PRECLINICAL ASSESSMENT OF INTRAVENOUS GENE THERAPY FOR GM2 GANGLIOSIDOSIS

by

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Abstract

GM2 Gangliosidoses are a group of severe neurodegenerative lysosomal storage disorders characterized by the inability to catabolize GM2 Gangliosides, leading to a neurotoxic accumulation of the GM2 lipids within the central nervous system. The Hexosaminidase A (HexA) enzyme is a heterodimeric protein composed of an α- and a β- subunit, which are coded by the *HEXA* and *HEXB* genes respectively. Mutations in either of these genes lead to Tay Sachs Disease and Sandhoff Disease, respectively, through the disruption of the HexA enzyme. These debilitating diseases are fatal by the age of 4 in the infantile version and have no effective treatment available to patients other than palliative care. Herein we assessed the efficacy of two distinct hexosaminidase gene therapy treatment strategies in a Sandhoff mouse model – the 'hexosaminidase hybrid' and 'bicistronic' vector treatments. We found significant extensions in survival, significant improvements in motor coordination, as well as drastic decrease in the accumulation of the GM2 lipid for both strategies. Specifically, in the assessment of the hexosaminidase hybrid, the low dose cohort and the remaining high dose mice had a significant survival benefit with an average/median survival of 40.6/34.5 and 55.9/56.7 weeks, respectively, over that of untreated controls whose humane endpoint is ~16 weeks. The bicistronic vectors also showed significant improvements in survival, where all vector treatments provided at all ages of administration conveyed improvements in the SD mice in their survival compared to vehicle-treated controls ($p<0.001$). Mice were treated at 0, 2, 4, and 6 weeks with the human bicistronic vector survived to a median age of 59, 68.4, 43.6, and 43.8 weeks and mice treated with the murine bicistronic vector survived to median age of 42.2, 76.8, 65.8, and 68.14 weeks, respectively. Mice treated with the ssAAV9/*mHexb* vector only at 0 and 6 weeks and they survived to a median age of 65.72 and 70.71 weeks respectively, with one mouse from the 0-week ssAAV9/*mHexb* group surviving to the 104-week study endpoint. Significant behavioral, biochemical, and molecular benefit were also observed in both the hybrid and bicistronic strategies. These findings are significant steps forward in the search for a viable treatment for GM2 Gangliosidosis.

Co-Authorship

Chapter 2 has been submitted to Molecular Therapy:

> Karlaina JL. Osmon, Patrick Thompson, Evan Woodley, Subha Karumuthil-Melethil, Clifford Heindel, John G. Keimel, William Kaemmerer, Steven J. Gray, and Jagdeep S. Walia. Correction of GM2 Gangliosidosis in Adult Sandhoff Mice using a Hybrid Hexosaminidase Vector – Efficacy and Dosage Study with Intravenous Administration. MTJ-19-4214

The research described herein was conducted by Karlaina Osmon under the supervision of Dr. Jagdeep S. Walia. All data within this thesis was collected by Karlaina Osmon, with the exception of a subset of behavioural testing timepoints and blood collections, which were performed by Evan Woodley, Meera Vyas (Figure 2.4). Patrick Thrompson, Evan Woodley, and Meera Vyas (all from the Walia Lab) also contributed significantly to the humane endpoint collections for this study. Additionally, the vector production and the biodistribution data (Figure 2.7) collected through qPCR analysis by Subha Karumuthil-Melethil and Clifford Heindel under the supervision of Dr. Steven Gray at the University of North Carolina, Chapel Hill. John Keimel and William Kaemmerer, from the New Hope Research Foundation, contributed intellectually and provided biostatistics expertise.

Chapter 3 - Impact of the Age of Administration of AAV9 Murine and Human Bicistronic Hexosaminidase Vectors in Sandhoff Mice on the Correction of GM2 Gangliosidosis

The research described herein was conducted by Karlaina Osmon under the supervision of Dr. Jagdeep S. Walia. All data within this thesis was collected and analysed by Karlaina Osmon (Survival, Hexosaminidase Activity, GM2 Ganglioside Storage, Behavioural Testing, Biodistribution, Immunological Assays) with the exception of a subset of behavioural testing timepoints and blood collections, which were performed by Natalie Deschenes, Shalini Kot, Alex Ryckman, Eminet Bogale, and Melissa Mitchell due to Karlaina developing a severe allergy to the experimental animals (Figure 3.5 - Figure 3.18). Natalie Deschenes, Shalini Kot, Alex Ryckman, Brianna Quinville, and Zhilin Chen also contributed significantly to the humane endpoint collections for this study. Additionally, the vector

production was performed by Subha Karumuthil-Melethil and Clifford Heindel under the supervision of Dr. Steven Gray at the University of North Carolina, Chapel Hill. The qPCR analysis for biodistribution data was performed by both Karlaina and Zhilin Chen (Figure 3.25). Brianna Quinville assisted Karlaina in the anti-HexA ELISAs and ELISPOT (Figure 3.26). John Keimel and William Kaemmerer (from the New Hope Research Foundation) contributed intellectually and provided biostatistics expertise.

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To my friends within the CNS and Kingston community: Thank you for keeping me sane and grounded throughout grad school! To "the key-chain ladies" – you three are some of the best friends I have ever had. Words cannot represent what having each of you as friends means to me. To 'my person' thank you for being the Meredith to my Cristina! To C&K, w(h)ine nights may need too be had via facetime, but I'll be there on the other end of the line if you need me to coach either of you through to the end of your respective PhDs! And to all my friends back home, thank you for your love and support!

Lastly, to my family, my ohana, both immediate and extended. Thank you for loving, supporting, teasing, consoling, celebrating, commiserating, and advising me throughout not only my PhD, but my life! Mom and Dad, although you guys still stand in separate corners, thank you for always being in my corner! To L&L, you're both welcome in Matante's Library anytime!

Dedication

To mouse 802,

Although you stubbornly hit the 104-week endpoint,

This is dedicated to you,

and the tens of thousands before her

that have been sacrificed in the name of scientific and medical progress.

-Thank you

Statement of Originality

I hereby certify that all the work described within this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

- *Karlaina JL Osmon, PhD*

(September 2019)

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List of Abbreviations

AAV	Adeno-associated Virus
AMP	2-amino-2-methyl-1-propanol
ANOVA	Analysis of Variance
BBB	Blood-brain barrier
BMP	Bis(monoacylglycero)phosphate
CB	Caudal Section of the Brain
CLEAR	Coordinated Lysosomal Expression and Regulation
CNS	Central Nervous System
DAB	3'-Diaminobenzidine
DNA	Deoxyribonucleic Acid
ERT	Enzyme Replacement Therapy
GE	Gene Editing
GFP	Green Fluorescent Protein
GM2	GM2 Gangliosides
GM2AP	GM2 Activator Protein
GT	Gene therapy
HD	High Dose
HexA	Hexosaminidase A protein
HSC	Hematopoietic Stem Cells
HSP70	Heat shock protein 70
IV	Intravenous
LD	Low Dose
LSD	Lysosomal Storage Disease
MB	Midsection of the Brain
MU	4-methylumbelliferone
MUG	4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside
MUGal	4-Methylumbelliferyl β-D-galactopyranoside
MUGS	4-Methylumbelliferyl-7-6-sulfo-2-acetamido-2-deoxy-bD-glucopyranoside
OFT	Open Field Test
PBS	Phosphate buffered saline
PFA	Paraformaldehyde

rAAV	Recombinant AAV
RB	Rostral Section of the Brain
RPM	Rotations Per Minute
RR	Rotarod
scAAV	Self-complementary AAV
SD	Sandhoff Disease
SRT	Substrate Reduction Therapy
ssAAV	Single-Stranded AAV
TFEB	Transcription factor EB
TSD	Tay Sachs Disease

Chapter 1

Introduction

The Search for a Treatment – Gangliosides, GM2 Gangliosidosis, Potential Treatments, and Gene Therapy

1.1 General Introduction to the Current Thesis – Intravenous Gene Therapy for GM2 Gangliosidosis

GM2 Gangliosidosis is a group of recessive lysosomal storage diseases characterized by the inability of the Hexosaminidase A (HexA) enzyme to catabolize the GM2 ganglioside (GM2) lipid. This enzyme dysfunction leads to the mass neurotoxic accumulation of GM2 across the brain and spinal cord, leading to a widespread neurodegeneration. The HexA enzyme is a heterodimeric enzyme composed of an α- and a β-subunit, coded by the *HEXA* and *HEXB* genes respectively. The HexA enzyme must then complex with the GM2 activator protein (GM2AP), coded by the *GM2AP* gene, in order to degrade GM2. Mutations in any of the genes required for the HexA-GM2AP complex results in one of three types of GM2 Gangliosidosis; Tay-Sachs Disease (TSD; α deficiency), Sandhoff Disease (SD; β deficiency), and the AB-variant (GM2AP deficiency). The progressive neurodegeneration in these diseases produces symptoms such as loss of motor control, sensory impairments/losses, ataxia, and seizures. These diseases are fatal with no current curative gold standard treatment, other than palliative care.

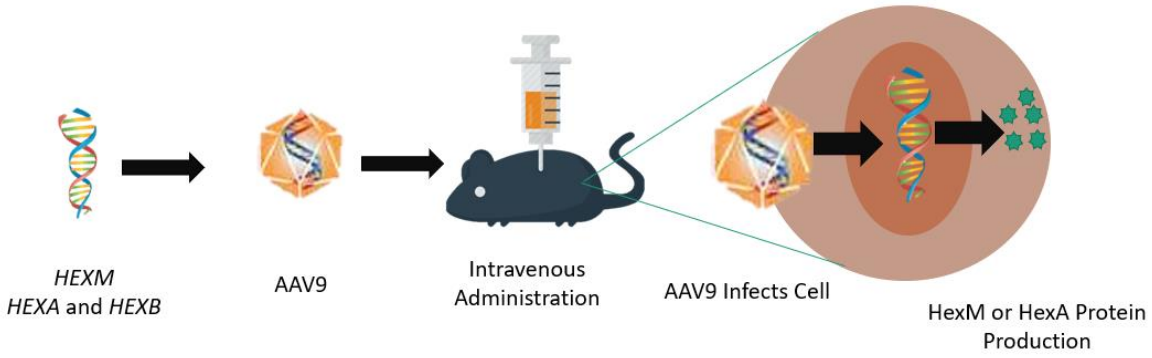

Figure 1.1 Gene Therapy and Overall Strategy for the Current Studies

The current studies in this thesis investigate the use of a hexosaminidase hybrid enzyme (HEXM transgene) and the use of a bicistronic hexosaminidase construct (HEXB-HEXA transgene). These genes were packaged into adeno-associated viral vector serotype 9 (AAV9) and intravenously delivered into Sandhoff mice. AAV9 crosses the blood-brain barrier to infect the brain and spinal cord, leading to therapeutic protein production of either HexM or HexA.

1.1.1 Research Objectives

The overarching goal of this study is to preclinically assess intravenous gene therapy GT treatments for GM2 Gangliosidosis. Chapter 1 outlines the current knowledge of GM2 Gangliosidoses diseases and potential treatments. Many types of treatment strategies have been attempted, each with varying levels of success. However, a gold standard treatment has yet to be established for this devastating and fatal group of diseases. This thesis outlines two studies that continue the search for an effective treatment for GM2 Gangliosidosis (Figure 1.1). We argue that GT treatments have the potential to become the most promising treatment for GM2 Gangliosidosis due to long lasting effects of gene transfer using AAV vectors. The treatments assessed in this thesis could theoretically be used to treat either TSD or SD. Chapter 1 then summarizes the key aspects of GT and ends in a discussion surrounding the ethics of GT.

Chapter 2 examines the investigation into the use of a Hexosaminidase hybrid gene, *HEXM*, in the intravenous treatment of Sandhoff Mice. The human HexM hybrid has previously been shown to functionally replace the HexA enzyme in its ability to catabolize GM2, because it

is a combination of the catalytic properties α-subunit with the stabilization properties of the β-subunit of the HexA heterodimer. This study further investigates the *in vivo* use of the hybrid HexM treatment by evaluating the efficacy of intravenous administrations of therapeutic doses to adult and neonatal SD mice. We hypothesized that a higher dose of gene therapy would produce more favourable outcomes, and that a tandem injection of mannitol would increase the bioavailability of the vector to the central nervous system.

Chapter 3 investigates the use of bicistronic Hexosaminidase vectors administered intravenously at different ages. Previous studies have shown a heterospecific immunological response to GT treatments, and unpublished data from our lab showed a heightened immune response in the SD mice to the human HexM hybrid enzyme. Therefore, we investigated the use of both a murine and a human-bicistronic hexosaminidase treatment in the SD mice in comparison to a GT vector containing only the murine *Hexb* gene. This study evaluated the efficacy of these three GT treatments in the SD mice at neonatal, juvenile, and adult ages of administration and preliminarily investigated the immunological response in the SD mice against the human HexA protein (from the human bicistronic vector). This study set out to investigate which of the three experimental vectors was superior, and which age of administration leads to the most beneficial outcomes. We hypothesize that: (1) earlier intervention will produce more favorable survival and other outcomes, (2) the human and murine B-A bicistronic vectors will produce similar beneficial outcomes, and (3) the human and murine B-A bicistronic vectors will provide an increase in beneficial outcomes compared to AAV9/*mHexb* alone.

The thesis is then concluded in Chapter 4 where we compare the effectiveness of the *HEXM* hybrid GT treatment to that of the bicistronic treatments. Furthermore, we discuss the idea of polypharmacological treatment interventions that may synergistically work towards curing GM2 Gangliosidosis. The original research within this thesis could lead to a potential gold standard treatment for GM2 Gangliosidosis disorders.

1.2 The Role of Gangliosides in Human Physiology and Disease

1.2.1 Gangliosides

Gangliosides are glycosphingolipids containing sialic acid and are a key component in the plasma membrane of mammalian cells (Schnaar, Gerardy-Schahn, & Hildebrandt, 2014; Yu, Tsai, Ariga, & Yanagisawa, 2011). There are an estimated 188 different gangliosides, each differing in their carbohydrate chain (Yu et al., 2011). The ceramide portion of gangliosides is embedded in the plasma membrane, while the glycan extends into the extra-cellular milieu (Schnaar et al., 2014) (See Figure 1.2). Gangliosides differ based on the complexity of their glycan chains, in both number of carbohydrate and sialic acid moieties. The glycan chain determines the specificity and function of the ganglioside (Schengrund, 1990; Schnaar et al., 2014).

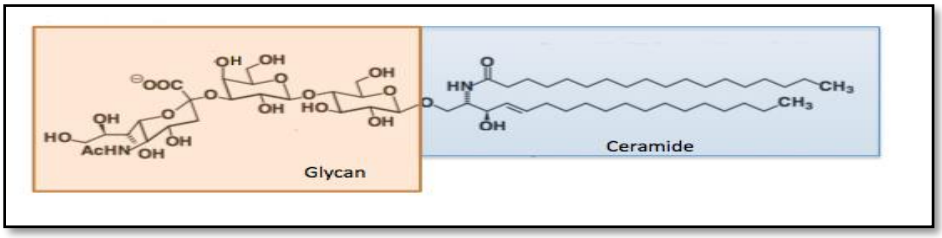

Figure 1.2 G_{M3} Ganglioside.

The ceramide portion is highlighted in blue, and the glycan portion is highlighted in orange. G_{M3} is one of the simplest gangliosides, based on size and complexity of the glycan chain. Figure Adapted from (Schnaar et al., 2014) and (Kolter & Sandhoff, 2010).

Gangliosides are found ubiquitously throughout the body, in all cell types, however they are most abundantly found in the central nervous system (Schnaar et al., 2014; Yu et al., 2011). The composition of gangliosides in certain tissues changes over the course of development and maturation (Yu et al., 2011).

1.2.2 Ganglioside Biosynthesis and Metabolism

Ganglioside biosynthesis occurs predominantly in the endoplasmic reticulum and final modifications are made in the Golgi apparatus of the cell (Yu et al., 2011). Many enzymes are involved in the synthesis and catabolizes of gangliosides, which are too extensive for this review, however overall sialyltransferases and sialidases add and remove sialic acid residues from a growing ganglioside, respectively (Schnaar et al., 2014). Some of the main gangliosides of the human body are depicted in Figure 1.3, showing the different types of gangliosides and their precursors. Figure 1.3 also depicts the 0-, a-, and b- series of gangliosides.

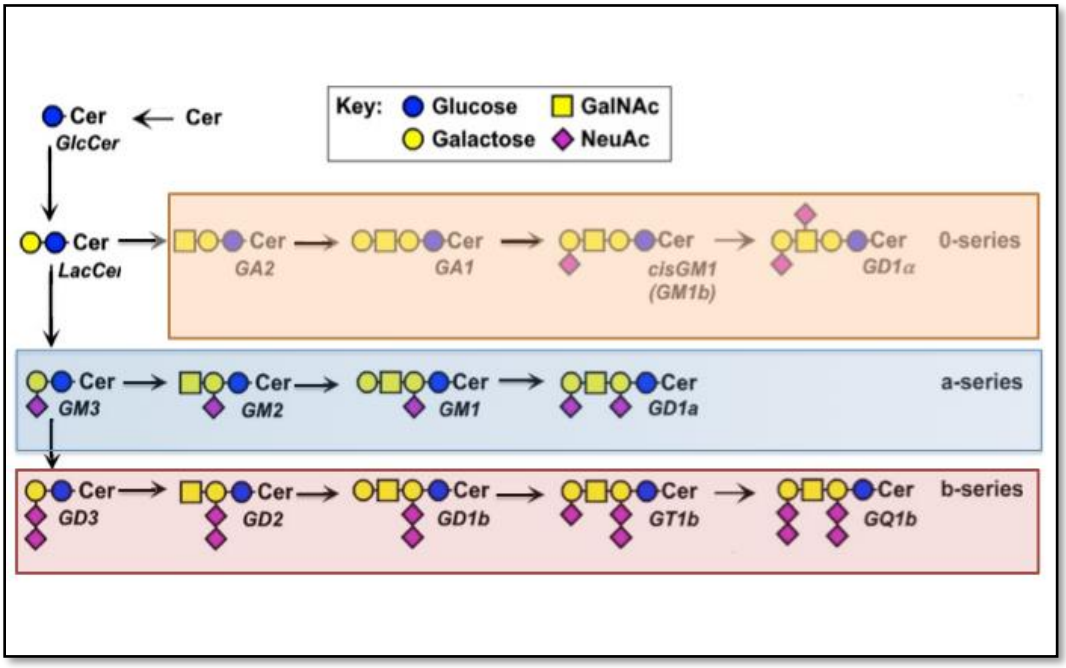

Figure 1.3 Ganglioside Biosynthesis and Metabolic Pathways

The synthesis and catabolizes of gangliosides occur in stepwise fashions that mirror each other. They originate from a simple ceramide that progressively obtains additional carbohydrates and sialic acid residues. The catabolism of gangliosides removes these residues in the reverse order. Adapted from (Schnaar et al., 2014)

1.2.3 Gangliosides in main cellular functioning

Scientists have uncovered a variety of mechanisms and have been able to compare the malfunctioning mechanism to the wild type in order to elucidate the components roles in both normal as well as disease human physiologies. Some of the roles of gangliosides have been elucidated this way, and these roles shall be discussed in this section.

1.2.3.1 Gangliosides are Lateral Regulator of Plasma Membrane Microenvironment

The mechanism for gangliosides' lateral regulatory effects on their microenvironment in the plasma membrane has yet to be completely explained. However, many of the roles gangliosides play in many cellular functions, such as differentiation, and interactions with protein kinases and receptors (Lopez & Schnaar, 2009; Schengrund, 1990; Schnaar et al., 2014), are due the their modulatory role in the placement of other membrane elements in their surroundings. Gangliosides are known to modulate the placement of neutrophil receptors, protein kinases, cholesterols and many other glycolipids and glycoproteins, not to mention fellow gangliosides (Schnaar et al., 2014). Through the modulation of membrane component placement, the gangliosides can control many things, including the activity of intracellular processes by blocking or enhancing binding to kinases (Abad-Rodriguez & Robotti, 2007; Schnaar et al., 2014), or grouping together to enable bacterial tetanus neurotoxin entrance into the cell (Chen, Fu, Kim, Barbieri, & Baldwin, 2009). These two processes will be further discussed in the following sections. It is also known that gangliosides can modulate insulin sensitivity and responses as well as angiogenesis through the placement of and modulation of insulin receptors and vascular endothelial growth receptors, respectively (Lopez & Schnaar, 2009).

1.2.3.2 Role in Differentiation

Ganglioside involvement in neural differentiation has been much more extensively researched than the roles of ganglioside in the differentiation of other cell types (and will be discussed later in this review). However, the role of GD1a in the differentiation of human

mesenchymal cells into osteoblasts has been studied (Yang et al., 2011). Increased levels of GD1a lead to the differentiation of mesenchymal cells into osteoblasts through the modulation of the activation of human ependymal growth factor receptor and kinase activity (Yang et al., 2011).

1.2.3.3 Role of Gangliosides in Immune Responses

Guillain-Barré syndrome causes patients to have acute neuromuscular paralysis, and it has been shown that this syndrome is caused, at least in part by anti-ganglioside antibodies (Schnaar et al., 2014). In short, previous infection of patients with *C. jejuni* elicited an immune response to the epitopes on the bacteria, which mimic GM1 and GD1a (Schnaar et al., 2014). These antibodies then begin attacking the gangliosides maintaining the axon-myelin interactions. The role of gangliosides in axon-myelin maintenance will be discussed in 1.2.4.2. The ability for pathogens to elicit immune responses to normal cellular molecules is called 'molecular mimicry' (Schnaar et al., 2014).

Many cancers are known to shed gangliosides and through this process, they evade the immune system (de Leòn, Fernández, Mesa, Clavel, & Fernández, 2006; Péguet-Navarro et al., 2003; Shen, Falahati, Stark, Leitenberg, & Ladisch, 2005). Normal human gangliosides contain NAc sialic acid residues, and not NGc sialic acid residues (de Leòn et al., 2006). A modified version of GM3, containing the glycolylated end (NGc) strongly suppresses the immune system in cancers through the suppression of CD4 leukocyte activity (de Leòn et al., 2006). The expression of GD1a has been tied to the suppression of inflammatory response by altering the interleukins that the CD4 cells release, switching them from the pro-inflammatory IL-12 to the anti-inflammatory IL-10 (Shen et al., 2005). The complete mechanisms behind these changes in inflammatory response have not been easy to assess, and further work needs to be done to determine the mechanism of action. Other roles of gangliosides in cancers are further discussed in the next section.

1.2.3.4 Role of Gangliosides in Cancer

It has recently been shown that GD3 plays an important role in the adhesion of cancer cells with synergistic effects when specialized growth factors are present (Furukawa et al., 2014). GD3 is highly expressed in melanomas and contributes to the malignancy of the cancer (Furukawa et al., 2014). GM3 has well documented inhibitory effects on cell growth and proliferation, but the decreased amounts of GM3 found in many tumours may contribute to the malignancy and growth rate of these cancers (Hakomori & Handa, 2015; X. Huang et al., 2013). The inhibition of growth is due to GM3's inhibition of the growth receptors on the cell surface (X. Huang et al., 2013).

Infantile and childhood neuroblastomas also have aberrant ganglioside expression, where increased malignance is due to an increase in a-series gangliosides and a decrease in b-series gangliosides (Kaucic, Etue, LaFleur, Woods, & Ladisch, 2001) (see Figure 1.1 for a- and b-series). Neuroblastomas that are diagnosed before the age of 1 are typically less malignant and contain mainly b-series gangliosides, suggesting that after 1 year of age, the transition to a-series gangliosides, or the loss of b-series gangliosides, results in the malignancy of these tumours (Kaucic et al., 2001). The role of gangliosides in the nervous system is reviewed in the next section.

1.2.4 Gangliosides in the Nervous System

As previously mentioned, the nervous system, specifically the brain is where the majority of the gangliosides of the human body are located (Schnaar et al., 2014; Yu et al., 2011). The abundance of gangliosides in the neural tissue indicates that gangliosides may have an important role in the development, maintenance and overall wellbeing of the nervous system. The roles of some of the major gangliosides in the nervous system are discussed in this section.

1.2.4.1 Neural Development and Differentiation

It has been shown that over the course of development, gangliosides of the nervous system begin as the simplest gangliosides (GM3 and GD3) during the first half of gestation, and develop to more complex gangliosides (GM1, GD1a, GD1b, and GT1b) over the second half of gestation and through to adulthood (Schnaar et al., 2014). Over the course of development, the total concentration of gangliosides in the nervous system increases as the complexity of the neural connections increase (Schnaar et al., 2014). The different types of gangliosides in the brain are not all ubiquitously found throughout the brain, but some are located in specialized structures, such as a-series gangliosides being found predominantly on cholinergic neurons, or GM1 and GD1a being concentrated in white and gray matter, respectively (Schnaar et al., 2014). Lastly and of great importance is the role of acetylated-GD3 in the role of neuroblast migration through the rostral migratory stream during the process of brain development (Schnaar et al., 2014). The effects of acetylation on ganglioside function have yet to be completely elucidated, however the requirement of acetylated-GD3 in the role of neuroblast migration is essential (Schnaar et al., 2014).

Differentiation of the cells in the central nervous system is accomplished by the regulation of gangliosides on both the plasma membrane and nuclear membrane of the cell. The majority of the research concentrates on the role of plasma membrane gangliosides, however there has been a subset of gangliosides found on the nuclear membrane that have been hypothesized to have a role in neuronal differentiation (Ledeen, Wu, Lu, Kozireski-Chuback, & Fang, 1998). Increases in GM1 on the nuclear membrane have been shown to decrease the amount of nuclear calcium ions, contributing to a neuronal differentiation pathway (Ledeen et al., 1998). Gangliosides GD3, GD1a, and GM3 play a role in regulating and inducing neuronal differentiation (Kwak et al., 2006; Moussavou et al., 2013; Ryu et al., 2009). GM1 has been shown to have a regulatory role during the course of neurogenesis and regeneration (Stojiljković

et al., 1996), as well as protecting the neuron from apoptosis (Cavallini, Venerando, Miotto, & Alexandre, 1999; Ferrari, Anderson, Stephens, Kaplan, & Greene, 1995).

It has been postulated that gangliosides may play a role in synaptic plasticity and memory formation. Increases in G_{T1b} augmented the amount of synapses in the brain (Kotani, Kawashima, Ozawa, Terashima, & Tai, 1993), as well as enhancing dendritic growth through increasing actin (Higashi & Chen, 2004). Figure 1.4 ties in many of the aspects of neurogenesis, differentiation and regeneration herein discussed, and hypothesizes gangliosides effects on memory formation (Wang & Brand-Miller, 2003).

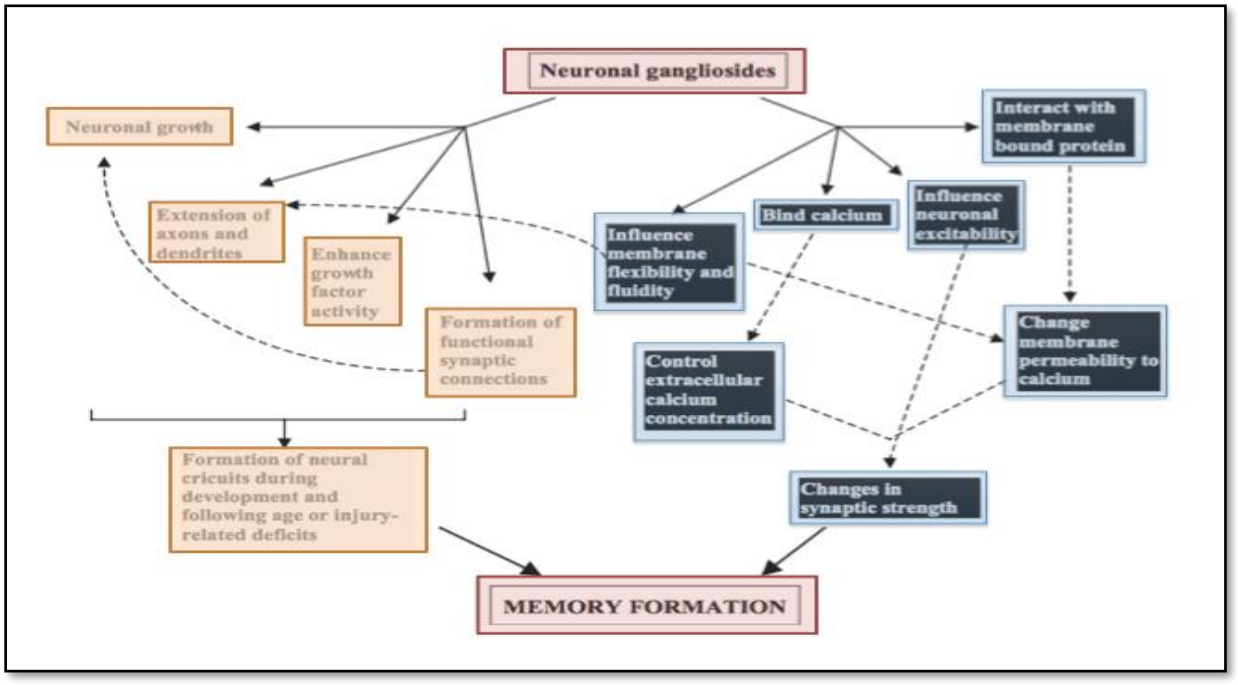

Figure 1.4 Summary of the possible mechanisms in the brain to which gangliosides may contribute.

Solid lines are the mechanisms proven to be true, while the dashed lines remain speculation. This pictorial hypothesis ties in many of the aspects known about gangliosides and their roles in neuronal tissues. Adapted from (Wang & Brand-Miller, 2003).

1.2.4.2 Mediator of Axon-Myelin Interactions and Regeneration

Gangliosides play crucial roles in axonal formation, maintenance and regeneration. In axon development, GM1 interacts with protein kinases to modulate the growth of axons and enhances the binding of neurotrophic factors and growth factors to the neuron (Abad-Rodriguez & Robotti, 2007). GT1b is a key determinant in the position of specific neurotrophic receptors in the plasma membrane, and along with G_{M1} helps protein kinases distinguish which action to carry out, depending on the neurotrophin binding to the receptor (Abad-Rodriguez & Robotti, 2007). G_{T1b} is also an important component in the inhibition of axonal growth through its interactions with the inhibitory complex disrupting myelin-associated protein's (MAG) function (Abad-Rodriguez & Robotti, 2007; Lopez & Schnaar, 2009). MAG works to stabilize myelin-axon interactions and regulate axonal growth during regeneration and repair (Lopez & Schnaar, 2009; Schnaar et al., 2014). MAG expressed on the myelin interacts with GDa1 and GT1b presented on the axon to stabilize the myelin binding to the axon (Lopez & Schnaar, 2009; Schnaar et al., 2014). MAG has been proposed to protect axons from neurotoxins, possibly through the increased, strong binding between the MAG-myelin and the axon (Schnaar et al., 2014).

The regeneration of nervous system tissues is possible in the peripheral nervous system, but extremely unlikely in the central nervous system. It has been found that the conversion of GD1a and GT1b to GM1 enables the peripheral nervous system to regenerate nerves, but this conversion is absent in central nervous system neurons (Kappagantula et al., 2014).

1.2.4.3 Receptors for Bacterial Neurotoxins

Gangliosides have long been known to be receptors for many bacterial neurotoxins (Schnaar et al., 2014), of which two will be discussed herein. GM3 is a receptor for the botulism neurotoxin deriving from *Clostridium botulinum type C*, whereas type C was previously thought to simply bind to GD1b and GT1b (Sugawara et al., 2015). Botulism toxins cause flaccid paralysis at the neuromuscular junction (Sugawara et al., 2015), whereas tetanus neurotoxin

produces paralysis by inhibiting neurotransmitter release in the central nervous system (Chen et al., 2009). The tetanus toxin requires both GM1a and GD3 in the correct spatial orientation to properly bind to the cell (Chen et al., 2009). This high affinity binding of tetanus to neural cells is mediated solely through the toxins interactions with these two gangliosides that comprise of its receptor (Chen et al., 2009).

1.2.4.4 Lysosomal Storage disorders – Ganglioside accumulation

There is a collection of neurodegenerative disorders characterized by an accumulation of specific types of gangliosides within the lysosome. A non- or mal-functioning catabolic enzyme responsible for the degradation of gangliosides causes these diseases. The build-up of GM1, and GM2 gangliosides in the neurons is caused by the malfunctioning β-galactosidase and Hexosaminidase A enzymes, respectively, leading to progressively rapid neurodegeneration and ultimately death (Sandhoff & Harzer, 2013; Schnaar et al., 2014). The accumulation of GM1 gangliosides is termed GM1 gangliosidosis and the accumulation of GM2 is referred to as GM2 gangliosidosis (Sandhoff & Harzer, 2013). Neimann-Pick's disease is also a lysosomal storage disorder resulting from the accumulation of GM2 and GM3 gangliosides in the neurons, leading to neurodegeneration (Schnaar et al., 2014).

1.2.5 Dietary Gangliosides for Mothers and their Babies

Not much is known about dietary gangliosides for both adults and infants, however, it has been shown that supplementing the amount of gangliosides in babies' formula increases the overall amount of brain gangliosides in their developing brain, increasing cognitive development (Ryan, Rice, & Mitchell, 2013). In addition human colostrum and breast milk have increased amounts of gangliosides, GD3 and GM3, respectively, providing infants with the additional building blocks they require for further growth (Pan & Izumi, 2000; Ryan et al., 2013). The maternal diet is the only externally derived source of gangliosides a fetus and baby have,

therefore, the intake of gangliosides is important for pre-, peri-, and post-natal care (Ryan et al., 2013).

Another role for dietary gangliosides has been seen in the protection of the intestinal tract of infants through the binding of dietary gangliosides to pathogenic bacteria (Rueda, Maldonado, Narbona, & Gil, 1998; Salcedo, Barbera, Matencio, Alegría, & Lagarda, 2013). As previously stated in section 1.2.4.3, gangliosides are often receptors for bacterial toxins (Schnaar et al., 2014). Gangliosides such as GM1, GM3, and GD3, of the milk in the gastro-intestinal tract bind to the epitopes presented on many bacterial toxins and species that have previously been involved in newborn diarrhea, inhibiting the bacterial binding to the colon (Fishman, 1982; Rueda et al., 1998; Salcedo et al., 2013). The bacterial invasion can then be safely excreted without harmful side effects (Rueda et al., 1998; Salcedo et al., 2013).

1.2.6 Gangliosides in the Blood

1.2.6.1 Role in Erythrocytes and in Serum

Gangliosides have been found circulating in human serum, and have been hypothesized to be bound to lipophilic proteins, since the hydrophobic ceramide moiety would not readily mix with the aqueous blood (Senn, Orth, Fitzke, Wieland, & Gerok, 1989). They found the main gangliosides circulating in serum were GD3 and GM3, however the role of gangliosides in the serum has yet to be ascertained (Senn et al., 1989). The study did conclude that ganglioside content and composition in the serum was not affected by age or sex of the healthy participants, and therefore, gangliosides in human serum must be regulated to stay constant in adults (Senn et al., 1989).

Much more interestingly is the role that gangliosides play in the determination of blood type. There are specialized gangliosides that are the cell surface marker on erythrocytes for the type-A blood type (Kushi et al., 2001). There are two slightly different A-active gangliosides (I

and II) that can present on the cell surface of erythrocytes. There are also B-active gangliosides that denote Type-B blood (Kushi et al., 2001).

1.2.6.2 Role in Leukocytes and Leukemia

It has been shown that gangliosides, specifically GM3, GD1a, GD3, and GT1b, in the environment of leukocytes increase the efficacy and rate of leukocytic phagocytosis (Yamaguchi, Miyazaki, Oka, & Yano, 1997). This was accomplished by coating heat killed *S. Aureus* bacteria with different gangliosides and observing the different phagocytic rates (Yamaguchi et al., 1997).

An increase in the overall amount of gangliosides in myelocytic leukemia compared to non- leukemic patients had been observed in the 1970s (Hildebrand, Stryckmans, & Vanhouche, 1972). The same study also revealed that lymphatic leukemia only had small traces of gangliosides, as compared to normal leukocytes (Hildebrand et al., 1972). To further investigate, subsequent study showed that lymphatic leukemia cells, both acute and chronic, contained increased amounts of GD3 compared to normal leukocytes, which showed no detectable amount of GD3 (Siddiqui, Buehler, DeGregorio, & Macher, 1984). Acute myeloid leukemic cells also contained increased amounts of GD3, but chronic myeloid leukemia cells did not (Siddiqui et al., 1984). It was hypothesized that ganglioside expression in leukocytes and leukemia is dependent on a variety of factors, including cell lineage, stage of differentiation, as well as leukemogenic state (Siddiqui et al., 1984).

1.2.7 Gangliosides in the Liver

The main gangliosides found in the hepatic tissues is an abundance of G_{M3} followed by a variety of minute concentrations of GM2, GM1, GD3, GD1a, GD1b, GT1a, and GT1b (Nilsson & Svennerholm, 1982; Tanno, Yamada, Shimada, & Oshashi, 1988). The ganglioside content and composition in the liver has been shown to remain relatively constant, regardless of the presence of cirrhosis, but the composition of the gangliosides changed in the carcinoma, decreasing in overall GM3 while increasing the concentration of all the other gangliosides (Tanno et al., 1988).

As for the functional role of gangliosides in the liver, some may contribute to the maintenance of blood-sugar levels through controlling the insulin sensitivity of hepatic cells through the modulation of sialidase in the cellular membrane (GM1's modulation on the insulin receptor) (Yoshizumi et al., 2007). It has also been suggested that all gangliosides secreted into the serum are produced in the liver (Kivatinitz, Miglio, & Ghidoni, 1991; Senn et al., 1989).

1.2.8 Gangliosides in the Human Eye – Cataracts

Over the course of time, it is natural for the composition of gangliosides in certain tissues to change, and accumulate (Yu et al., 2011). However, the aberrant accumulation of gangliosides can cause extensive damage to tissues and trigger the beginning of a disease. Cataracts, the lens of the eye growing more opaque over the course of time as humans age, is one such disease (Ogiso et al., 1990). Increases in GM3, GM1, and GD1a, were identified, as well as increases in unidentified gangliosides (Ogiso et al., 1990). Ogiso et al. showed that increased accumulation of gangliosides in the lens leads to an increase in opaqueness of the lens (Ogiso et al., 1990).

1.2.9 Summing up the Role of Gangliosides in Human Physiology and Disease

This review of gangliosides and their role in the human body is by no means an extensive list. The topics presented herein were interesting to the reader, however, with 188 different gangliosides, a review of all of them and their functions would be a monumental undertaking. The vast array in the functional roles of the gangliosides mentioned in this review is varied, depending on a multitude of factors, such as cell type, microenvironment and modifications to the gangliosides.

1.3 GM2 Gangliosidosis

GM2 Gangliosidoses are a group of recessive neurodegenerative lysosomal storage disorders characterized by the inability to properly degrade the GM2 ganglioside (GM2) lipid substrate (Sandhoff, 2012; Sandhoff & Harzer, 2013). This disruption in catabolism leads to the neurotoxic accumulation in the central nervous system, and ultimately leads to the death of the patient. Below we discuss the history, clinical presentation, biochemistry, and therapeutic options for the GM2 Gangliosidoses diseases.

1.3.1 Clinical presentation of GM2 Gangliosidosis

The clinical presentation of the GM2 Gangliosidoses diseases are nearly indistinguishable (Sandhoff, 2012; Sandhoff & Harzer, 2013). TSD, SD and the AB-variant are caused by a mal- or non-functional HexA-GM2AP complex, through mutations in the genes encoding the α- or β-subunit of the HexA protein, or the GM2AP, respectively (Sandhoff & Harzer, 2013). These diseases are characterized by mass neurodegeneration of the CNS leading to symptoms such as but not limited to the loss of motor control, sensory losses, paralysis and seizures (Bley et al., 2011; Sandhoff, 2012; Sandhoff & Harzer, 2013). TSD and SD have an estimated incidence of 1 in 222,000 and 1 in 422,000, respectively, however the incidence of TSD and SD are substantially higher in some populations, such as the Ashkenazi Jewish or French Canadian populations, or populations from Northern Saskatchewan (Andermann, Scriver, Wolfe, Dansky, & Andermann, 1977; Fitterer et al., 2014; Meikle, Hopwood, Clague, & Carey, 1999; Myrianthopoulos, 1962). Mutations to the GM2AP leading to the AB-variant of GM2 Gangliosidosis are very rare.

There are three distinct classifications of GM2 Gangliosidosis based on the age of disease onset. The age of disease onset is inversely correlated to the amount of residual HexA enzymatic activity retained by the patient (Bley et al., 2011; Conzelmann & Sandhoff, 1991; Leinekugel, Michel, Conzelmann, & Sandhoff, 1992a). The mutations resulting in a nullified (completely

non-functional or non-existent) HexA protein leads to the most severe and acute type of GM2 Gangliosidosis, the *infantile* presentation. Infants born with these diseases begin developing symptoms around 6 months of age, with a progressive decline in health where these patients do not typically survive past 4 years of age (Bley et al., 2011). The *juvenile* and *adult* ages of onset begin in childhood or early adulthood, respectively, where these ages of onset retain of approximately 6-10% HexA activity (Conzelmann & Sandhoff, 1991). The symptomology of these two classifications mimic that of the infantile version, but over a longer timeframe, since the residual enzyme activity was not sufficient to maintain proper GM2 degradation, leading to a much slower accumulation of GM2, hence delaying disease onset (Conzelmann & Sandhoff, 1991). It has been estimated that an increase in HexA activity to 10-15% of the activity observed in a healthy subject would provide significant therapeutic and possibly curative effects (Leinekugel et al., 1992a).

1.3.2 Genetics and Biochemistry of GM2 Gangliosidosis

The HexA enzyme complex is composed of an α- β-subunit heterodimer, which must complex with the GM2 activator protein (GM2AP) for proper functioning. These subunits and cofactors are coded by the *HEXA*, *HEXB*, and *GM2AP* genes respectively, which are located on chromosome 15 (*HEXA*) and chromosome 5 (*HEXB* & *GM2AP*) (Ferreira & Gahl, 2017; Don J. Mahuran, 1999). Severe mutations in any of these genes can lead to GM2 Gangliosidosis.

The formation of the HexA enzyme is accomplished through the heterodimerization of the α-subunit and the β-subunits (See Figure 1.5.A) (D. J. Mahuran, Neote, Klavins, Leung, & Gravel, 1988; D J Mahuran, Tsui, Gravel, & Lowden, 1982; Sinici et al., 2013). The HexA enzyme then interacts with the GM2 activator protein (GM2AP) to remove the terminal N-acetylgalactosamine residues from GM2, converting it to GM3 ganglioside (Sandhoff, 2012; Sandhoff & Harzer, 2013; Sinici et al., 2013). GM2 Gangliosidosis arises due to the inability of the HexA enzyme to catabolize GM2, leading to a neurotoxic accumulation of the substrate

within neurons of the central nervous system (CNS). This mass accumulation of GM2 triggers inflammatory responses ultimately concluding in the neurodegeneration seen in TSD and SD (See Figure 1.5.B-C) (M. Jeyakumar et al., 2003; Sandhoff & Harzer, 2013).

The precise molecular mechanisms underlying the neurodegeneration observed in SD and TSD has yet to be fully elucidated. However, recent work has found a bi-phasic immunological response in the brain, beginning with microgliosis, followed by astrogliosis, which ultimately contribute to and aggravate the neurodegeneration in the surrounding neural tissue(Hooper & Igdoura, 2016). Additionally, synaptic dysfunction has also been tied to the dysregulation of synaptic proteins and receptors, also contributing to the inevitable neurological decline (Hooper, Alamilla, Venier, Gillespie, & Igdoura, 2017).

Gene therapy (GT) is a remedial strategy where a therapeutic transgene is encapsulated in a viral vector, where upon successful cellular infection, the therapeutic protein is transcribed and translated into the therapeutic enzyme. For GM2 Gangliosidosis, delivering the genes for both the α- and β-subunits (each ~ 1.6kb) is somewhat challenging when using a self-complimentary AAVs (scAAV) owing to their limited packaging capacity of ~2.4 kb (although it is possible when using a single stranded AAV (ssAAV)) (Grieger & Samulski, 2005; Z. Wu, Yang, & Colosi, 2010a). With the intention of using scAAVs, a hybrid subunit, μ-subunit, was designed that incorporated the key elements of both the α- and β-subunits, which enabled the μ-subunit to homodimerize and form a stable enzyme, HexM, capable of interacting with GM2AP and catabolizing GM2 (Figure 1.5.D) (Tropak et al., 2016a). Specific changes in the amino acid sequence of the α-subunit enabled the inclusion of the β-subunit-like stable linkages, as well as the β-subunit GM2AP binding site to the α-subunit sequence (Tropak et al., 2016a).

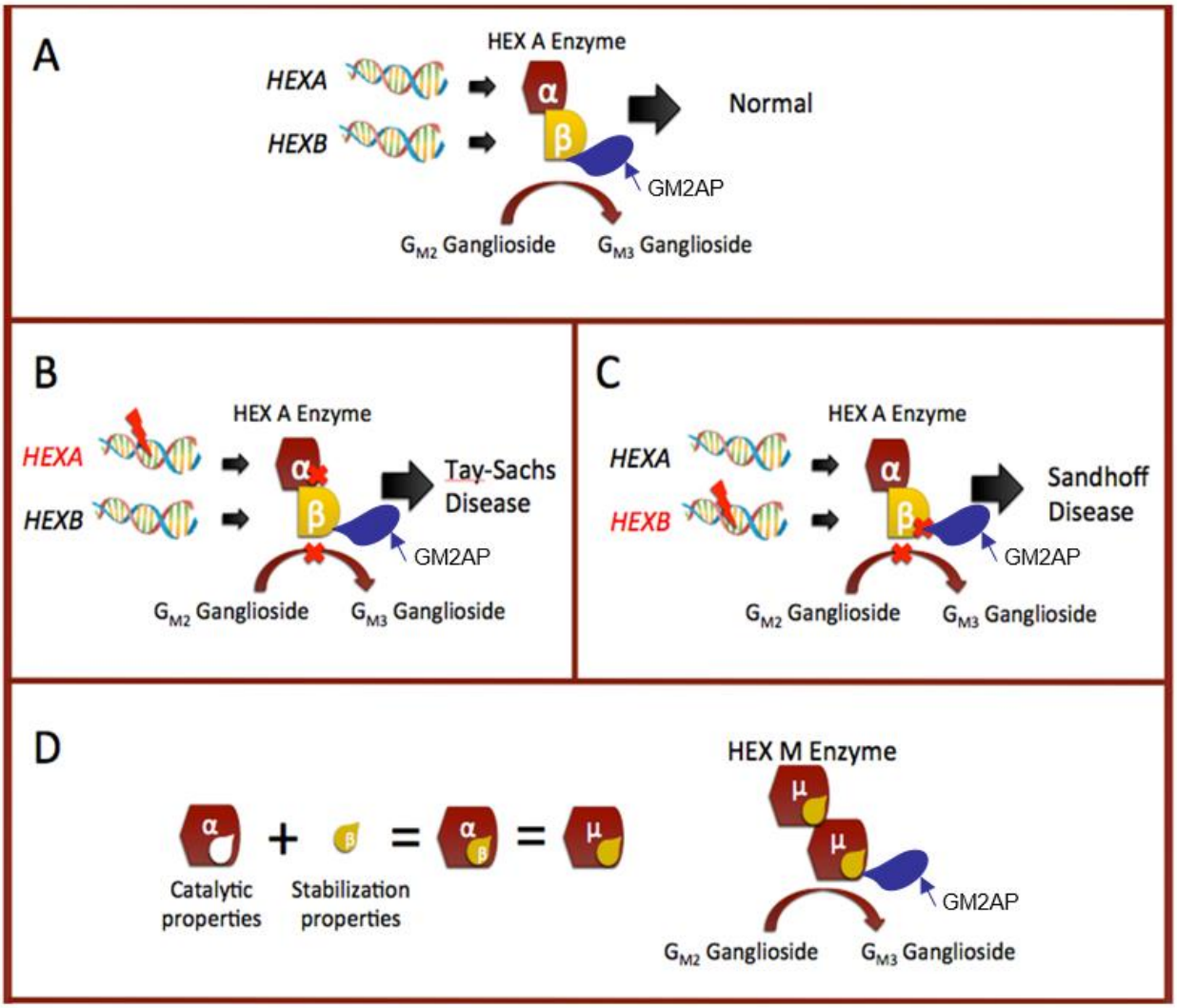

Figure 1.5 Biochemistry of the Wild Type Hexosaminidase A and the Hybrid Hexosaminidase M Enzymes

(A) The *HEXA* and the *HEXB* genes code for the α- and β- subunits, respectively. When both are wild type, individuals are unaffected and live normal healthy lives, where the HexA enzyme catabolizes GM2 ganglioside to GM3 ganglioside with the help of the GM2-activator protein (GM2AP). (B) However, when there are mutations in the *HEXA* gene, this causes completely or mildly non-functional α-subunit, leading to Tay Sachs Disease. (C) When there are mutations in the *HEXB* gene, this leads to completely or mildly non-functional β-subunit of the HexA enzyme, leading to Sandhoff disease. (D) Our collaborators designed the μ-subunit to contain the catalytic properties of the α-subunit, and the stabilization properties of the β-subunit. This μ-subunit can homodimerize and replace the non-functional HexA enzyme in its role in the catabolism of G_{M2} gangliosides to GM3 gangliosides. HexM is fully capable of interacting with the GM2APto degrade GM2.

1.4 Therapeutic Options for Lysosomal Storage Disorders

The treatment of lysosomal storage disorders (LSDs) is challenging due to the unique complexity of each of the +70 disorders (Parenti, Andria, & Ballabio, 2015; Platt, 2018). This section discusses the normal functioning of the lysosome, the pathological cascade of events that lead to LSDs, followed by the current therapeutics under investigation or being used to treat LSDs. Two main groups of LSDs - Mucopolysaccharidosis, and GM2 Gangliosidosis- and their treatment options will be discussed as examples.

1.4.1 The Normal Functioning Lysosome and Lysosomal Storage Disorders

Found in nearly all eukaryotic cells, lysosomes have been classically viewed as being the digestive organelle, or the recycling station of a cell (Luzio, Pryor, & Bright, 2007; Platt, 2018). Lysosomes contain multiple catalytic hydrolases maintained at acidic pH levels enabling the catabolism, however in recent years, it has been shown that the lysosome plays a key role in cellular homeostasis, including cell membrane repair (Andrews, 2002; Castro-Gomes, Corrotte, Tam, & Andrews, 2016; Luzio et al., 2007), energy metabolism (Mansueto et al., 2017; Pastore et al., 2017), cholesterol equilibrium (Castellano et al., 2017), and autophagy (Settembre et al., 2011).

The cellular maintenance of lysosomes has been linked to a network of genes designated the coordinated lysosomal expression and regulation (CLEAR) network (Palmieri et al., 2011; Settembre, Fraldi, Medina, & Ballabio, 2013). The activation of these CLEAR genes is accomplished by the transcription factor EB (TFEB), which is considered the master regulator for the CLEAR network, allowing for the proper homeostasis of lysosomal degradation within the cell (Palmieri et al., 2011; Sardiello & Ballabio, 2009; Settembre et al., 2011, 2013).

Normal lysosomal degradation of biomolecules begins with the activation of the genes encoding lysosomal membrane proteins or hydrolases (Appelqvist, Wäster, Kågedal, & Öllinger,

2013; Luzio et al., 2007; Parenti, Andria, & Ballabio, 2015; Parenti, Pignata, Vajro, & Salerno, 2013; Platt, 2018). The transcription and translation of these genes allow the production of the functional, properly folded lysosomal membrane proteins or hydrolases. These proteins are then transported to the lysosome in order to fulfill their intended role, either as a part of the lysosomal plasma membrane, or as one of the enzymes responsible for biomolecular degradation (Parenti, Andria, & Ballabio, 2015; Parenti et al., 2013; Platt, 2018). LSDs arise when these normal processes cannot efficiently occur.

It has been shown that the transcription and translation of the *HEXA* and *HEXB* genes are conducted separately, and that the α- and β-subunits only interact within the Endoplasmic Reticulum of the cells, where the subunits have the N-linked oligosaccharide and Mannose 6-phosphate (M6P) moieties attached (Cachón-González, Zaccariotto, & Cox, 2018; Conzelmann & Sandhoff, 1991; Hubbes, Callahan, Gravel, & Mahuran, 1989; Little, Lau, Quon, Fowler, & Neufeld, 1988; D. J. Mahuran et al., 1988; Quon, Proia, Fowler, Bleibaum, & Neufeld, 1989; Solovyeva et al., 2018; Sonderfeld-Fresko & Proia, 1989). After the addition of the M6P, disulfide bonds between the α- and β-subunits form, binding the entire HexA enzyme (Proia, d'Azzo, & Neufeld, 1984). The HexA enzyme then is transported through the Golgi Apparatus and is converted to the mature form of HexA upon entering the lysosome (Hubbes et al., 1989; Proia et al., 1984; Weitz & Proia, 1992).

LSDs are a collection of over 70 rare but distinct disorders, each stemming from a mutation in a gene encoding a lysosomal protein (Cox & Cachón-González, 2012; Parenti, Andria, & Ballabio, 2015; Parenti et al., 2013; Platt, 2018). LSDs are broadly characterized by the accumulation and storage of glycolipid and biomolecule substrates in the lysosome as a result of the production of dys- or non- functioning lysosomal proteins, caused by the aforementioned genetic mutation (Cox & Cachón-González, 2012; Parenti, Andria, & Ballabio, 2015; Parenti et al., 2013; Platt, 2018). The lysosomal proteins involved in LSDs typically maintain lysosomal

homeostasis and include not only catabolic enzymes, but lysosomal membrane proteins, lysosomal trafficking proteins, and the activator proteins that interact with the substrate and hydrolases for the proper catabolism to occur. Nearly 70% of LSDs affect the central nervous system (CNS) as a neurodegenerative disease, however the peripheral tissues and organs are still affected by the accumulation of substrate (Cox & Cachón-González, 2012). Although individual LSDs are considered rare, they collectively represent approximately 1 in 5000- 7000 births (Cox & Cachón-González, 2012; Platt, 2018).

There are three clinical levels of severity for LSDs, each named for the age of symptom onset and characterized by the amount of residual enzyme activity retained following the genetic mutation leading to the disease (Conzelmann & Sandhoff, 1983, 1991; Neufeld, 1991; Parenti, Andria, & Ballabio, 2015; Platt, 2018; Rapola, 1994). The infantile onset LSDs are the most commonly seen, where the patient retains no or undetectable residual enzymatic activity and begin developing symptoms during infancy. Patients affected with infantile LSDs generally do not survive past early childhood (Bley et al., 2011; Conzelmann & Sandhoff, 1983; Parenti, Andria, & Ballabio, 2015; Platt, 2018). The juvenile- and adult- onset LSDs retain a slightly higher percentage of residual lysosomal enzymatic activity, delaying the point at which the substrate storage becomes noxious (Neufeld, 1991; Platt, 2018; Rapola, 1994). This delays the age of symptom onset and prolongs the disease progression.

Genetic screening for newborns is one of the best ways to achieve an early diagnosis. Newborn screening is much easier to do when family history about LSDs is known, however many *de novo* occurrences of LSDs do not get diagnosed until well into symptom presentation (Platt, 2018). With LSDs, interventions that take place before the accumulation of substrates becomes toxic leads to more favourable clinical outcomes because there is a greater retention of healthy cells across all tissues, especially those in the CNS (Cox & Cachón-González, 2012; Parenti et al., 2013; Platt, 2018). A complete successful treatment of these diseases will depend

on the therapy's ability to treat the peripheral organs and cross the blood-brain barrier to treat the hallmark general progressive neurodegeneration of LSDs. LSDs are unique, in that residual activity above 15% of normal functioning activity is enough to prevent all symptoms (Conzelmann & Sandhoff, 1983, 1991; Leinekugel et al., 1992a; Parenti, Andria, & Ballabio, 2015; Parenti et al., 2013). Any treatment therefore should aim to improve residual or introduce enzymatic function to the patient in order for their overall activity to be increased above 15%.

As previously discussed, LSDs are caused by the aberrant expression of mutated non- or mal-functional lysosomal proteins, leading to the accumulation of the catabolic substrates within the organelle (Parenti, Andria, & Ballabio, 2015; Parenti et al., 2013; Platt, 2018). This substrate accumulation causes an increase in the size and number of lysosomes in the cells, which often leads to intra-cellular dysfunctions (Parenti, Andria, & Ballabio, 2015; Parenti et al., 2013; Platt, 2018). An inflammatory response typically follows, which activates immunological responses from the macrophage or microglia(M. Jeyakumar et al., 2003; Platt, 2018; Wada, Tifft, & Proia, 2000). The resulting overall cellular response is typically cell death (Parenti et al., 2013; Platt, 2018). The dysfunctional cascading process of LSDs is described in Figure 1.6. At nearly each step of the pathological cascade there is the possibility of therapeutic intervention (Figure 1.6). Therapeutic interventions at the beginning of the process would include GT or gene editing (GE), followed by small molecule chaperones, proteostasis regulators, enzyme replacement therapy (ERT), substrate reduction therapy (SRT), and lastly, anti-inflammatory drugs.

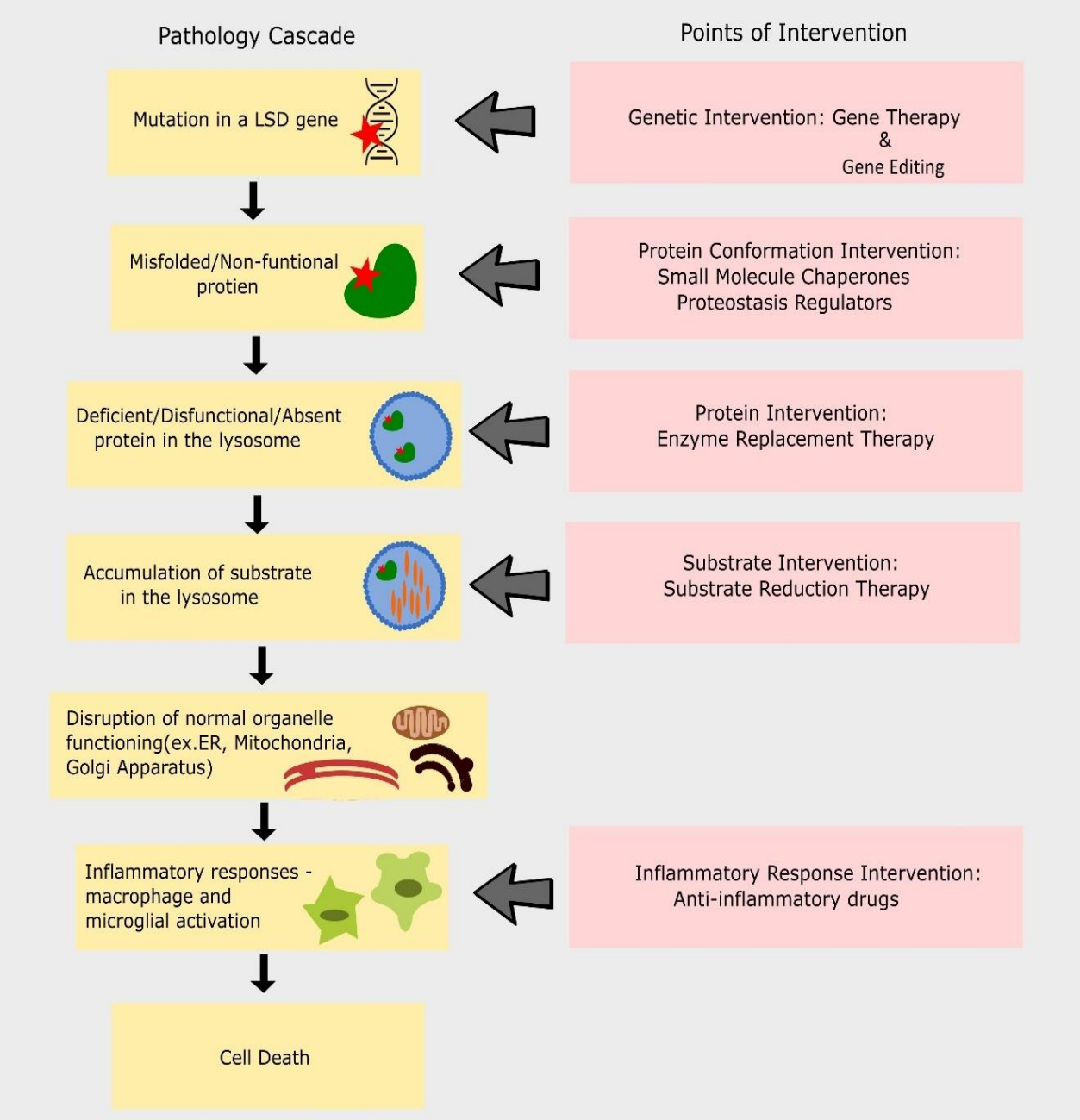

Figure 1.6 Pathological Cascade of LSDs with Therapeutic Points of Intervention

Adapted from (Platt, 2018). An overview of the pathological cascade of LSDs and their associated points of intervention, such as a genetic, protein conformation, protein level, substrate level, and inflammatory response points of intervention.

1.4.2 Gene Therapy and Gene Editing

Within the pathological cascade of disease (Figure 1.6), GT and GE would be the earliest point of intervention, at the genetic level. GT utilizes viral vectors as a conduit to introduce the wild type gene into the cell, ultimately replacing the mutated gene (Haskins, 2009; Herzog, Cao, & Srivastava, 2010; Michael Hocquemiller, Giersch, Audrain, Parker, & Cartier, 2016), whereas GE also uses viral vectors to transport CRSPR/Cas9 editing machinery to the area of interest in order to modify or add genetic information (H.-C. Fan et al., 2018). GT strategy works by delivering new genes into the nucleus of the cell and relies on the cellular machinery to properly express these genes. The transcription and translation of the newly inserted gene leads to the functional protein expression and localization to the lysosome for the enzyme to fulfill its metabolic function (Haskins, 2009; Michael Hocquemiller et al., 2016) (Figure 1.6). Classically, GE works either to correct a genetic mutation at the site of mutation, however in order to get broad change to a single genetic mutation across an organ, let alone the body, nearly each cell would need to be infected with both the CRISPR guide RNA as well as the specialized Cas9 in order to provide therapeutic benefit ("ASGCT 22nd Annual Meeting Abstracts," 2019; H.-C. Fan et al., 2018; Ou et al., 2019). Very recently, there has been research done into using the CRISPR/Cas9 system to insert entire wild type genes (or hybrid therapeutic genes) into the intron space of a constitutively expressed gene (expressed under the albumin promoter), which would provide long term undiluted genetic change into the treated organism (Ou et al., 2019).

Many viral vectors have been used in preclinical GT assessments for LSDs, with a few even advancing to clinical trials (Haskins, 2009; Herzog et al., 2010; Michael Hocquemiller et al., 2016; Parenti, Andria, & Ballabio, 2015). Some of the viruses commonly used for these trials include lentivirus, adenovirus, adeno-associated virus, and herpes simplex virus (Nayerossadat, Maedeh, & Ali, 2012; Robbins & Ghivizzani, 1998; Thomas, Ehrhardt, & Kay, 2003). The lentivirus, herpes simplex virus, and the adenovirus have large packaging capacities, which is useful when treating diseases with large genes, but have been shown to illicit strong

immunological responses in the gene therapy recipient (Chirmule et al., 1999; Thomas et al., 2003). These immunological responses included macrophage and microglial recruitment, which produces an inflammatory response to the treatment vector (Chirmule et al., 1999; Thomas et al., 2003). AAVs have been shown to be less immunogenic, however they are restrictive in their nucleic packaging capacity, as compared to the aforementioned viruses (Michael Hocquemiller et al., 2016; Thomas et al., 2003; Z. Wu, Yang, & Colosi, 2010b). Nonetheless, each of these viruses has proved to be successful in the search for gene therapy treatments for LSDs.

One advantage to GT is the capability of cellular cross correction (Arfi et al., 2005; Fratantoni, Hall, & Neufeld, 1968; Neufeld, 2011). It has been shown that soluble hydrolases tagged with M6P are capable of being secreted from the cell it was produced in and can travel to surrounding cells (Arfi et al., 2005; Fratantoni et al., 1968; Neufeld, 2011). Once in the neighbouring cell, the hydrolase proteins can localize to the lysosomes where it can break down its substrate. This process is called cross correction (Arfi et al., 2005; Fratantoni et al., 1968; Neufeld, 2011). GT often results in high levels of local protein expression, which is beneficial for surrounding cells that may benefit from cross correction (Platt, 2018). Another advantage GT treatments hold is the potential to have long lasting therapeutic effects with only one treatment dose(Parenti et al., 2013; Platt, 2018). The potential risk for oncogenic occurrences, due to the viral vector used in the therapy, is one drawback of GT (Donsante et al., 2001; Hacein-Bey-Abina et al., 2008; Walia et al., 2015). Another potential drawback to this therapy is that GT for the LSDs would need to be specialized to the particular disease (Platt, 2018). A single gene would not be able to treat all lysosomal storage disorders, thereby requiring the use of separate gene therapy treatments for each LSDs. (For instance, treating Pompe Disease, caused by a mutation in the *GAA*, with the *HEX* genes wouldn't work, the *HEX* genes would be used to treat Tay-Sachs and Sandhoff disease). However, the advantages of using GT for the treatment of LSDs outweigh the drawbacks to the therapy.

1.4.3 Small Molecular Chaperones and Proteostasis Regulators

Slightly downstream from where GT can be used to intervene at the genetic level, small molecule chaperones and proteostasis regulators intervene at the protein conformational level of the LSD pathology (Figure 1.6). Small molecule chaperone drugs work on the lysosomal proteins while they are undergoing post-translational modification in the endoplasmic reticulum of the cell (Banning, Gülec, Rouvinen, Gray, & Tikkanen, 2016; Maegawa et al., 2007; Parenti, 2009; Parenti, Andria, & Valenzano, 2015; Parenti, Fecarotta, et al., 2014; Parenti, Moracci, Fecarotta, & Andria, 2014). Normally, dysfunctional protein can be flagged and subsequently degraded, however these chaperone drugs circumvent this by helping the malfunctional lysosomal proteins fold into their proper conformation. These small molecule chaperones are pharmacological drugs that act as competitive inhibitors to the substrate (Parenti, Moracci, et al., 2014; Platt, 2018), meaning that the drug enters the active site of the protein and stabilizes both the protein and the catalytic active site. Once the enzyme is in the lysosome and encounters the substrate it is required to metabolize, the drug must then leave the active site, allowing the main substrate entry. This means that the affinity of the pharmacological chaperone to the active site must be high enough to elicit the stabilization and conformational changes required to retain enzymatic activity, but the affinity must remain lower than the affinity of the natural substrate (J.-Q. Fan, 2007; J.-Q. Fan, Ishii, Asano, & Suzuki, 1999; Platt, 2018). Conversely, in order to decrease the association of a drug acting in the active site of the lysosomal protein, drug libraries are being screened for possible allosteric modulators, where the potential allosteric modulator would stabilize the enzymatic structure, by binding somewhere other than the active site of the protein (Parenti et al., 2013; Platt, 2018).

Proteostasis regulators are pharmaceutics that increase the production of endogenous heat shock proteins, which in turn act as protein chaperones, and aid in protein folding (Platt, 2018; Calamini et al., 2011; Kirkegaard et al., 2016; Petersen & Kirkegaard, 2010; Kirkegaard et al., 2010). Specifically, the upregulation of the heat shock protein 70 (HSP70) has been linked to

assisting proper protein folding during post-translational modifications and has also been shown to interact with bis(monoacylglycero)phosphate (BMP) (Kirkegaard et al., 2010; Platt, 2018; Sandhoff, 2016). BMP is a lipid component in the lysosomal membrane, whose presence facilitates the breakdown of sphingolipid molecules (Platt, 2018). The drug Arimoclomol is one such proteostasis regulator that works to upregulate HSP70 in cells already under stress from the accumulation of the LSDs substrate, and has the potential to be used to treat multiple LSDs (Kirkegaard et al., 2016; Platt, 2018).

1.4.4 Enzyme Replacement and Hematopoietic Stem Cell Transplant Therapies

The third point of intervention in the LSD cascade is at the protein level (Figure 1.6). Enzyme replacement therapy (ERT) was one of the first successful therapies investigated for the treatment of a LSDs (Brady, 2006; Ries, 2017). In this therapy, the recombinant protein a patient is deficient in is administered, normally through intravenous injections (Brady, 2006; Platt, 2018). The protein is then absorbed into the cells of the body, where it can complete the catabolism of the accumulated substrates (Brady, 2006; Ries, 2017). This type of treatment is difficult to sustain, requiring multiple visits a week to medical practitioners for the ERT sessions (Platt, 2018). Although ERT can efficiently correct enzyme deficiencies in the peripheral body, a potential drawback is the inability of many full-sized enzymes to cross the blood brain barrier and enter the CNS (Brady, 2006; Platt, 2018; Ries, 2017). As many of the LSDs have peripheral organ and neurologic pathologies, finding a way to treat both systems will be crucial to the successful treatment of these diseases.

ERT remains a viable treatment option for those patients who produce some sort of partial mutated proteins, because the innate immune system has been tolerized to the enzyme administered through ERT (Gelder et al., 2015; Platt, 2018). However, patients who produce no enzyme whatsoever typically demonstrate a high immune response to the therapeutic protein (Gelder et al., 2015).

Another strategy to increase the amount of wild type enzyme available in the body for the treatment of LSDs includes the use of hematopoietic stem cell (HSC) transplants, through the transplantation of bone marrow or donor stem cells (Krivit, 2002; Krivit, Peters, & Shapiro, 1999; S. U. Walkley & Dobrenis, 1995). By administering HSCs to a patient, these cells will secrete the functioning enzyme into the bloodstream for systemic treatment. However, the issues with this therapy is the need to find a donor match, followed by the potential risk of transplanted tissue/cell rejection by the patient's immune system (Krivit, 2002; Krivit et al., 1999; Platt, 2018; S. U. Walkley & Dobrenis, 1995).

1.4.5 Substrate Reduction Therapy

Next, intervention at the substrate level in the LSD pathological cascade has shown effective (Figure 1.6). Substrate reduction therapy (SRT) aims at decreasing the biosynthesis of the substrates before they can accumulate to devastating levels in the cell (Coutinho, Santos, & Alves, 2016; Platt & Jeyakumar, 2008). Small molecule drugs that inhibit the anabolism of the substrates can help treat or even reverse the accumulation of the substrates, especially when the patient retains residual enzyme activity (Coutinho et al., 2016; Platt & Jeyakumar, 2008). The drugs currently approved being used for the SRT of glycosphingolipids that accumulate in LSDs are miglustat, migalastat and eliglustat (Andersson et al., 2004; Belmatoug et al., 2017; M. Jeyakumar et al., 2003; Platt et al., 1997). These drugs work by inhibiting the catalytic glucosylceramide synthase enzyme from forming the precursor backbone to all glycosphingolipids, glucosylceramide (Platt & Jeyakumar, 2008; Platt et al., 1997). By inhibiting this initial step in biosynthesis of glycosphingolipids, there is a reduced amount of glucosylceramide available to produce the more complex glycospingo-substrates (Platt & Jeyakumar, 2008).

1.4.6 Anti-inflammatory Therapies

One of the hallmarks of cellular dysfunction is marked by an increased immune response feeding into an increased inflammatory response (M. Jeyakumar et al., 2003; Mylvaganam Jeyakumar et al., 2004; Wada et al., 2000), which provides us with the last point of intervention (Figure 1.6). The pathogenesis of LSDs suggests that the inflammatory response that occurs post-substrate accumulation is also a potential target for therapy (M. Jeyakumar et al., 2003; Mylvaganam Jeyakumar et al., 2004; Wada et al., 2000; Williams et al., 2014). The use of anti-inflammatory drugs to treat LSDs have shown some success in alleviating some of the phenotypic and biological markers of the diseases, however, disease progression still occurred (M. Jeyakumar et al., 2003; Mylvaganam Jeyakumar et al., 2004; Wada et al., 2000). This shows that the use of anti-inflammatories alone is not enough, although if combined with another therapy may show beneficial synergistic effects.

1.4.7 Combinatorial Therapies - Polypharmacology

Polypharmacology is the use of multiple therapeutic or pharmaceutics to treat a particular disease at multiple target points (Platt, 2018). A prime example of polypharmacology arises from the use of ERT in tandem with small molecule chaperones (Porto et al., 2009, 2012) or anti-inflammatories (Mylvaganam Jeyakumar et al., 2004). In addition to stabilizing any mutated enzymes that retain some residual activity, the small molecule chaperones have been shown to naturally increase the efficiency with which the enzymes from ERT catabolize their respective substrates (Porto et al., 2009, 2012). Combining SRT and anti-inflammatory drugs (Williams et al., 2014), or theoretically combining SRT and GT or GE could also lead to beneficial outcomes for many LSDs. In an effort to treat these complex diseases that affect multiple bodily systems and compartments, a successful course of treatment will likely entail the combination of two or more of the aforementioned therapies.

1.4.8 Clinical Trials for the Treatment of GM2 Gangliosidosis

GM2 Gangliosidosis is a group of neurodegenerative LSDs whereby mutations in any of the hexosaminidase complex genes (*HEXA*, *HEXB* or the *GM2AP*), produce dysfunctional α-subunit, β-subunit, and the GM2 activator protein products, respectively, leading to Tay Sachs disease (TSD), Sandhoff disease (SD), or the AB-Variant (Sandhoff & Harzer, 2013). These diseases are characterized by a dysfunctional hexosaminidase A (HexA) hydrolytic enzyme complex, normally composed of the GM2 activator protein, α- and β- subunits (Sandhoff & Harzer, 2013). Dysfunction in HexA leads to the accumulation and storage of the GM2 ganglioside substrate (Sandhoff & Harzer, 2013). Clinically, the symptoms of all three GM2 Gangliosidosis diseases are indistinguishable. Symptoms typically include motor weakness, ataxia, spastic movements, loss of motor control, macular red-cherry spot, seizures, and paralysis, culminating in the death of the patient (Sandhoff & Harzer, 2013).

In the investigation into successful treatments for GM2 Gangliosidosis, intravenous ERT was attempted, but as the recombinant HexA protein was unable to cross the blood-brain barrier, there was no clinical improvement observed (Johnson et al., 1973). This led to further investigation into alternative therapies. In recent clinical trials, pyrimethamine, a small molecule chaperone drug is being used for the treatment of both TS and SD, which have shown some success (Chiricozzi & Niemir, 2013; Maegawa et al., 2007). Currently, pre-clinical GT trials are underway utilizing animal models of GM2 Gangliosidosis (Bradbury et al., 2013; Osmon et al., 2016; Tropak et al., 2016a; Walia et al., 2015). These GT trials have had some success in prolonging the life of murine SD and ovine TSD models (Gray-Edwards et al., 2017; Osmon et al., 2016).

1.4.9 Conclusion

LSDs are a collection of substrate storage disorders stemming from a non- or dys-functional lysosomal protein. There are a variety of treatment options for LSDs, however the most beneficial outcomes will likely arise form polypharmacological treatments that provide the necessary supportive treatment to both the peripheral organs, as well as to the CNS (Platt, 2018). Although there has been a collection of approved treatments for a handful of LSDs, there is a long road ahead of researchers and clinicians for the successful treatment of LSDs.

1.5 Animal Models of GM2 Gangliosidosis

GM2 Gangliosidosis has been naturally identified in a variety of animals. The three main animal models of GM2 Gangliosidosis are the ovine, feline, and canine models. The canine model of SD has been discovered in breeds; however this model has not been fully characterized and therefore haven't been used in the search for a therapeutic (Cummings, Wood, Walkley, Lahunta, & DeForest, 1985; Tamura et al., 2010; Yamato et al., 2002). The ovine model is a model of TSD and is occasionally used for the preclinical assessment of treatments for GM2 Gangliosidosis (Porter et al., 2011; Torres et al., 2010; Wessels et al., 2014). The feline model of SD have been fully characterized and is often used in preclinical research working towards a therapy for GM2 Gangliosidosis (Cork, Munnell, & Lorenz, 1978; Cork et al., 1977; Martin et al., 2004; McCurdy et al., 2015; Muldoon, Neuwelt, Pagel, & Weiss, 1994; Neuwelt et al., 1985; Rattazzi, Appel, & Baker, 1982; Steven U. Walkley, 1982).

The three animal models for GM2 Gangliosidosis discussed above are solely large animal models, for which the maintenance, husbandry, and breeding can be quite expensive and require much more space than smaller rodent models. The transgenic TSD and SD mouse models were produced in order to provide researchers with a more cost-effective and manageable animal model for pre-clinical research (Phaneuf et al., 1996; Sango et al., 1995).

1.5.1 Murine Models of GM2 Gangliosidosis

Sango et al. described the TSD and SD murine mouse models in 1995. The transgenic SD mouse model was created by the insertion of the MC1NeopolyA cassette into exon 13 of the murine *Hexb* gene (Sango et al., 1995). The production of chimeric mice that transmit the *Hexb* mutation through the germline was done by inserting the mutated exonal construct into murine embryonic stem cells, allow for homologous recombination and select only the positive clones for insertion into a mouse blastocyst (Sango et al., 1995).

TSD mice, caused by a mutation in the murine *Hexa* gene, do not produce minimal human TSD-like symptoms, due to the redundancy in their proteome that allowed for an alternative sialidase pathway for the catabolism of GM2 (Phaneuf et al., 1996; Sango et al., 1995; Tifft & Proia, 1997) (this sialidase pathway will be further discussed below in Section 1.5.1.2). These TSD mice still store a reduced level of GM2, but these levels are not significant enough to functionally impair the mice or impact survival (Phaneuf et al., 1996; Sango et al., 1995; Tifft & Proia, 1997). Unlike the TSD mouse model, the SD mouse model produced by the exon 13 mutation in the *hexb* gene develop human SD-like motor symptoms (motor tremors, ataxia, spasticity, muscular rigidity) and have a significantly short life-expectancy (15-18 weeks) all due to the neurotoxic accumulation of GM2 in the murine CNS (J.-Q. Huang et al., 1997; Phaneuf et al., 1996; Sango et al., 1995; Tifft & Proia, 1997). Therefore the SD mouse model is an ideal animal model with which to conduct pre-clinical research towards an effective treatment for GM2 Gangliosidosis (Andersson et al., 2004; Arfi et al., 2005; Cachón-González et al., 2006; Cachón-González, Wang, Ziegler, Cheng, & Cox, 2014; Osmon et al., 2016; Walia et al., 2015; Woodley et al., 2019). However, the SD mouse model retains approximately 2% of the HexA activity found in a normal mouse (Sango et al., 1995). The buildup of GM2 begins accumulating at birth, but the symptom presentation is delayed until roughly 12 weeks, by which time sufficient GM2 has accumulated to illicit enough neurodegeneration to affect motor control (Phaneuf et al., 1996; Sango et al., 1995). The neurodegeneration and symptoms worsen over the following few weeks and mice reach their humane endpoint around 16 weeks of age. After symptom onset, the disease course is naturally fatal to the mice within 6 weeks (Phaneuf et al., 1996; Sango et al., 1995).

As the molecular mechanisms underlying the pathogenicity of GM2 Gangliosidosis have yet to be fully elucidated, further investigation into the altered proteomics in the SD mouse model have shown that astrogliosis and microgliosis also play a part in the pathogenesis of SD in mice (Abo-Ouf et al., 2013; Hooper & Igdoura, 2016; Kyrkanides et al., 2012). Additionally, changes

to neuronal synaptic proteins and receptors may also contribute to the degeneration of the neurons in SD (Hooper et al., 2017).

1.5.1.1 Behavioural Characterization of the SD Mouse Model

Behaviourally, the SD mice begin exhibiting a decline in motor performance at approximately 12 weeks of age, where a progressive decline in coordination and general locomotion are observed until their humane endpoint (Sango et al., 1995). When measuring general locomotion, it's been noted that untreated SD *Hexb*$^{-/-}$ mice move significantly less than normal littermates (Walia et al., 2015). The SD *Hexb*$^{-/-}$ mice also show significant impairments in coordination compared to normal heterozygote controls (Kyrkanides, Miller, Brouxhon, Olschowka, & Federoff, 2005; Sango et al., 1995).

1.5.1.2 Disparity in the Catabolism of GM2 Gangliosides - The Alternative Murine Sialidase Pathway

Upon the creation of the murine models for TSD and SD, a discrepancy in the expected phenotypes was observed. The murine model for TSD did not present with any TSD-like symptoms and biochemically stored very little GM2 as compared to the SD model, which developed severe symptoms and GM2 accumulation consistent with the human SD (Phaneuf et al., 1996; Sango et al., 1995; Tifft & Proia, 1997). This discrepancy is due to a unique sialidase pathway in the mice that can degrade GM2 to GA2 ganglioside, where GA2 can be further catabolized by the murine HexB β-subunit homodimer into the lactosylceramide (LacCer; see Figure 1.7) (Phaneuf et al., 1996; Sango et al., 1995; Tifft & Proia, 1997). This alternative pathway circumvents the need for the HexA enzyme in the mice. This alternative catabolism of GM2 was confirmed by the elevated levels of both GM2 and GA2 found in the brains of the SD mouse model (Sango et al., 1995).

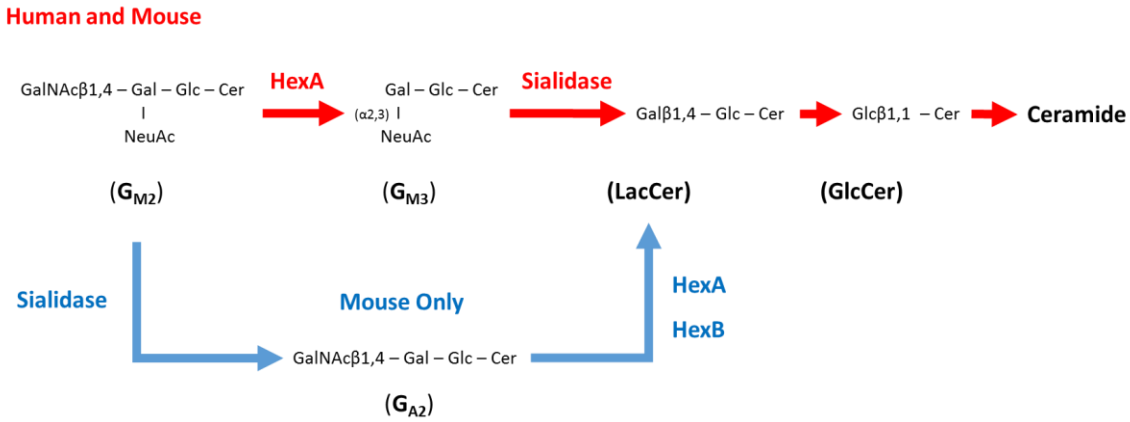

Figure 1.7 Differential catabolism of GM2 Gangliosides between Humans and Mice.

Figure from (Suzuki, Proia, & Suzuki, 1998).

1.6 Gene Therapy

In the search for a viable long-lasting treatment, many therapies have been assessed with varying success. Gene Therapy (GT) is a promising treatment strategy that utilizes transgenes packaged into viral vectors to facilitate the delivery of genetic information into affected tissues in order to restore proper cellular functioning through transgene expression (Figure 1.8). GT works best when treating monogenic recessive diseases due to the requirement of replacing a single wild type (WT) gene as opposed to polygenic or dominant genetic disorders, where more than one gene or treatment strategy would be required to fully treat the diseases. In addition, GT could potentially be a one-time treatment for these diseases due to it's long-lasting effects.

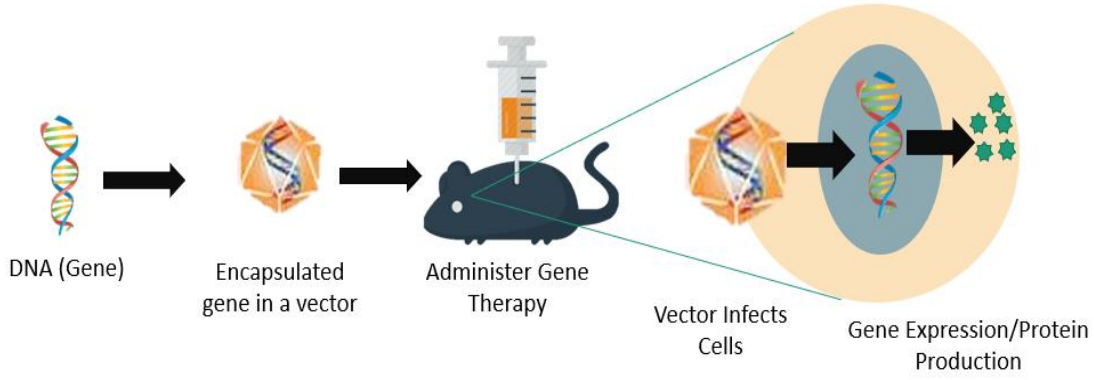

Figure 1.8 Gene Therapy Conceptual Outline

A gene is encapsulated into a viral vector and delivered to an organism in order for the virus to infect the cells of the target organ. This leads to the expression and protein production of the treatment construct.

1.6.1 Viral Vectors used in Gene Therapy

A variety of viral vectors have been used in both pre-clinical and clinical GT trials over the last few decades. As technology advanced, so did the use of different types of viruses. There are two main types of viruses used in GT studies, enveloped viruses whose entry into the target cell is mediated by the fusion of the viral capsule-membrane with the cellular phospholipid membrane (Plemper, 2011), whereas the non-enveloped viruses rely on receptor mediated entry into the cell (Bajaj, Dey, Bhukar, Kumar, & Banerjee, 2016). Each type of virus possesses a distinct combination of limitations and advantages (Thomas et al., 2003) (Table 1.1).

Of the enveloped viruses, the herpes simplex virus (HSV) contains a double stranded DNA genome and has the largest packaging capacity from all the viruses used in GT studies. HSV has a strong neuronal tropism, and remains episomal, however it also produces a high inflammatory response and may not be useful for the treatment of any other cell type other than neuronal. The genomes of the lentiviral and retroviral vectors are composed of RNA, their packaging capacity is 8kb, and although they both have a low inflammatory profile after administration, both of these viruses integrate into the genome and therefore have oncogenic potential. The main difference in utility between the lentivirus and the retrovirus arises in their differential tropism, where retroviral particles only successfully infect dividing cells, where the lentivirus infects a broad range of cell types at any mitotic stage.

The non-enveloped viruses include the adenovirus and the adeno-associated virus. The adenovirus is another double stranded DNA virus whose 8kb genome remains episomal post-infection. Although the adenovirus is adept at infecting most tissue types, the high inflammatory response elicited post-administration is extremely problematic (Thomas et al., 2003). Alternatively, AAVs can successfully infect both quiescent and dividing cells, while maintaining sustained expression in each (Büning, Perabo, Coutelle, Quadt-Humme, & Hallek, 2008; Chirmule et al., 1999; Foust et al., 2009; Thomas et al., 2003). The best characterized AAV is serotype 2, which has been shown to infect neurons when injected intracranially, but it cannot

cross the BBB like AAV9 (Duque et al., 2009; Foust et al., 2009; McCown, 2005). Additionally, a strong immunogenic response against AAV2 is observed in the majority of the human population, thus making the AAV2 an unsuitable vector for GT treatments (Blacklow, Hoggan, & Rowe, 1968; Blacklow et al., 1971; Boutin et al., 2010; Chirmule et al., 1999). Further discussion about the AAV virus can be found below in section 1.6.1.1.

Virus	Genome	Packaging Capacity	Tropism	Inflammatory Response	Episomal/ Integrated Genome	Disadvantages	Advantages
Enveloped							
Herpes Simplex Virus (HSV)	dsDNA	40kb	Strong neuronal tropism	High	Episomal	Inflammatory response; low transgene expression in non-neuronal cells	Large packaging capacity; strong neuronal tropism
Lentivirus	RNA	8kb	Broad	Low	Integrated	Possibly oncogenic due to integration into host genome	Continuous transgene expression in most cells
Retrovirus	RNA	8kb	Dividing cells	Low	Integrated	Infects only dividing cells; possibly oncogenic due to integration into host genome	Continuous transgene expression in infected (dividing) cells only
Non-Enveloped							
Adenovirus	dsDNA	8kb	Broad	High	Episomal	Potent Inflammatory response	Very efficient transduction of most tissues
Single-stranded Adeno-associated Virus (ssAAV)	ssDNA	<5kb	Broad, but serotype specific	Low	Episomal (90%) Integrated (10%)	Limited packaging capacity; hepatotoxicity	Non-inflammatory; non-pathogenic; serotype specific tropism
Self-complementary Adeno-associated Virus (scAAV)	dsDNA	<2.5kb	Broad, but serotype specific	Low	Episomal (90%) Integrated (10%)	Limited packaging capacity; hepatotoxicity	Same as ssAAV with the addition of quicker transgene expression post-treatment

Table 1.1 Viral Vectors used in Gene Therapy

Overview of the characteristics of the viral vectors used in gene therapy studies. Table apadted from (Thomas et al., 2003).

1.6.1.1 Adeno-Associated Viruses

AAV viral vectors are abundant in nature and were discovered as a contaminant to adenoviral vectors (Atchison, Casto, & Hammon, 1965; Bowles, Rabinowitz, & Samulski, 2006). The AAV is a non-pathogenic helper parvovirus that is replication deficient without the co-infection of an adenovirus (Bowles et al., 2006). The infection profile of the wild type AAV includes both a lytic and latent infection (Daya & Berns, 2008). The lytic infection occurs in the presence of adenoviruses, where both viruses are replicated and released to further infect the surrounding area, whereas the latent infection occurs in the absence of an adenovirus, where the wild type AAV genome is typically inserted into the adeno-associated integration site of chromosome 19 (Daya & Berns, 2008; Kotin, Linden, & Berns, 1992; Linden, Ward, Giraud, Winocour, & Berns, 1996; Rossi & Salvetti, 2016; Weitzman, Kyöstiö, Kotin, & Owens, 1994). The wild type AAV genome consists of inverted terminal repeats (ITRs) flanking the replication and capsid genes (Agbandje-McKenna & Kleinschmidt, 2011; Büning et al., 2008; Naso, Tomkowicz, Perry, & Strohl, 2017) (*rep* and *cap*, respectively; see Figure 1.9). However, the recombinant AAVs (rAAV) used in GT and GE research are replication deficient, due to the removal of the *rep* and *cap* genes, where these genes are then replaced by the elements comprising the GT treatment construct, such as a promoter, transgene, and enhancer elements (Büning et al., 2008; Lykken, Shyng, Edwards, Rozenberg, & Gray, 2018; Naso et al., 2017) (Figure 1.9). Although the wild type AAV genome preferentially inserts in chromosome 19, the recombinant AAVs used in GT trials show a decrease in insertions at this adeno-associated integration site, but have a broader spectrum of insertion across the genome (Hüser, Gogol-Döring, Chen, & Heilbronn, 2014; Hüser et al., 2010; Janovitz et al., 2013; Rossi & Salvetti, 2016). Although a small percentage of the AAV viruses used in GT studies integrate, the majority remain as extrachromosomal episomes and therefore lack the ability to replicate (McCown, 2005; Thomas et al., 2003). The inability of the episomal treatment to replicate indicates that the therapeutic levels aimed for by the study will persist if the infected cells do not divide and that

their transcriptional machinery remains active. However, the therapeutic benefit to the GT treatment can be lost due to immunological clearance or silencing of the transgene, promoter silencing, or if the infected cells divide (diluting the vector genomes by half with each mitotic division).

In order to make these replication deficient rAAV viral vectors for use in GT and GE, a triple co-transfection is required using the plasmid encoding the treatment construct, an AAV virus encoding the *rep* and *cap* sequences for the chosen serotype of interest, as well as a helper virus (such as the adenovirus) (Bowles et al., 2006; Naso et al., 2017). With this co-infection, the therapeutic genetic construct is encapsulated in the chosen rAAV serotype(Bowles et al., 2006; Büning et al., 2008; Naso et al., 2017). Serotypes will be further discussed in Section 1.6.1.1.2.

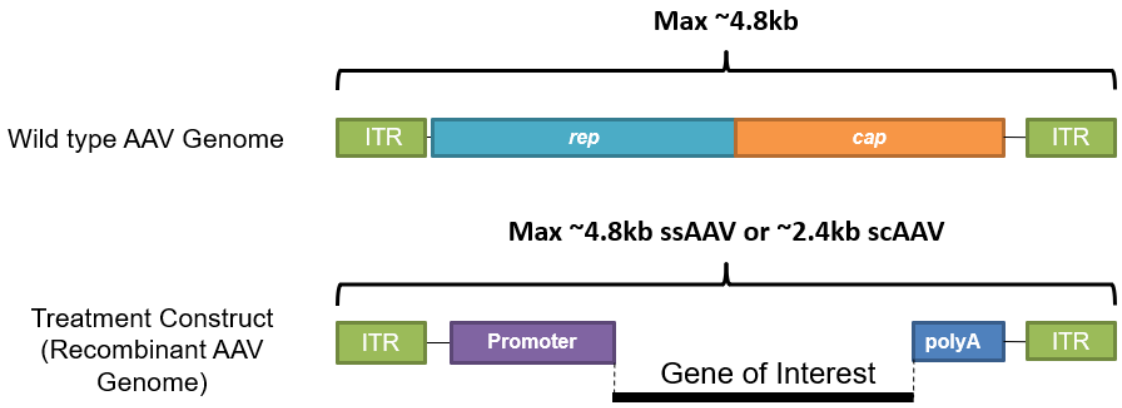

Figure 1.9 Wild Type and Treatment Construct AAV Genomes

Figure adapted from (Büning et al., 2008; Naso et al., 2017)Wild type AAV genomes consist solely of the rep and cap genes, which code for the replication and capsid proteins, allowing for further viral particle production and eventual spread as the cell infected cell undergoes apoptosis. The recombinant AAV genome has the rep and cap genes replaced with the promoter, transgene, and regulatory elements of a treatment construct.

1.6.1.1.1 Single Stranded and Self-complementary Adeno-Associated Viruses

Due to the small size of the AAV, only a finite amount of genetic information can fit within vector (Grieger & Samulski, 2005; J. Wu et al., 2007; Z. Wu et al., 2010b). The maximum length of nucleotides that can be encapsulated by the AAV capsid is ~4.8kb (Grieger & Samulski, 2005; McCarty, 2008; J. Wu et al., 2007; Z. Wu et al., 2010b). The single stranded (ss) AAV genome is one continuous strand of DNA that spans the entire treatment construct from ITR to ITR (Naso et al., 2017; Z. Wu et al., 2010a). During AAV production, the ss treatment construct genome is replicated through normal DNA synthesis, but both the 'plus' and 'minus' strands of the treatment construct are equally packaged within the chosen serotype (Berns, 1990; Berns & Muzyczka, 2017; McCarty, 2008). However, once the target cells have been infected, these single strand genomes must be converted to double stranded DNA within the host cell in order for the therapeutic genes to be expressed (McCarty, 2008). This conversion of ss to double stranded DNA takes time and delays the production of the therapeutic protein. In some cases of infection, if the ssAAV that contained the plus strand of the therapeutic construct infected the same cell as a ssAAV that packaged the minus stand, strand annealing can occur, which negates the need for second strand synthesis (Berns, 1990; Nakai, Storm, & Kay, 2000). Yet, this co-infection is random and therefore cannot be relied upon to provide an effective increase in treatment efficiency.

The second type of AAV genome is the self-complementary genome (scAAV), that exploits the formation of dimeric inverted repeat genomes during DNA replication (Carter & Rose, 1972; McCarty, 2008; McCarty, Monahan, & Samulski, 2001). The length of these complete dimers must be below ~4.8 kb in total because the dimers can fold and form the fully dsDNA required for protein production (McCarty, 2008; J. Wu et al., 2007). Therefore the therapeutic construct must be below ~2.4kb in length (McCarty, 2008; J. Wu et al., 2007). scAAVs provide more efficient and persistent transgene expression than the ssAAV expression attributable to the ability of these sc genomes to begin therapeutic protein expression without

having to create a second strand for these genomes (Gonçalves, 2005; McCarty, 2008; T. Wu et al., 2012).

1.6.1.1.2 Adeno-Associated Virus Serotype 9

Capsid structure determines the serotype of the AAV, and thereby contributes to the tropism of that specific AAV (Lykken et al., 2018; Murlidharan, Samulski, & Asokan, 2014; Zincarelli, Soltys, Rengo, & Rabinowitz, 2008). The tropism of the different serotypes can be altered by directed evolution of the capsids (Gray et al., 2010). The AAV serotype 9 effectively crosses the BBB and transfects both neurons and astrocytes, making AAV9 one of the most efficient vectors for the treatment of the CNS diseases (Cearley et al., 2008; Dayton, Wang, & Klein, 2012; Duque et al., 2009; Foust et al., 2009; Murlidharan et al., 2014). The primary receptor for the AAV9 serotype is N-terminal galactose (Dayton et al., 2012; Foust et al., 2009; Lykken et al., 2018; Murlidharan et al., 2014).

1.6.1.2 The Advantages and Drawbacks of AAV Gene Therapy for Central Nervous System Diseases

Various GT viral vectors have been assessed for their ability to treat the CNS disorders, and although many proved efficient at gene transfer, there are still major drawbacks associated with each virus to be considered. Some of these limitations to their use include the oncogenic potential of the retro- and lenti-viral vectors due to their integration into the host genome, as well as the potent immune response elicited by the adenoviral and HSV vectors (McCown, 2005; Thomas et al., 2003). AAVs are able to sustain transgene expression over long periods of time in both mitotic and post-mitotic cells, while seldomly integrating, and they rarely elicit strong inflammatory responses (Thomas et al., 2003). Both ss and sc AAV genomes typically remain as an extrachromosomal episome in the nucleus of the infected cells (Thomas et al., 2003). However, the episomal nature of the AAV genome may contribute to a dilution of the therapeutic treatment over time as the treated organism ages, where when the organism grows and cells

divide, an increasingly smaller percentage of the cells in the target organs contain the genetic treatment. Moreover, the tissue targeting tropisms of the different AAV serotypes combined with the efficiency of the non-mitotic selectivity of the AAV is a useful tool when selecting a potential vector for a therapeutic (Büning et al., 2008). AAV9 efficiently transduces the CNS, as it is readily capable of crossing the BBB when delivered intravenously, transfecting both neurons and glia (Duque et al., 2009; Foust et al., 2009). The large uptake, or 'sink' of the AAV viruses to the liver is a drawback to systemic AAV delivery (Chandler et al., 2015a; Daya & Berns, 2008; Donsante et al., 2001; McCown, 2005; Pulicherla et al., 2011), and leads to a diminished amount of viral particles available to cross the BBB. The resultant increased integration and, may be related, hepatocellular carcinomas that arise from the sink of the AAV to the liver has been noted when AAV treatments are administered to neonatal mice (Chandler et al., 2015a; Walia et al., 2015). The recent advances in AAV technology have enabled the creation and testing of chimeric AAV capsids combining aspects from multiple serotypes in order to achieve greater target specificity and efficiency (Asuri et al., 2012; Gray et al., 2010; McCown, 2005; Thomas et al., 2003). A variant of AAV serotype 9, the AAV9.47 capsid, was made to de-target the liver (Pulicherla et al., 2011), which can potentially offer a safer approach in the application of AAV9 to treat CNS disorders. In addition, there has been evidence to show promoter silencing and antibody responses that decrease the overall production of therapeutic protein over time. Despite this, AAVs have a favourable safety profile and track record in clinical trials.

1.6.2 Gene Therapy Treatments for GM2 Gangliosidosis

The different viruses presented above have each been utilized in the search for a viable GT treatment for GM2 Gangliosidosis. In comparison to the small packaging capacity of the AAV vectors, the packaging capacity of the lentivirus enabled the investigation into a bicistronic hexosaminidase treatment for GM2 Gangliosidosis (Kyrkanides et al., 2005). This study used the classic IRES linker as the bridge between the *HEXA* and *HEXB* genes, and found that with an

intraperitoneal neonatal administration of the bicistronic lentivirus yielded decreased levels of GM2 accumulation, better behavioural scores, a high HexA enzyme production, and the attenuation of any neuroinflammatory pathogenesis (Kyrkanides et al., 2005). The utility of the HSV virus was assessed in the TSD mouse model and demonstrated no adverse side effects for the 4 weeks following treatment administration (study endpoint; Martino et al., 2005)). Furthermore, adenoviral vectors have successfully treated the SD and TSD murine models with few if any adverse effects (Bourgoin et al., 2003; Guidotti et al., 1999). The lack of adverse side effects in many of these studies stems from the intracranial route of administration, where the therapeutic was directly injected into the brain, circumventing the peripheral immune system. Systemic administrations of the HSV and adenoviral viruses would elicit the classic inflammatory responses, and both lenti- and retro-viruses still may be oncogenic (Thomas et al., 2003).

Many of the recent GT studies have focused on the utilization of different serotypes of the AAV virus in order to exploit the advantages of the virus and the tropism specificity of the serotypes. Many studies have found success when treating GM2 Gangliosidosis with AAV serotypes 1, 2, 8 and 9 in animal models of SD (Bradbury et al., 2013; Cachón-González et al., 2006, 2012, 2014; Osmon et al., 2016; Sargeant et al., 2011; Walia et al., 2015; Woodley et al., 2019), however the majority of these studies entailed invasive intracranial therapeutic delivery.

1.6.3 Routes of Administration into the CNS

GT has been administered in a multitude of different methods, including intramuscularly, intravenously, intrathecally, as well as intracranially (Cardone, 2007; Michaël Hocquemiller, Giersch, Audrain, Parker, & Cartier, 2016; McCown, 2005; Murlidharan et al., 2014). Each route of administration has its advantages and drawbacks, especially when trying to find a suitable route of delivery for the CNS. For this reason, intramuscular or subcutaneous injections would be ill-advised due to the inability for the therapeutic to be moved or circulate to the CNS organs. Intrathecal and intracranial injections have been efficient at treating CNS diseases, but are highly

invasive (Cardone, 2007; Michaël Hocquemiller et al., 2016; McCown, 2005; Murlidharan et al., 2014). IV injections are systemic and can deliver a therapeutic to the CNS given that the vector selected is capable of traversing the blood-brain barrier in sufficient quantities (Murlidharan et al., 2014). This method is also the least invasive method available to traffic GT treatments into the CNS (Michaël Hocquemiller et al., 2016; McCown, 2005; Murlidharan et al., 2014). However, a systemic delivery of any viral vector can elicit an immunological response as well as has the chance of off-target side effects. Although more localized GT delivery would be ideal, the physical barriers that were evolutionarily designed to protect the most valued organ system prove formidable, and direct injections into the brain, spinal cord, or the cerebral spinal fluid require skill and are quite invasive (Cardone, 2007; Michaël Hocquemiller et al., 2016; McCown, 2005; Murlidharan et al., 2014).

1.6.4 Dosage Effects

Determining the correct dose of a therapeutic is a multifactorial process, where the type of therapeutic vector, the size and weight of the animal model, route of administration, timing of intervention, and the efficiency of infection all need to be carefully considered. Adenoviral vectors have been used in preclinical GT studies for GM2 Gangliosidosis with doses ranging between $4x10^6$ – $1.5x10^9$ vector genomes (vg) per mouse (vg/mouse) (Bourgoin et al., 2003; Guidotti et al., 1999), whereas lentiviral vectors have also been used at a dose of $5x10^6$ – $1x10^7$ vg/mouse (Kyrkanides et al., 2005). Another GT trial assessed the efficiency of doses of HSV between $2.5x10^6$ – $5x10^6$ plaque forming units per mouse (Martino et al., 2005). The dosages of AAV vector used in previous intracranial murine GT studies has ranged between $9.9\ x10^9$ – $4.7x10^{10}$ vg/mouse (Cachón-González et al., 2006, 2012, 2014; Sargeant et al., 2011). The intracranial AAV GT injections given to the feline SD mouse model ranged between $3x10^{10}$ – $4.2x10^{12}$ vg/cat (Bradbury et al., 2013). The systemic IV delivery of AAV vectors ranged between $2.04x10^{10}$ - $2.5x10^{11}$vg/mouse (Osmon et al., 2016; Walia et al., 2015; Woodley et al.,

2019). The challenge with systemic injections is that they require higher dosages of the GT intervention in order to obtain the correct infection rate in the target organs despite the systemic dispersal of the treatment.

1.6.5 Age of Delivery

It has long been thought that when dealing with any disease, earlier intervention is preferable and may be able to reverse or at least halt and maintain disease severity. This has been shown to the case for the treatment of SD in mice (Cachón-González et al., 2014; Walia et al., 2015). Previous studies have mainly focused on neonatal and adult IV delivery (Walia et al., 2015), or intracranial delivery at juvenile and adult ages (Cachón-González et al., 2014). Cachon-Gonzalez et. al (2014) investigated the latest possible therapeutic age of intracranial GT intervention by assessing at which point the treatment could no longer delay or reverse the pathogenesis of GM2 Gangliosidosis. Other intracranial treatments for LSDs have determined that earlier treatment in life has more beneficial outcomes than delaying administration (Cabrera-Salazar et al., 2007; Cachón-González et al., 2006, 2012). Lastly, systemic injections have also shown a benefit to having been treated at a neonatal age in comparison to waiting 6 weeks and treating the SD mice as adults (Walia et al., 2015).

1.6.6 Immunological Effects of Gene Therapy

The discovery of a suitable GT viral vector and treatment that efficiently infects and produces sufficient therapeutic proteins while maintaining minimal immunogenicity and toxicity is crucial for the successful treatment of these disorders. The safety profile of the viral vector is of utmost importance and will be widely investigated prior to clinical trials, since the oncogenesis of the retroviral GT vector used in previous clinical trials became apparent when the patients began developing leukemia-like symptoms (Hacein-Bey-Abina et al., 2008, 2003). Furthermore, the strong, acute, and ultimately fatal immunogenic response exhibited by Jesse Gelsinger after an IV adenoviral GT treatment has been a set back in the field of GT research (Somia & Verma, 2000).

1.6.6.1 Immunological Responses – Innate and Adaptive Immune Responses

The immune system is comprised of two main types of immunological responses (Chaplin, 2010; Hoebe, Janssen, & Beutler, 2004; Janeway Jr, Travers, Walport, & Shlomchik, 2001; Medzhitov & Janeway Jr, 1998). First, the innate immune system entails the physical barriers that protect the body from foreign pathogens, such as the skin and mucosal barriers, as well as the initial phagocytic and inflammatory properties of macrophages that recognize foreign pathogens and release general inflammatory signals (Chaplin, 2010; Janeway Jr et al., 2001). Phagocytic cells engulf the foreign pathogen, destroy it, and then present pieces of the pathogen on their cell surface (antigen-presenting) in order to inform the secondary level of the immune system (Chaplin, 2010; Hoebe et al., 2004; Janeway Jr et al., 2001). Whereas innate immunity is non-specific and essentially the initial line of defense for the body, the second level of the immune system, the adaptive immune system, is 'learned' or 'acquired' immunity targeting pathogens the body has previously encountered (Chaplin, 2010; Hoebe et al., 2004; Janeway Jr et al., 2001; Medzhitov & Janeway Jr, 1998). The adaptive immune response can be further subdivided into a humoral and a cellular immune response (Chaplin, 2010; Janeway Jr et al., 2001). Lymphocytes are the main cells responsible for adaptive immunity, and are further subdivided into B- and T- cells (Chaplin, 2010; Janeway Jr et al., 2001). The humoral immune response entails the recruitment of B-cells interacting with the antigen-presenting phagocytic cells from the innate immune system to produce antibodies towards pathogens that the body has previously encountered, as well as maintaining a 'long-term record' of the pathogens in the form of memory B-cells (Chaplin, 2010; Janeway Jr et al., 2001). The cellular immune response is mediated through a variety of T-cells. Upon activation, the natural killer T-cells (also known as cytotoxic T-cells) search the body for cells infected with a pathogen and induce apoptosis in them through the release of toxins, whereas helper T-cells secrete cytokines in order to attract other immune cells to the infected area (Chaplin, 2010; Janeway Jr et al., 2001).

In the case of GT, both the innate and adaptive levels of the immune system must be taken into account, where any virus known to cause human disease, such as the adenovirus, would surely elicit a strong inflammatory response from the innate immune system, triggering a strong adaptive response to the treatment and the cells already infected by the treatment once the adaptive immune system is fully tuned to the therapeutic viral construct and it's payload (Chaplin, 2010; Thomas et al., 2003).

1.6.6.2 Vector Safety Profile

The favourable safety profile of the AAV vectors make it an ideal candidate for use in a GT treatment. AAV genomes rarely integrate into the genome, reducing the chances of oncogenesis (Douglas M. McCarty, Young, & Samulski, 2004; Murlidharan et al., 2014; Thomas et al., 2003).

1.7 Ethical Views of Gene Therapy

Over the last few decades, researchers, clinicians, ethics boards, and legislative authorities have been investigating the use of gene therapy for the treatment of severe, debilitating diseases. Pre-clinical research has primarily been focused on the efficacy and safety of gene therapy treatments, and while clinical research, ethics boards and legislative authorities are also concerned about the feasibility of gene therapy in treating disorders, they are also concerned with the ethics and risk-benefit profiles dealing with gene therapy trials in humans. The first draft of the Declaration of Helsinki was introduced by the World Medical Association in 1964, outlining the ethical principles that would govern medical research using human participants ("WMA - The World Medical Association-Declaration of Helsinki," n.d.). Along with the extensive ethics regulations outlined in the International Ethical Guidelines for Health-related Research Involving Humans (2016) prepared by the Council for International Organizations of Medical Sciences and the World Health Organization ("International ethical guidelines for health-related research involving humans • COUNCIL FOR INTERNATIONAL

ORGANIZATIONS OF MEDICAL SCIENCES," n.d.), the latest version of the Declaration of Helsinki ("WMA - The World Medical Association-Declaration of Helsinki," n.d.), revised in 2008, remains the main sources for biomedical ethics used to govern clinical trials. These documents address the necessity for voluntary human participation in clinical research in order to spur progress in the field of medicine, but also outlines the need for medical research to minimize the risks to human participants. Under these documents, no clinical trial would gain approval from a research ethics committee if the benefits of the clinical research did not outweigh the associated risks ("International ethical guidelines for health-related research involving humans • COUNCIL FOR INTERNATIONAL ORGANIZATIONS OF MEDICAL SCIENCES," n.d.; "WMA - The World Medical Association-Declaration of Helsinki," n.d.). Gene therapy is no exception, where the benefits for the use of gene transfer must outweigh the risks of the treatment before being approved for clinical investigation in humans.

Gene therapy is a therapeutic in which diseases, such as neuropathological disorders, are treated by replacing a dysfunctional gene with a therapeutic (endogenous wildtype or hybrid) gene through viral vectors (Cotrim & Baum, 2008). Like any treatments, there are significant benefits and risks associated with every aspect of gene therapy treatment. However, there is an ongoing debate throughout the scientific community and society of whether the benefits of gene therapy outweigh the risks. On one side, it is argued that the **benefits associated with gene therapy treatments outweigh their risks** due to the possibility of replacing dysfunctional genes, eradicating diseases, and creating cures that were not previously possible. On the other hand, it is argued that **these benefits are not enough to outweigh the risks** associated with these treatments because of the possibility of changing the human gene pool and creating other detrimental disorders that previously never existed. For a **cohesive discussion of each viewpoint**, the pros and cons of medical research findings, societal, moral, and religious views of gene therapy treatments shall be discussed.

1.7.1 Do the benefits of gene therapy to treat disorders of the central nervous system outweigh the risks associated with applications of the technology?

The benefits of having gene therapy treatments for neuropathological disorders far outweigh the potential associated risks, as treating or potentially curing neuropathological disorders improves both the quality of life of the patient and their families, and the ability of medical professionals to aid patients. To illustrate this, the development of gene therapy can be compared to the historical development of other types of therapies, such as blood transfusion technology. In the early 1900s, blood transfusions were administered to individuals, without prior knowledge of crucial information, such as blood types or rhesus factors; which were discovered after post-inflammatory complications (Giangrande, 2000). However, the benefits of having the option to save lives through the use of blood transfusions outweighed the costs of allowing thousands die during the great wars (Giangrande, 2000). Furthermore, with the advancements in medical sciences over the last century, blood transfusions have now become a routine medical procedure (Giangrande, 2000). Similar to blood transfusions, with the recent advances in the fields of virology, neuroscience, and medicine, gene therapy is currently on its way to becoming a potential routine procedure for genetic diseases.

The ability to devise and implement a treatment strategy for diseases that have had no successful treatments is the paramount benefit to the application and use of gene therapy. Clinical gene therapy trials for disorders of the central nervous system (CNS), including many lysosomal storage disorders, Alzheimer's Disease, and Parkinson's Disease, are currently being conducted (Piguet, Alves, & Cartier, 2017). The results from these clinical trials will demonstrate the feasibility of gene therapy use for the treatment of neuropathological disorders. Furthermore, the propensity for each of these clinical trials to produce favourable treatment outcomes for these neurological disorders far outweighs the potential medical risks associated with gene therapy. In the case of treating severe or fatal CNS disorders, the extension of a life at the risk of potential oncogenic, immunological, or undetermined side effects prevails, since the alternative for the

patient is either severe neurological impairments or ultimately death ("Gene Therapy Benefits and Potential Risks · Guardian Liberty Voice," 2014; Kimmelman, 2005, 2008; Naldini, 2015; Piguet et al., 2017; Robillard, Roskams-Edris, Kuzeljevic, & Illes, 2014; L. Walters, 1986; LeRoy Walters & Palmer, 1997). In addition, the foreknowledge that there is increased potential for adverse side effects allows medical professionals to closely monitor the patient volunteers in a gene therapy trial for hallmarks of cancer or a heightened immune response and intervene as soon as possible to manage these deleterious side effects.

Advancements in immunology and oncology have granted clinicians the capability to cope with the known detrimental side effects of gene therapy, such as oncolytic tumour formation or immunologic responses. These side effects can be managed through medical interventions, such as chemotherapy (Hacein-Bey-Abina et al., 2008) or immunosuppressants (Arruda, Favaro, & Finn, 2009), respectively, whereas in the majority of cases, the original disease treated by gene therapy could not be medically treated (Kimmelman, 2008; Naldini, 2015; Piguet et al., 2017). In addition, the heightened risk or potential for adverse effects to occur to a patient does not necessarily equate to these side effects occurring in every single patient of a clinical trial.

In some ways, gene therapy holds just as many detrimental side effects as other modern-day medical procedures. Although gene therapy treatments have been shown to promote an array of side effects, many of the side effects are reminiscent of those observed in organ transplant patients, where without proper immunological intervention, the innate immune response towards the foreign transplanted organ forces the body to reject the transplant ("Potential risks of Transplant Surgery—UC Davis Health System Transplant Center," n.d.). The transplant patient is also at risk for developing cancer or contracting other viruses from the donor organ ("Potential risks of Transplant Surgery—UC Davis Health System Transplant Center," n.d.). This is analogous to the innate immune response a patient may exhibit to either the viral vector, or the transgene used in gene therapy. However, the use of anti-inflammatories (Arruda et al., 2009)

and/or specialized non-immunogenic viruses, such as adeno-associated viruses or newly synthesized viral capsids (Thomas et al., 2003), can negate the issue of immune enabled rejection of the gene therapy treatment.

Another benefit to having gene therapy as a potential treatment for CNS disorders is the ability for clinicians to provide at least one treatment option to a patient, where none previously existed (Noguchi, 2003). Especially where pediatric patients are concerned, parents and the legal guardians of patients would prefer to be given the option to risk a treatment, despite the adverse side effects, as opposed to having no option to aid their child (Noguchi, 2003). This view in other words is that it is better to try something, than to not try anything at all. In addition, it has been considered to be more ethical to provide gene therapy treatments to a paediatric patient born with a fatal or severe disease, than it would be to electively abort the pregnancy, since the life of the child is preserved by the use of gene therapy (Post, 1991; LeRoy Walters & Palmer, 1997).

Moreover, gene therapy has the theoretical capability of treating, and possibly curing a neurological disorder with a one-time treatment. The single dose of gene therapy could potentially negate the lifelong use of pharmaceutics and medical care in many of these patients, vastly increasing the quality of life of a patient, as well as those of their families who accompany them to their treatments (Kimmelman, 2008). This one-time therapeutic intervention, although costly, may also have powerful economic benefits, where the health care system may no longer be burdened by the cost to care for patients with prolonged neurological diseases.

An additional benefit to using gene therapy to treat CNS disorders relates to the unique immune privileges experienced by the CNS (Carson, Doose, Melchior, Schmid, & Ploix, 2006; Louveau, Harris, & Kipnis, 2015; Piguet et al., 2017). As the majority of gene therapy trials for CNS disorders centre around treatment administration directly into the immune privileged CNS (NCT02725580; NCT00087789; NCT01973543) (Piguet et al., 2017), there is a reduced risk for immunological complications for these treatments as well as off target gene therapy effects.

However, some research is being conducted where instead of an invasive intraparenchymal or intrathecal route of administration, the treatment is administered intravenously ("Gene Transfer Clinical Trial for Spinal Muscular Atrophy Type 1—Full Text View—ClinicalTrials.gov," n.d.; "Phase I/II Gene Transfer Clinical Trial of scAAV9.U1a.hSGSH - Full Text View—ClinicalTrials.gov," n.d.). This intravenous administration may lead to off target gene therapy transduction. Therefore, the possibility of inadvertently transducing the germline cells must be considered while providing intravenous gene therapy treatment for CNS disorders. While between the 1980s and 1990s, the majority of legislation, government organizations, professional organizations and religious bodies supported the use of gene therapy to treat the somatic cells for hereditary diseases, there was not unanimous support for germline gene therapy (Leroy Walters, 1991). Some now argue that the likelihood of significant changes to the germline while undergoing gene therapy, where these alterations affecting future generations, could be equated to the amount of germline cells being genetically altered through exposure to radiation and transposons over a lifetime (Jr, 1999; Kimmelman, 2008). In addition, the utility of gene therapy to treat the disease in the present, and deal with the future side effects is also what is seen with other modern day medical procedures (Kimmelman, 2008). However, the prevention of devastating neurological diseases through gene therapy of germline cells, potentially before they become a burden to the patient, family, medicine and society, could potentially be a benefit for the use of gene therapy.

Societal views on medical treatments have evolved over the last few decades. With the advent of technological advancements accessible to the majority of the population, such as the internet, many of the fears once associated with gene therapy have been assuaged through self-education about how gene therapy works, and what it can be used for (Robillard et al., 2013). A recent survey published in 2014 has shown that over 90% of respondents are in support of gene therapy use for the treatment of severe or fatal neuropathological disorders, such as Alzheimer's

Disease, where a lower percentage of respondents, pulled form adults in the general population, felt gene therapy a suitable potential therapy for less severe neurological disorders, such as attention deficit hyperactivity disorder (79%) (Robillard et al., 2014). This data is consistent with the perceptions towards gene therapy two decades ago, where 75% of those surveyed supported gene therapy use in the treatment of disorders in order to save lives and improve quality of life (Macer et al., 1995). In the recent survey, over 70% of respondents agreed that gene therapy will be beneficial to society as it will reduce suffering and increase the health of the population, and in the future gene therapy will be used to provide a cure for a large number of diseases (Robillard et al., 2014).

From a religious standpoint, gene therapy has classically been viewed as medical professionals attempting to 'play God', by altering and manipulating nucleic information for the treatment of diseases ("Moral and Ethical Issues in Gene Therapy | Society, Religion and Technology Project," n.d.). A small percentage (17%) of the population currently abides by their religious beliefs opposing gene therapy (Robillard et al., 2014). However, philosophically, whatever deity has granted us life has also enabled humans to learn, discover and create in order to facilitate their lives. Would it be remiss for clinicians and researchers to not pursue scientific and medical innovations in gene therapy, when said deity allowed humans the capacity to imagine and create such a treatment for the betterment of a fellow human? In addition, since most religions acknowledge life to be precious, it stands to reason that this belief should support the use of gene therapy to extend the lifespan and quality of life of patients with severe neuropathological disorders. Regardless, the role that religious beliefs plays in the viewpoints of the general population when it comes to using gene therapy as a treatment for neurological disorders has decreased over the last few decades (Robillard et al., 2014).

For the above reasons, the use of gene therapy for the treatment of neuropathological disorders should be considered a medical, social, and religious benefit. In summary, the life-

altering and lifesaving gene therapy technology provides patients with incurable or debilitating diseases a viable therapeutic option for their respective disorders, which immeasurably overshadows the potential risks of gene transfer treatments.

1.7.2 Do risks associated with gene therapy to treat disorders of the central nervous system outweigh the potential benefits of the technology?

The risks of treating CNS disorders with gene therapy outweigh the benefits of the technology, as the oncological, immunological and unknown long-term risks of gene therapy are too high to outweigh the benefits. The risk of inflicting more medical issues onto a patient already diagnosed with a severe or terminal disease is unethical. For example. the devastating oncological side effects of gene therapy were exposed when one patient died after developing leukemia-like symptoms due to the insertional mutagenesis of the virus used in the gene therapy treatment for Severe Combined Immunodeficiency (Hacein-Bey-Abina et al., 2008, 2003). Three of the other patients used in the same gene therapy clinical trial also developed leukemia, but were successfully treated with chemotherapy (Hacein-Bey-Abina et al., 2008, 2003). This clinical trial for severe combined immunodeficiency saw that four infants developed leukemia, from which, one of these infants died (Hacein-Bey-Abina et al., 2008, 2003).

In addition to the oncogenic side effects of gene therapy, the immunological and inflammatory risks of gene therapy were highlighted when a clinical trial participant was given a dose of adenoviral gene therapy that proved to be lethal (Lehrman, 1999; Raper et al., 2003; Savulescu, 2001; Sibbald, 2001). The death of Jesse Gelsinger, a result of the immune response to the high amount of virus used in his gene therapy treatment, prompted the temporary halt all clinical trials (Sibbald, 2001). Although the majority of gene therapy trials for CNS disorders directly inject the treatment into the immune privileged brain or cerebrospinal fluid (Piguet et al., 2017), treatment trials using an intravenous systemic route, in order to assess the effectiveness of this route of administration, are ongoing (NCT02122952) (Mendell et al., 2017). Although these

intravenous gene therapy trials use a virus that is less immunogenic, it could lead to similar immunological complications observed in Jesse's case (Lehrman, 1999; Raper et al., 2003; Savulescu, 2001), or oncogenic effects like the leukemia incident (Hacein-Bey-Abina et al., 2008).

Though Jesse was a legal adult consenting to the gene therapy treatment at age 18, in the court procedures from the lawsuit of his death, it was later revealed that neither Jesse, nor his family were properly informed about the potential lethal risks of the gene therapy treatment, or that the Phase I clinical trial was never designed to be of clinical benefit to the volunteers (Fox, 2000; Somia & Verma, 2000). This raises legal and ethical concerns about the proper procedures of informed consent in these gene therapy clinical trials. Informed consent has been a topic of ethical debate for all gene therapy trials, where the onus falls on the clinical researchers and medical professionals to ensure that the patient and their families understand all the benefits and risks they will encounter in the gene therapy trial well before treatment administration, and that the patients' wellbeing is the responsibility of the researchers conducting the clinical trial (Dettweiler & Simon, 2001). Clearly, informed consent was not properly undertaken in the case of the Gelsigner gene therapy trial, and although clinical trial participants can choose to voluntarily enroll themselves, it is not always guaranteed that they are given all the information required to make informed decisions. Is it right to use the lives of a group of individuals as a means to an end? The patients in Phase I clinical trials are not enrolled to combat their disease, but to be used to test the safety of the treatment (Dettweiler & Simon, 2001). In addition, can patients fully trust that the next generation of clinical researchers are indeed providing information about all the potential known risks to gene therapy treatments?

Moreover, the long-term effects of gene therapy are still unclear. As gene therapy treatments have yet to be fully assessed over the entire lifespan of higher mammals, the potential long term unknown risks of the treatment may be extreme (Kimmelman, 2008). Any latent side

effects may not present themselves until years after the end of a clinical trial for gene therapy (Kimmelman, 2008). Latent side effects could include anything from developing cancer years after the gene therapy treatment, to the inadvertent activation of dormant genes in the human genome, or the development of a new disease resulting from the overexpression of the therapeutic gene. For example, the occurrence of amyotrophic lateral sclerosis (ALS) has been attributed to the reactivation of a viral genome that has laid dormant inside the human genome for millions of years ("Dormant viral genes may awaken to cause ALS," 2015; Li et al., 2015). If gene therapies begin activating ancient dormant genes, there could be an influx of devastating unknown diseases. Additionally, the unethical inadvertent modification of the germline cells could also have unknown and unforeseeable latent consequences (Kimmelman, 2008).

In a survey comparing the views of medical professionals to those of the patients or non-professionals, it was shown that many patients derive a sense of self from their disease, viewing their disease as an integral portion of their identity (Scully, Rippberger, & Rehmann-Sutter, 2004). This is especially true when the disease is minimal to moderate in severity. Although ~75% of the population surveyed indicated their support for the gene therapy treatment of Attention Deficit Hyperactivity Disorder (ADHD) (Robillard et al., 2014), which has recently been discussed as a potential therapeutic for ADHD ("MIT research suggests possibility of gene therapy to treat ADHD | Center for Genetics and Society," n.d.), this may greatly affect the self-identity of patients with ADHD.

Risks to the society in general cannot be understated. Economically, the extremely high cost associated with the research and development of a gene therapy treatment for one disorder, let alone the production and implementation of said treatment is exorbitant (Kent & Spink, n.d.; Kimmelman, 2008; Naldini, 2015). Will these high costs be evenly spread across health care systems, or will the patient be required to pay for the treatment? If patients are required to purchase the gene therapy treatment, it stands to reason that only the rich and elite will prosper

and benefit from this potential treatment to the detriment of those who cannot afford it (Kent & Spink, n.d.; Kimmelman, 2008; Naldini, 2015). In addition, will the economy itself be able to be maintained with a change in resource allocation to the use of gene therapy? The exorbitant costs to produce even a single treatment for only one individual may prove to be impossible to sustain (Kent & Spink, n.d.; Kimmelman, 2008).

Hypothetically, if gene therapy has been approved to combat neuropathological diseases at their genetic causes, where does future gene therapy turn to? Which ailments will science and society decide to tackle next? The current investigation into gene therapy could eventually lead to the use of gene transfer to 'enhance' the human population with whatever trait is desirable, such as cognitive enhancements (Kimmelman, 2008). Gene therapy could be enticing to supporters of eugenics-like changes to society, which could potentially lead to societal upheaval, where the 'elite-enhanced' humans are a class above those born to parents who decided against enhancement or could not afford such luxuries (Blomkamp, 2013; Kimmelman, 2008; Niccol, 1997).

Although religious beliefs may no longer be a heavily weighted factor towards the rejection of gene therapy as being permissible, 17% of the general population surveyed indicated that their religious beliefs oppose gene therapy treatments for neuropathological diseases (Robillard et al., 2014). gene therapy is considered unnatural (Robillard et al., 2014), where sentient human beings, made up of deoxyribonucleic acid (DNA), are going back and re-writing the genetic code that makes humans human (Naldini, 2015). Scientists are 'playing God' for this meddling with the genetic code for human life, and potentially altering human evolution (Macer et al., 1995; "Moral and Ethical Issues in Gene Therapy | Society, Religion and Technology Project," n.d.). This is especially true when germline gene therapy occurs, whether it be inadvertent or purposefully done ("Moral and Ethical Issues in Gene Therapy | Society, Religion and Technology Project," n.d.). Genetic alterations to the heritable DNA in the germ cells could

have direct consequences on the future generations, and perhaps to human evolution. Bans on the alteration to germline cells are in place in many countries, including Canada ("Bill C-6: Assisted Human Reproduction Act (LS-466E)," n.d.; Kimmelman, 2008).

In conclusion, the medical, social and religious risks associated with the attempts to find GT treatments for neuropathological disorders greatly outweigh the potential benefits.

1.7.3 Overall Ethical Conclusions

As with any treatments, there are several benefits and risks involved. As addressed above, each stance about the risks and benefits to gene therapy being used for the treatment of CNS disorders provide valid points of concern and reasoning to support their perspectives.

Chapter 2

Correction of GM2 Gangliosidosis in Adult Sandhoff Mice using a Hybrid Hexosaminidase Vector – Efficacy and Dosage Study with Intravenous Administration

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Statement of Authorship:

The research described herein was conducted by Karlaina Osmon under the supervision of Dr. Jagdeep S. Walia. All data within this thesis was collected by Karlaina Osmon, with the exception of a subset of behavioural testing timepoints and blood collections, which were performed by Evan Woodley, Meera Vyas (Walia lab, Queen's University) (Figure 2.4). Patrick Thrompson, Evan Woodley, and Meera Vyas also contributed significantly to the humane endpoint collections for this study. Additionally, the vector production and the biodistribution data (Figure 2.7) collected through qPCR analysis by Subha Karumuthil-Melethil and Clifford Heindel under the supervision of Dr. Steven Gray at the University of North Carolina, Chapel Hill. John Keimel and William Kaemmerer (New Hope Research Foundation) contributed intellectually and provided biostatistics expertise.

2.1 Abstract

GM2 gangliosidosis is a neurodegenerative, lysosomal storage disease caused by a deficiency of β-hexosaminidase A enzyme (HexA), an α/β-subunit heterodimer. A novel variant of the human hexosaminidase α-subunit, coded by *HEXM*, has previously been shown to form a stable homodimer, HexM, that hydrolyzes GM2 in vivo. Here we assessed the efficacy of intravenous (IV) delivery of a self-complementary AAV9 vector incorporating *HEXM* in a mouse model of GM2 gangliosidosis, hexb-/-, Sandhoff disease (SD). Six-week old SD mice were injected with either 2.5 x 10^{12} vector genomes (low dose, LD; ~1.25 x 10^{14} vg/kg) or 1.0 x 10^{13} vg (high dose, HD; ~5 x 10^{14} vg/kg). Assessments included survival, behavioral outcomes, vector biodistribution, and enzyme activity within the central nervous system. Toxicity was observed in the HD cohort, with 8 of 14 mice dying within one month of the injection. As compared to untreated SD mice, which have typical survival of 16 weeks, the LD cohort and the remaining HD mice had a significant survival benefit with an average/median survival of 40.6/34.5 and 55.9/56.7 weeks, respectively. Significant behavioral benefit was also observed. These results demonstrate both the potential benefit and critical limitations of the treatment of GM2 gangliosidosis using IV delivered AAV vectors.

2.2 Introduction

GM2 gangliosidosis is a group of autosomal recessive genetic neurodegenerative diseases characterized by a deficiency of β-hexosaminidase A enzyme (HexA) to catabolize GM2 ganglioside (GM2) thereby causing GM2 accumulation within cellular lysosomes (Sandhoff, 2012; Sandhoff & Harzer, 2013). In humans, HexA is the sole enzyme capable of catabolizing GM2 ganglioside (Sandhoff & Harzer, 2013). The lysosomal accumulation of GM2 ganglioside, which primarily occurs within neurons, leads to widespread neurodegeneration throughout the brain and spinal cord. HexA is composed of two subunits, α and β, coded by the *HEXA* and *HEXB* genes, respectively (Sandhoff & Harzer, 2013). The hydrolysis of GM2 requires alignment of HexA with a complex consisting of a GM2 Activator Protein (GM2AP) and GM2 ganglioside.

Genetic mutations resulting in deficient activity of HexA or GM2AP lead to the development of GM2 gangliosidosis diseases: Tay Sachs disease (TSD), a HexA α-subunit deficiency; Sandhoff disease (SD), a HexA β-subunit deficiency; or the ultra-rare Tay Sachs AB-variant, a GM2AP deficiency. Numerous allelic combinations of mutations to either *HEXA* or *HEXB* have been identified that result in various degrees of HexA deficiency and thereby present a spectrum of phenotypic severity (Don J. Mahuran, 1999). The Infantile form of TSD and SD present with shakiness, rigidity, lethargy, loss of motor control, seizures, and paralysis (Sandhoff & Harzer, 2013), and result in death by four years of age. *Juvenile* and *Adult-onset* forms of GM2 gangliosidosis have a delay in symptom onset resulting from mutations that still allow low-level residual HexA activity (~2-10% of normal activity) (Conzelmann & Sandhoff, 1983, 1991; Leinekugel, Michel, Conzelmann, & Sandhoff, 1992b). There are no curative treatments available for GM2 gangliosidosis.

In vivo gene transfer is a promising option for the treatment of neurological disorders caused by enzyme deficiencies (Michaël Hocquemiller et al., 2016; Weinberg, Samulski, & McCown, 2013; Z. Wu, Asokan, & Samulski, 2006). Multiple studies have successfully utilized

adeno associated virus (AAV) vectors due to its ability to transduce neurons, demonstrated long-term expression within the central nervous system (CNS), low immunogenicity, and a small capsid size enabling better transport within tissue. These studies have primarily used direct intraparenchymal administration of the vector (Bradbury et al., 2013; Cachón-González et al., 2006, 2012, 2014; Sargeant et al., 2011) and have been shown to be effective in treating mouse models of GM2 gangliosidosis. While this method of delivery appears to distribute adequately across the small mouse brain, the distribution of the AAV vector in larger animals, even utilizing intraparenchymal convection enhanced delivery, is primarily limited to the volume immediately surrounding the site of injection. This limited vector distribution could potentially impact the therapy effectiveness in larger animals and humans (Bourgoin et al., 2003; Brown, Egleton, & Davis, 2004; Carty et al., 2010; Mastakov, Baer, Xu, Fitzsimons, & During, 2001).

Achieving adequate gene biodistribution and cellular uptake is a major challenge for treating neurological diseases that affect broad regions of the central nervous system. Several AAV serotypes have been reported to cross the blood brain barrier (BBB) enabling transduction of CNS neurons and astrocytes (Büning et al., 2008; Duque et al., 2009; Foust et al., 2009; McCown, 2005; Thomas et al., 2003). The potential to obtain broad distribution throughout the CNS makes this minimally invasive intravenous (IV) route of delivery desirable. Recent studies have investigated IV delivered vector in SD mice, but found long-term survival benefit only with neonatal injected mice in which the BBB has not yet fully formed (Niemir et al., 2018; Osmon et al., 2016; Walia et al., 2015). The low permeability of the mature BBB limits the amount of vector from entering the CNS, and alternative viral vectors or methods of delivery appear to be needed to maximize the effectiveness of IV delivery.

The efficiency of IV delivery of AAV vectors to the CNS has been reported to be significantly improved by the use of self-complementary (sc) DNA versus single-stranded (ss) DNA (Gray et al., 2011). The sc construct increases the transduction efficiency by eliminating the

need for host-cell-mediated synthesis of double-stranded DNA and has demonstrated to be especially beneficial in applications, such as IV delivery, that result in a low multiplicity of infection. The disadvantage of a sc construct is that it further limits the maximum AAV payload to approximately 2150 base pairs for the transgenes plus the associated vector regulatory sequences (McCarty et al., 2003). With the hexosaminidase α- and β-subunits each having a gene size of ~1600 base pairs, sc AAV packaging of both genes in one vector is not possible.

To create a sc construct, the vector utilized in this study expresses the previously described hexosaminidase μ-subunit, coded by *HEXM*, which is a synthesized variant of the human α-subunit (Tropak et al., 2016b). This engineered variant of the human α-subunit incorporates enhanced dimer interface stability and a GM2AP binding site, which normally exists only on the hexosaminidase β-subunit. The μ-subunit homodimerizes to form a highly stable HexM enzyme, previously shown to interact with the human GM2AP-GM2 complex and catabolize GM2 (Tropak et al., 2016b). HexM enzyme has been shown to hydrolyze GM2 in human cells at approximately twice the *Vmax* rate of HexA.

In the current study, we investigated the efficacy of IV delivery of a scAAV9/*HEXM* vector to six-week old adult Sandhoff mice. The mice were injected with one of two doses of the vector to investigate its potential benefit in terms of survival and behavioral outcomes. We hypothesized that a higher dose of gene therapy would produce more favourable outcomes, and that a tandem injection of mannitol would increase the bioavailability of the vector to the central nervous system. Survival and vector distribution were also investigated with the vector injection preceded by a fast IV injection of mannitol (3g/kg) intended to disrupt the BBB, temporarily increase its permeability, and enhance vector delivery to the CNS.

2.3 Methods

2.3.1 Experimental Animals

The murine model of SD was obtained from Jackson Laboratory (stock number: 002914; B6;129S4-*hexb*^{tm1Rlp}/J) and a colony for this study was maintained at Queen's University. Experimental mice were bred using female *Hexb*+/- heterozygous and male *Hexb*-/- or female *Hexb*-/- heterozygous and male *Hexb*-/- crosses. Genotype verification was performed through PCR amplification of deoxyribonucleic acid (DNA) extracted from ear punches ("Jackson Laboratoryâ□"B6;129S4-Hexb<tm1Rlp>/J," n.d.; Sango et al., 1996). All protocols were approved by the Queen's University Animal Care Committee.

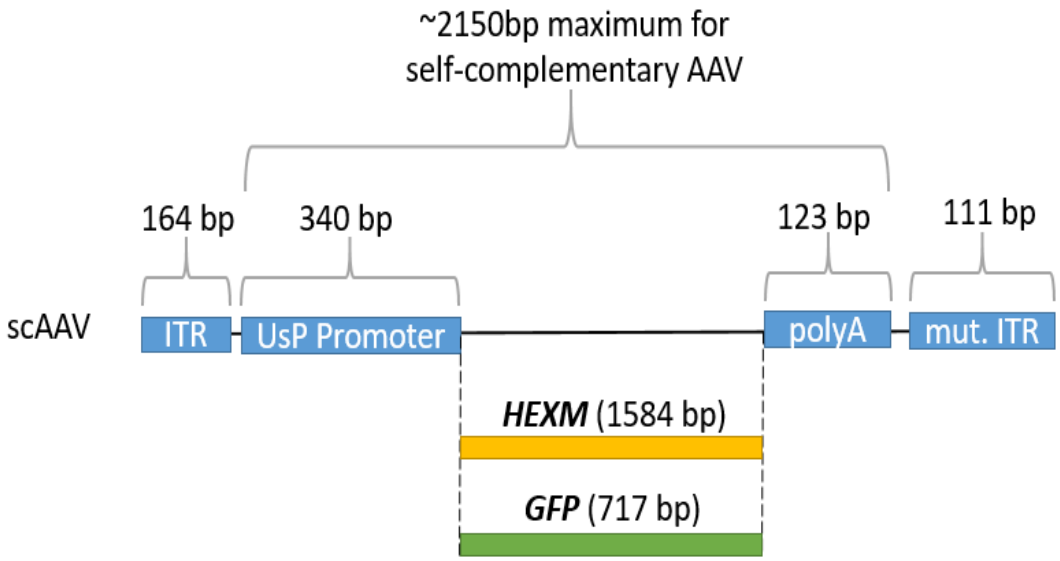


Figure 2.1 scAAV/*HEXM* & scAAV/*GFP* genome design.

Includes UsP promoter, as previously described (Osmon et al., 2016).

2.3.2 rAAV Vector and Injections

The scAAV9/*HEXM* vector (see Figure 2.1) was produced at the vector core facility at the University of North Carolina as previously described (Gray, Nagabhushan Kalburgi, McCown, & Jude Samulski, 2013; Karumuthil-Melethil et al., 2016; Osmon et al., 2016). The scAAV9/*HEXM* or scAAV9/*GFP* viruses were diluted to the correct volume in vehicle (PBS with 350 mM total NaCl and 5% sorbitol) as previously described (Woodley et al., 2019). Neonatal mice (postnatal day 0-1) received 50 μL with 2.5E+11 vg per mouse delivered through the temporal vein. Six-week old mice received 100 μL of vector solution containing $1x10^{13}$ vg/mouse (high dose, HD; ~5 x 10^{14} vg/kg) or $2.5x10^{12}$ vg/mouse (low dose, LD; ~1.25 x 10^{14} vg/kg) delivered via tail vein injections. Injections with an LD and HD of scAAV9/*GFP* (see Table 2.1) and PBS injections were also performed as sham and untreated controls. A separate cohort of scAAV9/*HEXM* LD mice received a rapid injection of 25% mannitol at 3g/kg via the tail vein prior to the virus injection. See Table 2.1,**Error! Reference source not found.** for study design a nd cohort sizes.

Genotype	Treatment	Age of Administration	AAV Serotype	AAV dose (vg/mouse)	Number of Mice Terminated at	
					16- weeks	Long Term Humane Endpoint
Heterozygotes (+/-)	None	N/A	None	0	6	10
Sandhoff (-/-)	*PBS*	Adult	None	0	5	0
Sandhoff (-/-)	*GFP*	Adult	AAV9	1×10^{13}	4	0
Sandhoff (-/-)	*GFP*	Adult	AAV9	2.5×10^{12}	4	0
Sandhoff (-/-)	*HEXM*	Adult	AAV9	1×10^{13}	0	14
Sandhoff (-/-)	*HEXM*	Adult	AAV9	2.5×10^{12}	5	12
Sandhoff (-/-)	*HEXM* & Mannitol	Adult	AAV9	2.5×10^{12}	4	12
Heterozygotes (+/-)	None	N/A	None	0	6	6
Sandhoff (-/-)	*PBS*	Neonatal	None	0	3	0
Sandhoff (-/-)	*GFP*	Neonatal	AAV9	2.5×10^{11}	4	0
Sandhoff (-/-)	*HEXM*	Neonatal	AAV9	2.5×10^{11}	6	8

Table 2.1 Study Design

The study design for the neonatal, adult, and adult & mannitol co-injectuions.

2.3.3 Euthanizations and Tissue Processing

Mice were humanely euthanized either at 16 weeks of age or at their humane survival endpoint, defined by >15% weight loss and/or the loss of the righting reflex. All PBS and GFP cohorts were euthanized at 16 weeks to allow age matched comparisons of hexosaminidase activity and GM2 ganglioside accumulation between treated groups and these controls. Terminal cardiac punctures were done to obtain serum in addition to the monthly serum collections. Visceral and nervous system organs were collected post-carbon dioxide asphyxiation, cardiac puncture, and perfusion using 10 mL of 1xPBS until clear. Visceral organs collected included the heart, lung, liver, kidney, spleen, gonad, and arm muscle. CNS tissue was grossly subdivided into rostral, mid, and caudal brain as shown in Figure 2.2, as well as the cervical and lumbar spinal cord. Samples taken for histological purposes were deposited into 4%PFA for 24 hours, after which they were put into 70% ethanol until tissue embedding in paraffin. Samples taken for molecular and biochemical analysis were stored at -20ºC for future processing. For further biochemical assessments, the MB samples were homogenized with 700µL of PBS through three 10 second bursts of sonication. The samples were then spun down at 13000 RPM for 20 minutes at 4ºC. 300µL of the supernatant was separated for hexosaminidase activity assays, and the remainder of the supernatant and the precipitate was stored for future GM2 ganglioside storage analysis.

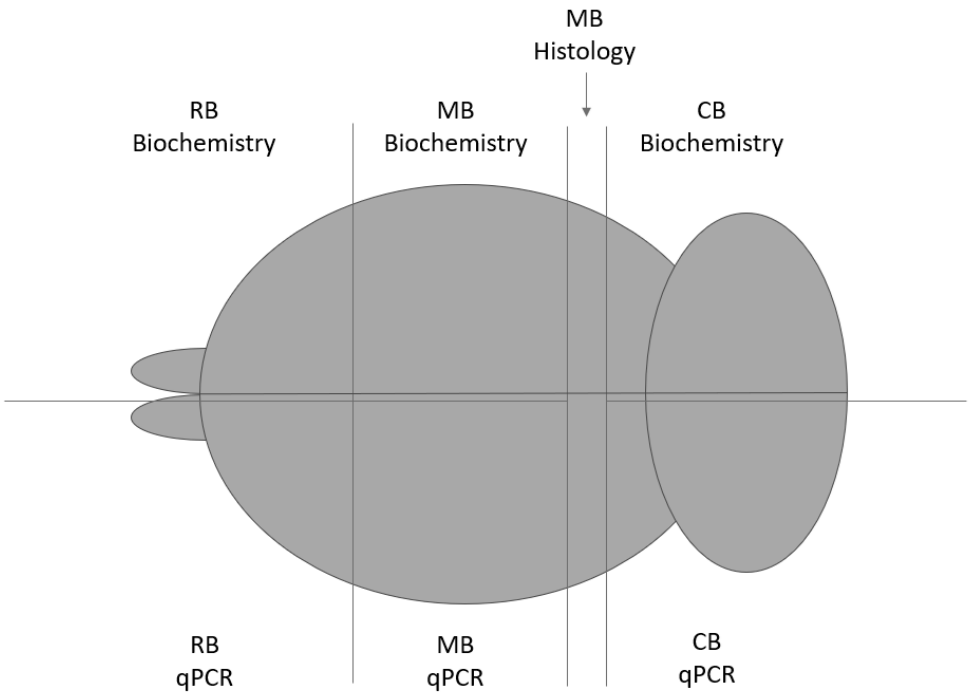


Figure 2.2 Gross Sectioning of the Brain for Further Processing.

Figure adapted from (Woodley et al., 2019). RB- Rostral Section of the brain; MB- Midsection of the brain; CB- Caudal Section of the brain.

2.3.4 Behavioral Testing

Behavioral assessments were performed weekly from 9 through 15 weeks of age, then monthly from 18 weeks until the humane endpoint of the animal. The Open Field Test (OFT) and Rotarod (RR) were conducted as previously described (Osmon, Vyas, Woodley, Thompson, & Walia, 2018; Osmon et al., 2016). Briefly, during the OFT mice were monitored for general locomotion for 5 minutes with time moving being recorded by instrumentation (ActiMot; TSE Systems). In the RR, an accelerated protocol was used to assess coordination and strength in the mice over the course of a 5 minute trial, where the RPM at the time the mouse fell from the rod was recorded as "end RPM" (IITC Life Sciences, Series 8).

2.3.5 Hexosaminidase Activity Assay

Hexosaminidase activity assays were performed on MB and serum samples utilizing the fluorogenic 4-methylumbelliferone (MU) substrates 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG) and 4Methylumbelliferyl-7-6-sulfo-2-acetamido-2-deoxy-bD-glucopyranoside (MUGS) as previously described (Maegawa et al., 2007; Osmon et al., 2016; Tropak, Reid, Guiral, Withers, & Mahuran, 2004). Samples of homogenized MB supernatant were diluted 1:50µl with PBS. The 4MU substrates were incubated with the diluted samples for 1 hour at 37ºC, at which time the reaction was stopped using a 0.1M 2-amino-2-methyl-1-propanol (AMP) solution at a pH of 10.5. All hexosaminidase activity was normalized to galactosidase activity as measured by the 4-Methylumbelliferyl β-D-galactopyranoside (MUGal) substrate.

2.3.6 Vector Distribution Analysis

Vector biodistribution was determined through qPCR analysis as previously described (Gray et al., 2010). Briefly, DNA extraction was performed on all organs collected. Isolated DNA was then quantitatively analyzed using primers specific for the *GFP* and *HEXM* transgenes. Data are reported as the number of double stranded target gene molecules per two double stranded copies of murine *LaminB2*, approximating the amount of target gene per diploid cell. Primer

sequences were as follows: *GFP* (forward: 5'-AGCAGCACGACTTCTTCAAGTCC-3'; reverse: 5'-TGTAGTTGTACTCCAGCTTGTGCC-3'); *HEXM* (forward: 5'-ACATCTACACCGCCCAAGAC-3'; reverse: 5'-TTGACAGGGCCAAAAGTACC-3'); *LaminB2* (forward: 5'-GGACCCAAGGACTACCTCAAGGG-3'; reverse: 5'-AGGGCACCTCCATCTCGGAAAC-3').

2.3.7 GM2 Storage Assay

Extraction and separation of gangliosides from homogenized MB samples were performed as previously described (Folch, Ascoli, Lees, Meath, & LeBaron, 1951; Folch, Lees, & Stanley, 1957; Osmon et al., 2016; Tropak et al., 2010, 2016b; Wherrett & Cumings, 1963). Briefly, mixed gangliosides were extracted through a series of dilutions and evaporations under nitrogen in methanol and chloroform, then separated with a thin layer chromatography plate using a 50:45:10 chloroform: methanol: 0.2% calcium chloride mobile phase. Visualization of the ganglioside bands was achieved by adding 0.4% orcinol solution in 25% sulfuric acid and baking the plate at 120ºC for approximately 10 mins. Densitometry analysis of the bands was performed using ImageJ software. The intensity of the GD1A band served as an internal loading control for the intensity of the GM2 bands.

2.3.8 Histology

Histological analysis of the processed MBs was conducted as previously described (Osmon et al., 2016; Walia et al., 2015). Briefly, the paraffin embedded samples were cut into 4-6µm sections and deparaffinized, then subjected to citrate buffer antigen retrieval. Slides were blocked for endogenous peroxidase activity and nonspecific binding, followed by incubations in primary and secondary antibodies. DAB stain was then applied prior to the dehydration and mounting of the slides. GM2 gangliosides were visualized through a 1:1000 dilution of the KM966 chimeric murine-human IgG1 anti-GM2 ganglioside antibody (Yamada et al., 2011)

(Kyowa Hakko Kirin Co., Ltd.), followed by incubation with a goat anti-human biotinylated secondary antibody.

2.3.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism and NCSS 12 statistical software. One-way ANOVAs using Tukey-Kramer post-hoc tests for multiple pairwise comparisons were performed on the MB hexosaminidase activity assays and Bonferroni post-hoc tests on the GM2 storage assays. Two-way repeated measure ANOVAs with Bonferroni post-hoc tests were used on behavioral measures and the serum hexosaminidase activity assay. The behavioral analysis past 16 weeks was based only on the surviving mice. Kaplan-Meier curves were used to visualize improvement in the survival of the treated SD mice, with the significance of the observed improvements assessed by Log-Rank (Mantel Cox) test. Mixed factors ANOVAs were performed to assess the biodistribution of the vectors.

2.4 Results:

2.4.1 Survival

To verify vector transport across a more permeable, immature BBB, a cohort of neonatal mice were intravenously injected with scAAV9/*HEXM* via the temporal vein at a dose of 2.5×10^{11} vg/mouse. The average survival of this cohort (n=8) was 44.7 weeks and their median survival was 46 weeks, with a range of 30-56 weeks of age (Supplementary Figure 2.1). The neonatal administration showed a highly significant survival benefit compared to the typical lifespan of approximately 16 weeks (R= 15-21; (Phaneuf et al., 1996; Sango et al., 1995; Suzuki et al., 1998)) (p<0.001).

Six-week old mice received 100µL of vector solution containing $1x10^{13}$ vg/mouse (high dose, HD; ~5×10^{14} vg/kg) or $2.5x10^{12}$ vg/mouse (low dose, LD; ~1.25×10^{14} vg/kg) delivered via tail vein injections. Injections with an LD and HD of scAAV9/*GFP* (Figure 2.3) and phosphate buffered saline (PBS) injections were also performed as sham and untreated controls. A separate cohort of scAAV9/*HEXM* LD mice received a rapid injection of 25% mannitol at 3g/kg via the tail vein prior to the virus injection. See Table 1 for study design and cohort sizes.

In the HD injected adult mice ($1x10^{13}$ vg/mouse; ~5×10^{14} vg/kg), 8 of the 14 mice (~57% of the cohort) died shortly after the IV scAAV9/*HEXM* administration with their age at death ranging from 8 weeks to 10.5 weeks (see Figure 2.3A). These mice were then necropsied and their liver enzymes were tested. Table 2.2 shows the elevated liver enzymes in the HD cohort as compared to the vehicle controls. There was also abnormal liver morphology observed during the histopathological analysis (data not shown). The survival of the remainder of this cohort and mice in the other experimental cohorts is depicted in Figure 3B. The average survival of the Adult HD (n=6) and Adult LD ($2.5x10^{12}$ vg/mouse, n=12) mice was 56.0 and 40.6 weeks of age, respectively. These Adult HD and LD groups had median survival of 56.7 weeks and 34.5 weeks, and a range of 18-89 weeks and 32-62 weeks, respectively. Excluding the mice in the HD group

that died shortly after vector administration, there was a highly significant increase in survival ($p<0.001$) of mice receiving either the HD or LD adult administrations as compared to the vehicle and GFP treated cohorts. In addition, there was a significant increase in the lifespan of the adult HD injected mice compared to the LD injected cohort ($p<0.05$).

Excluding the 3 mice that did not tolerate the mannitol co-injection (one of which developed ascites; Figure 2.3A), the survival of the LD & Mannitol cohort (n=9) ranged from 39.5 to 78.3 weeks of age, with an average of 41.6 weeks and a median survival of 44.8 weeks (Figure 2.3.B). This was a significant increase in survival compared to untreated cohorts ($p<0.01$). However, co-administration of mannitol with the low dose of scAAV9/*HEXM* vector did not significantly increase the survival time of the mice compared to the mice receiving the low dose of scAAV9/*HEXM* alone ($p = 0.0759$).

	Assay:	**ALT**	**AST**
	Units:	**U/L**	**U/L**
Untreated Mice	137	19	65
	147	20	63
	148	23	70
	149	19	49
HD Toxicity	Necro 144	5	309
	Necro 150	1701	5828
	Necro 153	204	330
	Necro 154	6	68
	Necro 157	149	246
	Necro 158	17	177

Table 2.2 Liver Enzyme Function Necropsy Results on high dose cohort toxicity.

Liver function parameters as measured through the Alanine Transaminase (ALT) and Aspartate Transaminase (AST) activity. Many of the necropsied mice from the HD cohort show elevated liver enzyme levels, indicative of liver disfunction, possibly due to liver toxicity.

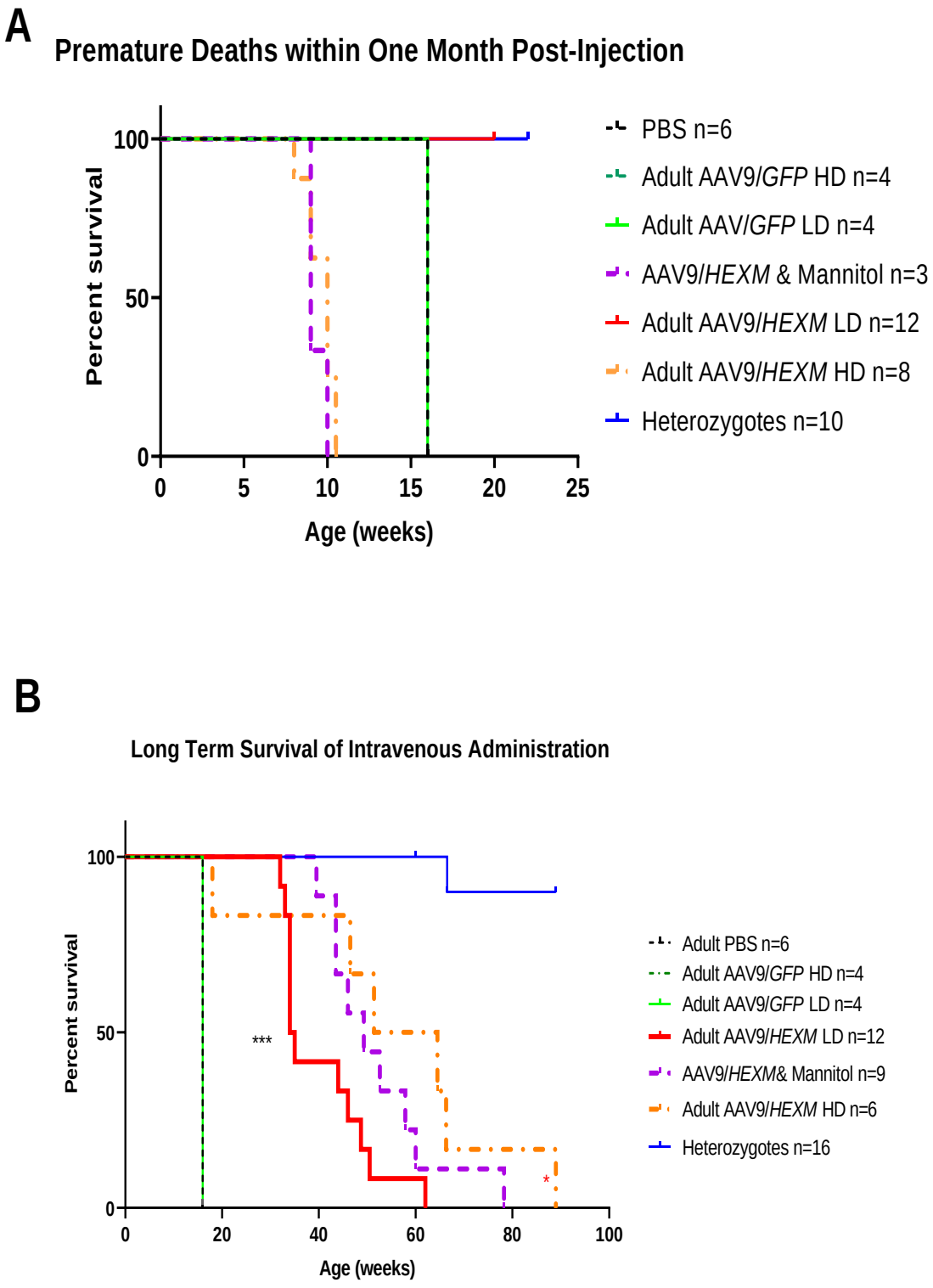


Figure 2.3 Survival of the adult scAAV9/*HEXM* administrations.

Caption for Figure 2.3 - Some of the treatments displayed a bimodal survival curve, with either early death or prolonged survival. These have been separated into panels A and B. (A) Premature deaths within one-month post-injection. The adult HD administration resulted in 57% lethality (8 of 14 mice) from acute toxicity in the month following the intravenous administration. In addition, 19% of the mice that received a co-injection of IV mannitol (3 of 16 mice) required euthanization within a month following injection. (B) Long term Survival. The average survival of the HD group (n=6, excluding the 8 that died acutely) was 56 weeks, a median of 56.7 weeks, with a range of 18 to 89 weeks. The average survival of the Adult LD (n=12) was 40.6 weeks, a median of 34.5 weeks, and a range of 32 to 62 weeks. The increase in survival of both the HD and LD adult cohorts was highly significant compared to the humane endpoint of the untreated mice (p<0.001), as per the Queen's University Animal Care Committee. The increase in survival of the HD group (excluding the acute toxicity) as compared to the LD administration was significant (p<0.05). The cohort of SD mice that received the co-infusion of the LD of scAAV9/*HEXM* and mannitol showed a significant increase in survival (mean of 41.6 weeks and range of 9.0 to 78.3 weeks) as compared to the natural survival of SD mice. However, this increase in survival was not significantly different from the cohort that received only the LD of the scAAV9/*HEXM* vector. (* = p<0.05, *** = p<0.001)

2.4.2 Behavioral Outcomes

Behavioral locomotion and coordination were assessed in the adult scAAV9/*HEXM* SD cohorts through Open Field Test (OFT) and Rotarod (RR), respectively.

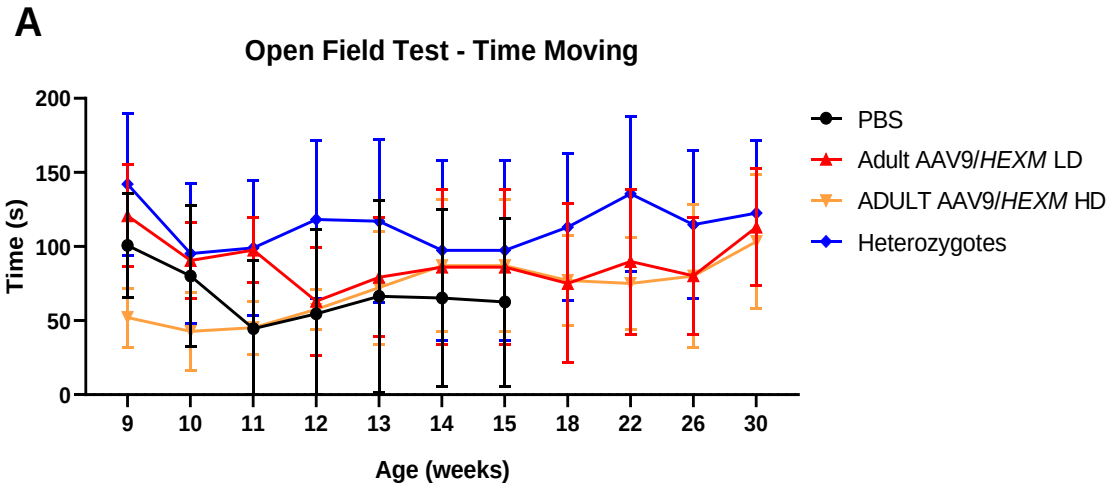
2.4.2.1 General Locomotion

In the measure of general locomotion from an age of 9 weeks through 15 weeks, there was a significant difference observed among treatment groups in the OFT time moving ($F_{6, 35}$=4.02, p<0.0001; Figure 2.4A). The adult HD group at 9 weeks spent less time moving compared to heterozygous controls (p<0.001). The adult HD group also spent less time moving than the adult LD group at 9 weeks (p<0.05). Over the time-course from 18 to 30 weeks of age, there were no statistically significant differences between the locomotion of the remaining adult scAAV9/*HEXM* treatment groups (HD and LD) in the OFT.

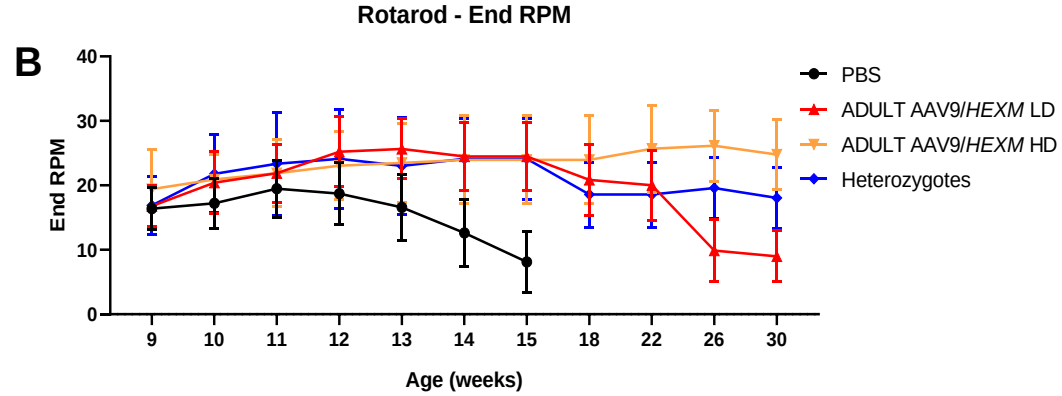
2.4.2.2 Coordination

The coordination of the scAAV9/*HEXM* treated cohorts was assayed through the accelerated protocol on the RR ($F_{5, 33}$=2.98, p=0.0250; Figure 2.4B). From the week 9 to week 22 of the behavioral testing time-course, the performance of the adult HD and LD cohorts approximated that of the heterozygote cohort. However, at the testing performed at the 26- and 30-week timepoints, the adult LD cohort diverged from the heterozygote group, showing a significant decline in performance (p<0.01, p<0.05, respectively). The adult LD group also showed decreased coordination at 26 and 30 weeks compared to the adult HD cohort (p<0.001, p<0.001, respectively). The adult HD group outperformed the PBS cohort at 14 and 15 weeks (p<0.01, p<0.001, respectively). Likewise, the mice of the adult LD group were also more coordinated than the PBS group at 13, 14 and 15 weeks (p<0.05, p<0.01, p<0.001, respectively). The coordination of the heterozygote controls was significantly better than the untreated PBS cohort at 14 and 15 weeks (p<0.01, p<0.001, respectively).

The treated neonatal cohort also demonstrated increased general locomotion and coordination on the OFT and RR compared to the untreated cohorts (see Supplementary Figure 2.2).



	9	10	11	12	13	14	15	18	22	26	30
Adult HD vs Adult LD	*										
Adult HD vs Het	***										
PBS vs Het	*			*							



	9	10	11	12	13	14	15	18	22	26	30
Adult LD vs Adult HD										***	***
Adult LD vs Het										**	*
Adult LD vs PBS					*	**	***				
Adult HD vs PBS						**	***				
PBS vs Het						**	***				

Figure 2.4 Behavioral Analysis of the adult scAAV9/*HEXM* administrations

Caption for Figure 2.4 - (A) Open Field Test - Time Moving. From 9-15 weeks, there was a significant difference observed among treatment groups in the OFT ($F_{6, 35}$=4.02, p<0.0001). All scAAV9/*HEXM* treated animals did not have significant changes in their general locomotion compared to heterozygous controls (p>0.05), except for the Adult HD group at 9 weeks with a highly significant decrease in movement compared to Heterozygotes (p<0.001). Over the long-term time-course (18-30 weeks) there were no statistical differences between the locomotion of the treatment groups in the OFT. (B) Rotarod - End RPM. There was a significant effect of treatment observed ($F_{5, 33}$=2.98, p=0.0250). Over the time-course the coordination of all scAAV9/*HEXM* treatment groups approximated the activity of the heterozygous control mice until 26 and 30 weeks, where the Adult LD cohort diverged, showing a significant decline (p<0.01, p<0.05, respectively). The Adult LD group also showed decreased coordination at 26 and 30 weeks compared to the Adult HD cohort (p<0.001, p<0.001, respectively). In addition, the heterozygous controls performed significantly better than the untreated PBS cohort at 14 and 15 weeks (p<0.01, p<0.001, respectively). Likewise, the Adult HD group outperformed the PBS cohort at 14 and 15 weeks (p<0.01, p<0.001, respectively), where the Adult LD group were also more coordinated than the PBS group at 13, 14 and 15 weeks (p<0.05, p<0.01, p<0.001, respectively). (* = p<0.05, ** = p<0.01, *** = p<0.001)

2.4.3 Biochemical Measures

2.4.3.1 Enzymatic Activity Assay

Hexosaminidase enzyme activity in the midsection of the brain (MB) homogenates or serum was quantified through the use of the synthetic fluorescent MUGS substrates relative to galactosidase activity measured with MUGal substrate. The analysis of variance of measures of hexosaminidase enzyme activity in MB tissue samples taken at termination showed a significant main effect of group ($F_{6,18}$=63.06, p<0.0001) (Figure 2.5A; ratios were log-transformed for the statistical analyses to achieve homogeneity of variance among groups). At their long-term (humane) endpoint, the mice receiving scAAV9/*HEXM* at either the low dose or high dose had significantly greater hexosaminidase enzyme activity in their MB samples than control group mice receiving the scAAV/*GFP* ($p < 0.001$). There was also a dose effect, with the high dose group mice having significantly greater hexosaminidase enzyme activity in their MB samples than mice receiving the lower dose ($p < 0.05$). The activity was also assessed in serum samples, where no increase in enzymatic activity was observed (data not shown).

See Supplementary Figure 2.1B for the MB hexosaminidase activity analysis of the neonatal cohorts.

2.4.3.2 Ganglioside Storage Analysis

There was a significant effect of treatment on the amount of GM2 ganglioside stored ($F_{7,61}$= 40.12, p<0.0001) (Figure 2.5B). The Adult AAV9/*HEXM* LD 16-week cohort had significantly less GM2 storage compared to the PBS untreated group (p<0.001). The amount of GM2 gangliosides stored in the MB samples collected at the humane long-term endpoint (LT timepoint) of the Adult scAAV9/*HEXM* LD cohort was significantly greater than that observed in the samples from the mice terminated at 16 weeks of age of this cohort (p<0.001). GM2 ganglioside storage in MB samples taken at the LT timepoint was significantly lower in the samples from the Adult HD group compared to the Adult LD cohort (p<0.0001). Compared to the

heterozygous controls, the PBS, Adult scAAV9/*HEXM* LD LT, and the Adult scAAV9/*HEXM* HD LT each showed a greater amount of GM2 gangliosides stored ($p<0.001$), as did the Adult scAAV9/*HEXM* LD group samples taken at 16 weeks ($p<0.01$). Supplementary Figure 2.1C shows the GM2 storage in the neonatal cohort.

Histological analysis showed noteworthy reductions of the GM2 ganglioside storage in the MBs of the adult LD and HD scAAV9/*HEXM* treatment groups (Figure 2.6). Supplementary Figure 2.3 depicts the histological analysis of the neonatal cohort.

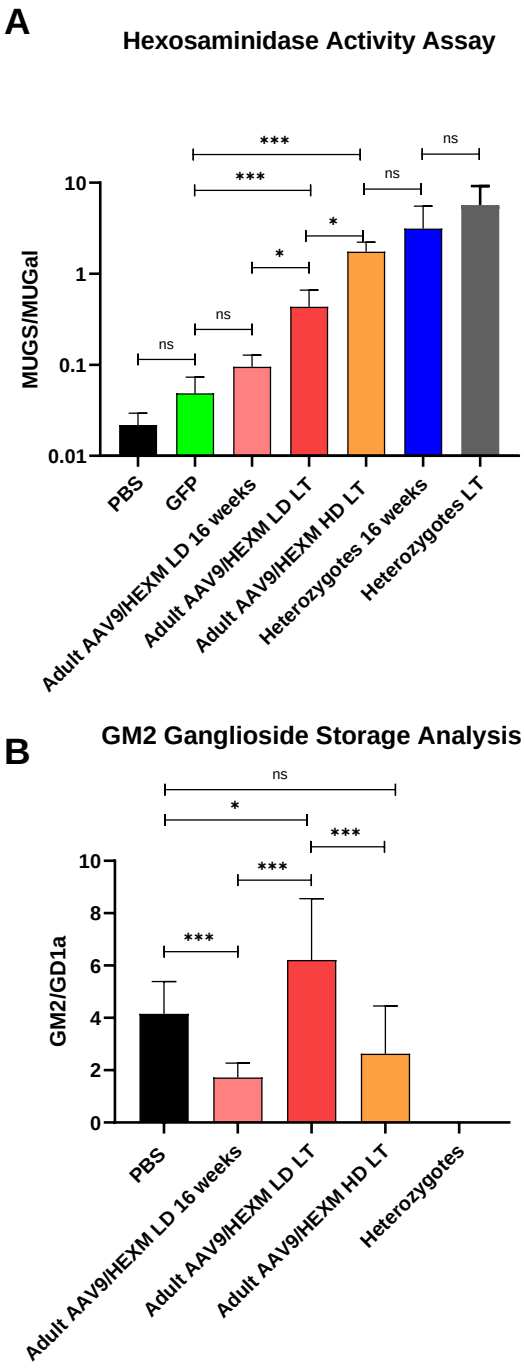


Figure 2.5 Biochemical Analysis of the adult scAAV9/*HEXM* administrations.

(A) Midsection of the Brain (MB) Hexosaminidase Activity Assay. There was a significant difference among treatment groups in the amount of hexosaminidase activity measured in MB samples ($F_{6,18}$ = 63.06, p < 0.0001). The graph shows the samples' activity against the MUGS substrate as a ratio to activity with the MUGal substrate. The differences between pairs of treatment groups shown in the graph and discussed in the text are based on the Tukey-Kramer method for multiple pairwise comparisons. (B) Ganglioside Storage Analysis. There was a significant effect of treatment group on the amount of GM2 ganglioside stored ($F_{7,61}$= 40.12, p<0.0001). The adult scAAV9/*HEXM* LD 16-week cohort showed a significant decrease in GM2 storage as compared to the PBS untreated group (p<0.001). A significant increase in the amount of GM2 stored was observed between the 16 week and LT timepoint for the adult scAAV9/*HEXM* LD cohort (p<0.001). As compared to the heterozygous controls, the PBS, GFP, adult scAAV9/*HEXM* LD LT, and the adult scAAV9/*HEXM* HD LT showed a highly significant increase in the amount of GM2 gangliosides stored (p<0.001), whereas the Adult scAAV9/*HEXM* LD 16 week group showed a significant increase (p<0.01). (* = p<0.05, *** = p<0.001, ns = not significant)

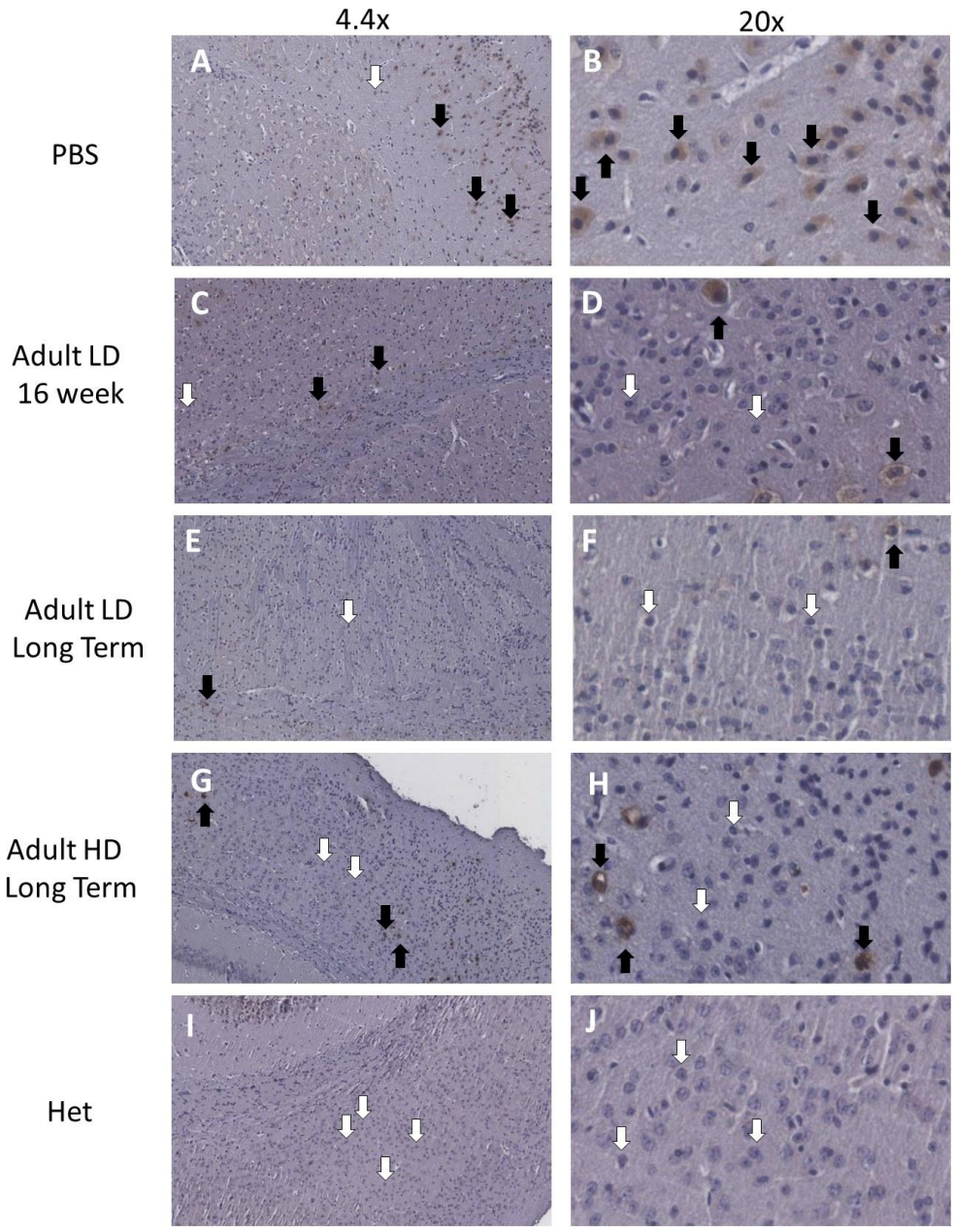


Figure 2.6 Histological Ganglioside Analysis of the adult scAAV9/*HEXM* administrations.

Sections of the murine neocortex at 4.4x and 20x magnification. PBS cohort (A, B), the AAV9/*HEXM* adult LD 16 week (C,D) and long term (E,F) cohorts, the AAV9/*HEXM* HD adult cohort (G,H), and the heterozygote controls (I,J). Black arrows indicate GM2 ganglioside filled neurons, which were found predominantly throughout the PBS and adult long-term cohorts. The white arrows indicate similar neuron soma lacking the GM2 ganglioside accumulation.

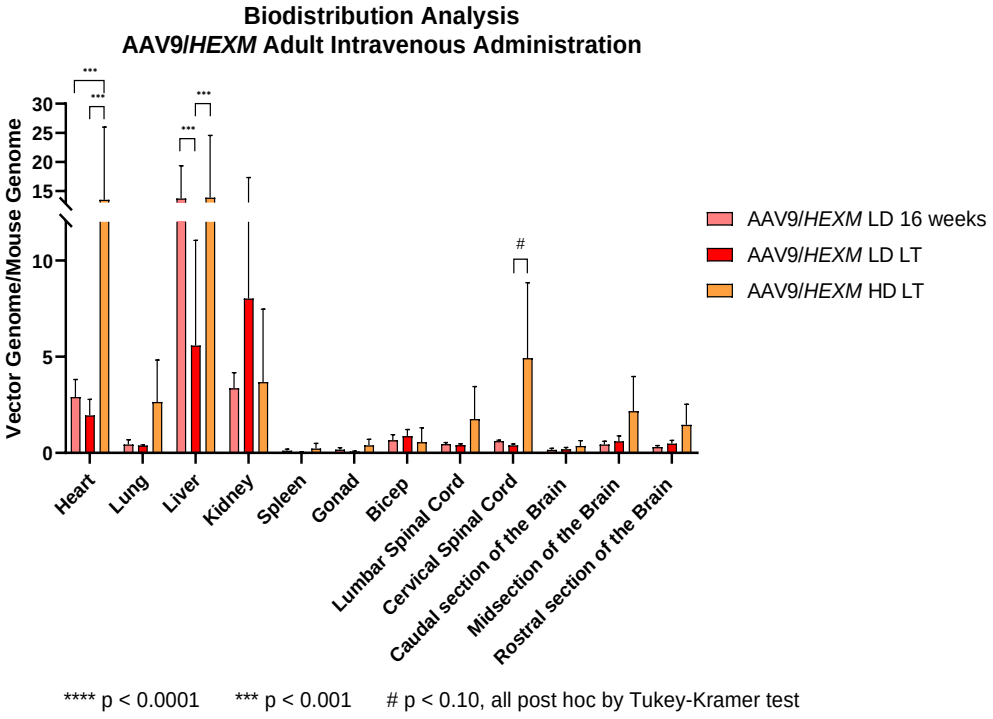
2.4.4 Biodistribution Analysis of IV scAAV9/*HEXM* treated mice

The scAAV9/HEXM vector biodistribution for the adult intravenous administration cohorts was through qPCR analysis (Figure 2.7A). Data is presented as the number of vector genomes per mouse genome. The biodistribution analysis of the 16-week and LT LD and HD scAAV9/HEXM administrations showed similar distribution of the vector throughout the central nervous system. In addition, high copy numbers were seen in the heart and liver samples of all injected animals, similar to previous reports (Gray et al., 2010, 2011). Supplementary Figure 2.1D shows the biodistribution analysis of the neonatal cohort.

Compared to the LD cohort, the cohort that received a co-injection of mannitol with the vector showed a significant increase in the amount of vector uptake in all regions of the central nervous system ($p<0.001$) (Figure 2.7B). When assessing the biodistribution to peripheral organs, there was a significant interaction between the pattern of transgene detection across tissues depending upon the use of mannitol ($p<0.05$), with mannitol co-administration resulting in relatively greater HEXM DNA detected in the bicep and lung, and less in the heart and kidney, compared to the LD of vector alone.

Caption for Figure 2.7 - Presented as the number of vector genomes per diploid mouse genome. (A) The biodistribution for the LD cohort at 16 weeks and long term, as well as the HD long term cohort. There was a significant effect of scAAV9-HexM dose on the copy numbers of *HEXM* DNA found in the heart ($p < 0.001$) and liver ($p < 0.01$) of mice at their humane endpoint, with greater copy numbers found in the higher dose group. A repeated measures ANOVA showed a significantly higher number of copies in the CNS of mice receiving the higher dose versus those receiving the lower dose ($p < 0.05$) when considering the CNS regions collectively. Post hoc tests on a region-by-region basis were not significant; however, the data suggest that the dose effect was likely greatest in the region of the cervical spinal cord ($p < 0.10$). (B) The inclusion of the mannitol infusion with the LD scAAV9/*HEXM* significantly increased the vector biodistribution across the central nervous system compared to the LD virus alone (ANOVA with one between animal factor [mannitol or not] and one within animal factor [region of CNS], mannitol main effect: $p < 0.001$). However, no *post hoc* test for mannitol effect at individual CNS regions achieved significance). When assessing the non-central nervous system organs, there was a significant interaction between the pattern of transgene detection across tissues depending upon the use of mannitol ($p<0.05$), with mannitol co-administration resulting in relatively greater *HEXM* DNA detected in the bicep and lung, and less in the heart and kidney, compared to the LD alone.

A



**** p < 0.0001 *** p < 0.001 # p < 0.10, all post hoc by Tukey-Kramer test

B

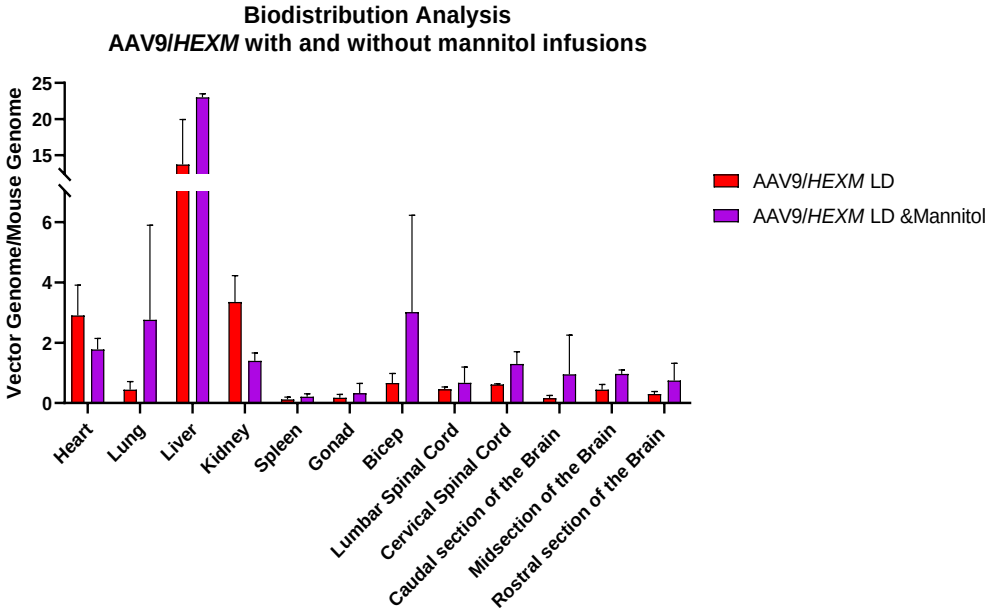


Figure 2.7 Copy Number Analysis for the Biodistribution of the scAAV9/*HEXM* Vector.

2.5 Discussion

This study demonstrates the efficacy of intravenously delivered scAAV9/*HEXM* gene therapy in adult SD mice. Overall, the neonatal and adult LD and HD cohorts each survived significantly longer than untreated controls. The scAAV9/*HEXM* treated neonatal and adult administrations showed improved behavioral performance as compared to the untreated controls. This improved phenotypic performance and survival was accompanied by significantly decreased GM2 ganglioside storage and a significant increase in hexosaminidase activity in the MB tissue samples. This study also assessed whether the biodistribution of the scAAV9/*HEXM* vector was altered when partnered with mannitol, an agent that temporarily increases the passage of large molecules into the brain across the blood-brain barrier. The LD & Mannitol group showed an increase in vector genomes detected in all regions of the central nervous system as compared to the cohort that received only the LD of vector alone. That this increase was not associated with longer survival of the mice may be due to the systemic nature of the disease in the Sandhoff mouse, whereby morbidity and mortality may be determined by a broader pathology than central nervous system effects alone.

Previous preclinical gene therapy studies have had varying success in treating animal models of GM2 gangliosidosis (Arfi et al., 2005; Bourgoin et al., 2003; Bradbury et al., 2013; Cachón-González et al., 2006, 2012, 2014; Gray-Edwards et al., 2017; Guidotti et al., 1999; Karumuthil-Melethil et al., 2016; Kyrkanides et al., 2005; Martino et al., 2005; McCurdy et al., 2015; Niemir et al., 2018; Osmon et al., 2016; Rockwell et al., 2015; Sargeant et al., 2011; Tropak et al., 2016b; Walia et al., 2015; Woodley et al., 2019). These previous studies used a variety of viral vectors, routes of administration, and treatment constructs, making a direct comparison to the current study using HexM difficult. Nevertheless, it may be noted that other studies delivered both hexosaminidase genes (α-subunit and β-subunit) in order to avoid depleting the supply of the endogenous complementary subunit (Guidotti et al., 1999). The

creation and use of the *HEXM* transgene has previously been reported by our team to circumvent this issue (Karumuthil-Melethil et al., 2016; Osmon et al., 2016; Tropak et al., 2016b). The expressed μ-subunit, which homodimerizes to form the functional HexM enzyme, would allow for the treatment of both TSD and SD in a one-time dose administration. The need to include only the gene of this subunit allows the AAV packaging of a self-complementary construct (Karumuthil-Melethil et al., 2016; Osmon et al., 2016).

Transfer of the murine β-subunit transgene alone has also been shown to be effective in rescuing mouse models of SD (Niemir et al., 2018; Osmon et al., 2016; Walia et al., 2015). However, unlike humans, mice have an alternate biochemical pathway whereby a combination of sialidase and HexB (the homodimer of the hexosaminidase β-subunit) catabolizes GM2 (Igdoura, Mertineit, Trasler, & Gravel, 1999). Because HexB is unable to catabolize GM2 through this pathway in humans, the dose response of gene transfer studies in mice that involve expression of the murine hexosaminidase β-subunit could result in overly optimistic expectations for the translation for human clinical trials. Studies in mice involving gene transfer of both the murine α-subunit and the murine β-subunit are also subject to this uncertainty. It would be expected that in this case the two expressed subunits would form the desired HexA (heterodimer), but similar levels of HexB would be formed due to the higher dimer stability of HexB.

HEXM gene transfer, has been previously shown to effectively and significantly extend the lifespan and ameliorate phenotypic and biochemical measures in neonatal SD mice, and consequently, it has also been confirmed that this human HexM enzyme effectively functions with the mouse GM2AP-GM2 complex to hydrolyze GM2 (Osmon et al., 2016). This neonatal treatment of SD mice with a dose of $5x10^{10}$vg/mouse of scAAV9.47/*HEXM* showed an increase in survival by a factor of 2.5x in the treated animals, achieving a median survival of 40 weeks (Osmon et al., 2016). In comparison, the survival of the SD mice in the current study show a median survival of 46 weeks in neonatal mice injected with 2.5E11vg/mouse of scAAV9/*HEXM*,

(using a 5-fold greater dose and an AAV9 serotype which is different than the AAV9.47 serotype previously used). Adult stage injections of scAAV9/*HEXM* had not been studied previously. In this study, the increase in survival of the treated adult SD mice demonstrates the effectiveness of the scAAV9/*HEXM* vector to be distributed within the CNS. Excluding the deaths that occurred within one month following the vector injection, the adult HD cohort had a 3.5-fold increase in survival benefit, while the adult LD cohort showed an increased survival by a factor of 2.5x, as compared to the natural SD mouse survival. However, this increase in survival came at a cost of acute toxicity in the month following administration of the HD of the vector. The percentage of animals succumbing to the acute toxicity implies that the $1x10^{13}$ vg/mouse (~5×10^{14} vg/kg) dose is approximately the lethal dose (LD50) for this vector. Although it was investigated, the cause of death of these mice was not conclusively determined. The timing of the acute toxicity coincides with the timing of maximal gene expression, potentially implicating the HexM protein itself. Alternatively, the timing is consistent with the cause being an immune response to either the AAV9 capsid protein or overproduced HexM protein, or both. There was an increase in liver enzyme levels in samples from animals, but histopathological analysis did not identify liver damage. Analysis of serum samples for liver enzyme levels was not possible in all animals, because some were found to have died overnight preventing the collection of useful samples. There were no early deaths or observed clinical toxicity in the four mice injected with the HD of the scAAV9/GFP vector or in the 17 mice injected with the LD of the scAAV9/*HEXM* vector. Other studies have reported severe immune responses to high doses of AAV administered by intravenous infusions (Hinderer et al., 2018) or by direct brain intraparenchymal injections (Golebiowski et al., 2017). The lack of increased hexosaminidase levels in the blood (while tissue levels are increased) may be suggestive of an antibody response against HexM (data not shown). HexM is a variant of the human HexA protein, and is secreted from transduced cells, which may make it more immunogenic than GFP. A study investigating the possibility of an anti-HexM

immune response, and whether a prophylactic immune suppression regimen may ameliorate such acute toxicity, is underway in our lab.

GM2 ganglioside accumulation begins weeks or months before the primary symptoms of SD become apparent (Cachón-González et al., 2014; Steven U. Walkley & Vanier, 2009). Administration of the AAV9/*HEXM* vector at a later timepoint still conveys significant benefit i.e. Neonatal (0-week) vs Adult (6-week) administration.

Previous behavioral studies have demonstrated that SD mice exhibit a decrease in measures of strength, coordination and general locomotion (Osmon et al., 2016; Phaneuf et al., 1996; Sango et al., 1996, 1995). The current study also found a decline over time in phenotypic performance consistent with previous findings for the untreated SD mice (Kyrkanides et al., 2005; Sango et al., 1995; Walia et al., 2015). In contrast, the adult LD and HD scAAV9/*HEXM* treated mice exhibited a long-lasting improvement in performance on the Rotarod. The HD scAAV9/*HEXM* cohort exhibited significantly better performance than untreated SD mice, and sustained this level of performance throughout the entire 30-week testing period, whereas the LD group performed better than untreated SD mice and did not begin exhibiting a decline in coordination and strength as measured on the rotarod until 26 week testing (see Figure 2.4B). Then, the decline in performance of the LD group approximated the drop-off in performance seen earlier in the untreated SD mice.

The GM2 ganglioside storage analysis measured in the current study is consistent with that of previously reported levels (Osmon et al., 2016; Walia et al., 2015). The adult LD groups showed decreased GM2 ganglioside storage compared to both age-matched untreated groups (GFP/PBS) and to their LD counterparts assessed at the long-term timepoint (humane endpoint). This demonstrates that the increase in enzyme activity in adulthood was able to degrade GM2 and reduce GM2 accumulation, and therefore affect the course of the disease.

It has been theorized that raising the cellular enzyme activity levels in lysosomal storage disease above a 'critical enzyme threshold', roughly above 10-15% of the normal enzyme activity level, will be sufficient to cure the disease due to the phenomenon of metabolic cooperation or cross-correction (Conzelmann & Sandhoff, 1991; Leinekugel et al., 1992b). Metabolic cooperation occurs when the therapeutic enzymatic product is secreted by successfully transduced cells and can then be taken up by surrounding non-transduced cells (Conzelmann & Sandhoff, 1991; Leinekugel et al., 1992b). In this study, mice receiving the low dose of scAAV9/*HEXM* had a level of hexosaminidase enzyme activity in their MBs averaging 13.9% of the level found in the MBs of heterozygous mice at 16 weeks of age. Mice receiving the high dose (and surviving the acute toxicity period) had a level of hexosaminidase enzyme activity in their MBs averaging 55.9% of the level of 16-week-old heterozygotes. The increase in these average levels of HexA activity was sufficient to provide a survival benefit, but it was not sufficient to enable the mice to live to a normal lifespan (Figure 2.5 A&B, Supplementary Figure 2.1 A&B). However, it should be noted that these are the average activity levels across a varied population of cells from the MB samples and may not be indicative of the enzyme activity within individual cells or more discrete CNS regions.

The biodistribution of the scAAV9/HEXM treatment in the adult mice was noted to be similar between animals terminated at 16 weeks and those terminated at long-term (humane endpoint) timepoints. This suggests there was little to no elimination of vector genomes from animals from the 16-week time-point onwards. In addition, similar presence of the vector genome throughout the brain and spinal cord tissues of all adult and neonatal treated mice was observed, indicating that the scAAV9/HEXM vector crossed both the mature (in 6-week old mice, Figure 2.7A) and immature blood brain barrier (in neonates, Supplementary Figure 2.1D). There was a significant effect of scAAV9/HEXM dose on the copy numbers of HexM DNA found in the heart ($p < 0.001$) and liver ($p < 0.01$) of mice at their humane endpoint, with greater copy numbers

found in the higher dose group. Among mice receiving the lower dose of scAAV9/HEXM, there were significantly fewer copies of HexM DNA found in the livers of mice at their humane endpoint, compared to the copies in the livers of the mice that were terminated at 16 weeks of age. A repeated measures ANOVA showed a significantly higher number of copies in the CNS of mice receiving the higher dose versus those receiving the lower dose ($p < 0.05$) when considering the CNS regions collectively. Post hoc tests on a region-by-region basis were not significant; however, the data suggest that the dose effect was likely greatest in the region of the cervical spinal cord ($p < 0.10$). A significant increase in uptake and persistence of the scAAV9/HEXM vector by a factor of 2.34x throughout the central nervous system was seen when the IV LD administration was paired with mannitol (Figure 2.7B). This is consistent with the expected effect of mannitol on the blood-brain barrier (Bourgoin et al., 2003; Brown et al., 2004; Carty et al., 2010; Mastakov et al., 2001).

Intracarotid delivery of a high dose of a hyperosmotic agent has been shown to disrupt the ipsilateral blood brain barrier and enable transport of therapeutic agents into the CNS from the blood stream (Bourgoin et al., 2003; Brown et al., 2004; Carty et al., 2010; Mastakov et al., 2001). Both mannitol and arabinose are commonly used as hyperosmotic agents in animal studies. Intravenous mannitol is currently approved for human use by the US Food and Drug Administration (FDA) and is labeled for reduction of intracranial pressure and brain mass. For the reduction of intracranial pressure in adults, the FDA labeling for IV mannitol recommends a dose range of 0.25 to 2.0 g/kg body weight infused over a period of 30 to 60 minutes. This does not mean that the use of mannitol is without risk. Complications for intravenous mannitol include severe hydration, hyperkalemia, hemolysis, and acute renal failure.

The use of mannitol to reduce intracranial pressure has also been performed in the clinical and pre-clinical setting (Archer, Freymond, & Ravussin, 1995; Brown et al., 2004; Fandino, 2017; Foley et al., 2014; Mastakov et al., 2001; D M McCarty, DiRosario, Gulaid,

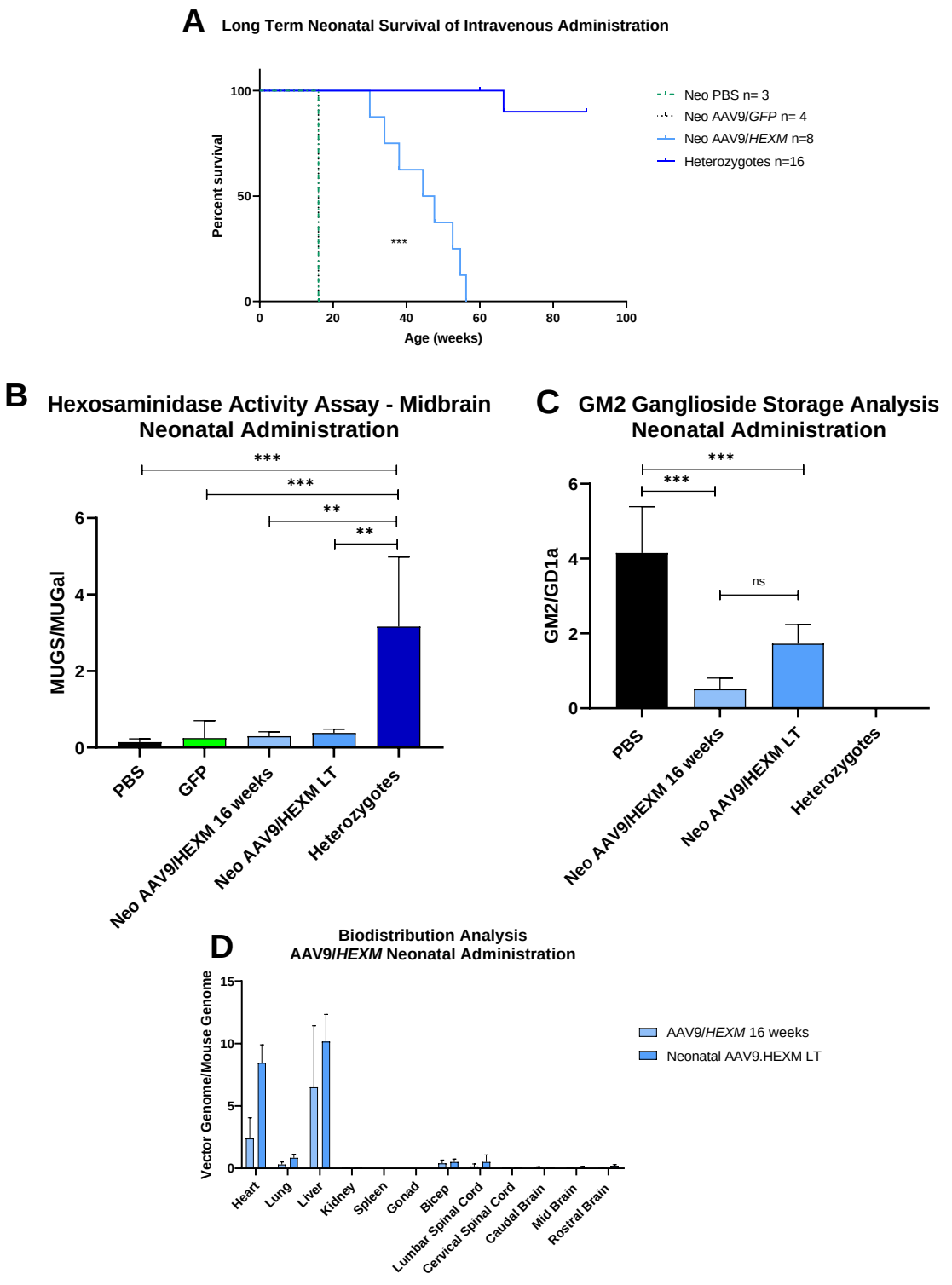
Muenzer, & Fu, 2009; Winkler & Munoz-Ruiz, 1995). One such preclinical study investigated the administration of an intra-arterial injection of mannitol followed by the gene therapy treatment 2 minutes later (Foley et al., 2014). The optimal time in which to deliver a therapeutic after the mannitol has been delivered to disrupt the BBB is 0-5 minutes. This study, as many others, used a concentration of 25% wt/vol of mannitol and determined that there was a minimum period of hyperosmolarity required for sufficient BBB disruption. Severe adverse events have been observed clinical studies for the treatment of brain tumors using unilateral vertebral or carotid artery infusion of 25% mannitol at 5 to 12 mL/s for 30 seconds to disrupt the BBB. These include non-reversible neurological damage (~1%) and seizures (~14%) (Rapoport, 2000).

While the current study showed an increase in CNS vector uptake when preceded by a mannitol injection, the rapid intravenous injection, the total fluid volume of 25% mannitol, plus the hyperosmolarity of the vector vehicle may have been excessive for the three mice that required humane euthanizations within a month following vector injection. A similar study used 25% mannitol at a dose of 1-2mg/g of mouse to facilitate the movement of a gene therapy treatment across the BBB (McCarty et al., 2009), which is lower than the dose used in the current study (3g/kg=3mg/g mouse). Further investigation of the dose and route of mannitol delivery (e.g., intracarotid) is required to identify a safe and effective means of enhancing AAV vector transport across the BBB.

Previous studies utilizing AAV gene therapy have reported high incidences of hepatic or lung tumors (Bell et al., 2006; Donsante et al., 2001; Russell, 2007; Walia et al., 2015). Throughout the course of this study, we did not encounter any tumors. This may be due to the synthetic promoter used in the scAAV9/*HEXM* treatment that confers moderate levels of expression, since it has been shown that tumorigenicity can be promoter dependent correlating with stronger expression (Chandler et al., 2015b).

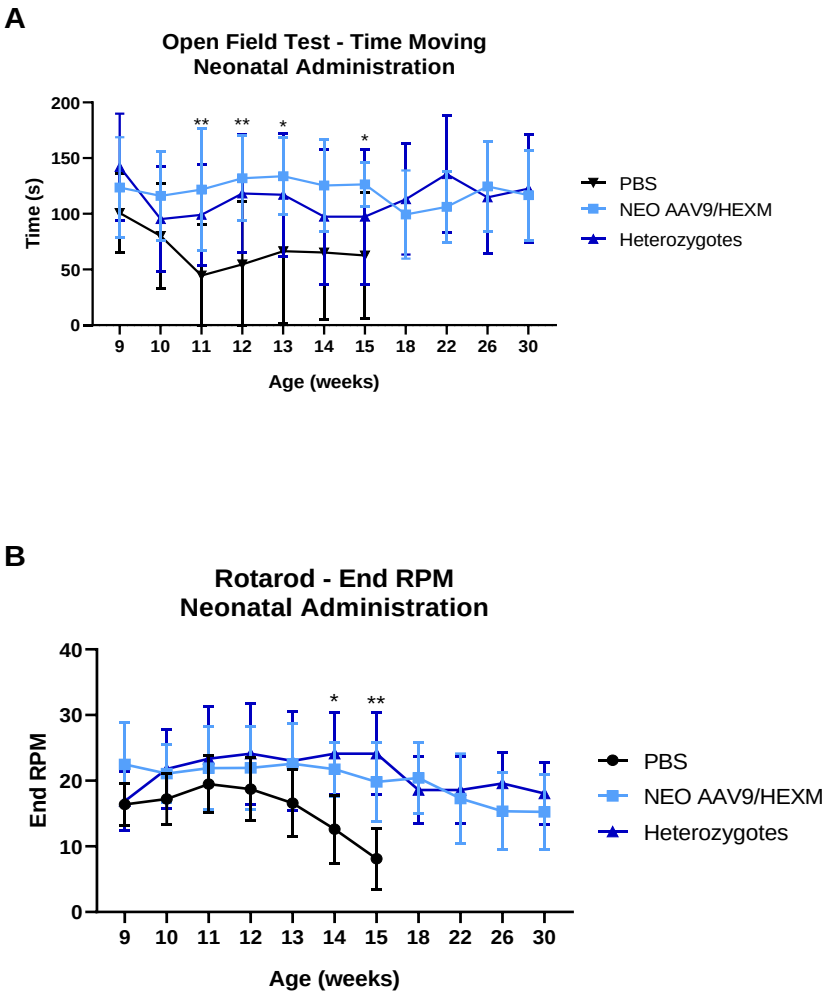
In the current study, we investigated the efficacy of 2 different doses on the adult administration of AAV9/*HEXM* in the SD mice. The data obtained support our hypothesis of a dose-response to scAAV9/*HEXM*; namely, that a higher dose can confer a greater benefit than a lower dose. However, this support was limited to mice that did not have a toxic acute reaction to the higher dose. With a dose of $1x10^{13}$ vg/mouse (HD) there was an approximate 57% lethality as shown in Figure 2.3A. Excluding the mice lost to acute toxicity from the adult HD cohort, there was an increase in survival, behavior, biochemical and molecular outcomes when comparing HD and LD adult injected groups. Excluding the acute toxicity, we noted that in these adult mice, with a 4x dosage increase, there was a 1.44x increase in survival benefit and an increase in brain hexosaminidase activity. The adult LD and HD cohorts showed 2.5-fold and 3.7-fold increases in survival over the natural lifespan of SD mice. The adult HD group outperformed the adult LD cohort throughout the behavioral testing, including the 26- and 30-week testing timepoints, when the LD cohort began declining in its coordination measures on the RR (Figure 2.4B). There was substantially less GM2 ganglioside in both the neonatal and adult scAAV9/*HEXM* recipients, as compared to the accumulation seen in the PBS treated group (Supplementary Figure 2.1C & Figure 2.5B). Molecular analyses demonstrated the vector's ability to cross the blood-brain barrier and distribute across the brain and spinal cord (Figure 2.7).

While the current study clearly shows that the scAAV9/*HEXM* vector can be effective in treating SD mice, further investigations are warranted in optimizing a method of delivering gene vectors to the CNS to safely maximize treatment efficacy. Nevertheless, this study provides a solid step towards the translation and justification of a future clinical trial of scAAV9/*HEXM* gene therapy for TSD and SD.



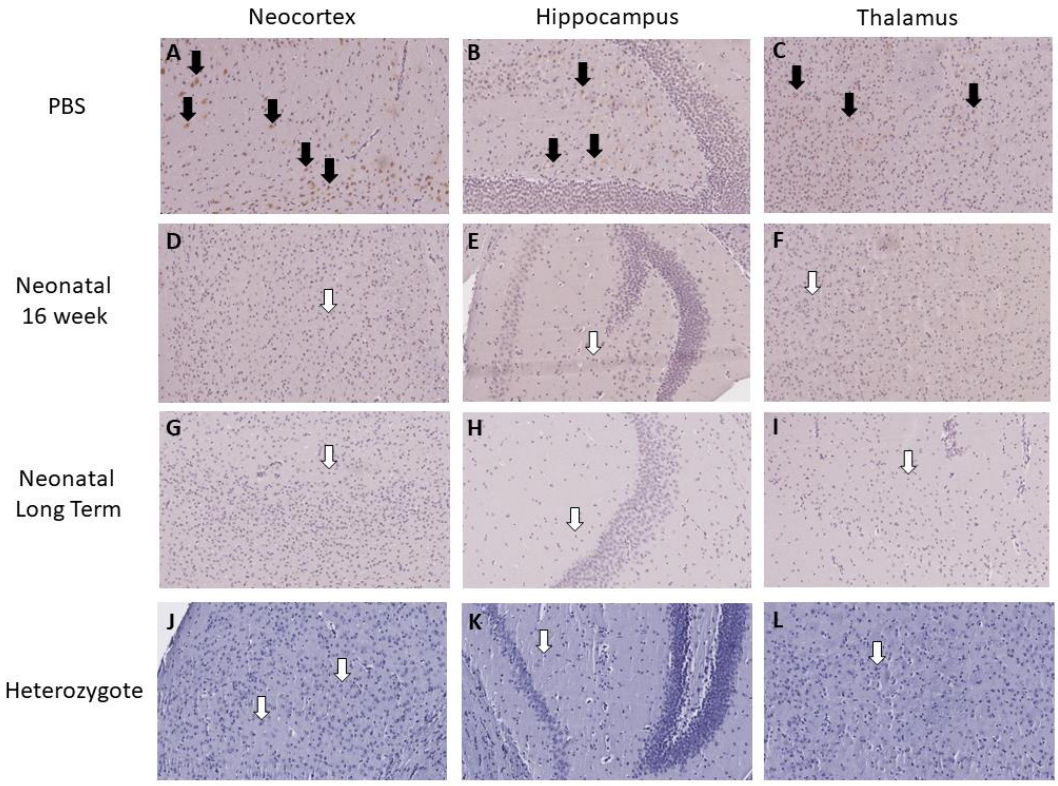
Supplementary Figure 2.1. Survival, Biochemical and Molecular Outcomes of the Neonatal IV scAAV9/*HEXM* Administration.

Figure Caption Supplementary Figure 2.1: (A) Survival of the AAV9/*HEXM* neonatal cohort. The neonatal administration showed a highly significant survival benefit compared to the typical lifespan of approximately 16 weeks (R= 15-21) ($p<0.001$). (B) Midsection of the Brain (MB) Hexosaminidase Activity Assay. There was a significant effect of treatment group on the amount of hexosaminidase activity measured ($F_{7, 33}=8.752$, $p<0.0001$). The 16-week MB homogenates from the neonatal scAAV9/*HEXM* cohort showed a slight non-significant increase in HEXM activity when compared to untreated PBS and GFP cohorts. The long-term scAAV9/*HEXM* cohort also showed a nonsignificant increase in the amount of HEXM activity as compared to untreated controls. The heterozygous cohort served as controls for normal hexosaminidase activity levels, therefore the normal levels of activity in this cohort differs significantly from the GFP and PBS cohorts ($p<0.001$ for both), the neonatal 16 week and LT mice ($p<0.01$ and $p<0.01$, respectively). (C) Ganglioside Storage Analysis. There was a significant effect of treatment group on the amount of GM2 ganglioside stored ($F_{7,61}= 40.12$, $p<0.0001$). When compared to the PBS and GFP groups, there was a significant decrease in the ganglioside storage of the neonatal scAAV9/*HEXM* cohort at 16 weeks ($p<0.001$, $p<0.05$). There was no significant difference between the amount of GM2 stored in the 16 week and LT timepoints of the neonatal scAAV9/*HEXM* cohort. As compared to the heterozygous controls, the PBS and neonatal scAAV9/*HEXM* LD LT showed a highly significant increase in the amount of GM2 gangliosides stored ($p<0.001$), and the Neo scAAV9/*HEXM* 16-week group showed no significant increase in storage. (D) Molecular Analysis- Biodistribution Analysis of the Neonatal scAAV9/*HEXM* Administration, presented as the number of vector genomes per diploid mouse genome. The central nervous system retained similar biodistributions of the scAAV9/HEXM vector from the 16-week analysis to the long-term endpoint.



Supplementary Figure 2.2. Behavioral Outcomes of the Neonatal IV scAAV9/*HEXM* Cohort

(A) Open Field Test - Time Moving. From 9-15 weeks, there was a significant difference observed among treatment groups in the OFT ($F_{6, 35}$=4.02, p<0.0001). The OFT showed significance in the general locomotion of the neonatal AAV9/*HEXM* group at 11, 12, 13, and 15 weeks of age as compared to PBS controls (p<0.01, p<0.01, p<0.05, p<0.05, respectively). In addition, all scAAV9/*HEXM* treated animals did not have significant changes in their general locomotion compared to heterozygous controls (p>0.05). Over the long-term time-course (18-30 weeks) there were no statistical differences between the locomotion of the treatment groups in the OFT. (B) Rotarod - End RPM. There was a significant effect of treatment observed ($F_{5, 33}$=2.98, p=0.0250). Over the time-course as measured in the RR, the coordination of all scAAV9/*HEXM* treatment groups approximated the activity of the heterozygous control mice. In addition, the heterozygous controls performed significantly better than the untreated PBS cohort at 14 and 15 weeks (p<0.01, p<0.001, respectively). The neonatal cohort outperformed the PBS controls at both 14 and 15 weeks of age (p<0.05, p<0.01, respectively).



Supplementary Figure 2.3. Histological Analysis of the Neonatal IV scAAV9/*HEXM* Cohorts.

Histological ganglioside analysis of the neonatal scAAV9/*HEXM* administrations. Sections of the murine neocortex, hypothalamus, and thalamus, from the PBS cohort (A-C), the scAAV9/*HEXM* neonatal 16-week (D-F) and long term (G-I) cohorts. Black arrows indicate GM2 ganglioside filled neurons, which were found predominantly throughout the PBS and adult long-term cohorts. The white arrows indicate similar nuclei lacking the GM2 ganglioside accumulation. GM2 gangliosides were completely cleared in the intravenous neonatal administration of AAV9/*HEXM* (D-I), approximating the heterozygous controls (J-L).

Chapter 3

Impact of the Age of Administration of AAV9 Murine and Human Bicistronic Hexosaminidase Vectors in Sandhoff Mice on the Correction of GM2 Gangliosidosis

Karlaina JL. Osmon, Natalie M. Deschenes, Brianna M. Quinville, Shalini Kot, Alex Ryckman, Eminet Bogale, Zhilin Chen, Melissa Mitchell, Clifford Heindel, John G. Keimel, William F. Kaemmerer, Steven J. Gray, and Jagdeep S. Walia

Statement of Authorship:

The research described herein was conducted by Karlaina Osmon under the supervision of Dr. Jagdeep S. Walia. All data within this thesis was collected and analysed by Karlaina Osmon (Survival, Hexosaminidase Activity, GM2 Ganglioside Storage, Behavioural Testing, Biodistribution, Immunological Assays) with the exception of a subset of behavioural testing timepoints and blood collections, which were performed by Natalie Deschenes, Shalini Kot, Alex Ryckman, Eminet Bogale, and Melissa Mitchell (from Walia Lab, Queen's University) due to Karlaina developing a severe allergy to the experimental animals (Figure 3.5 - Figure 3.18). Natalie Deschenes, Shalini Kot, Alex Ryckman, Brianna Quinville, and Zhilin Chen also contributed significantly to the humane endpoint collections for this study. Additionally, the vector production was performed by Subha Karumuthil-Melethil and Clifford Heindel under the supervision of Dr. Steven Gray at the University of North Carolina, Chapel Hill. The qPCR analysis for biodistribution data was performed by both Karlaina and Zhilin Chen (Figure 3.25). Brianna Quinville assisted Karlaina in the anti-HexA ELISAs and ELISPOT (Figure 3.26). John Keimel and William Kaemmerer (New Hope Research Foundation) contributed intellectually and provided biostatistics expertise.

3.1 Abstract

GM2 Gangliosidoses are severe neurodegenerative diseases caused by the progressive accumulation of GM2 gangliosides (GM2) due to a deficient Hexosaminidase A (HexA). HexA enzymes are comprised of an α- and β- subunit and complexes with the GM2 activator protein to breakdown GM2. Mutations in the HEXA, HEXB, or GM2AP genes cause deficiencies in the α-, β- subunit, or GM2AP giving rise to severe neurodegenerative diseases, such as Tay Sachs Disease (TSD), Sandhoff Disease (SD), or the AB-variant disease, respectively. The infantile forms of GM2 Gangliosidosis are fatal by 4 years old, and there are no current treatments. The progressive neurodegeneration of the brain leads to a variety of symptoms including motor and sensory impairments, paralysis and seizures. The current standard of care for these patients is palliative support. The investigation into alternative treatment options for GM2 Gangliosidosis is ongoing. Here, we evaluated the impact of the age of administration of gene therapy vectors in a SD mouse model. AAV9 vectors containing either one or two hex subunits - a human bicistronic (human *HEXB-HEXA*; hB-A), a murine bicistronic (murine *Hexb-Hexa*; mb-a), or a murine Hexb only (*mHexb*), were administered intravenously to SD mice at 0 weeks of age (at $2.5x10^{11}$vg/mouse or ~$1.25\text{-}2.5x10^{14}$ vg/kg) or 2, 4 or 6 weeks of age (at $1x10^{12}$ vg/mouse, $1.88x10^{12}$ vg/mouse, and $2.5x10^{12}$ vg/mouse (respectively) or ~1.25E14 vg/kg) (N=10 for each vector at each age of administration). Half of the mice in each cohort were euthanized at 16 weeks, equivalent to the humane endpoint for untreated controls. The rest were observed until their humane endpoint. All of the vector treatments conveyed improvements in the SD mice in their survival compared to vehicle-treated controls ($p<0.001$). Mice were treated at 0, 2, 4, and 6 weeks with the human bicistronic vector survived to a median age of 59, 68.4, 43.6, and 43.8 weeks and mice treated with the murine bicistronic vector survived to median age of 42.2, 76.8, 65.8, and 68.14 weeks, respectively. Mice treated with the ssAAV9/*mHexb* vector only at 0 and 6 weeks and they survived to a median age of 65.72 and 70.71 weeks respectively, with one mouse

from the 0-week ssAAV9/*mHexb* group surviving to the 104-week study endpoint. A significant increase in behavioural performance was observed at 16 weeks in all treatment groups across all ages of administration ($p<0.01$). At all ages of administration, analyses showed slight non-significant increases of midsection of the brain HexA activity across all treatment groups, significant decreases in GM2 storage in the mHexb, hB-A and mb-a groups ($p<0.001$), and constant hB-A biodistribution across the midsection of the brain. Humoral response was checked in mice receiving hB-A at 6 weeks of age. An increased humoral (anti-hHexA antibodies; $p<0.0001$), cellular response against the human HexA peptides was noted ($p<0.05$). A cross-species antibody response was expected, however the human bicistronic vector may not elicit an immune response in patients. Study limitations include the existence of an alternative sialidase pathway for GM2 catabolism in mice, the differential affinity of the subunits, the immature blood-brain barrier in mice at birth, and the possible interaction of the human HEXA enzyme with the murine GM2AP, each of which may have enhanced efficacy of the bicistronic treatment. Nevertheless, this data demonstrates that a bicistronic hexosaminidase AAV9 vector may be a viable option for the treatment of SD and TSD in humans.

3.2 Introduction

GM2 Gangliosidosis is a group of devastating neurodegenerative lysosomal storage disorders characterized by deficiencies in the Hexosaminidase A (HEXA) enzyme complex (Sandhoff, 2012; Sandhoff & Harzer, 2013). In humans, HEXA is the sole enzyme capable of catabolizing GM2 Gangliosides (GM2) (Sandhoff & Harzer, 2013; Sandhoff & Kolter, 2003). The HEXA is a heterodimeric enzyme comprised of an α- and a β- subunit, coded by the *HEXA* and *HEXB* genes, respectively. The HEXA enzyme complexes with the GM2 activator protein which is responsible for shuttling the GM2 lipids to the lysosome for degradation (Sandhoff, 2012). Mutations in the genes encoding the α-, β- subunit or the GM2 activator protein results in the formation of one of three forms of GM2 Gangliosidosis: Tay Sachs Disease (TSD), Sandhoff Disease (SD), or the AB-variant, respectively (Don J. Mahuran, 1999; Sandhoff & Harzer, 2013). As GM2 is a predominant component of the neuronal cells, the toxic accumulation of GM2 in the neuronal lysosomes of the central nervous system (CNS) leads to vast neurodegeneration (Sandhoff, 2012; Sandhoff & Harzer, 2013; Sandhoff & Kolter, 2003).

The incidence of TSD and SD are 1 in 422, 000 and 1 in 222,000, respectively, throughout the general population, however the incidence increases in those of Ashkenazi Jewish, French Canadian, or Northern Saskatchewan descent (Andermann et al., 1977; Fitterer et al., 2014; Myrianthopoulos, 1962). Clinically, there are three distinctive divisions of GM2 Gangliosidosis based on age of onset and severity (Bley et al., 2011; Conzelmann & Sandhoff, 1983, 1991; Leinekugel et al., 1992a; Sandhoff & Harzer, 2013). The infantile version presents around 6 months of age in which an infant begins regressing through the developmental milestones (i.e., they can no longer sit up or support their head), and they progressively develop motor, sensory, and neurological impairments, such as loss of motor control, ataxia, vision or hearing loss, and seizures etc. Infantile GM2 Gangliosidosis arises from mutations causing a complete non-functional HEXA enzyme complex (Bley et al., 2011; Sandhoff & Harzer, 2013).

Despite the best palliative care, most of these infants do not survive past the age of four. The juvenile and adult onset of GM2 Gangliosidosis occurs around 8-10 years of age and ~27-30 years of age, respectively, in which the age of onset is inversely correlated with the amount of residual activity retained by the mutated subunit or activator protein in the HEXA complex (Conzelmann & Sandhoff, 1983, 1991; Leinekugel et al., 1992a). As with other lysosomal storage disorders, it has been hypothesized that increasing the enzymatic activity above a critical threshold (~15% of normal wild-type levels) will provide a curative therapeutic benefit. These diseases are fatal with no current curative treatment (Conzelmann & Sandhoff, 1991; Leinekugel et al., 1992a).

The search for a gold standard treatment for these devastating neurodegenerative diseases has been ongoing for the past few decades (Cachón-González et al., 2018; Mylvaganam Jeyakumar, Dwek, Butters, & Platt, 2005; Platt, 2018). Studies have shown some success in treating lysosomal storage disorders through a variety of strategies, including small molecular chaperones, proteostasis regulators, enzyme replacement therapy, stem cell replacement therapy, substrate reduction therapy, and anti-inflammatory therapies (Cachón-González et al., 2018; Platt, 2018). Many of these strategies are viable for some of the affected individuals but are not a complete treatment for more than one division of GM2 Gangliosidosis or GM2 Gangliosidoses. Small molecular chaperones (Banning et al., 2016; Chiricozzi & Niemir, 2013; Clarke et al., 2011; J.-Q. Fan, 2007; J.-Q. Fan et al., 1999; Maegawa et al., 2007; Parenti, 2009; Parenti, Fecarotta, et al., 2014; Parenti et al., 2013) and proteostasis regulators (Calamini et al., 2011; Kirkegaard et al., 2016; Petersen & Kirkegaard, 2010; Platt, 2018) would only aid those patients with residual enzymatic activity. Enzyme replacement therapy has been shown to work, but since the majority of these treatments are administered intravenously, the main potential drawback is the inability for large proteins to the trafficked across the blood-brain barrier (BBB) (Brady, 2006; Gelder et al., 2015; Johnson et al., 1973; Matsuoka et al., 2011; Platt, 2018; Tsuji et al.,

2011; van Gelder, Vollebregt, Plug, van der Ploeg, & Reuser, 2012). In addition, enzyme replacement may be an easier option for those who retained some level of mutated protein since the immune system would have been partially tolerized to the enzyme being replaced(Gelder et al., 2015). Stem cell replacement has the same drawbacks as enzyme replacement therapies in addition to needing to find a donor match and the risks of transplanted tissue rejection (Krivit, 2002; Krivit et al., 1999; S. U. Walkley & Dobrenis, 1995). Substrate reduction therapy aims to decrease the biosynthesis of the substrate that once accumulated leads to the neurodegeneration of the CNS (Andersson et al., 2004; Belmatoug et al., 2017; Coutinho et al., 2016; Platt & Jeyakumar, 2008; Platt et al., 1997). These drugs would help halt the progression of the lysosomal storage disease, but not reverse any previous accumulation. Lastly, anti-inflammatories target in inflammatory response to the neurotoxic buildup of the substrate but does not help negate the root cause of the disease (M. Jeyakumar et al., 2003; Mylvaganam Jeyakumar et al., 2004; Wada et al., 2000). Gene therapy however has the potential to be a lasting treatment for GM2 Gangliosidosis with a one-time administration (Haskins, 2009; Herzog et al., 2010; Michaël Hocquemiller et al., 2016; Thomas et al., 2003).

Gene therapy is a viable treatment option for GM2 Gangliosidosis for a variety of reasons. Mainly, GM2 Gangliosidosis disorders are monogenic (Don J. Mahuran, 1999; Sandhoff & Harzer, 2013), meaning there is one single gene causing the disease, and the addition of the correct, wild type gene could theoretically treat or cure the disease. Secondly, although gangliosides are predominantly found in the CNS, they do play a role in other cell types throughout the body, and therefore a systemic gene therapy treatment could have few off target effects by eliciting a treatment for the peripheral organs (Sandhoff & Harzer, 2013). In addition, an overexpression of hexosaminidase enzymes has not been shown to be detrimental. Gene therapy would also enable to treatment of all ages of onset and depending on the transgene(s) used, could treat both SD and TSD patients with one vector and one treatment. Next, the HEXA

enzyme is a secreted protein, which, through cross correction, would enable cells successfully transfected by the gene therapy to produce and secrete the therapeutic HEXA protein to be taken up by the surrounding cells (Conzelmann & Sandhoff, 1983; Leinekugel et al., 1992a). Lastly, the requirement of increasing the enzymatic activity above a critical threshold of ~15% requires partial correction for treatment efficacy (Conzelmann & Sandhoff, 1983; Leinekugel et al., 1992a).

Neural-based gene therapy trials have largely centered around adeno-associated viruses (AAVs) for a variety of reasons (Berns & Muzyczka, 2017; Cachón-González et al., 2018; Foust et al., 2009; Hocquemiller et al., 2016; Mendell et al., 2017; Thomas et al., 2003). AAVs are non-pathogenic small viruses capable of infecting, dividing, and non-dividing mammalian cells (Berns & Muzyczka, 2017; Cachón-González et al., 2018; Foust et al., 2009; Hocquemiller et al., 2016; Mendell et al., 2017; Thomas et al., 2003). Additionally, AAV serotype 9 (AAV9) has been shown to cross the BBB and transfect both neuronal and astrocytic cells in the murine CNS. Although AAVs do have a limited packaging capacity, which constrains the length of genetic information able to be packaged into an AAV. A single stranded AAV (ssAAV) encapsulates single stranded DNA as their genomes enabling the packaging of ~4.8kb, whereas the self-complementary AAVs (scAAV) encapsulates a double stranded genome, meaning their genomes are half the size of the ssAAV (~2.4kb) (Grieger & Samulski, 2005; J. Wu et al., 2007; T. Wu et al., 2012; Z. Wu et al., 2010b). ssAAVs must undergo the process of double strand synthesis before therapeutic transcription and translation can occur, while the scAAV does not require this initial double strand synthesis and can proceed to expressing the therapeutic gene much quicker, therefore providing more efficient transgene expression (Gray et al., 2011; McCarty, 2008; J. Wu et al., 2007). However, to accommodate a larger treatment construct, such as a bicistronic hexosaminidase construct, concessions on therapeutic timing of the treatment can be made.

Like humans, the murine model for SD is also born asymptomatic and is characterized by a progressive accumulation of GM2 Gangliosides across the CNS (Phaneuf et al., 1996; Sango et al., 1995). In the SD mice, symptoms begin appearing around 12 weeks of age, and the humane endpoint for these mice is 16 weeks of age (Phaneuf et al., 1996; Sango et al., 1995). The humane endpoint for these SD mice is typically characterized by the loss of 15% body weight or the inability to right itself but may include seizures. SD mice exhibit motor impairments similar to that seen in human symptomology (Phaneuf et al., 1996). Unlike humans, the murine model of TSD has no overt symptoms and stores very little GM2 due to their alternative sialidase degradation pathway not found in humans (Phaneuf et al., 1996; Sango et al., 1995; Suzuki et al., 1998), as discussed below.

Previous studies have investigated the use of gene therapy for the treatment of GM2 Gangliosidosis (Arfi et al., 2005; Bourgoin et al., 2003; Bradbury et al., 2013; Cachón-González et al., 2006, 2012, 2014; Guidotti et al., 1999; Karumuthil-Melethil et al., 2016; Kyrkanides et al., 2005; Niemir et al., 2018; Osmon et al., 2016; Sargeant et al., 2011; Tropak et al., 2016b; Walia et al., 2015; Woodley et al., 2019). Many studies have focused on the administration of the gene mutated in that particular disease (ie HEXA to treat TSD, and HEXB to treat SD) with moderate success (Bourgoin et al., 2003; Cachón-González et al., 2006; Guidotti et al., 1999; Martino et al., 2005; Niemir et al., 2018; Walia et al., 2015). However, it has been shown that treating with only one subunit can elicit a rate limiting situation for the endogenous subunit (Guidotti et al., 1999). These findings may be due to the expression of the treatment vector being considerably higher than endogenous levels, for example in SD, only providing the *HEXB* gene will result in an excess of the β-subunit at the cellular level as compared to the endogenous α-subunit, and thus an inability for the heterodimeric HEXA enzyme to form as it favours the formation of the HEXB (β-subunit homodimer) when in high concentrations (Guidotti et al., 1999). Therefore, treating with both the HEXA and HEXB genes would negate this rate limiting situation by equally over-

expressing both subunits. There are many strategies with which both *HEXA* and *HEXB* genes can be used simultaneously to treat GM2 Gangliosidosis, but the use of two separate vectors for each transgene could lead to increased viral toxicity and an immunogenic response possibly negating the treatment. Moreover, there will always be uncertainty that both transgene constructs were delivered in pairs to all the cells. However, the use of a bicistronic construct encapsulated in one viral vector negates the requirement of excess viral dosages and could ensure an equal over expression of both the α- and β-subunits in all cells infected. This was shown to the true in a study utilizing a lentiviral hexosaminidase bicistronic construct that utilised the IRES linker in order to accommodate both the HEXA and HEXB genes (Kyrkanides et al., 2005). The drastic improvements in the treated SD mice included improved locomotion, increased HexA activity as well as a decrease in GM2 accumulation. This study also noted the halt of neuroinflammation due to their neonatal intervention (Kyrkanides et al., 2005).

An additional drawback to the studies assessing a HEXB gene therapy treatment in the SD mice is the added complication of the alternative murine sialidase pathway that is not always considered (Niemir et al., 2018). The alternative sialidase pathway uses the sialidase enzyme followed by the β- homodimer, HEXB, to degrade GM2 (Suzuki et al., 1998). The treatment of mice with only the HEXB gene can lead to data that overstates the recovery of the mice due to the potential treatment of the SD mice with both the normal GM2 degradation through the HexA enzyme as well as through the alternative sialidase pathway (Niemir et al., 2018). This sialidase pathway is not found in humans (Sango et al., 1995; Suzuki et al., 1998), and therefore must be taken into consideration when investigating treatments in a pre-clinical setting. Therefore, a treatment incorporating both Hex subunits would theoretically decrease the amount of HEXB made in favour of the HEXA heterodimer, as long as the subunits are expressed at relatively equal levels. This could be accomplished by a variety of means, including previous attempts at co-delivering an AAV9/*HEXA* and AAV9/*HEXB* treatment (Bradbury et al., 2013; Cachón-

González et al., 2006, 2012, 2014), or as in this current study, the delivery of one bicistronic treatment encoding both the *HEXB* and *HEXA* genes. Another alternative strategy to treating with both subunits has been explored, where the use of a hybrid hexosaminidase subunit (μ-subunit) capable of homodimerizing to form the HEXM enzyme to functionally replace HEXA showed promise in neonatal SD and TSD mice, as well as adult SD mice (Karumuthil-Melethil et al., 2016; Osmon et al., 2016; Tropak et al., 2016b, Chapter 2 above).

Previous studies have investigated the effect of age of administration using an AAV2/1 vector containing either the human *HEXA* or *HEXB* transgenes (Cachón-González et al., 2014). This study utilized intracranial stereotaxic injections of a mixture of both *HEXA* and *HEXB* constructs at 4- 8- 10- and 12-weeks of age. They concluded that earlier intervention leads to more favourable outcomes and that 10-weeks is the latest possible point of intervention for the treatment of the SD mice using the intracranial injections of both viral constructs (Cachón-González et al., 2014). This strategy proved to be successful with 4-week injected mice surviving to a median of 87.85 weeks, with just over 20% of the cohort surviving up to the 2-year study endpoint. Although these findings are promising, intracranial injections are considered extremely invasive and may not translate well to human clinical trials. This study aims at investigating the effects of an intravenous injection, a considerably less invasive route of administration using ssAAV9 vectors that have been shown to cross the BBB and effectively infect neurons and astrocytes. In addition, to our knowledge, no previous study, other than the one discussed below from our laboratory, has investigated the use of a bicistronic hexosaminidase construct encapsulated in an AAV9 vector for the treatment of GM2 Gangliosidosis. The use of a bicistronic *HEXA* and *HEXB* construct diminishes the viral load by half compared to using two separate viruses for each hex gene.

Preliminary work done on the human bicistronic vector utilized in the current study has found that the ssAAV9/hB-A treatment expressed the complete HEXA enzyme *in vitro* and *in*

vivo (Woodley et al., 2019). Woodley et al. investigated the use of the human bicistronic construct at doses well below therapeutic levels, but still showed an increase in lifespan and improvements in behavioural parameters of the treated SD mice. It was also shown that the human bicistronic vector was able to make viable HexA protein via western blot and diethylaminoethyl analysis, which separates the three separate hexosaminidase isoenzymes through fractionation (Woodley et al., 2019). The current study expands upon this knowledge base with the use of a human and murine bicistronic vectors being delivered at therapeutic doses to mice at a variety of ages, but also explored the differences, if any, for cross species genes used in the vectors in bicistronic form.

The objective of the current study was to observe the in vivo effects of the HEX gene therapy treatments – the human bicistronic ssAAV9/h*B-A* vector, the murine bicistronic ssAAV9/m*b-a* vector, and (3) a ssAAV9/*mHexb* vector - in SD mice at neonatal, juvenile and adult ages (administrations at 0-week, 2- and 4-week, and 6-weeks, respectively). This study set out to investigate which of the three experimental vectors was superior, and which age of administration leads to the most beneficial outcomes. We hypothesize that: (1) earlier intervention will produce more favorable survival and other outcomes, (2) the human and murine B-A bicistronic vectors will produce similar beneficial outcomes, and (3) the human and murine B-A bicistronic vectors will provide an increase in beneficial outcomes compared to AAV9/*mHexb* alone.

3.3 Methods

3.3.1 Vector Design

This study utilizes the ssAAV due to the length of the hexosaminidase genes (~1.6kb), and the requirement of a linker for the bicistronic construct since expression cassettes exceeding 4.8kb are often truncated around 4.8kb in order to fit into the capsid of the AAV virus (Grieger & Samulski, 2005). Therefore, in the current study, the bicistronic treatment designs could not exceed ~4.8kb. The 2A linkers has been shown to be effective linkers able to cleave two connected genes during translation. The 2A linkers are compact and require much less room than conventional hand-foot and mouth disease linkers. Considering the small packaging capacity of the AAV vectors, it was imperative that the linker take up minimal space in the expression cassettes but still linked the entire *HEXB* and *HEXA* genes (~1.6kb, each) in order to get relatively equal expression of each subunit. The P2A linker was shown to be superior at the cleavage of two genes in vitro and in vivo and was therefore used to link the human and murine hex genes in their respective plasmids. In addition, the bicistronic treatment vectors could not contain the WPRE enhancer element due to the packaging restraints, however the GFP and murine Hexb only plasmids contained the WPRE enhancer element.

The bicistronic plasmids were designed to incorporate the full length murine or human *HEXA* and *HEXB* genes, linked by a P2A linker. The murine hexb plasmid was also designed and all three constructs were synthesized by Biobasics, a commercial company in Toronto, ON. The cDNA for the human *HEXA* and *HEXB* genes, as well as the cDNA for the murine *Hexa* and *Hexb* genes were obtained from the central NCBI data base (NM_000520.4, NM_000521.3, NM_010421.4, NM_010422.2, respectively). The GFP control plasmid and the three experimental plasmids were then prepped and sent to the University of North Carolina's vector core facility to be packaged into ssAAV9 capsids as previously described (Gray et al., 2013;

Karumuthil-Melethil et al., 2016; Osmon et al., 2016). See Figure 3.1 for AAV genome design schematics.

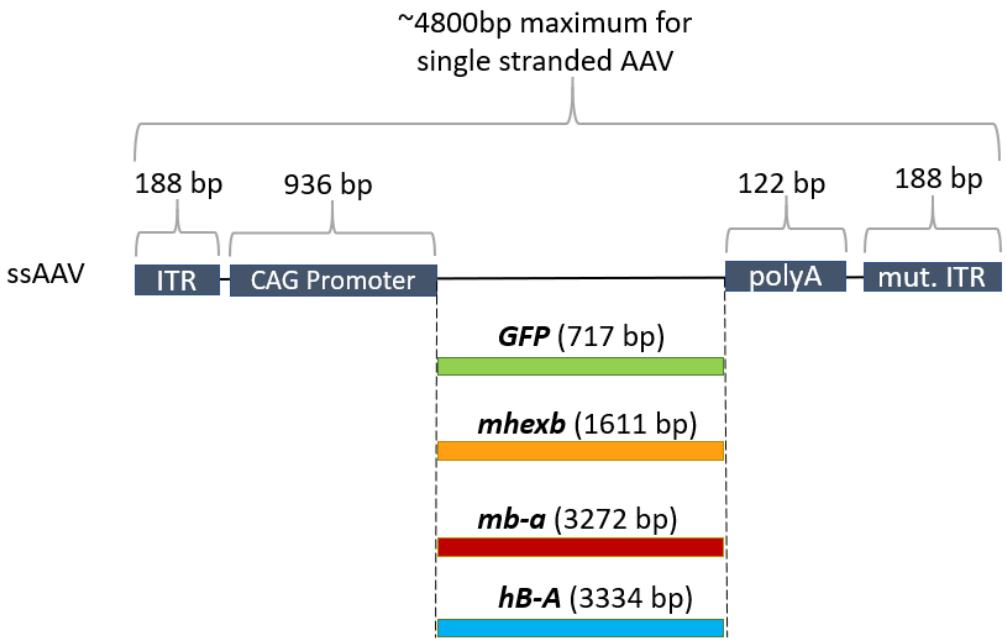


Figure 3.1 Gene therapy treatment constructs.

All transgenes were expressed under the ubiquitous CAG promoter and were packaged into recombinant AAV9 viral particles. The GFP and m*hexb* constructs also contained the WPRE enhancer element.

3.3.2 Animal Model & Injections

The Sandhoff mouse model was obtained from Jackson Laboratories (Phaneuf et al., 1996; Sango et al., 1995), where heterozygote breading established the breeding colony at Queen's University. All animal work was approved and done in accordance with the Queen's University Animal Care Committee. Experimental animals to be injected at 0, 2 or 4 weeks of age were obtained through homozygous KO crosses, whereas the experimental animals to be injected at 6 weeks of age were obtained through KO: KO or KO: H crosses.

Intravenous injections were performed on all mice in accordance with the Queen's University Animal Care Standard Operating Procedures. The vehicle used for the injections was PBS with 350nM NaCl containing 4% sorbitol (Woodley et al., 2019). The temporal vein was used for the 0-week or neonatal administration of $2.5x10^{11}$vg/mouse (~$2.5\ x10^{11}$vg/g). At the 0-week administration, mice were divided into cohorts and received either a vehicle, ssAAV9/h*B-A*, ssAAV9/m*b-a*, ssAAV9/*mHexb*, or AAV9/GFP injection. The 2- and 4-week administrations received retro-orbital intravenous injections of $1\ x10^{12}$ and $1.88\ x10^{12}$ vg/mouse, respectively (~$1.25\ x10^{12}$ vg/g, each). At 2- and 4- weeks, mice received a vehicle, ssAAV9/h*B-A*, or ssAAV9/m*b-a* injection. Lastly, the tail vein was used to administer the 6-week age of delivery at a dose of 2.5E12vg/mouse (~$1.25\ x10^{12}$ vg/g). The 6-week administrations received either a vehicle, ssAAV9/h*B-A*, ssAAV9/m*b-a*, or ssAAV9/*mHexb* injection. (See Table 3.1 for the Study Design).

The immunological effects of the bicistronic treatments were assessed through a preliminary intravenous study. Mice were intravenously administered the human bicistronic, murine bicistronic, or murine *Hexb*-only vector at 6 weeks ($2.5\ x10^{12}$ vg/mouse). Immunosuppression was administered orally to the mice via gavaging daily from 5-9 weeks of age. Mice received an oral daily dose of 24µg of prednisone diluted in 1x phosphate buffered saline (PBS) and 100µg rapamycin diluted in 0.9% saline, dissolved in the Pierce dimethylsulfoxide (DMSO) (Thermo Frisher Sceintific) vehicle. (See Table 3.2 for Study Design).

Genotype	Age of Administration (weeks)	Injection Type	Treatment	Dose (vg/mouse)	Euthanizations	
					16 week	Long Term
Heterozygote	/	/	None	0	7	4
Sandhoff	0	Temporal Vein	Vehicle	0	6	/
Sandhoff	0	Temporal Vein	ssAAV9/*GFP*	2.5 x10^{11}	4	/
Sandhoff	0	Temporal Vein	ssAAV9/h*B-A*	2.5 x10^{11}	5	5
Sandhoff	0	Temporal Vein	ssAAV9/m*b-a*	2.5 x10^{11}	6	9
Sandhoff	0	Temporal Vein	ssAAV9/*mHexb*	2.5 x10^{11}	5	4
Sandhoff	2	Retro-orbital	Vehicle	0	3	/
Sandhoff	2	Retro-orbital	ssAAV9/h*B-A*	1 x10^{12}	5	5
Sandhoff	2	Retro-orbital	ssAAV9/m*b-a*	1 x10^{12}	5	5
Sandhoff	4	Retro-orbital	Vehicle	0	6	/
Sandhoff	4	Retro-orbital	ssAAV9/h*B-A*	1.88 x10^{12}	5	5
Sandhoff	4	Retro-orbital	ssAAV9/m*b-a*	1.88 x10^{12}	5	5
Sandhoff	6	Tail Vein	Vehicle	0	5	/
Sandhoff	6	Tail Vein	ssAAV9/h*B-A*	2.5 x10^{12}	5	5
Sandhoff	6	Tail Vein	ssAAV9/m*b-a*	2.5 x10^{12}	5	5
Sandhoff	6	Tail Vein	ssAAV9/*mHexb*	2.5 x10^{12}	5	5

Table 3.1 Age and Vector Study Design

Injections of the human and murine bicistronic vectors were performed at 0-, 2-, 4-, and 6- weeks of age, and the murine *Hexb* only vector was administered at 0- and 6- weeks of age.

Genotype	Mice per cohort	Age of Admin. (weeks)	Injection Type	Gene Therapy Treatment	Dose (vg/mouse)	Immunosuppressant Regiment (Gavaging)	Dose (µg)
Sandhoff	4	6	Tail Vein	Vehicle	0	Vehicle	0
Sandhoff	4	6	Tail Vein	ssAAV9/h*B-A*	2.5 x10^{12}	Prednisone & Rapamycin	24 (P) 100 (R)
Sandhoff	4	6	Tail Vein	ssAAV9/h*B-A*	2.5 x10^{12}	Vehicle	0
Sandhoff	4	6	Tail Vein	ssAAV9/m*b-a*	2.5 x10^{12}	Vehicle	0
Sandhoff	4	6	Tail Vein	ssAAV9/*mHexb*	2.5 x10^{12}	Vehicle	0

Table 3.2 Immunosuppressant Study Design.

All gene therapy administrations performed at 6 weeks of age, and all mice euthanized three weeks later at the 9-week study endpoint.

3.3.3 Tissue Processing and Euthanizations

All experimental mice underwent monthly blood collections as previously described (Osmon et al., 2016; Woodley et al., 2019). Blood was collected through the saphenous or sub-mandibular vein using serum collection tubes (KMIC-SER, Kent Scientific) and was then spun down at 3500rpm for 10 minutes in order to separate the serum from the red blood cells. Aliquots of serum were stored at -20˚C for further processing.

Vehicle and ssAAV9/*GFP* mice were humanely euthanized at 16 weeks in due to symptom severity and accordance with the standards of care of the Queen's University Animal Care Committee. A cohort of ~5 mice per injection type and age were also euthanized at 16 weeks in order to facilitate treatment comparison at that timepoint. The remaining 5 mice per experimental gene therapy cohort were left for long-term survival, where mice were euthanized at their humane endpoint of 15% weight loss, the inability to right itself within 10 seconds, or if they survived to the study endpoint of 104 weeks (2 years). Cardiac punctures were performed to obtain terminal serum and to facilitate the whole-body perfusion with 10mL of 1xPBS. Blood obtained from the cardiac punctures was processed as described above for the serum extraction. Visceral organs and the central nervous system were collected for further molecular and biochemical analysis and were stored at -20˚C. The midsection of the brain was fixed in 4% PFA upon extraction for histological analysis. A portion of the midsection of the brain was also homogenized from frozen (-20˚C) for 10 second three times at an amplitude of 15 in 700µL of 1xPBS. Homogenized midsections of the brain were then centrifuged at 13000rpm for 20 minutes at 4˚C. 400µl of the supernatant was aliquoted for the enzyme activity assay, where the remainder of the homogenized midsection of the brain was set aside for the GM2 storage assay. Both homogenized midsections of the brain were stored at -20˚C prior to the assays. See Figure 3.2 for the division of the brain samples.

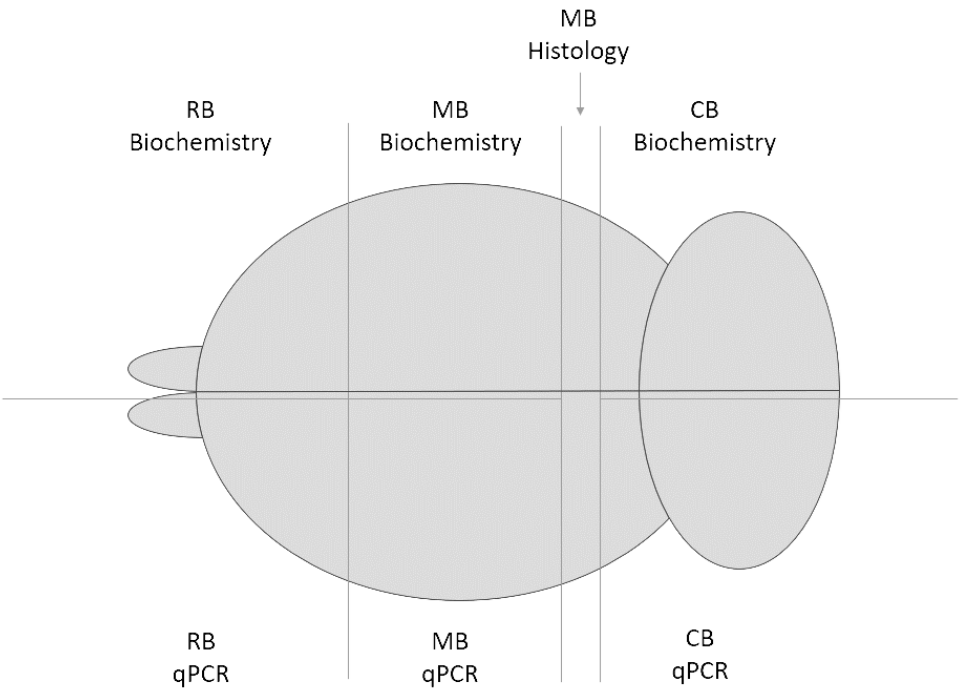


Figure 3.2 Brain sectioning for biochemical and molecular analysis.

RB- Rostral Section of the brain; MB- Midsection of the brain; CB- Caudal Section of the brain.

Mice on the immunosuppressant regimen were all euthanized 3 weeks post-vector administration (9 weeks of age) to facilitate biochemical comparisons. Prior to euthanization, saphenous vein blood collections were performed on the mice that underwent the immunosuppressive regiment in order to obtain a complete blood cell count (CBC) using heparin coated blood collection tubes (KMIC-LIHEP, Kent Scientific). Serum from cardiac punctures (obtained as described above), was used to acquire the biochemical parameters of the experimental mouse's serum composition. After the cardiac perfusion, the spleens were extracted, and underwent a splenocyte isolation protocol in advance of the ELISPOT assay. Briefly, in a sterile hood, spleens were manually homogenized through a 70µ cell strainer into 10ml of RPMI-1640 medium using the rubber end of a 10ml syringe plunger, followed by centrifugation (200g for 5 mins) and resuspension in red blood cell lysis buffer (10x stock, Biolegend). The RBC lysis

solution incubated with the splenocytes for 5 minutes, upon which time 5ml of RPMI-1640 was added to stop the lysis, and the cells were again centrifuged at 200g for 5 mins. Cells were then counted and were ready for the ELISPOT Assay.

3.3.4 Behavioral Testing

Previously described in **Chapter 2**.

The general locomotion of the experimental mice was assayed through the open field test (OFT), as previously described (Osmon et al., 2018; Osmon et al., 2016). Briefly, mice were monitored in the Acti-Mot system (TSE systems) for 5 minutes, were the distance traveled was recorded. Next, the coordination of the mice was evaluated by the accelerated rotarod (RR) protocol, as previously described (Osmon et al., 2018; Osmon et al., 2016). Briefly, the mouse is placed on a rotating rod that accelerates from 4rpm to 40rpm over the 5-minute trial (Rotarod, IITC Life Sciences). If a mouse completes the entire 5-minute trial, they have completed the entire RR test for that timepoint. If the mouse was unable to remain on the rotating rod for the complete 5 minutes and fell, it's end RPM score is recorded, and the mouse is given a 10-minute recovery period before subsequent trials. If a mouse does not attain the 5 minutes, then it must undergo 3 separate attempts.

3.3.5 Hexosaminidase Activity Assay

Hexosaminidase activity assays were performed as previously described (Maegawa et al., 2007; Osmon et al., 2016; Tropak et al., 2004) and seen in **Chapter 2**, except for being diluted in CP buffer and not 1xPBS.

Briefly, homogenized midsections of the brain, homogenized liver, or serum samples were diluted to 1:50, 1:400, or 1:35 in CP buffer, respectively. The diluted samples were then incubated with the fluorogenic 4-Methylumbelliferyl-beta-D-N-acetylglucosamine-6-sulfate (4-MUGS; M335000, Toronto Research Chemicals) substrate at 37˚C for 1 hour in order to assess the amount of HexA enzyme activity is within the sample. The plates were read with an

excitation of 365nm and emission of 450nm. Total protein concentration was assessed in the midsection of the brain and liver homogenates using the Pierce BCA Protein Assay Kit (Thermo Scientific, cat#23225). Final enzyme activity levels are reported as fluorescent 4MU units per μg of protein per hour, or fluorescent 4MU units per μl of serum per hour.

3.3.6 GM2 Storage Analysis - Column and Thin Layer Chromatography

The ganglioside storage analysis was conducted as previously described (Folch et al., 1951, 1957; Osmon et al., 2016; Tropak et al., 2010, 2016b; Wherrett & Cumings, 1963) and seen in **Chapter 2**.

Column and think layer chromatography analysis enabled the quantification of the GM2 gangliosides. Briefly, homogenized midsection of the brain was incubated at 4˚C overnight with chloroform and methanol and evaporated under nitrogen. Samples were then exposed to a series of dilutions of chloroform and methanol solutions and underwent ganglioside isolation and extraction through column chromatography (C-18, Phenomenex). Finally, the gangliosides were further separated through thin-layer chromatography (TLC), where the solvent front was composed of 55:45:10 chloroform: methanol: 0.22% calcium chloride. TLC plates were then sprayed with oorcinol dissolved in 25% sulfuric acid and baked for 10-15mins at 120˚C for the appearance of the visual output. Densitometry analysis of the plates was performed using image J software, and data is represented a normalized as amount of GM2 ganglioside per amount of GD1A ganglioside in the sample.

3.3.7 Biodistribution Analysis

Biodistribution of the human bicistronic vector was obtained through qPCR analysis of individual organs as previously described (Gray et al., 2010). The liver and central nervous system divisions were assayed to observe the biodistribution. CNS divisions were homogenized using ceramic beads and the homogenizer, whereas the liver samples were manually homogenized prior to genomic DNA extraction using the gSYNC DNA Extraction Kit (Geneaid,

cat# GS004). Genomic DNA was then diluted and plated for the qPCR analysis. Vector distribution was obtained using primers unique to the human *HEXA* gene (F: 5'- TATGGCAAGGGCTATGTGGT - 3', R: 5'-TGATTGTGTCTGGCTGAATCTT - 3'). The endogenous control used to normalize the biodistribution was murine *LaminB2* (F: 5'- GGACCCAAGGACTACCTCAAGGG - 3', R: 5'- AGGGCACCTCCATCTCGGAAAC- 3'). The biodistribution of the vector is represented as the copy number of the experimental human bicistronic treatment per diploid cell (2 copies of m*LaminB2*).

3.3.8 ELISpot Assay - Enzyme Linked Immune Absorbent Spot Assay

An enzyme linked immune absorbent spot assay (ELISpot) assay was performed as previously described on the isolated splenocytes from the mice that underwent the immunosuppressant regimen. Briefly, splenocytes were incubated for 48 hours with peptides encoding the human *HEXA* or *HEXB* genes, or the P2A linker on a PVDF-plate (MSIPS4510, Millipore Corp) prepped with the coating antibody (AN18) from the Mabtech ELISpot IFN-γ Kit (3321-2H, Mabtech). Upon completion of the incubation, the cells and stimulants are washed from the plate, and the secondary antibody from the Mabtech ELISpot kit (R4-6A2-biotin) is incubated in the plate for 2 hours. The plate is then washed, and wells are incubation with the streptavidin-HRP provided in the Mabtech ELISpot kit. Lastly, to develop the colorimetric spots, the AEC (3-amino-9methyl carbazole) substrate solution is added. In order to stop the color development, the plate is extensively washed with deionized water and left to dry before being shipped to ZellNet Consulting, Inc for spot counting.

3.3.9 ELISA – Enzyme Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay was performed as previously described on the diluted serum (1:10000) from the mice that were administered daily immunosuppressants. Briefly, the wells of a NUNC-Immuno 96 microwell plate were coated with the human HexA antigen overnight at 4˚C. After a series of washes (1xPBS with 0.025% Tween-20) and blocking

with casein in 1xPBS (Thermo Fisher), diluted mouse serum (1:10000) was incubated at 4˚C overnight. Following the overnight incubation and a series of washes, the donkey anti-mouse (AP192P, Millipore) conjugated secondary antibody was added and the plates incubated for 2 hours at room temperature, followed by the use of the 1-step Turbo TMB-ELISA substrate solution (Thermo Scientific) to determine the colorimetric change. The reaction was quenched using a 0.2M sulphuric acid stop solution and read at 450nm absorbance.

3.3.10 Statistical Analysis

Previously described in **Chapter 2**.

Statistical analysis was performed using Graph Pad Prism 8. Kaplan Meyer curves were performed to analyze the significance of the survival outcomes. The behavioural testing, and serum hexosaminidase activity time-course were analyzed through repeated measures 2-way ANOVAs with Tukey post-hoc tests. One-way ANOVAs with accompanying Tukey post-hoc tests were performed to analyze the midsection of the brain hexosaminidase activity assay, and the chromatography GM2 storage. A 2-way ANOVA followed by a Tukey post-hoc test was performed on the vector biodistribution.

3.4 Results

3.4.1 Survival

As seen in Figure 3.3 and demonstrated by the median survival in Table 3.3, significant increases in SD mouse survival are observed across all gene transfer treatments at all ages of administration ($p<0.01$). Below we represent the significance of these findings by assessing the vectors across the ages of administration as well as the ages of administration across the vectors.

3.4.1.1 Vectors Across the Ages of Administration

The **0-week** age of administration of the vectors (Figure 3.3A) showed that both bicistronic treatments and the ssAAV9/m*Hexb* treatment all had significant increases in survival as compared to vehicle and ssAAV9/GFP treated controls ($p<0.01$, each). The human bicistronic treatment did not differ significantly from the ssAAV9/m*Hexb* vector treatment ($p= 0.0746$), but it did show a significant increase in survival compared to the murine bicistronic treatment ($p<0.05$). In addition, both bicistronic treatments differed significantly from the heterozygous control group ($p<0.01$, each). The ssAAV9/m*Hexb* treatment did not differ from the heterozygous control mice ($p=0.3091$).

The assessment of the survival of the **2-week** administration (Figure 3.3B) of the vectors showed significant survival benefit as compared to vehicle controls ($p<0.01$ and $p<0.01$, respectively). The human bicistronic treatment had significantly lower survival than the murine bicistronic treatment ($p<0.01$), but neither human nor murine bicistronic treatments differed significantly from the heterozygous controls ($p=0.0569$, $p=0.8109$, respectively).

At the **4-week** age of administration (Figure 3.3C), both the human and murine bicistronic vector administrations showed significant increases in mouse survival as compared to vehicle controls ($p<0.01$ and $p<0.01$, respectively). The human bicistronic treatment showed intermediate survival, differing significantly from the murine bicistronic vector ($p<0.05$) as well as the

heterozygous controls (p<0.01). The murine bicistronic treatment showed survival data comparable and not statistically different from heterozygotes (p= 0.1208).

All vector treatments administered at **6 weeks** (Figure 3.3D) significantly improved survival compared to vehicle controls (p<0.01, each). The human bicistronic treatment also showed a significant reduction in survival compared to the murine *b-a*, the *hexb* only vector, and heterozygous controls (p<0.05, p<0.01, p<0.01, respectively). The survival of the mice administered the murine bicistronic treatment and the murine *hexb* only vectors did not differ significantly from each other or the heterozygous controls (p=0.1792, p= 0.2516, respectively).

Age of Administration	0-week		2-week		4-week		6-week	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
ssAAV9/h*B-A*	59	40.1-60	68.4	63.2-70.3	43.6	43.4-63.8	43.8	32.7-63.5
ssAAV9/m*b-a*	42.2	32-46.4	76.8	72.4-95.3	65.8	46-73.1	68.1	43.6-91.3
ssAAV9/m*Hexb*	65.7	49-102.3	/	/	/	/	70.7	67.9-76.4

Table 3.3 Median Survival and Survival Range of each vector at all ages of administration in weeks.

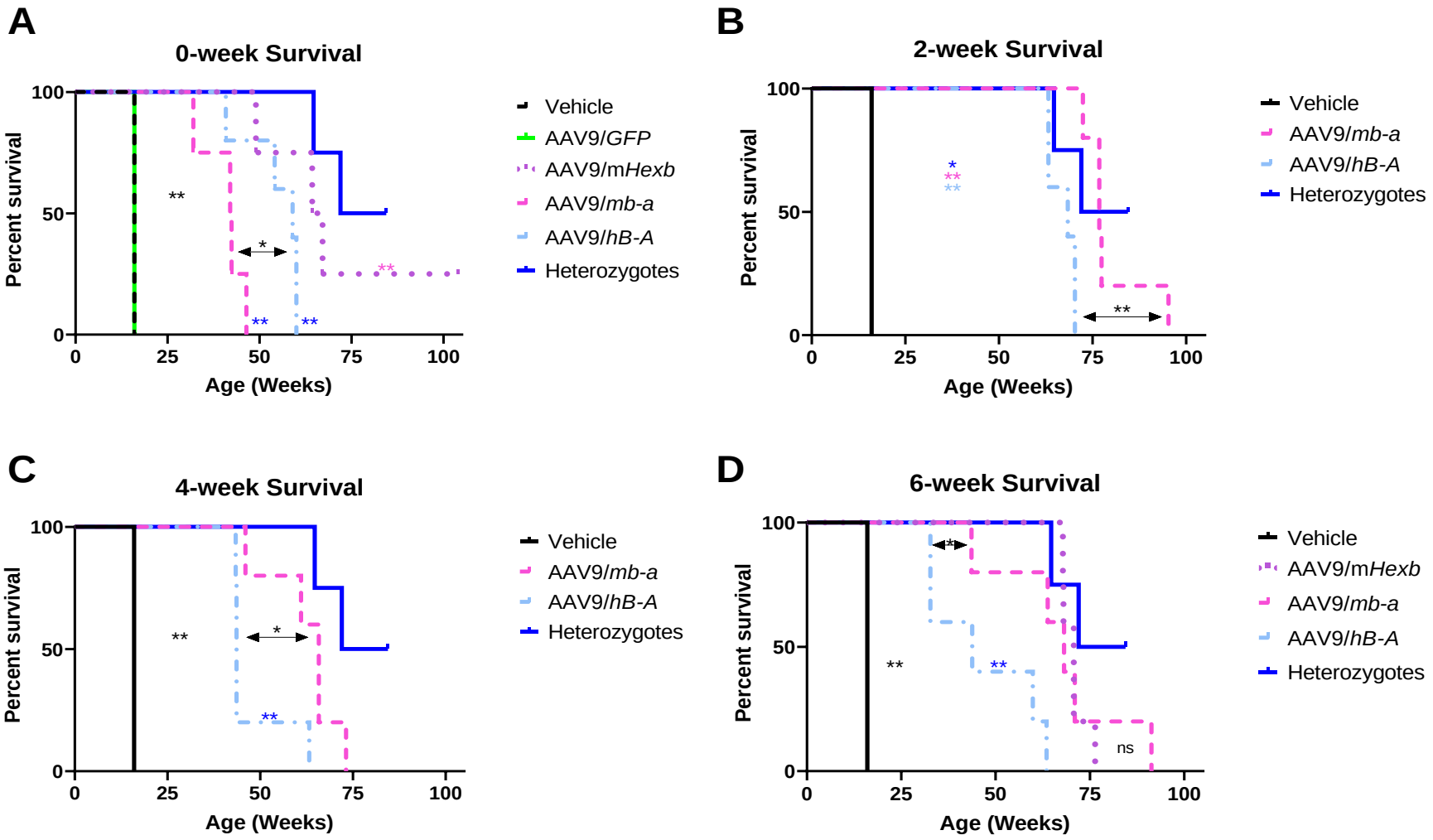


Figure 3.3 Survival of the SD treated mice – Murine Age at which the Treatment was Administered.

Caption for Figure 3.3: The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. (A) Survival of the 0-week treated SD mice. At the 0-week age of intervention, the human and murine bicistronic cohorts survived to a median of 59 and 42.2 weeks, with survival ranging between 40.1- 60 and 32 – 46.4 weeks, respectively. The murine Hexb cohort had a median survival of 65.7 weeks and a range of 49-102.3 weeks of age. (B) Survival of the 2-week treated SD mice, where the human and murine bicistronic cohorts survived to a median of 68.4 and 76.8 weeks, with survival ranging between 63.2 – 70.3 and 72.4 – 95.3 weeks, respectively. (C) Survival of the 4-week treated SD mice, where the human and murine bicistronic cohorts survived to a median of 43.6 and 65.8 weeks, with survival ranging between 43.4 – 63.8 and 46 – 73.1 weeks, respectively. (D) Survival of the 6-week treated SD mice. At the 6-week age of intervention, the human and murine bicistronic cohorts survived to a median of 43.8 and 68.1 weeks, with survival ranging between 32.7 – 63.5 and 43.6 – 91.3 weeks, respectively. The murine Hexb cohort had a median survival of 70.7 weeks and a range of 67.9 –76.4 weeks of age. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

3.4.1.2 Ages of Administration Across the Vectors

The administration of the **ssAAV9/h*B*-*A* treatment** at all ages of administration (Figure 3.4A) showed highly significant increases in survival as compared to vehicle controls ($p<0.001$, each). The 0-week administration of the human bicistronic treatment showed intermediate survival, having a significantly lower survival rate than the 2-week administration ($p<0.01$), but not differing significantly from the 4- or 6-week injections ($p= 0.7957$, $p= 0.8279$, respectively). The 2-week IV administration of the human bicistronic vectors showed significant increases in survival compared to both the 4- and 6- week administrations ($p<0.01$, $p<0.05$, respectively). The 2-week age of administration did not differ significantly from the heterozygotes ($p=0.0569$). The 4- and 6- week administrations of the human bicistronic treatment did not differ significantly from each other ($p= 0.6694$), but both differed significantly from the heterozygous controls ($p<0.01$ and $p<0.01$, respectively).

The **ssAAV9/m*b*-*a* treatment** showed significant increases in survival at 0-, 2-, 4-, and 6- week administrations as compared to the vehicle controls ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$, respectively; Figure 3.4B). The 0-week age of administration of the murine bicistronic treatment showed significantly inferior survival compared to the 2-, 4-, and 6- week administrations ($p<0.01$, $p<0.05$, and $p<0.05$, respectively). Additionally, the 2-week injection of the ssAAV9/m*b*-*a* showed an increase in survival compared to the 4-week administration ($p<0.01$), where it did not differ significantly from the 6-week administration ($p= 0.1356$). The 4- and 6-week administrations did not differ significantly ($p= 0.5150$). Lastly, the 2-, 4- and 6- week ssAAV9/mb-a injections showed no significant difference in survival as compared to the heterozygous controls ($p=0.2792$, $p= 0.1208$, and $p= 0.2516$, respectively).

The survival of the **ssAAV9/m*Hexb* vector** treated SD mice (Figure 3.4C) showed significant amelioration in lifespan when administered at either 0- or 6-weeks ($p<0.01$, and $p<0.001$, respectively). The survival of the 0 week and the 6-week administrations were not

significantly different from the survival of the heterozygous controls (p= 0.3091, p=0.3839, respectively).

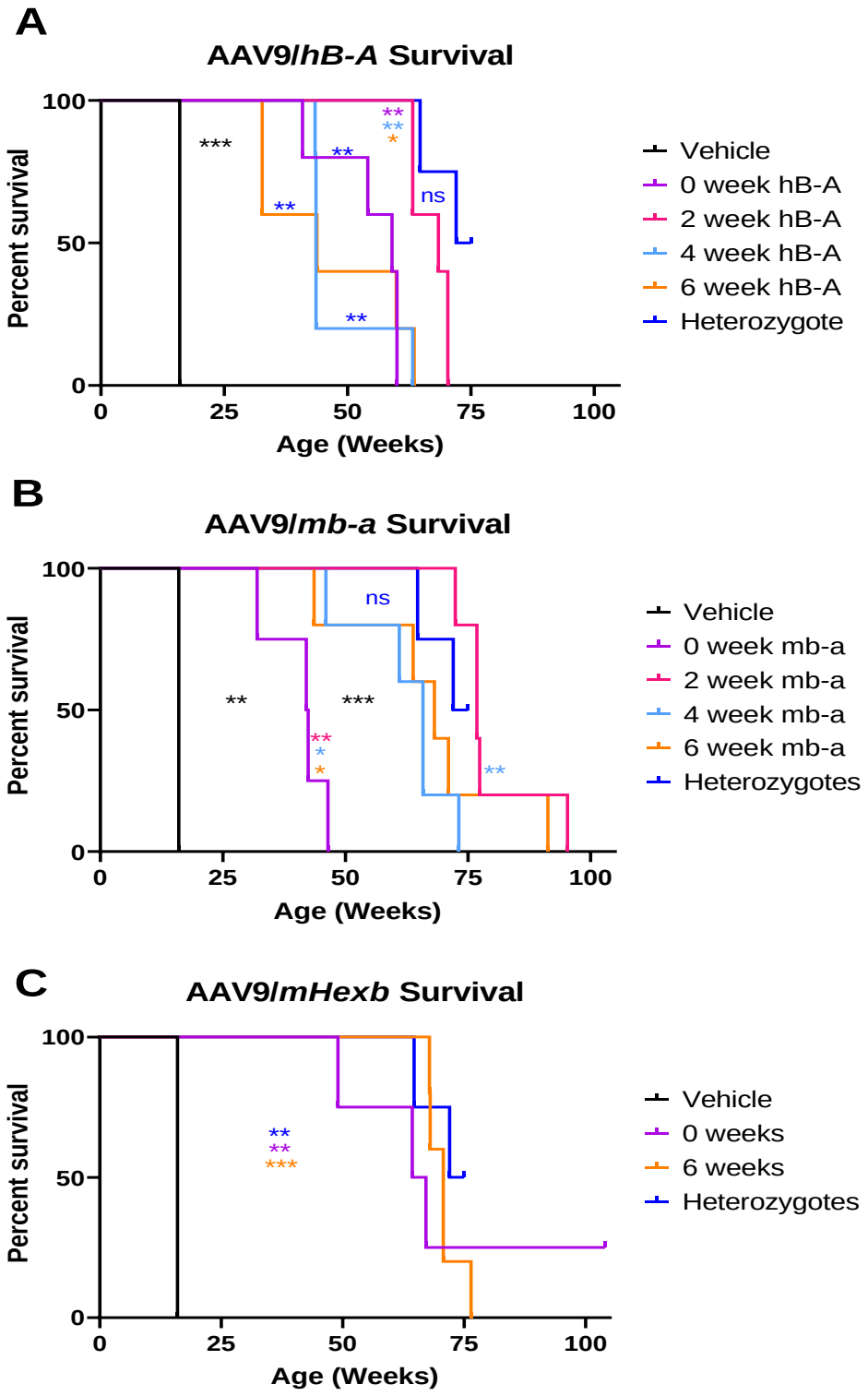


Figure 3.4 Survival of the SD treated mice - Vectors.

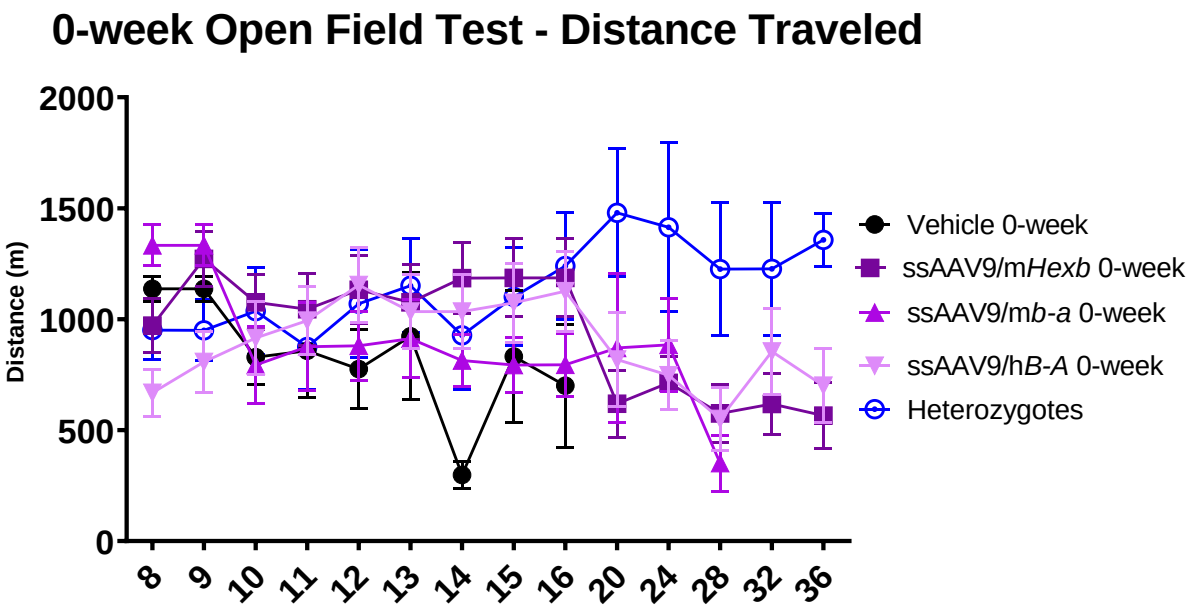
Caption for Figure 3.4: The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. (A) Survival of the SD mice treated with the ssAAV9/h*B-A* vector. The human bicistronic cohorts treated at 0-, 2-, 4-, and 6-weeks of age survived to a median of 59, 68.4, 43.6, and 43.8 weeks, respectively. (B) Survival of the SD mice treated with the ssAAV9/m*b-a* vector, where the 0-, 2-, 4-, and 6-week cohorts survived to a median of 42.2, 76.8, 65.8, and 68.1 weeks, respectively.. (C) Survival of the SD mice treated with the ssAAV9/*mHexb* vector. The ssAAV9/*mHexb* vector cohorts treated at 0- and 6-weeks of age survived to a median of 65.7 and 70.7 weeks, respectively. See Table 3.3 for range in survival. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

3.4.2 Behavioural Outcomes

The general locomotion and coordination of the SD treated mice were assessed through the Open Field Test (OFT; Figure 3.5- Figure 3.11) and the Rotarod Test (RR; Figure 3.12- Figure 3.18), respectively. There was a near significant effect of treatment in the OFT ($p<0.0557$, $F_{(14,115)}=4.839$, and a significant effect of treatment was observed in RR ($p<0.0001$, $F_{(14,115)}=1.746$). For each behavioural test, the results below are broken down into assessments of the different vectors at each age of administration, followed by the examination of the ages of injection by vector type.

3.4.2.1 Open Field Test - Vectors Across the Ages of Administration

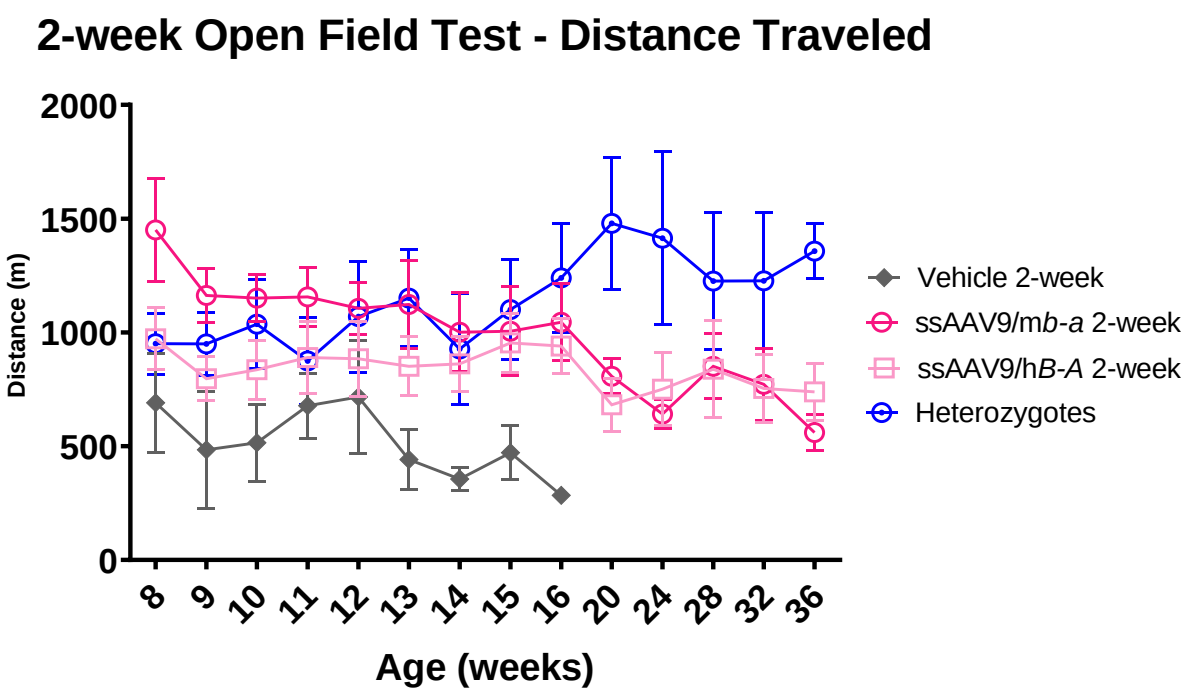
The OFT assessment of general locomotion in the treated SD mice at different ages of administration are depicted in Figure 3.5-Figure 3.8. The SD mice that were treated at the **0-week** age of administration showed little benefit compared to the vehicle controls (Figure 3.5). The **2-week** murine and human bicistronic treatments showed an increase in general locomotion compared to the vehicle controls at the 16-week assessment ($p<0.05$, each; Figure 3.6), and the ssAAV9/hB-A vector cohort nearly significantly outperformed the 2-week vehicle controls at the 14-week assessment ($p=0.08$). The mice treated with the murine and human bicistronic vectors at the **4-week** age of administration showed a significant benefit in general locomotion compared to the vehicle controls at the 16-week assessment ($p<0.01$, $p<0.05$, respectively; Figure 3.7). In the assessment of the **6-week** age of administration (Figure 3.8), the human bicistronic treatment group outperformed the vehicle controls at 16 weeks of age ($p<0.05$).



	8	9	10	11	12	13	14	15	16
ssAAV9/*mhexb* vs ssAAV9/m*b-a*							**		
ssAAV9/m*b-a* vs ssAAV9/h*B-A*	*								

Figure 3.5 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test – 0-week Treated SD mice.

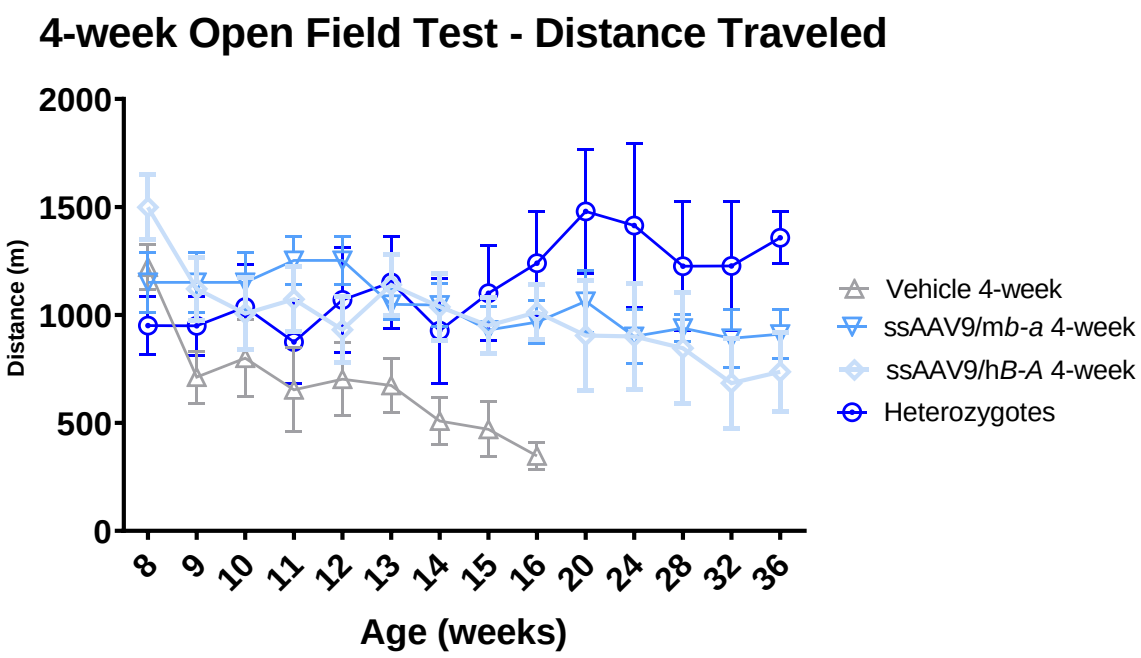
The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)



	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/m*b-a*									*
Vehicle vs ssAAV9/h*B-A*							†		*

Figure 3.6 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test – 2-week Treated SD mice.

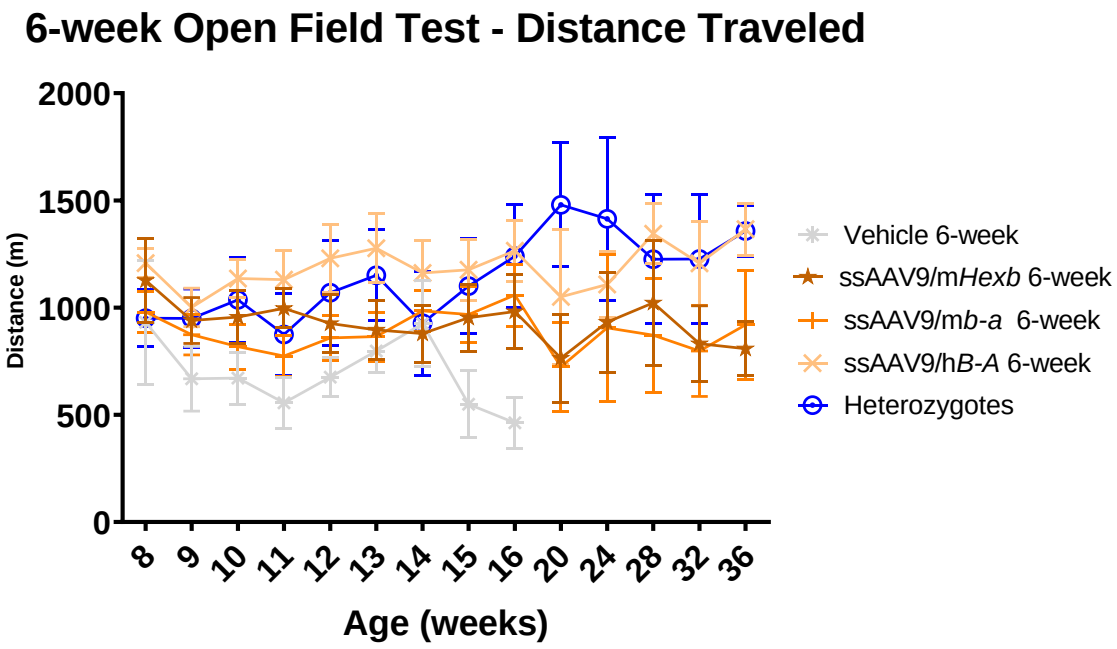
The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. Both the murine and human bicistronic cohorts outperformed the vehicle controls at 16 weeks of age. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)



	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/m*b-a*									**
Vehicle vs ssAAV9/h*B-A*									*

Figure 3.7 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test – 4-week Treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. Both the murine and human bicistronic cohorts outperformed the vehicle controls at 16 weeks of age. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)



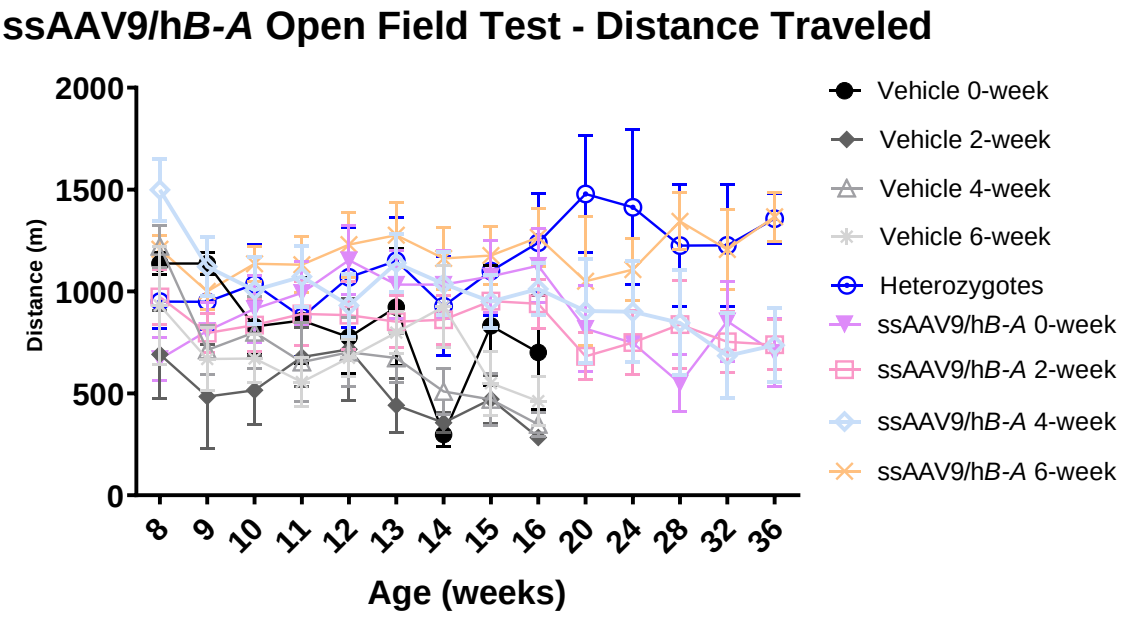
	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/h*B-A*									*

Figure 3.8 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test – 6-week Treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. The human bicistronic treatment significantly outperformed the vehicle treatment group at the 16-week timepoint. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)

3.4.2.2 Open Field Test - Ages of Administration Across the Vectors

The **human bicistronic** administration improved behavioural performance of the SD treated mice (Figure 3.9). The general locomotion of the 2- week human bicistronic treatment and the 4- and 6-week human bicistronic administrations were nearing significance and significantly increased compared to the 2-week vehicle control at 14-weeks of age ($p=0.08$, $p<0.01$, $p<0.01$, respectively). At the 15-week assessment, the behaviour of the mice in the 6-week human bicistronic intervention cohort showed a nearly significant increase in performance compared to the 6-week vehicle cohort ($p=0.08$). At 16-weeks, many of the human bicistronic treatment cohorts were significantly different from the vehicle controls. The performance of the mice administered the treatment at 0- and 4-week were significantly and nearly significantly different than the 0-week vehicle control cohort ($p<0.01$, $p=0.06$, respectively). The 2-week vehicle controls were significantly outperformed by the 0-week ($p<0.05$), 4-week ($p<0.01$), 6-week ($p<0.01$). The 4-week vehicle cohort was significantly outperformed by the 2-week ($p<0.05$), 4-week ($p<0.05$), 6-week ($p<0.01$) cohorts, and nearly significantly outperformed by the 0-week human bicistronic treatment ($p=0.08$). Lastly, the SD mice administered the human bicistronic vector at 6 weeks significantly outperformed the 6-week vehicle controls ($p<0.05$).

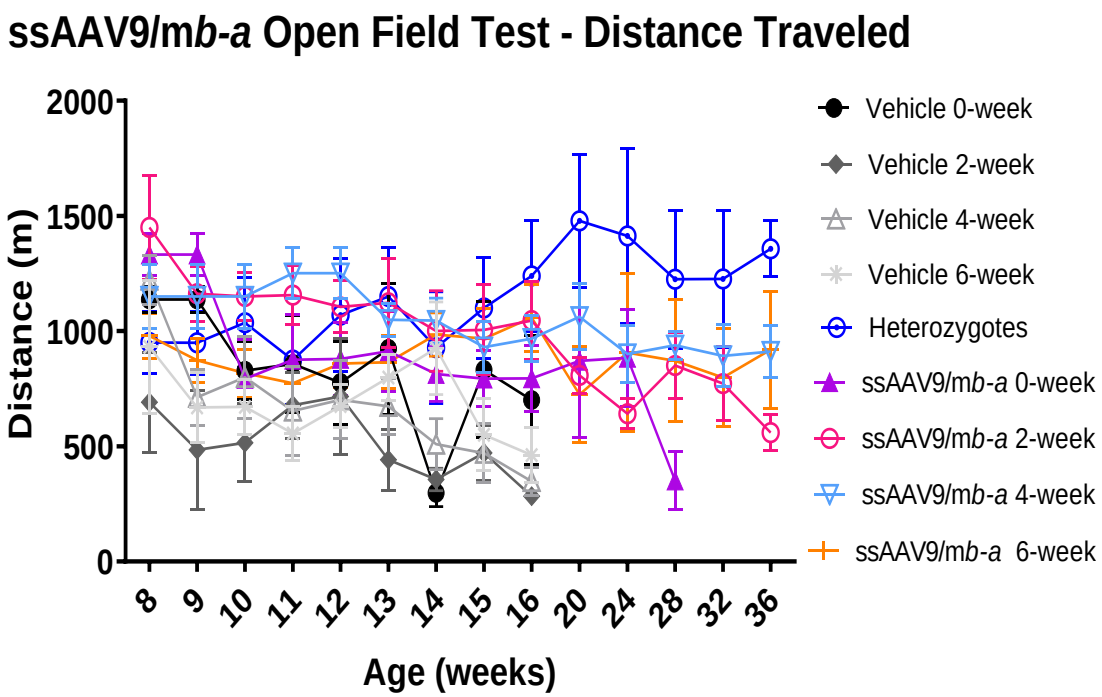


	8	9	10	11	12	13	14	15	16
ssAAV9/h*B-A* 0-week vs ssAAV9/h*B-A* 4-week	*								
ssAAV9/h*B-A* 0-week vs ssAAV9/h*B-A* 6-week	*								
Vehicle 2-week vs ssAAV9/h*B-A* 0-week									*
Vehicle 2-week vs ssAAV9/h*B-A* 4-week									**
Vehicle 2-week vs ssAAV9/h*B-A* 6-week									**
Vehicle 2-week vs Heterozygotes									†
Vehicle 4-week vs ssAAV9/h*B-A* 0-week									†
Vehicle 4-week vs ssAAV9/h*B-A* 2-week									*
Vehicle 4-week vs ssAAV9/h*B-A* 4-week									*
Vehicle 4-week vs ssAAV9/h*B-A* 6-week								†	**
Vehicle 6-week vs ssAAV9/h*B-A* 6-week									*

Figure 3.9 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test for the ssAAV9/h*B-A* treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. The vehicle 2-week cohort was significantly outperformed by the 0-, 4-, and 6-week human bicistronic administration cohorts at 16 weeks of age. The 4-week vehicle cohort was significantly outperformed by the 2-, 4-, and 6- week *hB-A* cohort, and nearly significantly outperformed by the 0-week human bicistronic cohorts at the 16-week timepoint. Lastly, the 6-week human bicistronic cohort also outperformed the 6-week vehicle cohort at 16-weeks of age. (*= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns= p>0.05, † = approaching significance)

The **murine bicistronic** vector conveyed a behavioural benefit to the treated SD mice (Figure 3.10). At the 14-week assessment, the 2-week vehicle cohorts were also outperformed by the 4- and 6-week murine bicistronic cohorts ($p<0.01$, each). Additionally, at 14 weeks, the performance of the mice treated with ssAAV9/mb-a at 0-weeks were outperformed by the 2-week ($p<0.05$), 4-week ($p<0.001$), and 6-week ($p<0.001$) murine bicistronic administrations. Lastly, the 14-week assessment showed that the 2-week vehicle cohorts were outperformed by the 4- and 6-week ssAAV9/mb-a cohorts ($p<0.01$, each). The 16-week assessment resulted in the 2-week vehicle controls being outperformed by the 2-week ($p<0.05$), 4-week ($p<0.01$), and 6-week ($p<0.05$) murine bicicstronic cohorts, and the 4-week vehicle cohort was outperformed by the 4- and 6- week administrations ($p<0.01$, $p<0.05$,respectively).

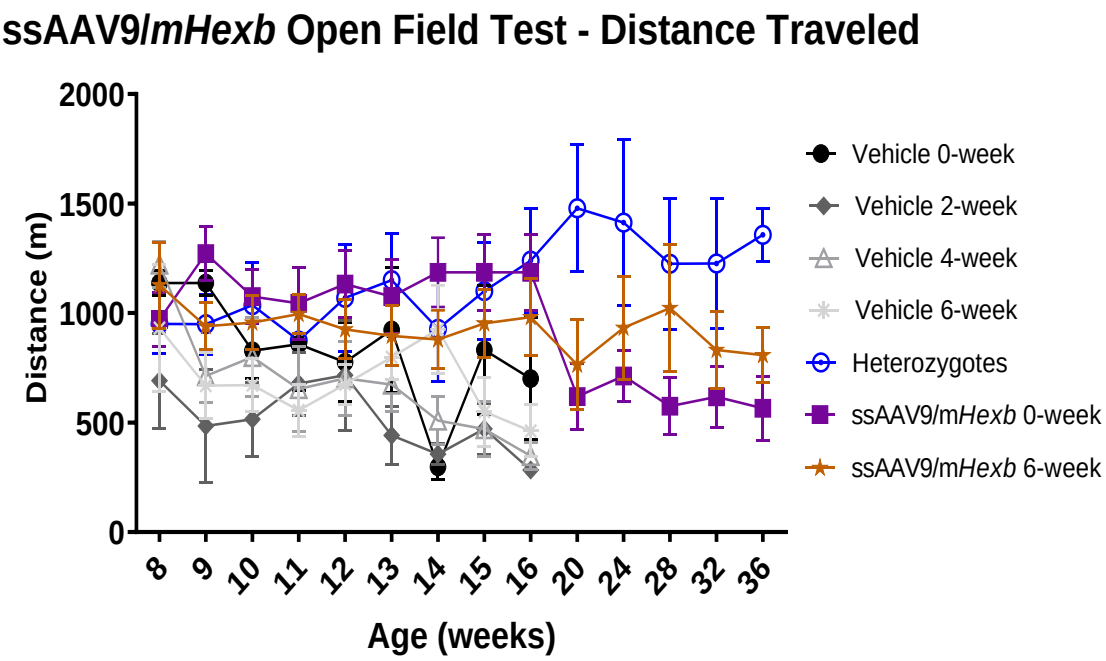


	8	9	10	11	12	13	14	15	16
ssAAV9/m*b-a* 0-week vs ssAAV9/m*b-a* 2-week							*		
ssAAV9/m*b-a* 0-week vs ssAAV9/m*b-a* 4-week							***		
ssAAV9/m*b-a* 0-week vs ssAAV9/m*b-a* 6-week							***		
Vehicle 2-week vs ssAAV9/m*b-a* 2-week									*
Vehicle 2-week vs ssAAV9/m*b-a* 4-week									**
Vehicle 2-week vs ssAAV9/m*b-a* 6-week									*
Vehicle 4-week vs ssAAV9/m*b-a* 4-week									**
Vehicle 4-week vs ssAAV9/m*b-a* 6-week									*
Vehicle 6-week vs ssAAV9/m*b-a* 4-week				†					

Figure 3.10 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test for the ssAAV9/m*b-a* treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. At 16 weeks of age, he 2- and 4- week vehicle cohorts were significantly outperformed by the 2-, 4-, and 6-, and 4- and 6- murine bicistronic cohorts, respectively. (*= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns= p>0.05, † = approaching significance)

Finally, the OFT assessments of the mice treated with the **ssAAV9/*mHexb*** vector (Figure 3.11) showed that the general locomotion of the SD mice treated with the ssAAV9/*mHexb* vector at 0-weeks showed significant behavioural improvements compared to the 2-week vehicle administrations at the 14-week OFT assessment ($p<0.05$), and compared to the 2- and 4-week vehicle cohorts at the 16-week timepoint ($p<0.05$, each).



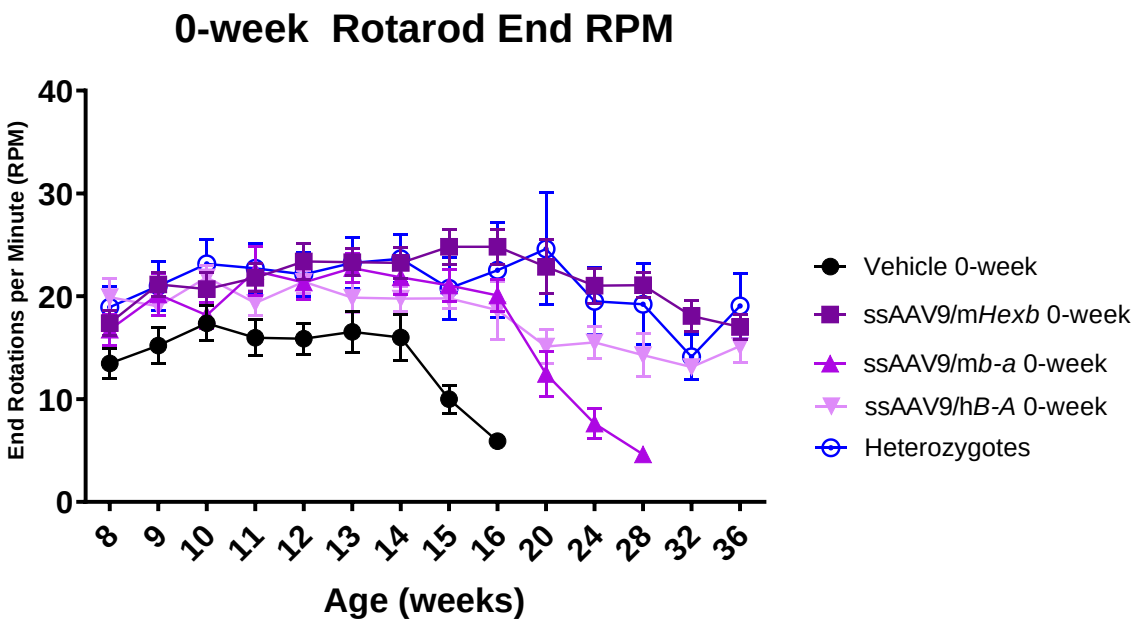
	8	9	10	11	12	13	14	15	16
Vehicle 2-week vs ssAAV9/*mHexb* 0-week									*
Vehicle 4-week vs ssAAV9/*mHexb 0*-week									*

Figure 3.11 Behavioural Testing for General Locomotion – Distance Traveled in the Open Field Test for the ssAAV9/*mHexb* treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. At 16 weeks of age, the 0-week administration of the AAV9/m*Hexb* outperformed the 2- and 4-week vehicle cohorts. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)

3.4.2.3 Rotarod - Vectors Across the Ages of Administration

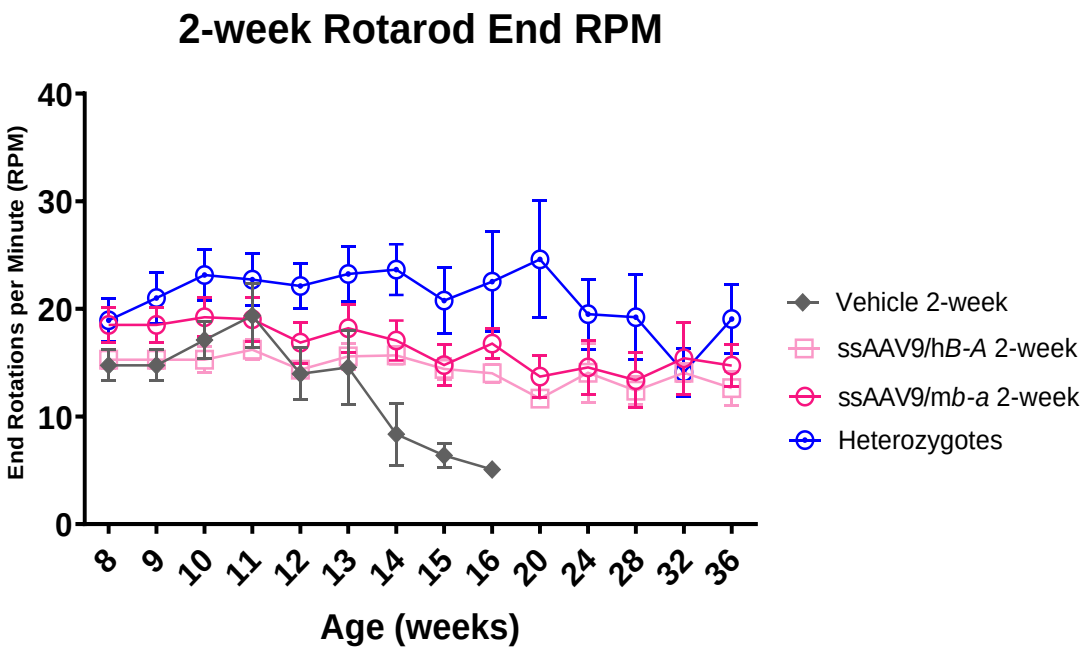
The evaluation of coordination through the RR in the mice treated at the different ages of injection is depicted in Figure 3.12-Figure 3.15. The **0-week** administrations (Figure 3.12) of the ssAAV9/*mHexb*, ssAAV9/h*B-A*, ssAAV9/m*b-a* cohorts showed highly significant increases in coordination compared to the vehicle control mice at the 15- ($p<0.001$, $p<0.01$, $p<0.01$, respectively) and 16-week assessments ($p<0.0001$, $p<0.001$, $p<0.0001$, respectively). The **2-week** age of administration showed a significant improvement of the murine and human bicistronic treated cohorts over the vehicle controls at the 15- ($p=0.095$, $p<0.05$, respectively) and 16-week ($p<0.001$, $p<0.0001$, respectively) assessments (Figure 3.13). At the **4-week** age of administration (Figure 3.14), compared to the vehicle mice, the murine bicistronic vector showed a nearly significant increase in coordination at 14-weeks ($p=0.08$), and a significant increase in performance at 15- and 16-weeks ($p<0.001$, $p<0.01$, respectively). The vehicle mice were also outperformed by the human bicistronic cohort at the 15- and 16-week assessments ($p=0.06$, $p<0.01$, respectively). In addition, the murine bicistronic cohort significantly outperformed the human bicistronic cohort at the 8-, 9- and 11-week assessments ($p<0.05$, $p<0.05$, $p<0.01$, respectively). Lastly, at the **6-week** administration, the vehicle controls were outperformed by the ssAAV9/*mHexb*, ssAAV9/h*B-A*, ssAAV9/m*b-a* cohorts at both the 15-week ($p=0.0547$, $p<0.05$, $p<0.01$, respectively) and 16-week ($p<0.01$, $p<0.001$, $p<0.0001$, respectively) timepoints (Figure 3.15).



	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/*mhexb*								***	****
Vehicle vs ssAAV9/m*b-a*								**	***
Vehicle vs ssAAV9/h*B-A*								**	****
Vehicle vs Heterozygotes									*

Figure 3.12 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for 0-week Treated SD mice.

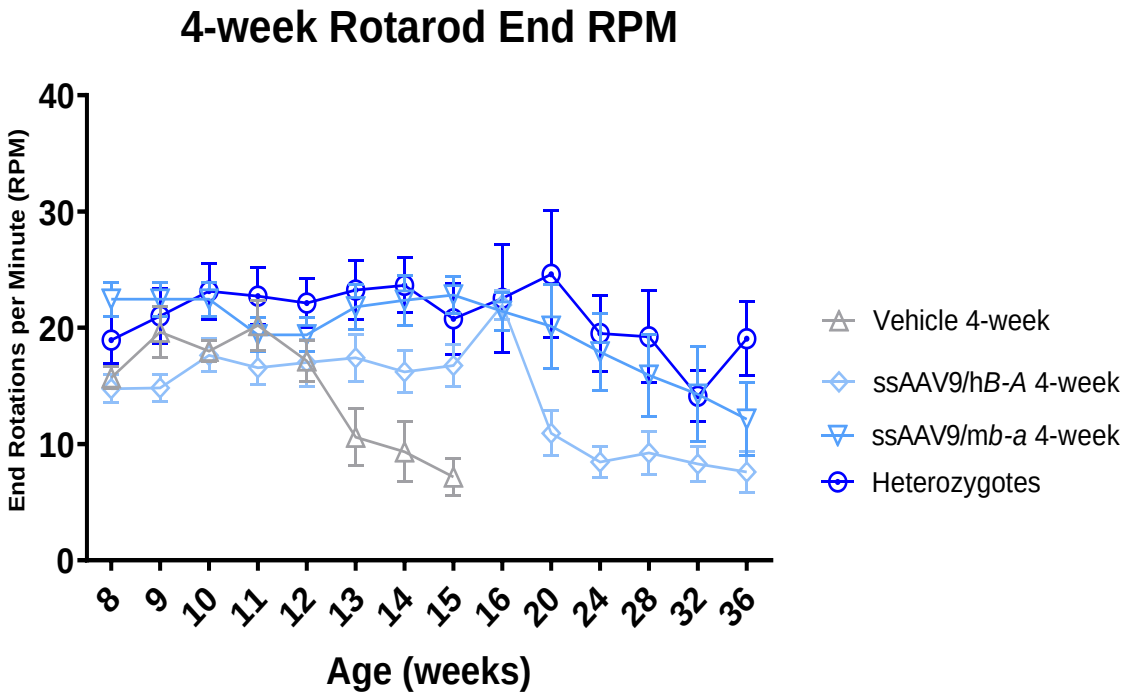
The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. The vehicle controls were outperformed by both the human and murine bicistronic constructs, as well as the m*Hexb* only group at both the 15 and 16 week assessments. (*= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns= p>0.05, † = approaching significance)



	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/m*b-a*								†	***
Vehicle vs ssAAV9/h*B-A*								*	****
Vehicle vs Heterozygotes									*

Figure 3.13 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for 2-week Treated SD mice.

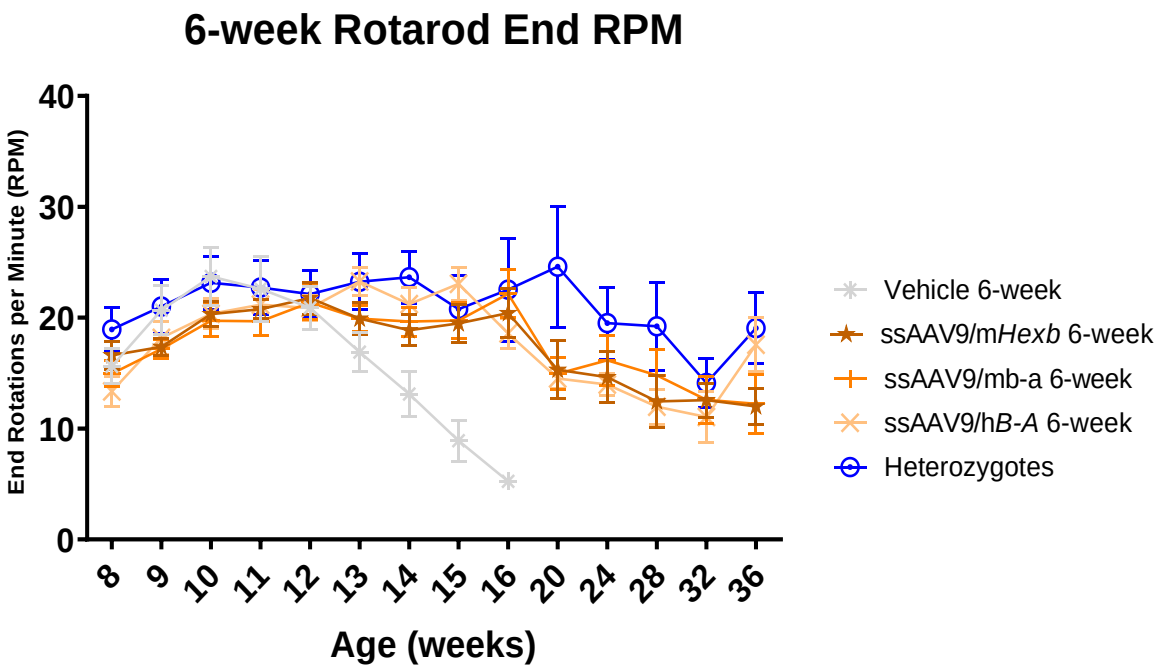
The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. Both the murine and human bicistronic cohorts outperformed the vehicle controls at 16 weeks of age. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)



	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/m*b-a*	†						†	***	**
Vehicle vs ssAAV9/h*B-A*								†	***
Vehicle vs Heterozygotes							†	†	†
ssAAV9/m*b-a* vs ssAAV9/h*B-A*	*	*		**					

Figure 3.14 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for 4-week Treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. Both the murine and human bicistronic cohorts outperformed the vehicle controls at 16 weeks of age. (*= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns= p>0.05, † = approaching significance)



	8	9	10	11	12	13	14	15	16
Vehicle vs ssAAV9/*mhexb*								†	**
Vehicle vs ssAAV9/m*b-a*								*	***
Vehicle vs ssAAV9/h*B-A*								**	****
Vehicle vs Heterozygotes									*

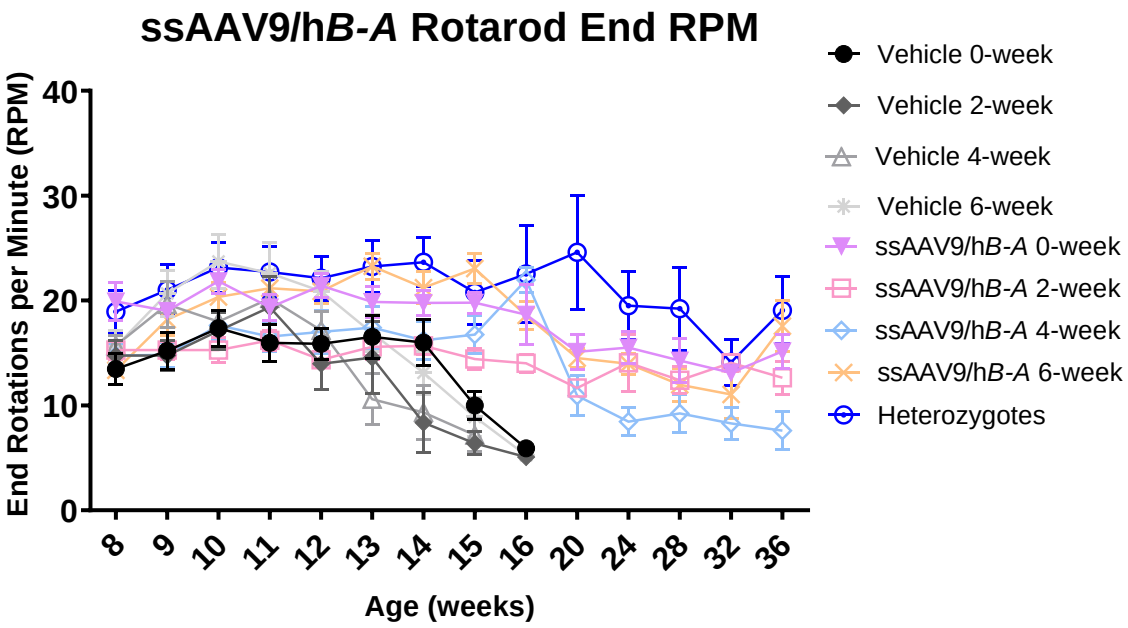
Figure 3.15 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for 6-week Treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. At 16-weeks of age, the cohorts of all GT treatment constructs assayed showed significantly better coordination than the vehicle controls. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)

3.4.2.4 Rotarod - Ages of Administration Across the Vectors

The RR test for coordination in the **human bicistronic** treated mice (Figure 3.16) showed that at 15 weeks of age, the 0-week vehicle controls were being outperformed by the 0- and 6-week human bicistronic cohorts ($p<0.01$, $p<0.001$, respectively), and the 2-week vehicle controls were being outperformed by the human bicistronic cohorts administered the gene therapy treatment at 0-, 2-, 4- and 6-weeks ($p<0.01$, $p<0.05$, $p<0.05$, $p<0.001$, respectively). At 15 weeks, the 4-week vehicle control was outperformed by the 0- and 6- week ssAAV9/h*B-A* treatment cohorts ($p=0.0629$, $p<0.001$, respectively), and the 6-week vehicle cohort was outperformed by the 6-week human bicistronic cohort ($p<0.01$). It also showed that the 0-, 2-, 4-, and 6- week vehicle mice were significantly outperformed by the SD mice treated with the ssAAV9/h*B-A* vector at any age of administration at the 16-week assessment for coordination ($p<0.05$, for each). Moreover, when comparing the ages of intervention of the human bicistronic vector, the SD mice that received the 2-week intervention were outperformed by the 0-week administration at the 10-, 12- and 15-week assessments ($p<0.05$, $p<0.01$, $p=0.0575$, respectively); the 4-week intervention at the 16 week assessment($p<0.01$), and the 6-week age of administration at the 12-, 13-, and 15-week assessments of coordination ($p<0.05$, $p<0.05$, $p<0.01$, respectively).

Figure Caption for Figure 3.16: The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. All vehicle control groups injected at any age were significantly outperformed by the human bicistronic cohorts administered the treatment at 0-, 2-, 4- or 6-weeks of age at the 16-week timepoint. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)

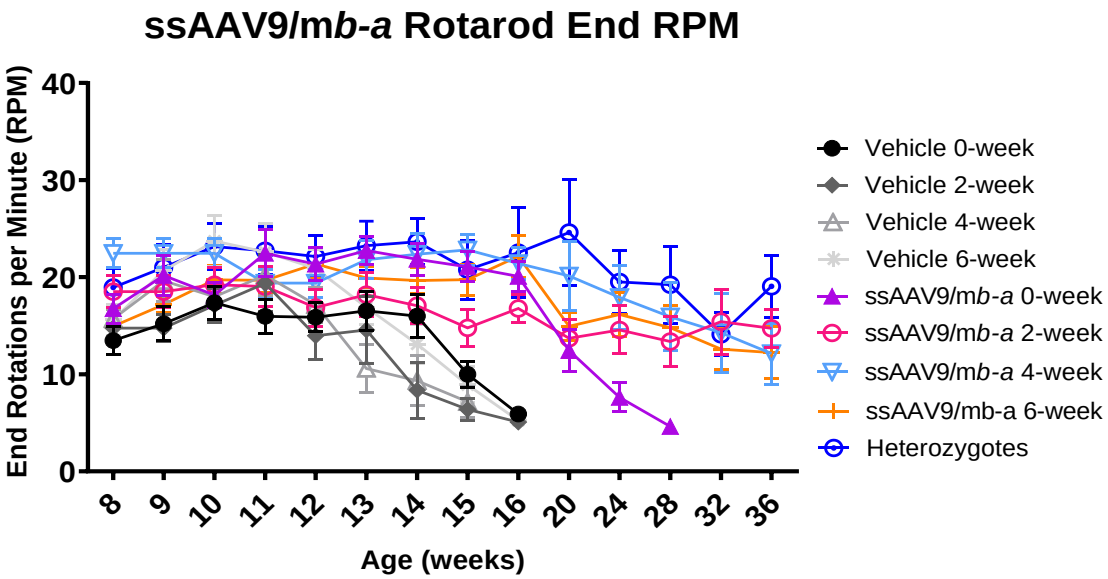


	8	9	10	11	12	13	14	15	16
Vehicle 0-week vs ssAAV9/h*B-A* 0-week								**	****
Vehicle 0-week vs ssAAV9/h*B-A* 2-week									****
Vehicle 0-week vs ssAAV9/h*B-A* 4-week									****
Vehicle 0-week vs ssAAV9/h*B-A* 6-week								***	***
ssAAV9/h*B-A* 0-week vs ssAAV9/h*B-A* 2-week			*		**			†	
Vehicle 2-week vs ssAAV9/h*B-A* 0-week								**	****
Vehicle 2-week vs ssAAV9/h*B-A* 2-week								*	****
Vehicle 2-week vs ssAAV9/h*B-A* 4-week								*	****
Vehicle 2-week vs ssAAV9/h*B-A* 6-week								***	***
ssAAV9/h*B-A* 2-week vs ssAAV9/h*B-A* 4-week									**
ssAAV9/h*B-A* 2-week vs ssAAV9/h*B-A* 6-week					*	*		**	
Vehicle 4-week vs ssAAV9/h*B-A* 0-week								†	**
Vehicle 4-week vs ssAAV9/h*B-A* 2-week									*
Vehicle 4-week vs ssAAV9/h*B-A* 4-week									***
Vehicle 4-week vs ssAAV9/h*B-A* 6-week							†	***	**
Vehicle 6-week vs ssAAV9/h*B-A* 0-week									****
Vehicle 6-week vs ssAAV9/h*B-A* 2-week									****
Vehicle 6-week vs ssAAV9/h*B-A* 4-week									****
Vehicle 6-week vs ssAAV9/h*B-A* 6-week								**	****

Figure 3.16 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for the ssAAV9/h*B-A* treated SD mice.

The assessment of coordination through the RR was assessed in the cohorts that received the **murine bicistronic** treatment (Figure 3.17). At 15 weeks of age, the ssAAV9/m*b-a* treated SD mice treated at 0-, 4-, and 6-weeks of age outperformed the vehicle cohorts treated at 0-weeks ($p<0.01$, $p<0.01$, $p<0.05$, respectively) and 4-weeks ($p<0.01$, $p<0.001$, $p<0.01$, respectively), and the 0-, 2-, 4-, and 6-week murine bicistronic vector administrations outperformed the 2-week vehicle controls ($p<0.01$, $p=0.095$, $p<0.001$, $p<0.01$, respectively). The 6-week vehicle cohort was also outperformed at 15 weeks by the 0- and 4-week ssAAV9/m*b-a* treatment ($p<0.05$, $p<0.01$, respectively). At 16 weeks, the 0-, 2-, 4-, and 6- week vehicle mice were significantly outperformed by the SD mice treated with the ssAAV9/m*b-a* vector at any age of administration ($p<0.05$, for each). At 11 weeks, the 4-week ssAAV9/m*b-a* cohort differed significantly from the 0-week cohort ($p<0.01$), the 2-week cohort ($p<0.05$), the 6-week cohort ($p<0.0001$), and the 4- and 6-week vehicle cohorts ($p<0.001$, and $p=0.089$, respectively).

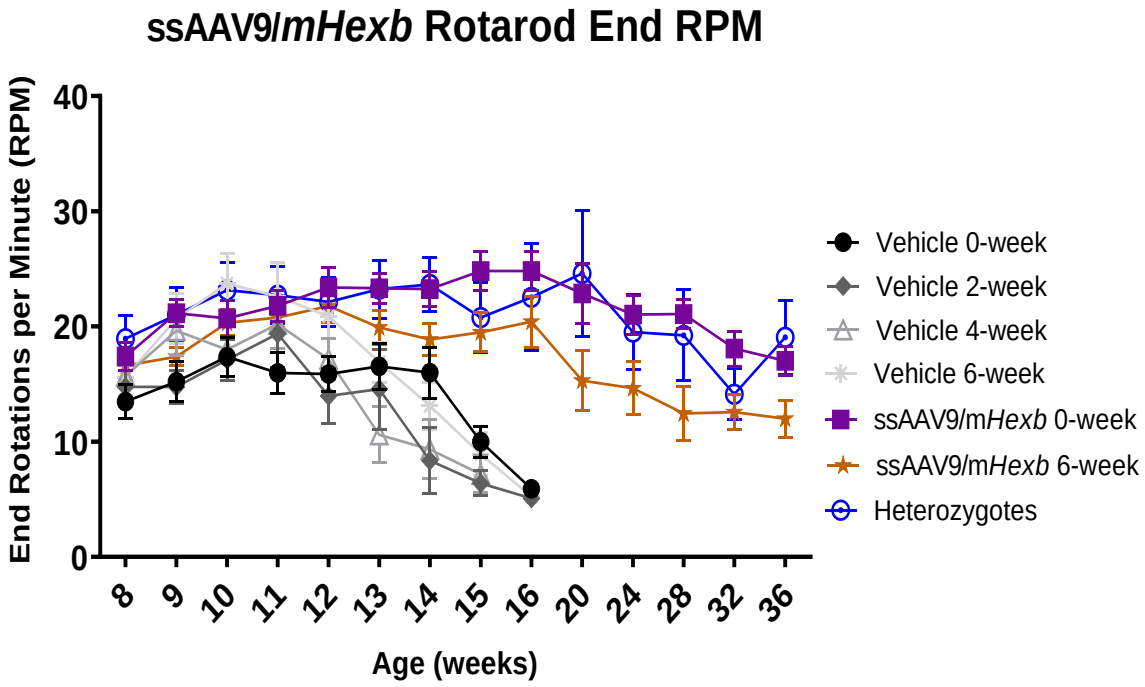
Figure Caption for Figure 3.17: The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. All vehicle control groups injected at any age were significantly outperformed by the murine bicistronic cohorts administered the treatment at 0-, 2-, 4- or 6-weeks of age at the 16-week timepoint. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)



	8	9	10	11	12	13	14	15	16
Vehicle 0-week vs ssAAV9/m*b-a* 0-week								**	***
Vehicle 0-week vs ssAAV9/m*b-a* 2-week									***
Vehicle 0-week vs ssAAV9/m*b-a* 4-week	*							**	***
Vehicle 0-week vs ssAAV9/m*b-a* 6-week								*	**
ssAAV9/ m*b-a* 0-week vs ssAAV9/m*b-a* 4-week				**					
Vehicle 2-week vs ssAAV9/m*b-a* 0-week								**	***
Vehicle 2-week vs ssAAV9/m*b-a* 2-week								†	***
Vehicle 2-week vs ssAAV9/m*b-a* 4-week								***	***
Vehicle 2-week vs ssAAV9/m*b-a* 6-week								**	***
ssAAV9/m*b-a* 2-week vs ssAAV9/m*b-a* 4-week				*					
Vehicle 4-week vs ssAAV9/m*b-a* 0-week							†	**	**
Vehicle 4-week vs ssAAV9/m*b-a* 2-week									*
Vehicle 4-week vs ssAAV9/m*b-a* 4-week	†			*			†	***	**
Vehicle 4-week vs ssAAV9/m*b-a* 6-week								**	**
ssAAV9/m*b-a* 4-week vs ssAAV9/m*b-a* 6-week	*			***					
ssAAV9/m*b-a* 4-week vs Heterozygotes				**					
Vehicle 6-week vs ssAAV9/m*b-a* 0-week								*	****
Vehicle 6-week vs ssAAV9/m*b-a* 2-week									***
Vehicle 6-week vs ssAAV9/m*b-a* 4-week				†				**	****
Vehicle 6-week vs ssAAV9/m*b-a* 6-week									***

Figure 3.17 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for the ssAAV9/m*b-a* treated SD mice.

Lastly, the coordination of the **ssAAV9/*mHexb*** treated SD mice was evaluated over the 16-week time course (Figure 3.18) and showed that at the 15-week assessment, the 0-week ssAAV9/*mHexb* cohort significantly outperformed all vehicle control administrations ($p<0.01$, each), and that the 6-week ssAAV9/*mHexb* cohort significantly outperformed the 0-, 2-, and 4-week vehicle administrations ($p<0.05$, $p<0.01$, $p<0.01$, respectively), and whose coordination was nearly significantly improved compared to the 6-week vehicle controls ($p=0.0547$). At the 16-week coordination assessment, the ssAAV9/*mHexb* mice treated at either 0- or 6-weeks significantly outperformed all vehicle control cohorts ($p<0.05$; Figure 3.18).



	8	9	10	11	12	13	14	15	16
Vehicle 0-week vs ssAAV9/*mHexb* 0week								***	****
Vehicle 0-week vs ssAAV9/*mHexb* 6-week								*	**
Vehicle 2-week vs ssAAV9/*mHexb* 0-week								***	****
Vehicle 2-week vs ssAAV9/*mHexb* 6-week								**	**
Vehicle 4-week vs ssAAV9/*mHexb 0*-week						†	*	***	***
Vehicle 4-week vs ssAAV9/*mHexb* 6-week								**	*
Vehicle 6-week vs ssAAV9/*mHexb 0*-week								**	****
Vehicle 6-week vs ssAAV9/*mHexb* 6-week								†	**

Figure 3.18 Behavioural Testing for Coordination – End Rotations per Minute (End RPM) in the Rotarod Test for the ssAAV9/*mHexb* treated SD mice.

The untreated vehicle group is represented in black, while the heterozygous normal controls are represented in royal blue. The vehicle controls of each age of injection were significantly outperformed by the 0- and 6- week mHexb cohorts at 15 and 16 weeks or age, except for where the 6-week treatment cohorts performance approached significance from that of the 6-week vehicle control group at the 15-week assessment. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$, † = approaching significance)

3.4.3 Biochemical Measures

3.4.3.1 Hexosaminidase Activity Assay

The HexA enzyme activity was assessed in the midsection of the brain (MB) (Figure 3.19 & Figure 3.20) and liver (Figure 3.21 & Figure 3.22) of the experimental SD mice. For each organ, the results below are broken down into assessments of the different vectors at each age of administration, followed by the examination of the ages of injection by vector type.

3.4.3.1.1 Midsection of the Brain - Vectors Across the Ages of Administration

In the comparison of enzyme activity across the MB samples of the treated cohort, there was an overall effect of vector and age of administration ($p<0.0001$, $F_{(26,110)}=8.211$). The assessment of Hex A production when administered **0-week** (Figure 3.19A), **4-week** (Figure 3.19C), and **6-week** (Figure 3.19D) resulted in no significant increases in HexA activity with any of the cohorts administered either of the bicistronic vectors or the ssAAV9/*mHexb* ($p>0.05$). However, at the assessment of the **2-week** age of administration (Figure 3.19B), the murine bisistronic vector showed increases in HexA activity in the LT timepoint that were significant compared to the ST human bicistronic treatment ($p<0.05$) and reaching significance when compared to the vehicle control cohort ($p= 0.0699$).

Caption for Figure 3.19: Hexosaminidase A activity in the Midsection of the Brain. – Age of Administration (A) Hexosaminidase A activity of the 0-week treated SD mice. No significant increases in Hexosaminidase A activity were detected across the 0-week age of administration. (B) Hexosaminidase A activity of the 2-week treated SD mice. A significant increase in Hexosaminidase A activity was observed in the LT murine bicistronic cohort compared to the ST human bicistronic cohort ($p< 0.05$). Additionally, the increase in enzymatic activity for the LT murine bicistronic group approached significance compared to the vehicle control group. (C) Hexosaminidase A activity of the 4-week treated SD mice. No significant increases in Hexosaminidase A activity were detected across the 4-week age of administration. (D) Hexosaminidase A activity of the 6-week treated SD mice. No significant increases in Hexosaminidase A activity were detected across the 6-week age of administration. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

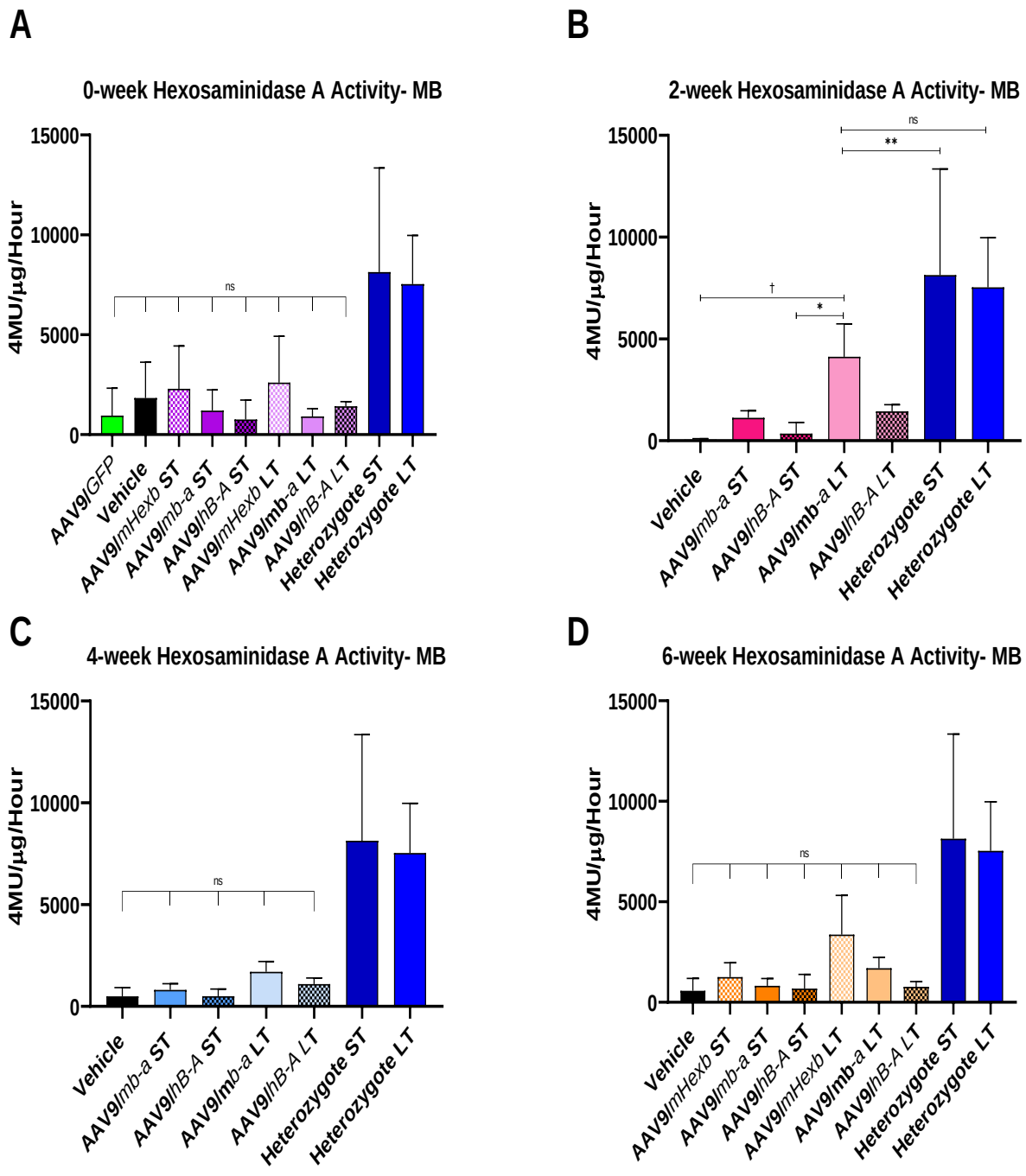


Figure 3.19 Midsection of the Brain Hexosaminidase A Activity Assay-Age of Administration.

3.4.3.1.2 Midsection of the Brain - Ages of Administration Across the Vectors

The analysis of the **ssAAV9/h*B-A*** vector across all ages of intervention showed no significant increases in enzymatic activity as compared to vehicle cohorts and ssAAV9/*GFP* controls (Figure 3.20A). The assessment of the **ssAAV9/m*b-a*** vector treatment across all ages of intervention (Figure 3.20B) showed an increase in HexA activity in the long-term 2-week administration as compared to the 4-vehicle controls ($p<0.05$), and approaches significance when compared to the 2- and 6- week vehicle controls ($p= 0.0699$, $p= 0.0666$, respectively). This increase in the long term murine bicistronic 2-week treatment group is not significantly different from the enzymatic activity in the LT heterozygous controls ($p>0.05$). As compared to vehicle controls, the analysis of the enzymatic activity in the mice administered the **ssAAV9/*mHexb*** vector (Figure 3.20C) showed non-significant increases in both the short-term and long-term 0-week administrations ($p>0.05$, each), as well as in the ST and LT 6-week administration ($p>0.05$, each). However, both the LT 0-week administration and the ST 6-week administration are not significantly different from the long-term heterozygous controls ($p>0.05$, $p=0.1499$, respectively).

Caption for Figure 3.20: Hexosaminidase A activity in the Midsection of the Brain. – Vectors (A) Hexosaminidase A activity of the SD mice treated with the ssAAV9/h*B-A* vector. No significant increases in Hexosaminidase A activity were detected across the human bicistronic ages of administration. (B) Hexosaminidase A activity of the SD mice treated with the ssAAV9/m*b-a* vector. The increase of HexA activity in the LT 2-week administration of the murine bicistronic vector is significant compared to the 4-week vehicle controls (*= $p<0.05$), and is nearly significant to the 2- and 6- week vehicle controls. The LT 2-week m*b-a* group showed no significant difference when compared to the LT Heterozygous normal controls. (C) Hexosaminidase A activity of the SD mice treated with the ssAAV9/*mHexb* vector. No significant increases in Hexosaminidase A activity were detected across the murine *Hexb* only ages of administration. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

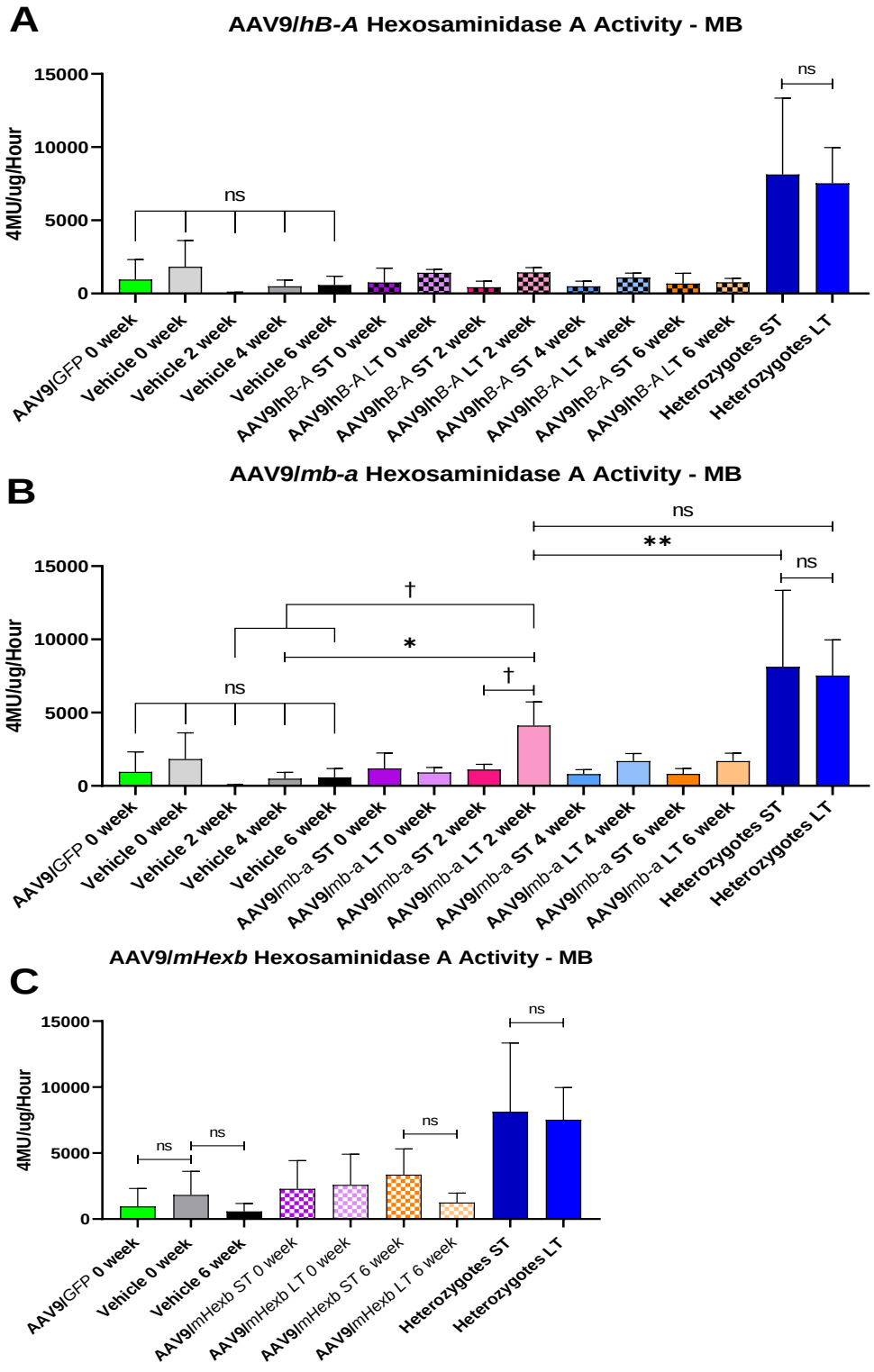


Figure 3.20 Midsection of the Brain Hexosaminidase A Activity Assay - Vectors.

3.4.3.1.3 Liver Samples - Vectors Across the Ages of Administration

The levels of HexA activity in the livers of the treated cohorts also showed a highly significant effect of age of administration and vector used ($p<0.0001$, $F_{(26,81)}=9$). Although there were no significant increases observed among either the human and murine bicistronic vector and the ssAAV9/*mHexb* treatment groups at **0-week** (Figure 3.21A) and **2-week** (Figure 3.21B) administrations, the **4-week** administration (Figure 3.21C) showed a huge increase in HexA activity observed in the liver samples of the murine bicistronic ST 4-week treated cohort that was significantly different from that of every other experimental cohort injected at 4-weeks including the heterozygote control groups ($p<0.0001$, each). Furthermore, the increase in HexA activity observed in the **6-week** assessment of the different vectors (Figure 3.21D) showed the massive highly significant increase in the ST murine bicistronic timepoint that was differed significantly from the 6-week vehicle, heterozygote cohorts, the ST and LT human bicistronic administrations, as well as the murine bicistronic and ssAAV9/*mHexb* treated cohorts ($p<0.0001$, each). Next, the HexA enzyme activity in the ST ssAAV9/*mHexb* treatment was meaningfully increased compared to that measured in the LT human bicistronic group ($p<0.0001$), but did not differ significantly from the ST or LT murine bicistronic cohorts, the ST human bicistronic cohorts as well as the LT ssAAV9/*mHexb* group.

Caption for Figure 3.21: (A) Hexosaminidase A activity of the 0-week treated SD mice. No significant increases in Hexosaminidase A activity were detected across the 0-week age of administration. (B) Hexosaminidase A activity of the 2-week treated SD mice. No significant increases in Hexosaminidase A activity were detected across the 2-week age of administration. (C) Hexosaminidase A activity of the 4-week treated SD mice. The murine bicistronic ST cohort had an extreme significant increase in Hexosaminidase A activity as compared to all other cohorts assayed. (****= $p<0.0001$). (D) Hexosaminidase A activity of the 6-week treated SD mice. The ST cohorts of the murine bicistronic cohorts also showed a very significant increase in HexA activity over all other cohorts assayed (****= $p<0.0001$). (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

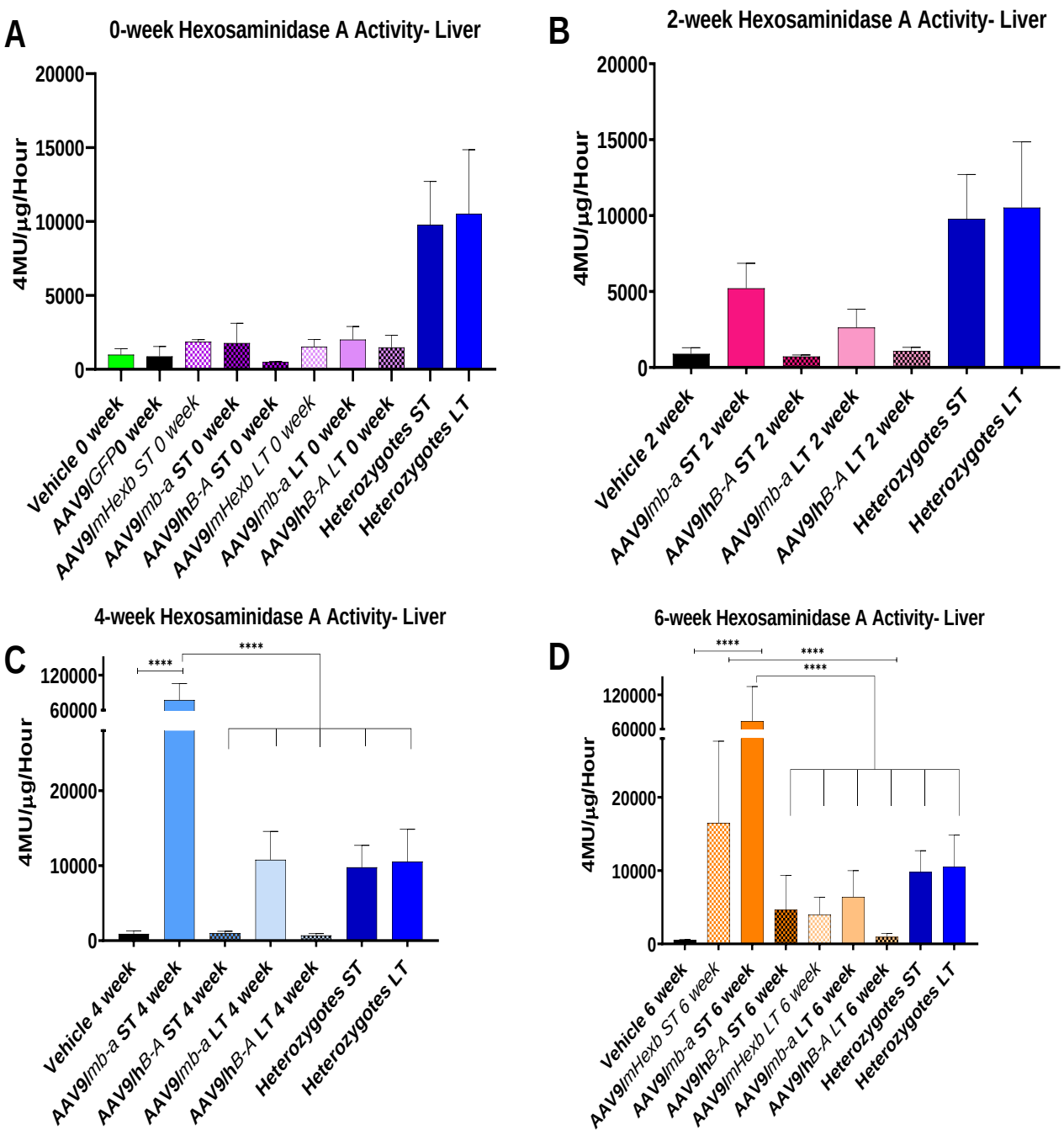


Figure 3.21 Liver Hexosaminidase A Activity Assay – Age of Administration.

3.4.3.1.4 Liver Samples - Ages of Administration Across the Vectors

When assessing the **human bicistronic** vector across all ages of administration, there were no significant differences detected (Figure 3.22A). However, within the assessment of the **murine bicistronic** group (Figure 3.22B), the increase in HexA activity in the 4-week and 6-week short-term assessments of the ssAAV9/mb-a vector were highly significant compared to every vehicle and heterozygote control, as well as to the 0- and 2- week administrations ($p<0.0001$, each). The 0- and 2-week ages of administration did not convey significant increases in HexA activity above those of the untreated vehicle controls. Finally, the enzymatic activity measured for the **ssAAV9/*mHexb*** (Figure 3.22C) showed no significance above vehicle control levels for the 0-week age of administration cohorts and for the LT 6-week cohort. However, the 6-week ssAAV9/*mHexb* ST cohort did show significant increases in enzyme activity compared to the vehicle controls and the 0-week administration ($p<0.0001$, each), which did not differ significantly from the heterozygous controls ($p>0.05$).

Caption for Figure 3.22: (A) Hexosaminidase A activity of the SD mice treated with the ssAAV9/h*B-A* vector. No significant increases in Hexosaminidase A activity were detected across the human bicistronic ages of administration. (B) Hexosaminidase A activity of the SD mice treated with the ssAAV9/m*b-a* vector. The 4-week ST and the 6-week LT murine bicistronic administrations showed highly significant increases in HexA enzyme activity compared to all other organs assayed. (****= $p<0.0001$). (C) Hexosaminidase A activity of the SD mice treated with the ssAAV9/*mHexb* vector. The LT 6-week administration of the m*Hexb* treatment showed significant increases in HexA activity over the vehicle and GFP controls. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

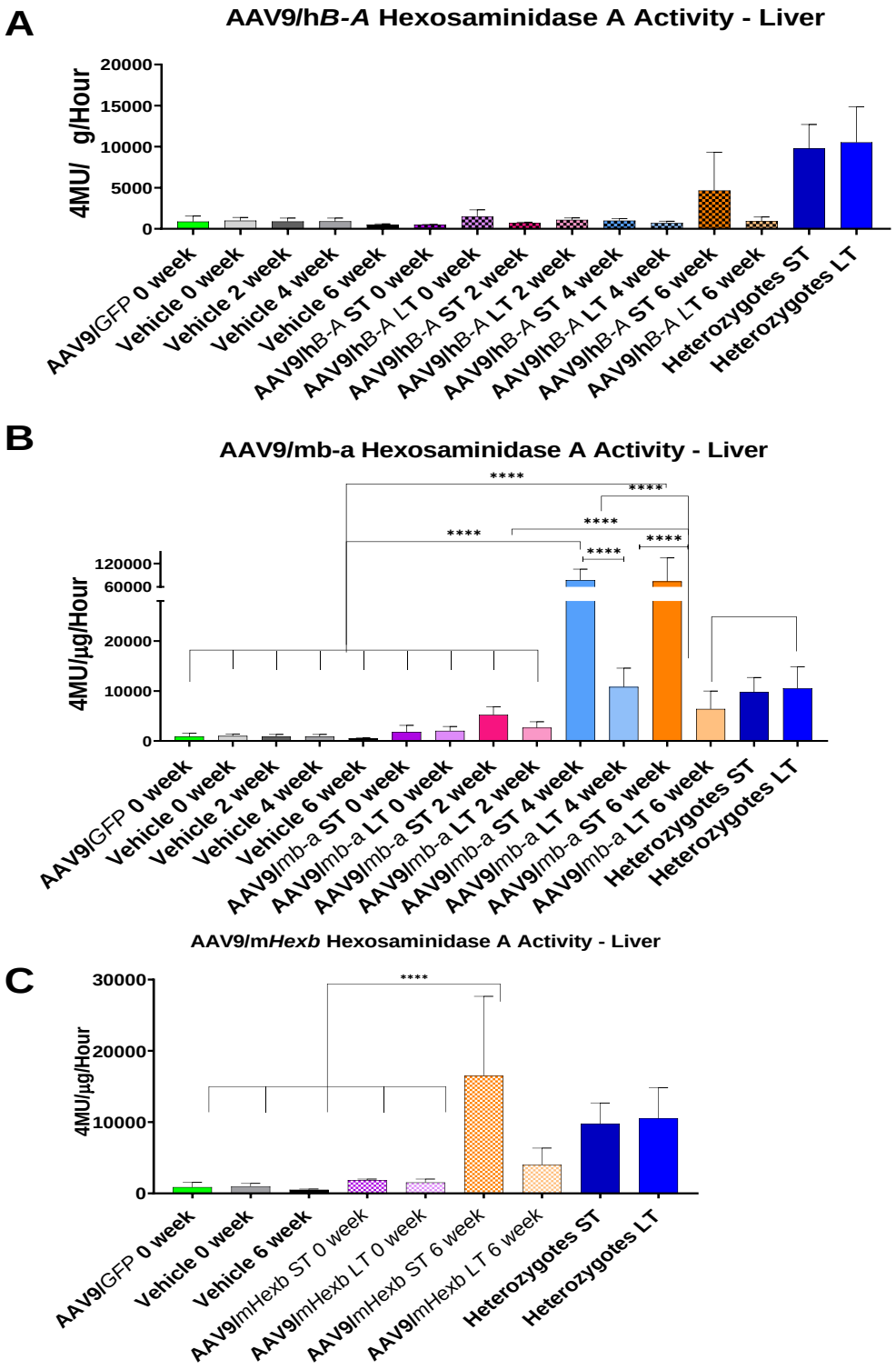


Figure 3.22 Liver Hexosaminidase A Activity Assay - Vectors.

3.4.3.2 GM2 Storage Analysis

A significant effect was observed in the reduction of GM2 gangliosides across the ages of administration and the vectors ($p<0.0001$, $F_{(26,111)}=1.503$; Figure 3.23 & Figure 3.24). Below the results are broken down into assessments about the different vectors at each age of administration, followed by the examination of the ages of injection by vector type.

3.4.3.2.1 Midsection of the Brain - Vectors Across the Ages of Administration

The storage of GM2 across the treatments delivered at the **0-week** administration (Figure 3.23A) showed a highly significant decrease in the ssAAV9/*mHexb* ST and ssAAV9/h*B-A* ST cohorts as well as the ssAAV9/*mHexb* LT cohort as compared to vehicle controls ($p<0.0001$, $p<0.0001$, $p<0.0001$, respectively). Although the clearance of GM2 observed in the ssAAV9/m*b-a* ST cohort was also significant compared to the vehicle controls ($p<0.05$), it was also significantly higher than the clearance observed in the ssAAV9/*mHexb* ST cohort ($p<0.01$). The accumulation of the murine and human long-term cohorts treated at 0-weeks did not differ significantly from the 0-week vehicle controls ($p>0.05$). Additionally, the clearance of the GM2 in the human bicistronic ST group and the ssAAV9/*mHexb* ST and LT cohorts did not differ significantly from that of either ST or LT heterozygous control cohorts.

The assessment of GM2 storage in the **2-week** administration cohorts (Figure 3.23B) revealed that both the murine and human bicistronic ST cohorts and the murine bicistronic cohort had significantly less GM2 accumulated than the 2-week vehicle control cohort. The accumulation in the ssAAV9/h*B-A* LT and 2-week vehicle cohorts did not differ significantly ($p>0.05$). Moreover, the clearance of GM2 observed in the ssAAV9/m*b-a* and ssAAV9/h*B-A* ST and the ssAAV9/m*b-a* LT cohorts does not differ significantly from that of either heterozygous control time-point ($p>0.05$).

The **4-week** age of administration (Figure 3.23C) showed significant GM2 clearance in the ssAAV9/m*b-a* ST cohort ($p<0.0001$) as compared to vehicle controls. This decrease observed

in the murine bicistronic group does not differ significantly from either heterozygous control time-point ($p>0.05$). The ssAAV9/m*b-a* LT and both the ST and LT human bicistronic 4-week administrations showed no significant decrease in GM2 storage as compared to the 4-week vehicle controls ($p>0.05$).

The clearance of GM2 from the **6-week** treated cohorts (Figure 3.23D) showed highly significant decreases in GM storage in the ssAAV9/*mHexb* ST and LT cohorts, as well as the ssAAV9/m*b-a* ST cohort compared to the 6-week vehicle control group ($p<0.0001$, $p<0.0001$, $p<0.0001$, respectively). The decrease observed in the 6-week ssAAV9/h*B-A* ST group and the levels measured in the ssAAV9/h*B-A* LT and ssAAV9/m*b-a* LT cohorts did not differ significantly from the levels accumulated in the vehicle controls. Furthermore, the decrease in GM2 storage seen in the ST murine bicistronic and both ssAAV9/*mHexb* cohorts were not significantly different from the levels measured in either heterozygous control cohort ($p>0.05$).

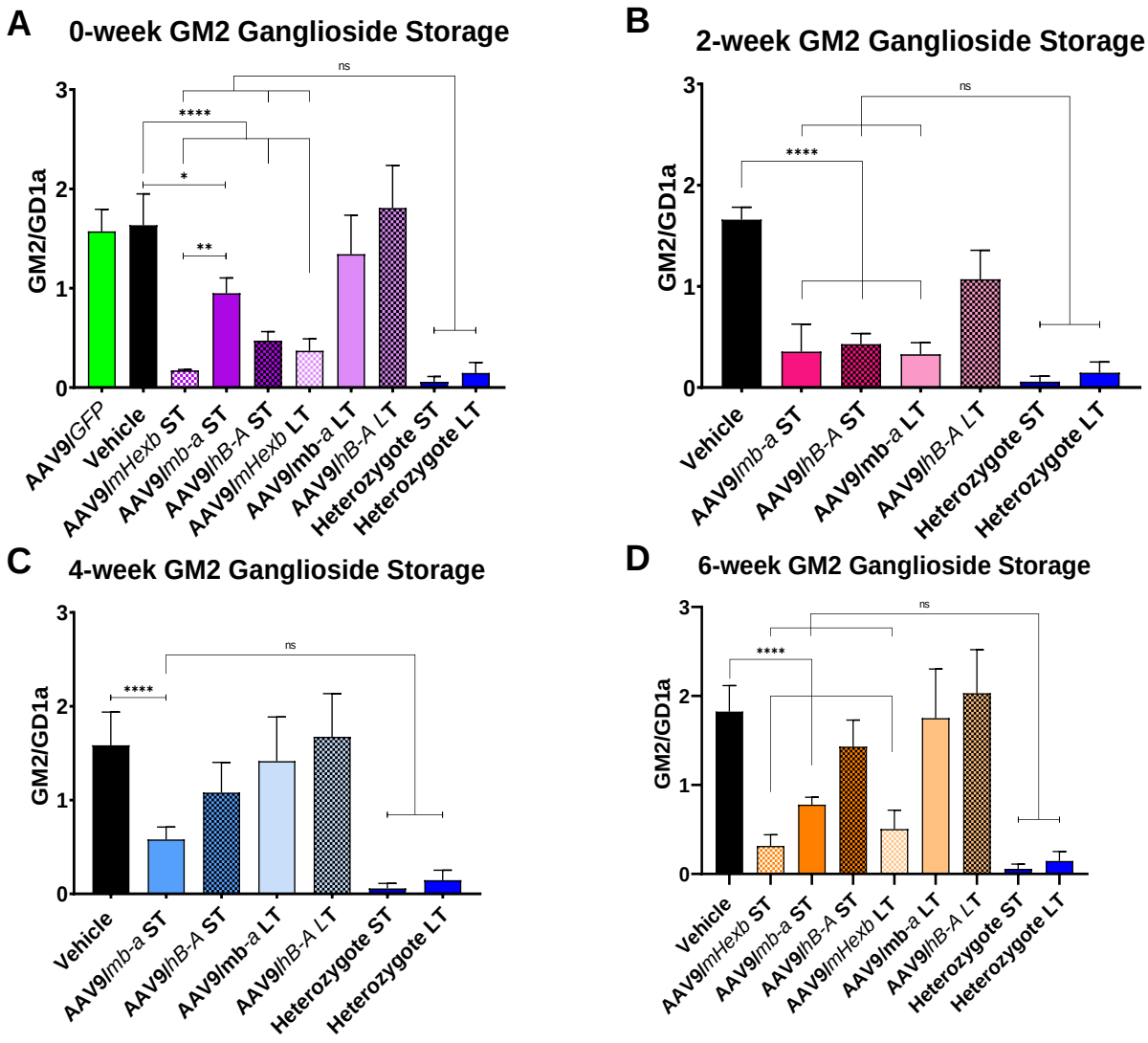


Figure 3.23 GM2 Ganglioside Storage Assay – Age of Administration

(A) GM2 accumulation of the 0-week treated SD mice. Highly significant decreases in GM2 storage were observed in the ST cohorts of both human and murine bicistronic groups as well as the mHexB cohort as compared to the vehicle controls. (****= $p<0.0001$). (B) GM2 accumulation of the 2-week treated SD mice. The ST and LT murine bicistronic group, and the ST human bicistronic group all showed highly significant decreases in GM2 storage as compared to vehicle controls (****= $p<0.0001$, each). (C) GM2 accumulation of the 4-week treated SD mice. The ST murine bicistronic cohort showed significantly less GM2 storage than the vehicle controls. (****= $p<0.0001$). (D) GM2 accumulation of the 6-week treated SD mice. A significant decrease in GM2 ganglioside storage was observed in the ST and LT m*HexB* cohorts, as well as the ST murine bicistronic cohort (****= $p<0.0001$, each). (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

3.4.3.2.2 Midsection of the Brain - Ages of Administration Across the Vectors

The accumulation of GM2 gangliosides in the midsections of the brain of the **human bicistronic** treatment (Figure 3.24A) showed that, as compared to the vehicle controls, there was a highly significant decrease in GM2 accumulation was seen in the short-term 0-week and 2-week administrations ($p<0.0001$, $p<0.0001$, respectively), as well as a significant decrease in GM2 storage at the short-term 4-week administration ($p<0.05$). As compared to vehicle controls, the GM2 storage in the long-term assessments were non-significant at the 0-, 4- and 6- week administrations ($p= 0.999$, each), but at the long-term 2-week administration there was a significant clearance of GM2 ($p< 0.05$). In addition, as compared to the short-and long-term heterozygous controls, no significant difference was observed in the GM2 accumulation between the short-term 0- and 2- week administrations ($p= 0.7908$, $p=0.9964$, $p= 0.9936$, $p> 0.9999$, respectively). The accumulation of GM2 stored in the short-term 0-week administration of the human bicistronic vector was not significantly different from the 2- and 4- week accumulation ($p> 0.9999$, $p= 0.2043$, respectively), but was significantly reduced as compared to the 6-week administration($p< 0.001$). In the short-term 2-week administration, the accumulation of GM2 was significantly decreased as compared to the 6- week administrations ($p<0.001$). There were no significant differences observed between the short- and long-term 4- and 6- week injections ($p= 0.2391$, $p= 0.2224$, respectively). The long-term 2-week administration showed significant clearance of GM2 as compared to the long-term 0- and 6- week administrations ($p<0.05$, $p<0.001$, respectively), but did not differ significantly from the 4-week injections ($p= 0.2133$).

When comparing the levels of GM2 clearance in the midsections of the brain of the **ssAAV9/m*b*-*a*** treated mice (Figure 3.24B), there was a highly significant decrease in the amount of GM2 accumulation seen in the short-term 0-, 2-, 4-, and 6-week administrations as compared to vehicle controls ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$, respectively). The long-term 2-week assessment of GM2 storage was also significantly decreased as compared to vehicle controls ($p<0.0001$), whereas there was no significant difference observed in the long-term 0-, 4-,

and 6-week administrations (p>0.05, each). There were no significant differences observed when comparing the short- and long-term heterozygote controls to the short- and long-term assessment of the 2-week administration (p> 0.05, each), as well as the short-term administrations of the 4-week administrations (p>0.05, each). The GM2 accumulation in the short-term 6-week administration did not significantly differ from the long-term heterozygous control (p>0.05). The short-term assessment of the GM2 accumulation in the 4- and 6-week administrations did not differ significantly from the short-term 2-week administration (p>0.05, each), nor did they differ significantly from each other (p>0.05). However, there is a highly significant decrease observed when comparing the long-term 2-week assessment of GM2 storage to the short- or long-term 0-week injections (p<0.0001, p<0.0001, respectively), and the long-term 4- and 6-week administrations (p<0.0001, p<0.0001, respectively).

The analysis of the GM2 storage in the **ssAAV9/*mHexb*** treatment (Figure 3.24C) showed that the short- and long-term assessments at the 0- and 6- week ages of administrations had highly significant decreases as compared to vehicle controls (all *p's* <0.0001). The GM2 accumulation observed in the short- and long-term 0-week administration (p> 0.05, each), as well as the short-term 6-week administration (p> 0.05, each) did not differ significantly from either short- or long-term heterozygous controls. The 0-week administration of the ssAAV9/*mHexb* treatment did not differ significantly from the 6-week administration at either the short-term or long-term administrations (p> 0.05, each).

Caption for Figure 3.24: Very significant decreases in GM2 storage across the ages of administration and vectors. (A) GM2 accumulation of the SD mice treated with the ssAAV9/h*B-A* vector. The 0- and 2- week ST cohorts showed and extremely significant decrease in GM2 storage as compared to vehicle controls (****= p<0.0001, each). (B) GM2 accumulation of the SD mice treated with the ssAAV9/m*b-a* vector. The 0- and 6- week ST assessments of the murine bicistronic vector revealed significantly less GM2 storage in the midsections of the brain (****= p<0.05, each), whereas the ST and LT 2-week administration and the ST 4-week cohort showed an highly significant decrease in GM2 accumulation (****= p<0.0001, each). (C) GM2 accumulation of the SD mice treated with the ssAAV9/*mHexb* vector. Both the ST and LT assessments of the 0- and 6- week administration of the m*Hexb* treatment conveyed highly significant decreases in GM2 storage compared to the vehicle controls (****= p<0.0001, each). (*= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns= p>0.05)

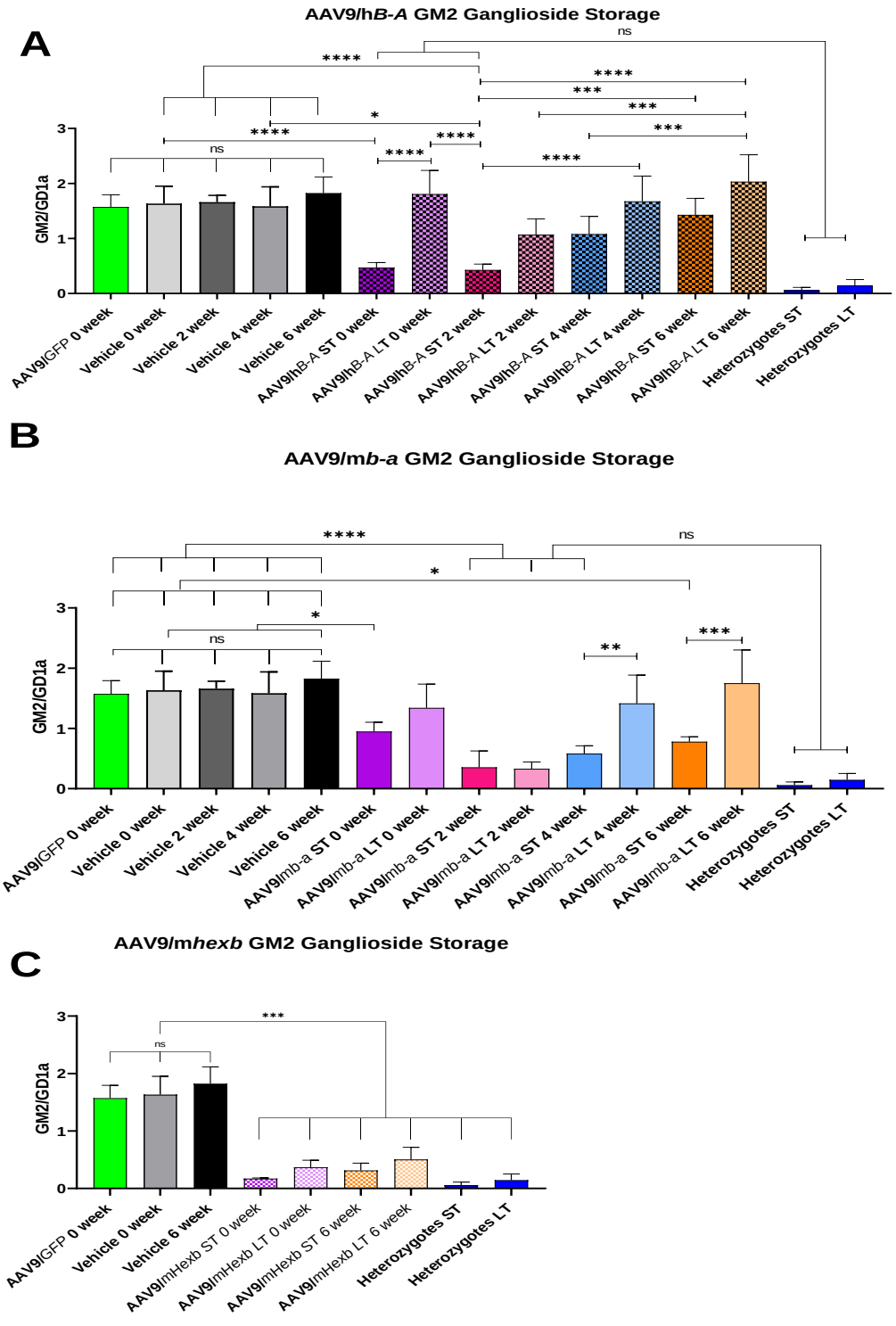


Figure 3.24 GM2 Ganglioside Storage Assay.

3.4.4 Biodistribution Analysis

At all ages of administration, the biodistribution analysis revealed movement of the ssVVA9/h*B-A* vector across the blood brain barrier (Figure 3.25). Beyond the 2-week age of administration, the biodistribution analysis showed uniform vector distribution across the brain and spinal cord regions. In addition, the copy number of the vector increases with the age of administration. There was a significant effect on the biodistribution across the organs ($p<0.001$, $F_{(5, 144)}=5.421$), however, upon further post-hoc analysis, there were no significant differences observed in the biodistribution data upon performance of a 2-way ANOVA.

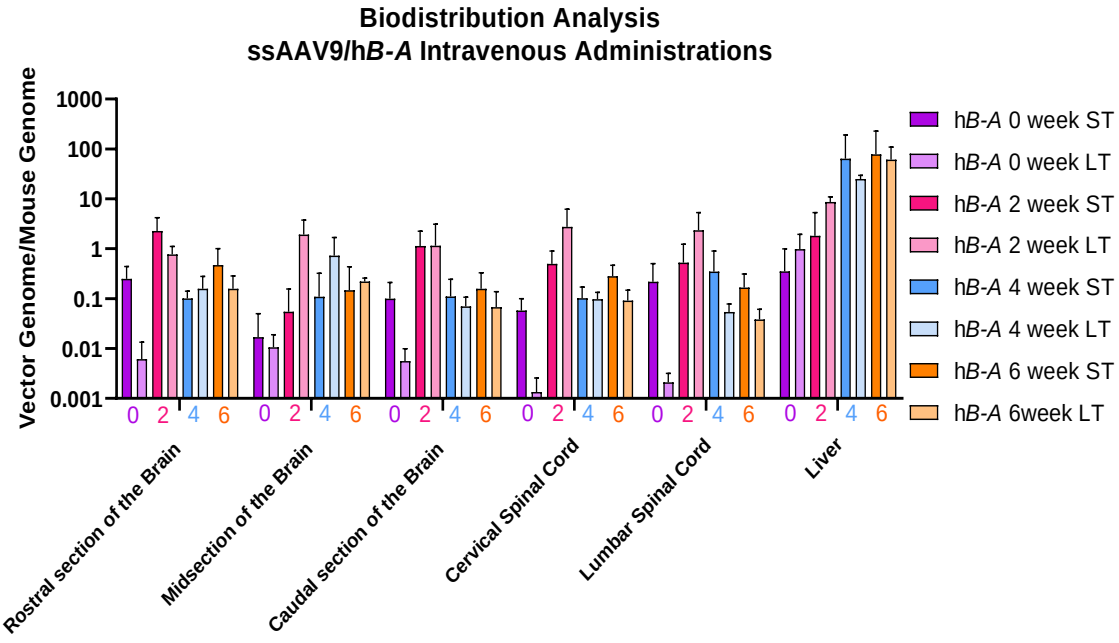


Figure 3.25 Biodistribution of the ssAAV9/h*B-A* vector across the CNS and in the Liver.

There was a significant effect on the biodistribution across the organs ($p<0.001$), however, upon further post-hoc analysis, there were no significant differences in the biodistribution observed across the different ages of administration for the human bicistronic gene therapy treatment. (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

3.4.5 Preliminary Immunological and Toxicological Outcomes

The anti-human HexA antibody response assessed in the experimental SD mice treated with the human bicistronic vector at 0-, 2-, 4-, and 6-week administrations determined that there was a significant increase in anti-hHexA antibody production as the age of administration is delayed (Figure 3.26A-D). Serum collected from the 8 week blood collection showed a significant effect of age of administration ($p<0.001$, $F_{(3,35)}=6.397$; Figure 3.26A). A significant increase in the 6-week ssAAV9/hB-A antibody response was observed compared to the 0-, 2-, 4-, and 6-week vehicle controls ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.05$, respectively). Additionally, there is an increase in anti-hHexA antibody production as the age of administration increases. The 6-week ssAAV9/hB-A cohort showed significantly higher levels of anti-hHexA antibody production as compared to the cohorts administered the human bicistronic treatment at 0-, 2-, and 4-week ($p<0.0001$, $p<0.0001$, $p<0.001$, respectively). At 8 weeks, there are also non-significant increases in antibody production in the 2- and 4-week administrations as compared to the 0-week administration of the human bicistronic treatment ($p=0.99$, $p=0.74$, respectively). The antibody response from the 16-week blood collection showed a highly significant effect of age of injection ($p<0.0001$, $F_{(3,36)}=11.55$; Figure 3.26B). The 16-week anti-hHexA serum analysis showed that the 6-week ssAAV9/hB-A cohort had a highly significant increase in total antibody concentration as compared to all vehicle and all other human bicistronic treated cohorts ($p<0.0001$, for each). The 16-week assessment of antibody response also showed non-significant increases in anti-hHexA antibody responses in the 2- and 4- week human bicistronic cohorts as compared to the 0-week cohort ($p=0.9841$, $p=0.5018$). The assessment of the anti-hHexA response in the long-term cohort at 20 weeks showed a significant effect of age of injection ($p<0.05$, $F_{(1.232,3.696)}=12.89$; Figure 3.26C), where the trend of increased antibody production with delayed ages of administration. Although there was a significant effect, individual post-hoc analysis showed that the difference in antibody production between the 0- and 6-week and the 4- and 6-week administrations were nearing significance ($p=0.0771$, $p=0.0646$, respectively), however none of

these increases were found to be significantly different($p>0.05$). Lastly, a 2-way RM ANOVA was performed on the timecourse of the anti-hHexA antibody response, showing a significant effect of age of injection ($p<0.0001$, $F_{(3,67)}=47.28$; Figure 3.26D), with the 6-week administration differing significantly from the 0-, 2-, and 4- week administrations at 8 weeks ($p<0.0001$, $p<0.0001$, $p<0.001$), 16 weeks ($p<0.0001$, $p<0.0001$, $p<0.0001$), and 20 weeks ($p<0.0001$, $p<0.01$, $p<0.01$).

The cellular immune response in the 6-week treated groups was assessed via enzyme linked immune absorbent spot (ELISpot) assay (Figure 3.26E), which determined that there was a significant effect of treatment group on the production of cytokines specifically targeting human HexA peptides ($p<0.01$, $F_{(2, 30)}=6.414$). Specifically, the number of spots (ELISpots) produced against the human HexA peptides in the ssAAV9/hB-A cohort is significantly higher than those produced in the vehicle controls ($p<0.05$) and is nearing significance than those produced by the ssAAV9/*mHexb* group ($p= 0.0551$). The number of cells secreting cytokines against the human HexA peptides in the human bicistronic group did not differ significantly from the cytokines produced in the murine bicistronic cohort ($p= 0.1022$). Lastly, there was no significant difference between the number of cells secreting cytokines against human HexA peptides in the ssAAV9/h*B-A* and the immunosuppressed ssAAV9/h*B-A* cohorts.

Figure 3.26 Caption: (A) Anti-hHexA levels measured at 8-weeks of age. The 6-week administration showed a significantly stronger antibody response to the foreign human HexA enzyme than the vehicles and human bicistronic treated SD mice at every other age of administration ($p<0.05$, for each age of administration and treatment). (B) Anti-hHexA levels measured at 16-weeks of age. The 6-week administration showed a highly significant increase in antibody production to the foreign human HexA enzyme than the vehicles and human bicistronic treated SD mice at every other age of administration ($p<0.0001$, for each age of administration and treatment). (C) Anti-hHexA levels measured at 20-weeks of age. The 6-week administration showed a trend towards increased anti-hHexA antibody production than the other ages of administration, however the difference was not significant ($p>0.05$). (D) Time course of the anti-hHexA levels in all the human bicistronic cohorts. The antibody response of the ssAAV9/h*B-A* SD cohort at 6-weeks had significantly higher levels of anti-human HexA antibodies compared to the 0-, 2-, and 4-week administrations at the 8, 16, and 20 weeks of age ($p<0.01$, for each age of administration at each timepoint). (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$, ns= $p>0.05$)

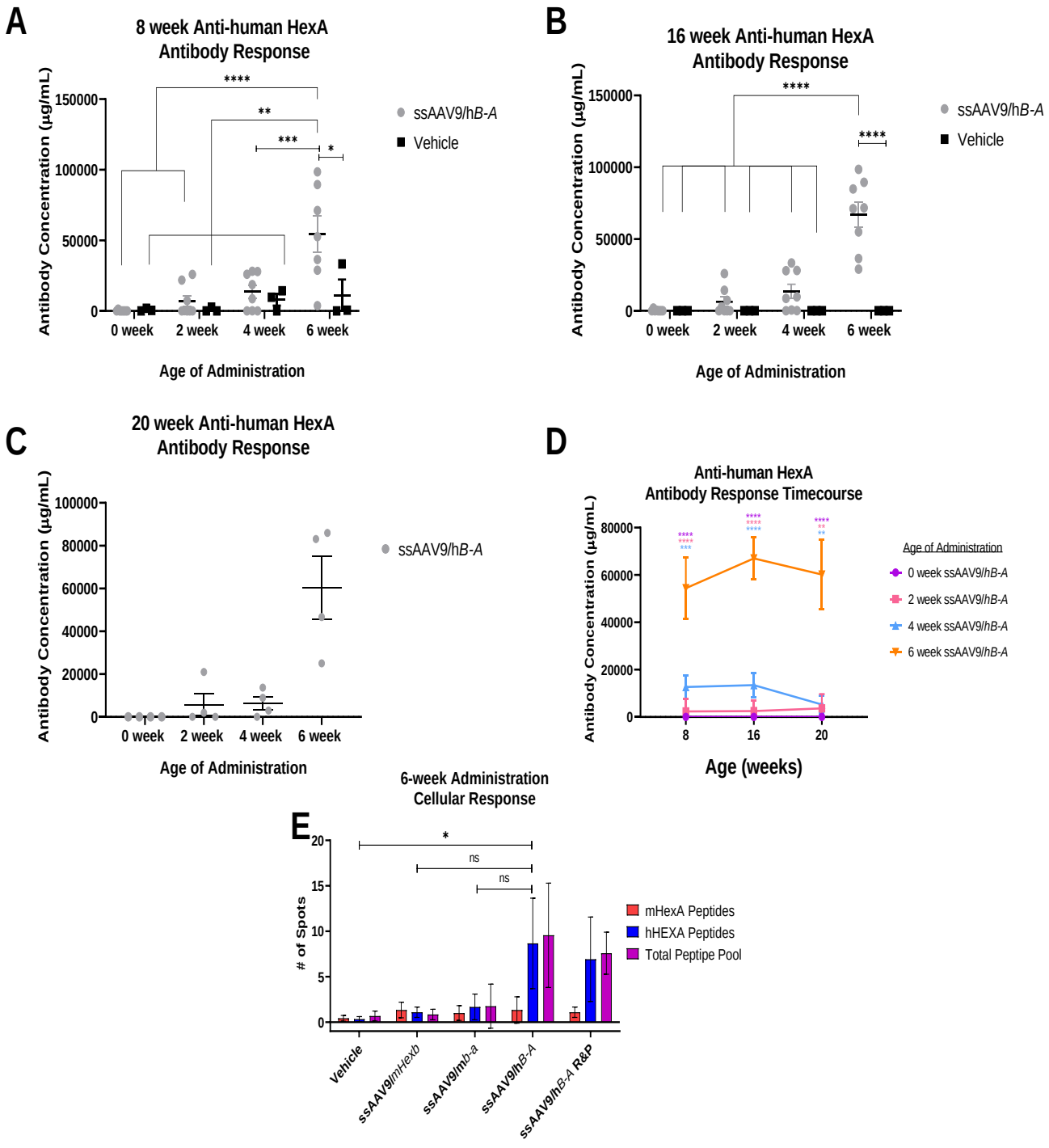


Figure 3.26 Immunological Response - Anti-hHexA Antibody Response and Cytokine Response.

In the toxicological parameters (Figure 3.27), there was no significant difference observed in white blood cell count between the treatment groups of the immunosuppressive study ($p=0.1309$, $F_{(5, 17)}=1.996$). All white blood cell counts in the treated mice hovered or were within the acceptable limits of the normal white blood cell counts in mice (Figure 3.27A). The alanine transaminase assay also showed no significant differences between treatment groups ($p=0.6264$, $F_{(5, 17)}=0.7067$). Other than one substantial outlier in the ssAAV9/m*b-a* group, all other mice hover around or within the normal murine range for alanine transaminase levels (Figure 3.27B).

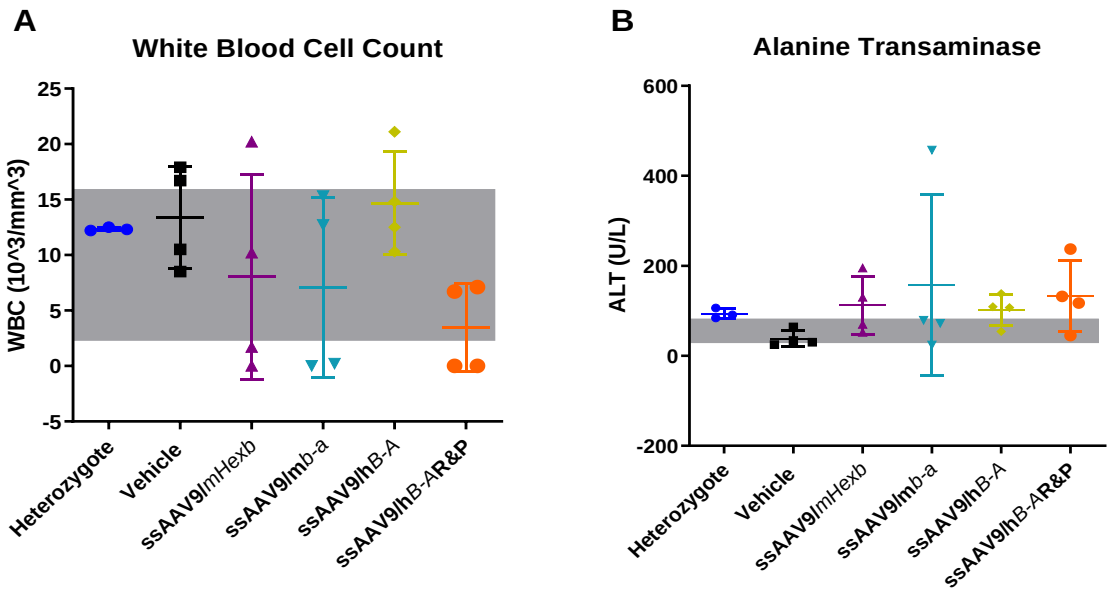


Figure 3.27 Preliminary Toxicological Parameters for mice euthanized at 9 weeks of age.

(A) White blood cell (WBC) counts. Normal average levels of WBCs are observed in all cohorts. (B) Alanine Transaminase (ALT) activity levels. ALT levels hovered around normal enzyme levels in all cohorts, other than one of the four mice in the murine bicistronic group.

3.5 Discussion

Although there have been previous attempts to treat GM2 Gangliosidosis using a gene therapy strategy, no previous study assessed the use of a bicistronic hexosaminidase construct at therapeutic levels. In the current study, we show that the bicistronic vectors are effective at treating GM2 Gangliosidosis in the SD mice. In addition, all other studies that assessed the best age of administration were conducted using an invasive intracranial injection, as opposed to the less invasive intravenous injections performed herein.

This study serves to further the investigation into the human bicistronic hexosaminidase gene therapy treatment published from our lab (Woodley et al., 2019). That proof-of-concept study focused on the feasibility of the human bicistronic treatment at a low dose in SD mice, whereas the current study investigates the *in vivo* effects of a clinically relevant dose of the human bicistronic, the murine bicistronic, and the ssAAV9/*mHexb* vectors administered in SD mice at neonatal, juvenile, and adult ages.

Overall, the primary hypothesis that earlier intervention would produce more favourable outcomes was supported, excluding the ssAAV9/mb-a 0-week administration. Treatment at or before 2 weeks of age proved to be the most beneficial based on the clearance of GM2 throughout the midsection of the brain, as well as the survival benefit. This is congruent with work done by Cachón-González et al. in 2014 where they performed intracranial injections on SD mice to investigate the latest age of intervention. The 0-week treatment is a hard technique and may have resulted in higher variability.

The secondary hypothesis stating that the murine and human bicistronic vectors would produce similar beneficial outcomes was not entirely supported. Although both human and murine bicistronic treatments showed highly significant increases in survival compared to the vehicle controls, there was a slight significant decrease in survival of the ssAAV9/hB-A vector. However, due to the heterospecific (cross-species) nature of the human transgene used im mice,

an immune response study is further warranted. The use of genetic information from a foreign species has been documented to produce an immunological response to the heterospecific proteins. This is true in the current study, where we observed anti-human HexA antibodies throughout the experimental SD mice administered the human bicistronic treatment at 2-, 4-, and 6-weeks.

Although the final hypothesis of the current study stated that the human and murine bicistronic vectors would provide an increase in beneficial outcomes compared to the ssAAV9/*mHexb* vector alone, the SD mice treated with the ssAAV9/*mHexb* vector matched or outlived either bicistronic treatment. This is likely due to the ssAAV9/*mHexb* treatment contributing to the increased availability of the β-subunits in the SD mice which contributes not only to the formation of the HexA enzyme, but also the HexB enzyme that also degrades GM2 in mice through the alternative sialidase pathway. We are quite confident that the availability of extra HexB provided the best results, but we also see that the survival benefit is not significantly different between m*Hexb* and m*Hexb-a* vector treatments at 6-week injections.

A recent study conducted by Niemir et al. (2018) utilized a similar monogenic treatment vector to the ssAAV9/*mHexb*, however their vector was scAAV and was delivered intravenously at a neonatal timepoint at a dose of $3.5x10^{10}$ vg/g (3.5×10^{13} vg/kg). This dosage is approximately a seventh of the neonatal dosage administered in the current study, however the endpoints approved by the Queen's University Animal Care Committee were much more stringent than those reported in the Niemir (2018) study. This study showed drastic improvements in survival, behavioral, and biochemical parameters of these treated SD mice, however their humane endpoints were much less stringent, requiring a 20% weight loss or the complete inability to right themselves to be considered at an endpoint (Niemir et al., 2018). In their analysis, this study also never accounted for the alternative sialidase pathway available to the mice, which is likely the reason for the extensive pre-clinical benefits seen in their results (Niemir et al., 2018). Indeed, the

ssAAV9/*mHexb* group in the current study that was injected at 0-weeks had a highly significant extension in survival, with one mouse also reaching the 104-week (2 year) study endpoint, matching the 2-year survival observed in the previous study (Niemir et al., 2018), however this increase in survival is likely due to the presence of both the HexA and HexB enzymes through the replacement of the murine β-subunit. In addition, the promoter used in this study was different – pgk promoter - as compared to our CAG promoter, so a direct comparison is hard to make (Niemir et al., 2018; Norrman et al., 2010; Qin et al., 2010).

Still, the experimental SD mice treated at any age of administration and with any of the vectors examined in this study showed drastic significant increases in survival, demonstrating that any treatment, no matter the delay, would prove to be beneficial (Figure 3.3). Table 3.4 shows the increases in survival conveyed by each experimental vector at the different ages of administration compared to the 16-week humane endpoint of the untreated vehicle controls. The age of administration that conveyed the best increase in survivability for the human and murine bicistronic vectors was the 2-week injection with an increased survival by a factor of 4.28x and 4.8x. The ssAAV9/*mHexb* treatments at 0- and 6- weeks of age did not show much of a fold increase in survival compared to each other, where the 0-week administration had an increased survival by a factor of 4.1x and the 6-week administration conveyed a 4.42x fold increase in survivability.

Age of Administration	0-week		2-week		4-week		6-week	
	Median	Increase	Median	Increase	Median	Increase	Median	Increase
ssAAV9/h*B-A*	59	3.68x	68.4	4.28x	43.6	2.73x	43.8	2.73x
ssAAV9/m*b-a*	42.2	2.64x	76.8	4.8x	65.8	4.11x	68.1	4.26x
ssAAV9/*mHexb*	65.7	4.1x	/	/	/	/	70.7	4.42x

Table 3.4 Median survival and the factor increase in survival over the vehicle cohorts.

Indeed, when comparing the ssAAV9/m*b-a* ssAAV9/*mHexb* administrations at 6-weeks, there were no significant increases in survivability conferred by one vector over the other (Figure 3.3D). This contradicts previous studies and hypotheses that treating earlier in life will provide more beneficial outcomes, possibly due to the alternative murine sialidase pathway.

When comparing the two murine vectors used in the current study, the increased survival and biochemical results from the midsection of the brain GM2 storage and Hexosaminidase activity assays are apparent and may be due to the alternative murine catabolic sialidase pathway, where GM2 gangliosides are degraded in the presence of functional murine HexB enzymes. This is supported by the overexpression of the HexA enzyme in the 4- and 6- week murine bicistronic liver samples compared to the ssAAV9/*mHexb* 6-week administration. The 4-week ST administration of the ssAAV9/m*b-a* vector showed an increase in HexA activity by a factor of 7.9x and 7.4x as compared to the ST and LT heterozygous controls, and the 6-week ST administration of the murine bicistronic vector showed a 7.5- and 7-fold increase in HexA activity compared to the ST and LT heterozygotes. In comparison, the 6-week ssAAV9/*mHexb* treatment only showed an increase in HexA activity by a factor of 1.5x and 1.6x as compared to the ST and LT heterozygous controls, respectively. This demonstrates the ability of the bicistronic vector to effectively produce high levels of the HexA enzyme, whereas the increase in survival and clearance of GM2 as compared to the bicistronic treatments can mostly be attributed to the increased activity and production of not only the murine HexA enzyme, but also the murine HexB enzyme which is also able to degrade GM2 through the alternative murine sialidase pathway.

Overall the 2-week administrations of the human and murine bicistronic hexosaminidase vectors conveyed significant survival benefits as compared to the 0-, 4-, 6-week, and the 0-, 4-week injections, respectively. However, species specific vectors do lead to better survival benefits, and in turn behavioural benefits. The murine bicistronic vector conveyed a significant

benefit in survival over the human bicistronic vector at 2-, 4-, and 6-week administration, which is reflected in the anti-human HexA antibody response observed in the experimental mice administered the ssAAV9/h*B-A* treatment at 2-, 4-, and 6-weeks.

While examining the general locomotor abilities, the ssAAV9/h*B-A* treated SD mice at 2-, 4-, and 6-weeks showed a significant increase in general locomotion compared to vehicle controls, whereas the ssAAV9/*mHexb*, ssAAV9/h*B-A* and ssAAV9/m*b-a* administrations all conveyed significant or near significant improvements in coordination to the mice at all ages of intervention. The recovery of coordination in the SD mice to that of the heterozygous control mice is promising, due to the ability of the mice to retain a higher level of motor control, rather than simple locomotion.

The comparison of the different ages of treatment administration within the same hexosaminidase gene therapy treatment produced interesting behavioural results. The behavioural improvement of the SD mice treated with the human bicistronic and ssAAV9/*mHexb* vectors showed little variation in behavioural performances across the different ages of administration. However, the behavioural murine bicistronic group demonstrated a quick decline in measures of coordination beginning at 20 weeks of age (Figure 3.17), as well as a deficiency in general locomotion at 28 weeks old (Figure 3.11). This contradicts the thought that earlier treatment intervention is best, however this finding will be further discussed below in the limitations section. Additionally, due to the variable results achieved through the 0-week administration, we concentrate most of our conclusions around the 2-, 4-, and 6-, week administrations herein.

In the short-term assessment of GM2 accumulation, there is an age of administration dependent decrease in amount of GM2 stored in the ssAAV9/*mHexb* and ssAAV9/h*B-A* treatments. Excluding the 0-week administration, the murine bicistronic vector also shows this trend in GM2 accumulation based on age of administration. The long-term assessments do not show any clear trend, as the long-term mice were euthanized at their humane endpoints, when

disease severity dictated their ability to remain on-study. Since the long-term mice were at an advanced stage of the disease course, it is expected that there were no differences between the GM2 accumulation in the long-term mice as compared to the untreated vehicle controls.

The midsection of the brain HexA activity levels weren't significantly affected other than at the short-term 2-week administration of the murine bicistronic vector (Figure 3.19B, Figure 3.20B). This may be due to insufficient amounts of the vectors that were able to cross the BBB and not sink directly to the liver, as AAV viruses tend to do. However, the assessment of the HexA activity levels in the liver showed a drastically significant increase in HexA activity in the short-term assessment of the 4- and 6- week murine bicistronic treatment cohorts (Figure 3.21C, Figure 3.21D, Figure 3.22B), as well as in the short-term 6-week ssAAV9/*mHexb* cohort (Figure 3.21D, Figure 3.22C). The drastic increase in HexA activity observed in the murine bicistronic treated groups at the later ages of delivery are consistent with the increased biodistribution observed in the human bicistronic administrations, where with the same approximate dosage delivered, the copy number of the vector increases in the liver increases when the age of delivery is delayed. This also shows the ability of the murine bicistronic vector to produce more functional HexA enzyme than the same dosage of the ssAAV9/*mHexb* treatment at 6 weeks of age (short-term analysis) (Figure 3.22D).

The assessment of the biodistribution of the human bicistronic vector showed that the administration of the vector beyond 2 weeks of age does not change the copy number taken up in the brain, showing sufficient movement of the vectors across the BBB (Figure 3.25). In addition, the vector copy number found in the liver increases with the age of administration, possibly contributing to the decreased availability of the virus treatments to the brain at later ages of administration. However, based on the results from the survival, behavioural, GM2 accumulation, and anti-body response data suggests that treatment at or before 2 weeks of age produces more favourable outcomes.

With the finding that the human bicistronic mice were reaching their long-term humane endpoints, the immunological and toxicological parameters were further investigated. There was a strong, significant heterospecific immunological response observed in the SD mice that received the ssAAV9/h*B-A* treatment at 6 weeks of age in both the anti-hHexA antibody level (Figure 3.26A-D) as well as the number of ELISpots in the presence of human HexA peptides (Figure 3.26E). The 2- and 4- week administration of the human bicistronic vector did show slight increases in anti-hHexA antibody production, however these increases are small and were not statistically significant (Figure 3.26A-D). The extremely low immunological response in the 0- and 2-week ages of intervention may be due to a tolerization effect since the therapeutic was delivered early in the lifespan of the mouse and these cohorts of mice survived the longest compared to the delayed ages of administration (4- and 6-weeks). The increase immune response in the 6-week treated mice may have contributed to their earlier disease progression of SD and human euthanization. This is reflected in the copy number analysis where despite receiving 0.75-fold more vector, the liver of the 4- week and 6-week human bicistronic cohorts had similar copy numbers (Figure 3.25 & Figure 3.26).

In a previous study using SD mice to investigate the best ages of administration of intracranial gene transfers, earlier intervention always produced more favorable outcomes than delaying treatment to a later age (Cachón-González et al., 2014). This study used the AAV2/1 virus that encapsulated either the HEXA or HEXB genes, and performed stereotaxic intracranial injections at 4-, 8-, 10- and 12- weeks of age. Their study also demonstrated a reversal in GM2 storage but not in the neuroinflammation that accompanies the disease (Cachón-González et al., 2014). The 4-week intracranial injection showed in survival by a factor of 4.6x compared to the untreated controls, whereas the intravenous injection in the of the human bicistronic treatment in the current study conveyed an increase in survival by a factor of 2.7x. This difference in survival can be attributed to both the different routes of administration, where the systemic injection of the

gene therapy treatment showed a amalgamate in the liver (Figure 3.25), where the intracranial injection is directly into the affected organ. Refining the route of administration for the treatment of GM2 Gangliosidosis is crucial for the advancement of the bicistronic treatment to clinical trials.

The main differences between the Cachón-González et al. study conducted in 2014 and the current study is the different route of administration, as well as ages of injection. With intravenous injections are much less invasive than the intracranial administrations, and therefore can be easily performed in mice younger than 4-weeks of age. Interestingly, in the current intravenous study, earlier intervention did not consistently lead to the most beneficial outcomes. where there is evidence for increased efficacy of the 2-week administration of the bicistronic vectors as compared to the 0-week injections. Disregarding human error on injection efficiency, there could be an anatomical difference for why the 2-week retro-orbital intravenous injections were superior to the 0-week temporal vein injections. Unlike the temporal vein that only drains into the body via the jugular vein, the capillary bed behind the eye partially drains into the pterygoid plexus, leading directly to the rest of the body via the jugular, but also drains into the cavernous sinus in the brain before draining into the body through the jugular. This may be why there is an increased amount of vector distribution in the 2-week administration cohort, which would then contribute to the increased behavioral, biochemical, and survival parameters.

Although the AAV viruses are considered low or non-pathogenic, the propensity for the virus to 'sink' to the liver where it may cause benign hypoplastic liver nodules has been well documented (Walia et al., 2015). The formation of hepatic nodules was observed in some of the ssAAV9/m*b-a* bicistronic and ssAAV9/*mHexb* treated groups, but only 1 of the 5 long-term ssAAV9/h*B-A* mice (and only in the 2-week administration) developed liver nodules. Mice from the 0- week and 6-week ssAAV9/*mHexb* administration developed liver nodules (1/4 and 3/5, respectively), where mice from the murine bicistronic administration also developed liver nodules

in the 2- , and 6-week administrations (2/5 and 1/5, respectively). Interestingly, no liver nodules were observed in the neonatal 0-week administrations of any vector. Interestingly, none of the 0-week bicistronic administrations developed nodules, and only 1 mouse from the ssAAV9/*mHexb* 0-week administration formed liver nodules, which is contradictive to previous literature showing that AAV induced hepatic nodule formation tends to happen in mice that received a neonatal treatment, and less in mice that received treatment at an adult (6-week) timepoint. In addition, neither of the bicistronic vectors contained the WPRE enhancer element, which may have synergistically enhanced liver nodule formation in the ssAAV9/*mHexb* treated group as previously described.

3.5.1 Limitations

One of the main limitations for the current study include the alternative sialidase pathway in mice and the differential subunit affinity (Sandhoff & Harzer, 2013; Sango et al., 1995; Suzuki et al., 1998). Unlike humans, in mice there exists an alternate pathway by which GM2 gangliosides can be degraded, the sialidase pathway. This pathway utilizes the a murine sialidase to initiate the degradation of GM2 to GA2, followed by the degradation of GA2 to lactosylceremide (LacCer) by the HexB enzyme (β-subunit homodimer) (Sango et al., 1995; Suzuki et al., 1998). This is the reason behind the asymptomatic murine model of TSD where there is an abundance of wild type β-subunit and is in stark contrast to the devastating murine model of SD, where there is no functional β-subunit (Phaneuf et al., 1996; Sango et al., 1995; Suzuki et al., 1998). Additionally, there is a slightly stronger affinity for the β-subunits to homodimerize as opposed to heterodimerizing with an α-subunit. In the bicistronic treatments, this may lead to the production of the HexB enzyme rather than the HexA enzyme. Moreover, its is a limitation that the real impact of survival due to HexA produced rather than HexB produced is hard to dissect in this study. A similar study on a double knockout mouse model made from

either SD or TSD mice with the sialidase pathway knocked out can possibly eliminate this limitation and shall be considered for future study.

The overall results seen from the neonatal ssAAV9/m*b-a* administration is another limitation to this study. There is evidence to support that earlier intervention with these diseases produces more favourable outcomes, however in the case of the 0-week ssAAV9/m*b-a* injections, this is not supported. It is evident in early behavioural timepoints that there was a benefit conveyed to the 0-week murine bicistronic treatment, however the treatment was not able to provide a long-term recovery of motor control or survival. This treatment simply delayed the age of onset of the motor impairments by approximately 4 weeks (Figure 3.17). All injections in this study were performed by the lead author, and it is unclear why such a deviation from the expected outcome of this 0-week murine bicistronic treatment. This observation was not seen in in any more injections in any age for any vector cohort (even the same vector at 2,4 and 6 weeks showed anticipated results). We have not tested the endotoxin levels of the vector preparations and there is a possibility that for that particular preparation there were high endotoxin levels or high number of empty capsids which made the preparation not well tolerated by the neonates but were acceptable to older ages.

In addition, the requirement of the presence of the GM2 activator protein to aid in the initiation of the catabolism of GM2 is another limitation to the study (Sandhoff & Kolter, 2003). Although the murine GM2 activator protein is functional and present in the SD mouse model, and although it has been shown to be a promiscuous activator protein, it is possible that the expression of an abundance of HexA from either bicistronic constructs may produce a rate limiting step, depleting the endogenous GM2 activator protein (Sandhoff & Harzer, 2013; Sandhoff & Kolter, 2003). Conversely, although the GM2 activator protein is slightly promiscuous in its affinity, it is possible that the murine GM2 activator protein will not work as efficiently with the human

HEXA enzyme, leading to an additional discrepancy between the efficiency of the human and murine constructs in treating the SD mice.

Another limitation to this study is the maturity of the BBB differs between neonatal mice, and juvenile or adult mice (Blanchette & Daneman, 2015; Daneman et al., 2010; Erdő, Denes, & de Lange, 2017; Percy et al., 2011). Unlike humans, who are born with a mature BBB, neonatal mice are born with an immature BBB that matures over the first few weeks after birth. Providing an intravenous gene therapy treatment to mice with an immature BBB does not fully recapitulate the ability of the treatment to functionally treat a mammal with an intact BBB. To that end, the 2-, 4- and 6- week injections would better represent the ability of the ssAAV9 viruses to cross a mature BBB and deliver the therapeutic to the CNS.

The dilution effect of the treatment in comparison to body size is another limitation to this study. AAV9 genomes are typically found episomally with a small percentage integrating into the host genome (Thomas et al., 2003). Although AAVs can infect both quiescent and dividing cells, the episomal nature of the AAV genome presents a problem when it comes to the propagation of the treatment during cell division. There is not only the risk that the episomal DNA is not copied and therefore not distributed to the daughter cells, but there is also the risk that the episomal DNA is degraded during the process of cell division.

The fixed dosage delivered to the mice was an additional limitation. Dosages were calculated based on the average weight of a C57/B6 mouse at that age, meaning that when administering the treatment to a group of mice at a certain age, every mouse in said cohort received the same amount vector genomes. For this reason, there is approximate dosages per kilogram of mouse included in this manuscript, however vector delivery wasn't strictly based on the weight of individual mice. Also, for 0-week treatment, the mice could have weighed 1-2 g and we chose a dose based on higher weight possibility and thus neonatal injections may have received more dose than 2,4,6-week time injected cohorts. That makes another argument that the

results of 2-, 4-, and 6- weeks cohorts can be compared to each other better than 0-week treatment results. The 0-weeks dose is same as what was used for neonatal treatment in chapter 2 and thus provided us an opportunity to compare the effect of the two human constructs in SD mice (discussed in more details in Chapter 4).

Lastly, the difference in enhancement elements in the vectors is also a limitation to this study. Both the human and murine bicistronic treatments do not contain the WPRE enhancer element, whereas the ssAAV9/*GFP* and ssAAV9/*mHexb* vectors do. This may also be a contributing factor to the improved outcomes in the ssAAV9/*mHexb* treated groups as compared to the murine bicistronic administrations. We also have not explored any integration events happening in the liver nodules. Random tumour formation in C67Bl/6 mice is reported with increasing age as well (Mori, Cardiff, & Borowski, 2019; Schile, Dion, Imai-Leonard, & Doty, 2018).

3.5.2 Future Directions

The future directions for this study should include assessing the immunological and toxicological effects of the bicistronic vectors, as well as exploring of alternative routes of administration, such as intrathecal injections. Furthermore, continuing to investigate the polypharmacological benefits of other treatments for GM2 Gangliosidosis paired with the bicistronic gene therapy treatments is another future direction for this promising treatment. Incidence and effect of these liver nodules can be studied with normal mice injected with these vectors in the future.

3.5.3 Conclusion

In conclusion, the current study demonstrated the efficacy of the murine and human bicistronic vectors in providing a long-term biochemical and clinical correction for SD in mice. It reconfirms that earlier intervention is better than delaying treatment. The differences observed between the human and murine bicistronic vectors can arguably be attributed to the

immunological responses that developed due to the heterospecific difference of the human gene/protein treatment in a murine model. The current study also demonstrated that the murine bicistronic vector was able to produce an increased amount of functional HexA compared to the ssAAV9/*mHexb* vector at the short-term assessment of the 4- and 6-week administrations, showing that the use of the bicistronic vector indeed increased the levels of HexA as opposed to increasing both HexA and HexB production. These promising results provide another steppingstone towards the discovery of a successful treatment for GM2 Gangliosidosis in clinics.

Chapter 4

General Discussion

Within Chapter 2 and Chapter 3, we have established that the scAAV9/HEXM and the ssAAV9 bicistronic vectors both have the potential to successfully treat GM2 Gangliosidosis in the SD mouse model. Below we discuss these findings in further detail, as well as the relevance of these findings and suggestions for future research projects.

4.1 Overall Findings

The primary goal of this thesis was to assess the efficacy of the intravenous administration of the human hexosaminidase hybrid and the bicistronic hexosaminidase constructs in treating SD in mice. The main overall finding of the current studies suggest that any of the two GT treatments provided to the SD mice results in significant increases in survival and behavioural outcomes as well as a significant decrease in the accumulation of GM2 within the MB of the treated mice, overall contributing to the correction of GM2 Gangliosidosis in the SD mice. The finding that the mice mount an immunological response to the HexM treatment (Kot, 2018, in preparation) spurred the preliminary investigation into the immunological responses elicited by the mice treated with the human bicistronic vector in Chapter 3.

In Chapter 2 we assessed the efficacy of the IV scAAV9/*HEXM* treatment in the SD mouse model. Six-week old SD mice were injected with either $2.5x10^{12}$ vg/mouse (low dose, LD) or $1x10^{13}$ vg/mouse (high dose, HD; ~5×10^{14} vg/kg). Assessments included survival, behavioral outcomes, vector biodistribution, and enzyme activity within the central nervous system. Toxicity was observed in the HD cohort, with 8 of 14 mice dying within one month of the injection. As compared to untreated SD mice, which have typical survival of 16 weeks, the LD cohort and the remaining HD mice had a significant survival benefit with an average/median survival of 40.6/34.5 and 55.9/56.7 weeks, respectively. Significant behavioral benefit was also observed.

These results demonstrate both the potential benefit and critical limitations of the treatment of GM2 Gangliosidosis using IV delivered AAV vectors.

Chapter 3 evaluated the impact of the age of administration of gene therapy vectors in a SD mouse model. ssAAV9 vectors containing either a human bicistronic (hB-A), a murine bicistronic (mb-a), or a murine hexb only (*mHexb*) transgene were administered intravenously to SD mice at 0 weeks of age (at 2.5 $x10^{11}$ vg/mouse or ~2.5 $x10^{14}$ vg/kg) or 2, 4 or 6 weeks of age (at 1 $x10^{12}$ vg/mouse, 1.88vg/mouse, and 2.5E12vg/mouse (respectively) or ~1.25 $x10^{14}$ vg/kg) (N=10 for each vector at each age of administration). Half the number of the mice in each cohort were euthanized at 16 weeks, equivalent to the humane endpoint for untreated controls. The rest were observed until their humane endpoint. The treatment of the SD mice with either bicistronic vector or the ssAAV9/*mHexb* vector showed significant increases in survival compared to vehicle controls ($p<0.001$). Mice treated at 0, 2, 4, and 6 weeks with the human bicistronic vector survived to a median age of 59, 68.4, 43.6, and 43.8 weeks and mice treated with the murine bicistronic vector survived to median age of 42.2, 76.8, 65.8, and 68.14 weeks, respectively. Mice treated with the ssAAV9/*mHexb* vector at 0 and 6 weeks survived to a median age of 65.72 and 70.71 weeks respectively, with one mouse from the 0-week mhexb surviving to the 104-week study endpoint. A significant increase in behavioural performance was observed at 16 weeks in all treatment groups across all ages of administration ($p<0.01$). At all ages of administration, analyses showed slight non-significant increases of midsection of the brain HexA activity across all treatment groups, significant decreases in GM2 storage in the m*hexb*, h*B-A* and m*b-a* groups ($p<0.001$), and constant h*B-A* biodistribution across the midsection of the brain. Anti-hHexA antibodies rose to significant levels in mice receiving h*B-A* at 6 weeks of age ($p<0.0001$), as did cellular immune response against the human HexA peptides ($p<0.05$). A cross-species antibody response was expected, however the human bicistronic vector may not elicit an immune response in patients. Study limitations include the existence of an alternative sialidase pathway for GM2

catabolism in mice, the differential affinity of the subunits, the immature blood-brain barrier in mice at birth, and the possible interaction of the human HEXA enzyme with the murine GM2AP, each of which may have enhanced efficacy. Nevertheless, this data demonstrates that a bicistronic hexosaminidase AAV9 vector may be a viable option for the treatment of SD and TSD in humans.

When comparing the median survival and increase in survival of the *HEXM* and bicistronic experimental mice compared to that of the 16-week humane endpoint, we see some interesting variances (see Table 4.1).

For the purpose of this discussion, we will compare the human scAAV9/*HEXM* treatments at the neonatal (Supplementary Figure 2.1) and adult (Figure 2.3B) administrations to the human ssAAV9/h*B-A* bicistronic administrations at the neonatal and adult ages of injection (Figure 3.3 & 3.4). Although this comparison isn't perfectly matched due to multiple reasons (sc vs ss AAV, presence of sialidase pathway in mice, unclear contribution of HexB and HexA in correction for bicistronic vector), we tried the comparison as both of these treatment vectors were delivered to the mice at the same dose at each age of intervention in Chapters 2 & 3.

Furthermore, both the hybrid and bicistronic vectors were derived from the human *HEXA* and *HEXB* genes, which are heterospecific genes being administered to the mice. Therefore, an immune response would be expected. Unpublished data from our lab confirmed a relatively high immune response was elicited in the SD mice administered the scAAV9/*HEXM* vector at 6-weeks of age (Kot, 2018). In this previous study conducted by Shalini Kot (2018), SD mice were injected with 1 x 10^{12} vg/mouse at 6-weeks of age and the cohort that was not provided any immunosuppression can easily be compared to the 6-week old LD (2.5E12vg/mouse) from Chapter 2. The survival of the scAAV9/HEXM mice not provided immunosuppression in the previous study averaged to ~27 weeks (Kot, 2018), the 2.5 fold increase in vector administered to the LD SD mice in Chapter 2 conveyed an increase in survival b a factor of 0.9x in the SD mice.

In Chapter 3, we did preliminary immunological analysis on the mice administered the human bicistronic vector at all ages of intervention. There we found evidence of both antibody and cellular responses to the human HexA, which may have contributed to the shorter survival of the human bicistronic vector treatment cohorts as compared to the murine bicistronic treated cohort. Despite increasing the dose by a factor of 2.5x, the immune response observed in the human bicistronic groups are quite a small (antibody: ~6000ug/ml, cytokine:10 spots; Figure 3.26) in comparison to the immunological scores for the mice administered 1 $x10^{12}$ vg of scAAV9/*HEXM* with no immunosuppression that developed a high level of antibody (~4000ug/ml) and cytokine (~225 spots) responses to the HexM enzyme (Kot, 2018). However, as seen in Table 4.1, the human bicistronic treatment groups at the neonatal and adult administrations show increased median survivals and fold increases in survival as compared to the neonatal and LD scAAV9/*HEXM* cohorts. This may be due to the presumed increase in immunogenicity of the scAAV9/*HEXM* vector without immunosuppression as compared to the human bicistronic vector, since a decrease by a factor of 2.5 in the dose of the HexM treatment elicited just under half the antibody response and 22.5 fold higher cytokine response (Kot, 2018).

Age of Administration Dosage	0-week 2.5E11 vg/mouse		2-week 1E12 vg/mouse		4-week 1.88E12 vg/mouse		6-week 2.5E12 vg/mouse	
	Median	Increase	Median	Increase	Median	Increase	Median	Increase
scAAV9/*HEXM* HD	/	/	/	/	/	/	56.7	3.7x
scAAV9/*HEXM* LD	/	/	/	/	/	/	34.5	2.5x
scAAV9/*HEXM*	46	2.875x	/	/	/	/	/	/
ssAAV9/h*B-A*	59	3.68x	68.4	4.28x	43.6	2.73x	43.8	2.73x
ssAAV9/m*b-a*	42.2	2.64x	76.8	4.8x	65.8	4.11x	68.1	4.26x
ssAAV9/*mHexb*	65.7	4.1x	/	/	/	/	70.7	4.42x

Table 4.1 Median survival of all experimental mice from both studies and the factor increases in survival over the vehicle cohorts.

When comparing the scAAV9/*HEXM* and human ssAAV9/h*B-A* groups, both treatment vectors showed similar behavioural outcomes as well as similar levels of GM2 clearance. In addition, for both of these human derived vectors, when treatment intervention was provided earlier in life, more beneficial outcomes were measured, which has previously been described in intracranial GT preclinical trials (Cachón-González et al., 2014).

Both the HexM hybrid treatment and the bicistronic treatments were all shown to effectively treat the SD mice and significantly delay the onset of the disease by a factor of 2.5x compared to the humane endpoint of untreated mice. However, the immunological response that the HexM treatment elicits in the SD mice as compared to the immune responses seen when treating with the human bicistronic vector are different and needs further exploration. A direct comparison is difficult due to the multiple aforementioned reasons. Overall, the ssAAV9/h*B-A* vector showed similar to slightly more beneficial outcomes across both the neonatal ($2.5x10^{12}$ vg/mouse) and adult ($2.5x10^{12}$ vg/mouse) age of administrations but may need further confirmation on a different mouse model with no sialidase pathway.

The increased survival seen in the murine bicistronic group can be attributed to having a lower chance of eliciting an immunological response due to the conspecific nature of the treatment vector and the mouse model. The additional benefit in survival observed in the ssAAV9/*mHexb* cohorts can also be attributed to the activation of the alternative murine sialidase pathway, which uses the HexB β-subunit homodimer to effectively degrade GM2 to lactosylceremide (Sango et al., 1995; Suzuki et al., 1998). Additionally, since previous work has shown that the transcription and translation of the α- and β-subunits are done separately, and the subunits only interact within the Endoplasmic Reticulum, where only after N-linked oligosaccharide and Mannose 6-phosphate (M6P) moieties are attached to each subunit, after which the subunits interact and form the disulfide bonds between the α- and β-subunits forming the HexA enzyme (Cachón-González et al., 2018; Conzelmann & Sandhoff, 1991; Hubbes et al., 1989; Little et al., 1988; D. J. Mahuran et al., 1988; Proia et al., 1984; Quon et al., 1989; Solovyeva et al., 2018; Sonderfeld-Fresko & Proia, 1989; Weitz & Proia, 1992), the equal production of both the α- and β-subunits from the bicistronic treatment could potentially lead to the formation of the HexA enzyme and not an overabundance of the HexB β homodimer, while the overexpression of only the murine β-subunit in the mice would then lead to the activation of both murine streams of the GM2 degradation pathway (Figure 1.7), thus contributing to survival of SD mice.

4.1.1 Implications and Clinical Relevance

A gold standard treatment for GM2 Gangliosidosis remains to be found, however the GT treatments herein show promise for the treatment of both main GM2 Gangliosidosis diseases, TSD and SD. Unfortunately, the immune response elicited in the mice to the human HexM hybrid treatment is problematic for future use in clinical studies, especially when compared to the significantly smaller immune response to the human bicistronic treatment. Barring any modifications and refinement of the HexM treatment, and based on the preliminary

immunological findings, the human bicistronic treatment vector may have a much better chance at successfully treating GM2 Gangliosidosis in humans with minimal side effects. However, with the use of a strong immune suppression regimen, AAV9/*HEXM* may prove to be a good therapeutic candidate as well.

The strategies used in the development of these therapies for GM2 Gangliosidosis can be applied to a variety of other diseases, particularly the other lysosomal storage disorders. Switching the hexosaminidase genes out and incorporating the appropriate gene that is mutated in another LSD may lead to an effective treatment for that disease. For example, removing the HEXM gene and replacing it with the GAA gene that encodes the acid alpha-glucosidase may be a potential treatment for Pompe Disease (Fraites et al., 2002; Kishnani et al., 2006; Mah et al., 2005), or the CLN6 gene for the treatment of Batten's disease (Cain et al., 2019; Katz et al., 2015).

The pre-existence of immunity towards AAVs is also a clinical limitation to consider for the success of a GT treatment (Gray et al., 2013; Lykken et al., 2018). It has been shown that pre-existing neutralizing antibodies occur throughout the human population, and the presence of these neutralizing antibodies in a patient requiring a GT treatment would prove useless, since these neutralizing antibodies would inhibit the therapeutic AAV from infecting target cells (Lykken et al., 2018). Systemic delivery could prove difficult for a patient with a high titre of neutralizing antibodies; however, an intrathecal administration of the GT treatment could be a viable solution for these patients since the relative abundance of antibodies in the cerebral spinal fluid is much less than the periphery (Gray et al., 2013; Lykken et al., 2018). Approximately 14% of the healthy adult human population have a high enough peripheral titre of AAV9 neutralizing antibodies (>1:200) that would interfere with peripheral GT (Boutin et al., 2010). Additionally, as compared to adults (90% of population have high titres to AAV2; (Boutin et al., 2010)), paediatric populations have a lower overall percentage of the population that have neutralizing

antibodies towards AAV2 (35%), and that those that have the neutralizing antibodies have a low titre (1:20) (Calcedo & Wilson, 2016). Therefore, from the perspective of neutralizing antibodies to the treatment vectors, the treatment of paediatric diseases are more feasible than delaying treatment until later in life.

Other neurological disorders could also benefit from a similar GT treatment strategy, such as Alper's disease. Alper's disease is an autosomal recessive neurodevelopmental disorder characterized by the dysfunction of the mitochondrial DNA polymerase gamma, *PLOG* (Copeland, 2010; Saneto, Cohen, Copeland, & Naviaux, 2013). The symptoms of Alper's disease include seizures, dementia and liver disease. The disease is typically fatal within the first 10 years of life, where the severity of the seizures or organ failure secondary to CNS dysfunction are often the cause of fatality (Copeland, 2010; *Homo sapiens DNA polymerase gamma, catalytic subunit (POLG), transcript variant 1, mRNA*, 2019; "POLG DNA polymerase gamma, catalytic subunit [Homo sapiens (human)]—Gene—NCBI," n.d.; Saneto et al., 2013). The GT treatment strategy using a minimal ubiquitous promoter, such as the UsP promoter used in the HexM treatment construct (340 kb), paired with the poly-adenylation sequence (122bp) after the *PLOG* cDNA (~4.5 kb), the treatment construct could then be packaged into AAV9 and delivered both intravenously and intrathecally in order to treat both the CNS and periphery.

Overall, we have identified GT strategies capable of treating neurodegenerative disorders through IV treatment administration. GT is a viable therapeutic option for genetic neurodegenerative and neurological diseases, especially those arising from monogenic recessive mutations. GT could also be a useful partner in any polypharmacological treatment intervention, as discussed below in section 4.2.

4.2 Future Directions – Where do we go from here?

The cyclic nature of basic and clinical research requires the amalgamation of several different types of studies (Figure 4.1). The progression from basic research to clinical research includes studies aimed at identifying both aberrant and normal molecular mechanisms, which inform the *in vitro* characterization of a concept, followed by pre-clinical animal model testing and ultimately ending in clinical trials (Figure 4.1). In the process of knowledge translation from basic to clinical research, each type of research informs both the preceding and following types and the cycle continues. Gaps in the knowledge of any type of research should be further investigated in order to completely elucidate the entire disease and treatment narrative. In the search for a clinical treatment for GM2 Gangliosidosis, there are still many aspects to be evaluated.

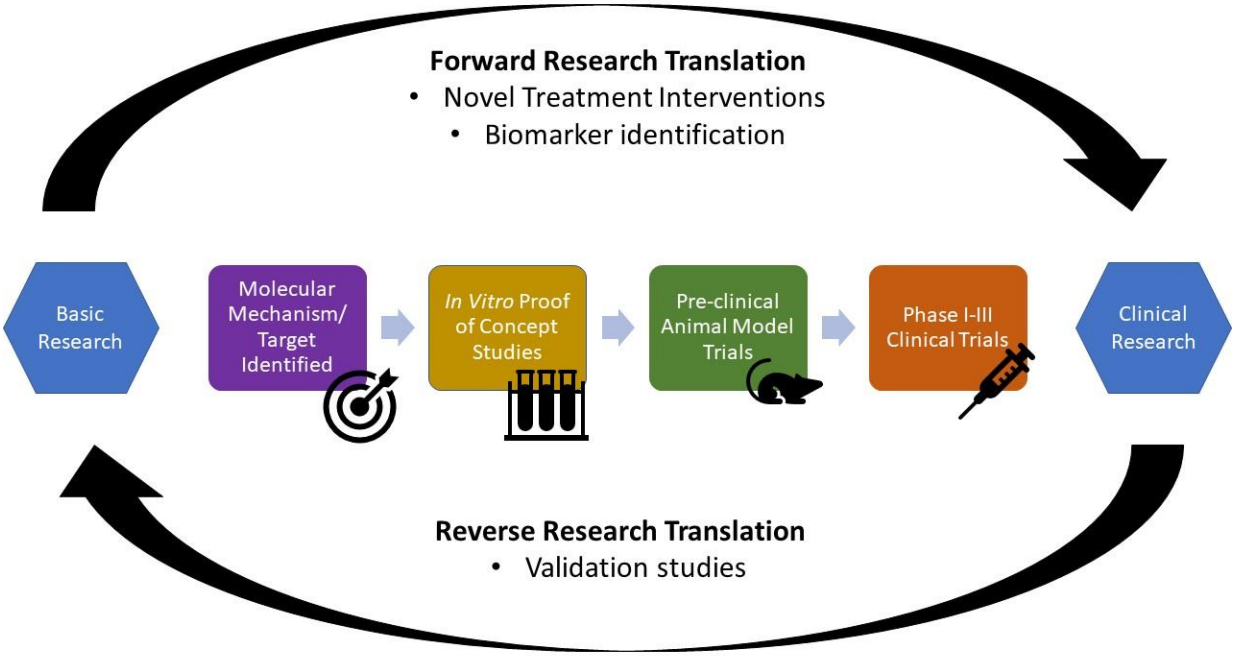


Figure 4.1 The Research Cycle Between Basic and Clinical Research.

Figure adapted from (Al Dahhan, De Felice, & Munoz, 2019) with author's permission.

At the cellular level, the molecular mechanisms leading to the neurodegeneration of the brain and spinal cord have yet to be fully elucidated. There have been recent studies attempting to identify specific targets within molecular cascades that have may have led to neuronal apoptosis (Abo-Ouf et al., 2013; Hooper et al., 2017, 2017). Another future study for basic research would combine the first three levels in Figure 4.1, where further investigation into a GT treatment for GM2 Gangliosidosis or any CNS disease should investigate to use of next generation AAV capsids. These capsids are designed and discovered through capsid shuffling or AAV barcoding. These designer next generation capsids would have a higher tropism for a more specific targeting of certain organs, they could also be more efficient at infecting the target cells, and they may have a better immunological and safety profile than the naturally occurring AAVs currently in use. Additionally, the incorporation of a regulatory element in the treatment construct could prove to be beneficial if the element could be activated or deactivated post-delivery in order to increase or decrease the therapeutic transgene expression as needed.

Further preclinical animal model studies using of any of the vectors in the current thesis for an IV GT delivery should look at increasing the dose provided to the mice above that of the neonatal dose of 2.5 $x10^{11}$ vg/mouse and the adult 2.5$x10^{12}$ vg/mouse in order to increase the amount of infected cells and therefore increasing the amount of the hexosaminidase enzyme expression. The immunological studies herein centered around the hexosaminidase enzymes as the antigen, whereas future studies should also investigate the immunological response elicited by the AAV9 vector.

An additional line of further exploration in the realm of preclinical research would be to assess the scAAV9/*HEXM* and ssAAV9/h*B-A* vectors head to head using intrathecal administrations. A study design that compares these two human based vectors using an intrathecal delivery would be beneficial in order to directly compare all aspects of both treatment vectors.

Additionally, pending the approval from the University Animal Care Committee, the administration of these intrathecal injections (either through lumbar or cisterna magna delivery) before the mice reach adulthood at 6 weeks of age would allow for the investigation into the effects of an earlier intervention.

The polypharmacological synergy between GT and a variety of other treatments for LSDs should also be further investigated at both the pre-clinical and then clinical trial steps. Although GT could theoretically treat these diseases with a single treatment administration this may not be the case. Using GT in combination with immunosuppressants, anti-inflammatories, and/or SRT would help determine which of these treatments would synergistically work together to treat GM2 Gangliosidosis, and which combinatorial therapies would be antagonistic.

The current thesis is only one pre-clinical piece of the puzzle toward the final goal of a gold standard treatment for GM2 Gangliosidosis. All the types of research and research techniques are required for the discovery of a least invasive treatment for GM2 Gangliosidosis, which could be a steppingstone to the treatment of other LSDs and neurodegenerative diseases.

4.3 Barriers to Cross-species Translational Research

There are a variety of barriers to the translation of pre-clinical research to a heterospecific clinical population. One such barrier is the assumption that molecular mechanisms are identical across species. The creation of the transgenic mouse models for TSD and SD disease are a prime example. The disruption of the *HEXA* gene in the mice did not elicit TSD-like symptoms or fatalities, which is in stark contrast to the human symptomology of TSD when the *HEXA* gene is mutated. In contrast the disruption of the *Hexb* gene in the mice did indeed elicit SD-like symptoms and fatality. The assumption that the disruption of the murine *Hexa* gene would provide a transgenic mouse model capable of recapitulating the human version of TSD proved to be false, but that finding, as well as the creation of the symptomatic transgenic SD mouse model also informed the molecular ganglioside degradation pathways in the mice. The creation of the

transgenic SD mouse model has enabled the research into potential therapeutics for SD and TSD, but there are still multiple cross-species barriers to be addressed.

A second barrier to the translation of preclinical research to clinical research lies in the differential genetic composition of the separate species (Martignoni, Groothuis, & de Kanter, n.d.; McGonigle & Ruggeri, 2014; Searls, 2003). Although mice, non-human primates, and humans all share versions of conserved mammalian genes, these genes are not all identical and could have evolved to perform other tasks in other species (Martignoni et al., n.d.; McGonigle & Ruggeri, 2014; Searls, 2003). In addition, the environment surrounding pre-clinical animal models is highly controlled and maintained at optimal conditions. This is not the case for human patients who are exposed to a constant bombardment of external factors that may hinder or affect the efficacy of the treatment (McGonigle & Ruggeri, 2014). Additionally, the differences in immunological responses between species is another barrier between the translation of preclinical research to the clinic.

These cross-species translational challenges must be taken into account when moving from pre-clinical animal research into the phases of human clinical trials.

4.4 Conclusions

Our works herein have established that both the scAAV9/*HEXM* and the ssAAV9 bicistronic vectors have the potential to successfully treat GM2 Gangliosidosis at any age of administration. Although we determined that any hexosaminidase GT treatment given to the SD mice produces significant beneficial outcomes, the use of the human bicistronic treatment for the correction of GM2 Gangliosidosis should be further investigated in order to move this potential treatment to clinical trials over that of the HexM hybrid treatment due to the elicited immune response to the HexM hybrid enzyme, unless a robust immunosuppression model is established. These findings contribute to the search for a viable treatment for GM2 Gangliosidosis and may be applicable to the treatment of other lysosomal storage disorders or neurodegenerative diseases.

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