Gene Transfer to the Central Nervous System for Treatment of GM2 Gangliosidosis

John G. Keimel1,2, Steven J. Gray1, Jagdeep S. Walia4, Patrick Thompson3, Karlina Osmorn, Sayun Yonekawa5, Subha Karumuthil Melethi5, Michael Kaytor1, Brian L. Mark2, Don Maharan5

1New Hope Research Foundation, 2University of Minnesota; 3University of North Carolina at Chapel Hill; 4Queen’s University, Kingston ON; 5University of Manitoba, Winnipeg MB; 6The Hospital for Sick Children, Toronto ON

PROGRAM SUMMARY
A key problem in treating central nervous system (CNS) diseases is the delivery of the therapy. The blood brain barrier (BBB) blocks most biologics and large molecules from freely entering the CNS. In this program, we are developing an in vivo gene therapy for GM2 gangliosidosis, a disease that affects tissue throughout the brain and spinal cord. Our program has engineered a gene transfer vector specifically designed for broad distribution within the CNS and has begun investigation on enhanced delivery methods utilizing convective transport of this vector.

Disease Overview:
• GM2 gangliosidosis is a rare, hereditary, neurodegenerative disease caused by a deficiency of a lysosomal enzyme, β-hexosaminidase A (HexA), which is required for hydrolysis of anionic GM2 ganglioside.
• HexA deficiency results in lysosomal ganglioside storage and eventual cell death.
• Disease severity varies with the degree of enzyme deficiency. Severe deficiencies cause death in childhood: milder deficiencies present clinically in adulthood with ataxia, muscle atrophy, severe psychiatric events, and cognitive decline.
• There is no cure for the CNS aspects of any lysosomal storage disease.

GENE VECTOR DESIGN
Requirements and Constraints:
• The small Adeno-associated virus (AAV) capsid size (~25 nm) is essential for transport across the BBB.
• Self-complementary adenovirus-associated virus (scAAV) vectors have been shown to have high transduction efficiency in applications with low multiplicity of infection, as would occur with broad vector distribution. scAAV vectors have been shown to be 10 to 100 times more efficient than traditional single stranded AAV vectors.
• The total gene carrying capacity of AAV capsids is only ~4700 bases. Minus the three inverted terminal repeats (439 bases), the regulatory and transgene sequences must total less than ~1210 bases to allow scAAV packaging.
• The Hex α- and β-subunits have a combined transgene length of ~3300 bases, and therefore, only one Hex sub-unit transgene can fit within a scAAV package.
• The scAAV packaging of one Hex subunit requires the promoter, intron, and polyA be less than a total of 460 bases (i.e., 2130 minus HexB transgene length, 1670).

Design of a New Hexosaminidase Subunit Capable of Forming Stable Homodimers that Hydrolyze GM2:

- Structure-based design of HexM
- β-Hexosaminidase subunit
- Highly stable interface

Model of engineered hexosaminidase homodimer (HexM, β-subunit) in complex with binding site on HexB

GM2 ganglioside

HexM: β-subunit 
HexB: α-subunit

HexB contains the proximal GM2 binding surface from the Fucα1-2Galactosyltransferase (Gal α1-3Gal)

GM2 binds to the β-subunit of HexM

HexB

GM2

GM2

Figure 2: Model of engineered hexosaminidase homodimer (HexM, β-subunit) in complex with binding site on HexB.

GM2 ganglioside

HexM

HexB

Figure 3: In vitro HPTLC assay of GM2 hydrolase by human Hex isozymes: HexB and HexM. In the absence (+) or presence (-) of human GM2AP.

Development of a Short Promoter Intron Combination:

- Comparison of alternative promoter-intron combinations on GFP expression in HeLaX3T7 cells
- ~210 bp maximum for self-complementary AAV

Figure 4: Comparison of Promoter-Intron combinations: A ubiquitous synthesized promoter (USP) plus short intron was developed (patent pending) to allow for scAAV packaging of Hex subunit transgenes. This chart shows the relative GFP expression level with this USP promoter with a short intron compared to the cytomegalovirus (CMV) promoter and the human ubiquitin C (hUbc) promoter with either a short or long intron. Relative GFP mRNA was assessed in HeLaX3T7 cells using real-time PCR normalized to levels of GAPDH.

IV VECTOR INJECTIONS IN NEONATAL SANDHOFF MICE
Study Design:

- 8 Week Cohorts of newborn C57BL/6 mice
- 8 Week Cohorts of newborn C57BL/6 mice
- Biochemical Analysis

Figure 5: Design of HEXM and GFP self-complementary AAV9.47 vectors. AAV9.47 is a variant of AAV9 that de-targets the liver. The USP-short intron was used to keep the total length of the HEXM and regulatory sequences less than the 2130 maximum.

RESULTS:

- Hexosaminidase Activity Assay
- Quantitative PCR
- Percentage survival
- PBS KO
- GFP KO
- HexM Treated KO
- Heterozygote

Figure 6: In-vivo study design. To determine the HexM functionality in vivo, neonatal Sandhoff mice were injected with 5×10⁶ tg mice and other terminated at 8 weeks or the humane end-point.

- Hexosaminidase Activity Assay
- Quantitative PCR
- Percentage survival
- PBS KO
- GFP KO
- HexM Treated KO
- Heterozygote

Figure 7: Biochemical Assays. Hexosaminidase (MUG5) activity increased (non-significant) with γ-Galactosidase levels are significantly reduced in the mid-brains of Sandhoff mice 8 weeks after neonatal, intravenous injection of AAV9.47-HEXM vector. GM2 levels are expressed as a ratio of the densities of the GM2 versus G01a spots from HPTLC plates (Y-axis). Mice injected with AAV9.47-HEXM, and AAV9.47-GFP were compared to untreated heterozygous, hexβ/ mice (Het). N= 6. *** p< 0.001

Neonatal Mice Study Limitations:
• The BBB does not mature in mice until several weeks after birth.
• Mice have an alternative pathway for hydrolyzing GM2 ganglioside by sequentially using sialidase and hexosaminidase enzymes. This alternative pathway is not present in humans. It is possible that the GM2 ganglioside reduction observed in mice injected with the HEMX vector was utilizing this alternative pathway.
• AAV particles (~25 nm diameter) have a low diffusion coefficient (Stokes-Einstein estimated D = 27 nm²/s) which has a calculated average diffusion distance of less than 2 mm during 6 hours in cerebrospinal fluid. Therefore, the distribution of the HEMX vectors in neonatal mice might not be applicable to large animals and humans due to the large differences in the transport characteristic length for the AAV capsid (i.e., differences in the Peclet number) between species.

NEXT STEPS
1. The transport of AAV vectors within the CNS of large animals is highly dependent on the cerebrospinal fluid and interstitial fluid flow. To better understand and utilize this flow in obtaining broad distribution of AAV vectors within the CNS, conduct an analysis of cerebrospinal fluid and interstitial fluid flow using computer modeling techniques (Fig. 9) in order to optimize delivery methods in large animals and man.
2. Validate the modeled delivery methods using injections of AAV9-GFP vector in normal sheep.
3. Develop assays to determine the immunogenicity of the AAV9-HEXM vector and the expressed HexM protein, and if needed, explore methods for inducing immune tolerance.

Figure 8: A multi-compartment model of fluid flow within the cranial and spinal column. The grey compartments represent the two phases (cells and ISF) of the parenchyma including the right and left brain, cerebellum, and spinal cord. Compartments outlined in blue represent CSF compartments: LV – lateral ventricle, 3V – third ventricle, 4V – fourth ventricle, CS – cisterna magna and basal cisterns, SAS – subarachnoid space. Spinal – spinal canal. The compartments with the blue background are depicted to be well-mixed. Blue arrows depict CSF and ISF flow between compartments. Dashed arrows represent flow that is distributed across a tissue surface. Compartments outlined in red are lumped blood vascular compartments distributed within the right and left hemispheres: Carotid Artery, Ar – arteries, A – arterioles, C – capillaries, V – veins, Ve – venous, Vs – venous sinus, and Jugular Vein. Red arrows depict the lumped flow between compartments. The green dashed arrows represent flow across the BBB. The directions of the arrows depict positive flow.

RESEARCH SUMMARY
A new β-hexosaminidase subunit has been developed capable of forming a stable homodimer, HexM (μ), and hydrolyzing GM2 ganglioside in conjunction with the HexA activator protein, GM2AP.

A new short promoter-intron combination has been developed that allows the construction of self-complementary AAV vectors with transgenes as large as ~1670 bp when used with a 120 base polyA. This has allowed the construction of a scAAV vector incorporating the HEXM transgene, scAAV9.47-HEXM.

Neonatal Sandhoff mice intravenously injected with scAAV9.47-HEXM show a significant reduction in GM2 ganglioside storage in the mid-brain and a significant increase in survival.

While this in vivo study has limitations, it shows the potential of using a relatively non-invasive method for treating GM2 gangliosidosis. Additional in vitro and in vivo research is required to enhance the delivery of vectors to broad regions of the CNS of large animals with an intact blood brain barrier.

REFERENCES