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Ganglioside-Cytokine Interaction in the Induction of Primary Brain Cell Death

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Dedication

To my family

GANGLIOSIDE-CYTOKINE INTERACTION IN THE INDUCTION OF PRIMARY BRAIN CELL DEATH

by

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DISSERTATION

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Abstract

Gangliosides have been implicated in multiple pathologies affecting the central nervous system (CNS) and recent research has implicated them in playing an active role in the pathogenesis of multiple sclerosis. Empirical studies and theoretical considerations have suggested the possibility of interactions between gangliosides, like GD3, and pro-inflammatory cytokines present in the nervous system. This study sought to investigate the possibility that either individual gangliosides acting alone or complexed with other species interact with the known immune response factor TNFα to initiate or facilitate cell death in the CNS. We examined the cellular viability and gene expression in primary brain cell cultures treated with either GD3, GD1b, or TNFα to observe relative changes that would provide evidence for a possible pathway involved in this form of cell death. We found that both GD3 and GD1b, but not TNFα led to up-regulation of gene expression for macrophage inflammatory protein 3 (MIP3A) and interleukin-1 receptor 1 (IL1R1), but down-regulation of fibroblast growth factor 13 (FGF13). The gene expression of the FGF receptor activating protein 1 (FRAG1) and interleukin-3 receptor alpha (IL3RA) was down-regulated by GD3 and GD1b for IL3RA, but only GD3 caused a down-regulation in FRAG1, while TNFα caused an up-regulated in the expression of both genes. All three treatments resulted in up-regulation of the gene for chemokine ligand 2 (CCL2). TNFα was also found to increase expression of the gene for N-acetyl-galactosaminyl transferase (GNT), a key enzyme in the synthesis of more complex gangliosides. These findings support the view that a pro-inflammatory stimulus, like TNFα, activates biosynthesis of gangliosides, which subsequently breaks down to forms not normally expressed in differentiated cells, like GD3, which then lead to or modulate cell death.

Table of Contents

List of Tables

List of Figures

Chapter I

INTRODUCTION

Gangliosides are membrane-bound glycolipids that are found in abundance in neuronal membranes. They occur predominantly in the central nervous system (CNS) and are commonly found sequestered in lipid raft domains of the membrane. Gangliosides have been associated with cell-cell communication, cell-macromolecule communication, and are targets for multiple pathogens such as *Clostridium botulinum*, *Clostridium tetani*, *Vibrio cholerae*, and influenza. In addition, they have multiple implications in several diseases including multiple sclerosis (MS) and Guillain-Barre syndrome, as evidenced by high amounts of anti-ganglioside antibodies associated with those conditions. They are most notably associated with diseases such as Tay-Sachs and Sandoff's disease, which are both caused by an accumulation of gangliosides within the neuron.

More recently gangliosides have also been thought to play a critical role in the pathology of MS and other autoimmune diseases. Anti-ganglioside antibodies have been produced *in vivo* and *in vitro* and are thought to recognize the carbohydrate portion of the glycolipid (Freimer, McIntosh et al. 1993; Zaprianova, Majtenyi et al. 2004; Marconi, Acler et al. 2006). This response may lead to the characteristic breakdown of the blood-brain barrier in MS, causing infiltration of T-cells into the brain.

The goal of this research was to investigate alternative mechanisms in the pathology of MS-induced neural cell death. I hypothesized that gangliosides, specifically GD3, play a role in specific cell death pathways that are implicated in the progression of neuronal death in the course of MS.

The completion of these experiments: 1) provided critical information on the putative role of gangliosides in neuronal apoptosis and MS pathogenesis, 2) generated potentially new candidate targets of MS pathogenesis, 3) and added to our overall knowledge base of what the normal function of gangliosides are in the CNS.

Neurodegeneration associated with autoimmunity is a well known dysfunction of the immune system, misrecognizing and targeting the "self" antigens rather than foreign pathogens/antigens. Multiple sclerosis is an autoimmune disease that involves T-cells of the immune system which recognize a component of the myelin sheath of neurons and cause massive cell death and ultimate dysfunction of the CNS. The myelin sheath acts as an electrical insulator which allows the propagation of bioelectrical signaling known as action potentials from neuron to neuron in the CNS and PNS. Loss of proper neuronal communication can lead to severe motor dysfunction, cognitive disabilities, and eventually death.

The etiology of MS is unknown; however genetic and environmental factors appear to play a role in the disease. Most patients with MS are Caucasians of European descent and the frequency of cases diagnosed increases as latitude increases north and south of the equator. One current hypothesis is that the Epstein-Bar virus may be a possible etiological agent, but there is not yet sufficient definitive evidence to support this claim (Ascherio and Munger 2007).

Approximately 2.5 million people suffer from this progressive neurodegenerative disease worldwide, and are treated with medications that target the immune system. These therapies act on the disease by inhibiting secretion of cytokines like interferon beta and tumor necrosis factor alpha (TNFα) by T-cells, thereby reducing the signal for cells to undergo inflammation and ultimately apoptosis. Myelin basic protein (MBP), myelin associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) are the most highly

investigated targets for the immune system against the nervous system in MS. However, the mechanisms of pathogenesis in this cruel disease remain vague and poorly understood.

Most of the available treatments for MS are immunosuppressant drugs that slow the progression of the disease but do not aid in the repair of the neuronal lesions caused by the immune response. Overall, however, the cellular and targeting mechanisms for MS are not well understood and proven effective alternative treatments are still lacking.

Current Research on MS

Immunological Research

Due to the lack of knowledge in MS disease progression and treatment, MS has been under intense investigation in the past few years to enhance our understanding of the biological processes that are present in its onset and progression. Novel experiments are underway to elucidate the potential targets that are involved in the progression of the disease by targeting the immune system and its dysfunction throughout the course of MS. One such target is the B-cell. The B-cell is important in the adaptive immune system because of its natural ability to make antibodies, activate T-cells, and its role in the formation of ectopic germinal centers in the intermeningeal spaces. For that reason research is being conducted on ways to use monoclonal antibodies to deplete B-cell populations by targeting CD20 with rituximab (Dalakas 2008). The rationale behind this treatment is to reduce the expression of two B-cell trophic factors, B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which are known to be up-regulated in the CNS and PNS, along with their receptors, in autoimmune disorders (Dalakas 2008).

Other areas of research are investigating T-cells and their ability to recruit or combine with other immune factors. Much of the research on MS concentrates on the T-cell because of

its ability to migrate throughout the endothelial system and lymphatic system. T-cells have the ability to migrate to sites of potential infection and traverse the endothelium in order to activate antigen presenting cells (APCs) like macrophages or dendritic cells to kill ingested cargo. T-cells can migrate back to lymph nodes to cross-talk with B-cells causing T-cell clonal expansion against the specific antigen peptide sequences, while simultaneously activating B-cells to secrete antibodies against the same peptide sequences. In human trials using MS patients' cerebral spinal fluid (CSF), Shi (2009) has seen an up-regulation of CD4(+)TNFα(+)IL-2(-)T-cells. This phenomenon occurs only during the relapse phase and not during the remission phase of relapsing-remitting multiple sclerosis (RRMS) suggesting an integrated, concerted, intrathecal immunological response in MS with an unknown initiating event or cause.

Not all of the research on MS is used to treat or cure the disease. Some of the research is based on the ability to predict the genetic susceptibility to MS or predict when a relapse may occur. Satoh (2008) has shown, using six human patients with MS, that during the acute relapse phase, when compared to the fully remitted phase, the CD3 T-cells have an aberrant regulation on the transcription factor NFκβ. This shows that activated T-cells are having problems controlling the expression of a transcription factor essential in inflammatory responses and proliferation.

The future of MS treatment and research is said to be patient-specific because of the single nucleotide polymorphisms (SNPs) found throughout the human genome. These one base-pair mutations can cause the loss or aberration of the function of a gene leading to the onset of a disease. Hoppenbrouwers (2008), mentions the genes that are well established to play a role in the susceptibility and progression of MS, which are human leukocyte antigen DRB1 (HLA-DRB1), interleukin receptor 2A (IL2RA) and interleukin receptor 7A (IL7RA), all of which are

involved in proper antigen presentation and immune function; but they also mention that other novel genes are being discovered as well. They found that ecotropic viral integration site 5 (EVI5) may also play a significant role in MS. Although the function of this gene is still relatively unknown and is thought to play a role in centrosome stability and dynamics, many more genes in the genome may play a role in MS in conjunction with or independent of other known targets as well.

Role of TNFα in Autoimmunity

TNFα is a known cytokine that is very influential in the initiation of inflammation, apoptosis and, in some cases, cell survival. Researchers have been trying to link TNFα mediated pathways with autoimmune diseases like MS. One of the potential findings is that TNFα interacts with the ganglioside GD3 causing release of reactive oxygen species (ROS) and cytochrome c, caspase 3 activation, and NFκβ suppression resulting in apoptosis of various cell types (Garcia-Ruiz, Colell et al. 2000; Colell, Garcia-Ruiz et al. 2001; Garcia-Ruiz, Colell et al. 2002). Kauffman (2007) tried to link a known SNP at position 376 on the TNFα gene known to increase the susceptibility of MS in patients from Spain (Fernandez-Arquero, Arroyo et al. 1999), but no correlation could be found in patients from the U.S. or the Netherlands, compared to patients from Argentina.

On the other hand, Favorova (2006) found a very distinct correlation between -238TNFB1 and -308TNFA2, with tri-allelic combinations, and a bi-allelic combination with TNFα9 in Russian patients with MS. This shows the complexity of genetic variation with different SNPs in alleles along with other allelic interactions or mutations and how treating the patient is key rather than a global treatment for MS. The next logical step would be to block TNFα signaling to prevent apoptosis during relapses. But anti-TNFα drugs have been given to

people with rheumatoid arthritis (RA) with very limited success. Mohan (2001) has reported that patients who have received the anti-TNFα drugs experienced confusion and difficulty walking, suggesting that patients with MS would not be good candidates for this type of treatment since RA patients mimic the symptoms of MS with this treatment. However, a recent finding (Kim and Moudgil 2008) has shown that TNFα and interferon-gamma (IFNγ) can reduce arthritic inflammation and even contribute to the resistance of arthritis. This shows that there is a fine balance when it comes to TNFα and its functionality in of against the cell.

Roles of Other Cytokines in MS

Many other cytokines and chemokines play a role in MS autoimmunity which leads to neurodegeneration. The most commonly known cytokine that has been implicated in MS is interleukin 12 (IL-12). Segal (1998) demonstrated that there is an IL-12/IL-10 axis involved in animals with the neurodegenerative model experimental autoimmune encephalomyelitis (EAE). They showed that IL-12 secretion is an essential cytokine for the production of Th1 auto-reactive cells. They also showed that IL-10 is a negative regulator of IL-12 production and that both cytokines are necessary for the initiation and termination of an immune response by Th1 cells. Some of the current treatments for MS use interferon-beta (IFNβ) as an immunosuppressant. IFNβ acts as an anti-inflammatory and can increase the integrity of the blood-brain barrier. Byrnes (2002) showed that patients taking IFNβ had a reversal of the IL-12/IL-10 axis meaning that the blood samples from the patients contained more IL-10 than IL-12. This has shown to be beneficial because IL-10 is negatively affecting Th1 autoimmune cells.

Another potential cytokine that may play a role in MS is IL-6. The normal functions for IL-6 is to initiate immune responses to trauma, induce fever, indirectly activate Toll-like receptors ((TLRs), receptors of the innate immune system that recognize structures of microbes

and activate an immune response)) , and respond to inflammation. (Serada, Fujimoto et al. 2008) showed that EAE animals treated with an IL-6r monoclonal antibody blocked the signal transduction of IL-6 and a consequent reduction of CD4+, CD8+, and Th17 lymph nodal T-cells specific for myelin oligodendrocyte glycoprotein (MOG) peptides.

Neurological Research on Neurodegeneration

Much research is being done by neuroscientists to better understand the underlying mechanisms in neurodegeneration. Some of the hot topics in the field of neurodegeneration deal with diseases like MS or Alzheimer's disease and the possibility of nerve regrowth and remyelination of damaged neurons. Others focus on the molecular mechanisms and pathways involving the cellular communication and apoptosis of neurons. Okun (2009) has suggested that toll-like receptors (TLRs) may play a role in the targeting and apoptosis of neurons. They found that glial cells (astrocytes, oligodendrocytes, and microglia) and lymphocytes that invade the CNS contain activated TLRs that may indirectly cause cell death or damage. This is achieved by the ability of TLRs to stimulate the immune cells, including microglia, to secrete pro-inflammatory cytokines and up-regulate cell adhesion molecules in response to inflammation.

Kaminska (2005) has suggested that the main role of p38 MAPK in activation is to produce and activate multiple inflammatory mediators. Others believe that lipids and their functions in the brain may play an important role in many diseases. Adibhatla (2008) have compiled a review implicating lipids, enzymes that control lipid metabolism, and lipoprotein transporters of lipids for numerous brain disorders like Parkinson's, Alzheimer's, Niemann-Pick disease, amyotrophic lateral sclerosis, and multiple sclerosis. They stated that the inhibition of the phospholipase A2 (PLA2) in EAE animals lessened the onset and progression of the model for MS. This makes sense because PLA2 increases cellular amounts of arachidonic acid which is

the precursor to several inflammatory intermediates like eicosanoids (prostaglandins and leukotrienes). Lipids probably play a major role in neurodegeneration due to the fact that the brain contains vast amounts of lipids. The most important area of research in this field, in my opinion, is in novel treatments and drug development. One recently published study strongly suggests that the neurohormone prolactin may be a potential therapeutic agent for MS because of its ability to remyelinate CNS neurons in pregnant rats afflicted with the experimental form of MS, EAE (Gregg, Shikar et al. 2007). Most of the current treatments in MS involve the manipulation of the immune system, as previously described.

Glial Cells

Glial cells (astrocytes, oligodendrocytes, and microglia) play a vital role in the maintenance and survival of neurons. Oligodendrocytes play a crucial role in the survivability and proper function of neurons. They wrap portions of their cell body around the neuron and, in essence, squeeze out the cytoplasm, forming an insulator around the axon of the neuron know as the myelin sheath. This allows the neuron to propagate action potentials, which are waves of bioelectrical currents that constitute cellular communication in neurons. This myelin sheath is the portion of the neuron that is attacked by the immune system during the onset and relapses of MS. The suspected targets for the immune system are three proteins: MBP, MAG, and MOG.

For neurons to maintain their proper functionality, astrocytes play an equally important role in the nervous system by supplying the neuron with nutrients (glucose). Astrocytes are numerically the most prevalent cell in the mammalian brain (Nair, Frederick et al. 2008). They also play a role in pH and ion balance in the CNS, as well as waste management and synaptic transmission by mediating the uptake of glutamate and converting it to glutamine which helps to prevent neuronal and oligodendrocyte damage (Nair, Frederick et al. 2008). It has also been

noted that in normal rat spinal cords, astrocytes express factors such as brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and nerve growth factor (NGF) -- all of which promote the survival of neurons (Nair 2008). Astrocytes even produce factors essential for oligodendrocyte progenitor cells (OPCs) to mature, which is essential for the remyelination process in CNS lesions (Nair, Frederick et al. 2008). This topic is controversial because glial scars form where MS lesions are found in the CNS. This process limits further spreading of CNS damage by astrocyte secretion of FGF-2, causing OPCs to proliferate and survive but not to mature, preventing the remyelination process.

Another critical function of astrocytes is the maintenance of the blood brain barrier which, when compromised, is another marker of neurodegenerative diseases like MS because of the influx of systemic immune factors like macrophages and T-cells. During injury or disease states, however, astrocytes are important mediators of inflammatory responses. Astrocytes can cause a condition known as reactive gliosis, in which a specific protein known as glial fibrillary acidic protein (GFAP) is highly up-regulated on the astrocyte's cell surface and is a marker used to diagnose MS (Nair, Frederick et al. 2008). GFAP seems to be tightly regulated as demonstrated in experiments with knockout mice and mice overexpressing GFAP, both of which show neurological dysfunction (Nair, Frederick et al. 2008). Astrocytes also cause the release of pro-inflammatory cytokines like IL-1 and IL-6 and can release cytotoxic cytokines like TNFα Fas ligand (FasL), and transforming growth factor beta (TGFβ) in response to neuronal damage (Kaminska, Gozdz et al. 2009).

Astrocytes also express Major Histocompatibilty Complex (MHC) I and II, which recognize specific antigen presentation by antigen presenting cells (APCs), and have the ability to activate CD4+ and CD8+ T-cells when treated with IFNγ (Nair, Frederick et al. 2008).

Although astrocytes are non-professional antigen presenting cells (APCs), they have been documented as forming immunological synapses with T-cells and secreting multiple types of cytokines and chemokines known to attract T-cells or cause inflammatory responses (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, and CXCL12). They also express cellular adhesion molecules like intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) which probably stabilize the immunological synapse formed by astrocyte-T-cell interactions as well as APC-T-cell interactions in the CNS (Nair, Frederick et al. 2008). Much of the present data is very controversial about the functions of astroglia in disease states like MS, indicating that a very specific time progression and consequent astrocyte reaction is involved in the disease.

Microglia have a relatively unknown normal physiological function in the brain but were first described in 1932 by Pio del Rio-Hortega as the macrophages of the nervous system. The main purpose of microglia in the CNS is to act as the immune cells of that system. When activated, they can up-regulate many different types of cellular receptors and secrete multiple factors employed by the immune system, including cell adhesion molecules, integrins, cytokines/chemokines and their receptors, toll-like receptors, matrix metalloproteinases, eicosanoids, growth factors, proteases, and cathepsins, just to name a few (Rock and Peterson 2006). Since microglia are the primary phagocytes of the brain and play a huge role in the activation of immune responses in the CNS, they have been targeted as one of the primary conductors in the pathology of MS. Gonsette (2008) has stated that one of the hallmarks of microglial activation in MS is oxidative stress and excitotoxicity. This leads to the compromise of the cells' integrity and apoptosis. They also seem to be involved in some type of cellular balancing act by being both protective and destructive at the same time. Muzio (2007) states that

there are multi levels of functionality of microglia, from an inflammatory phase they call "detrimental" to a protective, "non-detrimental" phase regulated by a feedback loop mechanism. They have vast amounts of cross over with astrocytes in function but are considered to be the main initiators and potentiators of the immune response in neurodegenerative diseases.

Gangliosides

Gangliosides are membrane bound, amphipathic molecules, with an unknown normal function, but their role in specific pathologies has been well defined. Gangliosides consist of a fatty acid tail, a ceramide portion, a lactose core, and a variable carbohydrate chain with sialic acid linkages to its galactose residues. This gives the ganglioside and its various isoforms an overall negative charge. Gangliosides seem to be evolutionarily conserved since they are found in all vertebrates and some in-vertebrates (echinoderms), with very high homology. Even bacterial lipopolysaccharide (LPS) has molecules that mimic ganglioside epitopes, which is important for the bacterium's ability to bind to other cells and evade the host's immune system. The most well known example of this is the role of ganglioside GM1 as the receptor for the cholera toxin which mediates the entry of the toxin into the cell.

While gangliosides are present in many tissue types, they occur in their more complex forms primarily in the CNS of vertebrates. The biosynthesis of gangliosides and their differentiation into the more complex series of gangliosides is catalyzed by the co-activation of the two key enzymes: sialyltransferase II (STII) and N-acetyl- galactosaminyl transferase (GNT), as shown in Fig. 1. If the activity of one of the enzymes is lost, the other becomes non-functional as well (Bieberich, MacKinnon et al. 2002), resulting in the over-expression of the most abundant ganglioside precursor, GM3, thereby causing it to build up in the cell. The inability to differentiate gangliosides into higher order forms or metabolize them into simpler

forms for cellular recycling contributes to multiple pathologies, including Tay-Sachs disease and numerous forms of lysosomal storage diseases.

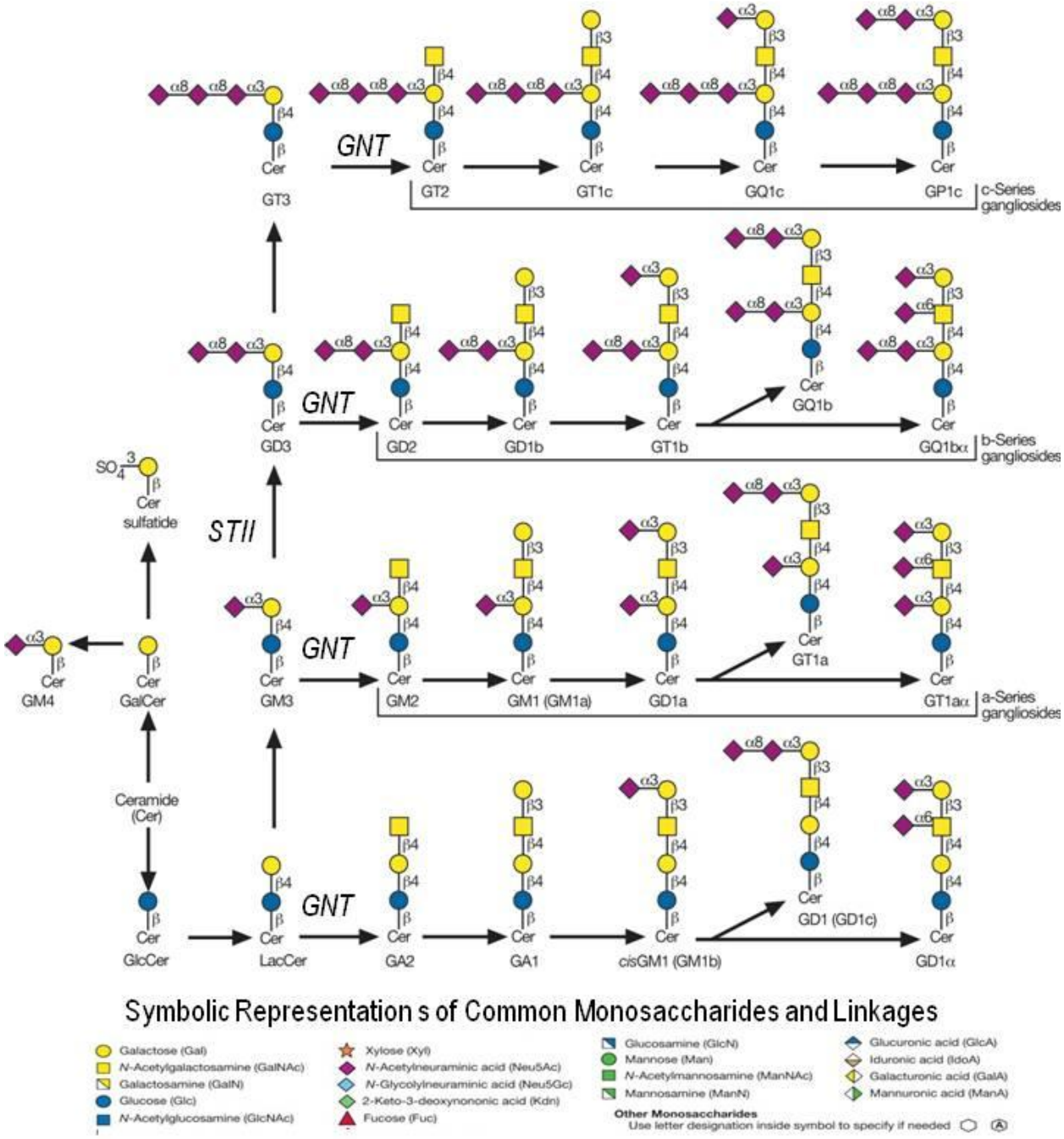

Fig.1. Glycosphingolipid Biosynthetic Pathway. STII catalyzes the addition of a second sialic acid to the first one, and GNT catalyzes addition of N-acetyl- galactosaminyl to galactose. Adapted from **Essentials of Glycobiology**, 2nd edition Copyright ©2009 by The Consortium of Glycobiology Editors, La Jolla, California.

Role of Gangliosides in Disease

The most classical neuropathology associated with gangliosides is Tay-Sachs disease. It is caused by mutation of the hexosaminadase A (Hex A) enzyme which converts GM2 into GM3, a genetic abnormality found mainly in people of eastern European Jewish descent. This leads to GM2 accumulation, neuronal dysfunction, and eventually death. A similar disease associated with gangliosides is Sandhoff's disease. Sandhoff's is almost identical to Tay-Sachs but the genetic deficiency affects the beta subunit of both the Hex A and Hex B enzyme and is more prevalent in non-Jewish populations. The disease causes the accumulation of GM2 in the lysosomes of the cell, ultimately causing cell death. Many of the diseases involving gangliosides exist because of a metabolic dysfunction in ganglioside synthesis and recycling resulting in the accumulation of specific gangliosides in the CNS.

Gangliosides also act as receptors for different types of pathogens as well. The classic example of this is the ability of cholera toxin to bind to ganglioside GM1 (Holmgren, Lonnroth et al. 1973; Holmgren, Lonnroth et al. 1973). GM1 acts as a receptor for cholera toxin by binding to its subunits and mediating the entry of the toxin into otherwise healthy cells. Gangliosides also bind and mediate the entry of other viruses and bacterial toxins like botulinum, influenza, and tetanus Mellanby, 1973 #40}. They have even become a potential target for biological warfare because of their ability to mediate the entry of viruses and toxins into cells (Singh, Harrison et al. 2000). This ability to mediate cellular infection and the lack of glycolipid metabolism by genetic dysfunction has drawn attention to the importance of gangliosides in biomedical research.

Gangliosides in Autoimmunity

It has been reported that gangliosides also play a role in the misrecognition of cancer cells by deactivating the T-cells. This occurs through massive shedding of gangliosides by tumor cells, allowing for the progression and metastasis of several types of cancers (Potapenko, Shurin et al. 2007). It has also been shown that exogenous gangliosides can act as antagonists against fibroblast growth factor receptors (FGFr) by binding to the COOH terminal of the receptor causing a reduction in the viability and proliferative functions of epithelial cells (Rusnati, Tanghetti et al. 1999). Many studies involving MS have determined that the targeting of the CD4+/CD8+ T-cells are specific for myelin basic protein (MBP) and myelin associated glycoprotein (MAG) (Musse and Harauz 2007; Quarles 2007). MBP is in the subgroup of the sialic acid-binding immunoglobulin superfamily whereas MAG may bind negatively charged lipids on the plasma membrane portions of glial cells, specifically oligodendrocytes, which provide the myelin sheath of neurons (Musse and Harauz 2007; Quarles 2007).

Gangliosides posses both of these characteristics; having both a negative charge and sialic acid sugars bound to their galactose molecules. This T-cell/microglial-neuron interaction may be mediated by the abundance of specific gangliosides found on the cell surface of antigen presenting cells (APCs), neurons, and glial cells. This further supports the idea that gangliosides may indeed play a role in the activation and mediation of T-cells and/or microglia in other autoimmune diseases such as MS in conjunction with the previously mentioned roles of MBP and MAG.

Further evidence of a link between MS and gangliosides has been provided by the demonstration that the initial onset of the disease is similar to hypersensitivity in allergic reactions in that activated B-cells release IgM and IgG autoantibodies against ganglioside

glycolipids (Zaprianova, Majtenyi et al. 2004; Marconi, Acler et al. 2006). This release of autoantibodies and their subsequent activation of T-cells may cause the breakdown of the blood brain barrier by using matrix metalloproteinase (MMP)-9, which is characteristic of MS, and allows for the infiltration of T-cells into the brain. This in turn causes TNFα proinflammatory cytokine secretion and apoptosis (Muroski, Roycik et al. 2008). The process by which the B/T-cells are presented with these gangliosides by APCs is still unknown, but much of the evidence supports the idea that gangliosides do play a role in the disease.

Recent promising research has implicated a long known gylcolipid in the apoptotic process of numerous cell types. This glycolipid is ganglioside GD3. It is believed that GD3 acts on mitochondrial membrane transport proteins to disrupt mitochondrial membrane potentials, which in turn causes a release of cytochrome c and caspase 3 activation (Garcia-Ruiz, Colell et al. 2002). Garcia-Ruiz and his colleagues (2002) have also shown that ganglioside GD3 is an important intermediate of TNFα mediated apoptotic cell death. They demonstrated that TNFα secretion caused dramatic re-localization of GD3 to the mitochondria during cell death in hepatocytes which, they concluded, occurred by neosynthesis. Melchiorri (2002) have also reported that increased levels of GD3 caused neuronal apoptosis. Other studies have suggested that gangliosides GM1 and GD2 may be potential targets for B and T-cells of the immune system due to the relatively high titers of autoantibodies in the serum of multiple sclerosis patients (Zaprianova, Majtenyi et al. 2004; Marconi, Acler et al. 2006). Some recently published papers have contradicted the theory that GD3, along with TGFβ, can actually produce some angiogenic affects by enhancing hepatocyte growth factor in glioma cell lines (Chu, Ma et al. 2007). Others have shown that GD3 can not only cause apoptosis by ROS formation and release, but by also inhibiting the binding of the transcription factor NFκβ which can be used to either cause the cell

to survive and proliferate or go into a cell death pathway for apoptosis (Colell, Garcia-Ruiz et al. 2001). Finally, TNFα receptor 1 has been reported to play a role in the negative proliferation of hippocampal neurogenesis in adult brains of mice (Iosif, Ekdahl et al. 2006).

Sialic Acid

The constituent that makes a glycolipid a ganglioside is the presence of sialic acid. Sialic acid is a negatively charged sugar attached to the galactose carbohydrates on the backbone of a ganglioside. Sialic acid is the main effector for many diseases like influenza, tetanus, botulism, and, when polysialylations occur, can protect the body from foreign pathogens, acting as part of the innate immune system by essentially distinguishing the self from possible pathogens, allowing for proper immune recognition (Schauer 2009). This helps to shield antigenic sites which can weaken immunoreactivity; but in cases of lost polysialylations, could possibly lead to autoantibody production at antigenic sites where prior bacterial and viral infections have occurred (Schauer 2009). Sialic acids also play a role in the antagonistic effects on receptors like (TrKA) FGF2, EGF, VEGF, LYVE-1, and B1 integrins which bind galectin-3 for anti-apoptotic effects (Schauer 2009).

Gangliosides have the ability to bind to galectins, specifically siglec 4 (MAG4) which plays an important role in the ability for myelin from oligodendrocytes to bind to the axons of neurons (Schauer 2009). The gangliosides involved in this association with the myelination of neurons are GT1b and GD1a but not GM1 or GD1b. When null mice lacking the complex gangliosides GT1b and GD1a were observed for their neuronal competence, the neurons displayed demyelination and axonal damage which was comparable to null mice lacking MAG (Lopez and Schnaar 2009). Siglec-7 has a very strong association with gangliosides from the b-series including GD3, GD1b, and GT1b (Lopez and Schnaar 2009). This interaction has been

shown to modulate natural killer cell responses and may lead to immune misrecognitions of the self (Lopez and Schnaar 2009).

Sialic acids found on glycoproteins known as selectins have also been studied for their involvement in the functional immune system. It has been shown that E, L, and P-selectins preferentially bind to the tetrasaccharides sialyl-Lewis$_x$ and sialyl-Lewis$_a$ (Schauer 2009). This allows for a point of entry for circulating lymphocytes by tethering themselves to the sialic acid moieties of these Lewis structures leading to the internalization and invasion of multiple immune system effector cells. TNFα has also been known to increase the expression of E and P-selectins during inflammation so this suggests a possible pathway involving sialic acid containing molecules working synergistically with the pro-inflammatory cytokines to achieve clearance of pathogens and apoptosis of dysfunctional cells.

Collectively, this evidence suggests a molecular mechanism in the progression of multiple sclerosis through neuronal apoptosis. This mechanism involves activated microglia which secrete TNFα or other pro-inflammatory cytokines and neurons which express MBP, MAG, growth factor receptors, and gangliosides. These factors are naturally occurring in the brain and have been previously investigated as potential mechanisms of neuronal cell death associated with MS. Since gangliosides have a ceramide moiety that can embed itself in the cell membrane, gangliosides can potentially affect the viability of the cell directly by interfering with the activity of the mitochondria via release of cytochrome c, activation of multiple caspases, and ultimately cell death. Additionally, gangliosides may reduce neuronal viability by interfering with growth factor receptors, reducing the signal needed for cells to remain viable and ultimately causing the neuron to die.

The hypothesis tested by this dissertation research is that TNFα instigates cell death of brain cells by interacting with gangliosides (especially GD3) in a way that promotes pro-inflammatory cell cascades and inhibits pro-growth and differentiation of cell processes.

Chapter II

VIABILITY OF NEUROBLASTOMA CELL LINES

TNFα Administration With and Without Gangliosides for Viability Testing

OBJECTIVE

To explore the possibility that a pro-inflammatory cytokine, TNFα, can initiate cell death to a degree dependent on the presence of a specific ganglioside profile, my initial project was to test the effect of TNFα on a family of neuroblastoma cell lines genetically modified to alter their ganglioside profiles. Neuroblastoma cells designated F11-A served as controls; lacking STII or GNT, they are unable to synthesize GD3 or any more complex ganglioside. The STII F11-A cell line consists of F11-A cells transfected with a coding insert for STII, so that GD3 can be synthesized. The GNT F11-A cell line consists of F11-A cells transfected with a coding insert for GNT, so that GM2 and GM1 can be synthesized, as well as all disialogangliosides, if STII is also present (see Fig. 1). All cells were tested for viability/cell death by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay, allowing a determination of whether and which gangliosides play a role in the signal transduction of cell death.

METHODS

F11-A Neuroblastoma cell culture and MTS viability assay

F11-A neuroblastoma cell lines (F11-A, STII, and GNT) were thawed from -140 Cº and plated in T-25 cell culture flasks with 5ml of DMEM culture media containing 10%FBS and 1% pen/strep. Flasks were grown to ~80% confluency and passaged to T-75 flasks containing 25ml of complete culture media. Cells were then grown to ~80% confluency and trypsinized. Cells were then collected in 50ml conical tubes and the trypsin was deactivated with complete media

containing FBS. 1 ml was retained for cell counting and the rest of the cell suspension was centrifuged at 1600 RPMs for 3 minutes to pellet the cells and the media was then poured off. The 1ml of the retained cells was counted using only 10µl of the 1ml cell suspension by hemocytometer and the centrifuged cells were resuspended to a final concentration of 100,000 cells per 1ml. 200ul (20,000 cells) were added to their appropriate wells in a 96 well cell culture plate and allowed to adhere for 24h. The cells were then treated with different concentrations of TNFα (1µM, 0.5µM, and 2µM) and incubated for 24h.The wells were then given 10µl of MTS assay reagent and incubated for 1-3h. The wells were read by a spectrophotometer at absorbance 490 to determine viability.

Assessment of ganglioside pattern

After detaching with a cell scraper, cells were aspirated to achieve a fine suspension, pooled from all flasks of each condition and centrifuged. The pellets were then resuspended and washed twice with 1X PBS, then spun down at 5000 x g for 5 minutes to remove cellular protein and debris. The pellets were then resuspended again in 2 ml chloroform/methanol (CM, 2:1) in 2 ml microfuge tubes and thoroughly homogenized to extract and isolate gangliosides from the cell membrane by the method of Irwin & Irwin (Irwin and Irwin 1979). The cells were incubated at 45º C for 15 minutes and spun down again at 10,000g for 5 minutes. The supernatant was applied to a 2 cm Unisil column pre-rinsed with 2 ml of CM (2:1) and flow-through then reapplied to the column. The non-gangliosides were be eluted with a 2 ml chloroform/methanol/ water (CMW, 65:25:4) rinse followed by elution of the gangliosides with 1 ml CMW (30:30:10). Gangliosides were eluted into clean, labeled 2 ml microfuge tubes and dried down by vacuum desiccation. Samples were resuspended into 25 µl of CM (2:1) and spotted on 5 mm lanes on HPTLC plates. The plates were ran in an organic solvent system containing CMCaCl$_2$

(50:40:10) to separate gangliosides of different polarity, based on their sugar and sialic acid contents. Plates were removed, dried, sprayed with resorcinol HCl, and placed on a pre-heated tempblock at 100 Cº for 20-25 min to visualize the bands. Gangliosides were identified by co-migration on the same plate with standards obtained from Matreya, Inc. (Pleasant Gap, PA).

RESULTS

Isolated F11-A cells did not express any complex gangliosides, while STII and GNT expressed complex gangliosides according to the function of the transfected enzyme (Fig. 2). STII cells did express GD3 (denoted by the red box), while GNT cells expressed monosialogangliosides and GD1a, but none of the more complex gangliosides for which GD3 is a required precursor.

Viability of the F11-A cells lacking STII and GNT enzymatic activity was substantially lower than those cells transfected with genes to code for the enzymes (Fig. 3). When treated with 0.5 or 1.0 μM TNFα for 24h, STII cells, which have GD3, did in fact show a significant reduction in viability, while GNT cells showed only a trend ($p<0.08$) toward a reduction in viability at the highest concentration of TNFα (Fig. 3).

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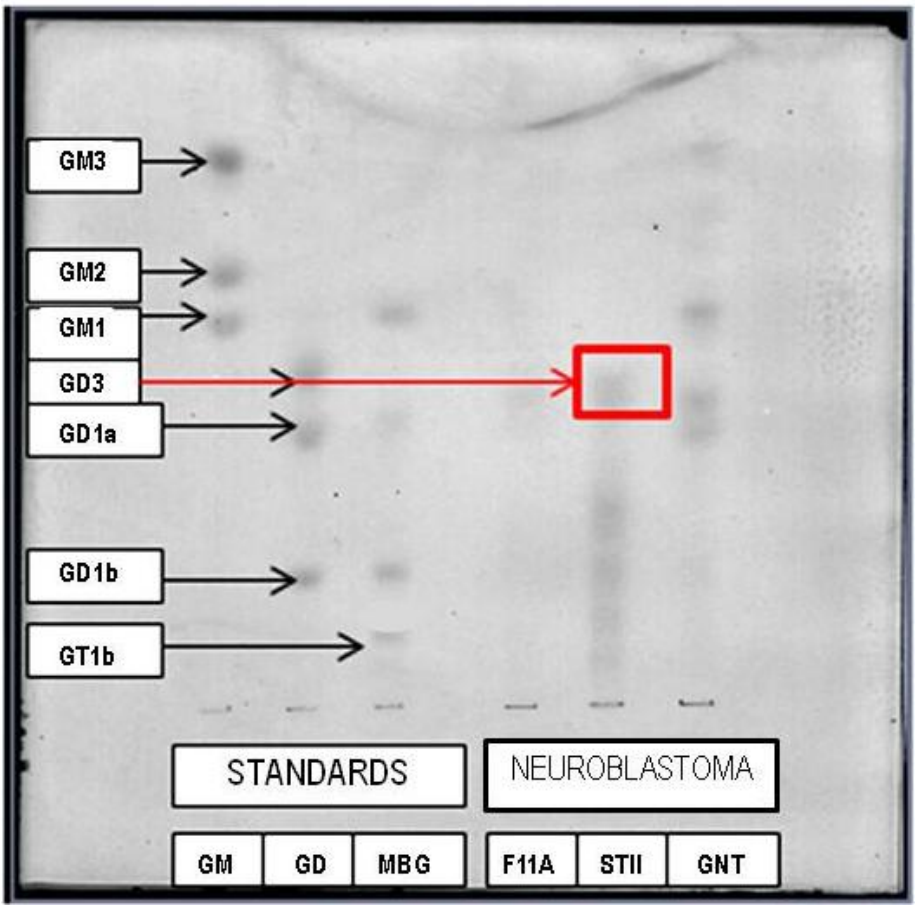


Fig. 2. HPTLC plate of F11-A, STII, and GNT cell lines for ganglioside expression. ~18,000,000 F11-A, 12,000,000 GNT, and 10,000,000 STII neuroblastoma cells were grown and gangliosides were isolated to obtain HPTLC banding patterns for each cell type. Aliquots of gangliosides (5 μg for standards) were spotted in each lane and developed in a solvent of CM0.2%$CaCl_2$ (50:40:10), then visualized with resorcinol reagent. Standards consisted of monosialogangliosides (GM), disialogangliosides (GD) and mixed brain gangliosides (MBG).

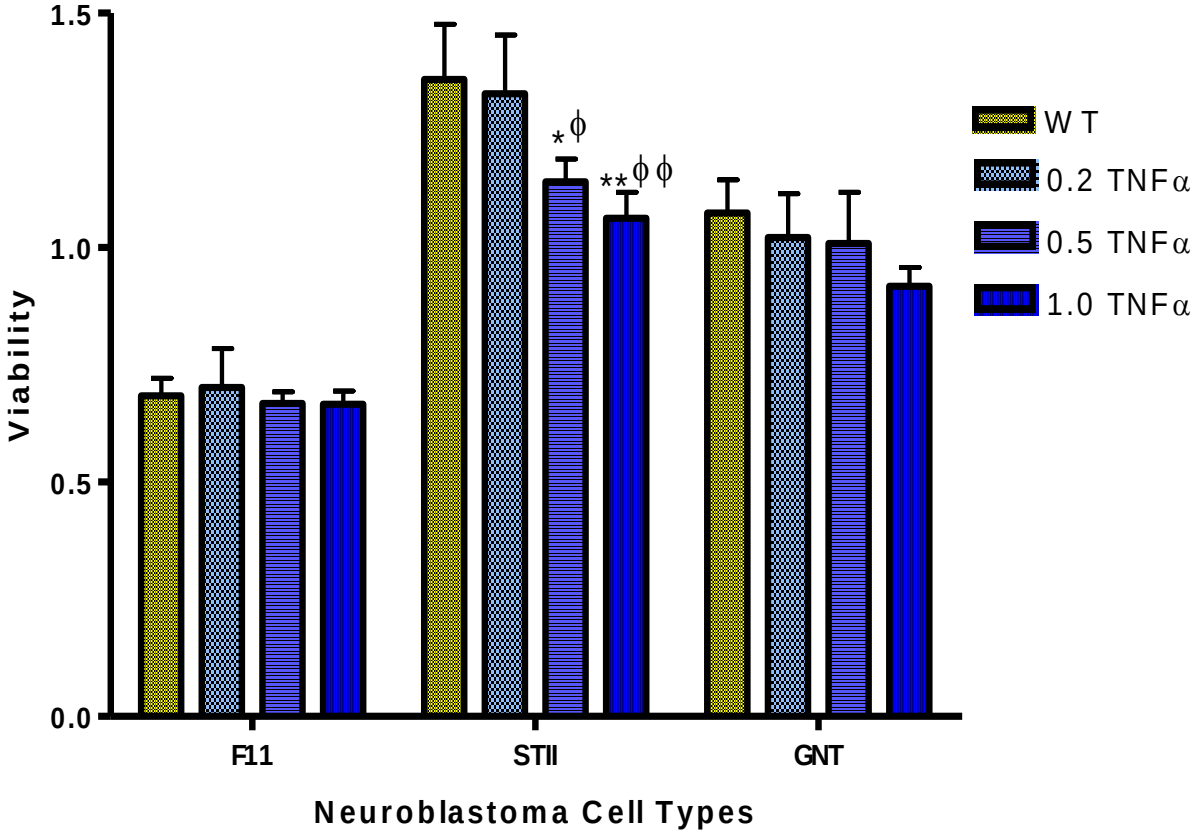


Fig. 3. Viability of F11-A, STII, and GNT neuroblastoma cell lines treated with variable concentrations of TNFα. 20,000 cells from each line were plated in 96 well cell culture plates and treated with 0.2μM TNFα for 24h. The significance of differences in viability within the two altered cell lines compared to wild type control (WT) cells was assessed by Tukey's multiple comparison test, with n=6 in all cases. *$p<0.01$, **$p<0.001$, ϕ$p<0.05$, ϕϕ$p<0.001$

DISCUSSION

The evidence that gangliosides play a role in cell death is continuing to mount. One way to test this hypothesis is to remove the gangliosides from the cell and try to stimulate the cell to undergo cell death by the administration of a pro-inflammatory cytokine like TNFα. One of the unique attributes of F11-A neuroblastoma cells is their lack of the enzymes STII or GNT, which means they should only express ganglioside GM3. In conjunction with having F11-A neuroblastoma cells, we have two other neuroblastoma cell lines that have been transfected with either enzyme, STII or GNT, both of which express a unique and distinct ganglioside banding pattern (Fig. 2).

This family of neuroblastoma cells allowed me to look at the possible association between specific gangliosides and TNFα, essentially acting as a knockout experiment for viability determination. Though substantially lower in viability than the cells expressing STII or GNT activity, F11-A cells treated with the varied concentrations of TNFα ranging from 0.2μM-1.0μM showed no noticeable decrease in viability. On the other hand, F11-A cells transfected with the STII gene showed a significant decrease in viability when stimulated with 0.5 or 0.1μM TNFα for 24h, compared to untreated, STII transfected F11-A neuroblastoma cells and STII transfected cells treated with 0.2μM TNFα (Fig. 3.) This is probably due to the increased amounts of GD3 brought on by the STII enzyme, which specifically adds a sialic acid residue to the precursor ganglioside, GM3. This suggested that gangliosides higher than GM3 may play a role in apoptosis when stimulated with a pro-inflammatory cytokine like TNFα.

.As for the GNT transfected F11-A neuroblastoma cells, there was a tendency toward decrease in viability at the highest concentration of TNFα, but this cannot be considered

significant ($p<0.08$). Thus, in the absence of GD3 or its catabolic precursors, GD1b and GT1b, no significant effect of TNFα is evident.

Chapter III

VIABILITY OF MIXED BRAIN CELL CULTURES

Experiment #1: Effect of Gangliosides and TNFα on Viability of Primary Brain Cells in Culture

OBJECTIVE

A cell culture system was established in order to determine whether individual gangliosides play a role in cytokine-mediated death of brain cells. The use of primary brain cell cultures represents a more physiologically relevant situation, as opposed to immortalized cells, like PC12 cells, or the neuroblastoma (F11-A) cell lines used in the first experiment.

Numerous studies have established that GD3 is a critical factor in apoptosis, but the same role for other gangliosides, with their different ceramide moieties and carbohydrate chains, has not been investigated. A stable, more physiologically relevant cell culture system was needed in order to study the mechanisms thought to be involved in neurodegeneration causing neuronal death. The purpose of this experiment was to determine which gangliosides, in the presence or absence of TNFα, contribute to cell death of primary brain cells in culture. The MTS viability assay revealed that TNFα and GD3, but not GD1b or GM1, caused substantial cell death in this *in vitro* system.

METHODS

Primary Cell Culture

Cultures of primary brain cells were established from pooled brain sections removed from post-natal day 1 (PND1) Sprague Dawley pups following quick decapitation. Under sterile culture conditions, cortical and hippocampal sections were dissected out and digested by 1mg/ml papain in 1.1mM EDTA, 0.067mM mercaptoethanol and 5.5mM cysteine-HCl. Tissues were

triturated by Pasteur pipette and the supernatant removed and centrifuged at 1000 RPMs for 2 minutes. The supernatant was then removed, and fresh Neurobasal™ (Invitrogen) medium containing 5ng/ml FGF, B27 supplement, 0.5mM glutamine, and 0.01% pen/strep was added to the cultures. The cells were finally resuspended to 1 x 10^6 cells/ml, added to T-25 Primaria™ (BD Falcon) flasks with a total volume of 5-6 mls of complete Neurobasal media, and incubated at 37° C under 5% CO_2 and 90% humidity.

Ganglioside and TNFα administration

Preliminary experiments were conducted in an attempt to reproduce the existing and sometimes controversial evidence that gangliosides are directly involved in neural cell death. Wells were exposed initially to mixed brain gangliosides (MBG), TNFα, and varying combinations of both together in a concentration gradient fashion (see Fig. 4 for plate setup). Mixed brain gangliosides were suspended in 10-fold dilutions, ranging from 10μg to 0.1μg, while TNFα treatments were serially diluted 10-fold to a final concentration range of 1μM to 0.01μM .

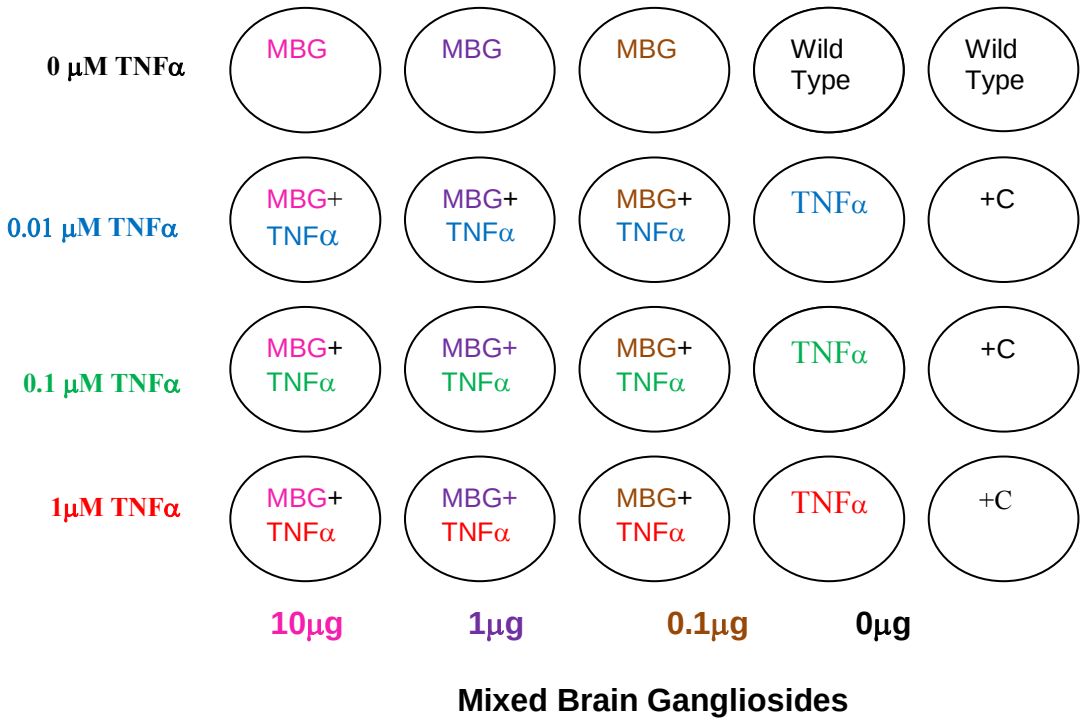


Fig. 4. Plate setup for performing Ganglioside-TNFα dose response assay. Wells contained ~100,000 cells and were treated with either 10μg, 1μg, or 0.1μg MBG in combination with 1μM, 0.1μM, or 0.01μM TNFα for 24h. Rows represent TNFα concentrations while columns represent ganglioside concentrations.

A second assay tested wild type controls and positive controls for cell death against 10 fold dilutions of GD3, from 1.6nM - 160μM, with a constant TNFα concentration (200nM) in order to determine a dose response effect for GD3 (Fig. 6a). In parallel, another plate was set up with the treatment conditions reversed -- 10 fold dilutions of TNFα starting with 200nM in wells containing a constant GD3 concentration of 160μM (Fig. 6b).

Later experiments involving gangliosides GD3, GD1b, and GM1 were tested individually at 160μM in 24 well plates containing ~100,000 neurons per well. Each ganglioside type at it's160μM concentration was tested in combination with 0.2μM TNFα for 24h. Three wells containing cells but neither gangliosides nor TNFα provided wild type controls. Three additional wells contained cells to which lysis buffer was added to provide a baseline measure for total cell death. Figure 7 shows the time courses study using specifically 160μM GD3 in combination with 0.2μM TNFα for 24, 48, and 72h.

Viability Assay/ Determination of Cell Death

Cell viability was assayed using 20μl of the MTS cell proliferation assay (Promega) on 100,000 primary neurons/well in a 24 well plate. Cells were incubated for 24h in complete Neurobasal medium in a total volume of 0.5ml per well. Plates were then removed from the incubators; 20 μl of MTS reagent were added to a total volume of 200μl, and the cultures were allowed to incubate for 2-4h. The solutions were then removed from the 24 well plates and transferred to 96 well plates and read at an absorbance of 490 nm, which provided a measure of cell viability.

This approach enabled us to test the effect of varying amounts and types of gangliosides with variable amounts of TNFα on (a) viability of brain cells in culture, and (b) the ganglioside

profile of the surviving cells (described later). This enabled us to determine if gangliosides were involved in the initiation of cell death.

RESULTS

Overall, exogenously administered gangliosides cause a significant decrease in primary brain cell viability (Fig. 5-7).

Within the concentrations tested for either mixed brain gangliosides or TNF□, the effect was not dose dependent, (Fig. 5). Ganglioside GD3 also depresses viability to essentially the same degree at all concentrations tested in combination with TNFα, with only a weak dose effect at the higher concentrations (Fig. 6a). When primary brain cell cultures were treated with a constant 160μM GD3 concentration in combination with 10 fold dilutions of TNFα (200nM-2pM), there was a significant decrease in viability when compared to wild type cells but not between the different combinatorial treatment groups.

After 24h, cell viability was significantly reduced by GD3+TNFα (Fig. 7a). After 48h, GD3 and TNFα alone, as well as in combination, significantly reduced viability (Fig. 7b). However, after 72h no significant reduction in cell viability was seen in response to all treatment conditions.

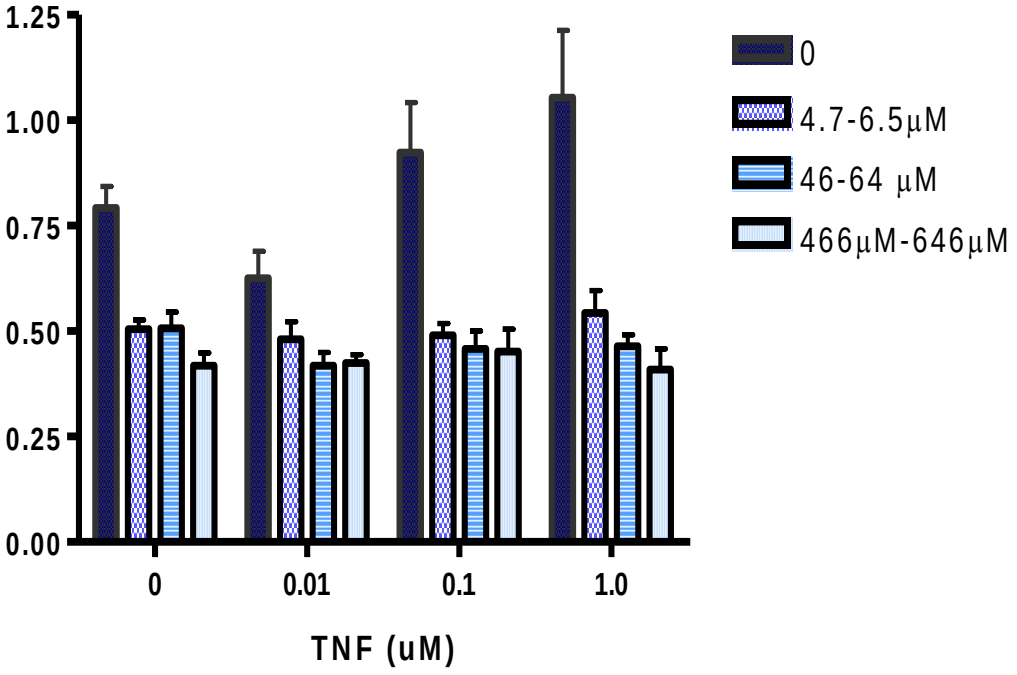
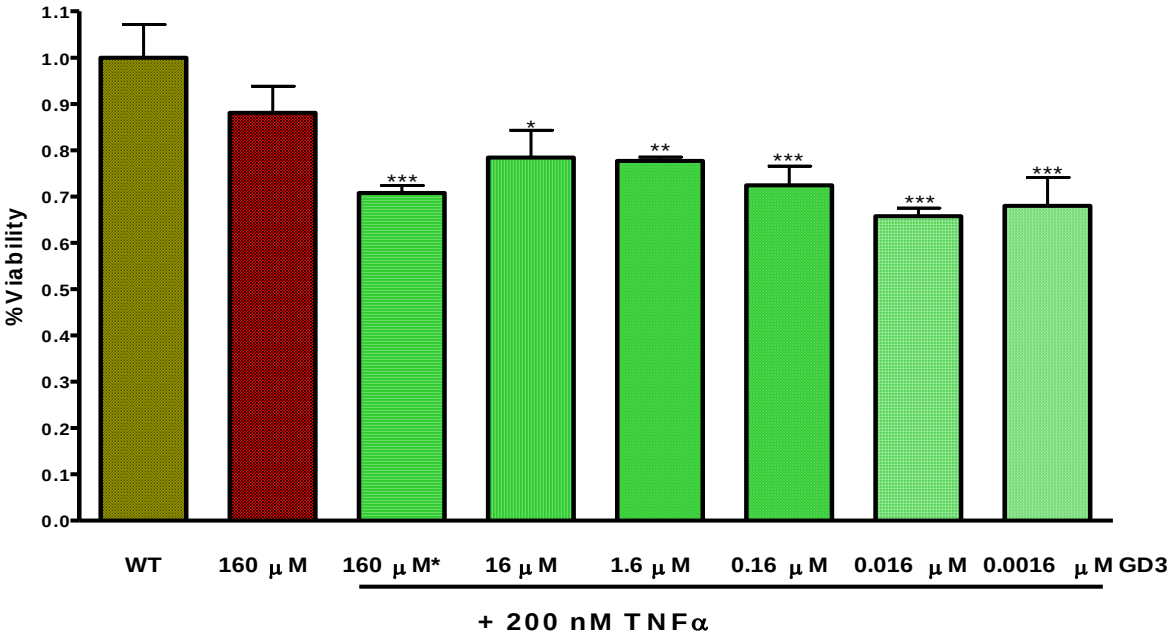


Fig. 5. Viability of primary brain cell cultures treated with mixed brain gangliosides (MBG) and TNFα. 100,000 primary brain cells/well in 24 well culture plates were treated with 0, 4.7-6.5μM, 46-64μM, and 466-646μM MBG in combination with 0, 0.01, 0.1, and 1μM TNFα for 24h. The effect of MBG concentration was assessed relative to cells untreated by MBG within each concentration of TNFα by One-Way ANOVA with Tukey's Multiple Comparison Post Test, with n=6 for each independent treatment. *p<0.01, **p<0.001

a)



b)

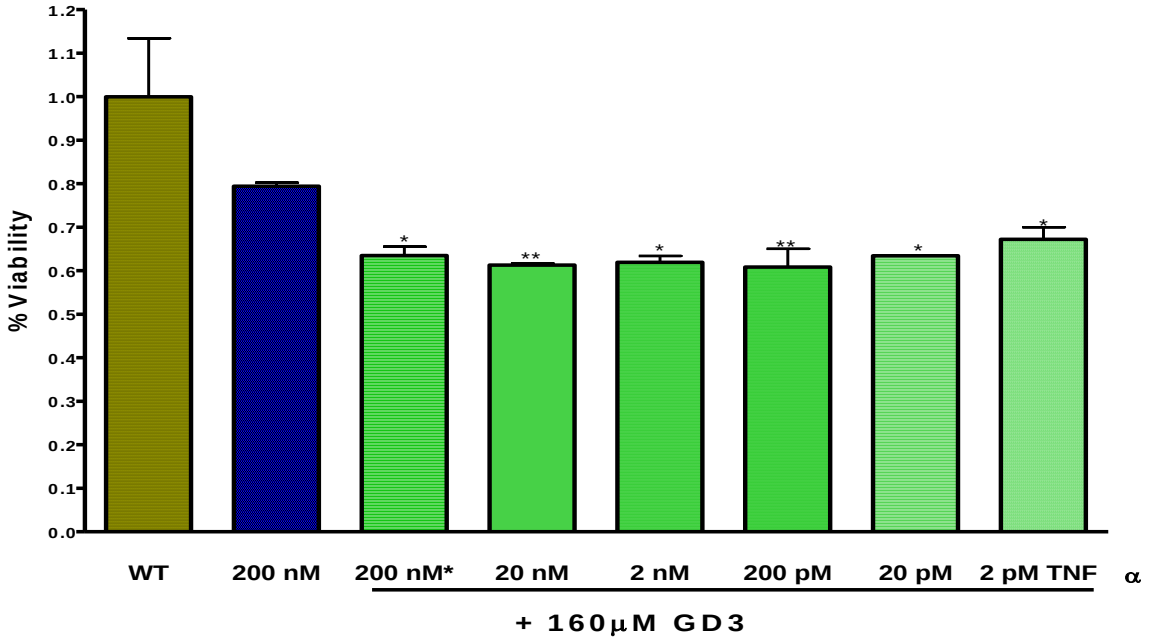


Fig. 6. Dose response for MTS viability assay for GD3+TNFα. 100,000 primary brain cells/well were treated in 24-well culture plates for 24h with (a) constant 0.2μM TNFα concentration in conjunction with varied concentrations of ganglioside GD3 ranging from 0.0016μM to 160μM, or (b) with a constant 160μM GD3 concentration with varied concentrations of TNFα ranging from 2pM to 0.2μM. Statisitcal significance of differences compared to WT controls was evaluated by a One-way ANOVA with Tukey's multpile comparison post test, with n=3 independent experiments. *p<0.05 **p<0.01, ***p<0.001

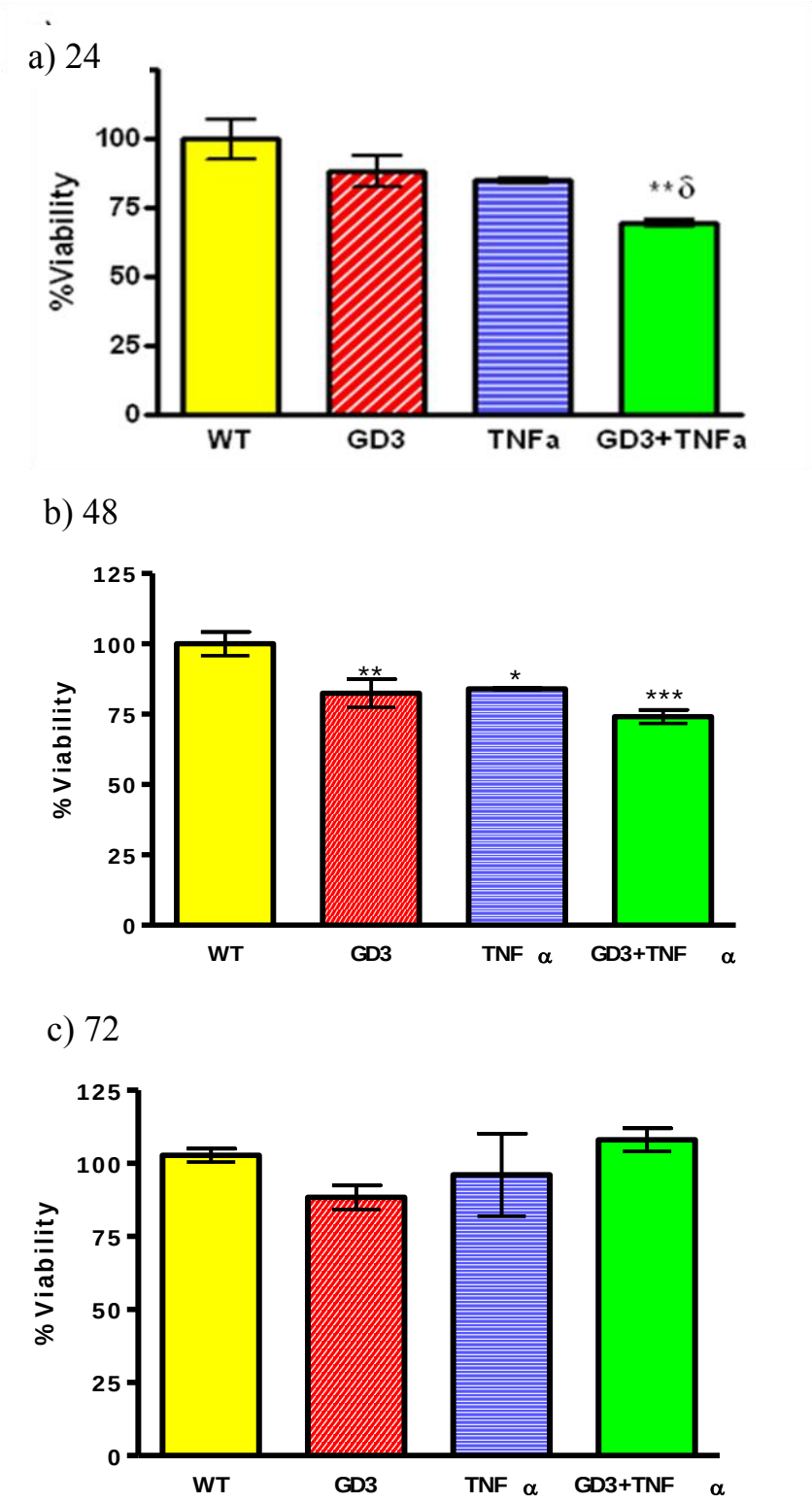


Fig. 7. Effect of ganglioside GD3 and TNFα administration on viability of primary brain cells over a 72h time course. 100,000 primary brain cells/well in 24 well culture plates were exposed to 160μM GD3+0.2μM TNFα for (a) 24h, (b) 48h, or (c) 72h Bars represent mean +/- s.e.m. The significance of differences across treatments was analyzed by One-way ANOVA, with Tukey's multiple comparison test for evaluating differences between specific treatments. *p<0.05,**p<0.01,***p<0.001, compared to WT; δp<0.05, compared to GD3.

DISCUSSION

Much evidence has appeared in the past decade suggesting that neuronal cell death is mediated in part by gangliosides, specifically GD3, or by some of the biosynthetic/metabolic pathways involved in ganglioside biosynthesis. Previous research has suggested that GD3 affects neuronal viability via mitochondrial pathways by (1) increasing the overall endogenous pool of GD3 within the neuron through the release of ROS that cause caspase activation, and by (2) activation of the specific sialidase enzyme Neu4L, which is mitochondrially located and brain cell specific (Garcia-Ruiz, Colell et al. 2002; Melchiorri, Martini et al. 2002; Hasegawa, Sugeno et al. 2007). A relatively easy way to analyze cell viability is by the MTS assay. If gangliosides and/or TNFα do cause a reduction in cell viability, MTS assays would be a fast way to obtain data for further hypothesis testing.

The literature states that the critical micelluar concentration (CMC) for disialo-gangliosides in an aqueous solution is in the range of 10^{-6} μM (Ulrich-Bott and Wiegandt 1984) but micelles are involved in direct interactions with cells and may interact in three different ways with their cellular counterparts (Schwarzmann 2001). Most studies have used exogenous gangliosides above their critical micelle concentration, ranging from molar concentrations at 10μM, to weight to volume amounts at 50μg/ml and higher in 6 well, 60mm^2, and 100mm^2 cell culture plates (Melchiorri, Martini et al. 2002; Simon, Malisan et al. 2002; Jou, Lee et al. 2006).

The first test combined 0, 4.7-6.5μM, 46-64μM, and 466-646μM of mixed brain gangliosides with 0.01, 0.1 and 1.0μM TNFα co-incubated for 24h to test whether gangliosides and TNFα could indeed reduce the viability of primary mixed brain cells in culture. The experiment was designed (Fig. 4) to determine if there was any interaction between gangliosides and TNFα. Overall, the MBG caused a significant loss of viability in all cultures treated with

and without TNFα. The results showed that (1) there is no media induced interaction with the MTS assay and (2) cell viability is significantly reduced primarily due to MBG rather than TNFα even when both substances are combined in any combination (Fig. 5). This also showed that there is no optimal concentration for ganglioside mediated cell death within the range tested, since all concentrations killed the primary brain cell cultures relatively equally (no significant differences between combinatorial conditions) when compared to wild types. Overall, in this experiment using MBG, there seemed to be an increase in the viability of the primary brain cell cultures treated with varied amounts of TNFα alone.

We then decided to test a concentration range of the implicated cell-death mediator GD3 ganglioside, ranging from 1.6 nM to 160 μM based on the vast array of concentrations tested above the CMC stated in the literature, and lack of lower concentrations tested, while using a constant TNFα concentration that would be within the range showing a decrease in viability according to the first MBG experiment (Fig. 5., 0.2μM TNFα). There was a significant decrease in viability in all conditions when compared to the wild type, but there was NO SIGNIFICANT decrease in viability amongst the 10-fold dilutions series of ganglioside GD3 conditions with a constant TNFα concentration of 200nM (Fig. 6a). The opposite holds true for 10 fold dilutions of TNFα ranging from 2pM-200nM with a constant GD3 concentration of 160μM (Fig. 6b).

In an attempt to determine if GD3, but no other ganglioside, decreases brain cell viability, more primary brain cell cultures were harvested and grown under the previously described conditions, and treated with ganglioside GD3, GD1b, and GM1 in the presence of 0.2μM TNFα for 24h.

GD3 exposure showed a trend toward reducing cell viability slightly more strongly than GD1b or GM1, but the difference was not statistically significant. Subsequent experiments were

carried out using GD3 only, while both GD3 and GD1b were used in the gene expression studies in the next section.

Fig. 7a shows that after 24h, the only condition that significantly reduced viability was the combination of GD3 and TNFα. After 48h, all conditions caused a significant reduction in viability when compared to the untreated wild type primary brain cells (Fig. 7b). After 72h, GD3 alone appeared to reduce viability, but the different was not statistically significant (Fig 7c).

These data give support to the possibility that gangliosides, specifically GD3, can alter primary brain cell viability. But GD3 in not normally expressed in the adult mammalian brain unless GD3 expression is up-regulated during pathological conditions. This increase in GD3 could be due to an increase in biosynthetic enzymatic activity needed to increase the overall endogenous ganglioside pools, to an increase in glycosidase enzyme activities that modify complex gangliosides to simpler forms like GD3, or to the neosynthesis of GD3 by the brain cells specifically for its role in cell death pathways. This led to a test of whether or not TNFα causes an increase in the mRNA expression of the biosynthetic enzymes responsible for ganglioside biosynthesis, STII and GNT.

Experiment #2 qPCR Analysis of Ganglioside Biosynthetic Enzyme GNT After TNFα Administration on Primary Brain Cell Cultures

OBJECTIVE

The ability of the pro-inflammatory cytokine, TNFα, to affect ganglioside biosynthesis was tested by analyzing its effect on the gene expression of GNT, which converts GD3 to GD2, and STII, which converts GM3 to GD3 in primary brain cell cultures.

METHODS

Primer Design and qPCR

To obtain a realistic measure of inter-animal variation, total RNA was extracted from each regional sample of individual rats. mRNA was then reverse transcribed into unlabeled cDNA templates using the Verso™ cDNA Kit (ABgene, Thermo Scientific). Specific forward and reverse primers for candidate genes were designed using Primer Designer™, Version 2.0 (Scientific & Educational Software), and synthesized by IDT, Inc. (Coralville, IA). The FastStart SYBR Green Master® (Roche Diagnostics) fluorescent labeling kit and custom primers (0.4 mM) were used to perform qPCR using the iCycler™ (Bio-Rad) and the following protocol: 40-60 cycles, each consisting of 30 sec for melting, 30 sec for annealing, and 20 sec for elongation. All samples were run in duplicate along with a standard curve to calculate copy numbers of mRNA for each gene, and normalized to ribosomal 18S RNA as an internal standard.

RESULTS

There was a significant increase in GNT expression but not in STII when TNFα was applied to primary brain cells in culture for 24h (Fig. 8).

Table 1. Sequences of forward and reverse primers used for qPCR

Gene	Primer	Oligo
GNT	F'	5'- GTA ACA CTG GTT GGT TGT
	R'	5'- CTT GAT GTC CGC CTG TC
STII	F'	5'- CAA CGA GAA GGA GAT TGT GC
	R'	5'- CTG AAG AGA CTG GCT GAC G

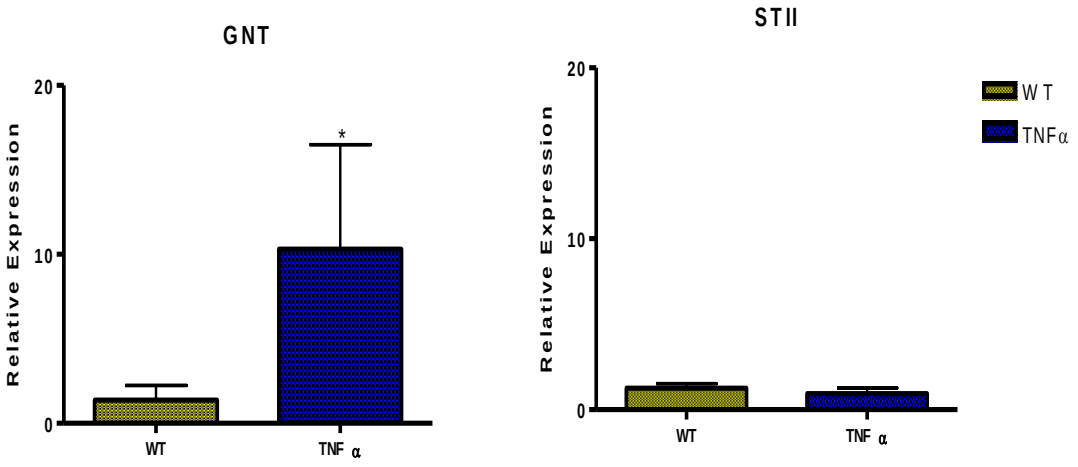


Fig. 8. Effect of TNFα on the gene expression of GM3/GD3 synthase (STII) and GD3/GD2 synthase (GNT) in primary brain cell cultures. 1,000,000 primary brain cells were treated with 0.2μM TNFα for 24h and mRNA was isolated for qPCR analysis. The graph represents the expression of the ganglioside biosynthetic enzymes GM3/GD3 synthase (STII) and GD3/GD2 synthase (GNT) in WT and TNFα treated primary brain cell cultures. Statistical analysis was performed by Mann-Whitney U test and significant values are represented by * ($p<0.0003$, $n=8$).

DISCUSSION

If the hypothesis that TNFα and gangliosides act together in some way to initiate cell death is correct, then TNFα should affect the gene expression of STII and/or GNT. The relative gene expression of STII was not affected when TNFα treated cultures were compared to normal wild type cultures. There was, however, a significant increase in the relative gene expression of GNT when TNFα -treated cell cultures were compared to wild type cultures (Fig. 8). Since most of the gene expression data (Section IV) show that GD3 and GD1b have similar effects on primary brain cell cultures, it is possible that GD1b may have a similar effect to that of GD3. It is possible that more time is needed to increase the overall pool of gangliosides, especially if STII and GNT work in conjunction with and activate one another to increase more complex gangliosides such as GD 1b, in order to increase endogenous GD3 by glycosidase cleavage of GD1b. GD3 is not normally expressed past gestational development of the brain and cannot be found in the adult brain, unless reactive gliosis or brain injury occurs, probably due to the concerted actions of these enzymes (STII and GNT). Thus it is plausible that cytokine stimulation increases ganglioside biosynthesis first, like the increase seen in GNT, and then therefore initiates a cell death cascade causing an increase in the normally expressed gangliosides like GD1b first, which may be needed to increase the overall pool of gangliosides that could eventually be modified by glycosidase cleavage, thus causing a downstream effect by increasing the cellular amounts of GD3. This could explain why STII- transfected neuroblastoma cells showed an immediate and significant decrease in viability when exposed to TNFα for 24 hours due to their already present GD3 supply and unneeded use for enzymatic modifications to produce GD3 while GNT transfected neuroblastoma cells had a smaller but marked decrease in viability but nothing that was significant (Fig. 3). GNT transfected cells do contain two isoforms

of GD1a and smaller amounts of GD1b which may account for the noted reduction in viability in these cells, but it is possible that more time is needed to see similar, significant decreases in viability like those observed in STII cells.

Experiment #3 Analysis of Ganglioside Profiles by Mass Spectrometry

OBJECTIVE

The ganglioside content of brain cell cultures following exposure to exogenous gangliosides and TNFα independently was analyzed in order to observe if any changes are made to the endogenous pools of gangliosides that may be associated with cell death.

PROCEDURE

Cell culture

Brains were taken from PND1 rat pups by live decapitation as previously described. Cortex and hippocampus were dissected out of each brain and digested in 1mg/1ml of papain for 30 minutes at 37 Cº. Cells were then transferred to 10ml of complete Neurobasal media containing 1ml B27, 1 ml Pen/Strep, 100µl glutamine, and 100µl FGF with as little papain as possible and titurated by pasteur pipette aspiration. The cell suspension was allowed to sit for 1 minute and then the supernatant was transferred to a new 50ml conical tube. 1ml of cell suspension was retained for counting by hemocytometer while the rest of the cell suspension was centrifuged at 1100 RPM for 3 minutes. The cells were then resuspended to a concentration of 1,000,000 cells per 1ml and plated into 6 well Primeria™ plates at 1,000,000 cells per well. Cells were allowed to grow until they were differentiated and then treated with 3µM GD3, GD1b, 200nM TNFα and controls were untreated, primary brain cell cultures. Cell cultures

were incubated for 24h and then collected for ganglioside separation and analysis by mass spectroscopy.

Ganglioside Extraction

Approximately 1,000,000 primary cells were removed by scraping, rinsed with 2ml of phosphate buffered saline (PBS), and centrifuged at 13,000 RPMs. All PBS was removed and the cells were lyophilized. Lipids were extracted 3× with chloroform/methanol (2:1, v/v). The suspensions were centrifuged for 30 minutes at 1,764 *g* and the supernatants were dried down under a stream of nitrogen. Sonication combined with Folch's partitioning four times with 1ml of each polar solvent: (1) 1:1 chloroform-methanol, (2 and 3) 55:25:20 isopropanol-hexane-water with removal of the upper phase by aspiration before use, and (4) 1:1 chloroform-methanol)) was then used to separated neutral and negatively charged glycosphingolipids. The supernatants were removed after centrifugation and dried under nitrogen gas. The samples were then resuspended in 5ml 100% methanol.

Separation of Gangliosides

Samples were run through a SAX column that was previously washed with 4ml methanol, 2ml 80% acetonitrile/0.05% trifluoroacetic acid, and equilibrated with 10ml 100% methanol. The 5ml samples were applied to the column and rinsed with 6ml 100% methanol, after which they were eluted with 6 ml of 250mM ammonium acetate in 100% methanol. Samples were dried under nitrogen stream and desalted in a 3ml reverse phase cartridge (Discovery DSC-18, Supelco, Bellefonte, PA, USA). Cartridges were washed with 4ml 100% methanol, equilibrated with 5ml deionized water, and the samples were loaded in 5ml 0.1M KCl. Cartridges were then washed with 10ml water and then eluted with 10ml methanol, after which

they were dried down under nitrogen stream. Samples were then permethylated, dried, and resuspended in 200μl 100% methanol in preparation for mass spectrometry.

RESULTS

Ganglioside profiles in the mixed brain cell cultures treated with exogenous GD3, GD1b and TNFα give no indication of altered overall endogenous ganglioside pools (Fig. 9). These samples were too small and had some contaminants within them.

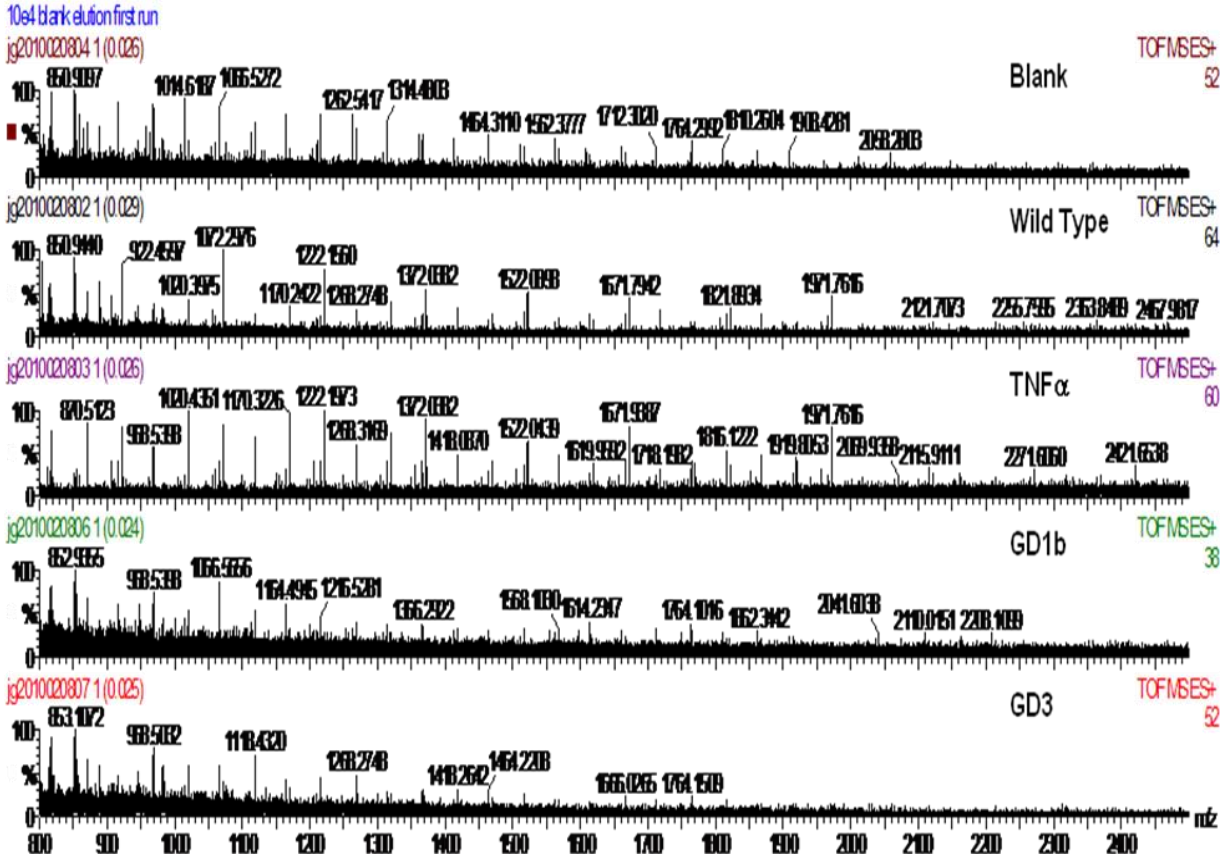


Fig. 9. Mass Spectrometry of gangliosides from primary brain cell cultures left untreated (wild type, WT) or Treated with TNFα, GD3, or GD1b. 1,000,000 primary brain cells were treated with 0.2μM TNFα and 3μM GD1b/GD3 for 24h. Gangliosides were extracted by Folch's partitioning and purified by a strong ion exchange column. Samples were permethylated and run through a QTOF mass spectrometer.

DISCUSSION

Gangliosides are constantly being recycled and modified at differential rates ranging from a few hours to about 48h. It is possible that brain damage or injury causes the release of gangliosides from other glial types, which in turn, modulate neuronal apoptosis. Nothing can be determined by the mass spectrometry data due to small sample amounts and contamination found within these samples. Future studies should included comparative standards for identification of specific ganglioside species and the use of more advanced mass spectrometers that can discriminate against background and possible contaminants.

Chapter IV

GENE EXPRESSION IN PRIMARY BRAIN CELLS IN CULTURE

Experiment # 1: Microarray Analysis of Primary Cultures Exposed to GD3, GD1b, and TNFα.

OBJECTIVE

The objective of these experiments was to determine which additional pathways and changes in gene regulation may be involved in the ganglioside-induced apoptotic pathway by using microarray analysis. Potential genes of interest were identified by their relative level of response in the cell during ganglioside-induced cell death using microarrays specifically targeted for cell signaling, cytokines, chemokines, and apoptosis. This yielded a number of novel and relevant targets involved in pathways of neural cell death governed by gangliosides that have yet to be identified.

At the completion of the cell viability experiments total RNA was collected from the cells, and cRNA probes were synthesized for microarray analysis of potential genes that may play a role in the apoptotic pathway. Those genes indicated as being differentially expressed by microarray assay were quantified by qPCR to determine their relative difference in mRNA expression over all conditions. The results suggested potential target genes for additional experiments on the apoptotic pathway possibly involved in neurodegenerative diseases, and enabled formulation of a hypothetical pathway involved in ganglioside mediated cell death.

METHODS

Microarray analysis of potential mechanisms and cRNA Probe Synthesis

Microarrays from SABioscience® for Chemokines and Receptors (ORN-22) and for Inflammatory Cytokines and Chemokines (ORN-11) were used to determine key role players in

this specific form of cellular death. One million primary brain cells were exposed to 3 μM exogenous GD3 and GD1b in separate wells, while other wells were exposed to only 0.2μM TNFα for 24hrs. Total RNA was isolated using the RNAqueous Micro™ (Ambion) kit and quantified using a Nanodrop® (ThermoScientific) spectrophotometer. RNA was reverse transcribed to cDNA which was then used to synthesize biotin-labeled cRNA probes using the Tru-Amp™ Labeling Kit (SABioscience). Labeled probes were then hybridized overnight to the microarray membranes and visualized by biotin excitation using the GEArray Chemiluminescent Detection Kit™ (Superarray). Images were taken of the arrays and transformed into TIF files. The images were scanned and normalized by Quanitity One™ according to the housekeeping genes at the bottom of the arrays. Percent differences were calculated and target genes were identified by their highly differential expression between control and treatment groups. This survey experiment was intended to identify potential molecular targets involved in ganglioside induced cell death.

RESULTS

Preliminary results (n=1) showed that GD3 and GD1b could in fact induce or repression the expression of multiple genes over a 24h incubation period (FGFR3 -21% for GD3, -19% for GD1b; TGFB2 -27% for GD3, -21% for GD1b) (Fig. 10) on the ORN-11 micro array. The preliminary data for the ORN-22 microarray also showed that GD1b could increase SCYE1 and NFκβ1 by 21% while increasing RDC1 by 30% over a 24h period (Fig. 11). Later studies were conducted in order to increase our sample size using the ORN-11 micro arrays (n=2). Control and treated cell cultures containing 1,000,000 primary brain cells (n=2) were lysed and the mRNA was isolated and collected for micro array studies. The ORN-11 micro array studies indentified 13 potential genes by housekeeping gene normalization using Quanitity One™, that

were affected by GD3, GD1b, or TNFα treatments (Fig. 12 & Table 2). The genes identified were: bone morphogenetic protein 2 (BMP2), transforming growth factor beta 2 (TGFB2), fibroblast growth factor 13 (FGF13), FGF receptor activating protein 1(FRAG1), transforming growth factor beta inducible early growth response (TIEG), interleukin 1 receptor 1 (IL1R1), chemokine ligand 2 (CCL2), interleukin 3 receptor alpha (IL3RA), interleukin 4 receptor (IL4R), interleukin 18 (IL18), Interferon-related developmental regulator 1 (IFRD1), Macrophage Inflammatory Protein-3 (MIP3A), and CCAAT/enhancer-binding protein beta (CEBPB).

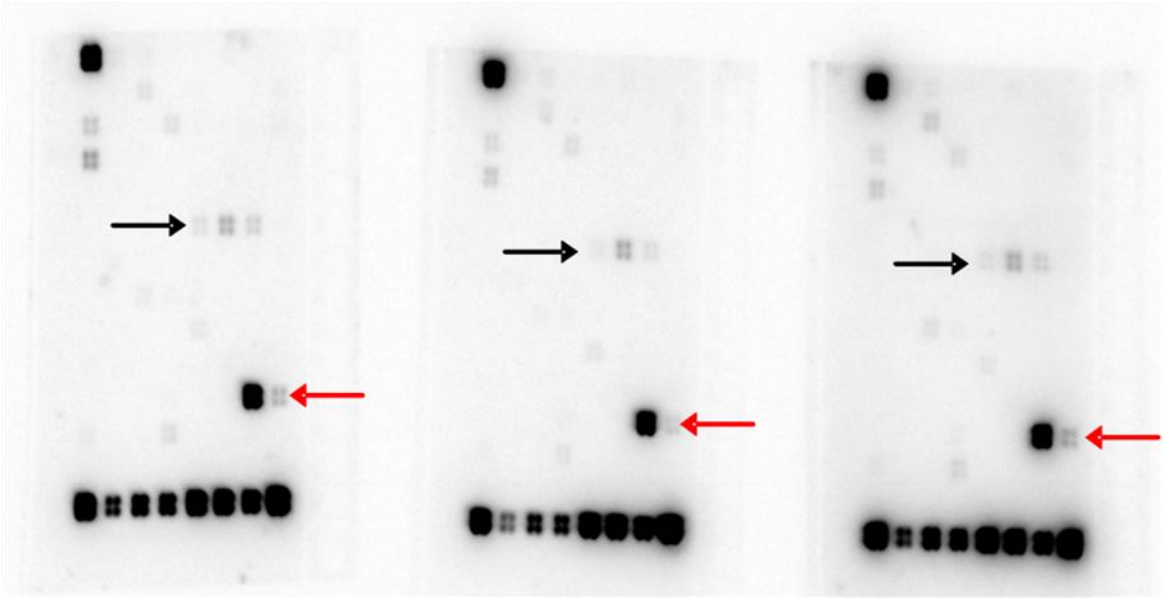


Fig. 10. Preliminary ORN-11 inflammatory cytokine microarray. The black arrows are indicating the relative gene expression of FGFR3. A 21% decrease was observed in the GD3 condition while the GD1b condition showed a 19% decrease in FGFR3 when compared to the WT control. The red arrows indicate the relative expression of TGFB2. A 27% decrease was seen in the GD3 condition whereas the GD1b condition observed a 21 decrease in the relative gene expression when compared to the WT control. Overall, both GD3 and GD1b showed a decrease in most of the genes but were too small of a decrease to be considered significant with an n=1.

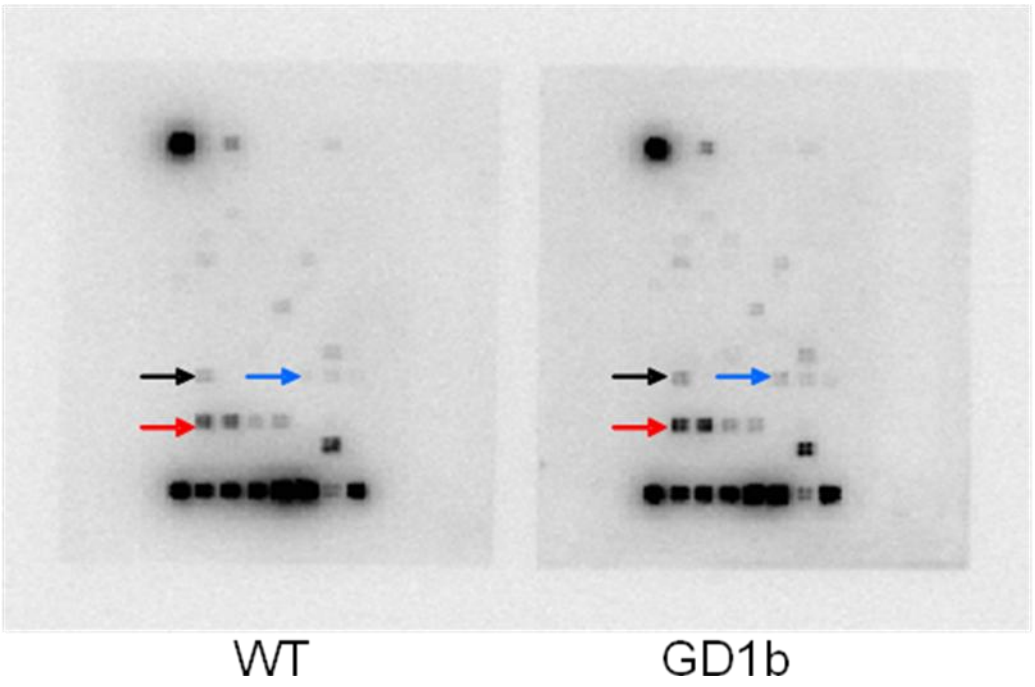
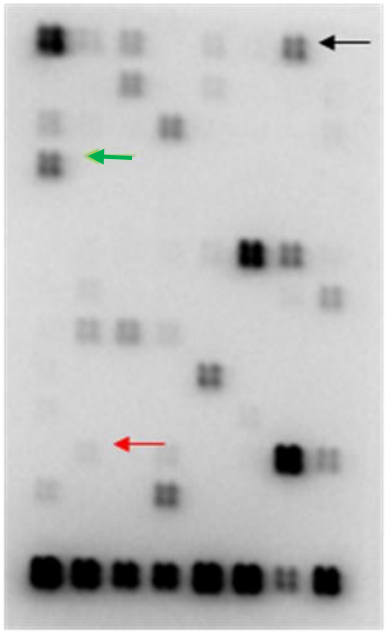
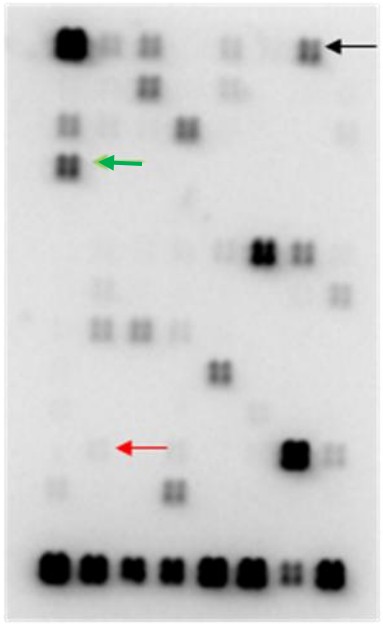


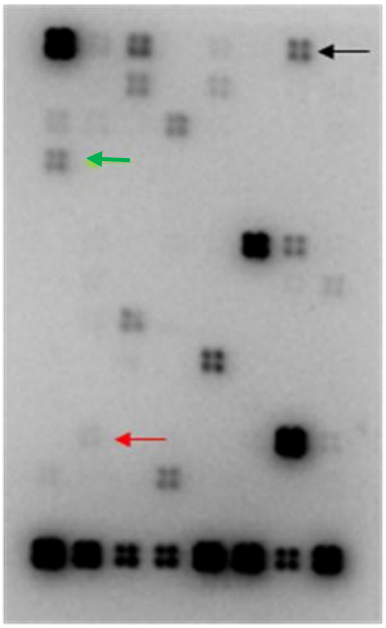
Fig. 11. Preliminary ORN-22 chemokines and receptors microarray. Microarray analysis of WT and GD1b treated primary neurons. Primary neurons were treated with ganglioside GD1b for 24hrs and their RNA was extracted for probe synthesis. The results were analyzed by normalizing the intensities of the scanned arrays and percent differences were performed. The black arrows are indicating the relative gene expression of NFκβ1. A 21% increase was observed in the GD1b treated neurons when compared to the wild type condition. The red arrows are indicating the relative gene expression of SCYE1 and a 21% increase was observed when compared to the wild type. The blue arrows indicate the relative gene expression of RDC1and a 30% increase was shown when compared to the wild type.



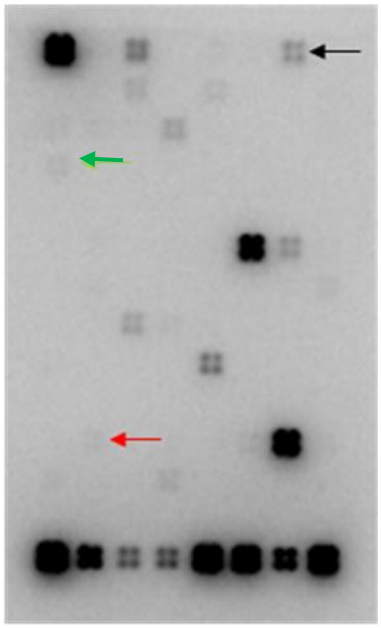
WT-1



WT-2



GD3-1



GD3-2

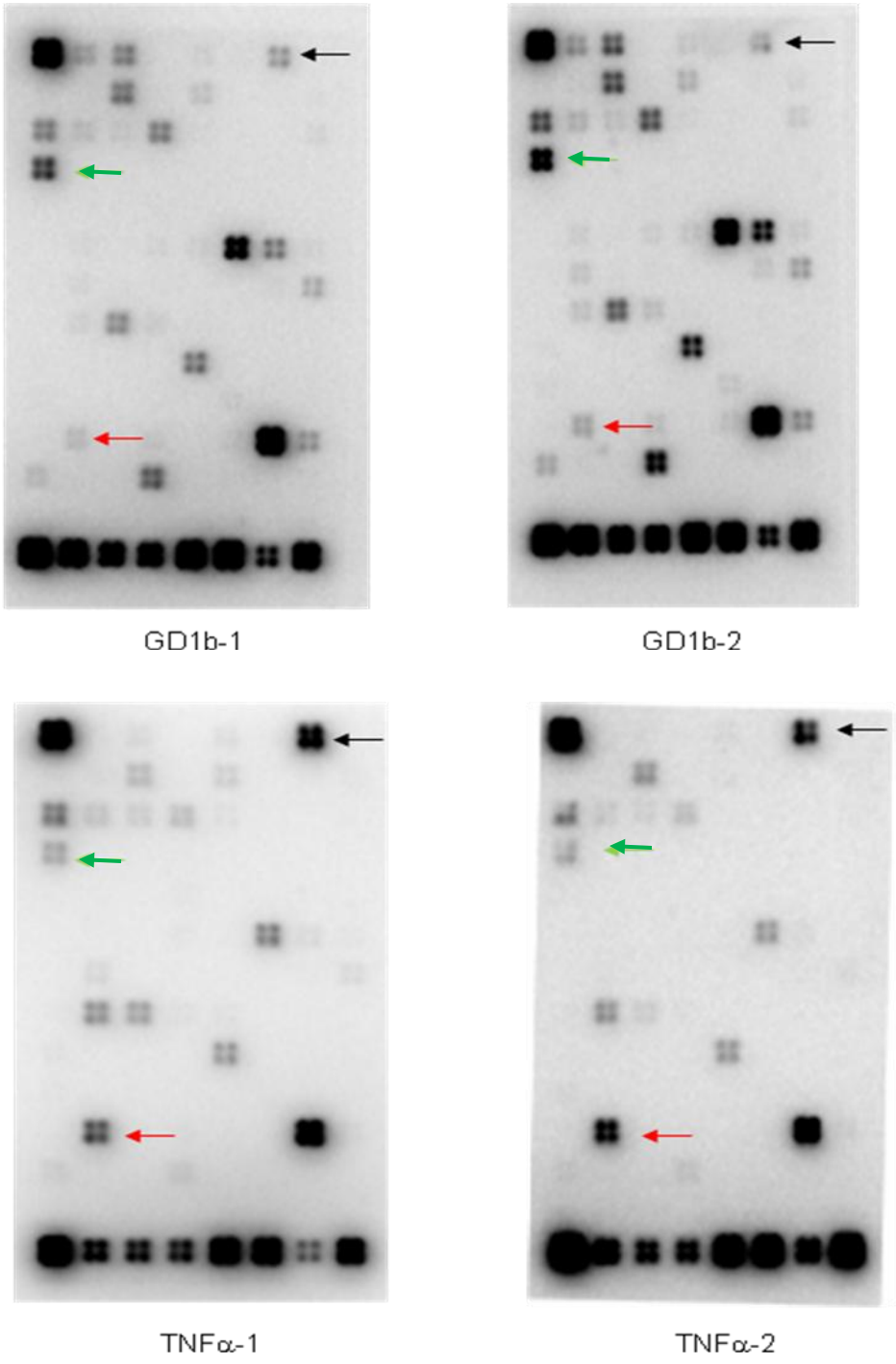


Fig. 12. Targeted ORN-11 microarrays for inflammation. Black arrows indicate CCL2, red arrows indicate MIP3A, and green arrows indicate FGF13. N=2 for each condition, housekeeping genes were normalized by Quanitity One™ (bottom row) and percent changes were calculated for all genes. Genes with the biggest percent difference between treatment conditions were chosen for qPCR quantification.

Table 2. Selected gene expression changes in primary brain cell cultures exposed to exogenous gangliosides and TNFα

Treatment Compared to WT			
GENES	**GD1b**	**GD3**	**TNFα**
Growth and Differentiation			
BMP2	16%	33%	-47%
TGFB2	-4%	-4%	-33%
FGF13	11%	-39%	-43%
FRAG1	-1%	21%	-59%
TIEG	11%	-15%	-44%
Immune System Effectors			
IL1R1	11%	1%	-16%
CCL2	-34%	-12%	73%
IL3RA	-5%	-17%	-44%
IL4R	0%	4%	-30%
IL18	-3%	-2%	-39%
IFRD1	2%	-10%	-61%
MIP3A	30%	31%	176%
Transcription Factors			
CEBPB	7%	-12%	-30%

Images from microarrays shown in Figs. 10-12 were quantified by scanning densitometry. This table represents micro arrays from Fig. 12. Numbers represent intensity differences reflective of mRNA levels relative to level of mRNA expression in WT controls. Results are based on one microarray membrane or the average of two membranes. Red text indicates genes that were significantly different when quantified by qPCR.

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Discussion

Based on these microarray results, 13 potential genes were picked for follow-up validation using qPCR (Table 2). The criterion for picking these genes was differential expression of at least 30% in at least one condition compared to controls. The use of microarrays was an important tool in the mining for potential genes involved in ganglioside-mediated cell death but was a less accurate method of analysis. Samples were pooled together which incorporates multiple animals, giving an overall look at changes in gene expression and allowed for enough mRNA to synthesize a probe for the microarray analysis. For Figures 10 and 11, the sample size was n=1, which isn't a big enough sample size to justify our findings. Figure 12 did have a sample size n=2 for each condition which gave a better indication of changes in gene expression. Microarray analysis is an excellent and fast method for finding large amounts of potential genes but does have some drawbacks. Microarrays are quantified by relative densitometry, which is relatively imprecise. If the microarrays membranes haven't been thoroughly washed, saturation and non-specific binding can become an issue when trying to quantify the relative densities of the microarrays. Another big drawback is the labeling process. This step is light sensitive and any exposure to light, or the length of time before scanning the membranes, can reduce the signal and give an inaccurate reading. This is what led to the use of qPCR, or quantitative polymerase chain reaction, which is an accepted method for microarray quantification. This gave a more precise look at the changes in gene expression, in real time, with better means of normalization.

Experiment #2-qPCR Quantification of Differential Gene Expression in Primary Brain Cells Exposed to GD3, GD1b, and TNFα

OBJECTIVE

The goal of this experiment was to quantify the differential expression of candidate genes isolated by microarray analysis in primary brain cells treated with gangliosides or TNFα using qPCR analysis.

PROCEDURE

Primer Design and qPCR

To obtain a realistic measure of inter-animal variation, total RNA was extracted from each regional sample of individual rats. mRNA was then reverse transcribed into unlabeled cDNA templates using the Verso™ cDNA Kit (ABgene, Thermo Scientific). Specific forward and reverse primers (Table 3) for candidate genes were designed using Primer Designer™, Version 2.0 (Scientific & Educational Software), and synthesized by IDT, Inc. (Coralville, IA). The FastStart SYBR Green Master® (Roche Diagnostics) fluorescent labeling kit and custom primers (0.4 mM) were used to perform qPCR using the iCycler™ (Bio-Rad) and the following protocol: 40-60 cycles, each consisting of 30 sec for melting, 30 sec for annealing, and 20 sec for elongation. All samples were run in duplicate along with a standard curve to calculate copy numbers of mRNA for each gene, and normalized to ribosomal 18S RNA as an internal standard.

Table 3. Sequences of Forward and Reverse Primers Used For qPCR

Gene	Primer	Oligo
BMP2	F' 395	5'- CAT CAC GAA GAA GCC ATC
	R' 801	5'- GAC CTG CTA ATC CTC ACA
CCL2	F' 84	5'- CTC TGT CAC GCT TCT G
	R' 362	5'- CTC ACT TGG TTC TGG TC
CEBPB	F' Pub Seq	5'- ACT TCC TCT CCG ACC TCT TC
	R' Pub Seq	5'- CAG CTT GTC CAC CGT CTT CT
IL3RA	F' 718	5'- CTG TGC TGG AGG AAG TC
	R' 828	5'- CTC GCA GTT CTC AGG TG
FGF13	F' 1365	5'- CCT CTC CTT CCT ACT GTC C
	R' 1606	5'- CAA TGC CAC TGT TCC AC
FRAG1	F' 1077	5'- CTC AAT GTG GTG GAG AAC
	R'1299	5'- CCG AGA AGA AGG AGA TGA AG
IFRD1	F' 427	5'- GAA GAC AAG ACA GGC AGC
	R' 1048	5'- GTC ACT CTC CAT TCC TC
IL18	F' 221	5'- GAA CAG CCA ACG AAT CC
	R' 296	5'- GTC ACA GCC AGT CCT CCT AC
IL4R	F' 2677	5'- GCT GAG GTC TGT GCT AAG G
	R' 3012	5'- CTA TGC CAG GAC TGC TGT G
MIP3A	F' Pub Seq	5'- AGA GTT TGC TCC TGG CTG
	R' Pub Seq	5'- GGA TGA AGA ATA CGG TCT GTG
TGFb	F' 1830	5'- CAG CAA CAA CAG CCA C
	R' 2072	5'- GCA GAG CAA TAC AGA GG
TEIG	F' 1121	5'- CCT TGC CTG TCA TCT G
	R'1451	5'- GTC TTG CCA CAT CCT G
IL1R1	F' 1766	5'-GCC AGT CAT CTG AAG AGC
	R'2091	5'-GCC AAG TGG TAA GTG TGT C

RESULTS

13 genes were identified by micro array analysis and primer sequences were designed for qPCR analysis (Table 3). Of the 13 genes tested, 6 showed significant changes in their expression in one or more treatment groups (13a-f). The expression of two genes, MIP3a and IL1R1, was up-regulated in cells treated with either GD3 or GD1b, but not by TNFα Conversely, the expression of FGF13 was down-regulated in cells treated with either GD3 or GD1b, but not by TNFα.

The expression of two other genes, FRAG1 and IL3RA, was down-regulated in cells treated with GD3 but not GD1b, and up-regulated by treatment with TNFα.

Cells subjected to all three treatments – GD3, GD1b, and TNFα – responded by up-regulation of the gene for CCL2. The effect was much stronger, however, in response to TNFα.

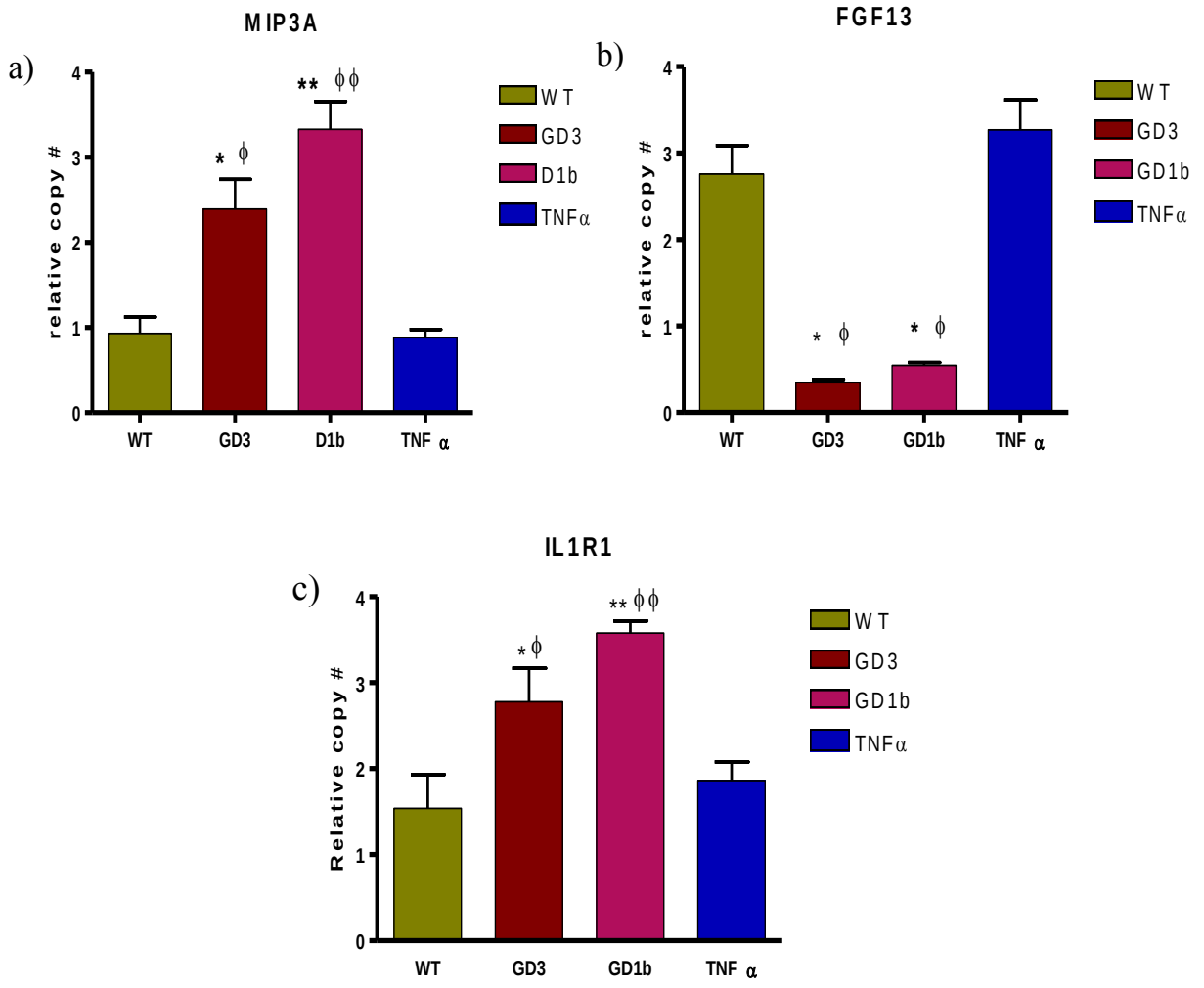
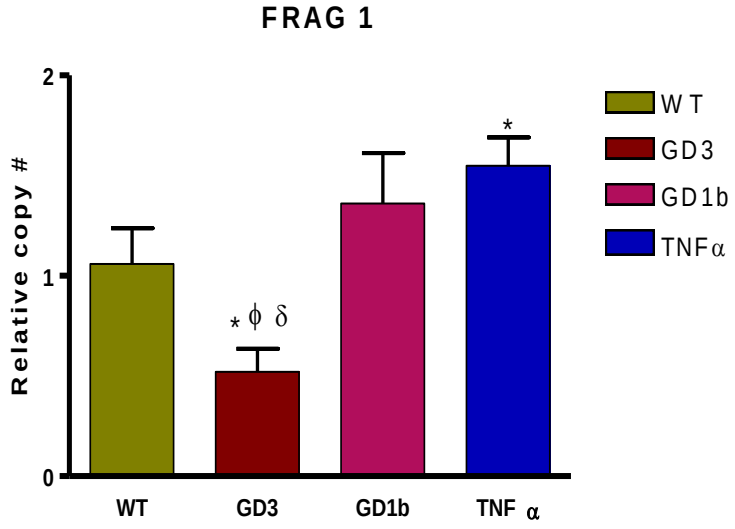


FIG. 13a. Effect of ganglioside GD3 and GD1b, and of TNFα, on gene expression of MIP3A. Cerebral tissue from rats 1-4 days postnatally was disrupted and cultured to a concentration of 1 x 10^6 cells per 2ml well, then exposed for 24h to 5 μg/ml gangliosides (3μm GD3 or 3μm GD1b), or to 200nm TNFα . Total RNA was extracted, mRNA was reverse-transcribed to cDNA, which was quantified for relative expression of individual genes by qPCR bars show means ± s.e.m for 8 experiments/treatment. Differences by unpaired t-tests were significant relative to untreated wild-type (WT) cells (WT *p<0.002, **p<0.0001; TNFα ϕp<0.0005,ϕϕp<0.0001; n=8 for all conditions).

FIG. 13b. Effect of ganglioside GD3 and GD1B, and of TNFα, on gene expression of FGF13. Experimental conditions as in Fig. 13b. Differences by unpaired t-tests were significant relative to untreated wild-type (WT) cells (WT* p<0.0001, TNFα ϕp<0.0001; n=8 for all conditions).

FIG. 13c. Effect of ganglioside GD3 and GD1B, and of TNFα, on gene expression of IL1R1. Experimental conditions as in Fig. 13c. Differences by unpaired t-tests were significant relative to untreated wild-type (WT) cells (WT* p<0.03, ** p<0.0001; TNFα ϕ p<0.03, ϕϕ p<0.0001; n=8 for all conditions).

d)



e)

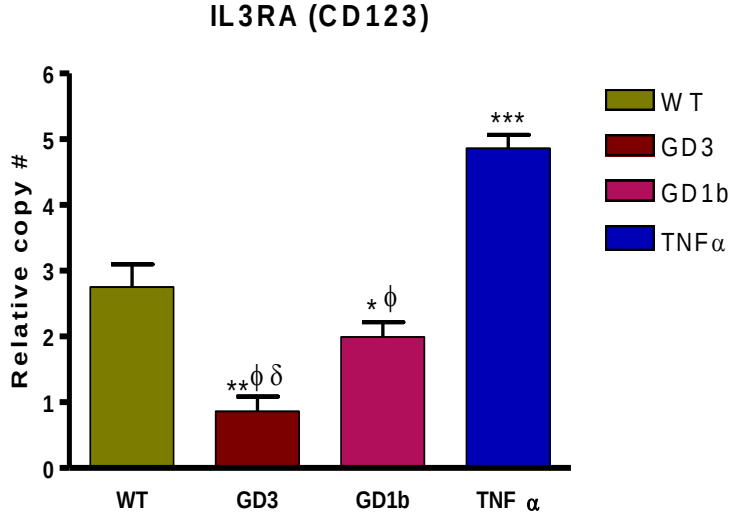


FIG. 13d Effect of ganglioside GD3 and GD1B, and of TNFα, on gene expression of FRAG1. Experimental conditions as in FIG. 13d. Differences by unpaired t-tests were significant relative to untreated wild-type (WT) cells (WT* $p<0.03$, TNFα ϕ $p<0.0001$, δ $p<0.007$; n=8 for all conditions).

FIG. 13e. Effect of ganglioside GD3 and GD1B, and of TNFα, on gene expression of IL3RA. Experimental conditions as in Fig. 13e. Differences by unpaired t-tests were significant relative to untreated wild-type (WT) cells (WT* $P<0.05$, **$P<0.0002$ *** $p<0.0001$; TNFα ϕ $p<0.0001$, δ $p<0.002$; n=8 for all conditions).

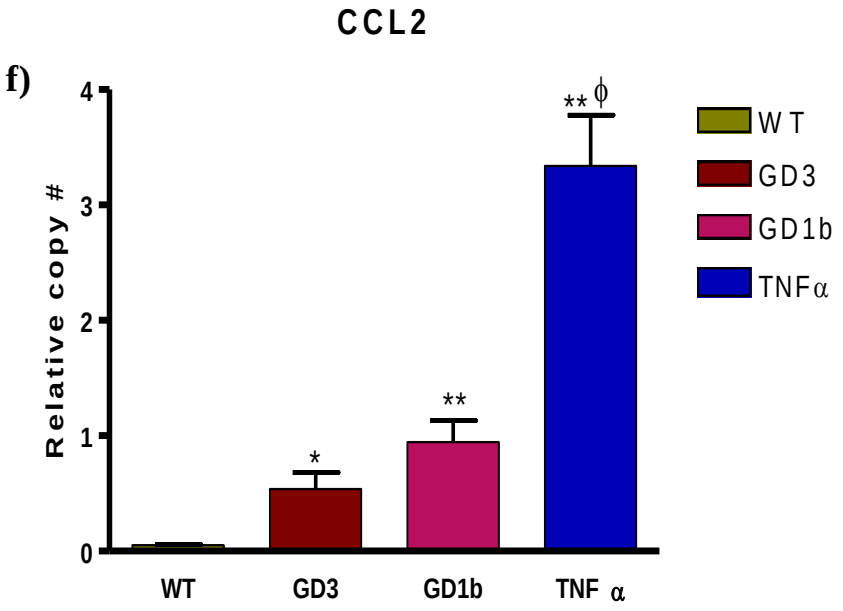


FIG. 13f. Effect of ganglioside GD3 and GD1B, and of TNFα, on gene expression of CCL2. Experimental conditions as in Fig. 13f. Differences by unpaired t-tests were significant relative to untreated wild-type (WT) cells (WT* $p<0.003$, ** $p<0.0001$; GD3 and GD1B ϕ $p<0.0001$; n=8 for all conditions).

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DISCUSSION

MIP3A (Macrophage inflammatory protein 3, CCL20) - The function of this protein is to serve as a chemotactic agent for lymphocytes. It attracts lymphocytes and dendritic cells eliciting its effects on the chemokine receptor CCR6. The expression of this protein can be initiated by LPS or by inflammatory cytokines like TNFα. MIP3A normally activates macrophages and recently has been described as having an impact on B-cells. It also plays an important role in the development of experimental autoimmune encephalomyelitis (EAE), the animal model of MS, during the sensitization phase (Krzysiek, Lefevre et al. 2000). Since MIP3A acts as a significant leukocyte chemoattractant to antigenic sites and also as a ligand for monocyte chemotactic protein-1 receptor (MCP-1), studies were performed on the blood samples of multiple sclerosis patients before and after intravenous methylprednisolone treatment (Michalowska-Wender, Losy et al. 2008). It showed a marked, but nonsignificant increase in the levels MIP3A (Michalowska-Wender, Losy et al. 2008). MIP3A has also been associated with TRAIL (TNFα superfamily member 10) in allergic airway diseases such as asthma in which symptoms manifest in an immediate response to a foreign pathogen. This results in immediate cytokine/chemokine release for inflammatory pathways, targeting myeloid dendritic cells and T-cells expressing CCR6 to activated areas, which is similar to the relapse initiation period seen in MS (Weckmann, Collison et al. 2007). Other research has provided evidence that Th-17 cells play an important role in the migration of lymphocytes into the brain by a mechanism involving a CCR6/MIP3A axis (Reboldi, Coisne et al. 2009). Th-17 cells are known to express CCR6 and when knocked out, caused the inability of mice to develop EAE. Once WT Th-17 cells were introduced into the mice with normal CCR6 expression, susceptibility to EAE was re-initiated in the choroid plexus, where MIP3A was constitutively expressed in the epithelial cells. (Reboldi, Coisne et al. 2009).

The data in Fig. 13a. shows a significant increase in the expression of MIP3A between both GD3 and GD1b treated primary brain cell cultures when compared to normal wild type and TNFα treated primary brain cell cultures. This may be due to the presence of glial cells in the cultures, specifically microglia, increasing their transcription of MIP3A. This response to ganglioside exposure may be due to their functional similarity to macrophages. Microglia only proliferate during times of infection and remain in their quiescent state under normal conditions suggesting that gangliosides may possibly play a role in their activation. .

FGF13 (Fibroblast growth factor 13) – The function of this protein and its family members is to enhance cell survival, proliferation, embryonic development, tissue repair, cell differentiation, and possibly play a role in cortical neuron differentiation. FGF proteins bind to FGF receptors. FGF13 gene expression was drastically down regulated in the GD3 and GD1b treated primary brain cultures when compared to normal wild type and TNFα treated cultures. This down regulation of FGF13 may be due to interference by ganglioside interaction with FGF13 or its receptor. Some of the isoforms for FGF13 are FGF2 or FHF2. FGF2 has been studied for its role in the recovery of multiple sclerosis, where it has been shown to initiate remyelination in animal models using MHV-A59, a murine hepatitis virus that causes demyelinating spinal cord lesions which spontaneously remyelinate (Messersmith, Murtie et al. 2000). Oligodendrocyte cell lines were generated and increased amounts of FGFRs were seen in the cell lines, specifically during times of remyelination, indicating that FGF2 may play a role in the recovery and remyelination process of MS. Other studies have shown that gene therapy using HSV-1 replication defective vectors containing the human FGF2 gene significantly reversed the clinical symptoms of mice with chronic EAE even after 60 days post-treatment. (Ruffini, Furlan et al. 2001).

. Gangliosides GD3 and GD1b caused a significant down regulation of the expression of FGF13 when compared to untreated, normal brain cell cultures and TNFα–treated cultures (Fig. 13b). TNFα treated cultures had a slight but nonsignificant increase on FGF13 expression when compared to the normal, untreated cultures. This provides evidence that gangliosides are impairing the recovery and remyelination of these cultures or are maintaining the cultures in a neurodegenerative state due to the significant reduction of FGF13 expression.

IL1R1 (Interleukin 1 receptor 1 aka CD121a) – This protein encodes a receptor for IL1a and IL1b, and is an important mediator of immune and inflammatory responses. IL1R1 forms a cytokine receptor complex with IL1R2, IL1RL2, and IL1RL1. This receptor has been shown to interact with other proteins such as MYD88, which is an activator protein for all TLRs, which in turn activate NFκβ. In a recent study, MS patients with the HLA-DRB1*1501 genotype were given one of the newer, immunotherapeutics, glatiramer acetate (Copaxone™), to determine its effects on and associations with the expression of 27 potential genes having 61 single nucleotide polymorphisms (SNPs). IL1R1 was one of the genes that had a significant response to the treatment along with 5 other genes, suggesting that these genes play a direct or indirect role in the mode of action of glatiramer acetate (Grossman, Avidan et al. 2007). IL1R1 seems to play an important role in recruiting CD3 T-cells to areas of neuronal injury. Studies in mice with axotomised facial motoneurons showed high levels of CD3+ T-cell recruitment to sites of injury causing neuronal death, but transgenic mice with IL1R1 knockout had a 54% reduction in recruitment of CD3+ T-cells (Raivich, Bohatschek et al. 2003).

Overall, there was a significant increase in IL1R1 expression in GD3 treated cultures when compared to normal, untreated cultures (Fig. 13c). But with GD1b treated cultures, there was a significant increase in relative IL1R1 expression when compared to normal, untreated and

TNFα treated cultures. There was no difference in normal, untreated cultures when compared to TNFα treated cultures.

FRAG 1 (FGF receptor activating protein 1) – This protein acts as a stimulator for the transforming activity and autophosphorylation of the FGF receptor. Not much is known about FRAG1 and its role in MS or apoptosis however it has been hypothesized that FRAG1 plays several roles as a surveillance protein during the S phase of the cell cycle: 1) monitoring replicative stress, 2) regulation of genomic stability, and 3) determination of apoptosis (Ishii, Inageta et al. 2005). When apoptosis occurs, it has been observed that the protein ATR down regulates FRAG1, causing RAD9/BCL2 to co-complex and lead to the induction of the pro-apoptotic protein Bax (Ishii, Inageta et al. 2005). Frag1 has also been recently associated with neuronal injury and death. In previous microarray studies, neurons with either proximal or distal axonal injury had increased expression of eight genes, including FRAG1, which was confirmed by RT-PCR. (Yang, Xie et al. 2006).

In this study there was a significant decrease in FRAG1 expression in only the GD3 treated primary brain cell cultures when compared to all other conditions (Fig. 13d). TNFα treated cultures had a significant increase in FRAG1 expression when compared to normal, untreated cultures.

IL3RA (Interleukin 3 receptor alpha aka CD123) – This is a cytokine receptor for interleukin 3 that is a multipotent cytokine which promotes the differentiation of hemopoietic progenitor cells into the myeloid (neutrophils, basophils, and eosinophils), erythroid (red blood cells), and lymphoid cell (Natural killer cells, T-cells, and B-cells) lineages. A popular medication or immunotherapeutic for the treatment of MS is IFN-β1α (trademarked as Avonex™). The mode of action for Avonex™ is to manipulate the IL10/IL12 axis, causing a

rise in IL10 therefore reducing or ablating the action of IL12, causing more of a TH2 anti-inflammatory environment. Recent research has shown that this can be achieved by increasing the amount of IL3RA (CD123). When IFN-β was given to human blood monocytes derived from myeloid dendritic cells, a dose dependent response was observed in the expression of CD123, which is traditionally a classic indicator of plasmacytoid dendritic cells, and is co-expressed with blood dendritic cell antigen 4+ (BDCA4+). However these dendritic cells were negative for another a traditional marker for plasmacytoid dendritic cells, BDCA2- (Huang, Adikari et al. 2005). The significance of these findings was that the IFN-β modulated dendritic cells were found to only produce IL-10 and IL-6. Furthermore, this IFN-β administration produced dendritic cells that predominantly secreted only IL-10 and were IL-12 deficient, causing the TH2 anti-inflammatory environment (Huang, Adikari et al. 2005). Other studies have seen similar outcomes in MS patients when it comes to IFN-β treatment and circulating blood levels of dendritic cells. It was confirmed that IFN-β induced the expression of CD123 in plasmacytoid dendritic cells and decreased the amount of circulating myeloid dendritic cells in the blood of MS, IFN-β-treated patients. These results also suggest that the circulating dendritic cells are being impaired in their maturation process allowing for a TH2 environment to prevail.

Fig. 13e. shows the relative gene expression of IL3RA in normal, untreated brain cell cultures, and in those treated with GD3, GD1b, and TNFα. GD3 treatment caused a significant reduction in IL3RA gene expression when compared to all other conditions. GD1β treated cultures showed a significant decrease in IL3RA gene expression when compared to TNFα treated cultures only. IL3RA gene expression increased significantly when treated with TNFα. This provides evidence that ganglioside administration reduces the likely hood of having an anti-inflammatory TH2 environment allowing for inflammatory processes to prevail.

CCL2 (chemokine ligand 2 aka MCP-1) – This protein, when bound to CCR2 or CCR4, functions to recruit monocytes, memory T-cells and dendritic cells to sites of infection or tissue injury. Recent studies have shown that chemokines, CCL2 being one of the four major chemokines, play an important role in the migration of leukocytes to the CNS (Subileau, Rezaie et al. 2009). Brain tissue samples from MS patients were taken and immunohistochemistry staining was performed on the samples identifying four main chemokines in the endothelial cells of the brain tissue samples (CCL2, CCL5, CXCL8, and CXCL10) (Subileau, Rezaie et al. 2009). This group also showed that these human brain endothelial cells constitutively released CCL2, but the release of CCL2 was increased in response to TNFα and/or IFNγ in the hCEMC/D3 cell line, a brain endothelial cell line (Subileau, Rezaie et al. 2009).

The chemokine receptor CXCR3 was up-regulated in cytokine stimulated cells while CXCR3 was down-regulated in non-inflammatory, remitted MS lesions linking its expression to times of relapse. There is evidence that glia-derived CCL2 plays an important role in the induction of demyelinating diseases like MS. It has been demonstrated that irradiated chimeric mice induced to have EAE had optimal disease states when glia cells produced CCL2, which in turn attracted iNOS dendritic cells and TNFα secreting macrophages to the brain initiating the entry of lymphocytes and leukocytes into the CNS (Dogan, Elhofy et al. 2008).

There is also evidence that kinin b2 receptors play a role in the induction and severity of EAE (Dos Santos, Roffe et al. 2008). This group, as well as the previous group, has shown that CCL2 plays a role in leukocyte adhesion in the brain, but the mechanism involving kinin b2 receptor is unknown. Mice that lacked kinin b2 receptors were shown to have a reduction in the chemokines CCL2 and CCL5 at day 14 in MOG induced EAE mice, lessening of disease severity, and a significant reduction of adherent leukocytes to the brain (Dos Santos, Roffe et al.

2008). This provides evidence that kinin b2 receptors play a role in the expression of CCL2 and CCL5 as well as regulation of leukocyte recruitment in inflammatory processes in the CNS. The natural receptor for CCL2 is CCR2. Recent advancements in research have suggested that CCR2 antagonists may be an effective therapeutic in inflammatory diseases (Xia and Sui 2009).

Fig. 13f shows the data for CCL2 gene expression between all groups. CCL2 gene expression was significantly increased in all conditions when compared to WT; but the increased expression associated with TNFα surpassed that of the ganglioside treatments to a statistically significant degree. This increase may be an effect of stimulation needed for glial activation and proliferation seen in reactive gliosis, thus causing an increase in the expression of CCL2.

Chapter V

DISCUSSION

Gangliosides in general, specifically GD3, have been implicated since the mid 1980's in the modulation of apoptosis in many neurodegenerative diseases. Seyfried (1985) was one of the many investigators to compile and review this lone ganglioside and its role in apoptosis in diseases such as multiple sclerosis, Creutzfeld-Jakob disease, subacute sclerosing leukoencephalitis, Huntington's disease, Alzheimer's disease, and many more in association with reactive gliosis. One of the hallmarks of reactive gliosis is the increase in GD3. Previous research has shown that GD3 can be an intermediate of cellular apoptosis and has a direct relationship with TNFα (Garcia-Ruiz, Colell et al. 2002). Most of the earlier studies focus on the interaction of ganglioside GD3 with the mitochondria, but do not thoroughly address the cellular interactions or responses that cause the cell to undergo apoptosis and the differential expression of additional potential targets involved in the GD3-mediated apoptotic pathway (Kristal and Brown 1999; Garcia-Ruiz, Colell et al. 2000; Garcia-Ruiz, Colell et al. 2002; Malisan and Testi 2002; Morales, Colell et al. 2004; Jou, Lee et al. 2006). It has been hypothesized that GD3-mitochondrial associations can lead to the inhibition of NFκβ expression in neurons and hepatocytes, thus stopping the activation of the survival pathway and activation of apoptosis (Morales, Colell et al. 2004). Many other genes in multiple brain cell types may be involved in this pathway with multiple effects on gene expression and in conjunction with other cytokine stimuli. Yet, little is known about the biosynthetic modifications made to the ceramide or carbohydrate moieties of the gangliosides once the initiation of apoptosis has begun. Even less information is known on the cellular pathways involved in this unique form of cell death that is initiated in the CNS without the initial involvement of the peripheral immune system. This

research sought to elaborate on the cellular cascade and possible cellular pathways involved in cell death initiated by gangliosides GD3 and/or GD1b, and their effects on gene expression in multiple brain cell types.

Viability

F11-A Neuroblastoma Family of Cell Lines

My first experimental approaches were designed to analyze the viability of cells that lack the ability to synthesize any complex gangliosides past GM3. This led to viability experiments using a family of F11-A neuroblastoma cell lines, in which one cell line does not express STII or GNT (F11-A), one cell line expresses only STII (STII), and one cell line expresses only GNT (GNT) (Fig. 2). If GD3/GD1b do in fact work together with TNFα to cause cell death, this method should isolate specific ganglioside patterns, or a particular series of gangliosides, which lead to an increase in cell death in conjunction with varied concentrations of TNFα.

Experiments with these immortalized cell lines did provide some justification to this hypothesis. Fig 2 shows the HPTLC of the F11-A, STII and GNT cell lines. Each cell line had a distinct and specific ganglioside pattern. The only cell line able to synthesize GD3 was the STII transfected cell line, while GNT cells had more GD1b and two GD1a isoforms while the F11-A cell line only had GM3. Varied amounts of TNFα (0-1.0μM) were given to the three cell lines and were analyzed for viability by MTS after 24h. Fig. 3 shows the results of the MTS assay (n=6). F11-A cells were unphased by the application of TNFα at any concentration while STII cells showed a significant and increasing reduction in viability as TNFα concentrations increased. GNT cells had a noted but insignificant decease in viability at the highest TNFα concentration ($p<0.08$). This data supports the theory that gangliosides, specifically GD3, cause a reduction in viability consistent with cell death.

GNT Gene Expression

Bieberich et. al.(2002) have shown that ganglioside biosynthesis is coordinated in a complex fashion, meaning that glycosyltransferases must complex together and, in essence, activate one another for proper ganglioside biosynthesis. This is important because GD3 is not normally seen in the developed brain. Ganglioside biosynthesis may be up-regulated in order to increase the endogenous pools of gangliosides, so that more complex gangliosides like GD1b can then be modified by glycosidase cleavage to produce the simpler gangliosides like GD3. Fig. 8 shows the comparison of wild type cells with TNFα treated primary brain cell cultures. There was a significant increase in GNT expression which provides evidence suggesting that ganglioside biosynthesis is up-regulated when cell cultures are exposed to a pro-inflammatory stimulus like TNFα.

Primary Brain Cell Viability

My next experiment was designed to test the viability of primary brain cell cultures based on the previous findings that gangliosides, specifically GD3, in conjunction with TNFα, can cause cell death in neuroblastoma cell lines. This allows for a more physiologically relevant system to test the hypothesis that gangliosides and TNFα work together to initiate cell death. Besides ganglioside GD3, previous studies have failed to fully investigate whether ganglioside GD1b can initiate neuronal apoptosis, since it is more highly expressed in normal adult brains than GD3 and can be modified by catalytic enzymes, like glycosidases, to become GD3 (Malisan and Testi 2002). GD3 plays a very important role in gestational development of the brain itself and the overall nervous system (Irwin, Michael et al. 1980), but is rarely seen in the adult brain **unless** a disease state like MS has been activated and reactive gliosis occurs, causing a shift in the readily available pool of gangliosides (Jou, Lee et al. 2006). This phenomenon may be

attributed to the role that GD3 plays during development; possibly for clearance of undeveloped or unnecessary cells formed during gestation. Experiments have been conducted by many groups to elucidate the mitochondrial processes that play a combined role with GD3 in neuronal apoptosis. A novel human sialidase enzyme, Neu4L, was isolated by biochemical fractionation and contained two isoforms, one with an N-terminal sequence and another that lack the sequence (Yamaguchi, Hata et al. 2005). The two proteins were labeled with a fluorescent tag and only the protein containing the N-terminal sequence was located in the mitochondria while the other isoform was located in intracellular membranes. Also, amino acid mutations of the N-terminal isoform, within the N-terminal region, showed the lack of mitochondrial localization, suggesting that this (1) N-terminal sequence is a tag sequence specific for trafficking to the mitochondria and (2) sialidase may play a role in apoptosis using GD3 specifically as a substrate (Yamaguchi, Hata et al. 2005). Neu4L has also been shown to be specifically expressed in the brain, have a dramatically decreased level of expression prior to the induction apoptosis, and to increase its expression while GD3 increases its localization in the mitochondria (Hasegawa, Sugeno et al. 2007). This, in conjunction with numerous other research on the subject, led me to perform MTS viability assays on primary brain cell cultures to support the view that gangliosides can mediate cell death, and to establish a baseline for future experiments involving gangliosides and TNFα.

The next objective of this research was to first establish a physiologically relevant cell culture system which incorporated primary, mixed brain cells, and then to treat them with mixed brain gangliosides plus a pro-inflammatory cytokine, TNFα, to establish a possible causal link integrating the nervous system and its immune function. The purpose for using mixed brain gangliosides is the fact that these gangliosides are normally expressed in the developed

mammalian brain. If there was an independent or interactive decrease in primary brain cell viability using mixed brain gangliosides with/or without a pro-inflammatory cytokine, this should support the theory that gangliosides can affect brain cell viability whether it be by direct cellular interactions or indirectly through enzymatic modification. Any form of these interactions, directly or indirectly, should lead to an alteration of the brain cell's gene expression. Fig. 5 shows that there is a strong possibility that gangliosides can cause cell death independently of TNFα. TNFα only seemed to have a marginal effect in conjunction with co-administered gangliosides, and, when administered by itself, had the opposite effect of causing an increase in the viability of the primary brain cell cultures. This could be due to the increased amount of glial cells to neurons in culture. The brain is composed of approximately 90% of brain tissue glial cells (Nair, Frederick et al. 2008), which is why co-cultures of cortex and hippocampus were used in hopes of increasing the neuronal population and elucidating the interactions of these two cellular subtypes (cortex with hippocampus). It has been stated that LPS, inactivated bacteria, and TNFα can cause an increase in the production of GD3 in microglia without reducing their viability (Simon, Malisan et al. 2002), therefore possibly causing the proliferation and migration of microglia and astrocytes to sites of injury or infection and initiating the beginning stages of cell death. This is possibly why an initial increase in viability in TNFα treated cultures was noticed. This phenomenon can be seen in the lesions of patients with neurodegenerative diseases such as multiple sclerosis, since the areas of injury are essentially filled in by glial cells. This functions to prevent further neuronal death in the brain, but at the same time prevents the regeneration of neurons and oligodendrocytes leading to permanent dysfunction. Simon (2002) has also shown that the attack by the self immune system can be targeted towards the oligodendrocytes, which could cause a secondary, downstream apoptotic cascade to occur in the

neurons themselves. One other interesting note is that peripheral lymphocytic cells produce GD3 during apoptosis, suggesting one causal link in the process of apoptosis (Simon, Malisan et al. 2002).

Since I had demonstrated that exogenously applied mixed brain gangliosides cause a definite reduction in primary mixed brain cell viability regardless of the presence of exogenously applied TNFα (Fig. 5, n=3), I then sought to support the theory that gangliosides GD3 and/or GD1b may be the responsible glycolipid/s in the mediation of this form of cellular death. These two gangliosides are very closely related in the ganglioside biosynthetic pathway, in that the only difference in their structures is the addition of the GalNac and terminal galactose to a GD3 precursor, while both maintain the two sialic acid moieties on the internal galactose carbohydrate (refer to Fig. 1). The amount and relative positioning of sialic acid on gangliosides, as well as other glycans, is of great importance, since they have been known to act as antigen binding sites for pathogens and can affect the overall cellular physiology during autoimmune dysfunction (Schauer 2009). Since GD3 is not normally expressed in differentiated, developed neurons, it is possible that ganglioside GD1b may be modified within the microglia by glycosidase cleavage into GD3, and may be secreted, causing a build-up of GD3 within oligodendrocytes and neurons. So additional experiments were performed on these primary brain cell cultures using varied amounts of GD3 in conjunction with varied amounts of TNFα to further investigate if the smaller, more primitive GD3 could be essential in the modulation of cell death. In the concentration range for GD3 of 1.6nM to 160μM, in the presence of 200nM TNFα, the viability of the primary brain cell cultures (compared to wild type) were consistently reduced, regardless of concentration (Fig. 6). Other experiments using a constant 160μM GD3 concentration with

varied amounts of TNFα (2 pM to 200 nM) showed the exact same results (Fig. 6). This led me to conduct more relevant time course studies involving this phenomenon.

Fig. 7 shows the time course study involving the combination of GD3 with TNFα. Fig. 7a shows that after 24h, the combination of GD3+TNFα caused a significant reduction in primary brain cell viability (n=3). Fig. 7b shows that neuronal viability was reduced after a 48h incubation period when GD3, TNFα, and the combination of the two were administered (n=3). Although, exposure to GD3 alone was the only condition to continue to have a marked decrease viability after 72h when compared to the combination of GD3+TNFα (Fig. 7c, n=3), this suggested that GD3 has a potent effect on viability with and without TNFα at this concentration (160μM), possibly on oligodendrocytes and neurons. This obscure TNFα phenomenon could be due to microglia/astrocytes needing a pro-inflammatory stimulus signal to cause their activation, not death since they are the resident immune cells in the CNS, which in turn would lead to an increase in their endogenous amounts of GD3 for secretion to neuronal or oligodendrocyte populations, thus causing cell death for the neuronal population. This phenomenon can be observed in MS lesions where increases in inflammation and neuronal death are eventually reduced, only to be followed by rapid astrocyte proliferation to fill in damaged areas thus causing scarring in the brain. Although GD1b didn't cause an initial decrease in viability over the 24h incubation period in conjunction with TNFα, it still doesn't exclude it from possibly modulating cellular apoptosis independently due to its homologous biochemical structure when compared to GD3, which might be the key to the initiation or the mediation of cellular death, and its noted reduction in viability with the F11-A-GNT MTS assay (Fig. 3). However, more mechanistic approaches are needed to determine the pathway involved in ganglioside-mediated cell death.

In order to elucidate a hypothetical pathway for ganglioside-mediated cell death, a microarray analysis of gene expression in mixed brain cell cultures treated independently with GD3, GD1b, and TNFα was carried out. This was based on the assumption that it is important to look at the individual effects first due to the fact the gangliosides by themselves can cause a decrease in cell viability, and determine a baseline function of the individual components when it comes down to gene expression. Later experiments should be conducted to look at the changes in gene expression when these components are added together.

Gene Expression

Figure 10 shows the preliminary results of the ORN-11 microarray data for inflammatory cytokines and receptors on wild type, GD3, and GD1b treated neurons. Overall, TGFB2, TGFB3, and TIEG, which are involved in the inflammatory pathways that cause apoptosis, were down regulated in the ganglioside-treated primary brain cultures. However FGF1, FGF2 and FGFR3, which are involved in cell proliferation, were down-regulated as well. This suggested that gangliosides may mediate apoptosis in a more complex manner than originally stated, and could lead to the discovery of novel cellular death pathways possibly involving more than one brain cell type. But this was a sample size of 1, so experiments were repeated with 2 arrays for all conditions, for a more reliable determination of target genes (discussed later).

Figure 11 shows the ORN-22 microarray data for chemokines and their receptors. The data shows that ganglioside GD1b causes an up-regulation of multiple targets: (1) MYD 88, the universal adaptor protein used by Toll Like Receptors to activate NFκβ 1; (2) NFκβ 1, which is involved in autoimmune diseases by using toll-like receptors (TLRs) and TNFα receptors; (3) RDC1, a chemokine receptor used in immune system targeting and functionality; (4) SCYE 1, a cytokine that induces apoptosis by TNFα; and (5) SDF 2, all of which are involved in apoptosis

through TLR/TNFα - mediated pathways. These targets suggested that (1) the immune system could interact with cells like microglia and astrocytes, (2) could be involved in the inflammatory death of these primary brain cell cultures and (3) that gangliosides play a role in these concerted, initial events leading to cell death.

These initial findings suggested the need to concentrate on the ORN-11 microarray data specific to inflammatory targets, so I chose to repeat this experiment using WT, GD3, GD1b, and TNFα treated cells with an n=2 for each condition (8 total arrays). Fig. 12 shows the results of the microarrays, quantified as the relative percent changes seen between potential target genes in Fig. 13a-e. The relative hybridization of the probes to the arrays were normalized by adjusting the relative intensity of the housekeeping genes on each array to one another using the Quantity One ™ software program and collecting numerical data points for each gene. Genes were then compared by relative percent change.

On the basis of the microarray results, 13 genes were selected for further study by qPCR. Out of the 13 genes whose expression was analyzed, only the 6 listed in Chapter 4 and 1 of the ganglioside biosynthetic enzyme genes (MIP3A, FGF13, IL1R1, FRAG1, IL3RA, CCL2, and GNT) showed a significant overall change in their gene expression (Fig. 8 &13a-13f).

In order to propose a pathway involving gangliosides and/or TNFα in cell death cascades, the initial events involved in this cellular orchestration have to first be defined. These initial events must lead to an increase in the pro-inflammatory cytokine TNFα and the release of gangliosides by injured brain cells. TNFα has been noted to cause the activation and the increase of GD3 in microglial cells and cause an overall increase in astrocytic populations. Once these factors have been released, all cell types involved in the cultures began to change their overall gene expression when compared to wild type. Most of the changes were seen in the

ganglioside-treated cells. All gene data and their known functions were discussed in Chapter IV. A further discussion on the possible functions and interactions of these genes within a proposed pathway leading to cell death is discussed below, incorporating additional targets and genes found in the literature.

Proposal for a Hypothetical Pathway Leading to Cell Death Involving Gangliosides/TNFα

TNFα/Ganglioside Functions in Cell Death in the CNS

TNFα is a known inflammatory cytokine that plays an important role in neurodegeneration and cell death. GD3 and TNFα interactions have been studied in various cell types with the emphasis that these interactions disrupt the mitochondrial membrane causing caspase activation (Garcia-Ruiz, Colell et al. 2000; Garcia-Ruiz, Colell et al. 2002). Microglia treated with gangliosides have been known to increase their expression of TNFα (Min, Pyo et al. 2004) and increase their expression of COX-2 and NO (Pyo, Joe et al. 1999) implicating gangliosides as a mediator of microglial activation. Activated microglia have also been known to secrete GD3 which they otherwise do not synthesize under non-pathological circumstances. Simon (2002) has shown that oligodendrocytes are especially susceptible to apoptosis induced by exogenous GD3. There is also evidence that gangliosides can disrupt the myelination of neurons by oligodendrocytes by interfering with myelin-associated glycoprotein (MAG4) (siglec4) binding to gangliosides with the terminal sequence of NeuAca2–3Galb1–3GalNAc, which include GD1a and GT1b (Lopez and Schnaar 2009). As of late, astrocytes have been found to modulate several processes in immune-mediated pathologies. Astrocytes increase their expression of TNFα once they undergo activation, and may be able to mediate TH2/TH1 cellular environments (Nair, Frederick et al. 2008). It has also been stated that when astrocytes release TNFα, there is an increased permeability at the blood-brain barrier allowing for easier entry of

lymphocytes into the CNS. These are just a few examples of how gangliosides, TNFα, and the combination of the two can play a role in pathologies within the CNS.

Microglia

Once microglia are activated by GD3/TNFα, they have to increase their intracellular amounts of GD3, which can possibly be attributed to the significant increase in GNT that we observed in our experiments (Fig 8). Once the microglia have built up their supply of GD3, there is a noticeable and significant decrease in the gene IL3RA Fig. 13e). This reduction allows for the modulation of a TH1 pro-inflammatory environment because the normal function of this gene is to increase IL10 which normally produces an anti-inflammatory environment creating TH2 type lymphocytes. This leads to an increase in IL-12 and thus increases the amount of TH1 lymphocytes causing inflammation. A reduction in IL-10 can also result in an increase in nitric oxide (NO) build up within the cell which causes an increase in NFκβ and iNOS expression.

More evidence is starting to mount for genes like CCL2 in diseases like multiple sclerosis. This gene could easily be attributed to microglial and astrocyte activation since both types of glial cells express it and can have the same pathways activated in relation to CCL2, but this section is theoretically related to microglial function. Samples of postmortem brain tissue lesions from human patients with multiple sclerosis have shown a high increase in CCR2, the receptor for CCL2, which can be found on foamy macrophages and activated microglia (Simpson, Rezaie et al. 2000).Kim (2002) has also shown that gangliosides can act to activate microglial cells, and possibly astrocytes, in the brain by means of the Jak/Stat pathway, causing an increase in the gene expression of CCL2, along with intracellular adhesion molecule (ICAM) and inducible nitric oxide synthase (iNOS). It is possible that the increase in CCL2 is coming from both glial cell types when exposed to gangliosides but it is also probable that both glial

types dramatically up-regulate CCL2 in the presence of TNFα (Fig. 13f). Furthermore, increases in CCL2 by microglia may be a function of toll like receptors complexing with IL1R1, forming a toll/interleukin receptor (TIR) domain which causes an increase in CCL2. This is discussed further below.

The gene data presented here also showed a significant increase in IL1R1 in the ganglioside-treated cultures. IL1R1 normally binds to IL1, which acts as an endogenous pyrogen, and is critical in the function of the immune system. Raivich (2003) has performed studies using the immunotherapy drug glatiramer acetate (Copaxone™) for the treatment of multiple sclerosis. This drug has been shown to significantly reduce IL1R1 in human trials, thus slowing the progression of MS. Raivich (2003) has shown that IL1R1, in conjunction with TNFR2 may possibly play an initial and critical role in the recruitment of lymphocytes to the CNS in a mouse motoneuron model, while IL1R1 also possesses the unique ability to complex with TLRs (Muzio, Polentarutti et al. 2000; O'Neill 2000; O'Neill 2002; Nyati, Prasad et al. 2009), which are known to bind to LPS, causing the activation of myeloid differentiation primary response gene (88) (MYD88) and eventually NFκβ which are all associated with IL1R1 activation. Other evidence has suggested that gangliosides themselves can activate TLRs (Jou, Lee et al. 2006; Yoon, Jeon et al. 2008), which may be the reason for the increase of IL1R1 in order to increase the amounts of TIR domains. Fig. 13c shows that only GD3 and GD1b cause an up-regulation of IL1R1. Since microglia come from the same cell lineage as macrophages, it would be highly likely that microglia would up-regulate the expression of IL1R1 in order to recruit or activate other macrophages, astrocytes, or other infiltrating lymphocytes to the CNS or increase its ability to capture gangliosides using TLRs, thus initiating the appropriate cellular pathways.

One other interesting side note is the expression of FRAG1. FRAG1 is known to activate FGF receptors and modulate mitogenic functions, but Ishii (2005) has shown that down-regulation of FRAG1 leads to the release of cell cycle checkpoint control protein RAD9 (RAD9) and allows for its association with BCL2. This then allows for the induction of the pro-apoptotic protein BAX and eventually leads to cell death. Fig. 13d shows that GD3 caused a significant decrease, while TNFα caused a significant increase in FRAG1 expression. This is probably due to the different responses of the various cell types within our primary mixed brain cell cultures. Since glial cells seem to need TNFα for activation and proliferation, it makes sense that the glial cells would up-regulate their expression of FRAG1 for increased survivability and activity. Conversely, GD3 would cause the down-regulation of FRAG1 in neurons by acting as an antagonist on FGF receptors.

All of this evidence together suggests that TNFα and ganglioside interactions cause an up-regulation cell death cascades through (1) an increase in the expression of GNT, leading to elevated levels of gangliosides like GD1b, which maybe modified to GD3, which induces death in neurons and oligodendrocyte upon its release from microglia. (2) The up-regulation of IL1R1 would increase the microglial ability to recruit or activate other immune cell types by increasing TIR domains for ganglioside-mediated activation, leading to increased CCL2 expression which would cause microglial/astrocyte survival and proliferation by activation of the TIR-Myd88-NFκβ pathway, indirectly increasing FRAG1 expression due to the glial cells ability to become activated by TNFα (3) A significant decrease in IL3RA production would cause inflammation within the CNS, and (4) an increase in CCL2 targeted to the blood brain barrier for peripheral immune cell recruitment/migration.

Astrocytes

Once gangliosides and TNFα have activated astrocytes, there is a noticeable and significant increase in the gene expression of MIP3A and CCL2. At the same time, gangliosides that have been released by activated microglia or by injured brain cells, are influencing pathways related to protein receptors at the cell surface. Many gangliosides have been known to influence the phosphorylation/activation of Jak 1 and 2 receptors (probably similar to the process in microglia) causing Stats to translocate into the nucleus and activate γ interferon activation site (GAS)/interferon stimulated response element (ISRE) promoter sites, leading to an increase in the transcription of CCL2, iNOS, and ICAM. It has recently been observed that glial-derived CCL2 plays an important role in the recruiting and attraction of dendritic cells that secrete iNOS and TNFα especially In the development of EAE. (Dogan, Elhofy et. al. 2008). Xia (2009) has compiled a review on the use of the CCR2 antagonists, which inhibit the binding of CCL2, and their possible use as a therapeutic agent due to their successful reduction of inflammation and monocyte chemotaxis.

MIP3A is of great significance in the breakdown of the blood brain barrier and in the modulation of infiltrating lymphocytes into the central nervous system. The blood brain barrier is composed mainly of astrocytes, which secrete MIP3A and modulate the increased migration of immature myeloid dendritic cells to the inflamed central nervous system (Ambrosini, Remoli et al. 2005). Ambrosini (2005) has also shown that CCL2 as well as MIP3A is increased in reactive astrocytes. This allows for circulating lymphocytes to tether to selectins and integrins, and use MIP3A and CCL2 as a molecular trail to enter the central nervous system. There is also evidence that astrocytes release pro-inflammatory cytokines like IL-6 and TNFα which cause an increased permeability at the blood brain barrier by affecting the endothelial cells and tight

junctions found there (Nair, Frederick et al. 2008). By using either the former or latter, or a combination of both mechanisms, it is clear that astrocytes mediate the influx of peripheral immune cells into the CNS.

Reboldi (2009) has shown that mice lacking CCR6, the natural receptor for MIP3A, are highly resistant to the induction of EAE. This is due to the lack of the ability to recruit TH-17 helper T cells, which is one of the defining characteristic of these helper T cells is the presence of CCR6 (Reboldi, Coisne et al. 2009). Activated astrocytes that produce MIP3A can affect microglia and cause them to produce IL-1β and iNOS (Terao, Ohta et al. 2009). Fig. 13a shows that both GD3 and GD1b cause a significant increase in MIP3A expression. This indicates the possibility that gangliosides themselves can influence the production of MIP3A, which could in turn then lead to an increase in TNFα production and secretion by these astrocytes {Nair 2008 #46}. Fig. 13f shows that TNFα caused a dramatic increase in CCL2, which could be a function of either microglia, astrocytes, or a combination of the two.

Another gene that was shown to be significantly decreased was FGF13. FGF13 acts as a growth factor for the support and survival of neurons. Although FGF13 has been described as similar to FGF2, FGF13 has recently been associated with proper axonal propagation and has even been linked to specific pathologies such as Borjeson-Forssman-Lehmann syndrome (BFLS) which is an X-linked form of mental retardation (Gecz, Baker et al. 1999). FGF13 associates with Na(v)1.6, a sodium channel found at the nodes of Ranvier on neuronal axons, and is important in the fast inactivation of these sodium channels in neuronal cells(Rush, Wittmack et al. 2006; Goetz, Dover et al. 2009). So it is logical that astrocytes act to support neurons in their growth, development, functionality, and differentiation. Fig. 13b indicates that only GD3 and GD1b cause a significant decrease in the expression of FGF13, whereas TNFα had no effect on

its gene expression. This means that astrocytes are playing a dual role in this ganglioside-mediated form of cell death by decreasing their secretion of growth factors like FGF13 while simultaneously increasing their amounts of MIP3A and CCL2 for the recruitment and invasion of peripheral lymphocytes into the central nervous system to aid in the immune response.

What all of this information suggests is that activated astrocytes can play a dual role in apoptotic events in the CNS. Astrocytes can be activated by gangliosides released from microglia or injured brain cells, causing an increase expression of the pro-inflammatory cytokines CCL2, MIP3A, and TNFα. These factors mediate lymphocyte entry and inflammation in the CNS, and activate microglia to produce IL-1β and iNOS. On the other hand, activated astroglia stop producing factors like FGF13 that enhance proliferation, differentiation, and functionality of neurons. So it seems that astrocytes play a mediating role in inflammation by activating glial cells, attracting peripheral lymphocytes, and by halting the proliferation and functionality of neurons.

Neurons and Oligodendrocytes

At this point, the neuronal population is being bombarded by GD3 and all of the other factors released by the microglia or astrocytes, and are not receiving any growth factor support from these cells. It has long been known that gangliosides bind to growth factor receptors such as FGF1 and FGF2, and antagonize any stimuli related to proliferation in neurons. I found a significant reduction in the gene expression of FRAG1, which aids in the phosphorylation and eventual activation of FGF receptors, in cell cultures treated with GD3. Ishii (2005) has shown that a subsequent loss of FRAG1 phosphorylation of FGF receptors can lead to an increase in the pro-apoptotic protein BAX. In my experiments, GD3 treated cells showed a significant decrease in FRAG1 expression (Fig. 13d), suggesting that the neuronal population is being killed off

while TNFα treated cultures are causing an increase in FRAG1 expression and the consequent thriving of the glial population.

IL3RA mediates the cellular environment by increasing the TH2 population, allowing for a more anti-inflammatory targeted immune system. It would only make sense that neurons would up-regulate their expression of IL3RA during normal physiological conditions while reactive glial cells would down regulate their expression of IL3RA. Huang (2005) has shown that approved medications for multiple sclerosis like Interferon-beta (IFN-beta 1 alpha), Avonex™, can act on dendritic cells by suppressing IL12 expression and increasing IL10 expression. IFN-beta has been shown (Lopez, Comabella et al. 2006) to increase the expression of IL3RA in peripheral dendritic cells (PDC) and decreased the number of circulating myeloid dendritic cells (MDC) Fig. 16e shows that cultures treated with TNFα increased their expression of IL3RA, which may be a mechanism that neurons use to protect against inflammatory stimuli. Cultures treated with GD3 or GD1b showed reductions in IL3RA expression, which is probably due to the glial populations converting a TH2 environment to a TH1 environment.

Oligodendrocytes can be grouped with neurons due to their intimate support of neuronal function and viability. The so-called "glue" that holds the myelin sheath to the axon of the neuron involves a very special interaction between MAG-4 (siglec 4, a sialic acid binding galectin) and gangliosides, specifically those that contain the specific terminal sequence of NeuAca2–3Galb1–3GalNAc, including GD1a and GT1b (Lopez and Schnaar 2009). If the secreted GD3 was to bind and enter into the membrane of the myelin sheath, this could cause the myelin sheath to slough off, causing a neurodegenerative state and essentially killing off the neuron. Lopez (2009) has stated that mice lacking GM3 synthase, STII, and GNT have all displayed axonal degeneration and dysmyelination similar to that found in MAG-null mice.

Besides its role in lymphocyte recruitment, CCL2, which is released by microglia or astroctyes and governed by the Jak/Stat pathway, as well as TNFα secretion, and/or TIR domain activation can cause direct activation of caspase activity through cytochrome c release. This could lead to an increase in the transcription of BAX, causing cell death (Kim, Park et al. 2002) which can lead to the decreased viability of neurons and oligodendrocytes.

All of this information taken together suggests that neurons and oligodendrocytes are being injured or killed by the influx of gangliosides, which can induce cell death by (1) the antagonistic effect gangliosides have on FGF receptors, (2) the lack of FGF13 secretion to help support neuronal viability and function, (3) the loss of FRAG-1 phosphorylation of FGF receptors leading to BAX induction, (4) ganglioside uptake by oligodendrocytes, causing MAG-4-ganglioside interactions to fail, resulting in demyelination, with (5) IL3RA increasing as a result to prevent inflammation and hopefully cell death.

Below is a compilation of pathways showing how gangliosides and TNFα might interact to mediate cell death (Fig. 14). This is a summary of the gene expression data obtained from my experiments, combined with what is known in the literature about the subject. Most of the model is based on information from the literature about the functionality of specific brain cells. However, my research on gene expression, as reported here, adds a new level of insight into pathways likely to be involved in the apoptosis of brain cells.

Gangliosides/TNFα Cell Death Pathway

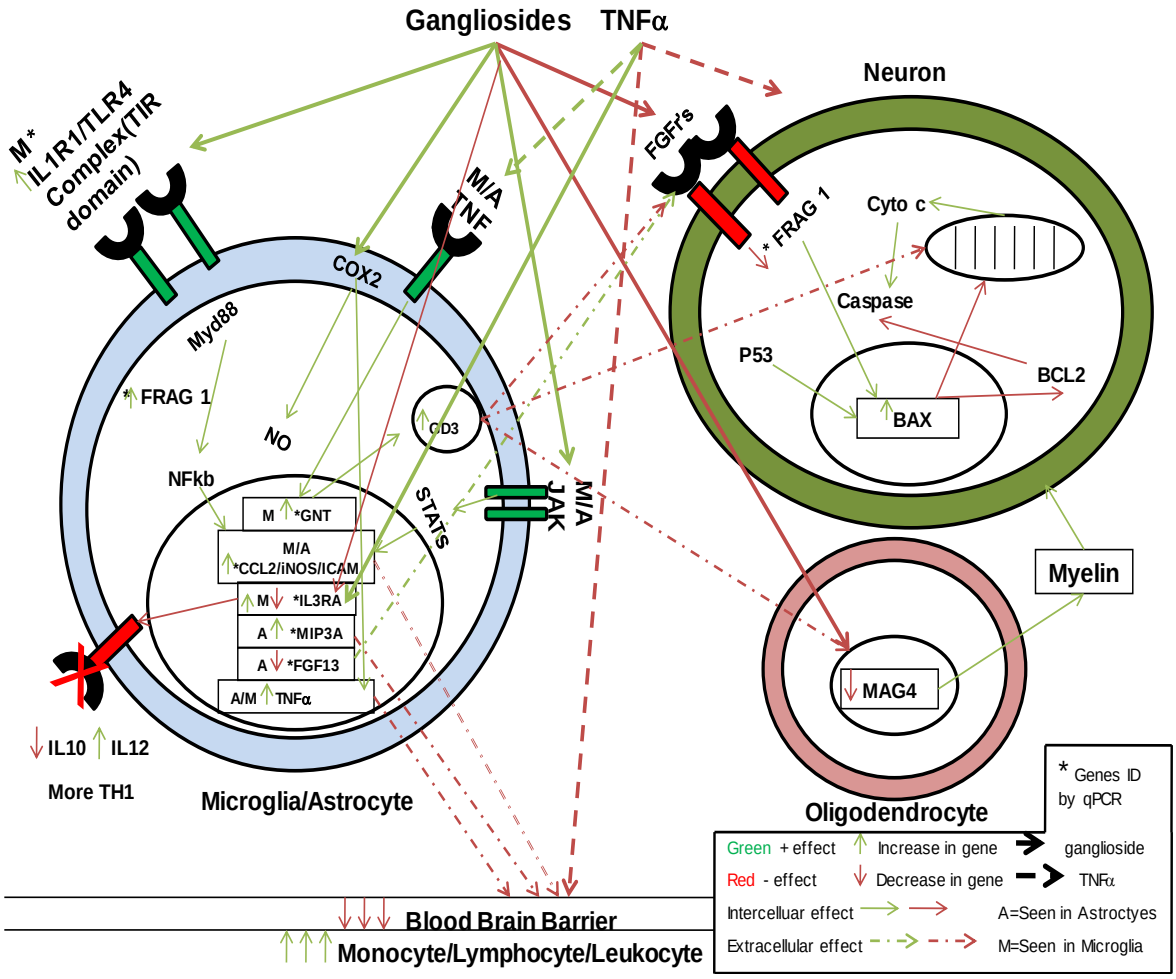

Fig. 14. Proposed Hypothetical Pathway Involving Ganglioside/TNFα Mediated Brain Cell Death. This diagram shows the possible effects of the genes found by micro array analysis (*) that were quantified by qPCR, along with some of the other known genes/targets that have been found in the literature to play a role in ganglioside/TNFα mediated cell death.

CONCLUSION

Gangliosides have been studied for many years and still have a relatively unknown normal function. They are best known for their roles in disease and pathology but are still highly debated as legitimate players in cell death. I believe that my research has shed a little more light on the legitimacy of gangliosides as efficient cell death effectors.

I demonstrated that gangliosides, specifically GD3, can cause significant reductions in primary brain cell viability with TNFα exposure for up to 48 hours. I have also shown that gangliosides can induce the expression of pro-inflammatory cytokines involved in many neurological pathologies. It may also be possible that exogenous gangliosides can change the overall global ganglioside pattern, in culture, resulting in changes in gene expression leading to cell death.

This experiment was designed to elucidate some of the genes being expressed during the initiation of cell death within the central nervous system caused by gangliosides and TNFα. Much of the literature emphasizes the combination of the two, but my approach was to first determine the individual effects of each component when studying a pathway as misunderstood and complex as this one. Future gene expression experiments should be carried out by treating primary brain cell cultures with the combination of gangliosides and TNFα in order to determine if their interaction induces any other genes to play a role in this pathway. This information should lead to pathologically direct pathways involving autoimmune disease within the CNS, uncover processes involved in neuronal degeneration, and uncover potentially new therapeutic targets for diseases such as Alzheimer's, Parkinson's, and Multiple Sclerosis.

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Curriculum Vita

John Charles Gorbet was born and raised in El Paso, Texas. John graduated from Eastwood High School in 1999 and started at EPCC in the fall of 2000. John then transferred to UTEP in the fall of 2002 and graduated with my B.S. in the fall of 2005. His undergraduate GPA was a 3.45. He then entered the Ph.D. program in spring 2006 and began his research on gangliosides and their involvement in neuropathology. He has presented posters on gangliosides at numerous scientific meetings; his first was while he was an undergraduate at the meeting for the Social Advancement for Chicanos and Native Americans in Science (SACNAS) in Denver, Colorado, 2005. He has also presented at the Society for Neuroscience meeting in Atlanta, Georgia in 2006 and at the American Society for Neurochemistry meeting in San Antonio, Texas 2008 and in 2010 in Santa Fe, New Mexico. Currently, he has one paper in review for publication entitled "Gene Expression Analysis for Cell Death in Primary Brain Cell Cultures Treated with Exogenous GD3, GD1b, and TNFα".

He has been a teaching instructor since 2005, starting when he was an undergraduate, and has taught molecular cell biology, anatomy and physiology, and vertebrate physiology laboratories. He received a fellowship in 2008 through the university to teach middle school children science, the GK-12 fellowship. He is currently a member of the Society for Neuroscience, American Society for Neurochemistry, and the National Scholars Honor Society. He graduated in May 2010 with his Ph.D. in Biological Sciences with a 3.83 GPA.

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