TUMOR NECROSIS FACTOR ALPHA (TNFα) in SANDHOFF DISEASE PATHOLOGY

DELETION OF TUMOR NECROSIS FACTOR-ALPHA (TNFα) AMELIORATES NEURDEGENERATION IN SANDHOFF DISEASE MICE

By

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TITLE: Tumor Necrosis Factor-Alpha (TNFα) Ameliorates Neurodegeneration in Sandhoff Disease Mice

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Abstract

Sandhoff disease (SD) is a monogenic lysosomal storage disorder caused by a lack of a functional β-subunit of the beta-hexosaminidase A and B enzymes. The clinical phenotype of *Hexb*$^{-/-}$mouse model recapitulates the symptoms and signs of Tay-Sachs and Sandhoff diseases in human. To gain insight into the neuropathology of Sandhoff disease, we defined the role of TNFα in the development and progression of Sandhoff disease pathology in mice, by generating a *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ double knock-out mouse. Behavioural testing and immunostaining data revealed the neurodegenerative role of TNFα in disease pathology. Double knock-out mice showed ameliorated clinical course, with prolonged life span. TNFα-deficient Sandhoff mice also demonstrate decreased levels of astrogliosis, and reduced neuronal cell death. Deletion of *Tnfα* in Sandhoff mice inhibited JAK2/STAT3 pathway, implicating its role in glia cell activation. This result points to TNFα as a potential therapeutic target to attenuate neuro-pathogenesis.

To investigate whether blood-derived or CNS-derived TNFα has the major impact on neurological function, we transplanted *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ with bone marrow from either *Hexb*$^{-/-}$*Tnfα*$^{-/-}$or *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice donors. Neurological tests shows a significant clinical improvement for Hexb$^{-/-}$*Tnfα*$^{-/-}$ compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient, regardless the genotype of donor cells. These findings highlight the importance of resident-derived TNFα during the robust neurodegenerative consequences in Sandhoff disease. To understand of the role of microRNAs in Sandhoff pathology, we investigated the miRNA profile in Sandhoff brains. A pattern of dys-regulated microRNAs was evident in Sandhoff CNS. Microarray identified miR-210 and miR-96 dys-regulated pattern in the

CNS of Sandhoff mice. Strikingly, neuronal pentraxin, a putative target gene for miR-210, was induced in Sandhoff brains.

Taken together, this work establishes the proinflammatory role of TNFα in Sandhoff pathology, leading to massive neuro-apoptosis. Importantly, our studies propose that neuronal pentraxin as a novel target gene for microRNA-210 in Sandhoff brain samples, providing a potential modulator of neurodegeneration.

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List of Abbreviations

ACK	Ammonium-chloride-potassium lysing buffer
AD	Alzheimer's disease
BMDMs	Bone marrow-derived macrophages
BMT	Bone marrow transplantation
cIAP-1	Cellular inhibitor of apoptosis protein1
CNS	Central nervous system
DKO	Double knock-out
ELISA	Enzyme-linked immunosorbent assay
GFAP	Glial fibrillary acidic protein
Gsr	Glutathione reductase
HIF1α	Hypoxia inducible factor 1
JAK2	Janus kinase 2
LPS	Lipopolysaccharide
LSDs	Lysosomal storage disorders
Mac-1α	Macrophage antigen alpha
Mcp-1	Monocyte chemotactic protein 1
MEF2C	Myocyte enhancer factor 2C
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
Nptx1	Neuronal pentraxin I
NSCs	Neural stem cells
Nxn	Nucleoredoxin
PD	Parkinson's disease
Prdx1	Peroxiredoxin 1
Ptgs1	Prostaglandin-endoperoxide synthase 1
RIP	Receptor-interacting protein
SD	Sandhoff disease
STAT3	Signal transducer and activator of transcription 3
TLC	Thin layer chromatography
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF2	TNF receptor-associated factor 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	Wild type

Chapter 1

Introduction

1.1.1 Overview

Since their discovery in late 1800s, glycosphingolipids remain ambiguous, mainly due to their intricate biological functions and specificities (d'Azzo, Tessitore, & Sano, 2006). However, glycosphingolipids have vital cellular roles, therefore, resulting in detrimental diseases from dys-regulation of their catabolism (d'Azzo et al., 2006). One striking feature of these molecular species in human is evident in devastating neurodegenerative diseases associated with their excessive subcellular storage (Kolter, Proia, & Sandhoff, 2002). Much attention has been given to find such therapeutic strategies that can treat these diseases.

1.1.2 Glycosphingolipid and ganglioside structure

Glycosphingolipids (GSLs) are a large group of lipids conserved from the simple forms of eukaryotic organisms to human cells and are found as an integral part of cell membranes (Degroote, Wolthoorn, & van Meer, 2004). To note, glycosphingolipids bind with cholesterol and proteins to create micro domains called lipid rafts (Degroote et al., 2004). However, GSLs display great structural heterogeneity (Karlsson, 1970). Furthermore, glycosphingolipids consist of a hydrophobic ceramide backbone attached to

a sugar head group (Degroote et al., 2004). Ceramide moiety acts as a membrane anchor, while the hydrophilic oligosaccharide chain varies in sugar composition and mostly faces extra-cytosolic space (Degroote et al., 2004). The vast existence of different head and backbone groups renders their combinations to generate several thousands of unique molecular structures (Degroote et al., 2004). In addition, the sphingoid bases show variant structures in terms of length, branching and fatty acid saturation (Karlsson, 1970) .

Based on the first oligosaccharide group bound to the ceramide group, GSLs are classified into two main categories- glucose (Glcbeta1-) or galactose (GalBeta1-) classes (Degroote et al., 2004). It is interesting to note that gangliosides constitute about 10 percent of the lipids in neuronal cell membrane (Ledeen, 1984). Another distinctive characteristic of neuronal tissues is the prevalence of lipid-bound sialic acid over the protein-bound sialic acid that exists in peripheral tissues (Ledeen, 1984). Ceramide is considered a robust signaling mediator and functions as the precursor of gangliosides, a sialic acid containing GSLs (d'Azzo et al., 2006). It is noteworthy that Gangliosides are present mostly in the outer leaflet of neuronal cell membranes and a small percentage (10 percent) is localized in the cytoplasmic organelles such as mitochondria and endoplasmic reticulum (d'Azzo et al., 2006). Furthermore, it is now widely accepted that a concentration of gangliosides in various cytoplasmic compartments is totally dependent on the parallel control of their synthesis, degradation and recycling (d'Azzo et al., 2006).

1.1.3 Ganglioside metabolism

The biosynthesis process of gangliosides occurs primarily in the ER and Golgi apparatus (Sandhoff & Klein, 1994). In fact, both membrane bound sialyltransferase and glycosyltransferase mediate the addition of oligosaccharide to the sphingolipid (Huwiler, Kolter, Pfeilschifter, & Sandhoff, 2000). At the cytosol-ER interface, UDP glucose (Glc) Cer glucosyltransferase attaches Glc molecule to Cer moiety to form the basic product of glycolipid that is glucosylceramide (GlcCer). Subsequently, Lactosylceramide (LacCer) is formed by adding galactose (Gal) moiety to GlcCer (Kolter et al., 2002). Lactosylceramide (LacCer) acts as a precursor of GSL series (Sandhoff & Klein, 1994). A step-wise glycosylation process of LacCer takes place to form complex gangliosides (d'Azzo et al., 2006). Particularly, GM3 is transformed to a series of gangliosides by sequential addition of Gal, N-acetylgalactosamine (GalNac), or Sialic acid residues in Golgi complex (Yu, Bieberich, Xia, & Zeng, 2004). Eventually, the final products of ganglioside biosynthesis are transported within budding vesicles to reside in plasma membranes and other intra-cellular membranes (Yu et al., 2004). Similarly, GD3 and GT3 are synthesized through step-wise linking of sugar moieties to LacCer (d'Azzo et al., 2006). Sequential addition of Gal, N-acetylgalactosamine or Sialic acid molecules to GM3, GD3 and GT3 generates a-, b- or c-GSL series, respectively (d'Azzo et al., 2006).

Ganglioside degradation occurs under the control of non-redundant hydrolytic enzymes in lysosomes that function optimally at lower pH (d'Azzo et al., 2006). Saposines or SAPs act as physiological detergents facilitating interactions between the

hydrolytic enzyme and its ganglioside substrate for ideal degradation (Kolter & Sandhoff, 2005). For degradation, it was found that gangliosides which are localized in the cell membrane undergo internalization to reach endocytic-lysosomal system and their sugar chains are exposed to the hydrolytic enzymes (d'Azzo et al., 2006). Hence, specific exoglycosidases start cleaving ganglioside sugar moieties in a sequence (Tettamanti, 2004). For example, multi-sialogangliosides such as GM1 can be degraded to GM2 mono-sialoganglioside (Sano et al., 2009). Furthermore, a specific lysosomal enzyme namely β-N-acetylhexosaminidase A cleaves the GalNac moiety to produce GM3 (d'Azzo et al., 2006). Lysosomal sialidase (neu1) removes sialic acid residues from GM2 to generate its asialo derivative, LacCer (d'Azzo et al., 2006). Sequential removal of sugars from LacCer leads to Cer, which is further processed to form its building blocks: Sphingosine and fatty acids (d'Azzo et al., 2006). Gal- series of gangliosides are found predominantly and represent the main form of lipid in neuronal myelin sheath in humans (Simons & van Meer, 1988).

Besides biosynthesis and degradation process, recent studies have demonstrated that gangliosides can be reused and recycled to form the neuronal cell membrane that indicates the presence of a salvage pathway (Tettamanti, Bassi, Viani, & Riboni, 2003). The latter salvage process explains how neurons save energy and accommodate sharing of gangliosides during cell division (d'Azzo et al., 2006). A balance between ganglioside synthesis and degradation is essentially required to maintain a level of steady-state for gangliosides in various subcellular domains (d'Azzo et al., 2006). The observation that ganglioside homeostasis is maintained under tight control in subcellular spaces, can be

seen as a result of the accurate and timely degradative bioactivity using non-redundant lysosomal hydrolases (Sano et al., 2009). Deficiency of GSL hydrolytic enzymes leads to the storage of lipids in lysosomes and is often manifested by neuronal pathology (Tettamanti, 2004).

1.1.4 Glycosphingolipid trafficking

Perhaps catering to Glycosphingolipids (GSLs) origin and destination, the vesicular transport system would be the main trafficking pathway for GSLs from Golgi to plasma membrane (Degroote et al., 2004). One of the main mechanistic models, that explain the preferred transport of GSLs from Golgi network to plasma membrane, is the lipid raft hypothesis (van Meer, Stelzer, Wijnaendts-van-Resandt, & Simons, 1987). In this hypothesis, GSLs assemble certain proteins such as SNARE and caveolin molecules that introduce transport preference after budding into transport vesicles (Baumgart, Hess, & Webb, 2003). It was demonstrated that ceramide synthesis, the common GSLs precursor, is sequestered between ER and Golgi (Futerman, 2006). Glycosylation of ceramide starts in the Golgi apparatus producing GlcCer and GalCer which moves into transport vesicles moving toward the plasma membrane (van Meer, Wolthoorn, & Degroote, 2003). The retrograde pathway of gangliosides that enable gangliosides to move back from plasma membrane to reach cytoplasmic organelles is still under investigation (Malisan & Testi, 2002).

1.1.5 Glycosphingolipid functions

The observation that the mice deficient of GlcCer-derived GSLs die within the first few days after birth, points to the essential role of GSLs during early development (Yamashita et al., 1999). Furthermore, GM3 synthase knocks out the exhibition of abnormal insulin sensitivity in mice due to decreased phosphorylation of insulin receptors (Yamashita et al., 2003). Interestingly, mice lacking GM2 synthase enzyme show defects in the nervous system and improper spermatocyte differentiation (Takamiya et al., 1996). These results manifest the important role of GSLs during the development of the nervous system, specifically myelin formation (Degroote et al., 2004). Owing to the protein incorporation of GSLs at plasma membrane lipid rafts, GSLs can interact specifically with key molecules to impose crucial functional roles in cell recognition and signaling processes (Brown & Rose, 1992). For example, specific epitopes in the carbohydrate chains of GSLs participate in cell-to-cell interaction, blood grouping antigenicity, and cell-pathogen recognition processes (Karlsson, 1986).

GSLs can modulate signal transduction pathways by binding with membrane receptors, such as receptor-associated protein kinases, and ultimately regulate their underlying signaling cascades (Degroote et al., 2004). For instance, GM3 can inhibit the epidermal growth factor receptor dimerization which is essential for triggering receptor kinase signaling pathway (Zurita, Maccioni, & Daniotti, 2001). Of note, GSL intermediates such as ceramide and Sphingosine-1-phosphate can modulate many intracellular signaling pathways including the apoptotic pathway (Maceyka & Machamer,

1997). In summary, Glycosphingolipids and its ganglioside subclass are vital and integral components of neuronal plasma membranes (Degroote et al., 2004). Glycosphingolipid species play an essential role in cell adhesion (Varki, 1993), membrane permeability (Wertz & van den Bergh, 1998), and the immune system (Hanada, 2005).

1.2.1 Lysosomes and epidemiology of lysosomal storage diseases

All eukaryotic cells contain membrane-bound lytic organelles that were first described by de Duve, which he termed lysosomes (Jalanko & Braulke, 2009; Sandhoff & Kolter, 1996). Lysosomes contain a range of digestive hydrolases that are soluble and optimally active at acidic pH (Futerman & van Meer, 2004). These acid hydrolases, such as proteases, glycosidases, and lipases, reside in the lysosomal lumen (Journet, Chapel, Kieffer, Roux, & Garin, 2002). The lysosomal system is completely backed by autophagosomal and ubiquitin-proteosomal systems, acting as an essential degradative and recycling cell machinery to maintain cellular homeostasis (Walkley, 2009).

Lysosomal storage diseases are caused by deficiency in enzymes required for the normal functioning of lysosomal system and they include a wide range of rare inherited autosomal recessive disorders (Walkley, 2009). It is now evident that about one in 5000 live births will suffer from a lysosomal storage disorder (Vitner, Platt, & Futerman, 2010). Although, of more than 50 known lysosomal storage diseases, the majority can be caused by defects in lysosomal hydrolases. Some LSDs were identified as resulting from the deficiency of non-enzymatic integral membrane proteins (Vitner et al., 2010). The

identification of numerous genetic mutations has become a milestone in observing most of lysosomal storage diseases (Futerman & van Meer, 2004). Indeed, LSDs are considered essentially monogenic inherited disorders in which one protein is typically affected (Futerman & van Meer, 2004).

1.2.2 Organ involvement (CNS pathology) and clinical picture

The issue of predicting the severity of the LSD condition was elucidated by the finding that the degree of residual enzyme activity influences the course of the disease (Futerman & van Meer, 2004). For instance, null mutations lead to severe forms of diseases In-Utero or in early infancy. On the other hand, milder mutations result in juvenile or adult-onset pathology (Vitner et al., 2010). The majority of lysosomal diseases demonstrate widespread organ involvement which includes storage of accumulated substrates in the central nervous system as well as in peripheral viscera (Walkley, 2009). Neurological symptoms are considered a common hallmark of LSDs manifestations, including mental retardation, motor system dysfunction, tremors, behavioural disturbances and blindness (Buccoliero, Bodennec, Van Echten-Deckert, Sandhoff, & Futerman, 2004). Of note, neuropathological manifestations are evident in two thirds of all lysosomal storage diseases (Buccoliero et al., 2004).

1.2.3 Pathogenesis of lysosomal storage diseases

The fact is there are no two lysosomal storage diseases that have same clinical manifestations. This might indicate that various secondary biochemical and cellular pathways play some role in the pathogenesis of LSDs (Buccoliero et al., 2004). It was reported that accumulated lysosomal storage leads to expansion of the endosomal and lysosomal system that would eventually collapse cellular homeostasis (Desnick, Thorpe, & Fiddler, 1976). There appear to be many factors that add to the complexity of neuronal LSDs pathogenesis. The essential role of the lysosomal system in controlling the molecular signaling pathways in neurons indicates the importance of lysosomal function for neuronal cell pathology (Walkley, 2009). LSD classification is mainly based on the biochemical nature of build-up material such as gangliosidosis in which gangliosides are the primary accumulating substrate (Vitner et al., 2010).

1.2.4 Lysosomal storage diseases (LSDs)

Lysosomal storage disorders (LSD) are all characterized by the intra-lysosomal build-up of un-degraded substrates due to deficiency of one or more lysosomal enzymes and/or their cofactors (Hodges & Cheng, 2006). Classified as a GM2 gangliosidoses, Sandhoff disease is a less common form than Tay-Sachs and is caused by a lack of the β -subunit that results in loss of two enzymes β -hexosaminidases A and B bioactivity (Igdoura, Mertineit, Trasler, & Gravel, 1999; Sakuraba et al., 2006). These deficiencies lead to an accumulation of GM2 and asialo-GM2 in neural tissues as well as an

accumulation of globoside, an aminoglycolipid, in viscera (Futerman & van Meer, 2004). Sandhoff and Tay-Sachs patients display similar clinical pictures of neurodegeneration including motor function disturbances, seizures, visual loss, and deafness, which usually end with death in early childhood. Neuronal apoptosis is evident in patient brain samples and in mouse models (Huang, Trasler, Igdoura, Michaud, Hanal, & Gravel, 1997; Wada, Tifft, & Proia, 2000). Although it is clear that the build-up of un-degraded ganglioside substrates is the primary insult to neurons, the exact molecular sequel that converts this primary insult to neuronal apoptosis remains poorly understood (Myerowitz et al., 2002).

1.2.5 Animal models of Gangliosidosis

Generations of knock-out animal models, via targeting the genes of specific hydrolases regulating ganglioside degradation, have been instrumental in enhancing our understanding of the complex pathogenic mechanisms underlying gangliosidosis (Tessitore et al., 2004). For example, Sandhoff human disease has been reproduced in mice which show very similar neurological manifestations to the human disease (Igdoura, Mertineit, Trasler, & Gravel, 1999). However, Tay-Sachs mouse model displays slight neurological pathology in contrary to its human counterpart (Yamanaka et al., 1994). Upon further investigation, it was shown that mouse sialidase can evidently remove sialic acid groups from GM2 generating its asialo derivative, GA2, hence alleviating the GM2 accumulation (Yamanaka et al., 1994). Thus, generating mouse models for gangliosidosis

are considered important tools to reveal molecular mechanistic pathways associated with ganglioside degradation defects (d'Azzo et al., 2006) .

1.3.1 Physiology of the central nervous system

The nervous system is categorized into the central (CNS) and peripheral nervous system (PNS) (Schmidt & Leach, 2003). Collectively, the brain, spinal cord and special sense systems constitute the central nervous system (Schmidt & Leach, 2003). The peripheral nervous system includes the cranial and spinal nerves (Schmidt & Leach, 2003). While peripheral nerves transmit sensory and excitatory signals to and from the spinal cord, the main function of the central nervous system is to interpret and conduct these signals (Schmidt & Leach, 2003). There are two major cell types in the nervous system; neurons and glia (Schmidt & Leach, 2003). Neurons are morphologically classified into unipolar, bipolar and multipolar cells (Fuentes, Canovas, Berndt, Noctor, & Kukuljan, 2012). Glia maintains homeostasis providing support and protection to neurons (Torres et al., 2012). Glia includes many cell types such as astrocytes, microglia, oligodendrocytes, satellite and Schwann cells (Jessen & Mirsky, 2005). While glial cells are abundant and can divide, neuronal cells in the CNS cannot normally undergo mitosis under their native conditions (Schmidt & Leach, 2003). Considering the poor regenerative capacity of the neurons in the central nervous system, it is most fruitful to reduce the damage caused by neuronal death (Rossi & Cattaneo, 2002).

1.3.2 Neurodegenerative diseases

Neurodegenerative diseases are characterized by marked gliosis followed by massive neuronal cell death (Jana, Hogan, & Pahan, 2009). Most importantly, neuronal apoptosis underlies the pathology of several human neurological diseases such as Alzheimer's, Huntington's and Parkinson's (Mattson, 2000). Besides the intrinsic mechanism, which induces neuronal apoptosis, it was found that other cascades of biochemical changes result in neuronal death (Faden, 1996). However, any death cascade is typically opposed by several survival mechanisms that act to regain neuronal homeostasis (Mattson, 2000). The observed commonalities in disease pathology might lead to multimodal neuro-protective treatment in human neurodegenerative disorders (Vajda, 2002).

Neurological manifestation of glycolipid storage diseases shows wide diversity that ranges from adult delayed-onset to devastating acute picture (Jeyakumar, Dwek, Butters, & Platt, 2005). The neurological signs include seizures, ataxia, spasticity, movement disorders, visual loss and developmental delay (Jeyakumar et al., 2005). Indeed, this considerable heterogeneity of clinical picture points to the possibility of the involvement of genetic factors in the etiology of such disorders (Jeyakumar et al., 2005). While delayed-onset mild disease variants result from genetic mutations resulting in relatively stable and functioning residual enzyme, infantile-onset variants are typically due to severe mutations that result in complete absence of residual enzyme activity (Jeyakumar, Butters, Dwek, & Platt, 2002). Currently, the best prognostic indicator of

clinical course and outcome of disease is the level of residual enzyme activity (Walkley, 1995). Furthermore, the wide diversity of the clinical manifestations is explained partly by a perspective geared from an anatomy-function correlation exclusively related to the CNS (Vajda, 2002). For example, lesions of extrapyramidal tract lead to movement disorders, but cortical damage results in seizures and cognitive deterioration (Nicotera & Lipton, 1999). Based on their clinical manifestations, GM2 gangliosidoses are subcategorized into infantile, juvenile and adult-onset variants (Jeyakumar et al., 2002).

1.3.3 Apoptosis and necrotic cell death

Cell death has been typified using specific histological and biochemical criteria (Yakovlev & Faden, 2001; Friedlander, 2003). Unlike necrosis, which is often an integral part of any pathologic process, apoptosis is a programmed cell death that occurs in specific pathological and physiological conditions (Friedlander, 2003). The histologic signatures of necrotic cell death are cellular and nuclear swelling as well as disintegration of organelles. Subsequently, the nuclear and cytoplasmic membrane rupture and DNA degrades (Martin, 2001). In contrast to necrosis, apoptotic cells are characterized by preservation of cell membrane and organelle integrity, cell shrinkage, membrane blebing and cleavage of genomic DNA (M. Li et al., 2000). Furthermore, necrotic process is rapid and extremely difficult to reverse. On the other hand, apoptosis, as a feature of physiology, could be regulated and even prevented (Friedlander, 2003).

Apoptosis is a vital mechanism by which multicellular organisms remove un-required cells to maintain normal cell turnover and homeostasis (Elmore, 2007; Sadowski-Debbing, Coy, Mier, Hug, & Los, 2002). Inappropriate apoptosis is associated with various human disease pathologies such as neurodegenerative diseases (Sadowski-Debbing et al., 2002). Renehan et al deduced that a huge number of cells (around 10 billion cells) have to undergo mitosis to compensate apoptotic cells every single day in the human body (Renehan, Booth, & Potten, 2001). More recently, neuronal cells, that fail to establish functional synaptic networking, have been found to undergo apoptosis (Nijhawan, Honarpour, & Wang, 2000; Opferman & Korsmeyer, 2003). Furthermore, apoptosis is required to eliminate activated inflammatory cells that are involved in tissue healing (Greenhalgh, 1998). Currently, there is no doubt that apoptosis is under tight regulation (Elmore, 2007).

Neuronal apoptosis is a common feature in disease pathology of patients with Alzheimer's, Amyotrophic lateral sclerosis, or Parkinson's diseases (Sadowski-Debbing et al., 2002). Perhaps owing to that fact that apoptotic process lasts only several hours, it is quite difficult to capture many apoptotic cells at any one time (Sadowski-Debbing et al., 2002). For this reason, cell culture models of neurodegenerative disorders were used extensively to demonstrate the apoptotic model of neuronal cell death (Sadowski-Debbing et al., 2002). Furthermore, it was demonstrated that, beside the only few cells showing the classic features of apoptosis, there were other dying cells that did not display apoptosis, suggesting apoptosis is only one of many mechanisms upstream of neurodegeneration (Troncoso, Sukhov, Kawas, & Koliatsos, 1996). For example, in

Alzheimer's disease, a common neurodegenerative disease, neuronal death occurs in the limbic regions of the cerebral cortex (Hyman & Yuan, 2012). Recent findings have revealed that amyloid-β can lead to apoptosis in cultured Alzheimer's neurons (Plesnila et al., 2001). In addition, excessive DNA fragmentation and caspase activity have been shown in apoptotic neurons associated with aggregates of hyper-phosphorylated tau protein and β-amyloid plaques in Alzheimer's disease CNS pathology (Cai, Frey, Sanna, & Behnisch, 2010). Moreover, neuroprotection from Alzheimer's associated apoptosis has been achieved by utilizing neurotrophic factors and cytokines (Gagliardini et al., 1994).

1.3.4 Mechanisms of apoptosis

The enhancement of our understanding of the molecular mechanisms of apoptosis in neurodegenerative diseases has become a milestone requirement for effective therapeutic strategies (Friedlander, 2003). There are many cellular events that can initiate neuronal apoptosis including excitotoxicity, oxidative stress, and neuroprotective factors withdrawal (McKay, Purcell, & Carew, 1999). Excitotoxicity is thought to play a role in neuronal apoptosis (Vajda, 2002). Glutamate buildup at postsynaptic receptors, enhanced sodium and calcium influx, and subsequently membrane depolarization induces calcium influx, as a result mediating neuronal death cascade (Olney, 1969). In fact, excitotoxicity has been found as a common apoptotic initiator mechanism in both acute and chronic

neurodegenerative diseases, in particular, Alzheimer's and motor neuron disorders (Wong, Rothstein, & Price, 1998).

Interestingly, apoptotic process in one neuronal cell can alter neighboring cell homeostasis (Friedlander, 2003). During a stroke, neurons exposed to ischemia produce proapoptotic factors (tumor necrosis factor-α, interleukin-1 β, and reactive oxygen species) (Yuan & Yankner, 2000). These diffusible factors mediate cell death cascade in neighboring cells that eventually die (Ona et al., 1999). The same phenomenon occurs in amyotrophic lateral sclerosis (ALS), a chronic neurodegenerative disease (Friedlander, 2003). Hence, a promising therapeutic avenue would be to adapt an anti-apoptotic approach, not only to limit cell death process in initial neurons but also to inhibit proapoptotic mediators that may trigger apoptotic cascade in neighboring cells (Friedlander, 2003).

In addition to neuronal cell death, neuronal dysfunction such as altered synaptic function has been observed in a number of chronic neurodegerative diseases, including Parkinson's, Alzheimer's and Huntington's (Scheff & Price, 2003). Of note, an anti-apoptotic approach was not sufficient to rescue the neurological manifestations of prion disease in mice, suggesting that, the addition of therapeutics that prevent synaptic dysfunction would result in a more effective therapeutic impact on prion disease pathology (Chiesa et al., 2005).

Another trigger of neuronal apoptosis is excessive oxidative stressors that inflict direct damage on DNA and cellular proteins (Sastry & Rao, 2000). Metabolic stress, due

to aging or glycolipid storage disorder, can initiate neuronal cell death (Beal, 1995). Of note, defective mitochondrial function is increasingly implicated as an important factor in the etiology of neuronal apoptosis (Beal, 1996). The observation that patients with mitochondrial disorders have pathogenic neuronal death and glial activation, might be seen as an evidence for involvement of mitochondria in the causality of apoptosis (Vajda, 2002). Most importantly, upregulation of inflammatory genes and subsequently increased expression of inflammatory cytokines were identified as molecular changes associated with neuronal apoptosis in brain and spinal cord disorders (Wada, Tifft, & Proia, 2000).

1.4.1 Inflammation and immune system

Inflammation is a major host protective response characterized by an excessive invasion of circulating immune cells and activation of CNS resident microglia (Lucas, Rothwell, & Gibson, 2006; Zipp & Aktas, 2006). Clinically, peripheral inflammation has classic features such as swelling, redness and pain (Niederkorn, 2006). Inflammation is a cardinal component of a physiological response that is essential during damage repair (Chavarria & Alcocer-Varela, 2004). Nevertheless, inappropriate, prolonged or massive inflammatory response deviates the response from its protective function to act as a detrimental factor (Niederkorn, 2006). Thus, neuroinflammation is a two-edged sword that requires tight control because it might be a harmful process if it is excessively or deficiently implemented (Minghetti, 2005). Indeed, CNS regulates several aspects of neuroinflammatory response (Lucas et al., 2006). It is now widely accepted that brain

cells can express proinflammatory mediators such as cytokines and chemokines, which can rapidly induce inflammatory response and mediate recruitment of immune cells into brain parenchyma respectively (Rothwell & Luheshi, 2000; Kulkarni, Kellaway, Lahiri, & Kotwal, 2004). Neuroinflammation is currently defined to comprise any pathophysiological condition that includes molecular neuro-immune crosstalk, regardless of its etiological factor (Lenzlinger, Morganti-Kossmann, Laurer, & McIntosh, 2001).

1.4.2 Cytokines and neuroinflammation

It was found that a potent inducer of fever, interleukin (IL-1β) can act centrally via thermoregulatory and neuro-endocrine mechanisms (Lucas et al., 2006). Furthermore, it is noteworthy that IL-1 affects memory, feeding and sleep (Pollmacher, Haack, Schuld, Reichenberg, & Yirmiya, 2002). There is increasing evidence that IL-1β is released from microglia and it has been involved in a number of neurodegenerative diseases (Lucas et al., 2006). TNFα has been described as a major mediator of several neuroinflammatory conditions and its action is mediated via CNS expressed TNFR1 and TNFR2 receptors (Lucas et al., 2006). Moreover, microglia, astrocytes and neurons secrete TNFα which binds to TNFR1 receptors and can mediate neuronal apoptosis (Lucas et al., 2006). Like TNFα cytokine, IL-6 is able to exert both deleterious and beneficial actions in neurons (Van Wagoner & Benveniste, 1999). TNFα can also induce the secretion of IL-6, high lightening the intricate nature of role of cytokines on neuropathology (Lucas et al., 2006).

1.4.3 Cellular components of neuroinflammation

Activated T cells have been recognized to trigger inflammatory responses in brain parenchyma that lead to BBB damage with subsequent recruitment of other immune cells (Chavarria & Alcocer-Varela, 2004). It has been shown that activated T cells are the source of cytokine profile including TNFα and INF-γ, as well as chemokines such as MIP-1α/CCL3 and MIP1β/CCL4) (Hickey, 1999). Other white blood cells including NK cells, dendritic cells, mast cells and B lymphocytes, can infiltrate BBB to contribute to the pathology of neuroinflammation (Hickey, 1999). The observation that myelin auto-antibodies were found in the context of MS disease indicates that B lymphocytes participate in neuroinflammation (Genain, Cannella, Hauser, & Raine, 1999). Moreover, mast cells have been identified in the CNS of EAE patients (Benoist & Mathis, 2002). NK brain cells can secrete IFN-γ in response to IL18 and 1L12 interleukins secreted by activated macrophages (Pagenstecher et al., 2000). Since Matyszak and Perry work, it is known that dendritic cells (DCs), migratory CNS cell population, can process and present antigens to T cells. Dendritic cells were found dispersed around brain vessels of healthy mice and humans (Matyszak & Perry, 1996; Serot, Foliguet, Bene, & Faure, 1997). Furthermore, DCs have been found infiltrating brain parenchyma as a part of many neuroinflammatory responses (McMahon, Bailey, Castenada, Waldner, & Miller, 2005).

1.4.4 The participation of the CNS cells in neuroinflammation

It is noteworthy that CNS cells have been demonstrated to be essential players during the pathology of neurological inflammatory conditions (Figure 1.1).

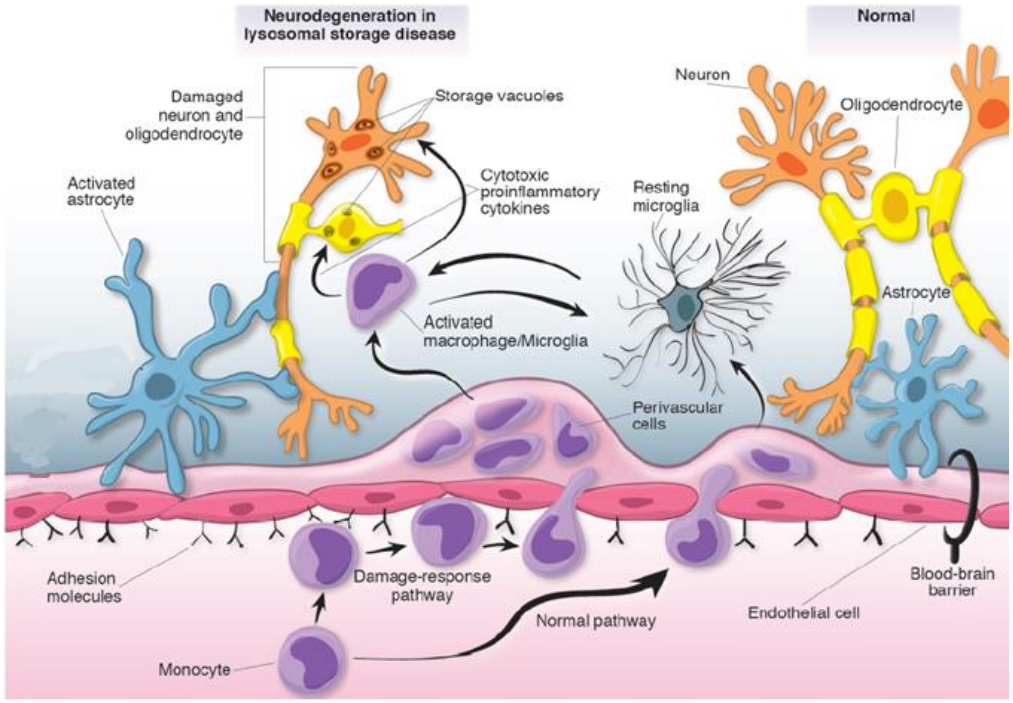

Figure 1.1 Role of the CNS cells in lysosomal storage disease

Microglia cells are normally recruited from blood-derived monocytes**.** In lysosomal storage diseases, lipid storage is associated with extensive activation and proliferation of microglia and astrocytes. Activated glia cells secrete proinflammatory cytokines, resulting in massive neuronal apoptosis. Image taken from Wu & Proia, 2004.

Microglia and astrocytes as well as neurons have the capacity to secrete a wide array of immunological proteins such as cytokines, chemokines, and complement molecules (McGeer & McGeer, 2001). Microglia was found to play a fundamental role in the pathogenesis of neuroinflammatory conditions (Zipp & Aktas, 2006). Resident glia has similar morphology to activated peripheral tissue macrophages and display high capacity to be highly immunoreactive after brain insult (Farber & Kettenmann, 2005). There is a large body of evidence indicating that microglia is differentiated from extravasated monocytes (Bechmann et al., 2005). Whether activated microglia has deleterious or beneficial consequence on neuroinflammatory condition, is still under debate (Minghetti, 2005).

It is well known that cultured microglia secrete vast number of proinflammatory molecules such as TNFα, upon its stimulation with LPS (Akiyama et al., 2000; Minghetti & Levi, 1998). However, persistent LPS stimulation of cultured microglia could render these microglia insensitive to further stimulation or even display anti-inflammatory capacity (Fassbender et al., 2004). Furthermore, neuro-microglial crosstalk has a major impact on determining the prominent aspect of the microglial dual function (Polazzi & Contestabile, 2002). It was demonstrated that co-culturing microglia with apoptotic neuronal cells inhibits proinflammatory cytokine release and enhances the secretion of anti-inflammatory molecules such as transforming growth factor-β (Minghetti, Ajmone-Cat, De Berardinis, & De Simone, 2005). Modulating glial function may represent a novel therapeutic approach of reducing neuroinflammatory conditions (Minghetti, 2005).

Astrocytes are the most prevalent cell population in the CNS which exhibit essential regulatory role on the neurotransmitter concentrations in the synapse (Philips &Robberecht, 2011). Although astrocytes are not considered as immune cells per se, they exert immunological functions upon certain conditions (Philips & Robberecht, 2011). Moreover, astroglial activation is associated with significant increase in the expression level of glial fibrillary acidic protein (GFAP) (Philips & Robberecht, 2011). While the role of neurons during neuroinflammation remains poorly understood, neurons can secrete inflammatory molecules such as cytokines, suggesting a possible role in modulating CNS inflammation (Flugel et al., 2000). Furthermore, it was observed that brain endothelial cells have an important role in maintaining the integrity of BBB and regulating the flow of blood immune cells into brain parenchyma (Wong, Prameya, & Dorovini-Zis, 1999). Taken together, neurons, astrocytes and microglia participate in an intricate assembly to regulate the intensity and fate of the neuropathological inflammation (Chavarria & Alcocer-Varela, 2004).

1.4.5 Secondary inflammation in brain diseases

Stroke and Alzheimer's disorders are common examples of neuropathological conditions in which non-inflammatory insult initiates neuronal damage (Zipp & Aktas, 2006). It was found that inflammation can occur as a relevant secondary reaction in those disorders (Babcock, Kuziel, Rivest, & Owens, 2003). Although immune response has a central role to protect the organism from invading harmful agents and promote healing,

there is increasing body of evidence pointing to the detrimental role of secondary inflammation in CNS (Schwartz, Shaked, Fisher, Mizrahi, & Schori, 2003; Kerschensteiner & Hohlfeld, 2003). Currently, there is a large body of evidence suggesting the involvement of immune cells in the development of neuronal inflammation. In adrenoleukodystrophy (ALD) pathology, although the primary insult is non-inflammatory, the brain shows massive infiltrate of immune cells including T cells and microglia (Saleh et al., 2004). To take another example, higher concentrations of inflammatory cytokines and chemokines were identified in brain CSF (Akiyama et al., 2000). Thus, CNS disorders, with divergent etiology, might have common pathological mechanisms (Zipp & Aktas, 2006).

1.5.1 Cytokines in the CNS

Cytokines comprise a number of cellular polypeptides including tumor necrosis factors-alpha (TNFα), interleukins (IL-1β, IL-6, and IL-10), chemokines, interferons (IFN) and growth factors (Allan & Rothwell, 2001). They have been implicated in diverse biological processes such as immune response, cell death and tissue injury (del Zoppo et al., 2000). Several cytokines, such as TNFα, are expressed as precursors that must be cleaved to be biologically active (Karkkainen, Rybnikova, Pelto-Huikko, & Huovila, 2000). Intricate networking has been described for cytokine bioactivities including ligand/receptor overlapping, potentiation and antagonistic actions (Allan & Rothwell, 2001). For example, while individual cytokine is less likely to kill the cell, a

mixture of cytokines shows a synergetistic neurotoxic effect (Hu, Peterson, & Chao, 1997). However, in-vitro experiments show that TNFα alone can directly invoke cell death (Reimann-Philipp, Ovase, Weigel, & Grammas, 2001).

It is noteworthy that a peripheral pro-inflammatory cytokine could act as an anti-inflammatory in the CNS (Allan & Rothwell, 2001). Changes of cytokine expression have been demonstrated in the context of neurodegeneration suggesting indirect evidence of cytokine participation in neuronal cell death (Allan & Rothwell, 2001). Moreover, it was shown that the level of cytokine expression is highly correlated with the severity of disease (Griffin et al., 1994). While rapid TNFα upregulation was observed within one hour after brain injury, other cytokines have delayed expression profile (Buttini, Sauter, & Boddeke, 1994). This finding may explain in part the complexities of cytokine bioactivities in CNS in which their temporal profile is required to accurately comprehend their discrete actions in neurodegeneration (Allan & Rothwell, 2001). It is likely that cell-specific cytokine expression can determine neuronal cell fate (Allan & Rothwell, 2001). For example, astrocytic expression results in neuronal pathology, not the neuronal overexpression of TNFα (Akassoglou, Probert, Kontogeorgos, & Kollias, 1997). Paradoxically, recent in-vitro studies show that TNFα may be neuroprotective (Bruce et al., 1996).

One plausible reason for this contradiction is the heterogeneity of CNS cell populations where cytokine dose and duration is highly relevant to pathology outcome (Allan & Rothwell, 2001). In general sense, TNFα and IL-1 are considered pro-

inflammatory while IL-10 is neuroprotective (Allan & Rothwell, 2001). There is increasing body of evidence that cytokines are involved indirectly in the development of neuronal cell death (Allan & Rothwell, 2001). For example, potent inhibition of endogenous TNFα, using neutralizing antibodies, highly dampens the levels of neuronal cell death in brain of ischemic mice (Mayne et al., 2001). However, a neuroprotective role of endogenous TNFα was concluded in TNFR1 knock-out mice in which a significant increase in brain injury is observed (Bruce et al., 1996). In contrast, TNFα knock-out mice displayed rapid recovery from acute brain trauma (Scherbel et al., 1999). Moreover, TNFα administered before but not at the time of brain insult shows neuroprotective actions (Ohtsuki, Ruetzler, Tasaki, & Hallenbeck, 1996). These accumulating data indicate the dual function of TNFα that might aggravate early neuronal damage or enhance recovery (Allan & Rothwell, 2001).

1.5.2 TNFα Biology

Many immune related cell types can produce TNFα including macrophages, T cells, NK cells, neutrophils, mast cells, and granulocytes (Smookler et al., 2006). However, it was shown that non-immune cells can also express TNFα including fibroblasts, neurons, keratinocytes, endothelial and smooth muscle cells (Tracey, Klareskog, Sasso, Salfeld, & Tak, 2008). In the CNS, it is likely that all cells can produce TNFα including neurons, microglia, astrocytes and endothelial cells (Chung et al., 2005). Significantly, most cells show at least some TNFα responsiveness affecting cell

proliferation, survival, differentiation, apoptosis and immune organ developments (Kollias, Douni, Kassiotis, & Kontoyiannis, 1999).

TNFα is expressed as a cell surface-bound precursor (a homotrimer of 26-kDa monomers) which has to undergo proteolytic cleavage by TNFα converting enzyme (TACE) to produce soluble non glycosylated protein (tmTNFα, a homotrimer of 17-kDa monomers) (Moller & Villiger, 2006). Both soluble and membrane-bound TNFα ligands are biologically active and can bind to their cognate receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Tracey et al., 2008). TNFα forms exert their biological activities through interaction with their related membrane receptors, TNFR1 (TNF receptor type 1; CD120a) and TNFR2 (TNF receptor type 2; CD120b) (Wajant, Pfizenmaier, & Scheurich, 2003; Locksley, Killeen, & Lenardo, 2001). In contrast to TNFR1 being expressed in most cells, TNFR2 expression is highly controlled thus found primarily in immune system cells. Subsequently, TNFR1 is recognized as the key player in TNFα signaling in most tissues while TNFR2 plays a major role in the lymphoid system (Arsenijevic et al., 2000). However, both TNFα receptors could bind not only the membrane-bound TNFα and the cleaved soluble mature TNFα, but also the lymphotoxin-a (LTa) (Marino et al., 1997). The extracellular domain of each TNFR1 and TNFR2 contains four “Cysteine-Rich-Domains” (CRDs) which fit in the lateral grooves between the protomers of the ligand trimer (Arsenijevic et al., 2000). In the absence of the ligand, these domains mediate the homophilic interaction of the receptor molecules thus keeping the receptors in a silent, homotrimerized status 1. However, the cytoplasmic domains of

the death containing TNFR1 and TRAF-interacting TNFR2 function as a docking site for signaling adaptor molecules (Wajant et al., 2003).

The binding of TNFα to death-domain containing TNFR1 triggers a series of intracellular signaling events beginning with dissociation of the silencer of death domain (SODD) protein from TNFR1's intracellular domain (ICD) (Wajant et al., 2003). Subsequently, the aggregated TNFR1-ICD is recognized by the adaptor protein TNFα receptor–associated death domain (TRADD), which functions as an assembly platform for recruiting and binding additional adaptor proteins: receptor-interacting protein (RIP), TNFR–associated factor 2 (TRAF2), and Fas-associated death domain (FADD) (Chen & Goeddel, 2002). As a result, key enzymes recruited to TNFR1 could initiate a cascade of signaling pathways (Chen & Goeddel, 2002). For instance, FADD-mediated recruitment of caspase 8 and 10 facilitates their autoproteolytic activation and then initiates a protease cascade that leads to apoptosis (Wajant et al., 2003). Thus, TRAF2, functioning as a receptor proximal adaptor (via TRADD binding) recruits not only cellular inhibitor of apoptosis protein-1 (cIAP-1), two anti-apoptotic proteins but also recruits the IKK complex to the TNFR1 signaling platform. In addition, it recruits and activates a cascade of kinases resulting in the activation of c-Jun NH2-terminal kinase (JNK) which could phosphorylate c-Jun to serve as a transcription factor (Wajant et al., 2003).

Lastly, the protein kinase RIP activates the transcription factor NF-kB via its ability to interact with NEMO which is crucial to the functioning of a third line of the

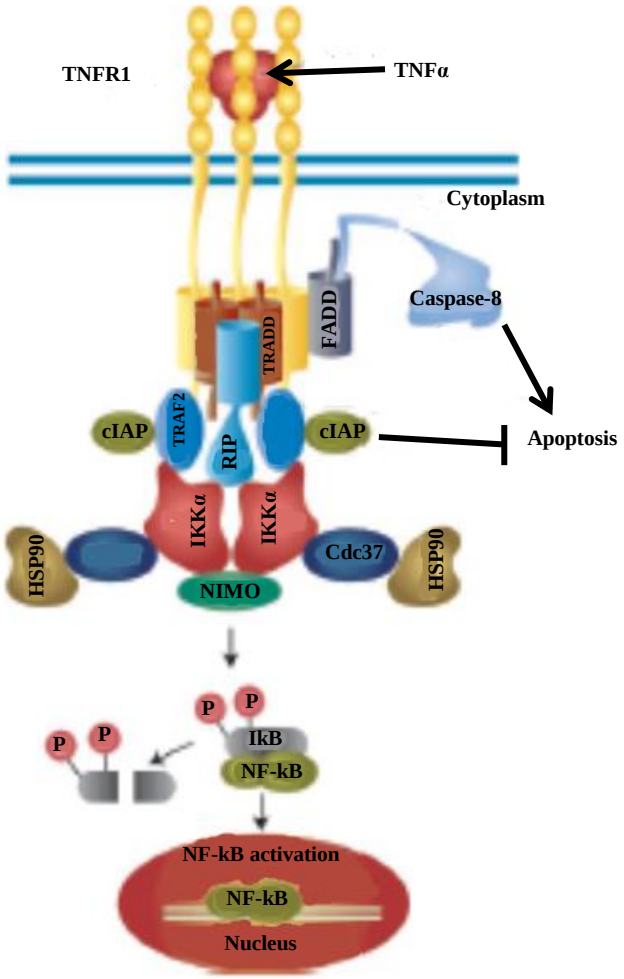

Figure 1.2 TNFR1 signaling

The binding of TNFα to death-domain containing TNFR1 triggers a series of intracellular signaling events. TNFR1's intracellular domain (ICD) binds to adaptor molecules including TRADD, FADD, and RIP. FADD recruits caspase-8, resulting in apoptosis. TRAF2 recruits an anti-apoptotic cIAP molecule. RIP activates IKK complex, ending with NF-kB activation. Image reproduced from Chen & Goeddel, 2002.

TNFR1 signaling network (Wajant et al., 2003). Although, TNF-induced NF-kB activation mechanism is quite understood comparable to the termination mechanism, TNF-induced NF-kB termination mechanism could be mediated via either internalization of sTNFα and TNFR1 complexes and their secondary lysosomal degradation or via NF-Kb dependent upregulation of NF-kB inhibitory proteins (Wajant et al., 2003). Consistent with its being a non-death domain containing receptor, TNFR2 shows a neuroprotective function as recently demonstrated in a murine model of retinal ischemia (Fontaine et al., 2002). However, the use of agonistic TNFR2 specific antibodies triggers an apoptotic pathway via indirect mechanisms including upregulation of tmTNFα and depletion of TRAF2 and TRAF2-associated protective factors (Chen & Goeddel, 2002). Recently, it has been demonstrated that there is a close crosstalk between TNFR2 and TNFR1 through TRAF2 (Wajant et al., 2003).

1.5.2.1 Tumor necrosis factor in the immune system

Most of knowledge regarding the role of TNFα ligands and receptors in innate and adaptive immunity is based on studies involving animal models (Pasparakis et al., 1997). Since Pasparakis's (Pasparakis et al., 1997) work demonstrating that TNFα ligand-deficient mice have partially disorganized B-lymphoid follicles, it is known that TNFα is required but not essential for immune cell follicular formation. TNFα can, moreover, up-regulate the expression of a variety of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and C-C

chemokine ligands (CCL) such as CCL19 and CCL21 (Aloisi & Pujol-Borrell, 2006). Furthermore, the discovery that TNFα enhances dendritic cell migration and ultimately antigen processing, has bolstered the role of TNFα in modulating adaptive immunity (Kim & Teh, 2004). One striking feature of TNFα is its dual role, either stimulatory or inhibitory of immune system, based on timing and levels of TNFα concentration (Tracey et al., 2008). For instance, TNFα can augment immunoreactivity, if administered early, but not after 4 weeks of age, in Type-1 diabetes-prone mice (Yang et al., 1994).

1.5.2.2 Tumor necrosis factor in inflammation and disease

During the last decades, the implication of TNFα in neuroinflammatory conditions has been largely studied, essentially when TNFα expressed in high concentration (Tracey et al., 2008). However, the plethora of these studies demonstrates that TNFα is a central mediator to immunity, inflammation and apoptosis via its NF-kB regulation (Balkwill & Coussens, 2004). For example, it was shown that TNFα can induce expression of a cascade of inflammatory cytokines such as IL-1β, IL-6 and C- reactive protein, ultimately augment inflammation in Crohn's disease (Tracey et al., 2008). It is noteworthy that TNFα exerts its activity within an intricate interplay of inflammatory mediators in which potentiating and inhibiting feedback loops orchestrate the duration and pathogenic fate of inflammation (Tracey et al., 2008). Thus, it is now widely accepted that TNFα is an early and cardinal mediator of downstream inflammatory processes (Tracey et al., 2008). In

vivo, TNFα is best defined for its ability to contribute to neuronal apoptosis in brain ischemia and viral HIV-1 infection (Venters, Dantzer, & Kelley, 2000).

1.5.2.3 TNFα and apoptosis

TNFα has been implicated as both mediator and inhibitor for cellular apoptosis, depending on complex networking between the surrounding microenvironment of the cell and its metabolic condition (Tracey et al., 2008). For instance, tmTNFα can inflict neuronal cell damage by binding to neighboring TNFα receptors, or promote cell survival by acting as a receptor to enhance apoptotic inhibitory signaling into tmTNFα bound cell (Eissner et al., 2000; Park et al., 1998). TNFα, in addition, can promote apoptosis via its interaction with receptors that can bind to Fas associated protein with death domain (FADD) (Park et al., 1998).

1.5.3 Regulation of cytokine bioactivity

The actions of cytokine bioactivities are regulated at several post-transcriptionally processes including cleavage, cellular release and receptor signaling (Allan & Rothwell, 2001). For example, TNFα binding protein (TBP), a physiological TNFα inhibitor, suppresses neuronal apoptosis in mice via competing with cell surface bound TNFα receptor (Shohami, Bass, Wallach, Yamin, & Gallily, 1996). Furthermore, TNFα can

initiate auto-regulatory feedback loop by evoking expression of IL-10 which itself reduces TNFα expression (Zhai, Futrell, & Chen, 1997a).

Recent findings have revealed that the regulation mechanism of cytokine expression and release is closely overlapping (Lucas et al., 2006). For instance, both TNFα and IL-1β can induce IL-6 and TGF-β (Chao, Hu, Sheng, Tsang, & Peterson, 1995). Most importantly, it was shown that the principal trigger for cytokine expression is the immense neuronal activity which itself manifested before actual neuronal death (Jander, Schroeter, Peters, Witte, & Stoll, 2001). Following brain injury, it was described that massive release of excitatory peptides, such as post-synaptic density protein-95 (PSD-95) can modulate the expression of inflammatory cytokines including TNFα, IL-1, IL-6 and INF α/γ (Savinainen, Garcia, Dorow, Marshall, & Liu, 2001).

1.5.3.1 Mechanism of TNFα action

The data from Venters and colleagues that TNFα kills neurons because it silences the survival signaling of insulin-like growth factor 1, might shed light on another cytokine mechanism to mediate neuronal cell death (Venters et al., 1999). Glia cells, the main target of cytokines including TNFα, can affect neuronal survival by exerting neurotoxic and neuroprotective bioactivities (Giulian, Vaca, & Corpuz, 1993; Venters et al., 1999). For instance, TNFα can invoke astrocytes to produce a number of neuroprotective factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF). TNFα, on the other hand,

can induce microglia to express neurotoxic molecules (Raivich et al., 1999). TNFα can modulate neuronal synaptic transmissions by increasing astrocytic intracellular Ca^{2+} and ultimately augmenting depolarization (Koller, Trimborn, von Giesen, Schroeter, & Arendt, 2001). While TNFα has shown multiple indirect mechanisms to mediate neuronal cell death, in-vivo experiments have shown that TNFα acts directly on neurons and induce expression of BCL proteins via NF-kB activation mechanism (Tamatani et al., 1999).

1.5.3.2 Other actions of TNFα

TNFα has been implicated as mediator of blood brain barrier (BBB) disruption, neurotoxin release from vascular endothelium and upregulation of adhesion molecules (Bonmann, Suschek, Spranger, & Kolb-Bachofen, 1997). Probably these effects of TNFα contribute to leukocyte invasion into brain parenchyma and ultimately participate in modulating CNS neurodegeneration (D. Wong & Dorovini-Zis, 1992). Furthermore, TNFα has ancillary indirect effects on neurodegeneration which is manifested by its actions on hypothalamus to induce fever (Rothwell & Hopkins, 1995).

1.5.4 Genetic modification of TNFα in animal model

Much evidence has accumulated from overexpression or knock-out studies implicating the involvement of TNFα in CNS inflammation (Moller & Villiger, 2006).

Wide array of TNF-transgenic mice experiments were developed mostly on a C57BL/6 background (Moller & Villiger, 2006). Mice that express a macrophage-specific human TNFα develop inflammatory polyarthritis (Keffer et al., 1991). It is noteworthy that human TNFα exerts its bioactivities entirely upon binding to murine TNFR1 (Moller & Villiger, 2006). In contrast, murine TNFα can bind to both receptors: TNFR1 and TNFR2 (Moller & Villiger, 2006). These findings may explain in part murine, but not human, TNFα overexpression results in spondylitis and inflammatory bowel disease (P. Li & Schwarz, 2003).

Other mice models including mice deficient in TNFα and TNFRs have produced contradicting results (McCoy & Tansey, 2008). While some studies show that single or double TNFR knock-out mice are not protected from motor neuron loss (Rousselet et al., 2002), other experiments show that double knock-out genetic approach for TNFRs is neuroprotective (Sriram et al., 2002). For example, it was found that TNFR deficiency results in aggravated brain infarction and increased oxidative stress in middle cerebral artery occlusion (MCAO) mouse model (Waage & Bakke, 1988). Furthermore, it was shown that TNFR1 receptor can signal both apoptotic and protective pathways (de Waal Malefyt et al., 1993). This divergent action of TNFR1 signaling transduction bifurcates at TRADD which interacts with either FADD to induce NF-kB mediated neuroprotection or TRAF2 to induce cell death (de Waal Malefyt et al., 1993). The issue of dissecting the role of TNFα and its receptors using solely genetic approach is complicated by the recognition of TNFα ligand by other receptors. In addition, TNFα receptor deficient mice might develop compensatory mechanisms due to protracted removal of TNFα effects

(Shohami, Ginis, & Hallenbeck, 1999). Most importantly, TNFα lacking mice show reduced neuronal dysfunction but not neuronal loss, in MPTP brain intoxication experiments (Ferger, Leng, Mura, Hengerer, & Feldon, 2004). Along the same line, induction of brain trauma in TNFR deficient mice leads to faster recovery compared to control mice (Gerard et al., 1993). These conflicting results can be explained by the assumption that different brain injury approaches might have various distinct influences upon the role of TNFα and its receptors in mediating neurodegeneration (McCoy & Tansey, 2008). One plausible reason for these conflicting results might be due to the fact that TNFα has a biphasic response in which TNFα exerts neurotoxic effect during only the early (24-48 h) post-injury phase (Shohami et al., 1999). In brief, the final outcome of disease pathology is highly dependent on the exact concentration and timing of TNFα (Shohami et al., 1999).

1.5.5 Blockade of TNFα in human disease

Many TNFα blockade drugs were identified as an effective treatment for peripheral inflammatory conditions such as rheumatoid arthritis (RA) (Moller & Villiger, 2006). The mode of actions of these drugs is either reducing the levels of soluble TNFα ligand, or binding to membrane bound TNFα (D'haens et al., 1999). A number of pharmacological therapeutics has been introduced as potential treatments for post-traumatic and ischemic CNS inflammatory conditions (Shohami et al., 1999). Administration of cyclosporine A, an inhibitor of TNFα production, has been reported as

a neuroprotective in CNS injury (Shohami et al., 1999). Thalidomide, another drug that acts post-transcriptionally to selectively reduce the synthesis of TNFα by enhancing the degradation of TNFα mRNA, is currently used in the treatment protocol of HIV-diseased patients (Moreira et al., 1993).

It is currently believed that TNFα -induced expression of IL-10 is providing a negative feedback for TNFα production (Zhai, Futrell, & Chen, 1997b). Thus, IL-10 administration short time (1 hour) before traumatic brain injury was found neuroprotective in rats (Knoblach & Faden, 1998). Neutralization of TNFα action by using either soluble antibody polypeptide or receptor is considered as a prominent therapeutic strategy for CNS inflammatory conditions (Shohami et al., 1999). Pretreatment of cerebral ischemic and CNS traumatic injury with monoclonal anti TNFα antibodies improved neurological outcome (Lavine, Hofman, & Zlokovic, 1998). It is possible that this clinical outcome has been achieved due to decreased expression of intracellular adhesion molecule -1 (ICAM-1). Preconditioning rat models with brain traumatic injury by TNFα binding protein (TBP), another TNFα neutralizing agent, shows attenuated neuronal loss and enhanced motor neuron function (Shohami et al., 1996).

1.6.1 Sandhoff disease

Sandhoff disease is a lysosomal storage pathology that induced by the absence of both β-hexosaminidases A and B (Myerowitz et al., 2002; Arthur, Lee, Snyder, &

Seyfried, 2012). Since GM2 ganglioside is catabolized only by β-hexosamindase A hydrolase, GM2 ganglioside accumulates in the deficient cells (Myerowitz et al., 2002). Lysosomal hydrolase, β-hexosamindase A consists of two subunits- α and β (Kyrkanides et al., 2012). Each subunit is encoded in humans by different genes, i.e., HEXA and HEXB, on chromosome 15 and 5 respectively, while the murine HexA and Hexb genes are located on chromosome 9 and 13 respectively(Kyrkanides et al., 2012). Thus, three isoforms of β-hexosamindase originates from various combinations of these subunits. β-hexosamindase isoforms include HEXA (α/β subunits), HEXB (β/β subunits) and HEXS (α/α subunits) (Kyrkanides et al., 2012).

1.6.1.1 Phenotypic variation between affected Sandhoff mice and patients

Deletion of the murine Hexb gene leads to a Sandhoff clinical picture which is closely comparable to its human counterpart with aggressive neurological symptoms (Sango et al., 1995). Unexpectedly, HexA deletion in mice resulted in an ameliorated clinical symptoms with minimal neurological signs (Sango et al., 1995). This phenotypic difference pointed to the presence of an alternative degradative pathway for GM2 in Tay-Sachs mice. Recent findings have revealed that murine GM2 can be catabolized by sialidase enzyme to asialo-GM2, in the absence of Hexosamindase A (Phaneuf et al., 1996). Thus, only the deletion of Hexb gene in mice leads to GM2 gangliosidosis and this mainly explains the wide utilization of *Hexb*$^{-/-}$ mice to investigate GM2 gangliosidosis pathology (Kyrkanides et al., 2012).

1.6.1.2 Neuronal susceptibility to Sandhoff lysosomal storage

Although GSLs are present in all cell types, neurons are found more susceptible to GM2 lysosomal storage (Arthur et al., 2012). One plausible reason for this higher susceptibility of neuronal tissue to lysosomal storage disorders is the presence of higher concentrations of GSLs in neurons (Jeyakumar et al., 2002). In support of this idea, gangliosides derived from glucosylceramide have been reported to have essential roles in myelin homeostasis (Jeyakumar et al., 2002). In addition, ganglioside synthesis performs essential role during neuronal development in mice (Yamashita et al., 2005).

1.6.1.3 Clinical features of Sandhoff disease

Based on the severity of clinical picture and age of onset, Sandhoff disease is classified into three categories; infantile, juvenile and late-onset (Bley et al., 2011). The infantile variant is considered the most severe and most prevalent form of Sandhoff disease (Hendriksz et al., 2004). Although, affected infants appear normal after birth, disease symptoms begin early before nine months of age (Bley et al., 2011). The clinical suspicion for the affected infants depends on the presence of seizures, cherry-red macula, hepato-splenomegaly and skeletal deformities (Jeyakumar et al., 2002). Similar to Sandhoff mice, affected infants develop slow developmental milestones, muscle weakness, spasticity and deterioration of motor function (Kyrkanides et al., 2008). As the

disease progresses, affected children display vision and auditory loss, mental retardation and progressive paralysis, and eventually die usually at 1-2 years of age (Bley et al., 2011). While late-onset form occurs in adult patients with mild symptoms, juvenile form starts approximately at 3-year old (Huang, Trasler, Igdoura, Michaud, Hanal, & Gravel, 1997). It is noteworthy that disease manifestation in *Hexb*$^{-/-}$ mice, which die at 4 months of age, almost mimics the clinical picture noticed in that of affected human infants with GM2 gangliosidosis (Cachon-Gonzalez et al., 2012).

1.6.2 Pathological features of Sandhoff disease

As in other GM2 gangliosidosis, one of the first microscopically identified features in Sandhoff pathology is the abnormal morphology of neurons (Volk, Adachi, & Schneck, 1975). The accumulated GM2 storage initiates an extended membranous intracellular lysosomal network of bodies that may shift the nucleus from its normal central location (Cachon-Gonzalez et al., 2012). Furthermore, recent findings have uncovered some of the mechanisms underlying the gain of the brain weight in Sandhoff pathology which might be caused by massive intracranial gliosis (Cachon-Gonzalez et al., 2012; Kyrkanides et al., 2012). It is now widely accepted that activated microglia and astrocytes are prominent pathological features in Sandhoff brains (Kyrkanides et al., 2007). One striking finding of Sandhoff pathology is the involvement of impaired autophagy pathway which results in intracellular build-up of ubiquitinized proteins and ultimately cell death (Di Malta, Fryer, Settembre, & Ballabio, 2012). Eventually, the

disease is manifested by axonal myelin defects and followed by extensive neuronal apoptosis (Huang, Trasler, Igdoura, Michaud, Hanal, & Gravel, 1997) .

In the case of neurodegenerative Sandhoff disease, several lines of evidence, including focal expansion of axonal spheroids along with neuro-axonal dystrophy, suggest that neurons are more vulnerable to GM2 storage injury (Cachon-Gonzalez et al., 2012). The arborized axonal spheroids in Sandhoff brains contain a network of membranous multivesicular bodies and expanded autophagosome that ultimately disturb neuronal networking and neuronal action potential (Walkley, Sikora, Micsenyi, Davidson, & Dobrenis, 2010). In addition, recent studies have reported that enhanced survival of Sandhoff mice transplanted with normal bone marrow cells without detecting any significant degree of increased enzyme activity or decreased storage level (Norflus, Tifft, McDonald, Goldstein, Crawley, Hoffmann, Sandhoff, Suzuki, & Proia, 1998). It is clear that GM2 storage per se cannot explain all CNS damage but it might be that other molecular pathways, underlying lysosomal biogenesis and autophagy regulation, participate greatly in the pathogenesis of Sandhoff disease (Sardiello et al., 2009). One group has identified increased titer of antiganglioside autoantibodies along with increased IgG deposition on neuronal cells in Sandhoff mice compared to wild-type, and subsequently suggested that uncontrolled inflammatory response against storage material may contribute to pathophysiological phenotype of Sandhoff mice (Yamaguchi et al., 2004). Others have described dys-regulated autophagy pathway manifested by significantly increased levels of LC3-II, an autophagy marker, contributes to the pathogenesis of Sandhoff disease (Vitner et al., 2010). Furthermore, reactive gliosis,

astrogliosis and microgliosis, have been described in several reports investigating Sandhoff disease pathology, suggesting their involvement in the development of neuronal damage in Sandhoff disease (Huang, Trasler, Igdoura, Michaud, Hanal, & Gravel, 1997;Wu & Proia, 2004;Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008).

1.6.3 Neuroinflammation and Sandhoff disease

Neuroinflammation has been implicated in a wide array of neuronal diseases including Alzheimer's and Parkinson's disorders (Barnum & Tansey, 2010; Vitner, Farfel-Becker, Eilam, Biton, & Futerman, 2012). Lysosomal storage disease is no exception as there is increasing evidence for the participation of inflammation in the pathogenesis of a number of lysosomal storage diseases such as Niemann-Pick C (Vitner et al., 2012; Smith, Wallom, Williams, Jeyakumar, & Platt, 2009), mucopolysaccharidosis I (Ohmi et al., 2003), and GM1 and GM2 gangliosidosis (Wada, Tifft, & Proia, 2000;Pelled et al., 2003). One of the cardinal signs of Sandhoff pathology, in both human patients and affected mice, is the recruitment of blood derived macrophages, proliferation of resident glia cells, and progressive secretion of inflammatory mediators such as tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β) (Wada, Tifft, & Proia, 2000). The involvement of inflammation in the pathogenesis of neurodegeneration can be extrapolated readily from the improvement of clinical picture upon introducing anti-inflammatory therapy to affected neurodegenerative subjects (Jeyakumar et al., 2004). For instance, Alzheimer's and Parkinson's mice models, treated with NSIADs,

display reduced microglial activation (Kurkowska-Jastrzebska et al., 2002). Furthermore, aspirin administration to amyotrophic lateral sclerotic mice enhanced their motor function (Barneoud & Curet, 1999) . Similarly, anti-inflammatory therapy increased significantly the survival of Sandhoff mice and delayed dramatically their disease progression (Jeyakumar et al., 2004). In addition, recent work shows that the diffuse microglial population in Sandhoff CNS is associated with increased astrocytic expression of macrophage-inflammatory protein 1 alpha, a leukocyte chemokine (Wu & Proia, 2004). This finding may explain reduced microglial activation and their associated pathology upon deletion of MIP-1α in Sandhoff mice (Wu & Proia, 2004). Astrogliosis is considered an essential feature in Sandhoff disease and many other neurodegenerative diseases (Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008). In fact, activated astrocytes were found to secrete toxic molecules, aggravate neuroinflammation and ultimately forming permanent CNS scars (Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008) .

Glial proliferation, microglial activation and expression of inflammatory cytokines, are all part of progressive and generalized inflammatory response that has been found to be associated with GM2 gangliosidosis (Jeyakumar et al., 2004). While Sandhoff mice show rapidly progressive inflammatory pathology, late onset Tay-Sachs (LOTS) mice do not display symptoms, suggesting a possible correlation between the severity of inflammation and degree of GM2 storage (Jeyakumar et al., 2003). Furthermore, a recent study, in which Sandhoff mice treated with anti-inflammatory drugs, has been performed to examine whether inflammation directly participate in Sandhoff pathology, the authors concluded that inflammation contribute directly to

Sandhoff disease progression (Jeyakumar et al., 2004). The non-steroidal anti-inflammatory drugs (NSAIDs) suppressed loss of neuronal motor function and reduced expression of histocompatibility complex class II (MHC II) in Sandhoff brains (Jeyakumar et al., 2004). Subsequently, treated mice show lower levels of macrophage infiltration and microglial activation (Jeyakumar et al., 2004).

1.6.4 Cytokines and Sandhoff disease

As neuroinflammatory disorders progress, it was found that brain macrophages and microglia secrete cytokines and express their cognate receptors dramatically within CNS damaged areas (McGeer, Rogers, & McGeer, 1994). Along this line, it was reported that certain level of storage has to be reached to induce microglia to produce several cytokines which is ultimately recruiting more blood immune cells into CNS (Jeyakumar et al., 2003). Particularly, CNS levels of TNFα and IL-1β increase in a graded fashion in correlation with the severity of Sandhoff disease pathology (Jeyakumar et al., 2003). In contrast, brain levels of growth factor (TGFβ1) were found prominent only at the end stage of Sandhoff disease (Jeyakumar et al., 2003). Of note, TGFβ1 displays an immune-inhibitory function via suppression of various cytokines including TNFα, IL-1β and IL-6 (Jeyakumar et al., 2003). This orchestrated manner of cytokine expression might suggest that TGFβ1 is produced to oppose the proinflammatory effects of TNFα and IL-1 in Sandhoff mice (Jeyakumar et al., 2003). Indeed, TNFα upregulation is transcriptionally controlled and predates Sandhoff disease symptoms, suggesting a temporal association

between TNFα cytokine release and the exacerbation of disease pathology (Wada, Tifft, & Proia, 2000).

1.6.5 Mechanism of immune cell activation in Sandhoff brain

Following GM2 storage in Sandhoff brains, microglial activation and cytokine production have become a landmark observation, although mechanistically they are still poorly understood (Jeyakumar et al., 2003). It was demonstrated that ganglioside can provoke microglia to release proinflammatory mediators including TNFα, NO and prostaglandins (Pyo, Joe, Jung, Lee, & Jou, 1999). These data firmly support the possible mechanism that phagocytosis of apoptotic neurons by brain microglia elicits an inflammatory response and ultimately leads to neurodegeneration in Sandhoff disease (Volbracht, Leist, & Nicotera, 1999). Thus, this might be seen as a contradiction to the controlled clearing function of normal apoptosis without provoking a powerful inflammatory response (Fadok et al., 1998). However, It is likely that GM2 storage pathology disturbs this normal immune-response regulating function of apoptosis (Jeyakumar et al., 2003). Furthermore, recent findings have revealed that there are useful implications of treating Sandhoff disease with N-butyldeoxynojirimycin (NB-DNJ), a drug that slows the rate of GSL accumulation into lysosomes via arresting the first step of GSL biosynthesis (Dwek, Butters, Platt, & Zitzmann, 2002). Similar favorable clinical outcome has been achieved via utilizing N-butyldeoxygalactonojirimycin (NB-DGJ), a more selective substrate reduction therapeutic compound, but with fewer side effects

(Andersson et al., 2004). Furthermore, GA2 accumulates in brain due to mouse sialidase bioactivity in the brains of Sandhoff mice along with GM2 storage (Jeyakumar et al., 2003). Neuroinflammation and subsequently neurodegeneration triggered by brain lipid storage is not a unique observation of GM2; this property was identified for other gangliosides including, GM1 (Huang, Trasler, Igdoura, Michaud, Hanal, & Gravel, 1997). In fact, GA2 accumulation showed a higher degree of correlation with Sandhoff disease progression and storage level, upon SRT application (Jeyakumar et al., 2001). Thus, there is now increasing evidence that asialo derivatives, such as GA2, participate in the pathology of Sandhoff disease (Jeyakumar et al., 2003).

1.6.6 Sandhoff disease and Blood-brain barrier

Blood brain barrier (BBB) represents a dynamic cross-talk between cerebral vascular endothelial layer and the CNS cells: mainly astrocytes, perivascular microglia and neurons (Abbott, 2005). Since the BBB tightly controls two dynamic junctional and trans-endothelial routes, it functions not only as an anatomical but also as a metabolic barrier regulating the uptake transport system in CNS (Deli, Abraham, Kataoka, & Niwa, 2005). One remarkable feature of Sandhoff pathology is the association of increased BBB permeability with immune cell activation and symptomatic progression (Jeyakumar et al., 2003). In addition, it was reported that increased cytokine CNS production disturbs BBB permeability (Anthony, Bolton, Fearn, & Perry, 1997). Along this line, TNFα has been shown to act on the blood endothelial cell layer to increase BBB permeability and

ultimately blood immune cell recruitment in Sandhoff disease (Anthony et al., 1997). In addition, bone marrow transplantation of normal cells into Sandhoff mice significantly dampens macrophage/microglial toxicity and shows less brain apoptosis (Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008). Intriguingly, neuronal cell death decreases in Sandhoff mice upon inhibiting recruitment of blood monocytes into CNS (Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008). Thus, neuroinflammation may impair the integrity of the BBB in Sandhoff pathology which might be seen as a vital step for neuronal apoptosis (Jeyakumar et al., 2003).

1.6.7 Therapeutic approaches of Sandhoff disease

GM2 gangliosidosis mouse models recapitulate phenotypes of their corresponding human disorders and were extensively utilized in the search for useful therapeutic strategies (Cachon-Gonzalez et al., 2012). Currently, therapeutics including bone marrow transplantation (BMT) (Jeyakumar et al., 2001), substrate reduction therapy (SRT) (Andersson et al., 2004), neuronal stem cell transplantation (Lacorazza, Flax, Snyder, & Jendoubi, 1996), anti-inflammatory compounds (Jeyakumar et al., 2001), gene therapy (Kyrkanides et al., 2007) and pharmacological chaperones (Tropak, Reid, Guiral, Withers, & Mahuran, 2004), have been tested with variable degree of favorable clinical outcomes.

1.6.7.1 Sandhoff disease and Gene therapy

Despite the relative success of current treatments to ameliorate Sandhoff disease progression and clinical outcomes, none has been shown to be completely curative (Byrne, Falk, Clement, & Mah, 2012). It is noteworthy that low concentration of residual enzymes is enough to prevent symptoms and renders affected individuals to be asymptomatic carriers (Byrne et al., 2012). In addition, it is well known that all LSDs are monogenic disorders resulted from recessive mutations (Byrne et al., 2012). Thus, all these characteristics render Sandhoff disease an ideal model for gene therapeutics (Byrne et al., 2012). Furthermore, cross correction, in which expressed proteins bind to mannose-6-phosphate receptor on distal cells to be internalized and targeted to lysosomal system, is considered a unique attribute of lysosomal enzymes (Byrne et al., 2012). Gene therapy using viral vectors has been reported to be the most practical approach in the preclinical setting in Sandhoff mouse model (Biffi & Naldini, 2005). Essentially, retroviral and adenoviral based strategies have been demonstrated to be capable to integrate into DNA genome, thus provide stable transduction (Mah, Byrne, & Flotte, 2002). Furthermore RV and AV vectors can efficiently infect non dividing neuronal cell population with a capacity to deliver a considerable size of DNA construct (Mah et al., 2002).

Recent findings have shown that intracranial injection of adeno-associated viral vectors (rAAV), containing a transgene that expresses the β-subunit of β-hexosamindase, prolonged survival and improved clinical picture in Sandhoff mice (Cachon-Gonzalez et al., 2006). Since effective gene therapy approach will necessitate simultaneous expression

of HexA (Guidotti et al., 1999), localized intracranial co-injection into cerebellum of adeno-associated viral vectors (rAAV) expressing HexA and HexB subunits prevented ataxia, a common Sandhoff symptom (Cachon-Gonzalez et al., 2012). Surprisingly, GM2 storage and immune cell activation were almost entirely resolved when gene therapy was administered to Sandhoff mice (Cox & Cachon-Gonzalez, 2012). It is likely that treating an infant by using AAV-mediated gene therapy will require more than one injection; one study estimated a requirement of more than 50 injections involved to achieve a thorough and an optimal expression of lysosomal enzymes (Passini, Lee, Heuer, & Wolfe, 2002). This might be impractical and hence, the development of rational therapeutics for intricate neurodegenerative LSDs such as Sandhoff disease would require a combinatorial strategy to restore normal CNS function (Cachon-Gonzalez et al., 2012). Moreover, although a number of phase 1 and phase 2 clinical studies have been reported as a gene therapeutic approach for LSDs, the unwanted hypersensitivity of immune system toward viral vehicles reduces the effectiveness of these trials (Byrne et al., 2012).

1.6.7.2 Sandhoff and bone marrow transplantation

Isolation of the brain from blood makes the delivery of neuro-therapeutics a highly challenging task (Gaillard, Visser, Appeldoorn, & Rip, 2012). In addition, invasive administration of drugs into brain is exceptionally difficult (Gaillard et al., 2012). First, the observed incompetency of neurons to regenerate requires cautious and delicate methods to access CNS during clinical trials (Su & Sinko, 2006). Second, the control

groups might be affected from the invasive methods (Su & Sinko, 2006). Thus, the utilization of placebo controls with invasive delivery of neuro-therapeutics to measure the clinical outcome would be subjective and less sensitive (Gaillard et al., 2012). Finally and particularly, there are a limited number of clinically reliable neuro-biomarkers which can be used to accurately assess the pathological progression of lysosomal neurodegenerative disorders (Su & Sinko, 2006). The acute urgency for new delivery methods into brain has initiated the arena for allogeneic bone marrow transplantation as a treatment option for LSDs (Hoogerbrugge et al., 1995). The therapeutic efficacy of transplantation of normal donor bone marrow cells into affected subjects is highlighted by the observation that donor bone marrow precursors can be recruited and differentiated into microglia to reside into recipient brains and then secrete lysosomal hydrolases to cross-correct distant neuronal cells (Krivit, Peters, & Shapiro, 1999). Recently, it has been reported that normal bone marrow transplantation (BMT) into Sandhoff mice inhibits activation of microglia and reduced neuronal cell death (Wada, Tifft, & Proia, 2000; Norflus, Tifft, McDonald, Goldstein, Crawley, Hoffmann, Sandhoff, Suzuki, & Proia, 1998). Another study demonstrated that BMT delayed onset of Sandhoff symptoms and prolonged survival rate of Sandhoff mice (Norflus, Tifft, McDonald, Goldstein, Crawley, Hoffmann, Sandhoff, Suzuki, & Proia, 1998).

It is noteworthy that normal microglia, capable of secreting β-hexosamindase enzyme, were identified within the same areas usually affected by neuronal death (Oya et al., 2000). In twitcher and α-mannosidosis storage disorders, it was found that, as expected, transplanted normal bone marrow donor cells infiltrated into affected recipient

brains and secreted normal lysosomal hydrolases which were able to cross-correct and reduce storage in remote neurons (Hoogerbrugge et al., 1988;Walkley et al., 1994). In normal bone marrow –transplanted Sandhoff disease mice, although normal microglia was defined in the brain parenchyma of affected mice, neither reduction of GM2 storage, nor β-hexosamindase positive neurons were detected in Sandhoff recipient mice (Wada, Tifft, & Proia, 2000;Oya et al., 2000). Thus, a disease pathology model was suggested in which neuroinflammation and microglia has a higher predominating role in triggering massive neuronal death over the primary insult of accumulated GM2 and GA2 storage (Wada, Tifft, & Proia, 2000). In this mechanism, the primary cause of neuronal apoptosis is the lipid accumulation and then, in a trial to resolve this primary neuronal death, enzyme deficient microglia engulf dying neurons (Wada, Tifft, & Proia, 2000). However, phagocytosis induces and aggravates neuroinflammation due to the inability of microglia to digest the internalized storage, hence recruiting more immune blood cells which ultimately lead to massive neurodegeneration (Minghetti & Levi, 1998).

The timing of introducing therapy to Sandhoff disease should be carefully considered because BMT shows effective outcome only if introduced in the pre-symptomatic stage of disease pathology (Jeyakumar et al., 1999). In summary, lessons from bone marrow transplantation (BMT) experiments put more light on the importance of neuroinflammation on disease pathology and may pave the way for a comprehensive therapy for the neurodegenerative group of lysosomal storage disorders (Jeyakumar et al., 1999). However, bone marrow transplantation as an advisable therapy should be taken with caution due to the associated complication to find a matching donor, the requirement

for prolonged usage of immunosuppression drugs, and the hazardous side-effects of myelo-ablation (Platt & Lachmann, 2009; Kasperzyk et al., 2004).

1.6.7.3 Sandhoff disease and substrate reduction therapy (SRT)

It is widely known that GSL synthesis occurs in step-wise fashion in which glucosylceramide (GlcCer) is the primary precursor of all gangliosides (Kasperzyk et al., 2004). Substrate reduction therapy (SRT) is a small molecule platform with a therapeutic rationale which aims to inhibit the activity of glucosylceramide synthase, the first catalytic enzyme in the glycosphingolipid (GSL) biosynthesis process (Platt & Lachmann, 2009). Apparently, being a small molecule that can cross BBB, a substrate reduction drug could act on brain cells and improve the pathology of neurodegenerative LSDs including Sandhoff disease (Martino et al., 2002). In addition, being an inhibitor of the first step of the GSL biosynthesis process, a substrate reduction molecule can benefit all lysosomal disorders that involve accumulation of any derived GSL lipids such as GM1 gangliosidosis, GM2 gangliosidosis, Gaucher and Fabry diseases (Walkley, 2004).

N-butyldeoxynojirimycin (Miglustat, NB-DNJ), an imino sugar derivative SRT drug has been shown to prolong the life span, improve the motor function and delay the onset of Sandhoff disease pathology (Jeyakumar et al., 2005). Miglustat (NB-DNJ) is approved as a pharmaceutical treatment in USA for the benign forms of Gaucher type 1 patients for which ERT is not an advisable treatment (Venier & Igdoura, 2012). In vitro, NB-DGJ can significantly reduce ganglioside synthesis with no signs of cell damage

(Brigande, Platt, & Seyfried, 1998). Recently, clinical trials, involving the utilization of SRT, have been implemented in a number of lysosomal storage diseases including GM2 gangliosidosis such as Tay-Sachs (Platt & Lachmann, 2009). However, serious side effects, such as body weight loss and irregular morphology of lymphoid tissue, have been noticed with the therapeutic utilization of Miglustat in high dosage (Andersson, Butters, Dwek, & Platt, 2000). Currently, N-butyldeoxygalactonojirimycin (NB-DGJ), the glucose analog of Miglustat, is showing high efficacy in inhibiting the neurological deterioration with little or no adverse effects (Andersson et al., 2004).

Favorable clinical outcome including longer survival with reduced pathogenesis was observed after treating adult Sandhoff mice with NB-DGJ (Andersson et al., 2004). Of note, starting SRT with NB-DGJ at early neonatal stage reduces GM2 storage in the CNS of Sandhoff mice (Kasperzyk et al., 2004). Recent studies have shown that combining SR and BM therapies had collective effects on Sandhoff disease pathology (Jeyakumar et al., 2001; Jeyakumar et al., 2004). More recently, NB-DGJ was more effective in treating juvenile Sandhoff mice than intracranial administration of neuronal stem cells (NSCs) (Arthur et al., 2012). In this study, NB-DGJ administration was alone enough to reduce both GM2 and GA2 significantly with no increase in Hex activity (Arthur et al., 2012). There was no synergistic effect observed when both NB-DGJ and NSCs therapy were utilized in Sandhoff mice (Arthur et al., 2012). In summary, early administration of SRT therapy might provide a novel therapy of controlling neurodegeneration in Sandhoff disease mice (Arthur et al., 2012).

1.6.7.4 Sandhoff disease and neuronal stem cell transplantation

Motor neuronal cell death was implicated in the pathogenesis of most neurodegenerative diseases including GM2 gangliosidosis (Chung et al., 2005). However, no applicable therapy, for most of neurodegenerative lysosomal diseases, is able to slow neuronal function deterioration in both mice and humans (Shihabuddin & Aubert, 2010). In general, it was found that stem cells can self-renew and ultimately differentiate to replace neuronal cells (Shihabuddin & Aubert, 2010). Although, embryonic stem cells are pluripotent that can differentiate into several cell types, neuronal stem cells show much limited pluripotency that can divide and replace mostly neurons and less frequently astrocytes and oligodendrocytes (Thomson et al., 1998). One striking feature of these neuronal stem cells is their capacity to replace and achieve neuronal circuit repair in the affected brains of recipients (Shihabuddin & Aubert, 2010). There is accumulating evidence for the involvement of both physiological and pathological factors in controlling the proliferation, migration, differentiation and integration of NSCs into adult brain neuronal circuits (Ming & Song, 2005).

Lately, it has been shown that components of CNS inflammation have a great impact on the fate of adult neurogenesis (Mathieu et al., 2010). Thus, the appropriate context of immune response may well influence the endogenous niche and ultimately alter the fate of transplanted NSCs into recipient brains (Mathieu et al., 2010). It was demonstrated that microglial activation had a negative impact on hippocampal neurogenesis (Monje, Toda, & Palmer, 2003). In addition, it was found that there is a

negative correlation between severity of microglial activation and the extent of proliferation of neuronal stem cells (Rock et al., 2004). One solid reason for this association was thought to be partly and significantly mediated by microglial secretion of proinflammatory cytokines such as interleukin 1β and TNFα (Rock et al., 2004). While TNFR1-mediated signaling reduced neuronal proliferation, signaling via TNFR2 enhanced neuronal survival and neurogenesis (Mathieu et al., 2010). Based upon data of TNFR1 and TNFR2 deletion experiments, the ability of TNFα to regulate neurogenesis is greatly dependent on not only its concentration but also on the differential TNFα signaling via its two receptors: TNFR1 and TNFR2 (Mathieu et al., 2010). Particularly for lysosomal enzyme deficiency subset of neurodegenerative diseases, neuronal stem cells can not only replace dying nerve cells but also traffic and secret the lacking enzyme to the rest of recipient CNS cells (Shihabuddin & Aubert, 2010). Thus, neuronal stem cell transplantation is considered an appealing therapeutic strategy because NSCs can bypass BBB, roam extensively, and give rise to multiple brain cell types (Jin, Carter, Huntley, & Schuchman, 2002). In Niemann Pick mice, it was found that genetically modified NSCs transplantation into CNS resulted in a significant increase of enzymatic activity in recipient mice brains (Lacorazza et al., 1996).

It was demonstrated that NSCs transplantation in Sandhoff neonatal mouse model improved motor function, increased mice longevity, and most importantly reduced disease inflammatory response (Lee et al., 2007). Comparable results have been achieved utilizing human NSCs transplantation into Sandhoff adult mice (Jeyakumar et al., 2009). Thus, the latter findings are related to opening of a promising therapeutic avenue in

which human NSCs were utilized to treat debilitated neurodegernative disorders such as Batten's disease (Hobert & Dawson, 2006). It is noteworthy that recipient brains did tolerate the injected human NSCs and showed improved pathological condition (Worgall et al., 2008). Despite the fact that the concept of cell replacement appears conceivable, it requires potentially high degree of customization to replace a certain cellular subtype for each neurodegenerative disease (Shihabuddin & Aubert, 2010). Some tailored applications of NSCs replacement have to be designed to enable NSCs to give rise to CNS glia cells and secrete neurotrophic factors (Shihabuddin & Aubert, 2010).

Although stem cell based therapeutics is still in its infancy, significant research progress has been accomplished that might provide a foundation for effective treatment (Shihabuddin & Aubert, 2010). Moreover, the development of effective stem cell therapies for LSDs will require early initiation before the emergence of the irreversible pathological signs (Shihabuddin & Aubert, 2010). Indeed, it should be borne in mind that the inflammatory pathology could spread to transplanted NSCs and subsequently this has to be investigated prior the utilization of NSCs as a potential therapy for GM2 gangliosidosis (Kordower & Brundin, 2009).

1.6.7.5 Sandhoff disease and Anti-inflammatory drugs

There is increasing evidence of the involvement of neuroinflammation in several neurodegenerative diseases (Kyrkanides et al., 2012) including Alzheimer's (Barnum & Tansey, 2010), Parkinson's (Vitner et al., 2012), GM1 and GM2 gangliosidosis (Wada,

Tifft, & Proia, 2000; Jeyakumar et al., 2003) and Gaucher's diseases (Vitner et al., 2012). It was reported that IL1β, IL-6 and TNFα levels are increased in a number of lysosomal storage diseases including Gaucher's and Sandhoff disease (Vitner et al., 2012), (Kyrkanides et al., 2012). Furthermore, the presence of pro-inflammatory chemotactic molecules such as CCL3 in nervous tissue of Sandhoff mice needs to be considered since this cytokine can mediate recruitment of blood immune cells into the brain parenchyma (Lopez, Klein, Hong, Dimbil, & Scott, 2012). Recent studies have shown that activated microglia and astrocytes are prominent in Sandhoff brains (Kyrkanides et al., 2007). Furthermore, transplantation of normal bone marrow cells into Sandhoff mice reduced neuroinflammation and improved the clinical picture (Wu & Proia, 2004). From the above data, it is possible that dys-regulated immune response contributes to neuronal cell death (Kyrkanides et al., 2012). In support of this idea, deletion of work utilizing macrophage inflammatory protein (MIP-1α) in Sandhoff mice showed that this experimental approach was able to ameliorate neurodegeneration (Wu & Proia, 2004).

Recent emergent clinical study involving intermittent administration of steroids to patients affected with juvenile neuronal ceroid lipofuscinosis (JNCL), a neurodegenerative disease caused by accumulation of lipofuscin in the body tissues, showed motor and cognitive beneficial effects (Aberg et al., 2008;Lim, 2011). However, it is established that neuroinflammatory response occurs long before the start of permanent neurological damage in most neurodegenerative diseases (Cooper, Russell, & Mitchison, 2006). This data greatly supports the concept that a successful treatment approach has to start early in the optimal therapeutic window and before acute

neurological loss (Lim, 2011). Utilizing glial activation biomarkers, as a diagnostic strategy to differentiate between beneficial and deleterious mode of glial activation, has recently shown significant promise (Ji et al., 2008). Hence, mitigating the glial upregulation would theoretically attenuate the aggressive innate immunity response associated with neurodegenerative LSDs, and ultimately gain clinical benefit (Lim, 2011). Similarly, reducing the recruitment molecules of adaptive immune response may be considered as a beneficial therapeutic strategy (Lim, 2011). Although, anti-inflammatory drugs might introduce various complications, relatively-safe non-steroidal anti-inflammatory agents incur beneficial outcome (Lim, 2011). Recent studies have shown that administration of NSAIDs attenuated microglial activation and protected neurons in Alzheimer's disease (Lim, 2011) as well as LSDs including Sandhoff disease (Jeyakumar et al., 2004). Furthermore, anti-TNFα therapy using minocycline has shown reduction of microglial toxicity and inflammation in neurodegenerative diseases such as Parkinson's, Huntington's and amyotrophic lateral sclerosis (Orsucci, Calsolaro, Mancuso, & Siciliano, 2009).

In LSDs, it was shown that neuroinflammation and neuronal loss spatially manifested in the same CNS areas (Farfel-Becker et al., 2011). Several animal studies provide large body of evidence implicating that administration of NSAIDs such as Ibuprofen would alleviate neuroinflammation and elongate survival in a number of LSDs including GM2 gangliosidosis (Hemsley & Hopwood, 2011), NP-C (Smith et al., 2009) and Krabbe diseases (Luzi et al., 2009).

1.7.1 Research Objectives

Sandhoff disease is an accurate prototype for common neurodengerative disorders, with severe clinical course for affected children including motor system dysfunction, tremors, and gait disturbance and even mental retardation. Multimodal therapeutics, such as bone marrow transplantation (BMT) (Jeyakumar et al., 2001) and anti-inflammatory drugs (Jeyakumar et al., 2001), are the current trend for treating those patients. Identification of potential inflammatory molecules, that play important role on the pathology of Sandhoff disease, is crucial for global and effective treatment, not only for Sandhoff disease but also for other common neurodegenerative disorders.

1.7.1.1 Objective One:

To investigate the role of TNFα in the development and progression of SD in mice, by creating a Hexb-/-Tnfα-/- double knock-out mouse, we will generate double knock-out mice lacking both Hexb and Tnfα genes. We will examine the neurological and pathological conditions of these mice.

1.7.1.2 Objective Two:

To assess the role of blood-derived TNFα on neurological function in vivo, we will transplant bone marrow cells from $Hexb^{-/-}Tnf\alpha^{-/-}$ into Sandhoff mice as well as we will examine the frequency and functions of peripheral immune cells.

1.7.1.3 Objective Three:

To define potential miRNA gene targets that play important role in Sandhoff disease pathology, we will isolate total RNAs and perform microRNA profiling for CNS tissue from Symptomatic Sandhoff mice.

Chapter 2

Deletion of tumor necrosis factor-α ameliorates neurodegeneration in Sandhoff disease mice

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Preface:

This chapter has been published in Human Molecular Genetics journal (Abo-Ouf et al., 2013). I performed the majority of the experiments described in this chapter. Dr. S. Igdoura, Alexander Hooper and I assembled the data and figures, and wrote the manuscript. Alexander Hooper provided immunohistochemistry data for the molecules described in the article. Considerable advice and direction for the experimental work, and contribution to the final version of the manuscript were given by my supervisor, Dr. S. Igdoura.

2.1 Abstract

Sandhoff disease (SD) is a lysosomal storage disorder caused by a lack of a functional subunit of the beta-hexosaminidase A and B enzymes, leading to accumulation of gangliosides in the CNS. The *Hexb*$^{-/-}$mouse model of SD shows a progressive neurodegenerative phenotype similar to the human equivalent. Previous studies have revealed that *Hexb*$^{-/-}$mice suffer from chronic neuroinflammation characterized by microglial activation and expansion. Tumor necrosis factor-α (TNFα), a key modulator of the CNS immune response in models of neurodegeneration, is a hallmark of this activation. In this study, we explore the role of TNFα in the development and progression of SD in mice, by creating a *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ double knock-out mouse. Our results revealed that the double knock-out mice have an ameliorated disease course, with an extended life span, enhanced sensorimotor coordination and improved neurological function. TNFα-deficient SD mice also show decreased levels of astrogliosis, and reduced neuronal cell death, with no alterations in neuronal storage of gangliosides. Interestingly, temporal microglia activation appears similar between the *Hexb*$^{-/-}$*Tnfα*$^{-/-}$and SD mice. Evidence is provided for TNFα activation of the JAK2/STAT3 pathway as a mechanism for astrocyte activation in the disease. This study reveals TNFα as a neurodegenerative cytokine mediating astrogliosis and neuronal cell death in Sandhoff disease, and points to Tnfα as a potential therapeutic target to attenuate neuro-pathogenesis.

2.2 Introduction

Lysosomal storage disorders (LSDs) are all characterized by the intra-lysosomal buildup of un-degraded substrates due to the deficiency of one or more lysosomal enzymes and/or their cofactors (Hodges & Cheng, 2006). GM2 gangliosidoses are inherited storage diseases resulting from a functional deficiency in the lysosomal hydrolase beta-hexosaminidase (Kolter & Sandhoff, 2006). Beta-hexosaminidase removes terminal N-acetylgalactosamine from the GM2 ganglioside. The two major isoforms of β-hexosaminidase, HexA and HexB, are made up of distinct combinations of homologous α and β subunits. The heterodimer, HexA (αβ), is capable of cleaving terminal N-acetylgalactosamine from GM2, while the HexB homodimer (ββ) is not (Hodges & Cheng, 2006; Meier, Schwarzmann, Furst, & Sandhoff, 1991). Mutations in the HEXA gene encoding the α-subunit lead to a deficiency in HexA, clinically known as Tay-Sachs disease. Mutations in the HEXB gene encoding the β subunit, on the other hand, give rise to a deficiency in both HexA and HexB isozymes, clinically known as Sandhoff disease (SD). Tay-Sachs and SD display similar clinical symptoms of neurodegeneration including motor function disturbances, spasticity, ataxia, seizures, visual loss, and deafness. The high turnover of gangliosides in the central nervous system (CNS) accounts for the primarily neurological phenotype associated with these diseases. Both diseases are generally terminal in early childhood (Kolter & Sandhoff, 2006). The age of onset and severity of the diseases depends upon residual enzyme activity, with null mutations giving rise to the more severe infantile phenotypes (Conzelmann, Kytzia, Navon, & Sandhoff, 1983; Hodges & Cheng, 2006). Our understanding of the GM2

gangliosidoses has been greatly advanced by the development of authentic animal models. In keeping with the progressive neurodegenerative course of SD, the Hexb$^{-/-}$mice develop severe motor impairments by 3 months of age, with death usually ensuing 4-6 weeks after symptom onset (Sango et al., 1995). Unexpectedly, however, the Hexa$^{-/-}$ mice have a normal lifespan and show no obvious neurological impairments until at least 1 year of age (Yamanaka et al., 1994). Currently, the Hexb knock-outs are used as a model of the acute forms of both GM2-gangliosidoses, while the Hexa knock-outs serve as a model for late onset variants of the diseases (Miklyaeva et al., 2004).

Several recent reports have implicated neuroinflammation as the driving force behind neurodegeneration in *Hexb*$^{-/-}$mice (Wu & Proia, 2004; Kyrkanides et al., 2008). Neuroinflammation is an intricate process which involves the participation of various cellular types of the immune system (macrophages, neutrophils, mast cells, lymphocytes, platelets, dendritic cells), localized CNS cells (microglia, astrocytes, neurons) and cellular products (adhesion molecules, cytokines and chemokines) (Niederkorn, 2006; Probert et al., 1997). Not only do neurons, astrocytes, microglia and oligodendrocytes generate inflammatory mediators but also they constitutively express cytokine receptors (Mrak & Griffin, 2005). Cytokines- including interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6)(Mrak & Griffin, 2005), transforming growth factor-beta (TGF-β), and tumor necrosis factor-alpha (TNFα)-are considered crucial players in the establishment of neuroinflammation (McGeer & McGeer, 2001; McGeer & McGeer, 2004). It is believed that activated microglia and astrocytes are the main sources of cytokines and chemokines. Activated microglia secrete multiple inflammatory factors

including TNFα, IL-1β, IL-6, interleukin-12 (IL-12), chemokines, proteases, glutamate, free radicals, and redox proteins via autocrine and paracrine mechanisms (Shie & Woltjer, 2007). In addition, glial cell derived neurotrophic factor (GDNF) is released from activated glia cells and serves as a potent inflammatory factor that supports neuronal repair. An extensive expansion in the activated microglia in the brain of SD mice is observed prior to a wave of apoptosis, and neuronal apoptosis is also evident in patient brain samples (Huang et al., 1997; Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008). Although it is clear that the build-up of un-degraded ganglioside substrates is the primary insult to neurons, the exact molecular sequence that converts this primary insult to neuronal apoptosis remains poorly understood (Myerowitz et al., 2002). The bioactivity of TNFα has beneficial or deleterious consequences on neuronal tissues, and expression of TNFα has been shown to increase sharply between 10 and 17 weeks of age in the brains of Sandhoff disease mouse models (Jeyakumar et al., 2003; Shishodia & Aggarwal, 2002; Ting & Endy, 2002). To further explore the potential role of TNFα in the neurodegenerative process, we generated a *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ double knock-out. Here, we present evidence that depletion of TNFα in SD mice results in improved neurological function, decreased levels of astrogliosis and reduced neural cell death. Using thin layer chromatography, we show that TNFα depletion had no discernible effect on the levels of accumulated GM2 and GA2 in the cerebrum and cerebellum of SD mice. We also present evidence that the NIK/non-canonical NF-κB pathway is activated in Sandhoff disease, and reduced in the absence of TNFα. Our findings demonstrate that neurological

improvement through reduced neuro-inflammatory condition can be achieved without correction of neuronal ganglioside storage.

2.3 Results

2.3.1 Deletion of TNFα improves the clinical course of Sandhoff Disease in mice

Previous studies suggested that TNFα is involved in the pathogenesis of Sandhoff disease (Kyrkanides et al., 2012). To elucidate the importance of TNFα in neurodegenerative course of SD mouse models, we generated *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ double knock-out mice. The lifespan and neurological functions of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and wild type mice were monitored. The mice were harvested when they reach a moribund endpoint state. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice show significantly higher body weights compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ mice (Figure 1A). *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ double knock-out (DKO) mice show longer lifespan to 152 days (Figure 1B). The life expectancy of DKO mice was 19 days longer than *Hexb*$^{-/-}$ mice. The *Hexb*$^{-/-}$*Tnfα*$^{+/-}$ mice followed a similar trend to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice where the loss of a single copy of Tnfα only extended their life expectancy by several days (Data not shown). To monitor motor function, four behavioural tests were performed, namely, rotorod, wire-hang, grip-strength righting reflex experiments. In this study, rotorod performance scores for *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$ mice were higher than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice after 90days. In fact, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ outperformed *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice in the rotorod test at 101 ($P<0.05$), 107 ($P<0.05$) and 110 ($P<0.05$), 113 ($P<0.05$), 116($P<0.05$), and 119 ($P<0.05$) days old (Figure 1C).

Furthermore, wire hang scores of Hexb$^{-/-}$Tnfα$^{-/-}$ mice were significantly higher at 86 (P<0.05), 95 (P<0.01) , 98 (P<0.01) , 101 (P<0.01) , 107 (P<0.01), 110 (P<0.05), and 116 (P<0.05) days old in comparison to scores of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice, indicating that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice show improved motor function relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice(Figure 1D). Performance on grip strength tests, which typically assess muscle strength, shows that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ had significantly better performance at 95(P<0.05),101(P<0.05),110(P<0.05),113(P<0.05), and116(P<0.05) days old (Figure 1E). Righting response of WT, SD, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice were performed to assess basic neurological reflexes of lower motor neurons. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice show a significant improvement in righting reflex time as compared to the *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice at 107(P<0.05), 110(P<0.05), 113(P<0.05), and116 (P<0.05) days old (Figure 1F). Analysis of behavioural test data revealed that the *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice experienced a reduced level of motor dysfunction as compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice.

2.3.2 TNFα does not change glycolipid storage within the central nervous system of SD mice

The beta-hexosamindase A and B deficiencies in SD produce significant accumulations in GM2 ganglioside and GA2 glycolipids in the CNS (Huang et al., 1997). In order to evaluate if the neurological improvement observed in double knock-out mice are attributed to reduced storage of GM2 and GA2 glycolipids in the CNS, the cerebrum and cerebellum from 17-week (~ 120 d) old WT, SD, and double knock-out mice were analyzed for glycosphingolipid content (Lee et al., 2007). In the cerebrum and cerebellum

from SD mice, the GM2 and GA2 storage was higher than their WT controls, but the degree of accumulation did not differ significantly from that seen in *Hexb*$^{-/-}$*Tnf*$\alpha^{-/-}$ mice (Figure 2A-C and 3A-C). These results indicate that the neurological improvement observed in *Hexb*$^{-/-}$*Tnf*$\alpha^{-/-}$ mice is independent of ganglioside storage.

Figure 1 TNFα alters body weights, lifespan and motor behavior in SD mice

(A)*Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice show significantly increased weights at 120 and 125 days old, compared with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ mice (n = 3–15 mice per group). **(B)** Influence of TNFα on the survival of SD mice (n = 11–31 mice per group). The absence of TNFα in SD mice significantly extended their lifespan by 19 days. **(C)** Rotorod test of WT, SD, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. Means of latency to fall are presented (n = 3–17 mice per group). The rotorod times for *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice were longer than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice at 101, 107, 110, 113, 116, and 119 days of age. **(D)** Wire hang times of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice were compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice. n = 3–18 mice per group. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ had significantly better times at 86, 95, 98, 101, 107, 110 and 116 days old, than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$. **(E)** Grip strength of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice were compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice. n = 3–17 mice per group. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ had significantly better performance at 95, 101, 110, 113, and 116 days old, than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$. **(F)** Righting response of WT, SD, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice (n = 6–12). *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice show a significant improvement in righting reflex time as compared to the *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice at 107, 110, 113, and 116 days old.*$P \leq 0.05$, **$P \leq 0.01$

Error bars = ±SE.

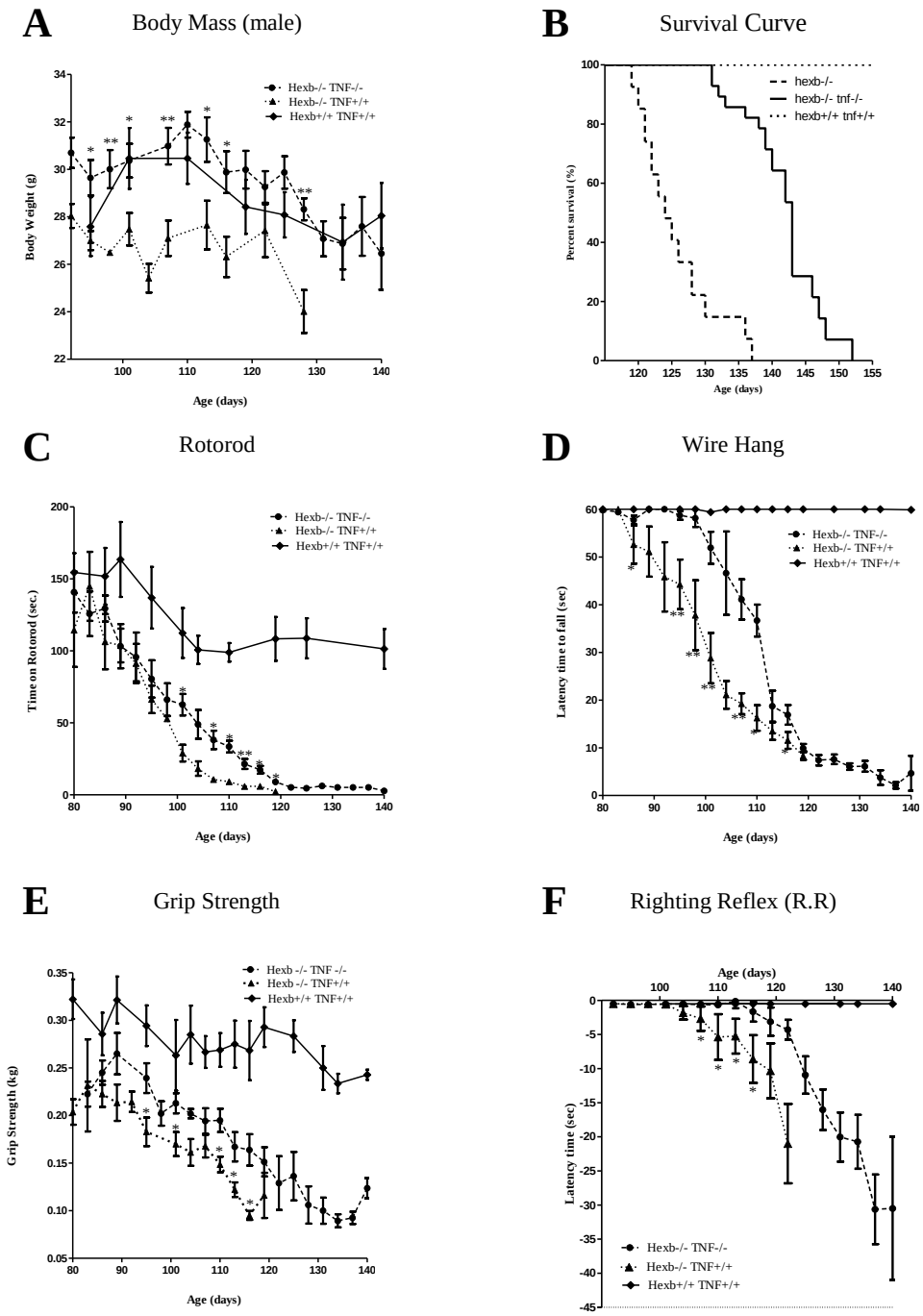

A
Body Mass (male)
Hexb-/- TNF-/-
Hexb-/- TNF+/+
Hexb+/+ TNF+/+
Body Weight (g)
Age (days)
B
Survival Curve
hexb-/-
hexb-/- tnf-/-
hexb+/+ tnf+/+
Percent survival (%)
Age (days)
C
Rotorod
Hexb-/- TNF-/-
Hexb-/- TNF+/+
Hexb+/+ TNF+/+
Time on Rotorod (sec.)
Age (days)
D
Wire Hang
Hexb-/- TNF-/-
Hexb-/- TNF+/+
Hexb+/+ TNF+/+
Latency time to fall (sec)
Age (days)
E
Grip Strength
Hexb -/- TNF -/-
Hexb -/- TNF+/+
Hexb+/+ TNF+/+
Grip Strength (kg)
Age (days)
F
Righting Reflex (R.R)
Age (days)
Latency time (sec)
Hexb-/- TNF-/-
Hexb-/- TNF+/+
Hexb+/+ TNF+/+

Figure 2 TNFα does not alter GM2 and GA2 gangliosidosis in the cerebrum of 17-week-old SD mice

Ganglioside fractions from cerebral lysates were analyzed by TLC. **(A)** Representative TLC distribution of ganglioside accumulation in the cerebrum (n = 3 experiments). The GM2 fraction was undetectable in WT mice. Sulfatides, cerebrosides and GM1 bands were not changed across the lanes. **(B)** Densitometric quantification of GA2 shows that it was significantly higher in SD and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$genotypes, relative to their WT controls. **(C)** Densitometric quantification of GM2 faction shows significantly higher levels in SD and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$genotypes (n = 3) when compared with control mice. *$P \leq 0.05$, **$P \leq 0.01$. Error bars, ±SE.

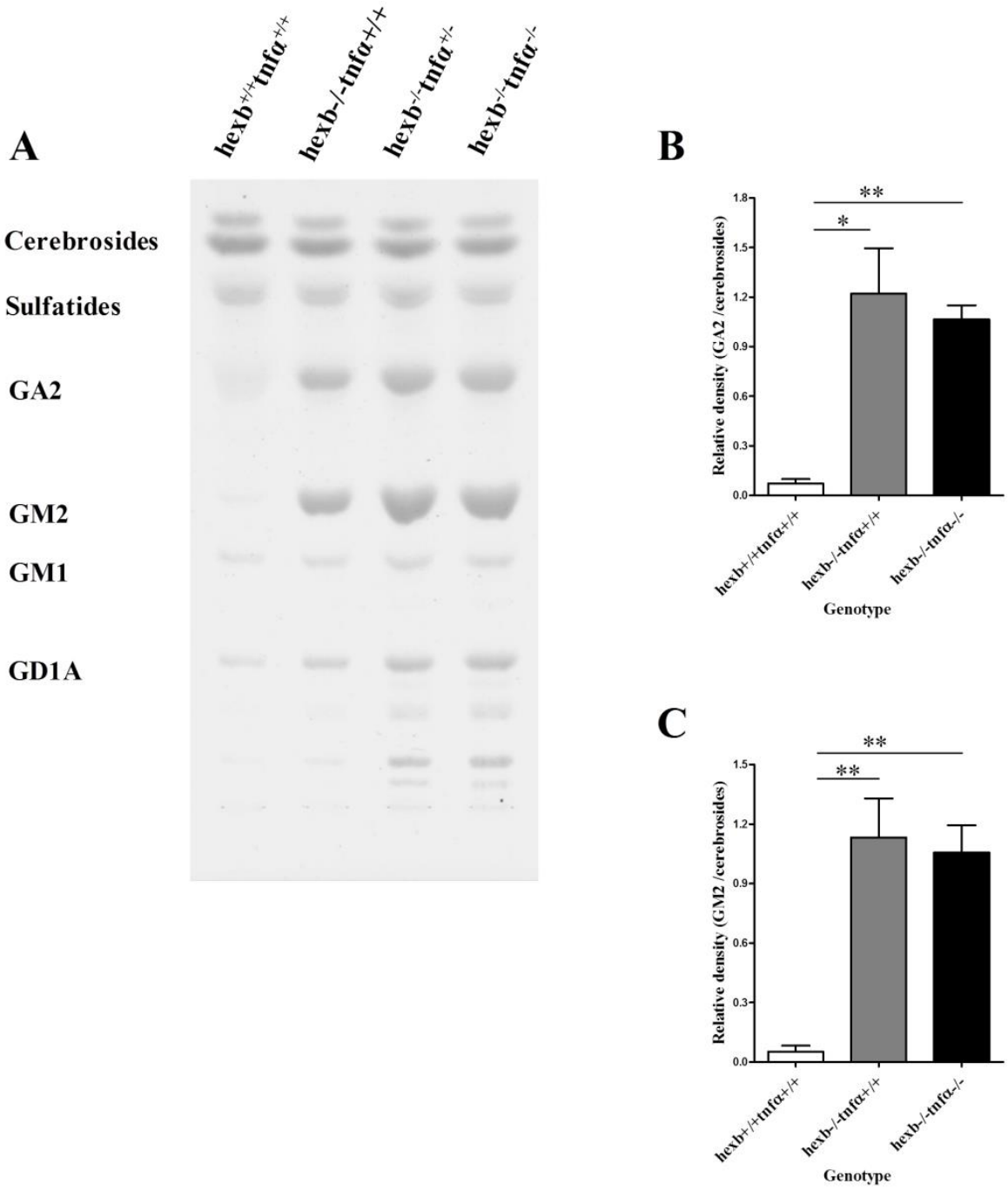
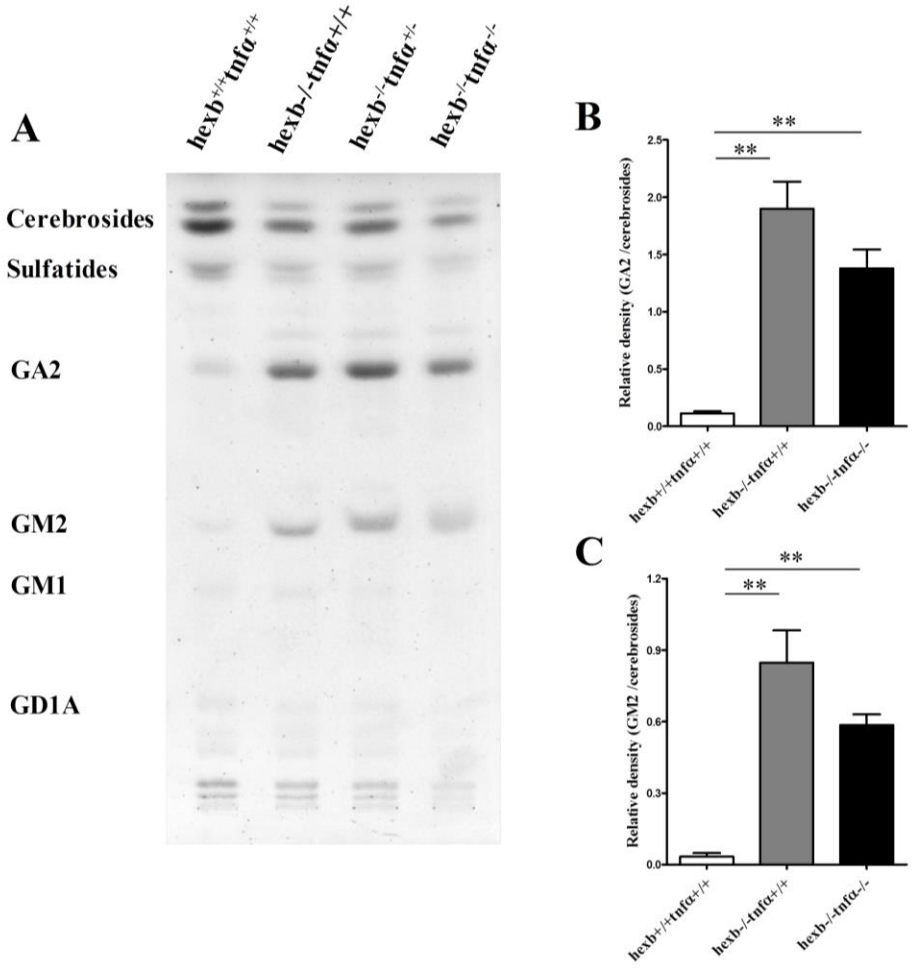

A
hexb+/+tnfα+/+
hexb-/-tnfα+/+
hexb-/-tnfα+/-
hexb-/-tnfα-/-
Cerebrosides
Sulfatides
GA2
GM2
GM1
GD1A
B
Relative density (GA2 /cerebrosides)
Genotype
C
Relative density (GM2 /cerebrosides)
Genotype

Figure 3 TNFα does not alter GM2 and GA2 gangliosidosis in the cerebella of 17-week-old SD mice

Ganglioside fractions from cerebellar lysates were analyzed by TLC. **(A)** Representative TLC photograph demonstrates the distribution of ganglioside accumulation in the cerebellum (n = 3 experiments). GM2 fraction was undetectable in WT mice. Sulfatides, cerebrosides and GM1 bands were not changed across the lanes. **(B)** Densitometric quantification of GA2 shows that it was significantly higher in SD and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$genotypes, relative to their WT controls. **(C)** Densitometric quantification of GM2 faction shows significant increases in SD and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice (n = 3 for each genotype) in comparison with WT control mice. *$P \leq 0.05$, **$P \leq 0.01$. Error bars, ±SE.

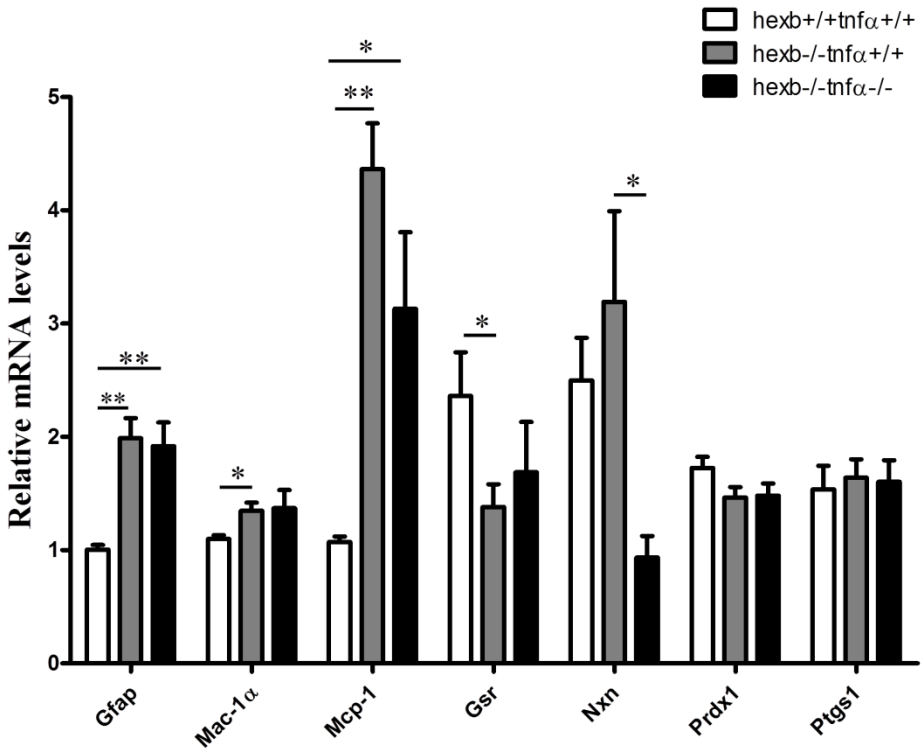

A
hexb+/+tnfα+/+
hexb-/-tnfα+/+
hexb-/-tnfα+/-
hexb-/-tnfα-/-
Cerebrosides
Sulfatides
GA2
GM2
GM1
GD1A
B
Relative density (GA2 /cerebrosides)
**
**
C
Relative density (GM2 /cerebrosides)
**
**

2.3.3 TNFα deletion attenuates the expression of markers of gliosis and oxidative stress

Utilizing mRNA isolated from the cerebella of *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was conducted to assess the expression level of the following genes: glial fibrillary acidic protein (*Gfap*), macrophage antigen alpha (*Mac-1α*), monocyte-chemotactic protein-1 (*Mcp-1*), glutathione reductase (*Gsr*), nucleoredoxin (*Nxn*), peroxiredoxin1 (*Prdx1*), and prostaglandin-endoperoxide synthase 1 (*Ptgs1*). Our data indicate *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice show significant increases in the mRNA levels of *Gfap*, *Mac-1α* and*Mcp-1* genes, a significant decrease in *Gsr* expression and no change in the expression of *Nxn*, *Prdx1* and*Ptgs1* genes (Figure 4). In comparison to *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ mice, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice show significant increases in the mRNA levels of *Gfap* and *Mcp-1* genes, a significant decrease in Nxn expression and no significant change in the expression of *Mac-1α*, *Gsr*, *Prdx1* and *Ptgs1* genes (Figure 4). The only gene product that appears to be affected by the absence of TNFα is *Nxn* which is significantly reduced in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice in comparison with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice.

Figure 4 Expression levels of the genes involved in gliosis and oxidative stress pathways

mRNA isolated from the cerebella of 15-week-old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice was assessed using qPCR for the level of the expression of the following genes: *Gfap*, macrophage-a antigen (*Mac-1α*), monocyte chemo-attractant protein-1 (*Mcp-1*), *Gsr*, *Nxn*, *Prdx1* and *Ptgs1*. The bar graph represents the mean expression measured in three independent quantitative real-time PCRs for each gene ± standard error bars. All plotted data represent gene expression levels relative to gene expression in the cerebella of age-matched *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ animals. The only gene that appears to be affected by the absence of TNFα in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice is *Nxn*, which was significantly reduced in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice in comparison with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice. *$P \leq 0.05$ and **$P \leq 0.01$. Error bars, ±SE.

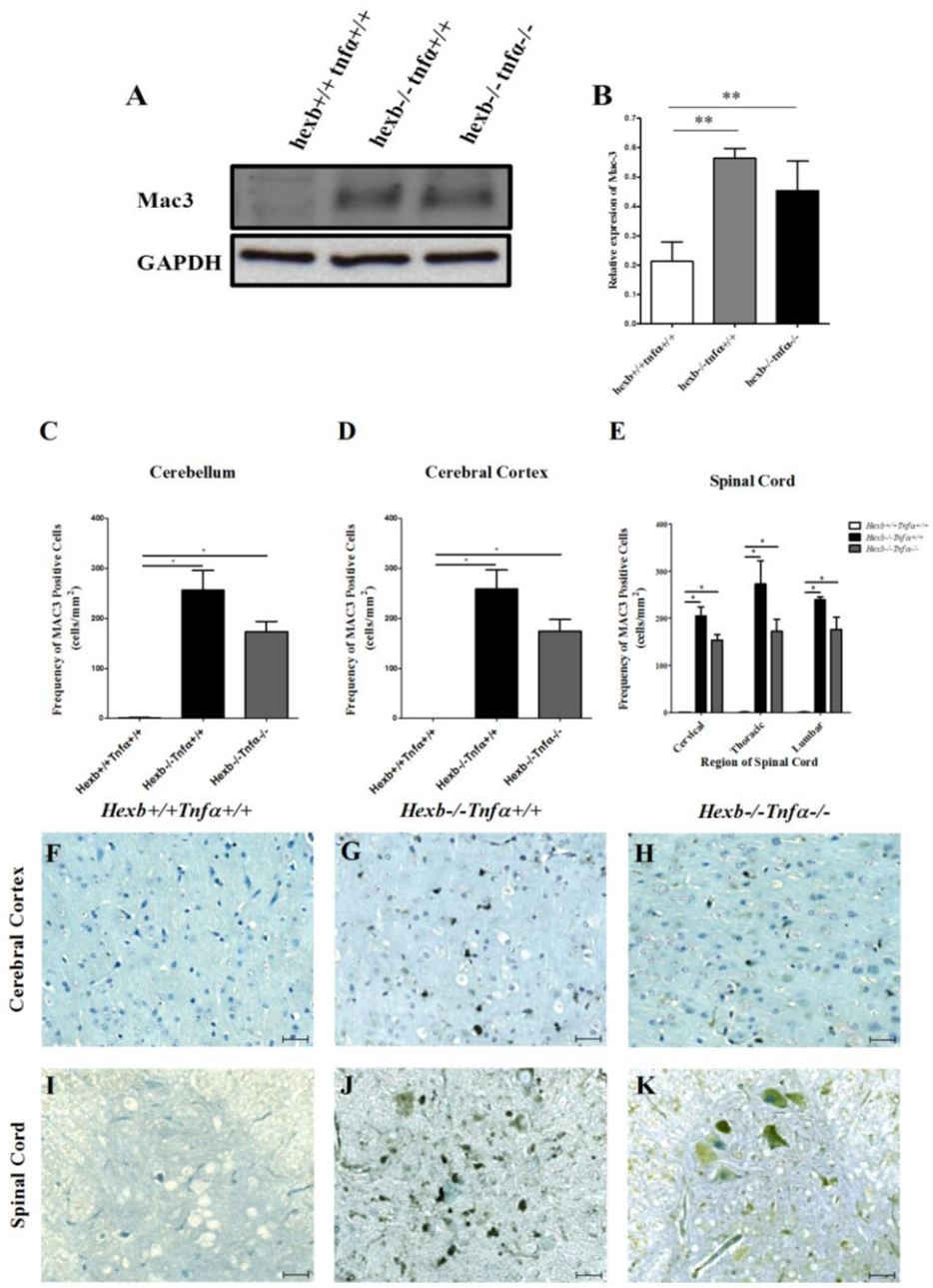

hexb+/+tnfα+/+
hexb-/-tnfα+/+
hexb-/-tnfα-/-
Relative mRNA levels
0
1
2
3
4
5
**
**
*
*
**
*
*
Gfap
Mac-1α
Mcp-1
Gsr
Nxn
Prdx1
Ptgs1

2.3.4 *Tnfα* deletion does not affect microgliosis in SD mice

In order to examine the molecular events leading to microglia activation, we examined the expression levels of MAC3 in the cerebellum of WT and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice, relative to *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice (at 120 days of age). Our results indicate significant upregulation of cerebellar MAC3 expression levels in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ in comparison to WT mice (Figure 5A and B). To investigate the role of activated microglia as a possible pathogenic mechanism that might be affected by TNFα deletion, we examined frequency of activated microglia (MAC3-positive cells), in the cerebellum, cerebral cortex and spinal cord. Our results indicate that microglia/macrophage numbers are similar in the cerebella and cerebral cortices of *Hexb*$^{-/-}$*Tnfα*$^{+/+,}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, despite a trend of lower levels in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice (Figure 5C, D and F-H). This suggests that either there is no difference in regulation of microglia in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, or that microgliosis occurs at a lower rate in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice, but by 17 weeks a maximal level of microgliosis is reached in the brain tissues of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice, and appears similar to levels observed in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice. In the spinal cord, microglia/macrophage numbers are not significantly different between *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice although there again appears to be a trend in the three spinal regions toward elevated levels in *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$mice (Figure 5E and F). Our findings indicate that TNFα may not be a major player during recruitment and/or activation of microglia/macrophages in the CNS to sites of neuroinflammation.

Figure 5 The effect of TNFα deletion on MAC3 expression in the CNS of SD mice

(A) MAC3 expression in the cerebellum was evaluated in 17-week-old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice by western blotting. An equal amount of sample protein was loaded in each lane. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(B)** Densitometric quantification of MAC3 expression in the cerebella of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice show significantly higher expression relative to *Hexb*$^{+/+}$*Tnfα*$^{+/+}$mice. Decreased MAC3 expression in the double knock-out mice is only a trend. **(C–E)** Quantification of MAC3-immunoreactive cells in the cerebellum, cerebral cortex and spine of *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. Macrophage numbers were similar in the cerebella, cerebral cortex and spines of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. **(F–K)** Images of the cerebral cortex and spinal cords illustrating differences in MAC3-immunoreactive microglia levels between *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. Images of spine sections illustrate MAC3 staining in the anterior horn. Scale bars indicate 20 μm. n = 3–5 mice per study. *P< 0.05. Error bars, ±SE.


A
hexb+/+ tnfα+/+
hexb-/- tnfα+/+
hexb-/- tnfα-/-
Mac3
GAPDH
B
Relative expression of Mac3
C
Cerebellum
D
Cerebral Cortex
E
Spinal Cord
Frequency of MAC3 Positive Cells (cells/mm²)
Region of Spinal Cord
Cervical
Thoracic
Lumbar
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
Cerebral Cortex
Spinal Cord
F
G
H
I
J
K

2.3.5 Astrogliosis is decreased with TNFα deletion in SD mice

To examine the level of GFAP expression as a measure of active astrogliosis in Sandhoff disease pathology, we used western blot analysis of cerebellum lysates from 17-week old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice (Figure 6A and E). GFAP expression in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice was significantly ($P<0.01$) higher than *Hexb*$^{+/+}$*Tnfα*$^{+/+}$mice. Notably, GFAP expression in the cerebellum of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice was significantly lower ($P<0.05$) than GFAP level in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice (Figure 6A and E). Little is known about the signal transduction pathways that are involved in the activation/proliferation of astrocytes (Sofroniew & Vinters, 2010). However, increased expression of GFAP in response to the activation of the IL-6/pStat3 pathway is considered the cardinal indication of reactive astrogliosis(Sofroniew & Vinters, 2010). To address the mechanisms underlying astrocyte activation, we examined the levels of IL-6 expression, JAK2 and STAT3 phosphorlyation and found no change in IL-6 or pJAK2 levels but a significant decrease in pSTAT3 in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ compared to SD mice (Figure 6A and C-E). To determine if STAT3 was present in the astrocytes of our mouse models, we double-labeled brains of 17-week old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice with immunofluorescent antibodies for STAT3 and GFAP (Figure 6F). Analysis of confocal slices reveals that STAT3 colocalizes with nuclei of astrocytes in the cerebellum of these mice, adding credence to its role in astrocyte activation. Astrogliosis, in the central nervous system, has typically been identified morphologically by the presence of hypertrophic astrocytes with increased process ramifications and immunohistochemically by the presence of increased expression of GFAP (Figure 7). The

number of GFAP-positive astrocytes in the cerebellum was found to be significantly higher in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice relative to WT (Figure 7A). Notably, there were significantly fewer GFAP positive astrocytes in the cerebella of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice. In the cerebral cortex, there was a trend toward lower numbers of GFAP positive astrocytes in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice (Figure 7B and D-F). These findings suggest that the onset of astrogliosis was delayed in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice. Additionally, astrocyte proliferation may occur at an accelerated rate in *Hexb*$^{-/-}$ mice due to up-regulated TNFα expression. Furthermore, we examined the GFAP expression in the spinal cord using sections from the thoracic, cervical and lumbar regions from *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice at 17 weeks of age (Figure 7C and G-I). Our results reveal that astrocyte numbers were significantly higher in the thoracic and lumbar spinal cord of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice compared to *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, with a similar trend visible in the cervical region. This indicated that TNFα was involved in increasing the recruitment or differentiation of astrocyte precursors in the spinal cord of SD mice. Thus, deletion of the *Tnfα* gene in *Hexb*$^{-/-}$ mice reduced the level of astrogliosis markers in the cerebellum and spinal cord. Our data indicates that the absence of TNFα activity in *Hexb*$^{-/-}$ mice results in a less severe neurodegenerative course that is associated with reduced astrogliosis.

Figure 6 Effect of TNFα deletion on neuroinflammation markers and phospho-STAT3 signaling in SD cerebellum

(A) Expression of IL-6, JAK2, STAT3 and GFAP, as well as the phosphorylation of JAK2 and STAT3 in the cerebellum were evaluated in 17-week-old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice by western blot analysis. GAPDH was used as a loading control. **(B)** Densitometric quantification of IL-6 expression shows no significant difference among all three groups. **(C)** Densitometric quantification of pJAK2 in the cerebellum of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice shows significantly lower phosphorylation relative to *Hexb*$^{+/+}$*Tnfα*$^{+/+}$mice. **(D)** Densitometric quantification of pSTAT3 in the cerebellum of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice shows significantly higher phosphorylation relative to *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ and significantly lower phosphorylation relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice. **(E)** GFAP expression was significantly increased in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice relative to *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ mice; however, GFAP expression in the Hexb$^{-/-}$Tnfα$^{-/-}$ mice was significantly lower than in Hexb$^{-/-}$Tnfα$^{+/+}$ mice. The data represent the mean ± SEM of three animals per group. *P ≤ 0.05 and **P ≤ 0.01. **(F)** Confocal immunofluorescence optical slice in the cerebellum of *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, showing double staining for GFAP-positive astrocytes (red) and STAT3 (green). Note that STAT3 is present in astrocytes and, in some cases, localizes with nuclei (arrows). Bars represent 10 µm.

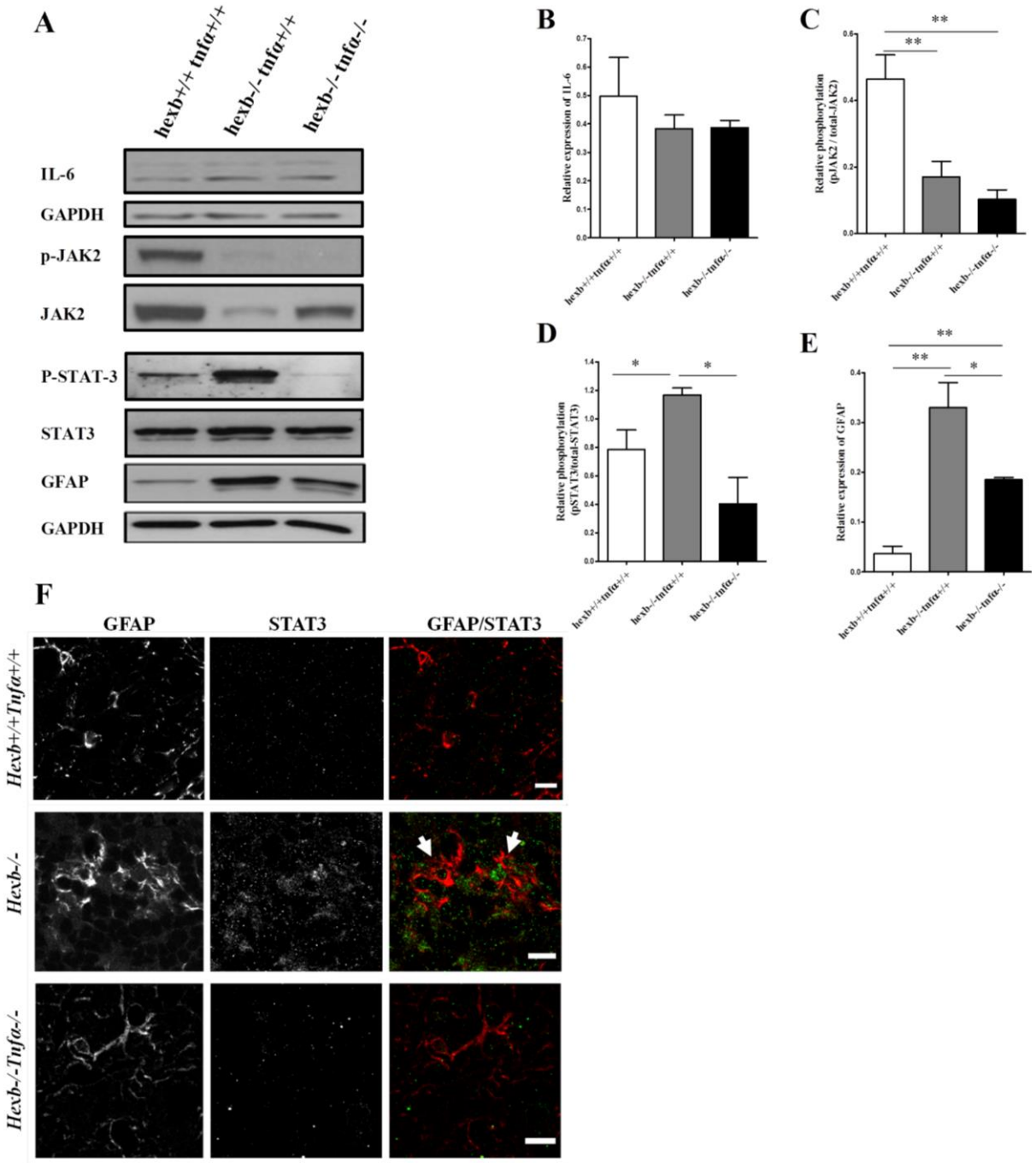
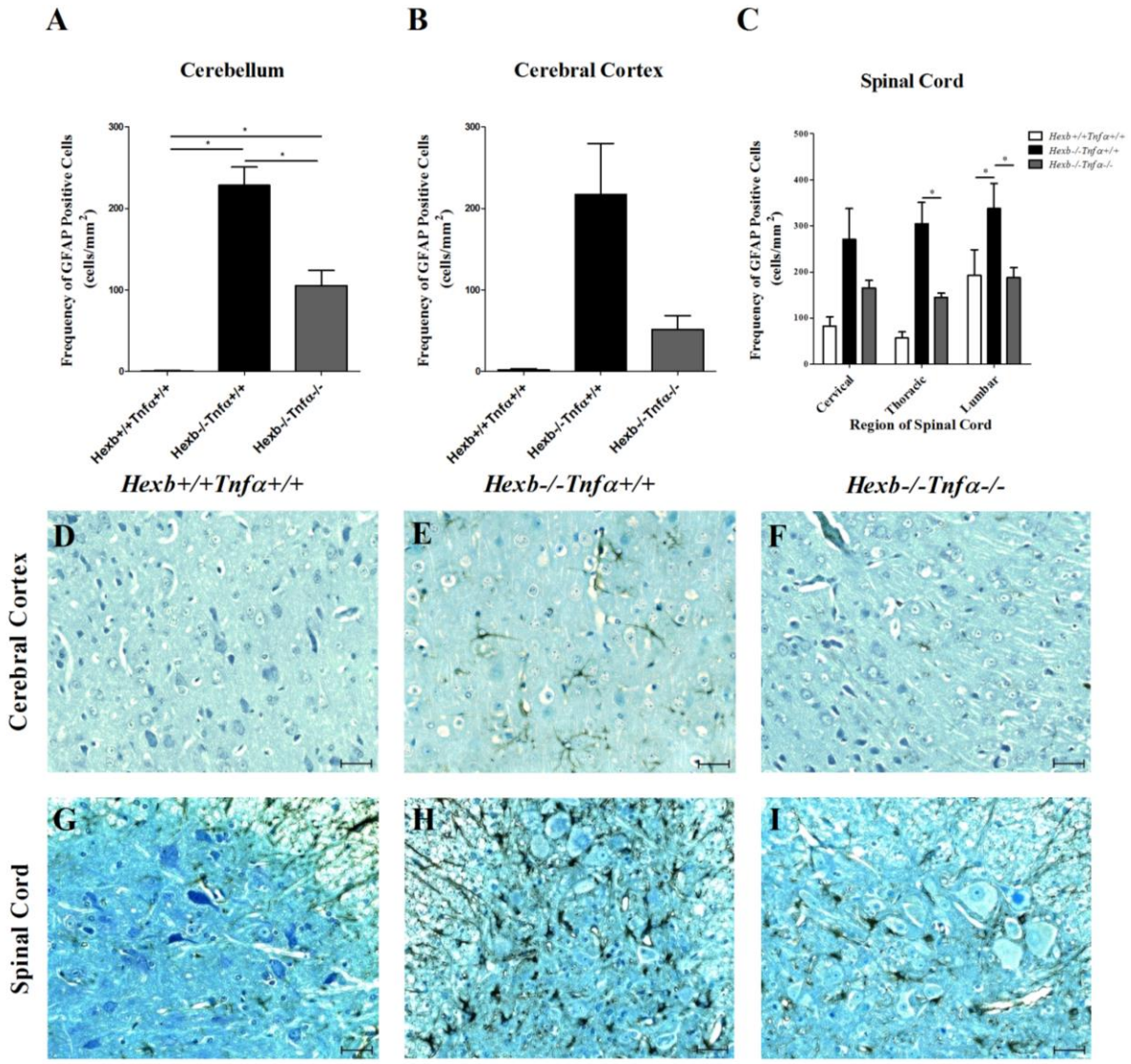

A
hexb+/+ tnfα+/+
hexb-/- tnfα+/+
hexb-/- tnfα-/-
IL-6
GAPDH
p-JAK2
JAK2
P-STAT-3
STAT3
GFAP
GAPDH
B
Relative expression of IL-6
C
Relative phosphorylation (pJAK2 / total-JAK2)
D
Relative phosphorylation (pSTAT3/total-STAT3)
E
Relative expression of GFAP
F
GFAP
STAT3
GFAP/STAT3
Hexb+/+Tnfa+/+
Hexb-/-
Hexb-/-Tnfa-/-

Figure 7 *Tnfα* deletion in SD mice reduces CNS GFAP immunoreactivity

(A–C) Quantification of GFAP positive cells in the cerebellum, cerebral cortex and spine of *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. The number of GFAP positively cells is significantly lower in the cerebella and lumbar region of the spine in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice. **(D–F)** Images of the cerebral cortex illustrating differences in GFAP-positive astrocyte levels between *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. **(G–I)** Spine images show GFAP staining in the anterior horn. Intense GFAP expression is clearly visible in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice and markedly reduced in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, indicating reduced astrogliosis. *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ mice show little to no GFAP staining across tissues of the CNS. Scale bars indicate 20 μm. n = 3–5 mice per study. *$P \leq 0.05$. Error bars, ±SE.


A
B
C
Cerebellum
Cerebral Cortex
Spinal Cord
Frequency of GFAP Positive Cells (cells/mm²)
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
Cervical
Thoracic
Lumbar
Region of Spinal Cord
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
D
E
F
G
H
I
Cerebral Cortex
Spinal Cord

2.3.6 Apoptotic neuronal death is decreased with deletion of TNFα in SD mice

We investigated if the deletion of *Tnfα* could modulate neuronal apoptosis in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice. There were significantly higher numbers of apoptotic cells in the cerebella of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice relative to both *Hexb*$^{+/+}$*Tnfα*$^{+/+}$and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice (included quantification numbers and stats) (Figure 8A and D). This significant difference was also observed in the cervical region of the spinal cord (Figure 8C and F), with similar trends in the thoracic and lumbar regions of the spinal cord, and in the cerebral cortex (Figure 8B and E). These data suggest that apoptosis was reduced in the CNS of *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$mice relative to SD mice. In agreement with our previous result that GFAP immunoreactivity is significantly reduced in the cerebellum and spine of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice in relation to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice, we detected reduced neuronal cell death in the same regions of the CNS in the *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice (Figure 8). Essentially, we found extensive apoptosis coincided with increased astrocyte activation. Our results raise the possibility that astrocytes residing in spinal cord and cerebellum might play an important role in the apoptosis-mediated pathogenesis of Sandhoff disease.

It has been shown that NF-kB activation in microglia can be detrimental to neuronal cell survival via release of excitotoxins and oxygen free radicals (Kettenmann, 2007). Nucleoredoxin, which we see to be down-regulated in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice (Figure 4), is shown to enhance TNFα and NIK induced activation of NF-κB pathways, suggesting reduction of the non-canonical NF-κB pathway in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice may be a factor in their improved survival/ behavioural performance (Hirota et al., 2000; Sun, 2011).To explore this, the relative expression of TRAF3, NIK and RelB- components of

the NIK/non-canonical NF-κB pathway- were determined by western blot (Figure 9). The results revealed a significant decrease in TRAF3 expression ($P<0.05$) in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mouse cerebella relative to SD mice (Figure 9A and B). Furthermore, NIK expression was significantly reduced in both *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ *mice* relative to SD mice (Figure 9A and C).The level of RelB was significantly lower in both *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice in comparison to SD mice (Figure 9A and D) ($P<0.05$). These data suggest that the NIK/non-canonical NF-κB pathway is activated in Sandhoff disease, and is limited by deletion of *Tnfα*.

Figure 8 Apoptosis is reduced in the CNS of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice

(A–C) Quantification of TUNEL positive cells in the cerebellum, cerebral cortex and spine of *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. There are significantly higher numbers of cells undergoing apoptosis in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$cerebella and cervical spine tissues, relative to both *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. A similar trend is seen in the cerebral cortex and thoracic and lumbar spine regions. **(D–F)** Images show examples of TUNEL positive cells in the cerebellum, cerebral cortex and spine, respectively. Apoptotic nuclei are small and stained brown. Scale bars indicate 20 μm. n = 3–5 mice per genotype. *P< 0.05. Error bars, ±SE.

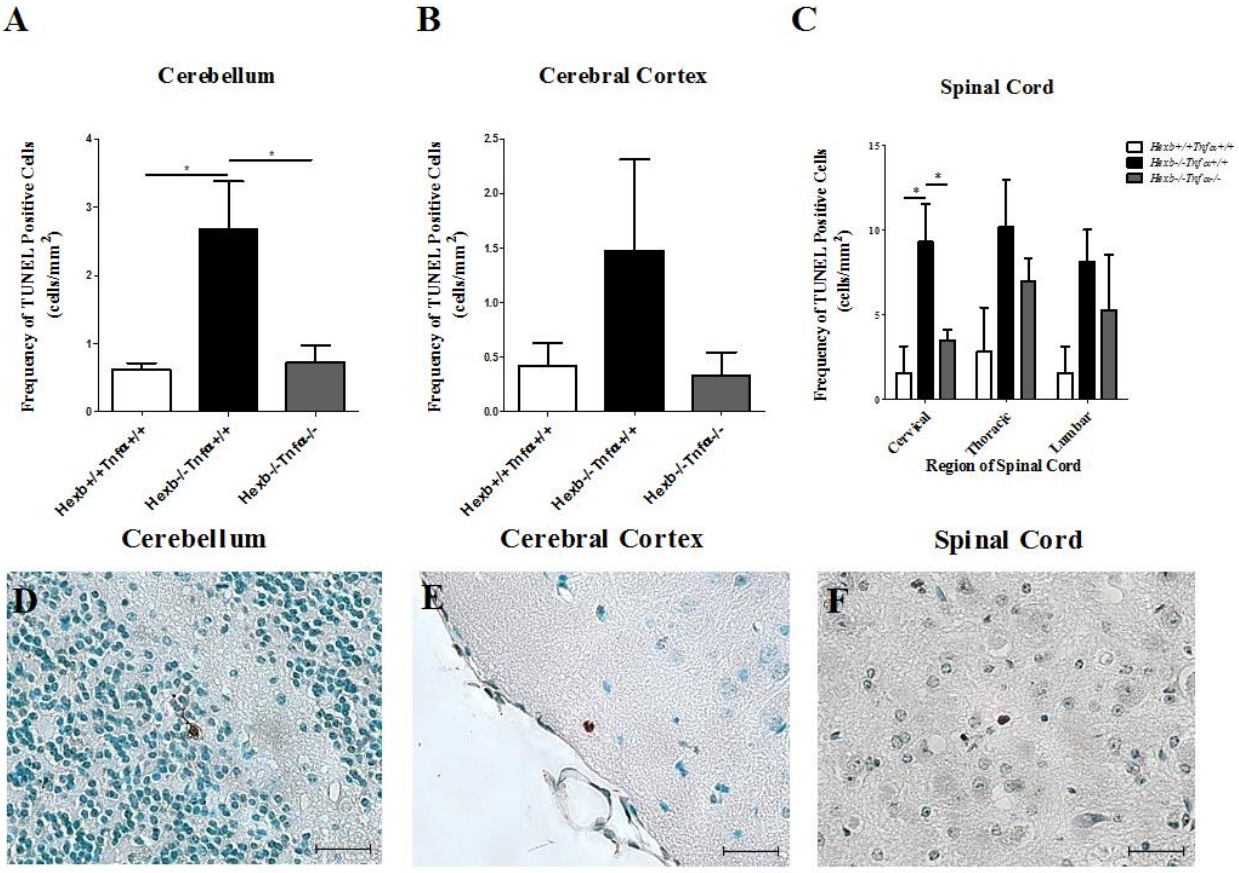
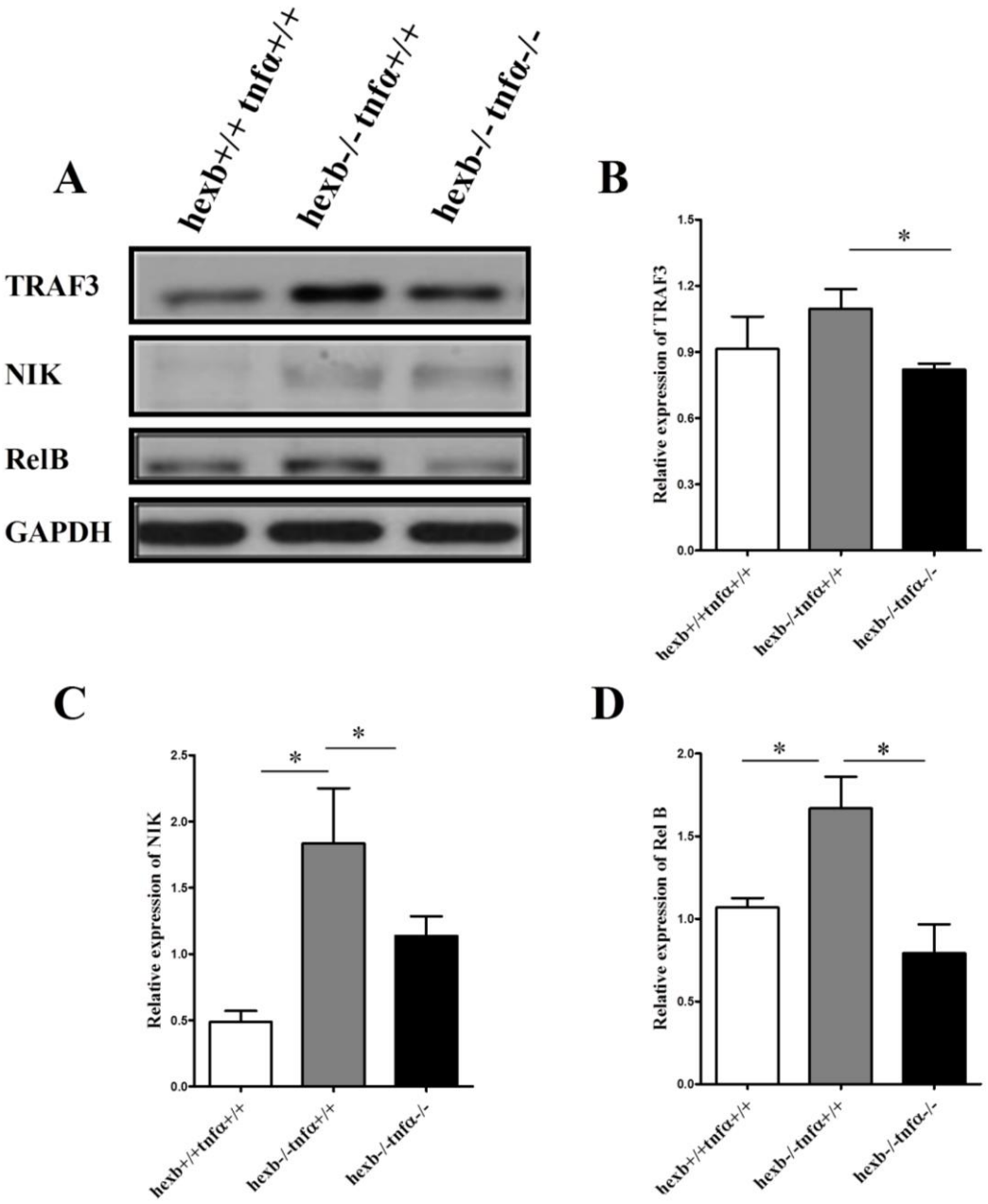

A
B
C
Cerebellum
Cerebral Cortex
Spinal Cord
Frequency of TUNEL Positive Cells (cells/mm2)
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
Cervical
Thoracic
Lumbar
Region of Spinal Cord
D
E
F

Figure 9 Differential activation of the NF-κB non-canonical pathway in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice

(A) TRAF3, NIK and RelB expression were assessed in the cerebella of 17-week-old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice using western blot analysis. GAPDH was used as a loading control. **(B)** Densitometric quantification of TRAF3 shows a modest trend of up-regulation in SD mice and down-regulation in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice in comparison with WT mice. TRAF3 expression was significantly reduced in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$cerebellum relative to SD cerebellum. **(C)** Densitometric quantification of NIK demonstrates a significant increase in its expression in the cerebella of SD and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice relative to WT mice. However, NIK expression shows a substantial reduction in the Hexb$^{-/-}$Tnfα$^{-/-}$cerebellum relative to SD cerebellum. **(D)** Densitometric quantification of RelB expression shows a significant reduction in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice relative to SD mice. RelB expression in SD cerebellum was highly up-regulated compared to WT controls. n = 3 mice per group. *$P \leq 0.05$. Error bars, ±SE.


A
hexb+/+ tnfα+/+
hexb-/- tnfα+/+
hexb-/- tnfα-/-
TRAF3
NIK
RelB
GAPDH
B
Relative expression of TRAF3
1.5
1.2
0.9
0.6
0.3
0.0
*
hexb+/+tnfα+/+
hexb-/-tnfα+/+
hexb-/-tnfα-/-
C
Relative expression of NIK
2.5
2.0
1.5
1.0
0.5
0.0
*
*
hexb+/+tnfα+/+
hexb-/-tnfα+/+
hexb-/-tnfα-/-
D
Relative expression of Rel B
2.0
1.5
1.0
0.5
0.0
*
*
hexb+/+tnfα+/+
hexb-/-tnfα+/+
hexb-/-tnfα-/-

2.4 Discussion

The pathogenesis of neurodegeneration in Sandhoff disease is complex and involves various cellular changes as well as a profound disturbance of neuronal and glial homeostasis (Wajant, Pfizenmaier, & Scheurich, 2003). Neuronal inflammation, glial activation and cytokine up-regulation are considered among the most prominent convergent points among various neurodegenerative diseases (McGeer & McGeer, 2001). TNFα is up-regulated in various neurodegenerative diseases including Sandhoff disease suggesting a significant role for this cytokine in modifying the neurodegenerative process (Fontaine et al., 2002; Murakami et al., 2005). A critical question has been the extent to which TNFα-mediated neuroinflammation contributes to the neurodegeneration in these disorders. In this study we have provided direct evidence that TNFα plays a major role in activation and expansion of the astrocytes populations, and that this process accelerates the neuronal cell death. Genetic deletion of TNFα revealed that the *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice gained neurological function leading to a delayed neurodegenerative cascade compared to SD mice (Abo-Ouf et al., 2013).

The Sandhoff disease mouse model is considered an ideal prototype of a sphingolipidosis pathology that ultimately leads to neurodegeneration (Wada, Tifft, & Proia, 2000; Akiyama et al., 2000). In Sandhoff mice, abnormal storage is detectable in multiple CNS locations, including cerebrum, cerebellum and spinal cord (Wu et al., 2008; Norflus et al., 1998). Whether a neurological improvement of Sandhoff pathology necessitates a detectable global or localized correction of glycolipid storage in CNS

region has been less clear. In this neurodegenerative model, it is expected that a critical threshold of GM_2 and GA_2 storage is the driving force behind neuronal apoptosis (Wada et al., 2000; Lee et al., 2007; Teng et al., 2002; Norflus et al., 1998). In fact, it was demonstrated that neurological amelioration has been achieved via reduction of the primary injury of neuronal storage (Lee et al., 2007; Norflus et al., 1998). However, some therapeutic strategies such as BMT were beneficial to suppress neurological deterioration without detectable reduction of GM_2 in Sandhoff brain cells (Jeyakumar et al., 2004). While it was demonstrated that TNFα increases GM_2 production via induction of GM_2 synthase in tumor cells(Raval et al., 2007), our study did not find differences in the levels of GM_2/GA_2 storage in SD and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$cerebella suggesting that the neuroprotective effect of TNFα depletion is not mediated via glycolipid storage.

The early signs of cerebellar dysfunction in SD mice, i.e., ataxia and tremors were monitored effectively using the behavioural tests conducted in our study. These neurological and behavioural tests made it possible to relate the effects of TNFα deletion in SD mice to cerebellar neuron function, neuronal signaling and motor behavior. Our data pointed to an improvement to the function of the basal ganglia and cerebellum neurons which reflect motor function as well as an improvement to muscle strength and coordination which reflect spinal motor neurons and possible neuromuscular junctions.

The mechanism by which the TNFα signal is translated into a pathophysiological effect remains to be determined. This study provides a new view of neurodegeneration in which TNFα may induce neuronal death indirectly through its effect on astrocytes. We examined the GFAP expression in spinal cord of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice.

Astrogliosis was significantly reduced in the spinal cord and cerebellum of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. A similar trend was observed in cerebral cortex. Our data indicated that the absence of TNF*α* resulted in a less severe neurodegenerative course that is associated with reduced astrogliosis. Microglia, the main producers of TNF*α*, have deleterious and/or protective dramatic effects on neurons in the course of neurodegenerative disease. Activated microglia have been shown to be involved in the progression of neuronal cell death in GM2 gangliosidosis (Myerowitz et al., 2002). To investigate the role of activated microglia as a possible pathogenic mechanism that might be affected by TNF*α* deletion, we immunostained CNS mice samples for the microglial marker, MAC3. Our data show a trend toward a reduction in the number of MAC3-immunoreactive macrophages in the cerebellum, cerebral cortex and spine of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice relative to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice, pointing to a reduction in the severity of inflammation in this region due to the absence of TNF*α*. The lack of statistical significance here may be due to a maximal threshold in microglial numbers reached early on by *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice that is approached at 17 weeks by *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, despite a lower overall rate of microglial activation/proliferation over the course of the disease.

Sandhoff Disease shares many features with other neurodegenerative disorders such as increased reactive astrocytic pathology and astrogliosis (Igdoura, Mertineit, Trasler, & Gravel, 1999). Recent studies have suggested that proinflammatory cytokines such as TNF*α* can trigger and modulate astrogliosis (Jeyakumar, Dwek, Butters, & Platt, 2005; Culmsee & Plesnila, 2006). The impact of TNF*α* on the induction of astrogliosis has been observed in several studies(Culmsee & Plesnila, 2006). It was reported that

TNF*α* microinjected into neonatal brain can elicit extensive GFAP immunoreactivity (Culmsee & Plesnila, 2006). Furthermore, astroglial response to MPTP neuronal injury was almost completely inhibited in mice deficient in both TNFR1 and TNFR2 (Hodges & Cheng, 2006). Besides these *in vivo* studies, TNF*α* is reported to increase GFAP expression *in vitro* (Zhang et al., 2000). It is possible that the regulatory JAK2/STAT3 axis of astrogliosis is broadly applicable to other neurodegenerative disorders. STAT3 is a crucial transcription factor involved in several molecular and cellular responses including neuroinflammation, cell proliferation and survival (Tsuda et al., 2011a). Recent study has reported that cytokine induced activation of STAT3 is controlled by glycogen synthase kinase-3 (GSK3β) in astrocytes (Sakuraba et al., 2006). Interestingly, recent work has reported that STAT3 can elicit an atypical cell death mechanism via lysosomal membrane permeabilization that leads to leakage of lysosomal proteases into the cytosol (Futerman & van, 2004). It has been found that STAT3 phosphorylation, accompanied by toxic gliosis, is associated with various neurodegenerative diseases such as spinal cord injury and neuropathic pain (Tsuda et al., 2011b). It has also been reported that activated JAK2-STAT3 signaling results in the increased expression of IL-6 and IL-10, and that IL-6 is considered an important STAT3 activator (Nilsson et al., 2010; Walkley, 2009). Thus, it was reasonable to hypothesize that decreased GFAP expression, as well as astrocytic proliferation, in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ brains is due to the decreased phosphorylation of STAT3. Our data provide a direct clue that inhibition of the JAK-STAT3 signaling pathway may prevent reactive astrogliosis in Sandhoff. Although, we found no difference in IL-6 or pJAK2 expression between *Hexb*$^{-/-}$*Tnfα*$^{+/+}$and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice, other

molecules, upstream to STAT3,and downstream of TNFα signaling- such as leukemia inhibitory factor (LIF) and oncostatin M (OSM)- could mediate this STAT3 activation (Suzuki et al., 2005; Park, Nozell, & Benveniste, 2012). Recent work has demonstrated that TNFR1 associates with JAK2 constitutively to form a complex and TNF recruits additional molecules of JAK2 (Guo, Dunbar, Yang, Pfeffer, & Donner, 1998). Furthermore, activation of STAT3 can be performed by the TNFR1/JAK2 complex (Pincheira, Castro, Ozes, Idumalla, & Donner, 2008). We also observe nuclear colocalization of STAT3 with nuclei of astrocytes, which is consistent with activation of STAT3. These findings may explain the increased phosphorylation of STAT3 in Sandhoff brains compared to the reduced levels of pSTAT3 observed in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice.

Unlike the canonical NF-κB pathway, which relies on IκBα activation, the non-canonical pathway relies on inducible processing of p100 (Hebert & De, 2009). We investigated the role of the non-canonical NF-κB pathway in SD mice in the presence or absence of TNFα. Our results show down-regulation of TRAF3, NIK and a trend towards down-regulation of RelB in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice compared to SD mice. It has been reported that TRAF3 degradation is essential to trigger TNFR2-receptor mediated activation of NIK and nuclear translocation of p52/RelB heterodimers into the nucleus (Vallabhapurapu et al., 2008; Rauert et al., 2010). Our results indicate that TNFα utilizes the NIK- mediated NF-κB pathway to elicit an immune response in Sandhoff disease but the stabilization of NIK appears independent of TRAF3 levels. Our results demonstrate that TNF*α* depletion in SD cerebellum down-modulates non-canonical NF-κB signaling.

Whether there is an IKKα-dependent feedback that controls the magnitude and kinetics of the pathway is still an open question.

An intriguing finding was the extensive level of apoptosis occurring in the spinal cord of SD mice which coincided with increased activation of astrocytes. Alongside the reduction in astrogliosis seen in the spinal cord of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice relative to *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$ mice, there was a reduction in apoptotic cells. The results indicate that astrocytes residing in spinal cord may play an important role in the apoptosis-mediated pathogenesis of Sandhoff disease.

In Summary, our results point to the cytokine, TNF*α*, as a potential therapeutic target to slow the rapid neurodegenerative process Sandhoff disease. Given that the multifaceted therapeutics to neurodegenerative diseases have gained a growing appreciation and provide a promising avenue to improve prognosis of neurodegeneration, our study also identifies the STAT3 pathway as a target to achieve a better outcome for disease pathology.

2.5 Materials and methods

Mice

The Sandhoff disease model mice (*Hexb*$^{-/-}$) were a generous gift from Dr. R. Gravel (University of Calgary, Canada).Tumor Necrosis Factor-α (TNF*α*) deficient mice purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and were backcrossed ten times onto a C57Bl/6 background in house. Since both transgenic strains are on a C57Bl6 genetic background, the *Hexb*$^{-/-}$ mice were crossed with *Tnfα*$^{-/-}$ mice to generate

doubly heterozygous (*Hexb*$^{+/-}$*Tnfα*$^{+/-}$) mice. These doubly heterozygous mice were mated with *Tnfα*$^{-/-}$mice to generate *Hexb*$^{+/-}$*Tnfα*$^{-/-}$ mice. Subsequently, *Hexb*$^{+/-}$*Tnfα*$^{-/-}$ mice were mated with each other to generate doubly null (*Hexb*$^{-/-}$*Tnfα*$^{-/-}$) mice. Genotypes were confirmed by PCR using tail DNA.

PCR Genotyping

The genotype of the *Tnfα* locus was determined by PCR from tail DNA. A single set of primers was used, 5- GAAAAGCAAGCAGCCAACCAG-3 and 5-GTCCAACCCACGGCTTC-3, yielding a 646bp product for the wild-type gene and a 1357 bp product for the knock-out gene. Primers for detection of *Hexb* wild-type sequence were primer 5'-GGTTTCTACAAGAGACATCATGGC-3 and primer yielding 141bp products for the wild-type gene and primer 5'-CAATCGGTGCTTACAGGTTTCATC-3 and primer 5'-GATATTGCTGAAGAGCTTGGCGGC-3 in neo to give 700bp for the *Hexb* knock-out gene.

Behavioural Tests

An Animal Utilization Proposal (AUP) was established which is in accordance with the Ontario Animals for Research Act requirements and the standards of the Canadian Council on Animal Care (CCAC). The lifespan, body weight, and neurological function of each group of mice were monitored by using modified Irwin (Irwin, 1968) and Moser (McDaniel & Moser, 1993) observational test batteries (Miklyaeva et al.,

2004). Motor function was evaluated by means of the rotorod, the wire hang, grip strength and righting reflex behavioural tests which were conducted weekly subsequent to weaning. ***Rotorod.*** The rotorod test measured the latency to fall off a rotating rod and infrared photo cells captured exact fall time (Miklyaeva et al., 2004). The Accuscan EZ-Rod and companion computer software EZ rod version 1.20 were used. Predetermined program involved placing the mice on pre-spinning rod, which gradually accelerated from 4 rpm to a maximum of 40 rpm over the 6 minute test. Each mouse was given 3 trials, the best of which was included in the data set. ***Wire Hang.*** A wire mesh was utilized to conduct the wire hang test. Mice were inverted 20cm above a padded surface and forced to use their four limbs to hold their body suspended upside down for a maximum of 60 seconds. The test was conducted in triplicate for each mouse and the highest time was recorded. ***Grip Strength.*** A digital force gauge meter (Chatillon Ametek Inc.) was used to measure the peak grip strength (Miklyaeva et al., 2004). This is the amount of force in kg exerted by the two front limbs of the mouse grasping a metal rod as the mouse is pulled away from the rod at a constant force. ***Righting Reflex.*** The righting reflex was conducted by placing a mouse on its back on a solid surface and recording the amount of time taken for the mouse flip back over to stand on all four limbs. Each mouse was given three reflex trials and the best of these was included in the data. Mice were left on their back to a maximum of 30 seconds which is indicative of Sandhoff endpoint in our AUP. Data were analyzed using student's t-test to evaluate statistical significance. A P-value <0.05 was considered statistically significant.

Brain Glycosphingolipid Isolation and Thin Layer Chromatography (TLC)

The *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice were sacrificed at 17 weeks of age. GA2 and GM2 extracts were determined in 430mg of cerebral cortex and 70mg of cerebellum, by thin layer chromatography (TLC). Gangliosides from mice brain tissue were extracted and analyzed as previously described (Irwin & Irwin, 1979). Briefly, brain samples were lysed and sonicated (Sonicator XL 2020, Heat systems Inc.) with chloroform/methanol/water (C: M: W, 10: 10: 1, v/v/v) for 1 hour. The upper phase containing the gangliosides was separated from the lower phase after mixing and low-speed centrifugation. The upper phase was applied slowly to a sephadex G-50 column at a steady rate. The collected elute was adjusted to C: M: W (30:60:8) and applied slowly to a column of DEAE-Sepharose. The eluted gangliosides and neutral glycolipids were subjected to dry air to evaporate all liquids then adjusted to C: M: 0.1M KCl (2: 48: 47). The mixture was applied to Sep-Pack C-18 reverse phase column and the collected eluate was evaporated and adjusted to C: M: W (10: 10: 1, 200ul). Using a Hamilton syringe, glycolipids brain extracts were spotted onto TLC Silica gel 60 F254 plates (EMD Chemicals Inc., Silica Gel 60, 5715-7). TLC plates were air-dried, and developed, using C: M: CaCl2 (60: 35: 8), for 2.5 hours then sprayed with orcinol/sulphuric acid [0.2% (w/v), 1M] and heat-treated (110°C for 20 min). The digital image of the TLC plate was captured using via flatbed scanner and Photoshop v6.0 (Adobe).

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR).

SYBR green assays were performed with SYBR green PCR master mix (Applied Biosystems, #4309155) using cDNA obtained from the cerebella of *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice.

Primers were as follows:

Gfap Forward 5'-CACGAACGAGTCCCTAGAGC

Reverse 5'-ATGGTGATGCGGTTTTCTTC

Mac-1α Forward 5'-GACTCAGTGAGCCCCATCAT

Reverse 5'-AGATCGTCTTGGCAGATGCT

Mcp-1 Forward 5'-ATGGTCAAGAGTTTGCAGCTT

Reverse 5'-CCTGAATTTTGGGAGAGTGTGAT

Gsr Forward 5'-GACACCTCTTCCTTCGACTACC

Reverse 5'-CACATCCAACATTCACGCAAG

Nxn Forward 5'-GTGGTAGCTTTGTACTTTGCGG

Reverse 5'-CCGTCTGCCGACACGAAAA

Prdx1 Forward 5'-AATGCAAAAATTGGGTATC

Reverse 5'-CGTGGGACACACAAAAGT

Ptgs1 Forward 5'-ATGAGTCGAAGGAGTCTCTCG

Reverse 5'-GCACGGATAGTAACAACAGGGA

Samples, master mix and primers were added to a MicroAmp Optical 96-well Reaction plate (Applied Biosystems, #4316813). The plate was spun down quickly to remove bubbles and placed in a 7900HT Sequence Detection System (Applied Biosystems).

Thermocycling conditions were as follows: 50°C, 2 min, 95°C , 10 min, 40 cycles of 95°C, 15s; 60°C, 1 min. SDSv2.3(SABiosciences) was used to analyze all gene expression data.

Western Blotting

Mice were anesthetized with ketamine/xylazine. Blood was obtained by cardiac puncture. Mice were perfused with phosphate-buffered saline (PBS) through the left ventricle of the heart. The cerebellum was harvested into lysis buffer containing protease inhibitors (Roche #14791200) and sonicated at 20% pulse for 10 sec. Laemmeli sample buffer (2x) was added to the lysate and boiled for 5 minutes before storage at -20°. Boiled samples were equally loaded into a 10% SDS-polyacrylimide (BioRad) gel and transferred at 120V for 70 minutes onto a nitrocellulose membrane. Membranes were blocked using 5% non-fat milk (Carnation) in Tris-buffered saline containing 0.5% Tween-20 (TBST) before probing with antibody suspended in 5% milk in TBST and incubated overnight at 4°C. After washing in TBST five times, membranes were blotted with secondary IgG-HRP conjugated antibodies in 5% milk in TBST (1:10000, Santa Cruz Biotechnology, goat anti-rabbit: #sc-2004, goat anti-mouse: #sc-2005, goat anti-rat: #sc-2006, donkey anti-goat: #sc-2020) for one hour at room temperature, followed by five washes in TBST. Blotted membranes were incubated with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, #RPN2106) and exposed on Amersham Hyperfilm ECL film (GE Healthcare, #28906839). Loading was normalized using anti-GAPDH antibody (1:1000, R+D Systems, #AF5718). Protein levels were analyzed using

the Bio Rad DC Protein Assay (#500-0116). Membranes were stripped of secondary antibodies between blotting using a stripping buffer at a pH 2.0 composed of 1.5% glycine, 0.12%SDS, and 0.1% Tween-20. The following antibodies were used in this study: anti mouse GFAP monoclonal antibody (1:5000, Sigma Aldrich, #G3893),anti-MAC-1α polyclonal antibody (1:300, Sigma Aldrich, #G9269),anti-IL-6 antibody (1:500, Santa Cruz Biotechnology, #sc-7920), anti-pSTAT-3 antibody (1:1000, Cell Signaling, #9145S), anti-STAT-3 antibody (1:1000, Cell Signaling, #4904S), anti-TRAF3 antibody (1:1000, Cell Signaling, #4729), anti-NIK antibody (1:1000, Cell Signaling, #4994P) and anti-RelB antibody (1:1000, Cell Signaling, #4922). For quantification of Western Blot analysis, X-ray films were scanned and saved as .JPEG files and analyzed using Image J (NIH). Image color was inverted and background was subtracted at 50 pixels. Using the free hand tool, bands were outlined, and the area, mean density, and integrated density were calculated. The integrated densities of each band were divided by the corresponding value for GAPDH to represent normalized protein levels. Student's t-test was used to determine significant difference of the means at $P<0.05$.

Immunohistochemistry

Hexb$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice were harvested at 17 weeks of age, perfused with PBS, and fixed with 3.7% formaldehyde. The brain and spine were harvested, and embedded in paraffin wax. Five-micrometre thick coronal brain sections and cross-sectional spine sections were cut from the blocks and mounted on slides. Samples underwent a xylene/ethanol rehydration series, quenching of endogenous

peroxidase activity with 1%H_2O_2, blocked with 10% goat serum for 15 min., and were immuno-labeled for 1.5hr at 37°C with primary antibodies– mouse monoclonal anti-GFAP (1:150, Sigma, #G3893) for reactive astrocytes, and rat monoclonal anti-MAC-3 (1:300, BD Pharmingen,#553322) for reactive microglia/macrophages. Biotinylated secondary antibodies were applied, followed by anavidin/HRP-bound biotin solution for signal amplification (Santa Cruz, Mouse ABC Staining System #sc-2017, Rat ABC Staining System #sc-2019). Samples were stained with 3', 3'- diaminobenzadinein the presence of 0.01% H_2O_2, and counterstained with methylene blue. Microwave antigen retrieval in 12 mM citric acid buffer was performed on MAC3 slides prior to application of the primary antibody. All washes were performed with Tris-buffered saline (0.1% Tween-20) (TWBS). Random field images were captured of the cerebral cortex and cerebellum at 400X magnification, via Zeiss Axiovert 200 microscope, equipped with a Zeiss Axio CamMRc Camera. Areas of images were calculated using AxioVision 4.7 software (Zeiss, Oberkochen, Germany). Positively stained cells were quantified from images. Images of spinal sections were captured, and areas measured with Axiovision 4.7, and positively stained cells were quantified fewer than 400X magnification. All statistical analyses were performed using SPSS v16 (SPSS Inc. Chicago, IL). One-way ANOVA performed onsets of data with 3 or more groups) followed by Tukey's post-hoc were used for all data with high normality and equal variance amongst groups. For groups of unequal variance, Dunnett's T3 test was used to determine significant difference of the means at $p < 0.05$.

TUNEL Analysis

Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) was performed on the previously described formalin-fixed, paraffin-embedded CNS samples, according to manufacturer's instructions using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, #S7100). Sections were subjected to a xylene/ethanol rehydration series, followed by treatment with proteinase K for 10 min. After blocking for endogenous peroxides activity with 3% hydrogen peroxide, the samples were incubated with terminal deoxynucleotidyltransferase (TdT) enzyme at 37 °C for 1 h. Sections were then treated with anti-digoxigenin-peroxidase and reacted with 3',3'-diaminobenzadine in presence of 0.01% H_2O_2. Nuclei were then counterstained with methyl green. All washes were performed with PBS. Areas of the cerebral cortex, and entire cerebella were measured using AxioVision 4.7 and positive cells were quantified at 400X magnification. Images of spinal sections were captured, and areas measured with Axiovision 4.7 (Zeiss, Oberkochen, Germany), and positively stained cells were quantified under 400X magnification. The number of all astrocytes in a specific spinal tissue type were counted and divided by total area of all sections of that type, for each mouse.All statistical analyses were performed using SPSS v16 (SPSS Inc. Chicago, IL). One-way ANOVA (Performed onsets of data with 3 or more groups) followed by Tukey's post-hoc were used for all data with high normality and equal variance amongst groups. For groups of unequal variance, Dunnett's T3 test was used to determine significant difference of the means at $p < 0.05$.

Immunofluorescence

Brains from *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice were harvested at 17 weeks of age, and embedded in paraffin as described above. Samples underwent a xylene/ethanol rehydration series, and microwave antigen retrieval was performed in 12 mM citric acid buffer. Tissues were blocked with 10% goat serum for 15 minutes and double-labeled with mouse anti-GFAP (1:150, Sigma, #G3893) and rabbit anti-STAT3 (1:150, Cell Signaling, #4904S) antibodies. Slides were then double-labeled with goat anti-mouse Alexa Fluor 594 (1:400, Invitrogen, #A-11005) and Goat anti-rabbit Alexa Fluor 488 (1:400, Invitrogen, #A-11008). Slides were stained with Hoechst, and mounted with ProLong Gold antifade reagent (Invitrogen, #P36930). Confocal microscopy optical slices were performed with argon 488, and HeNe 594 excitation lasers on a Leica TCS SP5 confocal microscope, using Leica Application Suite Advanced Fluorescence software.

Acknowledgements

H.A. is sponsored by a graduate scholarship from the Egyptian government. E.J.W. is the recipient of a Canadian Institutes of Health Research (CIHR) post-doctoral fellowship. This work was supported by a grant from the Canadian Institute of Health Research (CIHR) to S. I.

Chapter 3

Blood-derived TNFα contributes to inflammation in the pathogenesis of Sandhoff disease

Preface:

The results presented in this chapter were prepared as manuscript to be submitted for publication. Dr. Elizabeth White and I collaporated on this study. Dr. Elizabeth White performed flow cytometric characterization of peripheral immune cells in mice, presented in Figure 1. Dr. Elizabeth White performed enzyme linked immunosorbant assay of BMDMs presented in Figure 2 and 3. I generated and outbred Hexb$^{-/-}$ Tnfα$^{-/-}$ mice to be utilized in these studies. I performed bone marrow transplanation (BMT) expreiments and performed survival and behavioural testing presented in Figure 4 and 5 and 6 and 7. The article was co-written by both Dr. Elizabeth White and I with significant contribution to the final manuscript given by Dr. S. Igdoura. Considerable advice and intellectual contributions were provided by Dr. S. Igdoura.

3.1 Abstract

Mutations in the *Hexb* gene can produce a deficiency in the lysosomal enzymes β-hexosaminidase A and β-hexosaminidase B, which leads to the accumulation of ganglioside GM2 and related glycolipids in neurons (Huang et al., 1997). The *Hexb*$^{-/-}$ mouse provides a model of the inherited human disorder, known as Sandhoff disease, with progressive neurodegeneration leading to paralysis and seizures. In addition to the GM2 storage disorder that is intrinsic to disease progression, an increase in pro-inflammatory immune cell function contributes to the pathogenesis. Our previous work has demonstrated that the ablation of TNFα will delay the onset of neurodegeneration in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice. The aim of this study is to examine the impact of *Hexb* deficiency as well as *Hexb* and *Tnfα* combined deficiencies on the frequency and function of peripheral inflammatory cells, including their impact on neurological function in bone marrow transplantation experiments. Our findings indicate that *Hexb*-/- mice show a trend for a decreased frequency of CD11b^{+}Gr-1^{neg-lo} monocytes in peripheral blood compared to wild type mice. In addition, *Hexb* and *Tnfα* combined deficiencies result in decreases in the frequency of CD3ε^{+}CD4^{+} T helper cells, CD11b^{+}Gr-1^{neg-lo} monocytes and CD11b^{+}Gr-1^{hi} neutrophils. *Hexb*$^{-/-}$bone marrow-derived macrophages (BMDMs) showed lower sensitivity to LPS stimulation compared to wild type cells, with less secretion of IL-1β, IL-10, and MCP-1, while *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ macrophages produced less IL-1β and IL-10, but wild type levels of MCP-1. Stimulation with poly I: C only showed less MCP-1 secretion in *Hexb*$^{-/-}$ macrophages compared to wild type, while GM2 stimulation did not elicit

cytokine secretion in macrophages of any genotype tested. Finally, we utilized bone marrow transplantation to assess the role of blood-derived TNFα on neurological function in vivo. Our results indicate that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice transplanted with bone marrow from either *Hexb*$^{-/-}$*Tnfα*$^{-/-}$or *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice donors show a significant improvement in life span and neurological tests compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice transplanted with bone marrow from male *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ donors. Our data point to the effect of TNFα on the abundance of peripheral blood cells, their activation status, and neurological function during disease progression. Our findings highlight the importance of TNFα during the robust neuroinflammatory cascade in Sandhoff disease.

3.2 Introduction

GM2 gangliosidoses comprise three inherited disorders including Tay Sachs, Sandhoff and GM2 activator deficiency that are characterized by the accumulation of GM2 gangliosides and related glycolipids in neurons (Myerowitz et al., 2002). Sandhoff disease is caused by deficient Hexb gene product (β-subunit) that contributes to both β-hexosaminidase A (αβ) and B (ββ) enzymes (Huang et al., 1997). Accumulation of substrates such as GM2 gangliosides and GA2 glycolipids is particularly evident in neurons (Phaneuf et al., 1996). Consequently, the central nervous system is directly affected and develops progressive and debilitating neurodegeneration (Huang et al., 1997). Affected patients with Sandhoff disease display a progressive clinical picture of neurodegeneration resulting in death at an early age (Myerowitz et al., 2002). Currently, there is no curative treatment for lysosomal storage diseases that involve the CNS due to

the hindrance function of the blood brain barrier for the passage of effective therapeutics into the brain (Sly & Vogler, 2002). Furthermore, a better knowledge of the underlying mechanisms in lysosomal storage diseases is necessary to develop effective therapies (Wu & Proia, 2004).

Neuroinflammation and macrophage/microglia activation were invoked in disease progression and preceded neuronal apoptosis in these gangliosidoses (Wada, Tifft, & Proia, 2000). Recruitment of blood derived macrophages, proliferation of resident glia cells, and excessive secretion of tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β) were cardinal features of Sandhoff disease pathology (Wada, Tifft, & Proia, 2000). Furthermore, deletion of the chemo-attractant protein macrophage-inflammatory protein 1α has been shown to reduce peripheral blood mononuclear cell infiltration into the brain and ultimately can slow the progression of the disease in the Hexb-/- mouse (Wu & Proia, 2004; Kyrkanides et al., 2008). In addition, restoring HEXB enzyme in *Hexb*$^{-/-}$ neurons reduced TNFα expression in the brain suggesting that glial activation might be induced by GM2 accumulations (Kyrkanides et al., 2008). These reports led to the hypothesis that lipid storage results in macrophage/microglia expansion and activation, mainly via peripheral monocyte infiltration, ultimately ending in massive neuronal apoptosis (Wu & Proia, 2004).

It has been shown that lysosomal storage diseases that affect the CNS display irregular immune function (Castaneda, Lim, Cooper, & Pearce, 2008). It was established that lysosomal organelles are involved in crucial immune functions such as antigen presentation by antigen presenting cells (APCs) and secretion of proinflammatory

mediators by lymphocytes (Castaneda, Lim, Cooper, & Pearce, 2008). Furthermore, it was found that lysosomal glycolopid isoglobotrihexosylceramide (iGb3) controls peripheral immune response of natural killer T (NK) cells (Zhou et al., 2004). In Sandhoff disease mouse model, treatment with anti-inflammatory drugs prevented peripheral immune cell recruitment into the brain (Vitner, Platt, & Futerman, 2010). This accumulating evidence is indicating that several peripheral immune cells are participating in Sandhoff disease pathology. Hence, it is likely that preventing their expansion into CNS would provide a beneficial effect.

Upon an inflammatory neuronal damage occurring, glia cells were found to undergo excessive hypertrophy and activation that ultimately leads to the formation of permanent glial scars (Ramer, Harper, & Bradbury, 2000). Following brain injury, a massive recruitment of inflammatory blood cells including neutrophils and monocytes, occurs into brain parenchyma due to disruption of blood brain barrier (Wright, El, Osman, Chowdhury, & Johnson, 2011). Resident microglia and recruited macrophages secrete inflammatory cytokines along with protective molecules such as nerve growth factor (NGF) (Rabchevsky et al., 1998). These responses, together, contribute to the fate of CNS neurological function. Recently, it was reported that bone marrow cells have a wide plasticity that can yield not only blood cells but also cell phenotypes typical to neurons (Brazelton, Rossi, Keshet, & Blau, 2000). This neuronal trans-differentiation promotes the expansion of neurite projections and re-establishes neuronal circuits in central nervous system (CNS) of bone-marrow-treated mice (Wakitani et al., 2011). It has been reported that hematopoietic and mesenchymal stem cells in bone marrow can trans-

differentiate into glial cells (McGuckin, Forraz, Allouard, & Pettengell, 2004). Furthermore, bone marrow transplantation in spinal cord injury has demonstrated beneficial outcome that enhanced remyelination, axonal regeneration and neuronal functional recovery (Sasaki et al., 2001). Bone marrow (B.M.) transplantation allows the rapid generation of mice in which genes are either absent from or expressed specifically in BM-derived cells including monocyte-derived macrophages. This has become a powerful method to test the role of a gene of interest/mutation in BM-derived cells in the development of neurodegeneration. This led to bone marrow transplantation as a treatment option for LSDs (Hoogerbrugge et al., 1995). The therapeutic efficacy of transplantation of normal donor bone marrow cells into affected subjects is attributed to the recruitment of donor bone marrow precursors and their differentiation into microglia to reside into recipient brains and then secrete lysosomal hydrolases to cross-correct distant neuronal cells (Krivit, Peters, & Shapiro, 1999). Recently, it has been reported that normal bone marrow transplantation (BMT) into Sandhoff mice inhibited activation of microglia and reduced neuronal cell death (Wada et al., 2000; Norflus et al., 1998). Another study demonstrated that BMT delayed the onset of Sandhoff disease symptoms and prolonged the survival rate of Sandhoff mice (Jeyakumar et al., 2001; Norflus et al., 1998).

To understand the inflammatory processes occurring in the *Hexb*$^{-/-}$ mouse, we examined, in this study, the effect of TNFα deficiency on the frequency of immune cell populations and the function of bone marrow derived macrophages in *Hexb*$^{-/-}$ mice. Furthermore, we utilized bone marrow transplantation to examine the role of BM-derived

TNFα on the neurological functions of *Hexb*$^{-/-}$mice. Our results demonstrate that TNFα not only plays a role in the frequency of peripheral leukocyte populations but also is an important controller of cytokine secretion and cell activation in *Hexb*$^{-/-}$ mice. In addition, we have demonstrated that *Hexb*$^{-/-}$mice receiving *Hexb*$^{-/-}$*Tnfα*$^{-/-}$bone marrow developed neurodegeneration at a slower rate than *Hexb*$^{-/-}$mice receiving *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ bone marrow. This indicates that TNFα derived from blood cells enhances the pathogenesis of Sandhoff disease.

3.3 Results

3.3.1 TNFα deficiency reduces T helper cell, monocyte and neutrophil subsets in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice

To determine if TNFα deficiency reduces Sandhoff disease pathology by reducing the frequency of circulating inflammatory leukocytes, we examined peripheral blood of wild type, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$, and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice for the frequency of cell surface molecular markers for T helper lymphocytes (CD3ε^{+}CD4^{+}), cytotoxic T lymphocytes (CD3ε^{+}CD8^{+}), NK cells (CD3ε^{-}NK1.1^{+}), monocytes (CD11b^{+}Gr-1$^{neg\text{-}lo}$), neutrophils (CD11b^{+}Gr-1^{hi}) and B cells (CD19^{+}). No differences in peripheral blood leukocyte frequencies in T helper cells, cytotoxic T lymphocytes, NK cells, or B cells were observed between wild type and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice, although there was a trend for reduced monocyte frequency in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice (p=0.11). *Hexb*$^{-/-}$*Tnfα*$^{-/-}$mice exhibited reduced frequencies of T helper cells (P<0.05), monocytes (P=0.05) and neutrophils (P<0.05) compared to wild type mice (Figure 1).

3.3.2 Sandhoff disease decreases LPS-induced cytokine secretion in bone marrow derived macrophages

Since we observed that *Hexb* and TNFα deficiency influences the frequency of circulating monocytes, which potentially indicates their infiltration into the CNS, we decided to assess how *Hexb* deficiency affects the function of these cells once they become differentiated into macrophages. We tested whether bone marrow derived macrophages (BMDMs) from *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice respond differently to TL4 agonists (LPS, GM2) and TLR2 agonist (poly I: C) and to examine the impact of TNFα deletion on this response. One may expect that macrophages from *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice will be sensitized to activation due to lysosomal dysfunction and as a result their neuroinflammatory potential will be elevated. In order to mimic the *in vivo* situation, we exposed BMDMs to GM2 gangliosides, which bind TLR4 receptors (Gruber, Mancek, Wagner, Kirschning, & Jerala, 2004). In addition, we used LPS which represents a more efficient agonist of TLR4 receptors as well as poly I:C, which triggers signaling through TLR2 (Jerala, 2007). BMDM stimulation was determined by measurement of secreted cytokines, including TNFα, IL-1β, IL-6, IL-10 and MCP-1 (Figure 2A and B, 3A-C). In BMDMs incubated with media only, all genotypes show minimal secretion of cytokines.

Our data demonstrate a robust TNFα secretion in response to LPS in both wild type and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice, which was reduced to the limit of detection in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice (Figure 2A). Both *Hexb*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDMs secreted significantly less IL-1β following LPS stimulation compared to wild type BMDMs (Figure 2B). No differences in IL-6 secretion were observed between the genotypes following stimulation

with LPS, poly I: C or GM2 (Figure 3A). To understand the cellular mechanisms by which TNFα deletion in Sandhoff BMDMs might trigger an anti-inflammatory response, we measured the concentration level of IL-10 (Figure 3B). Upon LPS stimulation, we observed a reduction in the level of IL-10 secretion in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDMs compared to wild type cells. Interestingly, secretion of MCP-1, a cytokine critical for monocyte recruitment, was significantly reduced in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BMDMs compared to both wild type and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDMs following LPS and poly I:C treatment (Figure 3C). Only basal levels of cytokines were detected following GM2 stimulation. Overall, these results demonstrate that BMDMs cultured from *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice are less sensitive to LPS-induced inflammation.

Figure 1 Peripheral blood immunophenotypes from Hexb-/- and Hexb-/-TNFα-/- mice

Including CD3e+ T cells, CD4+ helper T cells, CD8+ cytotoxic T cells, CD3e+NK1.1+ NK T Cells, CD3e-NK1.1+ NK cells, CD11b+Gr-1hi inflammatory monocytes/granulocytes, CD11b+Gr-1lo monocytes, and CD19+ B cells. Data are expressed as % wild type run concurrently with the samples. While *Hexb*$^{-/-}$ mice show reduced quantities of circulating immune cells, additional TNFα deficiency increases the frequency of B cells, NK cells and cytotoxic T cells.

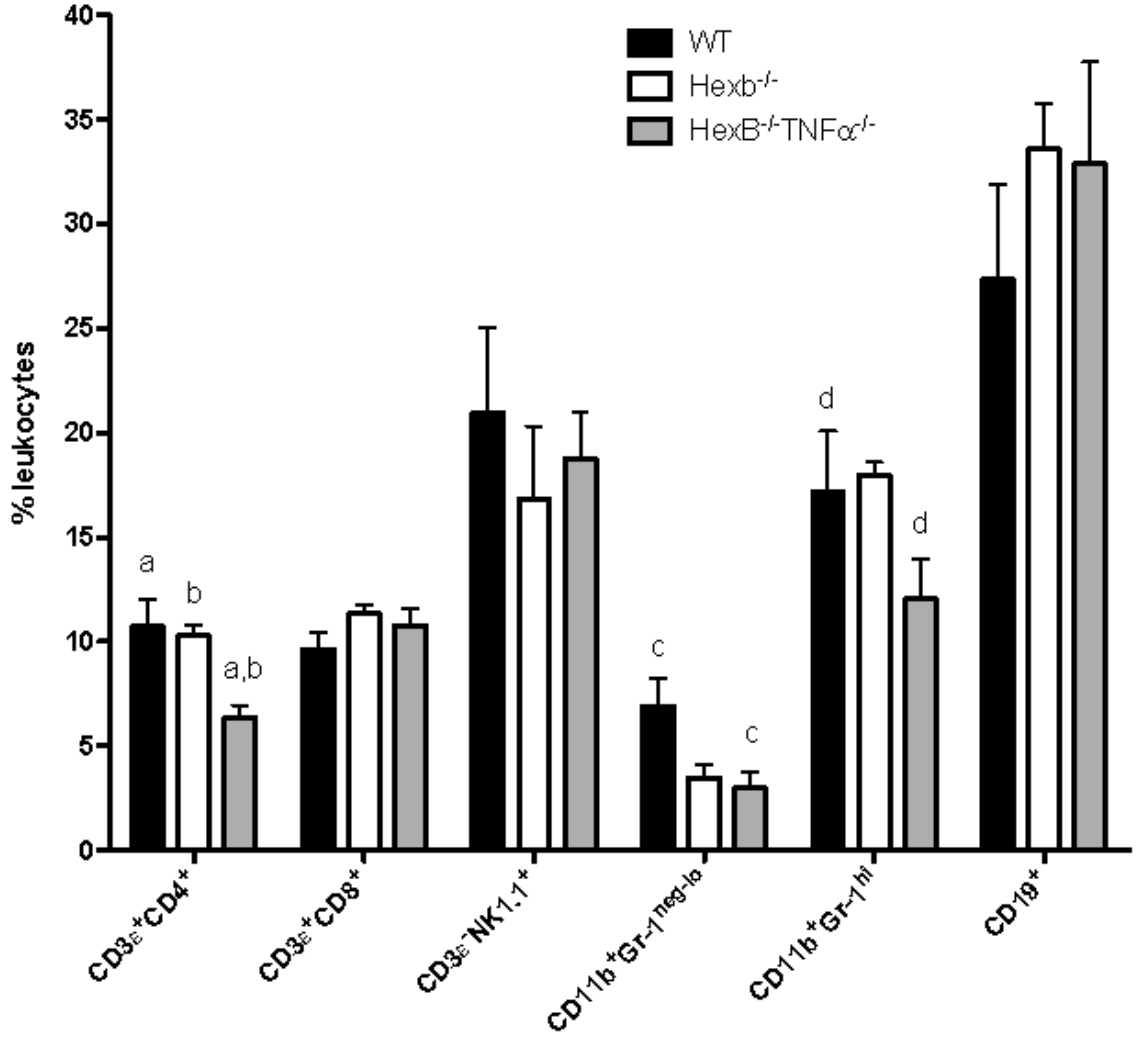
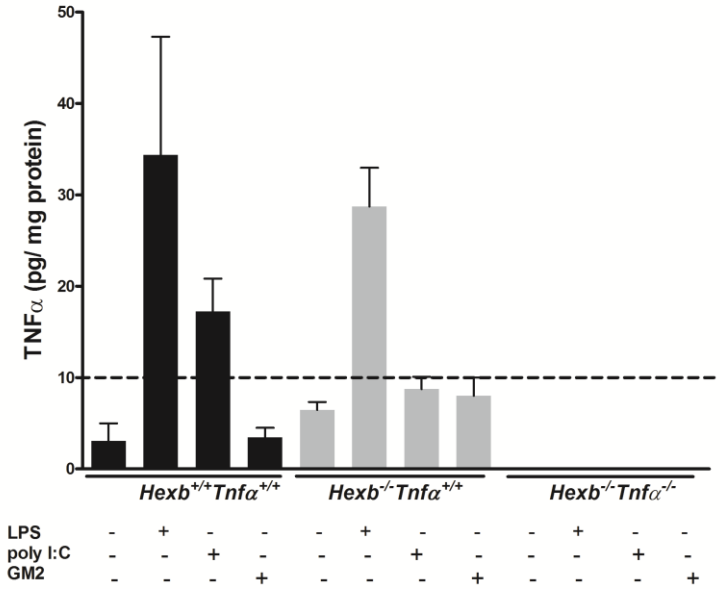

WT
Hexb-/-
HexB-/-TNFα-/-
% leukocytes
40
35
30
25
20
15
10
5
0
a
b
a,b
c
c
d
d
CD3ε+CD4+
CD3ε+CD8+
CD3ε-NK1.1+
CD11b+Gr-1neg-lo
CD11b+Gr-1hi
CD19+

Figure 2 Cytokine productions in *Tnfα*-lacking BMDMs in response to LPS

(A) To examine TNFα cytokine production in *Hexb*$^{+/+}$ *Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$,*Hexb*$^{-/-}$ *Tnfα*$^{-/-}$ mice, bone marrow derived macrophages (BMDMs) were stimulated with LPS, and after 24h TNFα was measured by enzyme-linked immunosorbent assay (ELISA). *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$macrophages shows higher levels TNFα secretion in response to LPS. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ macrophages produce substantially less TNFα compared to wild type and Sandhoff derived macrophages. Our results shows substantial reduction of LPS-induced TNFα secretion in double knock-out derived BMDM cells. **(B)**To examine whether IL-1β production is related to TNFα secretion in Sandhoff pathology, we used ELISA to determine the levels of IL-1β in *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ marrow derived macrophages (BMDM) in response to LPS, Poly I: C and GM2 treatment. IL-1β levels are reduced in LPS-stimulated *Hexb*$^{-/-}$ macrophages compared to wild type. *Hexb*$^{-/-}$*TNFα*$^{-/-}$ macrophages show further reduction in LPS-mediated IL-1β secretion. (a) denotes statistical difference from LPS-treated wild type BMDMs.

A


TNFα (pg/ mg protein)
50
40
30
20
10
0
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
LPS - + - - - + - - - + - -
poly I:C - - + - - - + - - - + -
GM2 - - - + - - - + - - - +

B

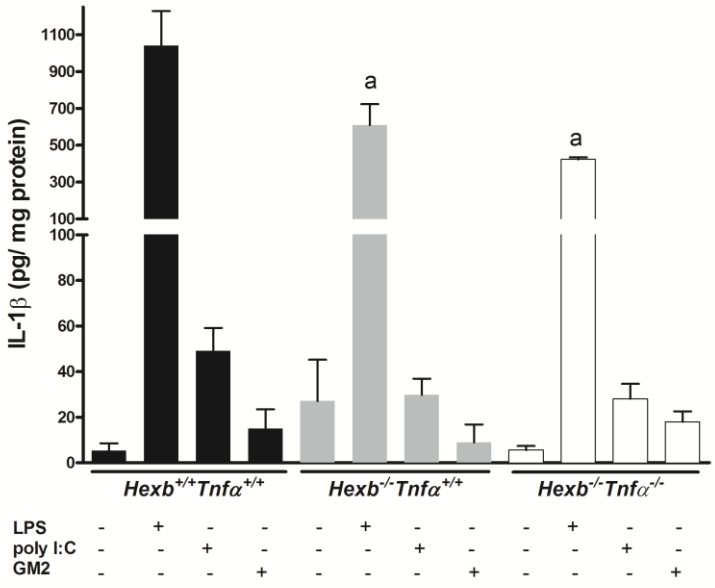
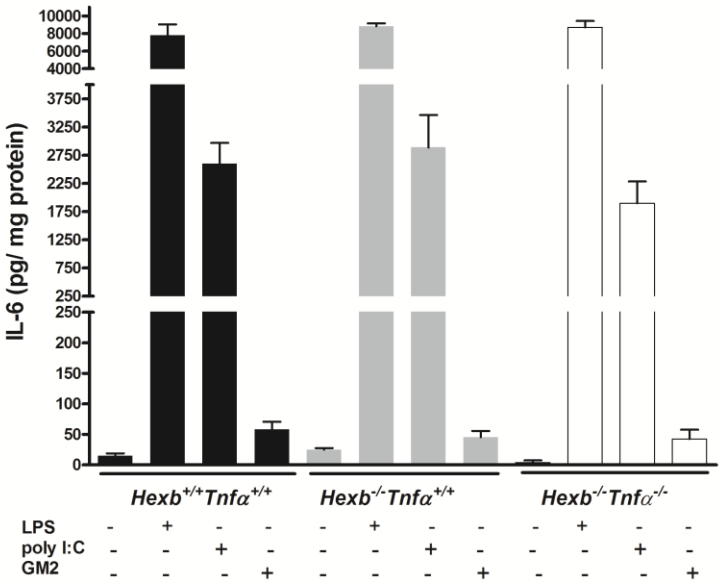

IL-1β (pg/ mg protein)
1100
900
700
500
300
100
100
80
60
40
20
0
a
a
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
LPS - + - - - + - - - + - -
poly I:C - - + - - - + - - - + -
GM2 - - - + - - - + - - - +

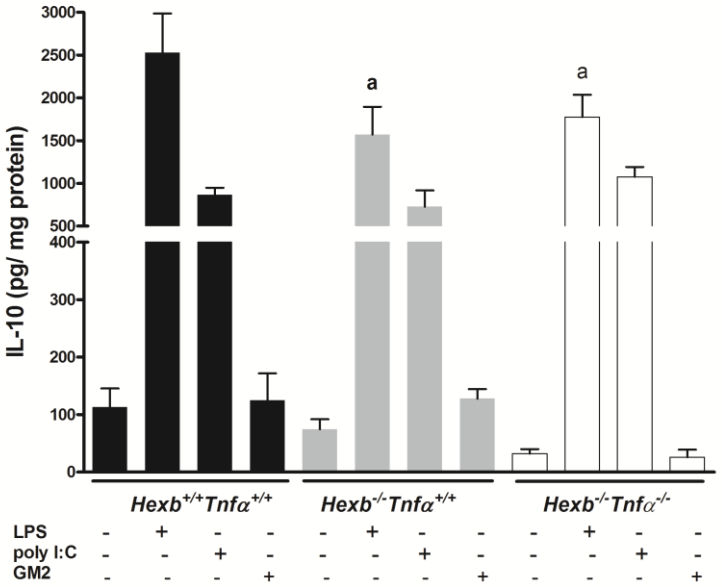
Figure 3 Cytokine productions in *Tnfα*-lacking BMDMs in response to LPS

(A) To examine whether IL-6 secretion is dependent on TNFα secretion, we utilized ELISA to measure IL-6 cytokine secretion levels in Hexb$^{+/+}$ Tnfα$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ marrow derived macrophages (BMDM) in response to LPS, Poly I: C and GM2 treatment. Secretion levels of IL-6 were higher in all genotypes in response to LPS. The data demonstrate that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDM cells produce IL-6 after stimulation with LPS. **(B)** To investigate the secretion of anti-inflammatory IL-10, we measured IL-10 cytokine production levels in *Hexb*$^{+/+}$*Tnfα*$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$ marrow derived macrophages (BMDMs) using ELISA in response to LPS, Poly I: C and GM2 treatment. BMDM cells secrete higher concentrations of IL-10 in all genotypes in response to LPS. No or minimal change of IL-10 production was observed among genotypes after LPS treatment. IL-10 levels are reduced in LPS stimulated *Hexb*$^{-/-}$and *Hexb*$^{-/-}$*TNFα*$^{-/-}$ macrophages compared to wild type. **(C)** To examine whether MCP-1secretion is dependent on TNFα secretion, we utilized ELISA to measure MCP-1 cytokine secretion levels in *Hexb*$^{+/+}$Tnfα$^{+/+}$, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ marrow derived macrophages (BMDM) in response to LPS, Poly I:C and GM2 treatment. MCP-1 levels are reduced in LPS and poly I:C stimulated Hexb$^{-/-}$ macrophages compared to wild type. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ macrophages produce more IL-10 after stimulation with LPS or poly I: C. (a) denotes statistical difference from LPS-treated wild type BMDMs. (b) denotes statistical difference from poly I:C-treated wild type BMDMs.

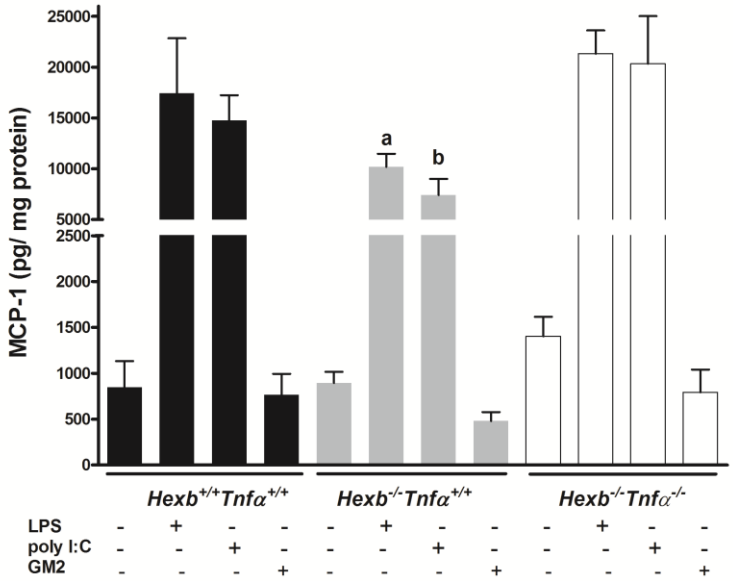
A


IL-6 (pg/ mg protein)
10000
8000
6000
4000
3750
3250
2750
2250
1750
1250
750
250
250
200
150
100
50
0
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
LPS - + - - - + - - - + - -
poly I:C - - + - - - + - - - + -
GM2 - - - + - - - + - - - +

B


IL-10 (pg/ mg protein)
3000
2500
2000
1500
1000
500
400
300
200
100
0
a
a
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
LPS - + - - - + - - - + - -
poly I:C - - + - - - + - - - + -
GM2 - - - + - - - + - - - +

C


MCP-1 (pg/ mg protein)
25000
20000
15000
10000
5000
2500
2000
1500
1000
500
0
a
b
Hexb+/+Tnfα+/+
Hexb-/-Tnfα+/+
Hexb-/-Tnfα-/-
LPS - + - - - + - - - + - -
poly I:C - - + - - - + - - - + -
GM2 - - - + - - - + - - - +

3.3.3 CNS-derived TNFα plays a greater role in the pathogenesis of Sandhoff disease compared to bone marrow derived TNFα

While levels of TNFα secreted by cultured BMDMs appear no different between wild type and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice in response to LPS, the levels of inflammatory cytokine MCP-1 appear to be increased in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDMs. This in fact pointed to effect of TNFα on the inflammatory status of BMDMs. In order to decipher the contribution of blood-derived versus CNS-resident TNFα in the neuroinflammatory cascade *in vivo*, we performed bone marrow transplantation in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. In each case, we implanted either *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ or *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow and exposed the recipient mice to a battery of neurological tests to assess the impact on neurological function as a measure of disease progression. Previously, we have shown than anti-inflammatory IL-10 levels were decreased in TNFα-lacking BMDMs. Furthermore, our previous study demonstrated that TNFα plays a negative role during the neurodegenerative cascade (Abo-Ouf et al., 2013). Therefore, BM-derived TNFα was expected to increase neurodegeneration in Hexb$^{-/-}$*Tnfα*$^{-/-}$ mice. Upon transplanting *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$ or *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow cells into *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice, we observed that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipients that received *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BM showed longer median survival (139.4) days compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipients that received *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ cells (121.7) days (Figure 4A). Upon transplantation with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ bone marrow cells, we detected a decrease in body weight of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice compared to *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice, after the age of 118 days (Figure 4B). The trend that was observed in the survival curves of bone marrow transplanted mice was

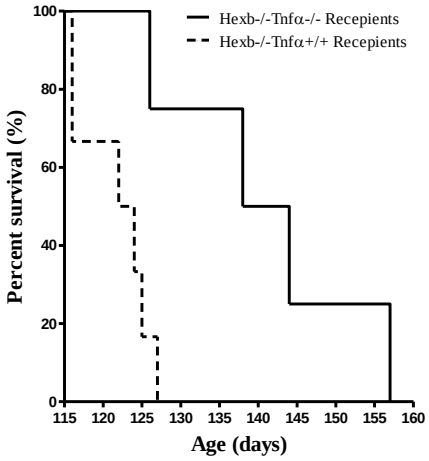
also reflected in the performance of the mice in the wire hang, rotorod and righting reflex behavior tests. Wire hang tests showed that lower motor function of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice was better than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$recipient mice, following transplantation with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ donor cells (Figure 5A). While *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice started to decline in wire hang performance at 121 days (51.3 s), *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipients started to decline at an earlier age of 91 days old (50.2 s) (Figure 5A). To measure the muscle/neuromuscular function of mice transplanted with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BM cells, we utilized grip strength test. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice showed better performance, during the testing period, than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice (Figure 5B). To determine whether mice transplanted with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$bone marrow cells show a decline in balance and coordination tests, we measured rotorod performance of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice (Figure 5C). The rotorod performance of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice declined at a faster rate than *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipients (Figure 5C). To investigate the postural adjustment as a measure of neuromuscular reflex in response to a stimulus, we performed a righting reflex test (Figure 5D). Using *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ donor cells, *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$ recipients were not different from *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice until 115 days of age (Figure 4D). After 115 days, we noticed that *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$ recipient mice showed a slower righting reflex compared to *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice (Figure 5D).

Figure 4 Survival and body weight of Hexb-/-Tnfα+/+ and Hexb-/-Tnfα-/- mice transplanted with Hexb-/-Tnfα+/+ bone marrow cells

(A) Survival of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice after BMT with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ bone marrow donor cells. The recipient mice were harvested when they reach the end-point. The median survival of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice was (139.4) days. The median survival of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice was (121.7) days. Hexb$^{-/-}$Tnfα$^{-/-}$ mice show prolonged life span compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice $P< 0.01$, n= 6. **(B)** Body weights of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ female recipient mice that transplanted with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ bone marrow cells. We did not see a detectable trend among recipient mice groups till the age of 118 days. After 118 days, we detected acute decrease of the body weight in *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ compared to *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice. n =3-5.

Donor: *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$

A



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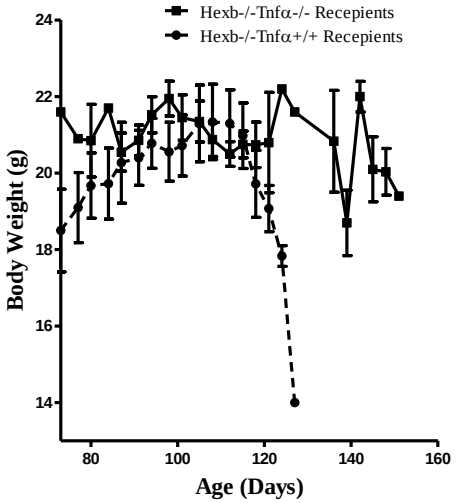
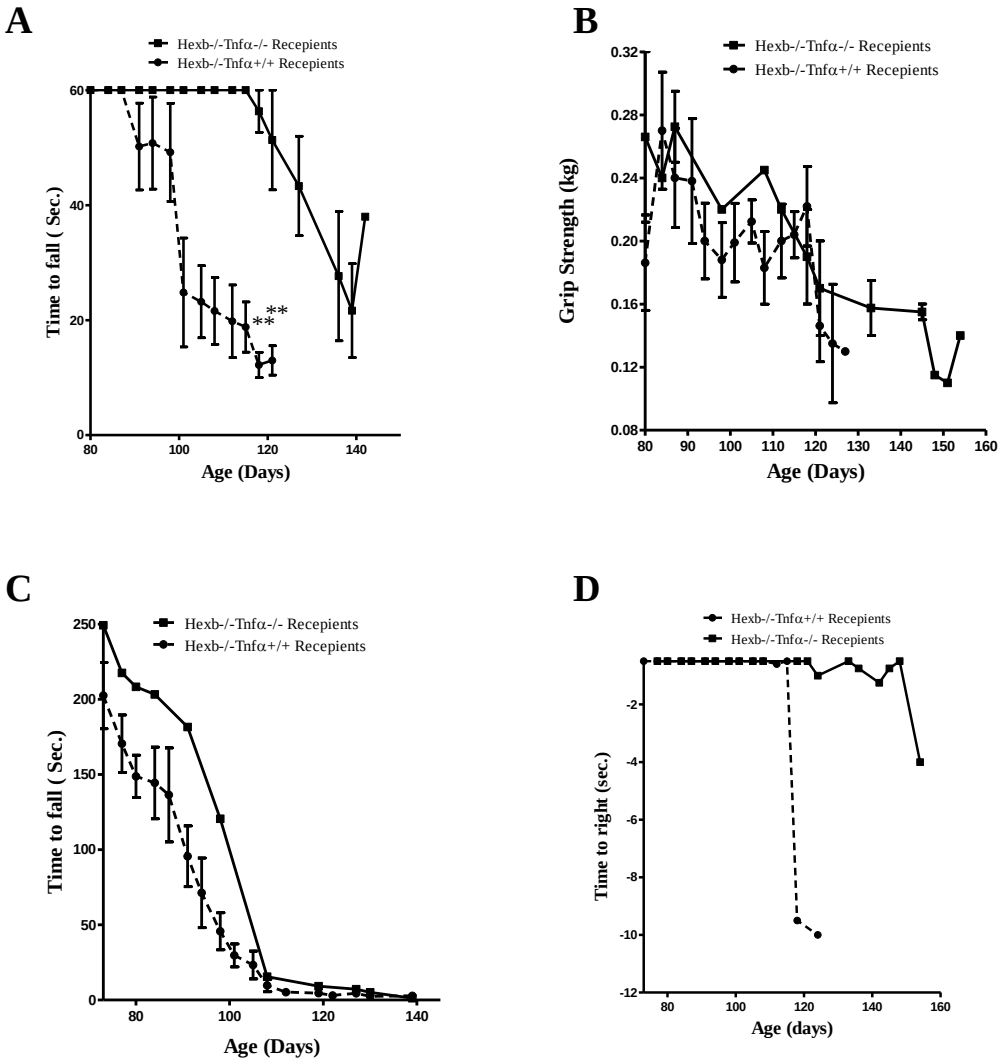


Figure 5 Behavioural testing of Hexb$^{-/-}$Tnfα$^{-/-}$ and Hexb$^{-/-}$Tnfα$^{+/+}$ transplanted with Hexb$^{-/-}$Tnfα$^{+/+}$ bone marrow cells

(A) Wire hang times of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BM cells. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipients had better performance at all the tested time points starting from 91 days. Note that, while *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice show mean latency times (51.3 seconds) at 121 days, the latency times of *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice was 50.2 seconds at 91 days. Hexb$^{-/-}$Tnfα$^{-/-}$ recipient mice showed significantly better performance at 118 and 121 days compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice. n = 3-5. **(B)** Grip strength latency times of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BM cells. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$recipient mice outperformed *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice at all the time tested points. n = 3. **(C)** Rotorod times of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BM cells. Note that the rotorod times for *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice show lower values than *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipients. n = 2-3. **(D)** The righting reflex of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ BM cells. Note that *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice started to show longer times to right themselves from 115 time point tested. n = 3-4.

Donor : *Hexb*$^{-/-}$ *Tnfα*$^{+/+}$



3.3.4 *Both, CNS-derived and bone-marrow-derived TNFα play key roles in the pathogenesis of Sandhoff disease*

Hexb$^{-/-}$*Tnfα*$^{-/-}$ recipients, which were transplanted with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow cells, showed longer median survival (145) days compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$mice that received *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ cells (130.5 days) (Figure 6A). Following transplantation with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow cells, we did not notice a detectable change in the body weight of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice till (130) days (Figure 6B).On wire hang experiment, we have found that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice did not start to decline until ~125 days of age, while *Hexb*$^{-/-}$*Tnfα*$^{+/+}$recipient mice started to decline at ~95 days of age, after transplantation with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow donors, (Figure 7A).*Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice, transplanted with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow donors, showed better grip strength compared to *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipients (Figure 7B). On Rotorod, Hexb$^{-/-}$*Tnfα*$^{+/+}$recipient mice declined at a faster rate compared to *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice (Figure 7C). The same trend has been detected on righting reflex that TNFα-deficient Sandhoff mice out performed Sandhoff recipients (Figure 7D). Using *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow donors, we found that *Hexb*$^{-/-}$*Tnfα*$^{-/-}$recipient mice started to outperform *Hexb*$^{-/-}$*Tnfα*$^{+/+}$recipients in the righting reflex test, starting from 115 days (Figure 7D). These findings support the notion that CNS-derived TNFα potentiates the main deleterious effect on Sandhoff pathology.

In table 1, we summarized the results from behavioural tests that were performed at 100 days, with the exception of righting reflex, which was measured at 120 days, near endpoint for *Hexb*$^{-/-}$mice. Recipient mice who expressed neither endogenous nor

transplanted TNFα (*Hexb*$^{-/-}$*Tnfα*$^{-/-}$into *Hexb*$^{-/-}$*Tnfα*$^{-/-}$) performed the best of all groups, on all tests. Those expressing both blood-derived and CNS-derived TNFα (*Hexb*$^{-/-}$*Tnfα*$^{+/+}$ into *Hexb*$^{-/-}$*Tnfα*$^{+/+}$) had the worst performance on all tests, as expected if TNF has a pathological effect. Interestingly, mice expressing only blood derived TNFα (*Hexb*$^{-/-}$ *Tnfα*$^{+/+}$ into *Hexb*$^{-/-}$*Tnfα*$^{-/-}$), had slightly longer life-spans relative to those expressing only CNS-derived TNFα (*Hexb*$^{-/-}$*Tnfα*$^{-/-}$ *into Hexb*$^{-/-}$*Tnfα*$^{+/+}$), as well as significantly better performance on rotorod and wire hang tests. This suggests that CNS- resident microglia play a larger role in TNFα secretion relative to than those migrating from the blood.

Figure 6 Survival and body weight of *Hexb*$^{-/-}$Tnfα$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice transplanted with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow cells

(A) Survival of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice after BMT with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow donor cells. The median survival of *Hexb*-/-*Tnfα*-/- recipient mice was (145) days. The median survival of *Hexb*$^{-/-}$*Tnfα*+/+ recipient mice was (130.5) days. *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$ recipient mice lived significantly longer than *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice. P< 0.001, n= (4–12). **(B)** Body weights of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ female recipient mice that transplanted with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow cells. We did not detect a change of body weights among recipient mice groups until 121 days. n=2-3.

Donor: *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$

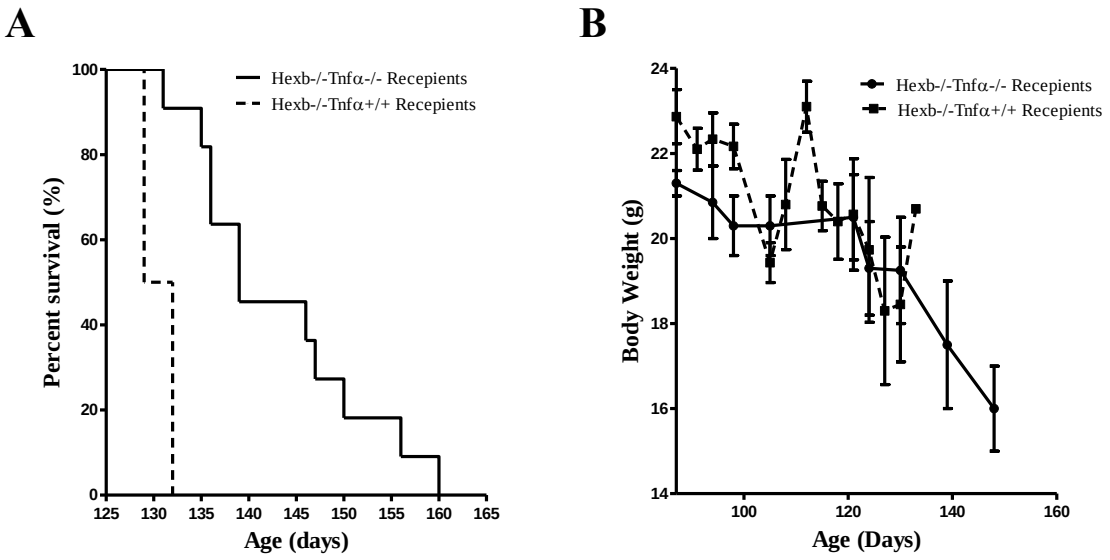


Figure 7 Behavioral testing of *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ transplanted with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ bone marrow cells

(A) Wire-hang times of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BM cells. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipients had better performance at all the tested time points starting from 91 days. Note that, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice started to show lower latency times starting from 118 days. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice show significant higher latency values at 101, 118 and 121 days compared with *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipients. *P < 0.05, **P < 0.01, n = 3-5. **(B)** Grip strength latency times of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BM cells. *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipient mice show higher values but without significance. n = 2-5. **(C)** Rotorod times of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BM cells. Note that the rotorod times for *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ recipient mice show lower values than *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ recipients. n = 1-6. **(D)** The righting reflex of transplanted mice with *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BM cells. Note that *Hexb*$^{-/-}$ recipient mice started to show longer times to right themselves from 118 time point tested. n = 3-5.

Donor : *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$

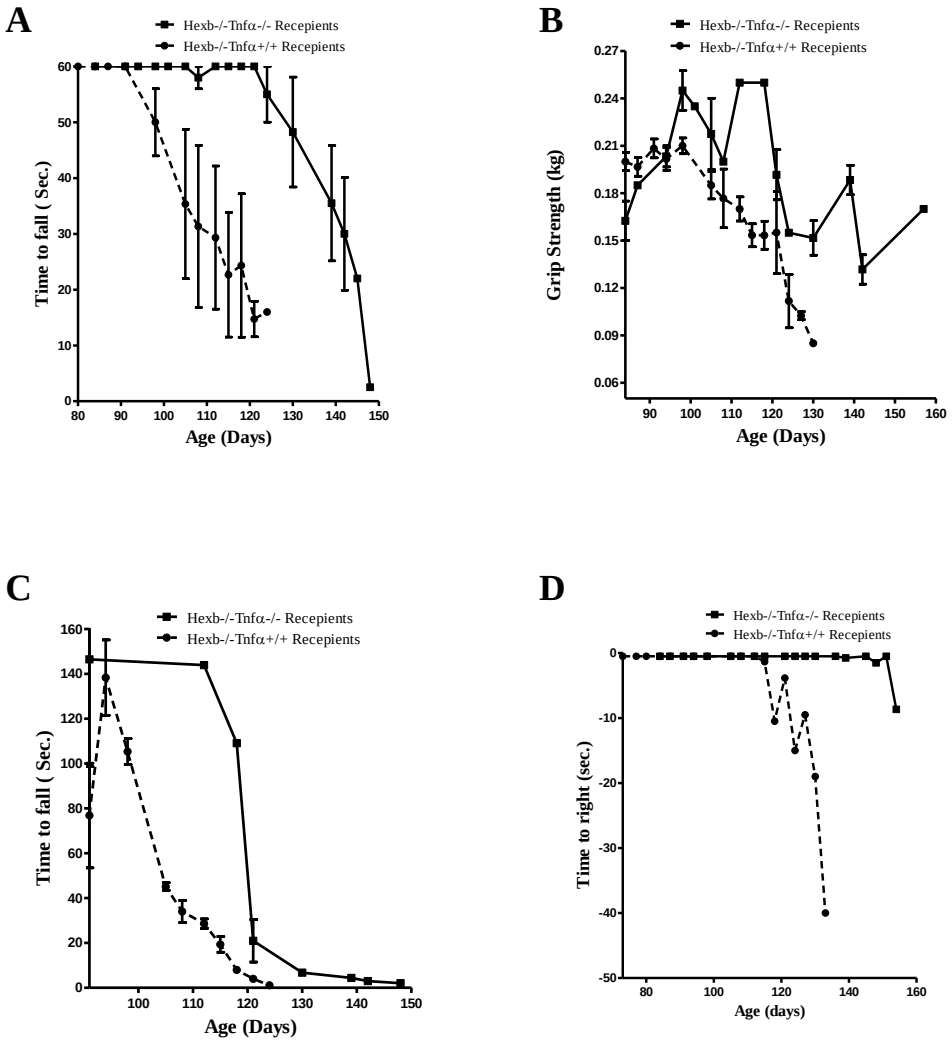


Table 1: Effect of CNS-resident and bone marrow-derived TNFα on survival and behavior of *Hexb*$^{-/-}$ mice

Tests were performed at 100 days, except for the righting reflex test which was performed at 120 days. Significant differences to other groups are indicated by superscript letters; P≤0.05.

		Bone marrow recepient mice			
		A	**B**	**C**	**D**
Genotype	**Donor**	Hexb$^{-/-}$Tnfα$^{-/-}$	Hexb$^{-/-}$Tnfα$^{+/+}$	Hexb$^{-/-}$Tnfα$^{-/-}$	Hexb$^{-/-}$Tnfα$^{+/+}$
	Recepient	Hexb$^{-/-}$Tnfα$^{-/-}$	Hexb$^{-/-}$Tnfα$^{-/-}$	Hexb$^{-/-}$Tnfα$^{+/+}$	Hexb$^{-/-}$Tnfα$^{+/+}$
Behavioral test	**Total N.**	12	4	4	6
		Mean ± SEM	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
	Survival (days)	145 ± 2.83	139.4 ± 5.96	130.5 ± 0.87	121.7 ± 1.8^{a,b}
	Rotarod (sec.)	145.4 ± 47.78	62.3 ± 5.56	45.17 ± 4.12	35 ± 9.96^{a,b,c}
	Wire-Hang (sec.)	60 (max.)	60 (max.)	45.8 ± 6.02	32.9 ± 13.2^{a,b}
	Righting Reflex (sec.)	0.5	0.5	6 ± 6.41	9 ± 8.93

Tests were performed at 100 days, except for the righting reflex which was performed at 120 days. $P \leq 0.05$.

[a]significantly different than group A.

[b]significantly different than group B.

[c]significantly different than group C.

3.4 Discussion

TNFα plays a significant role in the neuroinflammation generated during Sandhoff disease, as demonstrated by the delayed disease onset in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice compared to *Hexb*$^{-/-}$ mice. In this study, we investigated the importance of CNS-derived TNFα compared to BM-derived TNFα, with the anticipation that BM-derived TNFα could play a significant role following the infiltration of peripheral inflammatory cells into the brain. We first examined how TNFα deficiency could change peripheral inflammatory cell frequencies in *Hexb*$^{-/-}$ mice, and we revealed that TNFα deficient *Hexb*$^{-/-}$peripheral blood contains significantly lower frequencies of CD3ε^{+}CD4^{+} T helper cells, CD11b^{+}Gr-1^{neg-lo} monocytes, and CD11b^{+}Gr-1^{hi} neutrophils. TNFα has been implicated as important inducer of innate immune response in the CNS (Bhat & Steinman, 2009). In addition to its secretion by resident immune cells, it was found TNFα can breach the blood brain barrier and cause fever via its effect on the temperature regulating center within the hypothalamus (Steinman, 2013). It has been found that T-helper cells play a pathogenic role in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (Axtell et al., 2010; Wojno & Hunter, 2012). T-immune cells can indirectly induce microglia to secrete inflammatory cytokines (Butovsky et al., 2006), although an observation that T-cell infiltration is not one of the pathogenic features of Sandhoff disease (Lee et al., 2007), suggesting a minor role of T-helper cell in Sandhoff pathology. Neutrophils, as a subset of phagocytic cells that clearly affected by lack of catabolic HEXB enzyme (Pierce, Kosanke, Bay, & Bridges, 1976), can elicit peripheral immune response that indirectly affect Sandhoff CNS (Hopkins, 2007).

TNFα deficiency in mice has not been previously linked to changes in resting peripheral blood populations, but we observed a decrease in neutrophils in the *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$mice relative to both wild type and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice. There are many reports that TNFα, which is secreted in response to a number of Toll-like receptor stimuli, is important for inducing the production of leukocytes required to clear pathogens. This includes inducing the activity of myeloid progenitors(Mizrahi et al., 2013), therefore the decreased populations of circulating neutrophils in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice could reflect the absence of this phenomenon. Another possibility is that circulating neutrophils are recruited to the inflamed tissues, including the CNS, and are therefore less abundant in circulation, but we did not observe an increased infiltration of immune cells in *Hexb*$^{-/-}$ *Tnfα*$^{-/-}$ mice (Abo-Ouf et al., 2013).

Toll-like receptors (TLRS) are a class of innate immune receptors that signal in response to primarily pathogen-related ligands, such as bacterial lipopolysaccharide (LPS), and tissue damage-associated ligands, as reviewed in (Okun, Griffioen, & Mattson, 2011). It was found that TLRs are expressed in both, peripheral immune cells including B cells, mast cells, monocytes and natural killer cells, and also in brain cells, such as microglia, astrocytes and neurons (Leulier & Lemaitre, 2008; Olson & Miller, 2004). Ligand/TLR4 binding leads to activation of myeloid-differentiation factor 88 (MyD88)-dependent pathway ending in activated NF-kB to produce inflammatory cytokines such as TNFα and IL6 (Jung et al., 2005). The innate immune response of macrophages to bacterial LPS is a well-defined reaction and associated with secretion of inflammatory cytokines including TNFα and IL-6 (Takeda & Akira, 2005). Hence, these

cytokines mediate active recruitment and activation process to add more immune cells and aggravate inflammation (Liang et al., 2009). By stimulating bone marrow derived macrophages through TLR4 and measuring cytokine secretion, we discovered *Hexb*$^{-/-}$ macrophages were less sensitive to stimulation, and with combined TNFα deficiency we observed significantly reduced IL-1β but increased MCP-1 secretion. It is believed that monocyte chemo-attractant protein-1(MCP-1) is playing a major role to induce chemo taxis of blood monocytes to invade damaged CNS tissues (Wilms et al., 2003). In neurodegeneration, it was found that MCP-1 mRNA is elevated in the hippocampus (Bruccoleri & Harry, 2000). Furthermore, it was found that systemic administration of TNFα trigger intense MCP-1 expression in the brain (Thibeault, Laflamme, & Rivest, 2001). It is noteworthy that toll-like receptor 2 (TLR2) and (TLR4) agonists control MCP-1 expression in monocytes (Parker, Whyte, Vogel, Dower, & Sabroe, 2004). GM2 did not stimulate cytokine secretion from BMDMs. Deletion of TNFα in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDMs limits the LPS-induced secretion of cytokines, except for the up-regulation of MCP-1. MCP-1 is a crucial cytokine that has been implicated in the recruitment of peripheral monocytes to sites of neurodegeneration, in light of the evidence that the deletion of its receptor CCR2 significantly inhibits the infiltration of peripheral blood mononuclear cells (Kyrkanides et al., 2008). Although the up-regulation of MCP-1 in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ BMDMs could reflect up-regulation of MCP-1 in CNS-resident macrophages and possibly increased recruitment of peripheral blood mononuclear cells into the brain, there is only evidence of decreased neuroinflammation in *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ mice.

Over the years, bone marrow transplantation has been considered one of the routine therapeutic options for Gaucher lysosomal storage disease (Krivit, Aubourg, Shapiro, & Peters, 1999). Similar beneficial effects of bone marrow transplantation have been reported in other lysosomal storage diseases such as mucopolysaccharidosis type I and II and Krabbé disease (globoid cell leukodystrophy) (Peters & Krivit, 2000; Krivit, Peters, & Shapiro, 1999). It was found that early in life transplantation of bone marrow cells has a better effect on amelioration of neurodegeneration of these diseases as reviewed in (Malatack, Consolini, & Bayever, 2003). The beneficial effect of bone marrow transplantation is partly due to cross-correction phenomenon in which enzymes are secreted by donor cells to be recaptured and uptaken by affected neighboring neurons via mannose-6-phosphate receptor system (Hodges & Cheng, 2006). However, it was found that cross differentiation of mesenchymal stem cells into neurons is considered a good source to replace dying neurons and repairing neuronal circuits (Shihabuddin & Aubert, 2010).It has been shown that transplantation of normal bone marrow cells can improve the neurological symptoms in lysosomal storage diseases (Norflus et al., 1998). Whether this improvement is due to the infiltration of β-hexosaminidase-positive microglia into the apoptotic brain regions of the untreated mice (Hoogerbrugge et al., 1988; Walkley, 1998) or due to the reduction of the excessive inflammatory response leading to suppression of the activated microglial expansion in untreated mice (Oya et al., 2000), it is still controversial.

Our bone marrow transplantation experiments to identify sources of pathological TNFα have demonstrated that both CNS-derived and bone marrow-derived sources of

TNFα have similar pathological effects in the progression of Sandhoff disease in our mouse model. However, CNS-derived TNFα appears to be of larger influence and, therefore represents important potential targets for treatments in patients. Treatments with TNFα inhibitors should be designed to penetrate the blood-brain barrier for optimal effect. Previous studies involving bone marrow transplantation of wild-type bone marrow into *Hexb*$^{-/-}$mice have shown improvements in lifespan and neuromotor function as well as reduction in microgliosis, without changes in ganglioside accumulation (Jeyakumar et al., 2001). The clinical improvement of Sandhoff recipients, after transplanting *Hexb*$^{-/-}$ *Tnf*α$^{-/-}$bone marrow may implicate the importance of inflammation in the pathology of this disease, and provide further evidence that TNFα is an important detrimental factor in Sandhoff disease. After behavioural tests of bone marrow transplanted animals, we proposed a model in which both BM-derived and CNS-resident TNFα contributed to neurodegeneration in Sandhoff disease mice (Figure 8). Moreover, we found that CNS-derived TNFα had a greater effect on disease pathogenesis. These findings indicate that inhibiting TNFα signaling in macrophages, and their closely related CNS-resident microglia, is a viable target to slow the progression of Sandhoff disease.

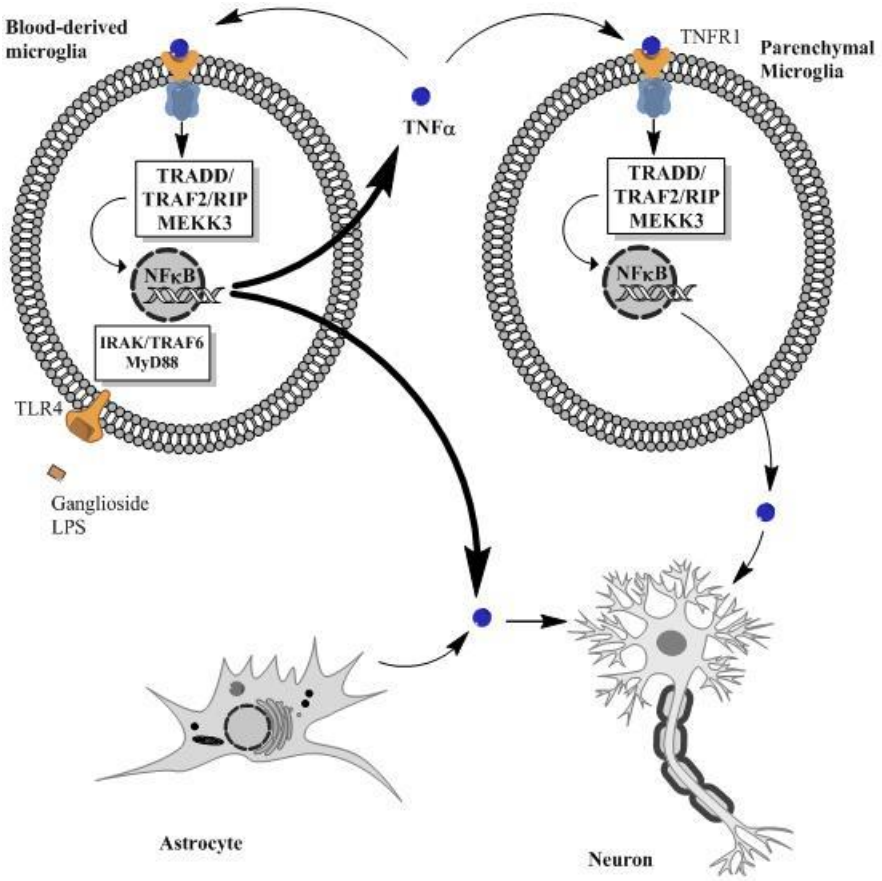


Figure 8 Model for neuronal and glial cell response to TNFα in Sandhoff mice model

In this model, blood-derived microglia secrete TNFα in response to ganglioside-mediated TLR4 activation. Secreted TNFα exerts its actions via autocrine and paracrine signaling pathways. Paracrine-activated resident microglia can secrete more TNFα to further activate glia cells. Ultimately, CNS neurons are affected by this massive secretion of TNFα, resulting in neurodegeneration

3.5 Materials and Methods

Mice

All procedures carried out on animals were approved by the McMaster University Animal Research Ethics Board, and were in compliance with the guidelines of the Canadian Council on Animal Care and the Ontario Animals for Research Act. *Tnfα*$^{-/-}$ mice were purchased commercially (The Jackson Laboratory, Bar Harbor, ME). Male Sandhoff disease model mice (*Hexb*$^{-/-}$) on a C57B/6 background were mated with female tumor necrosis factor-alpha (*Tnfα*$^{-/-}$) deficient mice to generate double heterozygous *Hexb*$^{+/-}$*Tnfα*$^{+/-}$ mice which were used to generate breeding colonies for double mutant mice. These doubly heterozygous mice were mated with *Tnfα*$^{-/-}$ mice to generate *Hexb*$^{+/-}$ *Tnfα*$^{-/-}$mouse. Subsequently, these *Hexb*$^{+/-}$*Tnfα*$^{-/-}$ mice were mated with each other to generate doubly null (*Hexb*$^{-/-}$*Tnfα*$^{-/-}$) mice. The genotypes of the *Hexb*, *Tnfα* loci were confirmed using polymerase chain reaction (PCR) utilizing isolated tail DNA.

Flow cytometry

Peripheral blood was collected from male *Hexb*$^{-/-}$, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ *and* *Hexb*$^{+/+}$*Tnfα*$^{+/+}$mice by terminal cardiac puncture with a heparinized needle. Erythrocytes were lysed using ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM Na_2EDTA), leukocytes were counted and cells were pre-incubated with rat anti-mouse CD16/CD32 (10 μg/ml, BD Pharmingen, Mississauga, Canada) and immunostained for cell surface markers using 1 μg of each antibody for 10^6 cells in FACS buffer (PBS, 0.2% BSA): hamster anti-mouse CD3e-APC-Cy7 (clone 145-2C11), anti-mouse CD11b-APC

(clone M1/70), rat anti-mouse CD19-V450 (clone 1D3), anti-mouse Gr-1-FITC (clone RB6-8C5), and mouse anti-mouse NK1.1-PE-Cy7 (clone PK136). Separate reactions were used to assess hamster anti-mouse CD3e-APC-Cy7 (clone 145-2C11), rat anti-mouse CD4-PE (clone GK1.5) and rat anti-mouse CD8a-Pacific Blue™ (clone 53-6.7). Samples were fixed with BD Cytofix™ Fixation Buffer (BD Biosciences, Mississauga, Canada) and run on a LSR II flow cytometer (Beckman Coulter).

Bone marrow isolation and culture

Bone marrow was isolated from male *Hexb*$^{-/-}$, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ *and* *Hexb*$^{+/+}$*Tnfα*$^{+/+}$mice, cultured for 9 days in RPMI-1640 supplemented with 10% FBS, 10 mM HEPES, penicilin/streptomycin, amphotericin B, and 5 ng/ml M-CSF. Macrophages were incubated with 10 μg/ml LPS, 25 μg/ml poly I: C, or 10 μg/ml GM2 ganglioside for 72 h before harvest of culture media for ELISA assay of cytokines and cell lysate to normalize protein levels.

Enzyme linked immunosorbant assay

Culture media harvested from stimulated macrophage cultures was assayed for TNFα, IL-1β, IL-6, IL-10, and MCP-1. Cytokine levels were normalized to cell protein measured using the DC Protein Assay (Bio-Rad, Mississauga, ON, Canada).

Bone Marrow Transplantation (BMT)

Bone marrow transplantation was performed as previously described (Norflus et al., 1998). Transplantations included *Hexb*$^{-/-}$*Tnfα*$^{+/+}$bone marrow donors into *Hexb*$^{-/-}$*Tnfα*$^{-/-}$recipients, *Hexb*$^{-/-}$*Tnfα*$^{+/+}$bone marrow donors into Hexb$^{-/-}$Tnfα$^{+/+}$ recipients, *Hexb*$^{-/-}$*Tnfα*$^{-/-}$bone marrow donors into *Hexb-/- Tnfα*$^{+/+}$recipients and *Hexb*$^{-/-}$*Tnfα*$^{-/-}$bone marrow into *Hexb*$^{-/-}$*Tnfα*$^{-/-}$recipients (n = 4-12). All recipient mice were females at ~2 months of age. Briefly, male donor mice were euthanized by cervical dislocation. Humiri, tibia, and femurs bones were dissected from each donor mouse under sterile conditions, and bone marrow was flushed with Iscove's medium (Lonza, 12-722F) and supplemented with L-glutamine (Gibco, 25030). Cells were passed through a cell strainer (0.45 µm) and red blood cells were lysed with cold ammonium chloride potassium (ACK) lysis buffer. Cells were stored on ice until injection. Bone marrow recipient mice were given sterile chow and Septra water for one week prior to irradiation. Irradiation of bone marrow recipient mice was broken into two treatments, with one dose of 7 Gy, followed by a second dose of 4 Gy within 4 hours. Under anesthesia, mice were injected intravenously with ~ 6-10 x10^{8} cells. The recipient mice recovered in autoclaved cages with sterile gel and Septra water.

Behavioural Tests

Bone marrow recipient mice were used to investigate lifespan, body weight, and neurological function. Each group of mice was monitored using modified Irwin (Irwin & Irwin, 1979; Irwin, 1968) and Moser (McDaniel & Moser, 1993) observational test

batteries (Miklyaeva et al., 2004). Motor function was evaluated using Rotorod, wire hang test, grip strength test and righting reflex, which were conducted weekly subsequent to weaning. To test neurological functions related to balance and sensorimotor coordination, indicators of cerebellar function, we utilized the Rotorod test to measure the latency to fall off a rotating rod where infrared photo cells captured exact fall time (Miklyaeva et al., 2004). The Accuscan EZ-Rod and companion computer software EZ rod (version 1.20) were used. The program involved placing the mice on pre-spinning rod, which was gradually accelerated from 4 to a maximum of 40 rpm over a 6 minute test. Each mouse was given 3 trials, the best of which was included in the data set. The wire-hang test was used to examine lower motor functions, i.e. peripheral nerves, motor-end plate and muscular functions, as previously described (Boyce-Rustay et al., 2006). For this test, the mouse gripped onto ~ 10 cm square wire mesh. The mesh was then inverted 20cm above a padded surface and the mice were forced to use their four limbs to hold their body suspended upside down for a maximum of 60 s. The latency for the mouse to lose its grip and fall was timed over a 60 s period. The test was conducted in triplicate for each mouse and the longest time was recorded. The grip strength test was conducted as previously described (Miklyaeva et al., 2004), using a digital force gauge meter (Chatillon Ametek Inc.). Force (in kg) was measured as the exertion of the mouse to grasp a metal rod as the mouse was pulled away from the rod at a constant force. The righting reflex test revealed any abnormalities in motor coordination and activity level as described previously (Nichols & Holmes, 2002). The righting reflex was conducted by placing a mouse on its back on a solid flat surface and the time taken for the mouse to

right itself on all four limbs was recorded. Each mouse was given three reflex trials and the best time of these was included in the data. Mice unable to right themselves after more than 45 seconds were used as a humane endpoint. Student's t-test was used to evaluate statistical significance. *$P < 0.05$, **$P < 0.01$ were considered statistically significant.

Chapter 4

MicroRNA profiling of Sandhoff mouse brain reveals novel roles for miR-210 and miR-96 in neurodegeneration

Preface:

The results presented in chapter 4 were prepared as a manuscript to be submitted for publication. I performed the majority of the experiments described in this chapter, gathered the data and figures, and wrote the manuscript. I isolated and prepared protein samples used by Helena Janse van Rensburg to perform and provide western blot data for some of the proteins described in the article including VEGF, α- Synaptophysin, PSD95 and MEF2C. Considerable advice and direction for the experimental work, and contribution to the final version of the manuscript were given by my supervisor, Dr. S. Igdoura.

4.1 Abstract

Sandhoff disease is a rare metabolic disorder caused by the absence of lysosomal enzymes called, beta-hexosaminidases A and B (Sandhoff, Andreae, & Jatzkewitz, 1968). This disorder leads to severe and profound neuromotor defect which ultimately results in death in early age(Gomez-Lira et al., 1995). Our objective was to define the miRNA profile in Sandhoff brains as they may be implicated in the pathophysiological changes associated with Sandhoff disease. Thus, a crucial step to understand of the role of microRNAs in Sandhoff pathology is the identification of their gene candidates. To investigate miRNA profile in Sandhoff brains, total RNAs were isolated from Sandhoff and wild-type cerebral tissues. Then, samples were assessed using Illumina microRNA Assay system. Putative target genes were predicted by using PicTar and miRanda algorithms. Our results show that there is pattern of dys-regulated microRNAs expressed in Sandhoff cerebrum. Microarray results show that miR-210 and miR-96 were amongst 19 dys-regulated miRNAs in the CNS of Sandhoff mice. Furthermore, we identified neuronal pentraxin 1 (*Nptx1*) and myocyte enhancer factor 2C (*Mef2c*) as putative target genes for miR-210 and miR-96, respectively, in Sandhoff disease. Results were validated using western blotting and TaqMan quantitative real time PCR. We have found that neuronal pentraxin expression levels were significantly induced in Sandhoff brains. Furthermore, our results demonstrated that MEF2C expression was decreased in Sandhoff mice. Our results might indicate that synaptic scaling could be regulated by *Nptx1*which in turn controlled by miR-210. Our study proposes that microRNA profiling of Sandhoff brain samples provides new gene targets as crucial modulators of neurodegeneration.

4.2 Introduction

microRNAs represent a newly emerging gene regulating network that add an additional layer of biological complexity involved in fine tuning of neuronal disease pathologic mechanisms (Wang et al., 2008). MicroRNAs are a family of non-coding, 19-23 nucleotides which post-transcriptionally regulate the expression of particular genes (Bartel, 2004). Up to date, the human genome contains around 1048 microRNAs (http://www.mirbase.org). MicroRNA genes can be found within or between coding regions (Borchert, Lanier, & Davidson, 2006). Furthermore, microRNA encoding genes are transcribed individually or as a cluster by RNA polymerase II to generate precursor microRNAs (pri-miRNAs) (Melino & Knight, 2010). In the nucleus, RNase III process pri-miRNAs to yield stem-loop-shaped pre-miRNAs that are transported to the cytoplasm for further processing by Dicer enzyme (Melino & Knight, 2010). Dicer cleaves the pre-miRNAs to generate 19–23 nucleotide double-stranded RNAs (Altar, Vawter, & Ginsberg, 2009). Subsequently, the double-stranded miRNAs unwind to provide the mature single-stranded miRNAs to for mmiRNPs (miR- protein complexes) (Melino & Knight, 2010). Down-regulation of targeted proteins via translational silencing or transcript degradation, is a result of incomplete complementarity of miRNAs with 3`UTRs of target mRNAs (Santos-Reboucas & Pimentel, 2010). Thus, these molecules are essential modulators of patho-physiological processes and, hence, might serve as possible therapeutic targets (Ozsait et al., 2010).

Normal brain development, and its relevant physiological processes, essentially require the integrity of microRNA machinery (Perkins et al., 2007). It has been firmly established that many specific aspects of neuronal function such as neurite growth and synaptic formation, are regulated by microRNAs (Gao, 2008). Much less insight has been gained regarding the microRNA network that regulates gene expression mediating neurodegeneration. While our knowledge of microRNA function in the context of human nervous diseases is still insufficient, experiments in mice have shown that microRNAs are expressed during the course of neuronal stress, suggesting microRNAs play an essential role during the pathophysiology of brain diseases (Bak et al., 2008). Evidence from recent work has demonstrated that dys-regulation of microRNA networks contributes to several neurological disorders, such as Down syndrome, Parkinson's disease (PD) and Alzheimer's disease (AD) (Bak et al., 2008). Recent studies have uncovered some of the mechanisms underlying microRNAs dynamics and provided critical glimpses into the functions of microRNAs. For instance, miR-133b and miR-132 have been implicated in the pathogenesis of Parkinson's disease (PD) and Alzheimer's disease (AD), respectively. Recent studies have shown that certain microRNAs have regulatory roles in lipid metabolism in lysosomal storage diseases (Ozsait et al., 2010). For instance, microRNA expression profiling of NPC fibroblasts has shown that down-regulated miR-143 expression was associated with lipid biosynthesis (Ozsait et al., 2010). Furthermore, the same study suggested a proinflammatory role of altered miR-98 in NPC fibroblasts (Jones et al., 2009). Interestingly, it was found that miR-128 can silence transcription elongation factor B (TEFB), a transcription factor that binds to coordinated

lysosomal storage expansion and regulation (CLEAR) promotor element (Sardiello et al., 2009). Thus, microRNAs are moving rapidly towards center stage as an additional guidance cue involved in deciphering neurodegeneration molecular mechanisms in neurodegenerative disease(Hebert & De Strooper, 2009).

Expression profiling has shed more light on the role of miRNAs in pathophysiological conditions (Wang et al., 2008). Microarray experiments have provided an extensive description on miRNA expression in both normal and diseased brain conditions (Babak, Zhang, Morris, Blencowe, & Hughes, 2004; Ciafre et al., 2005). The role of microRNAs in Sandhoff disease pathology is not yet clear. It is conceptually possible that glycolipid accumulation dys-regulates miRNAs expression which would provide mechanistic insights in regards to neurodegeneration in Sandhoff disease. However, it is important to distinguish the primary and secondary causes from effects to fully understand the microRNA pathways relevant to neurodegeneration. Expression profiling uncovers whether any miRNAs are differentially expressed in a mouse's CNS in the context of neuropathological states; this would reveal if microRNAs are involved in the mechanisms underlying these disorders (Bak et al., 2008).

In this report, we investigated whether miRNAs expression was differentially regulated during the symptomatic stage of Sandhoff neurodegenerative disease. Using microarray analysis, we examined the expression profiling of microRNAs in the brain of Sandhoff mice (*Hexb*$^{-/-}$) compared to a control microRNAs profile in wild-type mice brains. This is the first report unraveling the role of microRNA in controlling and modulating neurodegeneration in a mouse model of Sandhoff disease. Our findings set

the stage for therapeutic interventions targeting key microRNAs that may halt the progressive neuronal apoptotic events in Sandhoff disease.

4.3 Results

4.3.1 MicroRNAs are differentially expressed in the cerebrum of Sandhoff mice

In order to investigate if there was a distinct microRNA expression pattern associated with the symptomatic stage of Sandhoff disease in mice, we analyzed the differential expression of microRNAs from Hexb+/+ and Hexb-/- cerebral tissues (Figure 1). Compared to the microRNA expression level in the wild-type brains, and when we implemented a cut-off P-value lower than 0.05, we identified 19 putative miRNA targets that showed significant change in Sandhoff brains. Amongst the significantly up-regulated 14 miRNAs were miR-1190, miR-700, miR-193b, miR-96, miR-298, miR-712, miR-216b, miR-455, miR-881, miR-211, miR-300, miR-720, miR-703 and miR-874. Furthermore, we identified 5 microRNAs that showed significant down regulation in Sandhoff brains including miR-181c, miR-669e, miR-705, miR-210, and miR-592.

4.3.2 Functional analysis of microRNA targets

In order to examine and explore the functional significance of these miRNAs, we examined their roles in other diseases. To evaluate the potential molecular consequence of the compiled list of the significantly expressed miRNAs, we have focused on a subset of miRNAs which have been previously implicated in neurodegeneration. Recently, a long list of publications has shed new light into the important role of microRNAs during

neurodegeneration. We used bioinformatics algorithms, PicTar14 and miRanda to predict target genes. In table 1, we listed miRNAs along with their predicted target genes. miRNA-300 has several predicted target mRNAs that are relevant to CNS function such as synaptotagmin-11 (*Syt11*), huntingtin-associated protein 1 (Hap1) and potassium voltage-gated channel, Shaw-related subfamily, member 2 (*Kcnc2*). Furthermore, we identified myocyte enhancer factor 2 (*Mef2c*) as a novel predicted target for miRNA-96 in Sandhoff brains. Glutamate receptor, ionotropic, kainate 2 (*Grik2*) was among the list of genes predicted to be targets for microRNA-720. Another member of glutamate receptors, glutamate receptor ionotropic, NMDA3A (*Grin3a*) mRNA was predicted to have a binding site for microRNA-193b. Those predicted target genes of microRNAs in Sandhoff disease affect particular relevant pathways such hypoxia-inducible factor alpha, interleukin receptor, tumor necrosis factor, purinergic receptor P2Y and myocyte enhancer factor C. In addition, we have detected a list of predicted genes, associated with excitotoxicity such as neuronal pentraxin 1, potassium voltage-gated channel (*Kcna4*), glutamate receptor, ionotropic, AMPA2 (*Gria2*) were influenced by the highly expressed microRNAs in Sandhoff samples. Interestingly, we have detected a number of target genes related to neuronal tissue homeostasis such as neuronatin (*Nnat*), neuro-oncological ventral antigen 1(*Nova1*) and Synaptophysin-like 2 (*Sypl2*). We have focused onmiRNA-210 and miRNA-96 since they have been found to be significantly altered in Sandhoff brains and found to target neuronal pentraxin 1(*Nptx1*) and myocyte enhancer factor 2 (*Mef2c*), respectively.

Figure 1 Differential expression of microRNAs in the cerebrum of normal and Sandhoff mice

Cluster heatmap analysis of 8 cerebral samples from *Hexb*$^{+/+}$ and *Hexb*$^{-/-}$mice. Levels of nineteen miRNAs were significantly different ($P < 0.05$). Fourteen microRNAs show significantly higher expression across all Sandhoff brains compared to wild-type mice brains. The heatmap diagram includes 5 microRNAs that are significantly down-regulated in Sandhoff mice compared to wild-type samples. In the heatmap, each row represents each miRNA, and each column represents each sample. Red implies high relative expression, green implies low relative expression, and black indicates no change. Arrows points to miRNA-96 and miRNA-210.

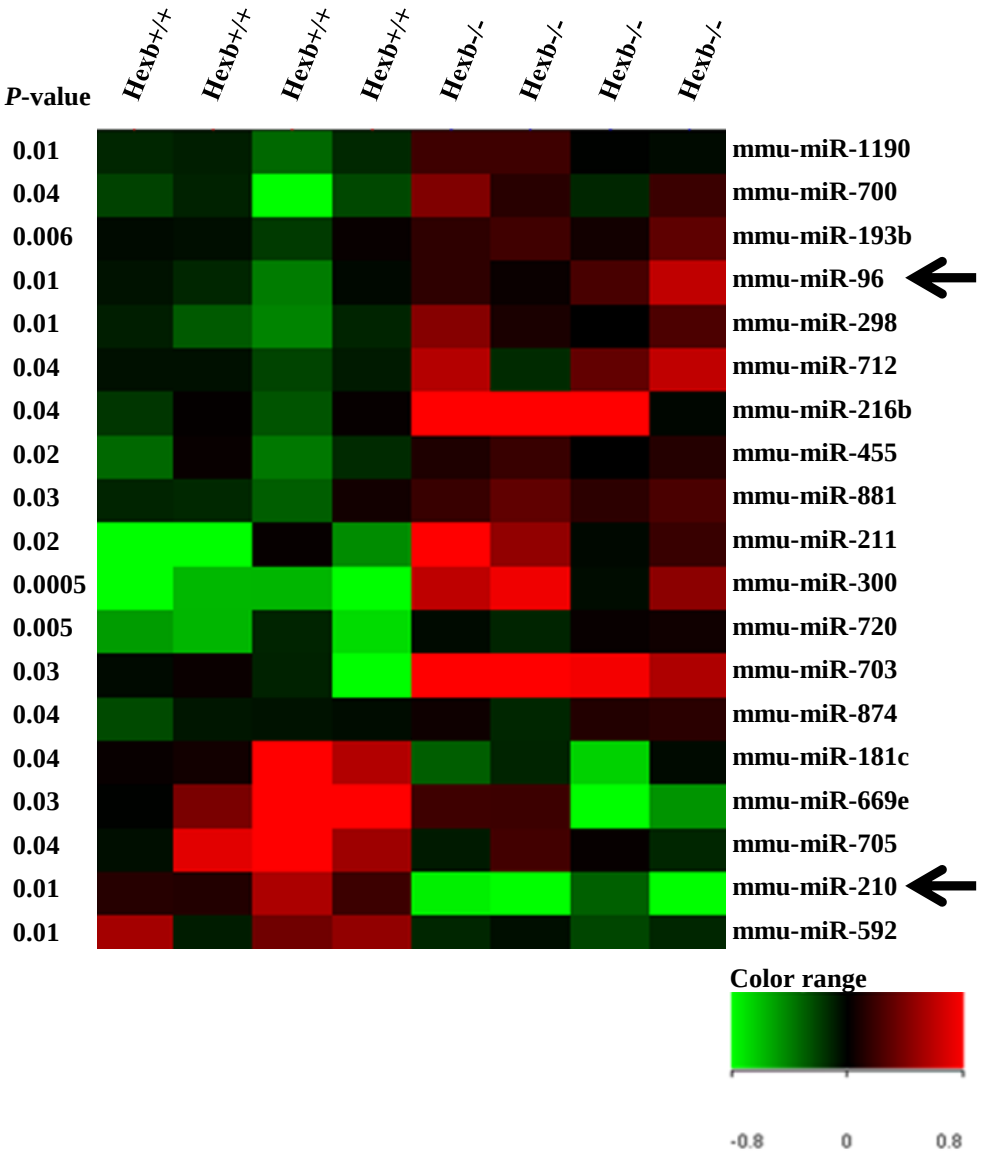

Hexb+/+
Hexb+/+
Hexb+/+
Hexb+/+
Hexb-/-
Hexb-/-
Hexb-/-
Hexb-/-
P-value
0.01
0.04
0.006
0.01
0.01
0.04
0.04
0.02
0.03
0.02
0.0005
0.005
0.03
0.04
0.04
0.03
0.04
0.01
0.01
mmu-miR-1190
mmu-miR-700
mmu-miR-193b
mmu-miR-96
mmu-miR-298
mmu-miR-712
mmu-miR-216b
mmu-miR-455
mmu-miR-881
mmu-miR-211
mmu-miR-300
mmu-miR-720
mmu-miR-703
mmu-miR-874
mmu-miR-181c
mmu-miR-669e
mmu-miR-705
mmu-miR-210
mmu-miR-592
Color range
-0.8
0
0.8

Table 1: Functional analysis of differentially expressed subset of miRNAs showing their putative target genes

Putative target genes for miRNAs were predicted using Pictar and miRanda prediction algorithms. Note that *Hif1α* and *Mef2c* have been shown as potential gene targets for miR-210 and miR-96, respectively.

microRNA	mouse RefSeq Id	Prediction tool	Targeted mRNA	Annotation
mmu-miR-210				
	NM_133195.3	PicTar	Brunol4	Bruno-like 4, RNA binding protein
	NM_008730.2	PicTar	Nptx1	Neuronal pentraxin 1
	NM_033144.2	PicTar	Sept8	Septin 8
	NM_010431.2	miRanda	Hif1a	Hypoxia inducible factor 1
	NM_031168.1	miRanda	Il6	Interleukin 6
	NM_011307.2	miRanda	Uimc1	Ubiquitin interaction motif containing 1
	NM_010570.4	miRanda	Irs1	Insulin receptor substrate 1
	NM_010923.2	miRanda	Nnat	neuronatin
mmu-miR-96				
	NM_020009.2	miRanda	Mtor	Mechanistic target of rapamycin
	NM_001170537.1	miRanda	Mef2c	Myocyte enhancer factor 2C
	NM_025278.5	miRanda	Gng12	Nucleotide binding protein, gamma 12
	NM_177914.3	miRanda	Dgkk	Diacylglycerol kinase kappa
mmu-miR-300				
	NM_018804	PicTar	Syt11	Synaptotagmin XI
	NM_010404	PicTar	Hap1	Huntingtin-associated protein 1
	NM_001025581	PicTar	Kcnc2	Potassium voltage gated channel, 2
	NM_008467	PicTar	Kpna4	Karyopherin alpha 4
	NM_031202	miRanda	Tyrp1	Tyrosinase-related protein 1
mmu-miR-720				
	NM_010349	miRanda	Grik2	Glutamate receptor, ionotropic, kainate 2
	NM_175130	miRanda	Trpm4	Transient receptor potential cation channel,4
mmu-miR-193b				
	NM_007936	miRanda	Epha4	Eph receptor A4
	NM_001025381	miRanda	Gpr17	G protein-coupled receptor 17
mmu-miR-592				
	NM_008364.2	miRanda	Il1rap	Interleukin 1 receptor accessory protein
	NM_146146.2	miRanda	(Lepr)	leptin receptor
mmu-miR-669e				
	NM_177371.3	miRanda	Tnfsf15	Tumor necrosis factor,15
	NM_018752.3	miRanda	Trpm1	Transient receptor potential cation channel,1
mmu-miR-705				
	NM_023517.2	miRanda	Tnfsf13	Tumor necrosis factor,13
	NM_175495.2	miRanda	Gpr150	G protein-coupled receptor 150
mmu-miR-181c				
	NM_021361.1	miRanda	Nova1	Neuro-oncological ventral antigen 1
	NM_013540.2	PicTar	Gria2	Glutamate receptor, ionotropic
	NM_021275.4	PicTar	Kcna4	Potassium voltage-gated channel

4.3.3 Neuronal pentraxin 1 (Nptx1), a newly identified miRNA-210 target in Sandhoff disease, is modulated by HIF1α

After taking the advantage of target prediction bioinformatics solutions, we recognized a set of genes, including *Nptx1*, as direct targets of miR-210. Here, we detected that miRNA-210 is significantly under expressed in Sandhoff brains (Figure 2A). Upon using miRanda alignment web interface, we were able to confirm that *Nptx1* is a putative candidate for miR-210 (sequence alignment is schematized in Figure 2C). To investigate whether NP-1 protein expression level was changed in correlation with miR-210, we performed a western blotting experiment (Figure 3A and D). Results showed that a significant increase of *Nptx1* expression in Sandhoff mice compared to wild-type. Previous reports showed that HIF1α might regulate miR-210 expression (Ivan, Harris, Martelli, & Kulshreshtha, 2008). To investigate whether HIF1α is involved in miR-210 regulation in Sandhoff disease, we used western blotting to assess the expression level of HIF1α and VEGF, a well-established HIF1α-responsive protein. Our results revealed that HIF1α was significantly down-regulated in Sandhoff compared to wild-type brains (Figure 3A and B). VEGF expression showed similar trend in response to lower HIF1α expression. The expression level of VEGF protein was substantially reduced in Sandhoff compared with wild-type controls (Figure 3A and C).

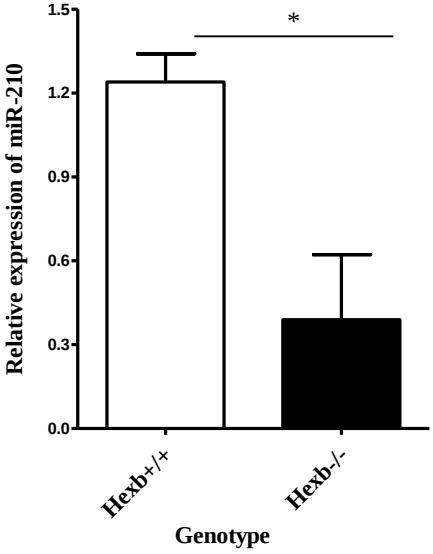
4.3.4 miRNA-210 is involved in the regulation of proteins affecting excitotoxicity in Sandhoff brains

Recently, it was shown that neuronal pentraxin 1 (*Nptx1*) is associated with excitotoxicity and synaptic scaling molecular events (Xu et al., 2003). To further analyze the role of miRNA-210 and *Nptx1* in Sandhoff disease pathology, we investigated the expression of a number of proteins which are closely associated with *Nptx1* and excitotoxicity. We have utilized western blotting analysis to determine complement component 1 (C1q), glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), α-Synaptophysin and postsynaptic density protein 95 (PSD-95) protein levels in the brain of Sandhoff mice. We have found a significant increase in the expression of C1q in Sandhoff cerebellum (Figure 4A and B). While we did not observe a significant alteration in the expression level of GluR2 (Figure 4A and D), we detected a trend of decreased GluR1 expression in Sandhoff brains, suggesting a possible role of glutamate receptor in Sandhoff pathology (Figure 4A and C). Furthermore, western blot analysis revealed that the protein level of α-Synaptophysin was strongly decreased in Sandhoff compared with wild-type mice (Figure 4A and E). In addition, we did not find significant change in the expression level of PSD-95 in Sandhoff compared with wild-type mice (Figure 4A and F). These results suggest that *Nptx1* is a potential gene target which might be regulated by miRNA-210. Taken together, we hypothesize a model in which HIF1α regulates miRNA-210 which in turn degrades *Nptx1*mRNA and hence decreases *Nptx1* protein level in the brains of normal mice. Furthermore, we have observed dys-regulation of the expression levels of C1q and α-Synaptophysin in the brain of Sandhoff mice. Furthermore, these results were associated with NP-1 upregulation.

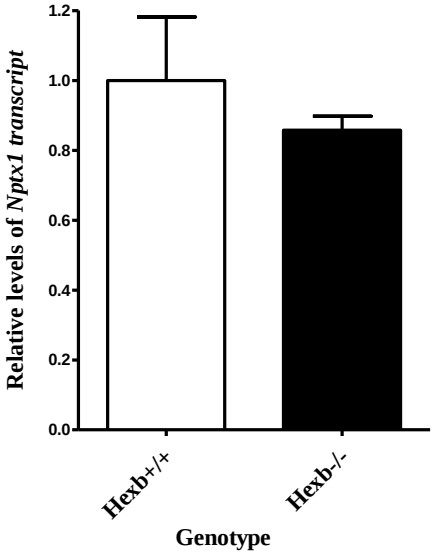
Figure 2 Level of miRNA-210 transcript in the CNS of Sandhoff mouse

Microarray analysis of the level of miR-210 and *Nptx1* in Sandhoff disease and normal mice. **(A)** The expression level of miR-210 is significantly decreased in *Hexb*$^{-/-}$ compared with wild-type samples. n = 4, * < 0.05. **(B)** Relative expression of *Nptx1* mRNA in *Hexb*$^{-/-}$ and *Hexb*$^{+/+}$ cerebral tissues. Bars represent quantified microarray signals normalized to wild-type. **(C)** miR-210/*Nptx1* alignment provided by miRanda alignment web interface (http://www.microrna.org).

A



B



C

```
3' agUCGGCGACAGUGUGCGUGUc 5' mmu-miR-210
     :||| ||| ||||||||||
5' auGGCCCCUG-CACACGCACAc 3' Nptx1
```

Figure 3 Neuronal Pentraxin1 (*Nptx1*) gene as a direct target of miR-210

(A) Western blot analysis of HIF1α, VEGF and Nptx1 expression in the cerebellum of 17-week old *Hexb*$^{+/+}$ and *Hexb*$^{-/-}$ mice. GAPDH was used as a loading control. (B) Densitometric quantification of HIF1α expression, performed as described in Experimental procedures, shows significant reduction of HIF1α expression in Sandhoff relative to wild-type mice. (C) A significant decrease of VEGF protein level was detected in Sandhoff compared to wild-type samples. Data are represented by the means ± SEM of three separate animals per group. **$P < 0.01$, *$P < 0.05$, unpaired t test.

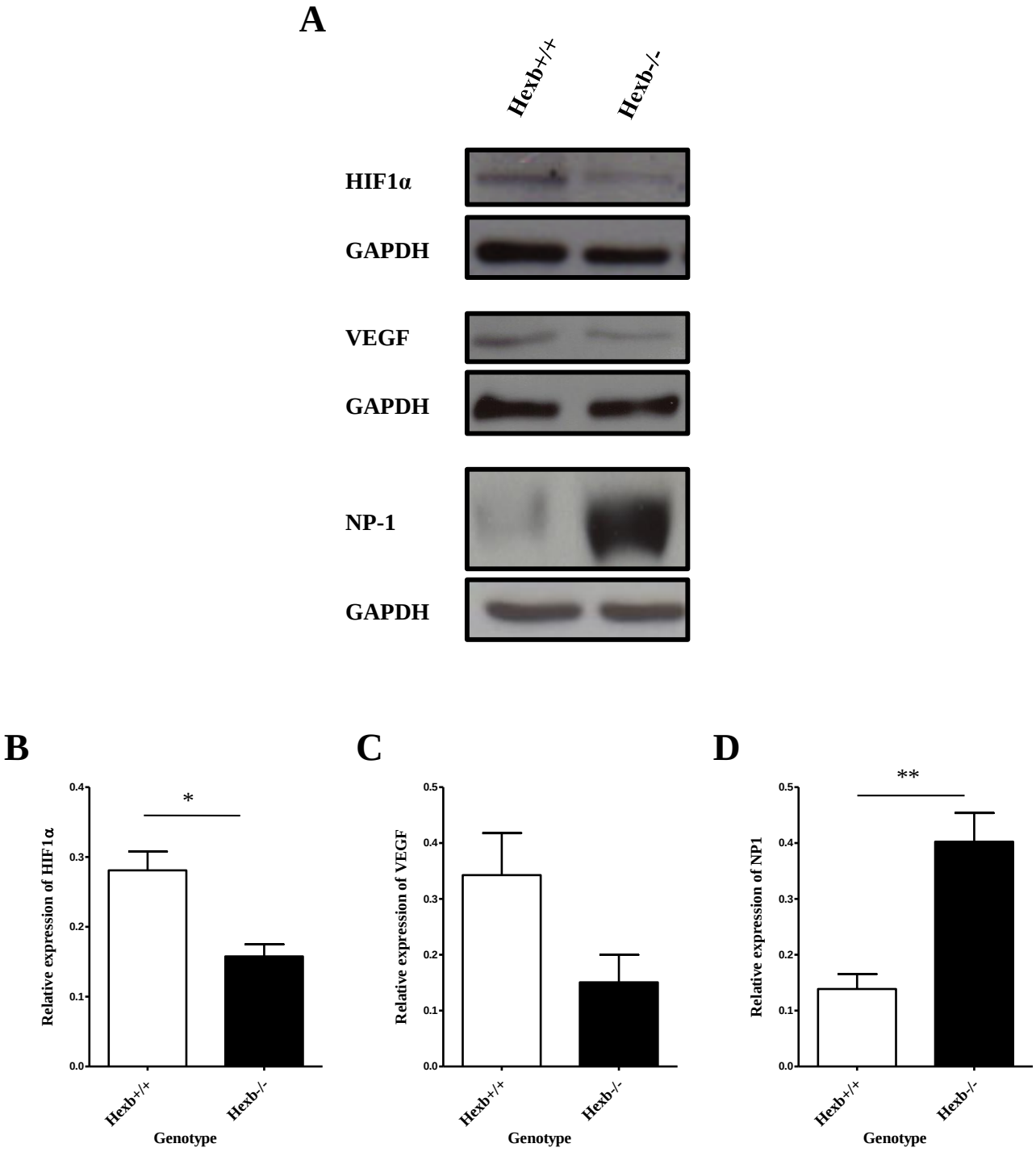
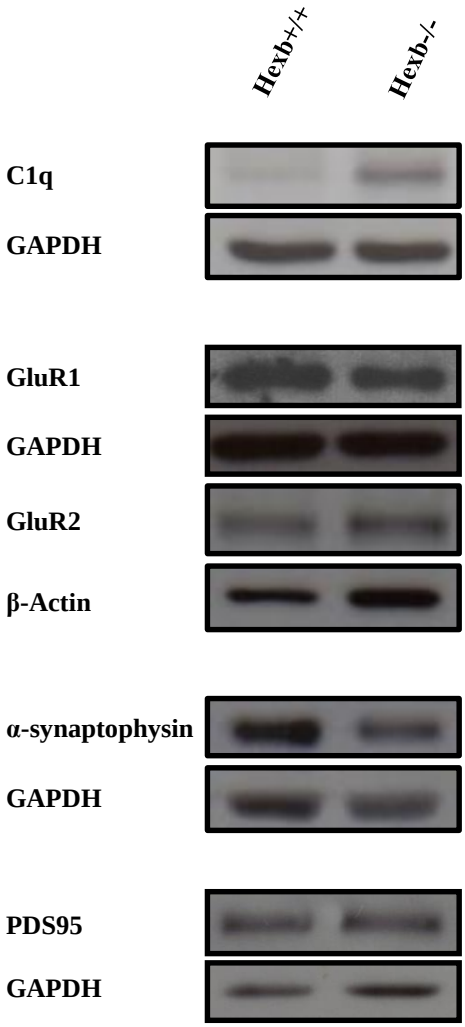

A
Hexb+/+
Hexb-/-
HIF1α
GAPDH
VEGF
GAPDH
NP-1
GAPDH
B
Relative expression of HIF1α
*
Hexb+/+
Hexb-/-
Genotype
C
Relative expression of VEGF
Hexb+/+
Hexb-/-
Genotype
D
Relative expression of NP1
**
Hexb+/+
Hexb-/-
Genotype

Figure 4 C1q, GluR1 and α-Synaptophysin involvement in Sandhoff disease pathology in mice

(A) Western blot analyses of C1q, GluR1, GluR2, α-Synaptophysin and PSD-95 in the cerebellum of wild-type and Sandhoff mice. (B) C1q protein expression was significantly increased in Sandhoff compared with wild-type mice. (C) A trend of decreased GluR1 expression in Sandhoff and double knock-out is observed compared to wild type mice. (D) The expression level of GluR2 was not changed in Sandhoff disease mice. (E) The protein level of α-Synaptophysin was significantly attenuated in Sandhoff cerebellum compared with wild-type samples. (F) A trend of increased PSD-95 protein levels was detected in Sandhoff compared with wild-type mice. The data represent the means ± SEM of three separate animals per group.*$P \leq 0.05$.

A


Hexb+/+
Hexb-/-
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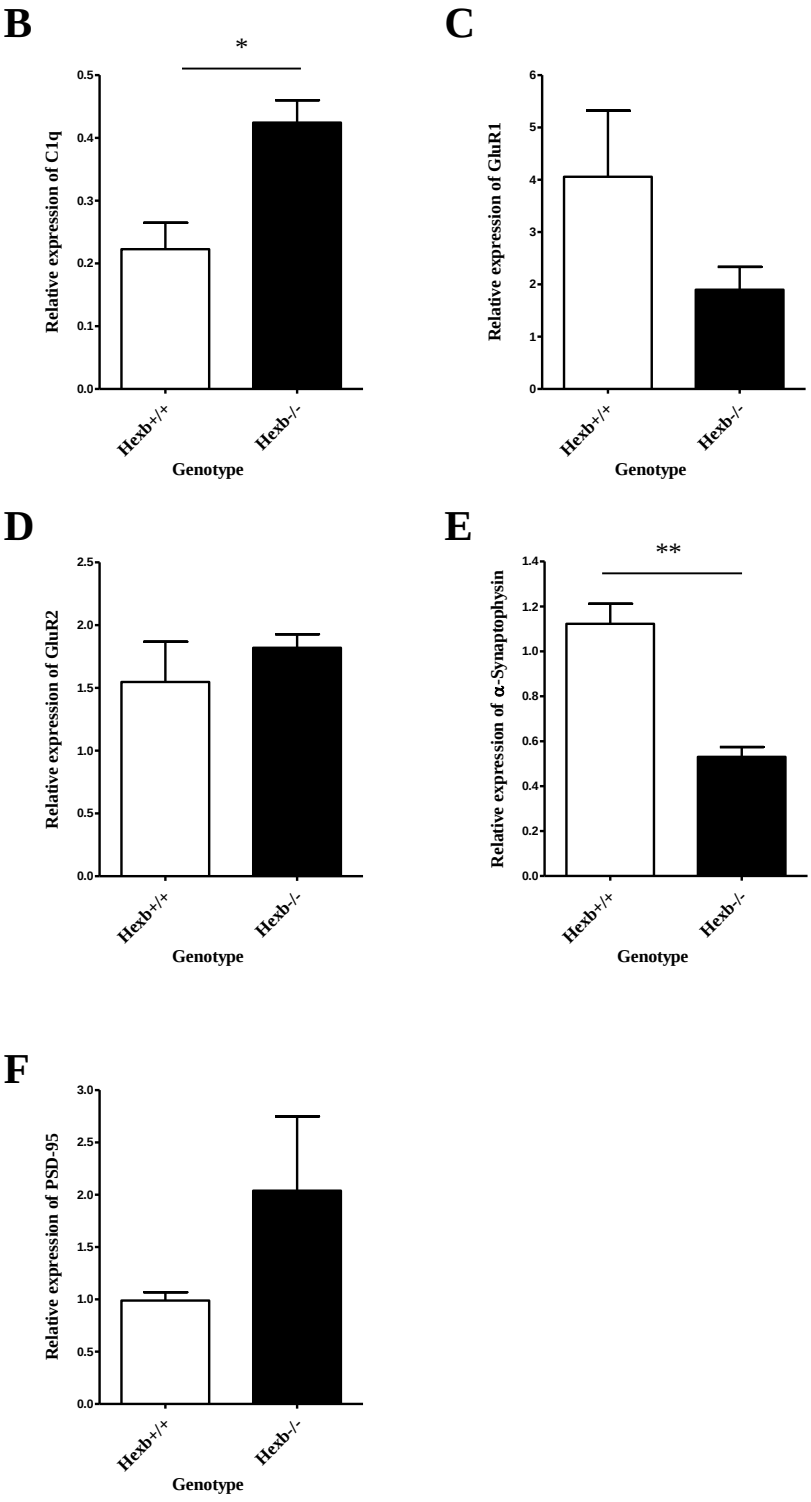
GAPDH
GluR1
GAPDH
GluR2
β-Actin
α-synaptophysin
GAPDH
PDS95
GAPDH


B
*
Relative expression of C1q
0.0
0.1
0.2
0.3
0.4
0.5
Hexb+/+
Hexb-/-
Genotype
C
Relative expression of GluR1
0
1
2
3
4
5
6
Hexb+/+
Hexb-/-
Genotype
D
Relative expression of GluR2
0.0
0.5
1.0
1.5
2.0
2.5
Hexb+/+
Hexb-/-
Genotype
E
**
Relative expression of α-Synaptophysin
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Hexb+/+
Hexb-/-
Genotype
F
Relative expression of PSD-95
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0.5
1.0
1.5
2.0
2.5
3.0
Hexb+/+
Hexb-/-
Genotype

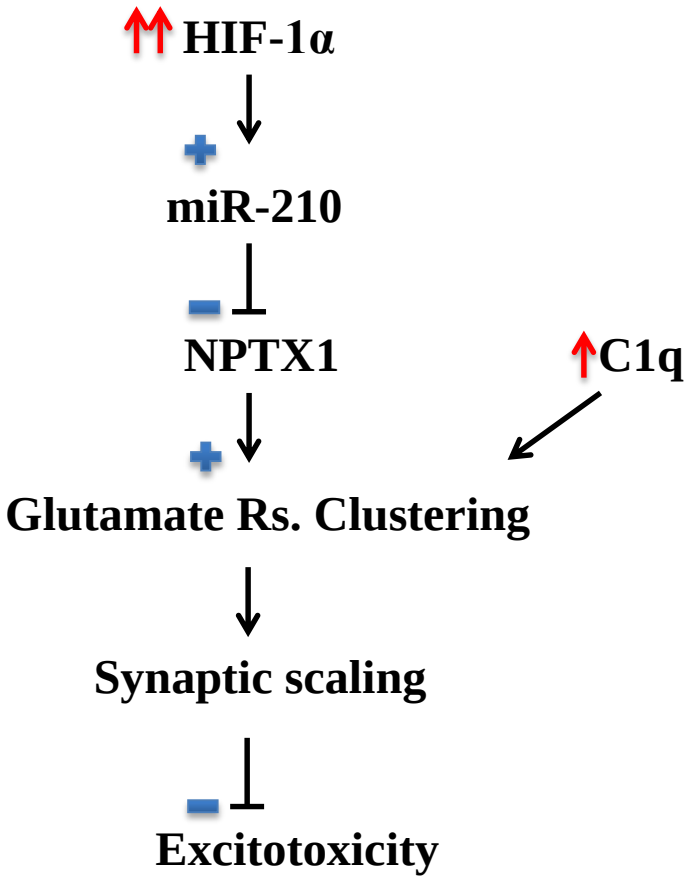


Figure 5 Schematic diagram of potential Nptx1 and C1q-mediated excitotoxicity in Sandhoff mice

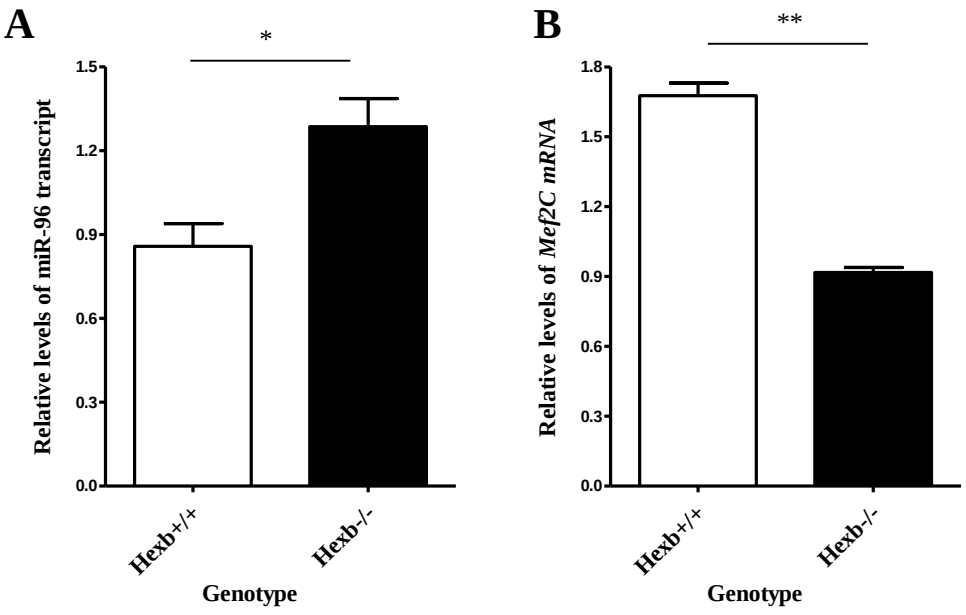
Proposed pathway through which synaptic scaling could be regulated by *Nptx1*and C1q proteins. Solid lines denote physical potential interactions that are supported by experimental evidence. Red arrows denote increased expression. (+) sign denotes upregulation or induction. (-) sign denotes down-regulation or inhibition.

4.3.5 Mef2c gene is a potential target for miRNA-96 in Sandhoff mice

Our expression results have shown that miRNA-96 was significantly up-regulated in Sandhoff compared to wild type brains (Figure 6A). Furthermore, we have shown that *Mef2c* gene was among the target gene list for miRNA-96. *Mef2c* mRNA expression significantly decreased in Sandhoff compared with wild-type mice (Figure 6B). Furthermore, we have found that *Mef2c* gene displayed 13 pairing motifs that were also identified in miR-96 sequence (sequence alignment is schematized in Figure 6C). In order to confirm that *Mef2c* is a putative target gene of miR-96 in Sandhoff mice, miRNA expression was assessed using quantitative RT-PCR (Figure 6D). We used western blotting to measure MEF2C protein expression in Sandhoff brains. We have detected a significant decrease in the expression level of MEF2C protein in Sandhoff brains (Figure 7A and B). Together, our data show that miRNA-96 regulates the expression of *Mef2c* gene. A potential model in which excitotoxicity is mediating synaptogenesis via miRNA-96 mediated MEF2C expression could be implicated in Sandhoff disease pathology (Figure 8).

Figure 6 The expression levels of miRNA-96 and *Mef2c* in the CNS of Sandhoff mice

(A) The expression level of miRNA-96 was significantly increased in the cerebrum of *Hexb*$^{-/-}$ mice compared with wild-type controls. **(B)** The expression level of Mef2c was significantly decreased in the CNS of *Hexb*$^{-/-}$ mice compared with wild-type controls.**(C)** miR-96/Mef2c Alignment provided by miRanda alignment web interface.*, and ** denote statistically significant difference with $P< 0.05$ and $P< 0.01$, respectively, n=3-5.



B

```
3' ucGUUUUUACACGAUCACGGUUu 5' mmu-miR-96
     |||||:    |   ||||||
5' gaCAAAAGGAAACAUUUGCCAAa 3' Mef2c
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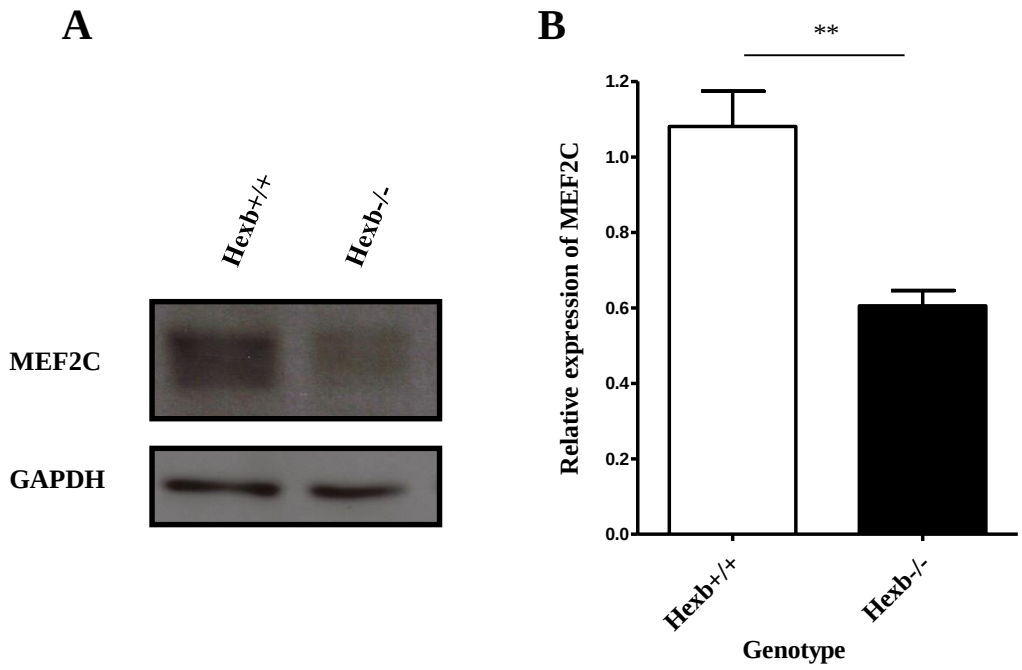
Figure 7 MEF2C protein involvements in Sandhoff disease pathology

(A) MEF2C protein expression in the cerebellum was evaluated in 17-week old *Hexb*$^{+/+}$ and *Hexb*$^{-/-}$mice by western blotting. GAPDH was used as a loading control. **(B)**Western blot analysis shows that MEF2C protein levels were significantly decreased in Sandhoff cerebellum compared with wild-type controls. The data represent the means ± SEM of three separate animals per group. ** denote statistically significant difference with $P< 0.01$.


A
Hexb+/+
Hexb-/-
MEF2C
GAPDH
B
**
Relative expression of MEF2C
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Hexb+/+
Hexb-/-
Genotype

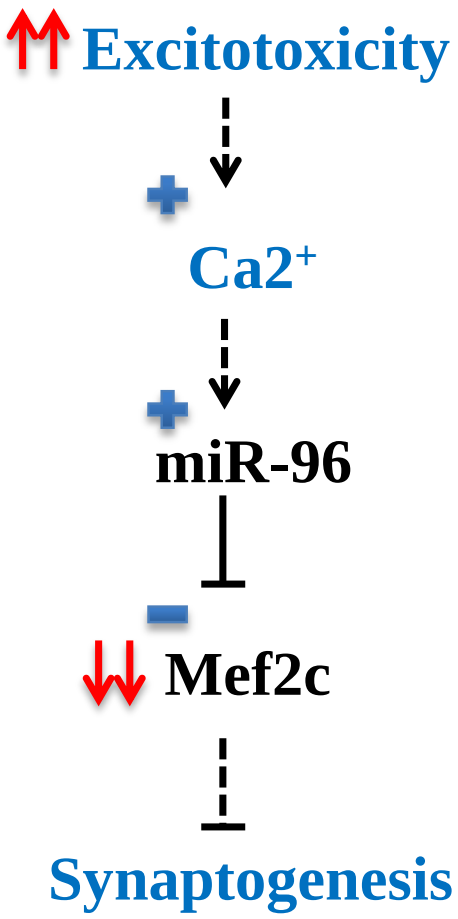


Figure 8 schematic diagrams depicting a potential involvement of miRNA-96 and MEF2C in synaptogenesis in Sandhoff disease pathology

Diagram shows cross-talk between increased intracellular calcium concentration and synaptogenesis mediated by miRNA-96 and its target, MEF2C protein. Solid lines denote physical potential interactions that are supported by experimental evidence. Red arrows denote increased expression. (+) sign denotes upregulation or induction. (-) sign denotes down-regulation or inhibition.

4.4 Discussion

The present study demonstrates dys-deregulated miRNA expression in Sandhoff mouse model. It is noteworthy that 70 percent of detectable human microRNAs are expressed in the brain to regulate several physiological processes such as synaptic formation, neuronal cell differentiation, and memory development (Santos-Reboucas & Pimentel, 2010). Dys-regulation of CNS microRNAs is thought to play a significant role in the development of neurodegenerative diseases including LSDs (Eacker, Dawson, & Dawson, 2009; Robberecht & Philips, 2013; Maes, Chertkow, Wang, & Schipper, 2009). However, despite the magnitude of data published on neurodegeneration, the question whether CNS microRNAs contribute to the pathogenesis of Sandhoff disease has not yet been investigated. Furthermore, identification of underlying molecular pathways associated with impairment microRNA machinery would provide attractive upstream candidates for proper diagnosis, prognosis and treatment of Sandhoff disease, possibly extended to other neurodegenerative disorders.

Gene results show that miRNA-210 and miRNA-96 were amongst 19 deregulated miRNAs in the CNS of symptomatic Sandhoff mice. These findings suggest that miRNA-210 and miRNA-96 possess relevant pathophysiological properties during neurodegeneration in Sandhoff mice. Consistent with this data, we found that both certain miRNAs might be implicated in the regulation of neurodegenerative conditions (Bak et al., 2008). Amongst these miRNAs, miRNA-210 was previously found having a regulatory role in the pathology of brain ischemic conditions (VanGilder, Huber, Rosen, & Barr, 2012). Furthermore, progressive hearing loss was found associated with

mutations affecting miRNA-96 (Wang et al., 2010), and neural induction of human embryonic stem cells (Du, Ma, Phillips, & Zhang, 2013). A major challenge to understand the dynamic mechanisms of microRNAs in the context of neurodegeneration is to identify the dys-regulated subset of miRNAs in corresponding to their target genes (Bak et al., 2008). Alzheimer's disease, progressive hearing loss and impairment of neural tube development are a few instances of neurodegenerative diseases where dys-regulated miRNA expression has been reported (Schonrock et al., 2010; Satoh, 2010; Boissonneault, Plante, Rivest, & Provost, 2009; Wang, Huang, Hu, Stromberg, & Nelson, 2011). For instance, expression of miRNA-300, miRNA-298, miRNA-210, miRNA-211 and miRNA-181c has been reported to be altered in Alzheimer's disease. Furthermore, miRNA-96 down-regulation has been associated with progressive hearing loss via targeting transmembrane channel-like 1 (Tmc1), protein tyrosine phosphatase, receptor type, Q (Ptprq) and growth factor independent 1 transcription repressor (Gfi1) genes(Lenz & Avraham, 2011).

We identified several genes, as potential targets for microRNA-210 in Sandhoff disease, participating in relevant neurodegenerative pathways such as; Neuronal pentraxin 1 (Nptx1), interleukin 6 (Il-6), (Yin, Lee, Cho, & Suk, 2009; Lee et al., 2009; Niyadurupola et al., 2013). Furthermore, we have detected a list of predicted genes, associated with synaptic scaling and neuro-excitotoxicity such as potassium voltage-gated channel (Kcna4) and AMPA2 (Gria2), which were correlated with the highly expressed microRNAs in Sandhoff samples (Modic, Ule, & Sibley, 2013).

It was demonstrated that neuronal stem cells (NSC), as an effect of hypoxic-ischemic insult, migrate to the lesion site and differentiate into mature neurons (Park et al., 2006). Furthermore, hypoxia was described as one of the most important factors causing neurodegeneration (Lindholm, Wootz, & Korhonen, 2006; Huang et al., 1997). It was established that miR-210 is a significant player of VEGF-mediated endothelial cell differentiation, hypoxia-associated cell response, and cell survival (Fasanaro et al., 2009). Furthermore, it was reported that HIF1α regulate miR210 in hypoxic pathologies (Ivan et al., 2008). Consistent with these reports, we identified that miR-210 is significantly down-regulated in Sandhoff brains compared to healthy controls. Importantly, we found also that HIF1α is down-regulated in Sandhoff mice brains. Recent in-vitro study has demonstrated that hypoxia induces neural pentraxin 1 expression via down-regulation of miRNA-210 (Pulkkinen, Malm, Turunen, Koistinaho, & Yla-Herttuala, 2008). Here, we have found that neuronal pentraxin 1 (Nptx1) expression was significantly induced in Sandhoff brains compared to wild-type. Together, these results indicate that Sandhoff brain hypoxia mediates a change in the expression level of miRNA-210, which in turn has a regulatory effect on the protein level of neural pentraxin in Sandhoff CNS.

It has been reported that miRNA-210 is involved in vascular endothelial growth factor (VEGF) biology (Liu et al., 2012). As a further step of validation, we found that VEGF protein shows a similar decrease pattern of HIF1α expression in Sandhoff brains. It is worth mentioning several studies reported that VEGF expression is controlled by cellular hypoxia (Slomiany, Black, Kibbey, Day, & Rosenzweig, 2006). In the last years, it has been shown that over-expression of neuronal pentraxin 1 regulates neuro-

excitotoxicity via modulating glutamate receptors (Hossain, Russell, O'Brien, & Laterra, 2004). Although our study was not designed to bring a comprehensive biological picture of microRNA-210, we decided to examine the expression levels of excitotoxicity-related proteins in Sandhoff pathology. It was reported that C1q binds to pentraxin receptors at the post-synaptic membrane. This in turn, results in complement activation and hence microglial phagocytosis of the synapse (Perry & O'Connor, 2008). This mechanism of synaptic scaling and refinement was found dependent on the ability of pentraxin to stabilize glutamatergic transmission (Perry & O'Connor, 2008). We found a significant increase in the expression of C1q in Sandhoff cerebellum suggesting a possible role of complement activation in Sandhoff pathology. It was reported that synaptic markers, including α-Synaptophysin, is decreased in patients with neurodegenerative disorders (Kim, Rapoport, & Rao, 2010). Our results revealed that the protein level of α-Synaptophysin was significantly decreased in Sandhoff mice. Therefore, we hypothesized a model in which excitotoxicity and synaptic scaling are mediated through a number of biomarkers such as neuronal pentraxin and C1q. All together, our results provide strong evidence that excitotoxic effects of Nptx1 might be regulated by miRNA-210 and C1q.

Recent studies have shown that myocyte enhancer factor 2C (MEF2C) is a key player in neurogenesis and neuronal cell differentiation (Li et al., 2008). Interestingly, our microarray experiment (data not shown) has demonstrated that MEF2C is down-regulated in Sandhoff brains compared to healthy controls. It was reported that intracellular calcium flux is associated with the expression level of miR-96 in platelets (Kondkar et al., 2010). Furthermore, it was reported that deletion of MEF2C transcription factor leads to

negative regulation of synaptogenesis in mice (Barbosa et al., 2008). We addressed whether MEF2C is regulated by certain microRNA from those previously shown as significantly changed in Sandhoff brain. We found that MEF2C is a putative target for miR-96. Furthermore, we found decreased MEF2C expression in Sandhoff brains. It was reported that miRNA is expressed preferentially in auditory hair cells (Li et al., 2008). It is noteworthy that Sandhoff infants display hearing loss, as the disease progresses. Moreover, Genome-wide study has revealed that Mef2c regulates the expression of several genes including, protocadherins 9, 10 and 17 that control multiple synaptic functions such as excitatory synaptic weakening and maturation (Flavell et al., 2008). Indeed, our data points at MEF2C, as a relevant candidate of miRNA-96 that, might be implicated in Sandhoff pathology

In conclusion, microRNA machinery in the CNS might provide a fine tuning tool to the underlying mechanisms of neurodegeneration. Hence, this would provide upstream targets for new treatments to modify and ameliorate the progressive nature of neurodegenerative disorders. Our study identified a novel subset of microRNAs that are dys-regulated in cerebrum of Sandhoff mice. Many of these dys-regulated microRNAs, such as miR-210 and miR-96, are implicated in essential biological process required for normal brain function. To our knowledge, this is the first report providing a biologically relevant microRNA profiling for Sandhoff CNS pathology.

4.5 Materials and Methods

Mice

Sandhoff disease model mice (Hexb-/-) on a C57B/6 background. The genotype of the Hexb locus was determined by PCR from tail.

Total RNA isolation and MicroRNA array

Three to five *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice brains at 15 week-old were homogenized in Trizol reagent (Invitrogen) and total RNA prepared according to the recommended protocol. Total RNA was purified by using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Total RNA samples were labeled ("A" for wild type, "B" for Hexb-/- and "C" for *Hexb*$^{-/-}$*Tnfα*$^{-/-}$ group) and sent to UHN Microarray Centre facility (http://www.microarrays.ca/) for microarray analysis. The RNA quality was determined by nanodrop assessment to A260/280 values of 2.0 to 2.1. Two hundred nanograms of your 12 RNA samples were label following Illumina microRNA Assay kit using Mouse microRNA panel V2.0. The labeled samples were then hybridized to a Universal -12 BeadChip. The BeadChip was incubated at 45°C, for 18.0 hrs for hybridization. The BeadChip was then washed and stained as per Illumina protocol and scanned on the iScan (Illumina) at UHN Microarray Center facility. The data files were quantified in BeadStudio Version 3.3.8 (Illumina).

MicroRNA target prediction and functional analysis

Predicted mRNA targets for the significantly differentially enriched microRNAs were identified using Pictar. Additional target prediction algorithm miRanda was utilized

to confirm and complete target gene predictions. The predicted microRNA patterns were listed in tables 1.

SDS-PAGE and Western Blotting analysis

Cerebella were dissected from 17-week old *Hexb*$^{+/+}$*Tnfα*$^{+/+}$ and *Hexb*$^{-/-}$*Tnfα*$^{+/+}$ mice and snap frozen on liquid nitrogen. Frozen brain tissues were homogenized in homogenizing RIPA buffer containing protease inhibitors (Complete mini, Roche, 04693124001). Samples were centrifuged at 14,600 xg for 5 minutes at 4°C and the supernatant was kept for western blot analysis. Protein concentrations were estimated using the DC protein assay (BioRad, 500-0111). Samples were mixed with 6x Laemmle sample buffer and boiled for 10 min before being separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and subsequently blocked with 5% non-fat milk in TBS-Tween for 1 hour before probing with various antibodies. Primary antibodies were incubated with the nitrocellulose membrane in blocking solution overnight at 4°C with rocking. The following day, blots were washed with TBS-Tween and incubated with peroxidase-conjugated secondary antibody for 1 hour. Signal was detected using Amersham ECL detection reagents and hyperfilm (GE healthcare, RPN2106 and 28906839). Protein levels were quantified using integrated band density estimated by ImageJ software. The following antibodies were used in this study: Rabbit monoclonal anti-MEF2C (Cell Signaling, 5030), mouse monoclonal anti-NP-1 (BD Transduction Laboratory, 610369), rabbit polyclonal anti-HIF-1α (Santa-Cruz, SC10790). Statistical comparisons were made by Student's t test. $P < 0.05$ was considered significant.

Quantitative Real-Time PCR

SYBR green assays were performed with SYBR green PCR master mix (Applied Biosystems, 4309155) using cDNA obtained as described above. Primers were as follows:

Nptx1 forward 5'-ACAGCCGCCTCAATTCTTCC-3',

Reverse 5'GCTCTCGATCTTGGCCCTTT-3',

Samples, master mix and primers were added to a MicroAmp Optical 96-well Reaction plate (Applied Biosystems, 4316813). The plate was spun down quickly to remove bubbles and placed in the 7900HT Sequence Detection System (Applied Biosystems). Thermocycling conditions were as follows: 50°C, 2 min, 95°C , 10 min, 40 cycles of 95°C, 15s; 60°C, 1 min. SDS (v.2.3) (SABbiosciences) was used to analyze all gene expression data. Statistical comparisons were made by Student's t test. $P < 0.05$ was considered significant.

MicroRNA Isolation and TaqMan Gene Expression Assays

Total RNA from cerebellar tissues of five to seven, 17 week-old $Hexb^{+/+}Tnf\alpha^{+/+}$ and $Hexb^{-/-}Tnf\alpha^{+/+}$ mice were homogenized in Trizol reagent (Invitrogen) and total RNA prepared according to the recommended protocol. Then, total RNA was purified using Purelink RNA Isolation Kit (Ambion, 12183018A). RNA was reverse transcribed with specific RT primers for miR-210 and miR-96 according to the Superscript III protocol (Invitrogen Superscript III, 18080-093). We used a thermocycler program (16° 30 min, 42° 30 min, 85° 5 min). TaqMan assays were performed with TaqMan gene expression

master mix (Applied Biosystems, 4370048) using cDNA obtained as described above. cDNA was mixed with master mix, and gene specific probes and added to a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, 4316813). The plate was spun down quickly to remove bubbles and placed in the 7900HT Sequence Detection System (Applied Biosystems). Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 60°C for 1 min. A standard curve was used and final values are expressed as relative gene of interest divided by the miRNA assay control, snoRNA202 (Applied Biosystems, Cat. # 4427975, control sequence):

5'-GCTGTACTGACTTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG-3'

mmu-miRNA-210 (Applied Biosystems, Cat. # 4427012, mature miRNA sequence: AGCCACUGCCCACCGCACACUG)

Statistical Analysis

Samples were grouped into A and B at UHN Microarray Centre facility (http://www.microarrays.ca/). Expression filter was applied and probes which at least 2 out of 9 samples have values between 20 and 100th percentile in the raw data were kept for further analysis. Unpaired t-test was performed between the two groups, and Benjamini & Hochberg FDR method was used for multiple testing correction. There was no significant result using corrected $P < 0.05$ as cut-off. Unpaired t-test was performed between the two groups with no multiple testing correction. Heatmap result list with $P < 0.05$ as cut-off and the probes with fold change > 2 were marked red.

Chapter 5

Summary and Conclusions

The Sandhoff disease mouse model is considered an ideal prototype of a sphingolipidosis pathology that ultimately leads to neurodegeneration (Wada, Tifft, & Proia, 2000; Akiyama et al., 2000). In Sandhoff mice, glycolipid storage is detectable in multiple CNS tissues, including cerebrum, cerebellum and spinal cord (Norflus et al., 1998; Wu, Mizugishi, Bektas, Sandhoff, & Proia, 2008). Whether a neurological improvement of Sandhoff pathology requires a detectable general or localized correction of glycolipid storage in CNS region is still not clear. Our results showed that neurological improvement of the double knock-out mice. This might be referred to as attenuated storage of glycosphingolipid (GSL) gangliotriaosylceramide (GA2), and monosialoganglioside (GM2) in the CNS(Lee et al., 2007).It is expected that a critical threshold of GM2 and GA2 storage is the driving force behind neuronal apoptosis(Teng et al., 2002;Lee et al., 2007). However, it was demonstrated that neurological amelioration has been achieved via reduction of the primary injury of neuronal storage (Jeyakumar et al., 2001). Furthermore, it was shown that suppression of neurological deterioration without detectable reduction of GM_2 can be achieved in Sandhoff brain cells (Andersson et al., 2004). Glycolipid pattern analysis revealed there is no decline in the storage level in the CNS of double knock mice. We have tested both the technical loading and the degradative pathway by comparing the storage with cerebrosides/sulphatides and GM1, respectively. Our observations clearly suggest that the improvement of clinical

manifestations in DKO mice is not associated with reduction of glycolipid storage (Chapter 3). Hence, it is most probably a consequence of downstream mechanistic pathway of GM2/GA2 accumulation.

Astrocytes, though previously underappreciated and of a role not greatly comprehended, have an essential role in the pathogenesis of LSDs (Di Malta, Fryer, Settembre, & Ballabio, 2012). Astrocytes have been found actively participating in modulating neuronal networking, releasing neurotransmitters and expressing cytokines and transporters (Belanger & Magistretti, 2009). Recent studies uncovered important mechanisms underlying astrogliosis suggesting that proinflammatory cytokines such as TNFα can trigger and modulate astrogliosis (Persidsky et al., 2001). Furthermore, it was reported that microinjected TNFα to the neonatal brain can elicit extensive GFAP immunoreactivity (Balasingam, Tejada-Berges, Wright, Bouckova, & Yong, 1994). Although neurodegeneration is a cardinal hallmark in Sandhoff pathology (Wu & Proia, 2004), the relevant role of astrocytes, in the absence of TNFα, is still unknown. We examined the GFAP expression in the spinal cord of Sandhoff and DKO mice. By comparison, astrogliosis appeared to be significantly reduced in the spinal cord of the double null mice. The same trend was observed in the cerebral cortex and cerebellum. Moreover, astrocytes were highly activated in the thoracic region of spinal cords in Sandhoff mice. Our results displayed a similar trend in cervical and lumbar regions suggesting that TNFα is involved in activation of astrocytes in the spine. Collectively, it appeared that the absence of *Tnfα* gene in Sandhoff mice is mediating a reduction of

astrogliosis in the spinal cord which is ultimately associated with alleviated neuronal manifestations (Chapter 3).

Activated microglia has been shown to be involved in the neuronal cell death progression of GM2 gangliosidosis including Sandhoff disease (Myerowitz et al., 2002).Microglia, the main TNFα secreting cell in CNS, could have deleterious and/or protective dramatic effects on neurons in the course of a neurodegenerative disease (Minghetti, 2005). An extensive expansion in the activated microglia in the brain of Sandhoff mice was observed prior to the massive end apoptosis (Wada et al., 2000). Moreover, it was reported that microglia, via TNFα-signaling pathway, can mediate neuronal cell death (Lucas, Rothwell, & Gibson, 2006). Interestingly, it was demonstrated that neuro-microglial crosstalk can determine whether microglia play a beneficial or deleterious role in disease pathology (Polazzi & Contestabile, 2002). Although, we previously reported the presence of a significant astrocytic role in Sandhoff pathology, we cannot exclude the possibility that other CNS cells such as microglia could modulate neurodegeneration in the lack of TNFα. Hence, it is possible that, in the absence of *Tnfα* gene, Sandhoff neurodegeneration is inflicted by microglia. To investigate whether microglia have deleterious or beneficial role, we examined MAC3 immunostaining in the spinal cord, cerebrum and cerebellum. We have found lower MAC3-postive cells in the cerebral cortex of DKO mice. Our data demonstrated that TNFα may be participating in enhancing the recruitment and/or differentiation of precursors to microglia/macrophages, in an autocrine/paracrine fashion, to sites of pathology in Sandhoff disease mice.

Neuroinflammation is one of the hallmarks of Sandhoff disease pathology (Wada et al., 2000). A recent report has uncovered some of the processes such as progressive gliosis and secretion of inflammatory molecules, underlying neuroinflammation in Sandhoff disease (Wu & Proia, 2004). These findings led us to examine the hypothesis that reduced inflammatory markers upon deletion of TNFα in Sandhoff mice are underlying mechanisms of clinical improvement. Upon testing inflammatory markers, we have shown that TNFα deficient Sandhoff mice have diminished levels of GFAP in CNS. However, TNFα and IL-6 were found in many neuropathological conditions as co-released in response to inflammatory events (Carrasco, Hernandez, Bluethmann, & Hidalgo, 1998). Both cytokines have several converging characteristics that would imply that deficiency of one mediator may be compensated by the presence of the other. Can a compensatory mechanism release IL-6 upon lacking TNFα in Sandhoff CNS? To answer this question we used western blot analysis and clearly showed IL-6 levels were not increased in cerebellum as a response of TNFα deficiency. In agreement with our previous immunostaining results, we found the microglial inflammatory marker; MAC3was increased in Sandhoff mice. Furthermore, we found a trend of substantial decrease in the expression of MAC3 in double knock-out mice. Altogether, these results might indicate that TNFα exerts its neurodegenerative effect on Sandhoff neuropathology by increasing levels of GFAP and MAC3 in the CNS and ultimately inducing excessive gliosis.

Although apoptosis is a physiological process by which the body maintains normal cell turnover, excessive apoptosis leads to pathology (Elmore, 2007). The end

stage of Sandhoff disease is characterized by progressive neuronal death as well as excessive glial cell activation (Jana, Hogan, & Pahan, 2009). We investigated if deletion of TNFα in Sandhoff mice affects neuronal cell death. We detected an obvious trend of reduced apoptotic cells in DKO mice. A striking observation was that extensive apoptosis process was occurring in the spines of Sandhoff mice; the same region showed increased activated astrocytes. The results raise the possibility that astrocytes residing in the spinal cord might play an important role in the apoptosis-mediated pathogenesis of Sandhoff disease. Thus, our data suggest that TNFα molecule has a neurodegenerative role in Sandhoff disease pathology. These results point to the cytokine, TNFα, as a potential therapeutic target to slow the rapid neurodegenerative process in the neurodegenerative disorder (Chapter 3, Figure 8).

Tumor necrosis factor-alpha (TNFα) has been demonstrated as a key modulator of Sandhoff pathology (Jeyakumar et al., 2004). A variable net effect of pleotrophic molecule such as TNFα on the pathogenic process may be attributed to the profile of glia-cell distribution and their inflammatory mediators. In this study we have unequivocally reported Sandhoff mice without TNFα showed ameliorated disease course. Furthermore, we demonstrated that TNFα deficient Sandhoff mice show reduced levels of neural cell death and decreased levels of astrogliosis and microgliosis. Our results reveal TNFα as a broadly neurodegernative cytokine in the progression of Sandhoff Disease and as a potential therapeutic target to attenuate neuro-pathogenesis. However, it will be important to determine the exact molecular mechanism or mechanisms underlying this clinical improvement in Sandhoff mice upon TNFα deletion.

There is convincing evidence that TNFα binding to its receptors can trigger many downstream signaling pathways such as NF-kB (Wajant, Pfizenmaier, & Scheurich, 2003). It has been demonstrated that TNFα-mediated NF-kB activation in microglia can be detrimental to neuronal cell survival via release of excitotoxin and oxygen free radicals (Kettenmann, 2007). Moreover, NF-κB genes can be activated via canonical and non-canonical pathways (Sun, 2011).Unlike in canonical NF-kB pathway of NF-kB activation which relies on IκBα activation, non-canonical pathway relies on inducible processing of P100 (Sun, 2011). However, the exact TNFα-mediated NF-kB pathway that affect neurodegeneration in Sandhoff disease remains to be firmly established. We evaluated the phosphorylation and expression levels of the molecular players of non-canonical NF-kB pathway in TNFα-deficient Sandhoff mice. Our results demonstrated that TRAF3 was down-regulated in the CNS of Sandhoff mice upon deletion of TNFα. It was reported that adaptor proteins; TRAF2 and TRAF3, recruit IKK complex to TNFα receptor signaling platform and hence enabling RIP to activate downstream cascade of kinases (Wajant et al., 2003) . Therefore, we measured NIK and RIP expression in the CNS of double knock-out mice. It is noteworthy that NIK may directly contribute to activation of deleterious NF-κB mediated genes (Sun, 2011).In our study, although NIK expression was not reduced significantly in double knock-out mice, RelB expression was significantly down-regulated. Taken together, it appears that non-canonical pathway of NF-kB pathway is abrogated in TNFα-lacking Sandhoff mice. These findings clearly suggest a novel role of TNFα in the activation of NF-kB transcriptional activity in Sandhoff mice.

Sandhoff disease shares many commonalities, such as astrogliosis, with other neurodegenerative disorders (Igdoura, Mertineit, Trasler, & Gravel, 1999). Recent studies uncovered important mechanisms underlying astrogliosis suggest that proinflammatory cytokines such as TNFα can trigger and modulate astrogliosis (Jeyakumar, Dwek, Butters, & Platt, 2005; Culmsee& Plesnila, 2006). It was demonstrated that astrogliosis was almost completely inhibited in mice deficient in both TNFα receptors (Sriram et al., 2006). Moreover, TNFα was reported to up-regulate GFAP in-vitro (Zhang et al., 2000).Astrocytes are considered the most abundant cell type in the CNS (Sofroniew & Vinters, 2010). There is little known about the signal transduction pathways that participate in their activation and proliferation in Sandhoff disease. It is noteworthy that phosphorylation of STAT3 associated with toxic gliosis was demonstrated in spinal cord injury (Shishodia & Aggarwal, 2002). The mechanisms for the regulatory role of TNFα on STAT3-mediated astrogliosis in Sandhoff disease remains to be studied. In this study, we extend our previous finding that astrogliosis was diminished greatly in the CNS of TNFα-lacking Sandhoff mice and demonstrate a significant reduction of STAT3 phosphorylation in double knock-out mice. It was demonstrated that IL-6 is considered a potent STAT3 activator (Sriram et al., 2002). However, we provide evidence that IL-6 is not the relevant upstream STAT3 activator in Sandhoff context. Overall, these observations suggest the hypothesis that TNFα-mediated STAT3 activation plays a role in regulating GFAP in Sandhoff brains. Furthermore, recent work has demonstrated that TNFR1 associates with JAK2 constitutively to form a complex and ultimately this complex activates STAT3 pathway (Guo, Dunbar, Yang, Pfeffer, & Donner, 1998;

Pincheira, Castro, Ozes, Idumalla, & Donner, 2008). These findings may explain the increased phosphorylation of STAT3 in Sandhoff brains compared to TNFα-deficient samples. It is likely that targeting STAT3 pathway might achieve a beneficial outcome on disease pathology.

The massive expansion of activated immune cells in brains of Sandhoff mice has been interpreted to indicate that neuroinflammation is a cardinal sign in Sandhoff disease pathology (Wada, Tifft, & Proia, 2000). Furthermore, it was demonstrated that utilizing anti-inflammatory therapeutics in Sandhoff disease mice resulted in significant decrease of immune cell infiltration into mice brains (Vitner et al., 2010). The exact mechanism for aggravating CNS inflammation in Sandhoff mice remains unclear but may require the peripheral blood immune cell recruitment into brain parenchyma of Sandhoff mice. Interestingly, a recent study has shown that improved neuronal condition of Sandhoff mice on the deletion of CCR2 chemo-attractant receptor (Wu & Proia, 2004; Kyrkanides et al., 2008). Moreover, Kyrkanides et al. reported that TNFα secretion was greatly reduced after restoring HEXB enzyme in Sandhoff mice brains (Kyrkanides et al., 2008). These studies suggest that lipid accumulation in Sandhoff mice is associated with macrophage and microglia proliferation which might be partly interpreted as a result of peripheral monocyte infiltration into Sandhoff brains (Wu & Proia, 2004). In our previous study, we have demonstrated that TNFα plays an important neurodegenerative role in Sandhoff pathology in mice (see Chapter 1). Furthermore, it was demonstrated that lymphocytes and natural killer cells displayed impaired immune function in lysosomal storage disorders (Castaneda et al., 2008; Zhou et al., 2004). Although the previous

studies present salient evidence that peripheral immune cells are involved in Sandhoff pathology, there are still many gaps to be filled. We studied whether CNS-derived TNFα or Blood-derived TNFα plays the major role in Sandhoff neuropathology.

First of all, we found reduced immune cell frequency, including $CD3\varepsilon^{+}CD4^{+}$ T helper cells, $CD11b^{+}Gr\text{-}1^{neg\text{-}lo}$ monocytes, and $CD11b^{+}Gr\text{-}1^{hi}$ neutrophils, in the brain of TNFα-lacking Sandhoff mice (Chapter 3, Figure 1). These results are consistent with previous studies that showed TNFα induced innate immune response (Bhat & Steinman, 2009). Furthermore, we observed a significant reduction of neutrophils frequency in TNFα-lacking Sandhoff mice. These results thus implicate TNFα. Several studies suggest a role of toll-like receptors (TLRs) in production of inflammatory cytokines, including TNFα and IL-6 from activated immune cells (Jung et al., 2005). Interestingly, it was found that TLRs are expressed in a wide array of immune and brain cells, including monocytes and microglia (Leulier & Lemaitre, 2008; Olson & Miller, 2004). On stimulating bone marrow derived TNFα-deficient Sandhoff macrophages with LPS, we demonstrated significant reduction of IL-1β secretion levels. Interestingly, we found that GM2 did not induce cytokine secretion in BMDMs (Chapter 3, Figure 2). Moreover, it was found that transplantation of normal bone marrow cells into affected mice has a beneficial effect on Sandhoff disease pathology (Norflus et al., 1998). In the same previous study, the authors found that he improved neuronal condition was associated with reduced inflammatory markets in the brains of recipient mice (Norflus et al., 1998). In addition, another study demonstrated that BMT ameliorated Sandhoff disease pathology and prolonged life span of recipient mice (Jeyakumar et al., 2001; Norflus et

al., 1998). We have conducted bone marrow transplantation experiments to identify whether blood derived TNFα or CNS-derived TNFα is more detrimental in Sandhoff pathology. We found that CNS derived TNFα has a greater effect on Sandhoff pathology (see Table 1).

After behavioral tests of bone marrow transplanted animals, we determined that BM-derived TNFα contributed to neurodegeneration in Sandhoff disease mice, although CNS-derived TNFα had a greater effect on disease pathogenesis. These findings indicate that inhibiting TNFα signaling in macrophages, and their closely related CNS-resident microglia, is a viable target to slow the progression of Sandhoff disease. Furthermore, we found that recipient mice lacking both CNS-derived and blood-derived TNFα outperformed Sandhoff recipients, regardless of the genotype of the donor bone marrow cells. Taken together, these findings support a simple model in which CNS-resident microglia are more implicated in the secretion of TNFα in brain than the blood-derived microglia (see Chapter 3).

To develop our understanding of the neuro-pathology of Sandhoff disease, the immediate goal of this research project was to detect impaired microRNAs specifically expressed in Sandhoff mice brains. Neuronal cell and synaptic development were found regulated by a wide array of normally CNS expressed microRNAs (Santos-Reboucas & Pimentel, 2010). In particular lysosomal storage diseases (LSDs), massive neuronal apoptosis occurs in association of impaired brain microRNAs (Eacker, Dawson, and Dawson 837-41). Taken together, these studies proposed the hypothesis that dys-regulation of CNS microRNAs is implicated in the pathogenesis of Sandhoff disease. As

described in Chapter 4, microRNAs array of Sandhoff mice brains was exploited to identify the expression pattern of microRNAs associated with Sandhoff disease pathology. Importantly, the expression pattern of microRNAs revealed a number of microRNA gene targets including, miR-210 and miR-96, demonstrating significant expression level change in Sandhoff cerebrum. The expression of microRNAs in Sandhoff brains supports the hypothesis that microRNAs regulate neuronal cell function in Sandhoff mice. Upon functional analysis of microRNA targets, we have identified a short list of putative genes, namely; neuronal pentraxin 1(*Nptx1*) and myocyte enhancer factor 2 (Mef2c), as immediate targets for miRNA-210 and miRNA-96, respectively. Furthermore, we have detected a number of predicted genes, related to CNS excitotoxicity, synaptic formation and neuronal development, as a direct target of dys-regulated microRNAs in Sandhoff pathology. Future experiments deciphering the role of these genes expressed in Sandhoff brain detected in this study will be extremely valuable for characterizing the mechanisms that control neuronal cell function in Sandhoff pathology.

The expression level of neuronal pentraxin 1 (*Nptx1*) gene was up-regulated in Sandhoff brains (see Chapter 4). Moreover, we identified *Nptx1*, as putative targets of miR-210 which was down-regulated in Sandhoff brains. Early studies showed that miR-210 is implicated in several physiological cell responses such as hypoxia-associated cell response (Fasanaro et al., 2009). Furthermore, a study by Ivan et al. demonstrated that HIF1α control the expression of miR210 in hypoxic conditions (Ivan et al., 2008). Based on these studies and results, we suggested the hypothesis that Sandhoff brain HIF1α

regulates the expression of miR-210. Interestingly, we have detected significant down-regulation of HIF1α in Sandhoff brains. Together we suggested a model in which HIF1α regulate miR-210, which ultimately it down-regulate the expression level of neuronal pentraxin (see Figure 5, Chapter 4). Consistent with this model, the expression level of C1q was significantly up-regulated in Sandhoff brains. C1q molecule has been reported as an important player in complement activation and synaptic scaling synapse (Perry & O'Connor, 2008). The same study proposed a model in which C1q binds to pentraxin receptors at post-synaptic regions resulting in synaptic phagocytosis by activated microglia synapse (Perry & O'Connor, 2008). With the identification of these novel Sandhoff disease markers such as neuronal pentraxin and HIF1α, it may be useful to use the expression level of these genes to determine the neurological pathology in Sandhoff brains. Other synaptic markers, such as α-synaptophysin, were found decreased in neurodegeneration (Kim et al., 2010).

miR-96 was among the 14 up-regulated miRNAs in Sandhoff brains. As demonstrated in chapter 4, we identified myocyte enhancer factor 2 (*Mef2c*) as a novel putative target gene for miR-96 in Sandhoff pathology. A decrease in miR-96 expression was found associated with progressive hearing loss (Lenz & Avraham, 2011). miR-96 was found, in the same study, mediating its associated pathology through its effect on expression of targeting transmembrane channel-like 1 (Tmc1) and growth factor independent 1 transcription repressor (Gfi1) genes(Lenz & Avraham, 2011). In addition, expression of *Mef2c* gene was significantly down-regulated in Sandhoff brains (Figure 6 and 7, Chapter 4). Interestingly, a study by Wang et al. found that progressive hearing

loss was associated with mutations affecting miRNA-96 (Wang et al., 2010). These experiments points to the essential role of miR-96 and its impact on the regulation of *Mef2c*gene in Sandhoff disease pathology.

In summary, this body of work shows that TNFα as an important bio-marker in Sandhoff disease pathology. Importantly, we demonstrated that TNFα induces neurodegeneration in one of the accurate prototypes of neurodegenerative disorders, Sandhoff disease. This discovery has led to a number of significant downstream works addressing the exact molecular mechanism by which TNFα exerts its bioactivity in Sandhoff pathology. Some topics remain to be addressed with respect to the upstream and downstream molecular pathways of TNFα. Moreover, it is essential to exploit these studies by investigating the utilization of FDA approved anti-TNFα therapeutics on Sandhoff mice. Indeed, a promising avenue to improve the clinical picture and quality of life for patients with lysosomal storage disorders, essentially Sandhoff disease, would be a direct consequence of this study.

Reference List

Abo-Ouf, H., Hooper, A. W., White, E. J., van Rensburg, H. J., Trigatti, B. L., & Igdoura, S. A. (2013). Deletion of tumor necrosis factor-alpha ameliorates neurodegeneration in Sandhoff disease mice. Hum Mol Genet.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M. et al. (2000). Inflammation and Alzheimer's disease. Neurobiol.Aging, 21, 383-421.

Altar, C. A., Vawter, M. P., & Ginsberg, S. D. (2009). Target identification for CNS diseases by transcriptional profiling. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 34, 18-54.

Axtell, R. C., de Jong, B. A., Boniface, K., van der Voort, L. F., Bhat, R., De, S. P. et al. (2010). T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. Nat.Med., 16, 406-412.

Babak, T., Zhang, W., Morris, Q., Blencowe, B. J., & Hughes, T. R. (2004). Probing microRNAs with microarrays: tissue specificity and functional inference. RNA., 10, 1813-1819.

Bak, M., Silahtaroglu, A., Moller, M., Christensen, M., Rath, M. F., Skryabin, B. et al. (2008). MicroRNA expression in the adult mouse central nervous system. RNA (New York, N.Y.), 14, 432-444.

Barbosa, A. C., Kim, M. S., Ertunc, M., Adachi, M., Nelson, E. D., McAnally, J. et al. (2008). MEF2C, a transcription factor that facilitates learning and memory by negative regulation of synapse numbers and function. Proc.Natl.Acad.Sci.U.S.A, 105, 9391-9396.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116, 281-297.

Bhat, R. & Steinman, L. (2009). Innate and adaptive autoimmunity directed to the central nervous system. Neuron, 64, 123-132.

Boissonneault, V., Plante, I., Rivest, S., & Provost, P. (2009). MicroRNA-298 and microRNA-328 regulate expression of mouse beta-amyloid precursor protein-converting enzyme 1. The Journal of biological chemistry, 284, 1971-1981.

Borchert, G. M., Lanier, W., & Davidson, B. L. (2006). RNA polymerase III transcribes human microRNAs. Nature structural & molecular biology, 13, 1097-1101.

Boyce-Rustay, J. M., Wiedholz, L. M., Millstein, R. A., Carroll, J., Murphy, D. L., Daws, L. C. et al. (2006). Ethanol-related behaviors in serotonin transporter knockout mice. Alcohol Clin.Exp.Res., 30, 1957-1965.

Brazelton, T. R., Rossi, F. M., Keshet, G. I., & Blau, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. Science, 290, 1775-1779.

Bruccoleri, A. & Harry, G. J. (2000). Chemical-induced hippocampal neurodegeneration and elevations in TNFalpha, TNFbeta, IL-1alpha, IP-10, and MCP-1 mRNA in osteopetrotic (op/op) mice. J.Neurosci.Res., 62, 146-155.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S. et al. (2006). Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Mol.Cell Neurosci., 31, 149-160.

Castaneda, J. A., Lim, M. J., Cooper, J. D., & Pearce, D. A. (2008). Immune system irregularities in lysosomal storage disorders. Acta Neuropathol., 115, 159-174.

Ciafre, S. A., Galardi, S., Mangiola, A., Ferracin, M., Liu, C. G., Sabatino, G. et al. (2005). Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem.Biophys.Res.Commun., 334, 1351-1358.

Conzelmann, E., Kytzia, H. J., Navon, R., & Sandhoff, K. (1983). Ganglioside GM2 N-acetyl-beta-D-galactosaminidase activity in cultured fibroblasts of late-infantile and adult GM2 gangliosidosis patients and of healthy probands with low hexosaminidase level. Am.J.Hum.Genet., 35, 900-913.

Culmsee, C. & Plesnila, N. (2006). Targeting Bid to prevent programmed cell death in neurons. Biochem.Soc.Trans., 34, 1334-1340.

Du, Z. W., Ma, L. X., Phillips, C., & Zhang, S. C. (2013). miR-200 and miR-96 families repress neural induction from human embryonic stem cells. Development.

Eacker, S. M., Dawson, T. M., & Dawson, V. L. (2009). Understanding microRNAs in neurodegeneration. Nat.Rev.Neurosci., 10, 837-841.

Fasanaro, P., Greco, S., Lorenzi, M., Pescatori, M., Brioschi, M., Kulshreshtha, R. et al. (2009). An integrated approach for experimental target identification of hypoxia-induced miR-210. J.Biol.Chem., 284, 35134-35143.

Flavell, S. W., Kim, T. K., Gray, J. M., Harmin, D. A., Hemberg, M., Hong, E. J. et al. (2008). Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. Neuron, 60, 1022-1038.

Fontaine, V., Mohand-Said, S., Hanoteau, N., Fuchs, C., Pfizenmaier, K., & Eisel, U. (2002). Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. J.Neurosci., 22, RC216.

Futerman, A. H. & van, M. G. (2004). The cell biology of lysosomal storage disorders. Nat.Rev.Mol.Cell Biol., 5, 554-565.

Gao, F. B. (2008). Posttranscriptional control of neuronal development by microRNA networks. Trends in neurosciences, 31, 20-26.

Gomez-Lira, M., Sangalli, A., Mottes, M., Perusi, C., Pignatti, P. F., Rizzuto, N. et al. (1995). A common beta hexosaminidase gene mutation in adult Sandhoff disease patients. Hum.Genet., 96, 417-422.

Gruber, A., Mancek, M., Wagner, H., Kirschning, C. J., & Jerala, R. (2004). Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. J.Biol.Chem., 279, 28475-28482.

Guo, D., Dunbar, J. D., Yang, C. H., Pfeffer, L. M., & Donner, D. B. (1998). Induction of Jak/STAT signaling by activation of the type 1 TNF receptor. J.Immunol., 160, 2742-2750.

Hebert, S. S. & De Strooper, B. (2009). Alterations of the microRNA network cause neurodegenerative disease. Trends in neurosciences, 32, 199-206.

Hebert, S. S. & De, S. B. (2009). Alterations of the microRNA network cause neurodegenerative disease. Trends Neurosci., 32, 199-206.

Hebert, S. S., Horre, K., Nicolai, L., Papadopoulou, A. S., Mandemakers, W., Silahtaroglu, A. N. et al. (2008). Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. Proc.Natl.Acad.Sci.U.S.A, 105, 6415-6420.

Hirota, K., Matsui, M., Murata, M., Takashima, Y., Cheng, F. S., Itoh, T. et al. (2000). Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. Biochem.Biophys.Res.Commun., 274, 177-182.

Hodges, B. L. & Cheng, S. H. (2006). Cell and gene-based therapies for the lysosomal storage diseases. Curr.Gene Ther., 6, 227-241.

Hoogerbrugge, P. M., Suzuki, K., Suzuki, K., Poorthuis, B. J., Kobayashi, T., Wagemaker, G. et al. (1988). Donor-derived cells in the central nervous system of twitcher mice after bone marrow transplantation. Science (New York, N.Y.), 239, 1035-1038.

Hopkins, S. J. (2007). Central nervous system recognition of peripheral inflammation: a neural, hormonal collaboration. Acta Biomed., 78 Suppl 1, 231-247.

Hossain, M. A., Russell, J. C., O'Brien, R., & Laterra, J. (2004). Neuronal pentraxin 1: a novel mediator of hypoxic-ischemic injury in neonatal brain. J.Neurosci., 24, 4187-4196.

Huang, J. Q., Trasler, J. M., Igdoura, S., Michaud, J., Hanal, N., & Gravel, R. A. (1997). Apoptotic cell death in mouse models of GM2 gangliosidosis and observations on human Tay-Sachs and Sandhoff diseases. Hum.Mol.Genet., 6, 1879-1885.

Igdoura, S. A., Mertineit, C., Trasler, J. M., & Gravel, R. A. (1999). Sialidase-mediated depletion of GM2 ganglioside in Tay-Sachs neuroglia cells. Hum.Mol.Genet., 8, 1111-1116.

Irwin, C. C. & Irwin, L. N. (1979). A simple rapid method for ganglioside isolation from small amounts of tissue. Anal.Biochem., 94, 335-339.

Irwin, S. (1968). Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia., 13, 222-257.

Ivan, M., Harris, A. L., Martelli, F., & Kulshreshtha, R. (2008). Hypoxia response and microRNAs: no longer two separate worlds. J.Cell Mol.Med., 12, 1426-1431.

Jerala, R. (2007). Structural biology of the LPS recognition. Int.J.Med.Microbiol., 297, 353-363.

Jeyakumar, M., Dwek, R. A., Butters, T. D., & Platt, F. M. (2005). Storage solutions: treating lysosomal disorders of the brain. Nat.Rev.Neurosci., 6, 713-725.

Jeyakumar, M., Norflus, F., Tifft, C. J., Cortina-Borja, M., Butters, T. D., Proia, R. L. et al. (2001). Enhanced survival in Sandhoff disease mice receiving a combination of substrate deprivation therapy and bone marrow transplantation. Blood, 97, 327-329.

Jeyakumar, M., Smith, D. A., Williams, I. M., Borja, M. C., Neville, D. C., Butters, T. D. et al. (2004). NSAIDs increase survival in the Sandhoff disease mouse: synergy with N-butyldeoxynojirimycin. Ann.Neurol., 56, 642-649.

Jeyakumar, M., Thomas, R., Elliot-Smith, E., Smith, D. A., van der Spoel, A. C., d'Azzo, A. et al. (2003). Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis. Brain, 126, 974-987.

Jones, S. W., Watkins, G., Le, G. N., Roberts, S., Murphy, C. L., Brockbank, S. M. et al. (2009). The identification of differentially expressed microRNA in osteoarthritic

tissue that modulate the production of TNF-alpha and MMP13. Osteoarthritis.Cartilage., 17, 464-472.

Jung, D. Y., Lee, H., Jung, B. Y., Ock, J., Lee, M. S., Lee, W. H. et al. (2005). TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-beta as a decision maker. J.Immunol., 174, 6467-6476.

Kettenmann, H. (2007). Neuroscience: the brain's garbage men. Nature, 446, 987-989.

Kim, H. W., Rapoport, S. I., & Rao, J. S. (2010). Altered expression of apoptotic factors and synaptic markers in postmortem brain from bipolar disorder patients. Neurobiol.Dis., 37, 596-603.

Kolter, T. & Sandhoff, K. (2006). Sphingolipid metabolism diseases. Biochim.Biophys.Acta, 1758, 2057-2079.

Kondkar, A. A., Bray, M. S., Leal, S. M., Nagalla, S., Liu, D. J., Jin, Y. et al. (2010). VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J.Thromb.Haemost., 8, 369-378.

Krivit, W., Aubourg, P., Shapiro, E., & Peters, C. (1999). Bone marrow transplantation for globoid cell leukodystrophy, adrenoleukodystrophy, metachromatic leukodystrophy, and Hurler syndrome. Curr.Opin.Hematol., 6, 377-382.

Krivit, W., Peters, C., & Shapiro, E. G. (1999). Bone marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy,

metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux-Lamy, and Sly syndromes, and Gaucher disease type III. Curr.Opin.Neurol., 12, 167-176.

Kyrkanides, S., Miller, A. W., Miller, J. N., Tallents, R. H., Brouxhon, S. M., Olschowka, M. E. et al. (2008). Peripheral blood mononuclear cell infiltration and neuroinflammation in the HexB-/- mouse model of neurodegeneration. J.Neuroimmunol., 203, 50-57.

Lee, D. W., Rajagopalan, S., Siddiq, A., Gwiazda, R., Yang, L., Beal, M. F. et al. (2009). Inhibition of prolyl hydroxylase protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity: model for the potential involvement of the hypoxia-inducible factor pathway in Parkinson disease. J.Biol.Chem., 284, 29065-29076.

Lee, J. P., Jeyakumar, M., Gonzalez, R., Takahashi, H., Lee, P. J., Baek, R. C. et al. (2007). Stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. Nat.Med., 13, 439-447.

Lenz, D. R. & Avraham, K. B. (2011). Hereditary hearing loss: from human mutation to mechanism. Hear.Res., 281, 3-10.

Leulier, F. & Lemaitre, B. (2008). Toll-like receptors--taking an evolutionary approach. Nat.Rev.Genet., 9, 165-178.

Li, H., Radford, J. C., Ragusa, M. J., Shea, K. L., McKercher, S. R., Zaremba, J. D. et al. (2008). Transcription factor MEF2C influences neural stem/progenitor cell differentiation and maturation in vivo. Proc.Natl.Acad.Sci.U.S.A, 105, 9397-9402.

Liang, X., Lin, T., Sun, G., Beasley-Topliffe, L., Cavaillon, J. M., & Warren, H. S. (2009). Hemopexin down-regulates LPS-induced proinflammatory cytokines from macrophages. J.Leukoc.Biol., 86, 229-235.

Lindholm, D., Wootz, H., & Korhonen, L. (2006). ER stress and neurodegenerative diseases. Cell Death.Differ., 13, 385-392.

Liu, F., Lou, Y. L., Wu, J., Ruan, Q. F., Xie, A., Guo, F. et al. (2012). Upregulation of microRNA-210 regulates renal angiogenesis mediated by activation of VEGF signaling pathway under ischemia/perfusion injury in vivo and in vitro. Kidney Blood Press Res., 35, 182-191.

Maes, O. C., Chertkow, H. M., Wang, E., & Schipper, H. M. (2009). MicroRNA: Implications for Alzheimer Disease and other Human CNS Disorders. Current Genomics, 10, 154-168.

Malatack, J. J., Consolini, D. M., & Bayever, E. (2003). The status of hematopoietic stem cell transplantation in lysosomal storage disease. Pediatr.Neurol., 29, 391-403.

McDaniel, K. L. & Moser, V. C. (1993). Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. Neurotoxicol.Teratol., 15, 71-83.

McGeer, P. L. & McGeer, E. G. (2001). Inflammation, autotoxicity and Alzheimer disease. Neurobiol.Aging, 22, 799-809.

McGeer, P. L. & McGeer, E. G. (2004). Inflammation and neurodegeneration in Parkinson's disease. Parkinsonism.Relat Disord., 10 Suppl 1, S3-S7.

McGuckin, C. P., Forraz, N., Allouard, Q., & Pettengell, R. (2004). Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp.Cell Res., 295, 350-359.

Meier, E. M., Schwarzmann, G., Furst, W., & Sandhoff, K. (1991). The human GM2 activator protein. A substrate specific cofactor of beta-hexosaminidase A. J.Biol.Chem., 266, 1879-1887.

Melino, G. & Knight, R. A. (2010). MicroRNAs meet cell death. Cell death and differentiation, 17, 189-190.

Miklyaeva, E. I., Dong, W., Bureau, A., Fattahie, R., Xu, Y., Su, M. et al. (2004). Late onset Tay-Sachs disease in mice with targeted disruption of the Hexa gene: behavioral changes and pathology of the central nervous system. Brain Res., 1001, 37-50.

Mizrahi, O., Sheyn, D., Tawackoli, W., Kallai, I., Oh, A., Su, S. et al. (2013). BMP-6 is more efficient in bone formation than BMP-2 when overexpressed in mesenchymal stem cells. Gene Ther., 20, 370-377.

Modic, M., Ule, J., & Sibley, C. R. (2013). CLIPing the brain: Studies of protein-RNA interactions important for neurodegenerative disorders. Mol.Cell Neurosci..

Mrak, R. E. & Griffin, W. S. (2005). Glia and their cytokines in progression of neurodegeneration. Neurobiol.Aging, 26, 349-354.

Murakami, Y., Saito, K., Hara, A., Zhu, Y., Sudo, K., Niwa, M. et al. (2005). Increases in tumor necrosis factor-alpha following transient global cerebral ischemia do not contribute to neuron death in mouse hippocampus. J.Neurochem., 93, 1616-1622.

Myerowitz, R., Lawson, D., Mizukami, H., Mi, Y., Tifft, C. J., & Proia, R. L. (2002). Molecular pathophysiology in Tay-Sachs and Sandhoff diseases as revealed by gene expression profiling. Hum.Mol.Genet., 11, 1343-1350.

Niederkorn, J. Y. (2006). See no evil, hear no evil, do no evil: the lessons of immune privilege. Nat.Immunol., 7, 354-359.

Nilsson, C. L., Dillon, R., Devakumar, A., Shi, S. D., Greig, M., Rogers, J. C. et al. (2010). Quantitative phosphoproteomic analysis of the STAT3/IL-6/HIF1alpha signaling network: an initial study in GSC11 glioblastoma stem cells. J.Proteome.Res., 9, 430-443.

Niyadurupola, N., Sidaway, P., Ma, N., Rhodes, J. D., Broadway, D. C., & Sanderson, J. (2013). P2X7 receptor activation mediates retinal ganglion cell death in a human retina model of ischemic neurodegeneration. Invest Ophthalmol.Vis.Sci., 54, 2163-2170.

Norflus, F., Tifft, C. J., McDonald, M. P., Goldstein, G., Crawley, J. N., Hoffmann, A. et al. (1998). Bone marrow transplantation prolongs life span and ameliorates neurologic manifestations in Sandhoff disease mice. J.Clin.Invest, 101, 1881-1888.

Okun, E., Griffioen, K. J., & Mattson, M. P. (2011). Toll-like receptor signaling in neural plasticity and disease. Trends Neurosci., 34, 269-281.

Olson, J. K. & Miller, S. D. (2004). Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J.Immunol., 173, 3916-3924.

Oya, Y., Proia, R. L., Norflus, F., Tifft, C. J., Langaman, C., & Suzuki, K. (2000). Distribution of enzyme-bearing cells in GM2 gangliosidosis mice: regionally specific pattern of cellular infiltration following bone marrow transplantation. Acta Neuropathologica, 99, 161-168.

Ozsait, B., Komurcu-Bayrak, E., Levula, M., Erginel-Unaltuna, N., Kahonen, M., Rai, M. et al. (2010). Niemann-Pick type C fibroblasts have a distinct microRNA profile related to lipid metabolism and certain cellular components. Biochem.Biophys.Res.Commun., 403, 316-321.

Park, K. I., Hack, M. A., Ourednik, J., Yandava, B., Flax, J. D., Stieg, P. E. et al. (2006). Acute injury directs the migration, proliferation, and differentiation of solid organ stem cells: evidence from the effect of hypoxia-ischemia in the CNS on clonal "reporter" neural stem cells. Exp.Neurol., 199, 156-178.

Park, K. W., Nozell, S. E., & Benveniste, E. N. (2012). Protective role of STAT3 in NMDA and glutamate-induced neuronal death: negative regulatory effect of SOCS3. PLoS.One., 7, e50874.

Parker, L. C., Whyte, M. K., Vogel, S. N., Dower, S. K., & Sabroe, I. (2004). Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. J.Immunol., 172, 4977-4986.

Perkins, D. O., Jeffries, C. D., Jarskog, L. F., Thomson, J. M., Woods, K., Newman, M. A. et al. (2007). microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. Genome Biol., 8, R27.

Perry, V. H. & O'Connor, V. (2008). C1q: the perfect complement for a synaptic feast? Nat.Rev.Neurosci., 9, 807-811.

Peters, C. & Krivit, W. (2000). Hematopoietic cell transplantation for mucopolysaccharidosis IIB (Hunter syndrome). Bone Marrow Transplant., 25, 1097-1099.

Phaneuf, D., Wakamatsu, N., Huang, J. Q., Borowski, A., Peterson, A. C., Fortunato, S. R. et al. (1996). Dramatically different phenotypes in mouse models of human Tay-Sachs and Sandhoff diseases. Human molecular genetics, 5, 1-14.

Pierce, K. R., Kosanke, S. D., Bay, W. W., & Bridges, C. H. (1976). Animal model of human disease: GM2 gangliosidosis. Am.J.Pathol., 83, 419-422.

Pincheira, R., Castro, A. F., Ozes, O. N., Idumalla, P. S., & Donner, D. B. (2008). Type 1 TNF receptor forms a complex with and uses Jak2 and c-Src to selectively engage signaling pathways that regulate transcription factor activity. J.Immunol., 181, 1288-1298.

Probert, L., Akassoglou, K., Kassiotis, G., Pasparakis, M., Alexopoulou, L., & Kollias, G. (1997). TNF-alpha transgenic and knockout models of CNS inflammation and degeneration. J.Neuroimmunol., 72, 137-141.

Pulkkinen, K., Malm, T., Turunen, M., Koistinaho, J., & Yla-Herttuala, S. (2008). Hypoxia induces microRNA miR-210 in vitro and in vivo ephrin-A3 and neuronal pentraxin 1 are potentially regulated by miR-210. FEBS Lett., 582, 2397-2401.

Rabchevsky, A. G., Weinitz, J. M., Coulpier, M., Fages, C., Tinel, M., & Junier, M. P. (1998). A role for transforming growth factor alpha as an inducer of astrogliosis. J.Neurosci., 18, 10541-10552.

Ramer, M. S., Harper, G. P., & Bradbury, E. J. (2000). Progress in spinal cord research - a refined strategy for the International Spinal Research Trust. Spinal Cord., 38, 449-472.

Rauert, H., Wicovsky, A., Muller, N., Siegmund, D., Spindler, V., Waschke, J. et al. (2010). Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). J.Biol.Chem., 285, 7394-7404.

Raval, G., Biswas, S., Rayman, P., Biswas, K., Sa, G., Ghosh, S. et al. (2007). TNF-alpha induction of GM2 expression on renal cell carcinomas promotes T cell dysfunction. J.Immunol., 178, 6642-6652.

Robberecht, W. & Philips, T. (2013). The changing scene of amyotrophic lateral sclerosis. Nat.Rev.Neurosci., 14, 248-264.

Sakuraba, H., Sawada, M., Matsuzawa, F., Aikawa, S., Chiba, Y., Jigami, Y. et al. (2006). Molecular pathologies of and enzyme replacement therapies for lysosomal diseases. CNS.Neurol.Disord.Drug Targets., 5, 401-413.

Sandhoff, K., Andreae, U., & Jatzkewitz, H. (1968). Deficient hexozaminidase activity in an exceptional case of Tay-Sachs disease with additional storage of kidney globoside in visceral organs. Life Sci., 7, 283-288.

Sango, K., Yamanaka, S., Hoffmann, A., Okuda, Y., Grinberg, A., Westphal, H. et al. (1995). Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism. Nat.Genet., 11, 170-176.

Santos-Reboucas, C. B. & Pimentel, M. M. (2010). MicroRNAs: macro challenges on understanding human biological functions and neurological diseases. Current Molecular Medicine, 10, 692-704.

Sardiello, M., Palmieri, M., di, R. A., Medina, D. L., Valenza, M., Gennarino, V. A. et al. (2009). A gene network regulating lysosomal biogenesis and function. Science, 325, 473-477.

Sasaki, M., Honmou, O., Akiyama, Y., Uede, T., Hashi, K., & Kocsis, J. D. (2001). Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. Glia, 35, 26-34.

Satoh, J. (2010). MicroRNAs and their therapeutic potential for human diseases: aberrant microRNA expression in Alzheimer's disease brains. Journal of pharmacological sciences, 114, 269-275.

Schonrock, N., Ke, Y. D., Humphreys, D., Staufenbiel, M., Ittner, L. M., Preiss, T. et al. (2010). Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. PloS one, 5, e11070.

Shie, F. S. & Woltjer, R. L. (2007). Manipulation of microglial activation as a therapeutic strategy in Alzheimer's disease. Curr.Med.Chem., 14, 2865-2871.

Shihabuddin, L. S. & Aubert, I. (2010). Stem cell transplantation for neurometabolic and neurodegenerative diseases. Neuropharmacology, 58, 845-854.

Shishodia, S. & Aggarwal, B. B. (2002). Nuclear factor-kappaB activation: a question of life or death. J.Biochem.Mol.Biol., 35, 28-40.

Slomiany, M. G., Black, L. A., Kibbey, M. M., Day, T. A., & Rosenzweig, S. A. (2006). IGF-1 induced vascular endothelial growth factor secretion in head and neck squamous cell carcinoma. Biochem.Biophys.Res.Commun., 342, 851-858.

Sly, W. S. & Vogler, C. (2002). Brain-directed gene therapy for lysosomal storage disease: going well beyond the blood- brain barrier. Proc.Natl.Acad.Sci.U.S.A, 99, 5760-5762.

Sofroniew, M. V. & Vinters, H. V. (2010). Astrocytes: biology and pathology. Acta Neuropathol., 119, 7-35.

Steinman, L. (2013). A Tip leads cytotoxic T cells to the crime scene in neuroinflammation. Nat.Immunol., 14, 196-197.

Sun, S. C. (2011). Non-canonical NF-kappaB signaling pathway. Cell Res., 21, 71-85.

Suzuki, S., Yamashita, T., Tanaka, K., Hattori, H., Sawamoto, K., Okano, H. et al. (2005). Activation of cytokine signaling through leukemia inhibitory factor receptor (LIFR)/gp130 attenuates ischemic brain injury in rats. J.Cereb.Blood Flow Metab, 25, 685-693.

Takeda, K. & Akira, S. (2005). Toll-like receptors in innate immunity. Int.Immunol., 17, 1-14.

Teng, Y. D., Lavik, E. B., Qu, X., Park, K. I., Ourednik, J., Zurakowski, D. et al. (2002). Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc.Natl.Acad.Sci.U.S.A, 99, 3024-3029.

Thibeault, I., Laflamme, N., & Rivest, S. (2001). Regulation of the gene encoding the monocyte chemoattractant protein 1 (MCP-1) in the mouse and rat brain in response to circulating LPS and proinflammatory cytokines. J.Comp Neurol., 434, 461-477.

Ting, A. Y. & Endy, D. (2002). Signal transduction. Decoding NF-kappaB signaling. Science, 298, 1189-1190.

Tsuda, M., Kohro, Y., Yano, T., Tsujikawa, T., Kitano, J., Tozaki-Saitoh, H. et al. (2011a). JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. Brain, 134, 1127-1139.

Tsuda, M., Kohro, Y., Yano, T., Tsujikawa, T., Kitano, J., Tozaki-Saitoh, H. et al. (2011b). JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. Brain, 134, 1127-1139.

Vallabhapurapu, S., Matsuzawa, A., Zhang, W., Tseng, P. H., Keats, J. J., Wang, H. et al. (2008). Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat.Immunol., 9, 1364-1370.

VanGilder, R. L., Huber, J. D., Rosen, C. L., & Barr, T. L. (2012). The transcriptome of cerebral ischemia. Brain Res.Bull., 88, 313-319.

Vitner, E. B., Platt, F. M., & Futerman, A. H. (2010). Common and uncommon pathogenic cascades in lysosomal storage diseases. J.Biol.Chem., 285, 20423-20427.

Wada, R., Tifft, C. J., & Proia, R. L. (2000). Microglial activation precedes acute neurodegeneration in Sandhoff disease and is suppressed by bone marrow transplantation. Proc.Natl.Acad.Sci.U.S.A, 97, 10954-10959.

Wajant, H., Pfizenmaier, K., & Scheurich, P. (2003). Tumor necrosis factor signaling. Cell Death.Differ., 10, 45-65.

Wakitani, S., Okabe, T., Horibe, S., Mitsuoka, T., Saito, M., Koyama, T. et al. (2011). Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. J.Tissue Eng Regen.Med., 5, 146-150.

Walkley, S. U. (1998). Cellular pathology of lysosomal storage disorders. Brain pathology (Zurich, Switzerland), 8, 175-193.

Walkley, S. U. (2009). Pathogenic cascades in lysosomal disease-Why so complex? J.Inherit.Metab Dis., 32, 181-189.

Wang, W. X., Huang, Q., Hu, Y., Stromberg, A. J., & Nelson, P. T. (2011). Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. Acta Neuropathologica, 121, 193-205.

Wang, W. X., Rajeev, B. W., Stromberg, A. J., Ren, N., Tang, G., Huang, Q. et al. (2008). The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. The Journal of neuroscience : the official journal of the Society for Neuroscience, 28, 1213-1223.

Wang, X. R., Zhang, X. M., Zhen, J., Zhang, P. X., Xu, G., & Jiang, H. (2010). MicroRNA expression in the embryonic mouse inner ear. Neuroreport, 21, 611-617.

Wilms, H., Sievers, J., Dengler, R., Bufler, J., Deuschl, G., & Lucius, R. (2003). Intrathecal synthesis of monocyte chemoattractant protein-1 (MCP-1) in amyotrophic lateral sclerosis: further evidence for microglial activation in neurodegeneration. J.Neuroimmunol., 144, 139-142.

Wojno, E. D. & Hunter, C. A. (2012). New directions in the basic and translational biology of interleukin-27. Trends Immunol., 33, 91-97.

Wright, K. T., El, M. W., Osman, A., Chowdhury, J., & Johnson, W. E. (2011). Concise review: Bone marrow for the treatment of spinal cord injury: mechanisms and clinical applications. Stem Cells, 29, 169-178.

Wu, Y. P., Mizugishi, K., Bektas, M., Sandhoff, R., & Proia, R. L. (2008). Sphingosine kinase 1/S1P receptor signaling axis controls glial proliferation in mice with Sandhoff disease. Hum.Mol.Genet., 17, 2257-2264.

Wu, Y. P. & Proia, R. L. (2004). Deletion of macrophage-inflammatory protein 1 alpha retards neurodegeneration in Sandhoff disease mice. Proc.Natl.Acad.Sci.U.S.A, 101, 8425-8430.

Xu, D., Hopf, C., Reddy, R., Cho, R. W., Guo, L., Lanahan, A. et al. (2003). Narp and NP1 form heterocomplexes that function in developmental and activity-dependent synaptic plasticity. Neuron, 39, 513-528.

Yamanaka, S., Johnson, M. D., Grinberg, A., Westphal, H., Crawley, J. N., Taniike, M. et al. (1994). Targeted disruption of the Hexa gene results in mice with biochemical and pathologic features of Tay-Sachs disease. Proc.Natl.Acad.Sci.U.S.A, 91, 9975-9979.

Yin, G. N., Lee, H. W., Cho, J. Y., & Suk, K. (2009). Neuronal pentraxin receptor in cerebrospinal fluid as a potential biomarker for neurodegenerative diseases. Brain Res., 1265, 158-170.

Zhang, L., Zhao, W., Li, B., Alkon, D. L., Barker, J. L., Chang, Y. H. et al. (2000). TNF-alpha induced over-expression of GFAP is associated with MAPKs. Neuroreport, 11, 409-412.

Zhou, D., Mattner, J., Cantu, C., III, Schrantz, N., Yin, N., Gao, Y. et al. (2004). Lysosomal glycosphingolipid recognition by NKT cells. Science, 306, 1786-1789.