BRAIN LIPID PROFILING OF TRIPLY MOUSE MODEL WITH THE DEFICIENCIES OF SIALIDASE NEU1, NEU4 AND β-HEXOSAMINIDASE A ENZYMES

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ABSTRACT

BRAIN LIPID PROFILING OF TRIPLY MOUSE MODEL WITH THE DEFICIENCIES OF SIALIDASE NEU1, NEU4 AND β-HEXOSAMINIDASE A ENZYMES

Tay-Sachs disease is a severe lysosomal storage disorder caused by mutations in the HEXA gene coding for α subunit of lysosomal β-hexosaminidase A enzyme, which converts G_{M2} to G_{M3} ganglioside. $HexA^{-/-}$ mice, depleted of β-hexosaminidase A enzyme, remain asymptomatic to 1 year of age, so it was thought there is a difference between human and mice lipid degradation. Previously identified a novel ganglioside metabolizing sialidase, Neu4, is abundantly expressed in mouse brain neurons. It was demonstrated that mice with targeted disruption of both HexA and Neu4 genes ($HexA^{-/-}$ $Neu4^{-/-}$) show accumulating G_{M2} ganglioside and epileptic seizures with 40% penetrance. Since all mice didn't show symptoms, it was suggested that Neu4 is not the only sialidase contributing to the metabolic bypass in $HexA^{-/-}$ mice (Seyrantepe et al. 2010). Therefore, we studied the role of another sialidase Neu 1 in glycolipid degradation. We profiled brain glycolipid content of triple deficient mouse model with the deficiency of β-Hexosaminidase A (0% activity), sialidase Neu4 (0% activity) and sialidase Neu 1 (10% activity) (NeoIn) by thin layer chromatography. Analysis of both double ($HexA^{-/-}NeoIn^{-/-}$) and triple ($HexA^{-/-}Neu4^{-/-}NeoIn^{-/-}$) mice models showed that sialidase Neu 1 deficency causes not significant difference in brain lipid profile and though also other sialidase/sialidases might have role in glycolipid degradation pathway in mice.

ÖZET

SİALİDAZ NEU1 VE NEU4 İLE β-HEKZOSAMİNİDAZ A ENZİM EKSİKLİĞİ OLAN FARE MODELİNDE BEYİN LİPİD PROFİLİNİN ÇIKARILMASI

Tay-Sachs hastalığı G_{M2} gangliosidinden sialik asidi uzaklaştırarak G_{M3}'e dönüştüren β-Hekzosaminidaz A enziminin α alt ünitesini kodlayan HEXA genindeki mutasyonların sebep olduğu ölümcül bir lizozomal depo hastalığıdır. β-Hekzosaminidaz A eksikliği olan Tay-Sachs hastalığının fare modeli ($HexA^{-/-}$) yaratılmış, bu farelerde kısıtlı bir G_{M2} birikimi olmasına rağmen hastalık bulguları gözlenmemiştir. Bu durumda insan ile fare arasında lipidlerin yıkım yolaklarının farklı olduğu, $HexA^{-/-}$ farelerde bir ya da daha fazla sialidaz enziminin yer aldığı bir bypass mekanizması ile G_{M2}'nin yıkılarak birikmediği ileri sürülmüştür (Sango et al. 1995). Bu hipotezi test etmek amacı ile yaratılan β-Hekzosaminidaz A ve sialidaz Neu 4 eksikliği olan farelerin ($HexA^{-/-}$ $Neu4^{-/-}$) %40'ının beyinlerinde belirgin miktarda artan G_{M2} ile bağlantılı epileptik krizler gözlenmiştir (Seyrantepe et al. 2010). Krizler tüm farelerde gözlenmediği için başka sialidaz ve/veya sialidazların bu yolakta yer alabileceği düşünülmüştür. Çalışmamızda bu hipotezi test etmek için üç enzim eksikliği olan (β-Hekzosaminidaz A- (%0 aktif), sialidaz Neu4- (%0) aktif ve sialidaz Neu1- (%10 aktif) (NeoIn) farelerin beyin lipid profilleri karşılaştırılmıştır. İnce tabaka kromotografisi ile yapılan analizlerde hem ikili ($HexA^{-/-}NeoIn^{-/-}$) hem de üçlü ($HexA^{-/-}Neu4^{-/-}NeoIn^{-/-}$) enzim eksikliği olan farelerde, sialidaz Neu 1 eksikliği, beyin gangliosid içeriklerinde çok hafif bir farklılığa yol açmıştır. Bu da sialidaz Neu 1'in ve ayrıca diğer sialidaz/sialidazların fare gangliosid yıkım yolağında rol alabileceğini düşündürmektedir.

To my wonderful daddy…

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CHAPTER 1

INTRODUCTION

1.1. Glycolipids

Glycolipids are glycosylated form of lipids that derive from a hydrophobic moiety such as ceramide by the addition of one or more monosaccharide residues (Chester 1997). Glycolipids are found in all animal cell plasma membranes, and constitute 5% of the lipid molecules in the outer leaflet of the membrane. Glycolipids in the plasma membrane bind extracellular matrix, serve as receptors for external molecules, protect the membrane integrity from environmental affects, such as low pH and degradative enzymes, and in neurons glycolipids may also play a role in electrical insulation (Hakomori 1986; Quinn and Cherry 1992). When needed, glycolipids are synthesized in the lumen of Golgi apparatus by the sequential addition of sugar groups to the newly synthesized lipid molecules. Later, they are degraded in lysosomes by enzymes that remove sugar residues (James 2007).

1.2. Sphingolipids

Sphingolipids are complex glycolipids having the core structure of sphingosine which is an 18-carbon amino alcohol with an unsaturated hycdrocarbon chain (Carter et al. 1947). Sphingolipids are divided into three groups; I) ceramide, II) sphingomylein and III) glycosphingolipid (Klenk and Fauenstein 1951; Yamakama and Suzuki 1951). (Figure 1.1) These three differ in the groups that attached to sphingosine. Sphingolipids are found in the external layer of the membrane (Feizi 1985) and build cell surface by anchoring carbohydrate groups to plasma membrane (Kolter and Sandhoff 1999).

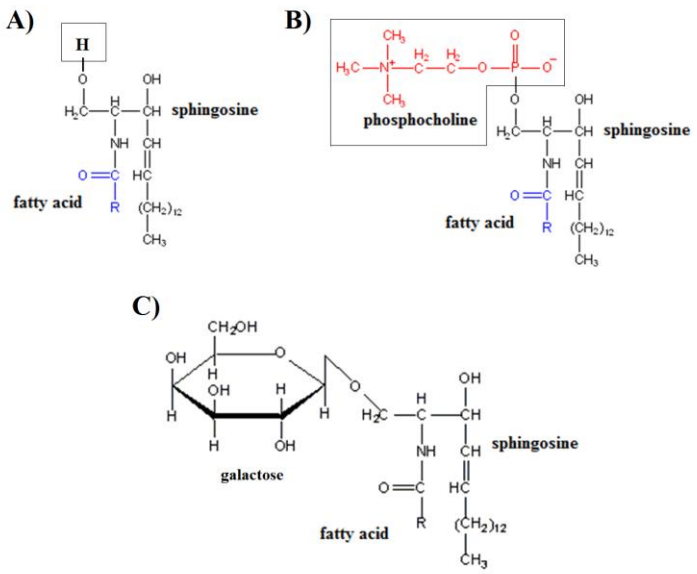

Figure 1.1. Structures of (A) ceramide, (B) sphingomyelin (have phosphocholine group) and (C) glycosphingolipid (have sugar group)

1.2.1. Glycosphingolipids

Glycosphingolipids (GSLs) are subgroup of glycolipids that has ceramide as the hydrophobic lipid moiety (Stults et al. 1989). They derived from glucosylceramide (GlcCer) or galactosylceramide (GalCer) (Coetzee et al. 1998). Amoung all tissues, the highest concentration of GSLs are found in the central nervous system tissues mostly contributing to the formation and stabilation of myelin protein which covers neurons (Yu and Saito 1989; Coetzee et al. 1998).

GSLs are mostly found on the outer leaflet of the plasma membrane and have roles in cell to cell interaction (Roseman 1971; Hakomori 1970), malignant transformation of cell (Hakomori 1973; Fishman 1975), cell differentiation (Varki 1993), signalling pathway (Hakomori 1990; Nagai and Tsuji 1994), cell adhession (Hidari et al. 1996), and cell death (Kohyama-Koganeya et al. 2004; Segui et al. 2006). GSLs are also important for intracellular protein trafficking, for instance protein transport from Golgi to melanosomes (intracellular melanin pigment containing membraneous structure) (Sprong et al. 2001). Since they have varies roles in different cell types, the pattern of glycosphingolipids varies especially during cell differentiation (Yu 1994). GSLs have a high melting temperature because of high saturation of hydrocarbon chains (Hoekstra et al. 2003). This property let them to self-associate in the

fluid plasma membrane (Ramstedt and Slotte 2006) to function in counted cellular processes (Simons and Ikonen 1997; Hancock 2006).

GSLs are highly heterogeneous and they show a big diversity depending on their carbohydrate structures and lipid moieties (Lingwood 2000). There are two families of glycosphingolipids that derived from glycosylceramide; the neutral glycosphingolipids and the acidic glycosphingolipids.

1.2.1.1. Neutral Glycosphingolipids

Neutral glycosphingolipids are glycosphingolipids without sialic acid or sulfate groups on their carbohydrate chains. Glucosylceramide, galactosylceramide, lactosylceramide, trihexosylceramide, phosphatidyletanolamine, phosphatidylcholine, plasmalogens, cholesterol, ceramide, sphingomyelin and cerebroside (Denny et al. 2007) are all in this group.

1.2.1.2. Acidic Glycosphingolipids

Acidic glycosphingolipids include gangliosides (the ones with sialic acid group attached to carbohydrate chain), cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidic acid and sulfatides (the ones with sulfate group attached to carbohydrate chain) (Lloyd and Furukawa 1998; Denny et al. 2007).

1.2.1.2.1. Gangliosides

Gangliosides are known as being complex glycosphingolipid which characteristically phosphorous free and contain sialic acid (*N*-acetylneuraminic acid) (NANA or SA or Neu5Ac or NeuAc) (Varki and Schauer 2001) (Figure 1.2), on their carbohydrate chain that consists of glucose, galactose and N-acetylgalactosamine residues (Klenk 1942) (Figure1.3). Gangliosides were firstly purified (Klenk 1935) and investigated from the brain of a patient with the Niemann-Pick disease (Klenk 1939) which is caused by the deficiency of sphingomyelinase enzyme resulting in the accumulation of sphingomyelin, cholesterol, and other kinds of lipids within the cells

and tissues of affected individuals (Schneider and Kennedy 1967). However, whole ganglioside structure became clear almost 3 decades later (Kuhn and Wiegandt 1963), after elucidation of sphingosine (Carter 1947) and sialic acid structures (Gottschalk 1955). Gangliosides are found in all vertebrate cells (Yu and Saito 1989; Hakomori 2001) as well as invertebrate cells (Sugita 1979; Smirnova and Kochetkov 1980; Zvezdina et al. 1989; Huwiler et al. 2000; Saito et al. 2001). Although they are abundant in brain (Leeden and Yu 1982; Svennerholm 1984; van Echten and Sandhoff 1989), they also exist in red blood cell stroma (Yamakawa 1951; Klenk and Wolter 1952), spleen (Klenk and Rennkamp 1942; Klenk 1959), human liver (Nilsson and Svennerholm 1982), human kidney (Rauvala 1976), porcine testis (Suzuki et al. 1975), rat testis (Keenan et al. 1972) and bovine testis (Bushway et al. 1977). They are not determined in some plants and microorganisms (Sperling and Heinz 2003; Warnecke and Heinz 2003; Lynch and Dunn 2004).

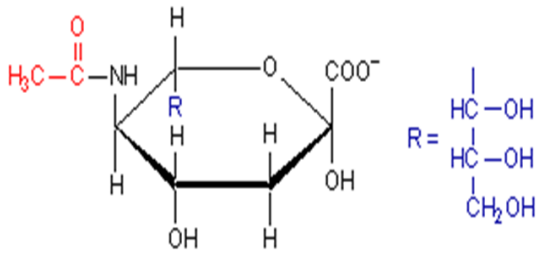

Figure 1.2. Structure of sialic acid

With a big saccharidic head group and double hydrophobic tails, gangliosides are categorized as complex lipids. The core molecule of gangliosides is ceramide which is found in all sphingolipids (Karlsson 1970). Sialic acids can be attached to core structure of glycosphingolipid in several positions which causes the heterogeneity amoung gangliosides. Additional diversity of the gangliosides arises from their hydrophobic components, fatty acids, connections, sequence and long chain bases as well as their carbohydrate moieties (Sonnino et al. 2006). Also the ceramide part of the lipid contributes this diversity by variations of alkyl chain length, the hydroxylation degree and desaturation (Lingwood 2000). 188 different ganglisosides have been identified in vertebrates so far (Yu et al. 2007).

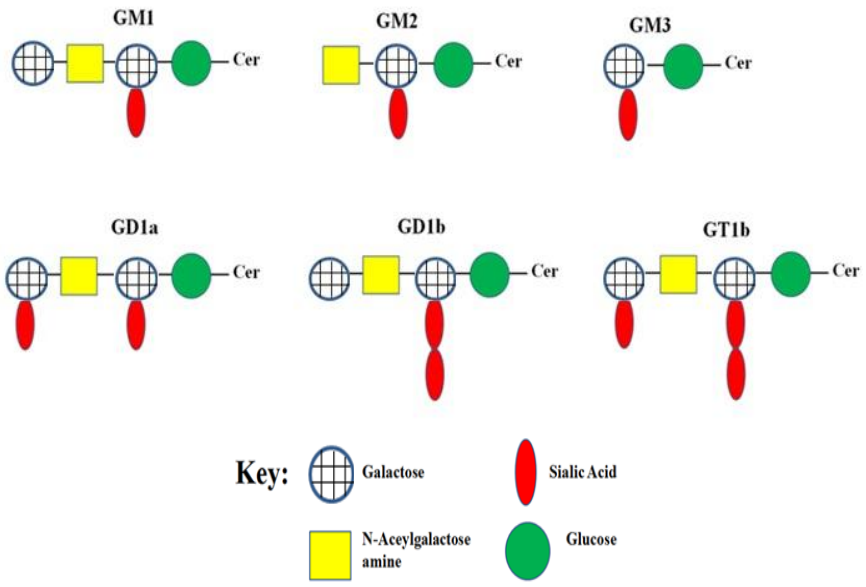

Figure 1.3. Schematic presentations of major gangliosides
(Adapted from: Woods and Jackson 2006)

1.2.1.2.1.1. Metabolism of Gangliosides

Synthesis of all glycosphingolipids including gangliosides is revealed at intracellular membranes (Kolter and Sandhoff 1999; Riboni et al. 1997). The synthesis is started by enzymes that are active at the cytosolic face of the endoplasmic reticulum membrane (Mandon et al. 1992) with the formation of ceramide (Kolter and Sandhoff 1999; Merrill 2002) and further modifications take place in the Golgi apparatus as a stepwise addition of extra carbohydrate groups (Maccioni 2007). Newly synthesized gangliosides are then transported to plasma membrane by the help of exocytotic membrane flow (van Meer and Lisman 2002). Beside this flow a possible transport of vesicles that contain gangliosides from the Golgi apparatus to plasma membrane and from plasma membrane to the lysosomes was also speculated (Forman and Ledeen 1972; Landa et al. 1981; Miller-Prodraza and Fishman 1982).

According to their sialic acids groups gangliosides are classified into asialo-, a-, b-, and c- series which have no, one, two and three sialic acid residue(s) linked to their inner galactose, respectively (Figure 1.4). Also there are α-series gangliosides which have sialic acid(s) in their inner N-acetylgalactosamine residue instead of galactose residue (Nakamura et al. 1988). The nomenclature of gangliosides is done according to

Svennerholm. In this perspective ganglioside is shown as Gxyz. G indicates ganglioside, x indicates the number of sialic acid residues, y indicates the migration order in a certain chromatographic system and z indicates the position of sialic acid(s) linkage (a, b or c) (Svennerholm 1963, 1994).

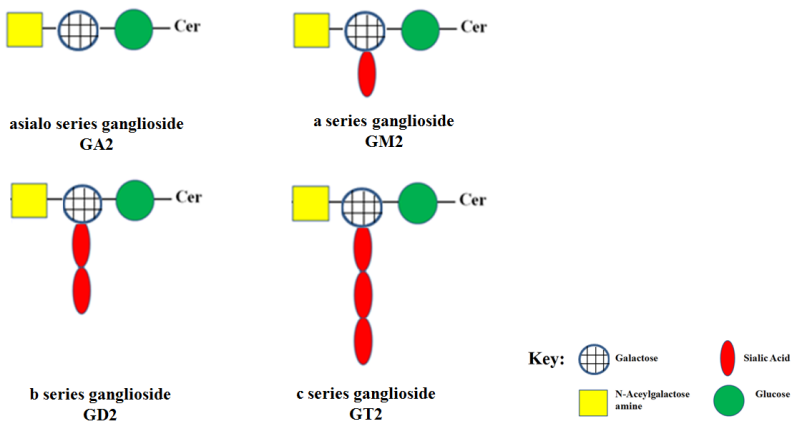

Figure 1.4. Example of asialo, a- , b- and c- series gangliosides

Ganglioside synthesis in lumen of the Golgi apparatus is cathalized by numerous glycosytransferases and galactosyltransferases that are specific to substrates. Because addition of monosaccharides is revealed in the lumen of the Golgi apparatus, these carbohydrate groups outstretch into the extracellular space where is topologically equivalent to the Golgi lumen (Kaufman et al. 1968; Roseman 1970; Keenan et al. 1974; Lloyd and Furukawa 1998; Kolter and Sandhoff 1999; Maccioni et al. 1999). Most gangliosides are synthesized from lactosylceramide (LacCer) while ganglioside G_{M4} is derived from galactosylceramide (GalCer).

First reaction is revealed by enzyme lactosylceramide sialyltransferase (ST-I or G_{M3} synthase) with the addition of a sialic acid to LacCer. This reaction forms the simplest ganglioside, G_{M3} (monoasiloganglioside). G_{D3} (diasiloganglioside) and G_{T3} (triasiloganglioside) are synthesized from G_{M3} and G_{D3} by stepwise addition of sialic acid with the enzymes of G_{M3} sialyltransferase (ST-II or G_{D3} synthase) and G_{D3} sialyltransferase (STIII or G_{T3} synthase), respectively. These synthesized gangliosides, G_{M3}, G_{D3} and G_{T3}, act as precursors and form complex gangliosides by addition of sugar groups with different galactosyltransferase (GalT), sialyltransferase (ST) and N-acetylgalactosamineyltransferase (GalNAcT) enzymes (Yu et al. 2011) (Figure 1.5).

There is a balance between anabolism and catabolism of gangliosides as seen in all biomolecules' metabolism in the cell. Gangliosides are transported to acidic compartments, lysosomes, of cells by endocytosis (Kolter and Sandhoff 1996) and degraded by a complex mechanism that involves enzymes, activator proteins (sphingolipids activator proteins –SAPs) and negatively charged lipids (Kolter and Sandhoff 1999). Dolicol phosphate found in late endosome and lysosomes membrane (Cojnacki and Dallner 1988; Kobayashi et al. 1998) is an example of negatively charged lipid which was shown to enhance the degradation of glycosylceramide (Wilkening et al. 1998). Until now, five SAPs have been determined which are encoded from two different genes. One gene produces G_{M2} activator protein (G_{M2}-AP) (Fürst and Sandhoff 1992; Swallow et al. 1993), and second gene produces prosaposin protein which is further processed to four homologous proteins (SAP-A, -B, -C, -D) by proteolytic cleavage (Nakano et al. 1989; Fürst and Sandhoff 1992; Sandhoff and Kolter 1996; Kolter and Sandhoff 1999). The anabolism and catabolism reactions of gangliosides differ in one aspect. In anabolism of gangliosides, both substrates of glycolipids and enzymes are membrane-bound, but in catabolism of gangliosides, enzymes are soluble in lysosome. This shows the requirement of activator proteins that have an important role in catabolism reactions but not in anabolism reactions (Kolter and Sandhoff 1999).

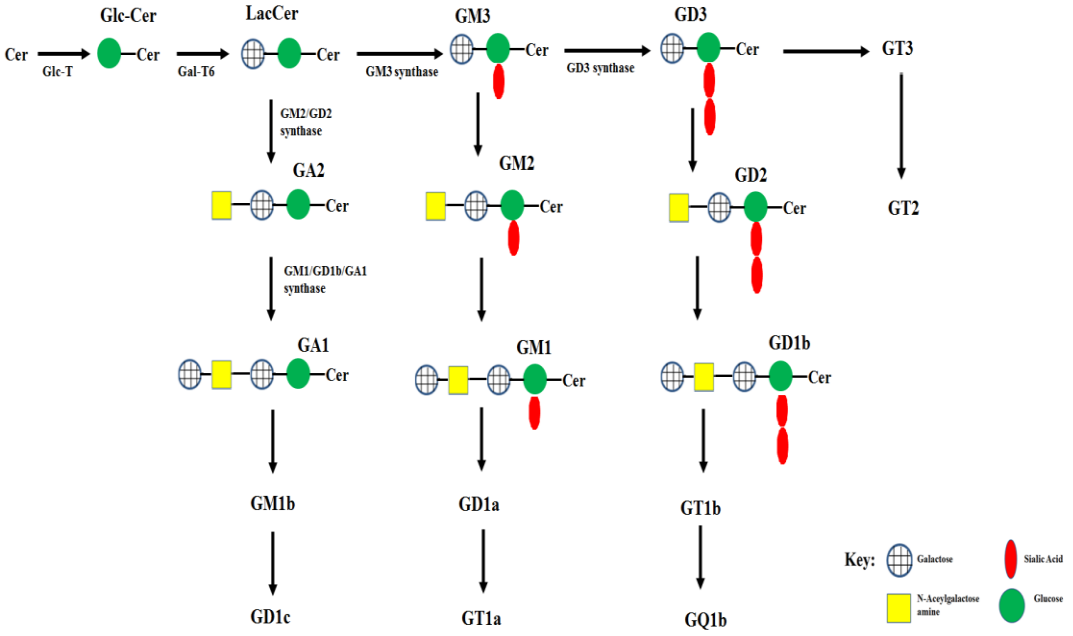

Figure 1.5. Synthesis pathways of gangliosides
(Adapted from: Yu et al. 2011)

1.2.1.2.1.2. Functions of Gangliosides

In cells, gangliosides are mostly found on the outer leaflets of the plasma membranes (Steck and Dawson 1974; Ledeen 1978) (Figure 1.6). Their concentrations on neuronal membranes are ten times higher than concentrations in non-neuronal cells (10-20 % of the total lipid on neuronal plasma membrane consists of gangliosides) (Ledeen 1985).

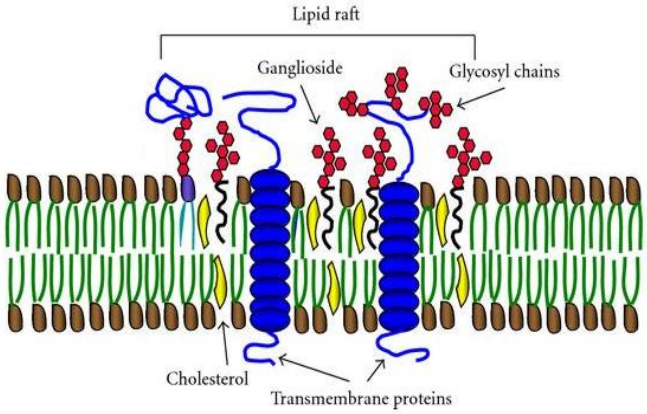

Figure 1.6. Schematic representation of cell membrane site which is enriched in gangliosides and cholesterol (Source: Malchiodi-Albedi et al. 2011)

Gangliosides are mostly found in dynamic membrane structures in plasma membrane, called lipid rafts (Simons and Ikonen 1997; Simons and Toomre 2000; Kasahara et al. 2000; van der Goot and Harder 2001; Vyas et al. 2001; Prinetti et al. 2001; Pike 2006; Fujita et al. 2007; Hanzal-Bayer and Hancock 2007) and caveolae (Anderson 1998) which are characterized by high concentration of glycosphingolipids and cholosterols (Hakomori et al. 1998). Gangliosides can affect the physico-chemical properties of the plasma membrane such as fluidity (Bertoli et al. 1981; Uchida et al. 1981), or thermotropic features (Masserini and Freire 1986) and the activity of some plasma membrane bound-enzymes such as kinases (Partington and Daly 1979; Davis and Daly 1980; Leon et al. 1981; Goldenrig et al. 1985; Kreutter et al. 1987; Chan 1988, 1989; Yates et al. 1989; Bassi et al. 1991). They also involve in cell signaling (Roisen et al. 1981; Rybak et al. 1983; Tsuji et al. 1983; Bremer et al. 1984), cell to cell interactions (Hakomori and Igarashi 1995), and binding of viruses, toxins and hormones to cell (Wolley and Gommi 1965; Haywood 1974; Van Heyningen 1974; Mullin et al. 1976; Meldolesi et al. 1976). Because they are enriched in neurons it was also suggested

they have a role in neurotransmitter release (Zitman et al. 2010) which involves the function of voltage gated Ca^{2+} channels (Kaja et al. 2007). Colocalization of Ca^{2+} channels with gangliosides in lipid raft (Chamberlain et al. 2001; Lang et al. 2001; Salaun et al. 2004; Taverna et al. 2004; Davies et al. 2006) has been reported. In this aspect it was shown that plasma membrane localized GM1 also influences the Ca^{2+} homeostasis by different mechanism(s) (Fang et al. 2002; Wu et al. 2004).

Beside plasma membrane, it has been reported that rat liver hepatocytes' nuclear membrane has 10 % of ganglioside concentration to plasma membrane; dominantly having G_{M1} and G_{M3} (Matyas and Morre 1987) while others reported that G_{M1} and G_{D1a} are the major gangliosides found in nuclear envelope (Ledeen and Wu 2006). It also was reported that G_{M3}, G_{D3}, and G_{T1b} gangliosides exist in the nuclei of bovine mammary gland which inhibit both the activity of protein kinase C (histone phosphorylation) and the phosphorylation of nuclear substrate (Katoh et al. 1993). In addition to plasma membrane, the association of G_{M1} with nuclear Na^{+}/Ca^{2+} exchanger has been shown that nuclear G_{M1} possess an important role in Ca^{2+} homeostasis (Ledeen and Wu 2006; Ledeen and Wu 2008).

Although gangliosides are abundant in neurons, they are found in different patterns and levels in different nervous systems (Schwarz and Futerman 1996; Ogawagoto and Abe 1998). For example G_{M1} is expressed highly in ventral comparing to dorsal root nerves myelin (Ogawa-Goto et al. 1992), G_{Q1b} is high in oculomotor nerve (Chiba et al. 1997), G_{Q1b}, G_{T1a} and G_{D1b} is found in neuromuscular junction of human extra ocular muscles but not found in axial and limb muscles (Liu et al. 2009). Some gangliosides are shown in dendritic and somatic sites of cerebellar Purkinje cells and retina neurons but they are not found in axons and presynaptic terminals of nerves (Schwarz and Futerman 1996). These also suggest gangliosides to have region specific functions (Svennerholm 1994).

Gangliosides are involved in pathology of many disorders such as lysosomal storage diseases. These disorders occur via distrupted gangliosides synthesis or break down and autoimmunity against gangliosides (Plomp and Willison 2009). When gangliosides are recognized as foreign by immune system these reactions cause neurological symptoms (Willison and Yuki 2002). Guillain-Barre syndrome is an autoimmune disease that results from the activation of immune system to cell surface

gangliosides (Kaida et al. 2009). In addition, influenza A viruses recognize sialic acid residues of ganglioside to enter the host cell and cause disease (Suzuki 2005).

Some other studies show that there is also disrupted ganglioside metabolism in Huntington's disease (Desplats et al. 2007), multiple sclerosis (Marconi et al. 2006) and Alzheimer's disease (Ariga et al. 2008). In Huntington's disease, studies with transgenic mice models revealed that there is a reduction in G_{M1} ganglioside, decrease in cerebrosides and sulfatides but no change in cholesterol level (Desplats et al. 2007). These reduced lipids are enriched in myelin content of neurons (Sandhoff et al. 1971; Seyfried and Yu 1980; Kaye et al. 1992) and these differences was thought to lead detected abnormalities in myelin content of mice model of Huntignton's disease. Also reduced gene expression of G_{M2}/G_{D2} synthase and $G_{M1b}/G_{D1a}/G_{T1b}$ synthase and lower levels of their products were observed. All these differences seen in gangliosides may cause apoptosis and distruption of Ca^{2+} signaling, which are hallmarks of Huntington's disease (Desplats et al. 2007). Beside Huntignton's disease, in Alzheimer's disease, specific G_{M1} ganglioside bound form of amyloid-β (Aβ) peptide, the known cause of disease pathology, were identified. G_{M1} ganglioside bound Aβ peptides were thought to be responsible for the early changes in brain of patients (Yanagisawa et al. 1997). This involment of gangliosides in accumulation and aggregations in Aβ peptide was also supported by other studies (Bernardo et al. 2009; Matsuzaki et al. 2010).

Noteworthy evidences of gangliosides having role in cancer are also reported. It was shown that ganglioside G_{D1a} induces angiogenesis and promotes cell survival, growth and migration on respond to growth factor (Mukherjee et al. 2008) whereas ganglioside G_{M3} supresses angiogenesis (Kanda et al. 1994). In neuroblastoma cells it has been reported that there are gangliosides with highly hydroxylated ceramide (Ladisch et al. 1989). In another study it was suggested that concentration of ganglioside G_{M3} and G_{D3} can enhance integrin-dependent adhesion which may promote tumor cell growth (Zheng et al. 1993; Cheresh et al. 1987). Some other studies revealed that G_{M3} inhibits endothelial growth factor receptor kinase whereas N-acetyl G_{M3} enhances activity of this kinase (Hanai et al. 1988) and serine kinase which together induce cell proliferation (Zhou et al. 1994). It was also reported that tumor cells have greater gangliosides than normal cells (Sjoberg et al. 1995). Therefore, high concentration of gangliosides in blood is associated to inhibition of patients' immune responses that cause tumor growth (Ladisch et al. 1994).

1.2.1.2.1.3. Sialidases and Degradation of Gangliosides

Sialidases also called neurominidases are a family of glycohydrolytic enzymes that function in catabolism of sialoglycoconjugates by removing sialic acid residues (Saito and Yu 1995). By their activity against sialiated biomolecules, sialidases are important for many biological processes such as cell proliferation/ differentiation, cell adhesion, clearance of plasma proteins and modification of receptors beside catabolism of gangliosides and glycoproteins (Schauer et al. 1995; Saito et al. 1995; Reuter and Gabius 1996).

Mammalian sialidases are classified into different groups according to their subcellular localization; cytosolic (Miyagi and Tsuiki 1985), lysosomal (Miyagi and Tsuiki 1984; Seyrantepe et al. 2004), lysosomal membrane associated, plasma membrane associated (Miyagi et al. 1990) and mitochondrial (Yamaguchi et al. 2005). Sialidases also differ in substrate specificity and immunological properties (Miyagi and Tsuiki 1985; Miyagi et al 1990). Mammalian sialidases are encoded by four different genes called Neu 1, Neu 2, Neu 3 and Neu 4. The quantitative real time PCR analysis shows that Neu 1 has highest expression level, 10-20 times higher that those of Neu 3 and Neu 4 whereas Neu 2 has really low expression level, only four-to ten-thousandth of the Neu 1 (Yamaguchi et al. 2005).

Sialidase Neu 1 is the lysosomal sialidase (Miyagi and Tsuiki 1984) which is encoded by the gene located in short (p) arm of chromosome 6 at position 21.3 (Oohira et al. 1985). Neu 1 makes complex with β-galactosidese (β-GAL) and lysosomal carboxypeptidase Cathepsin A (CathA). CathA protect Neu 1 and β-GAL against proteolytic degradation in lysosome and activate Neu 1 (Pshezhetsky and Ashmarina 2001). It was shown with immunoelectron microscopy analysis that sialidase Neu 1 is present on the plasma membrane and in intracellular vesicles in addition to lysosome membrane and lysosomal lumen (Vinogradova et al. 1998). Deficiency of sialidase Neu 1 results in sialidosis (mucolipidosis I) which is autosomal recessive lysosomal storage disorder (d'Azzo et al. 2001; Thomas 2001). Sialidosis patients are divided into two subgroup, type I is a relatively mild, late onset form whereas type II is early onset, severe and neuropathic form (Thomas 2001). In the patients due to deficiency of sialidase enzyme, accumulation of undegraded sialylated oligosaccarides and glycoproteins in lysosomes and excretion in urine is seen (Thomas 2001). Since some

patients with accumulation of G_{M3} and G_{D3} gangliosides in systemic organs (Ulrich-Bott et al. 1987) and in the brain (Yoshino et al. 1990) was reported, it was suggested that sialidase Neu 1 is also involved in the degradation pathway of gangliosides beside its known substrates. Clinically similar disease called galactosialidosis resulting from the secondary deficiency of Neu 1 that is caused by the genetic defects in CathA gene (d'Azzo et al. 2001).

Sialidase Neu 2 is mostly found in skeletal muscle. Neu 2 is a cytosolic protein and active against glycopeptides and gangliosides (Miyagi and Tsuiki 1985; Monti et al. 1991; Tringali et al. 2004). Although its exact function is not known, it was reported in several studies that Neu 2 has selective activity against G_{M3} ganglioside (Sato and Miyagi 1996; Akita et al. 1997; Fanzani et al. 2003).

Sialidase Neu 3 is integral membrane protein which is found in caveolae microdomains of plasma membrane (Wada et al. 1999; Monti et al. 2000; Wang et al. 2002). Highest expression is seen in adrenal gland, skeletal muscles, heart, testis and thymus (Wada et al. 1999; Monti et al. 2000). Neu 3 is mostly active against G_{M1} and G_{D1a} gangliosides (Schneider-Jakob and Cantz 1991). In addition, sialidase Neu 3 desialates GM2 and G_{M3} gangliosides to asilo derivatives *in vitro* (Igdoura et al. 1999; Li et al. 2001). It is thought that Neu 3 involves in the modulation of gangliosides' oligosaccharide chains on the cell surface. These modulations of gangliosides are important for transformation, cell contact formation (Kopitz et al. 1996; Kopitz et al. 1998), differentiation (Wu and Ledeen 1991), insulin signaling (Sasaki et al. 2003), carcinogenesis and apoptosis (Kakugawa et al. 2002).

Sialidase Neu 4 is recently identified sialidase located in 2^{nd} chromosome, having broad substrate specificity against glycoproteins, oligosaccharides and sialylated glycolipids. Also elimination of undigested substrates of Neu 1 and restoration of normal morphological phenotype in Neu 1 deficient sialidosis fibroblasts after the overexpression of Neu 4 revealed that Neu 4 is active against a majority of endogenous substrates of sialidase Neu 1 (Seyrantepe et al. 2004). This enzyme was shown to be located both in lysosomal lumen by the mannose 6-phospate receptor (Seyrantepe et al. 2004) and inner and outer membranes of mitochondria (Yamaguchi et al. 2005). In contrast to sialidase Neu 1, sialidase Neu 4 does not require additional accessory proteins for its enzymatic activity (Seyrantepe et al. 2004). Neu 4 has two isoforms which differ from each other with terminal amino acid residues that target enzyme to

mitochondria. Also isoforms are expressed in a tissue-spesific manner; brain, muscle and kidney contain both isoforms whereas the liver and colon contain dominantly the short form of sialidase Neu 4. The mitochondrial targeted long form of Neu 4 is thought to be involved in cell apoptosis or neural differentiation by regulating the apoptosis-related ganglioside G_{D3} level (Hasegawa et al. 2007).

Although Neu 2 and Neu 3 were shown to desialylate glycolipids *in vitro* (Li et al. 2001; Tringali et al. 2004) they are not involved in ganglioside catabolism in lysosomes because of their localization. In addition, sialidase Neu 4 was shown to clear undegraded sialylated glycoconjugates in cultured fibroblasts of sialidosis and galactosialidosis patients, it was thought that Neu 4 is the enzyme responsible for sialylated glycolipids degradation (Seyrantepe et al. 2004).

1.3. Lysosomal Storage Diseases

Lysosomes are vesicular systems which have low internal pH with many hydrolytic enzymes (De Duve et al. 1955). Lysosomes are responsible for the breakdown of substrates (De Duve and Wattiaux 1966). The membranes of lysosomes have transport systems to carry biomolecules between inside of the organelle and the cytosol, and also have proton pumps to maintain asidic pH in the lumen (Arai et al. 1993). The lysosomal enzymes are glycoproteins (proteins that have carbohydrate groups attach to them) which are synthesized in rough endoplasmic reticulum. Since these enzymes are degradative enzymes, they are firstly synthesized in inactive form and stay inactive until they reach to the lysosomes. The translocations of these enzymes from endoplasmic reticulum to Golgi and from Golgi to lysosomes require the signal sequences and sugar residues called mannose 6-phosphate on their N-terminal sites (Tabas and Kornfeld 1980; Hasilik et al. 1980).

Lysosomal storage diseases (LSDs) are a group of rare inherited metabolic disorders that result from a distruption in the lysosomal degradation pathway of biomolecules. With three exceptions (Hunter's disease, Fabry's disease and Danon disease), all LSDs are inherited in autosomal recessive manner (Sugie et al. 2003). To date, more than 40 lysosomal storage diseases have been recognized and the incidences of these disaeases are approximately 1 in 7000 - 8000 live births (Winchester et al. 2000) (Table 1.1.).

Distruption in lysosomal degradation can occur due to the deficiency of a specific lysosomal enzyme which has acid hydrolase function (Neufeld 1991; Kolter and Sandhoff 1998), a cofactor protein, a protein that involved in the post-translational modification, or a protein that involved in the transport of lysosomal protein (Winchester et al. 2000). As a consequence of dysfunction, uncatabolized substrate of associated enzyme accumulates in lysosomes. The accumulation (storage) of substrate may cause cellular toxicity (Neufeld 1991; Winchester et al. 2000). The storage is progressive so the disease symptoms worsen over time (Wraith 2002). Storage can occur in various cell types and affected individuals can show mild or severe abnormalities or can die at an early age (Barton et al. 1990, 1991). The severity of the phenotype is also related to the residual activity of the responsible enzyme. It was proposed that there is a "critical threshold" of the enzyme activity which determines the storage of the substrate in lysosomes. When the activity of the enzyme decreases below the threshold level, it can not degrade all substrates and storage occurs (Conzelmann and Sandhoff 1983). In general, lower residual activity causes earlier age of onset or more severe phenotype of the disease. The accumulated subsrates form typical histochemical and ultrastructural changes in the cell. These structures are characterized as vacuoles (lysosomes) which contain undegraded subsrates (biomolecules), and they are the hallmarks of the storage in related disorders. The first storage bodies were defined in Tay-Sachs' disease (Terry and Weiss 1963).

1.3.1. Tay – Sachs Disease

Tay-Sachs disease is one of G_{M2} gangliosidosis. It is caused by mutations in HEXA gene (Sandhoff et al. 1989) that is located in the long arm of 15^{th} chromosome (Gilbert et al. 1975). HexA gene encodes α-subunit of lysosomal β-hexosaminidase A (HexA) enzyme which removes N-acetyl-galactosamine residues from G_{M2} gangliosides converting to ganglioside G_{M3} for further degradation. Sandhoff disease is another G_{M2} gangliosidosis which results from the mutations in HEXB gene encoding β subunit of β-hexosaminidase A enzyme located in long arm of 5^{th} chromosome (Gilbert et al. 1975) as well as G_{M2A} gene encoding G_{M2} activator protein (Gravel et al. 1995). These two genes' products (α and β subunits) constitute iso-forms of HexA enzyme; αβ, ββ and αα (Figure 1.7). Because G_{M2} can only be degraded by αβ isoenzyme; deficient of both

HEXA and HEXB gene result in accumulation of G_{M2} ganglioside (Gravel et al. 1995). When this accumulation in membranous cytoplasmic bodies (MCB) reaches critical values in cells, this disturbs cytoarchitecture and causes apoptosis of neurons (Huang et al. 1997).

Table 1.1. Some of the diseases that result from enzyme defects in ganglioside degradation pathway

Disease	**Defected enzyme(s)**	**Affected lipid(s)**
Farber's disease	Acid ceramides	Ceramide
Niemann-Pick Disease (types A and B)	Sphingomyelinase	Sphingomyelin
Krabbe's Disease	Galactosylceramidase	Galactosylceramide Galactosylsphingosine
Metachromatic leukodystophy	Sulfatidase (Arylsulfatase A)	Sulfatide
Gaucher's Disease	Glucosylceramidase	Glucosylceramide Glucosylsphingosine
Fabry's Disease	α-Galactosidase A (Trihexosylceramidase)	Trihexosylceramide (gal-gal-glc-cer) Digalactosylceramide
G_{M1} gangliosidosis	G_{M1}-ganglioside β-galactosidase	G_{M1} ganglioside Galactose rich fragments of glycoproteins
Tay-Sachs Disease	**β-Hexosaminidase A**	**G_{M2} ganglioside**
Sandhoff's Disease	**β-Hexosaminidase A and B**	**G_{M2} ganglioside** **Asialo-G_{M2} ganglioside** **Globoside**

In the patients of Tay-Sachs disease progressive neuronal degeneration, muscle weakness, blindless and epilepsy are seen. Tay-sachs patients die in the second to fourth year of their life (Gravel et al. 2001). Sandhoff disease is more severe that Tay-Sachs, because of the absence of ββ isoenzyme that degrades some other substrates such as oligosaccharides and glycosaminoglycans in other organs (Gravel et al. 1995). Less frequently juvenile and adult forms of the disease are seen and characterized with later onset and milder symptoms (Sandhoff et al. 1989).

1.3.1.1. Mouse Models of G_{M2} Gangliosidosis

Mouse models of diseases are indispensable tools both to study the pathogenesis of the diseases and to improve therapeutic approaches to cure the disease. Mouse models mostly mimic the human diseases; besides spontaneous models (mucolipidosis in cat) (Bosshard et al. 1996), there are also induced mutants such as β-Hexosaminidase β-subunit (Hexb) deficiency (Sango et al. 1996; Phaneuf et al. 1996), galactosialidosis (Zhou et al. 1995) and total sphingolipids activator deficiency (Fujita et al. 1996). However, some mouse models of the human diseases such as β-Galactosidase deficient mouse model of G_{M1} gangliosidosis (Hahn et al. 1997; Matsuda et al. 1997) and β-hexosaminidase deficient mice of Tay-sach's disease (Yamanaka et al. 1994; Cohen-Tannoudji et al. 1995; Phaneuf et al. 1996) because some degradation pathways of substrates are different in mouse and human (Suzuki and Mansson 1998).

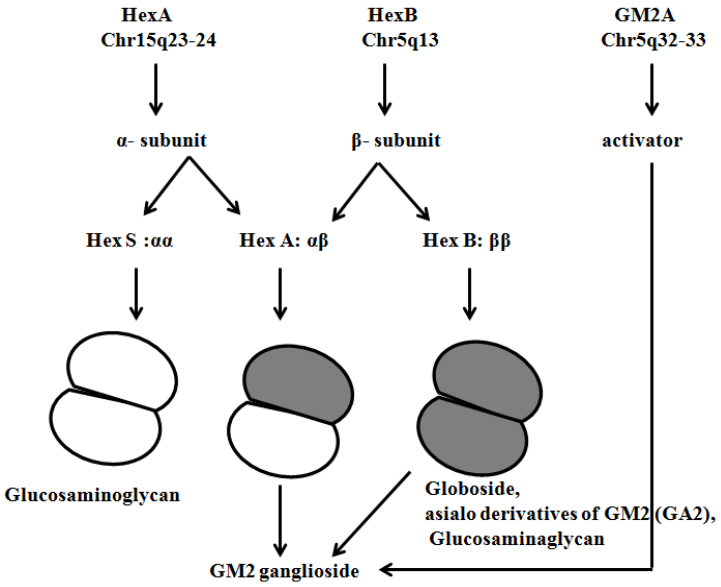

Figure 1.7. Formation of hexosaminidase isoenzymes from HexA and HexB gene products

In the deficiency of lysosomal β-Galactosidase (β-GAL) enzyme in humans G_{M1}-gangliosidosis occur (Suzuki et al. 1995). G_{M1}-gangliosidosis is a neurological disorder but it affects not only the central nervous system but also the peripheral organs. Only G_{M1} ganglioside is accumulated in patient's tissues. However, beside G_{M1} ganglioside, a significant level of accumulated G_{A1} glycolipid (asialyted form of G_{M1}

ganglioside) was also observed in the brain of mouse model of G_{M1} gangliosidosis (Suzuki and Chen 1967; Suzuki and Kamoshita 1969). These observations suggest that some murine neurominidases are active toward the G_{M1} ganglioside which is not detected in human. Responsible neurominidases convert G_{M1} ganglioside to G_{A1} ganglioside by removing its sialic acid residue, and cause accumulation of these two biomolecules in mice as distinct from human (Hanh et al. 1997).

Similarly β-Hexosaminidase deficient mouse model ($HexA^{-/-}$) generated by homologous recombination in embryonic stem cells to study Tay-Sachs disease, unexpectedly didn't show disease phenotype (Yamanaka et al. 1994; Cohen-Tannoudj et al. 1995; Sango et al. 1995; Sango et al. 1996; Phaneuf et al. 1996). The behavioral and motor coordination of these mice were the same with wild type mice ($HexA^{+/+}$) at least 1 year of life span. All neurons were affected in Tay-Sachs patients, however, only a limited accumulation of G_{M2} ganglioside and the membranous cytoplasmic bodies were present in the brain of HexA mice. Most brain sites such as cerebellum and spinalganglia were storage free (Yamanaka et al. 1994; Cohen-Tannoudji et al. 1995; Phaneuf et al. 1996). Another difference was that while human Tay-Sachs samples were positive for increased apoptosis, HexA deficient mice showed no change in apoptosis. This apoptosis difference was associated with the G_{M2} accumulation, because when there was no accumulation and apoptosis; symptoms of the disease were not observed (Huang et al.1997). This inequality revealed that there may be differences in the metabolic pathway of G_{M2} ganglioside catabolizing G_{M2} ganglioside via glycolipid G_{A2} (asialyted form of G_{M2} ganglioside) bypassing G_{M3} routed degradation in mice (Yamakana et al. 1994) (Figure 1.8). It has been proposed that the activities of sialidases which remove sialic acid residue of G_{M2} converting to G_{A2} which is further degradated by β-Hexosaminidase A enzyme to lactosylceramide by passing degradation pathway (Sango et al. 1996; Phaneuf et al. 1996). Previously HexB activity was thought to be responsible for this G_{M2}-degradation (Burg et al. 1983). Then it was shown that HexB was not able to degrade G_{M2} to G_{M3}. Instead it had an activity towards G_{A2} (which is formed by the activity of sialidases), convert it to lactosylceramide by removing N-acetyl galactosamine residue, only in the presence of an extra protein; mouse G_{M2} activator protein (mM2act). The same report showed that G_{A2} was not hydrolyzed by either human or mouse HexA and the human G_{M2} activator protein (hM2act) did not function as mouse G_{M2} activator protein (Yuziuk et al. 1998). This alternative pathway

was also supported by HexB deficient mice. In the HexB deficient mice (the model of Sandhoff disease), accumulation of G_{M2} ganglioside and G_{A2} ganglioside can not be stopped because the alternative pathway was also blocked (Sango et al. 1996). Depending on the absence of HexB activity (both αβ and ββ isoenzymes are deficient) symptoms of the diseases seen in human was observed (Huang et al.1997). In HexA deficient mice, there was no accumulation of either G_{M2} or G_{A2} (Riboni et al. 1995) suggesting the existence of an alternative pathway in mice which is different from human where sialidases have particular roles.

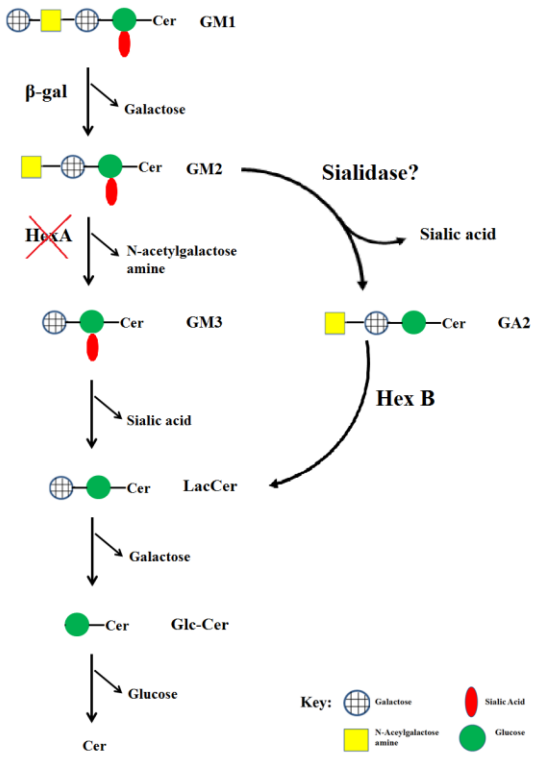

Figure 1.8. Metabolic by-pass in ganglioside degradation pathway in HexA deficient Tay-Sachs mouse model (Adapted from: Sango et al. 1995)

To enlighten the metabolic bypass pathway in HexA mice and to find which sialidase or sialidases have a role in converting G_{M2} to G_{A2}, several studies have been done. Since the *in vitro* activity through gangliosides, sialidase Neu 1 was thought as a candidate for catalyzing ganglioside into asialilayted form (Hiraiwa et al. 1987; Schneider-Jakob and Cantz 1991; Fingerhut et al. 1992; Hiraiwa et al. 1998). However it was not seen an accumulation of ganglioside either in galactosialidosis patients (d'Azzo et al. 2001; Thomas 2001) or knouckout mouse model of sialidosis (Zhou et al.

1995; de Geest et al. 2002). The role of sialidase Neu 4 has been investigated because of its *in vitro* degradative activity against several gangliosides including G_{M2} (Seyrantepe et. al. 2004). In mouse model with the Neu 4 deficiency, lysosomal storage was shown in lung and spleen with microscopic investigation. In addition, abnormal ganglioside pattern in brain was shown by thin layer chromatography analysis. In another study it was shown that overexpression of sialidase Neu 4 restores normal morphological phenotype and repaires the abnormal metabolism of G_{M2} via G_{A2} in the neuroglia cells from Tay-Sachs patients indicating that sialidase Neu 4 might have a role in desialylation of glycolipids in the HexA deficient mice (Seyrantepe et al. 2008). Therefore, mouse was generated with the deficiencies of both Neu 4 and HexA. The significantly higher level of G_{M2} ganglioside in lysosomes of neurons has been shown in double knockout mice (Figure 1.9). Most importantly mice with Neu 4 and HexA enzyme deficiency have epileptic seizures (hallmark of Tay-Sachs patients) which were not observed in mice with single HexA enzyme deficiency. Since only 40 % of mice with double enzyme deficiency have seizures, it has been proposed that Neu 4 is a modulator gene and it is not the only sialidase contributes the metabolic bypass seen in the HexA deficient mice (Seyrantepe et al. 2010).

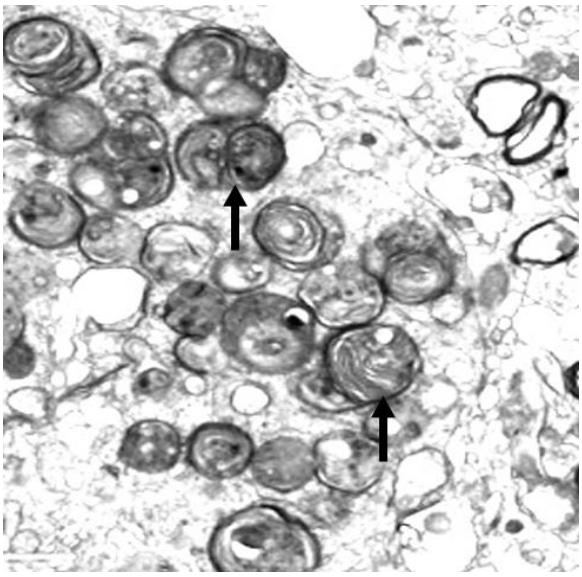

Figure 1.9. Hippocampal neurons electron micrograph of $Neu4^{-/-}HexA^{-/-}$ with seizures – accumulation of whorls of membranes in lysosomes is similar to Tay-Sach's disease pathology (Source: Seyrantepe et al. 2010)

1.4. Aim of the Study

The aim of my project is to profile brain glycolipids of mouse model (triply) with the deficiencies of three enzymes (β-Hexosaminidase A, sialidase Neu 4 and sialidase Neu 1). Although β-Hexosaminidase A and sialidase Neu 4 coding genes are completely knocked-out, there is about 10 % activity of normal sialidase Neu 1 in triply mouse model (Seyrantepe et al. 2010). The brain glycolipid profile of mutant mice with the deficiencies of two enzymes (β-Hexosaminidase A and sialidase Neu4) were previously reported (Seyrantepe et al. 2010). In this study we aimed to see the affect of additional sialidase Neu 1 on glycolipids, especially ganglioside degradation.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

Triply mice that have three enzyme deficiencies ($Neu4^{-/-}HexA^{-/-}NeoIn^{-/-}$) were generated by Assoc. Prof. Volkan Seyrantepe during his post-doctoral training and donated by Prof. Alexey V. Pshezhetsky (Centre Hospitaliere Universitaire Sainte-Justine, University of Montreal, Montreal, Quebec, Canada). These mice were generated by breeding double knockout mice with the deficiency of $Neu4^{-/-}$ and $HexA^{-/-}$ with hypomorphic mouse model with reduced sialidase Neu 1 activity (Seyrantepe et al. 2010). In hypomorphic mouse model the distrupted gene is not the sialidase Neu 1 gene (Seyrantepe et al. 2009). In these mice the Cathepsin A coding gene is changed and has neomycine cassette inserted in its noncoding region (Seyrantepe et al. 2008). This insertion caused a decrease in the CathA mRNA level consistent with the reported hypomorphic (partial loss of function) effects of Neo gene (Carmeliet et al. 1996; Meyers et al. 1998; Moran et al. 1999). Therefore the decrease of CathA protein expression caused highly reduction of normal sialidase Neu1 activity (Seyrantepe et al. 2009). In this study hypomorphic mouse of Neu 1 was named as NeoIn mouse since it has neo cassette in the CathA gene, and hypomorphic allele was named as NeoIn allele.

Triply mice were breeded with wild type mice strain C57/Black6 to obtain heterozygous females and males. We have breeded further to obtain single deficient ($Neu4^{-/-}$, $HexA^{-/-}$, $NeoIn^{-/-}$), double deficient ($Neu4^{-/-}HexA^{-/-}$, $HexA^{-/-}NeoIn^{-/-}$ and $Neu4^{-/-}NeoIn^{-/-}$) as well as triple deficient mice. In these crossings mostly brothers and sisters were mated with each other to have mice in the same genetic background (Table 2.1.).

Table 2.1. Crossing of mice

Breeding pairs	**Expected genotypes in F1 generation**
 ♀ $Neu4^{-/-}$ $HexA^{-/-}$ $NeoIn^{-/-}$	 $Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{+/-}$
 ♂ WT ($Neu4^{+/+}$ $HexA^{+/+}$$NeoIn^{+/+}$)	
Breeding pairs	**Expected genotypes in F2 generation**
♀ $Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{+/-}$	$Neu4^{+/+}$ $HexA^{+/+}$ $NeoIn^{+/+}$
♂ $Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{+/-}$	$Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{+/-}$
	$Neu4^{+/+}$ $HexA^{+/+}$ $NeoIn^{+/-}$
	$Neu4^{+/+}$ $HexA^{+/-}$ $NeoIn^{+/+}$
	$Neu4^{+/+}$ $HexA^{+/-}$ $NeoIn^{+/-}$
	$Neu4^{+/-}$ $HexA^{+/+}$ $NeoIn^{+/+}$
	$Neu4^{+/-}$ $HexA^{+/+}$ $NeoIn^{+/+}$
	$Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{+/+}$
	$Neu4^{+/+}$ $HexA^{+/+}$ $NeoIn^{-/-}$
	$Neu4^{+/+}$ $HexA^{+/-}$ $NeoIn^{-/-}$
	$Neu4^{+/-}$ $HexA^{+/+}$ $NeoIn^{-/-}$
	$Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{-/-}$
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	$Neu4^{-/-}$ $HexA^{+/-}$ $NeoIn^{-/-}$
	$Neu4^{-/-}$ $HexA^{-/-}$ $NeoIn^{+/+}$
	$Neu4^{-/-}$ $HexA^{-/-}$ $NeoIn^{+/-}$
	$Neu4^{-/-}$ $HexA^{-/-}$ $NeoIn^{-/-}$

We set five different pairs of breeding from the F1 generation. These heterozygous mice produce F2 generation but not the all expected genotypes were gotten in F2 generation. Further breedings were done and desired mice with deficiency were gotten in F3, F4 generations. During the process of reproducing mice, totally 274 mice were genotypyed for three alleles and 11 breedings to get Neu 4 deficient mice, 14 breedings to get HexA deficient mice, 2 breedings to get Neu 4, HexA double deficient mice, 2 breedings to get HexA, NeoIn double deficient mice, 2 breedings to get triple deficient mice and 16 breedings to get control mice were set from F2, F3, F4 generations.

2.2. Genotyping of Mice

The genotypes of mice were determined from the genomic DNA that was extracted from mice's tail. The isolation of genomic DNA is done by following steps. Tails taken from mouse were put in eppendorf tubes and 500µl lysis buffer (consist of 10% 1M Tris pH 7.6, 2.5% 0.2M EDTA, 20% SDS, 4% 5M NaCl) with 12µl Proteinase K (from 25µg/µl solution) were added. Samples were incubated overnight in incubator shaker at 55˚C at 70 rpm. The next day samples were centrifuged at 14.000 rpm for 10 minutes. Supernatant were transfered into new eppendorf tubes and same volume 100% isopropanol was added on each sample. DNA was collected and transfered into a new ependorf that contains 70% ethanol. After 1 minute centrifugation at 14.000rpm supernatant was removed and the remaining ethanol was totally air dried for 10 minutes. DNAs were dissolved in 200µl ultra pure water and incubated at 55˚C for 1 hour. Then concentrations of DNA were measured with Nanodrop spectrophotometer (ND-1000). The PCR for Neu4 and HexA were performed with 100ng genomic DNA in the 25µl reaction mix containing 50pmol of each primer, 10mM of each dNTPs, 1.5 units Taq polymerase (New England Biolab), 1.5mM $MgCl_2$, 10mM Tris-HCl and 50mM KCl buffer containing 10% DMSO. Allele specific primers for wild type and mutant allele were used for detection of Neu4 and HexA alleles (Table 2.1). Conditions for PCR are; 1 cycle 30 seconds at 95˚C; 30 cycles 30 seconds at 95˚C, 45 seconds at 60˚C, 45 seconds at 72˚C; and 1 cycle 5 minutes at 72˚C.

The PCR for NeoIn were performed with 100ng genomic DNA in the 50µl reaction mix containing 50pmol of each primer, 10mM of each dNTPs, 2.5 units Taq

polymerase (New England Biolab), 1.5mM $MgCl_2$, 10mM Tris-HCl and 50mM KCl buffer. A couple of primers were used for proliferate mutant and wild type NeoIn allele (Table 2.2). Conditions for PCR is; 1 cycle 5 minutes seconds at 92˚C; 30 cycles 45 seconds at 92˚C, 45 seconds at 57˚C, 45 seconds at 72˚C; and 1 cycle 5 minutes at 72˚C. Because mutant allele has an NdeI enzyme recognition site, genotype of NeoIn allele revealed after digestion of PCR product in a 20µl reaction mix containing 20 units NdeI (New England Biolabs) enzyme, 1X buffer by incubation overnight at 37˚C.

Table 2.2. Sequences of used primers in genotyping

Gene	Primer Name	Primer sequence
Neu4	Neu4F	CTCTTCTTCATTGCCGTGCT
	Neu4R	GACAAGGAGAGCCTCTGGTG
	NeoF	GCCGAATATCATGGTGGAAA
HexA	HexAF	GGCCAGATACAATCATACAG
	HexAR	CTGTCCACATACTCTCCCCACAT
	PGK	CACCAAAGAAGGGAGCCGGT
NeoIn	ScreenF	GGTGGCGGAGAACAATTATG
	ScreenR	AACAGAAGTGGCACCCTGAC

After PCR is completed all PCR samples were run on 1% agarose gel whereas digestion reaction of NeoIn was run on 2% agarose gel to visualize alleles.

2.3. Brain Tissues

Age matched single, double and triple deficient mice as well as control mice (2-3 months, 5-6 months, 8-9 months and 12 months) were sacrificed by servical dislocation. 100mg brain tissue from their right and left cerebral hemispheres (Figure 2.1) were removed, immediately frozen on dry ice and kept in -80˚C until needed.

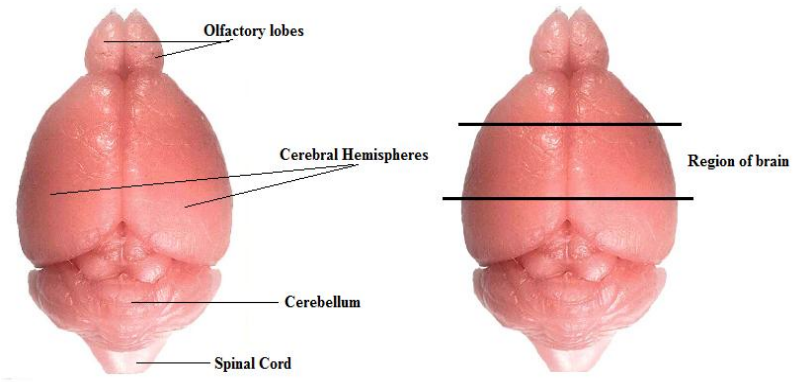

Figure 2.1. Main sites of brain and the region that samples were collected for lipid extraction

2.4. Lipid Extraction

To isolate total lipids from brain tissue an optimized form of Folch lipid extraction method was used (Folch et al. 1956, 1957). 100mg brain tissue was homogenizated in 1ml methanol using Heidolph SilentCrusher M Politron Homogenizer at 14.000rpm for nearly 20 seconds. After that additional 1.5ml methanol and 2.5ml chloroform (final chloroform: methanol concentration ratio is 1:1) are added to samples. It was mixed by vortex and kept in 4˚C for overnight. In the next day sample was centrifuged at 2000rpm at room temperature (RT) for 5 minutes. Pellet that contains cell debris, proteins etc was removed by centrifugation and supernatant which contains lipids was transferred into a new glass tube. This methanol/chloroform mixture was evaporated with N_2 flow with keeping tube in 55˚C water bath that eases evaporation. The pellet proceeded into the wash step that let us to separate acidic glycosiphingolipids from neutral glycosphingolipids. It was firstly dissolved into 1ml methanol, 1ml chloroform and 650µl 1X PBS, vortexed and centrifuged at 2000rpm at RT for 5 minutes. Upper phase was taken into a new glass tube. 650µl methanol and 650µl 1X PBS was added to the lower phase, it was vortexed and centrifuged at 2000rpm at RT for 5 minutes. The upper phase was added to the tube that contains previously taken solution. Then the lower phase was lastly washed with 650µl methanol and 650µl dH_2O, vortexed and centrifuged at 2000rpm at RT for 5 minutes. And the last upper phase was added to the tube that contains other upper phases. The remaining lower phase was kept +4˚C for other experiments (neutral lipid extraction). These collected upper phases contain polar lipids; sulfatides and gangliosides.

2.4.1. Isolation of Gangliosides from Total Lipids

To extract ganglioside from other lipids, Supelclean LC-18 column (Supelco) was used. Columns were placed on the Chromabond Vacuum manifold (Macherey-Nagel) and vacuum was fixed to 3-4Hg. For equilibration, each column was washed with firstly 2ml chloroform, then with 2ml methanol and finally with 2ml methanol: 1X PBS (1:1) solution. The previously collected upper phases of were applied to columns and after flowed through the columns were washed with 10ml ultra pure water. Samples were then eluted with 4ml methanol and 4ml methanol: chloroform (1:1) in low vacuum (<3Hg). Eluted samples were evaporated with N_2 flow before loading to thin layer chromatography plate.

2.4.2. Isolation Of Neutral Lipids

Neutral lipids were isolated from the lower phases that were left after upper phases were collected by sequential centrifugation in isolation of asidic lipids. The lower phase was evaporated with N_2 flow. 250µl chloroform and 250µl methanol containing 0,5N NaOH were added onto evaporated sample. The tube was covered and incubated 2 hours at 37˚C. Then 850µl chloroform and 250µl methanol containing 0,5N HCl and 430µl water were added into the same tube. It was mixed by vortex and phases were separated with centrifugation for 5 minutes at 2000rpm. The upper phase was discarded and the lower phase which contains non-hydrolysable lipids (glycosphingolipids and ceramides) was evaporated with N_2 flow before loading to thin layer chromatography plate.

2.5. Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a method used to separate biomolecules based on their structures and weights. It is used to analyse especially oligosaccharides and glycolipid content in different body fluids such as urine and tissues. There are two phases in TLC; mobile phase and a stationary phase. The mobile phase consists of organic and/or aqueous solvents where as the stationay phase is a solid adsorbent such

as silica and cellulose which is coated onto a thin layer support such as aluminium or glass (Fried and Sherma 2005).

Thin layer chromatography tank (Camag) was prepared 2.5 hours earlier than running. This time was needed for equilibrium of the tank to form a vaporous environment by the evaporaiton of solutions. Different solvent mixtures were used for different glycosphingolipids; chloroform: methanol: 22% $CaCl_2$ (55: 45: 10) mixture for acidic glycosphingolipids and chloroform: methanol: ammonia: water (65: 35: 2: 3) mixture for neutral glycosphingolipids. To load the samples to the silica covered glass thin layer chromatography plates (Merck), nitrogen dried samples were solved in 250µl methanol: chloroform (1:1) solution. 100µl sample were loaded to the TLC plate. The plate was put into the tank and the samples were runned until 3mm left for solution to reach to the top of the plate.

2.6. Staining with Orcinol / Resorcinol and Visualization

Both orcinol and resorcinol (Sigma) (Figure 2.2) dyes were prepared freshly. For 10ml orcinol dye solution 0,04g orcinol was solved in 10ml 25% sulfuric acid (2.52ml H_2SO_4 and 7.52ml deiyonised water). Orcinol forms a colorless solution and the stabilization of it took 5 minutes.

For resorcinol, firstly a stable stock solution was prepared. For this 1gr resorcinol was dissolved in 50ml ultrapure water. When resorcinol staining was needed this stock was used. For 20ml resorcinol reagent, 2ml resorcinol stable solution was mixed with 16ml pure HCl (37%) and 100µl 0,1M $CuS0_4$. And the volume was completed to 20ml with ultrapure water. The stabilization of resorcinol took 4 hours.

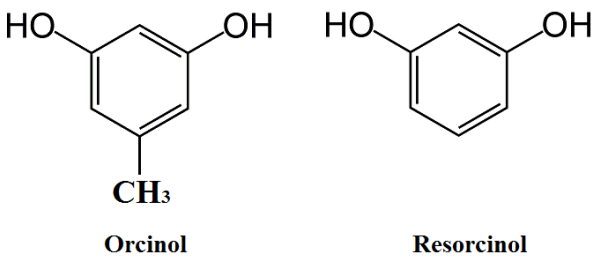

Figure 2.2. Structure of orcinol and resorcinol reagents

Orcinol and resorcinol reagents were prepared in 50ml glass TLC sprayer (Sigma). Dyes were sprayed on the plates after run was completed and the plate was completely dried by air flow. Then for both dyes the plates were put into the preheated oven. In orcinol staining plates were incubated at 120˚C for 10 minutes without a glass cover but in resorcinol staining plates were incubated in 120˚C oven for 30 minutes with a glass cover. After staining, lipids were identified by comparing with brain ganglioside standarts (Avanti Polar Lipids). Images of plates were taken with the VersaDoc™ Imaging System.

CHAPTER 3

RESULTS

3.1. Genotyping of Mice

The genotypes of mice for Neu4 and HexA alleles were determined by the PCR done with allele specific primers (Figure 3.1). For genotyping NeoIn allele, digestion reaction was also performed (Figure 3.2)

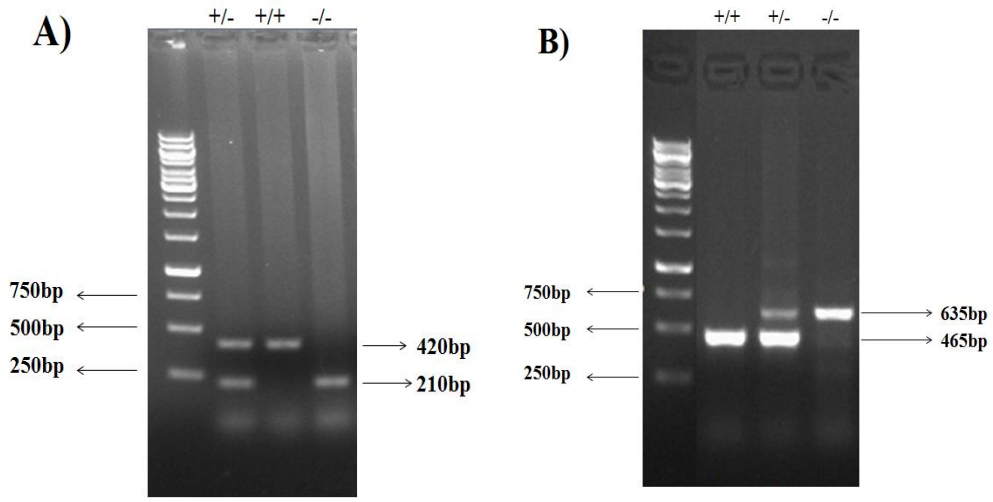

Figure 3.1. Gel images of (A) HexA PCR products and (B) Neu4 PCR products

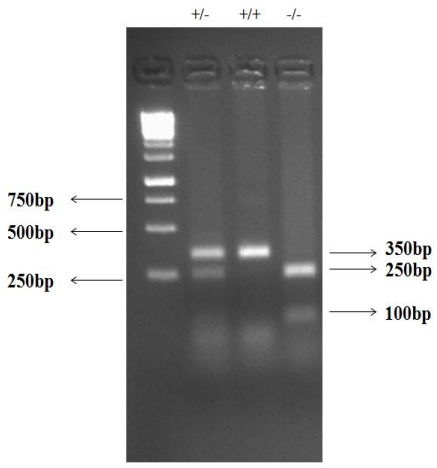

Figure 3.2. Gel image of NdeI digested PCR products of NeoIn allele

3.2. Thin Layer Chromatography

Thin layer chromatography analyses were optimized for our laboratory conditions and then all samples were analyzed in that consitions as decribed in the material method chapter.

Firstly age matched double deficient for HexA and NeoIn mice's gangliosides were analyzed with thin layer chromatography and orcinol staining as well as control and single deficient counterparts (Figure 3.3). TLC was then done for older mice with the same deficiencies (Figure 3.4).

Secondly age matched triple deficient mouse with its control, single HexA and Neu4 deficient and HexA Neu4 double deficient counterparts' gangliosides were analyzed with thin layer chromatography and orcinol staining (Figure 3.5). TLC was also done for older mice with the same deficiencies (Figure 3.6).

After gangliosides, neutral glycosphingolipids of each sample were isolated and analyzed with thin layer chromatography with orcinol staining (Figure 3.7A, 3.7B and Figure 3.8A, 3.8B).

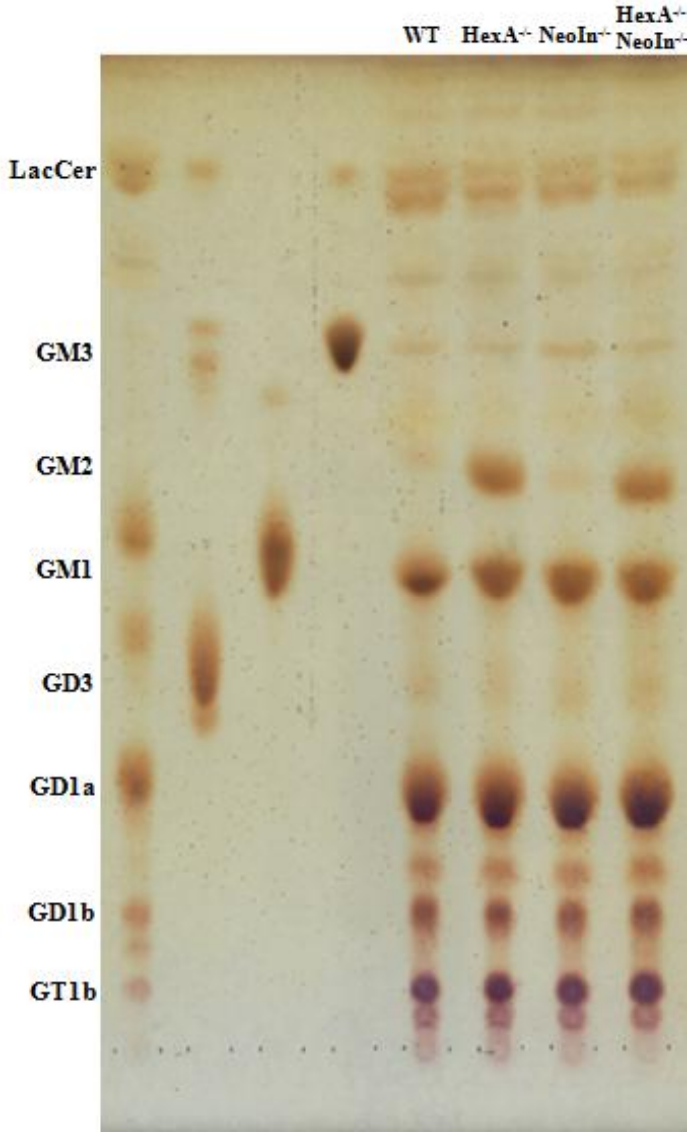

Figure 3.3. Thin layer chromatography and orcinol staining for gangliosides of 2.5 months HexANeoIn double deficient mice with its control and single HexA and NeoIn deficient counterparts (Samples are; wild type, $Neu4^{+/-}HexA^{+/-}NeoIn^{+/+}$, ♀, 2.5 months; HexA deficient $Neu4^{+/+}HexA^{-/-}NeoIn^{+/-}$, ♀, 2 months; NeoIn deficient, $Neu4^{+/+}HexA^{+/-}NeoIn^{-/-}$, ♀, 2.5 months; HexANeoIn double deficient, $Neu4^{+/-}HexA^{-/-}NeoIn^{-/-}$, ♂, 2 months, respectively.)

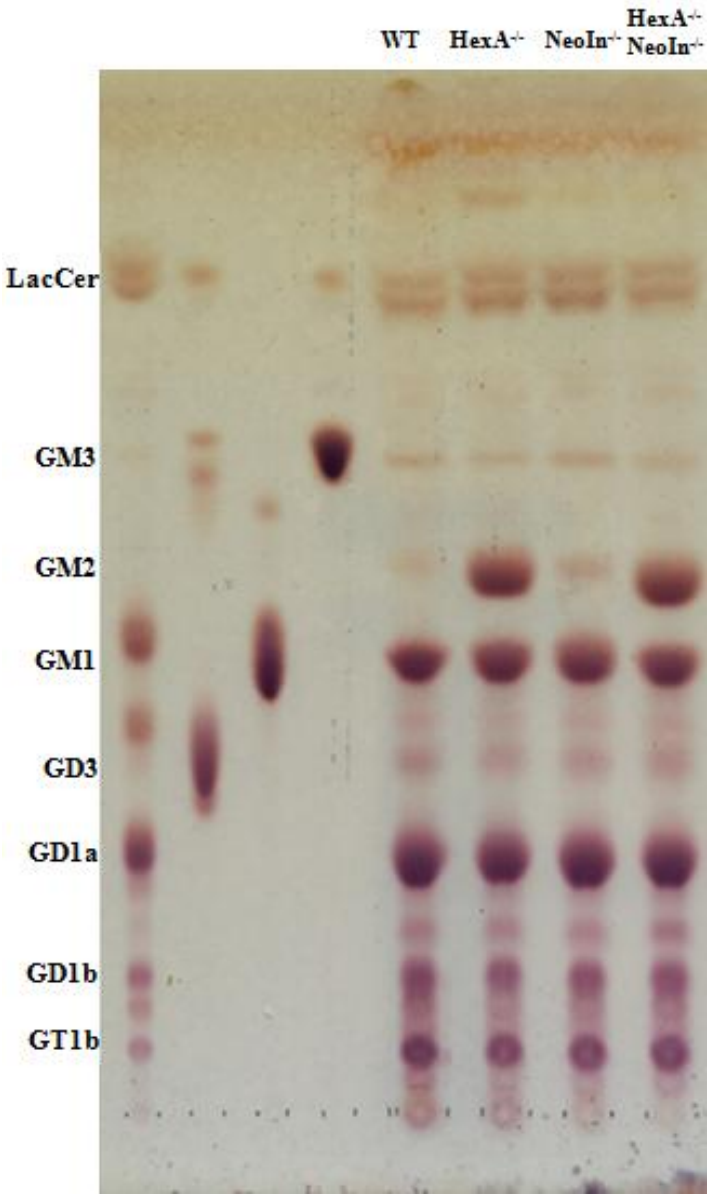

Figure 3.4. Thin layer chromatography and orcinol staining for gangliosides of 4.5 months HexANeoIn double deficient mice with its control and single HexA and NeoIn deficient counterparts (Samples are; wild type, $Neu4^{+/-}HexA^{+/-}NeoIn^{+/-}$, ♂, 4.5 months; HexA deficient $Neu4^{+/-}HexA^{-/-}NeoIn^{+/-}$, ♀, 4.5 months; NeoIn deficient, $Neu4^{+/+}HexA^{+/-}NeoIn^{-/-}$, ♂, 4.5 months; HexANeoIn double deficient, $Neu4^{+/-}HexA^{-/-}NeoIn^{-/-}$, ♀, 4.5 months, respectively.)

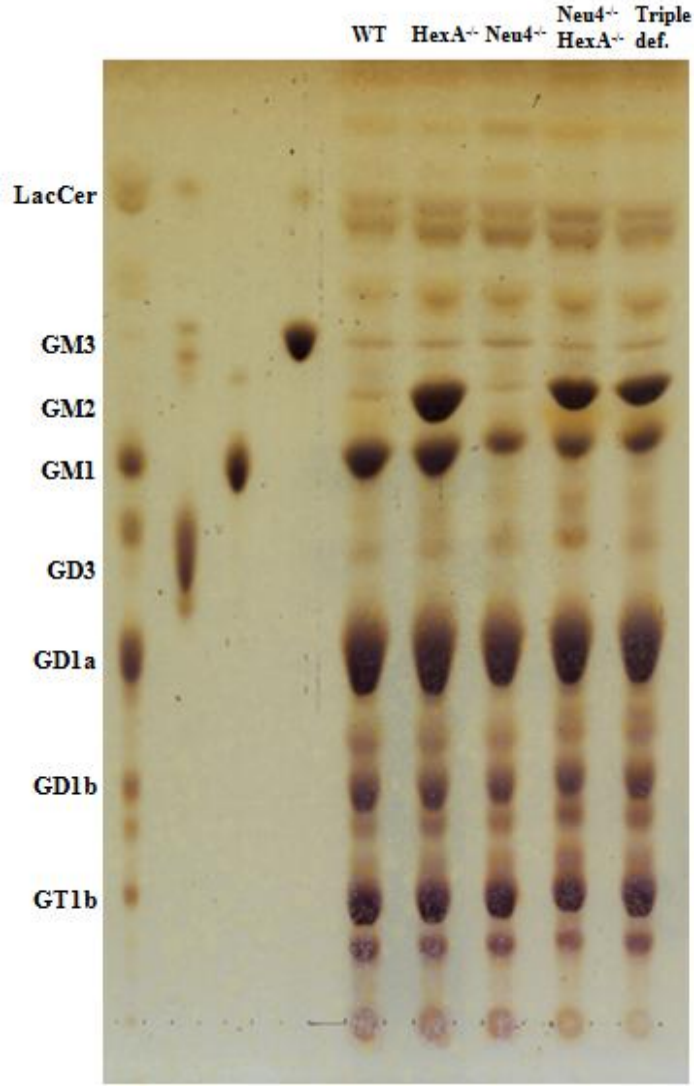

Figure 3.5. Thin layer chromatography and orcinol staining for gangliosides of 4.5 months triple deficient mice with its control, single HexA and Neu4 deficient and Neu4HexA double deficient counterparts (Samples are; wild type, $Neu4^{+/+}HexA^{+/-}NeoIn^{+/+}$, ♂, 4.5 months; HexA deficient, $Neu4^{+/+}HexA^{-/-}NeoIn^{+/+}$, ♀, 4.5 months; Neu4 deficient, $Neu4^{-/-}HexA^{+/+}NeoIn^{+/+}$, ♂, 5 months; Neu4HexA double deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{+/-}$, ♂, 4.5 months; triple deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{-/-}$, ♂, 4.5 months, respectively.)

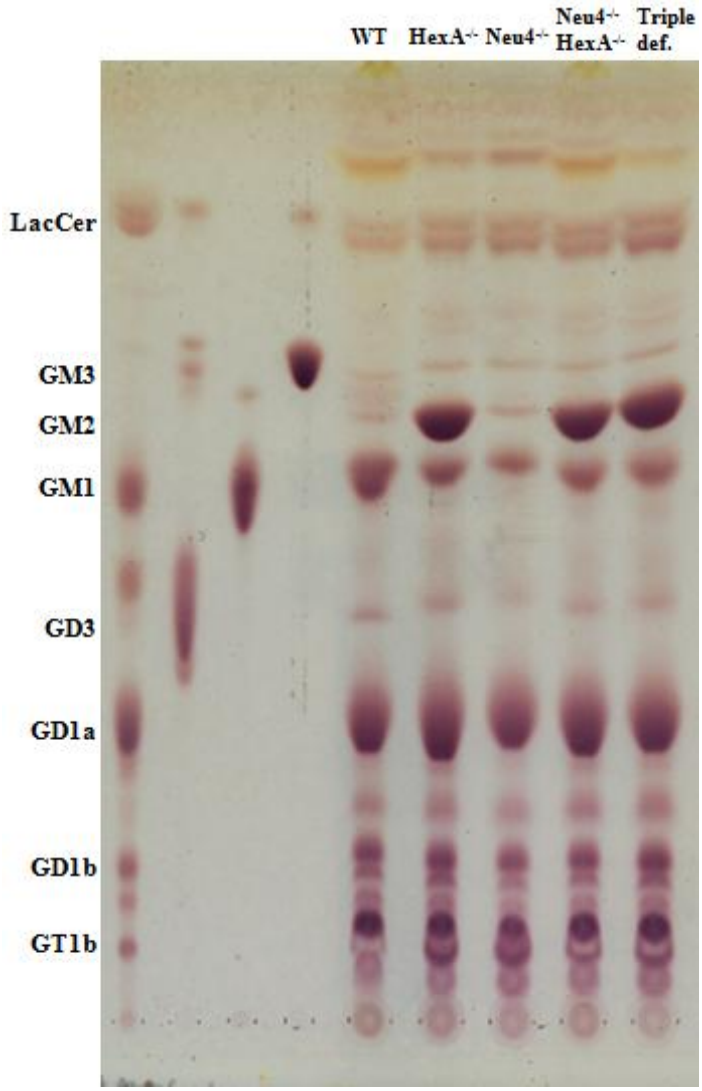

Figure 3.6. Thin layer chromatography and orcinol staining for gangliosides of 8 – 12 months triple deficient mice with its control, single HexA and Neu4 deficient and Neu4HexA double deficient counterparts (Samples are; wild type C57/Black6, $Neu4^{+/+}HexA^{+/+}NeoIn^{+/+}$, ♂, 12 months; HexA deficient, $Neu4^{+/-}HexA^{-/-}NeoIn^{+/-}$, ♀, 8.5 months; Neu4 deficient, $Neu4^{-/-}HexA^{+/-}NeoIn^{+/-}$, ♂, 8 months; Neu4HexA double deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{+/-}$, ♂, 8 months; triple deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{-/-}$, ♀, 12 months, respectively.)

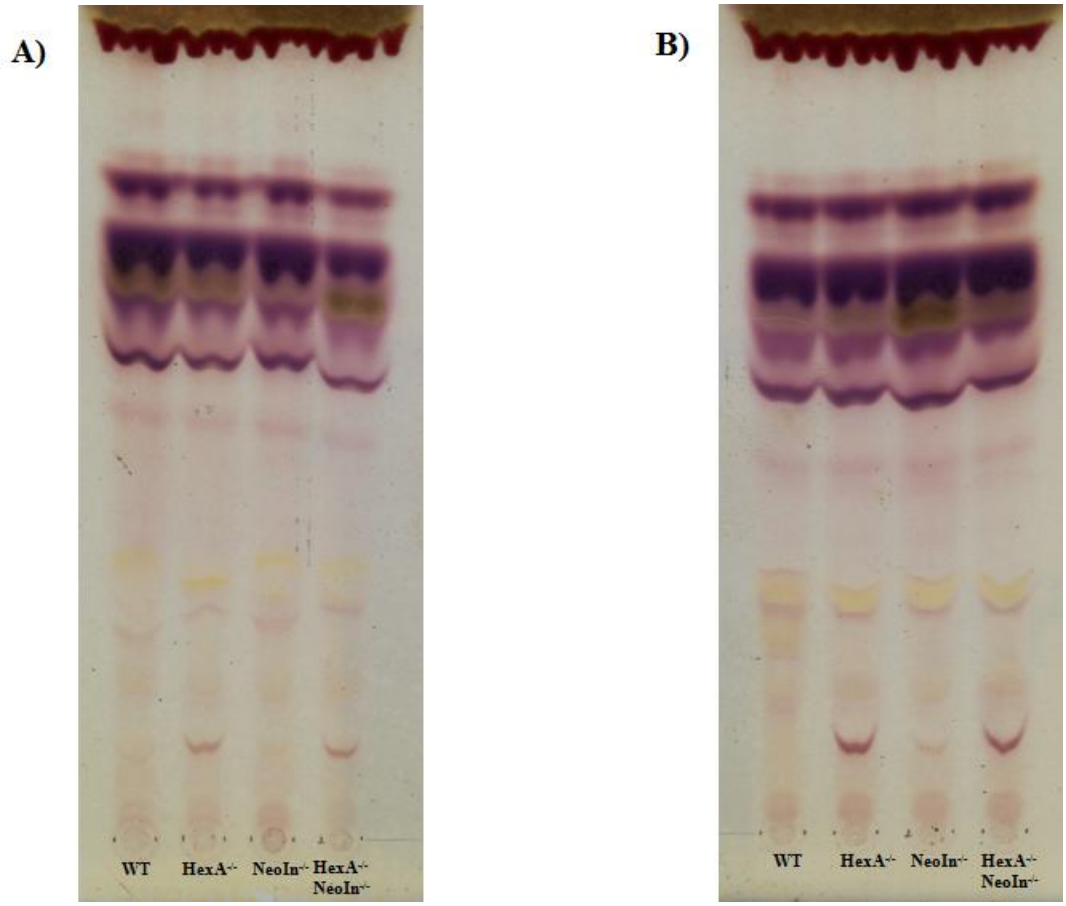

Figure 3.7. A) Thin layer chromatography and orcinol staining for neutal glycosphigolipids of 2.5 months HexANeoIn double deficient mice with its control and single HexA and NeoIn deficient counterparts (Samples are; wild type, $Neu4^{+/-}$ $HexA^{+/-}$ $NeoIn^{+/+}$, ♀, 2.5 months; HexA deficient $Neu4^{+/+}HexA^{-/-}NeoIn^{+/-}$, ♀, 2 months; NeoIn deficient, $Neu4^{+/+}HexA^{+/-}NeoIn^{-/-}$,♀, 2.5 months; HexANeoIn double deficient, $Neu4^{+/-}HexA^{-/-}NeoIn^{-/-}$, ♂, 2 months, respectively.) B) Thin layer chromatography and orcinol staining for neutal glycosphigolipids of 4.5 months HexANeoIn double deficient mice with its control and single HexA and NeoIn deficient counterparts (Samples are; wild type, $Neu4^{+/-}HexA^{+/-}NeoIn^{+/-}$, ♂, 4.5 months; HexA deficient $Neu4^{+/-}HexA^{-/-}NeoIn^{+/-}$, ♀, 4.5 months; NeoIn deficient, $Neu4^{+/+}HexA^{+/-}NeoIn^{-/-}$, ♂, 4.5 months; HexANeoIn double deficient, $Neu4^{+/-}HexA^{-/-}NeoIn^{-/-}$, ♀, 4.5 months, respectively.)

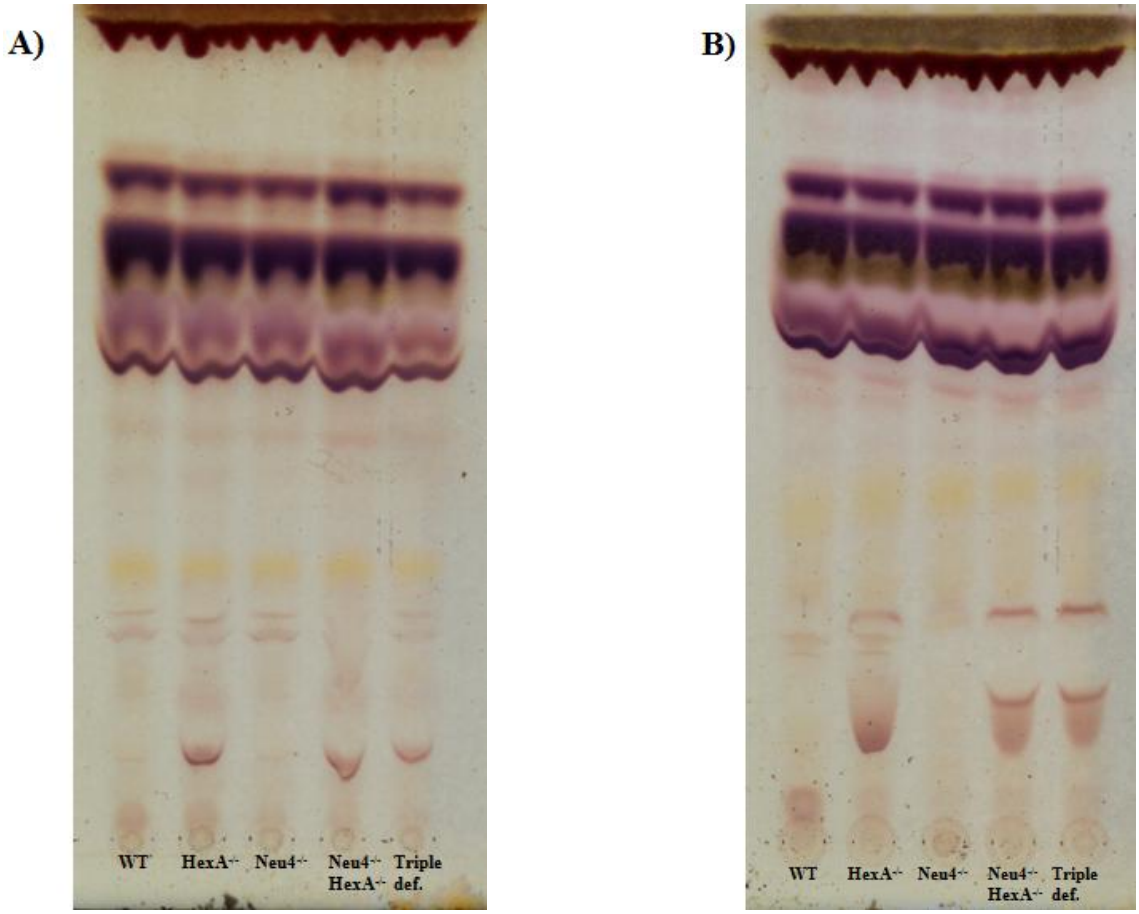

Figure 3.8. A) Thin layer chromatography and orcinol staining for neutal glycosphigolipids of 4.5 months triple deficient mice with its control, single HexA and Neu4 deficient and Neu4HexA double deficient counterparts (Samples are; wild type, $Neu4^{+/+}HexA^{+/-}NeoIn^{+/+}$, ♂, 4.5 months; HexA deficient, $Neu4^{+/+}HexA^{-/-}NeoIn^{+/+}$, ♀, 4.5 months; Neu4 deficient, $Neu4^{-/-}HexA^{+/+}NeoIn^{+/+}$, ♂, 5 months; Neu4HexA double deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{+/-}$, ♂, 4.5 months; triple deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{-/-}$, ♂, 4.5 months, respectively.) B) Thin layer chromatography and orcinol staining for neutal glycosphigolipids of 8 – 12 months triple deficient mice with its control, single HexA and Neu4 deficient and Neu4 HexA double deficient counterparts (Samples are; wild type C57/Black6, $Neu4^{+/+}HexA^{+/+}NeoIn^{+/+}$, ♂, 12 months; HexA deficient, $Neu4^{+/-}HexA^{-/-}NeoIn^{+/-}$, ♀, 8.5 months; Neu4 deficient, $Neu4^{-/-}HexA^{+/-}NeoIn^{+/-}$, ♂, 8 months; Neu4HexA double deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{+/-}$, ♂, 8 months; triple deficient, $Neu4^{-/-}HexA^{-/-}NeoIn^{-/-}$, ♀, 12 months, respectively.)

CHAPTER 4

DISCUSSION

Tay-Sachs disease is a severe lysosomal storage disorder caused by mutations in the HexA gene coding (Sandhoff et al. 1989) for α subunit of lysosomal β-hexosaminidase A enzyme, which converts G_{M2} ganglioside to G_{M3} ganglioside. Patients have progressive neuronal degeneration, muscle weakness, blindless and epilepsy. They die in the second to fourth year of their life (Gravel et al. 2001). However generated $HexA^{-/-}$ mice, depleted of β-hexosaminidase A enzyme, although there is G_{M2} accumulation remain asymptomatic to 1 year of age which suggests there is a metabolic bypass.

Previously identified a novel ganglioside metabolizing sialidase, Neu 4, is abundantly expressed in mouse brain neurons and has activity against gangliosides like G_{M2}. In mouse model with the Neu4 deficiency, lysosomal storage was shown in lung and spleen with microscopic investigation. In addition, abnormal ganglioside pattern (increased GM1a and decreased GM1 levels) in brain was shown by thin layer chromatography analysis (Seyrantepe et al. 2008). Then it was demonstrated that mice model with targeted disruption of both Neu4 and HexA genes ($Neu4^{-/-}HexA^{-/-}$) have multiple degenerating neurons in the cortex and hippocampus and multiple layers of cortical neurons accumulating G_{M2} ganglioside. Since not all mice did show symptoms such as epileptic seizures, it was suggested that sialidase Neu4 is modulator gene product and it's not the only sialidase contributing to the metabolic bypass in $HexA^{-/-}$ mice (Seyrantepe et al. 2010). Therefore, it was suggested that other sialidase and/or sialidases such as lysosomal sialidase Neu 1 might have a role in metabolic bypass. In this study we profiled brain ganglioside of mice models with the deficiencies of three enzymes (β-Hexosaminidase A, sialidase Neu4 and sialidase Neu1 (–NeoIn-)). In the mice that studied on, the enzymes β-Hexosaminidase A and sialidase Neu 4 have 0% enzyme activities according to wild type mice but sialidase Neu 1 enzyme activity is approximately 10% (Seyrantepe et al. 2010).

To isolate total lipids from brain tissue an optimized form of Folch lipid extraction method was used (Folch et al. 1956, 1957). Tissue was homogenized with

chloroform: methanol, cell debris was removed by centrifugation and the supernatant was washed first with different ratio of water and salt solution and then with the chloroform: methanol mix. The washing procedure of supernatant removes all the non-lipide contaminants from the samples (Folch et al. 1956). Polar lipids such as sulfatides and phosphatidyl serine cause contamination of the ganglioside mixture in the upper phase (Daun 1952; Svennerholm 1956) which was removed by using C18 columns that only retain gangliosides. The gangliosides were eluted from column with choloroform-methonol for further studies (Svennerholm 1957). The usage of chloroform: methanol in isolation of lipids, especially for membrane lipids, is cruial. Because chloroform seperates lipids from proteins in the membrane by disturbing the interactions and hydrogen bonds between lipids and proteins. And also polar lipids such as phospholipids and glycosphingolipids (gangliosides) can dissolve in such polar solvents (Schmid and Hunter 1971). Beside ganglioside, group of asidic lipids include sulfatides, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidyletanolamine, and phosphatidic acid as mentioned in the introduction part. Since we are mainly interested in gangliosides we need to separate gangliosides from other acidic lipids.

Thin layer chromatography analysis of gangliosides done with 2 – 2.5 months aged mice that are single deficient of HexA, NeoIn and double deficient of HexA and NeoIn (Figure 3.3) showed that HexA deficient mice and double deficient mice have increased level of G_{M2} in comparison to wiltype mouse. The accumulation of G_{M2} ganglioside was shown before in $HexA^{-/-}$ mice (Yamanaka et al. 1994; Cohen-Tannoudji et al. 1995; Phaneuf et al. 1996), but there was no significant difference between double deficient ($HexA^{-/-}$,$NeoIn^{-/-}$) mouse and the $HexA^{-/-}$ mouse in glycolipids content especially in G_{M2}. To see the affect of age on accumulation, 4 – 4.5 months mice's brain gangliosides were analyzed. In this TLC (Figure 3.4) it was seen that the pattern of gangliosides were similar to 2 – 2.5 months aged ones (Figure 3.3). $HexA^{-/-}$ and $HexA^{-/-}NeoIn^{-/-}$ double deficient had accumulation of G_{M2} as seen in 2 – 2.5 months aged ones. Also we observed no difference between single $HexA^{-/-}$ and $HexA^{-/-}NeoIn^{-/-}$ mice in the content of other gangliosides such as G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} in both ages (Figure 3.3, 3.4).

After comparing single $HexA^{-/-}$ with double deficient $HexA^{-/-}NeoIn^{-/-}$ mice we analyzed triple deficient $HexA^{-/-}Neu4^{-/-}NeoIn^{-/-}$ mice to see the additional affect of deficiency of sialidase Neu 1 on the ganglioside degradation pathway. In this analysis

mice with sialidase Neu 4 deficiency (single $HexA^{-/-}$, double $HexA^{-/-}Neu4^{-/-}$ or triple $HexA^{-/-}Neu4^{-/-}NeoIn^{-/-}$) showed a decreased level of G_{M1} ganglioside in comparison to wild type age-matched mouse (Figure 3.5). This is because of the activity of sialidase Neu4 on ganglioside G_{D1a} degradation and this role Neu 4 on ganglioside degradation pathway was previously shown (Seyrantepe et al. 2008). Also $Neu4^{-/-}HexA^{-/-}$ double deficient mice showed increased level of G_{M2} ganglioside compared to wildtype and single $HexA^{-/-}$ mice, as previously shown (Seyrantepe et al. 2010). In the triple deficient ($HexA^{-/-}Neu4^{-/-}NeoIn^{-/-}$) mice there was slightly difference in the content of ganglioside in comparison to double deficient $Neu4^{-/-}HexA^{-/-}$ mice. Moreover, triple mouse's ganglioside pattern was different from the single $HexA^{-/-}$ mice, but it was similar to the double deficient $Neu4^{-/-}HexA^{-/-}$ mouse's ganglioside pattern. This difference was the result of sialidase Neu 4 deficiency, not the result of the sialidase Neu 1 deficiency. To clarify whether the accumulation can be worsening over time or not, we also studied 8 – 12 months aged mice with the same deficiencies. Again we didn't observe different ganglioside pattern in the triple deficient mice when compared to the double deficient $Neu4^{-/-}HexA^{-/-}$ mice (Figure 3.6).

Besides orcinol staining all plates were also stained with resorcinol to analyze the asialo-series gangliosides such as G_{A1}, G_{A2}. Since G_{A2} is the key ganglioside in the bypass pathway (Seyrantepe et al. 2008), we wanted to determine whether G_{A2} levels differ between mice models or not.

In addition to the gangliosides, we aimed to analyze neutral glycosphingolipids but as seen in TLC results (Figure 3.7A, B and 3.8A, B) we didn't get affective separation on TLC. Therefore, we couldn't compare the neutral glycosphingolipids content in different deficient mice brain.

In this study we didn't observe significant affect of sialidase Neu 1 on ganglioside metabolism in mouse models. However according to all these results we can not exclude the possible role of sialidase Neu 1 in ganglioside degradation pathway in mouse. Since double deficient mice have 10% of normal sialidase Neu 1 activity, we speculate that sialidase Neu 1 is acting still enough to degrade G_{M2} in analyzed mice so there is no excessive accumulation is observed. It was reported that there is a "critical threshold" of the enzyme activity that determines the storage of the substrate in lysosomes (Conzelmann and Sandhoff 1983), so 10% activity might be enouh to degrade G_{M2}. Additionally, may be sialidase Neu1 is not the only sialidase and beside

sialidase Neu 1, other sialidases such as sialidase Neu 2 and/or sialidase Neu 3 may be involved in ganglioside degradation pathway in mouse.

4.1. Future Perspective

In the future, we aim further analyses of deficient mice ($Neu4^{-/-}HexA^{-/-}NeoIn^{-/-}$). We will focus on neutral sphingolipids to compare to see the role of sialidase Neu 1 in mice. Lipidomics based researches such as mass spectrophotometer profiling of lipid will be used to analyze these triple deficient mice to determine new lipids. Mass spectrophotometer is more sensitive method and can be used as an alternative to the TLC. Additionally, knockout mouse model of sialidase Neu 1 (enzyme activity will be 0% of the normal) can be generated and breeded with HexA deficient mice to obtain double ($HexA^{-/-},Neu1^{-/-}$) knockout mice. Since sialidase Neu 1 activity will be completely lost, these mice can reveal the potential affect of sialidase Neu 1 in that degration pathway in mice. Besides Neu 1, knockout mouse models of sialidase Neu 2 and sialidase Neu 3 can be generated and breeded with $HexA^{-/-}$ mice to obtain double ($HexA^{-/-},Neu2^{-/-}$ and $HexA^{-/-},Neu3^{-/-}$) and triple ($HexA^{-/-},Neu2^{-/-},Neu3^{-/-}$) knockout mice. Newly generated mouse models can be analyzed to enlight the role of sialidase in mice ganglioside degradation pathway that differs from human.

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