TRANSGENIC MOUSE MODEL OF HUMANIZED SIALIDASE

CHRACTERIZATION OF TRANSGENIC MOUSE MODEL OF HUMANIZED SIALIDSAE

By

Ghada A. Tarbah, B.Sc.

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AUTHOR: Ghada A. Tarbah, B.Sc.

SUPERVISOR: Suleiman Igdoura, PhD, Associate Professor, McMaster University

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ABSTRACT

Ganglosidoses are a group of fatal neurological disorders which primarily affect children. Pathologically, these disorders need more characterization. The recent development of mouse models of these diseases makes it possible to identify progressive pathological changes within the CNS at the molecular level. Tay Sachs disease is a lysosomal storage disorder characterized by accumulation of GM2 Ganglioside. Targeted Knockout of β-hexosaminidase A (hexa) was generated as a mouse model of Tay Sachs disease; however, the mouse showed little clinical phenotype. The molecular mechanism which allows the hexa-/- mouse to escape the disease involves a metabolic bypass facilitated by lysosomal Sialidase (neu1). In this study we hypothesize that the reason humans have the infantile Tay-Sachs whereas mice do not, is a difference in specificity between human and mouse sialidases. This specificity difference makes the human sialidase unable to hydrolyze GM2 to GA2, which leads to the accumulation of GM2 in neurons and consequently a resulting Tay-Sachs phenotype. In this project, a transgenic mouse expressing human Sialidase under the control of the mouse neu1 promoter was generated. Then a crossing of this mouse with hexa-/- mouse was done to generate a Tay Sachs mouse expressing human Sialidase. This double transgenic mouse was characterized phenotypically, biochemically and histologically. I found that the double transgenic mice are small in size, have hind limbs spasticity, peculiar posture with Kyphosis, and visual impairments; and around three months of age, which is very early comparing to Tay-Sachs late-onset animal model. Moreover the animals showed muscle weakness and clasping of their hind limbs, and they depicted weird behavior presented in vigorous combing of their faces while standing on their hind limbs and tails. However, these results indicate no significant difference between hexa-/- and the double transgenic (hexa-/-KI+) mice in gangliosides catabolic pathway.

DEDICATION

To the loving memory of my father .....and to my wonderful mother, I was very fortunate in God's decision that I be given to you as my father and my mother, for it was you who showed me how to fear God, how to have morals, how to be strong, and how to care. You are always in my thoughts and in my heart. I miss you every day dad and I can't wait to see you and be with you mom.

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LIST OF ABBREVIATIONS

cDNA	COMPLEMENTARY DEOXYRIBONUCLEIC ACID
CNS	CENTRAL NERVOUS SYSTEM
DNA	DEOXYRIBONUCLEIC ACID
DNTP	DEOXYNUCLEOTIDE TRIPHOSPHATE
EDTA	ETHYLENE DIAMINETETRAACETIC ACID
EGF	ENDOTHELIAL GROWTH FACTOR
GSLS	GLYCOSPHINGOLIPIDS
HCG	HUMAN CHORIONIC GONADOTROPIN
HEX A	β-HEXOSAMINIDASE A
HEX B	β-HEXOSAMINIDASE B
IU	INTERNATIONAL UNITS
Kd	KILO DALTON
KDN	2-KETO-3-DEOXYNONONIC ACID
LGG	LATE-ONSET GM2 GANGLOSIDOSES
mRNA	MESSENGER RIBONUCLEIC ACID
MU-NANA	4-METHYLUBELLIFERYL-N-ACETYLNEURAMINIC ACID
NEU	NEURAMINIDASE (SIALIDASE)
NEU1	LYSOSOMAL SIALIDASE
NEU2	CYTOSOLIC OR THE SOLUBLE SIALIDASE

NEU3	PLASMA-MEMBRANE-ASSOCIATED SIALIDASE
NEU 4	INTRACELLULAR-MEMBRANE-ASSOCIATED SIALIDASE
PAGE	POLYACRYLAMIDE GEL ELECTROPHORESIS
PBS	PHOSPHATE BUFFERED SALINE
PCR	POLYMERASE CHAIN REACTION
PMS	PREGNANT MARES SERUM
PPCA	PROTECTIVE PROTEIN/CATHEPSIN A
RDE	RECEPTOR DESTROYING ENZYME
RNA	RIBONUCLEIC ACID
RT	REVERSE TRANSCRIPTION
SAS	SIALIC ACIDS
SDS	SODIUM DODECYL SULFATE
SIALIDASE	LYSOSOMAL N-ACETYL-A-NEURAMINIDASE
TBS	TRIS BUFFERED SALINE
TLC	THIN LAYER CHROMATOGRAPHY
TSD	TAY-SACHS DISEASE

CHAPTER ONE: INTRODUCTION

1.1 Sialic acids

More than seventy years ago sialic acid was discovered as a major product released by mild acid hydrolysis of salivary mucins or brain glycolipid, since then several groups have studied and exposed the structure, chemistry, and biosynthesis of the obtained compound (N-acetyl-neuraminic acid or Neu5Ac, a 9-carbon, acidic α-keto sugar). In the 1940s, George Hirst and Frank Macfarlane Burnet have shown that sialic acid was the cellular receptor for influenza viruses. Shortly after that, Erwin Chargaff's group revealed that the (RDE) "Receptor destroying enzyme" operates as a sialidase, releasing sialic acids from macromolecules; another group has found a similar activity in bacteria.

In 1957, the name "neuraminidase" for this activity was suggested by Alfred Gottschalk. From the beginning, it was obvious that Neu5Ac was the most common member of a big family of connected molecules derived from neuraminic acid. This family was named the "sialic acids" partly because of its discovery in salivary mucins. More than 30 types of sialic acid have been described by the 1980s. The finding of (Kdn) 2-keto-3-deoxynononic acid as a desamino form of neuraminic acid further extended the sialic acids family, which now includes more than 50 members. Classically sialic acids (SAs) are found to be terminating branches of N-glycans, O-glycans, and glycosphingolipids (gangliosides) (and rarely capping side chains of GPI anchors). This one type of monosaccharide is remarkably diverse.

The primary level of diversity results from the different linkages that might be created between the C-2 of SAs and underlying sugars by distinct sialyltransferases, using CMP-SAs as high-energy donors. The most frequent linkages are to the C-3 or C-6 site of galactose residues or to the C-6 site of N-acetylgalac tosamine residues. Sialic acids can also reside in internal sites within glycans, most commonly at C-8 site when one SA residue is attached to another. Additionally, internal SAs can take place in the replicating units of some bacterial polysaccharides and echinodermal oligosaccharides.

The secondary level of diversity occurs from a range of natural modifications. C-5 site can contain an N-acetyl group (giving Neu5Ac) or a hydroxyl group (as in Kdn). Moreover, the 5-N-acetyl group can also be hydroxylated, giving N-glycolylneuraminic acid (Neu5Gc). A lesser amount of; the 5-amino group is not N-acylated, giving neuraminic acid (Neu). Neu5Ac, Neu5Gc, Kdn, and Neu are considered the four core SA molecules and they can hold one or more extra substitutions at the hydroxyl groups on C-4, C-7, C-8, and C-9 (O-acetyl, O-methyl, and O-sulfate, O-lactyl, or phosphate groups). At the C-1 the carboxylate group is usually ionized at physiological pH, but can also be condensed into a lactone with hydroxyl groups of nearby saccharides or into a lactam with a free amino group at C-5. Combinations of diverse glycosidic linkages with the massive amount of possible modifications create hundreds of ways in which SAs can present themselves. This distinct chemical diversity of SAs adds to the massive variety of glycan structures on cell surfaces and the distinctive form of different cell types. This, consequently, can determine and/or alter recognition by antibodies and by a range of SA-binding lectins of intrinsic or extrinsic source. Regardless of this complexity, it may be enough in some biological studies to simply know that a sialic acid residue is present at the terminal position and just label it with the broad abbreviation "SA" (Varki et al., 1999).

1.2 Sialidase Enzymes

Sialidase (neuraminidases) enzymes include a family of glycohydrolytic enzymes that hydrolyze terminal sialic acid residues from diverse sialo-derivatives, such as, glycolipids (gangliosides), glycoproteins, and oligosaccharides (Manzoni et al., 2007; Papini et al., 2004; Wang et al., 2002). These exoglycosidases are broadly spread in nature and they are expressed in viruses, bacteria, protozoa, mycoplasma, fungi, other micro-organisms and off course vertebrates. Cloning mammalian sialidases from diverse species have started around 1993 .Regardless of the high degree of homology and the presence of extremely conserved regions along the main structure, the cloned mammalian sialidases show remarkable differences in their substrate specificities and subcellular localization (Manzoni et al., 2007; Wang et al., 2002; Kakugawa et al., 2002; Yamaguchi

et al., 2005). The sialidase protein family can be divided into four major groups: the lysosomal sialidase (NEU1), the cytosolic or the soluble sialidase (NEU2), the plasma-membrane-associated sialidase (NEU3), and the intracellular-membrane-associated sialidase (NEU4) (Manzoni et al., 2007; Yamaguchi et al., 2005). In humans, lysosomal sialidase (NEU1) is part of a multienzyme complex which include galactosidase and cathepsin A (the protective protein) and is implicated in the lysosomal storage disorders, sialidosis and galactosialidosis.

It has been shown that its steady expression in the cell line of A431 human carcinoma leads to a reduced GM3 Ganglioside level and an improved tyrosine autophosphorylation of EGF (endothelial growth factor) receptor (Meuillet et al., 1999). In skeletal muscles, liver, and brain cells, the neu2 protein is expressed at low levels. Differentiation of rat L6 and mouse C2C12 from myoblasts to myotubes involves neu2 enzymes. The main change noticeable in neu2 transfected cells was a reduction in ganglioside GM3 levels concordant with an increase in lactosylceramide content (Chavas et al., 2005). The most studied member of the sialidase family is the plasma membrane-associated sialidase neu3 which is characterized by an elevated degree of specificity towards ganglioside substrates (Monti et al., 2002). The over-expression of neu3 noticeably alters the cell ganglioside content and is able to influence gangliosides exposed on the extracellular leaflet of the plasma membrane of neighboring cells in turn altering cell–cell interactions. The enzyme has been revealed to be linked with caveolin in lipid rafts and plays a crucial role in different cellular processes counting tumorigenic transformation and neuronal differentiation (Manzoni et al., 2007; Wang et al., 2002). NEU4 was initially described as a particulate enzyme associated with internal cell membranes.

Most recently, a human isoform with 12 extra amino acid residues at the N-terminus has been recognized and revealed to be associated with the mitochondrial membranes. Mysteriously, nue4 has been confirmed to localize in the lysosomal lumen and its over-expression in fibroblasts taken from sialidosis or galactosialidosis patients results in clearance of storage materials from lysosomes, which suggest a role for nue4 in lysosomal function (Manzoni et al., 2007; Seyrantepe et al., 2004). Up regulations of sialidase expression has been suggested as a possible treatment for Tay-Sachs disease (Champigny et al., 2003).

1.3 The human lysosomal sialidase (NEU1)

Human lysosomal sialidase is a glycoprotein that exists in two isoforms of 44 and 48 kDa (Bonten et al., 1996; Igdoura et al., 1998) and it can only be active as a component of a complex including β-galactosidase and the protective protein cathepsin A (Potier et al., 1990; Igdoura et al., 1998). This lysosomal sialidase is found to be involved in the severe lysosomal storage disorders, sialidosis and galactosialidosis (Manzoni et al., 2007) .In the late 1990s, three different groups reported the cloning of the first human lysosomal sialidase independently. Actually, in 1979 the naturally occurring partial neuraminidase deficiency in mouse strain SM/J was first revealed (Monti et al., 2002; Potier et al., 1979). The gene accountable was named NEU-1, then in 1981 the gene was mapped to the histocompatibility locus on the chromosome 17; which is syntenic to the human histocompatibility region on chromosome 6p21. This mapping data was supported by the recognition of the joint deficiency in sialidase and cytochrome P450 steroid 21-hydroxylase activities (Monti et al., 2002) an enzyme encoded by a gene that is sited, once more, in the histocompatibility locus on chromosome 6p21.These findings guided the way to a thorough search of the genes located in this locus, allowing the molecular cloning of the human sialidase called G9 (Monti et al., 2002; Milner et al., 1997).

These groups recognized some mutations in sialidosis patients, consequently indicating that the isolated cDNA encoded a lysosomal sialidase. By using the human cDNA as a probe the isolation of the mouse gene was possible, and the mutation accountable for the neuraminidase deficiency in SM/J mouse strain was recognized (Rottier et al., 1998; Monti et al., 2002). The lysosomal sialidase Neu-1 is suspected to have restricted substrate specificity. Natural substrate for this enzyme is indefinite and activity has consequently only been reported on simulated substrates such as 4-MU-NANA, 4-methylumbelliferyl-*N*-acetylneuramic acid, and nitrophenyl-NANA (WANG et al., 2004; Milner et al., 1997). Neu-1 mRNA is broadly expressed in a variety of tissues (Monti et al., 2002).

Lysosomal N-acetyl-α-neuraminidase (sialidase) activates the hydrolysis of gangliosides, oligosaccharides, glycolipids, and glycoproteins by confiscating their terminal sialic acid residues. The human enzyme favors α2→3 and α2→6 sialyl linkages and is thought to operate mainly on glycopeptides and oligosaccharide substrates, but can hydrolyze gangliosides with the aid of detergents or the sphingolipid activator Sap B. The biochemical characterization of lysosomal sialidase has been complicated because it is exceptionally labile on extraction and may be membrane bound.

Since first reported, several investigators have recognized that lysosomal sialidase activity can be recovered in mammalian tissues as an element of a big molecular mass compound that contain the glycosidase, β-galactosidase, and the carboxypeptidase protective protein/cathepsin A (PPCA). It is thought that by associating with PPCA, lysosomal sialidase and β-galactosidase obtain their active and steady conformation in lysosomes (Bonten et al., 1996).

1.4 Sialidosis and galactosialidosis

Sialidosis and galactosialidosis are two genetically distinct inborn errors of metabolism: sialidosis is caused by structural lesions in the lysosomal sialidase locus whereas; galactosialidosis is a combined deficiency of lysosomal sialidase and β- galactosidase caused by the absence of the complex PPCA.

Accumulations of sialylated oligosaccharides and glycopeptides in tissues and excretion of abnormal quantities of these compounds in body fluids and urine of sialidosis or galactosialidosis patients are found. Diverse clinical forms of sialidosis are well-known based upon the age of onset and the severity of the symptoms (Bonten et al., 1996).

1.5 Glycosphingolipids and Gangliosides

Glycosphingolipids (GSLs) are components of eukaryotic plasma membranes that form cell type-specific patterns on the cell surface. These patterns change with cell growth, differentiation and transformation (Metelmann et al., 2001). In addition, the interactions between GSLs with toxins, viruses and bacteria as well as with membrane-bound receptors and enzymes occur at the cell surface (Kollter & Sandhoff, 1998). Furthermore, many of the cellular function of GSLs complex such as neuronal and leukocyte differentiation, embryogenesis, cell adhesion and signal transduction can be attributed to sialic acid-containing ganglioside (Riboni at el., 1995). Degradation of the plasma membrane which contains GSLs occurs after it is endocytosed and trafficked through the endosomal compartments towards the lysosome. In the lysosome, hydrolyzing enzymes cleave sugar residues in a sequential fashion to produce ceramide, which is then deacylated to sphingosine. This product can leave the lysosome to re-enter the biosynthetic pathway, or to be further degraded (Kollter & Sandhoff, 1998).

Gangliosides are glycosphingolipids composed of a hydrophobic ceramide and hydrophilic oligosaccharide chain, bearing one or more sialic acid residues (Metelmann et al., 2001). Possible roles in neuronal recognition, myelination and synaptogenesis have been suggested due to an optimal period of ganglioside turnover in the central nervous system during neonatal development, a period just before myelination.

1.6 Tay-Sachs disease (the juvenile and the late onset form)

GM2-gangliosidosis is a group of relatively rare autosomal recessive human disorders, caused by genetically defective catabolism characterized by lysosomal accumulation of GM2 ganglioside (Liu et al., 1999; Lemieux et al., 2006; Peleg et al., 1994; Chavany& Jendoubi, 1998; Ribeiro et al., 1995).

These disorders can be distinguished as three major types which are: the B variant (Tay-Sachs disease), the O variant (Sandhoff disease), and the AB variant (GM2 activator). These three types are resulting from genetic abnormalities in the genes coding for the N-acetyl-β-hexosaminidase A, B and the gene coding the GM2 activator (A&B) (Tanaka et al., 1993). Hexosaminidase A (HexA) is a heterodimer. The two subunits of the heterodimer α, β are encoded on chromosomes 15q, and 5q correspondingly (Peleg et al., 1994; Young et al 1970). The catabolism of GM2 requires β- hexaminidase A (αβ) in the presence of a substrate-specific cofactor known as GM2 activator. The Human lysosomal hexosaminidase A (hexa) catalyze the release of terminal b-N-acetylgalactosamine residues of glycosaminoglycans, glycolipids, glycoproteins, and proteoglycans (Ozkara & Sandhoff, 2003; Yamanaka et al., 1994; Mahuran,1995; Massaccesi et al., 2007).

β- hexaminidase has two isoenzymes, including HexA and HexB, which are composed of subunits αβ and ββ respectively. HEXA encodes the α subunit of HexA, which is required for catabolism of the GM2/GM2 activator complex into GM3, and HexB encodes the β subunit of both HexA and HexB, with only the later enzyme effective in the further breakdown of GM2 cleavage product, GA2 (Zarghoonia et al., 2004; Maier et al., 2003; Chamoles et al., 2002; Peleg et al., 1995; Stirling et al., 1988). The sialidase (neuraminidase) pathway is also intrinsically involved in this process and helps to reduce levels of GM2 by hydrolysing it to GA2. However, this neuraminidase pathway works as part of a system and cannot clear gangliosides independently (Phaneuf et al., 1996; Huang at al., 1997). GM2 activator deficiency is a very rare member of the ganglosidoses, in which both α and β subunits are intact and the encoded hexaminidase enzymes remain unaffected. In this disorder, the GM2 activator is diminished, thus resulting in the pathogenic accumulation of GM2 (Yamanaka et al., 1994).

In humans, many mutations causing Tay Sachs and Tay Sachs-like diseases have been discovered such as: small insertions or deletions, base pair substitutions, and partial gene deletions (Ozkara & Topcu, 2004).

Mutations in any of the two genes encoding α & β subunits of hexosaminidase enzyme as well as a gene encoding GM2 activator can result in GM2 ganglosidoses; a group of clinically similar devastating neurological disorders, Tay–Sachs (hexa, a-subunit mutations), Sandhoff (hexb, b-subunit mutations), and the rare AB variant form (GM2 A, activator mutations). Up until now, there are 100 mutations in hexa, about 25 mutations in hexb and five mutations in GM2A that have been reported to cause GM2 ganglosidoses.

Over 50 mutations causing diverse forms of Tay-Sachs disease have been identified in the α-subunit encoding gene, HEXA. The majority of these mutations are in the coding sequence, and only small percentage is found in the sequences necessary for splicing (Richard et al., 1995). Exclusive biomedical research has been done to try to explain the observed phenotype based on the biochemistry of these well known mutations. The most common and devastating acute forms of GM2 ganglosidoses result from mutations which prevent any one of the three gene products from being made and/or reaching the lysosome. On the other hand, mutations that allow about 1–5% of wild-type Hex A activity produce a milder sub-acute or much milder chronic disease phenotype (Mark et al., 2003). The most severe ones are the mutations that cause mRNA instability or no RNA production, as they are described by very low or undetectable residual enzyme activity. There are considerable degrees of clinical heterogeneity associated with lysosomal storage diseases, with apparently insignificant changes in residual enzyme activity having an enormous impact on the disease severity and consequently prognosis.

The infantile onset forms of these diseases have lower levels or no residual activity and demonstrate the most severe pathology, whereas, adult onset forms have higher levels of residual enzyme activity and the disease develop slowly (Ozkara & Topcu, 2004).

Patients with classical infantile Tay-Sachs disease show normal development until 3-6 months of age, shortly after motor weakness, poor head control and hypotonia, occur. One of the initial signs that parents notice is an inflated startle reaction to sharp sounds. Around 10 months of age any motor skills will be lost. In the second year patients show head enlargement and their vision diminishes. Seizures and neurologic signs of both upper and lower motor neuron deterioration become progressively more obvious by 18 months. Additional deterioration in the second year of life leads to the completely vegetative state and eventually death (Mahuran, 1999, Li & Li 2001).

Late onset GM2 Ganglosidoses (LGG) is a rare genetic disorder of teenagers and young adults which may results in cognitive dysfunction, psychiatric disturbances, cerebellar dysfunction, and upper and lower motor neuron involvement. Infantile GM2 ganglosidoses, or Tay-Sachs disease, is infrequent, devastating encephalopathy that is found more often in people of Ashkenazi Jewish heritage (Wailoo& Pemberton, 1997; Chakravarti& Chakraborty, 1978; Tsuji et al., 2007) and causes a fast progressive cognitive and essential neurologic deterioration.

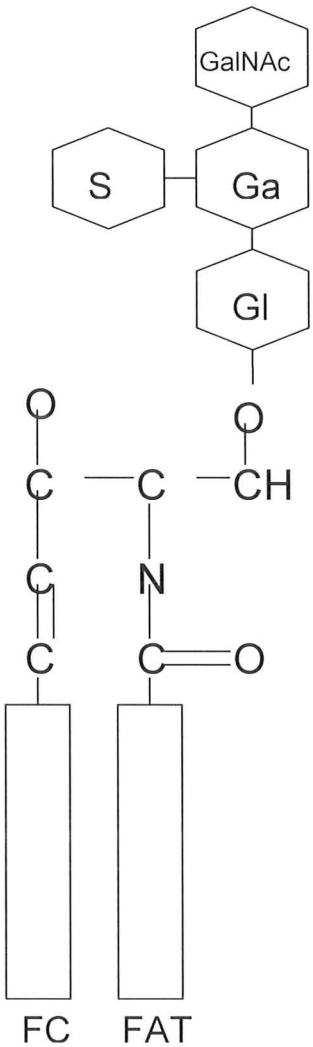

Figure 1.1, Structure of ganglioside GM2. GalNAc = N-acetylgalactosamine; Gal = Galactose; SA = Sialic Acid; Glc = Glucose; FC = Fatty Chain; FAT = Fatty Acid Tail

LGG differ from Tay-Sachs disease because of its prolonged disease course and its late- onset of neurologic dysfunction (Frey et al., 2005). Late onset GM2 Gangliosidosis (LGG) is not as well characterized, clinically or biochemically, as infantile Tay-Sachs disorder. Patients show signs of cerebellar and motor neuron involvement and, sometimes have psychosis but with typical sensory function. Those patients may have atypical Friedreich ataxia, atypical spinocerebellar degeneration, amyotrophic lateral sclerosis, or spinal muscular atrophy. Deterioration has been shown distinctly in cognition and intelligence in the juvenile form, but minimally in the chronic or adult-onset form. Since it has been mainly diagnosed in the population of Ashkenazi, occasionally in families with juvenile form of Tay-Sachs disease, it has been suggested that the variant GM2 gangliosidoses are due to composite heterozygosity of a rare mutation with a more common Tay-Sachs disease allele (Paw et al., 2005; Schneck et al., 1970).

1.7 Mouse models of Tay Sachs disease

Mouse models of Tay Sachs disease (hexa-/- mice) have been generated (Phaneuf et al., 1996). Mice deficient in β-hexosaminidase A , show a normal life span and are phenotypically normal until around 18 months of age. They accumulate moderate levels of GM2 Ganglioside in the CNS, but do not exhibit the neurological symptoms characteristic of human TS disease. The lack of symptoms in the TS mouse appears to results from the species differences in the ganglioside degradation pathway. In humans, GM2 Ganglioside is degraded primarily by β-hexosaminidase A and associated with the GM2 activator protein to yield GM3. The lack of β-hexosaminidase A in Tay Sachs disease blocks the pathway and results in the accumulation of GM2. Whereas in mice, GM2 ganglioside can be degraded by a pathway identical to that in humans, but also via a second pathway called the bypass pathway. In this bypass pathway, the sialidase cleaves GM2 to yield GA2. GA2 is then further degraded by Hex B, which is intact and functions normally in Tay Sachs disease (Jeyakumar et al., 2002; Phaneuf et al., 1996).

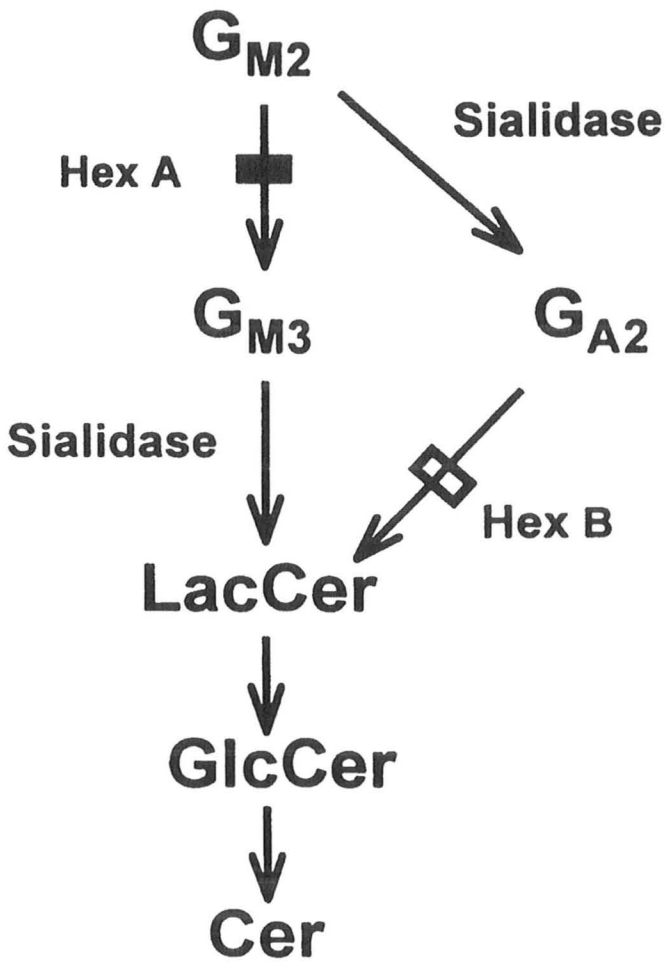

Figure 1.2 Schematic illustration of the neu1-mediated Tay-Sachs bypass in hexa-/- mice

1.8 Approaches to address why model of TSD mouse does not show symptoms

Mice deficient in HexA (Tay-Sachs mice) can escape the disease utilizing a bypass pathway in which, the mouse lysosomal sialidase converts GM2 to GA2. GA2 then will be cleaved by HexB to LacCer. If we introduced the human lysosomal Sialidase into the picture, we hypothesize that this later enzyme will interfere in the pathway, preventing mouse sialidase from converting GM2 to GA2. Therefore, an excessive accumulation of GM2 in the nerve cells occurs causing Tay-Sachs disease.

1.9 Objectives

In order to examine the role of sialidase in TSD, our objective was first to generate human sialidase transgenic on a hexa-/- deficient background, and second to assess the double transgenic mice biochemically and neurologically.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Targeting construct for human sialidase knockin

In order to generate a transgenic mouse expressing human sialidase under the control of mouse sialidase promoter, a targeting vector containing 1.8 kb was constructed, genomic 5'upstream region fused with a modified human sialidase cDNA. . First an introduction of intron I+loxP site in a human lysosomal sialidase cDNA was done as follows: Intron 1 was amplified by PCR (Platinum Taq, GIBCO). The product was subcloned into pCRII then confirmed by sequencing. LoxP sequence including a BamH1 site was introduced into the Acc1 site of intron 1 with synthetic oligos. The presence of the insert was confirmed by diagnostic BamH1 digest and by sequencing. Intron 1 was introduced back into the sialidase cDNA as a PshA1-BstEII restriction fragment and then confirmed by sequencing. In order to fuse a 1.8 kb upstream fragment from the mouse sialidase gene with the ATG of the human sialidase, then two silent mutations at the second and third codons proceeding the ATG were introduced to create a unique AgeI site by amplifying an upstream component of human sialidase cDNA using a sense primer creating NheI and AgeI and an anti-sense primer with intron 1.

The resulting product was digested with PshAI and NheI restriction enzymes. An upstream component of human sialidase cDNA was removed as a PshAI-NheI restriction fragment and replaced with the amplified PshAI-NheI fragment. Mouse sialidase 5' flanking region (1.8kb) was amplified by PCR introducing an AgeI site at the 3'end.the product was subcloned in pCRII vector. Mouse sialidase gene 5'UTR (1.8kb) region was introduced into the targeting vector as a NheI-AgeI fragment and was confirmed with a diagnostic BamH1 digest. A floxed pPGK-neo[1]was introduced in a SalI/NotI site then an ApaI genomic fragment (8 kb) containing exons 2-6 as well as 3'UTR sequence was introduced at the ApaI site of the vector. For all the PCRs involved in making the construct, the template used was an isolated SV129 genomic BAC clone containing the sialidase gene. This targeting vector and the transgenic mouse were generated previously in our laboratory (Figure 1).

2.2 Generation of human sialidase knock in transgenic mice:

To culture the plasmid, bacteria were transfected with the plasmid and grown overnight in 5 mL of LB media. DNA was extracted using the Noragen MiniPrep Kit. Female FVB mice, 6 weeks old, were injected with 5 International Units (IU) of Pregnant Mares Serum (PMS) interperitoneally (ip) 3 hours before the dark cycle. 46 hours later, 5 IU of human chorionic gonadotropin (hCG) was injected ip. Females were then being paired with males overnight. Simultaneously, 6 week old CD1 mice were paired with vasectomized males. Embryos were extracted from these females 7 hours post-insemination and kept in HEPES buffered KSOM media. Cumulus cells were dissolved with 10mg/mL hyaluronidase to isolate the embryos. Pronuclei of the embryos were injected with the linearized plasmid and injected using a microinjector apparatus. Embryos were then reimplanted into the infidibulum of the pseudopregnant CD1 females. Offspring were checked for incorporation of the vector using PCR using primers specific for the sialidase cDNA (Nagy et al, 2003).

2.3 Generation of double transgenic mice: (human sialidase knockin on a hexa-/- background)

The generation of the double transgenic mice was done in several steps. Base on the fact that we have the C57BL/6J hexa-/- mice generated on our lab previously as described in (Phaneuf et al., 1996) (Figure 2) the first step was to Crosse the C57BL/6J hexa+/- mice to generate the C57BL/6J hexa-/- mice and after identifying the targeted hexa-/- group, I then preceded by mating the previously identified human sialidase positive FVB mice (KI+) with the hexa-/- mice. The last step was to recognize the desired human sialidase positive KI+ /hexa-/- double transgenic mice. All the identifications were done using the PCR technique. Obviously these double transgenic mice have just one copy of the human sialidase gene at this point.

2.4 DNA extraction and genotyping reaction:

Tips of mice tails were excised into small pieces and digested each in 500µl lysis buffer (2 mM Tris-HCl, pH 8.0, 5 mM NaCl, 0.5 mM EDTA, 20mg/ml proteinase K, 20% SDS) by incubation at 55°C in water-bath overnight. The DNA was extracted with 500 µl buffer saturated phenol, precipitated at 16,400 rpm for 10 mins then top layer was removed and put into new tube. The same step was then repeated and the top layer was rinsed in 70% iced ethanol. The DNA was scooped with needle and resuspended in 500 µl autoclaved water. Mice containing the human sialidase cDNA were genotyped by PCR. The primers used for genotyping were: ML6522 (also called mhG9i15') human sialidase from intron one CTGTAGGGTTTGGGTGTTTG and ML6523 (also called mhG9il3') human sialidase from intron one TCAGCAAAGGCGAGAAGAGT: PCR annealing temperature was 55°C. The predicted PCR product is a 572 bp fragment of the human sialidase. (Figure 3.1)

PCR generated fragment was then digested with BamH1 restriction enzyme, since the loxP which is a part of the human sialidase insertion has a Bam H1 site. By digesting with Bam H1, two fragments resulted confirming the presence of human sialidase cDNA (Figure 3.2). In order to genotype hexa-/- mice we used the following primers: 1, 5'-GGCCAGATACAATCATACAG and primer 2, GGTTTCTACAAGAGACATGGC, and primer 3, CACCAAAGAAGGGAGCCGGT.

The DNA was diluted in TBE buffer, combined 1:5 with loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll Type 400 (Pharmacia) in H_2O] and subjected to electrophoresis on a 1.8% agarose gel containing ethidium bromide (0.4 [mu]g/ml) at 50 V. The DNA bands were visualized using a UV transilluminator and sized against a 1 kb ladder (fast runner)

2.5 RNA Extraction and Reverse-Transcriptase PCR (RT-PCR)

Total cellular RNA from about a 50-100mg of liver and kidney tissue was isolated using Triazol reagent, (Invitrogen) according to the manufacturer's instructions. The resulting RNA pellet was dissolved in DEPC-dH_20. RNA to be used in cDNA synthesis was heated to 65°C for 5 min., and then placed on ice. RT-PCR was carried out with 25 µg of RNA in 25µl of DEPC water incubated at $65C^o$ for 5 minutes then placed on ice immediately. After that (M1) mixture of (2µl) RNAse out, (1µl) oligedt, (1µl) DNTP, (10µl) 5X buffer, and (1µl) RT reveres- transcriptase was prepared. The mixture that contain RNA+ M1 was incubated at $42C^o$for one hour, upon completion of the RT-PCR reaction, separate PCR reactions were performed on 5 µl of the cDNA, one set of reactions using primers designed to amplify a **200** bp fragment of the cDNA forward primer ML6522,(CTGTAGGGTTTGGGTGTTTG) reverse primer ML6523, (TCAGCAAAGGCGAGAAGAGT), and annealing temperatures (55°C). The primers used were expected to bind to the mouse template; therefore, a diagnostic test was designed to distinguish the mouse gene from human cDNA. A restriction enzyme site for MspA1I is that can cut the human sialidase cDNA, but not the mouse sialidase cDNA. On the other hand, PvuII digests the mouse sialidase cDNA, but not the human cDNA. In addition a second RT-PCR using a ML6522 and a new primer- ACATCGCTCACTACTGCCCCAA- was performed by which I should have a fragment of 367 bp of the human sialidase cDNA. Upon completion of the PCR reactions, the resulting samples were run on a 1% agaros gel.

2.6 Antibodies

Rabbit polyclonal antibodies against recombinant human lysosomal sialidase were prepared as described previously (Igdoura, et al, 1998). Other primary antibodies used including secondary antibodies employed in immunocytochemistry included goat anti-rabbit peroxidase conjugated IgG, and goat anti-mouse peroxidase conjugated IgG (GE health care).

2.7 Western Blotting

Liver and kidney from mice with different genotypes were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, pH 7.4) and placed in a 1.5 ml microcentrifuge tube. The protein concentrations of the lysates were measured by Bradford assay (BioRad). An appropriate volume of 4× loading buffer was added to a 50 µl sample of each lysates and the samples were boiled for 10 min. The samples were loaded into the wells of a 7% SDS-polyacrylamide gel with a 5% stacking gel, such that each sample was represented by the same amount of protein, and run at 30 mA for ~ 2 hrs. Gels were blotted onto nitrocellulose membranes. Membranes were blocked for 1 hr at room temperature with 5% nonfat dry milk in TBS (10 mM Tris-Cl, pH 8.0, 150 mM NaCl). The membrane was then incubated with the rabbit-anti-human sialidase antibody at a dilution of 1:500 in 10% milk/TBS overnight at 4°C. The membrane was washed with TBS + 0.2% Tween 20 and then incubated with goat-anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1:5000 for 1 hr at room temperature.

The membrane was washed again with TBS + 0.2% Tween 20 and developed using the Western Lightning chemiluminescence reagent (GE Health care) according to manufacturers' instructions. Developed membranes were exposed to Kodak X-OMAT AR scientific imaging film. After this first exposure, membranes were stripped according to the Western Lightning protocol. Stripped membranes were then blocked again with 10% milk in TBS, this time for ½ hr. Following the block, membranes were incubated with the mouse-anti-histidine tag antibody at a dilution of 1:500 overnight at 4°C. Membranes were then washed with TBS + 0.2% Tween 20 and incubated with the secondary antibody, anti-mouse IgG conjugated to horseradish peroxidase, at a dilution of 1:5000 for 1 hr at room temperature. Following this incubation, membranes were washed, developed, and exposed as described above.

2.8 Assay of Sialidase Enzyme Activity

Liver and kidney tissues were weight then homogenized in distilled water. Tissue homogenates were assayed for lysosomal sialidase activity using 4-methylumbelliferyl-n-acetyl-α-D-neuramide (MUNANA) as a substrate, with incubation for 30 min at 37°C (Potier et al., 1979). Following incubation, reactions were stopped with 1.9 ml of 0.1M MAP, pH 10.5. Fluorescence was measured on the LS Reader Plate Fluorometer excitation at 360 nm and emission at 447 nm (Perkin Elmer). Protein concentrations of the cell lysates were measured using Bradford protein assay (BioRad). Data was presented with error bars representing standard deviation (n = 3).

2.9 Histological assessment

After cardiac perfusion of mice with PBS and then with phosphate buffered 3.8% formaldehyde, the brain, liver, kidney, spleen, heart, lung, muscle, and intestine were dissected and kept in fixative over night. Tissues were sent to the histology facility at McMaster University for paraffin embedding and sectioning. Sections were subsequently stained with Heamtoxalin and Eosin as well as Bielschawsky in order to examine and assess the histopathology of the tissues.

2.10 Ganglioside Labeling, Extraction and Thin Layer Chromatography (TLC)

Mice brain samples were dissected then weighed. For every 430mg of brain, 10ml of Chloroform/Methanol/dH_20 (C/M/H_20) at (10:10:1) ratio solutions were added. The samples were sonicated using a pulse sonicator (pulsate setting: 40 % duty cycle 4%, output control) for 10 sec on ice, and then using a water sonicator for 15 minutes (20sec on, 20sec off). Samples were centrifuged for 15min at 3000 rpm. Supernatant was designated 'S_1' from each sample and was collected. Extraction was repeated and' S_2' fraction from each sample was collected and then combined with 'S_1'. Five ml of C/M/NaAcetate (0.8M) at ratio (30:60:8) were used to extract pellet again to produce 'S_3' fraction. 'S_3' from each sample was collected and then combined with S_1 and S_2 fractions. The brain samples were incubated in a sand bath under a stream of air. Samples were re-dissolved in 10 ml of C/m/dH_20 at (30:60:8) ratio and then KOH was added to increase pH to11. Samples then were incubated in 50°C water bath for 4 hours; the pH was

checked periodically and drops of KOH were added to keep appropriate at pH 11. Specimens were then removed from water bath and left to cool, soon after 1-2 drops of glacial acetic acid were added to bring pH to 7. After that specimens were centrifuged at 3000 rpm for 10 min and the supernatant from each sample was collected then washed with 5 ml of C/M/H_2 at (10:10:1) ratio. Brain specimens were then centrifuged at 3000 rpm for 10 min and the supernatant was collected and combined.

The specimens were evaporated in sand bath with stream of air until approx. 500µl of each remained. Sephadex G-50 column were Prepared and the samples were added to the columns. The columns were centrifuged at 700 rpm for 2 min and the collected sample were transferred to glass tube and stored at 4°C until next column was completed. DEAE Sepharose Column was prepared by washing 2x with 10 ml of C/M/NaAc (0.8M) at (30:60:8) ratio to each 5ml of the DEAE Sepharose solution then 2x washing with C/M/H_20 at (30:60:8) ratio. Ten ml of C/M/H_20 at (30:60:8) ratio were added to rinse bottom of tube and pour DEAE Sepharose into column. After that the columns were washed with 5ml of C/M/H_20 at (30:60:8) ratio. The brain samples were added to the columns then the flow through (F_1) was collected. Columns were washed with 5ml C/M/H_20 at (30:60:8) ratio and flow through ($F_{2)}$ was collected. The columns were washed with 10ml of C/M/NaAc (0.8M) at (30:60:8) ratio to collect F_3 fraction. F_1, F_2 and F3 fractions were combined.

All liquids were evaporated in sand bath under air steam. Each dried sample was dissolved in 5ml of C/M/KCl (0.1M) at (3:48:47) ratio. Sep-PAK C-18 reverse phase column was prepared by washing first with 5ml pure methanol then washing with 10ml C/M at (2:1) ratio and finally washing with 5ml pure methanol; then equilibrate with 10ml C/M/KCl (0.1M) at (3:48:47) ratio. Sample were passed through columns then collected twice and the flows were wasted. Columns were washed with 15ml ddH_20 to elute salts then flows were discarded. Columns were washed with 15ml of C/M at (2:1) ratio to elute gangliosides out. All Liquids were evaporated then each dried was dissolved in 200µl C/M/H_20 at (10:10:1) ratio.

2.11 Thin Layer Chromatography

Serial dilutions of samples were made by 1part of a sample into 3 parts of C/M/H_20 at (10:10:1) ratio (1:4, 1:4, and 1:8). Specimens 30µl each were spotted on the plate with 1 cm apart. The plate was ran in acetone chamber until liquid reached 1 cm from the top of the plate, then the plate was removed from chamber and allowed to dry. C/M/$CaCl_2$ (0.2%) mixture was prepared in another chamber and sat for about 1 h before running the plate in it. After running the plate in the second chamber for about 2 h, the plate was removed and allowed to dry. The plate was sprayed with orcinol reagent: (0.2g orcinol dissolved in 10.60ml H_2SO_4, then Fill up with dH_2O to 100 ml). In order to visualize bands, the plate was baked in an oven at 110 C^o for 20 min.

2.12 Behavioral Assessment

Behavioral tests were performed in order to asses muscle strength of the animal models, especially in their rear limbs. These tests were (1) wire-hang test, (2) tail elevation test, (3) Open field_locomotion test, and (4) Rotarod test. In the wire-hang test, we measured muscle strength in the limbs of these animals. A normal animal is typically able to hang from a flipped wire for 60 seconds or more, while an animal with muscle weakness is not. In the second test, I hold the mice by their tails while videotaping their body posture (Video recording was done with Kodak Easy Share C533 camera). The third test was to evaluate abnormalities in locomotion; a 5-10 minute test session was carried out in an open field. The observation area was 30 cm x 30 cm and the animals were videotaped. Their movement was observed and notes were taken. In the fourth test, Rotarod was performed on a Rotarod machine with different animals and different genotypes for the same period of time. All mice were trained to walk on the rotating rod for 10 minutes. According to a predetermined program, the speed was gradually increased over the course of a 5-minute test from 4 rpm to a maximum of 40 rpm.

The speed at which mice fell off the rod was registered. The Rota Mex Capture RMSWIN software captured data, displayed it on the computer screen and saved it to files, which were then analyzed. Regular observation of the overall well being of mice was done in order to identify any symptoms of the disease or any abnormal behavior. Measuring body weights of animal have been done on different time points to detect any differences in genotypes.

CHAPTER THREE: RESULTS

3.1 Characterization of transgenic mice expressing human sialidase

Our laboratory has generated a transgenic mouse that expresses floxed human sialidase cDNA under the control of mouse sialidase promoter (Figure 3.1). The transgenic mice were designated KI+. The mice exhibited normal phenotype indistinguishable from wild type mice with no significant change in body weight or feeding behavior (Figure 3.2 and 3.3). The human sialidase has been previously shown to be active in mouse systems forming an active complex with mouse cathepsin A and β-galactosidase. The presence of loxP sites within the transgene provides a tool to remove part of the cDNA which should render the transgene inactive. The KI+ mice were genotyped using primers flanking the inserted first intron containing the first LoxP and a Bam HI site. Out of 24 samples in the first PCR reaction, 16 KI positive animals were identified. Because of the high percentage of positives, the slight difference in size between DNA fragments generated for human transgene and endogenous mouse gene was utilized, and to make sure that I was in fact genotyping for the human sialidase product, the PCR fragments were digested with Bam H1 enzyme. This procedure allowed us to identify true positives. Therefore, the validity of the genotyping method was verified by digestion with Bam HI which revealed the two expected fragments (Figure 3.1, 3.4 and 3.5).

3.2 Expression of human sialidase in transgenic mice:

In order to verify the expression of human cDNA, RNA was isolated from livers, and Kidneys using TRIZOL reagent and then the liver and kidney mRNA samples were used to perform RT-PCR. Although the samples were contaminated with genomic DNA fragment between 700-500 bp, PCR fragments around 200 bp corresponding to human cDNA were also observed.

To identify the human sialidase cDNA and to differentiate between it and the mouse sialidase cDNA, the 200pb PCR products were digested with two different enzymes MspA1I and PvuII. MspA1I is an enzyme that digests the human sialidase cDNA but not the mouse sialidase cDNA.

In addition, the restriction enzyme, PvuII digests the mouse sialidase cDNA but not the human sialidase cDNA. PCR digests were separated on an acrylamide gel for its high resolving power. Because the digested bands were very small and therefore very faint, it was difficult to obtain a clear image of the products. Nevertheless, it was apparent that the human sialidase mRNA was expressed in the positive sample along with the mouse sialidase mRNA.

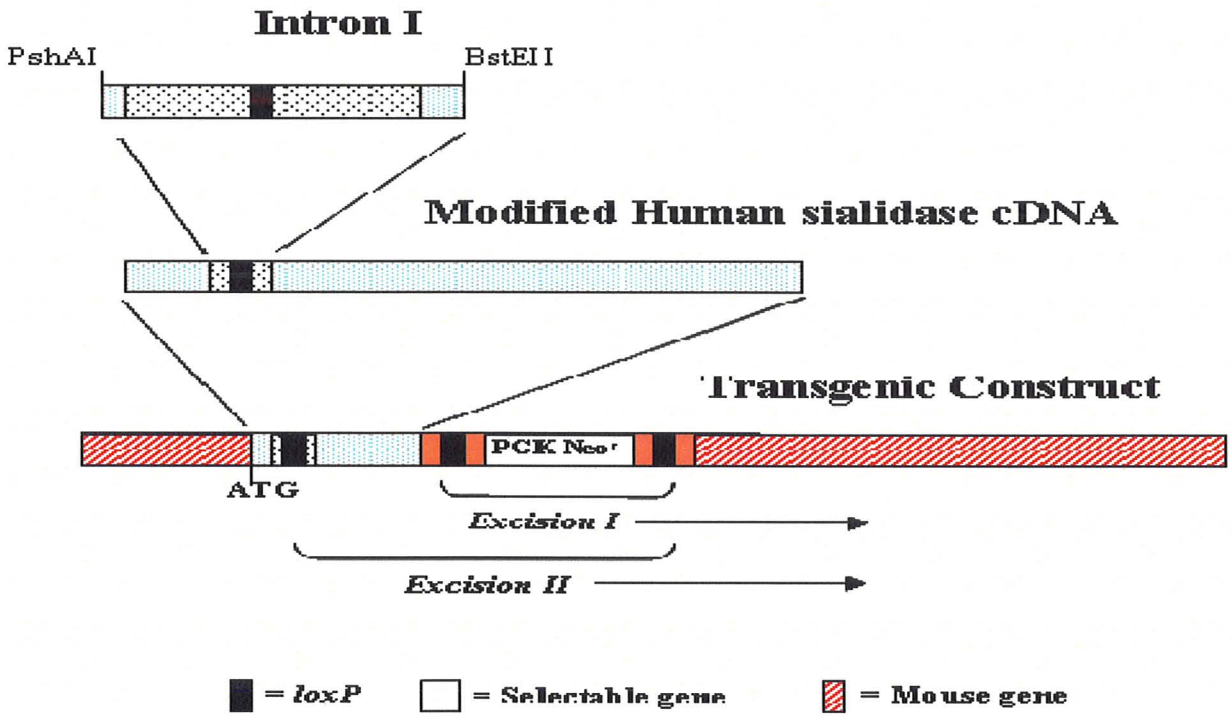

Figure 3.1 The human sialidase transgenic construct.

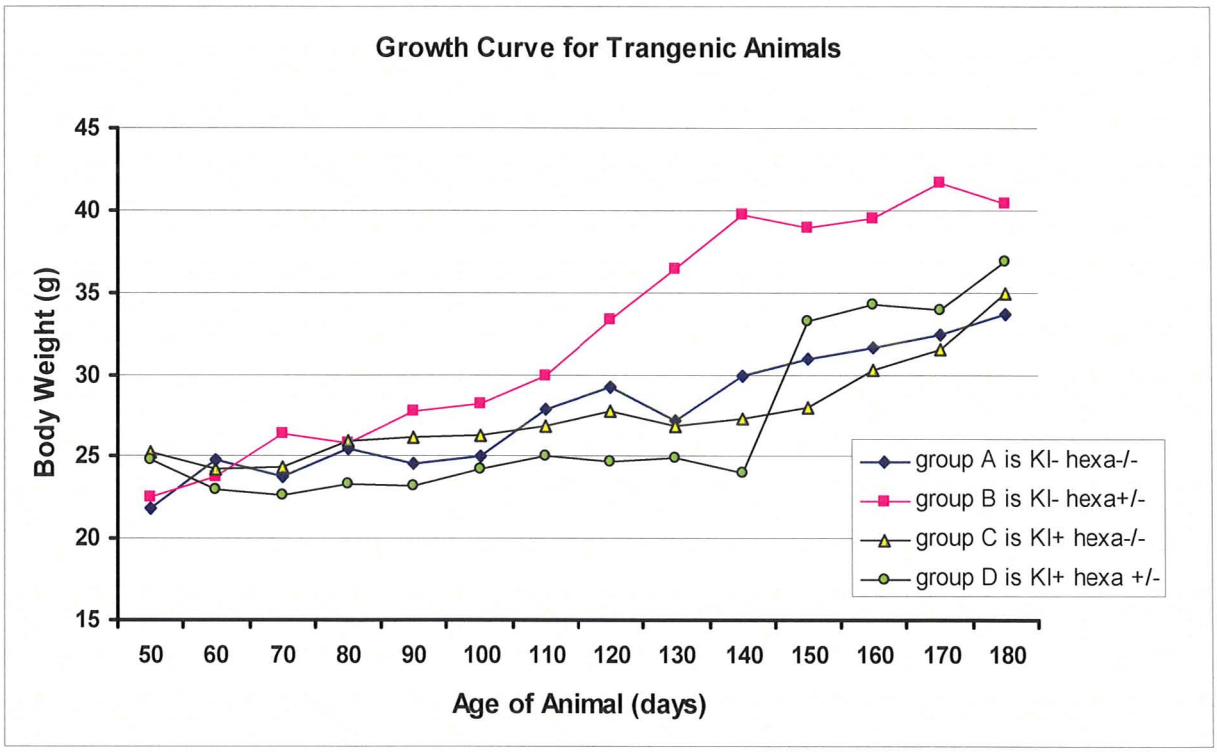

Figure 3.2 Body weight assessment of four different groups of animals: the A group is human sialidase negative and hexosaminidase deficient (hexa-/-), the B group is human sialidase negative and hexosaminidase heterozygote (hexa+/-), the C group is human sialidase positive and hexosaminidase deficient, and the D group is human sialidase positive and hexosaminidase heterozygote (hexa+/-).The C and D that are human sialidase positive show slightly decrease in body weight comparing with group A and B that are human sialidase negative group. The A group that its hexa-/- seems to be steady in its weight gain over time in comparison with the C group that has the same hexa genotyping.

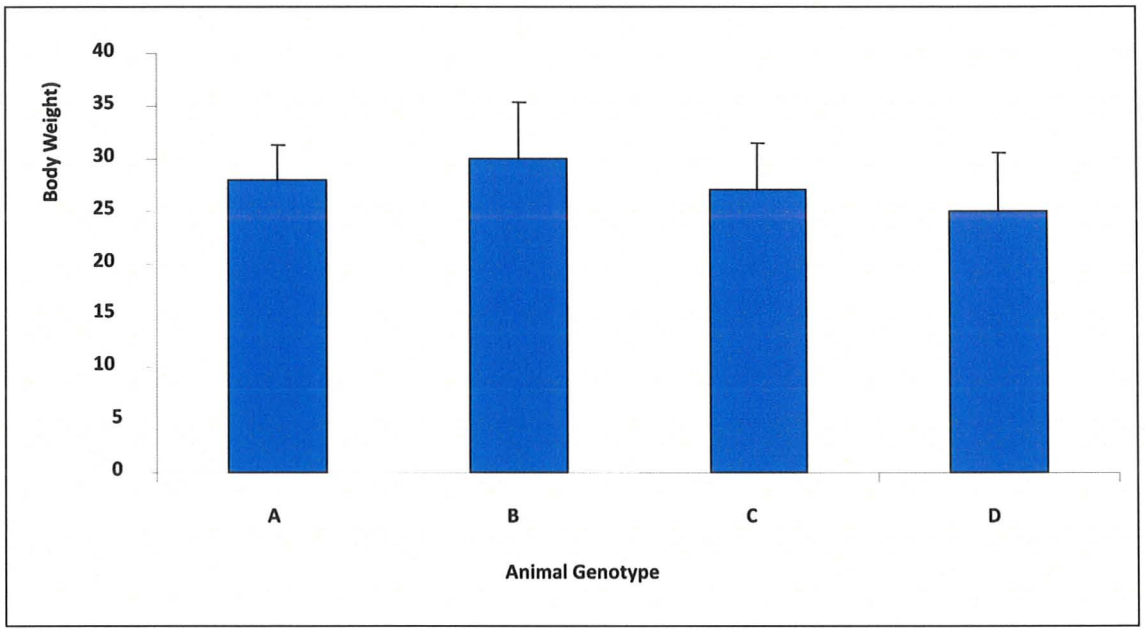

Figure 3.3 Mean body weight of 4 animals with different genotypes at age 110 days ±SD. the A group is human sialidase negative and hexosaminidase deficient (hexa-/-), the B group is human sialidase negative and hexosaminidase heterozygote (hexa+/-), the C group is human sialidase positive and hexosaminidase deficient, and the D group is human sialidase positive and hexosaminidase heterozygote (hexa+/-).The C and D that are human sialidase positive show slightly decrease in body weight comparing with group A and B that are human sialidase negative group. The A group that its hexa-/- seems to be steady in its weight gain over time in comparison with the C group that has the same hexa genotyping.

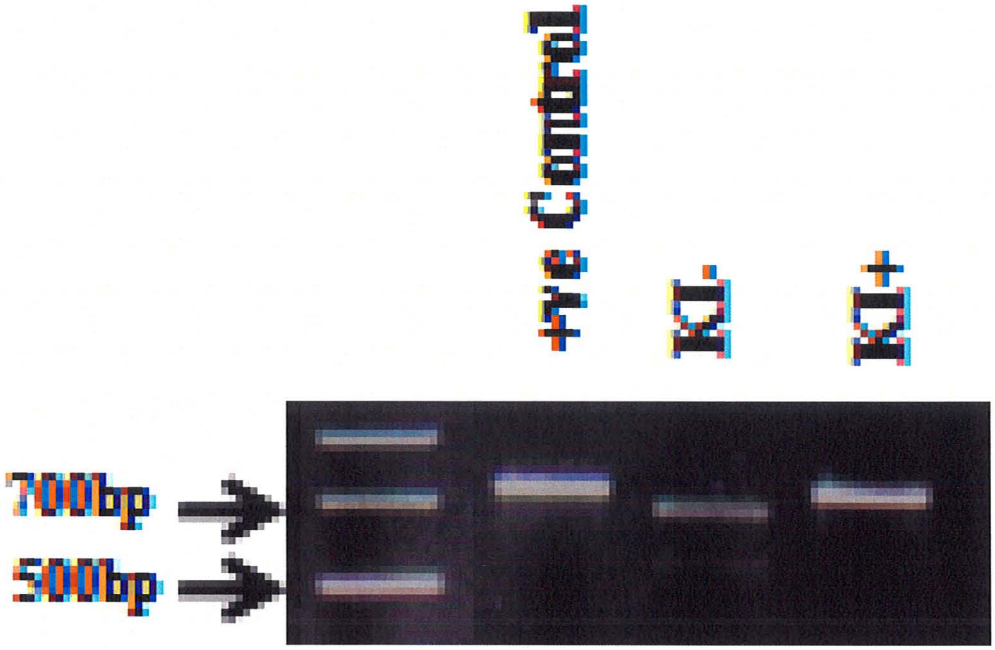

Figure 3.4 PCR genotyping of human sialidase knockin from transgenic mice DNA, PCR was performed using DNA template extracted from mice tails. The PCR product was separated by electrophoresis on 1% agarose gel. The third lane shows a band which is lower than the positive control band indicating a mouse sialidase derived product. The difference in band size between lane 2 and 4 versus lane 3 is due to the lox p site inserted in the 1st intron of the modified human sialidase cDNA in the knockin construct.

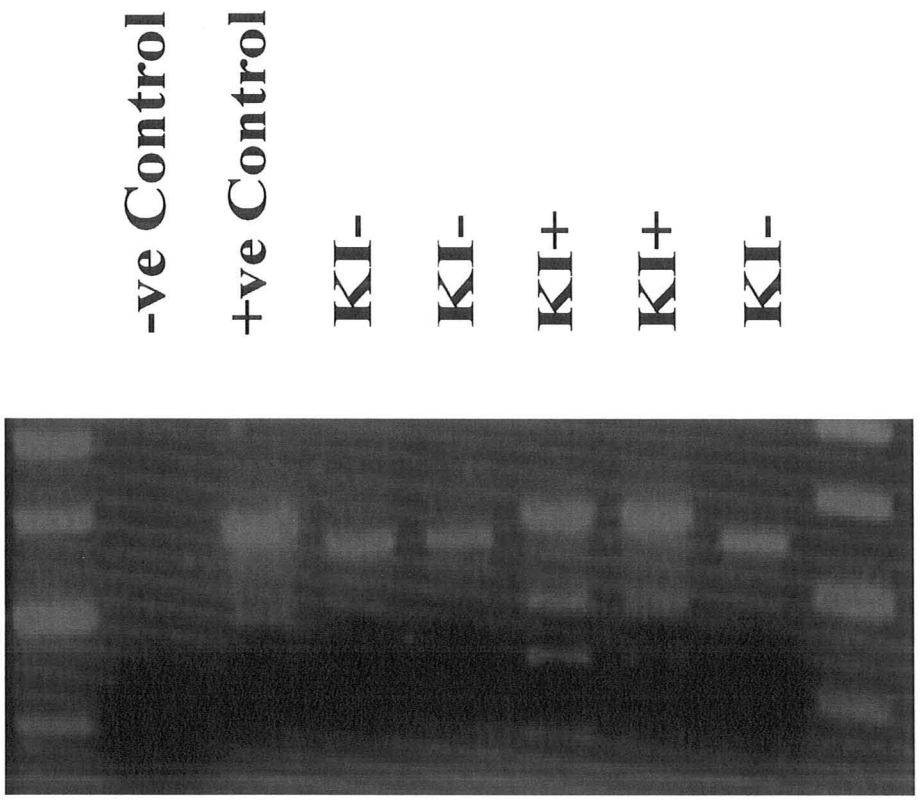

Figure 3.5 A partial digestion of the human sialidase DNA fragment KI+ comparing to the mouse sialidase DNA fragments KI-, after digesting with Bam H1 enzyme.

With the aim of examining transgene expression at the protein level, liver and kidney from KI positive mice (n=3) were dissected and then used in Western blotting analysis. The three mice were two males and one female at the same age. Using Rabbit polyclonal antibodies raised against recombinant human lysosomal sialidase as a primary antibody and a goat anti-rabbit peroxidase conjugated IgG as a secondary antibody, a clear band of the expected molecular size was observed in the kidney sample of the female (Figure 3.6).

3.3 Sialidase enzyme activity in transgenic animals:

Investigation of sialidase activity in organ extracts of transgenic mice expressing human sialidase indicated that the human neu1 is active and is tissue-specific. Lysates from liver, kidney, spleen and brain of 8-week old C57/Bl6 and KI mice were prepared and tested for neu1 enzymatic activity using 4-methylumbelliferyl-N-acetylneuraminic acid (MU-NANA) as a substrate. Sialidase (neu1) enzyme activity was indistinguishable in brain lysates of KI+ and KI- males and females (Figure 3.7). Homogenized liver and kidney tissues from four animals were used to monitor sialidase activity. Sialidase activity assay preformed on liver and kidney were done in triplicates. The kidneys samples of mice that have the human sialidase construct showed an increase in sialidase activity in comparison with samples from transgenic negative mice. One liver sample that tested positive for human sialidase showed slight increase in sialidase activity whereas the other positive sample showed the least amount of activity. The human sialidase negative liver samples showed lower enzyme activities (Figure 3.7).

3.4 Histological Assessment of transgenic mice:

In order to assess any pathological effects of human Sialidase expression, tissues from liver, muscle, kidney and lung were processed for histology. In the histology plate A-H are wild type mice tissue and I-P are from KI+ mice tissue. After carful observation of the histology samples, no histological difference was observed between the wild type samples and the human sialidase positive KI+ samples (Figure 3.8). Histological analysis

of transgenic mice further supported the cell-type specific nature of sialidase expression in these mice.

Examination at the light microscope level revealed that epithelial cells lining the proximal convoluted tubules were indistinguishable from wild type tissue. Control lungs from mice of the same age of the mutants did not present these morphological alterations. Hepatocytes and endothelial cells lining the sinusoids had a normal appearance. The liver did not present any accumulation of structures with lipid droplets and/or membranous material (Figure 3.8). Examination of the brain cortex and white matter of KI+ animals did not reveal any morphological differences with the brain of the normal control mouse with cortical neurons exhibiting normal cytoplasm and normal morphology (not shown).

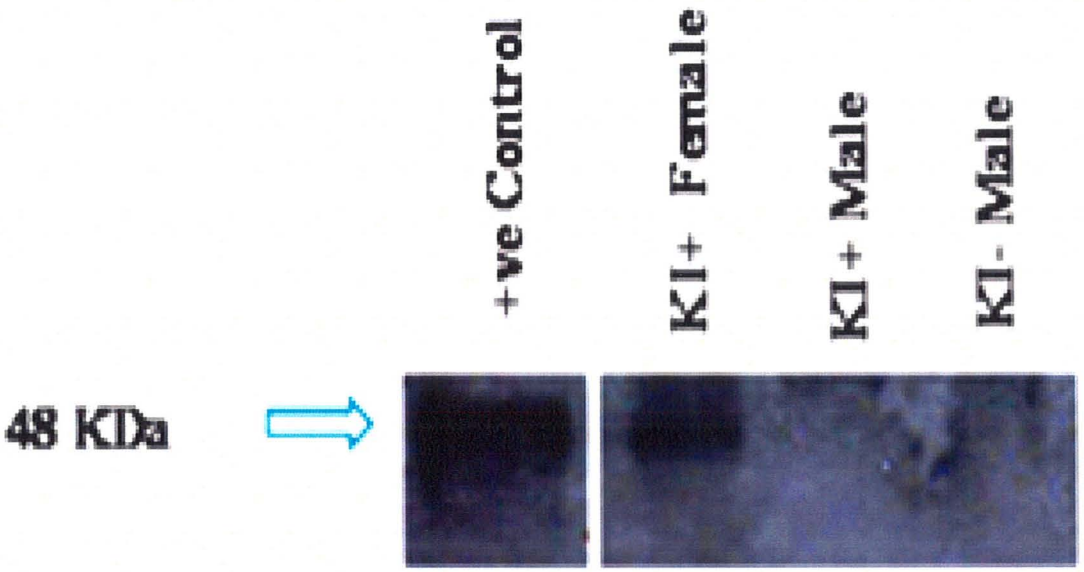

Figure 3.6 Human sialidase expression in the kidney of KI mice, Western blot analysis of kidney tissue from one KI positive female, KI+ male were blotted using rabbit polyclonal antibody against recombinant human lysosomal sialidase. The female kidney showed a clear band at the appropriate molecular size of 48KDa.

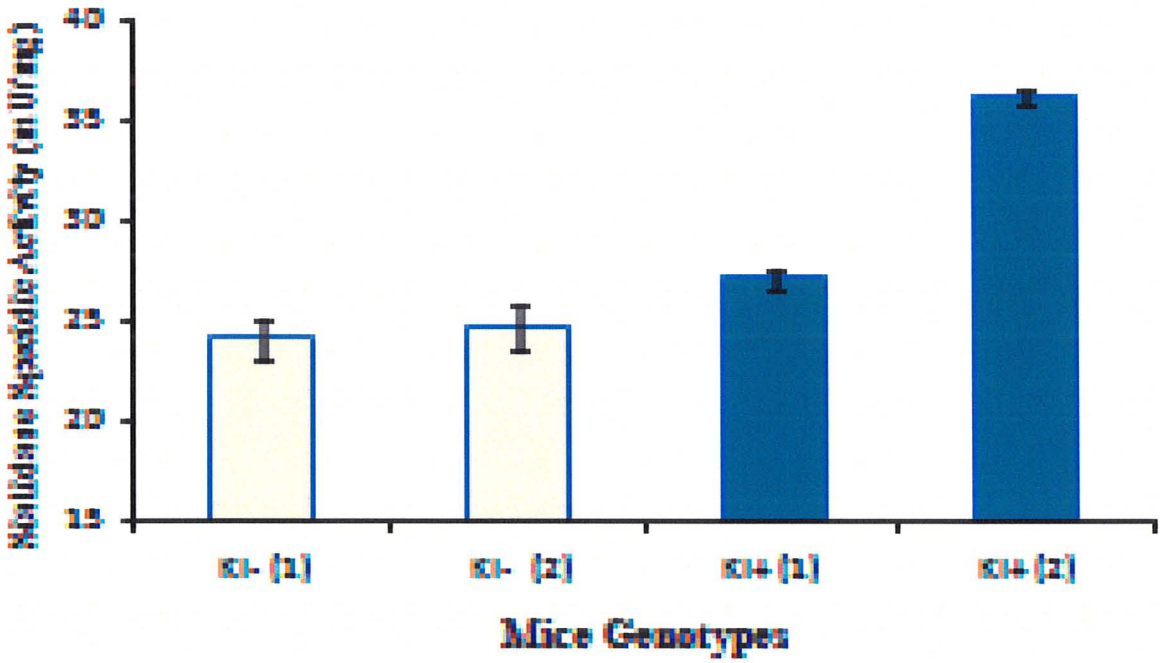

Figure 3.7 Sialidase activity assay preformed on kidney lysates indicated that kidneys samples of mice which have the human sialidase construct the first and the last one, show increased activities whereas; the samples that have just the mouse sialidase show much reduced activities.

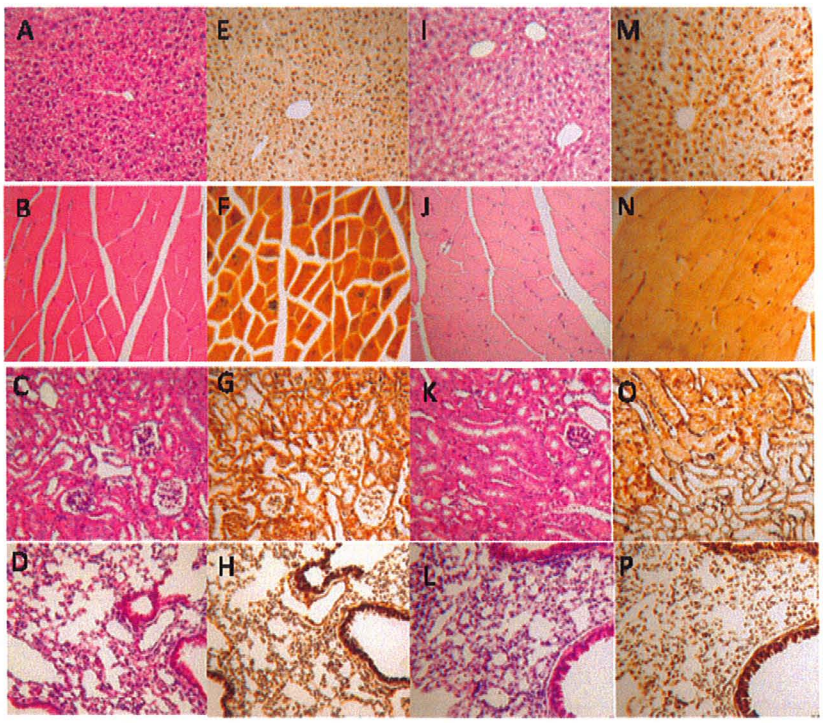

Figure 3.8 Cross-sections of liver, muscle, and kidney then lung of wild type and human sialidase mice stained with Bielschawsky and H&E, A to H samples are wild type mice tissues and I to P are human sialidase positive KI+ mice tissues.. No histological difference is observed between the groups.

3.5 Characterization of transgenic mice expressing human Sialidase on a hexosaminidase deficient background

This transgenic mouse was crossed with hexa$^{-/-}$ mice and after two crosses, a double transgenic mouse expressing human sialidase under the control of mouse promoter was generated. The first group of animals was generated by mating hexa-/- with KI mice (F1) were genotyped for KI gene. After identification of KI positive mice, homozygous hexa-/- mice positive for KI were generated by mating KI positive with each other (F2) the colony founders that were hexa heterozygote (hexa+/-) F2 generation were then genotyped in order to identify the transgenic mice, which are positive for the human sialidase insertion and heterozygote for hexa-/- (Figure 3.9).

3.6 Ganglioside analysis

Gangliosides isolation from total brain has been done on four different mice with four different genotyping, KI-/hexa+/-, KI-/hexa-/-, KI+/hexa+/- and KI+/hexa-/-. Acidic and natural gangliosides obtained from the four brain samples were pooled and then were resolved on TLC plate. The TLC plate showed a distinct accumulation of GM2 ganglioside in the KI-/hexa-/- and the KI+/hexa-/- mice samples. GM2 ganglioside accumulation is characteristic of hexosaminidase A deficiency. Our results unequivocally show that the presence of humanized sialidase did not change the number, intensity nor the character of any of the bands visualized on the TLC (Figure 3.10).

3.7 The behavior assessments

KI+/hexa -/- mice were indistinguishable from KI+/hexa +/-, KI-/hexa -/- and KI-/hexa+/- littermates at birth and grew normally to adulthood. Both male and female mice were fertile. With the aim of assessing tail elevation, four groups of mice KI+/hexa -/- , KI+/hexa +/- ,KI-/hexa -/- and KI-/hexa+/- suspended by the tail positioned their hind limbs at a slight angle away from the body with the distal bit of the limbs slightly flexed. Quick changes in limb position were observed commonly, even though the animals remained in the same position for several seconds.

In order to examine the neuromuscular status of the transgenic mice, Wire-hang test was conducted. Our results revealed no significant difference between the four groups of mice KI+/hexa -/- , KI+/hexa +/-, KI-/hexa -/- and KI-/hexa+/- in their hang time. Only the double transgenic mice KI+/hexa -/- that have double copies of the KI were unable to hang for 60 seconds.

Open field locomotion was observed and consistently showed little variation among the various transgenic strains. The noticeable exceptions were two three months old double transgenic mice (KI+/hexa -/-) which experienced hind limb dragging. While observing the subjects in the open field, it was noticed that one three months double transgenic mice (KI+/hexa-/-) was very small in size (half the size of its litter mates). One distinct feature of this mouse is that, it sits on its hind limbs while combing its face vigorously. It shows Kyphosis with one of its eyes almost white, potentially due to a cataract. A second mouse was found to share the same odd phenotype (Figure 3.11 and 3.12).

Rotarod analysis which assess the locomotive balance of mice and represent cerebellum function, were conducted on the various mice: KI+/hexa -/- , KI+/hexa +/- , KI-/hexa -/- and KI-/hexa+/-. The assessment revealed no major differences among the mice lines. This experiment was performed on the two symptomatic mice that show phenotypic variation at three months age (one was KI+/hexa-/- and the other was KI+/hexa+/-) as well as one KI-/hexa-/- mouse. All mice were able to walk on the rotating rod with constant speed. Upon Rotarod acceleration, the two symptomatic mice KI+/hexa-/- and KI +/hexa +/- were not able to keep up with the changing speed and fell at second whereas the KI-/hexa-/- group lasted longer and fell at a much longer time (Figure 3.13).

After setting up new mating to generate mice with double copies of the KI transgene, we observed some mice that showed some symptoms. Our observations of these mice are preliminary and need further work. The human sialidase positive with double copies with or without and hexa-/- were very small in size, have hind limbs spasticity and peculiar posture with Kyphosis. They were visually impaired. Furthermore, most of these symptoms were noticed around three months of age. Moreover the animals showed muscle weakness and clasping of their hind limbs.

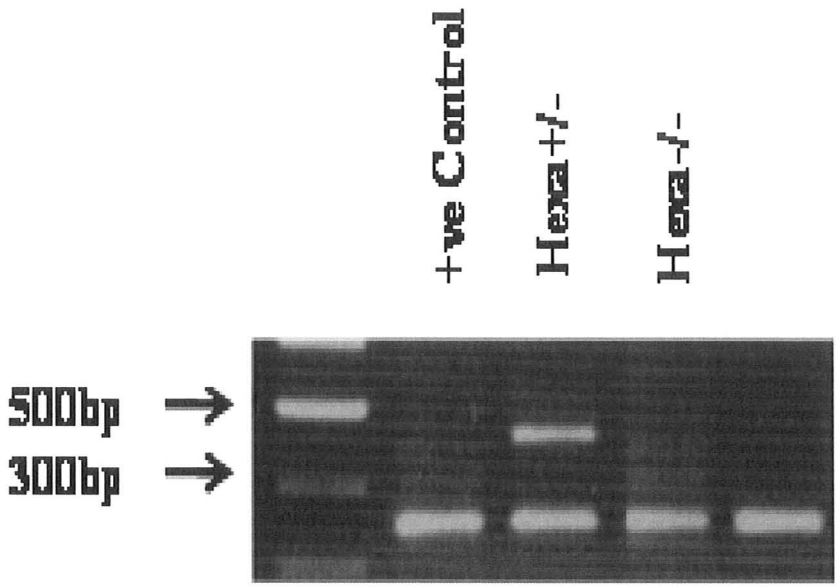

Figure 3.9 Genotyping for β-hexosaminidase α mutant alleles. PCR DNA fragments were amplified from tail genomic DNA and were separated by electrophoresis on 1 % agarose gel. Lane 1 contain molecular standards, lane 2, contains products amplified from genomic DNA for hexa-/- mice as a positive control. Lane 3 contains products amplified from heterozygote hexa+/- mice DNA. Lanes 4 and 5 show fragments amplified from hexa-/- genomic DNA.

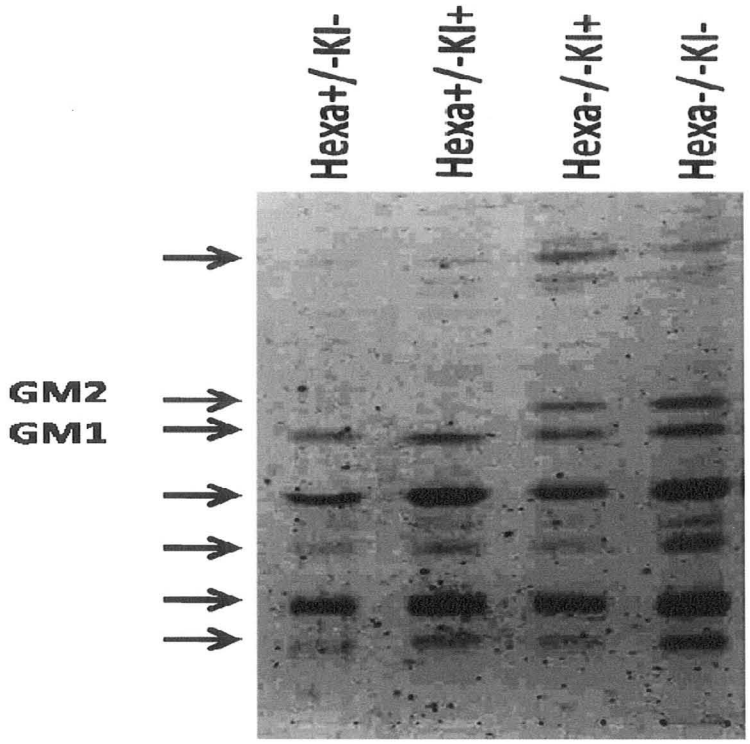

Figure 3.10 Ganglioside fractions were extracted from four the brains of mice from different genotypes. Samples were separated on TLC; there is no accumulation of GM2 in the hexa+/- brain samples with or without the human sialidase cDNA (the first two lanes from the left) namely, hexa+/-KI- and hexa+/-KI+. However, there is a significant accumulation of GM2 ganglioside in the hexaminidase deficient mice (last two from the left).

Figure 3.11 Behavioural assessment of symptomatic human sialidase positive mouse with two copies of the transgene showing clasping of the limbs which is characteristic of neurologically impaired mutant mice.

Figure 3.12 Behavioral assessment of the symptomatic human sialidase positive mouse with two copies of the transgene in which back curvature (Kyphosis) is apparent and the mouse is also blind with eyes almost completely shot.

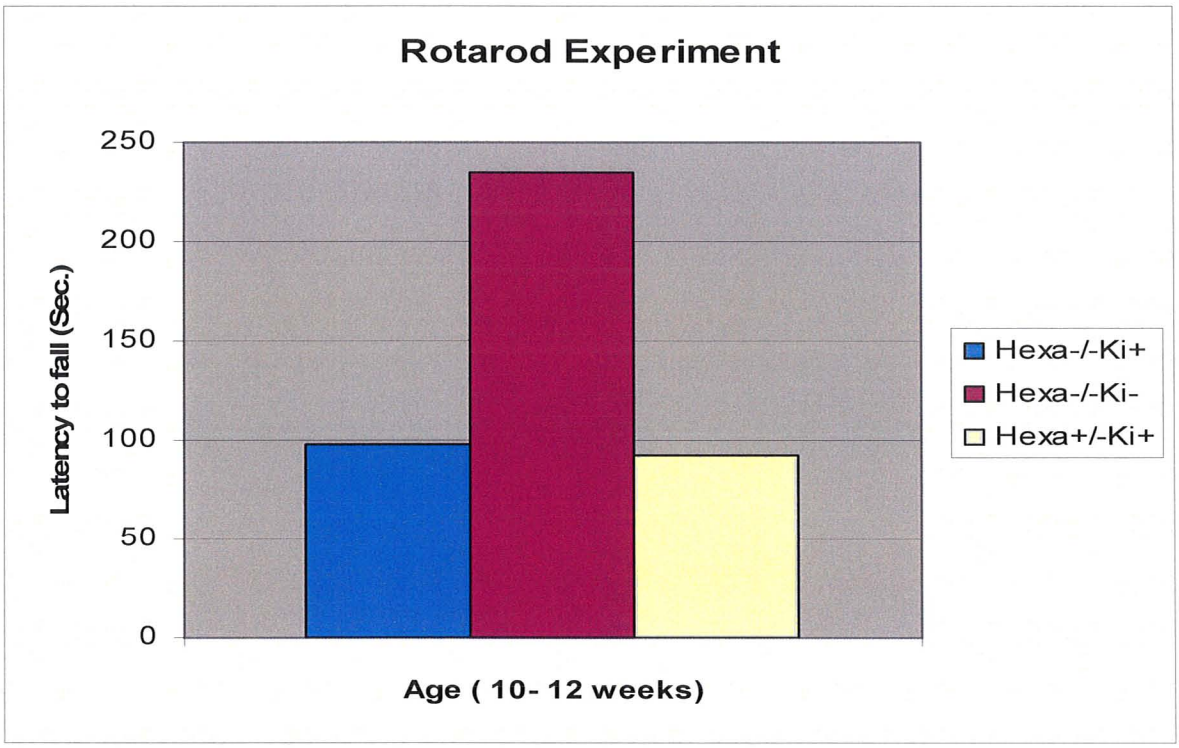

Figure 3.13 Rotarod performances of three different genotypes of animals at 10-12 weeks of age. While hexa-/- show the highest latency time, the mice that contained KI transgene appear to fall earlier indicating that presence of human sialidase may affect mouse performance on Rotarod.

A B

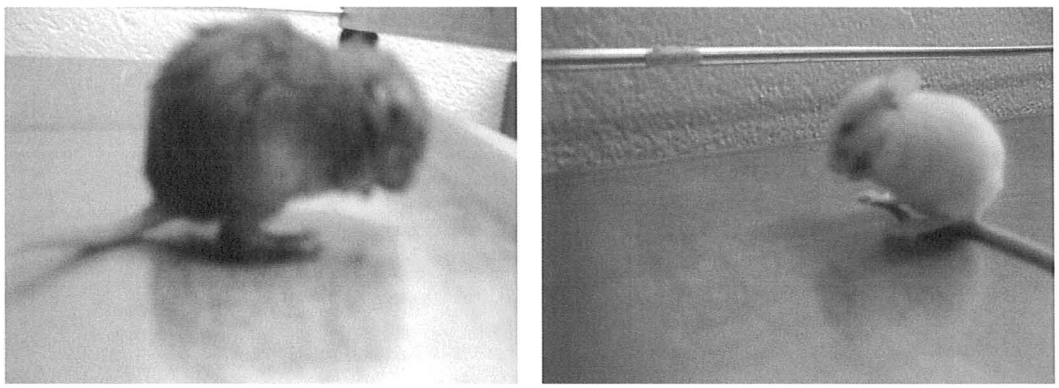

Figure 3.14 Phenotype of the symptomatic mice. (**A)** The first symptomatic mouse with one copy of the human sialidase transgene. (**B)** One of the symptomatic mice with two copies of the human sialidase transgene. The two mice were depicting weird behavior presented in vigorous combing of their faces while standing on their hind limbs and tails, also cataract in one eye; however, it is more obvious in the animal with black eyes.

CHAPTER FOUR: DISCUSSION

The overall objective of this work was based on the hypothesis that mice deficient in hexosaminidase A (Tay-Sachs mice) can escape the disease utilizing a bypass pathway in which sialidase can convert GM2 to GA2 then hexosaminidase B enzyme convert GA2 to lactosylceramide without the need for hexosaminidase A enzyme (Phaneufl et al., 1996). Therefore, by introducing the human lysosomal sialidase into the pathway, we hypothesized that this later enzyme will interfere in the bypass pathway preventing mouse sialidase from converting GM2 to GA2 leading to an excessive accumulation of GM2 in the nerve cells causing a phenotype similar to the juvenile form of Tay-Sachs disease.

The generation of the human sialidase transgenic mice was confirmed by PCR and human specific primers; however those primers primed the mouse sialidase as well. Therefore, a strategy to distinguish mouse sialidase from human sialidase PCR products was devised. This strategy was based on the fact that transgenic mice, which carry the human sialidase transgene, have Bam H1 site in their construct. So by digesting the PCR products with Bam H1 enzyme two small fragments from the human sialidase positive mice were expected to be obtained instead of one fragment from KI negative mice. After verifying the genotype of KI+ mice using PCR in conjunction with Bam H1 digestion, RT-PCR was utilized to test the RNA expression of human sialidase. Although the RT-PCR gels were difficult to make presentable, they demonstrated clearly that the RNA was expressed.

This was further confirmed using restriction enzymes diagnostic with MspA1I and PvuII. The MspA1I enzyme cut the human sialidase cDNA fragment, but not the mouse sialidase cDNA fragment. On the other hand, PvuII digested the mouse sialidase cDNA, but not the human cDNA. By means of rabbit polyclonal antibodies against recombinant human lysosomal sialidase and western blotting of kidney lysates, a 48 KD band was demonstrated in the kidney sample of a female KI+ mouse. However, the two other male kidney samples did not show any bands.

Lysosomal sialidase has been shown previously to be unstable and to show high turnover rate (Van der Spoel et al., 1998), this maybe the reason why spot the protein was difficult to be spotted in detectable steady state in the other samples of KI+ transgenic mice. In order to further confirm the presence of an expressible human sialidase cDNA, sialidase activity assays were carried out on homogenized liver and kidney samples. The results pointed to the presence of human sialidase, expressed along with the mouse sialidase. The kidneys samples of mice which are human sialidase positive, showed an increase in sialidase activity in comparison with samples from human sialidase negative samples, demonstrating a presence of an active human sialidase enzyme along with the mouse sialidase enzyme. One positive liver sample for human sialidase showed a slight increase in sialidase activity whereas the other positive sample showed minimum activity. The human sialidase negative liver samples showed decreased enzyme activity.

While it is still unclear how the mouse and human Sialidase enzymes cooperate, this putative cooperation could take the form of tissue-specific hetro dimerization, which would reflect the enzyme activity observed. Or as it was previously proven that the mouse lysosomal sialidase is capable of forming a catabolically active complex with the human cathepsin A and β-galactosidase proteins, (Igdoura et al., 1998) and given the high homology between human and mouse sialidase the human sialidase was expected to be able to form an active complex with the mouse cathepsin A and β-galactosidase proteins. Therefore, the human sialidase competes actively with the mouse sialidase and takes over its pathway.

Transgenic animals were weighed regularly and this was done along with the behavioral tests conducted. The human sialidase positive groups did not show significant changes in body weights in comparison with the human sialidase negative groups (Figure 6.1). The preliminary observations have also shown that the human sialidase positive individuals with two copies were very small in size comparing to their littermates at birth and throughout their short life span.

In the neurological examination of hexa-/- mice expressing human sialidase, some behavior abnormalities were noticed. However, all animals were able to remain in the flipped position for 60 seconds or more indicating that their muscles limbs were strong; which would not be the case if the animals were experiencing the early onset Tay-Sachs disease related symptoms.

The behavioral tests indicated that the presence of one copy of human sialidase enzyme on a hexa-/- background does not change the overall phenotype outcome. In the beginning it was unclear why only two symptomatic KI+ mice were found in the entire colony. Then, we thought maybe in order for these symptoms to show the mouse has to have two copies of the human sialidase (KI), so new matting groups were set up. The first matting groups were for the double transgenic mice (KI+/hexa-/-) with the double transgenic mice (KI+/hexa-/-), and the second groups were the (KI+/hexa+/-) with (KI+/hexa+/-); those were the control groups. The reason for setting up the control group was to make sure that these symptoms were KI+ and hexa-/- related symptoms not just KI+ related symptoms.

Sure enough an increase in the percentage of the symptomatic mice with these new doubles transgenic matting groups was possible; however, the control matting groups produced some symptomatic mice too. This finding confirmed that the double copied transgenic expressing human sialidase is the cause of the abnormal runty phenotype. Furthermore, this would suggest that human sialidase maybe functioning as a dominant negative. The late onset Tay-Sachs disease in mice is characterized by spasticity of the hind limb, tremors, weight loss upon onset, strange posture with curvature of the back (Kyphosis), possible visual impairment, and, after the disease progresses, weakness of the muscles, clasping of the limbs, and myoclonic twitches of the head. Specifically it was revealed that a tail elevation test, showing extended spastic hind limbs, is a dependable, early indicator of the late onset Tay-Sachs disease (Miklyaeva et al., 2004).

And since there is no infantile form of the Tay-Sachs disease yet discovered in mice, the phenotype of the late- Onset form was used along with the symptoms in human patients as a reference to our findings. Since the transgenic mice were produced in our lab prior to this project, in this study the work was divided into three stages. The first one was to identify the transgenic mice (the KI+ mice) and then to make sure that the human sialidase was expressed in molecular levels; in addition, these human sialidase positive mice were to be monitored for any abnormalities in their behavior due to the insertion.

The results showed that the human sialidase cDNA was successfully inserted into the mouse genome and was clearly expressed in molecular levels utilizing PCR, RT-PCR, western blotting, and sialidase enzyme assay as shown in the result section. Histological sections were obtained from different animals of different tissues of different genotypes. Histological sections from human sialidase positive mice and wild type mice stained with Bielschawsky and H&E and after carful observation of the histology, showed no histological difference. Moreover no behavior abnormality was observed. In this stage, the work was done on transgenic mice expressing human sialidase with one copy of the transgene. The second stage of the study was to characterize the double transgenic mouse model in which the human sialidase was bred on a hexa-/- background. The generation of the double transgenic mice required the generation of the hexa hetro mice (hexa+/-) first. After that mating the human sialidase positive mice with hexa+/- mice then identifying hexa+/- / KI+ mice via PCR those individuals were mated with hexa+/- KI- to generate the double transgenic with one copy of the human sialidase gene (hexa-/-/KI+ mice). The third stage of the study consisted of monitoring and characterizing the double transgenic mouse model hexa-/-/KI+. This model was hypothesized to show a phenotype similar to the juvenile form of the Tay-Sachs disease. At first, the double transgenic mice appeared to be asymptomatic to the naked eye with no abnormalities identified. The double transgenic performed normally in all of the behavioral tests. For example, the double transgenic mice showed a hang time of 60 second and up in the wire-hang test; there was no abnormal posture while elevated by their tails.

In addition, ganglioside analysis of the brain samples from KI+/hexa-/- and KI-/hexa-/- mice revealed the expected accumulation of GM2 ganglioside with little to no difference in their band intensity. These results indicate that the expression of human sialidase under the mouse promoter on a hexa-/- background has little effect on the GM2 accumulation. Overall the double transgenic mice demonstrated typical behavior and there was no sign of any Tay-Sachs related symptoms, up until the first appearance of the runty symptomatic two females around three months of age. The two symptomatic females described in the results section were small in comparison to their littermates.

They were visually impaired with obvious cataract in one eye. They appeared to have spinal curvature, and they depicted weird behavior presented in vigorous combing of their faces while standing on their hind limbs and tails (Figure3.14 a). First of all the small percentage of the symptomatic mice was concerning since out of all double transgenic only two were obtained. It was presumed that if the symptoms were Tay-Sachs disease related symptoms then all double transgenic mice should have them. One explanation might be that the human sialidase transgenic gene penetrance was low with the expression of one transgene copy not enough to impact the phenotype in all double transgenic mice. The second possible explanation is that the symptoms that the two mice have shown were caused by random mutation which is not disease related.

Base on the above we setup new matting pairs to generate double transgenic mice with two copies of the human sialidase gene (KI+). This was simply done by matting hexa-/-/KI+ with hexa-/-/ KI+ and another control matting in which the parents where KI+ /hexa+/+ (wild type). The investigation was further focused on the human sialidase transgene as an explanation for the observed symptoms because it was well established that the hexa-/- mice are asymptomatic before 12 months (Phaneufl et al., 1996; Miklyaeva et al., 2004) and because after confirming their genotyping by PCR, the two symptomatic mice were found to be KI+, but only one was hexa-/- where the other was hexa+/-.

This finding led me to believe that the symptoms were KI related, and not hexa related. Sure enough, the preliminary findings indicate that double copies of the KI transgene would produce more symptomatic mice. At this point, I ruled out any possibility that the symptoms were hexa related symptoms, i.e., Tay-Sachs disease related symptoms. Surprisingly, the new symptomatic mice were smaller in size and experienced sudden death around three months of age. Such growth impairment was also observed in PPCA-/- and neu1-/- mice, but the combination of growth impairment and sudden death phenomenon was observed only in neu1-/- mice (De Geest et al; 2002).

In summary, the human sialidase gene was successfully inserted in the mouse genome under the mouse promoter. In this study, its expression was confirmed at the molecular levels by means of PCR, RT-PCR, sialidase enzyme assay, and western blotting. In order to generate the Tay-Sachs mouse model carrying human Sialidase, the human sialidase gene was bred into hexa-/- background. Moreover, the double transgenic mice with one copy of the human sialidase mice were asymptomatic except for two atypical females; however, in the histological levels there was no difference between double transgenic and single transgenic mice. In addition, the double transgenic mice with two copies of the human sialidase cDNA depicted profound symptoms; such as very small size, hind limbs spasticity, and while suspended by their tails they depicted an abnormal clasping behavior which was associated with neurologically impaired mutant mice (Yamashita et al; 2005).

Moreover, these mice have peculiar posture with Kyphosis, visual impairment, and they depicted weird behavior presented in vigorous combing of their faces while standing on their hind limbs and tails (Figure 3.14 b) .Overall, this work set the stage for further analysis which may yield a mouse model useful as a therapeutic tool.

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