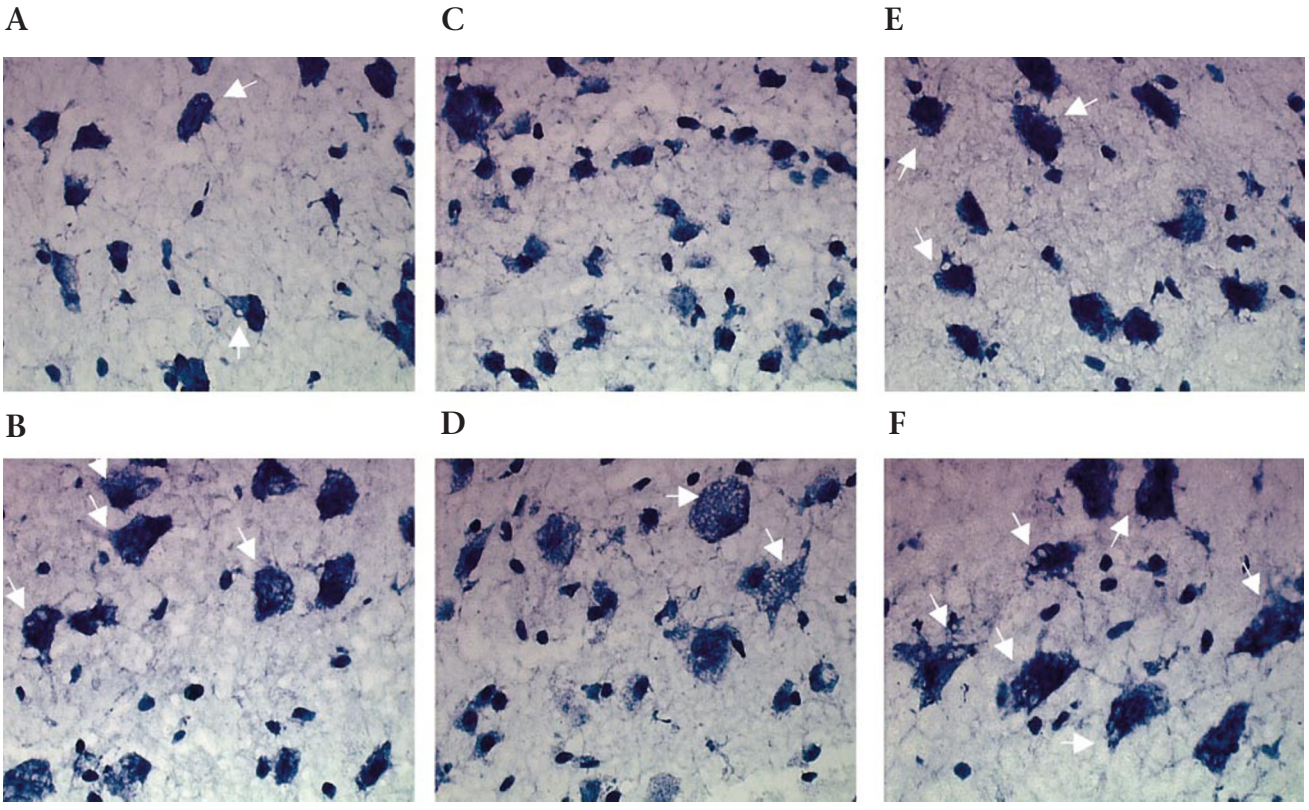

FIG. 5. *In vivo* correction of lysosomal storage in thalamic nuclei of MPSIIIB mouse brain by a single microinjection of AAV-NSE-hNaGlu: post-thalamic nuclear (A), ventral posteromed thalamic nuclear (C), and red nuclear (E) of injected thalamus, and their non-injected counterlateral nuclei (B, D, and F). The arrows indicate lysosomes in the cytoplasm of neurons.

for studying the correction of lysosomal storage in the CNS of MPS IIIB mice, using light microscopy.

Previous studies showed that intrathecal and intravenous delivery of AAV vectors containing a μ-glucuronidase gene into neonatal MPS VII mice resulted in the complete correction of GAG storage in the CNS, which carried to their adulthood [15,16,27]. However, in human the blood–brain barrier is closed before birth. Like other lysosomal enzymes, peripheral administered NaGlu would not be expected to cross the blood–brain barrier. Intravenous delivery of AAV vectors to the brain of patients with MPS would require disruption of the blood–brain barrier. We demonstrated here that direct delivery of the human NaGlu gene into adult MPS IIIB mouse brain can restore the NaGlu function in the injected and a limited neighboring area, by single injection of 1 μl of AAV vectors.

Our study also demonstrated that uptake of NaGlu is not species-specific and that both human and mouse NaGlu can efficiently catabolize the GAG accumulated in MPS IIIB mouse primary cell cultures. It supports the use of the MPS IIIB mutant mouse model as a tool for therapeutic studies of human MPS IIIB.

The MPS IIIB mutant mouse seems to be a useful model for gene therapy studies on neurological disorders of human MPS. Our studies suggest that recombinant AAV vectors can successfully deliver the NaGlu gene and restore the NaGlu activity in the CNS of adult MPS IIIB mice. However, more efficient means to obtain global brain delivery of the AAV viral vectors need to be developed.

MATERIALS AND METHODS

Construction of AAV-NaGlu expression plasmids. A plasmid containing human NaGlu coding sequence cDNA (pCMVhuNAGLU) was provided by Elizabeth F. Neufeld (UCLA). Two AAV serotype 2 (AAV2) derived plasmids, pTR-UF5 (containing a CMV promoter) and pTR-UF4 (containing a NSE promoter), were obtained from Nicholas Muzyzcka (University of Florida). A plasmid containing EGFP (pEGFP-N1) was obtained from Clontech.

Two AAV2 viral vector plasmids expressing hNaGlu have been constructed in our laboratory. A 2.5-kb *Eco*RI fragment of pCMVhuNAGLU, hNaGlu coding sequence cDNA, was inserted into the 4.2-kb *Not*I-*Sal*I fragment of pTR-UF5 or the 5.4-kb *Hin*dIII-*Not*I fragment of pTR-UF4 by blunt-end ligation, to construct pAAV-CMV-hNaGlu and pAAV-NSE-hNaGlu respectively.

Similarly, the pAAV-NSE-EGFP plasmid was constructed in our laboratory by blunt-end ligation of the EGFP gene, a 0.7-kb *Bsp*120I-*Not*I fragment of pEGFP-N1 (Clontech, 6085-1), with *Hin*dIII-*Not*I fragment of pTR-

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UF4. A pAAV-CMV-EGFP (Fig. 1) plasmid was provided by Douglas M. McCarty (Gene Therapy Center, University of North Carolina at Chapel Hill). These EGFP vectors were used to visualize AAV-mediated gene expression as a model for the expression of recombinant NaGlu (rNaGlu).

Production of rAAV viral vectors. The AAV viral vectors were produced in HEK293 cells using cotransfection and purified using heparin affinity chromatography by the Vector Core Facility, UNC Gene Therapy Center, following published methods [28].

These viral vectors contain either the hNaGlu or the EGFP gene, and the AAV2 terminal repeats, SV40 splice donor/acceptor, and SV40 polyadenylation (poly(A)) signals, driven by either a CMV or a NSE promoter. The structures of these AAV viral vectors are illustrated in Fig. 1.

Animals. The heterozygous breeding pairs (F2) of the MPS IIIB knockout mouse were provided by Elizabeth F. Neufeld (UCLA) [7]. The mice were bred on an inbred background by backcrosses of heterozygotes and maintained in the Laboratory Animal Facility of University of North Carolina at Chapel Hill. All the procedures were approved by IACUC at the university. All care and procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. (NIH) 85-23). The progeny mice were identified by PCR amplification of a 300-bp fragment of hNaGlu exon 6 and a 480-bp fragment of the neomycin gene.

Cell and tissue cultures. A human normal fibroblast cell line (GM00969D) and a human MPS IIIB fibroblast cell line (GM02931) were obtained from the NIGMS Human Genetic Mutant Cell Depository, NJ. They were grown at 37°C in MEM medium containing 10% fetal bovine serum (FBS).

Normal and MPS IIIB mouse brain primary cell cultures were generated using published methods [29]. Neonatal mice less than 24 hours old were identified by PCR as mentioned above and anesthetized with 2.5% Avertin (0.38–0.43 mg/g body weight). The cortices were collected using sterile procedures and used as the source of brain primary cell cultures. The cells were incubated at 37°C in growth medium (DMEM containing 10% FBS).

Similarly, normal and MPS IIIB mouse liver, kidney, and skin fibroblast primary cell cultures were also generated based on published procedures [30]. The liver, kidney, and skin tissues were collected using sterile procedure, dissected into the size of 1 cm^3 and rinsed with PBS (pH 7.2). The cells were dissociated using 0.25% trypsin at 37°C and filtered through a cell strainer. The cells were incubated at 37°C in growth medium (DMEM containing 10% FBS). The cells were mixed cell types.

Infection of MPS IIIB cells with AAV viral vectors. To study the AAV-mediated *in vitro* expression and secretion of rNaGlu, AAV-CMV-hNaGlu viral vectors (50–100 viral particles/cell in growth medium) were used to infect human MPS IIIB fibroblasts (on P-100 plates in 10 ml medium) and MPS IIIB mouse primary brain cell cultures (on P-60 plates in 5 ml medium). AAV-NSE-hNaGlu viral vectors were only used to infect MPS IIIB mouse brain primary cell cultures. The cells and the medium were collected and assayed for NaGlu activity after incubation at 37°C for 48 hours for human fibroblasts and 72 hours for mouse brain cell cultures. The harvested medium containing rNaGlu was stored at –20°C before being used in experiments for the correction of GAG storage in MPS IIIB cells. Normal human fibroblasts, normal mouse brain primary cell cultures, non-transduced MPS IIIB human fibroblasts, and MPS IIIB mouse brain primary cell cultures were used as controls. The FBS used in the experiments was incubated at 56°C for 6 hours, to inactivate the high level of bovine NaGlu in the serum.

Direct microinjection of AAV vectors into the brain of MPS IIIB mice. The stereotaxic (KOPF) microinjection was carried out following published techniques [18], using coordinates from a mouse brain atlas [31]. The mice at age 4 weeks were anesthetized with 2.5% Avertin (0.38–0.43 mg/g body weight) and 1 μl AAV viral vectors (10^7 particles/μl) were infused at a rate of 0.1 μl/min into brain tissues. The viral vectors were always injected into the right-side brain structures, one injection per site per mouse, four mice per experimental group. The injected brain structures were inferior colliculus, hippocampus, hypothalamus, cortex, and thalamus.

The mice, transduced with either AAV-CMV-hNaGlu or AAV-NSE-hNaGlu, were anesthetized with 2.5% avertin and sacrificed at 4 and 12 weeks after injection. The injected brain compartments as well as contralateral structures were collected and assayed individually for NaGlu activity. The whole brain samples were frozen on dry ice in OCT compound and stored at –808C for histopathology studies. Samples of normal and non-injected MPS IIIB mouse brain structures were used as controls.

AAV-CMV-EGFP and AAV-NSE-EGFP were also microinjected into the above brain sites of MPS IIIB mice using the same technique, to visualize AAV-mediated gene expression in mouse brain. Four weeks after AAV-vector infusion, the mice were anesthetized with 2.5% Avertin and perfused transcardially with cold 0.1 M phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). After being fixed in 4% paraformaldehyde overnight, the brains were sectioned (50 μm) using a vibratome. The sections were rinsed with PBS before being mounted on slides and visualized with a fluorescent microscope.

NaGlu enzyme activity assay. The NaGlu enzyme activity was assayed following a published method [32]. The tissue samples were homogenized with a motor-driven pestles before being suspended in sample buffer (0.15 M NaCl containing 0.1% Triton X-100). The cell samples and homogenized tissues were ruptured by freezing/thawing (on dry ice/at 37°C) 3–5 times. The cell debris was discarded by centrifugation at 10,000 rpm. Protein concentration of cells and tissue lysates were determined based on the method of Bradford (Bio-Rad Protein Assay).

The enzyme assay measures 4-methylumbelliferone (4MU), a fluorescent product formed by hydrolysis of the substrate 4-methylumbellireyl-*N*-acetyl-α-D-glucosaminide. The NaGlu activity is expressed as nmol of 4MU released/hour/mg protein for cellular samples and nmol of 4MU released/hour/ml for medium and reported as unit (U): 1 U equal to 1 nmol 4MU released/hour.

$^{35}SO_4$ labeling of GAG in tissue cultures. $^{35}SO_4$-labeling assay was carried out in human fibroblasts and mouse primary brain cell cultures, as described [32], to study the correction of GAG storage by AAV-mediated expression of rNaGlu.

The experiments were carried out at least in triplicate. The cells were grown on P100 tissue culture plates in Ham's F-12 growth medium (10% FBS, heat-inactivated at 568C for 6 hours) 48 hours before the addition of $^{35}SO_4$. The medium was then replaced with labeling medium (F-12 growth medium containing 4 μCi/ml of $H_2{}^{35}SO_4$, ICN), or labeling medium containing AAV-CMV-hNaGlu, AAV-NSE-hNaGlu, rNaGlu secreted by human MPS IIIB fibroblasts transduced with AAV-CMV-hNaGlu, natural human NaGlu (from HeLa cells), or natural mouse NaGlu (from mouse A9 cells). The $^{35}SO_4$-labeled materials were measured in Ecoscint A scintillation fluid and expressed as cpm/h/mg protein.

Histopathology. Histopathology was conducted following standard methods. The fresh whole brain samples of mice were embedded in OCT compound and frozen on dry ice. Thin transverse sections (8 μm) were obtained using a cryostat and fixed with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) at 4°C for 15 minutes and stained with 1% toluidine blue, which does not stain GAGs, at 37°C for 30 minutes. The sections were visualized under a light microscope.

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