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AAV-Mediated Gene Therapy for Glycosphingolipid Biosynthesis Deficiencies

Huiya Yang[1,2,3], **Robert H. Brown Jr**[2,3], **Dan Wang**[1,3,4], **Kevin A. Strauss**[5,*], **Guangping Gao**[1,3,6,7,*]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, USA

[2]Department of Neurology, University of Massachusetts Medical School, Worcester, MA, USA

[3]Li Weibo Institute for Rare Diseases Research, University of Massachusetts Medical School, Worcester, MA, USA

[4]RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA, USA

[5]Clinic for Special Children, Strasburg, PA, USA

[6]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, USA

[7]Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

Abstract

De novo glycosphingolipid (GSL) biosynthesis defects cause severe neurological diseases, including hereditary sensory and autonomic neuropathy type 1A (HSAN1A), GM3 synthase deficiency, and hereditary spastic paraplegia type 26 (HSPG26), each lacking effective treatment. Recombinant adeno-associated virus (AAV)-mediated gene therapy has emerged as a powerful treatment for monogenic diseases and might be particularly suitable for these neurological conditions.

Disorders of Glycosphingolipid Biosynthesis in Humans

Glycolipids contain a carbohydrate moiety attached via a glycosidic bond to fatty acid tails and are integral components of all eukaryotic cell membranes. GSLs, with the attached lipid being a sphingosine, are enriched in both neurons and oligodendrocytes, and represent the predominant glycolipid class of the mammalian central nervous system (CNS) [1]. They mediate both *cis* (e.g., architecture of membrane microdomains) and *trans* (e.g., ligand binding) membrane functions. Deficiencies in *de novo* GSL biosynthesis lead to severe neurological disorders. To date, three disorders have been identified as monogenic: HSAN1A, GM3 synthase deficiency, and HSPG26, caused by mutations in *SPTLC1,*

*Correspondence: kstrauss@clinicforspecialchildren.org (K.A. Strauss) and guangping.gao@umassmed.edu (G. Gao).

Declaration of Interests

G.G. is a scientific cofounder of Voyager Therapeutics, Adrenas Therapeutics, and Aspa Therapeutics, and holds equity in these companies. G.G. is an inventor on patents with potential royalties licensed to Voyager Therapeutics, Aspa Therapeutics, and ten other biopharmaceutical companies. The remaining authors declare no competing interests.

ST3GAL5, and *B4GALNT1*, respectively. Treatment for these disorders is directed at accommodating and/or alleviating symptoms. There are currently no disease-modifying therapies that address the underlying deficiency and maldistribution of cerebral GSLs.

Hereditary Sensory Neuropathy Type 1A

Patients with HSAN1A usually begin to experience symptoms when in their teens or later, starting with sensory loss in the feet followed by distal muscle wasting, skin damage, ulcers, and sensorineural hearing loss, and inheritance usually follows an autosomal dominant pattern [2]. The affected locus was mapped to the *SPTLC1* gene. Human *SPTLC1* contains a 1422-base pair (bp) cDNA, which encodes serine palmitoyltransferase long chain base subunit 1 (SPTLC1), which is widely expressed and catalyzes the formation of 3-ketosphinganine in *de novo* sphingolipid synthesis. Missense mutations, such as C133W, C133Y, and S331F, induce progressive neurological impairment by inhibiting serine palmitoyltransferase (SPT) activity and gaining toxicity via deoxysphinoid base (DSB) by-products. HSAN1A affects males and females in equal numbers, with an estimated prevalence of 1 in 500 000.

Transgenic Sptlc1^{C133W} mice overexpressing the SPTLC1 C133W mutant show impaired SPT activity accompanied by elevated DSBs [3]. They also develop age-dependent weight loss and sensory impairments at 10 months, which is in line with the disease pathology in patients with HSAN1A.

GM3 Synthase Deficiency

Simpson *et al.* identified that an infantile-onset symptomatic epilepsy syndrome in the Old Order Amish community was caused by a single premature stop codon mutation, p.R232X, in *ST3GAL5* [4]. The 1257-bp human *ST3GAL5* cDNA encodes GM3 synthase, the first enzyme in the ganglioside synthesis pathway. The nonsense mutation R232X in *ST3GAL5* abolishes GM3 synthase activity, as supported by the complete lack of GM3 and its ganglioside derivatives in patients with this disorder. GM3 synthase deficiency follows an autosomal recessive pattern, with an estimated prevalence of ~1 in 1200 births in the Amish community. All affected children start to have generalized tonic-clonic seizures and developmental milestone failures from 3 months of age. Brain magnetic resonance imaging at older ages shows generalized cerebral atrophy [5].

Homozygous *St3gal5*-knockout (*St3gal5*$^{-/-}$) mice show no abnormalities other than enhanced insulin sensitivity and complete hearing loss [6]. The milder phenotypes appear to be due to compensation by the remaining gangliosides in mice. Indeed, *St3gal5/B4galnt1* double knockout mice, which are unable to synthesize any ganglio-series GSL, phenotypically mirror patients with GM3 synthase deficiency [7]. They develop severe epilepsy, developmental delay, and neurodegenerative symptoms, and die soon after weaning. Biochemical analysis of postmortem brain tissue also confirmed the accumulation of lactosylceramide with the absence of all gangliosides.

Hereditary Spastic Paraplegia Type 26

Fishman *et al.* documented for the first time the accumulation of GM3 and the absence of complex gangliosides (GM1, GD1a, GD1b, and GT1b) in the brain and liver of a patient with spastic paraplegia [8]. This phenotype indicated the loss of function of B4GALNT1, which is responsible for the synthesis of gangliosides GM2, GD2, and asialo GM2. Later on, mutations were identified in *B4GALNT1* from 12 independent HSPG26-affected families [9]. HSPG26 is characterized by a slowly progressive spasticity of the lower extremities resulting from axonal degeneration, and mild to moderate intellectual impairment; some male patients exhibit low serum testosterone levels and hypogonadism.

B4galnt1$^{-/-}$ mice from the 129/Sv strain develop deficits in reflexes, strength, and balance starting from 14–16 weeks of age. Progressive gait disorder, tremors, and catalepsy are seen from 12 months, which are reminiscent of clinical features of patients with HSPG26. In addition, *B4galnt1*$^{-/-}$ male mice have progressive testicular atrophy with the presence of diffuse vacuoles in Sertoli cells and a severe reduction in serum testosterone [10].

Adeno-Associated Virus-Mediated Gene Therapy

AAV-mediated gene therapy has emerged as a powerful strategy for the treatment of monogenic CNS disease. To date, three AAV gene therapy products have been approved by the European Medicines Agency and the US FDA, and 17 AAV gene therapy clinical trials targeting the CNS are in progress (www.clinicaltrials.gov NCT03952637; NCT04273269; NCT04411654; NCT04133454; NCT03634007; NCT04120493; NCT01621581; NCT04167540; NCT03562494; NCT04127578; NCT02926066; NCT02852213; NCT02725580; NCT03770572; NCT03612869; NCT00151216; and NCT01161576).

Wild-type (wt)AAV has an icosahedral protein capsid encapsidating a 4.8-kilobase (kb) single-stranded DNA genome that is flanked by two inverted terminal repeats (ITRs). Recombinant AAV (rAAV) is an engineered AAV that retains the wtAAV capsid, but replaces the wtAAV genome, except for the ITRs, with therapeutic DNA. The rAAV genome can form a circularized episome that is stable and maintains long-term transgene expression in nondividing cells. This characteristic makes rAAV-mediated gene therapy a ‘one-time’ treatment possibility [11].

A successful CNS-directed rAAV-gene therapy requires a suitable delivery method and a well-designed vector genome. Direct CNS injection, such as intraparenchymal and intracerebroventricular (I.C.V.) administration, usually achieves local transduction in the CNS compared with intravenous (I.V.) injection, which delivers the vector systematically. By contrast, some naturally isolated serotypes, such as AAV9 and AAVrh10, are able to cross the blood–brain barrier and have wide-spread delivery throughout the CNS via I.V. injection, which might be advantageous for treating GSL biosynthesis deficiencies [12].

A well-designed vector genome aims to express the therapeutic genetic material at the appropriate level, location, and time. To have a potent and durable expression, self-complementary AAV that has a mutated ITR to help the vector genome bypass second-strand synthesis is favorable in some circumstances. In addition, the promoter strength

and codon usage of the transgene can be optimized to meet expression requirements. Additionally, applying tissue- or cell type-specific promoters or inclusion of miRNA binding sites can yield spatial control over transgene expression [13]. Finally, chemically inducible promoters can be switched ON or OFF by the administration of certain drugs to confer temporal control.

Application of AAV-Mediated Gene Therapy to GSL Biosynthesis Disorders

AAV-mediated gene therapy applications toward HSAN1A, GM3 synthase deficiency, and HSPG26 involve two major and distinct gene therapy strategies, namely gene silencing and gene replacement.

Due to the gain-of-function nature of *SPTLC1* mutations, gene silencing could be applied for HSAN1A. One strategy is artificial miRNA (amiRNA)-mediated allele-specific mutant *SPTLC1* knockdown. amiRNA expressing siRNAs in a natural pri-miRNA backbone reduces target mRNA expression. By silencing mutant *SPTLC1* and sparing the normal allele, DSB production would be reduced while maintaining the normal functions of wtSPTLC1. If achieving allele-specific knockdown is difficult, one alternative is to deliver a dual-function rAAV that expresses both amiRNA and a functional *SPTLC1* cDNA that function independently (Figure 1A) [14]. The former is recognized by the endogenous miRNA machinery and degrades the endogenous *SPTLC1* mRNA, while the amiRNA-resistant *SPTLC1* transcript produces wtSPTLC1 protein that executes its normal catalytical functions (Figure 1B).

By contrast, loss-of-function mutations in *ST3GAL5* and *B4GALNT1* could be resolved by rAAV-mediated gene replacement therapy. Due to alternative splicing and multiple start codons, human *ST3GAL5* expresses into multiple isoforms. Thus, the appropriate isoform(s) to choose for gene therapy needs to be determined. With the appropriate isoform, the rAAV-borne GM3 synthase is expected to restore the GSL profile and their downstream functions (Figure 2). One potential caveat is that *ST3GAL5* overexpression in periphery tissues might cause off-target toxicity, as evidenced by the association between overexpression of *ST3GAL5* and cancer or diabetes [15]. Therefore, expressing the exogenous *ST3GAL5* in the right cells and to the right extent is pivotal and challenging for gene therapy development. Similarly, the gene replacement therapy strategy and considerations can be applied for delivering *B4GALNT1* for HSPG26.

The aforementioned animal models can be used to evaluate therapeutic efficacy, based on both phenotypical and biochemical changes. However, substantial differences exist between murine models and humans, especially in brain size and anatomy. Large animal models, such as pig, can be developed to better understand disease pathology and serve as pre-clinical therapy assessment platforms.

Concluding Remarks

AAV vectors are capable of transducing a range of species and tissues *in vivo* with relatively low innate and adaptative immune responses and genotoxicity; therefore, they represent the leading gene delivery platform for the treatment of diverse human diseases. We expect that

developing such gene therapies could ultimately fulfil the unmet medical needs of patients with GSL biosynthesis deficiencies.

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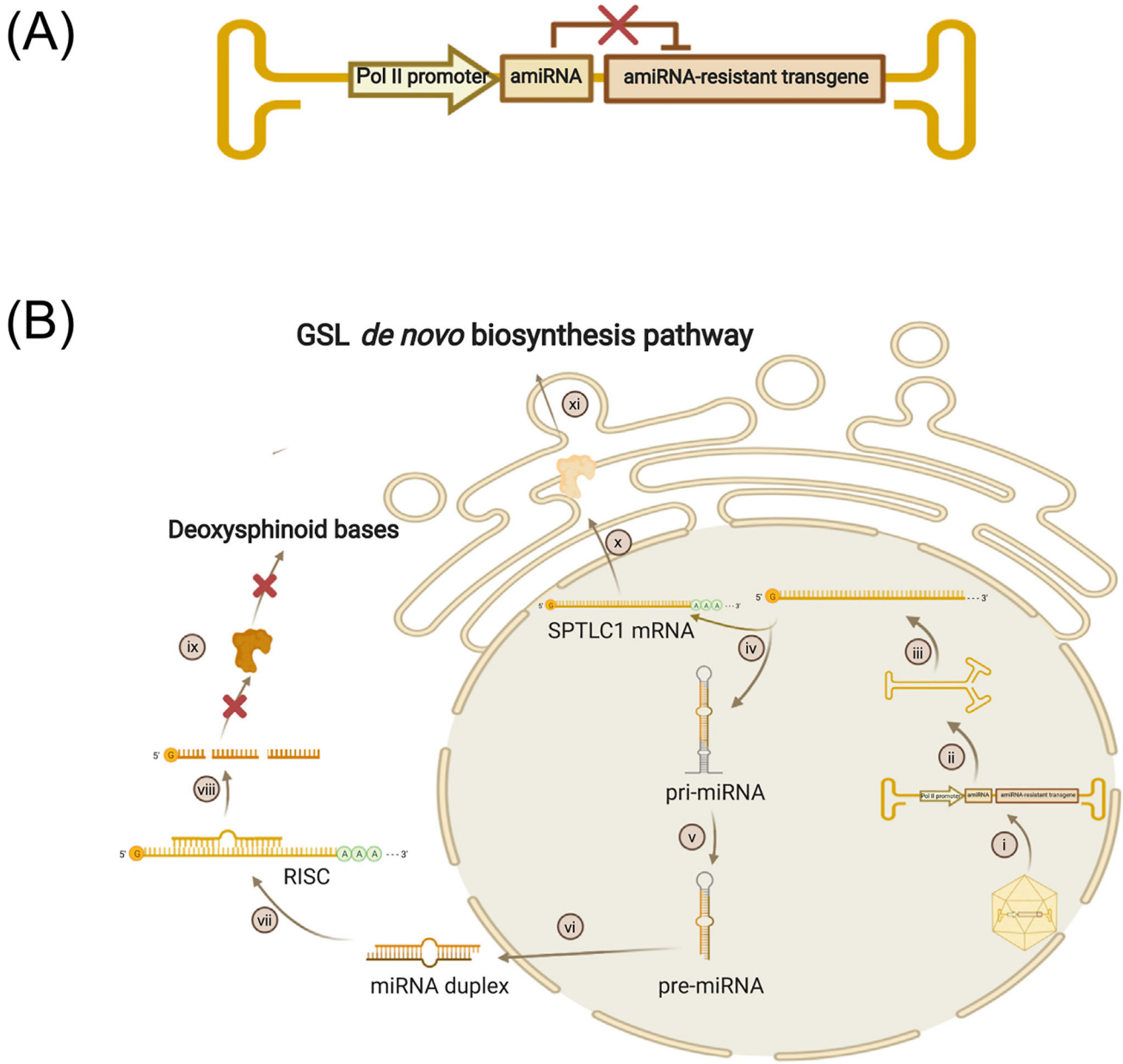

Figure 1. Dual-Function Recombinant Adeno-Associated Virus (rAAV) Vectors for Treating Hereditary Sensory and Autonomic Neuropathy Type 1A (HSAN1A) Caused by Serine Palmitoyltransferase Long Chain Base Subunit 1 (SPTLC1) Mutations.

(A) The rAAV genome contains an RNA polymerase II (pol II) promoter driving artificial miRNA (amiRNA) and amiRNA-resistant transgenes simultaneously. amiRNA-resistant transgenes encode the same amino acid sequence as the endogenous normal genes, but carry a different nucleic acid sequence that cannot be recognized by the amiRNA. (B) Dual-function rAAV vectors function in the cell. (i) AAV uncoating in the nucleus; (ii) second-strand synthesis; (iii) transcription and post-transcription modification; (iv) splicing into two independent transcripts: pri-miRNA and amiRNA-resistant *SPTLC1* mRNA; (v) cleavage; (vi) export; (vii) formation of RNA-induced silencing complex (RISC) with endogenous *SPTLC1* mRNA; (viii) endogenous *SPTLC1* mRNA cleavage; (ix) generation

of toxic deoxysphinoid bases prevented; (x) wild-type SPTLC1 protein translation; and (xi) SPTLC1 protein involved in normal glycosphingolipid (GSL) *de novo* synthesis. Created with BioRender.com.

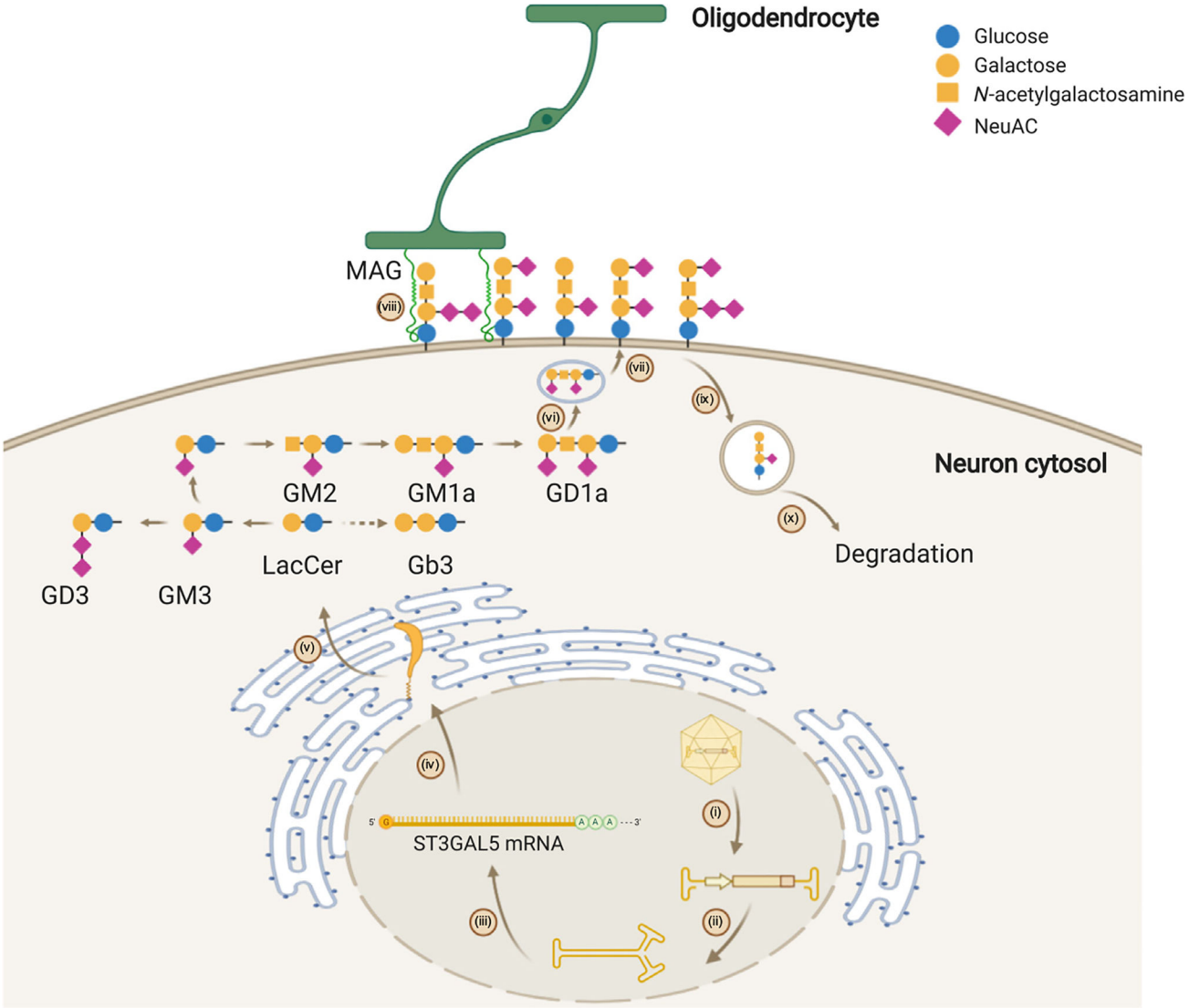

Figure 2. Recombinant Adeno-Associated Virus (rAAV)-Mediated Gene Replacement Therapy for Treating GM3 Synthase Deficiency.

(i) AAV uncoating; (ii) second-strand synthesis; (iii) transcription and post-transcription modification; (iv) protein translation and transport; (v) ST3GAL5 protein restores the ganglioside biosynthesis network through the initiation of GM3 synthesis in the Golgi; (vi) ganglioside transportation via vesicles; (vii) ganglioside integration on the cell membrane; (viii) restored cellular communication mediated by gangliosides, such as the interaction with myelin-associated glycoprotein (MAG) of oligodendrocytes; (ix) ganglioside recycling from cell membrane; and (x) ganglioside degradation. Abbreviation: NeuAC, N-acetylneuraminic acid. Created with BioRender.com.