Analyzing A-series gangliosides in neurons following exposure to glutamate

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Graduate Program in Anatomy and Cell Biology
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Abstract

Neurons within different brain regions have varying levels of vulnerability to external stress and therefore respond differently to injury. A potential reason to explain this may lie within a key lipid class of the cell’s plasma membrane called gangliosides. These glycosphingolipid species have been shown to play various roles in the maintenance of neuronal viability. The purpose of this study is to use electrospray ionization mass spectrometry (ESI-MS) technique and immunohistochemistry to evaluate the temporal changes in the expression profiles of various ganglioside species during the course of neurodegeneration in rat primary cortical neurons exposed to glutamate toxicity. Primary embryonic (E18) rat cortical neurons were cultured to DIV14. Glutamate toxicity was induced for 1, 3, 6 and 24 h. Immunofluorescence was used to stain for GM1 and GM3 species and ESI-MS was used to quantify the ganglioside species expressed within these injured neurons, which were compared to expression profiles of healthy neurons. Neurons were also pretreated with GM1 24 h before glutamate exposure to assess the level of neuroprotection conferred by GM1. Microglia were also activated using amyloid beta oligomer and stained for GM1 and GM3 expression. ESI-MS data revealed that d16:1 and d18:1 GM1 species were upregulated in neurons exposed to glutamate while no significant changes were observed for GM2 and GM3 expression. Furthermore, neurons that were pretreated with GM1 showed increased viability compared to untreated neurons when exposed to glutamate. Immunofluorescence revealed an elevated expression of GM3 in activated microglia compared to controls. These data suggests that different gangliosides and cells within the CNS play diverse roles in the process of neurodegeneration.

Keywords: Glutamate Toxicity, Neurons, Central Nervous System, Neurodegeneration, Gangliosides, GM1, GM2, GM3, Neuroinflammation, Electrospray Ionization Mass Spectrometry, Microglia
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<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
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<tr>
<td>b-FGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element-binding Protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIV</td>
<td>Days In Vitro</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>E__</td>
<td>Embryonic Day</td>
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<tr>
<td>EAA</td>
<td>Excitatory Amino Acids</td>
</tr>
<tr>
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<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipids</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HD</td>
<td>Huntington’s Disease</td>
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<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinase</td>
</tr>
<tr>
<td>MCAO</td>
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<tr>
<td>MPTP</td>
<td>1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine</td>
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<tr>
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<td>Neurobasal Media</td>
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<td>Platelet-Derived Growth Factor</td>
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<td>Retinal Ganglion Cell</td>
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<td>Spinal Cord Injury</td>
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<tr>
<td>TBI</td>
<td>Traumatic Brain Injury</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Section 1: INTRODUCTION
1.1 Brain Vulnerability

As humans age, they become more prone to injuries and diseases related to the brain. However, different regions of the brain have varying levels of vulnerability to external stress and injury. For example, in Alzheimer’s Disease (AD), the most severely affected areas of the brain include the cortex as well as the hippocampus\(^1\). With ischemic stroke, the CA1 region of the hippocampus is more vulnerable than the granule cells of the dentate gyrus\(^2\). Furthermore, in Parkinson’s Disease (PD), the neurons in the substantia nigra are primarily affected and die from oxidative stress\(^3\). Considering that the plasma membrane of the neurons is the first site of exposure to any changes to the external environment, its physical structure may hold the key to explaining the brain’s differing levels of vulnerability to toxic insults. Therefore studying the physical structure and composition of the plasma membrane and how it regulates itself during stress is important as it may give us an insight in explaining the differential vulnerability of neurons to toxic environments.

1.2 Plasma Membrane

The plasma membrane of cells is a heterogeneous structure composed of numerous proteins, phospholipids, cholesterol, and sugar molecules\(^4\). The plasma membrane is also a dynamic structure that is able to adjust its fluidity and density by changing its lipid composition depending on the extracellular environment\(^5\). It is believed that when animal cells undergo mitosis, half of the phospholipids of the plasma membrane are degraded and turned over for every one or two cellular divisions\(^6,7\). It is hypothesized that the reason for this significant turnover is linked to the cell’s viability.
and its attempt to repair any damages that occurred within the plasma membrane. Cells with inability to turnover their plasma membrane lipid compositions are associated with diseased states such as lysosomal-storage diseases, indicating that the integrity of the cell’s membrane is essential for survival. This bilayer structure also consists of lipid rafts that are rich in cholesterol and saturated lipid species such as sphingolipids that form an organized dense cluster that is distinct from the surrounding environment. These lipid rafts have various roles within the cell including signal transduction as well as sorting and trafficking of materials including cholesterol and proteins, through secretory and endocytic pathways. Within the lipid rafts, one of the major lipid species is gangliosides. Gangliosides are present in the lipid rafts of plasma membrane of virtually all cells, but are particularly abundant in neuronal cells.

1.3 Ganglioside Structure

Gangliosides have amphiphilic properties and are mainly located within the outer leaflet of the plasma membrane of all vertebrate cells. Their hydrophobic carbon tails are embedded within the membrane while their polar and bulky oligosaccharide moieties face the external environment. This property allows the gangliosides to interact with the soluble molecules as well as other hydrophilic components of other cells in the periphery. Although gangliosides and other glycolipids constitute small portions of total glycoconjugates, the levels of these sialic acid bound lipids are abundantly expressed in the central nervous system (CNS), comprising 5-10% of all lipid mass of neuronal membrane. Gangliosides are glycosphingolipids (GSL) – ceramide base and oligosaccharide chains – with differing numbers of sialic acid residues attached to the
sugar chains$^{14}$ (Fig. 1). In the lipid moiety, amino-alcohol is linked to the fatty carbon chain by an amide linkage$^{15}$. In the mammalian brain, the d18:1 and d20:1 sphingosine chains that contain a $trans$ double bond at position 4-5 in the ceramide component are the most commonly found$^{15}$ while the d16:1 gangliosides were only found in trace amounts in rat brains$^{16}$. Sialic acid residues are usually linked to other sialic acid residues, or galactose, at the terminal end of the molecule. The most common form of sialic acid that is found in mammals is the N-acetylneuraminic acid although the less common forms, N-glycolylneuraminic acid are also observed in some cases$^{17}$. The large sugar chains and the negative charge on the sialic acid forms a large shell of water, requiring a larger area in comparison to other neutrally charged phospholipid species$^{15}$. This allows gangliosides to form rigid domains of high density, called the lipid rafts, that are separated from other glycolipids. The lipid rafts are also stabilized by hydrogen bonds with the water and the amide group of the sphingosine$^{18}$ which further adds to the rigidity of the structure.

Gangliosides can also be further classified in to various series. Gangliosides with 0, 1, 2 or 3 sialic acid residues attached to the galactose unit are respectively termed asialo, a, b, and c-series, while those gangliosides that have sialic acid residues linked to the N-galactosamine unit are termed $\alpha$-series ganglioside$^{19}$. The expression pattern of gangliosides differs based on cell type and their quantities are also regulated based on the stage of embryonic development$^{20,21}$. 
1.4 Ganglioside Synthesis

Ganglioside expression profiles change depending on the cell type as well as the stage of their development\(^\text{22}\). Their expression profile is highly dependent on the balance of synthetic and catabolic processes as well as specific intracellular trafficking\(^\text{22}\). The ceramide tails, which are hydrophobic, act as anchors in the plasma membrane and are formed on the membrane of endoplasmic reticulum (ER). The ceramide is then further

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**Figure 1. Ganglioside structure.** Adapted from Schengrund 2015\(^\text{166}\)
modified and glycosylated within the Golgi apparatus by glucosyleramid synthase, which converts the ceramide into glucosyleramid. Complex GSL are further modified in stepwise fashion by the addition of various sugars such as galactose, and N-acetylalpha-galactosamine.

The precursor to all gangliosides is GM3 ganglioside which is produced by the CMP-sialic acid:LacCer α2-3 sialytransferase (ST1). This enzyme converts lactosylceramide by adding sialic acid in a α2-3 bond to lactosylceramide, forming GM3. Then GM3 can be further modified by stepwise addition of more sialic acids by cytidine-5'-monophosphate (CMP)-sialic acid: GM3 α2-8 sialytransferase (ST-2) and CMP-sialic acid: GD3 α2-8 sialytransferase (ST-3) forms GD3 and GT3 sequentially. GM3, GD3, and GT3 serve as precursors for a-, b-, and c-series respectively. From these molecules, various glycosyltransferases including UDP-GalNAc β1-4 N-acetylalpha-galactosaminyltransferase (GalNAcT), UDP-Gal β1-3 galactosyltransferase, CMP-sialic acid: α2-3 sialytransferase, CMP-sialic acid α2-8 sialytransferase produce more complex gangliosides in the pathway in a sequential manner.
Figure 2. Ganglioside biosynthesis pathway. Ganglioside synthesis starts with a ceramide base followed by a sequential addition of sialic acid and sugar residues by various enzymes. Cer: ceramide, GalNAc T: N-acetyl galactosaminyltransferase or GM2/GD2/GT2 synthase, Gal T1: galactosyltransferase I, Gal T2: galactosyltransferase II or GM1 synthase, Glc T: glucosyltransferase, ST1: sialyltransferase I or GM3 synthase, ST2: sialyltransferase II or GD3 synthase, ST3: sialyltransferase III or GT3 synthase, ST4: sialyltransferase IV or GD1a synthase, ST5: sialyltransferase V or GT1a synthase. Figure adapted from Yu RK et al. 2004.
1.5 Ganglioside Expression in the Nervous System

Gangliosides are the main glycolipids in the neurons and studies indicate that the expression patterns of gangliosides differ as the mammalian brain develops. For example, in mice, there is an 8-fold increase in total ganglioside in adult brains compared to those of embryonic mouse brains\textsuperscript{20}. There is also a turnover from an abundance of simple ganglioside such as GM3 and GD3 to more complex species such as GM1, GD1a, GD1b and GT1b as the mouse brain matures from embryos. Studies with matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) imaging demonstrated that GM3 is below detection threshold in healthy adult mouse brains \textit{in situ}\textsuperscript{27}. Similarly, the quantity of gangliosides increase 3 fold in humans from the level of gestational weeks to infantile stage. More complex species such as GM1 and GD1a levels are increased to 12-15 fold during this development as well\textsuperscript{28}. The main ganglioside species within adult human brain are GM1, GD1a, GD1b, and GT1b\textsuperscript{23}. The key to the change in the expression shift from simple to complex ganglioside lies in the changes in the activity of enzymes involved in the gangliosidic pathways. For example, the activity level of ST-II decreases during development while GalNAcT increases, which promotes expression of more complex ganglioside down the a-series\textsuperscript{29} pathway.

1.6 Ganglioside d18:1 vs d20:1 Sphingosine Moieties

The sphingosine moieties of gangliosides also vary within their carbon chain lengths. Specific lengths of these carbon chains have been shown to be expressed in different regions of the brain and may be associated with unique roles within the CNS. Specifically, GM1 d18:1 has been shown to be expressed ubiquitously in the healthy rat
brain while GM1 d20:1 was heavily concentrated within the dentate gyrus of the hippocampus. Studies indicate that d20:1 sphingosine moiety of gangliosides are primarily expressed only within the nervous systems. Furthermore, higher amounts of d20:1 are found in brains of older mammals than those of younger counterparts. For example, in rat cerebellum, the d20:1/d18:1 ratio from 8 day old animal to 2 year old animal increased for GQ1b, GT1b, GM1, GD1b and GD1a, indicating that d20:1 gangliosides are associated with aging of the brain. Total lipid extracts from human and many other mammalian fetuses contained mostly d18:1 species and a negligible amount of d20:1 species. As the brain matures, the levels of d18:1 remain relatively stable or decreases insignificantly. These findings may suggest that the ratio between the d18:1 vs d20:1 species may be markers of aging.

1.7 Ganglioside Degradation

Degradation of gangliosides occurs via the endosome and lysosome pathways. Each step requires an acidic environment and uses the H+ to reduce and hydrolyze the molecule, which enters into the organelles by proton pumps. Gangliosides are degraded by glycosidases, which are soluble enzymes in the lumen of endosomes and lysosomes, starting with the non-reducing terminal monosaccharide unit of the ganglioside sugar chains. Eventually, ceramide is degraded into its fatty acid component and sphingosine by ceramidase. When gangliosides are endocytosed into the cell, the endocytosed vesicle containing the gangliosides merges with the early endosome. The endosome further invaginates, which allows the gangliosides to face the external environment within the endosome. This vesicle is transported and merges with the lysosome. Through
the merging of the vesicle and the lysosome, the ganglioside is degraded by the lysosomal glycohydrolases (Fig. 3).

Figure 3. **Ganglioside degradation pathway.** Degradation of gangliosides occurs through endosomal and lysosomal enzyme pathway. Adapted from Schulze et al. 2009\textsuperscript{165}
In the initial step in the ganglioside degradation pathway, the more complex gangliosides with multiple sialic acid residues such as GT1b and GQ1b are converted to GM1. Then galactose is removed from GM1 to produce GM2 by β-galactosidase. Subsequently, the action of β-N-acetyl-hexoaminidase cleaves off N-acetyl-galactosamine from GM2 to produce GM3. Sialic acid residues can also be directly cleaved from GM1 or GM2 by specific sialidases which produces asialoganglioside GA1 and GA2 which are converted to Lac-ceramide\(^{38-40}\). Lac-ceramide is then further degraded by β-galactosidase and β-glucosidase into ceramide. Most of the degradation processes that occur in the lysosomes require lipid binding glycoprotein molecules called saposins\(^{37}\), which act as transfer proteins that dislodge the gangliosides from the membranes and deliver them to the hydrolases\(^{37}\).

### 1.8 Ganglioside Function

Gangliosides have demonstrated to play various roles within the mammalian body. For example gangliosides are believed to be receptors for microbial toxins; GM1 ganglioside within the brain and epithelial cells of the intestines bind to cholera toxin\(^{41}\). B-series gangliosides within the nervous tissue also act as receptors for tetanus toxin\(^{41,42}\).

Gangliosides have also shown to mediate cell adhesion in blood cells. One way in which this is mediated is through proteins such as lectin that bind through carbohydrates and recognize specific glycolipids. For example, one class of lectin known as selectins are able to recognize and bind to sialyl-fucosyl-LacNAc sugar sequences of glycolipids\(^{43,44}\). Gangliosides also affect growth of tumor cells by interacting with growth
factor and integrin receptors\textsuperscript{45}. Although the exact mechanism of growth modulation is not understood, ganglioside GM3 seems to interact with N-glycosylation of the epidermal growth factor (EGF)\textsuperscript{46} receptor. GM3 has also been shown to play roles in cellular motility. Integrin $\alpha$-3 and membrane protein tetraspanin CD9 forms a complex with GM3 that inhibits laminin-5 dependent motility. Studies on colorectal and bladder carcinoma cells showed inhibition in motility when GM3, CD9 and integrin $\alpha$-3 were co-expressed\textsuperscript{47,48}.

In the CNS, studies indicate that gangliosides are required for normal development of the brain for survival, proliferation and differentiation of the neurons. For example, mice that are deficient of GlcCer synthase, which converts the ceramide into glucosylceramide\textsuperscript{23}, suffers from embryonic lethality due to its inability to differentiate ectodermal layer of the germ layer\textsuperscript{49}. Furthermore, mice that were deficient in GlcCer synthase specifically in neurons showed loss of structural integrity of the cerebellum as well as disrupted function and die within three weeks of birth\textsuperscript{50}. Additionally, mice that were knocked out for ST-II enzymes, which synthesize b-series gangliosides, also showed delayed hypoglossal nerve regeneration after damage\textsuperscript{51}. When formation of more complex gangliosides was inhibited by GalNAcT knock-out, leaving mainly GM3 and GD3, myelin formation was defected, axons showed degeneration, and conduction velocity was decreased from the tibial nerve to the somatosensory cortex\textsuperscript{52,53}. When GalNAcT and ST-II were both knocked out in mice, which only expresses GM3, mice died in response to acoustic-induced seizures\textsuperscript{54}. Furthermore, in humans with mutation that had a defective ST-I gene which limited ganglioside expression to just the a-series showed infantile-onset epilepsy syndromes\textsuperscript{55}. A loss of function mutation in the GM3
synthase gene also lead to infantile progressive brain atrophy, epilepsy and chorea, which are all symptoms that resemble that of juvenile Huntington’s Disease (HD)\(^5\). All of these studies indicate that gangliosides play important roles in the maintenance of the structural integrity including axonal and myelination, stability, and signal transduction within the CNS.

1.9 GM1 Ganglioside and the Central Nervous System

Monosialic acid ganglioside GM1 is one of the more prominent gangliosides within the adult human brain\(^2\) and is a major lipid component within the lipid raft of many cell types\(^5\). GM1 has been linked to neuronal growth, protection, and repair in cell cultures and animal models of aging and injury\(^5\). GM1 has been shown to activate neurotrophic effects of nerve growth factor (NGF) \textit{in vivo} by interacting with its receptors\(^5\). Specifically, it has been shown to activate NGF receptor (Tyrosine Kinase Receptor A) by promoting phosphorylation and dimerization\(^5\). Through its neurotrophic like-activity, it is able to stimulate neurite growth, prevent degeneration, and has even been shown to have repair effects on motor spinal neurons after injury and aging\(^5\). When taken into clinical setting of spinal cord injury (SCI), intravenous administration of GM1 to patients of SCI showed a trend in the improvement of spinal cord activity including motor, light touch, and pinprick scores, bowel/bladder function, anal contraction, although it was not significant. The patients with the less severe form of the SCI showed the most benefit with GM1 treatment\(^5\).

Striatal neurons and fibroblasts attained from HD patients displayed lower levels of GM1 synthesis and expression and the cells were more susceptible to apoptosis\(^5\). When GM1 synthesis was inhibited in wild-type striatal cells, the level of apoptotic
susceptibility was increased to those of HD striatal cells\textsuperscript{65}. These findings suggest that reduced levels of GM1 might lead to the symptoms and progression of HD. Finally, when GM1 was re-administered to HD striatal cells, there were increased levels of phosphorylation of the Htt mutant protein that is responsible for the toxic properties of HD and improved the survival of the HD striatal cells\textsuperscript{65}. These findings indicate that GM1 may be used as a potential therapeutic target to treat HD due to its neuroprotective property.

GM1 also has the ability to interact with various proteins to promote neuroprotection. For example, growth-associated protein 43 (GAP43) is a protein that is expressed in the membrane of neurons which is highly expressed during axonal growth, and the development and regeneration of the CNS and peripheral nervous system (PNS). Specifically, GAP43 has been shown to be upregulated during spinal cord regeneration\textsuperscript{66}. When spinal cord neurons were injured with glutamate toxicity and treated with GM1 and NGF, the levels of GAP43 mRNA increased significantly more than when treated with NGF or GM1 alone, suggesting that GM1 acts synergistically with NGF to provide neuroprotection\textsuperscript{67}. Additionally, when the optic nerve is severed, the retinal ganglion cells (RGC) undergo degeneration from lack of neurotrophin (NT), altered gene expression, and increased oxidative stress\textsuperscript{68–72}. However, the RGC’s survival has been shown to increase by treatment with various neurotrophic factors such as brain-derived neurotrophic factor (BDNF), NGF, and NT3 or NT4\textsuperscript{73,74}; these neurotrophic factors activate the mitogen-activated protein kinase (MAPK) pathway\textsuperscript{75}. MAPK and phosphatidylinositol 3-kinase (PI 3-K) are known to target cAMP response element-binding protein (CREB)\textsuperscript{76} which is believed to be involved in intrinsic survival program
of the cells. Similar to how these growth factors increased the viability of severed RGC, exogenous GM1 treatment on the severed RGC increased their survival by enhancing the activation of MAPK and CREB. Furthermore, when these cells were co-treated with MAPK inhibitor PD98059, the GM1 protection was negated and phosphorylation and activation CREB was not observed\textsuperscript{77}. This suggests that the pathways in which GM1 provides neuroprotection is through the MAPK and/or the PI 3-K pathway\textsuperscript{78,79} (Fig. 4).

**Figure 4. MAPK pathway induced by GM1.** Mitogen activated protein kinase (MAPK) induces phosphorylation of cAMP response element binding (CREB) protein that promotes neuronal survival and plasticity. Figure adapted from Walton MR *et al.* 2000\textsuperscript{76}. 
GM1 has also demonstrated effective neuroprotection in various ischemic models. Rats that suffered from ischemic damage by the occlusion of left middle cerebral artery showed decreased infarct volume when GM1 was administered intravenously, compared to those who were not treated with GM1\textsuperscript{80}. Another study looked at the level of activity of Na\textsuperscript{+} and K\textsuperscript{+} ATPase to assess the level of ischemic damage done by ischemic injury in rat models. While the levels of Na\textsuperscript{+} and K\textsuperscript{+} ATPase activity decreased in the peri-ischemic areas by 42-59\%, there was no significant loss of this activity when treated with GM1. The primary ischemic area was also reduced compared to those that were not treated with GM1\textsuperscript{81}. When rats underwent ischemic damage, levels of various fatty acids was substantially depleted including palmitic, stearic, oleic, and arachidonic acids which indicate that ischemia irreversibly changes and damages the plasma membrane of neurons. However, the acute treatment with GM1 showed partial restoration and prevented loss of these species of fatty acids within the primary area of ischemic damage and complete restoration in peri-ischemic areas\textsuperscript{81}. Extending beyond rat models, when fetal sheep were pretreated with GM1 and given continuous infusion of GM1 after hypoxic insult, it showed reduction in the neuronal loss and improvements in electrical activity, primary and secondary edemas\textsuperscript{82}. However, when GM1 treatment was taken into clinical trial, no significant improvements were found on stroke patients who were treated with GM1. In some cases, patients suffered from adverse effects including skin irritation, and developed Guillain-Barre syndrome\textsuperscript{83}.

One disease in which GM1 had promising results in a clinical setting was PD. Prior to clinical trials on humans, animals models were used to test the effect of GM1 on PD. For example, mice that were treated with GM1 and 1-methyl-4-phenyl-1,2,3,6-
tetrahydropyridine (MPTP) showed an increase in L-dopa compared to those that were only given MPTP\textsuperscript{84}, suggesting that GM1 increases dopamine synthesis. Furthermore, primates who were given MPTP also improved their PD symptoms through treatment with GM1. Primates who were given GM1 treatment also showed increased levels of striatal dopamine levels and its metabolites, and also showed increased levels of dopaminergic innervation in the striatum. In summary, in animal models of MPTP-induced PD, GM1 treatment repaired damaged substantia nigra dopamine neurons, increased the levels of dopamine in the striatum and promoted dopamine synthesis\textsuperscript{85–88}. When GM1 treatment was taken to clinical stage, it showed promising results. When PD patients were given intravenous GM1 administration, they showed improvements in their motor symptoms according to the Unified Parkinson’s Disease Rating Scale (UPDRS)\textsuperscript{89}.

In AD, one of the hallmark features is accumulation of amyloid-\(\beta\)-peptide and evidence of oxidative stress, such as an increased levels of lipid peroxidation, and increased oxidation of DNA and proteins, which all cause injury and death of neurons\textsuperscript{90}. One way in which GM1 counteracts reactive oxygen species is by increasing catalase activity in the brain as shown in rat cortical brains \textit{in vivo}\textsuperscript{91}. Catalase is an enzyme that breaks down \(H_2O_2\) into water and molecular oxygen and plays an important role in reducing the levels of oxidative stress in the cells. However, some studies indicate that since amyloid-\(\beta\)-peptide is formed in the membranes, it can interact with GM1, which may facilitate the formation of toxic fibrils that accelerates the progression of the disease\textsuperscript{92–94}. On the other hand, many studies suggest neuroprotective roles of GM1 in AD models. When PC12 cells (cells derived from tumor of rat pheochromocytoma. When exposed to NGF, their phenotype changes to those of sympathetic neurons\textsuperscript{95}) were
transfected with Swedish-type mutant beta amyloid precursor protein (APPsw) mutation to model AD, the PC12 cells showed increased sensitivity to oxidative stress. Once the cells were exposed to H₂O₂ or amyloid-β protein, Na⁺, K⁺ ATPase was partially inactivated and there was a production of malonyldialdehyde, which is a marker for oxidative stress. However, this was prevented by treating the cells with GM1 ganglioside⁹⁶. When organotypic hippocampal neurons from rats were treated with as little as 10 µM of GM1, neuroprotection from Aβ25-35 toxicity was measured by decreased levels of propidium iodide (PI) uptake⁹⁷. This group also showed that pretreatment of the neurons with GM1 reduced the level of dephosphorylation of glycogen synthase kinase 3 beta (GSK3β) (activation) which is a pathway that triggers apoptosis in animal models of AD⁹⁸. GM1 has also been shown to have antioxidant properties by directly scavenging for hydroxyl radicals and superoxide anions⁹⁹.

1.10 GM2 Ganglioside

GM2 ganglioside is formed from GM3 by the addition of N-acetylgalactosamine by N-acetylgalactosaminyltransferase¹⁹. Previous studies with MALDI imaging showed that the levels of GM2 was below threshold of detection in healthy mice brains but was increased around the infarct region after mice suffered ischemic stroke through middle cerebral artery occlusion (MCAO)²⁷. In the ferret brain, GM2 has also been shown to be more prominent during development of the brain where it was detectable from post natal day 1 to 21, but disappeared by adulthood¹⁰⁰. Furthermore, in the brains of mouse models of osteopetrosis associated transmembrane protein 1(OSTM1)-dependent osteopetrosis that shows various forms of neuropathology including retinopathy, and cortical atrophy,
there was an accumulation of GM2 in the lysosomes, although, the ganglioside enzymatic activities were similar to control animals\textsuperscript{101}. Since the enzymatic activities were not affected, it suggests that GM2 accumulation may stem from degradation of GM1 in the neurons. It is yet unclear as to whether GM2 is responsible for the neurodegenerative phenotype. However there is an association of GM2 and neuronal death particularly because GM2 accumulation has been shown in lysosomal storage diseases including, Sandhoff and Tay-Sach’s disease, and Niemann-Pick C disease and in vesicular organelles in mucopolysaccharidoses\textsuperscript{101–104}, all of which show neuronal death.

Mice brains that were treated with ethanol showed increase in GM2 in the mitochondrial and lysosomal fractions. Immunohistochemistry also indicated increase in GM2 staining in the microglia of these brains\textsuperscript{105}. This opens the possibility that GM2 expression patterns observed in brain tissue may arise not from cortical neurons but other cells within the brain.

In AD models of mice that express APP Swedish and London mutations, levels of GM2 were markedly increased in the cortex\textsuperscript{106}. Similarly, GM2 levels were also elevated in the frontal and parietal cortex of AD patients\textsuperscript{107}. This may be due to an accelerated degradation in the lysosomes to simpler gangliosides in AD patients as fibroblasts from AD patients have been shown to increase the degradation of GM1 to produce more GM2\textsuperscript{108}.

### 1.11 GM3 Ganglioside

GM3 is the simplest ganglioside in the biosynthetic pathway and is a precursor to all gangliosides\textsuperscript{24}. In many different cell types including fibroblasts and cancer cells,
GM3 has been shown to interact with receptors that are linked to angiogenic pathways including insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (b-FGF), EGF, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), cell adhesion molecules such as integrins\textsuperscript{109–116}. Compared to other gangliosides such as GT1b, which promotes angiogenesis, GM3 inhibited endothelial proliferation in human tumor cells. When the tumor cells were re-incubated in media that lacked GM3, they showed regrowth of endothelial cells\textsuperscript{117}. In rabbit cornea, angiogenesis was repressed by GM3 while it was promoted by GM1 or GD3. Furthermore, motility of microvascular endothelium was significantly reduced with the addition of GM3, which was restored when GD3 was further added\textsuperscript{118}. GM3 has also shown to prevent cell proliferation by interfering with growth factor receptors. GM3 has been shown to interfere with binding of EGF with its receptor which is essential for cellular proliferation\textsuperscript{46}. When GM3 was introduced to keratinocytes, the cells showed inhibited growth and low levels of GM3 has been linked to various forms of proliferative skin disorders including psoriasis and squamous cell carcinomas\textsuperscript{119,120}. GM3 has been targeted as a potential therapeutic option for treating cancers due to its pro-apoptotic pathways. GM3 has been shown to prevent metastasis by disrupting cellular motility and induces apoptosis by interacting with CD82 and CD9 which are metastasis-suppressing genes\textsuperscript{121}.

In the CNS, GM3 has also been shown to selectively halt proliferation in dividing neural stem cell and astrocytes and promotes apoptosis by acting on cyclin-dependent kinase inhibitor, p27\textsuperscript{122}. GM3 has been shown to be particularly abundant in the brains during embryonic development\textsuperscript{20} but is below the threshold of detection in healthy adult brains\textsuperscript{27}. However, GM3 levels showed an increase within the infarct region of a brain.
after mice underwent MCAO treatment as detected by MALDI imaging in situ\textsuperscript{27}. GM3, unlike GM1, has been linked to apoptosis of cells. In \textit{vitro} studies showed that GM3 was upregulated in HT22 immortalized mouse hippocampal cells undergoing neurodegeneration by glutamate excitotoxicity. When GM3 was also added exogenously to these cell lines, increase in neuronal death was observed. Additionally, increasing the levels of GM3 by injection of GM3 synthase mRNA in zebra fish embryos resulted in increase in cell death\textsuperscript{123}.

GM3 also has been associated with the pathophysiology of AD in animal models. In mutant mice that overexpress GM3 due to GM2 synthase deficiency, there was a significant increase in the level of amyloid-\(\beta\) protein plaques deposited in the vascular tissues. These mice also showed signs of cerebral amyloid angiopathy, where amyloid-\(\beta\) protein extend from the blood vessels into the parenchyma\textsuperscript{124}.

Another disease associated with GM3 is the Niemann-Pick disease. Common signs of this disease are developmental delay, childhood onset of ataxia, vertical supranuclear gaze palsy (VSGP) dementia, and seizures\textsuperscript{125}. In this disease there is a built-up of various sphingolipids and GSL, mainly gangliosides that are stored in the brain. Among the gangliosides stored in the lysosomes, GM2 and GM3 are the predominant species\textsuperscript{104}. Accumulation of these gangliosides by defective enzymes in the lysosome ultimately leads to neurodegeneration\textsuperscript{126}. One group found that activity of sphingosine-1-phosphate (S1P), an antiapoptotic molecule, was increased by partially deleting the GM3 synthase gene in the Niemann-Pick Type C brain, suggesting that GM3 synthase inactivity can attenuate the pathology of neurodegeneration in Niemann-Pick Type C by decreasing the amount of GM3 available in the cells\textsuperscript{125}. 

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At the behavioral level, GM3 seems necessary to maintain normal cognitive function. When GM3 was knocked out of mice, they showed lower scores in the Y-maze test which is used for assessing cognition levels\textsuperscript{127}. When these mice were administered with nicotine, their behavior levels returned to normal, suggesting that GM3 may play a role in the nicotinic signaling pathways. At the same time, children who suffered from GM3 synthase mutation showed signs of refractory epilepsy, psychomotor delay, blindness and deafness\textsuperscript{128}. However, mice that were knocked out of GM2/GD2 synthase, which leads to build-up of GM3, showed reduction in weight, abnormal shape of the brain, dysfunction in locomotion tests and their fear and emotional behavior was also reduced, which all were exacerbated with aging\textsuperscript{129}.

\textbf{1.12 Glutamate Toxicity and Neuronal Death}

Glutamate is one of the most ubiquitous excitatory neurotransmitter within the brain that is present in millimolar concentrations at the presynaptic terminals at physiologically normal conditions in humans\textsuperscript{130}. Despite its abundance in the nervous system, abnormal quantities of this excitatory amino acids (EAA) can lead to injurious events in the brain\textsuperscript{131}, termed excitotoxicity. Glutamate excitotoxicity is one of the many consequences of various insults of the brain, such as traumatic brain injury (TBI), hemorrhage, ischemia, AD, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and PD\textsuperscript{132–138}, leading to degeneration of neurons. In normal conditions, glial cells actively uptake the excessive glutamate from the extracellular space before it reaches a harmful threshold\textsuperscript{139}. 

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One way in which excess glutamate causes neuronal death is through its ability to over-activate the postsynaptic receptors. Once glutamate is released into the synaptic cleft, it binds to ionotropic receptors such as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate receptors\textsuperscript{140}. Once excessive quantities of glutamate are released into the synaptic cleft, it over-activates the ionotropic receptors and causes abnormally large quantities of Ca\textsuperscript{2+} to enter the neurons\textsuperscript{141,142}. Although Ca\textsuperscript{2+} influx is required for normal neuronal function, irreversible damage and death may result from excessive amount of Ca\textsuperscript{2+} entering the cell as this activates various enzymes such as proteases, lipases, and nitric oxide synthase (NOS)\textsuperscript{143}. NOS activation produces nitric oxide (NO)\textsuperscript{144,145}, which produces oxidative compounds that causes nitrination and oxidation of proteins, lipid peroxidation, and DNA damage\textsuperscript{146,147}. Exposure to glutamate is one of many methods that can be used to induce neurodegeneration.

1.13 Rationale

Gangliosides are expressed abundantly in the nervous system and their roles have been implicated in brain development, injury and repair. However, the temporal expression profile of gangliosides (when and which gangliosides are up and down-regulated) or the distinct roles these molecules play within dying neurons is yet unclear. Therefore, an understanding of the key gangliosides and defining their roles in neurodegeneration may provide potential novel targets for therapeutic intervention.

Preliminary evidence suggests that GM2 and GM3 are increased while GM1 is decreased in rat and mice models of stroke\textsuperscript{27,148}. What is not clear, however, is whether
these changes occur in neurons or other cell types within the brain. Therefore, work in this thesis sets out to define the temporal expression profile of GM1, GM2 and GM3 following glutamate toxicity in neurons. Preliminary studies also suggest that GM1 may play protective roles in neuronal injury in vivo. Therefore the second component of this thesis sets out to determine if GM1 can protect against glutamate toxicity in neurons. We believe that there is a balance in the regulation of a-series gangliosides in neurons. As the neurons start to die, the expression levels of gangliosides shift to the simple species such as GM2 and GM3, leading to a decrease in GM1. By being able to restore the balance by pretreating the neurons with exogenous GM1, we believe that the neurons’ membrane integrity will be preserved and promote survival during stress induced by glutamate.

1.14 Hypothesis and Aims

Aim 1: Investigate the expression profile of gangliosides in rat cortical embryonic neurons undergoing neurodegeneration by glutamate exposure.

Hypothesis 1: Ganglioside expression profiles of cortical embryonic neurons will change following stress induced by glutamate exposure. Specifically, GM2 and GM3 ganglioside expression will increase while GM1 ganglioside expression will decrease over time.

Aim 2: Determine whether exogenous treatment of GM1 can protect neurons from undergoing neurodegeneration.
Hypothesis 2: Pretreating the rat embryonic cortical neurons with exogenous GM1 prior to glutamate exposure will restore the balance in the GM1 loss in the neurons and reduce damage and death to the cells exposed to glutamate.
Section 2: METHODS


Culturing cortical neurons from rat embryo

2.1 Animals

All experimental procedures were carried out aligned with the standards of Canadian Council on Animal Care and protocols were approved by Western University Animal Use Subcommittee. Pregnant female Wistar Rats (Charles River, Montreal, Quebec) were housed at a temperature of 22-24 °C and were provided food and water ad libitum.

2.2 Plate Preparation

To coat the plates, 7% poly-L-Ornithine (molecular weight 30,000-70,000 Sigma, St. Louis, MO, USA) solution was prepared with distilled water. Glass cover slips were placed in each of the 24-well plates and these were coated with 500 µL of the 7% poly-L-Ornithine solution. The 10 cm plates were coated with 10 mL of 7% poly-L-Ornithine solution. All of the plates were incubated with the solution for 1 h at room temperature in the bio-hood. The solution was then aspirated and washed three times with distilled water. They were left to dry overnight at room temperature.

2.3 Cell Culture

One pregnant female Wistar rat was sacrificed by cervical dislocation. Embryos were surgically removed from the female mother on embryonic day 18 (E18). The dissected uterus was taken out and was placed in a petri dish containing 10 mL of Hank’s balanced salt solution (HBSS, Wisent Bioproducts, St. Bruno, Quebec). Cortices from
each embryo were extracted and the meninges were carefully removed with tweezers. The cortices were placed in separate petri dish containing HBSS on ice.

**Following volumes are for 10 brains:**

Rat embryo brain cortices were placed in a 14 mL conical tube containing 1.8 mL of HBSS. The tube was centrifuged at 4000 x g for 1 min at room temperature. HBSS was aspirated and 1.8 mL of solution A containing 5 mL HBSS, 6 µL MgSO₄ (1 M) and 2 mL trypsin (Sigma, St. Louis, MO, USA) was added to the same conical tube. The tube was mixed well by shaking and rotating by hand, ensuring the neurons were free floating, and were rotated at 37 °C for 25 min in an automated rotator in a heated chamber. After rotation, 3.6 mL of solution B containing 7 mL HBSS, 8 µL MgSO₄ (1 M), 175 µL DNase1 (10 mg/mL) (Roche Life Sciences, Indianapolis, IN) and 112 µL trypsin inhibitor (100 µg/mL) (Roche Life Sciences, Indianapolis, IN) were added to the conical tube and mixed by shaking and rotating by hand for 2 min. The tube was then centrifuged at 4000 x g for 5 min at room temperature and HBSS was aspirated. Finally, 6 mL of a solution C containing 20 mL of HBSS, 48 µL MgSO₄ (1 M), 1.3 mL DNase1 (10 mg/mL), and 1 mL trypsin inhibitor (100 µg/mL) was added to the resulting cell pellet. These cells were transferred to 50 mL falcon tube and another 6 mL of solution C was further added. The cells were titrated 10-15 x until the solution appeared homogenous. Cells were then centrifuged at 4000 x g for 5 min and the supernatant was aspirated. The cell pellet were re-suspended in 36 mL of Neurobasal plating media containing 96 % neural basal media (NBM, Wisent Bioproducts, ST. Bruno, Quebec), 2 % B27 supplement (Invitrogen, Carlsbad, CA), 0.8 % N2 Supplement (Invitrogen, Carlsbad,
CA), 0.5 % penicillin/streptomycin (Invitrogen, Carlsbad, CA), 0.25 % Glutamax (Invitrogen, Carlsbad, CA), and 0.1 % Amphotericin B solution (Sigma, St. Louis, MO, USA). The cell suspension was kept on ice during cell counting. In order to count the cells, 20 µL of Trypan Blue Solution (Thermo Fisher Scientific, Waltham, MA, USA) and 20 µL of cortical cell suspension were prepared. The cells were counted in the following way on the hemacytometer (Thermo Fisher Scientific, Waltham, MA, USA), using a cell counter and a microscope:

**Calculation:** Cells/mL = Avg. count of 2 squares x dilution factor (2) x 10^4

i.e. avg. cells in 2 squares of Hemacytometer = 100 cells

100 x dilution factor (2) = 200

200 x 10^4 = 2 x 10^6 = 2,000,000 cells/mL

We have a 36 mL cell suspension, so total we have 7.2 x 10^7 cells

Using this calculation, media was added to the cortical cell suspension to create a stock suspension at 1.0 x 10^6 cells/mL. The cells were plated at density of 0.5 x 10^6 cells/well by adding 500 µL of the 1.0 x 10^6 cells/mL solution to the 24-well plates and 1.0 x 10^7 cells/plate by adding 10 mL of the 1.0 x 10^6 cells/mL solution to the 10 cm plates. Cells were kept in an incubator at 37 °C and 5 % CO₂. Half of the media was exchanged every 2 days until 14 days *in vitro* (DIV).

### 2.4 GM1 Treatment

GM1 stock solution was made by directly dissolving GM1 (Sigma, St. Louis, MO, USA) in NBM to achieve a final concentration of 100 µM. Neurons in the 24-well plates were pretreated with 0, 5, 10, 25 µM of GM1 24 h prior to exposure to glutamate.
2.5 Glutamate Exposure

10 mM of stock glutamate solution was made with phosphate-buffered saline (PBS). On DIV 14, cells were exposed to 100 µM of glutamate for 1, 3, 6, and 24 h for 24-well plates and 10 cm plates. 10 cm plates were used for lipid extraction and 24-well plates were used for immunohistochemistry analysis.

2.6 Cell Fixation & Immunofluorescence

The neurons in the 24-well plates were treated with 20 µL of 1 mg/mL PI solution 45 min before cell fixation. After these cells were exposed to 100 µM of glutamate for the time points, they were fixed with 4 % paraformaldehyde (PFA) for 15 min and washed with PBS three times. Cover slips containing neurons were washed with PBS 3 x and were incubated with either GM1 (Abcam, Toronto, Ontario) or GM3 (Amsbio, Milton Park, Abingdon, UK) at 1:100 diluted with 3 % bovine serum albumin (BSA) in 0.01 M PBS for 24 h at 4 °C. Cover slips were washed with PBS 3x and were incubated with FITC-conjugated dylight 488 secondary anti-mouse (1:200) (Thermo Fisher Scientific, Waltham, MA, USA) for GM3 and FITC-conjugated dylight 488 secondary anti-rabbit (1:200) (Thermo Fisher Scientific, Waltham, MA, USA) for GM1 for 24h at 4°C. The cover slips were washed with PBS 3 x and double distilled H₂O 2 x and were mounted onto microscope glass slides with Fluoroshield with 4’,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA).
2.7 Lipid Extraction

Lipids were extracted using a modified Folch method\textsuperscript{150}. Four 10 cm plates were used to create a single experimental replicate (n=1). Each plate was washed with PBS on ice before scraping. Following PBS washing, cells were scraped using a cell scraper and collected into a glass tube. To the 0.8 volume parts of aqueous cell extract, 2 parts of methanol was added and homogenized using an automated homogenizer. Subsequently, 1 part of chloroform was added to give one phase Chloroform:Methanol:Water (C:M:W) = 1:2:0.8. This mixture was left at room temperature for 1 h with periodical vortexing. 1.2 parts of water was added to the sample for phase separation to achieve a final composition of C:M:W = 1:2:2. Sample was centrifuged at 2000 rpm and the upper aqueous phase was collected. The lower phase in the glass tube was re-extracted with the theoretical upper phase of M:W = 2:2 and was centrifuged at 2000 rpm. The upper phase was added to the first extracted sample and KCl was added to achieve a final concentration of 0.1 M.

Prior to eluting the gangliosides using the Solid Phase Extraction C18 column (Waters, Mississauga, Ontario), the column was first washed with 10 mL of MeOH, 10 mL of CHCl\textsubscript{3}:MeOH (2:1), another 10 mL of MeOH, and 10 mL of MeOH:0.1KCl (1:1). Then the upper phase cell extract was passed through the column twice. 20 mL of H\textsubscript{2}O was applied through the column. The sample was eluted with 2 mL of MeOH and 10 mL of CHCl\textsubscript{3}:MeOH (1:1). Sample was evaporated to complete dryness with N\textsubscript{2} gas and stored in -80 °C until analysis. Before analyzing the sample with the mass spectrometer, the dried sample was re-dissolved in 1 mL of CHCl\textsubscript{3}:MeOH (1:1) and was put in the sonicator bath for 15 min.
2.8 Electrospray Ionization Mass Spectrometry Detection of Gangliosides

Liquid Chromatography Conditions

Samples were separated using a Luna 3 µm 100 Å NH₂ LC column (150 x 1 mm; Phenomenex, Torrance, CA). LC conditions were adapted from Ikeda et al. 2010\textsuperscript{151}. Mobile phase constituted of 2 solutions: A) acetonitrile/water (83:17) (1 mM ammonium formate) B) acetonitrile/water (1:1) (50 mM ammonium formate). Initial gradient was held for 5 min at solution A:B = 100:0, converted linearly to A:B=25:75 for 5 min, then converted linearly to A:B=10:90 for 5 min and held there for 10 min. The flow rate was set to 0.15 mL/min and the temperature of the column was set to 40 °C.

Mass spectrometry was performed with Waters QToF Micro (Waters, Mississauga, Ontario) and the spectra were analyzed by Mass Lynx v4.1 software (Waters). Flight tube voltage was set to 5630 V, MCP voltage was set to 2700 V. Source tube voltage was set to 3500 V and the sample cone was set to 40 V and ran on negative ion mode. The source temperature was set to 100 °C and desolvation temperature was set to 300 °C. Collision energy was set to 5 V. Each experimental replicate was analyzed three times to produce three technical replicates. NaCsI was used to calibrate the instrument and GM1 standards from bovine brain were purchased from Avanti Polar Lipids (Alabaster, Alabama).

Quantification of Gangliosides

Specific gangliosides of interest were searched in the chromatogram according to mass to see at which time points they were eluted. The chromatogram was extracted to analyze the mass spectrum of the eluants. The peak intensities of these gangliosides were recorded. To quantify these ganglioside species, the peaks were normalized in the
following way. Firstly, the peak intensities of all nine gangliosides were summed for a single run in one time point. This was done for every run within each time point. Then the average of total lipid peak intensities from all of the runs was calculated. In order to account for the different amount of lipids that were ran per sample, we calculated the quotient between the total lipid peaks relative to the average peak intensity of all runs. This number was divided by the peak intensities of each ganglioside peak. Furthermore, when neurons are undergoing apoptosis, some cells become detached from the plates and are washed away which also adds to the variation in the total lipids present in the sample. In order to account for this variation, DAPI cell counts were used to quantify the average number of cells present in each experimental settings. A factor was found relative to the control, and this number was multiplied to the peaks of each gangliosides as well.

Example:

All of the following are intensities from 1 h glutamate exposure groups

Run 1:

Ganglioside A – 400, Ganglioside B – 200 Ganglioside C – 600 Ganglioside D – 1000

Run 2:

Ganglioside A – 100, Ganglioside B – 50, Ganglioside C – 150, Ganglioside D – 250

Run 3:

Ganglioside A – 200, Ganglioside B – 100, Ganglioside C – 300, Ganglioside D – 500
Total Lipids from Each Run

Run 1: $400 + 200 + 600 + 1000 = 2200$

Run 2: $100 + 50 + 150 + 250 = 550$

Run 3: $200 + 100 + 300 + 500 = 1100$

Average of Three Runs $= (2200 + 550 + 1100)/3 = 1283$

Using this average, we found the quotient factor for each of the runs:

Run 1: $2200/1283 = 1.714$

Run 2: $550/1283 = 0.4286$

Run 3: $1100/1283 = 0.8574$

These values would be divided by each of the ganglioside peaks in the appropriate runs

Run 1
Ganglioside A $– 400/1.714 = 233$
Ganglioside B $– 200/1.714 = 117$
Ganglioside C $– 600/1.714 = 350$
Ganglioside D $– 1000/1.714 = 583$

Run 2
Ganglioside A $– 100/0.4286 = 233$
Ganglioside B $– 50/0.4286 = 117$
Ganglioside C $– 150/0.4286 = 350$
Ganglioside D $– 250/0.4286 = 583$

Run 3
Ganglioside A $– 200/0.8574 = 233$
Ganglioside B $– 100/0.8574 = 117$
Ganglioside C $– 300/0.8574 = 350$
Ganglioside D $– 500/0.8574 = 583$
MS/MS Conditions

Ganglioside eluants were collected from the Luna 3 µm 100 Å NH₂ LC column (150 x 1 mm; Phenomenex, Torrance, CA) using a syringe. The gangliosides in the syringe were directly injected into the Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) for tandem mass spectrometry (MS/MS) analysis. Negative ion mode was used and the gangliosides were fragmented using high-energy collisional dissociation at voltages ranging from 50 – 75 V. Each ganglioside was individually selected based on their specific mass as a precursor molecule. Each of the gangliosides were identified by detecting specific fragments: sialic acid, sugar molecules, and the ceramide base.

Culturing microglia from neonatal rat

2.9 Animals

All experimental procedures were carried out aligned with the standards of Canadian Council on Animal Care and protocols were approved by Western University Animal Use Subcommittee. Postnatal day 0-2 neonatal Wistar Rats (Charles River, Montreal, Quebec) were housed at a temperature of 22-24 °C and were provided food and water ad libitum.

2.10 Plate Preparation

To coat the T75 culture flask (Corning, Tewksbury, MA, USA), 5 µg/mL of Poly-D-Lysine (EMD Millipore, Billerica, MA, USA) solution was prepared with autoclaved water. The T75 plates were incubated with the Poly-D-Lysine solution for 3 h at 37 °C.
The Poly-D-Lysine solution was aspirated and the plates were washed once with autoclaved water. The plates were dried under UV light in the cell culture hood for 20 min. The plates were prepared just prior to the dissection process.

2.11 Cell Culture

Postnatal day 0-2 neonatal (P0-2) Wistar rat was sacrificed by hypothermia. The head was wiped with 70 % ethanol and Kimwipe (Kimberly-Clark Professional, Roswell, GA, USA). The head was removed with scissors and 4 pups were used per T75 culture flask (Corning, Tewksbury, MA, USA). The heads were placed in petri dish that contained ice-cold HBSS (Wisent Bioproducts, St. Bruno, Quebec). After removing the skin and the skull with forceps, the brain was removed with a spatula and placed in a fresh petri dish containing ice-cold HBSS. Using microforceps and dissection microscope, the cortex was isolated and the meninges were removed. These cortices were placed into 15 mL conical tubes with 14 mL of ice-cold HBSS on ice. HBSS was aspirated from the conical tubes containing the 4 brains and 4 mL of 1 x trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Fisher Scientific Company, Ottawa, Ontario) was added to the conical tube. The brain tissue was titrated with p1000 pipette tip and the tube was incubated for 15 min at 37 °C. To stop the enzymatic digestion, 4 mL of complete microglial media made from 1 x Dulbecco’s Modified Eagle Medium (DMEM, Wisent Bioproducts, St. Bruno, Quebec), 10 % fetal bovine serum (Fisher Scientific Company, Ottawa, Ontario), 1 % sodium pyruvate (Cellgro®Corning, Tewksbury, MA, USA), 0.08 % gentamycin (BioWhittaker®, VWR International,
Radnor, PA, USA) was added to the conical tube containing the brain tissue. The tube was shaken by hand to mix the contents and was spun at 1500 rpm for 5 min. The supernatant was aspirated and 4 mL of complete microglial media was added into the tube. The content in the tube was mixed again by mixing by hand and was centrifuged at 1500 rpm for 5 min. After aspirating the supernatant, the cells were resuspended in 10 mL of complete microglial media and filtered through a 40 µM mesh cell strainer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The cells were plated at density of 8 cortices per 10 mL of total microglial media in the T75 culture flask (Fisher Scientific Company, Ottawa, Ontario). The T75 culture flask was then incubated at 37 °C and 5 % CO₂. On day 3 of incubation, the media was exchanged with complete microglial media. On day 10, in order to detach the microglia, 400 µL of 60 mM lidocaine (Sigma, St. Louis, MO, USA) in HBSS was directly added into the T75 culture flask. The T75 culture flask was then incubated at room temperature for 10 – 15 min. The detached microglia in the T75 flask was collected in a falcon tube and the plate was washed with HBSS and the remaining microglia was collected in the same falcon tube. To the cell suspension in the falcon tube, 5 mM of EDTA (pH 8.0) was added to a final concentration of 50 µM. The falcon tube with the cell suspension was spun in the centrifuge at 15000 rpm for 5 min and re-suspended in 1 mL of DMEM with 1 % fetal bovine serum. Using a hemacytometer, a cell counter, and a microscope, viable cells were counted:

**Calculation:** Cells/mL = Avg. count of 2 squares x dilution factor (2) x 10⁴

i.e. avg. cells in 2 squares of Hemacytometer= 100 cells

100 x dilution factor (2) = 200

200 x 10⁴ = 2 x 10⁶ = 2,000,000 cells/mL
The microglia suspension was split and placed into 24-well plates with glass cover slips coated with 5 µg/mL of Poly-D-Lysine (EMD Millipore, Billerica, MA, USA) at density of 2.5 x 10⁴ cells per well. The 24-well plates with the microglia were kept in the incubator at 37 °C and 5 % CO₂.

2.12 Amyloid β 1-42 Oligomer Preparation for Microglia

Amyloid β-Protein (1-42) (Aβ peptide) was purchased from Bachem Americas, Inc. (Torrance, CA, USA) and was stored in -80 °C in a vial. When they were ready for use, Aβ peptide was equilibrated to room temperature for 10-15 minutes. Prior to re-suspending the Aβ peptide in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma, St. Louis, MO, USA), HFIP was placed on ice in the fume hood to cool. After HFIP was cooled, it was added to the vial containing solid Aβ peptide to achieve a final peptide concentration of 1mM. The vial was vortexed thoroughly. The peptide solution in the vial with HFIP was placed in room temperature until the peptide was completely dissolved. The vial containing the Aβ peptide solution was then placed onto ice for 10 min. Once the Aβ peptide solution in the vial was cooled, 10 µL was aliquoted into 0.5 mL microcentrifuge tubes. Parafilm was placed over the tubes containing the aliquoted Aβ peptide solution and holes were made with 27 G needle. The solution in the tubes were then lyophilized for 60 min until all traces of HFIP was removed and the resulting peptide appeared as a thin and clear film at the bottom of the tube. The dried Aβ peptide film was stored in -80 °C until use.

Dried Aβ peptide film was removed from -80 °C and placed on ice. 10 µL of fresh anhydrous dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) was added into the
microcentrifuge tubes containing the dried Aβ peptide. The DMSO/Aβ peptide solution was pipetted thoroughly to dissolve the Aβ peptide. The microcentrifuge tube containing the DMSO/Aβ peptide was then sonicated for 10 min at 37 °C. PBS added to the tubes to achieve a final DMSO/Aβ peptide concentration of 150 µM and the peptide was oligomerized by incubating for 24 h at 4 °C. This oligomer preparation was stored in -80 °C until use.

### 2.13 Aβ Toxicity on Microglia

On DIV 2, microglia were adhered to the glass coverslips and ready for exposure to Aβ oligomer. The microcentrifuge tube containing Aβ oligomer preparation was removed and placed at room temperature. From the microcentrifuge, 6.76 µL of Aβ oligomer preparation was added into the 24-well plates to achieve a final concentration of 2 µM of Aβ oligomer. Microglia were incubated with 2 µM of Aβ oligomer for 1 h to activate them.

### 2.14 Microglia Fixation & Immunofluorescence

After the cells on the coverslips were exposed to Aβ peptide, 500 µL of 4 % PFA was directly added into each of the 24 wells containing the media. After 2 min, 4 % PFA was removed and replaced with 2 % PFA and the cells on the coverslips were incubated for 20 min at room temperature. The coverslips were washed with PBS 3 x and were placed in the fridge at 4 °C until staining. Cover slips containing the microglia were washed with PBS 3 x and were incubated with either GM1 (Abcam, Toronto, Ontario) or
GM3 (Amsbio, Milton Park, Abingdon, UK) at 1:100 diluted with 3% BSA in 0.01 M PBS for 24 h at 4 °C. Cover slips were washed with PBS 3 x and were incubated with FITC-conjugated dylight 488 secondary anti-mouse (1:200) (Thermo Fisher Scientific, Waltham, MA, USA) for GM3 and FITC-conjugated dylight 488 secondary anti-rabbit (1:200) (Thermo Fisher Scientific, Waltham, MA, USA) for GM1 for 24 h at 4°C. Activated microglia that were also labeled with GM3 and FITC were also co-labelled with 1:100 glial fibrillary acidic protein (GFAP, Thermo Fisher Scientific, Waltham, MA, USA) with 3% BSA in 0.01 M PBS for 24 h. The cover slip was then washed with PBS 3 x and incubated with Alexa 594 (Thermo Fisher Scientific, Waltham, MA, USA) (1:200) secondary anti-rabbit for 24 h. The cover slips were washed with PBS 3 x and double distilled H₂O 2 x and were mounted onto microscope glass slides with Fluoroshield with DAPI (Sigma, St. Louis, MO, USA).

2.15 Imaging

Images were taken on Nikon Eclipse Ni (Nikon Instruments Inc, Melville, NY, USA) microscope. Analysis and quantification was done using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, USA)

Cortical Neurons:

A single cover slip was used to create a single experimental replicate (n=1) and three pictures from different regions of the cover slip were taken for triple technical replicates. In order to quantify the number of viable neurons that expressed GM1 at each time point of glutamate exposure, ImageJ (Wayne Rasband, National Institute of Health,
Bethesda, USA) was first used to merge the DAPI, PI and GM1 images. Then, the number of neurons that were positive for GM1 without PI positive nuclei was counted using Image J. This number was divided by the total cell counts based on DAPI and expressed as a percentage of total neurons. The percentage of neurons that express GM1 without PI nuclei from each time point of glutamate exposure was normalized to the control value.

To assess the level of neuronal viability, the total number of PI positive neurons within each picture was expressed as a percentage of total number of neurons present within the picture. The percentage of PI positive cells from three replicates gave an average of PI positive cells per n. This calculation was used to assess the level neuroprotective property of GM1 ganglioside in glutamate exposure.

DAPI images at 40 x was taken and stitched together using the Nikon Imaging Software Elements (Nikon). On ImageJ, using a grid setting of 500000 pixels per area, five random squares were chosen. Within each of these squares, the total number of DAPI positive cells were counted and averaged to represent the total number of cells present within the time frame. This was done for each glutamate exposure time points to account for the number of cells present for each time point.

**Primary Microglia:**

GM1 and GM3 optical densitometry on control (non-activated) and activated microglia was performed using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, USA).
2.16 Data Analysis

Averages of all data were calculated using Microsoft Excel. Statistical analysis was performed using Prism 6 (GraphPad). Multiple group comparisons were done by one-way ANOVA. Comparison of the intensity of different carbon chain ganglioside was done by two-way ANOVA. Statistical significance was done to compare experimental groups to the control groups using Dunn’s and Dunnett’s multiple comparisons post hoc test. To test the significance between two groups, Tukey’s multiple comparison test was performed. Data is presented as mean ± SEM. All significance level was set to p ≤ 0.05.
Section 3: RESULTS
3.1 Effects of glutamate exposure on cortical neurons

Glutamate has been previously shown to induce neuronal cell death\(^{152}\). It was nevertheless important for us to confirm whether glutamate was a viable method to induce neuronal death in our primary cell system. Jong et al\(^{149}\) used varying concentration of glutamate, from 10 µM to 2 mM and determined that 100 µM was an optimal dose to induce gradual neuronal death. Thus, this would permit us to track the possibility of gradual changes of ganglioside expression profiles in dying neurons. For this study, DIV 14 embryonic rat cortical neurons were exposed to 100 µM of glutamate for up to 24 h. These neurons were also labeled with PI 45 min before fixation with PFA to assess the level of dying neurons.

Healthy control neurons that were not exposed to glutamate (control) showed round cell bodies with discernible projections protruding out to the periphery. These cells were not accompanied by PI signal in the nucleus (Fig. 5 A). As early as 1 h following glutamate exposure, neuronal projections began to retract. At this stage, neurons showed uptake of PI into the nucleus indicating dying neurons (Fig. 5 A). By 3 h of glutamate exposure, there was a visible increase in the number of PI positive neurons. Following 6 h of glutamate exposure, the structural integrity of neurons was severely compromised and showed high levels of PI in the nuclei. By 24 h, levels of neuronal death were extensive as indicated by the cell debris as well as PI positive nuclei.

Neuronal viability was quantified by subtracting the number of PI positive neurons from total neuron cell counts using DAPI and expressed as a percentage of total cells. The percentage of living cells was then normalized to the controls (100 % ± 1.712). As early as 1 h following glutamate exposure, neurons showed a significant decrease (1
h: 71.1 ± 2.0 %) in viability compared to uninjured controls (Fig. 5 B, p ≤ 0.05). By 3 h of glutamate exposure, neuronal viability decreased significantly (3 h: 33.5 ± 4.0 %) and remained significantly reduced by 6 and 24 h compared to controls (6 h: 20.6 ± 3.9 %, 24 h: 26.8 ± 8.1 %) (Fig. 5 B, p ≤ 0.05).
A

Control

DIC

1 h 100 μM glutamate

PI

3 h 100 μM glutamate

DAPI

6 h 100 μM glutamate

Merged

24 h 100 μM glutamate
Figure 5. Quantification of viable cells after exposure to glutamate. (A) Photomicrograph of DIV 14 rat cortical neurons following exposure to 100 µM glutamate. Neurons were exposed to 100 µM of glutamate for 1, 3, 6, or 24 h and fixed with 4% PFA. These cells were labeled with DAPI and PI. (B) The number of PI positive neurons were subtracted from the total DAPI counts to assess the number of viable neurons. The number of viable neurons was divided by the total cell counts based on DAPI and expressed as a percentage of total cells. Data is normalized to the control. Data is presented as mean +/- SEM; data was analyzed and compared to the control by one-way ANOVA and Dunnett’s multiple comparisons test. * indicate p ≤ 0.05 (n=8 for each time point). Bar indicates 50 µm for each panel.
3.2 Ganglioside GM1, GM2, and GM3 Levels in Neurons Exposed to Glutamate

To investigate the changes of ganglioside GM1, GM2, and GM3 levels in neurons following exposure to glutamate, lipids were extracted using a modified Folch method. These cell lipid extracts were collected and filtered through a solid phase extraction C18 column and NH\textsubscript{2} hydrophilic column and analyzed through a Waters QTOF Micro mass spectrometer and Mass Lynx 4.1 (Fig. 6, 8, 10). The d16:1, d18:1, and d20:1 sphingosine moieties of gangliosides were analyzed as they are believed to play different roles with respect to neuronal function and death. In order to account for varying amount of lipids collected from different cell replicates, the peaks of each gangliosides were normalized to the sum of 9 lipid species of interest (d16:1, d18:1, and d20:1 for GM1, GM2, and GM3). To further adjust for the total starting lipid present in the sample, a numerical factor was found between the total number of neurons present in the 10 cm plate of each time point of glutamate exposure relative to the control 10 cm plates. This was done by dividing the number of neurons present in the coverslips of control group by the number of neurons present in the cover slips from each time points of glutamate exposure. The factors for each time point was multiplied to the ganglioside peaks for the corresponding time of glutamate exposure (Fig. 7 D). This process took into account of the neurons that died and detach from the plates, which resulted in reduced levels of starting lipids.

At 1 h following glutamate exposure, d16:1 GM1 did not show a significant change compared to control levels (Control: 100 ± 2.4 %, 1 h: 88.6 ± 7.8 %) (Fig. 7 A, p > 0.05). However, following 3 h of glutamate exposure, there was a significant increase (3 h: 135 ± 2.7 %) in the d16:1 GM1 compared to control neurons (Fig. 7 A, p ≤ 0.05).
6 h, d16:1 was significantly elevated (6 h: 145.4 ± 4.5 %) (p ≤ 0.05). However, at 24 h, there was no significant difference in the level of d16:1 compared to control group (p > 0.05). When d18:1 GM1 ganglioside level was analyzed in dying neurons, there was no significant change at 1 h and 3 h of glutamate exposure compared to control neurons (Fig. 7 B, p > 0.05). However, there was a significant increase of d18:1 GM1 ganglioside (6h: 149.8 ± 6.8 %) at 6 h of glutamate exposure compared to the controls (p ≤ 0.05). Following 24 h of glutamate exposure, d18:1 GM1 returned to control levels and there was no significant difference between the two groups (p > 0.05). Finally, when d20:1 GM1 was analyzed, there was no significant change between control at any time of exposure to glutamate (Fig. 7 C, p > 0.05).
GM1

**A**

- **GM1**
  - d18:1 - 18:0
  - m/z: 1544.8

**B**

- **GM1 d18:1 - 18:0**
  - m/z: 1544.8

- **GM1 d16:1 - 18:0**
  - (d18:1-d16:0)
  - m/z: 1516.8

- **GM1 d20:1 - 18:0**
  - m/z: 1572.9
Figure 6. Mass spectra of GM1 ganglioside from uninjured control neurons. Following the detection of gangliosides with a Waters QTOF ESI-MS instrument, chromatogram was extracted with Mass Lynx 4.1 to visualize GM1, GM2, GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties. Peaks of GM1, GM2 and GM3 are presented relative to each species (A). Higher peaks indicate higher quantity of gangliosides. (B) represents an enlarged spectrum of GM1 d18:1 and GM1 d20:1. (C) represents an enlarged spectrum of GM1 d16:1.
Figure 7. GM1 expression changes in rat embryonic cortical neurons exposed to glutamate. Normalized peak intensity from Waters QTOF ESI-MS instrument of (A) GM1 d16:1, (B) GM1 d18:1, and (C) GM1 d20:1 from DIV14 rat cortical neurons following exposure to 100 µM of glutamate for up to 24 h. Neurons were cultured in 10 cm plates and were exposed to glutamate for 1, 3, 6, and 24 h. Lipids were collected using a modified Folch method. Each biological replicate (n) was represented by four 10 cm plates and each n was analyzed with a Waters QTOF instrument in triplicates. Peak intensities from each run were normalized to the sum of d16:1, d18:1, and d20:1 sphingosine moieties of each GM1, GM2, GM3 gangliosides for total of 9 lipids from all the runs within glutamate exposure time point. Peaks were multiplied by a cell total number factor for each time point relative to control to account for the total number of cells present at different time points of glutamate exposure (D). Data is presented as mean +/- SEM; data was analyzed and compared to the control by one-way ANOVA and Dunnett’s multiple comparisons test. * indicate p ≤ 0.05. (for each time point n = 4).
None of the sphingosine moieties, d16:1, d18:1, or d20:1, of GM2 ganglioside showed significant changes throughout the time course of exposure to glutamate compared to uninjured control neurons (Fig. 9, p > 0.05). Similarly, none of the sphingosine moieties of GM3 gangliosides showed significant changes throughout the time course of exposure to glutamate compared to uninjured control neurons (Fig. 11, p > 0.05).
Figure 8. Mass spectra of GM2 ganglioside from uninjured control neurons. Following the detection of gangliosides with a Waters QTOF ESI-MS instrument, chromatogram was extracted with Mass Lynx 4.1 to visualize GM1, GM2, GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties. Peaks of GM1, GM2 and GM3 are presented relative to each species (A). Higher peaks indicate higher quantity of gangliosides. (B) represents an enlarged spectrum of GM2 d16:1, GM2 d18:1 and GM2 d20:1.
Figure 9. GM2 expression changes in cortical neurons exposed to glutamate. Normalized peak intensity from Waters QTOF ESI-MS instrument of (A) GM2 d16:1, (B) GM2 d18:1, and (C) GM2 d20:1 from DIV14 rat cortical neurons following exposure to 100 μM of glutamate for up to 24 h. Neurons were cultured in 10 cm plates and were exposed to glutamate for 1, 3, 6, and 24 h. Lipids were collected using a modified Folch method. Each biological replicate (n) was represented by four 10 cm plates and each n was analyzed with a Waters QTOF instrument in triplicates. Peak intensities from each run were normalized to the sum of d16:1, d18:1, and d20:1 sphingosine moieties of each GM1, GM2, GM3 gangliosides for total of 9 lipids from all the runs within glutamate exposure time point. Peaks were multiplied by a cell total number factor for each time point relative to control to account for the total number of cells present at different time points of glutamate exposure (D). Data is presented as mean +/- SEM; data was analyzed and compared to the control by one-way ANOVA and Dunnett’s multiple comparisons test. * indicate p ≤ 0.05. (for each time point n = 4).
Figure 10. Mass spectra of GM3 ganglioside from uninjured control neurons. Following the detection of gangliosides with a Waters QTOF ESI-MS instrument, chromatogram was extracted from Mass Lynx 4.1 to visualize GM1, GM2, GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties. Peaks of GM1, GM2 and GM3 are presented relative to each species (A). Higher peaks indicate higher quantity of gangliosides. (B) represents an enlarged spectrum of GM3 d16:1, GM3 d18:1, and GM3 d20:1.
Figure 11. GM3 expression changes in cortical neurons exposed to glutamate. Normalized peak intensity from Waters QTOF ESI-MS instrument of (A) GM3 d16:1, (B) GM3 d18:1, and (C) GM3 d20:1 from DIV14 rat cortical neurons following exposure to 100 µM of glutamate for up to 24 h. Neurons were cultured in 10 cm plates and were exposed glutamate for 1, 3, 6, and 24 h. Their lipids were collected using a modified Folch method. Each biological replicate (n) was represented by four 10 cm plates and each n was analyzed with Waters QTOF instrument in triplicates. Peak intensities from each run were normalized to the sum of d16:1, d18:1, and d20:1 sphingosine moieties of each GM1, GM2, GM3 gangliosides for total of 9 lipids from all the runs within glutamate exposure time point. Peaks were multiplied by a cell total number factor for each time point relative to control to account for the total number of cells present at different time points of glutamate exposure (D). Data is presented as mean +/- SEM; data was analyzed and compared to the control by one-way ANOVA and Dunnett’s multiple comparisons test. * indicate p ≤ 0.05. (for each time point n = 4).
Ganglioside GM1, GM2 and GM3 sphingosine moieties d16:1, d18:1 and d20:1 are believed to play unique roles in neurons. Comparing the level of these moieties between embryonic neurons and adult neurons may give an insight to their specific roles in neuronal regulation and death. In primary cortical neurons, d18:1 sphingosine moieties were the most abundant species of GM1, GM2, and GM3 (Fig. 12) while d16:1 and d20:1 were less abundant. The difference in the quantity between d16:1 and d20:1 for GM1, GM2, and GM3 was not significant in cortical embryonic neurons (p > 0.05). However, in 4-month old healthy rat cortices, there was an increased expression of d20:1 GM1 ganglioside which was significantly higher (d20:1: 695.6 ± 347.8) than d16:1 GM1 ganglioside (158.0 ± 79.0) (Fig. 12 A, p ≤ 0.05). Furthermore, d18:1 GM2 species in 4-month old healthy rat cortices was significantly lower (591.8 ± 295.9) than in control (3383.4 ± 1691.7) or glutamate exposed embryonic primary cortical neurons (1 h: 2161.3 ± 1080.6, 3 h: 3786.0 ± 1893.0, 6 h: 4433.4 ± 2216.7, 24 h: 5036.2 ± 2518.1) (Fig. 11 B, p ≤ 0.05). However, there was no significant difference in the expression levels of d18:1 GM3 gangliosides between control or glutamate exposed primary embryonic cortical neurons and 4-month healthy rat brains (Fig. 12 C, p > 0.05).
Figure 12. Comparison of peak intensities of sphingosine moieties of gangliosides GM1, GM2, and GM3. Peak intensity of (A) GM1, (B) GM2, and (C) GM3 and its d16:1, d18:1, and d20:1 moieties from rat adult and embryonic cortical neurons following exposure to 100 µM glutamate for up to 24 h. Neurons were cultured in 10 cm plates and were exposed to glutamate for 1, 3, 6, and 24 h. Lipids were collected using a modified Folch method. Each biological replicate (n) was represented by four 10 cm plates and each n was analyzed with Waters QTOF instrument in triplicates. Peak intensities from each run were normalized to the sum of d16:1, d18:1, and d20:1 sphingosine moieties of each GM1, GM2, GM3 gangliosides for total of 9 lipids from all the runs within glutamate exposure time point. Furthermore, the peaks were multiplied by a cell total number factor for each time point relative to control to account for the total number of cells present at different time points of glutamate exposure (D). Data is presented as mean +/- SEM; data presented as mean +/- SEM; data were analyzed and the d16:1, d18:1, d20:1 sphingosine moieties were compared by two-way ANOVA and tukey’s multiple comparisons test. Data of (D) was analyzed by one-way ANOVA and Dunnett’s multiple comparisons test. * indicate p ≤ 0.05. (for each time point n = 4).
3.3 GM1 and GM3 immunohistochemistry

In order to visualize the expression profile of GM1 ganglioside in dying neurons, cortical neurons were labeled with GM1 antibody following exposure to glutamate. These cells were also labeled with PI to understand the relationship between the time profile of neurodegeneration and GM1 expression.

In control neurons, there were only a few PI positive cells and only some neurons showed GM1 positive label, which was localized to the cell bodies (Fig. 13 A). By 1 h of glutamate exposure, there was an increase in the level of PI positive neurons. At this time point, some neurons showed GM1 expression without PI label in the nuclei. By 3 h of glutamate exposure, there was a detectable increase in the level of GM1 expressing neurons and an increase in PI positive nuclei. There was also an increase in the number of neurons that were positive for GM1 and negative for PI. By 6 h, GM1 was highly expressed in the neurons. Neurons positive for GM1 and negative for PI were also increased by 6 h. However, by 24 h, although there was an increased level of PI positive cells, the level of GM1 positive neurons was reduced. There was a reduction and the neurons that were positive for GM1 and negative for PI, presumably due to extensive cell death by this time point.

The number of viable neurons that express GM1 was quantified by dividing the number of neurons that were labeled GM1 positive without PI from total number of neuron counts by DAPI, and expressing it as a percentage of total neurons. By 1 h of glutamate exposure, there was no significant change in the number of neurons that were GM positive and PI negative compared to the control group (Fig. 13 B). However, by 3 h and 6 h following exposure to glutamate, there was a significant increase in the number...
of GM1 positive and PI negative neurons compared to the control group, with a peak at 6 h (control: 100.0 ± 20.5 %, 3 h: 220.1 ± 9.4 %, 6 h: 272.0 ± 38.1 %). By 24 h of glutamate exposure, the number of GM1 positive and PI negative neurons decreased back down to control levels (p > 0.05).
A

Control

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged

DIC

50 µm

1 h 100 µM glutamate

3 h 100 µM glutamate

6 h 100 µM glutamate

24 h 100 µM glutamate

GM1

PI

DAPI

Merged
Figure 13. Quantification of viable neurons that express GM1 after exposure to glutamate. (A) Photomicrograph of DIV 14 rat cortical neurons following exposure to 100 µM glutamate. Neurons were exposed to 100 µM of glutamate for 1, 3, 6, or 24 h and fixed with 4 % PFA. These cells were labeled with GM1, DAPI and PI. Arrows indicate neurons that are GM1 positive and PI negative. (B) The number of GM1 positive neurons that were PI negative were divided by total cell counts based on DAPI expressed as a percentage of total cells. Data is normalized to the control. Data is presented as mean +/- SEM; data was analyzed and compared to the control by one-way ANOVA and Dunnett’s multiple comparisons test. * indicate p ≤ 0.05 (n=8 for each time point). Bar indicates 50 µm for each panel.
To analyze the structural and temporal expression profile of GM3 in neurons undergoing neurodegeneration, primary cortical neurons were exposed to glutamate for 1, 3, 6, and 24 h and fixed with PFA. They were then stained for GM3 ganglioside, DAPI and PI.

As the exposure time to glutamate increased, neurons lost their structural integrity; showed neurite retractions, cell bodies lost their round healthy shape (Fig. 14 C). This was also accompanied by an increase in PI uptake in the nuclei (Fig. 14 A). However, GM3 ganglioside by immunofluorescence was undetectable in either the uninjured control cortical neurons or the neurons exposed to glutamate (Fig. 14 A).
Control
1 h 100 µM glutamate
3 h 100 µM glutamate
6 h 100 µM glutamate
24 h 100 µM glutamate

DIC
GM3
PI
DAPI
Merged

50 µm
Figure 14. Photomicrographs of rat embryonic cortical neurons labeled with antibody to GM3, DAPI, and PI following exposure to 100 µM of glutamate for up to 24 h. (A) Neurons were exposed to 100 µM of glutamate for 1, 3, 6, 24 h. These cells were fixed with 4 % PFA and stained with GM3 antibody, DAPI and PI. (B) Zoomed in DIC images of uninjured control neuron and (C) Zoomed in DIC images of neurons exposed to 3 h of 100 µM glutamate. Bar indicates 50 µm for each panel.
3.4 Effect of exogenous GM1 pretreatment of rat primary cortical neurons before exposure to glutamate

In order to assess whether exogenous GM1 pretreatment can provide neuroprotection to cortical neurons undergoing neurodegeneration, DIV 14 rat primary cortical neurons were treated with 5, 10, or 25 µM of GM1, 24 h prior to 100 µM glutamate exposure. These cells were also labeled with PI to assess the level of neuronal death.

Neurons without exposure to glutamate showed negligible levels of PI positive nuclei across different concentrations of GM1 pretreatment (Fig. 15 A). As the time of exposure to glutamate increased, there was an increase in PI positive nuclei across all groups. However, the neurons that were not pretreated with GM1 showed higher levels of PI positive nuclei (Fig. 15 A). By 24 h of glutamate exposure, virtually all cell nuclei were positively labeled for PI across all GM1 treatment groups.

Cell viability was quantified by subtracting the number of PI positive cells from total neuron cell counts using DAPI and expressed as a percentage of total cells. Viability values of neurons exposed to various times of glutamate were normalized to the GM1 pretreated neurons that were not exposed to glutamate. At 1 h of glutamate exposure, there was no significant difference in the number of viable neurons across the different GM1 treatment concentration groups (Fig. 15 B, p > 0.05). By 3 h of glutamate exposure, the neurons that were pretreated with GM1 showed significant increase of neuronal viability compared to neurons that were not pretreated with GM1 (no GM1: 33.5 ± 4.0 %, 5 µM: 68.8 ± 5.4 %, 10 µM: 56.7 ± 4.1 %, 25 µM: 90.2 ± 2.4 %) (Fig. 15 B, p ≤ 0.05). Pretreatment with 25 µL showed the greatest number of surviving neurons compared to the controls. Following 6 h of glutamate exposure, neurons that were treated with GM1
continued to show significant increase in neuronal viability (no GM1: 20.6 ± 3.9 %, 5 μM 55.8 ± 1.5 %, 10 μM 57.0 ± 4.5 %, 25 μM 72.5 ± 3.7 %) (Fig. 15 B, p ≤ 0.05), with 25 μM of GM1 showing the greatest survival. By 24 h, there was no significant difference in viable neurons across all groups (p > 0.05).
No GM1 pretreatment

No glutamate 50 μm

1 h 100 μM glutamate

3 h 100 μM glutamate

6 h 100 μM glutamate

24 h 100 μM glutamate

5 μM GM1 pretreatment

10 μM GM1 pretreatment

25 μM GM1 pretreatment
Figure 15. Effects of GM1 pretreatment on neurons exposed to 100 µM of glutamate. Cortical neurons were treated with 5, 10, or 25 µM of exogenous GM1 24 h before exposure to 100 µM glutamate. Following exposure to glutamate, neurons were fixed with 4% PFA and mounted onto cover slips and stained with DAPI (A). Total cell counts were measured using DAPI and PI positive cells were used to indicate dying neurons. To quantify cellular viability, neurons that were PI negative were expressed as a percentage over all neurons. Data was normalized to neurons that were not exposed to glutamate (B). Results are presented as mean +/- SEM; data were analyzed and compared to the control (control n=8, GM1 pretreatment group n = 4) by two-way ANOVA and tukey’s multiple comparisons test. * indicate p ≤ 0.05. Bar indicates 50 µm for each panel.
3.5 Ganglioside GM1 and GM3 expression pattern in activated microglia

Microglia are involved in the initial phase of inflammation in the brain and may contribute to the ganglioside expression profiles observed in in vivo and in situ models of neurodegeneration\textsuperscript{27}. Therefore, it was important to assess the ganglioside expression profiles solely in microglia in this study.

Primary rat microglia were cultured and on DIV2, they were activated by exposing them to amyloid beta oligomers for up to 1 h\textsuperscript{155–158}. They were fixed with 4 \% PFA and labeled with DAPI and antibody to GM1 or GM3.

Control microglia showed small and elongated cell bodies with projections and showed weak staining for GM3 (Fig. 16 A, B). When these cells were exposed to amyloid beta oligomer for 1 h, a known activator of microglia, they became activated, as indicated by spherical and phagocytic appearance (Fig. 16 C). They were also accompanied by GM3 staining that showed a punctate pattern within the cell bodies (Fig. 16 A). When optical densitometry for GM3 expression was performed on control and activated microglia, there was a significant increase in the pixel intensity (24.3 ± 5.0 \%) for GM3 in activated microglia compared to control (Fig. 16 E, p ≤ 0.05). Additionally, some coverslips exposed to amyloid beta oligomers were also labeled with GFAP (red) to ensure that the source of GM3 expression increase was due to activated microglia and not astrocytes. Although some GM3 expression were seen on astrocytes (red), most of the GM3 stain does not co-label with GFAP staining, indicating that most of GM3 increase observed in these cultures stemmed from microglia (Fig. 16 D).
Figure 16. GM3 expression measured in microglia after being activated with amyloid β oligomer for 1 h. Microglia was activated using 2 µM of amyloid β oligomer and fixed with 4 % PFA and labeled with DAPI, and GM3 (A). Zoomed DIC image of inactive microglia (B). Zoomed DIC image of microglia after exposure to 2µM of amyloid-β peptide for 1 h (C). Cover slips were also stained with GFAP after exposure to 2 µM of amyloid β oligomer (D red). Arrows indicate astrocytes. Pixel intensity of GM3 expression per microglia was measured using Image J (E). Average pixel intensity per cell of GM3 in microglia after 1 h of exposure to 2 µM of amyloid β oligomer was compared to control. Intensity was measured for up to 54 microglia in control and 46 microglia in 1 h of amyloid β oligomer exposure across 4 cover slips for each group(n=4). Results are presented as mean +/- SEM; data was analyzed by two-tailed t-test. * indicate p ≤ 0.05. Bar indicates 50 µm for each panel.
When GM1 staining was analyzed, control microglia showed positive GM1 staining within the cell body (Fig. 17 A). Once microglia were activated, GM1 expression was spread over the entire cell body which took on a round and phagocytic shape. When the optical density for GM1 was measured using Image J, there was no significant difference between the control and activated microglia (Fig. 17 B, p > 0.05). However, when microglia became activated, cell size increased and GM1 was expressed throughout the entire cell body. Although the expression density of GM1 did not change, overall production of GM1 increased (Fig. 17 A), presumably due to the increase in cell size.
Figure 17. GM1 expression measured in microglia after being activated with amyloid β oligomer for 1 h. (A) Microglia were activated using 2 µM of amyloid β oligomer and fixed with 4% PFA. Microglia were labeled with DAPI, and GM1. (B) Average pixel intensity of GM3 expression per microglia was measured using Image J. Intensity of GM1 in microglia after 1 h of exposure to 2 µM of amyloid β oligomer was compared to control. Intensity was measured for up to 40 microglia in control and 38 microglia in 1 h of amyloid β oligomer exposure across 4 cover slips for each group (n=4). Results are presented as mean +/- SEM; data was analyzed by two-tailed t-test. * indicate p ≤ 0.05. Bar indicates 50 µm for each panel.
Section 4: DISCUSSION
4.1 Changes in Ganglioside Expression in Dying Neurons

Gangliosides have been associated with various roles within the CNS including regulation of brain development, neuroprotection and repair, and neurodegeneration\textsuperscript{49,58,123}. Although ganglioside expression profiles in the injured brain have been studied through \textit{in vivo} models, it has never been studied at the level of the neurons within the brain. Through this study, we demonstrated, for the first time, the temporal expression profile of simple ganglioside GM1, GM2, and GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties in rat embryonic primary cortical neurons that are undergoing neurodegeneration through exposure to glutamate.

As expected, exposure to glutamate reduced neuronal viability in a time-dependent manner and suggested that glutamate was an appropriate reagent to induce cell death to study ganglioside expression profiles in steadily dying neurons. The transient increase of d16:1 GM1 at 3 h and 6 h and peak of d18:1 at 6 h was an interesting finding. A previous study by Whitehead \textit{et al.} 2011 showed through MALDI-MS that d18:1 GM1 increased at 24 h and peaked at 7 d and decreased by 14 d of MCAO reperfusion injury in mice brains. Their study showed that d20:1 also increased at 24 h and peaked at 3 d.

Furthermore, Caughlin \textit{et al.} 2015 demonstrated a significant increase in GM1 d18:1 at 3 d within brain infarct region from combined model of Aβ toxicity and endothelin-1 injection following treatment. These results are similar with this current study. The function of d16:1 relative to d18:1 and d20:1 is yet unclear but our study indicates that the it is being produced concomitantly with d18:1 in response to injury. Particularly noting the neuroprotective property of GM1 from this and previous studies, dying neurons may be increasing endogenous synthesis of GM1 in an attempt to self-
protect from glutamate exposure and cell death. Furthermore, although Whitehead et al. 2011 and Caughlin et al. 2015 showed increased expression of d20:1 in brain tissue following infarct injury, our study did not show significant change in dying neurons. This may be due to the fact that there are differences in the expression patterns of sphingosine moieties in embryonic brains and adult brains. Previous work showed that there are higher quantities of d20:1 sphingosine moieties found in adult mammal brains. For this reason, we also looked at the relative abundance of d16:1, d18:1 and d20:1 ganglioside species in embryonic neuron culture and compared to 4-month adult rat brain. Our study using ESI-MS accurately showed that d18:1 was the most abundant ganglioside both in embryonic neurons as well as 4-month adult brains which is consistent with previous studies. Furthermore, d20:1 GM1 was increased in adult brain in comparison to embryonic neuronal cultures which is also consistent with previous studies. These evidences suggests that there may not have been sufficient expression of d20:1 in embryonic cortical neurons in our study, to show a measurable response to injury. It is also interesting to note the immunofluorescence data that there was a significant increase in the GM1 expression within neurons exposed to glutamate that were not labelled with PI. This suggests that the increase in GM1 expression in dying neurons observed through ESI-MS stemmed from the few live neurons which up-regulate GM1 synthesis in order to counteract the damage induced by excess glutamate.

This study also investigated the expression profile of GM2 and GM3 in dying neurons as they are associated with cellular death. We predicted that these species would not be present in uninjured control neurons. However, ESI-MS revealed that both GM2 and GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties were
present in the control groups, indicating that GM2 and GM3 is not toxic at every stage of development. However, levels of GM2 in 4-month adult rat brains were significantly lower in comparison to embryonic neurons which is consistent with previous studies. Although in mature healthy adult mammalian brains, GM2 and GM3 were undetectable or were present in trace amounts\textsuperscript{27}, in the developing brain of mammals, GM2 and GM3 are prominently expressed as they are believed to play regulatory roles in development\textsuperscript{20,104}. As many studies in the literature indicate that expression of GM2 and GM3 is increased in injured brains, we predicted that levels of GM2 and GM3 will increase in cortical neurons exposed to glutamate. Interestingly and contrary to our predictions, GM2 and GM3 did not show significant difference in neurons exposed to glutamate compared to uninjured control neurons. In order to visually confirm that there was a lack of change in the expression of GM3 in dying neurons, immunofluorescence was performed to on the GM3 gangliosides. However, the antibody was not able to detect any GM3 expression in the rat primary embryonic cortical neurons in culture. This suggests that ESI-MS is far superior in sensitivity than the antibody’s ability to bind.

One possible explanation of the lack of significant change in the expression profile of GM3 and GM2 in the dying cortical neurons from this study may be that the variation in the ESI-MS peak intensities was too large between replicates to achieve statistical significance. By increasing the number of replicates, there might have been significant increase in the expression levels of GM2 and GM3 in injured neurons exposed to glutamate. Another potential explanation is that the increase in GM2 and GM3 expression reported in previous studies using \textit{in vivo} models are due to other cells within the brain that are responding to injury.
4.2 GM1 Pretreatment

In this study, pretreating the rat primary embryonic cortical neurons with exogenous GM1 showed increase in cellular viability when exposed to glutamate. Our hypothetical mechanism of GM1 neuroprotection was that as the level of GM1 decreases in the dying neurons, exogenous addition of GM1 would replenish the depletion of the endogenous GM1 within the membrane. Although ESI-MS data suggests that there was no significant decrease in the level of GM1 in dying neurons, by 1 h of exposure to glutamate, there was a trend towards a decrease in GM1 ganglioside in these neurons. Furthermore, Caughlin et al. 2015 also showed in their study that in small brain infarcts of rat brains treated with endothelin-1, there was a slight decrease in GM1. This suggests that our hypothesis is a potential mechanism in which GM1 provides neuroprotection in dying neurons. Overall, our data suggests that dying neurons may increase synthesis of GM1 as an inherent mechanism to counteract environmental insult. By adding exogenous GM1, it replenishes the depleted GM1 in the membrane of dying neurons to enhance cellular viability in neurons exposed to glutamate.

4.3 Ganglioside Expression in Activated Microglia

The lack of change in ganglioside GM3 was of particular interest considering Whitehead et al. 2011 showed an increase in GM3 in mice brain infarcts after 3 d and 7 d of MCAO reperfusion injury around the border of the infarct region. We were led to believe that the source of GM3 increase was from other cells in the brain. We were particularly interested to observe the GM3 ganglioside expression in microglia because the time frame of GM3 expression increase at 3d and 7d, and decrease at 28 d, in
Whitehead et al. 2011 study closely correlates with microglial activity as they are the first cells to migrate to CNS injury\textsuperscript{159} (Fawcett JW et al. 1999). The final stage of CNS injury is glial scarring, predominantly responsible by astrocyte activity\textsuperscript{159}. If astrocytes were responsible for GM3 production, the increase would have persisted until 28 d of MCAO damage, which it did not.

One way to activate microglia is through exposure to soluble amyloid beta oligomer 1-42 \textsuperscript{155–157}. Significantly increased expression of GM3 in activated microglia compared to inactivated controls was an intriguing finding. This confirmed our hypothesis that GM3 detected in injured brain is attributed to microglial activity. Furthermore, although astrocytes showed some GM3 staining, most of the GM3 staining were positive in cells that were not stained with GFAP. This indicated that GM3 increase happens primarily in microglia and not astrocytes. In peripheral tissues, macrophages have synonymous function as microglia in the CNS. A study by Bobryshev et al. 1997 showed that there was an increase of GM3 ganglioside expression within the inflamed regions of the atherosclerotic lesions\textsuperscript{160}. It was further investigated that macrophages that infiltrated the atherosclerotic plaques had increased expression of GM3 and GM3 synthase compared to those of non-diseased intima layers\textsuperscript{160,162}. It is believed that the role of GM3 in macrophages is a modulator that assists in the differentiation of monocytes to macrophages\textsuperscript{162}. This may also explain our observation with activated microglia in this study. A possible reason for the increase in the levels of GM3 in activated microglia compared to the inactive control might be that the synthesis of GM3 may have increased in order to differentiate and activate these cells as a response to inflammation. Similar to how active macrophages deposit GM3 in atherosclerotic lesions, active microglia may
also release GM3 as a product of activation into the surrounding brain tissues. This may explain the increase in GM3 levels observed within infarct regions of mice brains at 3 d and 7 d following MCAO treatment\textsuperscript{27} which was not observed in dying cortical neurons in our study. Toxic properties of GM3 to neurons may also exacerbate neuronal death in infarct regions.

It was intriguing to note that GM1 was detectable in both inactive and activated microglia. Although the intensity of GM1 expression through optical densitometry revealed no significant difference between inactive and activated microglia, the overall GM1 expression was increased in activated microglia. Moreno-Altamirano \textit{et al.} 2007 showed evidence of two subtypes of monocytes in which one subtype produced low GM1 and another subtype that expressed high levels of GM1\textsuperscript{163}. GM1 expression levels also correlated with the macrophage endocytic capabilities in which cells that expressed more GM1 were more endocytic. Furthermore, the percentage of monocytes that expressed more GM1 increased during differentiation from monocytes to macrophages, suggesting that GM1 may play a role in helping monocytes transform into active macrophages. This evidence suggests that the activated microglia in our study might be increasing synthesis of GM1 as it differentiates from inactive microglia to increase its endocytic capability in response to injurious stimuli; in our case amyloid beta oligomer.

### 4.4 Study Limitations

The first limitation to this study is the availability of antibodies used to label the gangliosides in rat embryonic cortical neurons. Although GM1 and GM3 were commercially available, a GM2 antibody that was suitable for immunofluorescence was
not available. A previous study using antibodies against GM2 ganglioside were custom made from supernatants from hybridoma clones\textsuperscript{105}, to which our lab did not have access. For this reason, we were unable to supplement the ESI-MS data with immunohistochemistry of GM2 expression profiles in dying neurons.

Another limitation to this study is that the antibodies used against gangliosides cannot distinguish between the d16:1, d18:1 or the d20:1 sphingosine moieties of gangliosides. Proteins that bind to specific gangliosides recognize the specific sugar chain combination\textsuperscript{27,43,44} of the molecule. Therefore distinguishing the sphingosine moieties based on different carbon lengths, which are embedded within the membrane by antibodies was not possible. Consequently, the immunofluorescence images of GM1 and GM3 antibody is not specific to a particular sphingosine moiety.

Another potential limitation of this study is the possibility of ganglioside antibodies cross reacting with another species of gangliosides. For example, anti-GM1 antibody collected from serum of patients with Guillain-Barré syndrome showed some cross reactivity with GD1b ganglioside\textsuperscript{164}. As a result, it is a possibility that the GM1 antibody used in this study showed some reactivity to GD1b. This was resolved by ESI-MS which is superior than immunohistochemistry as it has the ability to accurately distinguish the different species of gangliosides and its sphingosine moieties based on its mass to charge ratio.
4.5 Future Studies

This is the first study to analyze how gangliosides are regulated in pure cortical neurons undergoing neurodegeneration. We used glutamate to model neurodegeneration as it provides a basis in which how gangliosides are regulated in dying neurons. Now that we have a better understanding of simple ganglioside expression profile changes in dying neurons, we can investigate ganglioside expression profile changes in models that have more clinical relevance. A logical next step would be to analyze ganglioside expression profiles in neurons by inducing death by oxygen-glucose deprivation (OGD), exposure to Aβ oligomer, or MPTP, to mimic stroke, AD, and PD, respectively.

In this study, we have demonstrated the changes of GM1 and GM3 expression profiles in control and activated microglia through immunofluorescence. This data, combined with ganglioside expression changes in cortical neurons, suggests that the accumulation of GM3 in the brain of in vivo and in situ models likely stem from microglial activity. To avoid antibody cross reactivity and to selectively detect d16:1, d18:1, and d20:1 sphingosine moieties of gangliosides, ESI-MS should be performed on the control and activated microglia in the future. This would give us an accurate understanding of how gangliosides are regulated in microglia and its roles in inflammation and neuronal death. Once we have a full understanding of the ganglioside metabolism in activated microglia and its interaction with cortical neurons in neuroinflammation, perhaps we can manipulate certain pathways to reduce GM3 or GM2 accumulation to prevent or attenuate neuronal death in neurological diseases.
Section 5: SUMMARY AND CONCLUSIONS
Summary of Key Findings

1. Glutamate induced neuronal death in time-dependent manner.
2. Compared to uninjured control neurons, d16:1 GM1 increased in neurons exposed to 3 and 6 h of glutamate, d18:1 GM1 increased in neurons exposed to 6 h of glutamate, but there was no significant difference in the expression pattern of d20:1 GM1 in neurons exposed to glutamate.
3. Compared to uninjured control neurons, GM2 expression did not show significant difference in neurons exposed to glutamate.
4. Compared to uninjured control neurons, GM3 expression did not show significant difference in neurons exposed to glutamate.
5. d18:1 sphingosine moieties were the most commonly found gangliosides in rat embryonic cortical neurons.
6. There was a significant increase in the level of d20:1 GM1 gangliosides relative to d16:1 in healthy 4-month adult rat brain compared to rat cortical embryonic brain.
7. Immunofluorescence indicated a significant increase in the number of GM1 positive and PI negative neurons compared to the control group after exposure to glutamate.
8. Pretreatment of cortical embryonic neurons with exogenous GM1 24 h before glutamate exposure showed significant increase in cellular viability.
9. GM3 ganglioside was undetectable in inactive microglia. When microglia were activated with amyloid beta oligomer, levels of GM3 ganglioside expression increased.

10. Both inactive and activated microglia show expression of GM1 but there was an increase in the overall production of GM1 in activated microglia.

Conclusions

Primary rat cortical embryonic neurons that underwent neurodegeneration following exposure to glutamate showed increase in GM1 d16:1 and d18:1 while GM2 and GM3 did not show significant differences. When neurons were pretreated with GM1 before glutamate exposure, cellular viability increased. Together, these findings suggest that GM1 has neuroprotective properties and that neurons may be upregulating its GM1 synthesis to counteract the damage induced by glutamate exposure. Furthermore, when microglia were activated, GM3 expression was increased. These findings suggest that the increased levels of GM3 in injured brain models may stem from microglial activity (Fig 18).

This study provides critical evidence for ganglioside metabolism in neurodegeneration in pure neurons and the link between microglia to provide an overview of ganglioside expression profiles of in vivo and in situ models. Accumulation of GM3 has been shown in models of neurodegeneration including stroke, AD, and Niemann-Pick disease. By understanding that GM3 is primarily produced from activated microglia as this study suggests, we can use GM3 in the microglia as a target to ameliorate neurodegeneration in clinical settings.
Figure 18. Hypothetical schematic of ganglioside expression in the CNS during glutamate excitotoxicity. Once brain is exposed to glutamate, neurons become injured. In response, some neurons increase their synthesis of GM1 in order to protect themselves from further damage. In parallel, in response to neuronal injury, microglia are transformed into activated state and increase their synthesis and expression of GM3. GM3 ganglioside is released into the extracellular environment that acts in a paracrine manner to induce further damage to neurons.
References


151. Ikeda, K. & Taguchi, R. Highly sensitive localization analysis of gangliosides and sulfatides including structural isomers in mouse cerebellum sections by combination of laser microdissection and hydrophilic interaction liquid


163. Moreno-Altamirano, M. M. B., Aguilar-Carmona, I. & Sánchez-García, F. J. Expression of GM1, a marker of lipid rafts, defines two subsets of human


Appendix
MS/MS Spectra of Ganglioside GM1, GM2 and GM3

GM1 d16:1 – d18:0 (d18:1-d16:0)

GM1 d18:1 – d18:0

GM1 d20:1 – d18:0
GM2 d16:1 – d18:0 (d18:1 – d16:0)

GM2 d18:1 – d18:0

GM2 d20:1 – d18:0
AUP Number: 2014-016
PI Name: Whitehead, Shawn N
AUP Title: Role Of Vascular Risk Factors In Cognitive Decline
Approval Date: 09/19/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Role Of Vascular Risk Factors In Cognitive Decline" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2014-016::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care
Curriculum Vitae
Dae Hee Park

University Educational Background

2013-2015  Master of Science in Anatomy and Cell Biology (MSc.), Western University
Thesis: “Investigation of changes in the ganglioside expression profiles of rat primary cortical neurons undergoing neurodegeneration through glutamate excitotoxicity by electrospray ionization mass spectrometry technique”

2009-2013  Bachelor of Medical Sciences (BMSc.) with Distinction.
Honors Specialization in Basic Medical Sciences, Western University

Honours, Scholarships and Awards during University

2014  NSERC Canada Graduate Scholarship.
Awarded for strong academic and research experience. Competition: 238 NSERC applicants university-wide, 25 awarded across all faculties. Value: $17,500

2013 & 2014  Western Graduate Research Scholarship, Western University
Awarded for strong academic performance. Value: $1,500

2010-2013  Dean’s Honor List, Western University
Named for academic excellence in university

2012  NSERC Undergraduate Student Research Award, Western University.
Awarded for academic achievement. Competition: 54 applications to Schulich School of Medicine and Dentistry – Western University, 17 awarded. Value: $6,000

2009-2013  Continuing Admissions Scholarship, Western University
Awarded for academic average of 95%+ in high school
Value: $10,000 ($2,500 per year – renewable)

Research Experience

2013  Summer Research Student, Western University
Optimizing the sublimation of 1, 5-diaminonaphthalene matrix onto rat brain tissue for image processing through matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) technique
**Supervisor:** Dr. Shawn Whitehead

**2010**  
**Summer Clinical Research Student**, Baycrest Hospital, Toronto, ON  
INCAS - Integrated Neurocognitive Assessment System  
**Supervisor:** Dr. Jon Ween

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**Publications, Presentations and Abstracts**

2015  

2015  
Caughlin S, **Park DH**, Hepburn J, Jurcic K, Yeung KKC, Cechetto DF, Whitehead SN. **Poster** (May 1): Increases in simple gangliosides GM2 and GM3 detected using MALDI IMS in a rat model of AD and stroke. **Conference:** Southern Ontario Neuroscience Association (SONA), Hamilton, ON, Canada

2015  
**Park DH**, Lajoie GA, Whitehead SN. **Poster Presentation** (April 1): Electrospray ionization mass spectrometry detects changes in expression profile of gangliosides neurodegenerating rat primary cortical neurons. **Conference:** London Health Research Day, London, ON, Canada

2015  

2014  
**Park DH**, Lajoie GA, Whitehead SN. **Poster Presentation** (November 16): Electrospray ionization mass spectrometry reveals changes in expression profile of gangliosides in neurodegenerating rat primary cortical neurons. **Conference:** Society for Neuroscience (SFN), Washington DC, USA

2014  
Caughlin S, Hepburn J, **Park DH**, Jurcic K, Yeung KKC, Cechetto DF, Whitehead SN. **Poster** (Nov 14): Changes in gangliosides expression detected by MALDI IMS in a combined rat model of Alzheimer’s disease and stroke. **Conference:** Society for Neuroscience (SFN), Washington DC, USA

2014  
**Park DH**, Lajoie GA, Whitehead SN. **Poster Presentation** (October 23): Glutamate toxicity changes expression profile of gangliosides in neurodegenerating rat primary cortical neurons. **Conference:** Anatomy and Cell Biology Research Day, London, ON, Canada


Teaching Experience

2013-2015 Teaching Assistant, MEDSCIEN 4900G: Medical Sciences Laboratory, Western University

2013 Teaching Assistant, ANATCELL 4451F: Integrative Neuroscience, Western University

Extra Curricular Activities

2014 Volunteer in the dialysis Unit at Victoria Hospital
I helped patients on dialysis machine perform simple physical exercises. I was able to build rapport and provide emotional support.

2012-2013 VP Volunteer of Meal Exchange
I lead out and organized trips to various soup kitchens and food shelters across local London community. I also coordinated the leaders for the trips and recruited volunteers to help at the food shelters and soup kitchens.

**2010-2011**

**Delaware Residence Soph**
I guided first year students during orientation week and I lived in residence alongside first year students as their mentor. I provided first year students with a sense of comfort and emotional support when they were in need. I also organized floor dinners, lead floor meetings, etc.

**2011**

**Volunteer in Leprosy Colony in Guanzhou China**
I provided basic services to leprosy patients including but not limited to: feeding, washing, cleaning homes, taking vitals, basic medical treatments (such as cleaning wounds).

**2010**

**Medical Missionary to Zambia**
I assisted a medical doctor in providing various treatments to patients of Chilubi Island in Zambia. I also assisted an eye surgeon during cataract surgeries.

**2010**

**Hospital Volunteer in the ICU at Mount Sinai Hospital**
I was in charge of administrative duties including taking phone calls, refilling medical supplies, delivering equipment to its designated locations. I also interacted/comforted visitors of patients and offered Korean translation services to patients and families.