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(54) Titre : VECTEURS DERIVES DE VIRUS ADENO-ASSOCIES POUR LE TRAITEMENT DE TROUBLES DU STOCKAGE LYSOSOMAL
(54) Title: ADENOASSOCIATED VIRUS VECTORS FOR THE TREATMENT OF LYSOSOMAL STORAGE DISORDERS

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The present invention provides new adenoassociated virus vectors and pharmaceutical compositions containing the same for the treatment of lysosomal storage disorders and specially, for the treatment of mucopolysaccharidoses Type IIIB.

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(54) **Title:** ADENOASSOCIATED VIRUS VECTORS FOR THE TREATMENT OF LYSOSOMAL STORAGE DISORDERS

(57) **Abstract:** The present invention provides new adenoassociated virus vectors and pharmaceutical compositions containing the same for the treatment of lysosomal storage disorders and specially, for the treatment of mucopolysaccharidoses Type IIIB.



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ADENOASSOCIATED VIRUS VECTORS FOR THE TREATMENT OF LYSOSOMAL STORAGE DISORDERS

FIELD OF THE INVENTION

5

The present invention relates to vectors useful for the expression of proteins of interest and their utilization in gene therapy. The present invention also relates to vectors and nucleic acid sequences helpful for the treatment of mucopolysaccharidoses (MPS), and in particular, for the treatment of

10 mucopolysaccharidoses type III-B or Sanfilippo B syndrome.

BACKGROUND OF THE INVENTION

The lysosome is an organelle found in the cytoplasm of animal cells that

15 contains more than 50 hydrolases that break down biomolecules during the recycling of worn-out cellular components or after the engulfment of viruses and bacteria. This organelle contains several types of hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases and sulfatases. All enzymes are acid hydrolases.

20 Lysosomal storage diseases (LSDs) are caused by genetic defects that affect one or more lysosomal enzymes. These genetic diseases result generally from a deficiency in a particular enzyme activity present in the lysosome. To a lesser extent, these diseases may be due to deficiencies in proteins involved in lysosomal biogenesis.

25 LSDs are individually rare, although as a group these disorders are relatively common in the general population. The combined prevalence of LSDs is approximately 1 per 5,000 live births. *See* Meikle P, *et al.*, JAMA 1999;281:249-254. However, some groups within the general population are particularly afflicted

by a high occurrence of LSDs. For instance, the prevalence of Gaucher and Tay-Sachs diseases in descendants from Jewish Central and Eastern European (Ashkenazi) individuals is 1 per 600 and 1 per 3,900 births, respectively.

Type III mucopolysaccharidoses (MPSIII), known collectively as Sanfilippo syndrome, are LSDs caused by deficiency in one of the enzymes involved in the stepwise degradation of the glycosaminoglycan (GAG) heparan sulfate (HS), leading to its pathological accumulation. MPSIII is classified into four subtypes depending on the enzyme deficiency. Loss of N-acetylglucosaminidase, alpha enzymatic activity causes subtype IIIB. The resulting disease is characterized clinically as a childhood-onset progressive neuropathy of the CNS. The clinical course can generally be divided into three phases. In the first phase of the disease, after a symptom-free period that spans the first months of life, a slowing of mental development becomes apparent. This is followed by severe behavioral problems and progressive intellectual decline during the second phase of the disease. Finally, with the onset of severe dementia, the behavioral problems slowly disappear, and all motor functions start to decline, eventually resulting in dysphagia, complete loss of locomotion and pyramidal tract lesions. In addition to the neurological symptoms, MPSIIIB patients suffer non-neurological alterations, including recurrent ear, nose, throat and chest infections, frequent diarrhea and constipation, progressive joint degeneration and skeletal abnormalities which affect mobility, as well as hepato and splenomegaly. *See* Cleary and Wraith, Arch Dis Child. 1993;69(3):403-6, Neufeld and Muenzer, "The Mucopolysaccharidoses" in Scriver C, *et al.*, Eds., "The metabolic and molecular basis of inherited disease", McGraw-Hill Publishing Co., New York, NY, US, 2001, pp. 3421-3452, van de Kamp *et al.*, Clin Genet. 1981;20(2):152-60, Moog *et al.*, Am J Med Genet C Semin Med Genet. 2007;145C(3):293-301. Patients usually die at the end of the second or beginning of the third decade of life. *See* Neufeld and Muenzer, *supra*. A more slowly progressive form of the disease with later onset and longer survival, known as the attenuated phenotype, has been described in a subset of MPSIIIB patients. *See* Moog *et al.*, *supra* and Valstar *et al.*, Ann Neurol. 2010;68(6):876-87.

There is currently no available treatment for MPSIIIB. Therefore, the control of the disease is symptomatic and aimed at improving the quality of life of patients and their families. This is of special importance for those symptoms arising from CNS alterations. For example, intermittently raised intracranial pressure is a recognized cause of behaviour disturbance and it is not uncommon in MPSs. *See* Muenzer *et al.*, Genet Med. 2006;8(8):465-73. The thickening of the meninges due to mucopolysaccharide deposition would be the underlying cause for the raising of cerebrospinal fluid (CSF) pressure. Thus, when behavioural alterations are refractory to conventional medication, the insertion of a cerebrospinal fluid shunt is considered in order to alleviate the pressure. Neurological symptoms improved significantly in six Sanfilippo patients who had cerebrospinal shunts inserted. *See* Robertson *et al.*, Eur J Pediatr. 1998;157(8):653-5. On the other hand, melatonin administration is considered to be the best treatment for the sleep disorders characteristic of the disease, although it is not completely effective. *See* Fraser *et al.*, Arch Dis Child. 2005;90(12):1239-42. There is no specific treatment for the somatic pathology either, and only palliative therapies can be applied to each individual symptom.

New therapeutic approaches to MPSIIIB are being tested with different degrees of success. Substrate deprivation therapy (SDT) aims at reducing the rate of GAG synthesis, so that, if any residual enzymatic activity remains, excessive accumulation of GAGs is prevented or at least the rate of accumulation is slowed down. Genistein, a soybean isoflavone, has been suggested to act as an inhibitor of HS production by decreasing the kinase activity of the Epidermal Growth Factor receptor (EGFR). *See* Jakobkiewicz-Banecka *et al.*, J Biomed Sci. 2009;16:26, Piotrowska *et al.*, Eur J Hum Genet. 2006;14(7):846-52. Recent studies indicate that genistein inhibits synthesis of GAGs in fibroblasts of patients suffering from various mucopolysaccharidoses (types I, II, III-A and III-B) *See* Piotrowska *et al.*, *supra*. Short-term and long-term oral administration of genistein to a mouse model of MPSIIIB disease resulted in reduction of storage and better performance in motor skills tests. *See* Malinowska *et al.*, Mol Genet Metab. 2009;98(3):235-42, Malinowska *et al.*, PLoS One. 2010;5(12):e14192. When administered

intravenously, genistein is expected to be able to cross the blood-brain barrier (BBB), permitting the treatment of the CNS pathology. Supporting this notion, an open label pilot study in which a genistein-enriched soybean extract was administered to 5 MPSIIIA and 5 MPSIIIB patients for 12 months resulted in a
5 significant amelioration of both somatic and neurological parameters. *See* Piotrowska *et al.*, *Current Therapeutic Research*. 2008;69(2):166-179. However, subsequent studies did not show improvement in either the disability scales or the behaviour scores of MPSIIIA, MPSIIIB or MPSIIIC patients treated with genistein for 12 months. *See* Delgadillo *et al.*, *J Inherit Metab Dis*. 2011;34(5):1039-44,
10 study NTR #1826 registered at The Netherlands National Trial Register and de Ruijter *et al.*, *Ann Neurol*. 2012;71(1):110-20. Another molecule, rhodamine B, has also proven to be effective in decreasing GAG accumulation in preclinical studies, with an efficacy similar to that observed with genistein. *See* Hendriksz *et al.*, “Guidelines for the investigation and Management of Mucopolysaccharidosis type
15 III”, 2012. Rhodamine B is thought to suppress the synthesis of GAG chains by inhibiting the formation of sugar precursors and/or the activity of glycosyl transferases. *See* Roberts *et al.*, *Mol Genet Metab*. 2007;92(1-2):115-21.

Normal cells secrete significant amounts of mannose-6-phosphate (M6P)-tagged lysosomal enzymes, which can be subsequently taken up by other cells via
20 M6P receptors on the plasma membrane. This opens the possibility of treating LSDs caused by deficiency of soluble hydrolases by infusing the correct version of the missing enzyme. Although enzyme replacement therapy (ERT) is yet not available for Sanfilippo B patients, the intravenous administration of recombinant N-acetylglucosaminidase, alpha to 3-month-old MPSIIIB mice showed that the
25 recombinant enzyme distributed to several organs, mainly to the liver. An insignificant amount of enzyme was detected in the brain, which was attributed to the presence of the BBB that limits the entry of exogenously provided proteins to the brain parenchyma. *See* Yu *et al.*, *Mol Genet Metab*. 2000;71(4):573-80 and Hendriksz *et al*, *supra*. There is an early development program for an ERT product
30 for the treatment of neurologic disease in MPSIIIB patients. The HGT-3010

program (Shire) is an ERT based on intrathecal delivery of recombinant enzyme, and is nowadays in preclinical phase. Direct delivery of ERT to the CNS has reduced neurological pathology in MPSIIIA mice and is currently being tested in MPSIIIA patients. *See* Hemsley K, *et al.*, Genes Brain Behav. 2008;53(2):161-8, 5 Savas P, *et al.*, Mol Genet Metab. 2004;82:273-285, NCT01155778 and NCT01299727 at ClinicalTrials.gov which is a resource provided by the U.S. National Library of Medicine. However, the implantation of the permanent intrathecal delivery device that the therapy requires is associated with substantial risks and shortcomings, and the therapy itself has a very high economic cost per 10 patient/year.

Hematopoietic stem cell transplantation (HSCT) using bone marrow-derived stem cells (Bone marrow transplantation, BMT) has proven efficient in the treatment of both somatic and neurological pathology in patients with other MPSs *See* Peters *et al.*, Blood 1996;87(11):4894-902, Peters and Steward, Bone Marrow 15 Transplant 2003;31(4):229-39 and Yamada *et al.*, Bone Marrow Transplant 1998;21(6):629-34. Donor-derived myeloid cells are able to cross the BBB, enter the brain parenchyma, and differentiate into microglial cells that secrete the missing lysosomal enzyme, which is then taken up by surrounding cells leading to correction of GAG accumulation in the brain. *See* Krivit *et al.*, Cell Transplant. 20 1995;4(4):385-92. However, no clear benefit has been observed in Sanfilippo A or B patients who have undergone BMT. *See* Hoogerbrugge *et al.*, Lancet 1995;345(8962):1398-402, Vellodi *et al.*, J Inherit Metab Dis. 1992;15(6):911-8, Güngör and Tuncbilek, Turk J Pediatr. 1995;37(2):157-63, Sivakumur and Wraith, J Inherit Metab Dis. 1999;22(7):849-50 and Lange *et al.*, Arq Neuropsiquiatr. 25 2006;64(1):1-4. The main reason for BMT failure seems to be the slow pace of replacement of the microglial population by the hematopoietic cell progeny compared to the quick progression of the primary disease. *See* Rovelli, Bone Marrow Transplant 2008;41Suppl2:S87-9. Therefore, HSCT using BMT is currently not considered as a treatment option for MPSIII patients. *See* Boelens *et al.*, 30 *Pediatr Clin North Am.* 2010;57(1):123-145. HSCT using umbilical cord blood-derived stem cells improved the cognitive outcome in MPSIIIB mice but required repeated cell administration. *See* Willing *et al.*, Cell Transplantation 2013;epub of

ahead of print. This approach has recently been used to transplant several children with MPSIIIA and MPSIIIB; it is yet unclear whether it results in protection of the CNS from degeneration. See de Ruijter *et al.*, Curr Pharm Biotechnol. 2011;12(6):923-30.

5 Given the limitations of current therapeutic options for MPSIII, and particularly for MPSIIIB, alternative approaches are needed. In vivo gene therapy offers the possibility of a one-time treatment for MPS IIIB and other inherited diseases, with the prospect of lifelong beneficial effects. Several gene therapy approaches based on the use of different viral vectors combined with different
10 routes of administration have been tested in animal models of MPSIIIB disease.

 Lentiviral vectors coding for the human N-acetylglucosaminidase, alpha gene have been administered intravenously to young MPSIIIB mice, resulting in low levels of transgene expression in liver, spleen, lung and heart, which reduced but did not normalize GAG accumulation in these tissues. See Di Natale *et al.*,
15 Biochem J. 2005;388(2):639-46. The therapeutic potential of lentiviral vectors has also been tested by direct delivery of vectors to the brain parenchyma via intracranial administration. See Di Domenico *et al.*, Am J Med Genet A. 2009;149A(6):1209-18. MPSIIIB mice administered at a single brain site showed increased N-acetylglucosaminidase, alpha activity up to 6 months after treatment,
20 which only partially corrected lysosomal storage lesions.

 Adenoassociated virus (AAV) vector-mediated gene transfer, in particular, is rapidly emerging as the approach of choice for many *in vivo* gene therapy applications, due to the high transduction efficiency and the lack of pathogenicity of these vectors. AAV vectors can transduce post-mitotic cells and several pre-
25 clinical and clinical studies have demonstrated the potential of AAV vector-mediated gene transfer to efficiently drive sustained expression of therapeutic transgenes for a variety of diseases. See Bainbridge *et al.*, N Engl J Med. 2008;358(21):2231-9, Hauswirth *et al.*, Hum Gene Ther. 2008;19(10):979-90, Maguire *et al.*, N Engl J Med. 2008;358(21):2240-8, Niemeyer *et al.*, Blood
30 2009;113(4):797-806, Rivera *et al.*, Blood 2005;105(4):1424-30, Nathawani *et al.*,

N Engl J Med. 2011;365(25):2357-65 and Buchlis *et al.*, Blood 2012;119(13):3038-41.

When AAV vectors of serotype 2 coding for human N-acetylglucosaminidase, alpha were delivered to the brain parenchyma of MPSIIIB mice at a single location, N-acetylglucosaminidase, alpha expression and activity were restricted to the site of injection and only partial amelioration of the disease phenotype was achieved. See Fu *et al.*, Mol Ther. 2002;5(1):42-9, Cressant *et al.*, J Neurosci. 2004;24(45):10229-39. AAV2 vectors delivered intravenously to MPSIIIB mice following pre-treatment with mannitol to permeate the BBB, led to significantly extended survival, improved behavioural performance, and reduction of brain lysosomal pathology, although only partial correction of somatic disease was achieved. See McCarty *et al.*, Gene Ther. 2009;16(11):1340-52. On the other hand, a single administration of AAV2 vectors to the cerebrospinal fluid (CSF) by intracisternal injection led to restoration of N-acetylglucosaminidase, alpha activity and reduction of GAGs in the MPSIIIB mouse brain, although no detectable N-acetylglucosaminidase, alpha activity was observed in somatic tissues. See Fu *et al.*, J Gene Med. 2010;12(7):624-33. A combined therapy involving intracisternal and intravenous delivery of AAV2 has demonstrated significant long-term therapeutic efficacy in the CNS as well as partial somatic correction. See Fu *et al.*, Gene Ther. 2007;14(14):1065-77.

Other studies have taken advantage of the high tropism for neuronal cells shown by serotype 5 AAV vectors following intraparenchymal administration. These vectors have, however, a low distribution within the brain parenchyma, and the approach requires multiple injections. The delivery of AAV5 vectors to multiple sites of the brain of newborn MPSIIIB mice in combination with bone marrow transplantation, showed therapeutic efficacy similar to that obtained with serotype 2 vectors. See Heldermon *et al.*, Mol Ther. 2010;18(5):873-80. In animal models of larger brain size, the stereotactic administration of AAV5 vectors to four different brain locations in MPSIIIB dogs led to the detection of active N-acetylglucosaminidase, alpha in widespread areas of the brain. However, enzymatic

activity remained low or undetectable in the most rostral and most caudal regions, especially in the cerebellum. See Ellinwood *et al.*, Mol Ther. 2011;19(2):251-9. Lysosomal pathology was improved but not fully corrected in treated MPSIIIB dogs, indicating that the levels of enzymatic activity achieved with this approach
5 were insufficient to cope with GAG storage. Despite this partial efficacy, a clinical trial based on this approach has recently been initiated. See ISRCTN19853672 at ISRCTN register. The larger the brain the more difficult it becomes to cover the whole volume of the organ with intraparenchymal injections, and delivery to humans needs vector administration at several sites, making delivery technically
10 challenging and requiring the development of specific surgical procedures. See Souweidane *et al.*, J Neurosurg Pediatr. 2010;6(2):115-122.

To date, only one study that uses AAV vectors of serotype 9 for the treatment of MPSIIIB has been reported. The approach takes advantage of the ability of AAV9 vectors to transduce the CNS when systemically administered. See
15 Foust *et al.*, Nat Biotechnol. 2009;27(1):59-65 and Duque, *et al.*, Mol Ther. 2009;17(7):1187-1196. The intravenous delivery of AAV9-N-acetylglucosaminidase, alpha vectors to MPSIIIB mice, resulted in correction of lysosomal storage pathology in CNS and somatic organs, improvement of behavioural performance and extension of lifespan. See Fu *et al.*, Mol Ther.
20 2011;19(6):1025-33. However, this proposed course of action has several shortcomings. First, the CMV promoter utilized has been reported to silence. See Löser *et al.*, J Virol. 1998 Jan;72(1):180-90. Second, therapeutic efficacy was achieved at a very high dose of vector ($\geq 1 \times 10^{13}$ vg/kg). The use of such high doses would suppose a challenge for clinical translation from the manufacturing and
25 safety points of view.

None of aforementioned approaches has fully restored N-acetylglucosaminidase, alpha activity, achieved full eradication of intracytoplasmic inclusions in the CNS and somatic tissues, or corrected all clinical signs of MPSIIIB. Thus, there is a need for novel approaches to the treatment of MPSIIIB
30 that have better efficacy and safety profiles.

SUMMARY OF THE INVENTION

The present invention provides new recombinant vectors for the treatment
5 of diseases, in particular for the treatment of mucopolysaccharidoses type III
(MPSIII), especially MPSIIIB.

A first aspect, the invention relates to adenoassociated virus vectors (AAV)
containing a nucleotide sequence coding for N-acetylglucosaminidase, alpha
(Naglu) SEQ ID NO: 1. The AAV vectors according to the invention are of
10 serotype 9 (AAV9). These vectors proved to be very efficient to fully revert the
pathological GAGs storage in all regions of the brain and somatic tissues.

The AAV9 vectors according to the invention contain a nucleotide sequence
coding for Naglu that has at least 80 % sequence identity with SEQ ID NO: 2, and
preferably at least 84 % sequence identity with SEQ ID NO: 2.

15 The AAV9 vectors of the present invention further contain a promoter
linked to the coding nucleotide sequence in order to control the expression of
Naglu. A suitable promoter is the CAG promoter, SEQ ID NO: 4.

Another aspect of the invention relates to plasmids containing a nucleotide
sequence coding for N-acetylglucosaminidase, alpha (Naglu), and in particular a
20 nucleotide sequence having at least 80 % sequence identity with SEQ ID NO: 2 or
preferably at least 84 % sequence identity with SEQ ID NO: 2.

A further aspect of the present invention relates to a pharmaceutical
composition comprising a therapeutically effective amount of the AAV9 vectors of
the invention or a plasmid described therein.

25 The present invention further provides methods useful for delivering
polynucleotides, especially Naglu polynucleotides.

Still a further aspect of the invention relates to the AAV9 vectors of the invention or a plasmid described therein for use as a medicament, in particular for the treatment of mucopolysaccharidoses type III (MPSIII), especially MPSIIIB.

The present invention also provides method for the production of the AAV
5 9 vectors according to the invention.

In a further aspect the invention relates to isolated cells comprising the nucleotide sequence coding for N-acetylglucosaminidase, alpha (Naglu), and in particular a nucleotide sequence having at least 80 % sequence identity with SEQ ID NO: 2 or preferably at least 84 % sequence identity with SEQ ID NO: 2.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Hydrodynamic delivery of plasmids encoding for human N-acetylglucosaminidase, alpha (pAAV-CAG-hNaglu). Two month-old MPSIIIB
15 mice were hydrodynamically injected with 50 µg of the plasmid pAAV-CAG-hNaglu. Histograms depict N-acetylglucosaminidase, alpha (NAGLU) activity measured 1 week post plasmid administration in liver (A) and serum (B). NAGLU activity of saline-injected WT mice was set to 100%. Values are means ± SEM of 3-4 mice per group. *** P<0.001 vs. WT.

Figure 2. Intravenous (IV) delivery of AAV9 vectors coding for human N-acetylglucosaminidase, alpha (AAV9-CAG-hNaglu). N-acetylglucosaminidase,
20 alpha activity in the liver (A) and in serum (B) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an IV injection of 5×10^{11} vg of AAV9-CAG-hNaglu. (C) Glycosaminoglycan (GAG) quantification in
25 somatic organs. Values are means ± SEM of 5 to 8 mice per group. \$\$\$ P<0.001 vs. WT, ** P<0.01, *** P<0.001 vs. untreated MPSIIIB. *nd*: not detected.

Figure 3. Intravenous delivery of AAV9 vectors coding for human N-acetylglucosaminidase, alpha (AAV9-CAG-hNaglu). (A) N-acetylglucosaminidase,

alpha activity in different parts of the brain (I-IV) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an IV injection of 5×10^{11} vg of AAV9-CAG-hNaglu. (B) Glycosaminoglycan (GAG) quantification in the same parts of the brain. Values are means $\pm$ SEM of 5 to 8 mice per group. *** P<0.001 vs. untreated MPSIIIB. *nd*: not detected

Figure 4. Intravenous delivery of AAV9 vectors coding for optimized human N-acetylglucosaminidase, alpha (AAV9-CAG-cohNaglu). N-acetylglucosaminidase, alpha activity in the liver (A) and serum (B) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an IV injection of 5×10^{11} vg of AAV9-CAG-cohNaglu. (B) Glycosaminoglycan (GAG) quantification in somatic organs. Values are means $\pm$ SEM of 5 to 8 mice per group. \$\$\$ P<0.001 vs. WT, ** P<0.01, *** P<0.001 vs. untreated MPSIIIB. *nd*: not detected.

Figure 5. Intravenous delivery of AAV9 vectors coding for optimized human N-acetylglucosaminidase, alpha (AAV9-CAG-cohNaglu). (A) N-acetylglucosaminidase, alpha activity in different parts of the brain (I-IV) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an IV injection of 5×10^{11} vg of AAV9-CAG-cohNaglu. (B) Glycosaminoglycan (GAG) quantification in the same parts of the brain. Values are means $\pm$ SEM of 5 to 8 mice per group. *** P<0.001 vs. untreated MPSIIIB. *nd*: not detected.

Figure 6. Intracisternal (IC) delivery of AAV9 vectors coding for human N-acetylglucosaminidase, alpha (AAV9-CAG-hNaglu). (A) N-acetylglucosaminidase, alpha activity in different parts of the brain (I-IV) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an intracisternal injection of 9.3×10^9 vg of AAV9-CAG-hNaglu. (B) Quantification of glycosaminoglycans (GAGs) in the same brain areas. Values are means $\pm$ SEM of 5 to 8 mice per group. *** P<0.001 vs. untreated MPSIIIB. *nd*: not detected.

Figure 7. Intracisternal delivery of AAV9 vectors coding for human N-acetylglucosaminidase, alpha (AAV9-CAG-hNaglu). (A) N-acetylglucosaminidase,

alpha activity in the liver of wild-type (healthy) mice and untreated MPSIIIB mice and MPSIIIB mice treated with an intracisternal injection of 9.3×10^9 vg of AAV9-CAG-hNaglu. (B) Glycosaminoglycan (GAG) quantification in somatic organs. Values are means $\pm$ SEM of 5 to 8 mice per group. \$\$\$ $P < 0.001$ vs. WT, ** $P < 0.01$, *** $P < 0.001$ vs. untreated MPSIIIB. *nd*: not detected.

Figure 8. Intracisternal delivery of AAV9 vectors coding for optimized human N-acetylglucosaminidase, alpha (AAV9-CAG-cohNaglu). (A) N-acetylglucosaminidase, alpha activity in different parts of the brain (I-IV) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an intracisternal injection of 9.3×10^9 vg of AAV9-CAG-cohNaglu. (B) Quantification of glycosaminoglycans (GAGs) in the same brain areas. Values are means $\pm$ SEM of 5 to 8 mice per group. *** $P < 0.001$ vs. untreated MPSIIIB. *nd*: not detected.

Figure 9. Intracisternal delivery of AAV9 vectors coding for optimized human N-acetylglucosaminidase, alpha (AAV9-CAG-cohNaglu). (A) N-acetylglucosaminidase, alpha activity in the liver of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice treated with an intracisternal injection of 9.3×10^9 vg of AAV9-CAG-cohNaglu. (B) Glycosaminoglycan (GAG) quantification in somatic organs. Values are means $\pm$ SEM of 5 to 8 mice per group. \$\$\$ $P < 0.001$ vs. WT, ** $P < 0.01$, *** $P < 0.001$ vs. untreated MPSIIIB. *nd*: not detected.

Figure 10. Hydrodynamic delivery of plasmid encoding for optimized human N-acetylglucosaminidase, alpha-version2 (pAAV-CAG-cohNaglu-version2). Two-month-old MPSIIIB mice were hydrodynamically injected with 50 μ g of the plasmid pAAV-CAG-cohNaglu-version2. Histograms depict N-acetylglucosaminidase, alpha (NAGLU) activity measured 1 week post-plasmid administration in liver (A) and serum (B). NAGLU activity of saline-injected WT mice was set to 100%. Values are means $\pm$ SEM of 3-4 mice per group. *** $P < 0.001$ vs. WT.

Figure 11. Hydrodynamic delivery of plasmid encoding for optimized human N-acetylglucosaminidase, alpha-version3 (pAAV-CAG-cohNaglu-version3). Two-month-old MPSIIIB mice were hydrodynamically injected with 50 μ g of the plasmid pAAV-CAG-cohNaglu-version3. Histograms depict N-acetylglucosaminidase, alpha (NAGLU) activity measured 1 week post plasmid administration in liver (A) and serum (B). NAGLU activity of saline-injected WT mice was set to 100%. Values are means $\pm$ SEM of 3-4 mice per group.*** P<0.001 vs. WT.

Figure 12. Intracisternal delivery of AAV9 vectors coding for optimized murine N-acetylglucosaminidase, alpha (AAV9-CAG-comNaglu). Biodistribution of vector genomes following the administration to the cisterna magna of MPSIIIB mice of 3×10^{10} vg of AAV9-CAG-comNaglu. Vector genomes were quantified in brain (A) and somatic tissues (B) by quantitative PCR and referred to the quantity of DNA contained in a diploid genome. *ND*: not detected.

Figure 13. Intracisternal delivery of AAV9 vectors coding for optimized murine N-acetylglucosaminidase, alpha (AAV9-CAG-comNaglu). (A) N-acetylglucosaminidase, alpha activity in different parts of the brain (I-V) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice administered in the cisterna magna with either 3.9×10^{10} vg of control vector (AAV9-Null) or 3×10^{10} vg of AAV9-CAG-comNaglu. (B) Quantification of glycosaminoglycans (GAGs) in the same brain areas. Values are means $\pm$ SEM of 4 mice per group. ** P<0.01, *** P<0.001 vs. MPSIIIB-null. *nd*: not detected.

Figure 14. Intracisternal delivery of AAV9 vectors coding for optimized murine N-acetylglucosaminidase, alpha (AAV9-CAG-comNaglu). (A) Quantification of the signal intensity obtained in different areas of the brain following staining for the lysosomal marker LIMP-2, which gives an indication of the size of the lysosomal compartment, in wild-type (healthy) mice and MPSIIIB mice administered in the cisterna magna with either 3.9×10^{10} vg of control vector (AAV9-Null) or 3×10^{10} vg of AAV9-CAG-comNaglu. Values are means $\pm$ SEM of 4 mice per group. * P<0.05, ** P<0.01, *** P<0.001 vs. MPSIIIB-null. (B)

Electron microscopy analysis of the cerebral cortex and cerebellum of wild-type (left panels), untreated-MPSIIIB (middle panels) and MPSIIIB mice administered in the cisterna magna with AAV9-CAG-comNaglu (right panels). 1. neurons; 2. perineuronal glial cells; 3. purkinje neurons. (C) Activity of other lysosomal enzymes in brain extracts obtained from wild-type (WT) mice, untreated MPSIIIB mice (MPSIIIB) and MPSIIIB mice administered in the cisterna magna with either 3.9×10^{10} vg of control vector (AAV9-Null) or 3×10^{10} vg of AAV9-CAG-comNaglu. SGSH, *N-sulfoglucosamine sulfohydrolase*, GUSB, *glucuronidase, beta*, HEXB, *hexosaminidase B*. WT enzyme activities were set to 100%. Values are means $\pm$ SEM of 4 mice per group. *** $P < 0.001$ vs. MPSIIIB-Null.

Figure 15. Intracisternal delivery of AAV9 vectors coding for optimized murine N-acetylglucosaminidase, alpha (AAV9-CAG-comNaglu). Histograms represent the signal intensity measured following immunostaining for the astrocyte marker GFAP (A) and for the microglial marker BSI-B4 (B) in sections of frontal, parietal, and occipital cortex, superior colliculus, and thalamus from wild-type (healthy) mice, and MPSIIIB mice administered in the cisterna magna with either 3.9×10^{10} vg of control vector (AAV9-Null) or 3×10^{10} vg of AAV9-CAG-comNaglu. Values are means $\pm$ SEM of 2-3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. MPSIIIB-null. (C) Functional categorization based on Gene Ontology (GO) annotation. Pie chart indicating the fraction of transcripts associated with each biological process term. Clearly, most of the terms represent inflammation/immunity-related categories. (D) Hierarchical clustering of all experimental groups based on the set of genes that the CTEN software assigned to be representative of microglia. Each column represents a gene and each row represents an animal. The level of expression of each gene is depicted relative to the mean abundance for that gene across all samples in a grey scale shown at the bottom. The dendrogram of samples shown above the matrix represents overall similarities in transcript levels. Clearly, all MPSIIIB mice treated with AAV9-CAG-comNaglu have a gene expression profile close to that of healthy, age-matched WT littermates.

Figure 16. Intracisternal delivery of AAV9 vectors coding for optimized murine N-acetylglucosaminidase, alpha (AAV9-CAG-comNaglu). N-acetylglucosaminidase, alpha activity in the liver (A) and serum (B) of wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice administered in the cisterna magna with either 3.9×10^{10} vg of control vector (AAV9-Null) or 3×10^{10} vg of AAV9-CAG-comNaglu. (C) Glycosaminoglycan (GAG) quantification in somatic organs. Values are means $\pm$ SEM of 4 to 8 mice per group. \$ P<0.05 vs. WT, ** P<0.01, *** P<0.001 vs. untreated MPSIIIB. *nd*: not detected. (D) Electron microscopy analysis of the liver. Multiple electro-lucent vacuoles (arrows) can be observed in the cytoplasm of hepatocytes from MPSIIIB mice treated with null vector and these vesicles disappear in animals treated with AAV9-CAG-mNaglu. (E) Activity of other lysosomal enzymes in liver extracts obtained from the same cohorts of animals as in (A and B). IDS, *iduronate-2-sulfatase*, IDUA, *iduronidase, alpha-L-*, SGSH, *N-sulfoglucosamine sulfohydrolase*, GUSB, *glucuronidase, beta*, HEXB, *hexosaminidase B*. WT enzyme activities were set to 100%. Values are means $\pm$ SEM of 4 mice per group. ** P<0.01 and *** P<0.001 vs. MPSIIIB-Null.

Figure 17. Intracisternal delivery of AAV9 vectors coding for optimized murine N-acetylglucosaminidase, alpha (AAV9-CAG-comNaglu). Behavioural evaluation in naïve-tested wild-type (healthy) mice, untreated MPSIIIB mice and MPSIIIB mice administered in the cisterna magna with either 3.9×10^{10} vg of control vector (AAV9-Null) or 3×10^{10} vg of AAV9-CAG-comNaglu. Data correspond to the locomotor and exploratory activity recorded during the first 2 minutes, and are represented as the mean $\pm$ SEM of 10 to 15 animals per group. (A) Latency to centre, (B) Time in border, (C) Entries to centre, (D) Resting time, (E) Total distance travelled, (F) Total number of lines crossed. *P < 0.05 and **P < 0.01 vs. MPSIIIB-null. (G) Kaplan-Meier survival analysis in WT (n = 23), untreated MPSIIIB mice (n = 12), MPSIIIB mice injected with 3.9×10^{10} vg of AAV9-Null (n = 5) or 3×10^9 vg of AAV9-CAG-comNaglu (n = 8). Treatment with AAV9-CAG-comNaglu gene therapy considerably extended the lifespan of MPSIIIB animals. P = 0.0007 for AAV9-CAG-comNaglu-treated MPSIIIB mice vs. MPSIIIB-null.

Figure 18. Intracisternal delivery of AAV9 vectors coding for optimized canine N-acetylglucosaminidase, alpha (AAV9-CAG-cocNaglu) to Beagle dogs. (A) Follow up of anti-AAV9 Neutralising antibodies (Nab) titers in matched serum and CSF samples before and after intra-CSF delivery of AAV9-CAG-cocNaglu vectors to healthy Beagle dogs. Dogs 3 and 4 were pre-immunized by intravenous delivery of 1×10^{11} vg/kg of AAV9-null vectors one month in advance of intracisternal administration. (B) Follow up of N-acetylglucosaminidase, alpha activity in the CSF of healthy adult Beagle dogs following intra-CSF administration of 6.5×10^{12} vg of AAV9 vectors encoding for canine N-acetylglucosaminidase, alpha. Dogs 1 and 2 (open symbols) were naïve at the moment of administration whilst Dogs 3 and 4 (filled symbols) had pre-existing immunity against the AAV9 vector. The CSF white blood cell counts (WBC) for each time point for all dogs are depicted in the same graph (dashed lines).

Figure 19. Schematic representation of the plasmid pAAV-CAG-hNaglu (A) and schematic representation of the genome of the recombinant AAV vector AAV9-CAG-hNaglu (B).

Figure 20. Schematic representation of the plasmid pAAV-CAG-cohNaglu (A) and schematic representation of the genome of the recombinant AAV vector AAV9-CAG-cohNaglu (B).

Figure 21. Schematic representation of the plasmid pAAV-CAG-cohNaglu-version2 (A) and schematic representation of the genome of the recombinant AAV vector AAV9-CAG-cohNaglu-version2 (B).

Figure 22. Schematic representation of the plasmid pAAV-CAG-cohNaglu-version3 (A) and schematic representation of the genome of the recombinant AAV vector AAV9-CAG-cohNaglu-version3 (B).

Figure 23. Schematic representation of the plasmid pAAV-CAG-comNaglu (A) and schematic representation of the genome of the recombinant AAV vector AAV9-CAG-comNaglu (B).

Figure 24. Schematic representation of the plasmid pAAV-CAG-cocNaglu (A) and schematic representation of the genome of the recombinant AAV vector AAV9-CAG-cocNaglu (B).

5 DEPOSIT OF MICROORGANISMS

The plasmid pAAV-CAG-cohNaglu (SEQ ID NO: 6) was deposited on November 13th, 2012, under access number DSM 26626 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

The plasmid pAAV-CAG-hNaglu (SEQ ID NO: 5) was deposited on March 13th, 2014, under access number DSM 28568 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

The plasmid pAAV-CAG-cohNaglu-version2 (SEQ ID NO: 20) was deposited on April 29th, 2015, under access number DSM 32042 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

The plasmid pAAV-CAG-cohNaglu-version3 (SEQ ID NO: 23) was deposited on April 29th, 2015, under access number DSM 32043 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

DEFINITIONS

The term “nucleotide sequence” refers to a nucleic acid molecule, either DNA or RNA, containing deoxyribonucleotides or ribonucleotides respectively.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence.

The term “% sequence identity” refers to the percentage of nucleotides of a candidate sequence that are identical to the nucleotides in the sequence of reference, after aligning the sequences to achieve the maximum % sequence identity. The % sequence identity can be determined by any methods or algorithms established in the art, such as the ALIGN, BLAST and BLAST 2.0 algorithms. See Altschul S, *et al.*, Nuc Acids Res. 1977;25:3389-3402 and Altschul S, *et al.*, J Mol Biol. 1990;215:403-410.

Herein, the % sequence identity is calculated dividing the number of nucleotides that are identical after aligning the sequence of reference and the candidate sequence, by the total number of nucleotides in the sequence of reference and multiplying the result by 100.

The terms “codify” or “coding” refer to the genetic code that determines how a nucleotide sequence is translated into a polypeptide or a protein. The order of the nucleotides in a sequence determines the order of amino acids along a polypeptide or a protein.

The term “protein” refers to a macromolecule composed of one or more linear chains of amino acids or polypeptides. Proteins can suffer post-translational modifications, like the conversion of a cysteine residue to 3-oxoalanine, glycosylation or metal binding. Glycosilation of a protein is the addition of different carbohydrates that are linked covalently to the amino acid chain.

The term “effective amount” refers to an amount of a substance sufficient to achieve the intended purpose. For example, an effective amount of an AAV9 vector to increase N-acetylglucosaminidase, alpha (Naglu) activity is an amount sufficient to reduce glycosaminoglycan accumulation. A “therapeutically effective amount” of an expression vector to treat a disease or disorder is an amount of the expression vector sufficient to reduce or eradicate the signs and symptoms of the disease or disorder. The effective amount of a given substance will vary with factors such as

the nature of the substance, the route of administration, the size and species of the animal to receive the substance and the purpose of giving the substance. The effective amount in each individual case may be determined empirically by a skilled artisan according to established methods in the art.

5 The term “individual” refers to a mammal, preferably human or non-human mammal, more preferably mouse, rat, other rodents, rabbit, dog, cat, pig, cow, horse or primate, further more preferably human.

 The term “operably linked” refers to the functional relation and the location of the promoter sequence with respect to the gene of interest (e.g. a promoter or
10 enhancer is operably linked to a coding sequence if it affects the transcription of the sequence). Generally, a promoter operably linked is contiguous to the sequence of interest. However, an enhancer does not have to be contiguous to the sequence of interest to control its expression.

 The term “tropism” refers to the way in which different viruses have
15 evolved to preferentially target specific host species, or specific cell types within those species.

 The term “gene therapy” refers to the transfer of genetic material (e.g. DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition. The genetic material of interest encodes a product (e.g. a protein
20 polypeptide, peptide or functional RNA) whose production *in vivo* is desired. For example, the genetic material of interest can encode an enzyme, hormone, receptor, or polypeptide of therapeutic value.

 The terms “recombinant viral vector”, “viral vector”, “recombinant vector” or “vector” refer to an agent obtained from a naturally-occurring virus through
25 genetic engineering techniques capable of transferring genetic material (e.g. DNA or RNA) of interest to a cell, which results in production of the product encoded by that said genetic material (e.g. a protein polypeptide, peptide or functional RNA) in the target cell. In the context of the present invention, a “recombinant vector” or a “vector” is to be understood as being a capsid protein as well as the genetic material

contained within used to transfer said genetic material into a cell. By referring to a “recombinant vector” or to a “vector” through a nucleotide sequence, it means that it refers to a “recombinant vector” or a “vector” whose genome is as set forth in the corresponding sequence listing (SEQ ID).

5 The term “recombinant plasmid” or “plasmid” refers to a small, circular, double-stranded, self-replicating DNA molecule obtained through genetic engineering techniques capable of transferring genetic material of interest to a cell, which results in production of the product encoded by that said genetic material (e.g. a protein polypeptide, peptide or functional RNA) in the target cell.

10 Furthermore, the term “recombinant plasmid” or “plasmid” also refers to a small, circular, double-stranded, self-replicating DNA molecule obtained through genetic engineering techniques used during the manufacturing of viral vectors as carriers of the recombinant vector genome.

15

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention provides new recombinant vectors for the treatment of diseases, in particular for the treatment of mucopolysaccharidoses type III (MPSIII), especially MPSIIIB.

 Apart from the genetic material, the recombinant vector may also contain different functional elements that include control elements for transcription like promoters or operators, transcription factors binding regions or enhancers and control elements for the initiation or termination of translation.

25 The vectors according to the invention are adenoassociated vectors (AAV) that are used to transfer the gene of interest. They have proved to have a high efficiency in transducing post-mitotic cells in wide range of tissue. In the context of the present invention, the vectors are used to deliver the human N-

acetylglucosaminidase, alpha (hNaglu) polynucleotide (SEQ ID NO: 2) or a codon optimized human N-acetylglucosaminidase, alpha (cohNaglu) polynucleotide (SEQ ID NO: 3). An adenoassociated vector is a vector derived from the genome of an adenoassociated virus of the family of *parvoviridae*. The adenoassociated virus genome is built of single-stranded deoxyribonucleic acid (ssDNA). These vectors infect mammals but are non-pathogenic (i.e. do not cause disease). They can infect dividing or non-dividing cells, and their tropism changes depending on the serotype. The serotype is the classification of the viruses groups, depending on their capsid antigens. The serotype of adenoassociated virus, determined by its capsid protein, defines the virus tropism and allows its entry into a specific cell type. In the context of the present invention, the serotype 9 of the adenoassociated virus vectors (AAV9) shows the best ability to deliver the genetic material to the brain as well as to peripheral organs upon a single administration.

The inventors have surprisingly found that the association, in the same entity, of the AAV9 capsid with a nucleotide sequence coding for the N-acetylglucosaminidase, alpha, together with a specific CAG promoter allows a long-lasting expression of the missing enzyme in all areas of the brain. As a consequence the lysosomal accumulation of glycosaminoglycan (GAG) is corrected, preventing by that way the neurological alterations characteristic of the MPSIII diseases, and in particular of the MPSIIIB. This effect has been obtained even in the olfactory bulb, which is distant from the point of administration of the vectors. Further the AAV9 vectors according to the invention delivered into the cerebrospinal fluid were able to reach the systemic circulation to transduce the liver. The production and secretion of the enzyme by liver cells resulted in an increase of N-acetylglucosaminidase, alpha activity in serum, ultimately leading to the reduction of lysosomal pathology in many somatic tissues. This represents a clear advantage of the vectors according to the invention over the existing approaches that only partially corrected the clinical signs of the disease and usually exert their effect either in the brain or in the systemic circulation, but not in both.

Accordingly the present invention relates to recombinant AAV9 vectors containing a CAG promoter (SEQ ID NO: 4) linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1.

In particular, the recombinant AAV9 vectors of the present invention contain a CAG promoter (SEQ ID NO: 4) linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 80 % sequence identity to SEQ ID NO: 2. More preferably, the AAV9 recombinant vectors of the present invention contain a CAG promoter (SEQ ID NO: 4) linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 84 % sequence identity to SEQ ID NO: 2. In particular the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 has a 84 %, 87 %, 90 %, 95 %, 98 %, or 99 % sequence identity to SEQ ID NO: 2.

In another particular embodiment the recombinant AAV9 vectors of the present invention contains a CAG promoter (SEQ ID NO: 4) linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 85 % sequence identity to SEQ ID NO: 3. In particular the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 has a 85 %, 87 %, 90 %, 95 %, 98 %, or 99 % sequence identity to SEQ ID NO: 3.

In a preferred embodiment of the invention, the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 2.

In a further preferred embodiment of the invention, the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 3. The sequence as set forth in SEQ ID NO: 3 presents 84 % sequence identity with SEQ ID NO: 2.

In a still further preferred embodiment of the invention, the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 19.

In a still further preferred embodiment of the invention, the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 22.

The AAV9 vectors according to the present invention contain a promoter
5 that control the translation and transcription of the gene of interest. A promoter is a nucleotide sequence operably linked to said gene of interest. The promoter used in the present invention is the CAG promoter which refers to the combination comprising the cytomegalovirus early enhancer element and the chicken β -actin promoter. It further includes a portion of β -globin intron that confers stability to the
10 m RNA derived from the gene of interest, *See* Alexopoulou A, *et al.*, BMC Cell Biology 2008; 9(2): 1-11. The CAG promoter included in the AAV9 vectors of the present invention has a sequence SEQ ID NO: 4. In particular this CAG promoter proved to be more efficient than the CMV promoter usually used in the art.

In a further preferred embodiment of the invention, the recombinant AAV9
15 vector is chosen from AAV9-CAG-hNaglu (SEQ ID NO: 9), AAV9-CAG-cohNaglu (SEQ ID NO: 10) and AAV9-CAG-cohNaglu-version2 (SEQ ID NO: 21) and AAV9-CAG-cohNaglu-version3 (SEQ ID NO: 24). Preferably, the recombinant AAV9 vector is chosen from AAV9-CAG-hNaglu (SEQ ID NO: 9), AAV9-CAG-cohNaglu (SEQ ID NO: 10). More specifically, the recombinant
20 AAV9 vectors of the present invention are composed of the viral capsid of the serotype 9 of human adenoassociated virus and a modified genome containing the Inverted Terminal Repeats (ITRs) of human adenoassociated virus serotype 2, the CAG promoter, the Coding Sequence (CDS) of the human alpha N-acetylglucosaminidase (Naglu) gene and the polyA from the rabbit beta-globin
25 gene.

The present invention also related to plasmids that contain the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1. In particular the plasmids according to the present invention contain a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 80 %
30 sequence identity to SEQ ID NO: 2. Preferably, the plasmids according to the

present invention contain a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 84 % sequence identity to SEQ ID NO: 2. In particular the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 has a 84 %, 87 %, 90 %, 95 %, 98 %, or 99 % sequence identity to SEQ ID NO: 2.

In a preferred embodiment, the plasmids according to the present invention contain a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 85 % sequence identity to SEQ ID NO: 3. In particular the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 has a 85 %, 87 %, 90 %, 95 %, 98 %, or 99 % sequence identity to SEQ ID NO: 3.

These plasmids are useful to produce the recombinant AAV9 vectors of the present invention by transfection of HEK293 cells using methods known in the state of the art.

In a preferred embodiment of the invention, the nucleotide sequence contained in the plasmids of the invention and coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 2.

In another preferred embodiment of the invention, the nucleotide sequence contained in the plasmids of the invention and coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 3.

In another preferred embodiment of the invention, the nucleotide sequence contained in the plasmids of the invention and coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 19.

In another preferred embodiment of the invention, the nucleotide sequence contained in the plasmids of the invention and coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 is SEQ ID NO: 22.

In a more preferred embodiment, the plasmids of the invention are chosen from pAAV-CAG-hNaglu (SEQ ID NO: 5), pAAV-CAG-cohNaglu (SEQ ID NO: 6), pAAV-CAG-cohNaglu-version2 (SEQ ID NO: 20) and pAAV-CAG-cohNaglu-

version3 (SEQ ID NO: 23), and especially chosen from pAAV-CAG-hNaglu (SEQ ID NO: 5) and pAAV-CAG-cohNaglu (SEQ ID NO: 6), and preferably the plasmid is pAAV-CAG-cohNaglu (SEQ ID NO: 6).

5 The present invention further provides a method for the production of the adenoassociated viral recombinant vectors AAV9 according to the invention. The process comprises the steps of:

- 10 i) providing a first vector comprising the sequence coding for the protein of interest interposed between a first AAV terminal repeat and a second AAV terminal repeat, a CAG promoter operably linked to the sequence coding for the protein of interest ; a second vector comprising an AAV *rep* gene and a AAV *cap* gene from serotype 9; and a third vector comprising the adenovirus helper function gene;
- 15 ii) co-transfecting competent cells with the vectors of step i);
- iii) culturing the transfected cells of step ii); and
- iv) purifying the expression vectors from the culture of step iii).

 In a preferred embodiment, the AAV first and second terminal repeats of the first vector are ITRs from the AAV serotype 2. In another preferred embodiment,
20 the AAV *rep* genes of the second vector are from the AAV serotype 2. In another preferred embodiment, the competent cells are HEK293 cells.

 The invention also provides a method for the preparation of the plasmids according to the invention, comprising the step of:

- 25 i) excising the sequence coding for the protein of interest from the starting plasmid, by digestion, in particular using MluI/EcoRI,
- ii) cloning the sequence coding for the protein of interest between two restriction sites of the AAV backbone plasmid pAAV-CAG, hereby obtaining the corresponding plasmid including the sequence coding for the
30 protein of interest.

The present invention contemplates, in an additional aspect, pharmaceutical compositions containing a therapeutically effective amount of the AAV9 vectors described therein, or a therapeutically effective amount of the plasmids described therein.

Pharmaceutical compositions of the invention comprise the recombinant AAV9 vectors in a pharmaceutically acceptable carrier. The composition may also comprise at least one auxiliary substance. The auxiliary substances can be selected among carriers, excipients, solvents, diluents, or adjuvants. Acceptable carriers, diluent or adjuvants are non-toxic and are preferably inert at the dosage and concentrations employed and include buffers such as phosphate, citrate or other organic acids; antioxidants; low molecular weight polypeptides, proteins such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers; amino acids; monosaccharides, disaccharides and other carbohydrates including glucose, mannose or dextrans; chelating agents; sugar alcohols such as mannitol or sorbitol, salt forming counterions such as sodium; and/or non-ionic surfactants such as polyethylene-polyoxypropylene block copolymer (Pluronic F68®) polyethylene glycol (PEG).

In a preferred embodiment, the pharmaceutical compositions according to the invention are suitable for parenteral administration. Examples of parenteral administration are intravenous, subcutaneous, intracisternal and intramuscular injections. Preferably, the pharmaceutical composition according to the invention is suitable for intravenous or intracisternal administration. Compositions suitable for such parenteral administration include sterile aqueous solutions or dispersions, sterile powders for extemporaneous preparation of sterile solutions or dispersions. Advantageously the pharmaceutical compositions according to the invention are preserved from contaminating action of bacteria and fungi.

The daily dosage for humans and animals may vary depending on factors that have their basis in the respective species or other factors, such as age, sex, weight or degree of illness and so forth.

Another aspect of the present invention relates to the therapeutical use of the AAV9 vectors described hereinbefore, or the plasmids described hereinbefore. As mentioned above the recombinant AAV9 vectors according to the invention achieve an expression of the missing Naglu enzyme, thus correcting the lysosomal accumulation of GAGs. This allows correcting all clinical signs of the mucopolysaccharidoses type III (MPSIII) and especially MPSIIIB. In this respect the present invention also concern the recombinant AAV9 vectors described hereinbefore, or the plasmids described hereinbefore for use as a medicament.

In particular, the invention relates to the recombinant AAV9 vectors described hereinbefore, or the plasmids described hereinbefore for increasing the alpha N-glucosaminidase activity in the body.

In a further preferred aspect, the present invention relates to the recombinant AAV9 vectors described hereinbefore, or the plasmids described hereinbefore for treatment of mucopolysaccharidoses type III (MPSIII) and especially MPSIIIB.

In a still further embodiment, the present invention relates to the use of the recombinant AAV9 vectors described hereinbefore, or the plasmids described hereinbefore for the manufacture of a medicament useful for the treatment of mucopolysaccharidoses type III (MPSIII) and especially MPSIIIB.

Another embodiment of the present invention is directed to the method of treatment of mucopolysaccharidoses type III (MPSIII) and especially MPSIIIB, comprising the step of administering at least a recombinant AAV9 vector described hereinbefore, or at least a plasmid described hereinbefore to a subject in need thereof.

The present invention further provides an isolated cell comprising the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1. In

particular the cell according to the invention comprises a nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 80 % sequence identity to SEQ ID NO: 2. Preferably, the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 that has at least 84 % sequence identity to SEQ ID NO: 2. In particular the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 has a 84 %, 87 %, 90 %, 95 %, 98 %, or 99 % sequence identity to SEQ ID NO: 2. In a further particular aspect, the nucleotide sequence coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1 has at least 85 % sequence identity to SEQ ID NO: 3, and preferably 85 %, 87 %, 90 %, 95 %, 98 %, or 99 % sequence identity to SEQ ID NO: 3.

In a preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 2 coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1.

In another preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 3 coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1.

In another preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 19 coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1.

In another preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 22 coding for N-acetylglucosaminidase, alpha SEQ ID NO: 1.

The following examples are merely illustrative of certain embodiments of the invention and cannot be considered as restricting it in any way.

25 GENERAL PROCEDURES

1. Recombinant AAV Vectors

The AAV vectors described herein were obtained by triple transfection. The materials required for making the vectors were: HEK293 cells (expressing E1 genes), helper plasmid providing adenovirus function, plasmid providing AAV *rep* genes from serotype 2 and *cap* genes from the serotype 9 (AAV9) and, finally, the backbone plasmid with AAV2 ITRs and the construct of interest.

To generate N-acetylglucosaminidase, alpha-expressing AAV vectors, the non-optimized or optimized CDS of human, murine or canine N-acetylglucosaminidase, alpha were cloned into an AAV backbone plasmid under the control of the ubiquitous hybrid CAG promoter.

Vectors were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita T, *et al.*, Gene Ther. 1998;5:938-945 and Wright J, *et al.*, Mol. Ther. 2005;12:171-178. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by the viral ITRs of serotype 2 AAV (described above); 2) a plasmid carrying the AAV rep2 and the cap9 genes; and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using an optimized protocol as previously described. See Ayuso E, *et al.*, Gene Ther. 2010;17:503-510. Vectors were dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

The vectors of the present invention were constructed according to molecular biology techniques well known in the art.

2. Animals

A congenic mutant C57Bl/6J N-acetylglucosaminidase, alpha-deficient mouse (MPSIIIB) model was purchased from The Jackson Laboratory (Bar Harbor, ME, USA. Stock 003827). *See Li et al.*, Proc Natl Acad Sci. 1999; 96(25):14505-10. Affected MPSIIIB and healthy control mice were inbred from heterozygous founders. Genotype was determined on genomic DNA from tail-clipped samples with a PCR analysis that amplifies a sequence encompassing the targeted mutation. The sequences of the respective sense and antisense primers were: Forward Primer: 5'-GTC GTC TCC TGG TTC TGG AC-3' (SEQ ID NO: 13), Reverse Primer: 5'-ACC ACT TCA TTC TGG CCA AT-3' (SEQ ID NO: 14), Reverse Primer Mutation: 5'- CGC TTT CTG GGC TCA GAG-3' (SEQ ID NO: 15). Mice were fed ad libitum with a standard diet (Harlan, Tekland)) and maintained under a light-dark cycle of 12 h (lights on at 9:00 A.M.).

3. Hydrodynamic delivery of hNAGLU-encoding plasmids to mice

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For hydrodynamic delivery of pAAV-CAG-hNaglu, pAAV-CAG-cohNaglu-version2 and pAAV-CAG-cohNaglu-version3 plasmids, 2-month-old MPSIIIB and wild-type animals received through tail vein injection in <5 seconds a total dose of 50 µg of plasmid in a volume equal to 10% of the body weight of the animal. This technique results in expression of plasmid-encoded transgenes mainly in the liver. *See Liu et al.*, Gene Ther. 1990;6(7):1258-66. As control, a cohort of mice received an equal volume of saline solution. Mice were sacrificed either at 1 week after hydrodynamic injection of the plasmids. Organs were harvested as described in the following section.

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4. Vector administration and sample collection

For intracisternal delivery of AAV9-CAG-comNaglu vectors to mice, a total dose of 3×10^{10} vg were injected to the cisterna magna of 2-month-old MPSIIIB animals. A similar cohort of animals was injected with 3.9×10^{10} vg control non-coding (AAV9-null) vector. At 5 months of age, i.e. 3 months post vector administration, mice were anesthetized and then transcardially perfused with 10 ml of PBS to completely clear blood from tissues. The entire brain and multiple somatic tissues (including liver, spleen, pancreas, kidney, lung, heart, skeletal muscle and testicles) were collected and either frozen in liquid nitrogen and stored at -80°C or immersed in formalin for subsequent histological analyses.

For intravenous delivery of AAV9-CAG-hNaglu and AAV9-CAG-cohNaglu vectors, 2-month-old MPSIIIB animals received a total dose of 5×10^{11} vg through tail vein injection. At 4 months of age, i.e. 2 months post vector administration, mice were sacrificed and organs were harvested as described in the previous paragraph.

For intracisternal delivery of AAV9-CAG-hNaglu and AAV9-CAG-cohNaglu vectors, a total dose of 9.3×10^9 vg were injected in the cisterna magna of 2-month-old MPSIIIB animals. At 4 months of age, i.e. 2 months post vector administration, mice were sacrificed and organs were harvested as described in the previous paragraph.

For intracisternal delivery of AAV9-CAG-cocNaglu vectors to dogs, a total dose of 6.5×10^{12} vg was administered to healthy adult Beagle dogs via cisterna magna injection. Two of the animals received an intravenous injection of 1×10^{11} vg/kg of AAV9-null vectors 6 weeks prior to administration of Naglu vectors to pre-immunize them against AAV9. First weekly, and then monthly, CSF and serum samples were collected and stored at -80°C .

5. Quantification of vector genome copy number

Tissues (≈ 100 mg) were digested overnight (ON) at 56°C in 400 μl of Proteinase K

solution (0.2 mg/ml). Total DNA was isolated from supernatants by extraction using standard techniques. DNA was resuspended in distilled water and quantified using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). Vector genome copy number in 20 ng of total DNA was determined by quantitative real time PCR with primers and probe specifics for the murine N-acetylglucosaminidase, alpha transgene that do not amplify the endogenous genomic locus. Forward primer: 5'-GCC GAG GCC CAG TTC TAC-3' (SEQ ID NO: 16); Reverse primer: 5'-TTG GCG TAG TCC AGG ATG TTG-3' (SEQ ID NO: 17); Probe: 5'-AGC AGA ACA GCA GAT ACC AGA TCA CCC-3' (SEQ ID NO: 18). The final values were determined by comparing to a reference standard curve, built from serial dilutions of the linearized plasmid used for AAV vector production spiked into 20 ng non-transduced genomic DNA.

6. N-acetylglucosaminidase, alpha activity and glycosaminoglycan quantification

Liver and brain samples were sonicated in Mili-Q water. Serum was analysed unprocessed. N-acetylglucosaminidase, alpha activity was determined with a 4-methylumbelliferone-derived fluorogenic substrate (Moscerdam Substrates, Oegstgeest, NL), as described previously. *See* Marsh and Fensom, Clin Genet. 1985;27(3):258-262. Brain and liver activity levels were normalized against the total amount of protein, quantified using Bradford protein assay (Bio-Rad, Hercules, CA, US). Serum activity was normalized against volume.

For glycosaminoglycan (GAG) quantification, tissue samples were weighted and then digested with proteinase K and extracts were clarified by centrifugation and filtration. GAG levels were determined in tissue extracts with the Blyscan sulfated glycosaminoglycan kit (Biocolor, Carrickfergus, County Antrim, GB), using chondroitin 4-sulfate as standard. The levels of GAG were normalized to wet tissue weight.

7. Activity of other lysosomal enzymes

IDUA activity was measured in 15 µg of protein incubated for 1 h at 37°C with 4-methylumbelliferyl α-N-iduronide (Glycosynth). For IDS activity, 15 µg of protein were first incubated with 4-methylumbelliferyl-α-L-iduronide-2-sulphate (Moscerdam Substrates) for 4 h at 37°C, followed by a second 24 h incubation at 37°C with a pool of lysosomal enzymes from bovine testis (LEBT-M2, Moscerdam Substrates). SGSH activity was measured as previously described. See Haurigot *et al.*, J Clin Invest 2013;123(8):3254-71. For GUSB activity, 10 µg of protein were incubated with 4-methylumbelliferyl-β-D-glucuronide (Sigma) at 37°C for 1 h. HEXB activity was assayed by incubation of 0.1 µg of protein with 4-methylumbelliferyl N-acetyl-β-D-glucoaminide (Sigma) for 1 h at 37°C. After stopping reactions by increasing the pH, released fluorescence was measured with FLx800 fluorimeter (BioTek Instruments). All enzyme activities were normalized against total protein content quantified by Bradford (Bio-Rad).

8. Histological analyses

Tissues were fixed for 12-24 h in formalin, embedded in paraffin and sectioned. For immunohistochemical detection of LAMP1 in somatic tissues, paraffin sections were subjected to heat-induced epitope retrieval in citrate buffer, pH 6, and then incubated overnight at 4°C with rat anti-LAMP1 antibody (1D4B; Santa Cruz Biotechnology, Santa Cruz, CA, US) diluted at 1:100 and subsequently incubated with biotinylated rabbit anti-rat antibody (Dako, Glostrup, DK) at 1:300. For immunohistochemical detection of LIMP2 in the brain, paraffin sections were incubated overnight at 4°C with rabbit anti-LIMP2 antibody (NB400; Novus Biologicals, Littleton, CO, USA) diluted at 1:100 and subsequently incubated with biotinylated goat anti-rabbit antibody (31820; Vector Laboratories, Burlingame, CA, USA) at 1:300. For GFAP immunostaining in brain samples, paraffin sections were incubated overnight at 4°C with rabbit anti-GFAP antibody (Ab6673; Abcam,

Cambridge, UK) diluted at 1:1000 and subsequently incubated with biotinylated goat anti-rabbit antibody (31820; Vector Laboratories, Burlingame, CA, USA) at 1:300. LAMP1, LIMP2 and GFAP signals were amplified by incubating sections with ABC-Peroxidase staining kit (Thermo Scientific, Waltham, MA, US) at 1:100 dilution and visualized using 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, US) as a chromogen. Brightfield images were obtained with an optical microscope (Eclipse 90i; Nikon, Tokyo, JP).

To stain microglial cells in brain samples, paraffin sections were incubated overnight at 4°C with Bsi-B4 lectin (L5391; Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:100. Bsi-B4 signal was visualized using 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, US) as a chromogen. Brightfield images were obtained with an optical microscope (Eclipse 90i; Nikon, Tokyo, JP).

The NIS Elements Advanced Research 2.20 software was used to quantify LIMP2, GFAP, and Bsi-B4 signals in 3-5 images of each brain region (original magnification, $\times 20$) per animal, using the same signal threshold settings for all animals. Then, the percentage of positive area was calculated, i.e., the area, in pixels, with a positive signal over the total tissue area in the image.

9. Transmission electron microscopic analysis

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Mice were sacrificed by an overdose of isofluorane (Isofluo, Labs. Esteve, Barcelona, ES) and perfused via inferior vena cava with 1 ml of 2.5% glutaraldehyde and 2% paraformaldehyde. A small portion (approximately 1mm³) of the lateral lobe of the liver and of the cerebral cortex were sectioned and incubated for 2 hours at 4°C in the same fixative. After washing in cold cacodylate buffer, the specimens were postfixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, and then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (600–800 Å) from the resin blocks were stained

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using lead citrate and examined in a transmission electron microscope (H-7000; Hitachi, Tokyo, JP).

1. Transcriptomic analysis

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Half mouse brain (~250 mg) was mechanically homogenized and total RNA was isolated with mirVana™ (Ambion, Life Technologies). cDNA was synthesized and subsequently hybridized in the GeneChip Mouse Gene 2.1 ST 16 array plate (Affymetrix) by Progenika Biopharma (Spain); sample processing, hybridization and scanning were carried out following Affymetrix recommended protocols and equipment. Data normalization was done by RMA (Robust Multiarray averaging) method using Affymetrix® Expression Console™ tool, obtaining log2 transformed normalized values. Data were filtered to focus the analysis on known coding sequences, obtaining an initial list of 26688 altered genes, which were subsequently refiltered to remove genes with variance below the 75th percentile. This process generated a working list of 6672 genes. For differentially expressed genes, FDR (False Discovery Rate) criteria <0.1 with 80% confidence was established. For clustering analysis, data were standardized and represented as heatmap using the J-Express Pro software (jexpress.bioinfo.no). Functional analysis was performed using Genecodis Tool 2.0. Array data have been submitted to ArrayExpress database.

2. Behavioural assessment

25 Behavioural changes were assessed through the Open-field test. Animals were placed in the lower left corner of a brightly lit chamber (41 × 41 × 30 cm). The surface of the arena was divided in three concentric squares: centre (14 × 14 cm), periphery (27 × 27 cm) and border (41 × 41 cm). Exploratory behaviour and

general activity were recorded during the first two minutes using a video-tracking system (Smart Junior, Panlab). The test was always performed at the same time of day (9:00 am to 1:00 pm) to minimize influence of circadian cycles.

5 12. Statistical analysis

All results are expressed as mean $\pm$ SEM. Statistical comparisons were made using one-way ANOVA, and multiple comparisons between control and treatment groups will be made using Dunnett's post test. Statistical significance was
10 considered if $P < 0.05$. The Kaplan–Meier method will be used to analyze survival, and the log-rank test for comparisons.

EXAMPLES

15 **Example 1: Construction of pAAV-CAG-hNaglu**

The human N-acetylglucosaminidase, alpha coding sequence (CDS) was utilized as starting material (NCBI Reference Sequence: NM_000263) and chemically synthesized for this purpose (GeneArt; Life Technologies). The CDS
20 was received cloned inside the plasmid pMA (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3' ends, respectively. N-acetylglucosaminidase, alpha CDS was excised by MluI/EcoRI digestion and then cloned between the MluI and EcoRI restrictions sites of the AAV backbone plasmid pAAV-CAG (AmpR). The resulting plasmid was named pAAV-CAG-hNaglu (accession number DSM
25 28568). See SEQ ID NO: 5, and Figure 19 A.

The pAAV-CAG plasmid had been previously generated and contained the ITRs from the AAV2 genome, the CAG promoter, and the polyA signal from rabbit

β -globin, as well as a multicloning site for cloning of CDSs of interest. The CAG promoter is a hybrid promoter composed of the CMV early/intermediate enhancer and the chicken β -actin promoter. This promoter is able to drive a potent expression ubiquitously. See Sawicki J *et al.*, Exper Cell Res. 1998;244:367–369, Huang J *et al.*, J Gene Med. 2003;5:900–908, Liu Y *et al.*, Exp Mol Med. 2007; 39(2):170-175.

Example 2: Production of AAV9-CAG-hNaglu

10 Vectors AAV9-CAG-hNaglu (SEQ ID NO: 9 and Figure 19 B)) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid
15 carrying the expression cassette flanked by AAV2 ITRs (pAAV-CAG-hNaglu); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. See Ayuso, 2010, *supra*. Vectors were
20 dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

Example 3: Construction of pAAV-CAG-cohNaglu

25 Expression cassettes including an optimized version of the human N-acetylglucosaminidase, alpha CDS (cohNaglu) were designed and obtained. The sequence optimization (GeneArt®) was performed to maximize the efficiency of N-acetylglucosaminidase, alpha protein production in human beings through

elimination of cryptic splice sites and RNA destabilizing sequence elements for increased RNA stability, addition of RNA stabilizing sequence elements, codon optimization and G/C content adaptation, avoidance of stable RNA secondary structures amongst others changes. The optimized CDS was received cloned in the plasmid pMA-RQ (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The pMA-RQ-cohNaglu plasmid was digested with MluI and EcoRI to excise the optimized N-acetylglucosaminidase, alpha CDS. Subsequently, this fragment was cloned between the same restriction sites of the pAAV-CAG backbone plasmid to generate the pAAV-CAG-cohNaglu plasmid (accession number DSM 26626). See SEQ ID NO:6 and Figure 20 A.

Example 4: Production of AAV9-CAG-cohNaglu

Vectors AAV9-CAG-cohNaglu (SEQ ID NO: 10 and Figure 20 B) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by AAV2 ITRs (pAAV-CAG-cohNaglu); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. See Ayuso, 2010, *supra*. Vectors were dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

Example 5: Construction of pAAV-CAG-cohNaglu-version2

Expression cassettes including a second optimized version of the human N-acetylglucosaminidase, alpha CDS (cohNaglu-version2) were designed and obtained. The optimized CDS (DNA 2.0®) was received cloned in the plasmid pJ208 (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The pJ208-cohNaglu-version2 plasmid was digested with MluI and EcoRI to excise the optimized N-acetylglucosaminidase, alpha-version2 CDS. Subsequently, this fragment was cloned between the same restriction sites of the pAAV-CAG backbone plasmid to generate the pAAV-CAG-cohNaglu-version2 plasmid (accession number DSM 32042). See SEQ ID NO:20 and Figure 21 A.

Example 6: Production of AAV9-CAG-cohNaglu-version2

Vectors AAV9-CAG-cohNaglu-version2 (SEQ ID NO: 21 and Figure 21 B) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by AAV2 ITRs (pAAV-CAG-cohNaglu-version2); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. See Ayuso, 2010, *supra*. Vectors were dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

Example 7: Construction of pAAV-CAG-cohNaglu-version3

Expression cassettes including a third optimized version of the human N-acetylglucosaminidase, alpha CDS (cohNaglu-version3) were designed and
5 obtained. The optimized CDS (GenScript, Inc) was received cloned in the plasmid pUC57 (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The pUC57-cohNaglu-version3 plasmid was digested with MluI and EcoRI to excise the optimized N-acetylglucosaminidase, alpha-version3 CDS.
10 Subsequently, this fragment was cloned between the same restriction sites of the pAAV-CAG backbone plasmid to generate the pAAV-CAG-cohNaglu-version3 plasmid (accession number DSM 32043). See SEQ ID NO:23 and Figure 22 A.

Example 8: Production of AAV9-CAG-cohNaglu-version3

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Vectors AAV9-CAG-cohNaglu-version3 (SEQ ID NO: 24 and Figure 22 B) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning,
20 NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by AAV2 ITRs (pAAV-CAG-cohNaglu-version3); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using either a standard
25 protocol or an optimized protocol as previously described. See Ayuso, 2010, *supra*. Vectors were dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

Example 9: Construction of pAAV-CAG-comNaglu

5 The CDS for murine N-acetylglucosaminidase, alpha (NCBI Reference Sequence: NM_013792) was subjected to sequence optimization (GeneArt; Life Technologies). The optimized CDS was received cloned inside the plasmid pMA-RQ (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

10 The MluI/EcoRI optimized murine N-acetylglucosaminidase, alpha CDS fragment was excised from the pMA-RQ plasmid and subsequently cloned between the MluI and EcoRI restrictions sites of the AAV backbone plasmid pAAV-CAG. The resulting plasmid was named pAAV-CAG-comNaglu. See SEQ ID NO: 7 and Figure 23 A.

Example 10: Production of AAV9-CAG-comNaglu

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Vectors AAV9-CAG-comNaglu (SEQ ID NO: 11 and Figure 23 B) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by AAV2 ITRs (pAAV-CAG-comNaglu); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. See Ayuso, 2010, *supra*. Vectors were 25 dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

Example 11: Construction of pAAV-CAG-cocNaglu

The CDS for canine N-acetylglucosaminidase, alpha (NCBI Reference Sequence: XM_548088.4) was subjected to sequence optimization (GeneArt; Life Technologies). The optimized CDS was received cloned inside the plasmid pMA-RQ (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The MluI/EcoRI optimized canine N-acetylglucosaminidase, alpha CDS fragment was excised from the pMA-RQ plasmid and subsequently cloned between the MluI and EcoRI restrictions sites of the AAV backbone plasmid pAAV-CAG. The resulting plasmid was named pAAV-CAG-cocNaglu. See SEQ ID NO: 8 and Figure 24 A.

Example 12: Production of AAV9-CAG-cocNaglu

Vectors AAV9-CAG-cocNaglu (SEQ ID NO: 12 and Figure 24 B) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. See Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by AAV2 ITRs (pAAV-CAG-cocNaglu); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutive cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. See Ayuso, 2010, *supra*. Vectors were dialyzed against PBS, filtered, titred by qPCR and stored at -80°C until use.

Example 13: Hydrodynamic delivery of the plasmid pAAV9-CAG-hNaglu

A total dose of 50 µg of the plasmid pAAV9-CAG-hNaglu containing the wild-type human N-acetylglucosaminidase, alpha expressing cassette were administered to 2-month-old MPSIIIB mice via hydrodynamic tail vein (HDTV) injection. This technique targets expression of the delivered plasmid to the liver.
5 See Liu *et al.*, Gene Ther. 1990;6(7):1258-66.

One week post plasmid delivery, a considerable increase in N-acetylglucosaminidase, alpha activity over pre-treatment levels was documented in the liver and serum of all the animals administered with wild-type human N-acetylglucosaminidase, alpha-coding plasmids. See Figures 1A and 1B. No activity
10 was detected in MPSIIIB animals injected with saline solution. The levels of N-acetylglucosaminidase, alpha activity observed in the liver and serum of treated animals corresponded to 150% and 1700%, respectively, of the mean value of activity measured in the liver and serum of WT animals, which was set to 100%.
See Figures 1A and 1B.

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Example 14: Intravenous delivery of AAV9-CAG-hNaglu

A total dose of 5×10^{11} vector genomes of AAV9-CAG-hNaglu vectors was administered to 2-month-old MPSIIIB mice via tail vein injection.

20 Consistent with the high tropism of AAV9 vectors for the liver, two months after administration treated animals showed high levels of N-acetylglucosaminidase, alpha activity in this organ (700% of the activity levels observed in healthy animals), which completely eliminated or considerably reduced the pathological accumulation of GAGs observed in the somatic tissues of untreated
25 MPSIIIB mice. See Figure 2A-C. In addition, given the high efficiency with which AAV vectors of serotype 9 transduce the brain upon systemic administration, treated animals showed significant levels of N-acetylglucosaminidase, alpha activity (20-30% of healthy mice) in all brain regions analysed. See Figure 3A. This

partial restoration of N-acetylglucosaminidase, alpha activity was sufficient to clear GAG storage from all areas of the brain. *See* Figure 3B.

Example 15: Intravenous delivery of AAV9-CAG-cohNaglu

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A total dose of 5×10^{11} vector genomes of AAV9-CAG-cohNaglu vectors was administered to 2-month-old MPSIIIB mice via tail vein injection.

Two months after administration, treated animals showed high levels of activity of N-acetylglucosaminidase, alpha in the liver (600 % of healthy levels) and a moderate increase (7% of healthy levels) in the levels of activity in serum. *See* Figure 4A and 4B. N-acetylglucosaminidase, alpha production completely eliminated or considerably reduced the pathological accumulation of GAGs observed in the somatic tissues of untreated MPSIIIB mice. *See* Figure 4C. In addition, treated animals showed significant levels of N-acetylglucosaminidase, alpha activity (18-27% of healthy mice) in all brain regions analysed. *See* Figure 5A. This partial restoration of N-acetylglucosaminidase, alpha activity was sufficient to clear GAG storage from all areas of the brain. *See* Figure 5B.

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Example 16: Intracisternal delivery of AAV9-CAG-hNaglu

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A total dose of 9.3×10^9 vector genomes of AAV9-CAG-hNaglu vector was injected into the cisterna magna of 2-month-old MPSIIIB animals in a total volume of 5 μ l.

The intra-CSF administration of AAV9-CAG-hNaglu vectors led to high levels of N-acetylglucosaminidase, alpha activity in all brain areas analysed (50-100% of healthy mice); in the most forefront parts of the brain activity reached the

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levels observed in healthy animals. *See* Figure 6A. The excessive storage of GAGs was completely abolished in the brains of treated MPSIIIB mice. *See* Figure 6B. When delivered to the CSF, AAV vectors of serotype 9 leak into the bloodstream and transduce the liver. *See* Haurigot *et al.*, J Clin Invest 2013;123(8):3254-71. In agreement with this, N-acetylglucosaminidase, alpha activity was detected in the liver of treated MPSIIIB mice at levels of 32% of healthy animals. *See* Figure 7A. This increase in N-acetylglucosaminidase, alpha activity mediated the correction of GAG accumulation in liver, spleen, heart, lung, testis and urinary bladder and also significantly diminished GAG storage in kidney. *See* Figure 7B.

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Example 17: Intracisternal delivery of AAV9-CAG-cohNaglu

A total dose of 9.3×10^9 vector genomes of AAV9-CAG-cohNaglu vector was injected into the cisterna magna of 2-month-old MPSIIIB animals in a total volume of 5 μ l.

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Intracisternal administration of AAV9-CAG-cohNaglu vectors led a considerable increase in the levels of N-acetylglucosaminidase, alpha activity, which ranged from 22 to 45% of healthy values, in all brain regions analysed. *See* Figure 8A. Accordingly, pathological accumulation of GAGs was completely reverted in all brains regions of treated MPSIIIB mice. *See* Figure 8B. N-acetylglucosaminidase, alpha activity also increased in the liver of treated MPSIIIB mice to 25% of healthy animals. *See* Figure 9A. This increase in N-acetylglucosaminidase, alpha activity mediated the correction of GAG accumulation in liver, heart, lung and urinary bladder and also significantly diminished GAG storage in spleen, testis and kidney. *See* Figure 9B.

20
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Example 18: Hydrodynamic delivery of the plasmid pAAV9-CAG-cohNaglu-version2

A total dose of 50 µg of the plasmid pAAV9-CAG-cohNaglu-version2 carrying an expression cassette containing an optimized version (version2) of human N-acetylglucosaminidase, alpha were administered to 2-month-old MPSIIIB mice via tail hydrodynamic tail vein injection. As aforementioned, this technique targets expression of the delivered plasmid to the liver. *See Liu et al., supra.*

One week post plasmid delivery, N-acetylglucosaminidase, alpha activity was increased over pre-treatment levels in the liver and serum of all the animals that received a hydrodynamic injection of the plasmid containing the optimized version2 of N-acetylglucosaminidase, alpha-coding sequence. *See Figures 10A and 10B.* No activity was detected in MPSIIIB animals injected with saline solution. In treated animals, liver and serum N-acetylglucosaminidase, alpha activity reached levels that were 150% and 1500%, respectively, of the mean value of activity observed in WT animals (set to 100%). *See Figures 10A and 10B.*

Example 19: Hydrodynamic delivery of the plasmid pAAV9-CAG-cohNaglu-version3

A total dose of 50 µg of the plasmid pAAV9-CAG-cohNaglu-version3 containing the a codon optimized version (version3) human N-acetylglucosaminidase, alpha expressing cassette were administered to 2-month-old MPSIIIB mice via tail hydrodynamic tail vein injection. As aforementioned, this technique targets expression of the delivered plasmid to the liver. *See Liu et al., supra.*

One week post plasmid delivery, a considerable increase in N-acetylglucosaminidase, alpha activity over pre-treatment levels was documented in the liver and serum of all the animals administered with the plasmid carrying the expression cassette that contained the version3 of codon-optimized human N-acetylglucosaminidase, alpha-coding sequence. *See Figures 11A and 11B.* No activity was detected in MPSIIIB animals injected with saline solution. The levels of N-acetylglucosaminidase, alpha activity observed in the liver and serum of

treated animals corresponded to 170% and 1100%, respectively, of the mean value of activity determined in the liver and serum of WT animals, which was set to 100%. *See* Figures 11A and 11B.

5 **Example 20: Intracisternal delivery of AAV9-CAG-comNaglu**

A total dose of 3×10^{10} vector genomes of AAV9-CAG-comNaglu vector was injected into the cisterna magna of 2-month-old MPSIIIB animals in a total volume of 10 μ l.

10 AAV9 vector genomes could be detected in all brain areas analysed, as well as in the spinal cord. In peripheral tissues, vector genomes could be detected at considerable gene copy numbers only in the liver, and at low gene copy numbers in the lymph nodes in which the head drains (mandibular lymph nodes). *See* Figure 12A-B.

15 The intra-CSF administration of AAV9-CAG-comNaglu vectors led to very high levels of N-acetylglucosaminidase, alpha activity in all brain areas analysed; reaching levels that were several fold higher than those observed in healthy animals in all regions. *See* Figure 13A. Three months post vector delivery the lysosomal pathology characteristic of the disease was completely reverted in the brains of
20 treated MPSIIIB mice, as indicated by the normalization of GAG accumulation and of the signal intensity for the lysosomal marker LIMP-2+ in all brain areas analysed. *See* Figures 13B and 14A. Ultrastructural analysis of the occipital cortex and the cerebellum by transmission electron microscopy confirmed the reduction in lysosomal pathology, which was very evident in cortical perineuronal glial cells
25 that appeared distended and full of large storage vesicles in MPSIIIB animals that received control vector, while they had a normal appearance in animals treated with AAV9-CAG-comNaglu. *See* Figure 14B. In LSD, the activity of several lysosomal enzymes aside from that directly affected by the inherited mutation can be altered

secondary to perturbation of normal lysosomal homeostasis. *See Sardiello et al., Science. 2009;325:473–477.* In the brain of untreated or Null-treated male MPSIIIB mice aged 5 months, the activities of Iduronate 2-sulfatase (IDS), N-sulphoglucosamine sulphohydrolase (SGSH), β -glucuronidase (GUSB), and β -hexosaminidase (HEXB) were significantly increased. *See Figure 14C.* Consistent with the reduction in GAG storage observed 3 months after intra-CSF delivery of AAV9-CAG-comNaglu vectors, the activity of brain SGSH, GUSB and HEXB returned to healthy wild-type levels in treated animals. *See Figure 14C.*

In agreement with the correction of the lysosomal pathology, all signs of inflammation disappeared from the brains of treated MPSIIIB mice. The signal intensities for the stainings used to detect astrogliosis (GFAP) and microgliosis (BSI-B4) were similar in treated MPSIIIB mice and in healthy animals, as opposed to the signal documented in untreated MPSIIIB mice that showed a clear upregulation of these markers of neuroinflammation. *See Figures 15A and 15B.* To further assess the efficacy of intra-CSF AAV9-CAG-comNaglu gene therapy on CNS inflammation, a gene expression profiling study using the Affimatrix® microarray platform on total RNA isolated from WT and AAV9-CAG-comNaglu or AAV9-Null-treated MPSIIIB encephalon was performed. After data processing and filtering, 94 genes were found to be differentially expressed among the three groups. When Gene Ontology (GO) enrichment analysis was used to classify differentially expressed genes by biological process, 67 of the 94 genes were annotated with corresponding ontologies. *See Figure 15C.* Of these, the vast majority were associated with inflammation and innate immunity or functions that can be attributed to cells involved in these processes. *See Figure 15C.* To confirm this observation, we used Cell Type Enrichment (CTEN) analysis to assess the contribution of different cell types to the observed changes in transcript levels. This software considers there is enrichment of a specific cell type if a software-defined score is greater than 2. The highest score for our data set was obtained for microglial cells (score = 60), a result in agreement with the important role attributed to microglia in neurodegenerative diseases and in MPSIII. *See Ohmi et al., Proc Natl Acad Sci USA. 2003;100:1902–1907, McGeer et al. Alzheimer Dis*

Assoc Disord. 1998;12 (Suppl. 2):S1–E16, Derecki *et al.*, Nature. 2012;484: 105–109, DiRosario *et al.*, J Neurosci Res. 2009;87:978–990, Archer, *et al.*, J Inherit Metab Dis. 2014;37:1–12. When the effect of intra-CSF delivery of AAV9-CAG-comNaglu vectors was evaluated, a striking change in the profile of gene expression with respect to AAV9-Null-injected animals was observed. Three months after vector administration, the vast majority of genes in treated MPSIIIB mice had transcript levels that resembled those of healthy littermates. Almost 90% of the genes differentially expressed in untreated MPSIIIB mice showed a correction in their transcript levels of at least 50% following AAV9-CAG-comNaglu treatment and in 60% of them the correction was of 75%. Similar or slightly higher degrees of normalization in transcript levels were observed when the set of genes that the CTEN software had assigned to microglia was analysed separately. *See* Figure 15D. This observation supports the idea that microglia is largely responsible for the profile of gene expression observed in MPSIIIB and is in agreement with the complete reversal of microgliosis documented in treated animals.

AAV9 vectors administered to the CSF leak to the periphery and transduce the liver. *See* Figure 9 and Haurigot *et al.*, *supra*. Accordingly, an increase in N-acetylglucosaminidase, alpha activity was documented in the liver and serum of MPSIIIB mice treated with AAV9-CAG-comNaglu, reaching levels of approximately 800% of the levels observed in healthy animals. *See* Figures 16A and 16B. The levels of enzymatic activity in serum correlated well with those of activity in the liver of treated mice, suggesting that the liver was the main source of circulating enzyme. When the somatic efficacy of the therapy was evaluated through quantification of the GAG content in different organs, a full normalization was observed in most tissues, including liver, spleen, heart, and lung, with the exception of kidney, in which a >50% reduction of GAGs was observed. *See* Figure 16C. The ultrastructural analysis of the liver in the different experimental groups showed the complete disappearance of the storage vacuoles that are characteristic of the MPSIIIB disease from the hepatocytes of MPSIIIB mice treated with AAV9-CAG-comNaglu. *See* Figure 16D. As observed in CNS, the clearance of storage

material from the lysosomal compartment led to restoration of the activity of other lysosomal enzymes. In liver of untreated or Null-treated MPSIIIB mice, the activities of α -iduronidase (IDUA), iduronate 2-sulphatase (IDS), SGSH, GUSB and HEXB were altered. *See* Figure 16E. Three months following AAV9-mediated NAGLU gene transfer, the activity of all these enzymes returned to normal levels, further supporting the concept that CSF gene transfer of NAGLU through AAV9-CAG-comNaglu can reverse disease phenotype also in peripheral organs. *See* Figure 16E.

The impact of the intra-CSF administration of AAV9-CAG-comNaglu on behaviour was assessed with the open field test, which evaluates the general locomotor and exploratory activity of mice in unknown surroundings. Untreated and AAV9-null-treated MPSIIIB mice displayed reduced exploratory activity compared with healthy mice in terms of the latency to enter the centre, the time spent in the border, the number of entries to the centre, the resting time, the total distance travelled, and the number of lines crossed. Intracisternal administration of AAV9-CAG-comNaglu completely corrected behavioural deficits. *See* Figures 17A-F.

Furthermore, treatment with AAV9-CAG-comNaglu significantly extended the lifespan of MPSIIIB mice. By 15 months of age, all untreated MPSIIIB mice had died while 100% of the animals receiving intracisternal AAV9-CAG-comNaglu were still alive at 18 months of age. *See* Figures 17G. Treated MPSIIIB males showed a median survival of 21 months, compared with a 13.8 month median survival for AAV9-null MPSIIIB males; $P=0.0007$. Median survival for healthy male was 26.6 months. The normalization of behavioural responses and the greater survival of treated MPSIIIB mice further demonstrate the therapeutic efficacy of AAV9-CAG-comNaglu.

Example 21: Intracisternal delivery of AAV9-CAG-cocNaglu to dogs

The first step towards the clinical application of a gene therapy approach requires the demonstration of its feasibility in a large animal model. We previously demonstrated that the distribution of AAV9 vectors upon intra cerebrospinal fluid administration to Beagle dogs, an animal model with a brain size closer to that of humans, is very similar to that observed in mice receiving an equivalent dose of vector through the same route. *See Haurigot et al., supra.* Briefly, the administration of 2×10^{13} vg of AAV9 vectors encoding for a reporter protein GFP demonstrated widespread transduction of cells in the brain, cerebellum, meninges, spinal cord and dorsal root ganglia. Similar to the observations made in mice, GFP was also detected in the liver of Beagle dogs, where an average of 3.7% of hepatocytes was transduced. *See Haurigot et al., supra.* Importantly, the intra-CSF administration of AAV9 vectors encoding for the lysosomal enzyme sulfamidase, whose deficit causes MPSIIIA, led to sustained levels of enzyme in the CSF of treated dogs. The CSF bathes the CNS, making the enzyme available to different CNS structures. Indeed, the periodic delivery of recombinant enzyme to the CSF is a therapeutic strategy currently under clinical investigation for MPSIIIA. *See* NCT01155778 and NCT01299727, clinicaltrials.gov.

The same approach has been used to illustrate the potential efficacy of the AAV 9 vectors according to the present invention.

A total dose of 6.5×10^{12} vg AAV9-CAG-cocNaglu. vectors was administered to the cisterna magna of 4 adult Beagle dogs (Dogs 1-4). To evaluate the impact of preexisting immunity on the CSF levels of N-acetylglucosaminidase, alpha activity that could be achieved by the treatment, two of those dogs (Dogs 3 and 4) were immunized by systemic administration of 1×10^{11} vg/kg of non-coding AAV9-null vectors 6 weeks before CSF delivery. By the time the intracisternal administration of vectors was performed, naïve dogs had low titers of anti-AAV9 neutralizing antibodies (NAbs) in circulation and CSF, as it would be expected for animals that have not been previously exposed to the wild-type or recombinant virus. In contrast, pre-immunized dogs had high NAb titers in the circulation but low levels in the CSF, an observation compatible with the asymmetrical distribution

of NABs across the blood-brain barrier. *See* Figure 18A and Haurigot *et al.*, *supra*. The administration of AAV9-CAG-cocNaglu vectors to the cisterna magna led to a significant increase in the activity of the enzyme measured in the CSF samples from all 4 dogs, with no significant differences with respect to the presence or not of pre-existing immunity. *See* Figure 18B. Enzyme activity levels peaked between the 2nd and 3rd weeks and reached steady state levels thereafter. Importantly, this increase in the activity of N-acetylglucosaminidase, alpha in CSF was long-lasting (>4 months). *See* Figure 18B.

The following embodiments are provided:

- 10 1. A recombinant AAV9 vector containing a CAG promoter SEQ ID NO: 4 linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha, SEQ ID NO: 1, wherein the nucleotide sequence coding for N-acetylglucosaminidase, alpha, SEQ ID NO: 1 is SEQ ID NO: 3, sequence SEQ ID NO: 19 or sequence SEQ ID NO: 22.
- 15 2. A plasmid containing a CAG promoter linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha, SEQ ID NO:1, wherein said plasmid is pAAV-CAG-cohNaglu with accession number DSM 26626, containing the nucleotide sequence SEQ ID NO: 3 coding for N-acetylglucosaminidase, alpha, SEQ ID NO: 1, pAAV-CAG-cohNaglu-version2 with accession number DSM 32042, containing the nucleotide sequence SEQ ID NO: 19 coding for N-acetylglucosaminidase, alpha, SEQ ID NO: 1 or pAAV-CAG-cohNaglu-version3 with accession number DSM 32043, containing the nucleotide sequence SEQ ID NO: 22 coding for N-acetylglucosaminidase, alpha, SEQ ID NO: 1.
- 20 3. A pharmaceutical composition comprising the recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 and a pharmaceutically acceptable carrier.
- 25 30

4. The pharmaceutical composition according to embodiment 3 for intravenous or intracisternal administration.
- 5 5. Use of the recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for the manufacture of a medicament.
6. Use of the recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for the manufacture of a medicament for increasing N-acetylglucosaminidase, alpha activity in a subject.
- 10 7. Use of the recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for the manufacture of a medicament for the treatment of mucopolysaccharidosis.
- 15 8. Use according to embodiment 7, wherein mucopolysaccharidosis is mucopolysaccharidosis type IIIB.
9. Use of the recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for increasing N-
- 20 acetylglucosaminidase, alpha activity in a subject.
10. Use of the recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for the treatment of mucopolysaccharidosis.
- 25 11. Use according to embodiment 10, wherein mucopolysaccharidosis is mucopolysaccharidosis type IIIB.
12. The recombinant AAV9 vector according to embodiment 1 or the plasmid
- 30 according to embodiment 2 for increasing N-acetylglucosaminidase, alpha activity in a subject.

13. The recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for use in the treatment of mucopolysaccharidosis.
- 5
14. The recombinant AAV9 vector according to embodiment 1 or the plasmid according to embodiment 2 for use in the treatment of mucopolysaccharidosis type IIIB.
- 10
15. A method of producing the vector according to embodiment 1 comprising the steps of:
- 15
- i) providing a first vector comprising the CAG promoter operably linked to the nucleotide sequence coding for N-acetylglucosaminidase, alpha, interposed between a first AAV terminal repeat and a second AAV terminal repeat, a second vector comprising an AAV *rep* gene and an AAV *cap* gene from serotype 9; and a third vector comprising the adenovirus helper function gene;
- 20
- ii) co-transfecting competent cells with the vectors of step i);
- iii) culturing the transfected cells of step ii); and
- iv) purifying the expression vectors from the culture of step iii).
16. An isolated cell comprising the recombinant AAV9 vector according to embodiment 1.

CLAIMS

1. A recombinant AAV9 vector containing a CAG promoter SEQ ID NO: 4
linked to a nucleotide sequence coding for N-acetylglucosaminidase, alpha,
5 SEQ ID NO: 1, wherein the nucleotide sequence coding for N-
acetylglucosaminidase, alpha, SEQ ID NO: 1 is SEQ ID NO: 3, SEQ ID
NO: 19 or SEQ ID NO: 22.
2. A plasmid containing a CAG promoter linked to a nucleotide sequence
10 coding for N-acetylglucosaminidase, alpha, SEQ ID NO:1, wherein said
plasmid is pAAV-CAG-cohNaglu with accession number DSM 26626,
containing the nucleotide sequence SEQ ID NO: 3 coding for N-
acetylglucosaminidase, alpha, SEQ ID NO: 1, pAAV-CAG-cohNaglu-
15 version2 with accession number DSM 32042, containing the nucleotide
sequence SEQ ID NO: 19 coding for N-acetylglucosaminidase, alpha, SEQ
ID NO: 1 or pAAV-CAG-cohNaglu-version3 with accession number DSM
32043, containing the nucleotide sequence SEQ ID NO: 22 coding for N-
acetylglucosaminidase, alpha, SEQ ID NO: 1.
- 20 3. A pharmaceutical composition comprising the recombinant AAV9 vector
according to claim 1 or the plasmid according to claim 2 and a
pharmaceutically acceptable carrier.
4. The pharmaceutical composition according to claim 3 for intravenous or
25 intracisternal administration.
5. Use of the recombinant AAV9 vector according to claim 1 or the plasmid
according to claim 2 for the manufacture of a medicament.

6. Use of the recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for the manufacture of a medicament for increasing N-acetylglucosaminidase, alpha activity in a subject.
- 5 7. Use of the recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for the manufacture of a medicament for the treatment of mucopolysaccharidosis.
- 10 8. Use according to claim 7, wherein mucopolysaccharidosis is mucopolysaccharidosis type IIIB.
- 15 9. Use of the recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for increasing N-acetylglucosaminidase, alpha activity in a subject.
- 20 10. Use of the recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for the treatment of mucopolysaccharidosis.
- 25 11. Use according to claim 10, wherein mucopolysaccharidosis is mucopolysaccharidosis type IIIB.
- 30 12. The recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for increasing N-acetylglucosaminidase, alpha activity in a subject.
- 35 13. The recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for use in the treatment of mucopolysaccharidosis.
- 40 14. The recombinant AAV9 vector according to claim 1 or the plasmid according to claim 2 for use in the treatment of mucopolysaccharidosis type IIIB.

15. A method of producing the vector according to claim 1 comprising the steps of:
- 5 i) providing a first vector comprising the CAG promoter operably linked to the nucleotide sequence coding for N-acetylglucosaminidase, alpha, interposed between a first AAV terminal repeat and a second AAV terminal repeat, a second vector comprising an AAV *rep* gene and an AAV *cap* gene from serotype 9; and a third vector comprising the adenovirus helper function gene;
 - 10 ii) co-transfecting competent cells with the vectors of step i);
 - iii) culturing the transfected cells of step ii); and
 - iv) purifying the expression vectors from the culture of step iii).
16. An isolated cell comprising the recombinant AAV9 vector according to
- 15 claim 1.

Figure 1

HDTV – Human NAGLU

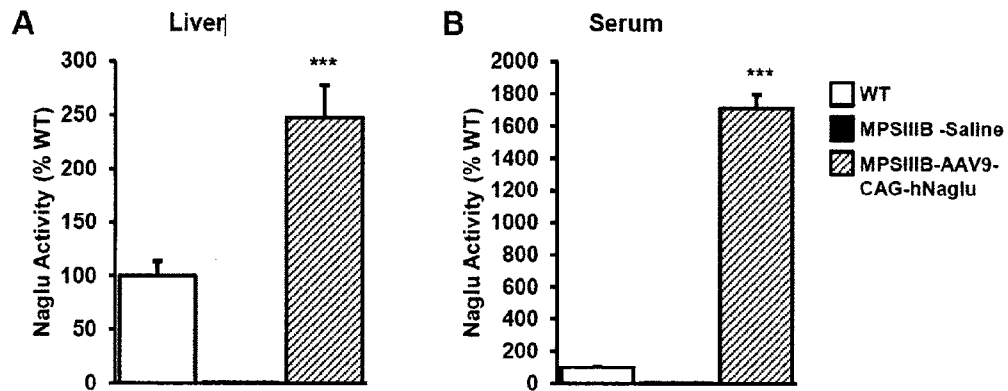


Figure 2

IV – Human NAGLU

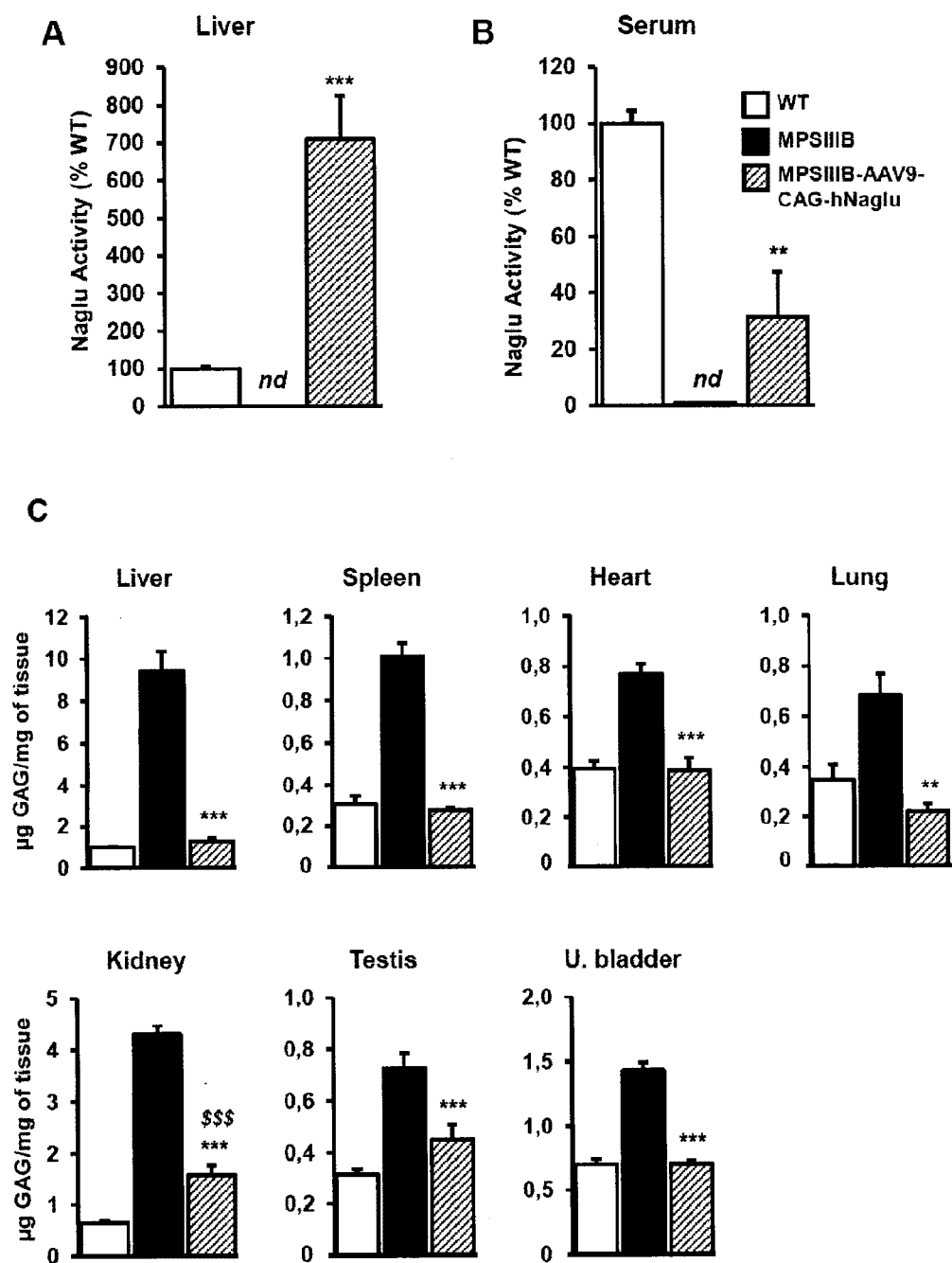


Figure 3

IV – Human NAGLU

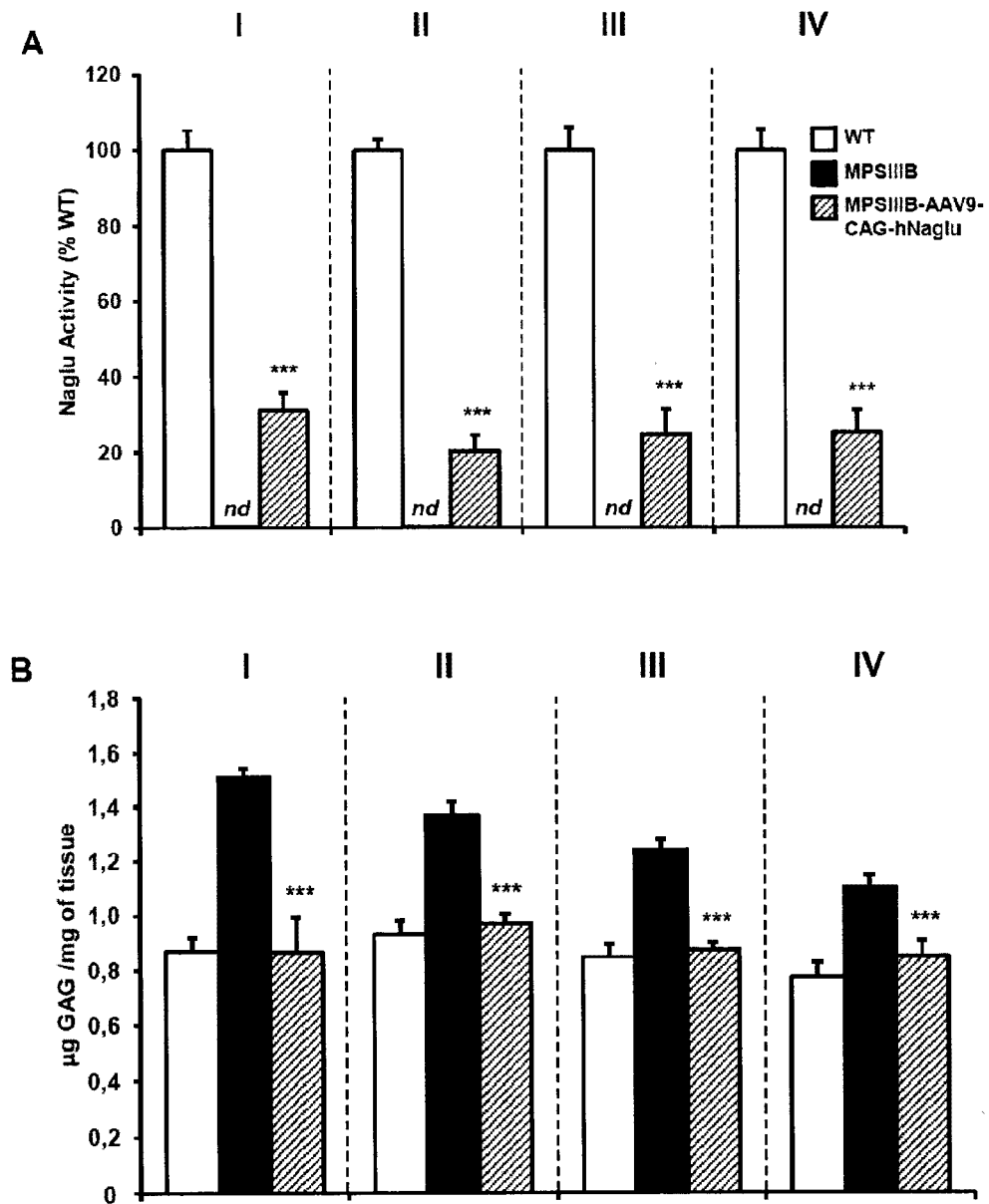


Figure 4

IV – Codon-optimized human NAGLU

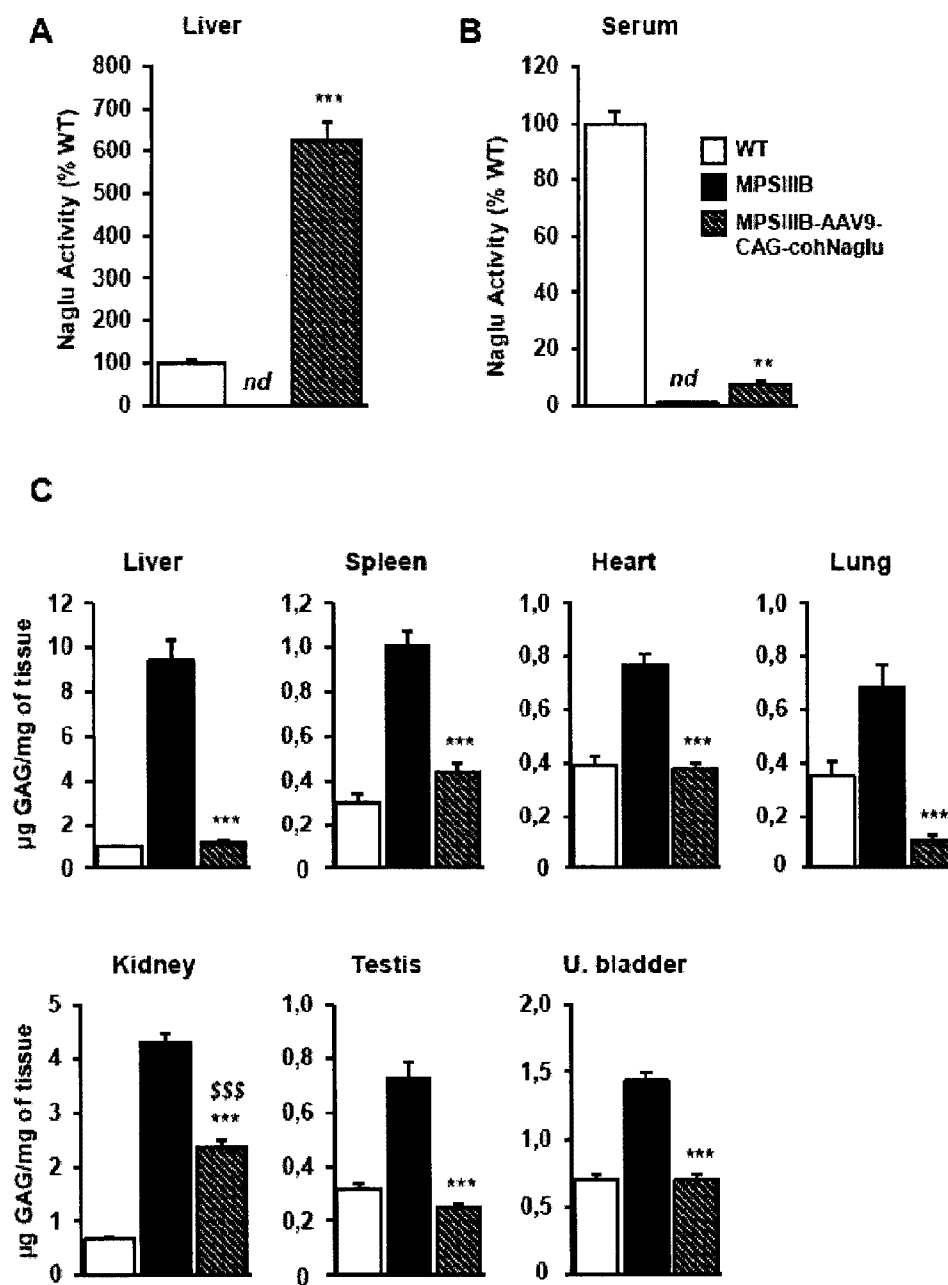


Figure 5

IV – Codon-optimized human NAGLU

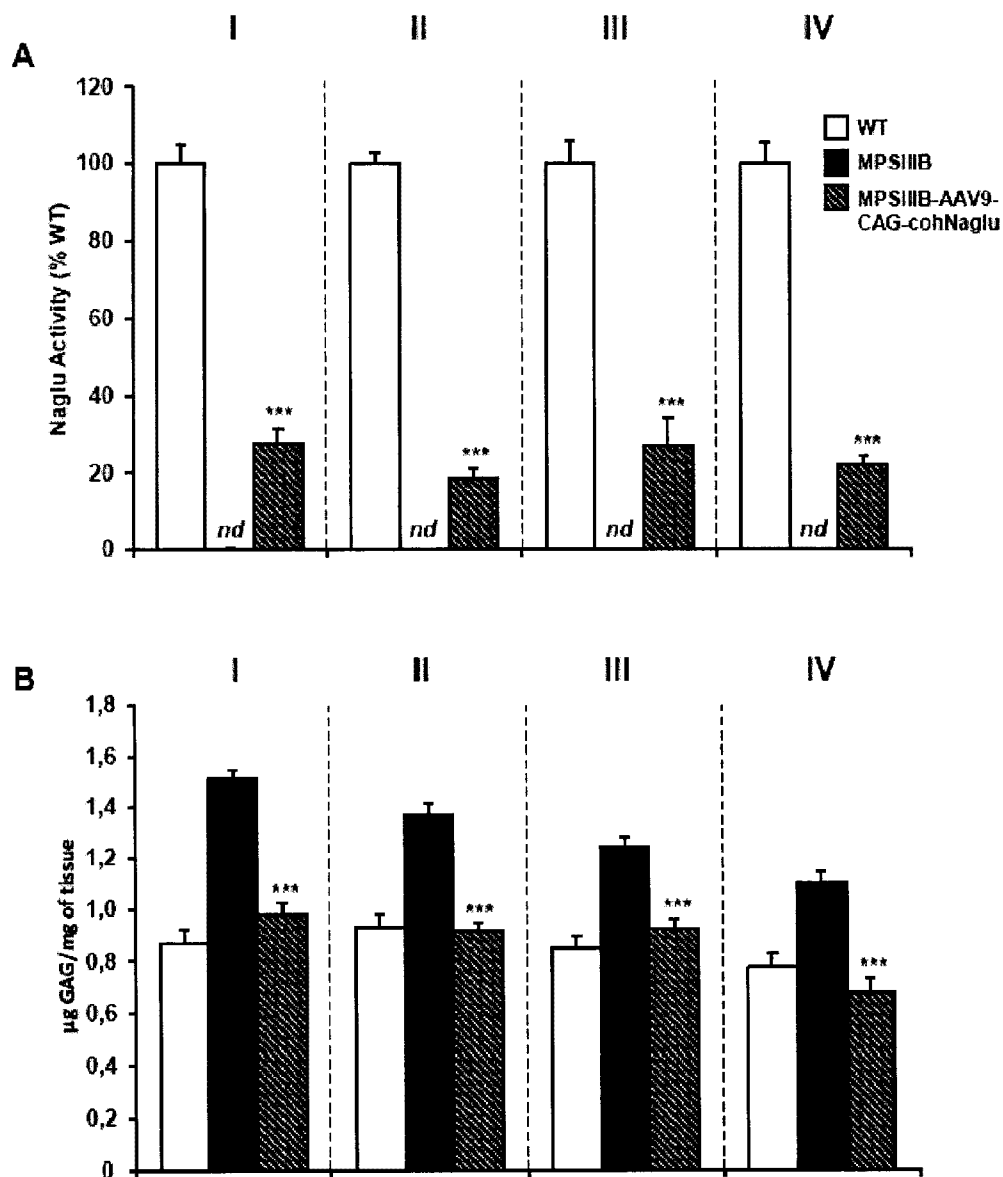


Figure 6

IC – Human NAGLU

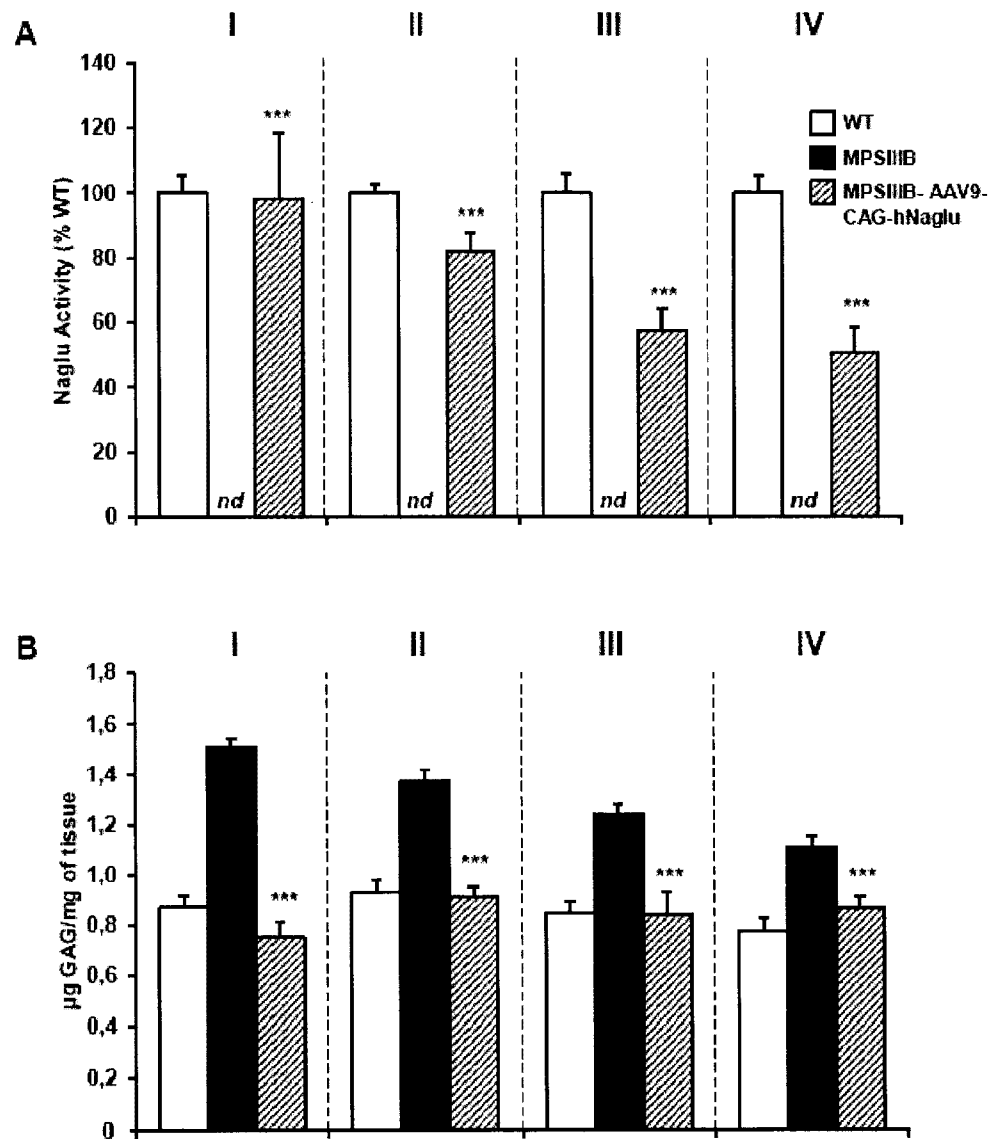


Figure 7

IC – Human NAGLU

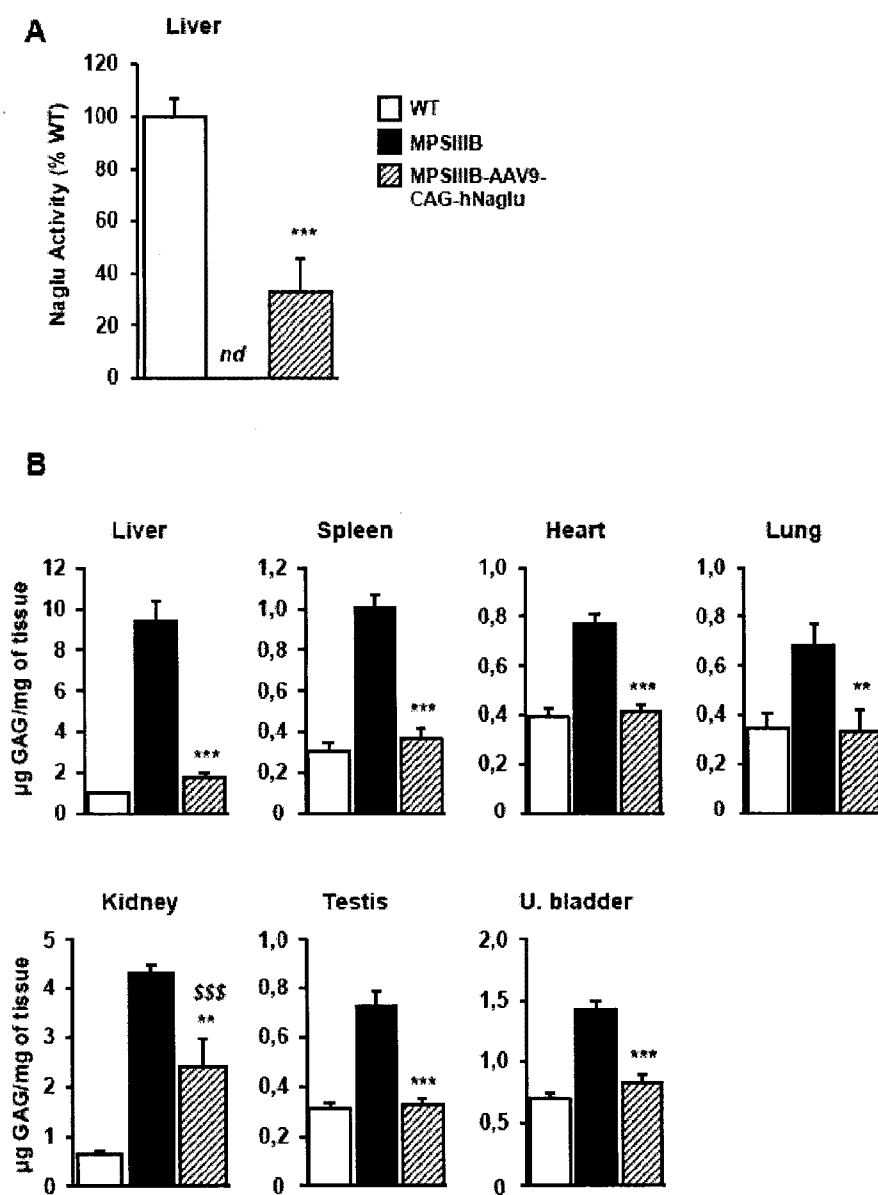


Figure 8

IC – Codon-optimized human NAGLU

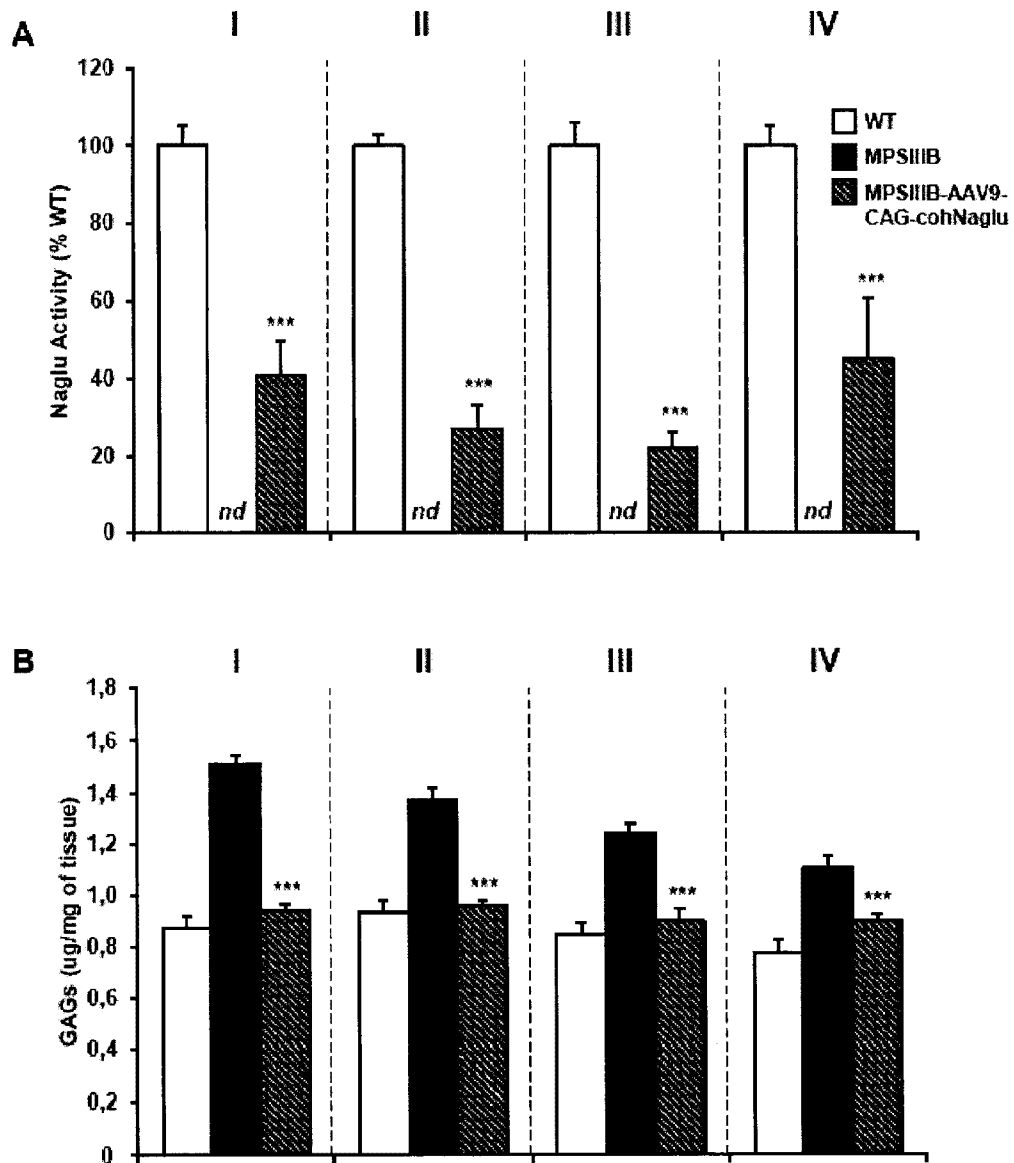
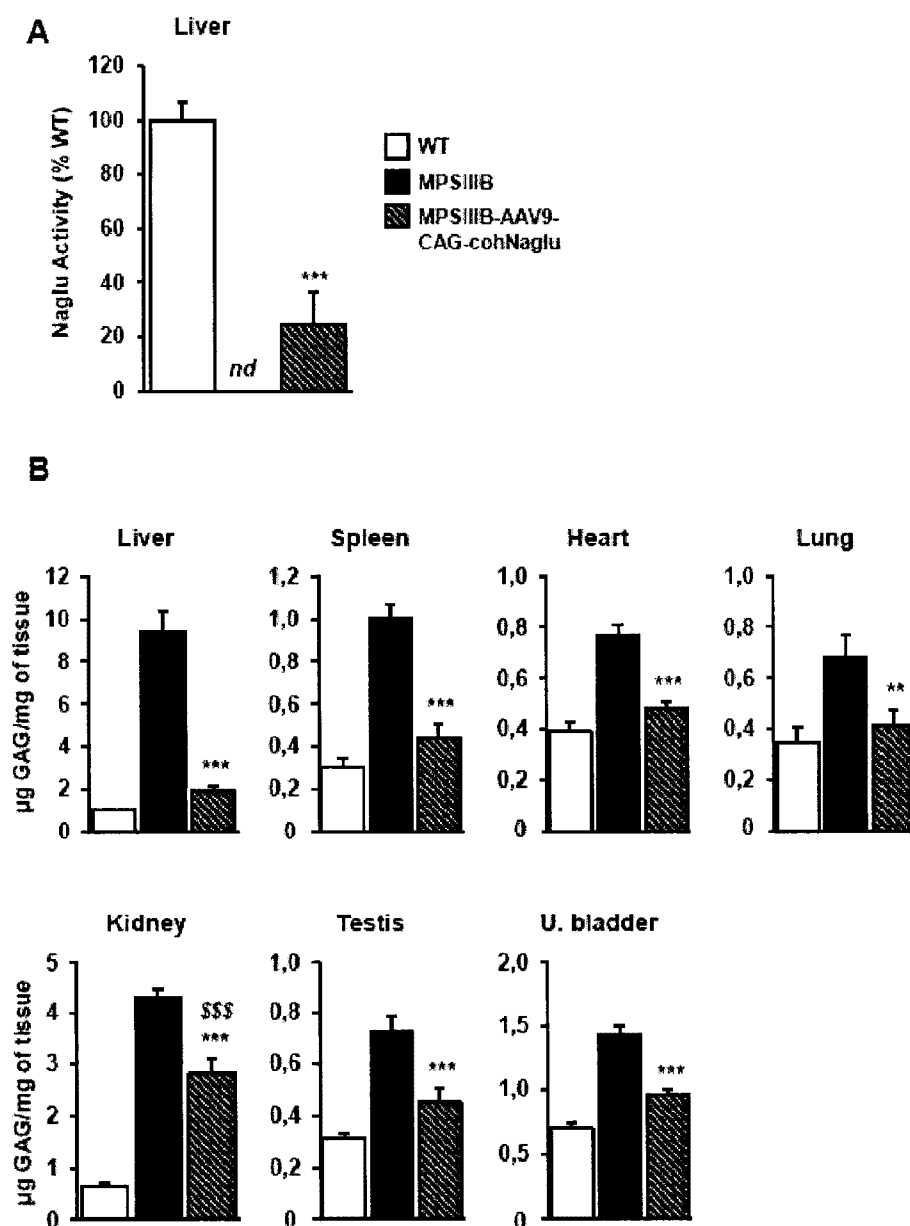


Figure 9

IC – Codon-optimized human NAGLU



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Figure 10

HDTV – Codon-optimized human NAGLU version2

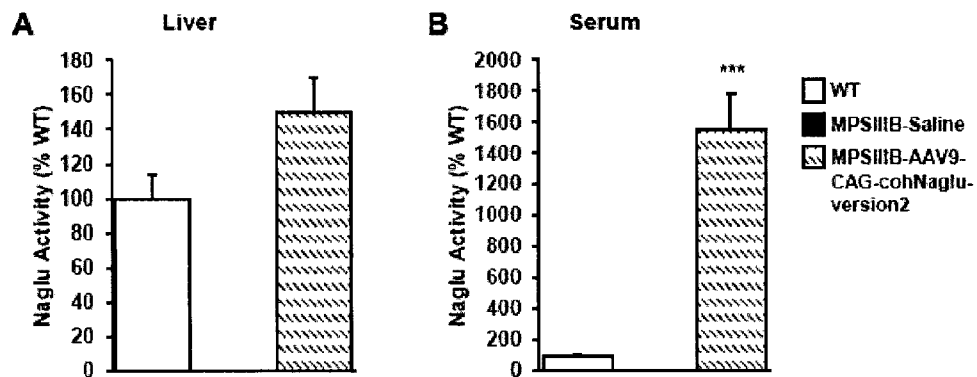


Figure 11

HDTV – Codon-optimized human NAGLU version3

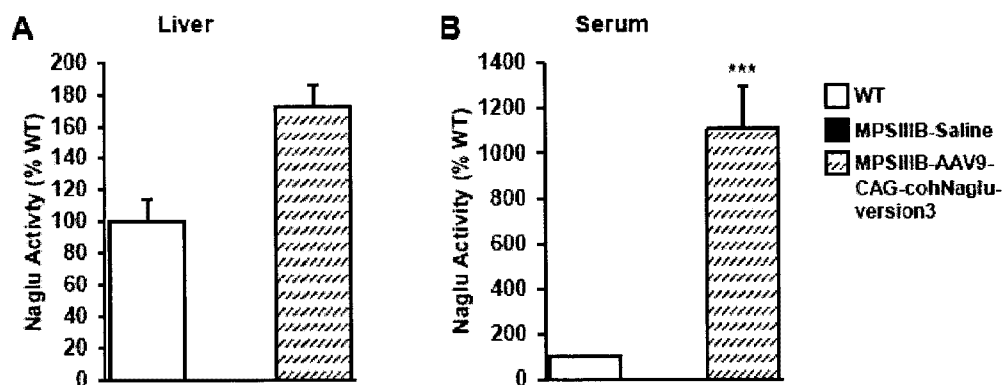


Figure 12

IC – Codon-optimized murine NAGLU**A**

Brain Section 	Vector genomes /diploid genome	
	<i>Males</i>	<i>Females</i>
I	0.14 ± 0.04	0.13 ± 0.03
II	0.09 ± 0.01	0.12 ± 0.04
III	0.13 ± 0.04	0.19 ± 0.10
IV	1.61 ± 0.73	0.80 ± 0.55
V	0.94 ± 0.15	1.35 ± 0.74
Spinal cord	0.14 ± 0.07	0.07 ± 0.02

B

Tissue	Vector genomes /diploid genome	
	<i>Males</i>	<i>Females</i>
Liver	0.78 ± 0.33	0.72 ± 0.33
Spleen	0.03 ± 0.02	0.02 ± 0.01
Heart	0.01 ± 0.00	0.01 ± 0.01
Kidney	0.01 ± 0.00	0.01 ± 0.00
Lung	0.01 ± 0.00	0.01 ± 0.00
Adrenal gland	0.04 ± 0.01	0.10 ± 0.01
Mandibular lymph node	0.12 ± 0.02	0.12 ± 0.02
Inguinal lymph node	0.01 ± 0.00	0.02 ± 0.01
White adipose tissue	0.02 ± 0.00	0.03 ± 0.01
Bone marrow	ND	ND
Skin	0.01 ± 0.00	ND
Thymus	ND	ND
Skeletal muscle	ND	ND
Intestine	ND	ND
Pancreas	ND	ND
Urinary bladder	ND	ND
Epidydimus	ND	ND
Testicle	ND	ND
Oviduct	ND	ND

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Figure 13

IC – Codon-optimized murine NAGLU

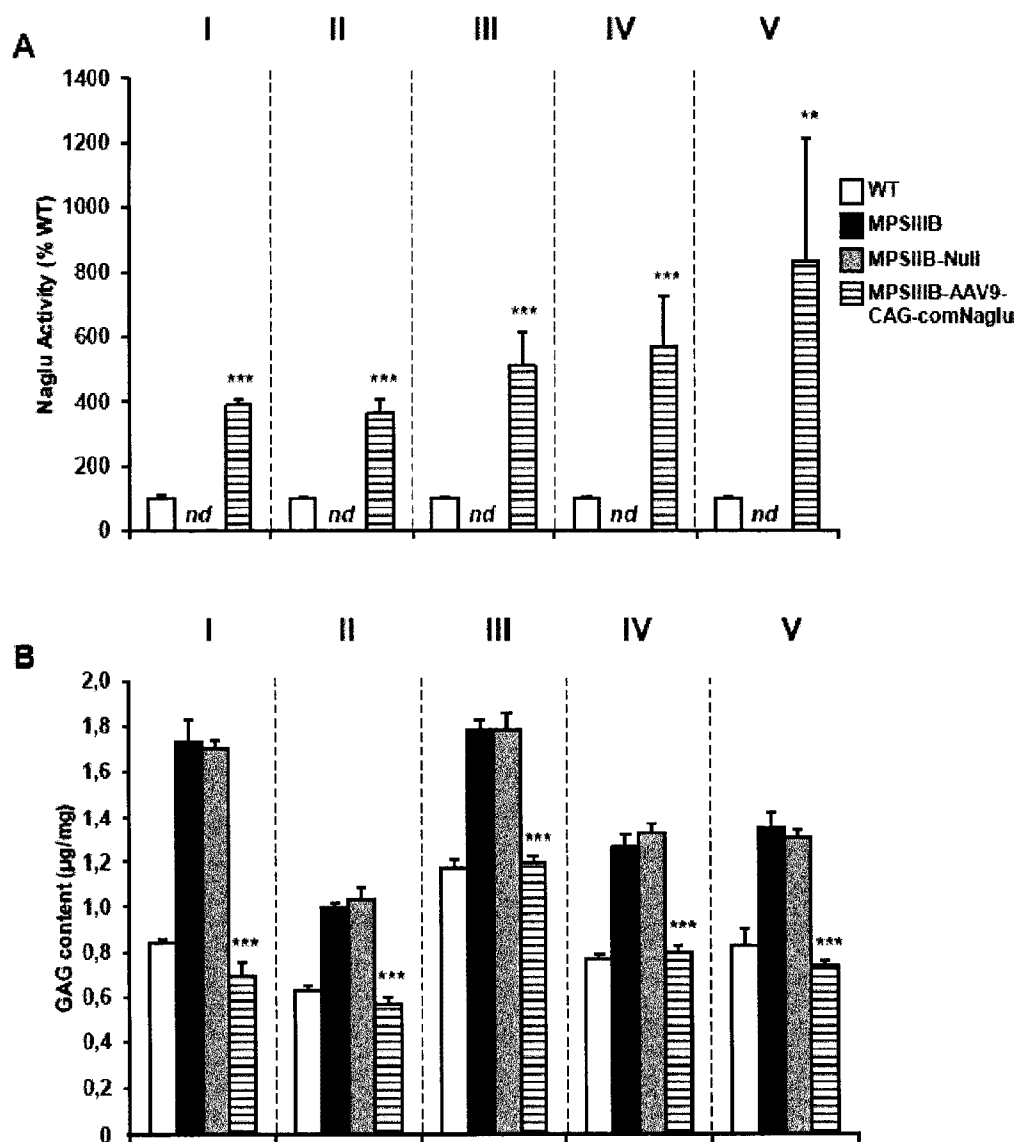
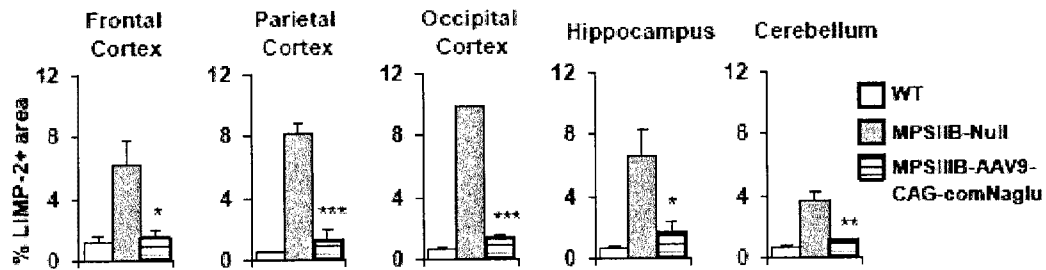


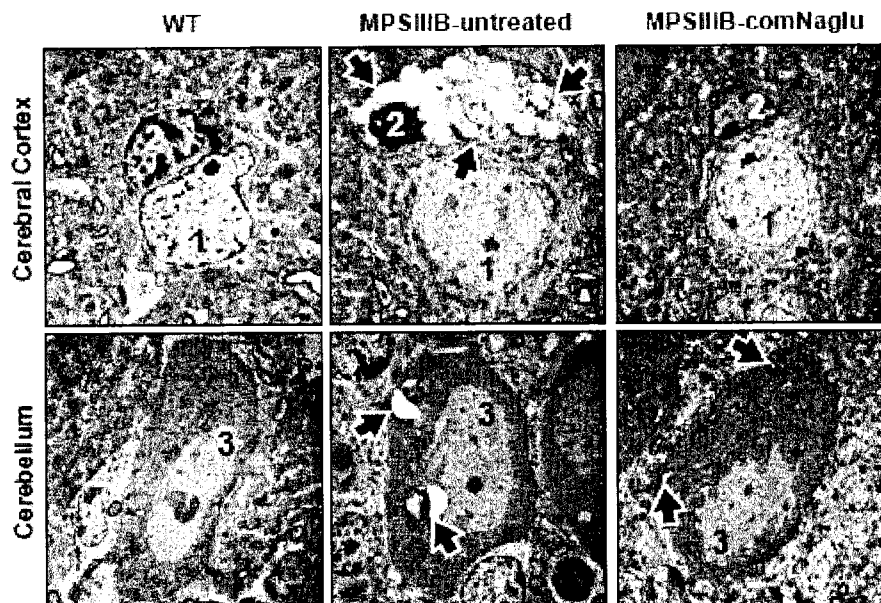
Figure 14

IC – Codon-optimized murine NAGLU

A



B



C

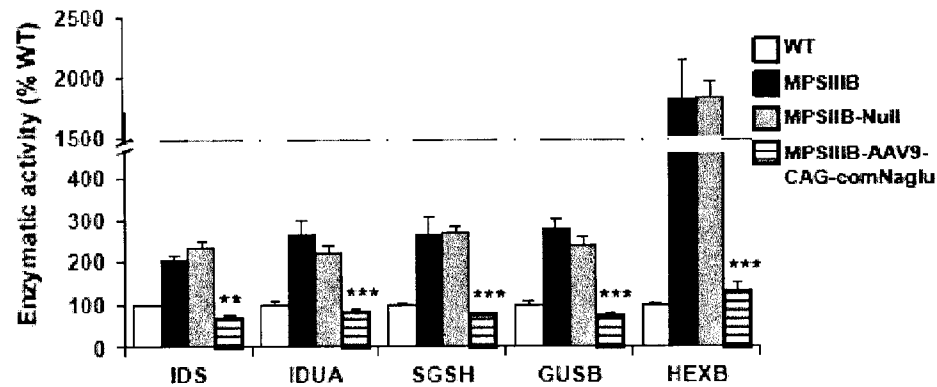


Figure 15 IC – Codon-optimized murine NAGLU

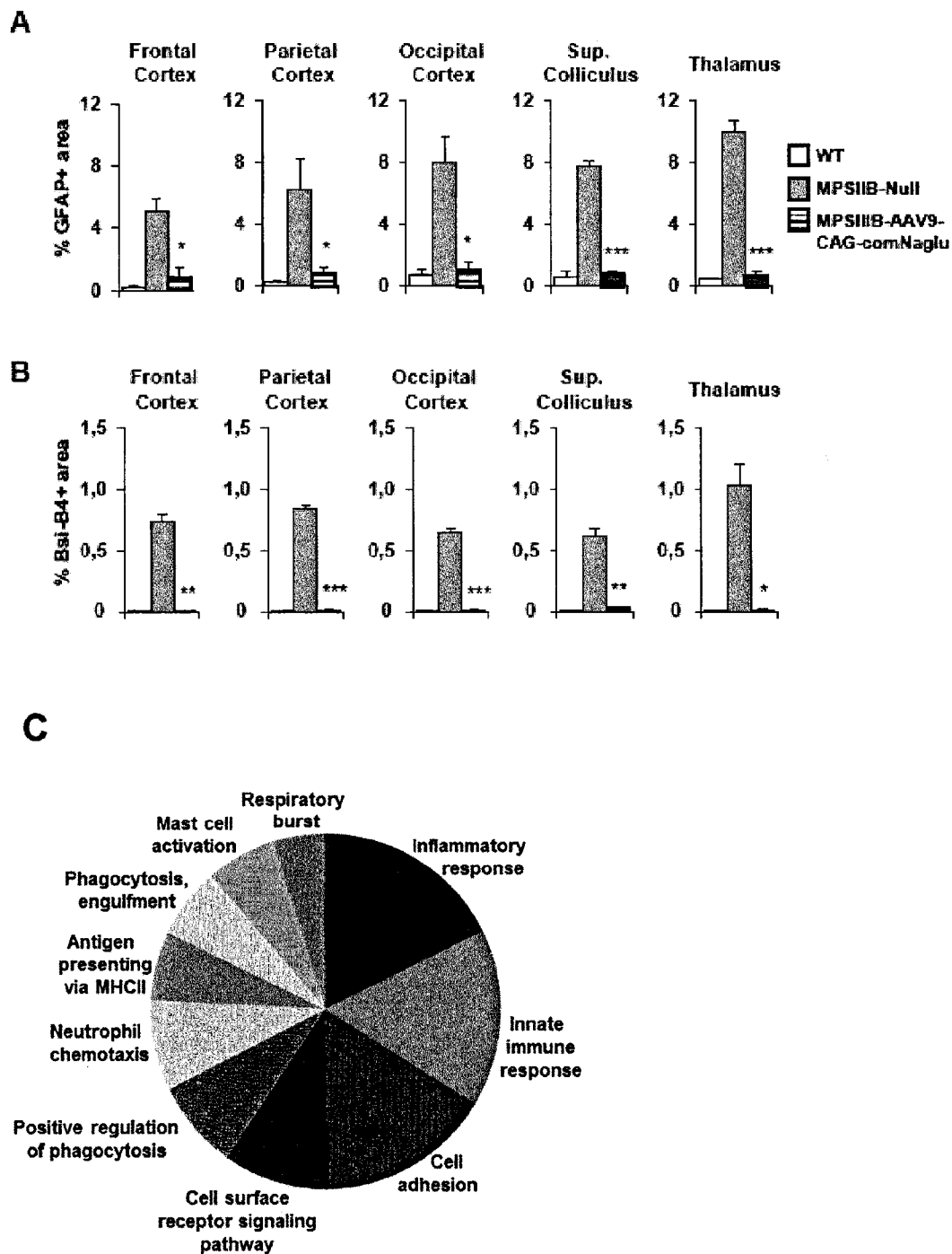


Figure 15

D

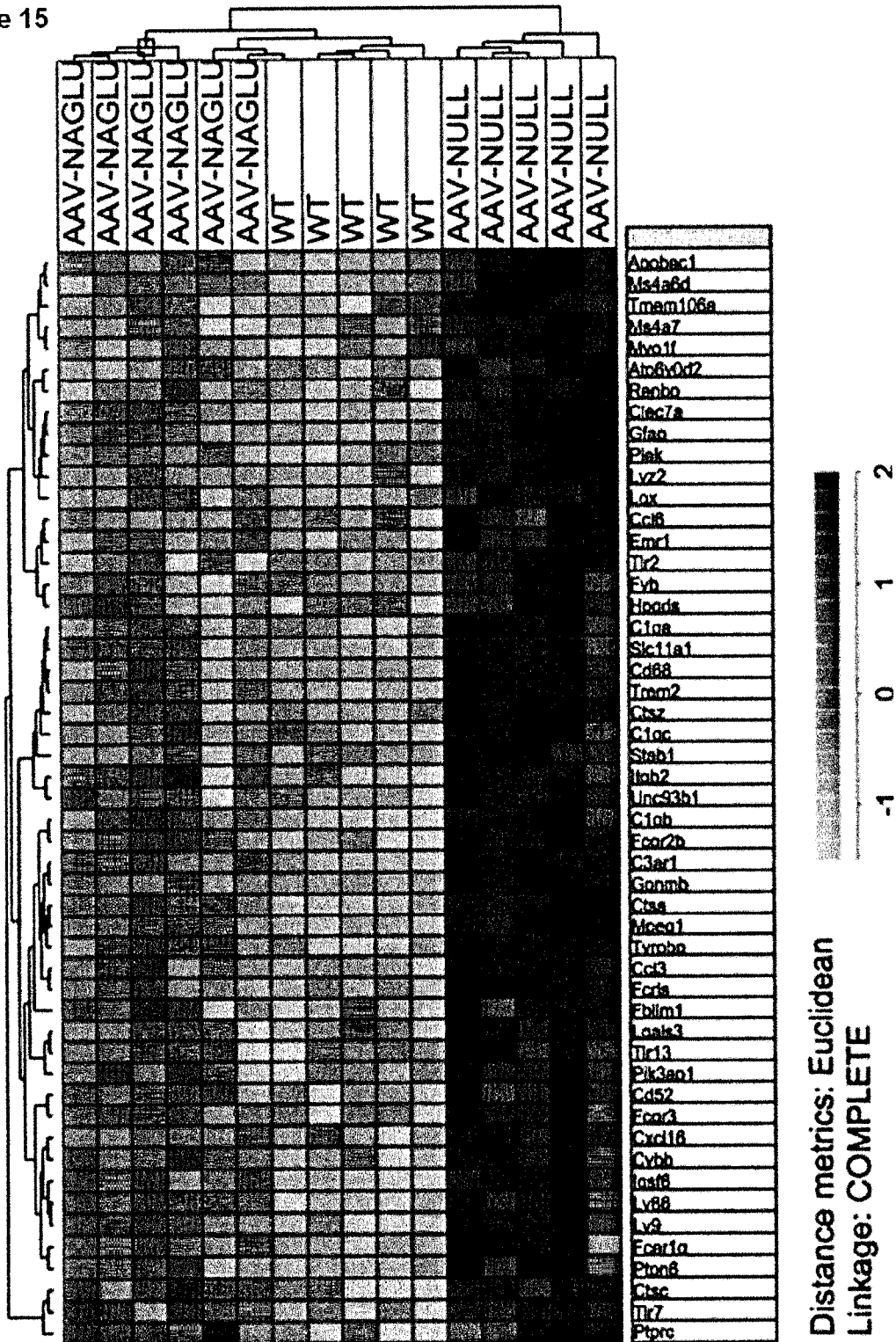


Figure 16

IC – Codon-optimized murine NAGLU

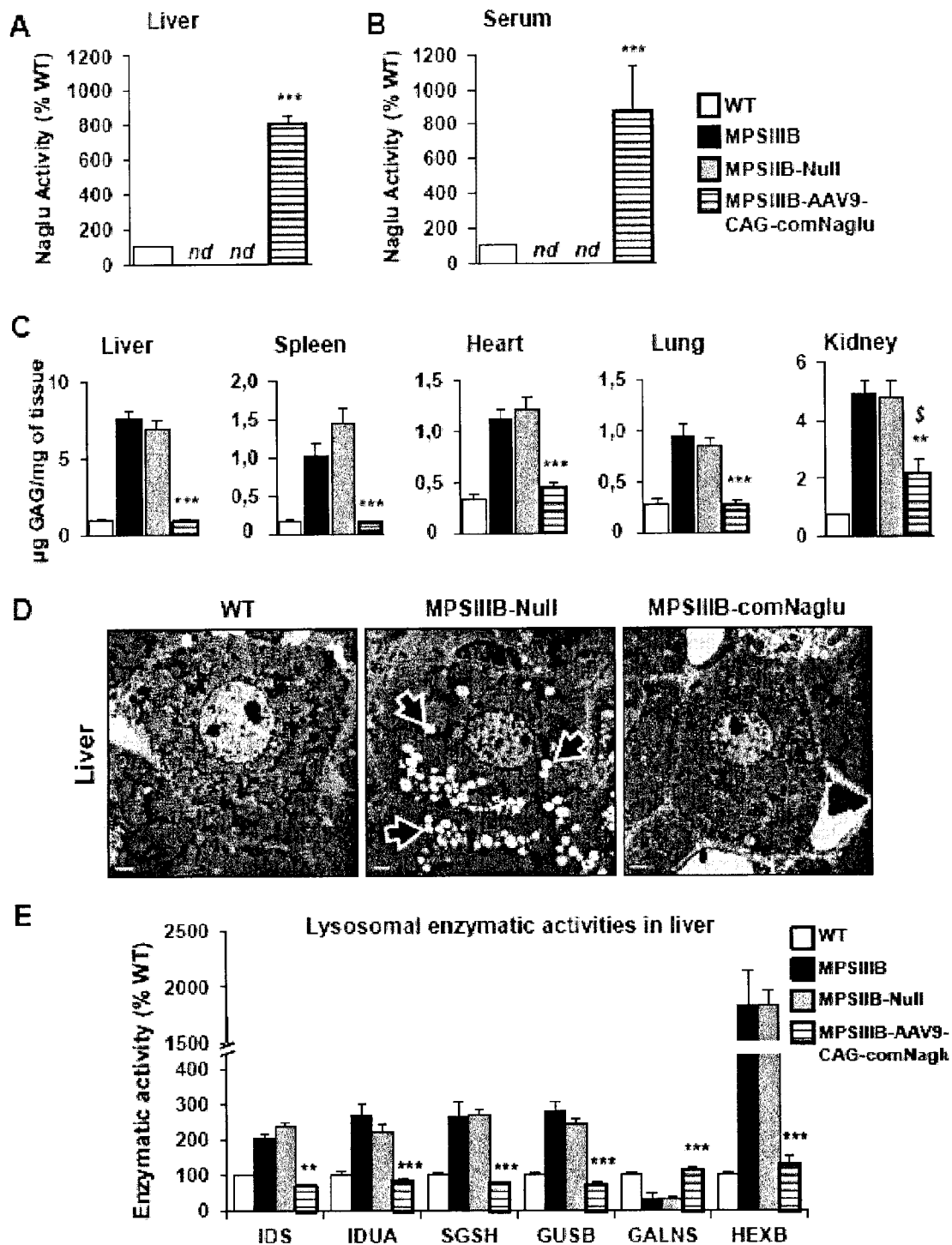


Figure 17

IC – Codon-optimized murine NAGLU

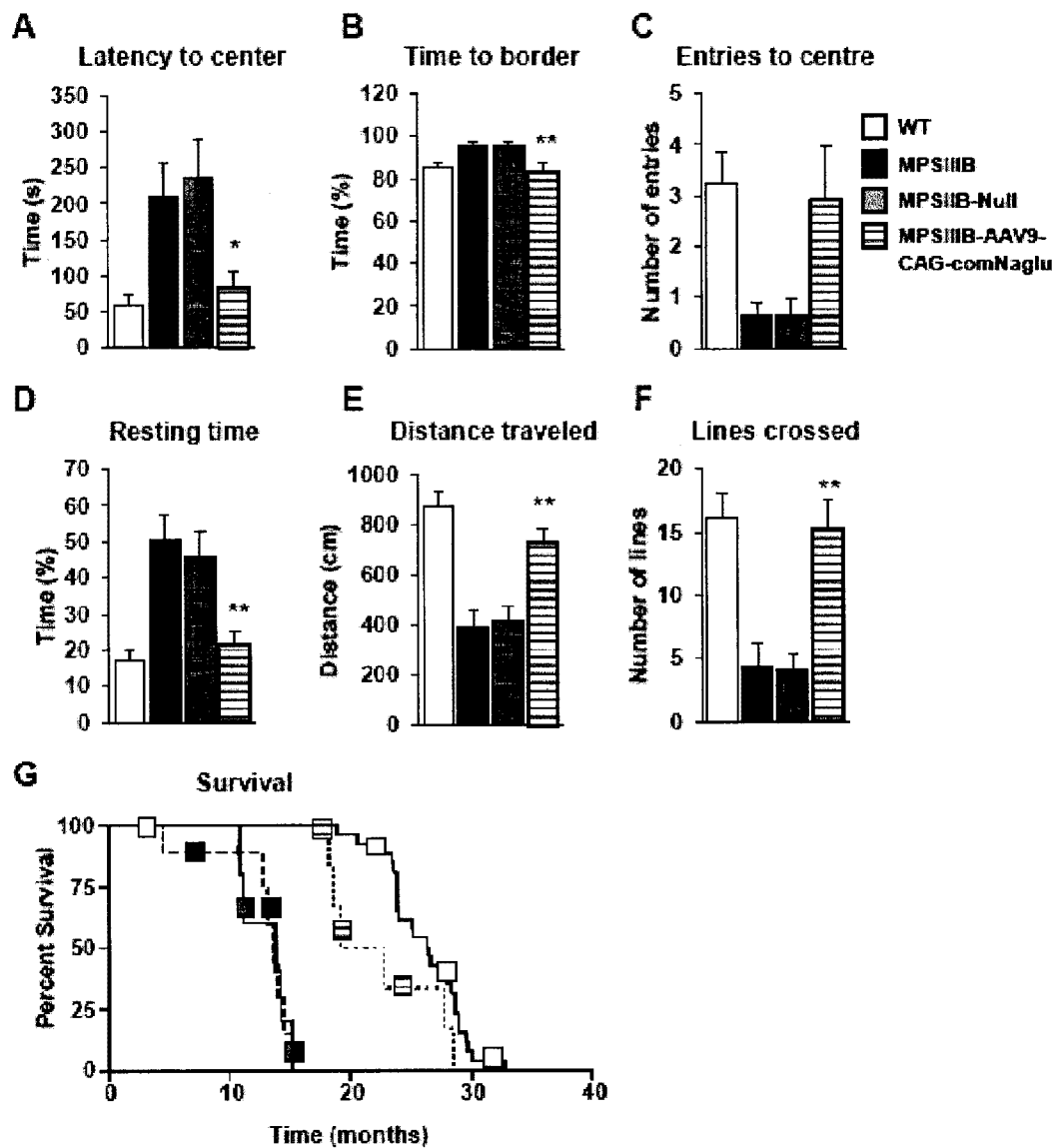


Figure 18

IC – Codon-optimized canine NAGLU

A

Anti-AAV9 Neutralizing antibodies								
Week	Non-immunized dogs				Immunized dogs			
	Dog 1		Dog 2		Dog 3		Dog 4	
	Serum	CSF	Serum	CSF	Serum	CSF	Serum	CSF
W -6	-	-	-	-	1:1	-	1:1	-
W -5	-	-	-	-	>1:316	-	>1:316	-
W -1	-	-	-	-	1:100	-	1:100	-
W 0	<1:1	1:1	<1:1	1:1	1:100	1:1	1:1000	1:1
W 1	>1:31,6	1:1	>1:31,6	1:1	1:10000	1:1	1:1000	1:1

B

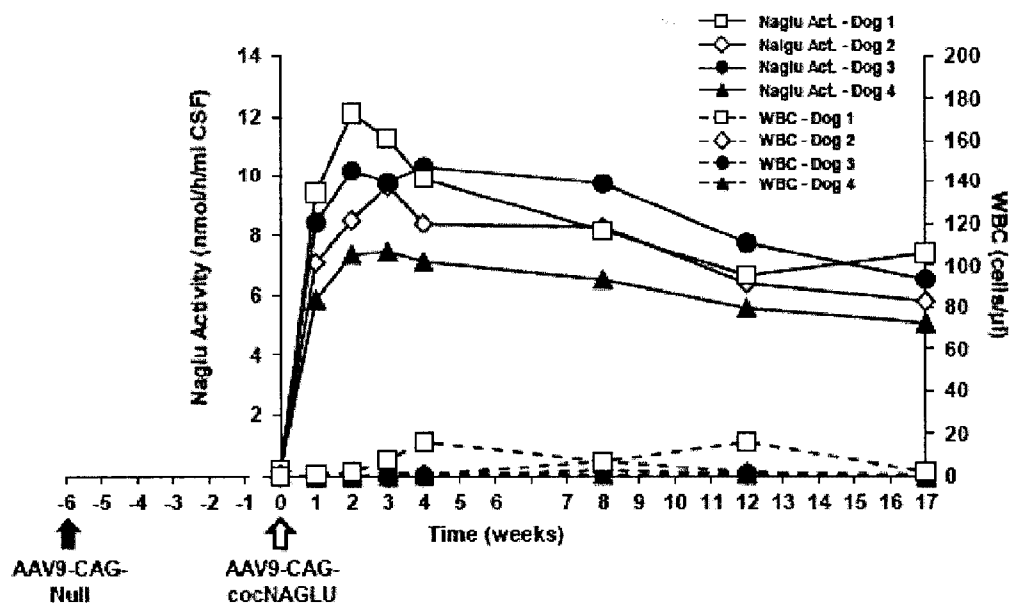
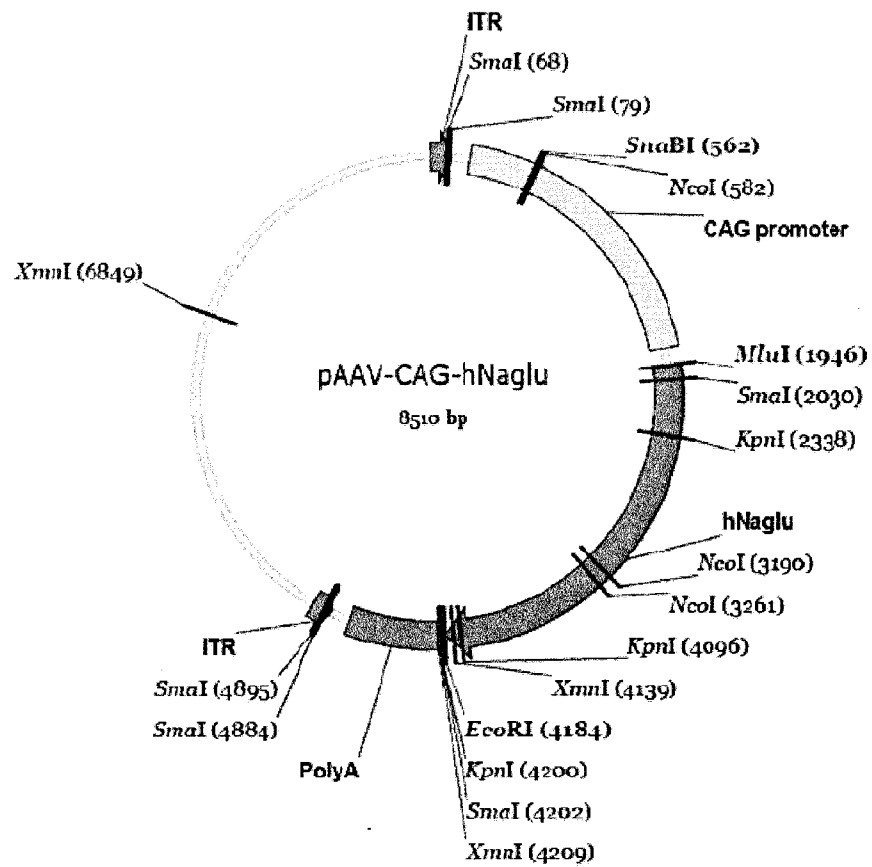


Figure 19

Human Naglu

A



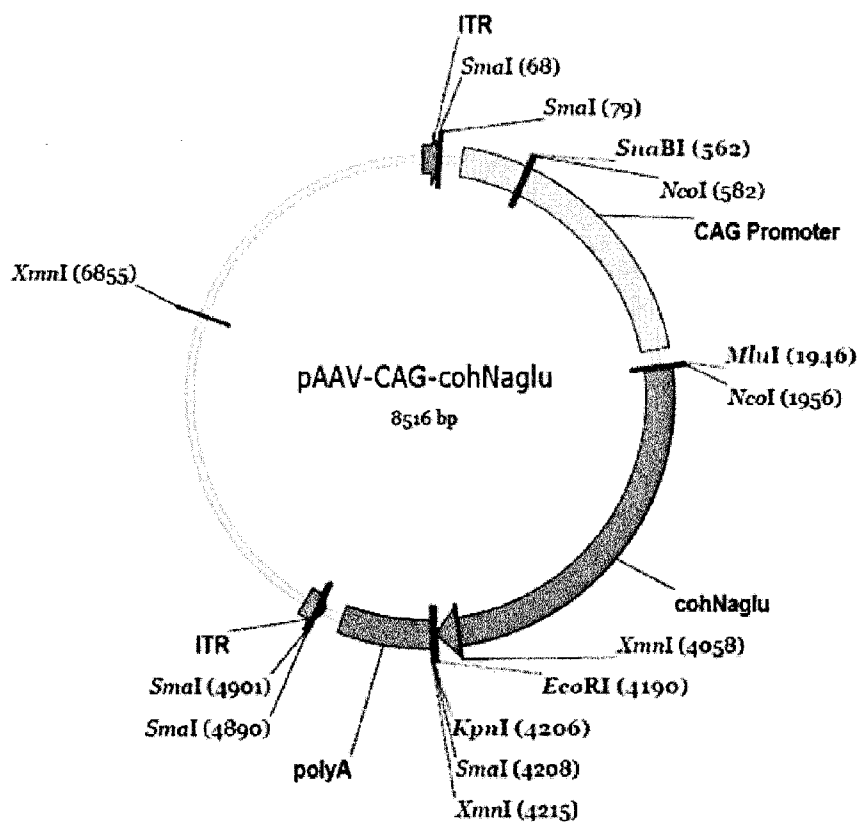
B



Figure 20

Codon-optimized human Naglu

A



B



Figure 21

Codon-optimized human Naglu version2

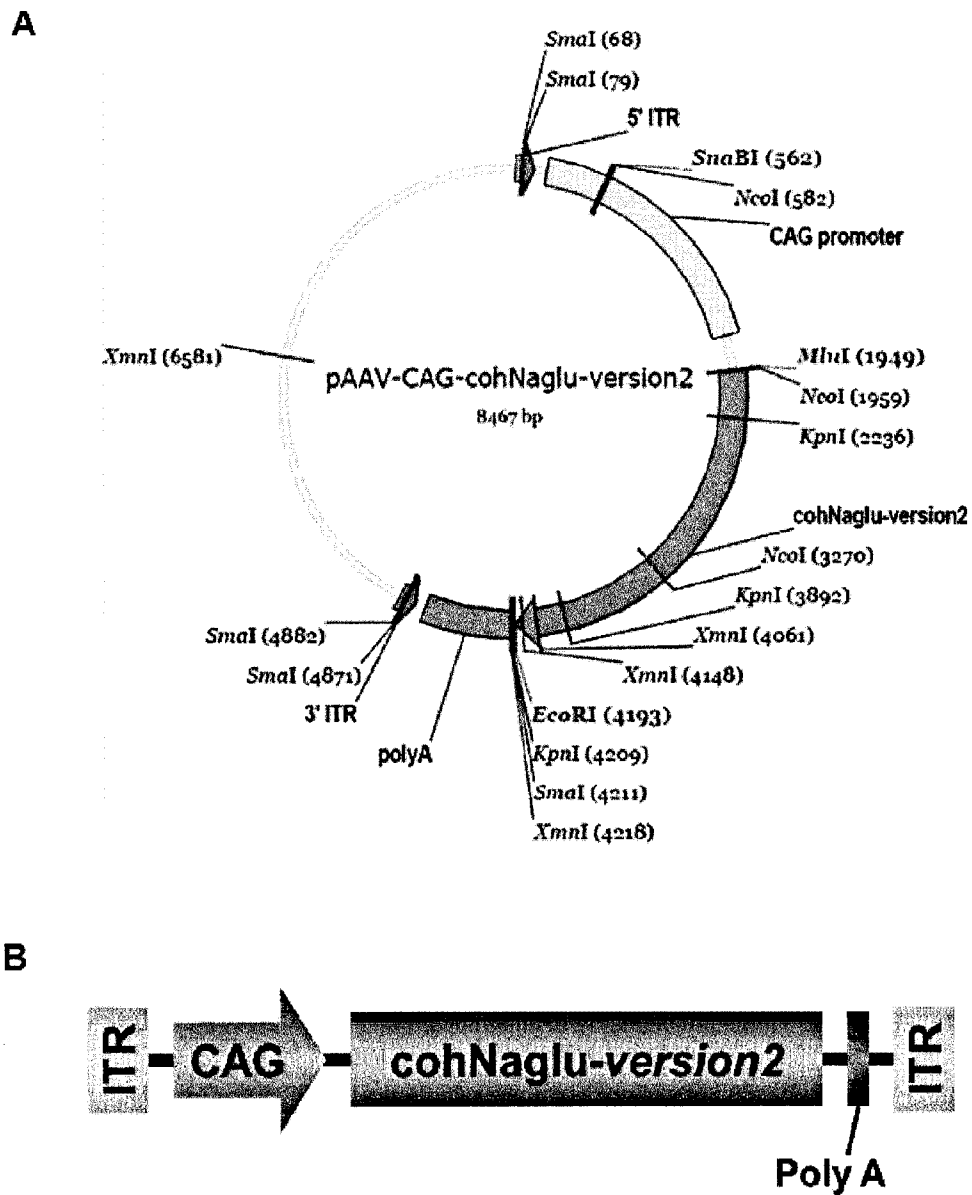


Figure 22

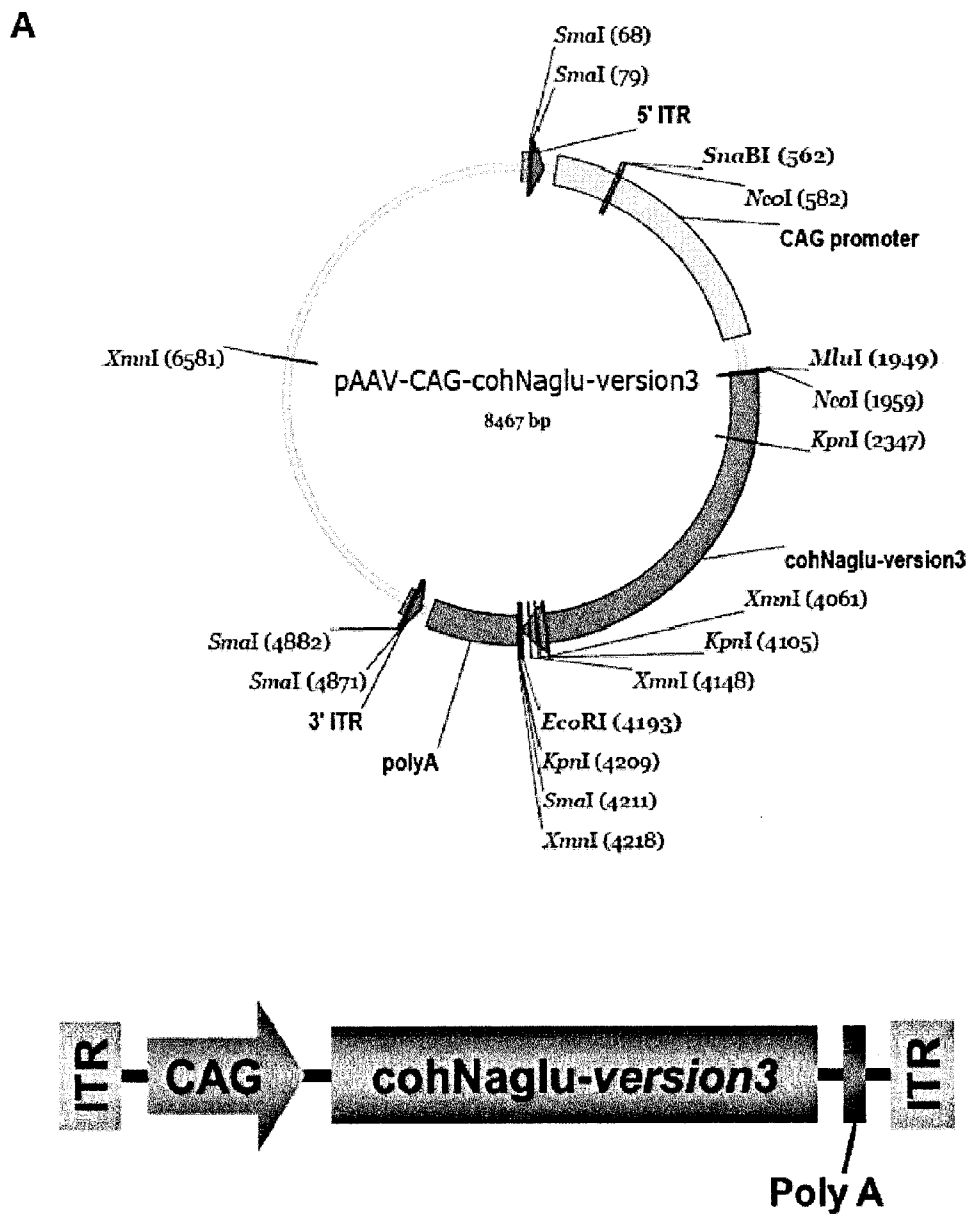
Codon-optimized human Naglu version3

Figure 23

Codon-optimized murine Naglu

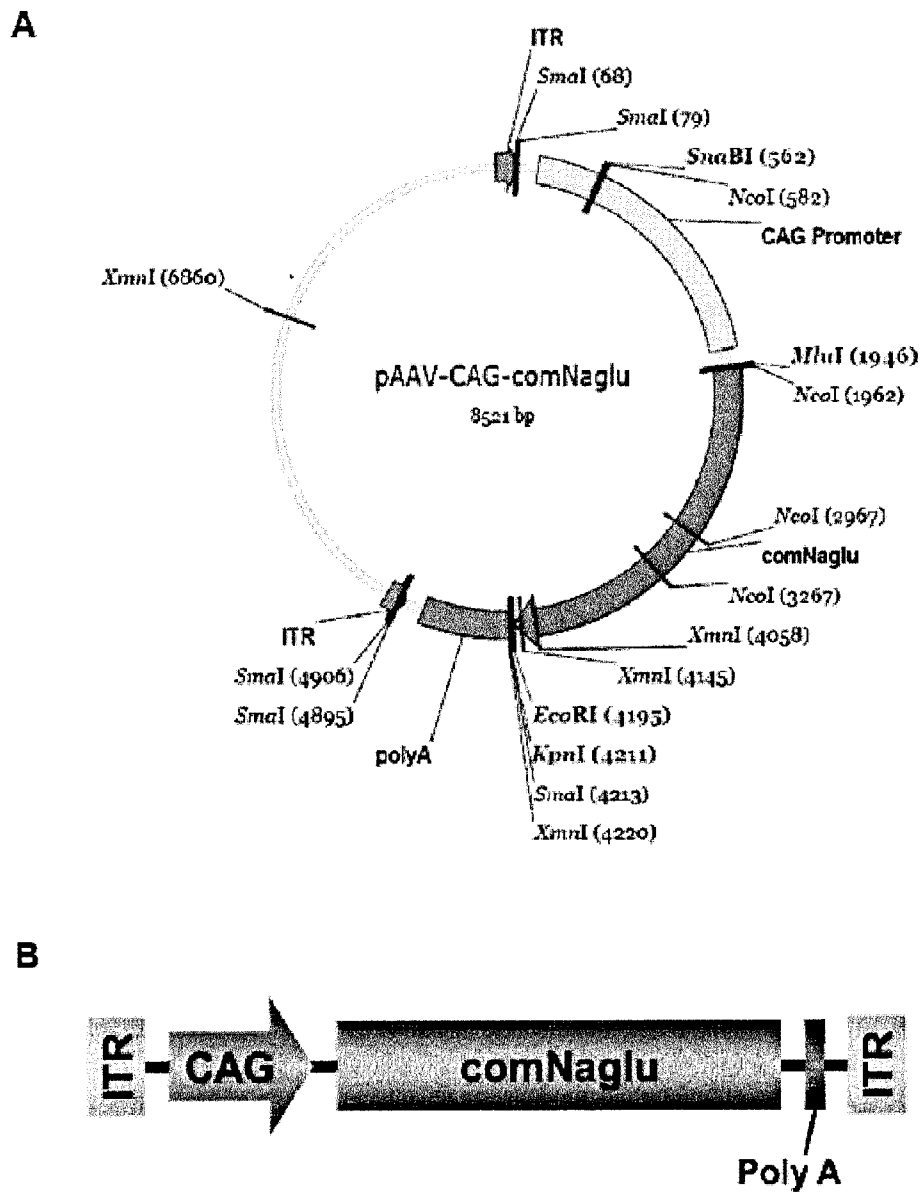


Figure 24

Codon-optimized canine Naglu

