IDENTIFICATION OF NOVEL GANGLIOSIDE-ASSOCIATED PROTEINS AND ADVANCES IN SINGLE-CELL GANGLIOSIDE METABOLOMICS IN NERVE CELLS

by

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Abstract

Gangliosides, glycosphingolipids that carry at least one sialic acid residue, are enriched in the plasma membranes of neurons. Despite their abundance, little is known about the molecules they interact with in the plasma membrane and how their metabolism differs from cell-to-cell. Rare human disorders of ganglioside biosynthesis and catabolism have devastating neurological defects and currently have no treatments. My thesis project aims to take a two pronged approach to help understand ganglioside function and ganglioside metabolic disorders. The first aim was to identify novel ganglioside interacting proteins by utilizing an unbiased screening method, mass spectrometric interaction analyses (iTRAQ) mass spectrometry. Primary rat cerebellar granule neuron cell surface proteins were isolated and ganglioside specific interacting proteins were collected via affinity chromatography on GT1b- and GM1-derivatized beads. iTRAQ and subsequent validation studies revealed reproducible differences between GT1b- and GM1-bound proteins from biological replicates. Rigorous statistical analyses revealed six GT1b-selective binding proteins, three of which suggested that GT1b participates in the regulation of glutamate-receptor surface expression. The second aim was to develop methods to quantify ganglioside metabolism in single cells. Fluorescently labeled gangliosides and various precursor glycosphingolipids were added to primary neuronal cultures. Separation of metabolites and ultrasensitive quantification was achieved using capillary electrophoresis coupled to a laser-induced fluorescence detection system with a sub-zeptomole (10e-21 mole) detection limit. Addition of labeled glycosphingolipids to primary cerebellar cultures allowed determination of anabolic and catabolic products at the single cell level. Neuronal and glial cells could be differentiated

with this method and heterogeneity was distinguishable among cells when analyzed on the single cell level. Analysis of metabolism in culture homogenates (thousands of cells) was consistent with metabolism averaged from a large number of single cells. Through understanding both ganglioside metabolism and the molecules that gangliosides interact with treatments could be developed for these rare human disorders.

Thesis advisor: Professor Ronald L. Schnaar

Thesis reader: Professor Daniel M. Raben

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Chapter 1: Introduction

Section 1.1: Gangliosides Structure and Metabolism

Glycans play roles in cell-cell interactions and signaling pathways (EGF, insulin), and defects in glycan expression play important roles in disease states (cancer, metabolism) [1-4]. Gangliosides are the dominant species of glycans on nerve cells in the mammalian brain with ~90% being GM1, GT1b, GD1a and GD1b [5]. They are glycosphingolipids (GSLs) that carry at least one sialic acid residue, and it is the variations in glycans that give GSLs their unique properties (Figure 1.1).

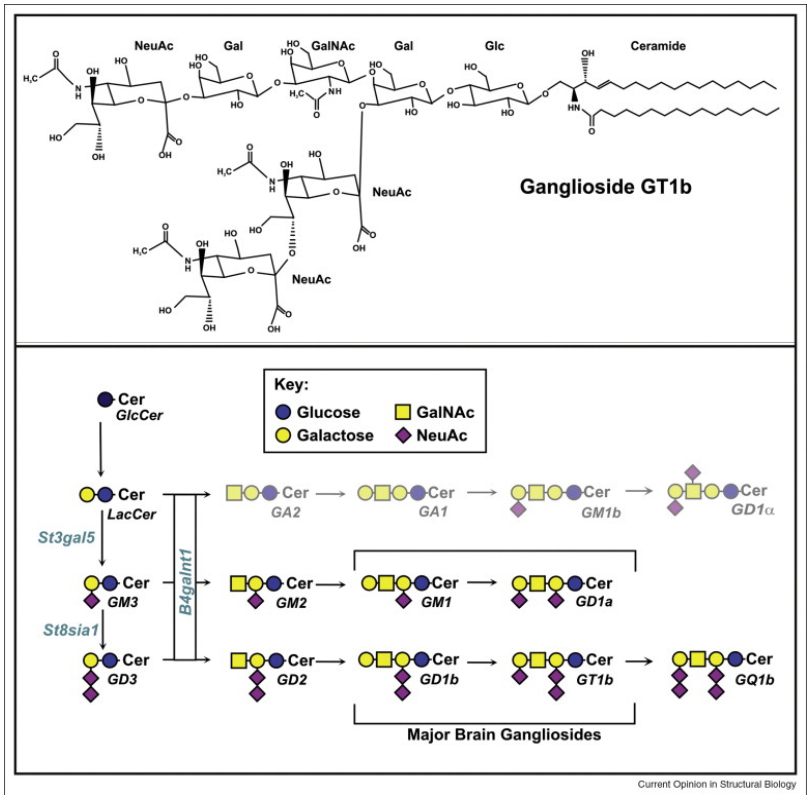


Figure 1.1 Ganglioside structure and metabolic pathway. GT1b structure is shown above the symbolic representation of the gangliosides biosynthesis pathway. Gangliosides are glycosphingolipids that carry at least one sialic acid residue. The number and linkage of the sialic acid(s) to the core glycan structure results in unique molecules with distinct properties [1,6].

Changes in ganglioside expression occur over development and they are highly spatiotemporally regulated. There is a shift from simple gangliosides in the developing

brain, such as GM3 and GD3, to the four major brain gangliosides GM1, GT1b, GD1a and GD1b. This increase in overall ganglioside expression, begins around the third trimester in humans [7,8]. The change occurs due to shifts in glycosyltransferases spatial and temporal expression levels over development [9]. The increase in ganglioside expression is also coincident with an increase in myelination [10].

Gangliosides are located in the plasma membrane with their ceramide potion inserted into the outer leaflet of the bilayer and their glycan head is extended into the extracellular space, this topology is conserved through biosynthesis. Gangliosides are created stepwise with specific glycosyltransferases adding various glycans to the ceramide core. This synthesis begins in the endoplasmic reticulum and then is modified in the Golgi before the final ganglioside product is transported to the plasma membrane. When glycolipids are internalized from the plasma membrane they are broken down in a step wise manner once they reach the lysosome [11]. The biosynthesis and degradation of gangliosides is a relatively rapid pathway, as evidenced by addition of a glycosphingolipid biosynthesis inhibitor (1*R*,2*R*)-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (P4) [12]. Within 24 h the cell surface is cleared of gangliosides due to P4 addition. The catabolic and biosynthetic pathways of gangliosides are well established, but cell-to-cell variability in these pathways is not well known.

Section 1.2: Ganglioside Interacting Proteins and the Glycosynapse

Gangliosides are known to play roles in diverse signaling pathways involved in the immune system, insulin resistance, cancer, and development and stability of axons [1-3]. They can interact both laterally (*cis*) within the same membrane and in *trans* with molecules on opposing cellular surfaces. This allows gangliosides to play a role in both

regulation and recognition pathways involved in cellular signaling and physiology [13]. Gangliosides can cluster in the cell membrane, this is believed to be due to their *cis* interactions because they typically have long unsaturated lipid chains that associate laterally via hydrophobic interactions and their head group can act both as a hydrogen bond donor and acceptor due to the presence of their hydroxyl and acetamide group [14]. It has been demonstrated by many scientific groups that gangliosides are found in lipid rafts with important signaling complexes [2,15,16]. This signaling platform is also referred to as the glycosynapse, a term coined by Dr. Sen-Itiroh Hakomori [15], where the glycan is not thought of as a passive marker for a signaling platform, but rather plays an active role in the signaling pathway. This term is analogous to the "immunological synapse" which controls adhesion and signaling between immunocytes [17].

When the normal glycolipid biosynthesis pathways are disrupted so too is the glycosynapse. Mice with disrupted glycolipid glycosyltransferase genes demonstrate defects in normal lipid raft formation. When ganglioside biosynthesis is blocked, gangliosides accumulate behind this block and reach the same total amount of ganglioside as in wild type, albeit with a different composition of glycolipids. Other lipid concentrations and distributions are not affected in these knockouts (i.e. cholesterol, phospholipids etc.), but common lipid raft markers are altered in these mice. For example, in GM2/GD2 (*B4galnt1*-null) and GD3 synthase (*St8gal1*) single or double knockout mice, common lipid raft markers like flotillin-1 and caveolin-1 are dispersed from the lipid rafts, but the same total protein concentration is maintained. It is believed that the disorganization of these lipid raft signaling platforms may lead to age related neurodegeneration and improper complement activation [18]. A better understanding of the

role of gangliosides in lipid rafts in both *cis* and *trans* relationships is crucial to piecing together important disease related signaling pathways.

Section 1.3 *Trans* interactions - Myelin Associated Glycoprotein (MAG) Structure and Function

One commonly studied ganglioside *trans* interaction is with MAG, which is a transmembrane glycoprotein that is found in the membranes of Schwann cells and oligodendrocytes [19]. In the CNS is only found on the inner most myelin wrap on the membrane directly apposing the neuronal membrane, it is not present in compact myelin. MAG is a minor component of myelin, only making up about 1% of the total protein in CNS myelin and 0.1% in PNS myelin [20]. Despite its low abundance, this protein plays a crucial role in cell-cell interactions which can stabilize the myelin-axon interface in healthy states, but it is a potent inhibitor of axonal regeneration in times of injury.

MAG is a 100-kDa transmembrane glycoprotein containing five extracellular Ig-like domains and is heavily glycosylated – about 30% of its weight is carbohydrate – this makes this glycoprotein very heterogeneous [21]. Myelin rich membrane fractions containing MAG can be isolated utilizing centrifugation and gradients for neurite inhibition growth assays [12,22]. MAG is highly conserved over evolution, with human and rat MAG 89% homologous at the nucleotide level and 95% homologous at the amino acid sequence level [23]

In rodents, myelination begins after birth when oligodendrocytes (central nervous system), or Schwann cells (peripheral nervous system), wrap axons. Myelination of axons is crucial for proper propagation of nerve impulses; it is analogous to the wrapping of electrical wires allowing for more efficient and further transmission of electricity. The

maximum rate of myelination in rats occurs at twenty days after birth, where myelin is created at the rate of 3.5 mg per day, and at this time only roughly 15% of adult levels of myelin has accumulated [24]. MAG expression begins very early in the process of myelination and is believed to play a role in the initial interactions of the membranes of oligodendrocytes/Schwann cells that helps to wrap axons. MAG expression remains high in adults, which reflects its role in axon-myelin maintenance [25]. Studies of MAG null mice reveal MAG's role in both initiation of myelination and maintenance, this phenotype is more pronounced in the CNS than the PNS [26]. This was not fully appreciated initially due to the late onset of these effects and variations due to the genetic background of animals.

Trans interactions of gangliosides GT1b and/or GD1a on nerve cells with MAG on oligodendrocytes/Schwann cells is important in cell-cell recognition. This interaction serves a dual purpose: it is necessary for the healthy maintenance of axons, but in injury states it is inhibitory, preventing axon regeneration. MAG is a lectin of the siglec family that binds the sialic acids on GD1a and GT1b, specifically the terminal sequence of NeuAc α2,3 Gal β1-3 GalNAc [13]. When nerve cells are grown *in vitro* on a MAG coated substrate, their axon outgrowth is significantly inhibited [12]. When sialidase is added to cell culture, the key terminal sialic acid is lost, converting GD1a and GT1b (MAG binding gangliosides) to GM1 (which does not bind MAG). In some types of nerve cells, sialidase allows for reversal of MAG mediated axon regeneration inhibition. Mice genetically engineered to lack these MAG binding gangliosides do not show inhibition on MAG coated surfaces, and they also have a similar phenotype to MAG knock-out animals [12,27]. MAG-ganglioside interactions are also known to play a role in inhibiting

axon regrowth after spinal cord injury *in vivo* [28]. Addition of sialidase in rat spinal cord injury models has been shown to improve both motor function and behavior. Combination of sialidase with chondroitinase ABC (ChABC), was not shown to improve recovery more so than sialidase alone[29]. ChABC cleaves chondroitin sulfate proteoglycans (CSPGs), another axon regeneration inhibitor present in the glia scar of a spinal cord injury [29]. The signaling pathway for MAG mediated inhibition is known to require RhoA activation, but the signal transuding molecules leading from MAG-ganglioside engagement to RhoA activation are not yet well established.

Section 1.4 – *Cis* interactions – Lipid Rafts, Membrane Proteins and Ganglioside Interactions

The plasma membrane separates extracellular and intracellular components, and molecules within the membrane are known to be organized in such a way to relay information between these segregated areas. The bilayer is a stable structure, but it does allow for some transmembrane mobility of molecules and for them to readily diffuse laterally within the membrane [3]. There are several states of the lipid bilayer that depend on both the temperature and lipids present. Lipid bilayers at physiological temperature often exist in the lipid-disordered (l*d*) phase which is characterized by high fluidity of the lipid acyl chains leading to high mobility of lipids within the membrane [3]. When the temperature is lowered, the lipid acyl chains straighten and become ordered, so their movement laterally is limited, this is referred to the liquid-ordered (l*o*) phase [3]. Glycosphingolipids have a tendency to form laterally separated liquid-ordered phases leading to glycolipid enriched areas that can influence the lateral movement of other

molecules within the membrane. The existence of lipid rafts is generally accepted, and these rafts are often thought of as signaling platforms that organize molecules within the membrane. Gangliosides are known to alter both the lipid composition and functionality of proteins within the membrane and lipid rafts [30-32]. Generally, 30-40% of the total lipid components of lipid rafts are sphingolipids and cholesterol, the remainder is generally made up of glycerophospholipids [33]. Ganglioside GM1 is a commonly used marker of lipid rafts, and it known to co-localize in raft fractions with various membrane signaling proteins, including GPI-linked proteins and lipid-linked signaling molecules such as (among many others) Src family kinases on the inner membrane leaflet and the glutamate receptor families NMDAR [34] and AMPAR [35] on the outer membrane leaflet.

In the mammalian brain, gangliosides are highly abundant membrane components. Their concentration is ten times higher than in many non-neuronal tissues [36]. Ganglioside interactions laterally (*cis*) with proteins within the same membrane is known to play a role in both regulation and recognition pathways involved in cellular signaling and physiology [13]. The *cis* interactions of gangliosides with receptor tyrosine kinases is well documented, and one notable example is GM3 interacting with the insulin receptor (IR). Lateral interaction of GM3 with IR alters its sensitivity to insulin by inhibiting its tyrosine kinase activity [37]. When GM3 levels are increased pharmacologically or genetically, insulin-stimulated phosphorylation of IR is suppressed and this leads to insulin resistance, which is a common indicator of type 2 diabetes. The insulin resistant phenotype can be rescued by reducing GM3 levels, thus manipulation of GM3 can be utilized to create a model system to study diabetes. The interaction of GM3 and IR involves movement laterally in the membrane to different lipid rafts. There is a

competition between caveolin-1 and GM3 to interact with IR, and it is this balance that can be altered in diabetes. When IR and caveolin-1 interact, the receptor is insulin sensitive, and when GM3 and IR interact, the receptor is insulin insensitive. The role of gangliosides in insulin resistance is now being investigated as a drugable target in diabetes, and glucosylceramide synthase inhibitors have been shown to be therapeutic in animal models[38].

Section 1.5 – Ganglioside biosynthesis and catabolic disorders in humans

Gangliosides are abundant cell surface determinants on all vertebrate nerve cells, but their functions are largely unknown. Human disorders of ganglioside biosynthesis and catabolism currently have no cure. These are rare human disorders with a poor prognosis, where the patient suffers severe neurological deficits and often a shortened lifespan. A better understanding of the ganglioside metabolism and identifying the molecules that interact with gangliosides at the cellular level could lead to novel treatments.

Human diseases of ganglioside catabolism have been characterized as lysosomal storage disorders. Defects in glycolipid degradation are known to cause various rare genetic diseases where the glycolipid accumulates in the lysosome [39,40]. Since the turnover of glycolipids is highest in neural tissues, these diseases often have severe neurological impacts. These diseases are generally the result of defective glycosidases, and are known as glycosphingolipid storage diseases. One example Sandhoff disease (a GM2 gangliosidosis disease) is caused by mutations in a hexosaminidase subunit and results in the buildup of GM2, which eventually leads to nervous system failure. Figure 1.2 shows the names of these metabolic disease and the names of enzyme(s) involved. The severity of these diseases depends on the level of enzyme impairment and these

neurologically affected enzymes generally have no available therapeutics [40]. Not all cells are affected equally in these diseases since ganglioside metabolism can be highly variable between cell types and even between the same types of cells [41]. A better understanding of ganglioside metabolism at the single cell level could be used to generate novel therapeutics.

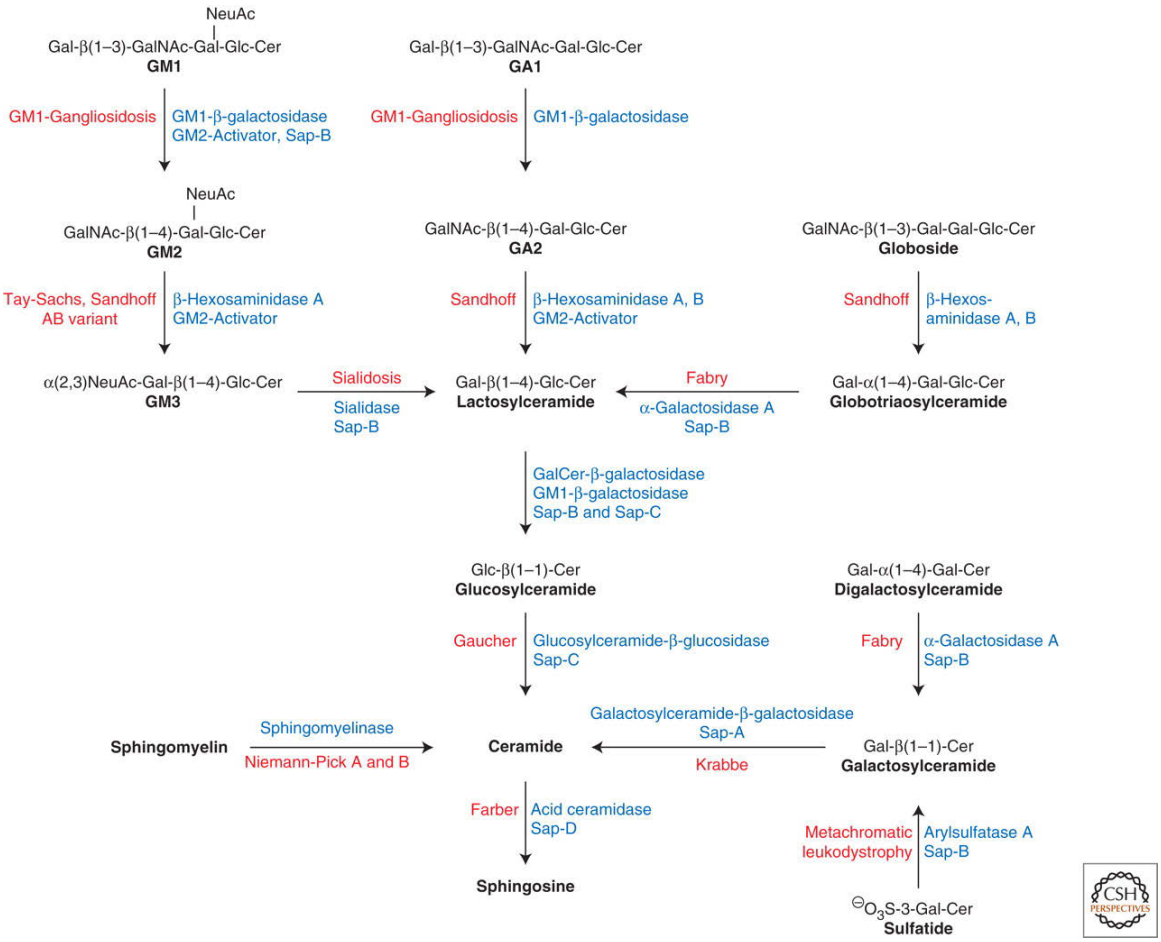


Figure 1.2: Glycolipid storage diseases. Various diseases of sphingolipid catabolism are listed here, listing both the involved mutated protein (blue) and the disease name (pink) [40].

With the advances in genome-wide linkage mapping and exome sequencing, rare ganglioside biosynthesis diseases have also been recently described. In these diseases, defective glycosyltransferases cause the complete absence of certain gangliosides (Figure 1.3). Since the same total level of gangliosides is maintained, the ratios of simpler gangliosides are increased behind the block. Glycosidases function normally in these patients and no defects are seen in the breakdown of gangliosides in the lysosome. Two glycosyltransferases have been identified in rare human neurological diseases, GM3 synthase (*ST3GAL5*, lactosylceramide α-2,3-sialyltransferase) and GM2/GD2 synthase (*B4GALNT1*, β-1,4-N-acetylgalactosaminyltransferase 1). Both of these diseases completely lack the four major brain gangliosides (GM1, GD1a, GD1b, GT1b). GM3 synthase diseases lack GM3 and all of its biosynthetic derivatives, with an increase in lactosylceramide (LacCer) and (based on animal models) the O-series gangliosides GA2, GA1, GM1b, GD1α[42]. GM2/GD2 synthase diseases lack GD2 and GM2, including all of their biosynthetic derivatives (including the major brain gangliosides), with an increase in gangliosides GM3 and GD3[43]. The severity of these diseases depends on the level of enzyme impairment; the described human diseases are often severe with null or nonsense mutations. Mutations in GM3 synthase cause severe infantile-onset epilepsy, developmental stagnation, blindness and death by the age of four[42]. Mutations in GM2/GD2 synthase cause variable early-onset spastic paraplegia and peripheral neuropathy, with neurological defects and sensitivity to seizures. The disease progression is highly variable with the mean of 32.2 +/- 19 years from onset to full peripheral paralysis and prognosis mortality is less severe than GM3 synthase diseases[43]. It is not

yet known why there is a difference in the severity of these two diseases; both lack the major brain gangliosides, but the outcome varies between them immensely. It is not known yet which contributes more to these diseases: the absence of complex gangliosides or the buildup of simpler gangliosides, or both. A fuller understanding of ganglioside interacting molecules could reveal which signaling pathways are impacted in these complex diseases.

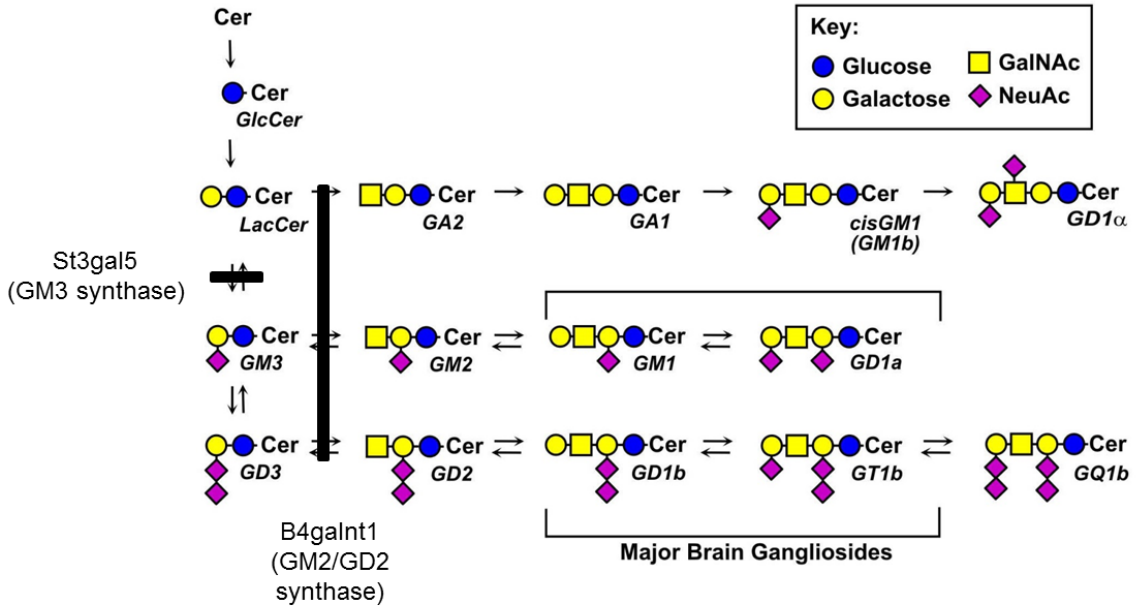


Figure 1.3: Glycolipid biosynthesis diseases. Defects in glycosyltransferases have been identified in rare human neurological diseases. Absence of GM3 synthase (*ST3GAL5*) or GM2/GD2 synthase (*B4GALNT1*) causes loss of the major brain gangliosides (GM1, GD1a, GD1b, GT1b) and other synthetic derivatives, coinciding with an increase in simpler glycolipids behind this defect.

Chapter 2: Novel Ganglioside-Interacting Proteins

Section 2.1 Introduction

Protein-protein interactions are often well studied, but protein-ganglioside interactions are not yet as thoroughly examined. Techniques to study gangliosides are not well-known outside of the field, and the tools to study glycolipids lag behind other technologies because of their highly complex structures and properties. Recently, there has been a large effort to make glycosciences accessible to the entire scientific community. The Nations Research Council of the National Academies recent roadmap report highlighted the field, and the committee recommended glycobiology as a high priority for future funding as its development has the possibility to impact multiple research disciplines [44,45]. More investigators are beginning to study glycolipids since they are known to play roles in diverse signaling pathways involved in the immune system, insulin resistance, cancer, and development and stability of axons [1-3].

The goal of this research project was to identify specific protein-ganglioside interactions and to study their functional significance. To enhance identification of structurally specific interactions, experiments were designed to detect differential nerve cell protein binding to two structurally distinct major brain gangliosides, GM1 and GT1b. As this project developed over time, nerve cell proteins used to study ganglioside interactions were first partially purified to enrich for membrane proteins, then membrane proteins at the cell surface. Although specific interactions of gangliosides with intracellular proteins are not discounted –it has been shown gangliosides are also expressed on the nuclear envelope and endoplasmic reticulum[46]– the focus of the current study was on interactions at the cell surface. The first experiments utilized a "medium"

stringency detergent, Brij-98, and a total membrane (all cellular membranes) protein sample. This was the least stringent method and relied on density centrifugation to isolate membranes in the presence of Brij-98 detergent (unpublished data, experiment 21, 2010); many non-plasma membrane proteins were isolated. In order to target the plasma membrane over total cellular membranes, we moved from relying on centrifugation methods to labeling and isolating cell surface proteins. The second iteration of this experiment utilized Brji-98 but paired it with a cell surface labeling method. Using a non-cell permeable and cleavable (disulfide-containing) protein biotinylation reagent, the extracellular lysine residues on membrane proteins were preferentially labeled before cell lysis. Once cell surface proteins were labeled, cells were lysed and biotin-tagged proteins collected on an avadin column. Cell surface proteins were specifically eluted using DTT since this biotin was cleavable. This surface protein-enriched fraction was then incubated with GT1b- or GM1-derivitized beads. This method did prove to be more stringent than the previous method, but due to the stringency of the detergent we believe some non-specific interactions occurred during lysis and obscured 'authentic' binders (unpublished data, experiments 26-29, 2010). The final iteration of this project involved utilizing both the cell surface labeling and Triton X-100, which is a more stringent detergent. We believe this method, paired with rigorous statistical analysis, has provided us with a protein complex that selectively interacts with GT1b- or GM1-derivitized beads (unpublished data, experiments 1-4, 2011). Statistical analyses revealed six GT1b-selective binding proteins, three of which suggested that GT1b participates in the regulation of glutamate-receptor surface expression. These findings reveal a unique

functional and physical protein-ganglioside interaction; gangliosides have not previously been directly linked to glutamate receptor recycling at the plasma membrane.

Section 2.2 Experimental Procedure

Cerebellar Granule Isolation and Preparation -- All experiments utilized cerebellar granule neurons (CGNs) from newborn rats. CGNs were isolated in large numbers (yield ~30 million cells per 4 animals) and represented a well-documented homogenous population of neuronal cells [33,47,48]. Successful protocols for cell isolation and cell culture were adapted from Mehta *et al*. [12]. CGNs were isolated from 5-6 day old rat pups. Cerebella were collected, and then dissociated using a papain dissociation kit (Worthington) following the supplier's protocol. Briefly, isolated cerebella were incubated in a solution of papain and DNase for 30 min at 37°C, cells were triturated with a fire-polished pipette a total of two times at 15 min intervals. Cells were collected by centrifugation and re-suspended in NS21-containing medium (Neurobasal Medium containing 25mM KCL, 2mM Glutamine, 100 units/mL penicillin, 100ug/mL streptomycin, 1:50 NS21) at 1 million cells per mL and plated 2mL per 35mm poly-d-lysine coated dishes [49]. Cells were cultured at 37°C and 5% CO2 atmosphere for 12 days with half medium changes twice per week.

Ganglioside-aldehyde beads – Gangliosides GT1b and GM1 were covalently bound to Dynabeads M-270 Amine beads (Invitrogen 14307D) after an ozonolysis reaction as described in Schnaar *et al*. [50]. Briefly, gangliosides were oxidized at the 4-5 carbon double bond in the sphingosine using ozone. This reaction resulted in an aldehyde-ganglioside derivative. The ganglioside-aldehyde was then linked to the beads via

reductive amination, covalently binding the ganglioside to the bead. Ozonolysis was improved through use of DMS to efficiently transform the gangliosides into the aldehyde derivative. Aldehyde-ganglioside structures were confirmed by TLC and MALDI-TOF before binding to Dynabeads.

Triton X-100 method: surface membrane proteins –To enrich surface membrane proteins, living 12 day old *in vitro* neurons were incubated with a non-cell permeable and cleavable biotinylation reagent using the Pierce Cell Surface Protein Isolation Kit #89881(EZ-Link® Sulfo-NHS-SS-Biotin) before collection for cell lysis. Neurons were labeled according to manufacturer's protocol using Sulfo-NHS-SS-Biotin for 30 min at 4C. During this incubation, cells were either incubated or not, with a final concentration of 1 mM of non-cleavable ATPγS (Calbiochem 119120) with 3 mM $MgCl_2$. If cells were treated with non-cleavable ATP this was included in all subsequent steps. This reaction was quenched and cells were scraped off plates, a slow spin was performed to remove nuclei (500 x *g*, 3 min), and the supernatant was collected and subjected to centrifugation at 4000 x *g* for 5 min at 4ºC. The pellet was resuspended in 1 mL of detergent free buffer (20 mM HEPES, 150 mM NaCl; pH=7.2, 1:100 Protease inhibitor P8340 SIGMA), homogenized using a Potter-Elvehjem homogenizer (10 strokes on ice) and after an 800 x *g* 5-min spin, the supernatant was collected. The supernatant was spun at 100,000 x *g* for 1 h at 4ºC. The pellet was solubilized in 0.5 mL 1% Triton X-100 buffer for 1 h at 4ºC with tumbling. After 1 h the sample was centrifuged at 20,000 x *g* for 20 min at 4ºC and the supernatant was collected. Boitin-labeled (surface membrane enriched proteins) were isolated by incubating the soluble fraction with an avidin column and the bound proteins were eluted via DTT cleavage of the biotin. The enriched surface membrane proteins

were then incubated with GT1b or GM1 conjugated Dynabeads overnight with tumbling at 4ºC. After allowing sufficient incubation time, several washes were performed and bound proteins were eluted using NuPAGE® MOPS SDS Buffer kit (#NP0050, Invitrogen). The proteins eluted from ganglioside beads were either used directly for SDS-PAGE with subsequent Western blotting or were cleaned up via TCA-acetone precipitation for iTRAQ.

TCA/Acetone protein precipitation for iTRAQ – The protein purification protocol was supplied by the Johns Hopkins University School of Medicine (JHUSOM) Mass Spectrometry and Proteomics. Briefly, after the protein sample was collected according to the NuPAGE® MOPS SDS Buffer kit (heating ganglioside-aldehyde protein bound beads at 70ºC for 10 min in the presence of DTT and LDS) a portion (2/3 total volume) was cleaned up for iTRAQ. An eight-fold volumetric excess of 10% TCA/90% acetone at -20ºC was added to the sample on ice and then vortexed, and incubated at -20ºC for 2-4 h. The pellet was collected by centrifugation at 14,000 x *g* for 10 min at 4ºC and washed with the same volume of acetone (-20ºC) on ice and vortexed and then incubated at -20ºC for 10 min. The pellet was centrifuged at 14,000 x *g* for 10 min at 4ºC and the supernatant was removed. The pellet was allowed to briefly dry at room temperature then stored at -80ºC for subsequent iTRAQ analysis.

Mass spectrometry, iTRAQ -- All mass spectrometry experiments were performed by the Johns Hopkins University School of Medicine (JHUSOM) Mass Spectrometry and Proteomics Facility. Samples were digested with trypsin and labeled using 8-plex iTRAQ kit according to the manufactures protocol (AB SCIEX). Samples were subjected to reduction (TCEP) and alkylation (MMTS) and subsequently proteolyzed with trypsin

(Promega) as previously described by Shevchenko *et al*. [51]. Labeled peptides were combined and fractionated with strong cation exchange chromatography using a polysulfoethylA SCX column. Digested peptides were cleaned up using Oasis reverse phase clean up and then dried via SpeedVac. Dried peptides were resuspended in 0.1% formic acid and loaded on a 75 μm x 2.5 cm trap packed with Magic AQ C18, 5 μm 100 Å material (Microm Bioresources), then fractionated by reverse phase HPLC using an acetonitrile gradient. The instruments utilized were an LTQ-Orbitrap Velos MS (Thermo Fisher Scientific) interfaced with a 2D nanoLC system (Eksigent). Peptide sequences were identified from the Rat Refseq database by using Mascot (www.matrixscience.com) software and further analyzed using Thermo Proteome Discoverer Program (Thermo Fisher Scientific, Inc.). A 1% False Discovery Rate threshold (FDR) was used for identification.

Satistical Analysis of iTRAQ data, Bonferroni correction – 446 proteins identified from iTRAQ were further analyzed by applying the Bonferroni correction by Dr. Ingo Ruczinski of Johns Hopkins School of Public Health. This statistical method is one of the simplest and conservative methods to control error due to multiple comparisons. This method is more stringent than only using 1% FDR, and thus increased the chances of identifying authentic ganglioside-specific interacting proteins. Whereas about half of the identified proteins were differentially expressed according to the FDR (q-values), only 39 proteins were differentially expressed between GM1 and GT1b protein binding according to Bonferroni ($P<0.05/446=0.00011$, see Section 2.3 Results).

Western blot – ganglioside-interacting eluted proteins – Three biological replicates of 12-day-old *in vitro* neurons were collected as described above in "Triton X-100 method:

surface membrane proteins." Proteins were diluted in NuPage LDS buffer with reducing agents, loaded onto 4-12% Bis-Tris NuPage gels (Invitrogen). After gel separation, proteins were transferred to PVDF membranes utilizing the iBlot system (Invitrogen). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween-20/1xPBS and subjected to Western Blot analysis using antibodies against Thorase (1:1000, Antibodies Inc.), GluR2 (1:1000, Antibodies Inc.), N-ethylmaleimide-sensitive fusion protein (NSF, 1:2000, Abcam), glutamate receptor interacting protein (GRIP, 1:1000, BD Biosciences), and Nicalin (1:1000, Millipore). The blots were incubated overnight at 4C, then thoroughly washed and probed with horseradish peroxidase-conjugated secondary antibodies (Cell signaling) and visualized using an enhanced chemiluminescence kit (GE Healthcare). Images were collected on a Carestream Gel Logic imager and bands were quantified using ImageJ analysis software (NIH). ImageJ band densities of ganglioside-interacting eluted proteins were normalized to the total density 'pre-loading' proteins that were not incubated with ganglioside beads prior to SDS-PAGE.

Western blot - brain homogenates -- Three age and sexed matched mice were used per genotype for whole brain homogenate Western blotting. Mice were anesthetized with isoflurane and sacrificed; their brains were rapidly removed and homogenized in CellLytic MT mammalian tissue lysis reagent (Sigma). After centrifugation to remove non-soluble material, the soluble protein concentration was determined using a BCA assay (Thermo Scientific Pierce). Equal amounts (15 μg) of brain homogenate proteins were diluted in NuPage LDS buffer with reducing agents, and then were loaded onto 4-12% Bis-Tris NuPage gels (Invitrogen). After electrophoretic separation, proteins were transferred to PVDF membranes utilizing the iBlot system (Invitrogen). Membranes were

blocked with 5% nonfat dry milk in 0.1% Tween-20/1xPBS and subjected to Western Blot analysis using antibodies against glyceraldehyde 3-phosphate dehydrogenase as control (GAPDH, 1:2000, Sigma), Thorase (1:1000, Antibodies Inc.), GluR2 (1:1000, Antibodies Inc.), N-ethylmaleimide-sensitive fusion protein (NSF, 1:2000, Abcam), glutamate receptor interacting protein (GRIP, 1:1000, BD Biosciences), Nicalin (1:1000, Millipore), and myelin associated glycoprotein (MAG, 1:500, kindly gifted by Dr. Norman, Cornell University, Ithaca, NY). The blots were incubated overnight at 4°C, then thoroughly washed and probed with horseradish peroxidase-conjugated secondary antibodies (Cell signaling) and visualized using an enhanced chemiluminescence kit (GE Healthcare). Bands were quantified using ImageJ analysis software (NIH). Western Blot guidance, data on St3gal2/3 ganglioside-null mice, and additional brain homogenates were kindly provided by Dr. Seung-Wan Yoo (Johns Hopkins University).

Animals – Two different genetic knockout mice models were utilized: *St3gal2/St3gal3* double nulls and *B4galnt1*-nulls. The mice were housed in a 12-h light/dark cycle with free access to food and water. All experimental procedures were approved by the Johns Hopkins Animal Care and Use Committee and were consistent with federal law and NIH regulations. Johns Hopkins Medical Institutions are accredited by the American Association for Accreditation of Laboratory Animal Care. The *St3gal2* and *St3gal3* enzymes are responsible for GD1a and GT1b synthesis. *St3gal2* enzyme is believed to be more strongly involved in glycolipid sialylation, while *St3gal3* has a stronger influence on protein sialylation[52]. It is only when both *St3gal2* and *St3gal3* enzymes are removed (*St3gal2/3* mice) that GT1b and GD1a are nearly completely knocked down and GM1 and GD1b are increased to compensate[52]. The other mouse model utilized was the

B4galnt1 mice that lack the enzyme GM2/GD2 synthase (UDP-N-acetyl-D-galactosamine:GM3/GD3 N-acetyl-D-galactosaminyltransferase, EC 2.4.1.92). They lack all the major brain gangliosides, GT1b, GD1a, GD1b and GM1, and instead have a buildup of the simpler gangliosides GM3 and GD3 [53].

Immunohistochemistry, Thorase staining of Ganglioside-null brains– Eight week old *St3gal2* and/or *St3gal3* wild type, single-, and double-null mice were intracardially perfused with PBS then 4% paraformaldehyde (PFA). Brains were dissected, post-fixed with 4% PFA, embedded in paraffin and sectioned to 5-µm thickness. Antigens were retrieved by boiling the sections in 10 mM sodium citrate (pH 6.0), this was followed by endogenous peroxidases inactivation with 3% hydrogen peroxide treatment. Sections were blocked in PBS with 10% serum, and then probed with antibodies for Thorase protein (1:200 Antibodies Inc.). The sections were then incubated with a biotin-conjugated secondary antibody (1:250, Vector Laboratories), and then developed using the DAB-ABC kit (Vector) with nickel enhancer following the manufacture's protocol. Slides were dehydrated and mounted using Kyrstalon mounting medium and imaged using a Nikon Eclipse 90 microscope and NIS image analysis software.

Immunohistochemistry, Ganglioside staining of Thorase-null brains– Mice were anesthetized with isoflurane and transcardially perfused with PBS then 4% PFA. Brains were removed and postfixed in 4% PFA overnight at 4°C. After 24 h brains were cryoprotected in 20% sucrose, followed by 30% sucrose and frozen in isopentate at -70°C until use. Frozen tissues were embedded in M1 matrix compound and 20-µm slices (*in situ* slide stain) or 35 µm slices (free floating stain) were cut. Age, sex and histological brain level matched sections were stained in situ for gangliosides GT1b (anti GT1b-1,

Seigaku) and GM1 (anti GM1-1, Seigaku). Sections were blocked with serum/bovine serum albumin (BSA), and then stained with primary antibody overnight (1:2000). Sections were washed, then incubated with either Cy3-labeled secondary antibody (in situ slide staining, 1:200 Jackson Immunochemistry) or a biotin-conjugated secondary antibody (floating sections, 1:250, Vector). For the floating sections, the avadin-biotin complex was developed using the DAB-ABC kit (Vector) with nickel enhancer following the manufacture's protocol. After development, floating sections were mounted and coverslipped on glass slides. In situ slides (Cy3) were also coverslipped after imaging using VectaSheild (Vector) and the edges were sealed with nail polish. Data on floating stained sections was graciously generated by Dr. Marija Heffer (Osijek, Croatia).

Cell surface GluR2 staining and imaging – Hippocampal neurons were collected from E18 rat pups as described in Makuch *et al* [54]. Neurons were cultured for 16 d and some were treated with 20 mU/mL sialidase for the last 24 h before fixation. After 16 d in culture, neurons were fixed with 4%PFA/4% sucrose PBS solution, and then surface staining for GluR2 was performed. Coverslips were incubated with mouse anti-GluA2 (15F1, a generous gift from Dr. Eric Gouaux, Oregon Health & Science University) at 1:500 in a detergent free gelatin dilution buffer for 2 h at room temperature. Coverslips were washed, then cells were permeabilized with Triton X-100 containing gelatin dilution buffer and incubated with 1:250 anti-GluR2/3 rabbit antibody (JH4854, a generous gift from Dr. Richard Huganir, Johns Hopkins University). Coverslips were washed, and incubated with secondary antibodies (Alexa fluor 488 anti-rabbit (total) and Alexa flour 546 anti-mouse (surface), Invitrogen) at 1:1000 in Triton X-100-containing gelatin dilution buffer for 1 h at room temperature. Coverslips were washed then mounted on

glass slides with Fluromount-G (Southern Biotech) and imaged using a Zeiss LSM 510 Meta Confocal microscope utilizing a 63x oil objective. Hippocampal collection and staining was kindly provided by Olof Lagerlöf of Dr. Richard Hunagir's laboratory (Johns Hopkins University).

Statistical analyses – All quantified data utilized for statistical analyses are the result of at least three biological replicates. Error bars represent the mean +/- the standard error. Statistical analyses on two experimental conditions were performed using a homoscedastic (equal variance) method via Student-T-test. One Way Analysis of Variance (ANOVA) and pairwise multiple comparison procedures (Holm-Sidak method) were utilized when comparing three or more experimental conditions (SigmaPlot, Systat Software Inc.).P values less than 0.05 are denoted with an asterisk (*).

Section 2.3 Results

A reproducible method was established for ganglioside conjugation to amino beads for use in affinity chromatography. Subsequently, four biological replicates of cerebellar granule neurons in culture had their surface proteins biotin tagged, solubilized with detergent, enriched via biotin/avidin pull down before affinity chromatography of equivalent samples over GT1b- and GM1-derivatized beads. Rigorous Bonferroni statistical correction for multiple comparisons revealed differential interacting proteins to both GT1b- and GM1-derivertized beads reaching a p value of ≤ 0.0001. Subsequent experiments validated the selective binding of Nicalin, NSF, and Thorase to ganglioside GT1b and revealed selective binding of AMPA receptors to GM1. The data support a model in which gangliosides contribute to the steady state surface expression and subunit composition of the major excitatory neurotransmitter receptors in the brain.

Gene	Protein name	GT1b/GM1
Napg	gamma-soluble NSF attachment protein	7.3
Hsd17b12	estradiol 17-beta-dehydrogenase 12	6.5
Ncln	nicalin precursor	5.4
Atad1	Thorase: ATPase family AAA domain-containing protein 1	5.2
Gnai2	guanine nucleotide-binding protein G(i) subunit alpha-2	4.2
Cct7	T-complex protein 1 subunit eta	4.1

Table 2.1: GT1b-associated proteins

The listed proteins bind selectively to GT1b, beating the Bonferroni statistical correction ($p < 0.0001$), which most conservatively accounts for multiple comparisons. Of these, Thorase, NSF and Nicalin (red font) were chosen for further study based on their proposed common roles in regulating plasma membrane protein recycling/fusion. Gangliosides are known to be in membranes of vesicles as well as at the surface of cells[55,56]. Subsequent studies (below) indicate that NSF, Nicalin and Thorase are in a complex. Two of these proteins were confirmed to selectively interact with GT1b via Western blotting (NSF and Thorase). NSF and Thorase were shown to interact with each other via immunoprecipitation. Immunopreciptation of Nicalin resulted in pull-down of Thorase but not NSF. Antibodies suitable for Western blotting were not available for Nicalin.

Gene	Protein name	GM1/GT1b
G3bp2	ras GTPase-activating protein-binding protein 2	16.1
Dlst	dihydrolipoyllysine-residue succinyltransferase	10.8
Canx	calnexin precursor	10.0
Rtn4	reticulon-4 (Nogo)	9.9
Prkcsh	glucosidase 2 subunit beta	8.2
Hsp90ab1	heat shock protein HSP 90-beta	7.5
Tmx4	thioredoxin domain containing 13	7.2
Hsp90aa1	heat shock protein HSP 90-alpha	6.4
Fam134c	hypothetical protein LOC360632	6.3
Psma1	proteasome subunit alpha type-1	6.0
Calr	calreticulin precursor	6.0
Fam82a2	regulator of microtubule dynamics protein 3	5.9
Vcp	transitional endoplasmic reticulum ATPase	5.5
Ppp1r9b	neurabin-2	5.5
Pdia6	protein disulfide-isomerase A6	4.1
Hspa5	78 kDa glucose-regulated protein precursor	4.0
Dap3	death associated protein 3	3.8

Table 2.2: GM1-associated proteins

Thirty-three total proteins beat the Bonferroni correction ($p < 0.0001$) for specifically associating with GM1 (the 16 ribosomal proteins are not listed). Ganglioside GM1 is a low abundance ganglioside in cerebellar granular neurons. Reticulon-4 (Nogo) has been extensively studied as an important inhibitor of neuronal regeneration after injury, and could be an interesting candidate for future studies. These proteins were not studied further, but are included here as reference to demonstrate there are also proteins that specifically interact with GM1.

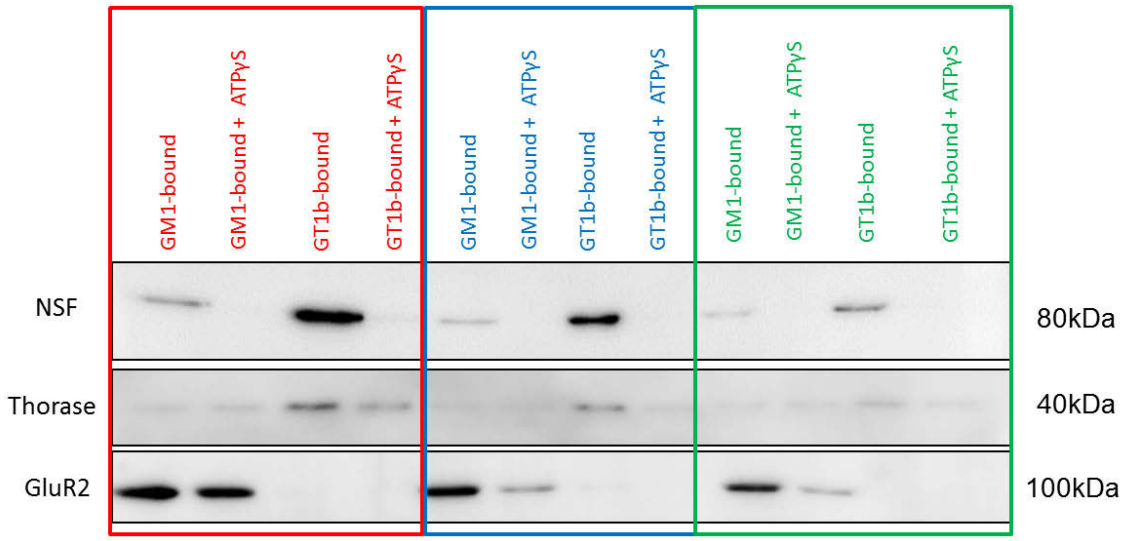


Figure 2.1: Selective association of NSF, Thorase and GluR2 with gangliosides and its abrogation by ATPγS

Biological triplicates are denoted by the three different colors. Differential binding is seen for these proteins between GM1 and GT1b. GluR2 specifically associates with GM1 and this is ATP state dependent. In the presence of non-cleavable ATP (ATPγS) GluR2 interaction is lessened. GluR2 does not associate with GT1b - this is the opposite of Thorase. GluR2 and Thorase are known to interact[57]. NSF is known to interact with Thorase and this plays a role in AMPAR recycling at the neuronal membrane (Ted and Valina Dawson, unpublished results).

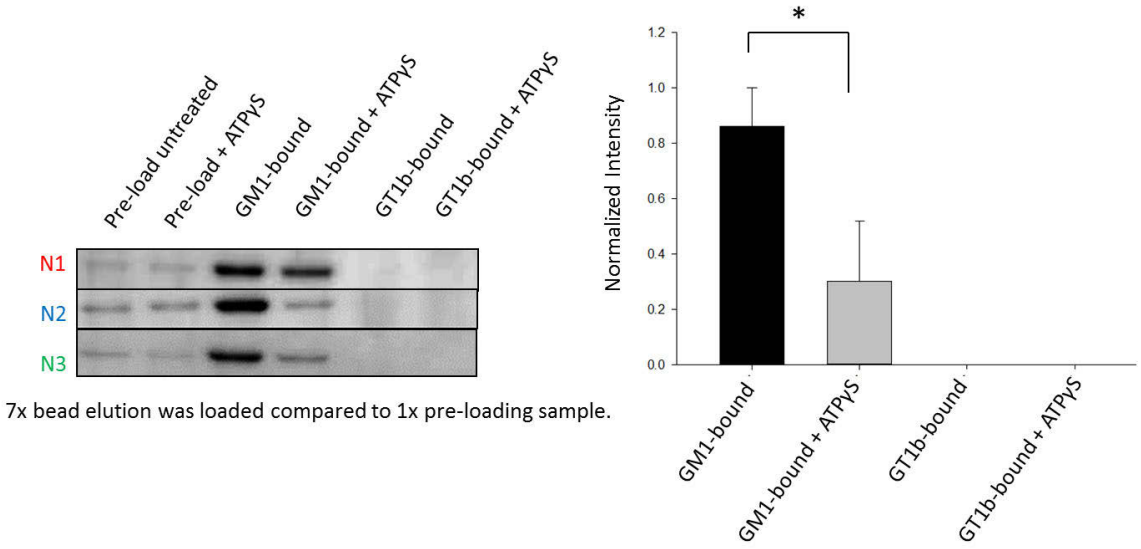


Figure 2.2: Quantification of selective ATP-regulated GluR2 binding to GM1

Biological triplicates were probed for GluR2 binding and normalized to pre-loading controls. GluR2 shows specificity for ganglioside GM1, and this association is diminished in the presence of ATPγS. GM1 is known to co-localize in raft fractions with various membrane signaling proteins, such as the glutamate receptor families NMDAR [34]and AMPAR [35]. While they have been shown to co-localize to the same lipid raft areas, glutamate receptors and gangliosides have never been formally linked in a signaling pathway. Statistical significance was determined via one-way ANOVA, p values less than 0.05 are denoted with asterisk (*).

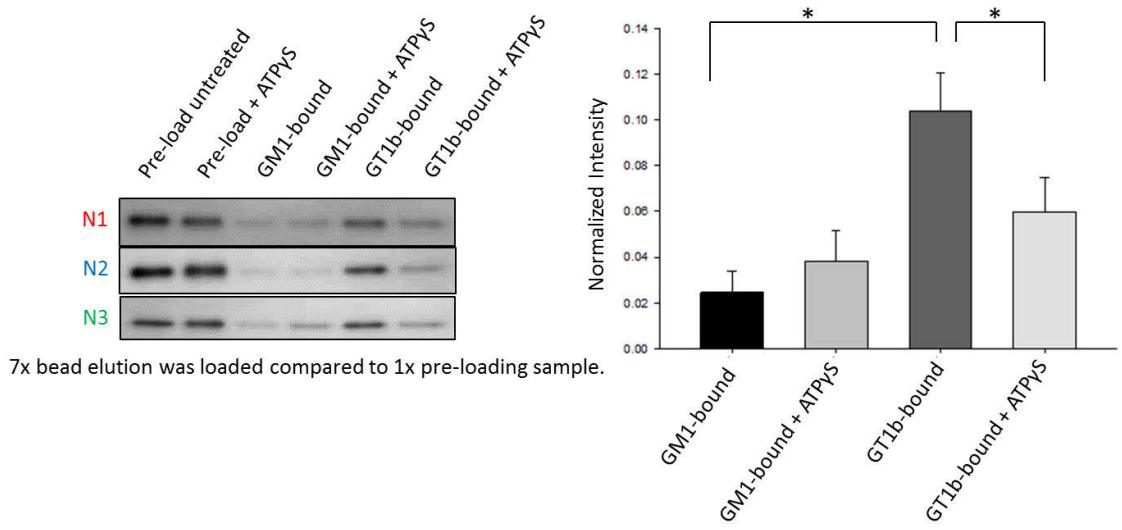


Figure 2.3: Quantification of selective ATP-regulated Thorase binding to GT1b.

Biological triplicates were probed for Thorase binding and normalized to pre-loading controls. Thorase preferentially associates with GT1b, and this interaction is diminished in the presence of ATPγS. Statistical significance was determined via one-way ANOVA, p values less than 0.05 are denoted with asterisk (*).

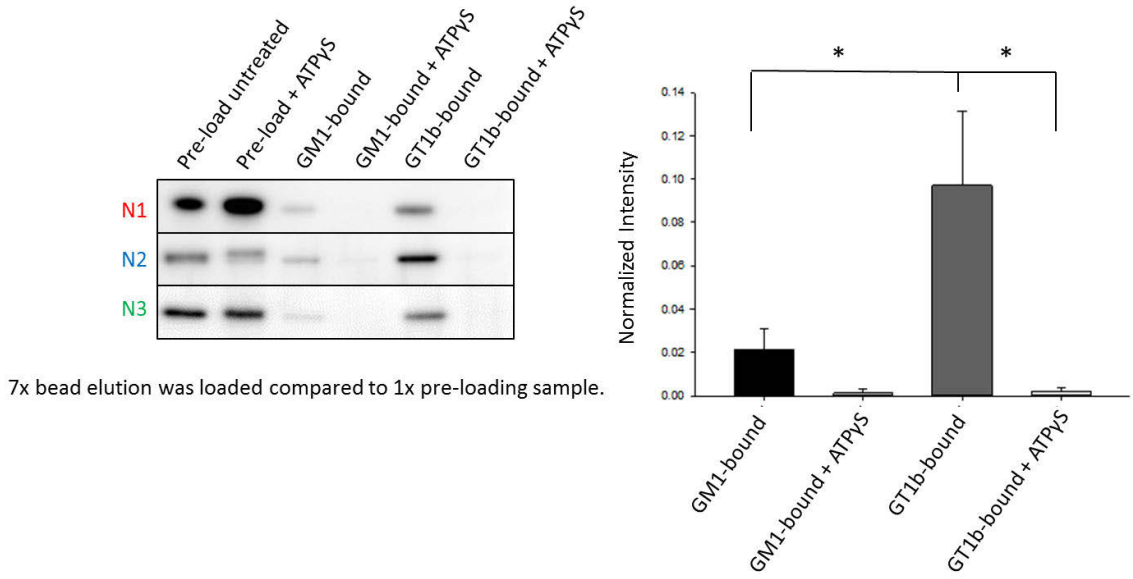


Figure 2.4: Quantification of selective ATP-regulated NSF binding to GT1b

Biological triplicates were probed for NSF binding and normalized to pre-loading controls. NSF preferentially associates with GT1b, and this interaction is diminished in the presence of ATPγS. Statistical significance was determined via one-way ANOVA, p values less than 0.05 are denoted with asterisk (*).

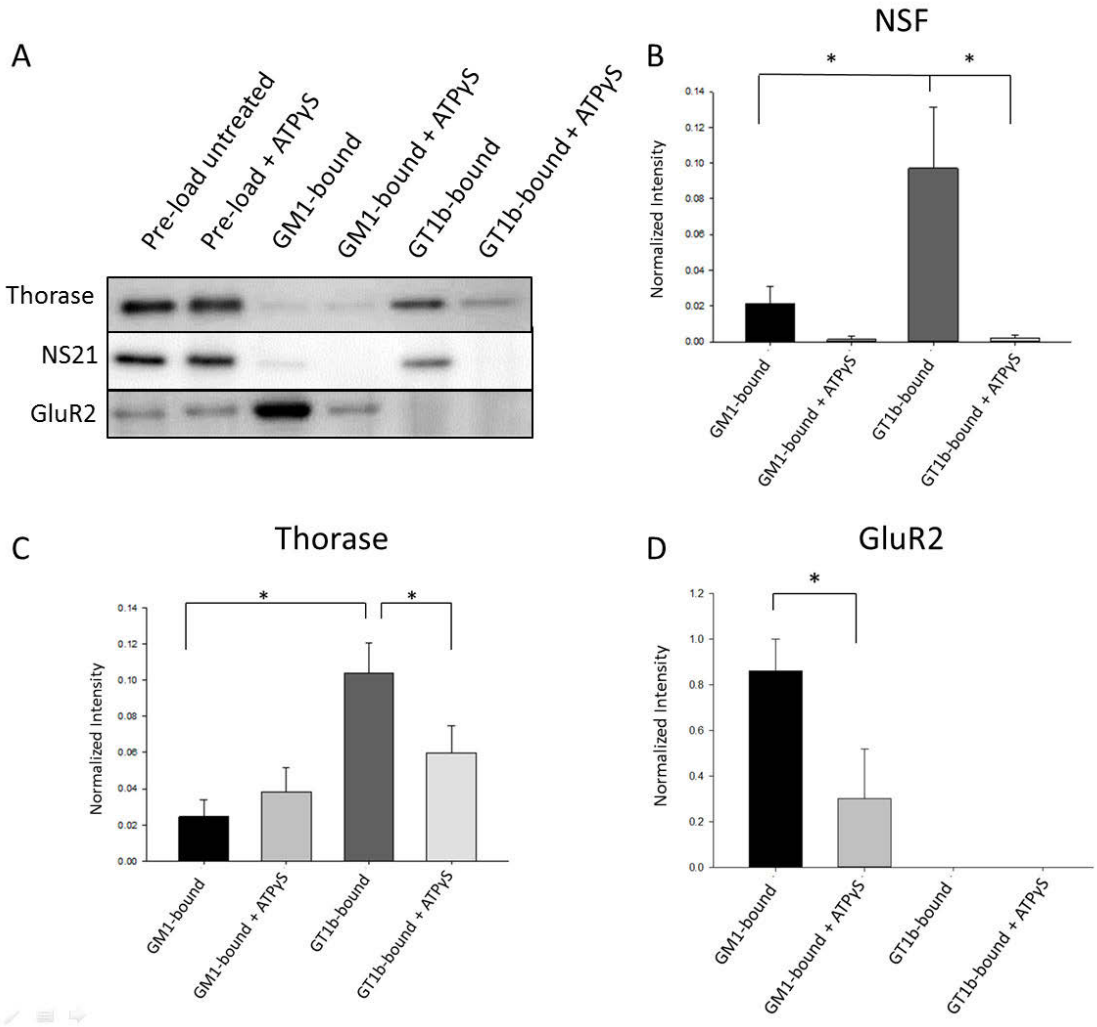


Figure 2.5: Selective ganglioside binding by Thorase, NSF and GluR2 is diminished in the presence of ATPγS.

A summary of Figures 2.2-2.4. Biological triplicates of cell surface ganglioside-interacting proteins were Western blotted for NSF, Thorase and GluR2. A representative blot from the replicates is shown in (A). NSF and Thorase (B, C) statistically and preferentially associate with GT1b, while GluR2 associates with GM1 (D). These interactions are significantly diminished in the presence of non-cleavable ATP (ATPγS). Statistical significance was determined via one-way ANOVA, p values less than 0.05 are denoted with asterisk (*).

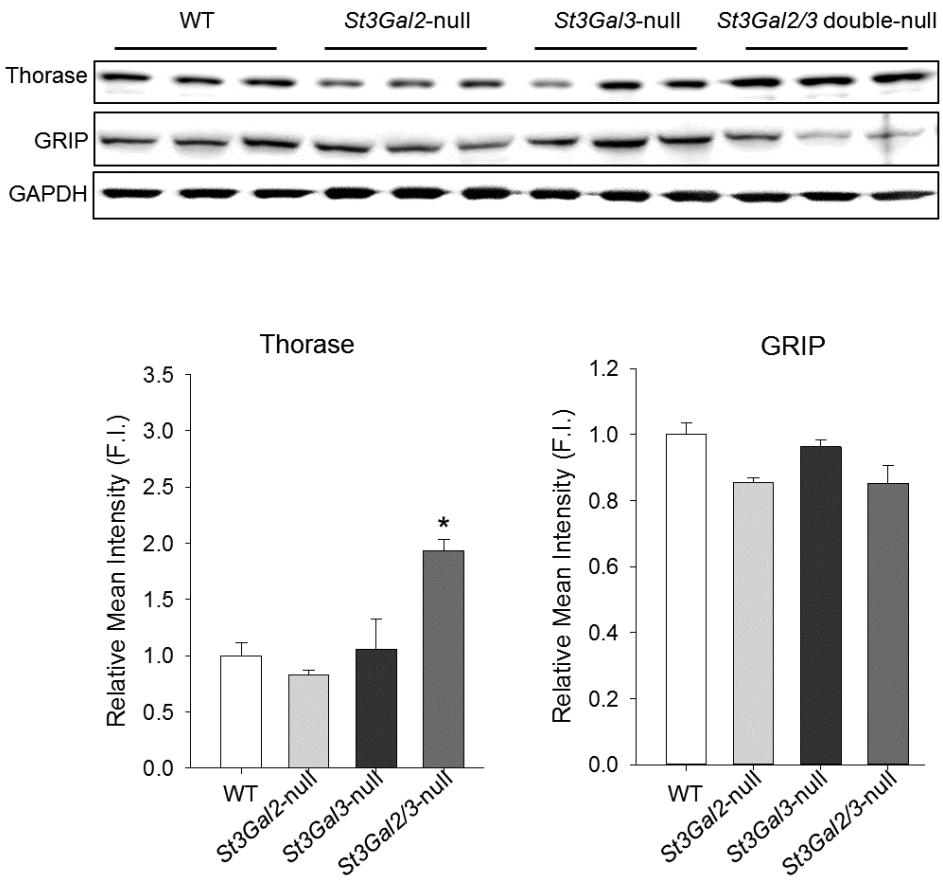


Figure 2.6: Expression of Thorase but not GRIP is increased in GT1b/GD1a deficient mouse brains.

Mice with knockouts of various sialyltransferases were studied. The double nulls *St3gal2/3* do not express GT1b (or GD1a), instead expressing an elevated level of GM1 (and GD1b). The single knockouts express normal (*St3gal3*-null) or ~50% diminished (*St3gal2*-nulls) GT1b. Both enzymes need to be knocked out to prevent GT1b expression. Thorase expression is significantly increased in the double-null mice, while another player in the Thorase pathway, GRIP, is unaltered. All intensity values were normalized to GAPDH levels to account for protein loading variations. Experiment performed by Dr. Seung-Wan Yoo, Johns Hopkins University. Statistical significance was determined via one-way ANOVA, p values less than 0.05 are denoted with asterisk (*).

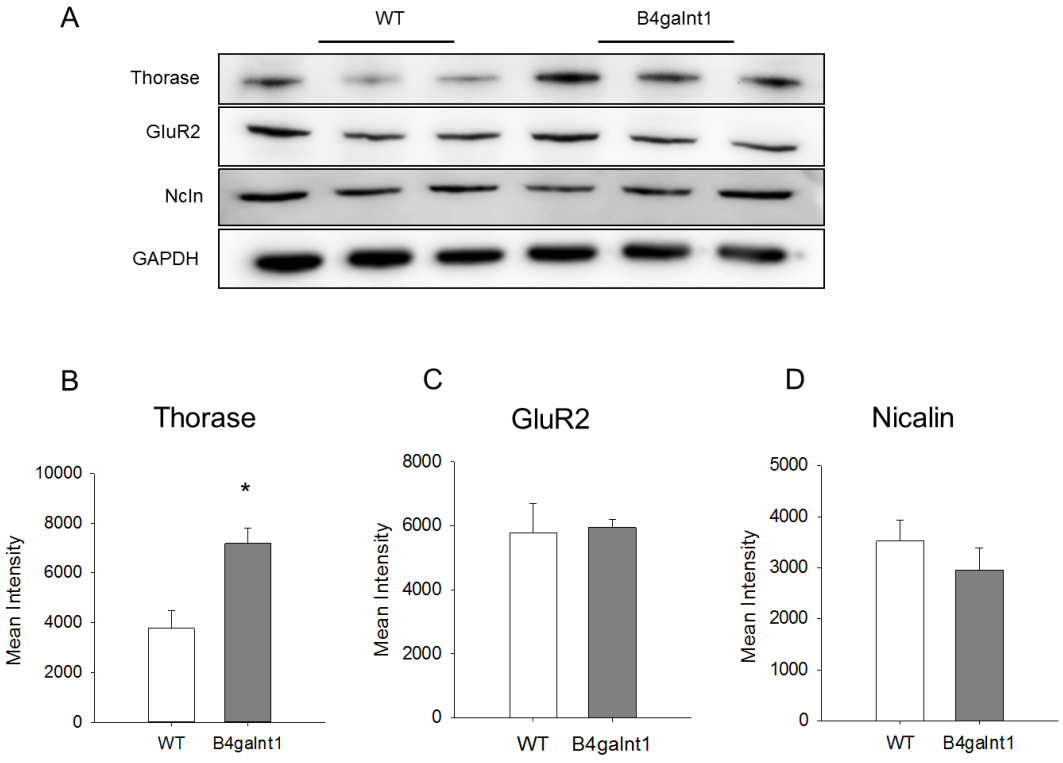


Figure 2.7: Expression of Thorase, but not GluR2 or Nicalin is increased in *B4galnt1*-null (complex ganglioside deficient) mouse brains.

Mouse brains from animals with a genetic knockout of the N-acetylgalactosaminyltransferase gene *B4galnt1* were probed for Thorase, GluR2 and Nicalin expression. These animals lack the four major complex gangliosides (including GM1 and GT1b) and instead have a buildup of the simpler gangliosides GM3 and GD3. Thorase (B), but not GluR2 (C) and Nicalin (D), shows a significant increase in expression in null brains. The impact of the increase in Thorase expression of ganglioside-null animals is not yet known. Since the same relative level of increased Thorase expression is seen in two different ganglioside-null genetic models (*St3gal2/3*- and B4galNT1-nulls) there is likely a link between the steady state expression of ganglioside and that of Thorase. All intensity values were normalized to GAPDH levels

to account for protein loading variations. Brain homogenates kindly provided by Dr. Seung-Wan Yoo (Johns Hopkins University). Statistical analyses were performed using a homoscedastic (equal variance) method via Student-T-test, p values less than 0.05 are denoted with an asterisk (*).

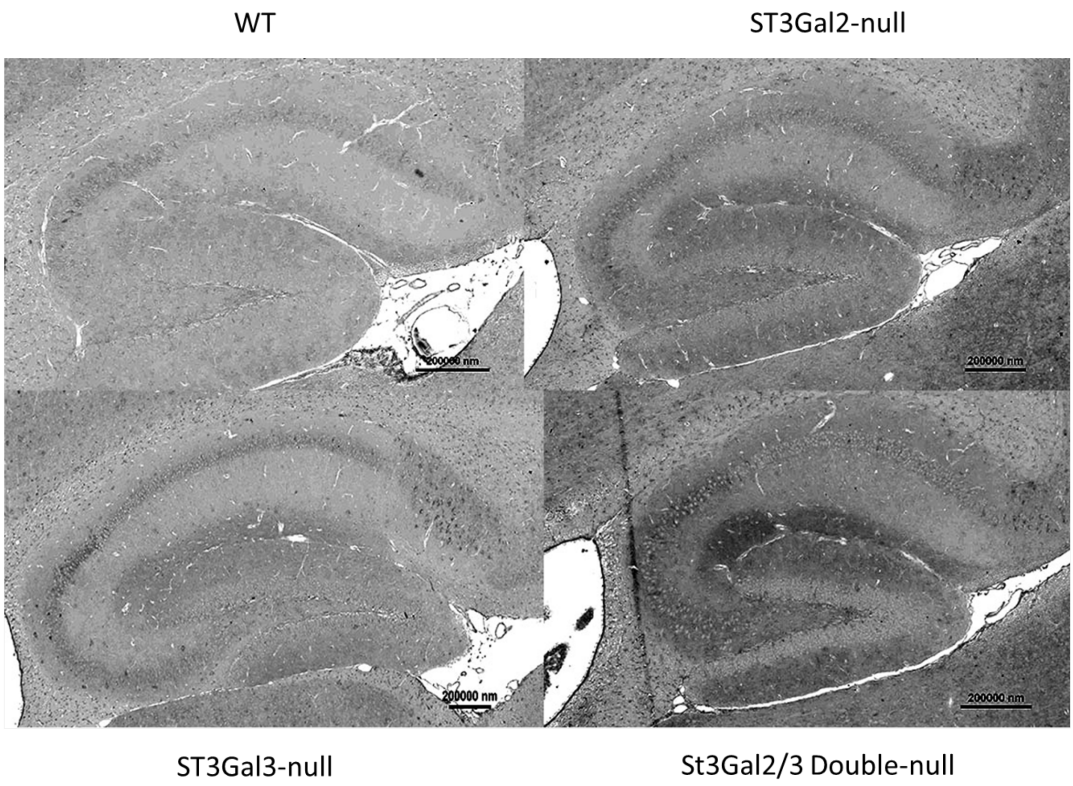


Figure 2.8: Thorase immunohistochemical staining in GT1b/GD1a knockout mouse brains.

Mice with knockouts of the indicated sialyltransferases were immunohistochemically stained for Thorase to study spatial expression. Thorase is a heterogeneously expressed protein with relatively high expression in hippocampal CA1 pyramidal cells[57]. The *St3gal3*-nulls and wild type (WT) do show some Thorase staining in the hippocampus, but this appears to be elevated in *St3gal2*-nulls and more so in *St3gal2*/3-double nulls. *St3gal3*-nulls defect is mainly in protein sialylation while *St3gal2*-nulls show more pronounced lipid sialylation defects[52]. Scale bar 0.2 mm. Brain sections were kindly provided by Dr. Seung-Wan Yoo (Johns Hopkins University).

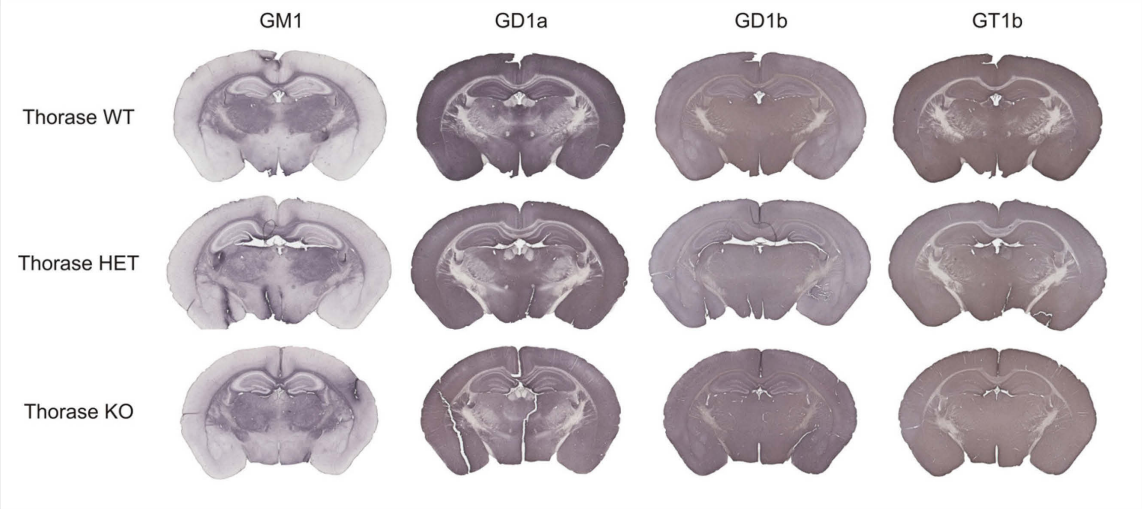
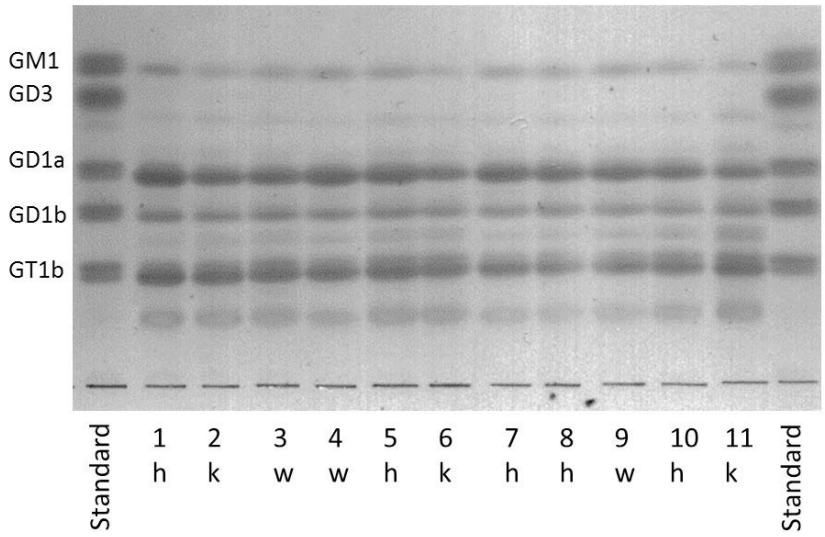


Figure 2.9: Immunohistochemical staining of gangliosides in Thorase knockout mouse brains

To investigate if there were differences in brain ganglioside distributions in Thorase-null mice, immunohistochemistry of the four major brain gangliosides, GM1, GD1a, GD1b and GT1b was performed. There were no gross changes in the localization of gangliosides in wild type and null brains, although intensity changes may be apparent (see Fig. 2.10). Staining was kindly performed by Drs. Senka Majic and Marija Heffer, Osijek, Croatia.



Ganglioside Series	Wild Type	Thorase-null	P Value
a-series	44%	36%	0.033
b-series	53%	60%	0.045
Other	3%	4%	NA

Figure 2.10: Thin layer chromatography of gangliosides in Thorase-null mouse brains.

To quantitatively establish if there are differences in brain gangliosides in Thorase-null animals thin layer chromatography (TLC) was performed. Wild type (W), null (K) and heterozygote (H) brain gangliosides were extracted from age matched mice. Gangliosides migrate in a well-defined manner during TLC and can be identified using standards as migration markers. Bands were identified and quantified using ImageJ software. Analysis revealed a statistically significant shift from a-series to b-series gangliosides in Thorase-null mice. Mouse brain samples were kindly provided by Dr. George Umanah, Ted Dawson and Valina Dawson, Johns Hopkins University. Statistical analyses were performed between wild type and null animals using a homoscedastic (equal variance) method via Student-T-test.

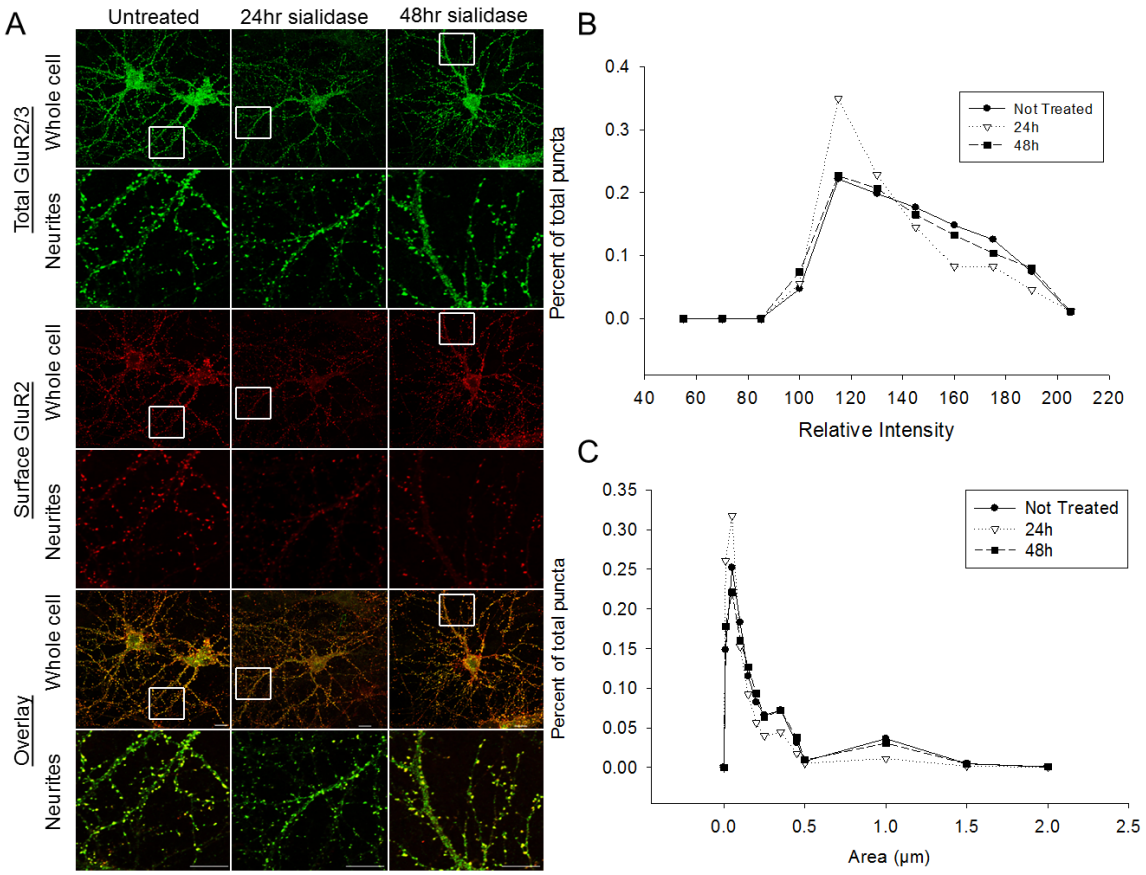


Figure 2.11: Sialidase treated hippocampal neurons show temporal loss of GluR2 subunits from the cell surface.

Wild type rat hippocampal cells were cultured for 16 days *in vitro,* and then 20 mU/mL sialidase was added to the media and incubated for 24 or 48 h. Sialidase (also called neuraminidase) is a robust enzyme that cleaves sialic acid residues from both gangliosides and sialylated glycoproteins[28]. Temporal changes can be seen in glutamate receptor expression on the surface due to sialidase treatment. After 24 h, there is an increase in smaller puncta and less intense GluR2 surface staining. This indicates loss of AMPA receptors at the surface. This trend is reversed by 48 h, indicating there is some compensatory mechanism (e.g. homeostatic plasticity) to restore AMPA receptors to non-treated levels despite the presence of sialidase at 48 h. Cell culture and staining was

completed by Olof Lagerlöf from Dr. Richard Huganir's laboratory, Johns Hopkins University.

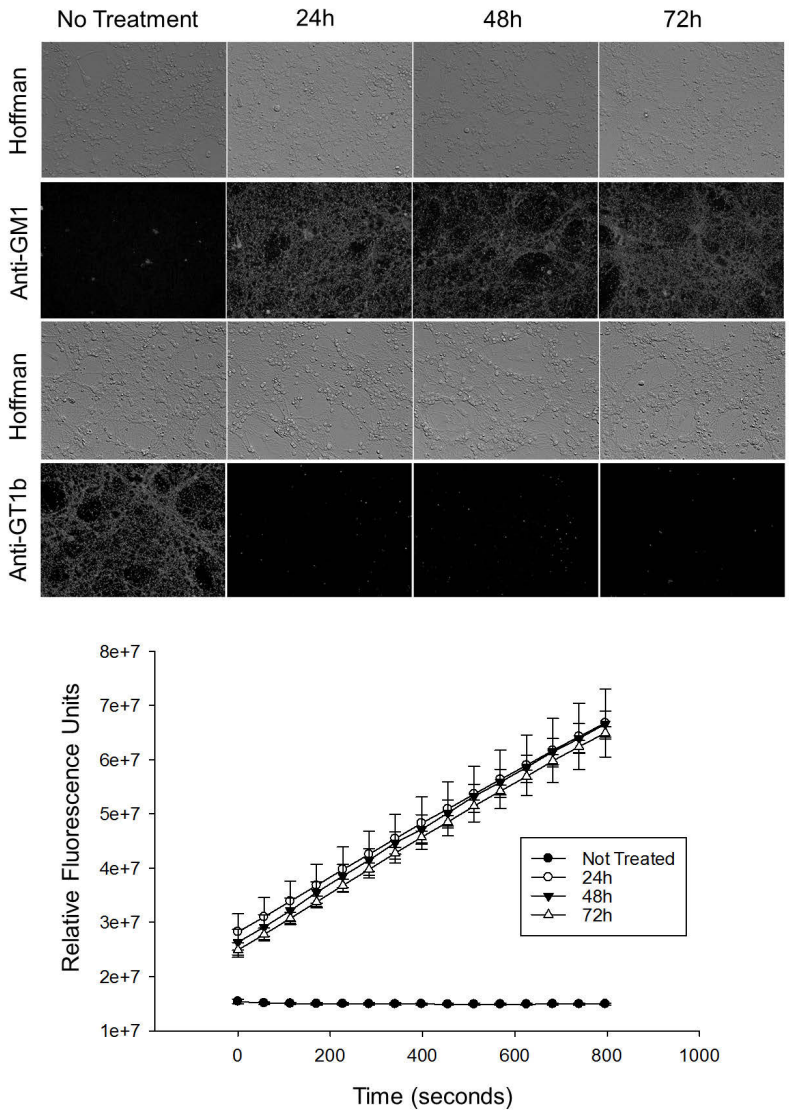


Figure 2.12: Sialidase shows no loss of activity even 72 h after addition to cultures.

To ensure the reversion of AMPA receptors from the 24 vs. 48 hour treatments seen in Figure 2.11 was not due to loss of enzyme activity, ganglioside immunohistochemistry and sialidase activity determinations using 4-methylumbelliferyl-alpha-D-N-acetylneuraminate as substrate were preformed[29]. Cerebellar granular neurons were grown for 12 days *in vitro* then 20 mU/mL sialidase was added to the media for 24, 48 or 72 h. At each time, media was collected for sialidase assays, and cells were fixed with

4% paraformaldehyde for ganglioside immunostaining. Cell density and health was similar between treated and non-treated cells as seen in Hoffman imaging. Sialidase treatment resulted in ganglioside conversion from GT1b to GM1, and this alteration was unchanged between 24 and 72 h. Sialidase activity in the medium was equivalent over the same time period. This suggests the changes seen in GluR2 surface expression in Figure 2.11 between 24 and 48 h sialidase treatment is not due to a loss of sialidase activity over time in culture, but occur via compensatory mechanisms.

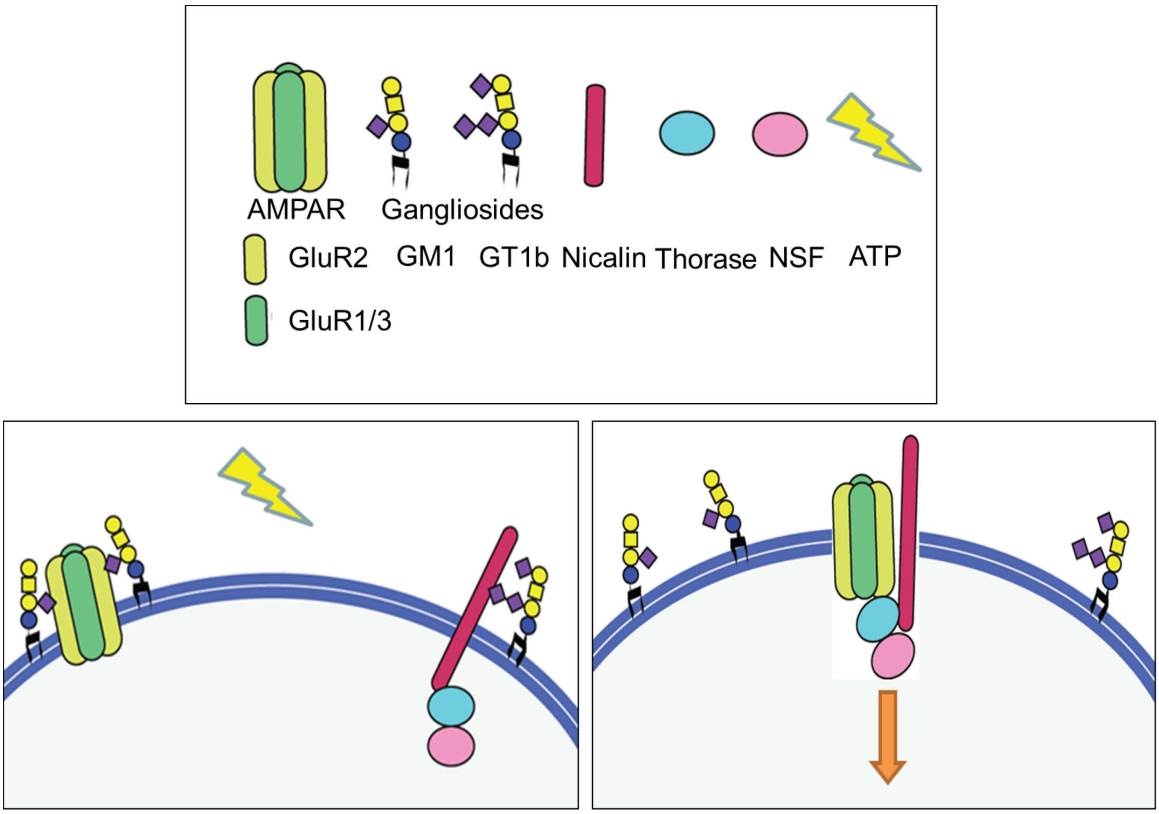


Figure 2.13 Model of ganglioside lipid raft segregation

Gangliosides are known to alter both the lipid composition and functionality of proteins within the membrane and lipid rafts [2,30,32] Our data support a model of different flavors of ganglioside lipid rafts segregating proteins away from each other. NSF, Nicalin and Thorase interact with ganglioside GT1b, most likely through Nicalin. GluR2 interacts with GM1. In the absence of ATP, Nicalin, Thorase and NSF are separated from AMPA receptors. Since these molecules are separated, this allows GluR2-containing AMPA receptors to remain at the plasma membrane. When ATP is added to the system these proteins dissociate from their ganglioside lipid rafts and can diffuse to interact laterally within the membrane. This causes Thorase to bind to GluR2 and internalize the GluR2-containing AMPA receptors. When GT1b is defective this disrupts the GT1b lipid rafts and leads to a decrease in surface AMPA receptors, as seen with treatment of nerve cells

with sialidase. Currently this model is speculative, and further research is needed to flesh out the specifics of this novel ganglioside-signaling pathway.

Section 2.4 Discussion

Despite their abundance at the plasma membrane, ganglioside signaling pathways are not well understood. Many gangliosides localize to raft fractions, and due to ease of identification GM1 is known to co-localize in raft fractions with various membrane signaling proteins, such as the glutamate receptor families NMDAR [34]and AMPAR [35]. While they have been shown to co-localize to the same lipid raft areas, glutamate receptors and gangliosides have never been formally linked in a signaling pathway. Our recent work suggests that gangliosides may have a direct link to regulating glutamate receptor recycling from the neuronal membrane.

Ganglioside interacting proteins at the plasma membrane

Gangliosides are known to interact with a variety of proteins at the plasma membrane both in *cis* and *trans* as described in sections 1.3 and 1.4 in the Introduction. These interactions can occur between the ganglioside and protein directly, or via ganglioside and N-glycan interactions. Despite the interaction mechanism, these interactions rely on the glycan moiety of the ganglioside. We discovered novel ganglioside specific interacting proteins that implicate gangliosides as regulators of neuronal excitability, and that could explain why human ganglioside metabolic diseases are associated with seizures.

AMPA receptor recycling, the role of NSF and Thorase

Synaptic plasticity is a crucial process in learning and memory, regulation of AMPA receptors (AMPARs, also referred to as Glutamate receptors or GluR/GluA) on the synaptic membrane plays a role in this process. AMPA receptors are the main ionotropic glutamate receptors that mediate fast excitatory synaptic transmission in the brain[58]. AMPA receptors are tetramers, made up of homologous subunits GluA1-4 (also

referred to as GluR1-4). These receptors are generally composed of two identical heterodimers; GluA1/2 is the most common in mature hippocampal pyramidal neurons followed by GluA2/3[58,59]. GluA4 generally forms homodimeric receptors and they are the main AMPARs expressed during synaptogenesis[60]. The GluR subunits mainly differ in their C-terminal domain and this is often where intracellular proteins interact with them. These intracellular proteins are indispensable for maintaining the proper subunit composition, numbers, and cellular localizations of AMPA receptors, and defects can lead to seizures and a variety of mental diseases such as autism and schizophrenia[61].

Trafficking of AMPA receptors to and from the synaptic membrane is regulated by subunit-specific AMPA receptor interacting proteins that either help stabilize or remove AMPA receptors from the membrane. Stability is achieved with the glutamate receptor interacting protein (GRIP) which delivers and stabilizes GluA2 at the synapse [58]. Other proteins drive synaptic removal of GluA2-containing AMPARs from the membrane, for example "protein interacting with C-kinase 1" (PICK1). Some proteins are known to alter the interactions of GluA2-GRIP or GluA2-PICK1 which also affects AMPA receptor localization. Thorase is a recently described AAA^+ ATPase protein that is both highly and heterogeneously expressed in the brain[57]. GluA2, GRIP, and Thorase form a complex in the presence of ATP where Thorase disrupts the GluA2-GRIP complex. This disruption causes a dissociation of the AMPAR complex, and leads to endocytosis and removal of AMPAR from the membrane[57]. A related AAA^+ ATPase, NSF, also plays a similar role, binding to GluA2 and disassembling GluA2-PICK1. This disruption results in stability at the membrane or recycling of AMPA receptor into postsynaptic membranes from intracellular compartments [62]. AMPA receptors are known

to be in lipid rafts, and when these rafts are disrupted AMPA receptor exocytosis and thus surface expression is greatly reduced[63]. Addition of AMPARs to the synapse membrane results in long-term potentiation (LTP), but removal from the membrane results in long-term depression (LTD). It is the balance of these two states that shapes neuronal synapses in the brain leading to the complex behaviors of learning and memory [58].

The wild card, Nicalin:

Our mass spectrometry studies revealed that Nicalin specifically interacted with ganglioside GT1b. Nicalin is a member of the aminopeptidase/transferrin receptor (TfR) superfamily, and is distantly related to the γ-secretase component Nicastrin. γ-Secretase is a protein complex that consists of four transmembrane proteins including Presenilin 1 or 2, Nicastrin, PEN-2 and APH-1. γ-Secretase is involved in the proteolytic processing of a wide variety of transmembrane proteins through cleavage within the membrane[64]. Nicalin is a type I transmembrane glycoprotein that forms a distinct high molecular weight complex from γ-secretase[65]. Components of this complex include at least two proteins: Nomo (Nodal modulator) and transmembrane protein 147 (TMEM147). Nomo is a 130 kDa protein with no recognizable functional motifs or previously known functions[66]. TMEM147 is an uncharacterized 22 kDa protein with seven transmembrane domains and a topology similar to APH-1[67]. The molecular mechanisms of this Nicalin-complex are not well understood, and there maybe still other proteins in this complex as its isolation is extremely sensitive to detergent isolation.

Significant sequence similarity between Nicalin and Nicastrin is confined to a region of 180 residues which corresponds to a previously described aminopeptidase domain[65]. Both Nicastrin and Nicalin lack the amino-acid conservation required for

predicted catalytically active aminopeptidases, instead they are believed to be important in the formation of their respective protein complexes[65]. Unassembled monomeric components of these complexes are unstable and rapidly degraded[67]. The activity of the Nicalin complex has only been tested for the cleavage of amyloid precursor protein (APP), a known substrate of the γ-secretase complex[65]. Nicalin did not show activity for APP cleavage, and it has not yet been tested for cleavage of other proteins (such as the glutamate receptor).

γ-Secretase cleaves substrates within their transmembrane domains, so it is possible that the lipid bilayer could alter activity and processivity of this complex, but this is not yet well established. Gangliosides are known to alter γ-secretase activity in a detergent-free lipid vesicle system. Addition of total ganglioside extract (a mixture of brain gangliosides including GT1b, GM1, GD1a, GD1b and others) to these vesicles results in increased activity and decreased processivity of γ-secretase causing an elevation of $A\beta_{42/40}$[68]. Neither the ganglioside(s) in this mixture, nor the molecular mechanisms responsible for gangliosides altering γ-secretase are known. In addition to *in vitro* systems, *in vivo* genetic mouse models of Alzheimer's disease have also been examined for ganglioside affects. Mice lacking GD3 synthase (*St8sia1*, lack b- and c-series gangliosides) when crossbred with double-transgenic (APP/PSEN1) mouse model of Alzheimer's disease demonstrate a reduced Aβ production and aggregation. These triple-null mice are indistinguishable from wild type mice, and show no evidence of Alzheimer's disease[69]. Both *in vitro* and *in vivo* models suggest that gangliosides could be a novel target for cognitive defects that affect Alzheimer's patients. Due to similarity of γ-secretase and the Nicalin-complex it is conceivable that the Nicalin-complex could also

be involved in cleaving proteins at synapses, such as AMPA receptors and this activity is altered by ganglioside GT1b as suggested by our work.

The Nicalin-complex has been described to be located in the endoplasmic reticulum (ER) based upon several criteria. The first being that Nicalin and Nomo are high mannose N-glycosylated proteins as demonstrated by degycoslyation experiments with N-Glycosidase F (PNGase F) and Endoglycosidase H (Endo-H)[66]. These experiments were carried out using HEK293T cell lysates in the presence of 0.1% SDS. Since no difference was seen in the bands created by PNGaseF (which cleaves most N-linked glycans) and EndoH (which cleaves only high mannose N-glycans), Nicalin and Nomo were described as high mannose proteins. This description would be appropriate if they were resident ER proteins as they do not have access to the enzymes that convert high mannose to complex N-glycans in the Golgi. The Nicalin complex was also localized to the ER through immunocytochemistry and density gradient centrifugation co-localization with the resident ER-protein calnexin[65,67]. Calnexin is involved in the folding and assembly of nascent proteins in the ER and recently a small fraction has also been demonstrated to be present on the plasma membrane[70]. These experiments were carried out in non-neuronal cell lines where often the Nicalin-complex components were overexpressed, so localization in nerve cells may be different. γ-Secretase is also described as an ER-protein, but small amounts are known to localize to GluR1 subunit AMPA receptor containing synapses, so it is possible that small amounts of the Nicalin-complex also localize to synapses. γ-Secretase operates on both sides of the synapse with metalloproteases and this complex is important in cleaving proteins implicated in synapse remodeling and maintenance, including EphRs, ephrins, and cadherins[71]. The activation

of synaptic NMDA receptors stimulates the cleavage of N-cadherin by a proteolytic complex composed of metalloproteases, but γ-secretase cleavage of N-cadherin is not affected by synapse activity[71]. Synaptic scaffolding proteins, such as glutamate receptor-interacting protein/AMPA receptor-binding protein (GRIP/ABP) are also known to associate with MT5-MMP proteases which are involved in the ectodomain shedding of γ-secretase substrates[71]. This suggests a possible complex between γ-secretase and AMPA interacting proteins.

Nicalin has been only previously studied in the context of zebrafish development, where the Nicalin-complex, through an unknown mechanism, was shown to antagonize Nodal/TGFß signaling during mesendodermal patterning[65]. Nicalin function in the adult mammalian brain is unknown, but in *Caenorhabditis elegans (C. elegans),* orthologs of the Nicalin-complex are involved in nicotinic acetylcholine receptor (nAChR) assembly[72]. nAChRs are homo- or heteropentameric ligand-gated ion channels involved in excitatory neurotransmission, similar to AMPA receptors, but they respond to different neurotransmitters (acetylcholine and glutamate, respectively). In *C. elegans* loss of the orthologs of Nicalin (nra-2) and Nomo (nra-4) caused defects in synaptic nAChR subunit composition and function; this was partially rescued by expression of human Nicalin[72]. The interpretations of the Nicalin-complex effect on the nAChR's in the literature are limited by their focus only on its ER-localization. Our studies suggest that Nicalin at the plasma membrane forms a complex with Thorase. This could be part of the yet unknown mechanism of Thorase regulation of surface proteins, where the Nicalin-complex could cleave the surface proteins and drive their internalization. Further investigation of this γ-

secretase-like complex could reveal the molecular mechanism and additional novel binding partners/substrates of Nicalin and Thorase.

Gangliosides and glutamate

Gangliosides have been described as being associated through unknown mechanisms with glutamate in various pathways. Exogenous addition of ganglioside GT1b to cell culture medium stimulates release of glutamate from neuronal-like cells (neuroblastoma cells and dorsal root ganglia-derived F-11 cells). This was specific and significant for GT1b only, other major brain gangliosides, like GM1, had no effect on glutamate release[73]. Gangliosides have also been linked to nociceptive responses mediated through glutamate-dependent pathways. *St8sia1*-knockout mice, which lack b- and c-series gangliosides (including GT1b) but not asialo- and a-series gangliosides, are grossly normal, but display both thermal and mechanical allodynia and reduced nociceptive behavior during formalin injections. During nociceptive responses, glutamate accumulates in the extracellular spaces in subcutaneous tissue. GT1b injection into tissue caused accumulation of glutamate, but this was not seen with other gangliosides like GM1. The GT1b injection pain response involved glutamate receptor activation and additional treatment with glutamate receptor antagonists or sialidase significantly reduced nociception and hyperalgesia[74]. This study suggested that GT1b mediated nociceptive responses were through a glutamate-dependent pathway. Gangliosides have also been linked to sensitivity to glutamate excitotoxicity. Cerebellar granule neurons from *B4galnt1*-null (GM2/GD2 synthase-null) mice that lack the major brain gangliosides are more prone to apoptosis induced by glutamate excitotoxicity and elevated KCl. These null cells can be rescued by addition of exogenous GM1 or a semi-

synthetic derivative of GM1 (LIGA20)[75]. Ganglioside involvement in glutamate pathways has a disjointed and unclear history, from being involved in glutamate release from synapses to being linked to glutamate excitotoxicity. Our research suggests that gangliosides interact with AMPA receptors, one of the major glutamate receptors.

Seizure phenotypes

Epilepsy is a complex disorder affecting as many as 60 million people worldwide with no cure[76]. It is characterized by spontaneous recurrent seizures due to improper regulation of excitatory synaptic molecules. Severe seizures occur in Thorase-, NSF-, and AMPA-receptor-null individuals[57,77-79]. Mutations in ganglioside biosynthesis, such as GM3 synthase, also cause severe epilepsy through an unknown mechanism[42]. Our research suggests a connected signaling pathway between gangliosides and these proteins, that when misregulated leads to seizures. A fuller understanding of how gangliosides are linked to these proteins could lead to novel therapeutics for seizures.

Section 2.5 Summary and Future Directions

Gangliosides are abundant cell surface determinants on all vertebrate nerve cells, but their functions are largely unknown. Rare human disorders of ganglioside biosynthesis (and corresponding mouse genetic models) link altered ganglioside expression to severe seizures and other neurological disorders. The molecular mechanism that connects ganglioside expression to seizures has not been established. An unbiased proteomic screen for ganglioside interacting proteins revealed a potential link between the expression of brain gangliosides and cell surface glutamate (AMPA) excitatory neurotransmitter receptors. AMPA receptors are key mediators of seizures and are an

emerging target for antiepileptic drugs in humans. Our data support a ganglioside-AMPA receptor interaction pathway that may regulate excitatory neurotransmission and whose dysregulation may result in seizure disorders.

Future studies should determine the molecular mechanism and demonstrate a definitive link between the Nicalin-complex, gangliosides and AMPA receptors. A suitable Nicalin antibody for Western blotting will need to be developed or purchased for future experiments. Immunoprecipitation experiments will be undertaken to determine the conditions in which AMPA receptors and Nicalin-complex directly interact. Soluble forms of Nicalin and AMPA receptors will be developed or purchased for assays. ELISA assays will utilize these soluble forms to determine their binding specificity to gangliosides. Cleavage assays will be performed to determine if the Nicalin-complex has protease activity against AMPA receptors and related glutamate-receptors. The binding of Thorase to Nicalin and Thorase to NSF will be studied in further depth, focusing on protein modifications (such as nitrosylation) and their effect on AMPA receptor trafficking. AMPA receptor trafficking in various ganglioside-null mice could also be explored through electrophysiology, immunohistochemistry and other assays to assess differences in protein expression patterns and/or synaptic activity due to loss of gangliosides.

Chapter 3: Ganglioside Metabolism in Single Primary Neurons.

Section 3.1: Introduction

Although the biosynthetic and catabolic pathways of gangliosides have been largely elucidated, there is further need for metabolic studies, especially on the single cell level. Diseases caused by incorrect metabolism of gangliosides are well established and animal models of some of these diseases have been reported, but not all cells are equally affected in these models. The ability to measure variations in ganglioside metabolism in single healthy and diseased cells could lead to better understanding of these disorders.

This project was a collaborative effort, with three laboratories working to develop novel methods to study ganglioside metabolism in single cells and cellular lysates. Tetramethylrhodamine (TMR) or boron dipyrromethene difluoride (BODIPY-FL) was conjugated to various gangliosides and glycosphingolipids by the Hindsgaul and Palcic labs at the Carlsberg Laboratories, Copenhagen, Denmark (Figure 3.0)[80]. These compounds allowed for very sensitive detection of the metabolic products down to the single cell level in the Dovichi lab (University of Washington, Seattle, WA and Notre Dame University, Notre Dame, IN) using capillary electrophoresis and laser fluorescence detection. This method has already been successfully used on various cell lines [81,82] and primary cultures [83,84].

This project addressed several technological limits of single cellular metabolism utilizing metabolic cytometry. This technology allows for highly sensitive (yoctomole, 10^{-24} mol, ~70 molecule range) analysis with an unprecedented dynamic range of nine orders of magnitude. This sensitivity allowed for the resolution of both small and large amounts of metabolites in the sub-picoliter volumes of the single cells. Great advances

were also made in multi-colored analysis of single cell metabolism through the addition of various BOIDPY/TMR compounds to cells and the creation of multi-colored analysis instruments. Further refinement of this technology could answer many questions of metabolic processes on the single cellular level in various metabolic disease states ranging from cancer to lysosomal storage diseases.

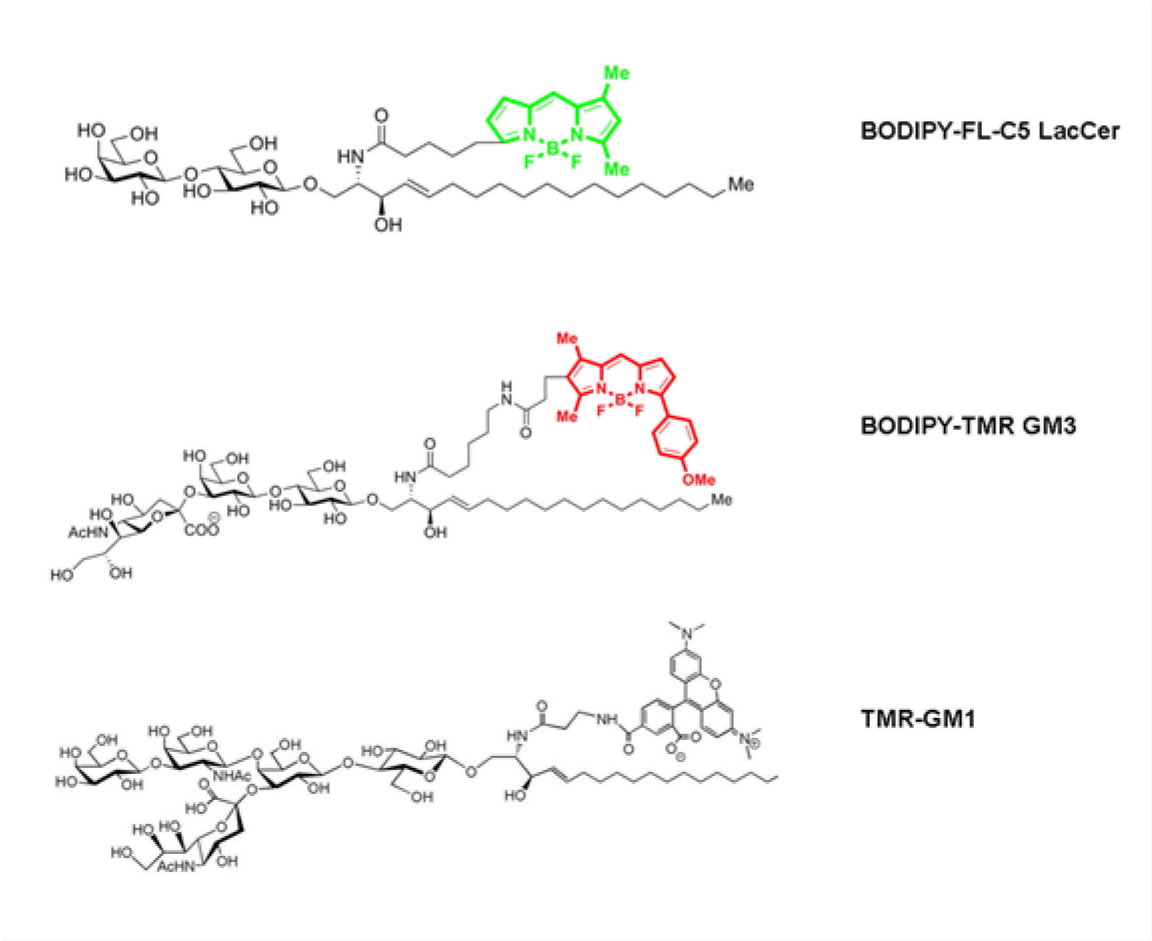


Figure 3.0 Examples of glycosphingolipid compounds tested. Over the lifetime of the metabolism project many compounds were tested, they fell into three generally categories: BODIPY, TMR or BODIPY-TMR conjugated. The sphingolipid core remained the same and the various glycan head groups were tested (i.e. GM1, GM3, LacCer).

Section 3.2: Experimental Procedure

Ganglioside compounds: 5-Carboxyl-tetramethylrhodamine (TMR) and boron dipyrromethene difluoride (BODIPY-FL) glycosphingolipids were created by the laboratories of Dr. Hindsgaul and Dr. Palcic utilizing the methods outlined in Larsson *et al.* [80].

Dorsal Root Ganglion Isolation and Preparation – Dorsal root ganglion (DRG) neurons were isolated from 5-6 day old rat pups utilizing an established protocol with a few changes [12]. Collagenase concentration was increased to 10mg/mL and an additional purification step using a Percoll gradient was added to this protocol [83].

Cerebellar Granule Isolation and Preparation -- Cerebellar Granule Neurons (CGNs) were isolated from 5-6 day old rat pups utilizing an established protocol [85]. Cerebellum were collected, dissociated using the papain dissociation kit (Worthington) following the supplier's protocol. Briefly, isolated cerebellum were incubated in a solution of papain and DNase for 30 min at 37°C, cells were triturated with a fire-polished pipetted a total of two times at 15 minute intervals. Cells were collected by centrifugation and re-suspended in NS21 containing medium (Neurobasal Medium containing 25mM KCL, 2mM Glutamine, 100 units/mL penicillin, 100ug/mL streptomycin, 1:50 NS21) at 1 million cells per mL and plated 2mL per 35mm poly-d-lysine coated dishes [49]. Cells were cultured at 37°C and 5% CO2 atmosphere for various time points depending on the experiment being performed (7-12 days). For some experiments, cells were incubated with 10 μM cytosine- b-D-arabinofuranoside (AraC, Sigma-Aldrich, St. Louis, MO) 18 h after plating, and kept in the medium during growth to deplete the dividing non-neuronal cells. Medium changes were performed every two days.

Addition of fluorescent compounds -- was done in protein free medium (Neurobasal Medium containing 25mM KCL, 2mM Glutamine, 100 units/mL penicillin, 100ug/mL streptomycin – no NS21). Various carrier molecules (TMR, BODIPY, BODIPY-TMR) were tested over the lifetime of this project for efficient delivery of fluorescently labeled glycolipid (Chapter 3 Introduction Figure 3.0). For fatty acid free bovine serum albumin (BSA) carrier, equimolar amounts labeled glycolipid and BSA were pre-complexed in an ethanol-aqueous mixture (2:1) and then diluted to final concentration in protein-free growth medium. For MGP/MMP11/MMP14/Methyl-β-cyclodextrin carrier: a 1.5 (carrier) to 1 (glycolipid) molar ratio was diluted to final concentration in protein-free growth medium. Cultures were washed, and incubated for various times with glycolipids (complexed with carriers) in protein-free medium, washed and cultured in protein-free medium and in some cases incubated for additional time before collection. Incubation time, glycolipid and carrier molecule utilized and final concentration depended upon experimental conditions; please see figure legends for exact conditions.

Single cell collection -- After incubation, cells were washed with 1xPBS and incubated with 2.5mg/mL trypsin and 0.9M EDTA (Invitrogen) for 15 min at 37°C to cause cells to lift off the plate. After 15 minutes, cells were removed from the plate via gentle pipetting and placed into a glass collection tube. An equal volume of 2.5mg/mL soybean trypsin inhibitor was added to quench the trypsin. The cells were collected via centrifugation (10 minutes at 240g), and washed with 1xPBS. A portion of the live cells were counted and imaged on the Nikon TE200 microscope before fixation in 4% paraformaldehyde (PFA) for 12 minutes at room temperature. Fixed single cells were collected via centrifugation, washed with 10mM glycine/PBS, and stored in a glass Reacti-Vial (Thermo Scientific) in

10mM glycine/PBS. Cells were stored and shipped at 4°C to the Dovichi laboratory for capillary electrophoresis analysis against migration of known fluorescently labeled glycosphingolipids.

Homogenate collection – Homogenate collection was carried out as single cell collection except cells were not fixed using PFA. After PBS washes, the supernatant was removed from the cell pellet and the pellet was suspended in a small volume (i.e. 100uL) of 1%SDS in water and stored at -80°C until shipped on dry ice to the Dovichi laboratory for analysis.

Capillary Electrophoresis – Capillary electrophoresis separation of labeled glycosphingolipids of single or homogenate cellular samples was carried out at the Dovichi laboratory according to established protocols [84]. The separation buffer and the capillary length were adapted over time and for single cell vs. homogenate measurements. Generally, the separation buffer utilized was composed of 10 mM sodium tetraborate, 35 mM sodium deoxycholate, and 5 mM methyl-β-cyclodextrin. This allowed for resolution of peaks of known glycolipid standards as well as cellular samples well above the background noise. Single CGN cells were picked from non-neuronal cells based upon size and morphological differences in a microscope attached to the capillary electrophoresis machine. Each cell was sandwiched between two plugs of a 0.4% Triton X-100 detergent solution which resulted in complete cell lysis and metabolite solubilization. Homogenates were loaded in a similar manner, minus the single cell injection. As the Dovichi laboratory developed this technology, two-color instruments were developed to allow for simultaneous analysis of dual labeled samples (such as TMR and BODIPY). Two-color labeled single cells and homogenates were loaded into the

machine in a similar manner to one color labeled samples. Data was visualized using a custom LabVIEW program which allowed for comparison of peak areas for quantification.

Section 3.3: Results

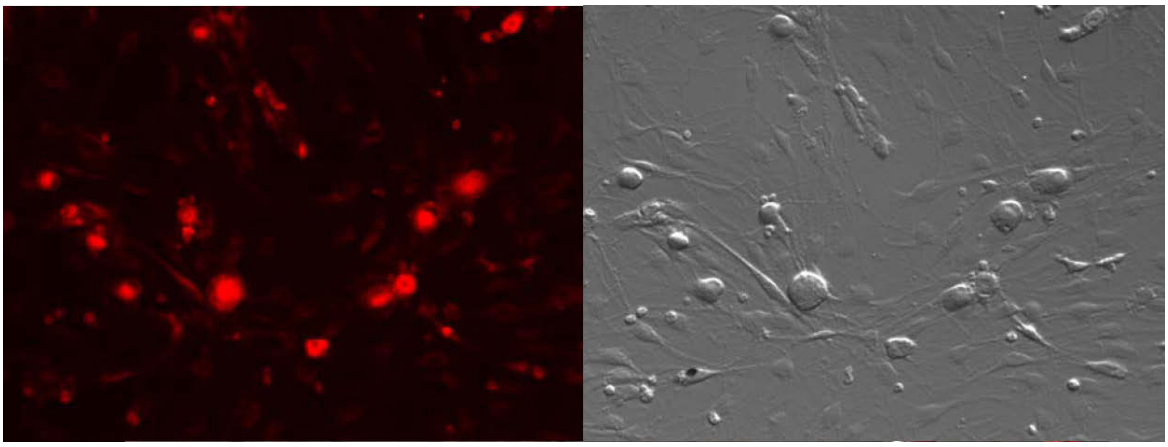


Figure 3.1: Dorsal root ganglion (DRG) neurons cellular uptake of GM1-TMR. DRGs were collected from P5-6 rat pups, and after growing for 24 h growth in serum containing medium, cells were was washed with serum free medium. Cells were then incubated with 5μM GM1-TMR pre-complexed with equal molar BSA in serum free medium for 24 h before collection. DRGs were imaged at 20x using Nikon TE200 microscope and images were collected via NIS Elements software. Differences in DRG cell size ranging from small to large cell bodies are evident in the Hoffman image and uptake of GM1-TMR as evidenced by fluorescence intensity also varies cell to cell [83].

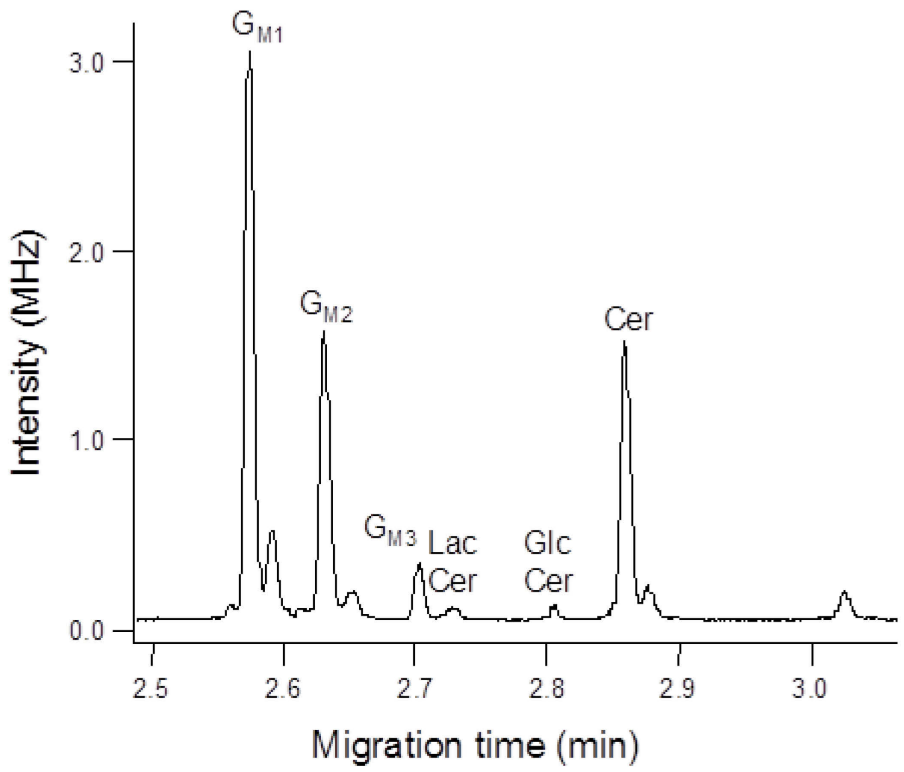


Figure 3.2: Homogenate DRG Electropherogram: A portion of DRGs treated with GM1-TMR from Figure 3.1 were collected as homogenates in 1% SDS as described in the method section. The largest peak is the substrate GM1, but this demonstrates these cells are capable of taking up our exogenously added compounds. Only catabolic (break-down) products are evident by capillary electrophoresis analysis [83].

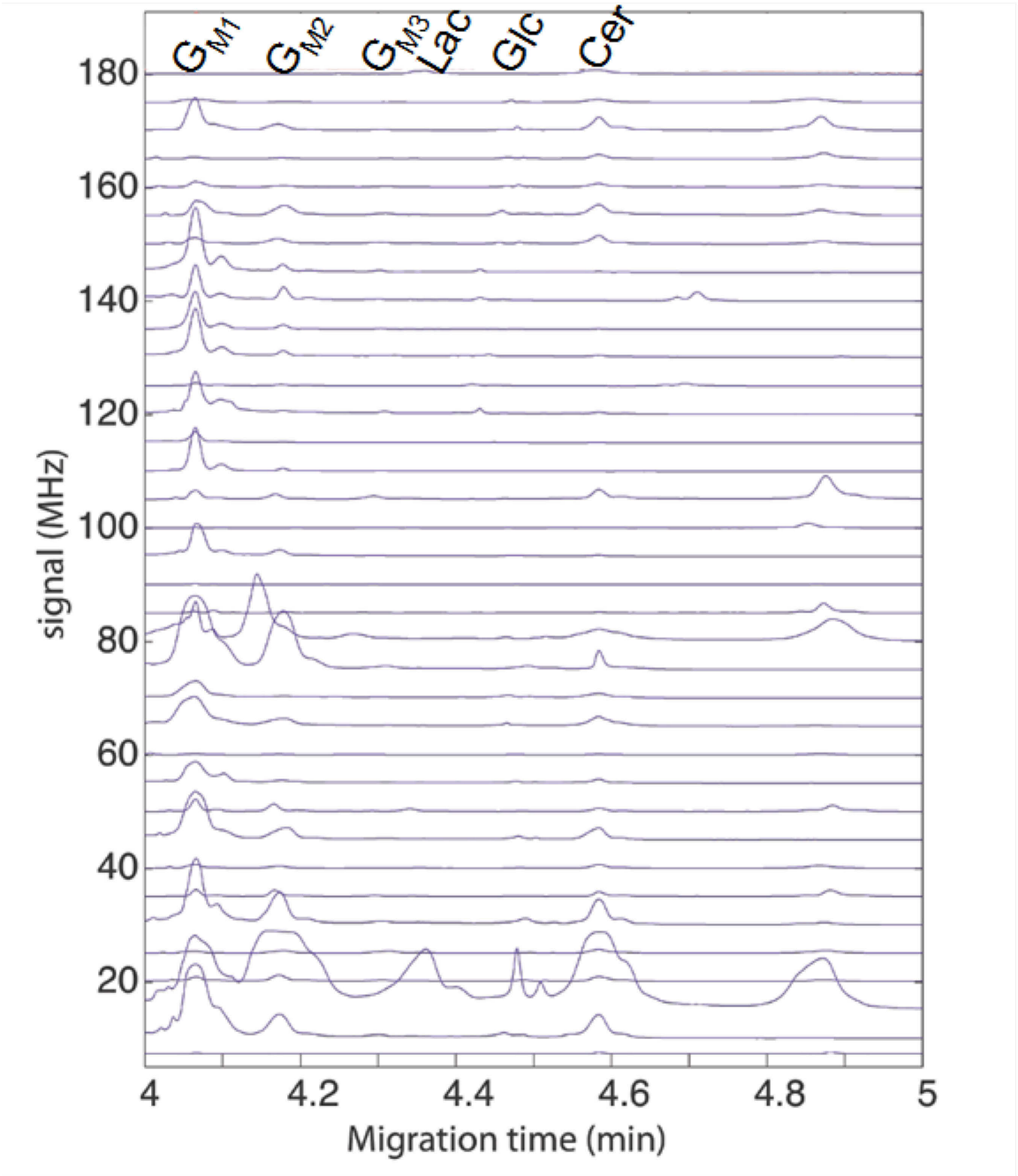


Figure 3.3: Single cell DRG Electropherograms: 36 single DRGs were collected and fixed with 4% PFA as described in the methods section from DRGs in Figure 3.1. Each line is a single DRG neuron, and a large variation in uptake efficiency (peak height) and distribution can be seen from cell to cell. Some DRGs had almost flat traces, and others were above the detector's linear range [83].

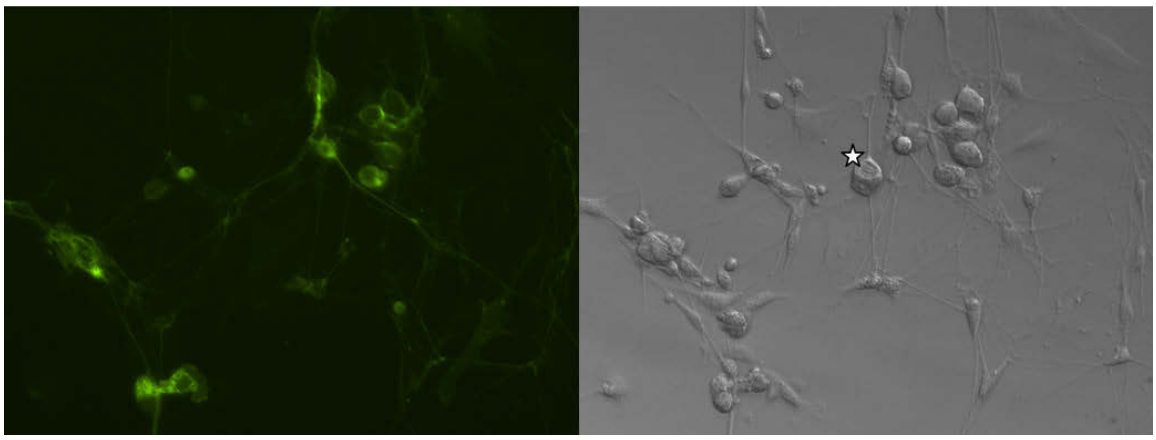


Figure 3.4: Labeling subsets of DRG neurons for analysis. DRGs were grown for 48 h and then fixed in 4% PFA and immunostained with IB4 (Griffonia simplicifolia isolectin B4-FITC (Vector FL-1201 at 1:200)). IB4 selectively labels small non-peptidergic neurons which is one subset of DRG neurons [41]. Large cells show no IB4 stain as expected (star) and various intensity of stain could be seen on other cell bodies. This could be a way to isolate a subpopulation of cells for injection by utilizing a biotin IB4 coupled with NANOGOLD streptavidin and a LI silver enhancement kit (Molecular Probes/Nanoprobes Co). Ideally this would have allowed enrichment of homogenous cells from a heterogeneous population. In the end, this method did not result in dark enough staining to be seen by phase microscopy, and also resulted in massive cell loss during the handling steps (starting with ~150,000 cells after IB4/silver stain there were often less than 3,000 cells). Despite its short falls it could be an interesting future endeavor with further development. (unpublished data Experiment 5, 2009).

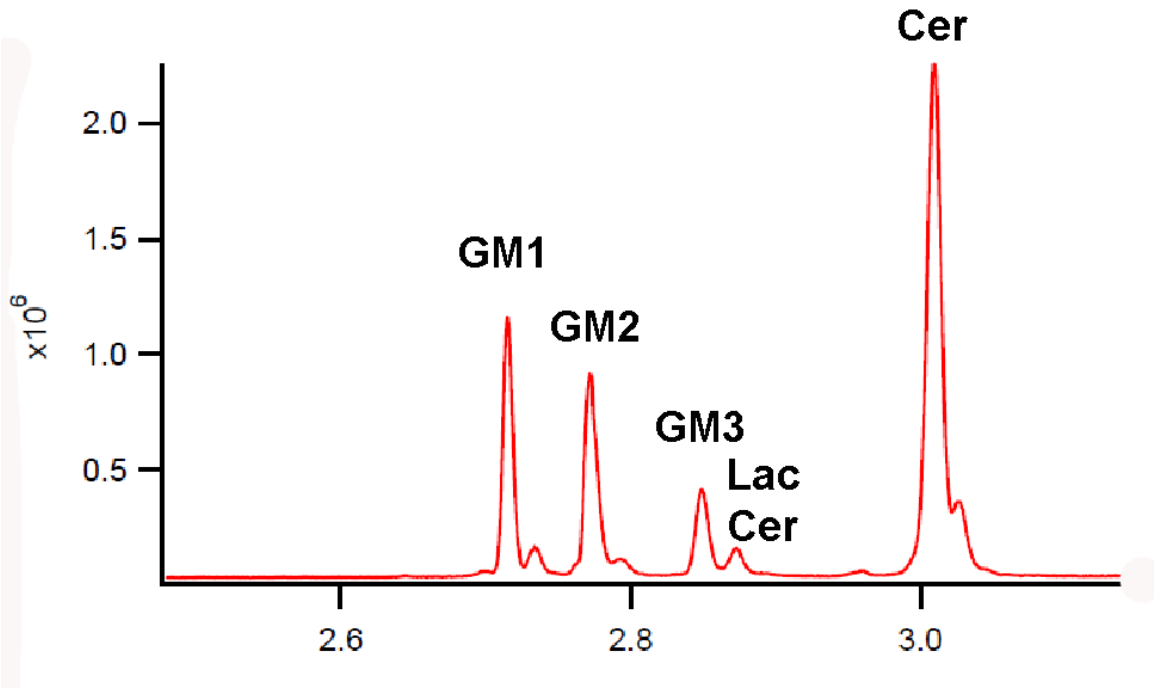


Figure 3.5: Homogenate CGN Electropherogram: Cerebellar granule neurons (CGNs) were grown for 10 days and then incubated with 5μM GM1-TMR pre-complexed with fatty acid free BSA for 24 h before collection for homogenate studies. Their homogenate electropherogram was similar to DRGs (Figure 3.2). Cerebellar granule neurons were chosen for all future studies because of their homogenous population (>90%), ease of isolation and uniform size (~10um). In addition, their ganglioside profile over many days in vitro is well established, and these cells express relatively high levels of the major brain gangliosides [13,33] (unpublished data experiment 19, 2009).

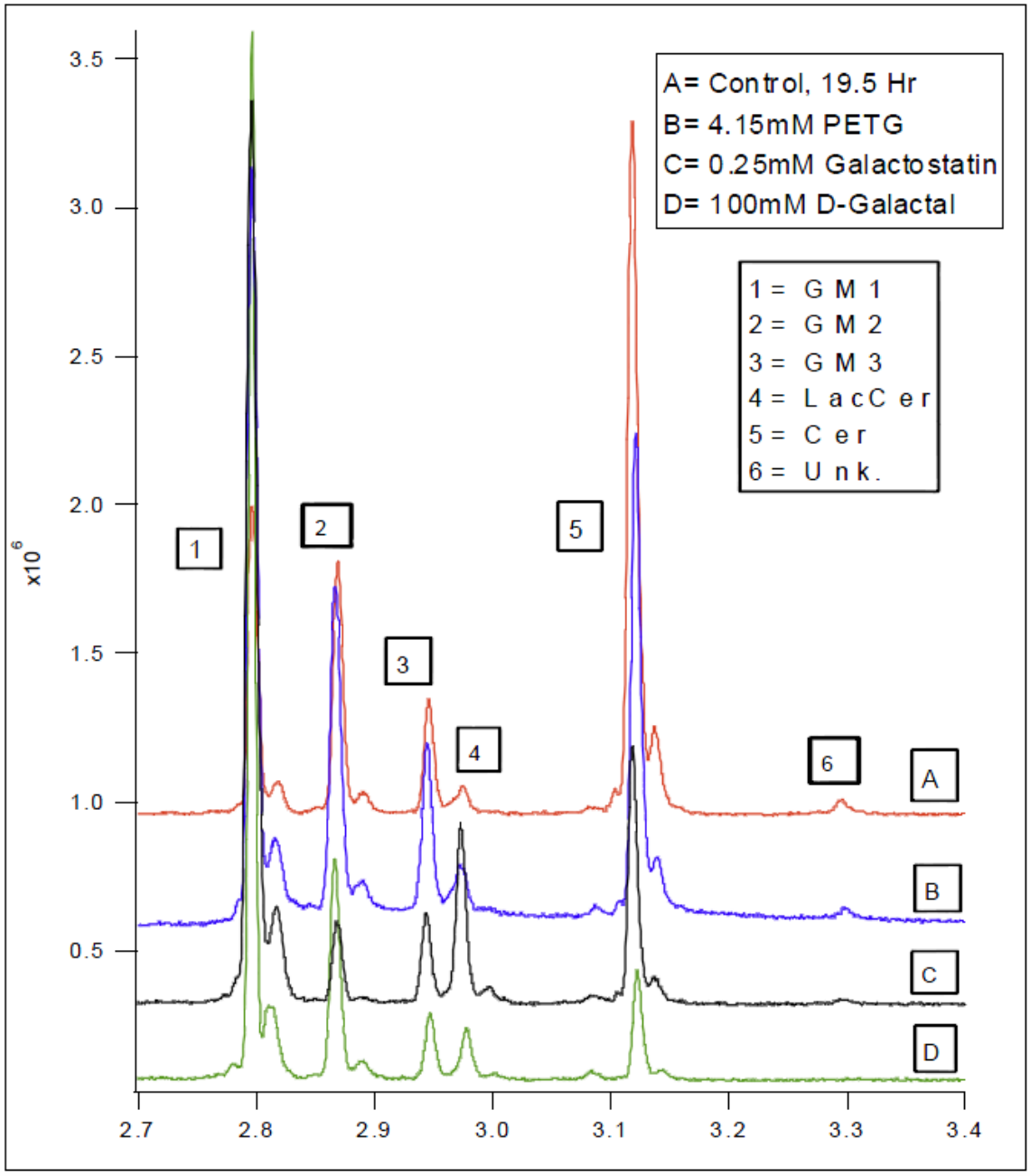


Figure 3.6: CGN β-galactosidase inhibitor testing. Since only catabolic products are seen, Galactostatin, Phenylethyl ß-D-thiogalactopyranoside (PETG), and D-galactal which are all β-galactosidase inhibitors were tested. It was expected that they should prevent the complete breakdown of GM1-TMR to allow for it to sample anabolic pathways. CGNs were grown for 11 days in NS21 containing medium, and then washed

with NS21 free medium. Cells were then incubated for 1 h with an inhibitor (100mM D-galactal, 0.25mM galactostatin bisulfate, or 4.15mM PETG), or NS21 free medium alone. After this hour cells were incubated for 19.5 h with 5μM TMR-GM1 which was pre-complexed with equimolar fatty acid free BSA. Cells that were treated with inhibitor the hour before also had this inhibitor in the medium with the TMR-GM1. After 19.5 h incubation, cells were removed from the plate via trypsin and lysate homogenates were collected with 1% SDS. Inhibitors decreased the ceramide-TMR peak along with increasing other catabolic peaks, but no anabolic products were seen with any inhibitor. D-galactal was the most promising inhibitor tested, with the largest reduction in the ceramide peak. All inhibitors were toxic at higher concentrations, so their concentration could not be increased further in an attempt to see anabolism. (Unpublished data experiment 3 2010).

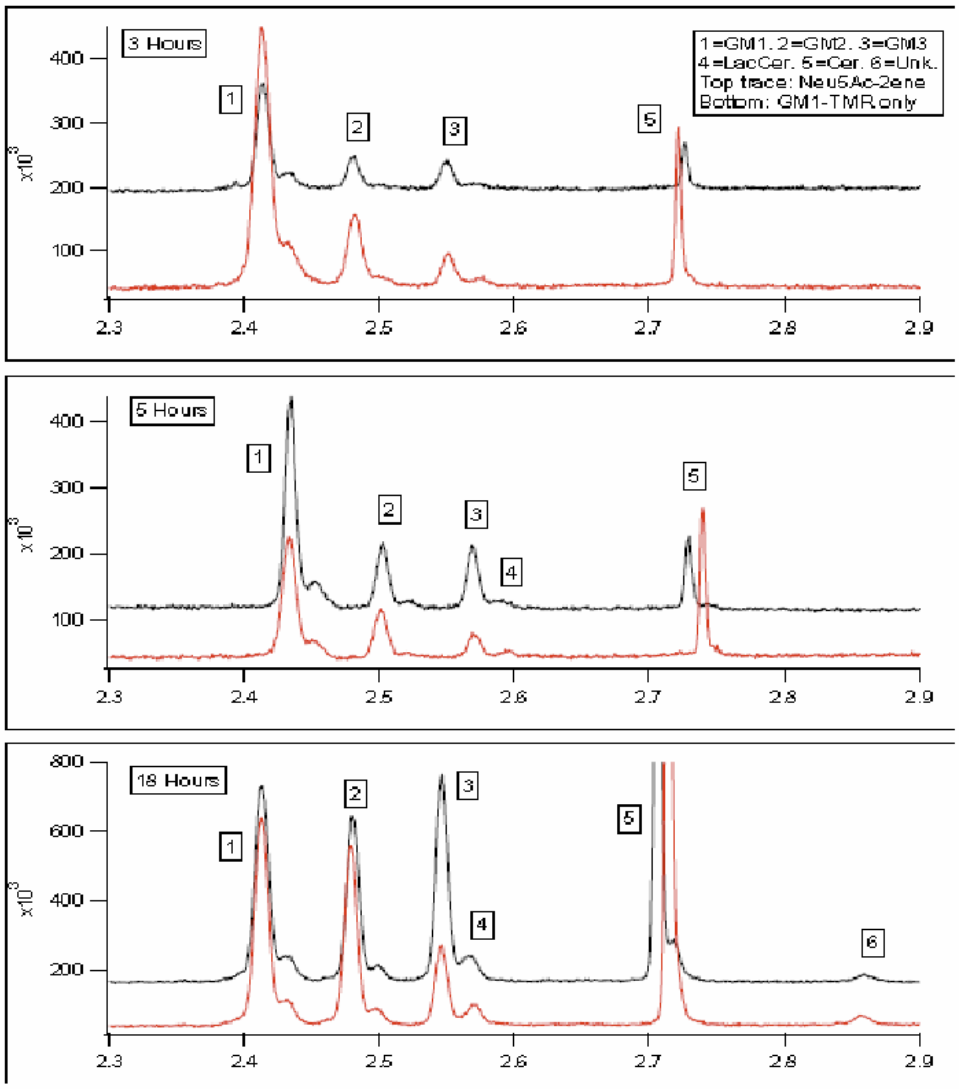


Figure 3.7: Time course experiment with CGNs and sialidase inhibitor testing. Since only catabolic products have been seen previously with TMR, the sialidase inhibitor N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (Neu5ac2ene Sigma, Aldrich D9050) and shorter incubation times with GM1-TMR were utilized to try to improve anabolic product creation. CGNs were grown for 11 days in NS21 containing medium. They were washed with NS21 free medium and either incubated for 1 h with 2.5mM Neu5ac2ene in NS21 free medium, or in NS21 medium lacking Neu5ac2ene. After this hour cells were washed with NS21 free medium and then incubated for various times (3, 5, or 18 h) with

5μM GM1-TMR/BSA with or without 2.5mM Neu5ac2ene. Lysate homogenate were collected after these time points using trypsin to remove the cells from the plates and lysis via 1% SDS. The inhibitor (black line) does increase the catabolic products and reduce the ceramide-TMR product, but no anabolic products are seen. Shorter incubation times lead to higher recovery of earlier catabolic peaks (GM1, GM2, GM3), as time goes on (18 h) all products in the absence of inhibitor shift heavily to ceramide-TMR. Shorter incubation time points may be best to study for better uptake of compounds. This inhibitor does not seem to increase anabolism and it cannot be increased without toxicity to cells (unpublished data Experiment 2, 2010).

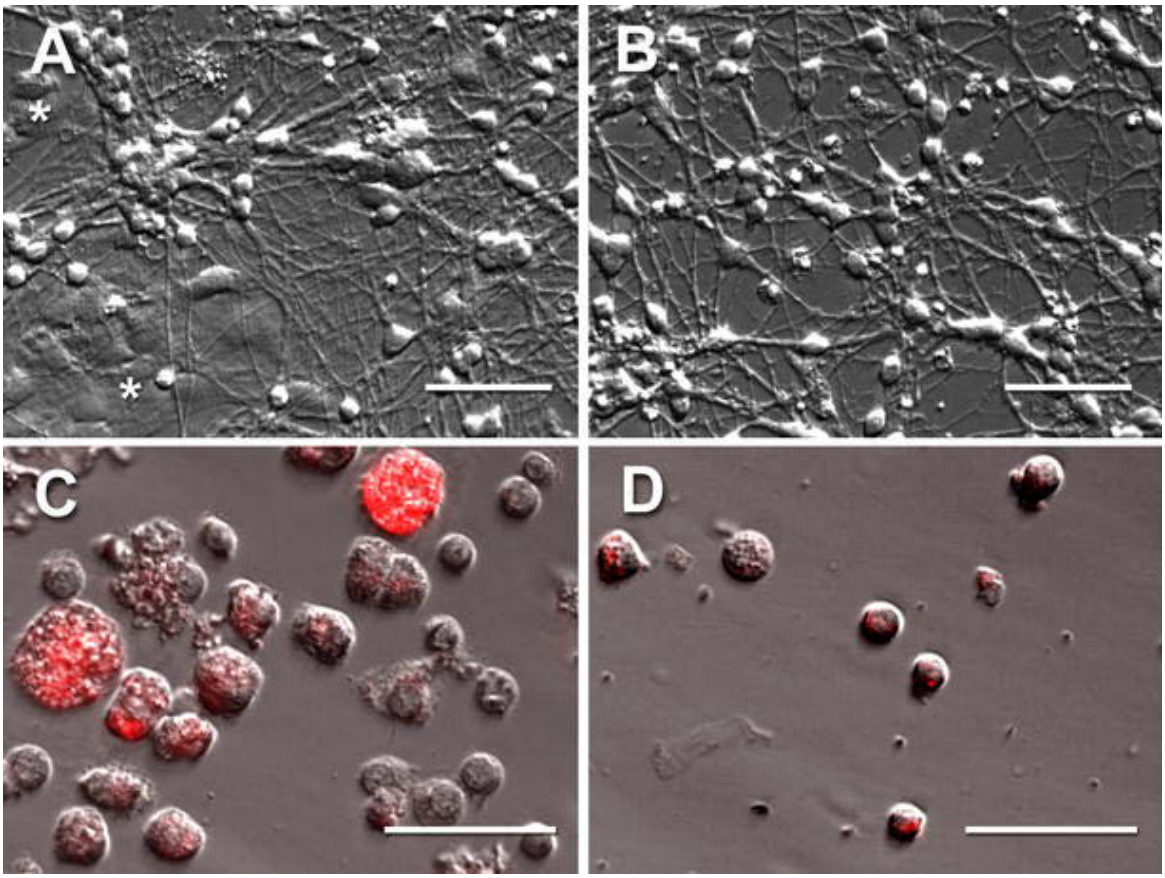


Figure 3.8: Neuronal and non-neuronal uptake of TMR-GM1. CGN cultures were grown in vitro for 7 days, with (B/D) or without (A/C) 10 μM cytosine-β-D-arabinofuranoside (AraC, Sigma-Aldrich, St. Louis, MO) added 18 h after plating and kept in the medium thereafter to deplete dividing non-neuronal cells. Equimolar amounts of TMR-GM1 and fatty acid free BSA were pre-complexed and diluted to a final concentration of 5μM in NS21-free medium. CGNs were incubated with GM1-TMR for 14 h and then collected via trypsin off the plate and either fixed in 4% PFA for 12 min at room temperature or lysed in 1% SDS. Hoffman images (A/B) demonstrate after 7 days in culture, CGNs have small refractile cell bodies that put out a mat of axons, with occasional flat non-neuronal cells as marked by an asterisk (*) which are more evident in the absence of AraC. After removing cells from the plate via trypsin, large non-neuronal

cells (>15μm) as seen in C, take up much more of the TMR compound compared to the smaller neuronal cells (C/D). Scale bar 50 μm [85].

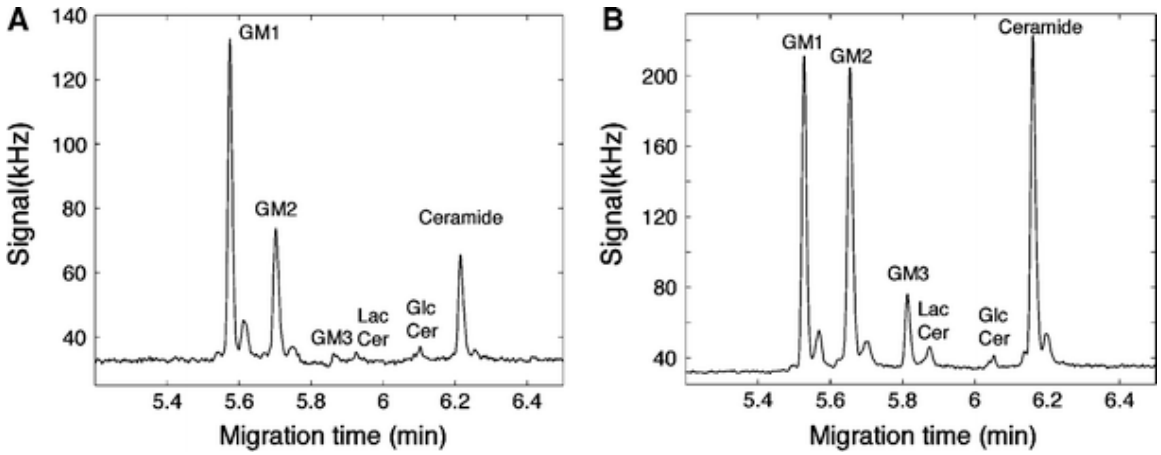


Figure 3.9: CGN homogenate alaysis. CGN cultures were grown in vitro for 7 days, with (A) or without (B) 10 μM cytosine-β-D-arabinofuranoside (AraC, Sigma-Aldrich, St. Louis, MO) added 18 h after plating and kept in the medium thereafter to deplete dividing non-neuronal cells. Equimolar amounts of TMR-GM1 and fatty acid free BSA were pre-complexed and diluted to a final concentration of 5μM in NS21-free medium. CGNs were incubated with GM1-TMR for 14 h and then collected via trypsin off the plate and lysed in 1% SDS and stored at -80ºC until analysis. The CGNs treated with AraC had a metabolic profile that was less catabolic than the non-AraC treated samples which mainly consisted of ceramide-TMR [85].

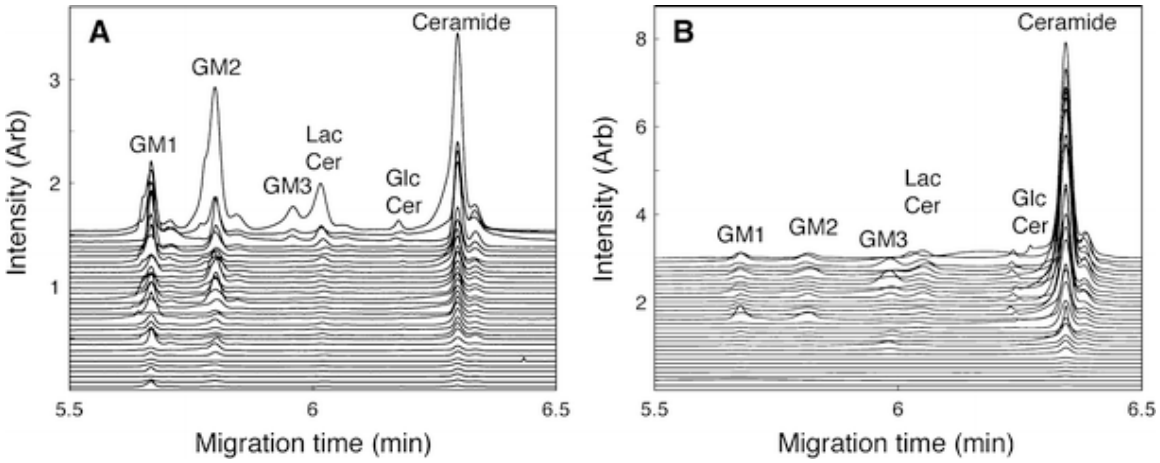


Figure 3.10: Capillary electrophoresis of single cells. CGN cultures were grown in vitro for 7 days. Equimolar amounts of TMR-GM1 and fatty acid free BSA were pre-complexed and diluted to a final concentration of 5µM in NS21-free medium. CGNs were incubated with GM1-TMR for 14 h and then collected via trypsin off the plate and fixed in 4% PFA for 12 min at room temperature. Cells were washed and collected in 10mM glycine-PBS and stored in 4ºC until analysis. Cells were chosen for analysis based upon size and morphology. 31 neurons (A) have a very different metabolic profile from the 30 non-neuronal cells (B). Neurons demonstrate more catabolic products, where non-neuronal cells mainly catabolize GM1-TMR to ceramide-TMR [85].

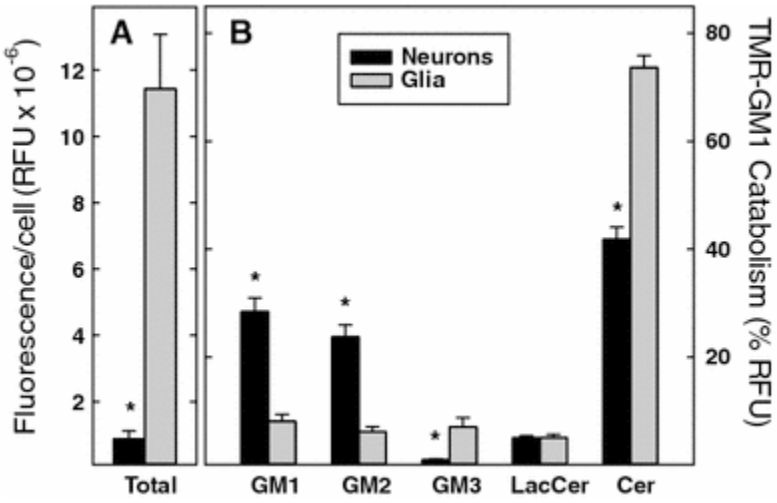


Figure 3.11: Summary of single cell analysis data. This figure is a summary of values from the cells analyzed in the previous figure. Average total fluorescence (TMR) uptake was much higher in non-neuronal cells compared to neuronal cells (A). The average intensity of the catabolic products for neurons vs. non-neuronal cells also showed marked differences in distribution (B). The dynamic range of the capillary electrophoresis machine allowed for quantification of TMR and its catabolites for each cell type. The average of the single cell analysis from Figure 3.10 was reflective of the homogenate study Figure 3.9 trends *$p<0.001$ (Student's t-test, neurons vs. glia) [85].

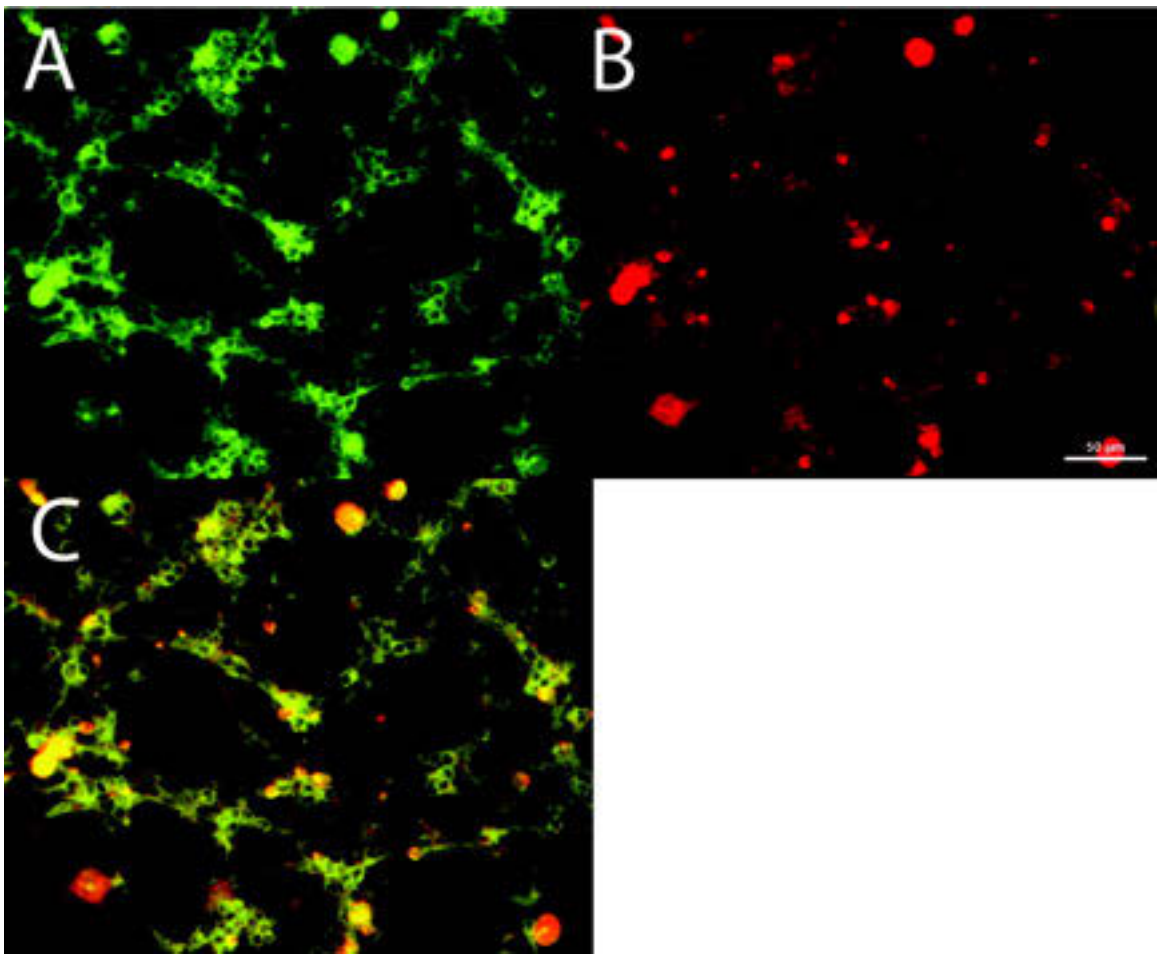


Figure 3.12: CGNs co-incubated with BODIPY-LacCer and TMR-GM1. CGN were grown for 7 days in vitro in NS21 containing medium. They were then washed with NS21 free medium, and Lac-Cer BODIPY and GM1-TMR was prepared by pre-complexing with equal molar fatty acid free BSA and diluted to a final concentration of 5µM of each compound. Cells were treated for 2 h with both compounds and then incubated for an additional 22 h in NS21 free medium to allow for incorporation of the BODIPY and TMR. Cells were imaged after 24 h total, cell bodies and axons were stained with BODIPY compound (green, A). Larger non-neuronal cells more readily took up the TMR-GM1 (red, B). Overlay of BODIPY/TMR channels seen in C. Scale bar 50µm [86].

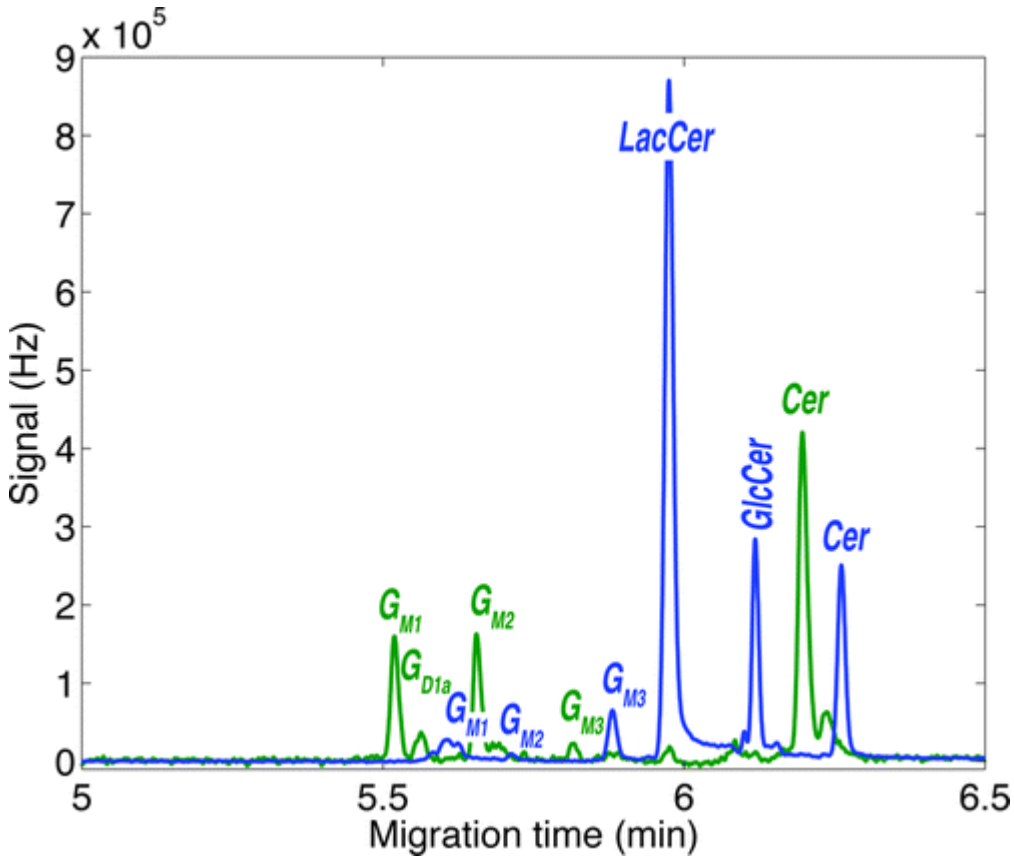


Figure 3.13: Two-color laser-induced fluorescence detection of CGN homogenate lysate co-incubated with LacCer-BODIPY and GM1-TMR. CGN were grown for 7 days in vitro in NS21 containing medium. They were then washed with NS21 free medium, and LacCer BODIPY and GM1-TMR was prepared by pre-complexing with equal molar fatty acid free BSA and diluted to a final concentration of 5µM of each compound. Cells were treated for 2 h with both compounds and then incubated for an additional 22 h in NS21 free medium to allow for incorporation of the BODIPY and TMR labeled glycosphingolipids. Cells were collected off the plate via trypsin and after washing were lysed in 1% SDS. GM1-TMR (green trace) showed only break down products, while the LacCer-BODIPY (blue trace) showed both anabolic and catabolic products [86].

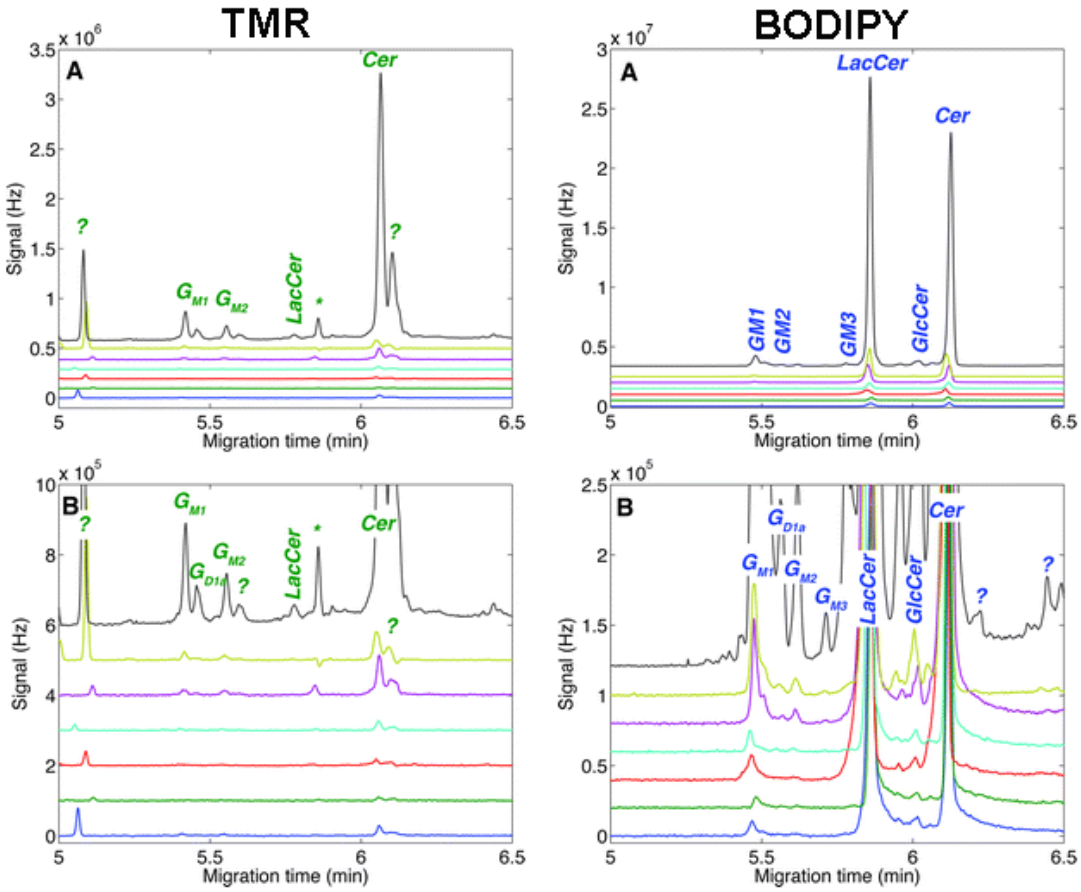


Figure 3.14: Single CGNs co-incubated with GM1-TMR and LacCer-BODIPY. CGN were grown for 7 days in vitro in NS21 containing medium. They were then washed with NS21 free medium, and LacCer BODIPY and GM1-TMR was prepared by pre-complexing with equal molar fatty acid free BSA and diluted to a final concentration of 5µM of each compound. Cells were treated for 2 h with both compounds and then incubated for an additional 22 h in NS21 free medium to allow for incorporation of the BODIPY and TMR. Cells were collected off the plate via trypsin and after washing were fixed with 4% PFA in PBS for 12 minutes at room temperature. Fixed cells were washed and stored in 10mM glycine-PBS at 4ºC until analysis. Seven cells were analyzed in the TMR or BODIPY channel (A) full channel, (B) expanded scale. Differences can be seen

in metabolism between the two compounds, TMR shows only break down products from the GM1-TMR added, with the highest peak being ceramide. BODIPY shows both anabolic and catabolic peaks, with the largest peak being the exogenously added LacCer. Peaks marked with "*" are artifacts, and peaks marked with "?" are unknown with no standard for reference [86].

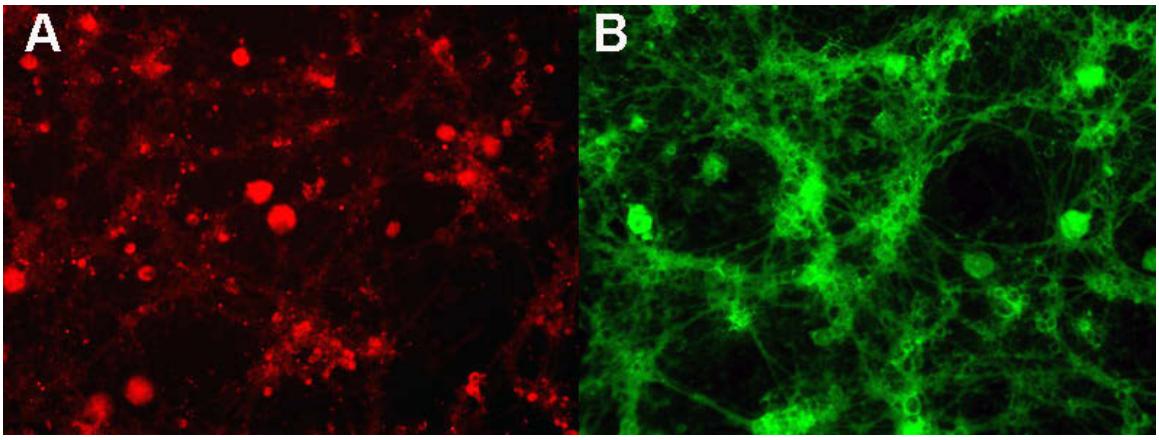


Figure 3.15: The fluorescent tag utilized alters CGN uptake and distribution of exogenously added molecules. CGNs were grown for 12 days in vitro with NS21 containing medium. After 12 days cells were washed with NS21 free medium and incubated for 2 h with 5µM TMR-GM1 (A) or 5µM BODIPY-GM1 (B) which were both pre-complexed with equal molar amounts of fatty acid free BSA. After 2 h incubation, cells were washed with NS21 free medium and incubated in NS21 free medium for an additional 22 h before collection. TMR-GM1 has bright staining of larger non-neuronal cells and is very punctate. BODIPY-GM1 stains the axons and smaller cell bodies of the CGNs in addition to some non-neuronal cells. This suggests that the fluorescent tag added to the same ganglioside GM1 core alters the uptake of these molecules. (Unpublished results, Experiment 2, 2011).

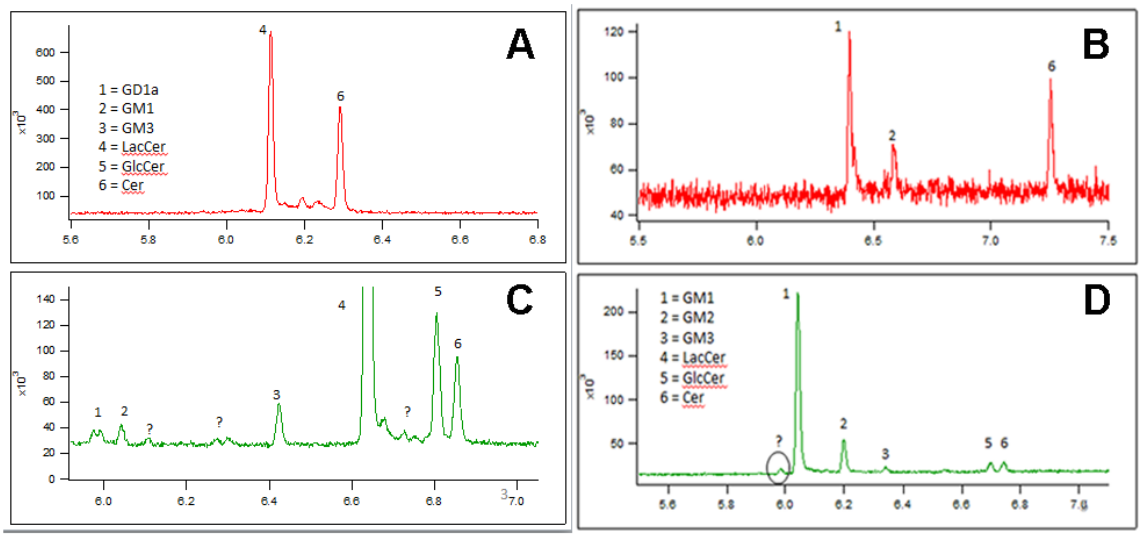


Figure 3.16: Electropherograms of CGNs treated with Lac-Cer-BODIPY and LacCer-TMR or treated with GM1-TMR and GM1–BODIPY. The importance of both glycosphingolipid core and fluorescence tag was explored to demonstrate the effects of these combinations on metabolic pathway sampling. CGNs were grown for 7 days in vitro in NS21 containing medium. Cells were washed with NS21 free medium and incubated for 30 minutes with a final concentration of 1.6uM glycosphingolipid pre-complexed to an equimolar concentration of BSA then incubated for an additional 23.5 h in NS21 free media to allow incorporation before homogenate collection. Both TMR and BODIPY compounds were incubated at the same time in one sample: Cells were treated with the same glycosphingolipid core: one sample with TMR-LacCer (A) and BODIPY-LacCer (C); the other with TMR-GM1 (B) and BODIPY-GM1 (D). Both the fluorescent tag (BODIPY (C/D) or TMR (A/B)) and also the starting glycosphingolipid (GM1 (B/D) or LacCer(A/C)) both make a difference in what metabolic pathways exogenously added molecules sample. BODIPY (green traces C/D) seems to be a superior molecule in terms of sampling both catabolic and anabolic pathways. TMR (red traces A/B) and GM1

(GM1-TMR B, GM1-BODIPY D) no matter what they are conjugated to only seem to sample catabolic pathways. (unpublished data experiment 10+11, 2011).

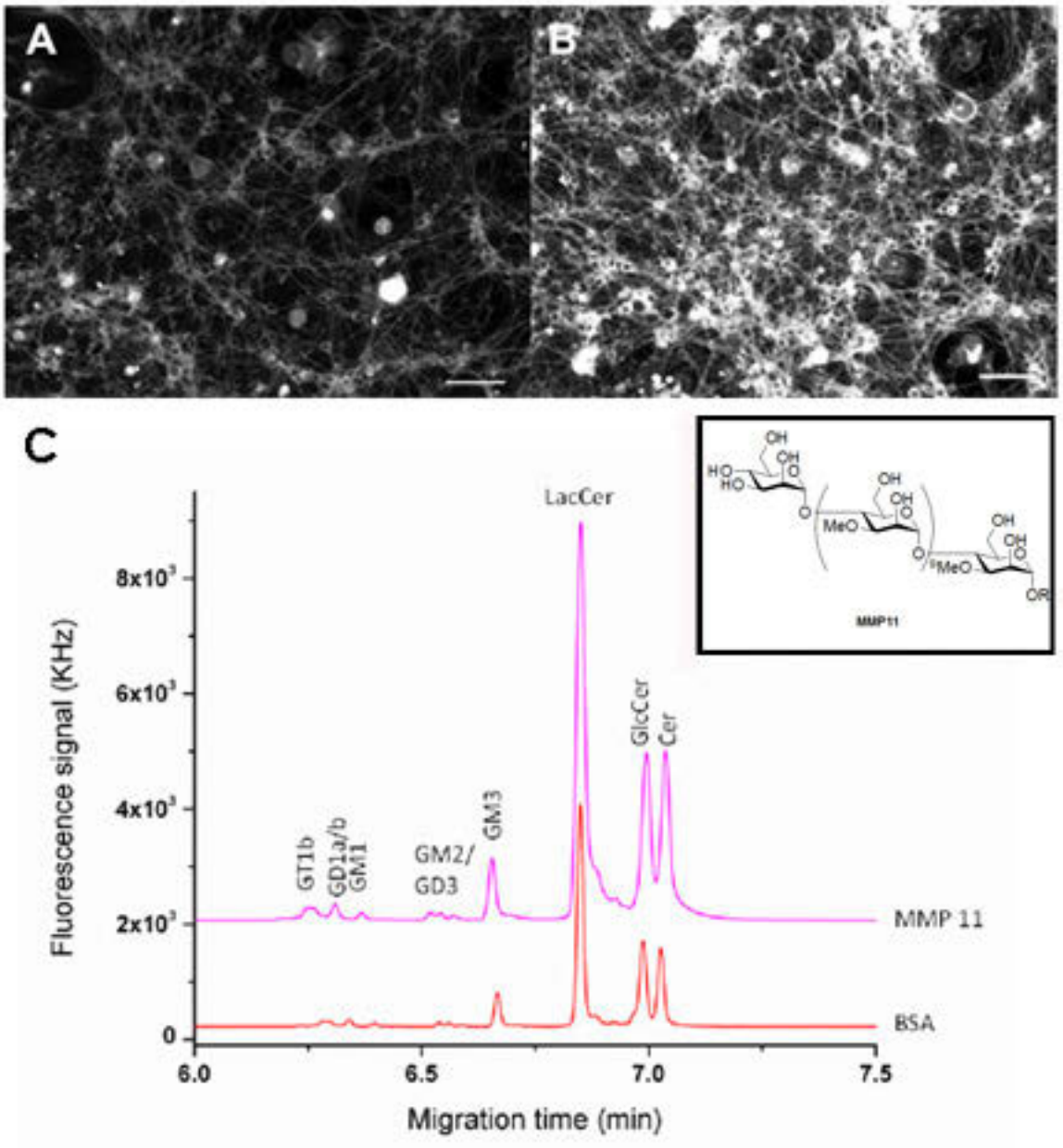


Figure 3.17: CGNs treated with LacCer-BODIPY and various carriers. CGNs were grown for 7 days in vitro in NS21 containing medium. They were washed with NS21 free medium and incubated with 1.6µM LacCer BODIPY pre-complexed with either equal molar BSA or 1.5x molar 3-O-methyl-mannose polysaccharide MMP11. MMP11 is a mycobacterial glycan that is known to be a lipid molecule carrier. MMP11 was a generous gift of Dr. Todd Lowary from the Alberta Glycomics Centre, Edmonton,

Canada [87]. Cells were incubated for 0.5 h with compounds then washed with NS21 free medium and incubated an additional 23.5 h in NS21 free medium before homogenate collection. A difference can be seen in fluorescent intensity, correlating to differences in uptake efficiently based upon the carrier utilized, with the classic carrier, BSA, being dimmer (A) and MMP11 (B) being much brighter when viewed at the same exposure, this is also reflected in the electropherogram (C). Scale bar= 50 μm. (unpublished data experiment 3, 2012).

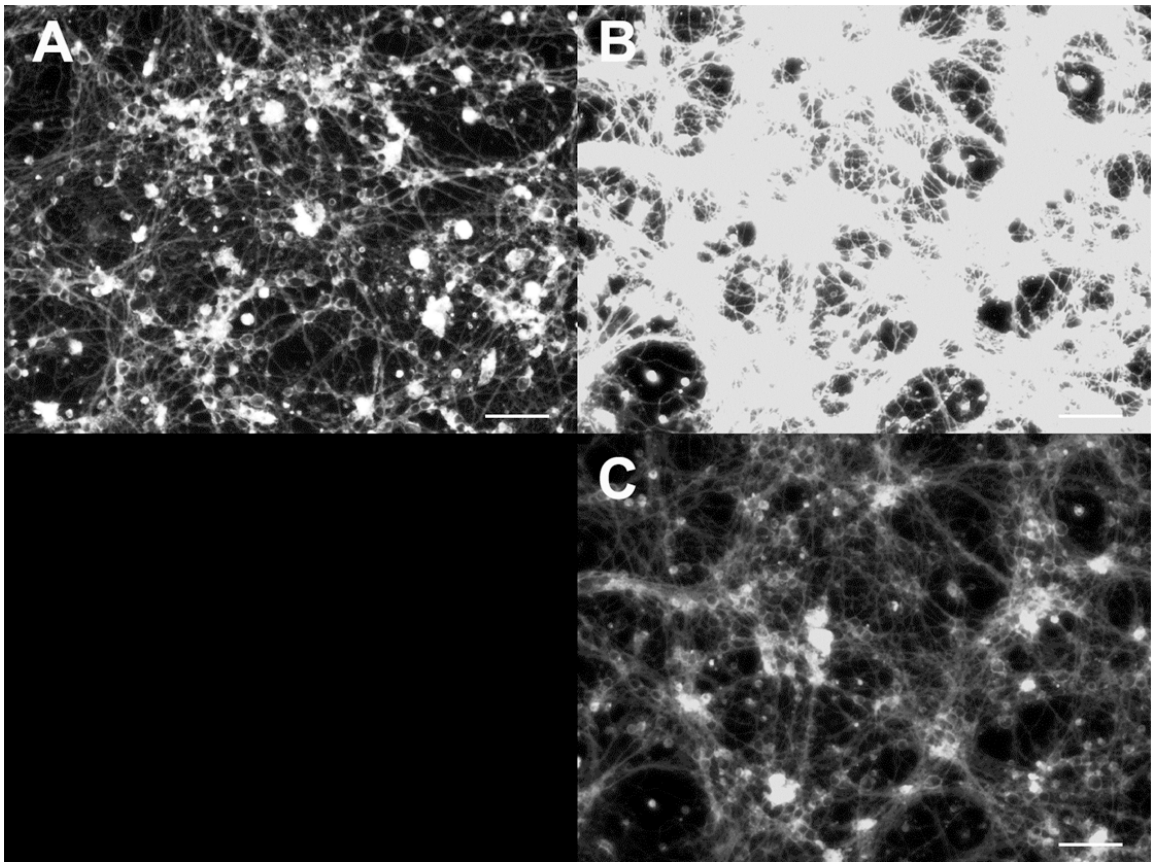


Figure 3.18: CGN incubated with LacCer BODIPY or GM3 BODIPY-TMR and MMP11. CGNs were grown for 7 days in vitro in NS21 containing medium. They were washed with NS21 free medium and incubated for 30 minutes with 1.6μM GM3-BODIPY-TMR or 1.6μM LacCer BODIPY final concentration; both were pre-complexed with 1.5x molar MMP11. After incubation cells were washed and incubated in NS21 free medium for an additional 23.5 h before single cell collection. Images A and B are taken at the same exposure, C is image B at a lower exposure time. Differences in fluorescent intensity, correlate to differences in uptake efficiently based upon the glycosphingolipid and fluorescent tag, with LacCer-BODIPY-FL-C5 being dimmer (A) than GM3-BOIDPY-TMR (B) at the same exposure time. Scale bar = 50 μm (unpublished data experiment 2, 2012).

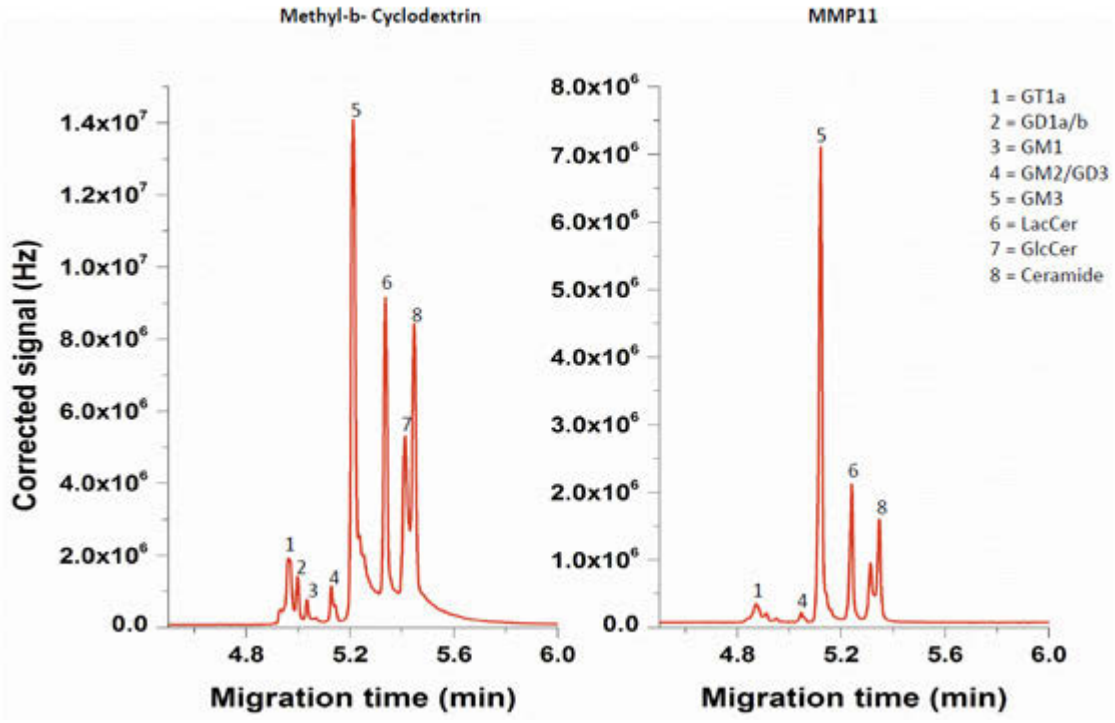


Figure 3.19: CGNs treated with GM3-BODIPY-TMR and various carriers. CGNs were grown for 7 days in vitro in NS21 containing medium. They were washed with NS21 free medium and incubated with 1.6μM GM3-BODIPY-TMR pre-complexed with 1.5x molar MMP11 or 1.5x molar methyl-β-cyclodextrin. Cells were incubated for 0.5 h with compounds then washed with NS21 free medium and incubated an additional 23.5 h in NS21 free medium before homogenate collection. Note the difference in intensity scale on the y axis. Methyl-β-cyclodextrin intensity was greater than MMP11, correlating to differences in uptake efficiently based upon the carrier utilized. Methyl-β-cyclodextrin was also brighter when imaged at the same exposure under fluorescence microscopy before collection (unpublished data experiment 4, 2012).

Section 3.4: Discussion

What is metabolic cytometry?

Metabolic cytometry is one way to study single cell metabolism. This method has been developed by Dr. Dovichi and it uses modern chemical instrumentation to monitor metabolism in single cells through exogenously added tagged molecules [88,89]. Immunostaining experiments such as Figure 3.20 have revealed that the distribution of glycosphingolipids can be highly heterogeneous within neuronal populations [41]. It has also been documented that not all cells are affected equally in glycosphingolipid metabolic diseases [40]. These differences in cellular expression cannot be studied quantitatively by methods like immunohistochemistry, but rather need to be studied on the single cell level. Metabolic cytometry methods are desirable over these affinity reagent (like antibodies or toxins) methods, because these reagents can interfere with the normal cellular processes. Affinity reagents are also not practical for quantitation, as labeling each individual metabolite would lead to a resolution nightmare. Each metabolite would be seemingly impossible to tease apart with the limited available secondary antibodies and it would be hard to avoid spectral cross talk, ultimately limiting resolution and dynamic range.

In order to view the metabolome in high resolution, we utilized metabolic cytometry. This method allows for all possible paths can be sampled given the pre-cursor added to cells samples that pathway, the dyes remain stable and the dyes can be separated using capillary electrophoresis (CE) and detected with high sensitivity laser-induced fluorescence. Detection limits are very fine, up to the yoctomole (10^{-24} mole, ~70 molecules) level [84]. Metabolic cytometry also allows for a large dynamic range to be

sampled, allowing for identification of both high and low abundance metabolites. Currently, our methods allow for an unprecedented dynamic range of nine orders of magnitude to be sampled [84]. The fine detection limit and dynamic range allows for quantification at the molecular scale. This could lead to understanding of metabolism at the molecular scale, allowing for novel quantifiable differences to be seen between normal and diseased neurons, possibly leading to new therapeutic breakthroughs.

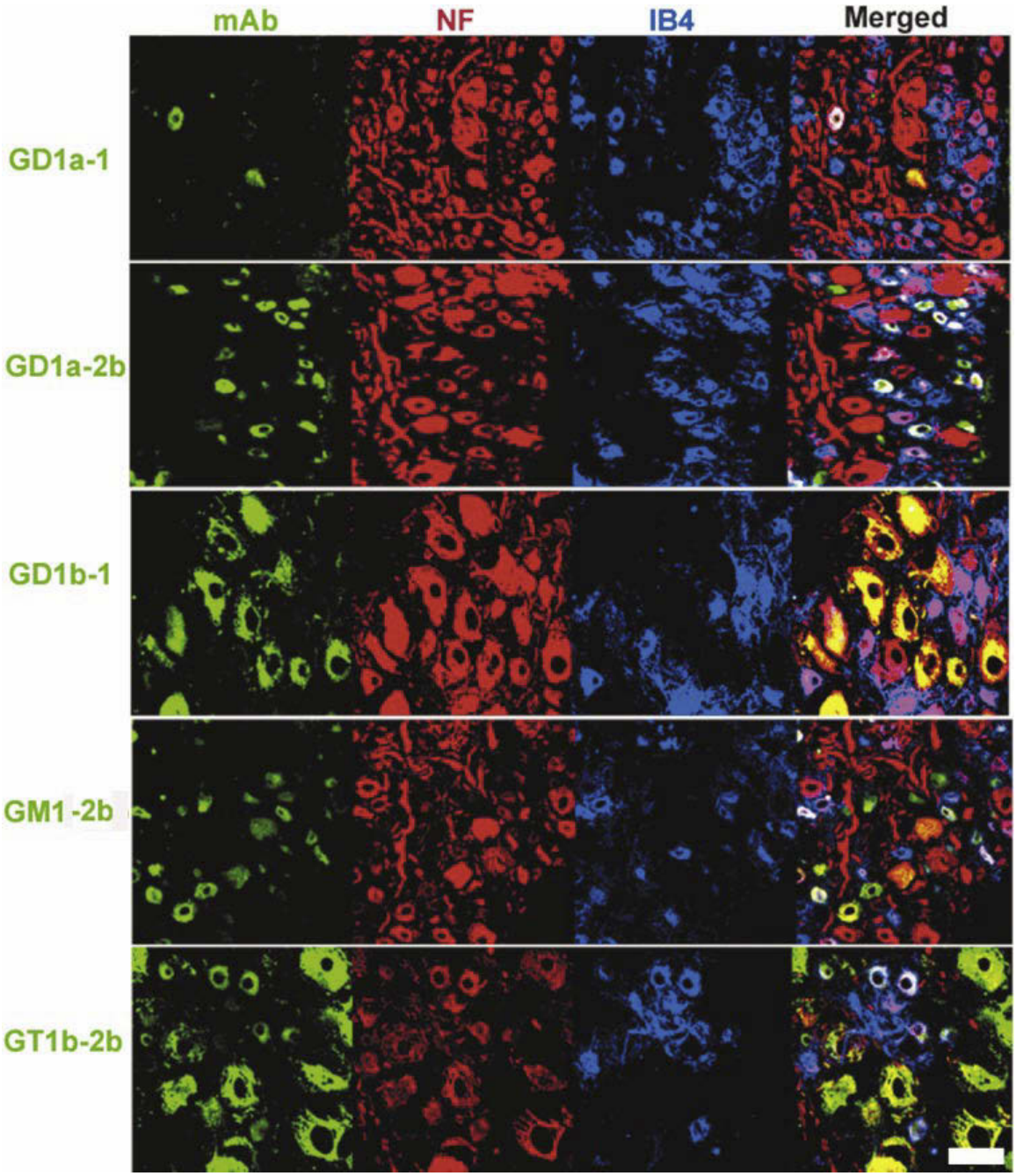


Figure 3.20: Rat DRGs triple-labeled demonstrate differential ganglioside localization. Sections were stained with anti-ganglioside mAb (green), neurofilament (red) and IB-4 (blue), co-localization of all three are shown in merged. This figure demonstrates the heterogeneity of ganglioside expression of neurons. Scale = 20µm. [41].

Fluorescent compound, carrier, and glycosphingolipid combination is crucial to metabolic pathways sampled

To visualize the metabolic pathways within a single cell exogenously added fluorescently tagged glycosphingolipids were added to cells. Since this was a tagged lipid, a carrier molecule had to be utilized to insure solubility and delivery. Both carrier, fluorescent tag, and glycosphingolipid used were altered over the lifetime of these experiments. The best combination so far that allowed for both maximum solubility, and sampling of catabolic and anabolic pathways, was a methyl-β-cyclodextrin carrier with a BODIPY tagged molecule with a GM3 glycosphingolipid core.

Fluorescent tag – Historically, these studies began with 5-Carboxyl-tetramethylrhodamine (TMR) compounds. TMR-GM1 and TMR-LacCer when complexed with BSA carrier, resulted in punctate staining in two different cultured nerve cell types (DRG/CGN) (Figure 3.1, Figure 3.15) which only demonstrated catabolism in electropherograms (Figure 3.2, Figure 3.5). TMR uptake was very high in non-neuronal cells both visually and by electropherograms, but also only showed catabolism (Figure 3.8-3.11). We ruled out the punctate staining of TMR as being lysosomal as it did not localized to lysosomes as co-visualized with lysosomal marker LysoTracker green (Invitrogen L7526) (unpublished results experiment 16, 2011). Inhibitors of glycosphingolipid catabolism were utilized with TMR tagged glycosphingolipids in an attempt to prevent catabolism, to sample anabolic pathways. Both β-galactosidase inhibitors (Galactal, Galactostatin, Galactostatin Bisulfate, PETG) and sialidase inhibitor Neu5ac2ene did not result in sampling of anabolic pathways (Figure 3.6, Figure 3.7). While these inhibitors did increase some of the earlier catabolic peaks and shifted the

trend away from primarily all ceramide, they did not address the lack of anabolic pathways sampling. In the end, TMR as a carrier was not effective in sampling anabolic pathways even with catabolic inhibitors, so other dyes were explored. Boron dipyrromethene difluoride (BODIPY-FL) was the first alternative dye tested. BODIPY compounds allowed for both anabolic and catabolic pathways to be sampled (Figure 3.13, Figure 3.14). Alterations to BODIPY changing its absorbance/emission spectrum allowed for even greater uptake by cells and we are able to utilized multiple BODIPY derivatives at the same time (i.e BODIPY-FL in green and BODIPY-TMR in red). BODIPY-TMR which is a red emitting BOIDPY compound (Chapter 3 Introduction Figure 3.0) allowed sampling of both catabolic and anabolic pathways at an even greater intensity than BODIPY-FL (Figure 3.18). Currently, BODIPY derivatives are being explored for multi-colored metabolic cytometry, and a three color system has already been created which could be utilized to study multiple channels of BODIPY derivatives simultaneously (personal communication Dr. Richard Keithley).

Glycosphingolipid – In addition to the fluorophore used, the core glycosphingolipid modified made a great difference what metabolic pathways were sampled. Over the course of these experiments, GM1, LacCer, and GM3 were the main glycosphingolipid cores studied. GM1-TMR and GM1-BODIPY-FL compounds only sampled catabolic products under the conditions tested, while LacCer-BODIPY-FL compounds sampled both catabolic and anabolic pathways (Figure 3.16). By adding a pre-cursor GM1 we may have limited ourselves to only sample catabolic metabolism. Since in the major ganglioside is GT1b in the CGNs tested, the exogenously added fluorescently tagged GM1 would have to be broken down into a pre-cursor like GM3 or LacCer to be able to

sample the beta ganglioside series which was dominant in these cells (see Figure 1.1 for the metabolic pathway). By studying LacCer, a precursor that can be made into either the alpha or beta gangliosides, we were able to sample both catabolic and anabolic products. LacCer uptake was more robust, and when paired with BIODIPY-FL we received the robust cellular uptake. The uptake was present in both neurites and neuronal cell bodies, as a strong, seemingly cell surface staining, with some non-neuronal cells taking up the dye as well. This was a stark contrast to the robust non-neuronal staining and mediocre punctuate staining of neurons by TMR compounds (Figure 3.15). Most recently, GM3 derivatives were explored. GM3 is the glycosphingolipid at the branching point between the alpha and beta gangliosides and this core has shown even greater promise than LacCer-BODIPY-FL compounds. GM3-BODIPY-TMR compounds resulted in improved signal to noise and much brighter uptake as seen by fluorescence microscopy (Figure 3.18).

Carrier – The exogenously added tagged glycosphingolipid had to have a lipid carrier molecule to insure solubility and delivery to cells. Historically, equal molar amounts fatty acid free bovine serum albumin (BSA) and tagged glycosphingolipid was utilized. This ran into a solubility issue since the tagged glycosphingolipid was solubilized in ethanol, and BSA can precipitate in ethanol. Alternatives to BSA were explored that allowed for greater solubility and delivery. Known lipid carrying bacterial compounds were explored first 3-O-methyl polysaccharide (MMP11 and MMP14) and de-acylated methyl glucopolysaccharide (MGP) [90]. MMP11 and MMP14 were a generous gift of Dr. Todd Lowary from the Alberta Glycomics Centre, Edmonton, Canada [87]. MMP11, MMP14 and MGP delivered the tagged glycosphingolipid more efficiently than BSA, as seen both by

brighter fluorescence imaging of the cells and increased electropherogram intensity (Figure 3.17-3.18, unpublished data experiment 1, 2012). MMP11 was more effective than MGP and MMP14, but all of these molecules are intensive to synthesize, so we looked into the readily available alternative of methyl-β-cyclodextrin. When MMP11 was compared to methyl-β-cyclodextrin, delivery of GM3-BODIPY-TMR was more efficient with methyl-β-cyclodextrin as demonstrated both by higher intensity microscopy fluorescence and electropherograms (Figure 3.19, unpublished data experiment 4, 2012). Methyl-β-cyclodextrin will be used in future experiments for delivery, as this is far easier to obtain and cheaper than synthesizing MMP/MGP compounds.

Section 3.5: Summary and Future Directions

Ganglioside biosynthesis pathways are well established, but cell-to-cell variability in these pathways is not well known. Utilizing metabolic cytometry, ganglioside metabolism can be studied at the single cell level. Currently, this method is highly sensitive allowing for quantifiable differences to be seen with as few as 70 molecules. Better understanding ganglioside metabolism on the molecular level could shed light on genetic deficits in these pathways which have known nervous system pathologies in animal models and humans. Historically, these metabolic cytometry studies began as a simple rotation project on dorsal root ganglion (DRG) homogenates that have now blossomed into an extensive and fruitful collaborative effort. Our experiments demonstrated that primary neuronal cells can be harvested, successfully labeled with fluorescent ganglioside derivatives, and can be fixed and shipped across the county for analysis. Perhaps more importantly, our studies have demonstrated that our metabolic

cytometry methods can distinguish subpopulations of cells in mixed cultures (neurons vs. glia) which could be translated into *in vivo* work.

Characterization of subsets of single cell types – Metabolic heterogeneity could be explored in cell types like DRGs, which are a known heterogeneous cell type. In our experiments, DRGs showed differences from cell to cell in uptake of the exogenously added fluorescent ganglioside derivatives [83]. The most appropriate methods to distinguish homogenous subpopulations in this heterogeneous cell type would need to be fleshed out. We already know that our current methods can distinguish neuronal from non-neuronal cell types, and both of these cell types have different metabolic profiles (Figure 3.8-3.11). Also, we can cluster homogenous cell types metabolic electropherograms to see trends in subsets of cells in a heterogeneous population, but we would need an additional identifier *in vitro* to make this more meaningful [83]. We have tried affinity reagents in the past by attempting to label homogenous subsets of DRGs with IB4, but this did not produce robust results (Figure 3.4). Further development to characterize subsets of homogenous cells (i.e. diseased vs. healthy cells) within a larger heterogeneous population could lead to interesting metabolome discoveries that could translate our *in vitro* to *in vivo* discoveries.

TMR vs. BODIPY – Over the course of this project two fluorophores were explored, BODIPY and TMR. BODIPY compounds often showed both catabolism and anabolism, while TMR compounds only showed catabolism. The differences we see in fluorescent tagged sampling of metabolic pathways are most likely due to their differences in structure. There is a difference in the charge, linker, and steric size of the fluorescent molecules utilized, and these all could affect the glycosphingolipid's insertion into

membrane. Recently, Dr. Richard Keithley in Dr. Dovichi's group found that the hydrophobicity of the dye plays a role in cellular uptake. Where order of most efficient uptake was: LacCer-BODIPY-FL, followed by LacCer-BODIPY-TMR, then LacCer-BODIPY-650/665 (personal communication Dr. Richard Keithley). This suggests that the hydrophobicity of the dye could plays a role in membrane insertion, where the more hydrophobic dyes insert more readily into the membrane resulting in higher efficiency of uptake into cells. In addition to the hydrophobicity of the dye, the sterics of the molecule and linker could also play a role in membrane insertion efficiency. A recent paper on cholera toxin uptake via tagged GM1 into cells showed that the saturation of the acyl chain of GM1 was extremely important in retrograde trafficking from the plasma membrane. Unsaturated acyl chain GM1 sorted form the plasma membrane to the trans-Golgi network and then to the endoplasmic reticulum, but if the acyl chain of GM1 was saturated it went to from the plasma membrane to endosomes [91]. Perhaps, the longer linker with an extra carboxamide group and bulkier structure of the TMR compounds mimicked a saturation of the acyl chain, and lead to predominantly endosomal sorting. We only saw catabolic sampling of TMR compounds, and staining was punctuate, but it did not localize with LysoTracker green (Invitrogen L7526). LysoTracker is a pH dependent dye, and should not stain endosomes well, if at all. So, perhaps TMR compounds could localize to endosomes in our experiments. Studies with endosomal markers such as Invitrogen's early or late endosomal markers (C10586 or C10588) which utilize GFP baculovirus expression vectors could co-localize with our punctuate TMR *in vitro*. This could be a great tie in with the literature, and explain why we see punctate

TMR staining, but not lysosomal co-localization. In the future, both the hydrophobicity and sterics should be considered in the design of new fluorescent compounds.

Continuing optimization of fluorophore, carrier, and glycosphingolipid core -- A large amount of effort went into optimizing the combination of fluorophore, carrier and glycosphingolipid to allow for efficient sampling of both catabolic and anabolic pathways. TMR compounds only demonstrated catabolism, while BODIPY compounds generally sampled both catabolic and anabolic pathways. We also found the glycosphingolipid core was essential in the pathways sampled, with GM1 only showing catabolic products, while LacCer and GM3 sampled both catabolic and anabolic pathways. The choice of carrier was also crucial for proper delivery of the tagged glycosphingolipid to cells; we found methyl-β-cyclodextrin to be a superior carrier to BSA. While various inhibitors of catabolism were tested, we ultimately found that the best way to sample both catabolic and anabolic pathways would be to alter fluorophore, carrier and glycosphingolipid choice. Currently, our favored combination is a BODIPY based tag on GM3 or LacCer and utilizing a methyl-β-cyclodextrin carrier. In the future new BODIPY derivatives, carriers and glycosphingolipid core combinations should be explored to maximize metabolome sampling.

Multicolor analysis – We have tracked two different dyes (TMR and BODIPY-FL) simultaneously, but the ability to sample more color spectrums would allow for a larger portion of the metabolome to be sampled concurrently. Currently, the Dovichi laboratory is working on a three color system that can measure BODIPY-FL, BODIPY-TMR, and BODIPY-650/665 (personal communication Dr. Richard Keithley). These dyes have already been tested on differentiated PC12 cells, and extending this research into primary

CGNs or other primary cells should be undertaken. Utilizing multicolored analysis would allow for a wider picture of the metabolome, and could assist technically in accelerating fluorophore, carrier, and glycosphingolipid core combination optimizations.

In vivo work- Ultimately our *in vitro* work could one day be translated into animal models of metabolic diseases. Utilizing single cell metabolic cytometry could reveal molecular differences in metabolism between diseased and healthy cell populations. Once we understand the disease on a single cell level, this could lead to novel targeted therapeutics these subsets of cells. We have begun work to bridge our *in vitro* work to *in vivo*. Tissue slice cultures offer the control of an *in vitro* environment, but supply tissue architecture that is more *in vivo* like than dissociated cultures. Yellow fluorescent protein (YFP) expressing motor neurons allow us to see a subset of cells in the motor cortex slice culture. Incubating the motor cortex slice with GM3-BODIPY-TMR allowed us to visualize both green motor neurons and cells that had taken up GM3-BODIPY-TMR. This method could eventually allow us to translate our single cell work into animal models but it needs to be optimized. Currently, the GM3-BODIPY-TMR is taken up by seemingly every cell type, and results in an overwhelming red stain. The combination of the thickness of the slice culture and intensity of BODIPY-TMR makes it hard to distinguish tissue architecture. The technical aspect of preparing the slices for imaging and optimal concentration and incubation of fluorescent glycosphingolipid needs improvement. Once the technical aspects of tissue preparations are optimized, there are many readily available animal models of metabolic diseases that could really show off the power of single cell metabolic cytometry.

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CURRICULUM VITAE

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Education

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Ganglioside-associated signaling molecules in primary neurons

- Unbiased methods identified novel ganglioside specific interacting proteins via mass spectrometry from primary neurons.
- Discovered potential new ganglioside signaling regulation pathway involving AAA+ ATPase Thorase.
- Experience in immunocytochemistry and protein extraction utilizing primary neuronal cell cultures, NIS-Elements software, preparation for mass spectrometry, and mass spectrometry data set analysis using Thermo Proteome Discoverer, Scaffold and ProteinCenter. Isolation of mouse brain and cryosectioning for immunohistochemistry. Extensive work with gangliosides including ganglioside extraction from primary cell culture, affinity chromatography, and thin layer chromatography.

Ganglioside metabolism in single primary neurons and glia

- Part of a collaborative effort to develop methods to analyze single cell glycolipid metabolomics from rat primary cerebellar neuronal cultures. Sensitivity with this method was achieved at the 100 yoctomole level (1 ymole = 10^{-24} mol) which corresponds to roughly 70 molecules.

- Established primary cell cultures and optimized synthetic lipid delivery methods to maximize uptake. Prepared samples for analysis via an ultrasensitive capillary electrophoresis carried out with our collaborators.
- Collaborative effort between the laboratories of Dr. Ronald L. Schnaar, Dr. Ole Hindsgaul, Dr. Monica Palcic, and Dr. Norman Dovichi

Spring 2008: Rotation Research Dr. Donald Zack
Department of Ophthalmology, Johns Hopkins School of Medicine Baltimore

- Assisted screening small molecule libraries for compounds that promote retinal ganglion cell and photoreceptor differentiation and survival, focusing on changes in gene expression. Tested small molecules both *in vitro* and *in vivo*. Preformed: retinal ganglion primary cell culture, optic nerve crush/cut, intraocular injection of small molecules, induced glaucoma via IOP elevation, RNA isolation from retinal tissue, real time PCR, Thermo Cellomics software and instrument calibration, cryosectioning and in situ staining of cryosectioned retinas.

Winter 2007: Rotation Research Dr. Xinzhong Dong
Department of Neuroscience , Johns Hopkins School of Medicine Baltimore

- Studied TRPV1 and TRPM8 receptor role in neuronal nociception and the novel role of sensory specific gene 1 (Ssg1) as a regulator of TRP channels in neurons. Experience in cloning, vector creation, co-transfection experiments in HEK cells, GST pull downs, gel electrophoresis and Western Blotting.

Fall 2007: Rotation Research Dr. Ronald L. Schnaar
Department of Pharmacology and Molecular Science, Johns Hopkins School of Medicine Baltimore

- Part of a collaborative effort to develop methods to analyze single cell and whole culture homogenate glycolipid metabolomics of dorsal root ganglion neuronal cells. Prepared samples for analysis via an ultrasensitive capillary electrophoresis carried out with our collaborators.
- Collaborative effort between the laboratories of Dr. Ronald L. Schnaar, Dr. Ole Hindsgaul, Dr. Monica Palcic, and Dr. Norman Dovichi.

Summer 2007: Rotation Research Dr. Deborah Andrew
Department of Cell Biology , Johns Hopkins School of Medicine Baltimore

- Studies focused on organogenesis in the Drosophila, examining the role of candidate genes Mipp1, VAM and Veli in tubular organ formation of the salivary gland and trachea. Experienced maintaining and creating Drosophila knockout lines, FlyBase, Drosophila embryo immunohistochemistry and in situ hybridization, PCR, and DNA electrophoresis.

Fall 2003-2007: Undergraduate Research Dr. Jennifer Normanly
Department of Biochemistry and Molecular Biology, University of Massachusetts Amherst

- Examined total protein synthesis in Taxus cell cultures, focusing on paclitaxel (Taxol) production. Extensive experience with protein extraction methods, Bradford Assay, isoelectric focusing, one- and two-dimensional gel electrophoresis, and various SDS-PAGE staining techniques.
- Performed honors research and honors thesis in this laboratory.

Spring 2003: Undergraduate Research Dr. Eric L. Bittman
Department of Biology , University of Massachusetts Amherst

- Examined circadian rhythms in Syrian Hamster, focusing on antibody labeling of brain tissue sections. Specifically focused on differential expression of various hormones and early response factors in multiple circadian rhythm hamster models. Gained experience with immunohistochemistry, brain tissue preparation and sectioning.

Honors and Awards

2013 Glycobiology of Cell Signaling - Best Poster Award
NIH&FDA Glyosciences Research Day May11, 2013

2012 The Sigma-Aldrich Glycobiology of Cell Signaling - Best Poster Award
NIH&FDA Glyosciences Research Day June 12, 2012

Spring 2012 Scheinberg Travel Award
Department of Pharmacology, Johns Hopkins School of Medicine

2006-2007 Junior Fellowship Program
University of Massachusetts Amherst

- Competitive science scholarship awarded to only fourteen University students with extensive research experience. Induction entails public presentations of student's research and organization of a multi-campus life-science symposium in the spring 2007.

2003-2004 BioTap Member
University of Massachusetts Amherst

- Program for incoming freshmen who show exceptional high school academics and are Biology/Biochemistry majors.

2003-2007 Deans List Scholar
University of Massachusetts Amherst

2003-2007 Member of Commonwealth College (Honors College)
University of Massachusetts Amherst

Bibliography

Publications

Essaka, D.C.*, **Prendergast, J. ***, Keithley, R.B., Hindsgaul, O., Palcic, M.M., Schnaar, R.L., and Dovichi N.J. (2012) "Single cell ganglioside catabolism in primary cerebellar neurons and glia," *Neurochem.Res.* **37**(6):1308-14. PMCID: PMC3673005
*Authors contributed equally to this work

Essaka D.C., **Prendergast J**., Keithley R.B., Palcic M.M., Hindsgaul O., Schnaar R.L., Dovichi N.J. (2012) "Metabolic Cytometry: Capillary Electrophoresis with Two-Color Fluorescence Detection for the Simultaneous Study of Two Glycosphingolipid Metabolic Pathways in Single Primary Neurons." *Anal. Chem*. **84**(6): 2799:804. PMCID: PMC3313455

Dada, O.O., Essaka D.C., Hindsgaul, O., Palcic, M.M., **Prendergast, J**., Schnaar, R.L., and Dovichi, N.J. (2011) "Nine orders of magnitude dynamic range: Picomolar to millimolar concentration measurement in capillary electrophoresis with laser induced fluorescence detection employing cascaded avalanche photodiode photon counters," *Anal. Chem.* **83**(7): 2748-2753. PMCID: PMC3072807

Whitmore C.D., **Prendergast J**., Essaka D., Hindgaul O., Palcic M.M., Schnaar R.L., Dovichi N.J. "Metabolic cytometry – the study of glycosphingolipid metabolism in single primary cells of the dorsal root ganglia" in "Chemical Cytometry, Ultrasensitive Analysis of Single Cells", Lu C. Ed.; Wiley-VCH 2010; Chapter 2, pp 21-29.

Research Presentations

Prendergast, J. Schnaar, R.L. (2012). "Ganglioside interacting proteins – bridging the gap between myelin associated glycoprotein (MAG) and RhoA." Invited speaker at Glycolipid & Sphingolipid Biology Gordon Research Conference. Italy.

Prendergast ,J. , Yoo, SW., Umanah, G. Dawson, T.M., Dawson, V.L., Schnaar, R.L. (2013). "Ganglioside-interacting proteins in nerve cells: Specificity and potential functions of GT1b- and GM1-binding proteins." Poster presented at the NIH & FDA 2013 Glycosciences Research Day. National Institutes of Health.

Prendergast ,J. , Yoo, SW., Umanah, G. Dawson, T.M., Dawson, V.L., Schnaar, R.L. (2013). "Ganglioside-interacting proteins in nerve cells: Specificity and potential functions of GT1b- and GM1-binding proteins." Poster presented at the Baltimore-Washington Glycobiology Interest Group (GIG). Johns Hopkins School of Medicine.

Prendergast, J., Essaka, D.C., Keithley, R.B., Palcic, M.M.,Hindsgaul, O., Dovichi, N.J., Schnaar, R.L. (2012). "Ganglioside metabolism in single cells." PSM11-06. Poster presented at the American Society for Neurochemistry (ASN) 43rd Annual Meeting. Baltimore. Abstract published in "Transactions of the American Society for Neurochemistry."

Prendergast, J. Schnaar, R.L. (2012). "Ganglioside interacting proteins – bridging the gap between myelin associated glycoprotein (MAG) and RhoA." PSM05-03. Poster presented at the American Society for Neurochemistry (ASN) 43rd Annual Meeting. Baltimore. Abstract published in "Transactions of the American Society for Neurochemistry."

Prendergast, J. Schnaar, R.L. (2012). "Ganglioside interacting proteins – bridging the gap between myelin associated glycoprotein (MAG) and RhoA." Poster presented at Glycolipid & Sphingolipid Biology Gordon Research Conference. Italy.

Prendergast, J., Essaka, D.C., Keithley, R.B., Tanaka H., Yoshimura Y., Palcic, M.M.,Hindsgaul, O., Dovichi, N.J., Schnaar, R.L. (2012). "Ganglioside metabolism in single cells." Poster presented at Glycolipid & Sphingolipid Biology Gordon Research Conference. Italy.

Prendergast, J., Essaka, D.C., Keithley, R.B., Tanaka H., Yoshimura Y., Palcic, M.M.,Hindsgaul, O., Dovichi, N.J., Schnaar, R.L. (2012). "Ganglioside metabolism in single cells." Poster presented at Baltimore-Washington Glycobiology Interest Group (GIG). Johns Hopkins School of Medicine.

Prendergast, J. Schnaar, R.L. (2012). "Ganglioside interacting proteins – bridging the gap between myelin associated glycoprotein (MAG) and RhoA." Poster presented at Baltimore-Washington Glycobiology Interest Group (GIG). Johns Hopkins School of Medicine.

Prendergast, J. Schnaar, R.L. (2012). "Ganglioside interacting proteins – bridging the gap between myelin associated glycoprotein (MAG) and RhoA." Poster presented at NIH & FDA 2012 Glycosciences Research Day. National Institutes of Health.