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Biochemical evaluation of intracerebroventricular rhNAGLU-IGF2 enzyme replacement therapy in neonatal mice with Sanfilippo B syndrome

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Abstract

Mucopolysaccharidosis IIIB (MPS IIIB, Sanfilippo syndrome type B) is caused by a deficiency in α-*N*-acetylglucosaminidase (NAGLU) activity, which leads to the accumulation of heparan sulfate (HS). MPS IIIB causes progressive neurological decline, with affected patients having an expected lifespan of approximately 20 years. No effective treatment is available. Recent preclinical studies have shown that intracerebroventricular (ICV) ERT with a fusion protein of rhNAGLU-IGF2 is a feasible treatment for MPS IIIB in both canine and mouse models. In this study, we evaluated the biochemical efficacy of a single dose of rhNAGLU-IGF2 via ICV-ERT in brain and liver tissue from *Naglu*$^{-/-}$ neonatal mice. Twelve weeks after treatment, NAGLU activity levels in brain were 0.75-fold those of controls. HS and β-hexosaminidase activity, which are elevated in MPS IIIB, decreased to normal levels. This effect persisted for at least 4 weeks after treatment. Elevated NAGLU and reduced β-hexosaminidase activity levels were detected in liver; these effects persisted for up to 4 weeks after treatment. The overall therapeutic effects of single dose ICV-ERT with rhNAGLU-IGF2 in *Naglu*$^{-/-}$ neonatal mice were long-lasting. These results suggest a potential benefit of early treatment, followed by less-frequent ICV-ERT dosing, in patients diagnosed with MPS IIIB.

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Author Contributions

Conceived and designed the experiments: SK, PID, JDC, RL, BEC

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Keywords

Sanfilippo syndrome type B; mucopolysaccharidosis IIIB; intracerebroventricular enzyme replacement therapy (ICV-ERT); heparan sulfate; neonatal mice

Introduction:

Mucopolysaccharidosis IIIB (MPS IIIB, Sanfilippo syndrome type B; MIM #252920) is an autosomal recessive, neurodegenerative lysosomal storage disease caused by a deficiency in α-*N*-acetylglucosaminidase (NAGLU; EC 3.2.1.50), a lysosomal enzyme that mediates the catabolism of glycosaminoglycan (GAG), heparan sulfate (HS). In patients with MPS IIIB, excess storage of partially degraded HS (intracellularly and extracellularly) in the central nervous system (CNS) leads to the clinical features of the disease. The patient's clinical course can be devastating, with progressive neurological deterioration resulting in dementia, behavioral disturbances, speech, mobility loss, and a decreased lifespan of 20–30 years[1–3]

The gene for human *NAGLU* was first identified in 1996 and since been localized to chromosome 17q21.1.[4] More than 100 genetic variants associated with *NAGLU* deficiency have been identified. Homozygous or compound heterozygous variants in the gene encoding *NAGLU* lead to different phenotypic manifestations of MPS IIIB Extensive allelic heterogeneity is postulated to contribute to the wide spectrum of clinical phenotypes seen in patients with MPS IIIB[2, 5, 6].

Current treatment for patients with MPS IIIB is limited to palliative care[7, 8]. Although hematopoietic stem cell transplantation is an effective option for some patients with different types of MPS (e.g., MPS I, II, VI, VII)[9], it does not prevent neurological deterioration in patients with MPS IIIB[10, 11]. Enzyme replacement therapy (ERT) is effective in some animal models of disease caused by deficiencies in soluble lysosomal enzymes. However, recombinant human NAGLU (rhNAGLU) produced in Chinese hamster ovary (CHO) cells is poorly taken up by cells due to its lack of mannose 6-phosphate (Man6-P)[12–14], which is an important post-translational modification for receptor-mediated endocytosis and targeting to lysosomes[15, 16]. In addition, the development of an effective treatment has been hampered by the need for the treatment compound to cross the blood-brain barrier (BBB) to reach the CNS, where MPS IIIB pathology is most severe[17]. Studies in mice have demonstrated that these issues may be overcome by introducing an insulin-like growth factor 2 (IGF2) motif to intracerebroventricular (ICV) ERT to form an rhNAGLU (rhNAGLU-IGF2) fusion protein[18,19]. The IGF2 motif peptide facilitates rhNAGLU's entrance into the cell and subsequent trafficking to the lysosome in a glycosylation-independent manner. Lysosomal targeting occurs even without Man6-P, as rhNAGLU utilizes a secondary binding site on the Man6-Preceptor[14,19,20]. rhNAGLU-IGF2 has also been shown to be taken up by neurons and astrocytes in *in vivo*18,19 and *in vitro*20,21. One study of adult *Naglu*$^{-/-}$ mice showed that the administration of rhNAGLU-IGF2 into the lateral ventricle resulted in the broad bio-distribution of enzyme, the elimination of disease-specific HS, and a reduction in secondary lysosomal HS accumulation to control levels[18,19]. This therapeutic approach is currently being tested in clinical trials investigating the delivery of rhNAGLU-IGF2 directly to the

lateral ventricle via an implanted ICV reservoir in patients suffering from MPS IIIB (NCT02754076 and NCT03784287). In this study, we further evaluate and monitor the efficacy and bio-distribution of ICV rhNAGLU-IGF2 administered to neonatal *Naglu*$^{-/-}$ mice. We found that NAGLU enzyme activity was still detectable in brain homogenates 12 weeks after a single initial injection from birth. HS accumulation and secondary elevation of β-hexosaminidase enzyme activity were also in the normal range in treated animals 4 weeks post-treatment. Moreover, ICV rhNAGLU-IGF2 led to NAGLU activity in liver that persisted up to 2 weeks post-treatment, and the normalization of secondary elevation of β-hexosaminidase in the liver was sustained for 4 weeks post-treatment, indicating the potential for some systemic effects following CNS delivery of this lysosomal enzyme. Our results suggest some prospective advantage for an early administration of treatment in order to lower the initial burden of storage accumulation and subsequently to administer less frequent ICV-ERT dosing.

Results:

NAGLU enzyme activity is stable and can be detected up to 12 weeks after a single dose of rhNAGLU-IGF2 given ICV at birth.

We first evaluated NAGLU enzyme activity in brain homogenate from *Naglu*$^{-/-}$ mice 2, 4, 8 and 12 weeks following a single dose of neonatally delivered ICV-ERT with rhNAGLU-IGF2, for comparison with samples from heterozygous littermates and untreated *Naglu*$^{-/-}$ age-matched controls. Mean levels of NAGLU enzyme activity in whole-brain homogenate from *Naglu*$^{+/-}$ carrier controls was 0.090–0.112 units/mg protein; as expected, there was no detectable NAGLU enzyme activity in untreated *Naglu*$^{-/-}$ mice. In ERT-treated *Naglu*$^{-/-}$ mice, mean NAGLU enzyme activity was 26.1 times higher (range, 0.942–43.0 times higher, n=3) than that in age-matched carrier controls at 2 weeks, 12.2 times higher (range, 3.60–20.9 times higher, n=3) at 4 weeks, 1.69 times higher (range, 0.219–2.87 times higher, n=4) at 8 weeks, and 0.75 times higher (range, 0.29–1.38 times higher, n=7) at 12 weeks (Fig. 1A). The calculated *in vivo* half-life of catalytic enzyme activity for rhNAGLU-IGF2 in mouse brain was 12.06 days (Fig. 1B).

Complete clearance of pathological HS after a single dose of rhNAGLU-IGF2 given ICV at birth

Elevated levels of total HS and disease-specific HS chain non-reducing ends (NRE) were detected in brain homogenate from untreated *Naglu*$^{-/-}$ mice, compared to age-matched *Naglu*$^{+/-}$ carrier controls, as early as 2 to 4 weeks of age. In *Naglu*$^{-/-}$ mice treated with ICV-ERT, total HS levels in brain decreased to normal levels, with no significant difference between ERT-treated *Naglu*$^{-/-}$ mice and carrier controls but a significant decrease ($p<0.001$) compared to untreated *Naglu*$^{-/-}$ mice at 2 and 4 weeks post-treatment. NRE were not detected in brain homogenate from ICV-ERT–treated *Naglu*$^{-/-}$ mice or carrier controls at 2 or 4 weeks post-treatment (Fig. 2).

Secondary elevation of ß-hexosaminidase activity in Naglu$^{-/-}$ mouse brain but not in treated mice

β-hexosaminidase is one of the most abundantly expressed lysosomal enzymes, and its activity is invariably increased in *Naglu*$^{-/-}$ mice[18, 19, 22]. A consistent 2.5- to 3-fold increase in β-hexosaminidase enzyme activity was observed in brain homogenate from *Naglu*$^{-/-}$ mutants, compared to age-matched carrier controls, at all time-points up to 12 weeks of age. In contrast, a significant reduction in β-hexosaminidase enzyme activity to levels seen in *Naglu*$^{+/-}$ carriers was observed in *Naglu*$^{-/-}$ mice given a single neonatally administered dose of ICV-ERT (p<0.001 compared to untreated *Naglu*$^{-/-}$ mice, and not statistically different from *Naglu*$^{+/-}$ carrier controls). This effect persisted from 2 to 8 weeks post-treatment. A slight elevation of β-hexosaminidase enzyme activity was observed in ICV-ERT–treated *Naglu*$^{-/-}$ brain, compared to *Naglu*$^{+/-}$ carrier controls, at 12 weeks post-treatment (p<0.01); notably, the levels in ICV-ERT–treated *Naglu*$^{-/-}$brain were 81% lower than in age-matched, untreated *Naglu*$^{-/-}$ mice (p<0.001).

Reversal of the disease-associated elevation in Lamp1 protein expression in Naglu$^{-/-}$ mouse brain after ICV-ERT with rhNAGLU-IGF2

Lysosomal membrane protein-1 (Lamp1) protein expression has been used as a surrogate marker for lysosomal storage burden in *Naglu*$^{-/-}$ mice, with higher levels of Lamp1 indicating greater size/volume of the lysosomal compartment[19]. Elevated Lamp1 protein expression was observed in brain homogenates from *Naglu*$^{-/-}$ mice, compared to heterozygous controls. A significant reduction in the magnitude of this elevated Lamp1 expression was observed in ICV-ERT–treated *Naglu*$^{-/-}$ brain at 4 and 8 weeks post-treatment (p<0.01). However, 12 weeks after a single neonatal dose of ICV-ERT, Lamp1 expression was similar to that detected in age-matched untreated *Naglu*$^{-/-}$ mice (p=0.95).

Effects in liver of a single dose of rhNAGLU-IGF2 administered ICV at birth

The distribution of ICV-delivered rhNAGLU-IGF2 in liver was determined by measuring NAGLU enzymatic activity within this organ. Two weeks after treatment, liver homogenates of ICV-ERT–treated *Naglu*$^{-/-}$ mice showed NAGLU enzyme activity that was 44% of carrier levels. In contrast, no detectable NAGLU activity was measured in liver homogenate from ICV-ERT– treated *Naglu*$^{-/-}$ mice harvested >4 weeks after treatment (Fig. 4A). β-hexosaminidase activity in these treated mutant mice was within normal limits at 2 and 4 weeks after treatment (no significant difference between ICV-ERT–treated *Naglu*$^{-/-}$ mice and *Naglu*$^{+/-}$ carrier controls; p=0.34 and 0.56 for 2 and 4 weeks post-treatment, respectively). In ICV-ERT–treated *Naglu*$^{-/-}$ mice sacrificed 8 weeks after treatment, β-hexosaminidase activity was 36% lower than in age-matched untreated *Naglu*$^{-/-}$ mutant mice (p<0.001), and at 12 weeks post-treatment, there was no statistically significant change in β-hexosaminidase activity.

Discussion:

In this study, measurements of NAGLU enzyme activity in brain homogenate from *Naglu*$^{-/-}$ mice that received a single injection of rhNAGLU-IGF2 at birth showed detectable levels of NAGLU enzyme activity for 12 weeks after treatment. HS accumulation was shown within

normal limits 4 weeks after treatment. Secondary elevation of β-hexosaminidase enzyme activity and Lamp1 protein expression both showed decreased accumulation up to 8 weeks after treatment, and the buildup of these markers were shown at 12 weeks. Moreover, normal levels of NAGLU activity were observed in liver samples from animals that received ICV rhNAGLU-IGF2; this effect persisted for up to 2 weeks post-treatment. Treatment with ICV rhNAGLU-IGF2 also normalized the secondary elevation of β-hexosaminidase in liver; this effect was sustained for 4 weeks post-treatment. These findings indicate the possibility of systemic effects after CNS delivery of this lysosomal enzyme. Our results point to possible advantages of early treatment with rhNAGLU-IGF2 for MSP IIIB. This therapeutic approach may lower the initial HS accumulation burden and subsequently allow for less frequent ICV-ERT dosing.

The ICV delivery of ERT is under active development as a potential therapy for MPS IIIB (NCT02754076 and NCT03784287). This approach delivers NAGLU protein directly to the most severely affected organ. The direct injection of enzyme into the CNS compartment produces protein levels in the brain that are orders of magnitude greater than can be achieved with intravenous ERT, even if the protein has been engineered to aid its transit across the blood-brain barrier. Preclinical studies of ICV administration of the research form of rhNAGLU-IGF2 to adult *Naglu*$^{-/-}$ mice have demonstrated pathological and biochemical treatment efficacy in the brain[18, 19]. In previous studies as well as the current investigation, NAGLU enzyme activity was also detected in liver, and enzyme-treated mutant mice showed lower levels of HS than vehicle-treated controls. It is not surprising that the NAGLU enzyme was able to reach the liver following an ICV injection. The CSF is replaced multiple times per day, so it is expected that therapeutics injected into CSF will also reach the systemic circulation[23]. Our data suggest that for MPS IIIB, which manifests primarily in the CNS, with milder somatic disease, ICV treatment may prove to be sufficient as a single route for effective enzyme replacement.

While previous pre-clinical studies in 16-week-old mice have administered 4 doses of ICV-ERT (100 μg rhNAGLU-IGF2 in 5 μL artificial CSF per dose) within 2 weeks[18, 19], our study administered the same amount of ICV-ERT (100 μg rhNAGLU-IGF2) as a single dose to neonatal mice. If one compares brain weight between humans and mice (1400 g vs. 0.5 g), the dose used in currently ongoing clinical trials is quite similar to the scaling of enzyme dose applied in the pre-clinical studies we have conducted (300 mg vs. 0.1 mg)[24, 25]. In this study, we demonstrated biochemical therapeutic efficacy following a single dose of rhNAGLU-IGF2, given ICV to neonatal *Naglu*$^{-/-}$ mice. The therapeutic effect persisted in brain tissue for up to 12 weeks, and in liver tissue for up to 4 weeks. One possible explanation is that earlier treatment led to a dose/body weight ratio that was higher than in previous studies, such that a relatively higher initial dose was administered in this study. The estimated *in vivo* half-life of rhNAGLU-IGF2 in brain is 12.06 days, which is slightly longer than the 7-day half-life that we previously reported in a study of adult mice[19]. In any case, the duration of the therapeutic effect was much longer than would be predicted by the half-life of the enzyme, supporting the hypothesis that accumulation of the HS substrate in MPS IIIB occurs slowly. Moreover, given that the accumulation of HS and other lysosomal storage material is progressive, it is reasonable to postulate that early treatment may

attenuate disease over the long term. The lower initial disease burden may also permit lower or less frequent dosing.

There have been two clinical trials of ERT for MPS IIIB to date. In one completed study, patients received rhNAGLU via intravenous (IV) infusion (NCT02618512). This treatment approach provided only a limited reduction in CSF levels of HS and only a minor improvement in neurocognitive function[26]. In another ongoing clinical trial, patients are receiving rhNAGLU-IGF2 via an implanted ICV port and reservoir (NCT02754076). Preliminary data in this trial have shown CSF HS reduction, normalization of liver and spleen sizes, and stabilization or improvement of the developmental quotient (DQ) in most treated patients[24, 25].

With the expanding availability of treatments for lysosomal storage disorders, newborn screening has become more widely adopted as a means to facilitate early therapeutic intervention[8]. Neonatal pathophysiology and the potential implications of early treatment are therefore of greater importance than ever before. Neonatal IV-ERT have been applied in mouse models of MPS I[27, 28], MPS IIIA[29], MPS IVA[30], and MPS VII[31]. Administering such treatments to animal models of MPS during the newborn period is certainly more effective than later interventions, resulting in the absence or near-absence of lysosomal storage disease and, in some cases, improved pathology in hard-to-treat organs such as the heart and bones[27, 30]. Neonatal treatment may also reduce the chances of an immune response to the delivered enzyme. For example, MPS I mice treated with recombinant human alpha-L-iduronidase via intravenous infusion every other week from 60 days after birth developed significantly higher levels of IgG antibodies against iduronidase ($p<0.05$) than mice treated from birth[27]. These findings may be attributed to the induction of immune tolerance at early stages of disease progression, which has also been described in studies of the canine model of MPS I[32]. As such, further quantification of IgG antibodies against the moiety of rhNAGLU in the blood of treated mice will need to be performed to elucidate whether the early introduction of rhNAGLU-IGF2 via ICV-ERT to neonates will similarly trigger immune tolerance. It is possible that the immune tolerance achieved via early treatment administration may even increase the effectiveness of subsequent ICV-ERT treatments.

We found that treatment with ICV rhNAGLU-IGF2 led to significantly lower biochemical disease burden in the CNS in neonatal MPS IIIB mice, compared to untreated mutant mice. Further pre-clinical studies of repeated ICV-ERT treatments in mice will evaluate the long-term efficacy of ICV-delivered rhNAGLU-IGF2. ICV delivery of rhNAGLU-IGF2 may also correct systemic manifestations of the disease, in addition to providing disease attenuation within the CNS. Future research is needed determine whether the benefits of early ICV-ERT in MPS IIIB mice may translate to patients with MPS IIIB.

Material and Methods:

Experimental Animals

The *Naglu*$^{-/-}$ knockout mouse was originally a gift from Dr. Neufeld at UCLA and back-bred onto a C57BL6/J background[22]. This colony has been maintained for at least 10 generations at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical

Center (LA BioMed, now Lundquist Institute). *Naglu*$^{+/-}$ females were crossed with *Naglu*$^{-/-}$ males to obtain homozygous affected (*Naglu*$^{-/-}$) mice and heterozygous (*Naglu*$^{+/-}$) controls. Genotyping was performed at birth with primer set NAG5′: TGGACCTGTTTGCTGAAAGC and NAG3′: CAGGCCATCAAATCTGGTAC for *Naglu* wild-type alleles, and primer set Neo5′: TGGGATCGGCCATTGAACAA; Neo3′: CCTTGAGCCTGGCGAACAGT for *Neor* mutant alleles. Experiments were performed on age-matched mice (usually littermates) of both genders. All animal experiments were approved by the Institutional Animal Care and Use Committee at LA BioMed, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Enzyme Replacement Therapy

For this study, 100 μg of purified rhNAGLU-IGF2 (research form, in 5 μl artificial CSF) was administered into the left cerebroventricles of *Naglu*$^{-/-}$ mice at postnatal day 1 or 2 under cryoanesthesia by Hamilton syringe with 32-gauge needles. The injection site for ICV injection was approximately 2.5 mm between bregma and eyes; 2 mm lateral and 2.5 mm deep to the sagittal suture[33].

Animals were sacrificed at 2, 4, 8, and 12 weeks after the initial administration of ERT. Brains and liver lobes were rapidly dissected and homogenized in PAD buffer (10 mM sodium phosphate, pH 5.8, 0.02% sodium azide, 0.1 mM dithiothreitol, and 0.1% Triton X-100) for biochemical analyses. Age-matched heterozygous (*Naglu*$^{+/-}$) littermates and untreated *Naglu*$^{-/-}$ were used as controls.

Enzyme Activity Assays

The catalytic activity of NAGLU was determined by hydrolysis of the fluorogenic substrate, 4-methylumbelliferyl-*N*-acetyl-α-glucosaminide (MilliporeSigma, Burlington, MA), with a final concentration of 0.1 mM substrate in the incubation mixture, as described previously[14]. The catalytic activity of β-hexosaminidase (combined A and B isoforms) was determined by hydrolysis of 4-methylumbelliferyl-*N*-acetyl-β-glucosaminide (MilliporeSigma, Burlington, MA) using 1.25 mM substrate in the incubation mixture14. A unit of activity was defined as the release of 1 nmol of 4-methylumbelliferone (4MU) per hour at 37°C. Protein concentration was estimated with the Bradford method, using bovine serum albumin (Bio-Rad Laboratories, Hercules, CA) as standard. Fluorescence measurements were obtained using an RF-1501 spectrofluorophotometer (Shimadzu Scientific Instruments, Columbia, MD) with excitation and emission wavelengths of 360 nm and 450 nm, respectively. Enzyme activity is presented as units/mg protein.

Quantification of Heparan Sulfate

An adaptation of the Sensi-Pro assay previously described[34, 35] was used to quantify both total HS and HS NRE specific to MPS IIIB. Tissue samples were homogenized in water in a bead homogenizer, and glycosaminoglycans were isolated by anion exchange chromatography and depolymerized with heparin lyases (IBEX Technologies, Inc., Montreal, Quebec, Canada). Digested samples were end-labeled by reductive amination with the fluorescent tag 2-aminoacridone (AMAC, Sigma-Aldrich, St. Louis, MO). Digestion

products were re-suspended in 10 μL of 0.3 M AMAC in dimethyl sulfoxide:glacial acetic acid (85:15 v/v). Ten microliters of 1 M sodium cyanoborohydride in dimethyl sulfoxide:glacial acetic acid (7:3, v/v) was then added to each sample. Reactions were carried out for 16 hours at 37°C. The reactions were then quenched with 10 μL of 1 M acetic acid, and samples were diluted with water before compositional quantitative analysis with UPLC-FLD.

AMAC-labeled HS digestion products were separated on an Acquity UPLC HSS C18 column (Waters Corporation, Milford, MA). Solvent A was 10 mM ammonium acetate in water. Solvent B was methanol. The initial solvent composition was 100% A at a flow rate of 0.1 mL/min. The column was kept at 50°C. The LC elution gradient was as follows: started at 100% A and immediately ramped to 90% A/10% B over 1 min, then ramped to 77.3% A/22.7% B over 14 min, changed to 20%A/80%B for 6 min; finally, the column was re-equilibrated for 9 min at 100% A. Samples were detected with the fluorescence detector set to an excitation wavelength of 435 nm and emission wavelength of 537 nm. Data analysis was performed with Empower software (Waters Corporation, Milford, MA).

All samples were quantified using an external standard calibration curve ranging from 2 to 125 pmol disaccharide/μL. The curve included six HS internal standards: D0A0, D0A6, D0S0, D2S0, D0S6, and D2S6 (Dextra Laboratories Ltd, Reading, UK) and the MPSIIIB-specific NRE trisaccharide (A0I2S0, synthesized for us by Alberta Innovates NuRx Services, Alberta, Canada). The quantity of disaccharide obtained by lyase digestion is a measure of total HS, whereas the quantity of trisaccharide with *N*-acetylglucosamine at the nonreducing end is a measure of MPS IIIB-specific NRE.

Quantification of Lamp1 Protein Expression

Lamp1 protein expression was detected by western blot and quantified by densitometry. In brief, protein from 10 μg of brain homogenate in PAD buffer was separated by 4–20% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) with a Trans-Blot-Turbo transfer system (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% skimmed milk, and primary antibodies were added to detect the cognate proteins. The following antibodies were used in this study: chicken anti-GAPDH (1:1000; SAB3500247; MilliporeSigma, Burlington, MA); rabbit anti-LAMP1 (1:1000; ab24710; Abcam, Cambridge, MA). After secondary antibodies (1:5000 goat anti-chicken IgY-HRP or goat anti-rabbit IgG-HRP, SouthernBiotech, Birmingham, AL) amplification, blots were developed with Immobilon Western Chemiluminescent HRP substrate (MilliporeSigma, Burlington, MA). Luminescence was detected with a Bio-Rad ChemiDoc MP system (Bio-Rad Laboratories, Hercules, CA), and densitometry was analyzed with BioRad Image Lab Software 6.01 (Bio-Rad Laboratories, Hercules, CA). Lamp1 protein band densities in brain homogenate were normalized to the amount of Gapdh house-keeping protein. Uncropped scans of representative blots are presented in Supplementary Fig. 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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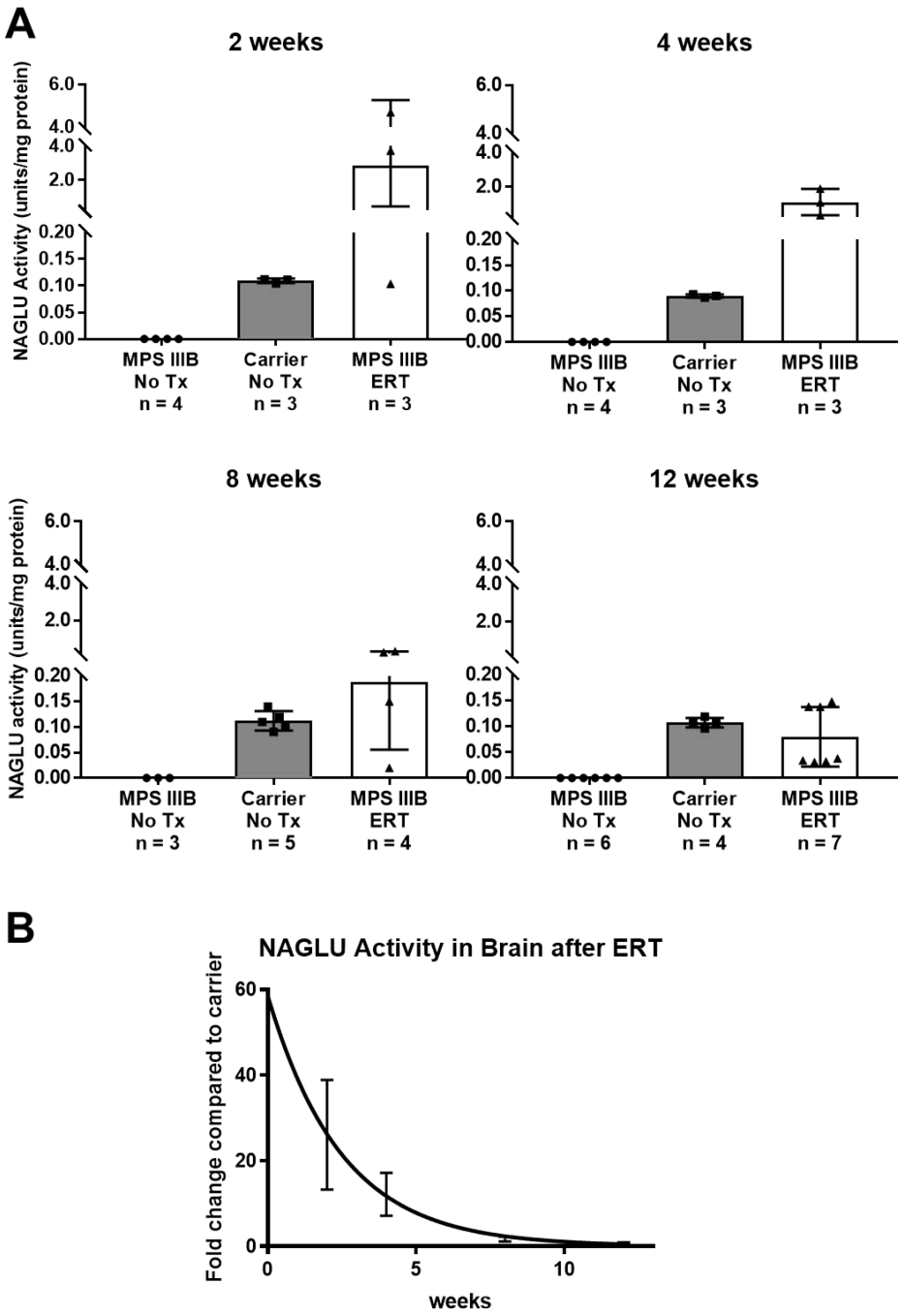

Fig. 1. NAGLU enzyme activity in brain homogenate after a single dose of rhNAGLU-IGF2 (research form) administered ICV at birth.

(A) Mean NAGLU enzyme activity in whole brain homogenate from heterozygous controls ranged from 0.090–0.112 units/mg protein (carrier; grey bars). In contrast, mean NAGLU activity in brain homogenate from ICV-ERT–treated *Naglu*$^{-/-}$ mice at 2, 4, 8, and 12 weeks following neonatal treatment was 2.84, 1.09, 0.19, and 0.080 units/mg protein, respectively (white bars). One unit of NAGLU activity was defined as 1 nmol of converted 4-methylumbelliferyl substrate per hour. Measurements were made in triplicate, expressed as

units per mg protein, and shown as mean ± standard deviation. **(B)** The average fold increases in NAGLU enzyme activity in whole brain homogenate from ICV-ERT–treated *Naglu*$^{-/-}$ mice, compared to heterozygote controls, were plotted versus time since neonatal treatment and expressed as a best fit to a nonlinear exponential decay module using *GraphPad Prism*. The calculated half-life of rhNAGLU-IGF2 is about 1.72 weeks, or ~12.06 days.

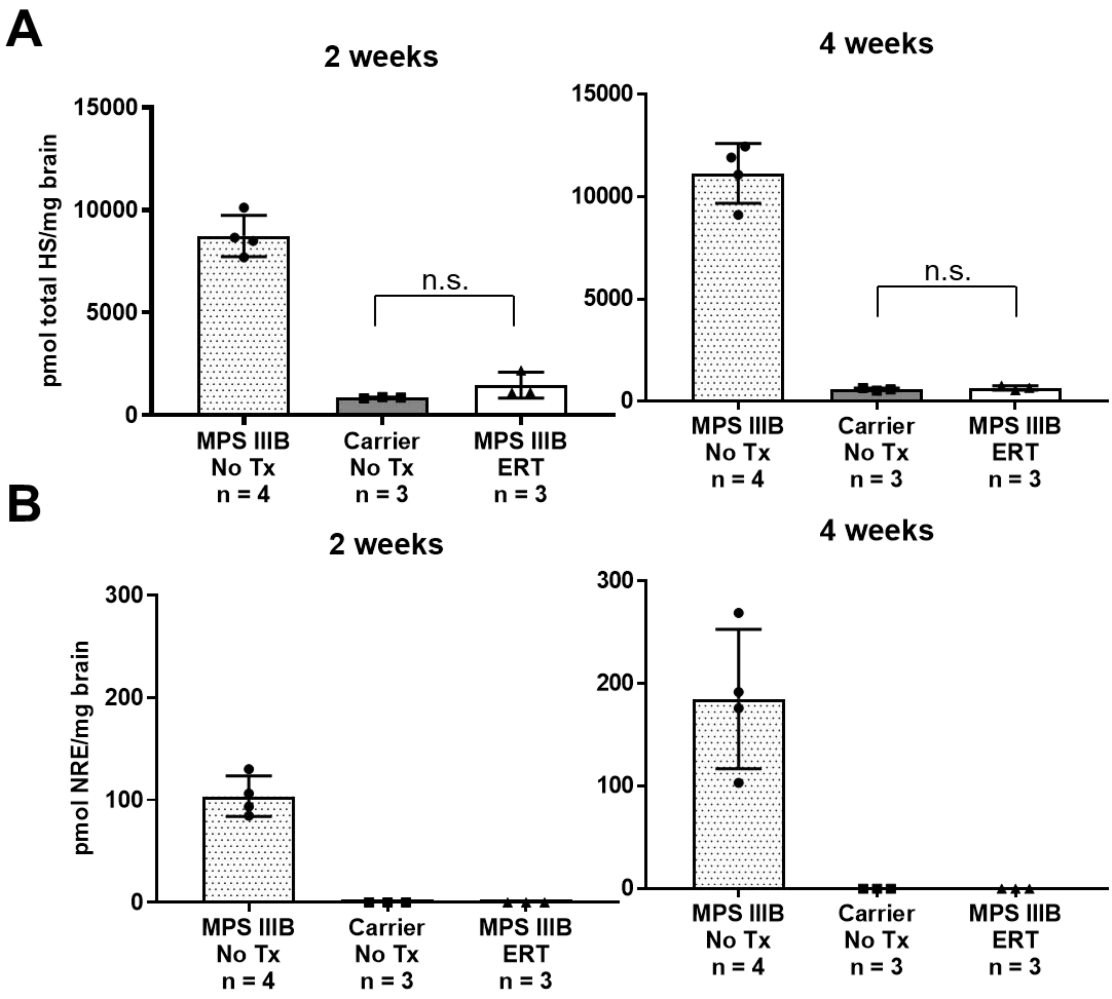

Fig. 2. Heparan sulfate (HS) in brain homogenate from *Naglu*$^{-/-}$ mice after a single dose of rhNAGLU-IGF2 at birth.

(A) Representative total HS levels in whole-brain homogenates from all groups. (B) Levels of HS trisaccharide with an *N*-acetylglucosamine residue at the non-reducing end (NRE) that is specific to MPS IIIB, measured in the same samples. Mouse brain samples used to measure HS at 2 and 4 weeks post-treatment were the same as those used for Fig. 1. Total HS is expressed in picomoles of disaccharide HS per milligram wet weight of brain tissue; the amount of MPS IIIB-specific HS NRE is expressed as picomoles of trisaccharide per milligram wet weight of brain tissue. Values are presented as mean ± standard deviation. n.s., not significant.

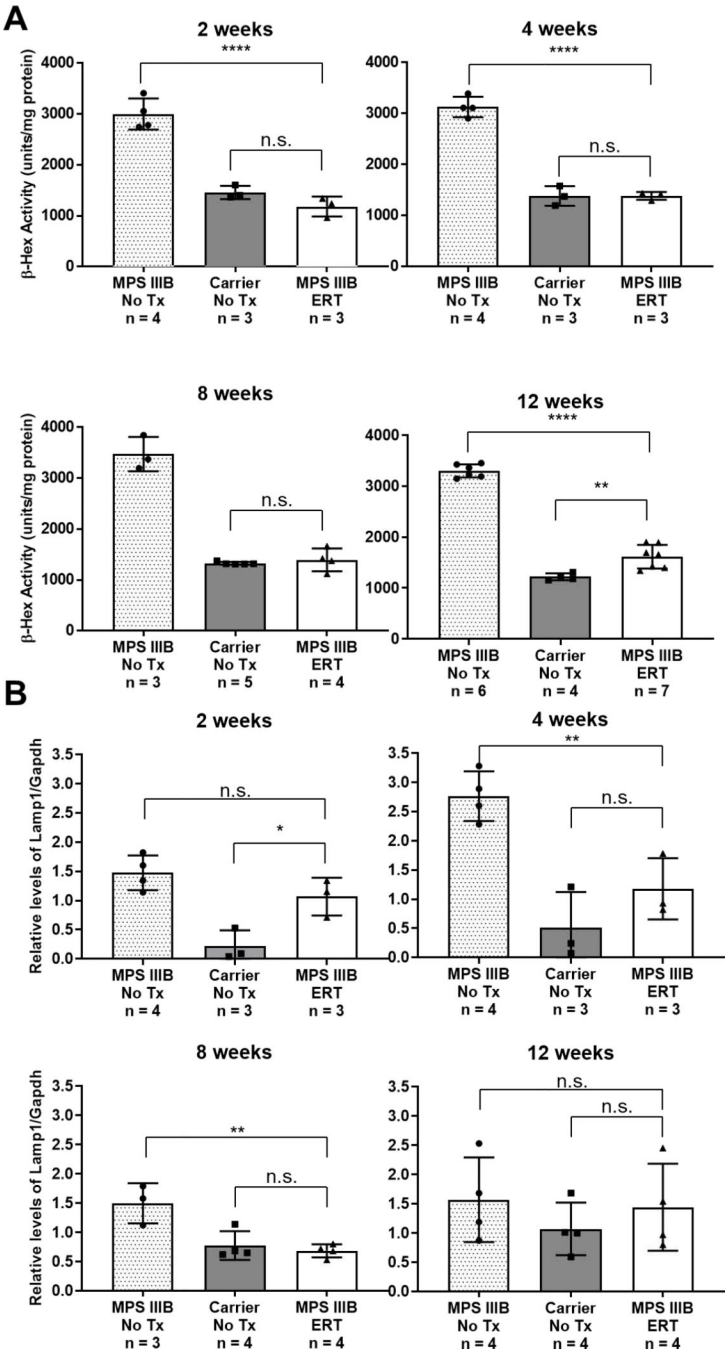

Fig. 3. (A) β-hexosaminidase enzyme activity and (B) Lamp1 expression in brain homogenate from *Naglu*$^{-/-}$ mice treated with a single dose of rhNAGLU-IGF2, administered ICV at birth.

(A)Elevated β-hexosaminidase activity was observed in the brain homogenate of untreated *Naglu*$^{-/-}$ mice (dotted bars), compared to heterozygous age-matched controls (carriers; grey bars). Mouse brain samples used for measurements were the same as those used for Fig. 1 and 2. One unit of β-hexosaminidase enzyme activity was defined as 1 nmol of converted 4-methylumbelliferyl substrate per hour. Measurements were conducted in triplicate. Values are presented as units per mg protein and expressed as mean ± standard deviation. (B) Western blot was performed to measure Lamp1 expression in brain homogenate. Gapdh was

used as a loading control. Protein band signals were quantified, and relative expression levels of Lamp1 were normalized to the level of Gapdh. The data shown are representative of three independently performed experiments. Elevated Lamp1 expression was observed in the brain homogenate of untreated *Naglu*$^{-/-}$ mice (dotted bars), compared to heterozygous controls (grey bars). Reduced Lamp1 protein expression was noted after 4 and 8 weeks in *Naglu*$^{-/-}$ mice treated with ICV-ERT (white bars). Values are presented as relative levels of Lamp1/Gapdh and expressed as mean ± standard deviation. Uncropped scans of representative blots are presented in Supplementary Fig. 1. *p<0.05; **p<0.01; ****p<0.001; n.s., not significant.

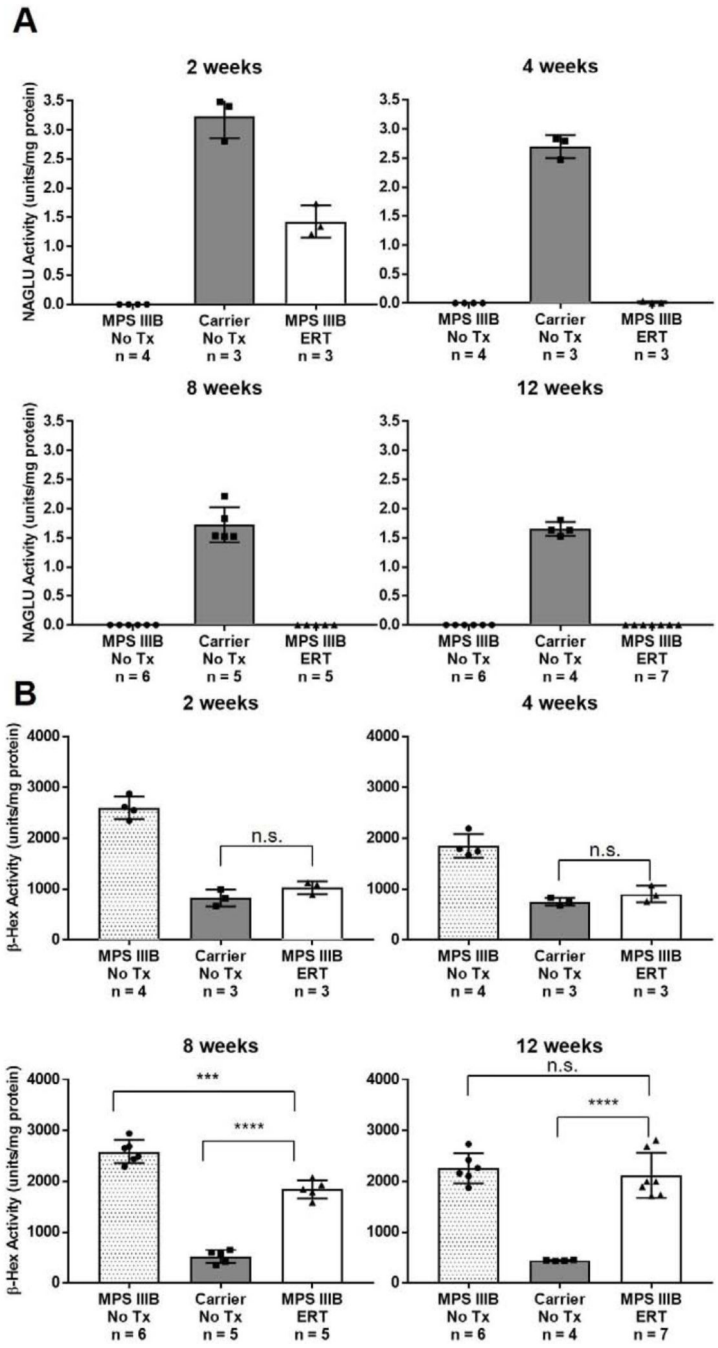

Fig. 4. Liver NAGLU and β-hexosaminidase activity in mice treated with a single dose of rhNAGLU-IGF2 ICV at birth.

Liver levels of NAGLU (A) and β-hexosaminidase (B) activity in *Naglu*$^{-/-}$ mice treated as neonates with a single dose of ICV rhNAGLU-IGF2 (white bars), compared to age-matched, untreated *Naglu*$^{-/-}$ (dotted bars) and *Naglu*$^{+/-}$ controls (grey bars). Liver tissue was harvested from the same animals used for Fig. 1–3. All bars show mean ± standard deviation ***p<0.005; ****p<0.001; n.s., not significant.