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Ganglioside Metabolism in Embryonic Chick Neurons

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GANGLIOSIDE METABOLISM IN EMBRYONIC CHICK NEURONS

by

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CHAPTER I

INTRODUCTION

Many studies have implicated gangliosides as playing an important role in the nervous system (Wiegandt, H., 1982). Nerve growth and differentiation are affected by gangliosides. Neuritogenesis occurs when gangliosides are added to neuronal tissue cultures (Ledeen, R., 1984). Neurite initiation, elongation, and branching differ in their response to different gangliosides. The possibility of using gangliosides for neuronal regeneration is being actively studied. During development of the nervous system there are changes in the concentration and type of gangliosides present which seem to correspond to changes in neuronal differentiation and synaptogenesis (Yates, A., 1986).

The location of gangliosides in the outer leaflet of the plasma membrane has led to several studies on the role that gangliosides may have in cellular interaction and recognition (Hakomori, S. 1981). The gangliosides may be important for cell adhesion, contact inhibition, and spreading or as markers for as yet unidentified functions. Oncogenic transformation has been found to be associated with ganglioside changes and may be involved in cell cycle signaling. Several gangliosides are thought to act as immune recognition factors and may in fact be surface antigens.

Gangliosides have been claimed to be important receptors for several classes of compounds. Initial studies which implicated gangliosides as possible receptors were the binding

of cholera toxin and tetanus toxin to specific gangliosides. Thyroid stimulating hormone, interferon, and neurotransmitters (eg. serotonin) have specificities for various gangliosides. Sendai virus interacts with gangliosides. Gangliosides may not be the actual receptor for many of these biological factors but may regulate the actual receptor indirectly. It is not surprising that gangliosides may be involved in so many different functions because of the wide variety of gangliosides found in nature. This variety is made possible because of the many potential variations in linkages, arrangement of sugars, and type of ceramide moiety in gangliosides. The specificity and the number of enzymes needed to accomplish this wide variety of ganglioside structures is intriguing since each addition of sugar is thought to require its own specific glycosyltransferase. In the intact neuron, gangliosides appear to be synthesized at the luminal surface of the Golgi cisternae and are then transported to the plasma membrane of the neuron via axonal transport. Gangliosides have been identified in all mammalian tissues but are found in highest concentration in the nervous system. The types of gangliosides in each tissue varies and may relate to a tissue specific function. Interestingly, gangliosides in the neuron are localized in the synaptic plasma membrane and may be important for electrical excitability of the membrane and synaptic transmission (Leskawa and Rosenberg, 1981). Control of the synaptic ionic environment may involve gangliosides

since it is known that gangliosides bind calcium close to the plasma membrane. Another speculative function of gangliosides involves their possible role in memory.

There has been no definitive function attributed exclusively to gangliosides despite the evidence presented above. One method of determining the function of gangliosides is to study their metabolism in detail and try to verify what controls their biosynthesis. Most studies on the metabolism of gangliosides to date have focused on identifying the metabolic steps through in vitro studies on homogenates but gives us no information on how they are metabolized and compartmentalized in vivo. Initial studies on C6 and N18 cell lines provided some evidence of the intracellular processing of gangliosides (Fishman and Prodraza, 1982 and 1984). Anabolic studies in fibroblasts provided key information on how glycosphingolipids are metabolized (Saito, M., et. al. 1985). These studies on C6, N18, and fibroblasts utilized a cationophore called monensin which has been found to specifically inhibit Golgi processing events. The treatment of C6 and N18 cells with monensin caused a quantitative increase in glucosylceramide and a decrease in lactosylceramide, other neutral glycolipids, and gangliosides. Treatment of fibroblasts with monensin resulted in an increase in quantity of both glucosylceramide and lactosylceramide and an increase in metabolic incorporation of [^{3}H]-galactose but a decrease in labeling of gangliosides. Many of the gangliosides found in

normal nervous tissue are not present in these cell lines and because these cell lines are transformed they may have altered biosynthetic and transport pathways. Primary neuronal cultures exhibit the polysialogangliosides present in normal tissue but absent in the cell lines and also are not transformed. Therefore the primary neuron should provide a better model for studying the processing pathways of gangliosides. The initial, central, and terminal glycosylations of the complex glycosphingolipids can be studied by using the monovalent cationophore monensin as a tool for disruption of the Golgi complex. The organization of the Golgi cisternae is thought to involve close interactions with the cytoskeleton. Inhibitors of the cytoskeleton can also provide a tool for studying glycosphingolipid metabolism. Colchicine, which causes depolymerization of the microtubules; vinblastine, which causes crystallization of microtubules; and cytochalasin D, which inhibits actin polymerization in microfilaments will be used to study the metabolism of gangliosides. Cobalt is also thought to interfere with intracellular transport events by inhibiting fusion of transitional elements with the Golgi complex and by inhibiting transport of vesicles exiting the Golgi and can be used as a specific tool for glycosphingolipid metabolism. Since calcium plays such a fundamental role in transport events and in cytoskeletal assembly the use of the calcium ionophore A23187 to perturb the intracellular calcium concentration will also be useful in this study. The various

inhibitors mentioned above shall be used on primary neuronal cultures to determine what role intracellular transport processes play in the metabolism of glycosphingolipids and to further understand the possible function of these interesting molecules.

CHAPTER II

REVIEW OF RELATED LITERATURE

Gangliosides

Structure and Metabolism

The structure and chemical characterization of gangliosides has been extensively studied (for review see Li and Li, 1982 and Wiegandt, 1982). Gangliosides are amphophilic in nature and consist of a lipophilic ceramide moiety and a hydrophilic carbohydrate moiety. The ceramide moiety is inserted into the outer leaflet of the plasma membrane and the carbohydrate moiety extends outward into the aqueous phase. Biological properties of gangliosides are closely associated with the carbohydrate moiety but the ceramide moiety is also important in orienting the oligosaccharide chain and allowing movement in the fluid phase of the plasma membrane.

Biosynthesis of ceramide is presented in Figure 1. Palmitoyl-CoA reacts with a pyridoxal-P-bound serine to form 3-ketodihydrosphingosine which results in the decarboxylation of serine. The 3-ketodihydrosphingosine is then reduced with NADPH to form dihydrosphingosine. A double bond is formed when a flavoprotein enzyme dehydrogenates dihydrosphingosine to sphingosine. The sphingosine residues of vertebrate nervous system gangliosides have predominantly 18 and 20

carbon atoms which depends on the initial acyl-CoA which condensed with serine. Ratios of C18-sphingosine and C20-sphingosine vary with age, localization, and ganglioside type (Avrova and Zabelinsky, 1971; Yohe et al., 1976). Sphingosine is acylated at the amino group with another acyl-CoA of a long chain fatty acid to form ceramide. This fatty acid can also vary according to age, localization, and ganglioside type but the predominant type (>80%) in mammalian brain gangliosides is stearic acid.

The carbohydrate moiety of gangliosides is attached to the primary hydroxyl of ceramide in straight or branched chains through glycosidic bonds. The most common sugars found in gangliosides are glucose, galactose, N-acetylgalactosamine, and N-acetylneuraminic acid (i.e. sialic acid). Nomenclature of gangliosides is based upon the chemical structure of the carbohydrate moiety. One of the most common systems of naming is that of Svennerholm, 1963. The capital letter "G" symbolizes gangliosides and the capital letters "M", "D", "T", and "Q" represent mono, di, tri, and quatro sialic acid residues, respectively. A "1" refers to the four residue backbone Gal-GalNac-Gal-Glc, the number "2" designates the 3 residue backbone GalNac-Gal-Glc, and the number "3" represents the 2 residue backbone Gal-Glc. The "a" and "b" notation refers to the position of the sialic acid residues when necessary. GD1a and GD1b both have two sialic acid residues but the residues are in a different configuration on each. An "a" or "b"

designation is given if there is one or two sialic acid(s) attached to the proximal galactose, respectively (the galactose closest to ceramide is referred to as the proximal galactose and the galactose farthest away is called the distal galactose).

Biosynthesis of gangliosides is represented in Figure 2 (for review see Fishman and Brady, 1976 and Wiegandt, 1982). Specific glycosyltransferases catalyze the transfer of sugars from activated sugar donors to ceramide or a growing oligosaccharide chain. The activated sugar substrates are UDP-glucose, UDP-galactose, UDP-N-acetylgalactosamine, and CMP-sialic acid. These glycosyltransferases show a high degree of specificity for the sugar and lipid substrates. Glucosyltransferase catalyzes the transfer of glucose from UDP-glucose to ceramide to form glucosylceramide (Basu et al.,1968 and 1973). The formation of lactosylceramide (LacCer) from UDP-galactose and GlcCer by lactosyltransferase has been shown (Basu et al., 1968). LacCer:sialyltransferase has been demonstrated to catalyze the transfer of sialic acid from CMP-sialic acid to lactosylceramide to form the ganglioside GM3 (for review see Samuelsson, 1987). Sialic acid is bound only to galactose or to another sialic acid residue. The addition of one or more sialic acid(s) distinguishes gangliosides from other glycosphingolipids. The biosynthetic pathway bifurcates at GM3 to follow two distinct proposed routes (the "a" series and the "b" series). GM3 is converted to GM2 by

N-acetylgalactosaminyltransferase which transfers a N-acetylgalactosamine from UDP-N-acetylgalactosamine (Steigerwald et al., 1975). N-acetylgalactosaminyltranferase is thought to regulate the synthesis of the more complex gangliosides (Fishman and Brady, 1976). GM1 can be formed from GM2 by the transfer of a galactose from UDP-galactose by a galactosyltransferase (Basu et al., 1965). The formation of GD1a from GM1 requires the transfer of a sialic acid from CMP-sialic to GM1 by a sialyltransferase (Kaufman et al., 1968). The formation of the trisialoganglioside GT1a from GD1a is catalyzed by a sialotransferase which transfers a sialic acid from CMP-sialic to give a disialo residue on the terminal galactose (Yohe, 1980). Synthesis of GD3 which begins the "b" series has been demonstrated (Kaufman, 1968). A sialyltransferase catalyzes the transfer of sialic acid from CMP-sialic acid to GM3 to form GD3. A GalNac (from UDP-GalNac) is then added to GD3 by a N-acetylgalactoseaminyltransferase to produce GD2 (Cumar et al., 1971). Synthesis of GD1b from GD2 and UDP-Gal by a galactosyltransferase has been described (Cumar et al., 1972). The structure of GM3 is given in Figure 3 to show the most common linkages found in gangliosides. Linkages shown in Figure 3 indicate that sialic acid is often found in an α glycosidic bond (several other linkages are known to exist) and the other sugars are usually in a ß glycosidic bond. The linkage designation for the four sugar backbone found in several of the gangliosides is Gal(ß1→3)-

GalNac(ß1→4)Gal(ß1→4)Glc(ß1→)Cer. When sialic acid is bound to a galactose the linkage is (α2→3) and when bound to another sialic acid is often a (α2→8) linkage but other linkages do exist. The transfer of sialic acid from CMP-sialic acid to GD1b to form GT1b is catalyzed by a sialyltransferase (Mestrallet et al., 1977). A tetrasialoganglioside, GQ1b, can be synthesized from GT1b and CMP-sialic acid by another sialyltransferase (Yohe et al., 1982). Higher polysialogangliosides have been identified but their enzymatic synthesis has not been demonstrated. An ordered precursor-product relationship provides good evidence for the pathway shown in Figure 2. There has been some speculation that multiglycosyltranferase complexes may exist (Roseman, (1970) and Landa et. al. (1977). These multienzyme complexes would contain two or more transferases and the final product would be released after the initial substrate was acted upon by each of the transferases in sequence. There has been some speculation that each ganglioside would require a separate multienzyme complex. Anabolism of gangliosides is thought to primarily reside in the Golgi complex (Yusuf et. al. (1984) and Landa et. al. (1979)) because studies have determined that several of these glycosyltransferases are primarily localized in the Golgi complex (Keenan, T. W., et. al. (1974); Wilkinson, F.E. et. al. (1976); Richardson, C. L., et. al. (1977); Eppler C.M., (1980); and Senn H.J. (1981 and 1983). Most of these glycosyltransferase studies were done on sub-

fractionations of rat liver (because of the difficulty of obtaining a Golgi subfraction from brain) so it is debatable if these results equate to the nervous system.

There is also evidence for ganglioside enzyme activities (sialidase-sialosyltransferase) in the outer leaflet of the plasma membrane that function to modify existing gangliosides (Schengrund C.L. and Rosenberg, A. (1970); Leskawa, K. and Rosenberg, A. (1981); Preti, A (1980). These surface enzymes could regulate the local environment and alter various synaptic events.

Degradation of gangliosides also occurs systematically (Tettamanti, 1984). Each sugar moiety is removed from the non-reducing terminus of the ganglioside by specific glycohydrolases starting with the terminal sugar. Ceramidase can then cleave ceramide to form sphingosine and fatty acid. These degradative enzymes are localized in the plasma membrane and in lysosomes. Interest in these degradative enzymes was driven by the fact that several diseases have been identified which result from a deficiency (loss of activity) of a particular glycohydrolase (Wiegandt, (1982); Kanfer, J.N. (1983)). Gaucher disease is autosomal recessive and is caused by a structural mutation of the lysosomal enzyme glucosylceramide β-glucosidase. This defect results in an accumulation of glucosylceramide in particular organs and can cause severe pathological changes. Tay-Sachs disease results from the inability to cleave N-acetyl-galactosamine from GM2 because of

a deficiency in hexosaminidase. Several other enzyme deficiencies have also been discovered which involves a loss of lysosomal degradative activities and can lead to neurological damage.

One exception to this is an identified loss of activity in an anabolic enzyme which resulted in the death of the patient. This anabolic enzyme is UDP-GalNac : GM3 N-acetylgalactosaminyl transferase which produces GM2 from GM3. The patient lost all higher gangliosides and only GM3 and GD3 were detectable. This GM3 gangliosidosis is the reverse of Tay-Sachs disease. The severe problems which can result from these changes in activities imply an important function for gangliosides.

Gangliosides exhibit distribution patterns which are species and tissue specific (Nagi and Iwamori, 1984). Gangliosides occur in all mammalian tissues studied. The greatest degree of complexity and the highest concentration of gangliosides is seen in the nervous system (particularly the gray matter) which suggests an important physiological role. There are also regional differences in ganglioside patterns found in the brain (Yates, 1986). In neurons there are higher concentrations of gangliosides found in the synaptosomal fractions than in the perikarya (Morgan, et. al. (1976); Burton, (1976); and Leskawa and Rosenberg, (1981)).

Changes occur in the ganglioside pattern during nervous system development in chick, rat, mouse, and human (Dreyfus, et. al. 1980 and Yates, 1986). These changes in ganglioside pattern appear to correspond to changes in cell division, synaptogenesis, myelination, and neuronal differentiation. In human and rat there is a significant increase in ganglioside content and there are changes in ganglioside pattern during development. Chick primary neurons display all the gangliosides associated with adult nervous system tissue. There is a large increase in ganglioside concentration and a significant changes in the ganglioside pattern during cell maturation of chick primary neurons. GD3 is the major ganglioside in chick primary cultures during cell division (days 1-3) but decreases during synapse formation and differentiation (days 3 and 4) while GD1a increases during synapse formation. This change in the ratio of GD3 to GD1a also is found during development of the retina and brain hemispheres (Dreyfus, et. al. 1975). The similarities between primary neuronal and whole tissue ontogenesis suggest that primary neurons would serve as a good model for the study of ganglioside metabolism.

Neuronal differentiation, contact interactions, trophic properties, and neuritogenesis coincide with changes in the distribution pattern and/or complexity of gangliosides (Ledeen, 1984 and Dreyfus, 1978). Gangliosides have been reported to cause neuritogenesis when added exogenously in neuronal cell lines and in primary neurons (Leskawa and Hogan,

1985 and Byrne, et. al. 1983). PC12 cells have increased neuritogenesis after ganglioside addition but only in the presence of nerve growth factor suggesting a modulation affect on neurotrophic factors (Matta, 1986). The possibility of using gangliosides as regenerative agents for nervous system lesions is being actively pursued. Neurite initiation, growth, branching, adhesion, target recognition, and synaptogenesis may each or may all be involved in the effect of gangliosides on neuritogenesis.

Many biological molecules have been found to interact with gangliosides at the cell surface. The initial discovery of the ability of gangliosides to bind various molecules at the cell surface was the binding of cholera toxin to GM1 (Van Heyningen, 1974). Tetanus toxin was also found to bind ganglioside (Van Heyningen, 1976). Since gangliosides do not normally bind toxins there has been an extensive search for other physiological important molecules which bind gangliosides. Many such molecules have been discovered (Wiegandt, 1982). Thyroid stimulating hormone (TSH) was one of the first effector molecules found to bind gangliosides (Mullin, 1976). The binding of TSH to membranes was inhibited by the addition of gangliosides. Interferon also can bind to ganglioside (Besancon and Ankel, 1974). Gangliosides also act as a possible receptor for serotonin (Ochoa and Bangham, 1976). Many other biologically significant molecules have been identified as binding to gangliosides suggesting the diverse roles

that gangliosides may have at the cell surface. There may also be a modulation effect of gangliosides on surface enzyme activities. GQ1b has been shown to stimulate the activity of ecto-protein kinase (Tsuji, 1988). Several of the glycosphingolipids have been identified as possible cell surface antigenic determinants and so may be important for immune recognition (Hakomori, 1981). Antibodies can be raised against gangliosides.

There is growing evidence to support the role of gangliosides in the biochemical events mentioned above but their actual *in situ* biological role is unknown. Much of the difficulty in establishing a role for gangliosides in the nervous system is the heterogeneous cell population present. One approach to the problem has been to produce clonal cell lines derived from the nervous system which provides a homogeneous cell population and are easy to propagate. It has been suggested that transformed clonal cell lines may not be adequate for studying ganglioside metabolism because of the deficiency of polysialogangliosides and that primary neuronal cultures possess gangliosides patterns which closely resemble tissue cell fractions (Dreyfus *et. al.*, 1978). Transformed cells are devoid of normal growth restraints and are missing the more complex gangliosides (Fishman and Brady, 1976). These higher gangliosides may be important for cell contact inhibitions and transformation may be a direct result of their loss.

Very little is known concerning the intracellular ganglioside biosynthetic pathways and their relationship to the Golgi apparatus in the nervous system and primary neuronal cells will provide an excellent model for studying these complex events. Knowledge of neuronal biosynthesis of gangliosides *in vivo* is limited because of the heterogeneous cell population and the difficulty in isolating Golgi from lipid rich cells (Yusuf *et. al.* 1978). Most studies on anabolism have come from *in vitro* studies on brain microsomes, liver subfractionations, and purified enzyme preparations (for review see Wiegandt 1982). The information obtained from *in vitro* studies has given us a good understanding of the steps involved in ganglioside biosynthesis. Studies *in vivo* are essential to provide us with a clearer picture of ganglioside metabolism and may provide clues as to the function of gangliosides.

Monensin is a carboxylic polyether monovalent cationophore which has been shown to specifically inhibit intracellular transport of several classes of proteins which are processed through the Golgi complex (apparatus) (Tartakoff , 1983a and 1983b). In biological membranes monensin acts as a diffusional carrier which mediates a cation exchange and results in an equilibration of the ionic environment. It is lipophilic and can presumably insert into membranes. Treatment of cells with monensin results in a rapid dilation of the Golgi cisternae and there is no apparent affect on other

intracellular organelles. The endoplasmic reticulum and the transitional elements which are in close association with the Golgi are unaffected. Nothing is known of the ion content of the Golgi so it is difficult to explain why monensin has this unique effect. Tartakoff (1983a) gives two possible explanations on how the swelling may occur.

1. The cisternae may contain a weak acid and passive water influx may occur if protons exchange with Na^+.

2. Ion pumps may control the efflux of water which results in the normal compressed form of the cisternae and any interference with these pumps will allow them to dilate.

Overall protein synthesis, ATP levels, and pH are unchanged after monensin treatment. The action of monensin appears to be a specific inhibitor of Golgi processing events. The Golgi subsite of transport inhibition has been postulated to be somewhere between the distal and proximal compartments of the Golgi complex. Monensin has provided researchers with a good tool for exploring the poorly understood area of protein processing and transport events of the Golgi. Gangliosides are another important class of compounds which are processed within the Golgi (Yusuf et. al. 1984). Monensin has recently been used to study its effect on ganglioside biosynthesis in fibroblasts (Saito et. al. 1984,1985), N18

neuroblastomas, and C6 gliomas (Miller - Prodraza and Fishman, 1984). These studies indicate that initial sites of glycosylation and final sites are distinct and that some type of subcompartmentation exists. Treatment of fibroblasts, N18 neuroblastomas, and C6 gliomas with monensin results in an increase in labeling (with [^{3}H]-Galactose added to the media) and concentration of glucosylceramide. An increase in labeling and concentration of lactosylceramide was seen in fibroblasts but not in N18 and C6 cultures which may indicate cell differences. Other gangliosides and higher neutral glycosphingolipids show a decrease in labeling and no change in concentration. Polysialogangliosides are absent from the N18 neuroblast (Dreyfus et. al. 1978) and the study of Golgi processing events may be altered in these cells. Primary neuronal ganglioside patterns are similar to tissue patterns and therefore may provide a better analysis of ganglioside anabolism.

Microtubules are thought to be important in maintaining the organization of the Golgi complex (Thyberg and Moskalewski, 1985) possibly by maintaining the three dimensional orientation of the cisternal stacks. Microtubules may interact directly with the cisternae by attachment to microtubule associated proteins. Colchicine and vinblastine are two microtubule inhibitors which cause disassembly of microtubules and a reversible disruption of the Golgi complex. Both of these inhibitors caused dilation of the cisternae much like

monensin. The depolymerization of microtubules by colchicine and the crystallization of microtubules by vinblastine can disrupt both Golgi transport and intraneurite transport. Secretion of several compounds (collagen, growth hormone, insulin, lipoproteins, etc.) has been shown to be inhibited by colchicine. Since colchicine and vinblastine act through different mechanisms it would be of interest to compare both inhibitors for their effect on glycosphingolipid transport processes.

Cytochalasins are inhibitors of actin polymerization which interfere with the contractile process of microfilaments (Goddett and Frieden, 1986) but are not thought to interfere with intracellular transport. The addition of actin subunits at the growing end of microfilaments is inhibited. The retraction of neurites in PC12 cells is prevented by cytochalasin D (Joshi, et. al. 1985) which actually appears to stabilize the neurite. It will be interesting to compare this microfilament inhibitor with the microtubule inhibitors mentioned above.

To determine if calcium plays a role in glycosphingolipid metabolism the calcium ionophore A23187 can be utilized. A23187 is an antibiotic which can bind divalent cations and transport them across membranes (Reed and Lardy, 1972). Divalent cations are important in formation of microtubules and microfilaments and perturbation of calcium concentrations by the ionophore A23187 will disrupt this formation and thus

may interfere with Golgi transport and intracellular transport.

Cobalt is a calcium antagonist which has been shown to interfere with the initial passage of new proteins from the endoplasmic reticulum to the Golgi complex and also to interfere with protein transport at a stage after the proteins have left the Golgi but before the proteins have entered the axon (Stone and Hammerschlag, 1981 and 1987). Sorting and vesicular delivery of proteins which are exiting the Golgi is thought to involve the trans-Golgi network (also referred to as GERL: Golgi-Endoplasmic-Reticulum-Lysosome)(Griffiths and Simons, 1986). The action of cobalt is thought to be due to the inhibition of the calcium mediated fusion of the transitional vesicles with the cis Golgi surface and to inhibit the vesicles exiting the Golgi by preventing fusion with transport organelles in the trans-Golgi network. Monensin and cobalt both interrupt intracellular transport of proteins but at different sites.

SPECIFIC AIMS

Ganglioside metabolism in vivo is poorly understood. There is good evidence that gangliosides synthetases are localized in the Golgi but how these synthetases are arranged and in what compartments gangliosides are synthesized is unknown. The aim of this research is to clarify the complex area of ganglioside biosynthetic processing in primary neurons. Various inhibitors are known to inhibit Golgi processing events. This research will compare the effects that monensin, colchicine, vinblastine, cytochalasin D, A23187 and cobalt have on neuronal development, Golgi function, and ganglioside biosynthesis in cultures of primary neurons. Hopefully by examining the in vivo processing of these gangliosides not just the biosynthetic pathways will be elucidated but a function for gangliosides may also be found.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

Eggs were obtained from Sharp sales (West Chicago, Il.) or Hall Brothers hatchery (Wallingford, Conn.) and were incubated at 38.5^{0}C in a humid atmosphere and turned automatically every 3 hours. Plastic petri dishes, Dulbecco's modified Eagles media (DMEM), Fetal Bovine Serum (FBS), penicillin (5000 U/ml) - streptomycin (5000 mcg/ml) (PS), and phosphate buffered saline (PBS) were obtained from Gibco. 41 µ nylon mesh (Spectramesh) and HPLC quality solvents were obtained from Fisher Scientific. Insulin 6.25 µg/ml- transferrin 6.25 µg/ml- selenium 6.25 ng/ml- bovine serum albumin 1.25 mg/ml- linoleic acid 5.35 µg/ml (ITS+) was obtained from Collaborative Research. Unisil (activated silicic acid 100-200 mesh) was obtained from Clarkson Chemical Company. Dialysis membrane was obtained from Spectrum Medical (Spectrapor MW cutoff 12,000-14,000). High performance silica gel 60 thin layer chromatography plates (HPTLC-10 X 20 cm) were obtained from E. Merck. Bovine brain gangliosides and ceramides from bovine brain sphingomyelin were obtained from Sigma. Purified GM1, GD1a, and GT1 were obtained from Supelco. DL-dihydrolactocerebroside, DL-dihydroglucocerebroside, and DL-dihydrogalactocerebroside were obtained from Calbiochem. Resorcinol and cupric sulfate were obtained from

Sigma and resorcinol spray was prepared fresh weekly (1.0 ml of 6% resorcinol in water (was stored at 4^0C and protected from light) + 8.0 ml concentrated HCl + 25 µl 0.1 M cupric sulfate + 1.0 ml water. Anthrone and thiourea were obtained from Sigma and anthrone spray was prepared fresh weekly (66 ml 95% sulfuric acid + 34 ml water + 0.5 g anthrone + 10 g thiourea (was stored at 4^0C and protected from light). P-10 gas (10% methane and 90% argon was obtained from Matheson Gas. Monensin and nigericin were obtained from Calbiochem Behring. Colchicine, vinblastine, cytochalasin D, A23187, and cobalt ($CoCl_2$-$6H_2O$) were obtained from Sigma.

METHODS

Primary Neuronal Cultures

Pure primary neurons were prepared from the telencephalon of 7 or 8 day chick embryos (Pettman et. al.). Plain plastic petri dishes were coated with poly-L-lysine by treating each dish with sterile filtered poly-L-lysine (15 mg/liter in boric acid 0.1 M pH 8.4 - 10 ml per 100 mm dish) and allowed to sit overnight. The polylysine solution was removed and each dish was rinsed once in phosphate buffered saline (10 ml per 100 mm dish). DMEM containing 15% FBS and 2% PS was then added to each dish (9 ml per 100 mm dish) and the dishes were placed in the incubator at 37^0C with 5% CO_2 - 95% air. All tools were sterilized with 70% EtOH and flamed before the removal of each embryo from its egg. All eggs were cleaned before beginning. All tissue culture work was performed under a laminar flow hood and standard sterilization techniques were used. 7 or 8 day old eggs were rinsed with 70% EtOH and the blunt end was chipped out with sterile forceps. The forceps were resterilized and the chorioallantoic membrane was peeled away. Forceps were again sterilized and the embryo was removed by grasping under the head. The embryo was gently placed in a 100 mm petri dish containing 10 ml DMEM. With sterile forceps the two telencephalon lobes were dissected out and any attached meninges

were removed. The lobes were placed in DMEM - 2% PS. Lobes were gently drawn into a 10 ml pipet and the lobes settled quickly into the tip and were blown out into 10 ml of media. The lobes settled quickly into the bottom of the tube and the overlaying media was immediately removed. Lobes were dissociated by passing them back and forth through the tip of a pipet several times until they were well dispersed. The dissociated cells were passed through a sterile 41 μ nylon mesh which was placed over the end of a glass tube (7 cm long and 14 mm internal diameter) and sterilized (the tube was dipped in 70% EtOH, sonicated for 5 minutes and then placed in a petri dish under the tissue culture hood to air dry before use). The mesh was rinsed with an additional 5 ml media. The cells were diluted and counted in a hemacytometer. Cell counting and viability was determined by trypan blue exclusion (Malinin and Perry, 1967). Cells were added to prewarmed media (6 X 10^6 cells per 100 mm dish). The cultures were incubated at 37^0C with 5% CO_2 - 95% air in a humidified atmosphere. After 3 or 4 days the media was changed and replaced with DMEM - 15% FBS - 2% PS or with defined media (DMEM - ITS+ - 2% PS).

Glycosphingolipid Isolation

Glycosphingolipids were extracted and analyzed (Saito et. al. 1984 and 1985). All solvents used were of HPLC grade. Incubation of the cultures was stopped by placing the dishes on ice. The media was then removed and cold phosphate buffered saline (PBS pH 7.4) was added to each dish. Cells were gently scrapped off with a rubber policeman and were placed in a 13 X 100 mm test tube which was on ice. A second aliquot of cold PBS was added to each dish. The dish was scrapped again to ensure removal of most of the cells and the cells were added to the first scraping. Cells were pelleted in a refrigerated centrifuge (1500 rpm for 5 minutes) and the supernatant was discarded. The cells were twice more resuspended and repelleted. The cell pellet was extracted immediately or frozen at -80^0C for later analysis. Total lipids were isolated from the cells by sequential extractions with 1 ml $CHCl_3$ - MeOH 2:1 (v:v), 1 ml $CHCl_3$ - MeOH 1:2 (v:v), and 1 ml $CHCL_3$ - MeOH 2:1 (v:v). After each addition of solvent the cells were sonicated and allowed to sit for 5 minutes and then centrifuged at 3000 rpm for 10 minutes and the supernatant from the three extractions was combined.

The total lipids and the remaining protein pellet were dried (evaporation of the solvent with nitrogen or the Savant centrifugal evaporator). The protein pellet was saved for protein analysis (Hess et. al. 1978).

Total lipids were separated into neutral lipids and gangliosides by silicic acid chromatography (Saito et. al., 1984 and 1985). Silicic acid columns were prepared by placing a small plug of glass wool in a small pasteur pipet and adding silicic acid to a depth of 2 cm. The total lipids were dissolved in 1.0 ml $CHCl_3$-MeOH 2:1 (v:v), sonicated, allowed to sit for 10 minutes, and then applied to the silicic acid column. Another 0.5 ml $CHCl_3$ - MeOH 2:1 (v:v) was added to each test tube in order to rinse it and then was also applied to the column. An additional 1.5 ml $CHCl_3$ - MeOH 2:1 (v:v) was used to wash the column. The eluant contained the neutral lipids. Each column was then placed over a second test tube and washed with 2.0 ml $CHCl_3$ - MeOH - H_2O 50:50:15 (v:v:v). The eluant, which contained the ganglioside fraction, was collected. The neutral lipid and ganglioside fractions were then dried.

Neutral glycolipids were separated from the neutral lipids with another silicic acid column (Saito et. al., 1984,1985). The neutral lipids were dissolved in 0.5 ml $CHCl_3$, sonicated, allowed to sit 5 minutes, and then layered on top of the column. Another 0.5 ml $CHCl_3$ was used to rinse the test tube and this was also applied to the column. The column was washed with an additional 1.0 ml $CHCl_3$ and the eluant was discarded. 3 ml acetone - MeOH 9:1 (v:v) was added to the column and this fraction which contains the neutral glycolipids was collected and dried. To each dried neutral

glycolipid and ganglioside fraction 150 µl H_2O was added and then sonicated and dialyzed overnight by attaching a dialysis membrane over the open end of the test tube and tightly fastening it with a rubber band. To dialyze, the tubes were inverted and placed in distilled water for 24 hours. The dialyzed samples were centrifuged at 1500 rpm for 5 minutes and then lyophilized (the dialysis membrane was removed and replaced with lens paper).

GLYCOSPHINGOLIPID THIN LAYER CHROMATOGRAPHY

Purified glycosphingolipid fractions were separated by high performance thin layer chromatography (HPTLC). Each sample or standard was dissolved in 20 µl $CHCl_3$ - MeOH 2:1 (v:v), sonicated, and applied with a glass capillary (was prepared by flaming a capillary and drawing it out until a very fine tip formed) as a thin streak (1mm thick x 1cm wide) 1 cm from the bottom of a HPTLC plate. Each sample tube was rinsed with another 20 µl $CHCl_3$ - MeOH 2:1 (v:v) and this was also applied. Ganglioside standards were applied to each plate for ganglioside analysis. Neutral glycolipid standards were applied to each plate for neutral glycolipid analysis. The developing solvent for separation of gangliosides was $CHCl_3$ - MeOH - 0.25% $CaCl_2$ 54:42:10 (v:v:v) and for neutral glycolipids was $CHCl_3$ - MeOH - H_2O 65:25:4 (v:v:v). The TLC development tank was equilibrated for at least 2.0 hours with 50 ml developing solvent (a filter paper saturation pad was placed in the tank to provide for a more even equilibration). The plates were placed in the tank and the solvent was allowed to rise to the top of the plate. The plate was removed and air dried. Separated bands were transiently marked with iodine vapor to determine position. Gangliosides were more permanently stained with resorcinol spray which splits off the sialic acid groups and then reacts with sialic acid to give a quantitative response. The plate was sprayed with resorcinol.

A clean cover glass was then clamped over the HPTLC plate and heated at 120^{0}C for 20 minutes (the silica layer was face down when heating). Neutral glycolipids were sprayed with anthrone reagent and heated at 140^{0}C for 15 minutes to give a quantitative response. Quantitation of the individual bands was determined by densitometric scanning. Gangliosides were scanned at 580 nm and neutral glycolipids at 625 nm using a Kratos model SD 3000 spectrodensitometer attached to an Hewlett-Packard model 3390A integrator. Standard linear plots were established for the standards (µg standard versus area under the densitometric peak) and the amount of unknown sample glycosphingolipid was determined from the plot. Identification of each individual sample band was determined by comparison to the retention time of the purified standards.

CERAMIDE ANALYSIS

Total lipids were isolated as previously stated. The lipids were dissolved in $CHCl_3$ - MeOH 9:1 (v:v), sonicated, allowed to sit for 10 minutes, and then applied to a silicic acid column (prepared as previously stated). The ceramide fraction was then eluted with three washes of 2.0 ml $CHCl_3$ - MeOH 9:1 (v:v). Samples were applied to a HPTLC plate along with ceramide standards. The samples were developed in $CHCl_3$ - MeOH - water 60:35:8 (v:v:v) until the solvent front reached 4.0 cm from the bottom of the plate (Selvam and Radin, 1981). The plate was removed and the solvent was evaporated from the plate in a vacuum desiccator for 20 minutes. The plate was chromatographed again with $CHCl_3$ - MeOH - acetic acid 90:2:8 (v:v:v) until the solvent front was 1.0 cm from the top of the plate then the plate was removed. Solvent was evaporated in a vacuum desiccator for 30 minutes. The lipids were visualized by charring the plate with 3% cupric acetate in 8% phosphoric acid (w:v) and heating at 180^0C for 15 minutes (Fewster et. al.,1969). Densitometric scanning was done as previously stated (350 nm).

RADIOACTIVE LABELING OF GLYCOSPHINGOLIPIDS

Gangliosides and neutral glycolipids were labeled with D-[1-^{3}H]-Galactose (1.0 mCi/1.0 ml ethanol:water 9:1 ; 11.5 Ci/mmole-American Radiolabeled Chemicals). D-[1-^{14}C]-N-Acetyl-mannosamine (0.1 Mci/1.0 ml ethanol:water 9:1 ; 55mCi/mmole-New England Nuclear) was also used to label gangliosides. [^{14}C]-Acetate (50 Mci/Mm) (American Radiolabeled Chemicals) was used to label ceramide. To each 100 mm dish, 10 µCi of [^{3}H]-galactose, 1.0 µCi [^{14}C]-N-acetyl-mannosamine, or 10 µCi [^{14}C]-acetate was added. The final concentration of ethanol (0.18%) present in treated and control cells was the same. The amount of label incorporated into each glycosphingolipid was determined by scrapping each individual band after HPTLC chromatography and quantitating by liquid scintillation. Each band was scraped and placed in a 7 ml scintillation vial and 0.5 ml water was added. The vial was sonicated and 5.0 ml Aquasol was then added and the vial was vortexed.

The labeling pattern was also observed using a radioactive thin layer chromatography scanner (RTLC - Radiomatic Instruments, Tampa, Fl) which is comprised of a position sensitive proportional counter and a multichannel analyzer. A TLC plate was placed very close to the analyzer and an inert gas (P-10) was injected between the plate and the analyzer. The analyzer detected the β-particles along an entire TLC lane and bands of greater than 1.0 mm were resolved and the results

were automatically analyzed by computer. Scans were usually run for 10 mins.

AUTORADIOGRAPHY

To detect radioactivity on TLC plates, they were first sprayed with ENHANCE (New England Nuclear). The plates were then placed on top of X-omat autoradiography film (Kodak Company) and exposed for one week at $-80^{0}C$.

INHIBITORS OF INTRACELLULAR TRANSPORT

All inhibitors used in this study were dissolved in pure ethanol except for cobalt ($CoCl_2$-$6H_2O$) which was dissolved in water. The concentrations of inhibitors used are presented in the results section. Whenever a drug was dissolved in ethanol an equal amount of ethanol was added to the control. The concentrations of inhibitors used was determined form dosages recommended in the literature.

MICROSCOPY/PHOTOGRAPHY

Microscopy was done with an inverted Olympus microscope (phase contrast) hooked up to an Olympus automatic exposure adapter. All photographs presented in this dissertation were done at a magnification of 100 X with the exposure set at 1 and reciprocity set at 2. Any glial cells present were identified by their characteristic flat polygonal shape. Most observable cells were rounded and extended several neurites which is characteristic of neurons. Many of these extended neurites had observable growth cones and many were touching other cells which is also characteristic of neurons.

DATA ANALYSIS

All experiments were performed on at least triplicate samples. The standard deviation was determined for all graphs and tables. Each experiment was repeated at least three times and similar results were obtained for all reported data. The monensin and the nigericin data were repeated 5 times. Statistical analysis was performed on all data points using the Student t test (unpaired, two tailed). The P values were calculated for all data points and given in figure legends.

CHAPTER IV

RESULTS

Dissociated neuronal cells prepared from 7 or 8 day chick embryonic telencephalon attached to poly-L-lysine coated dishes within 15 minutes of plating and were well dispersed. After 24 hours, most neurons displayed short unipolar, bipolar, or multipolar processes and remained dispersed. The cells began to form small aggregates after 48 hours and neurites could be observed contacting other cells. On the 3rd or 4th day the media was changed to a defined media without serum so that cells were not subjected to the variability of serum lots or to the presence of proteins and lipids which may interfere with analysis of the cells. At 6 days, in culture cell number was stabilized and there was a fine neuritic network with some of the neurites displaying fasciculation. A few of the neurites could be found suspended above the surface of the dish between two small aggregates. An initial cell concentration of $6x10^6$ per 100 mm petri dish was found to provide the best dispersion of cells on the dish so that large aggregates did not form. This allowed easy access for any drug treatment and also provided enough cells for lipid analysis. The purity of the neuronal cultures was high (>98 %) since there were very few observable glial cells at 6 days in culture. Glial cells were flat and polygonal in shape. Neurons had long thin neurites extending from the cell body.

Many of these neurites exhibited growth cones. Most of the neurites were extended and touched other cells. A majority of the cells, therefore, exhibited classical neuronal morphometry. Viability of the cultures remained >95 % as determined by trypan blue exclusion during all testing procedures. These 6 day old pure, viable, and cytodifferentiated neurons were used as standard conditions for all studies (Figure 6). The concentrations of all inhibitors used in this study were recommended in the literature as being effective.

Gangliosides, neutral glycolipids, and ceramide were extracted from the primary neurons by silicic acid chromatography as described in Methods. A new solvent system ($CHCl_3$-MeOH-0.25 % $CaCl_2$ 54:42:10 (v:v:v)) was developed to provide optimal high performance thin layer chromatographic (HPTLC) separation of the more complex gangliosides found in neurons. The resorcinol staining pattern of primary neuronal gangliosides separated by HPTLC is shown in Figure 7. All gangliosides were well resolved. Ganglioside standards showed linear standard curves. The gangliosides GD3 and GM2 were present as a doublet which was caused by differences in the long chain fatty acids. There is an unidentified resorcinol positive band just below GQ1b which may be a higher sialylated ganglioside so the band was given the designation GPX (G=ganglioside, P=polysialo, X=unknown). The GD2 band might contain small amounts of GT1a and GT3 because they comigrate in this region and could not be separated.

The densitometric scan of Figure 7 is shown in Figure 8. Integration of the densitometric scan and quantitation of the individual ganglioside species is presented in Table 1. Peak area was determined by cutting out each peak and weighing. Every ganglioside was quantitated by comparing each to ganglioside standards. The ganglioside standard graphs were linear for the concentrations studied.

TABLE 1

Quantitation of gangliosides present in primary neurons cultured for 6 days in culture.

GM3	1.6 ± 0.4
GM2	0.7 ± 0.2
GM1	0.7 ± 0.2
GD3	4.5 ± 1.1
GD1a	1.7 ± 0.2
GD2	1.9 ± 0.4
GD1b	0.6 ± 0.1
GT1b	12.2 ± 1.1
GQ1b	9.1 ± 2.3
GPX	8.8 ± 1.9

Ganglioside μg/mg Cellular Protein (n=3, ± SD)

The HPTLC separation of neutral glycolipids is shown in Figure 9. Glucosylceramide (Glucer) and lactosylceramide (Laccer) the ganglioside precursors were well separated from the other neutral glycolipids. Both Glucer and Laccer were found in low concentration and several dishes of cells were combined to provide enough neutral glycolipid for HPTLC analysis (lane 1). Laccer was difficult to see even after combining several plates and so could not be accurately quantitated. An unidentified band (probably trihexosylceramide-CTH) was also present in primary neurons. Near the solvent front was a band containing various phospholipids.

Ceramide, the precursor to glucosylceramide, was also analyzed by thin layer chromatography (Figure 10). There was a nice separation of ceramide from cholesterol, triglycerides and fatty acids, cholesterol esters, and the phospholipids.

RADIOACTIVE LABELING OF GLYCOSPHINGOLIPIDS

Gangliosides were labeled with [^{3}H]-galactose in order to analyze new biosynthesis. Incorporation of [^{3}H]-galactose into gangliosides was assayed by first separating the gangliosides by HPTLC then directly measuring the radioactivity in each band by using a radiochromatographic TLC scanner (Figure 11) or by autoradiography (Figure 12) or by scraping individual bands and determining the level of radioactivity using a liquid scintillation counter (Figure 16-see control). RTLC scans and autoradiograms were used only for qualitative analysis but are important in confirming the quantitative assessment by scraping. The autoradiogram is also important for determining the position of labeled bands. Quantitation was established by scraping individual bands separated by HPTLC (Figure 16-control). The highest incorporation of label was found to be in the ganglioside GD3 (both lower and upper bands of GD3 were combined). There was also significant uptake of label into GD1a, GD2, GT1b, GM3, and GQ1b. Lower amounts of label were found in GM1, GM2, GD1b and GPX. When you look at the specific radioactivity of each ganglioside, the higher gangliotetraosyl gangliosides (GT1b, GQ1b, and GPX) have a much lower specific activity then the other gangliosides (Figure 13-control). The specific activities of GD1b, GD2, GD1a, GD3, GM1, and GM3 were similar.

Neutral glycolipids were also examined for incorporation of [^{3}H]-galactose after incubation for 16 hours with the label. The radioactive TLC scan is shown in Figure 14. There was significant labeling of glucosylceramide, lactosylceramide, and trihexosylceramide. Glucosylceramide incorporated the most label. Scraping of the individual bands yielded the following results (Table 2).

Table 2

Incorporation of [^{3}H]-galactose into neutral glycolipids of primary neurons 16 hours after addition of label.

GLUCOSYLCERAMIDE	555 ± 80
LACTOSYLCERAMIDE	80 ± 10

(cpm/mg protein)(n=4, ± SD)

MONENSIN TREATMENT

Cultures of primary neurons were treated with monensin and were observed for any morphological changes (Figure 15). When compared next to a control culture it becomes evident that there was a significant reduction in the neuritic network after 18 hour treatment with 100 nM monensin. Neurite retraction began to occur after about 8 hours. Cells maintained greater than 95% viability for up to 25 hours treatment with monensin (100 nM). On close examination it could be seen that it was the fine neurites and the branching neurites which appeared to be most affected and that the larger longer neurites remained. Many of these longer neurites appeared to end in growth cones or to extend between two small aggregates.

The individual ganglioside concentrations after monensin treatment for 15 hours, compared to control, is shown in Table 3. There were no significant changes in the total ganglioside concentration after treatment with monensin. The loss of neurites does not effect the concentration of gangliosides present. The bulk of the gangliosides may be in the large longer neurites which remain after monensin treatment and this may support the evidence that gangliosides were found in highest concentration in nerve endings.

Table 3

Quantitation of gangliosides in primary neurons which have been treated with or without monensin.

GANGLIOSIDE	CONTROL	MONENSIN 0.1 μM
GM3	1.6 ± 0.4	1.7 ± 0.1
GM2	0.7 ± 0.2	0.7 ± 0.1
GM1	0.7 ± 0.2	0.8 ± 0.1
GD3	4.5 ± 1.1	4.5 ± 0.1
GD1a	1.7 ± 0.2	1.9 ± 0.3
GD2	1.9 ± 0.4	1.4 ± 0.2
GD1b	0.6 ± 0.1	0.6 ± 0.0
GT1b	12.2 ± 1.1	10.5 ± 1.1
GQ1b	9.1 ± 2.3	8.7 ± 0.8
GPX	8.8 ± 1.9	8.2 ± 0.5

μg ganglioside/mg total protein (n=3, ± SD)

No significant difference was found between control and monensin treated cultures.

Neuronal cultures were incubated with [^{3}H]-galactose and with or without (control) 100 nM monensin for 15 hours. Gangliosides were then extracted and the amount of label incorporated into individual gangliosides was determined (Figure 13-CPM/µg ganglioside or Figure 16-CPM/mg protein). Incubation of cells with monensin resulted in a selective decrease in labeling of individual gangliosides (Figure 16). The labeling of GM3 after monensin treatment was unaffected while all the other gangliosides had reduced labeling but to varying extents. Labeling of GT1b was reduced almost 80 % and GPX about 74% after monensin treatment. Monensin also significantly reduced the labeling for GD1a (69%), GD1b (66%), GM1 (52%), and GQ1b (52%). Labeling was reduced to a lesser extent in GD3 (40%) and GM2 (39%).

The rate of incorporation was examined by labeling ([^{3}H-galactose) of the gangliosides over an 18 hour time span (Figures 17 to 26). The uptake of label into individual gangliosides was found to exhibit a slow initial phase of label incorporation for about 4 to 6 hours. This slower phase was followed by a faster incorporation of label which leveled off after about 12-15 hours. Label was incorporated most rapidly into GD3 (Figure 23) but there was also significant labeling of GD1a (Figure 22); GD2 (Figure 21); GT1b (Figure 19); GQ1b (Figure 18); and GM3 (Figure 26). Lower levels of labeling were found for GD1b (Figure 20); GPX (Figure 17); GM1 (Figure 24); and GM2 (Figure 25). GM3 was the only ganglio-

side which showed no difference in the rate of incorporation of label in monensin treated cultures as compared to controls from 9 up to 18 hours treatment (Figure 26). There was a small decrease in labeling of GM3 after 3 and 6 hours treatment with monensin. In contrast the labeling of GT1b (Figure 19) was the ganglioside most significantly inhibited by monensin over time. Next to GM3, the ganglioside GD3 (Figure 23) had the least inhibition of the incorporation of label over all time points. The time courses for the incorporation of label into GD1a (Figure 22); GD2 (Figure 21); GQ1b (Figure 18); GD1b (Figure 20); and GPX (Figure 17) also showed significant reduction in labeling after treatment with monensin. Gangliosides GM1 (Figure 24) and GM2 (Figure 25) had intermediate inhibition of labeling when monensin was added.

Primary neurons were also treated with nigericin which has a close structural relationship to monensin. Neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 µM nigericin (Figure 51). The inhibition of the incorporation of label by nigericin was similar to that of monensin. Label incorporation into GM3 was unchanged after treatment with nigericin. Incorporation of label into GD3 was reduced about 40 % by nigericin. The gangliosides (GPX, GQ1b, GT1b, GD2, GD1b, GD1a, and GM1,) all had decreased labeling when nigericin was added.

Neutral glycolipids were also examined for incorporation of [^{3}H]-galactose with and without 0.1 μM monensin or nigericin added for 16 hours (Table 4).

TABLE 4

Effect of monensin and nigericin on the incorporation of [^{3}H]-galactose into neutral glycolipids of primary neurons after treatment for 16 hours.

	LACTOSYLCERAMIDE	GLUCOSYLCERAMIDE
CONTROL	80 ± 10	555 ± 80
MONENSIN 0.1μM	157 ± 13[a]	545 ± 65[b]
NIGERICIN 0.1μM	127 ± 1[a]	520 ± 64[b]

CPM/mg Protein (n=3, ± SD)([a]$p<.01$, [b]not significant)

Both monensin and nigericin caused almost a doubling in labeling of lactosylceramide. Glucosylceramide labeling in the presence of monensin or nigericin was not significantly affected.

Incorporation of [^{3}H]-galactose into neutral glycolipids was examined over a 24 hour period (Figure 27 and 28). Labeling of glucosylceramide (Figure 28) was very fast and no initial lag phase was observed but the labeling slowed

after 8 hours. Initial labeling of lactosylceramide (Figure 27) was slower for up to 4 hours and then increased. Monensin (0.1 μM) treatment resulted in a significant increase of lactosylceramide labeling over time. There was no inhibition of labeling of lactosylceramide up to 4 hours but after 8 hours treatment with monensin there was an increase in the incorporation of label. At longer time points (20 and 24 hours) there was a significant increase in labeling of lactosylceramide caused by monensin (Figure 27). There was no significant change in labeling of glucosylceramide caused by monensin treatment (Figure 28).

The precursor ceramide was analyzed by labeling primary neurons with [^{14}C]-acetate and incubating the cells in the presence or absence of 0.1 μM monensin (Figure 29). Monensin had no effect on the incorporation of label into ceramide when compared to control. When the ceramide band was scraped, and the amount of label incorporated was analyzed, the results confirmed the RTLC scan (Table 5).

TABLE 5

Effect of monensin on the incorporation of [^{14}C]-acetate into ceramide of primary neurons after the addition of label for 16 hours.

CONTROL	1943 ± 318
MONENSIN (0.1 μM)	1835 ± 259

(cpm/mg protein)(n=4, ± SD)

No significant difference was found between control and monensin treated cultures.

To further observe the effect that monensin has on ganglioside metabolism and to specifically label only gangliosides, the label D-[1-^{14}C]-N-acetyl-mannosamine was utilized. N-acetyl-mannosamine is the precursor to sialic acid and so will only label sialic acid groups in the gangliosides. Neutral glycolipids will not become labeled. This label was added with or without monensin (0.1 μM) and the incorporation was examined at 5, 10, and 15 hours (Figures 30 to 39). Label incorporation into most of these gangliosides is represented by a sigmoidal curve. Gangliosides with low label incorporation had an initial lag phase of incorporation and then a faster phase before leveling off (GM1, GM2, and GM3). When there was a high level of incorporation there was an initial fast incorporation of label followed by a leveling

off before increasing again (GPX, GQ1b, GT1b, GD1a). GT1b had the largest incorporation of label (Figure 32). GD3 (Figure 36); GQ1b (Figure 31); and GPX (Figure 30) were also highly labeled. Lower levels of label were seen in GD2 (Figure 34); GD1a (Figure 35); GM3 (Figure 39); and GD1b (Figure 33). The lowest labeling was found in GM2 (Figure 38) and GM1 (Figure 37). The labeling of GM3 by N-[^{14}C]-acetyl-mannosamine was unaffected by monensin treatment except at 5 hours where there was a small increase in labeling. The inhibition of labeling of GD3 by monensin (Figure 36) resembled the inhibition of labeling seen for [^{3}H]-galactose (Figure 23). The ganglioside GPX seems to be the most affected by monensin treatment (Figure 30). GD1b (Figure 33); GD1a (Figure 35); GT1b (Figure 32); GQ1b (Figure 31); GD2 (Figure 34); and GM1 (Figure 37) all exhibit similar inhibition of labeling profiles when treated with monensin. GM2 was the only ganglioside whose monensin inhibition differed greatly between [^{14}C]-N-acetyl-mannosamine and [^{3}H]-galactose labeling. There was a significant inhibition of [^{3}H]- galactose labeling of GM2 caused by monensin (Figure 25) but there was no change in labeling when [^{14}C]- N-acetyl-mannosamine was used to label cells (Figure 38).

Other pharmacological agents which are known to interfere with intracellular transport were examined for their effect on radiolabeling of glycosphingolipids. Colchicine, which causes a depolymerization of microtubules and thus interferes with transport processes, was added (1 μM) to primary neurons. Morphology of the neurons was altered by colchicine and the change was similar to that seen for monensin treatment (Figure 40). There was a decrease in size, number, and complexity of neurites when colchicine was added to primary neurons, but the long axonal like processes remained. Vinblastine, another agent which disrupts microtubules, also caused a reduction in the neuritic network (Figure 41). Vinblastine has been reported to cause crystallization of free tubulin which may result in the grainy appearance of the remaining neurites. Cytochalasin D is a cytoskeletal antagonist which blocks polymerization of actin molecules and thus inhibits microfilament contraction but is not thought to interfere with intraneurite transport. When cytochalasin D was added to primary neurons there seemed to be no reduction in the neuritic network as seen with the other cytoskeletal inhibitors but there actually appeared to be a slight increase in the neurite complexity when compared to control (Figure 42).

The effect of these three inhibitors (colchicine, vinblastine, and cytochalasin D) on the incorporation of [^{3}H]-galactose into gangliosides and neutral glycolipids was

examined. Primary neurons treated with or without colchicine (1.0 μM) are shown in Figure 43 and 44. The labeling of most of the gangliosides was inhibited by colchicine including GPX, GQ1b, GT1b, GD1b, GD2, and GD1a. GT1b and GD1a labeling was inhibited to the greatest extent by colchicine. Interestingly, labeling of GD3 and GM3 was unaffected by colchicine treatment.

The inhibitor cytochalasin D (1.0 μM) had little effect on the labeling of gangliosides by [^{3}H]-galactose (Figure 45). There was some decrease in labeling of GT1b when cytochalasin D was added and a small increase in GM1. This data agrees with the report that cytochalasin D does not interfere with intraneurite transport.

When vinblastine (1.0 μM) was added to primary neurons, the effect seen on labeling was similar to that of colchicine but with some distinct differences (Figure 46). GT1b was decreased in radioactive labeling the most followed by GD1a and then GQ1b. GD3 had a small increase in labeling and GM3 was unaffected by vinblastine treatment. Labeling of GM2 was slightly enhanced by vinblastine which was different from treatment with colchicine. Overall, the cytoskeletal inhibitors (colchicine, vinblastine, and cytochalasin D) caused less inhibition of labeling than did monensin. This may be due to the fact that monensin has a direct effect on Golgi transport whereas the cytoskeletal inhibitors only have an indirect effect on Golgi transport.

The effect of colchicine and cytochalasin D on neutral glycolipids is presented in Table 6.

TABLE 6

Effect of cytochalasin D and colchicine on the incorporation of [^{3}H]-galactose into neutral glycolipids of primary neurons when treated for 15 hours.

	LACTOSYLCERAMIDE	GLUCOSYLCERAMIDE
CONTROL	15 ± 1.5	106 ± 13
CYTOCHALASIN D	13 ± 2.3[b]	95 ± 15[b]
COLCHICINE	11 ± 0.7[a]	122 ± 9[b]

(cpm/mg protein) (n=3, ± SD)([a]$p<.05$, [b]not significant)

Drugs were added at a concentration of 1.0 μM.

Treatment of primary neurons with cytochalasin D or colchicine had no effect on the labeling of glucosylceramide has shown in Table 6. Cytochalasin D also had no effect on the labeling of lactosylceramide but colchicine caused a small decrease in its labeling.

Cobalt is thought to specifically inhibit transport between the endoplasmic reticulum and the Golgi apparatus and so was used to examine glycosphingolipid transport. The higher gangliosides GPX, GQ1b, and GT1b, had a significant

reduction in the incorporation of [^{3}H]-galactose when cobalt (0.5 mM $CoCl_2$-$6H_2O$) was added to the media which was similar to that of monensin (Figure 47a and 47b). Cobalt was also similar to monensin because GM3 had no reduction in label incorporation. GD3 incorporation of label was also unchanged after treatment with cobalt. There was a slight reduction in label incorporation of GD1a and no significant change in GD2 after treatment with cobalt. The most significant difference between cobalt and monensin treatment was the incorporation of label into GM2. Cobalt caused a large increase in the incorporation of label into GM2 but monensin did not. This is significant because GM2 is the first step of the "a" series after branching from GM3. Another significant difference between cobalt and monensin was that cobalt significantly enhanced the incorporation of label into glucosylceramide but reduced the label incorporation into lactosylceramide (Figure 48 and Table 7). This suggested that cobalt interfered at another earlier step in the biosynthesis of gangliosides than did monensin.

TABLE 7

Effect of cobalt on the incorporation of [^{3}H]-galactose into neutral glycolipids of primary neurons after treatment for 18 hours.

	LACTOSYLCERAMIDE	GLUCOSYLCERAMIDE
CONTROL	22 ± 1.0	66 ± 1.5
COBALT 0.5 mM	13 ± 1.2[a]	141 ± 8.7[a]

(cpm/mg protein) (n=3, ± SD) ([a]$p < 0.001$)

To discover if calcium has a role in glycosphingolipid biosynthetic pathways the calcium ionophore A23187 was used to increase the intracellular concentration of this ion. When A23187 (1.0 µM) was added along with [^{3}H]-galactose to primary neurons there was almost a complete abolition of the incorporation of label into GPX, GQ1b, GT1b and GD1a suggesting an important role of calcium in ganglioside transport (Figure 49a and 49b). Incorporation of label into GM3 remained unchanged. A23187 caused a significant enhancement of label incorporation into both GD3 and GM2. Both GD3 and GM2 are the first step in the "b" series and the "a" series respectively, therefore, calcium may play an important role in regulation of ganglioside biosynthesis. Interestingly, A23187 had no effect on the incorporation of label into both lactosylceramide and glucosylceramide (Figure 50 and Table 8).

TABLE 8

Effect of A23187 on the incorporation of [^{3}H]-galactose into neutral glycolipids of primary neurons after treatment for 15 hours.

	LACTOSYLCERAMIDE	GLUCOSYLCERAMIDE
CONTROL	36 ± 1.2	162 ± 4.5
A23187 1.0 µM	27 ± 2.5[a]	147 ± 14.0[b]

(cpm/mg protein)(n=3, ± SD)([a]$p<0.01$, [b]not significant)

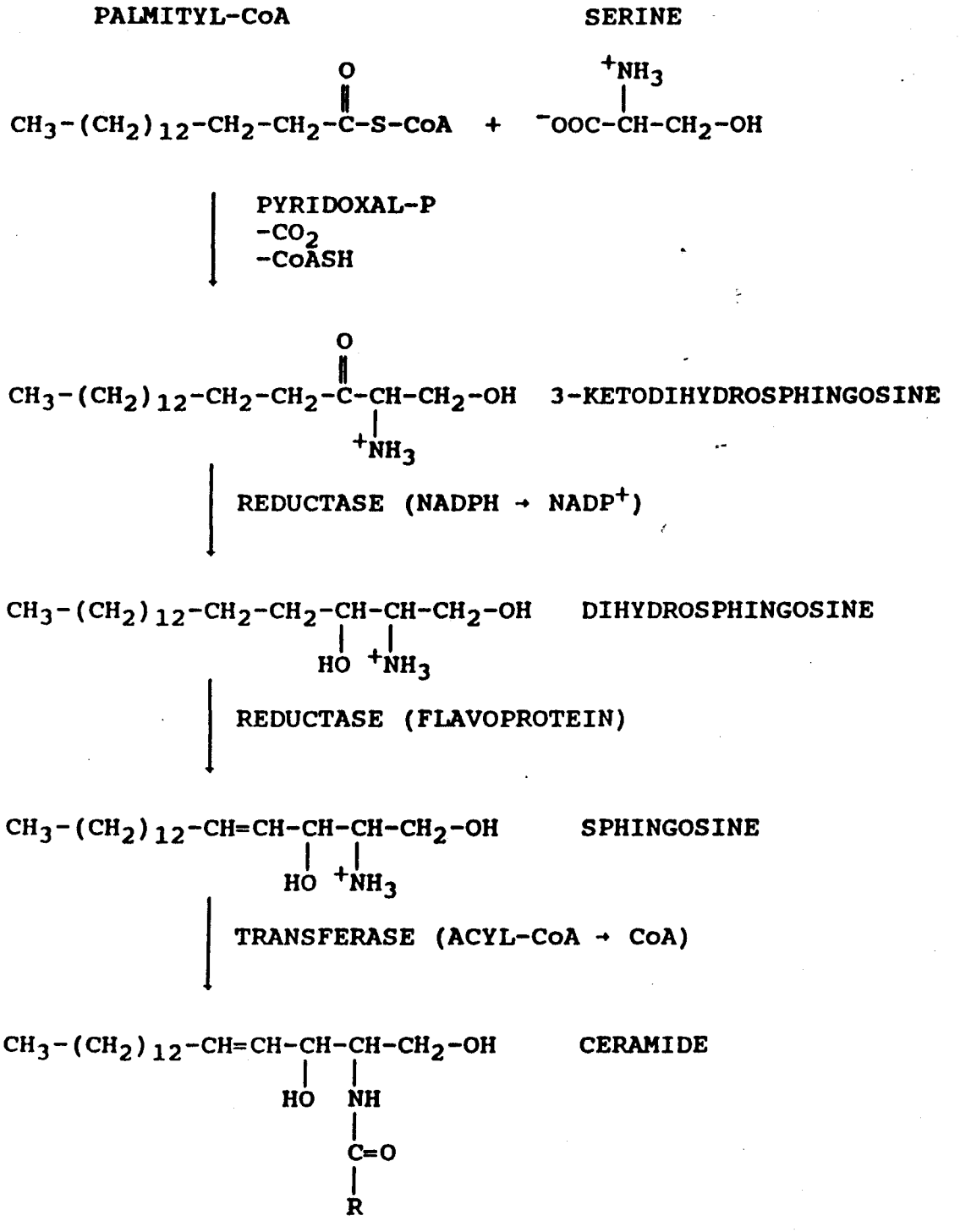
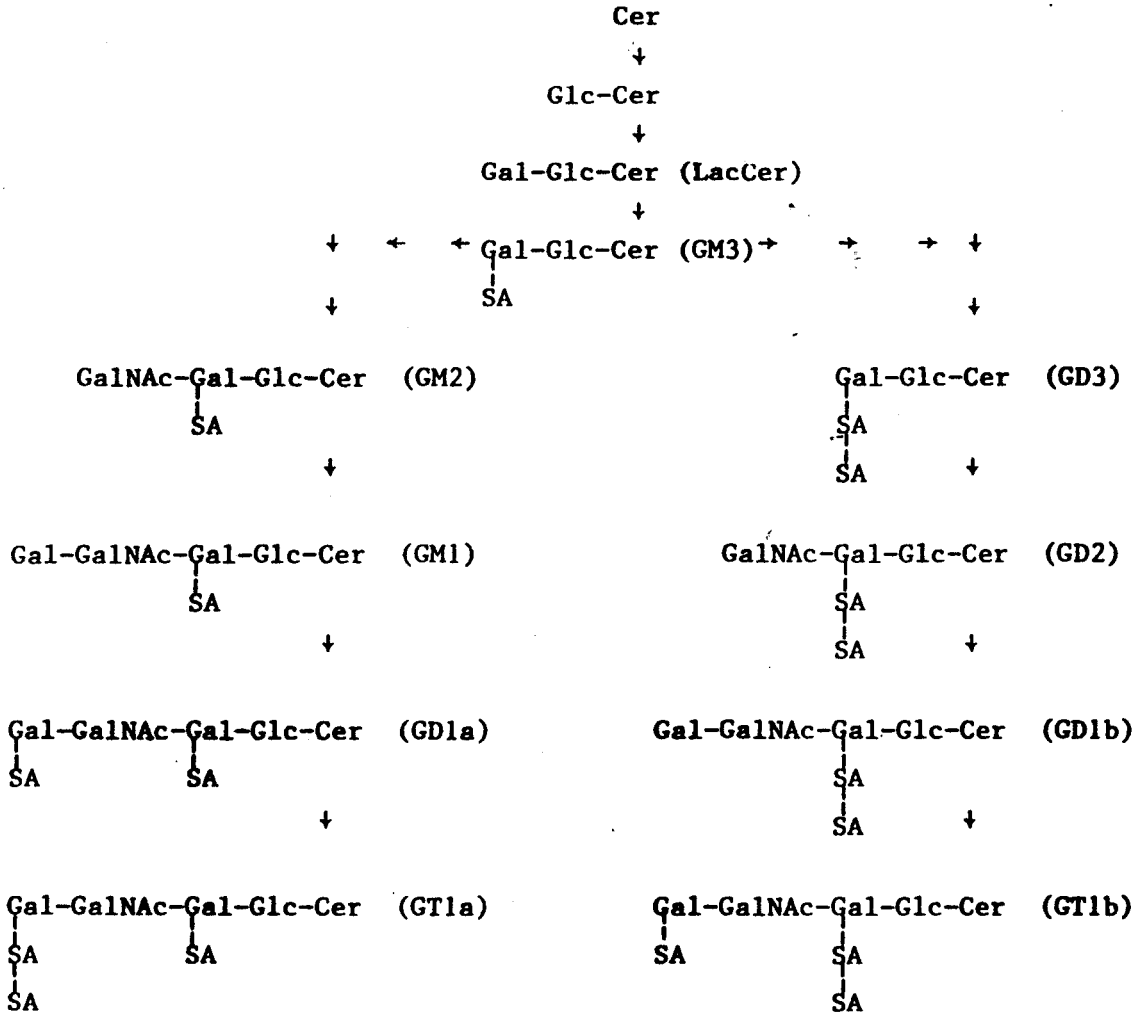


Figure 1

Biosynthesis of ceramide



Cer - Ceramide

Gal - Galactose

Glc - Glucose

SA - Sialic Acid

GalNAc - N-acetylgalactoseamine

Figure 2

Ganglioside biosynthesis

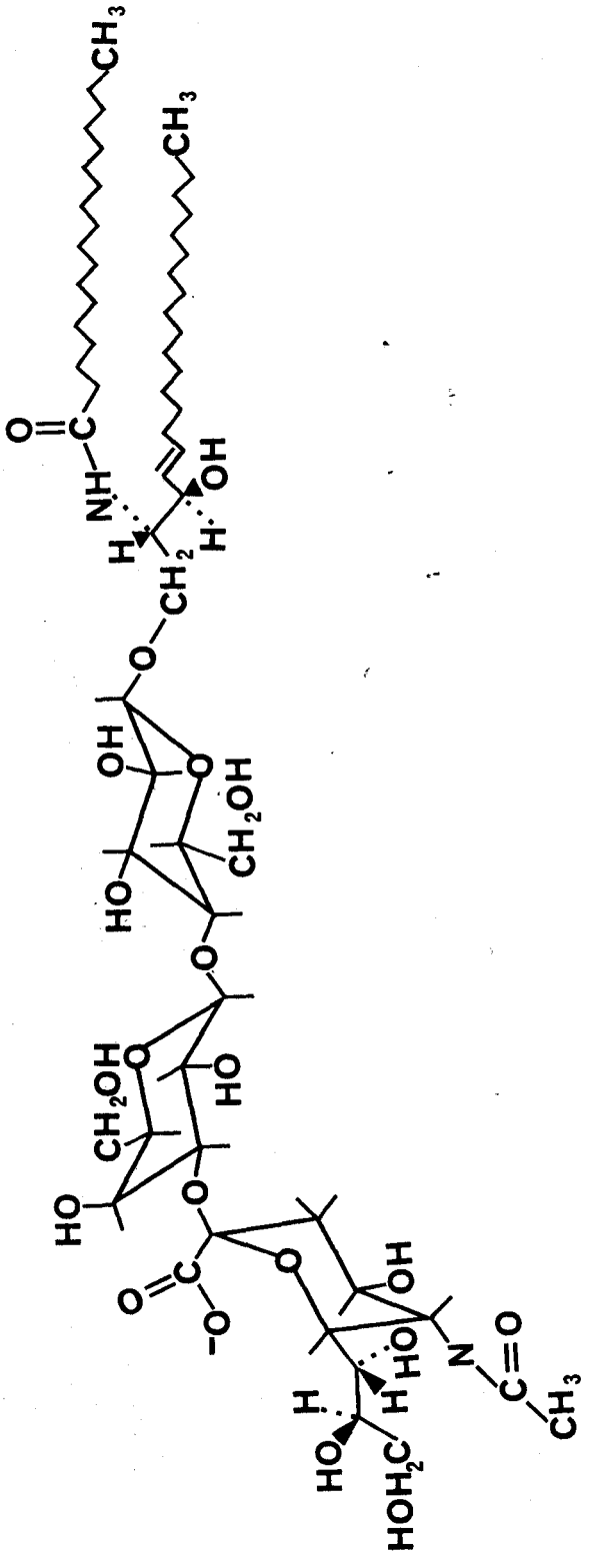


Figure 3

Structure of GM3

1. RECEPTORS
    A. HORMONES (TSH,HCG,etc.)
    B. CELL GROWTH FACTORS (NGF)
    C. DIFFERENTIATION FACTORS
    D. INTERFERON
    E. LECTINS
    F. NEUROTRANSMITTERS
    G. ETC.
2. NEURITOGENIC AND NEUROTROPHIC EFFECTS
3. CELL RECOGNITION
4. CELL ADHESION AND MIGRATION
5. DIFFERENTIATION
6. ELECTRICAL EXCITABILITY
7. CONTROL OF MEMBRANE IONIC ENVIROMENT
8. MANY OTHER MEMBRANE RELATED PHENOMENA

Figure 4

Functions attributed to gangliosides

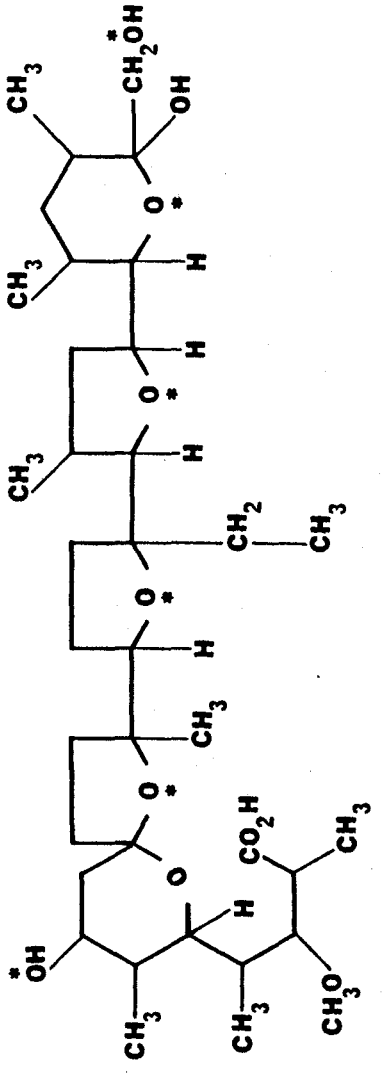


Figure 5

Structure of Monensin

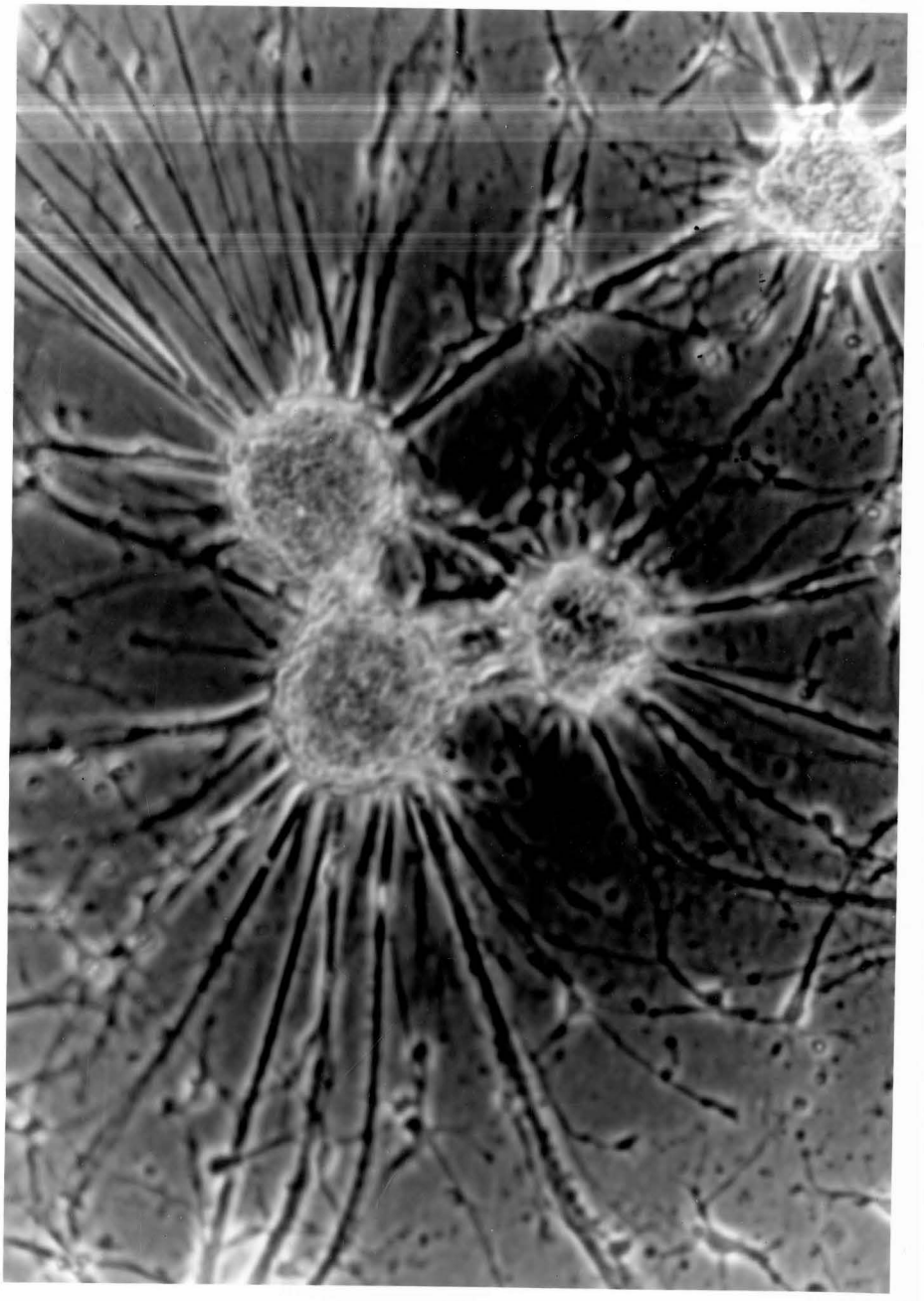


Figure 6

Chick primary neurons prepared from 7 day old embryo telencephalon and grown for six days in culture. (Magnification 100 X)

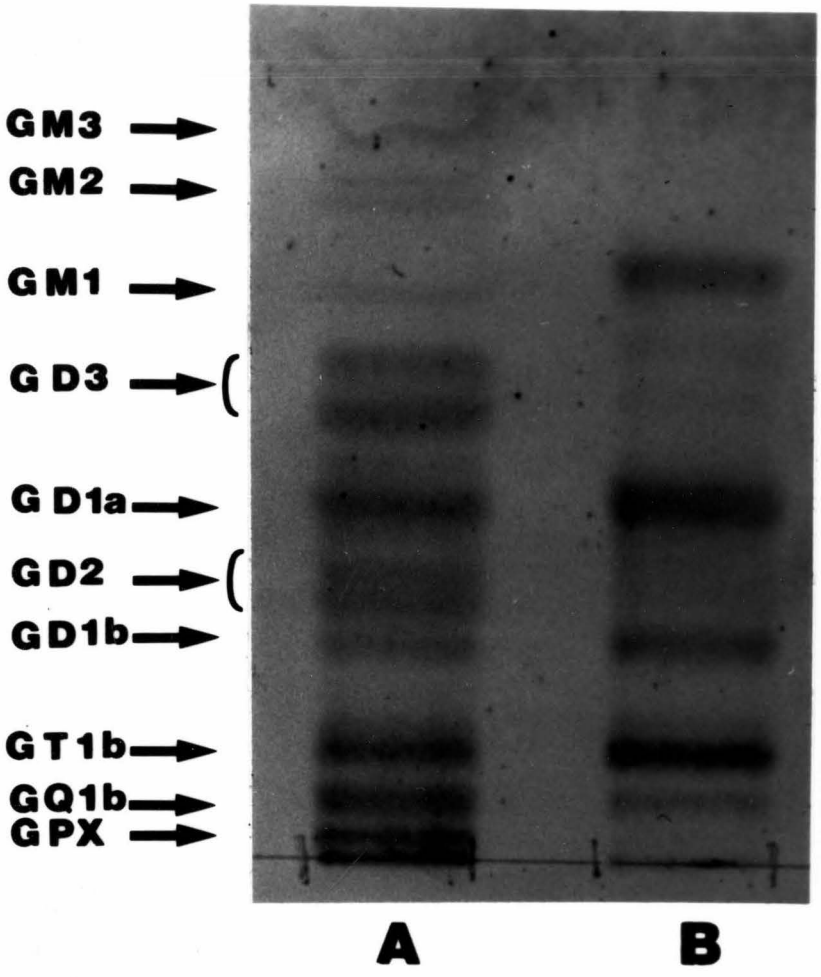


Figure 7

Gangliosides were isolated from 7 day primary neurons.
Individual gangliosides were separated by HPTLC.
The HPTLC plate was then sprayed with resorcinol.
A. Ganglioside pattern of chick primary neurons.
B. Ganglioside standards

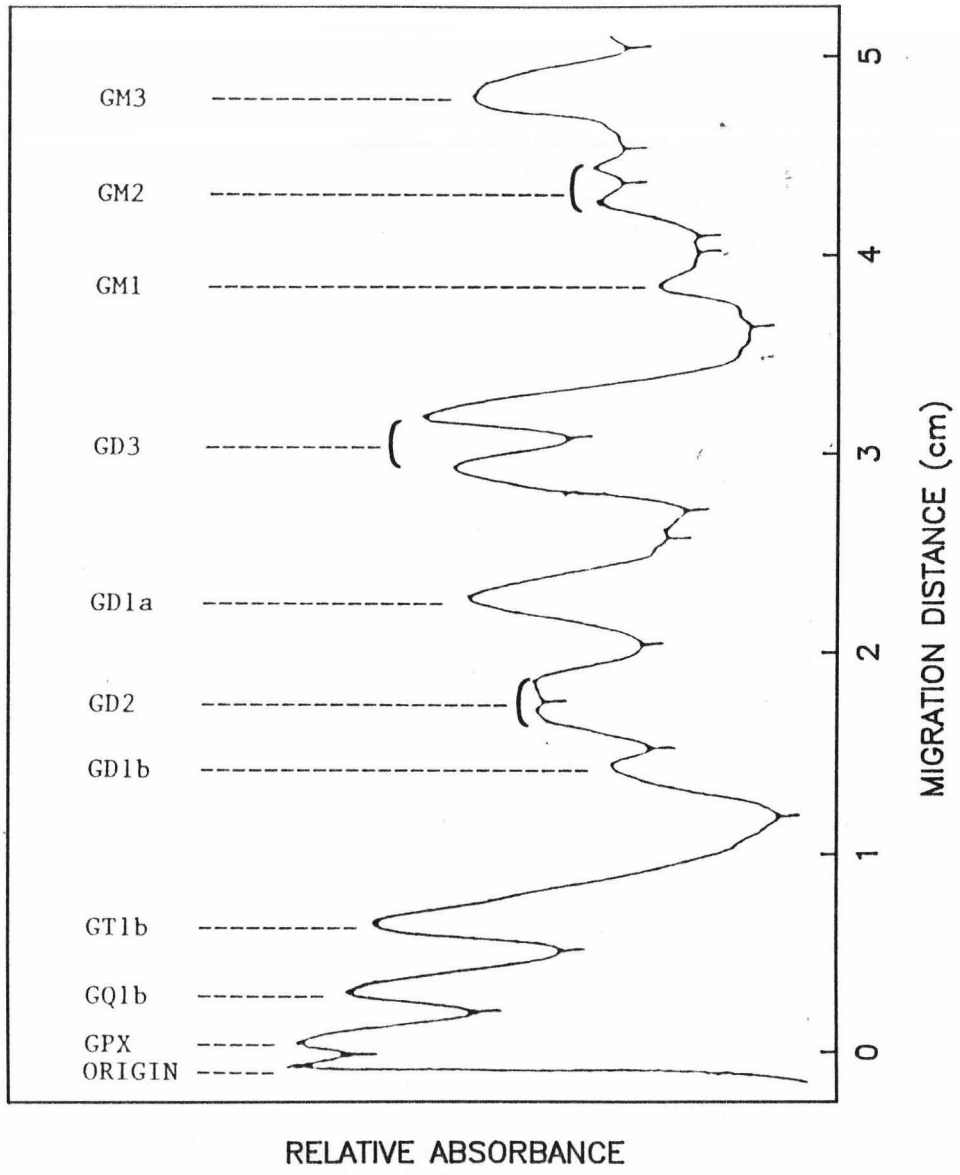


Figure 8

Densitometric scan of Figure 7. Peak area was quantitated by cutting out individual peaks and weighing each one.

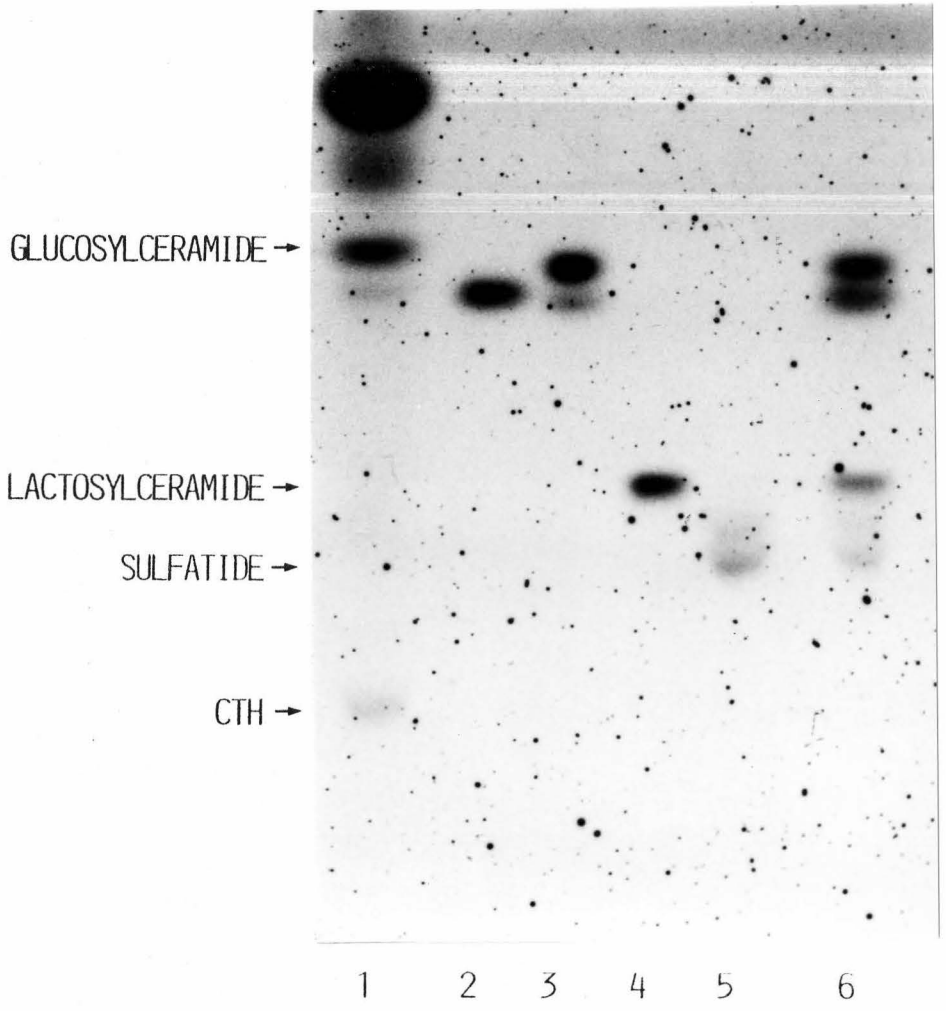


Figure 9

Neutral glycolipids were isolated from 7 day primary neurons. Individual neutral glycolipids were separated by HPTLC. The HPTLC plate was sprayed with anthrone reagent.

Lane 1 Neutral glycolipids of chick primary neurons
Lane 2 Galactosylceramide standard
Lane 3 Glucosylceramide standard
Lane 4 Lactosylceramide standard
Lane 5 Sulfatide standard
Lane 6 Mixture of neutral glycolipid standards
(CTH = Trihexosyl ceramide)

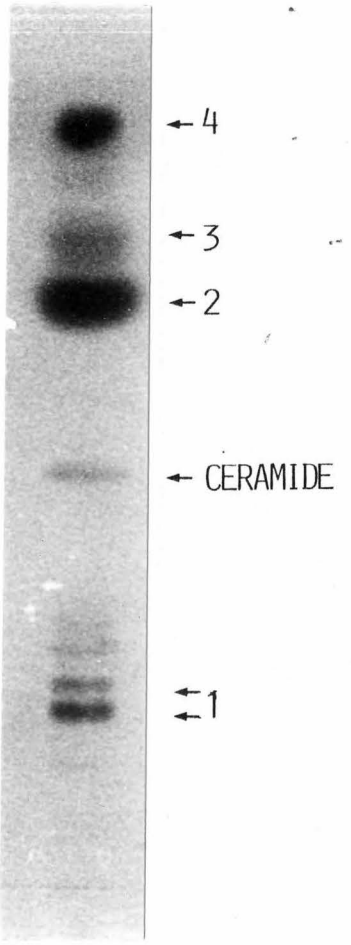


Figure 10

CERAMIDE ANALYSIS

Lipids were isolated from 7 day primary neurons. These lipids were separated by HPTLC. The HPTLC plate was charred with phosphoric acid/cupric acetate reagent.

Band 1 Phospholipids
Band 2 Cholesterol
Band 3 Triglycerides and fatty acids
Band 4 Cholesterol esters

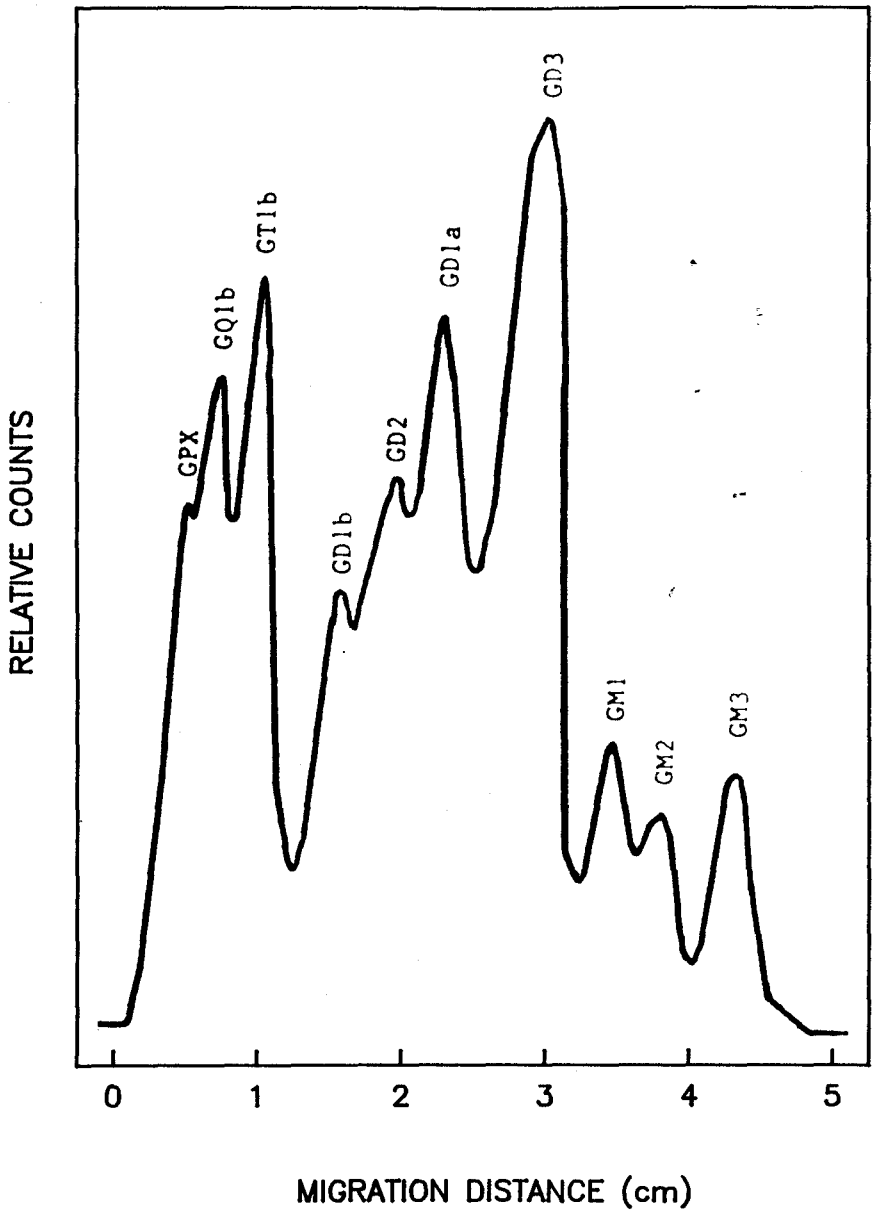


Figure 11

Primary neurons were labeled with [^{3}H]-galactose for 18 hours. Gangliosides were isolated and individual gangliosides were separated by HPTLC. The HPTLC plate was placed under the radioactive TLC scanner for 10 mins. The RTLC scan is presented.

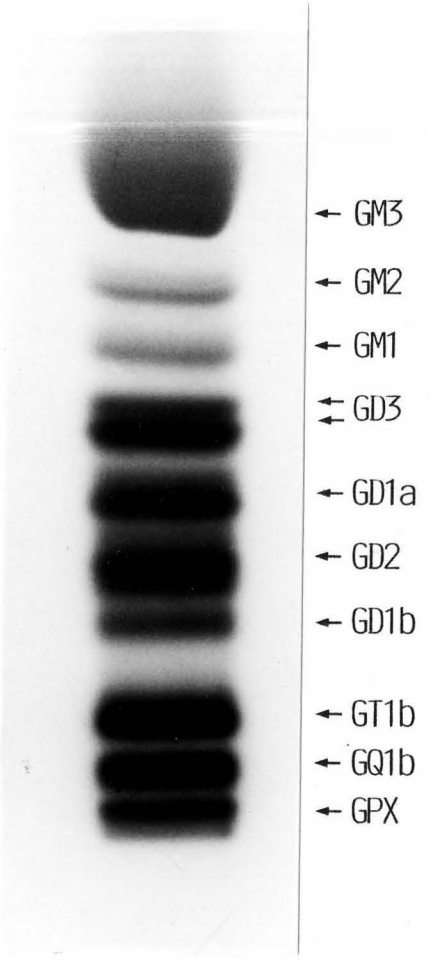


Figure 12

Primary neurons were labeled with [^{3}H]-galactose for 18 hours. Gangliosides were isolated and individual gangliosides were separated by HPTLC. The HPTLC plate was sprayed with scintillant and exposed to autoradiographic film for 5 days at -80 ^{0}C. The autoradiogram of the TLC plate is presented.

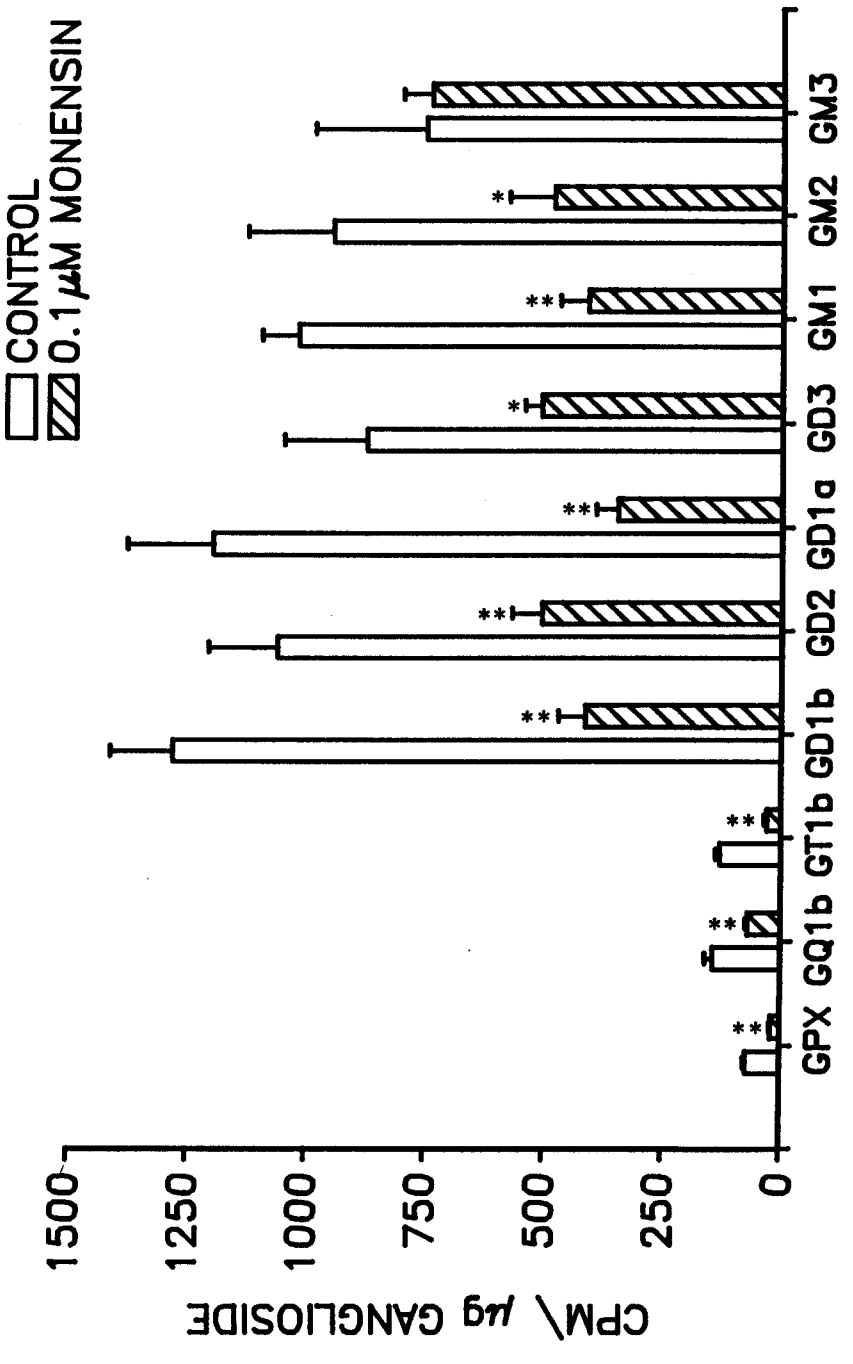


Figure 13 Primary neurons were labeled for 15 hours with [^{3}H]-galactose. The gangliosides were isolated then separated by HPTLC. The specific activity of labeled gangliosides both before and after monensin (0.1 μM) treatment for 15 hours was determined. Gangliosides were quantitated by densitometric scanning. Specific activity was calculated from the amount of radioactivity in each band (CPM) as compared to the amount of ganglioside (μg) present. (n=3, ± SD, *p<.05, **p<.01). GM3 was not significantly different from control.

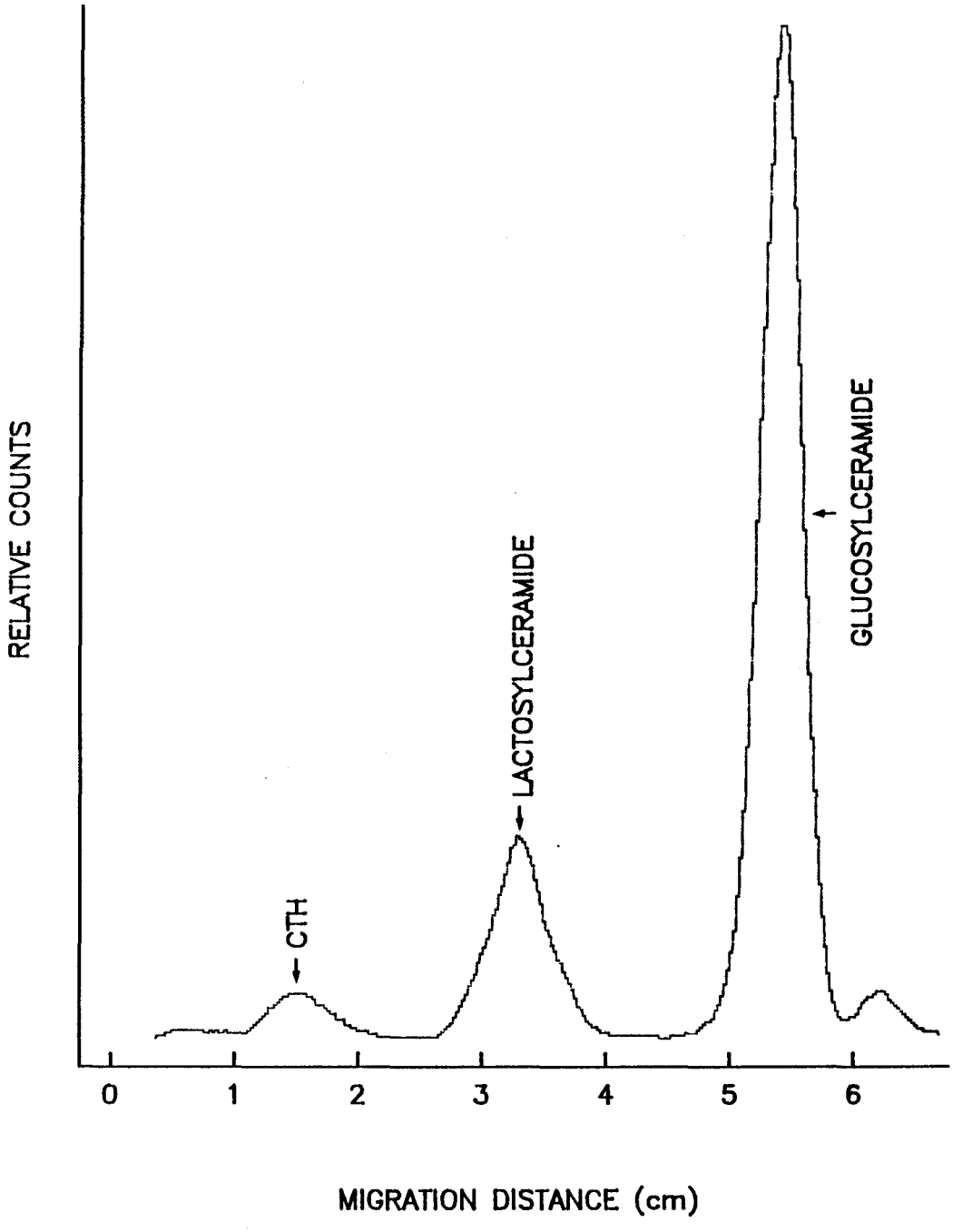


Figure 14

The neutral glycolipids of chick primary neurons were labeled with [^{3}H]-galactose for 18 hours. Neutral glycolipids were isolated and then separated by HPTLC. The HPTLC plate was placed under the radioactive scanner for 10 mins. The RTLC scan is presented.

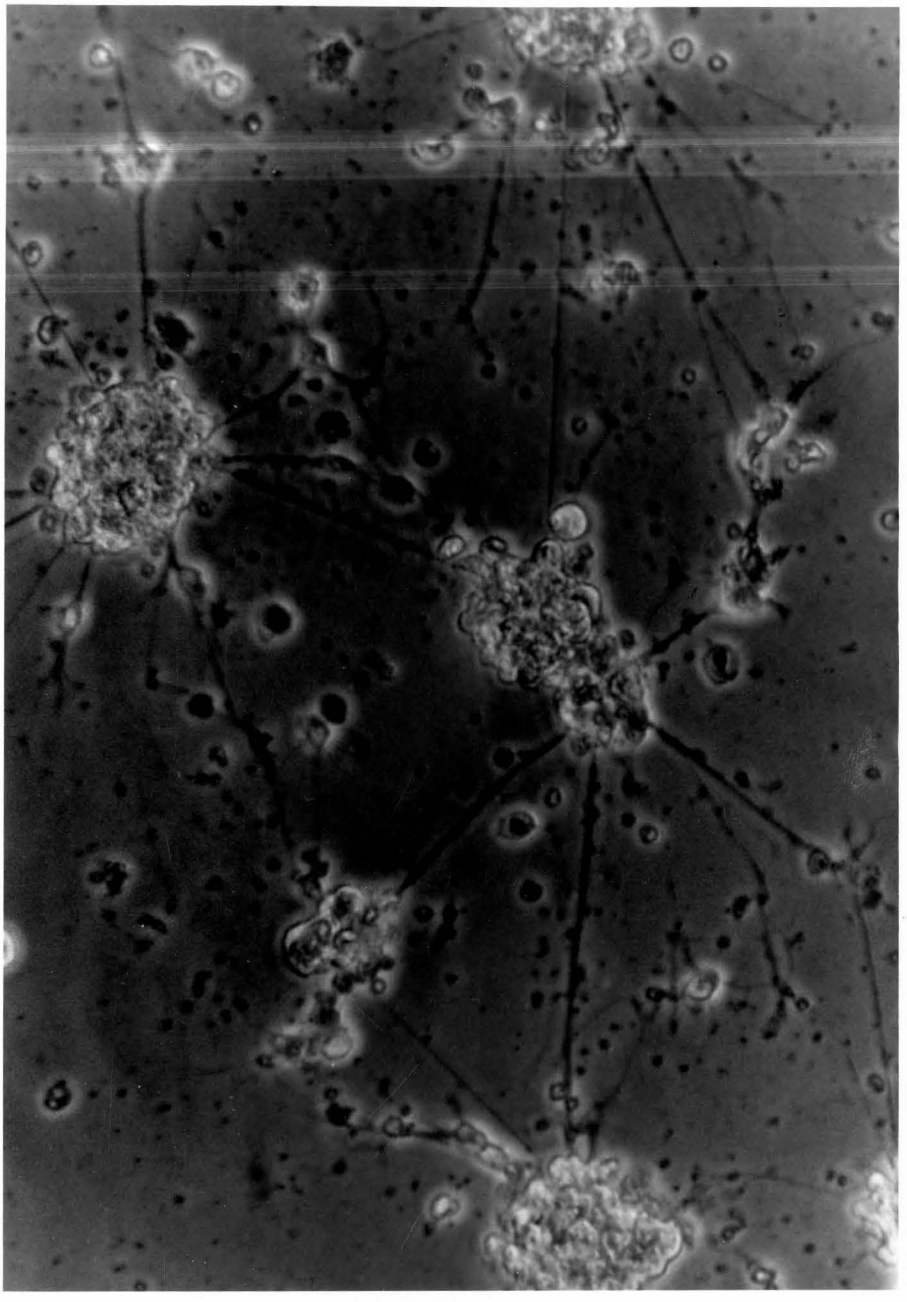


Figure 15

6 day primary neurons were exposed to 0.1 µM monensin for 18 hours. The photograph after monensin treatment is presented. (Magnification 100 X)

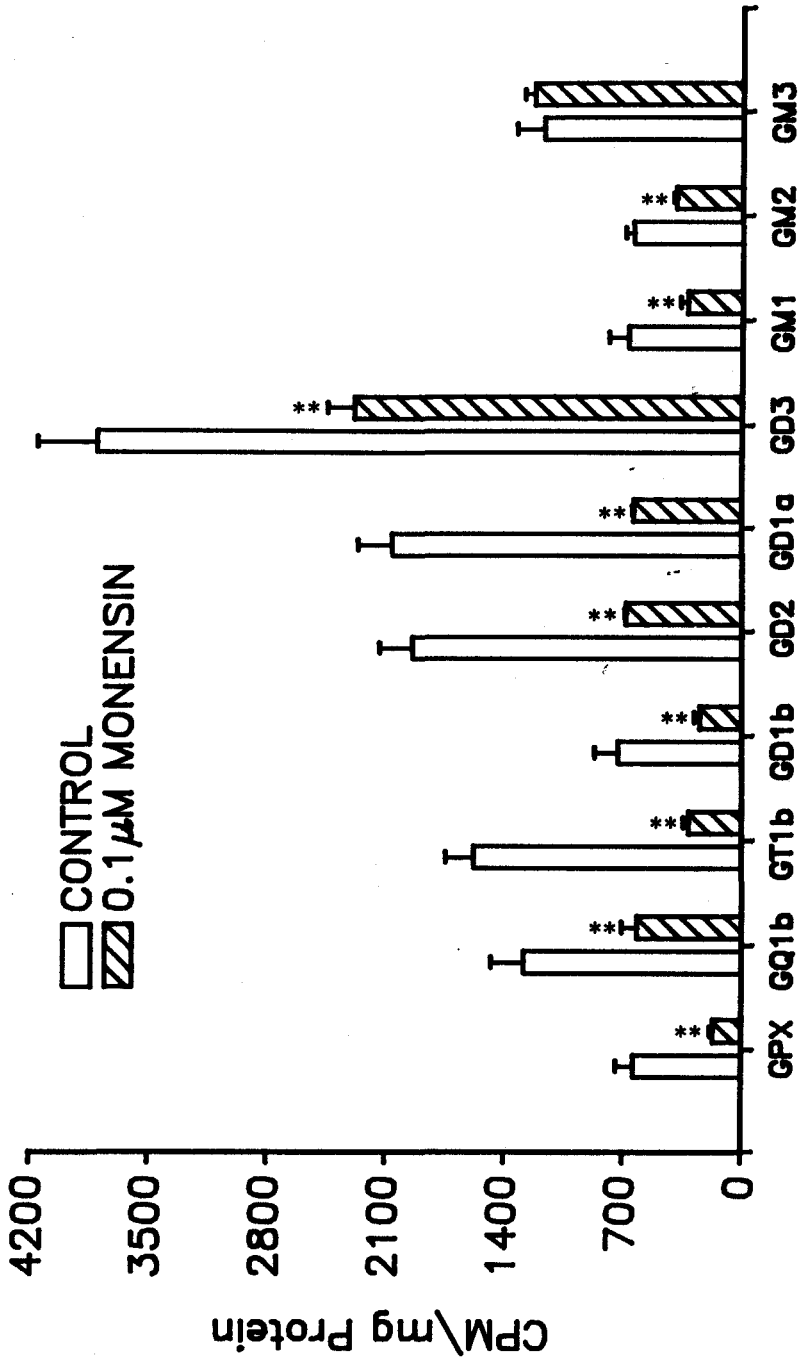


Figure 16

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for 18 hours. The gangliosides were isolated and then separated by HPTLC. Individual gangliosides were scraped and the amount of radioactivity in each band was determined. (n=3, ± SD, **p<.01). GM3 was not significantly different from control.

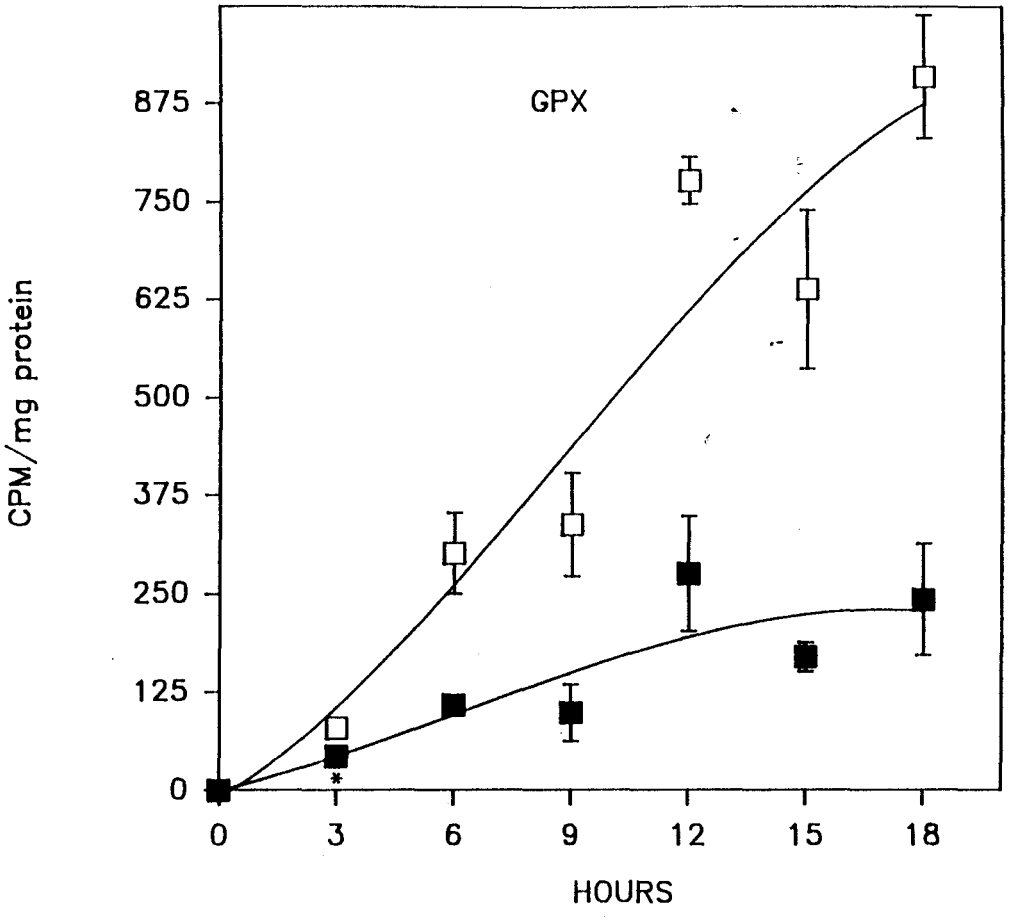


Figure 17

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GPX band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, *$p<.05$). All other time points $p<.01$.

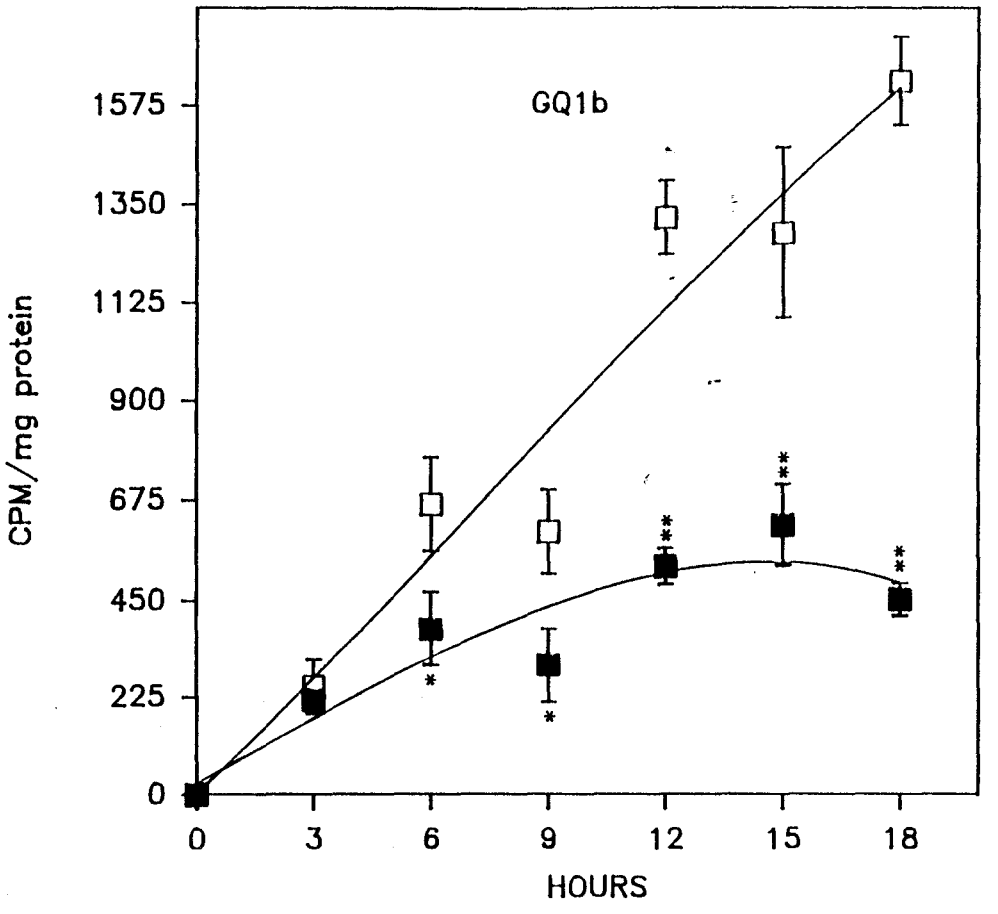


Figure 18

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GQ1b band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, *p<.05, **p<.01). The 3 hour time point was not significantly different from control.

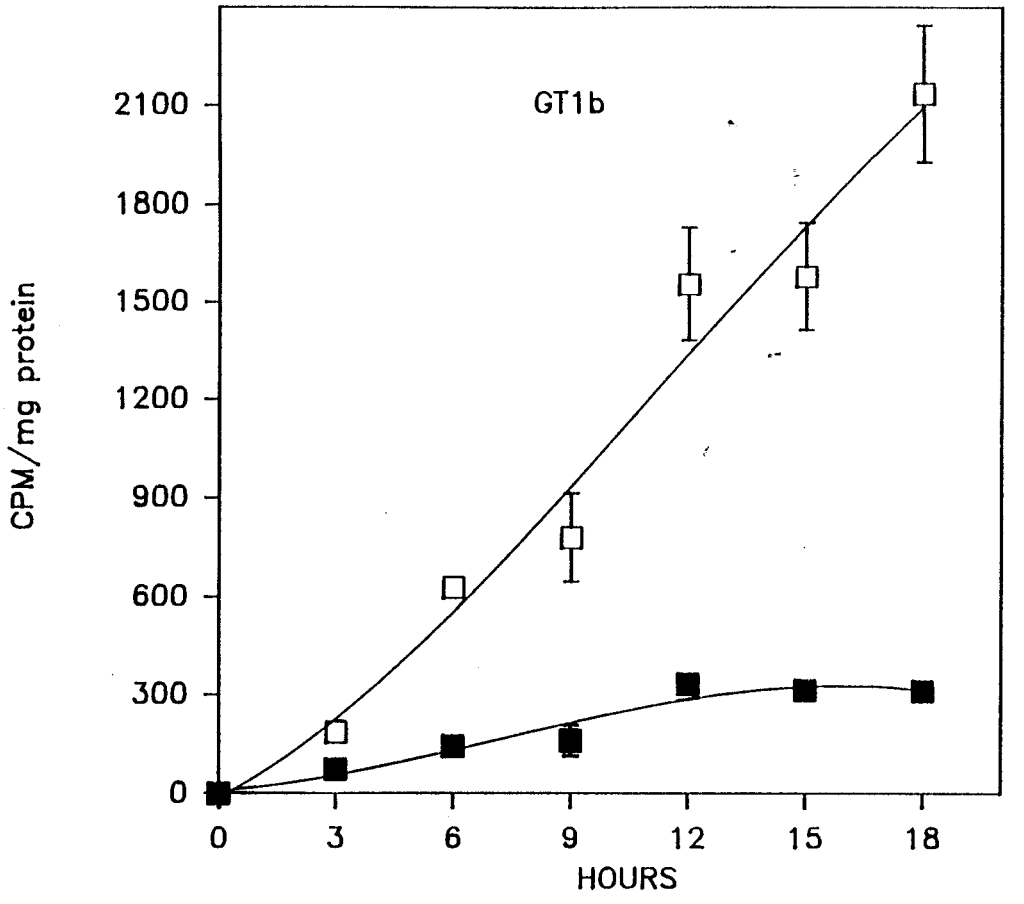


Figure 19

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GT1b band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, $^{**}p<.01$ for all timepoints when compared to control).

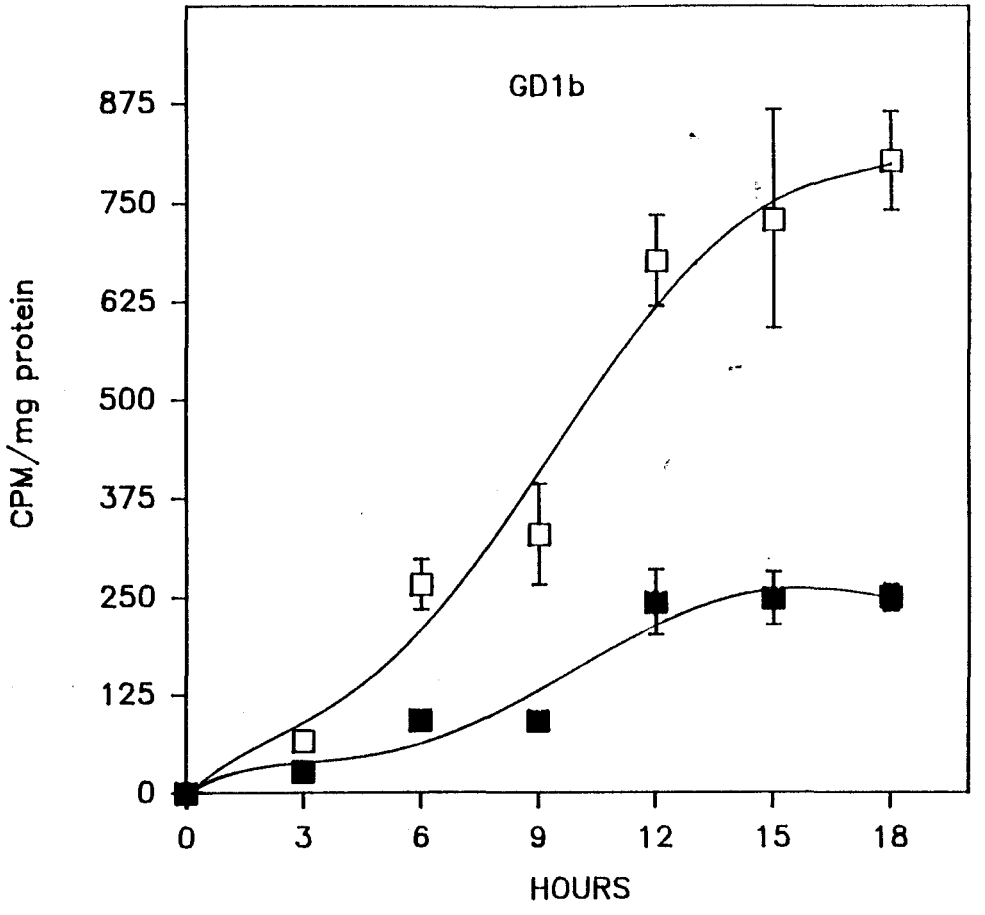


Figure 20

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GD1b band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, $^{**}p<.01$ for all time points when compared to control).

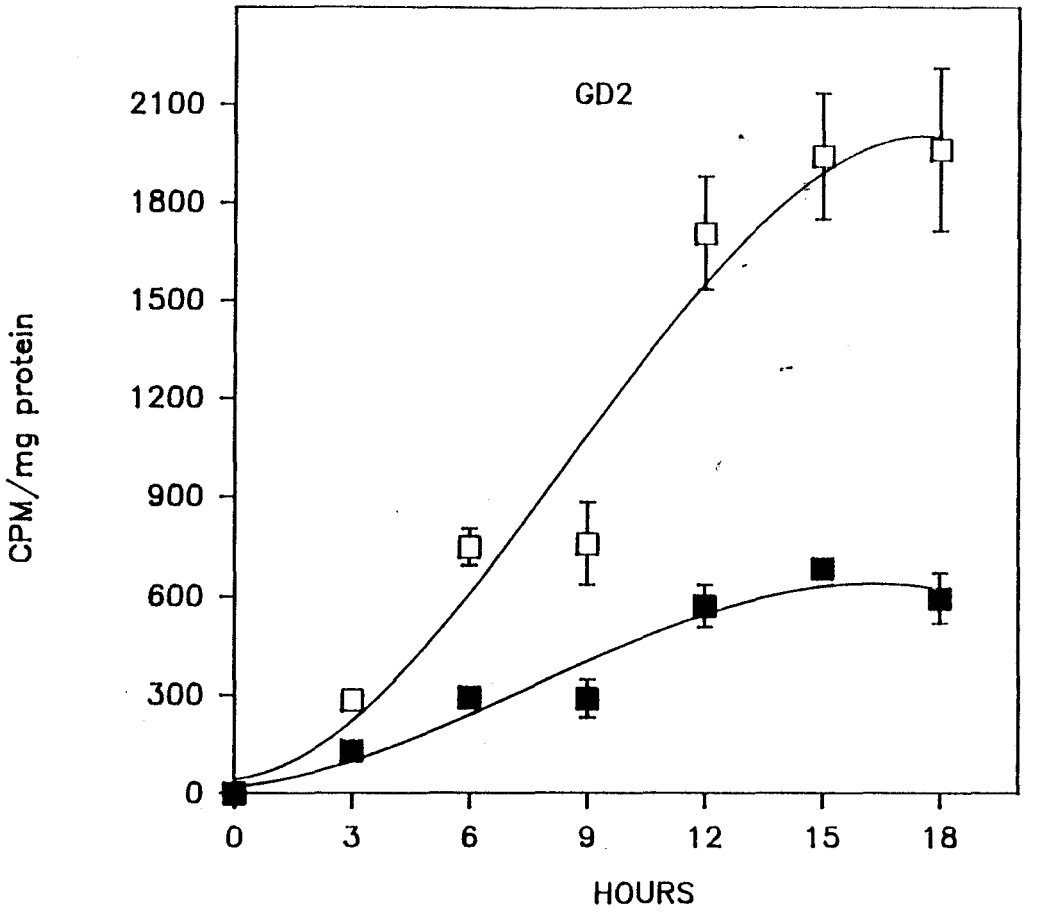


Figure 21

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GD2 band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, $^{**}p<.01$ for all time points when compared to control).

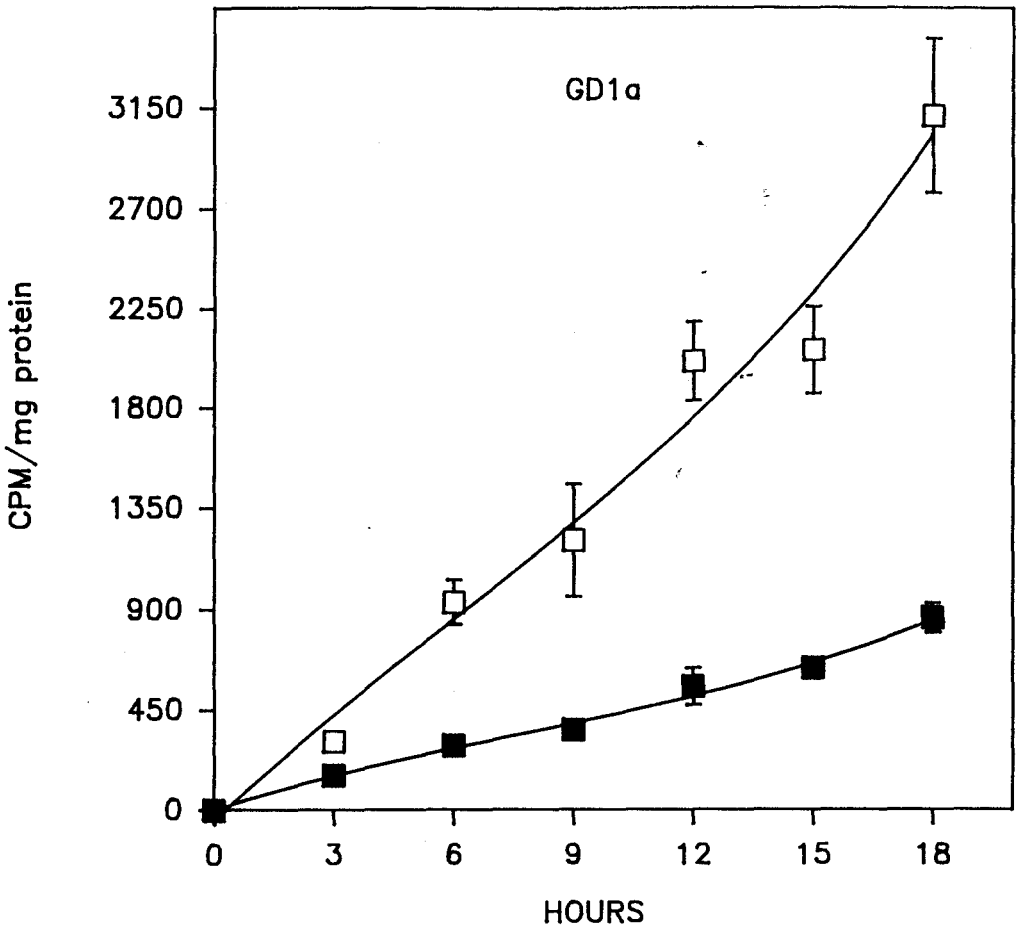


Figure 22

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GD1a band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, $^{**}p<.01$ for all time points when compared to control).

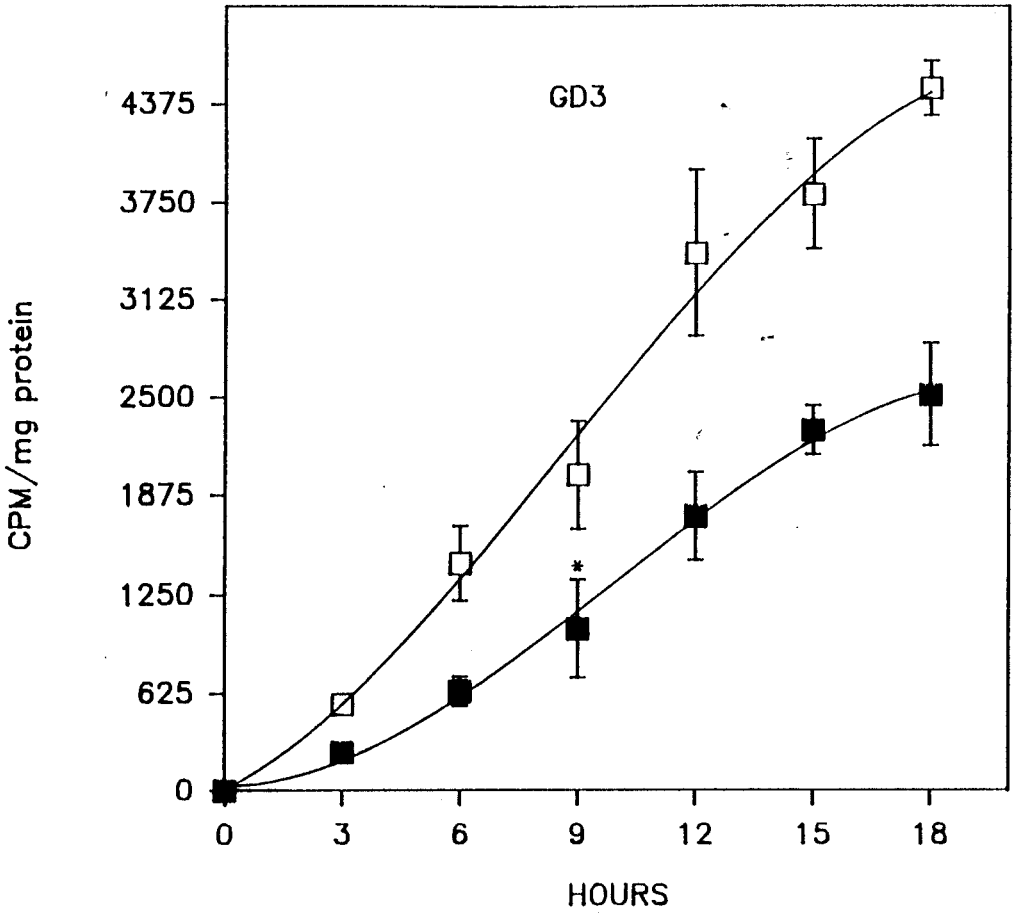


Figure 23

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GD3 band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, $^{**}p<.01$ for all time points except 9 hour ($^{*}p<.05$) when compared to control).

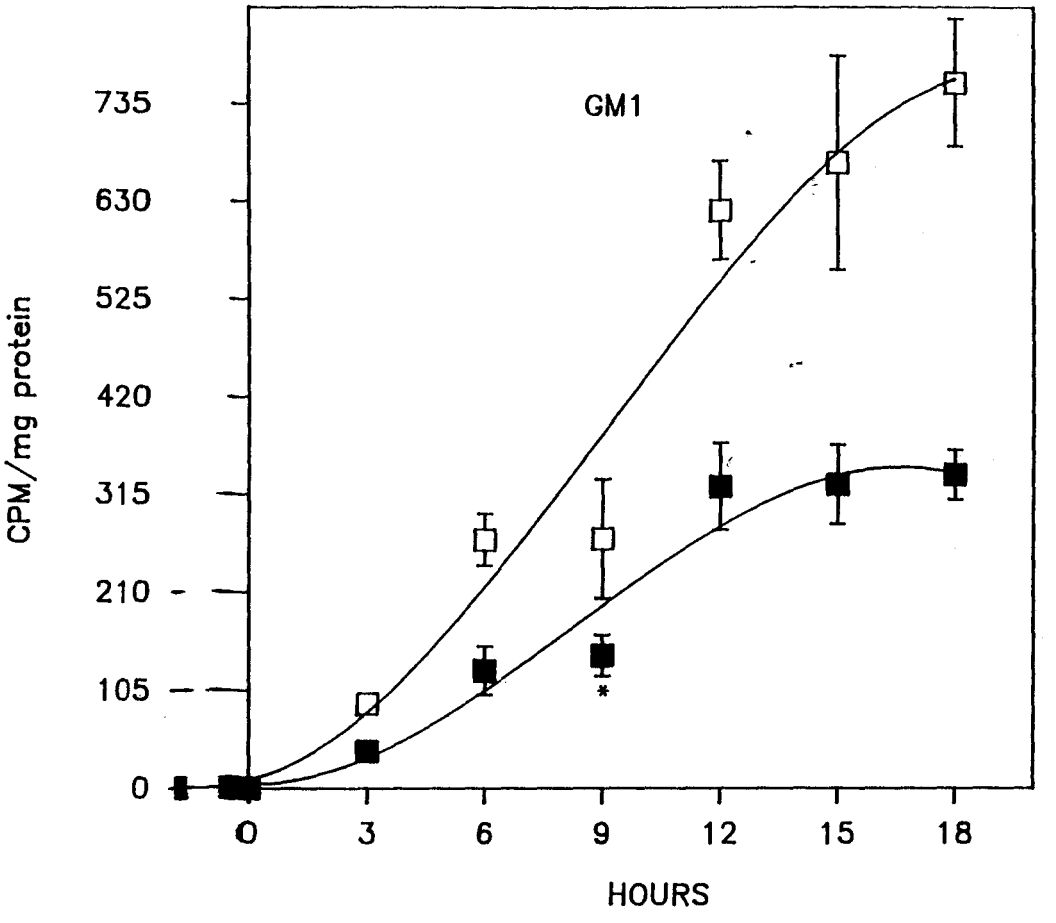


Figure 24

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GM1 band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, **p<.01 for all time points except 9 hour (*p<.05) when compared to control).

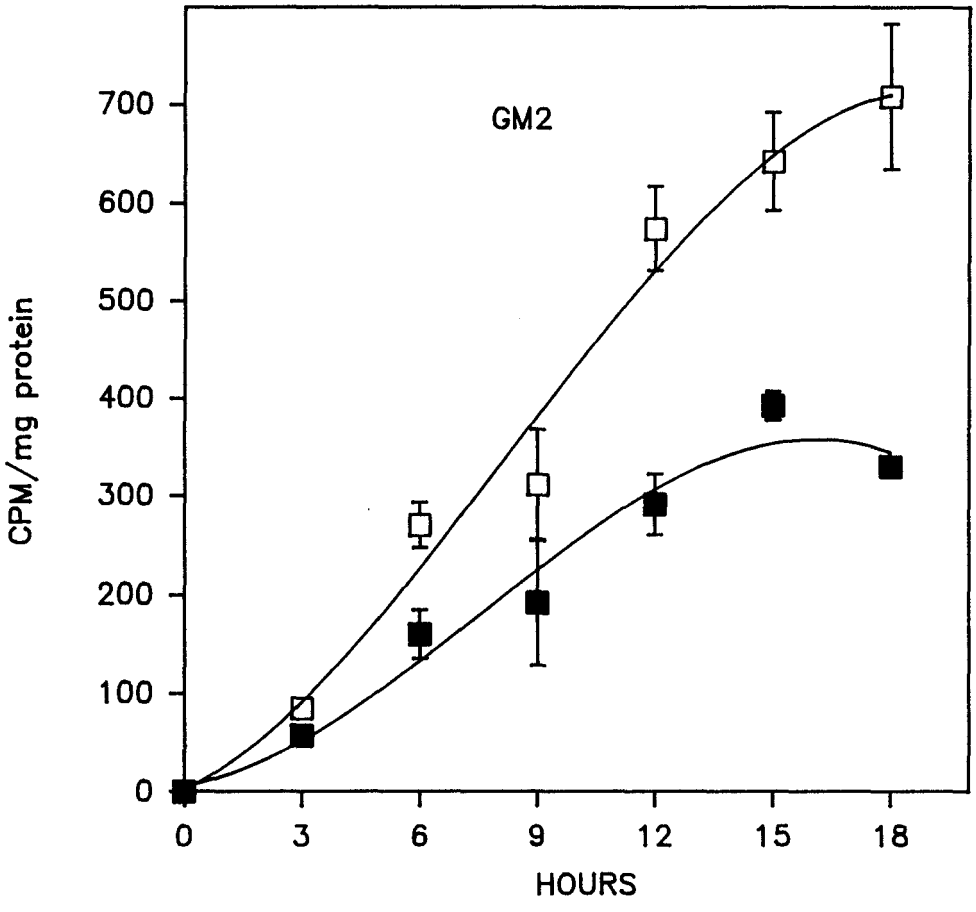


Figure 25

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GM2 band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, $^{**}p<.01$ for all time points except 9 hour (not significant) when compared to control).

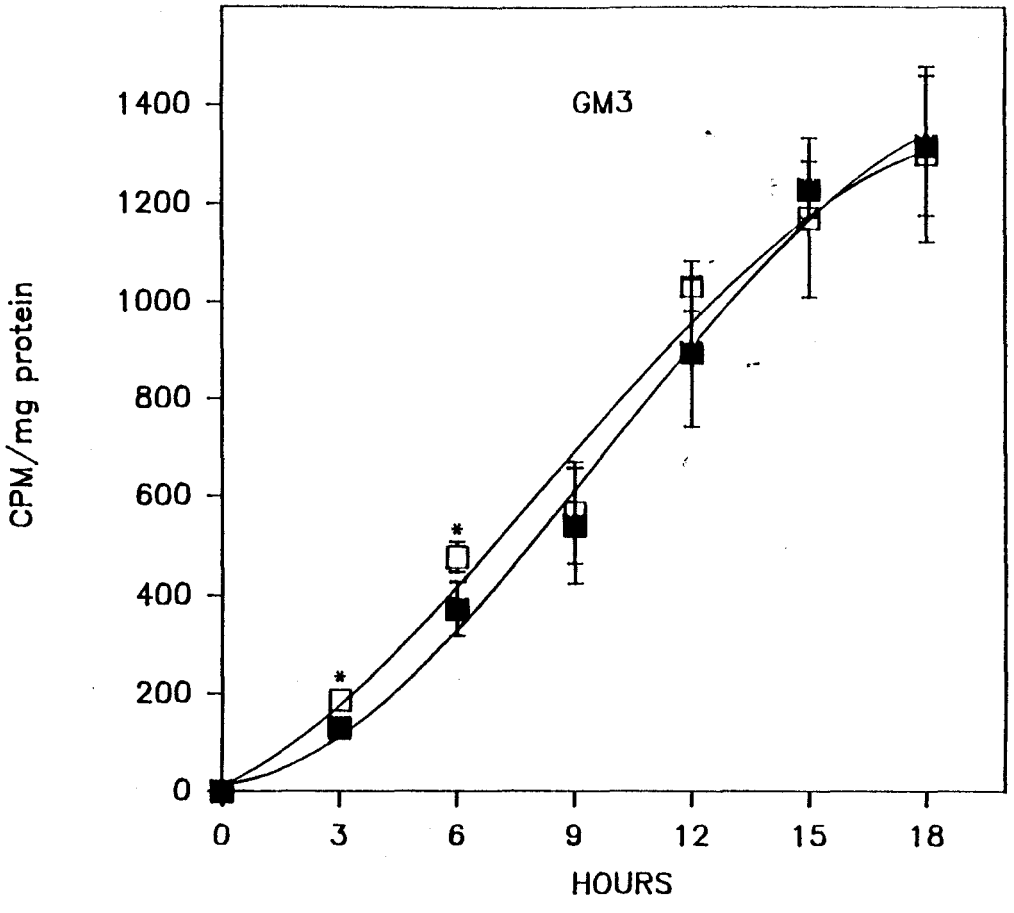


Figure 26

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Gangliosides were isolated and then separated by HPTLC. The GM3 band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated neurons. (n=3, ± SD, *p<.05, other time points were not significant when compared to control.

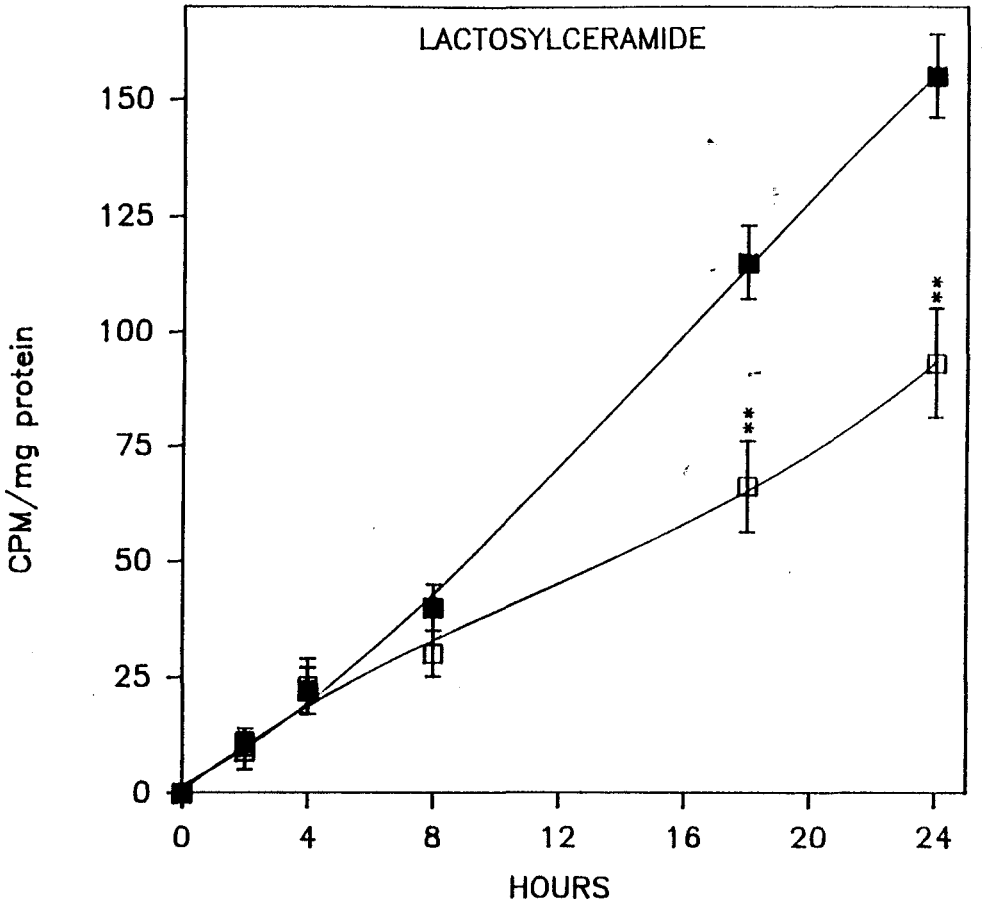


Figure 27

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 μM monensin for various times. Neutral glycolipids were isolated and then separated by HPTLC. The lactosylceramide band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated cells. (n=3, ± SD, **$p<.01$, other time points were not significant when compared to control.

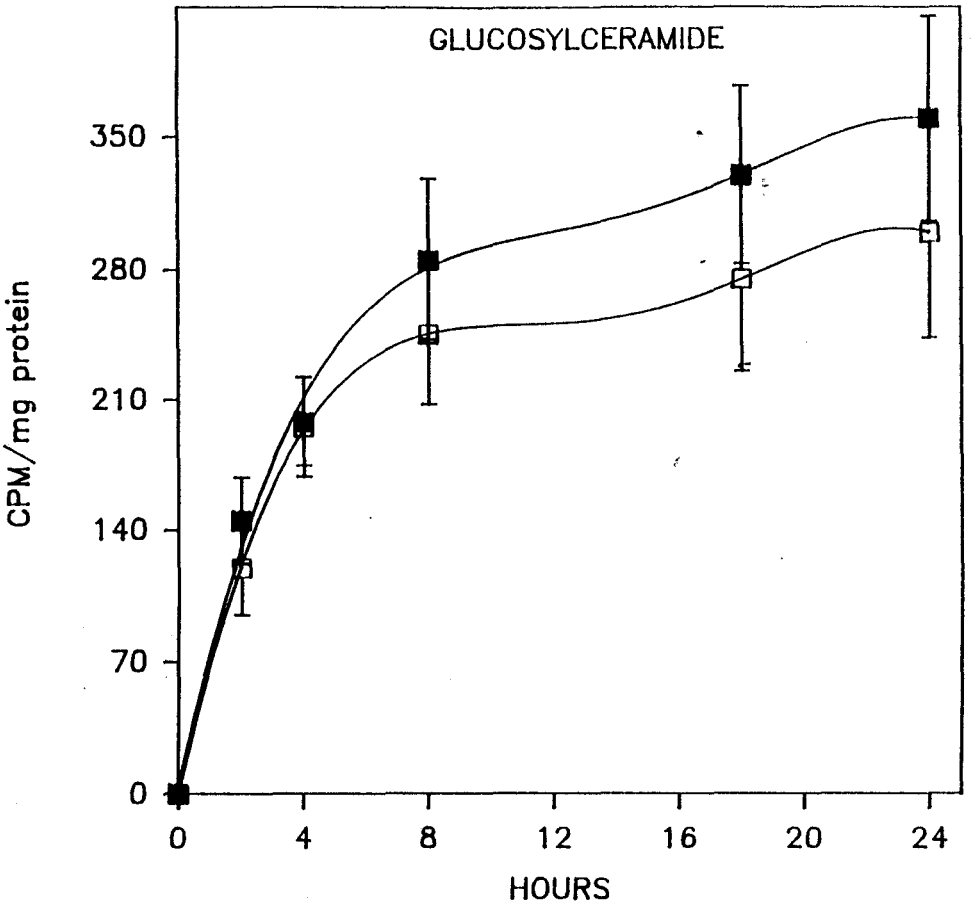


Figure 28

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 µM monensin for various times. Neutral glycolipids were isolated and then separated by HPTLC. The glucosylceramide band was scraped and the radioactivity was determined. Open squares represent control and filled squares represent monensin treated cells. (n=3, ± SD) All time points were determined to be not significant when compared to control.

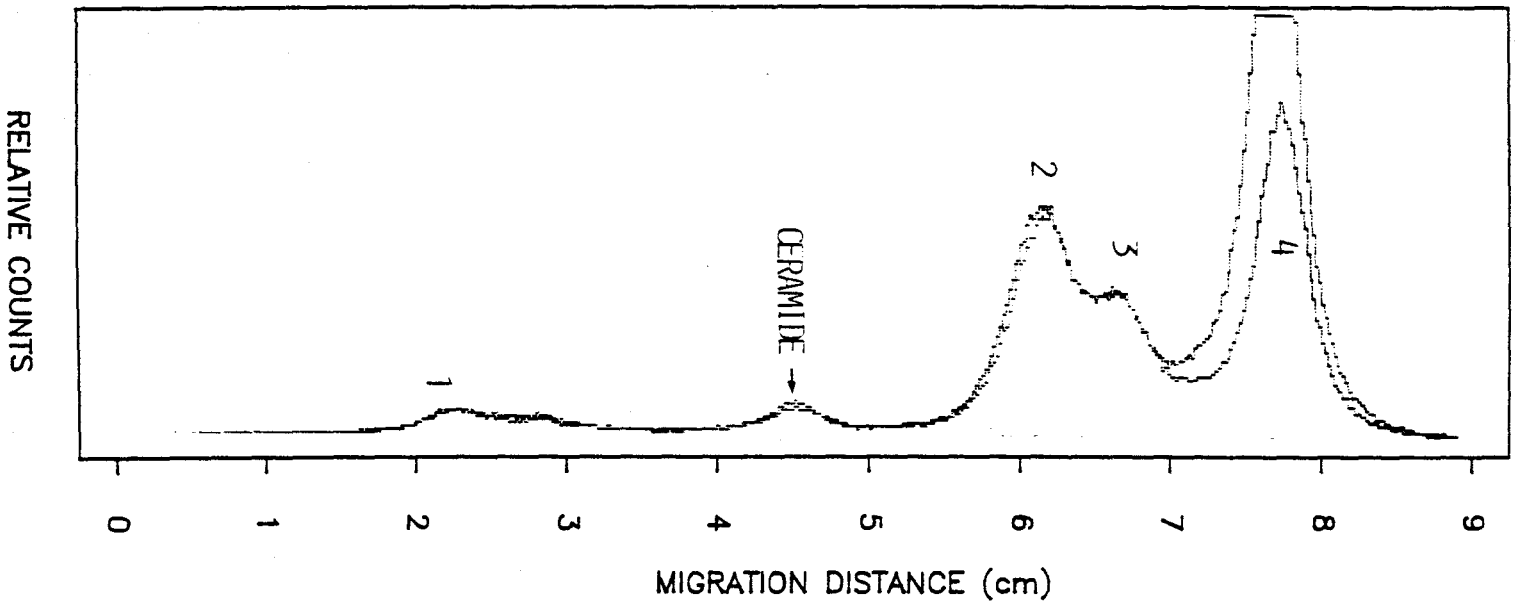


Figure 29

Primary neurons were labeled with [^{14}C]-acetate and treated with or without 0.1 µM monensin for 15 hours. Lipids were isolated and then separated by HPTLC. The HPTLC plate was placed under the radioactive TLC scanner for 10 mins. Peak 1 phospholipids, Peak 2 cholesterol, Peak 3 triglycerides and fatty acids, Peak 4 cholesterol esters

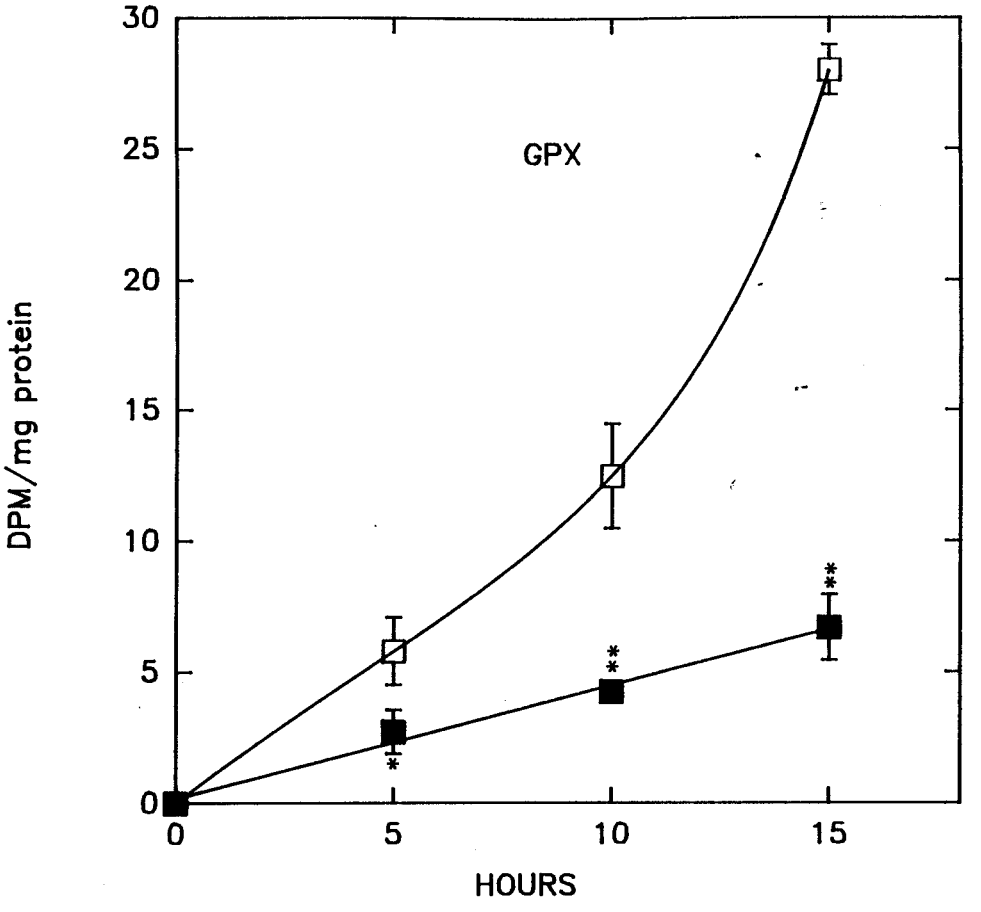


Figure 30

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GPX band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, *p<.05, **p<.01)

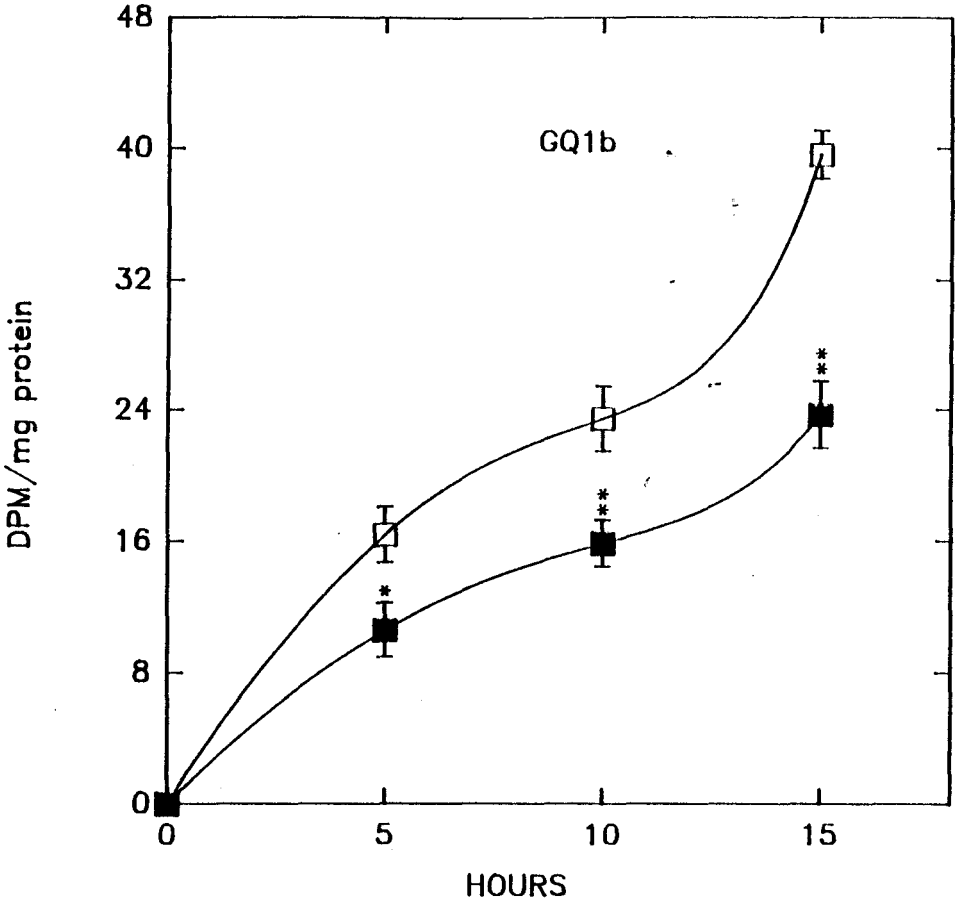


Figure 31

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GQ1b band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, *p<.05, **p<.01)

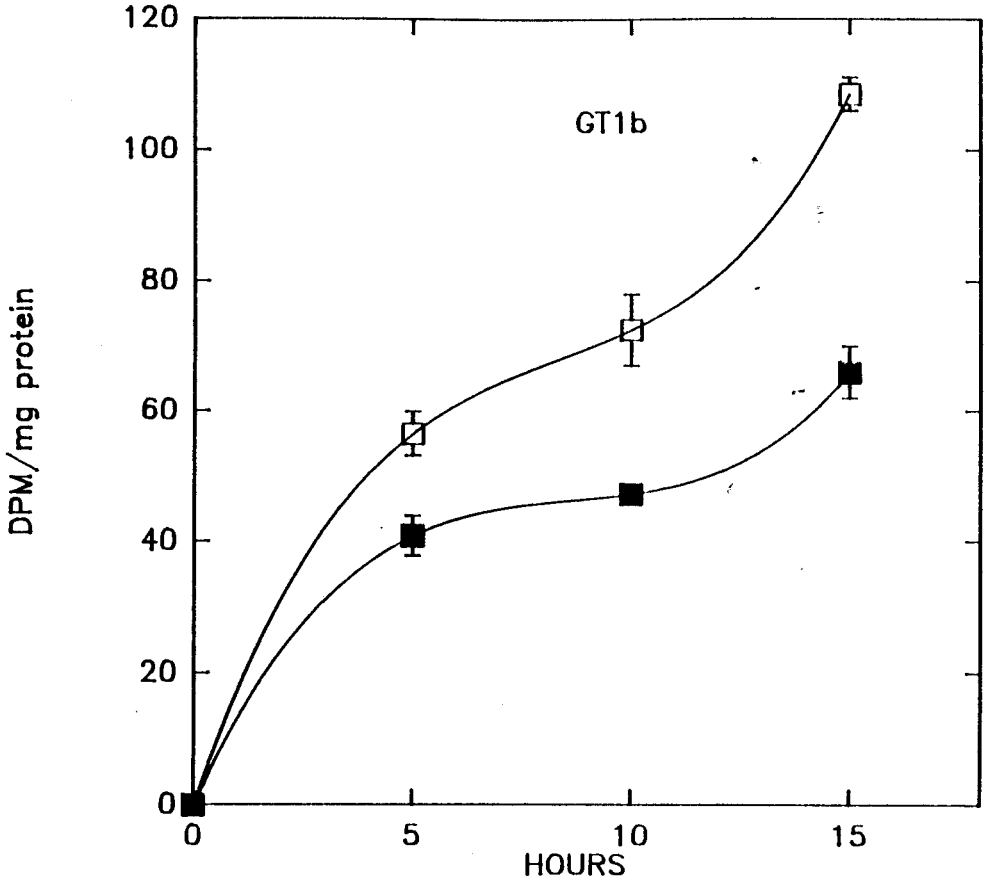


Figure 32

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GT1b band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, $^{**}p<.01$ for all time points when compared to control.

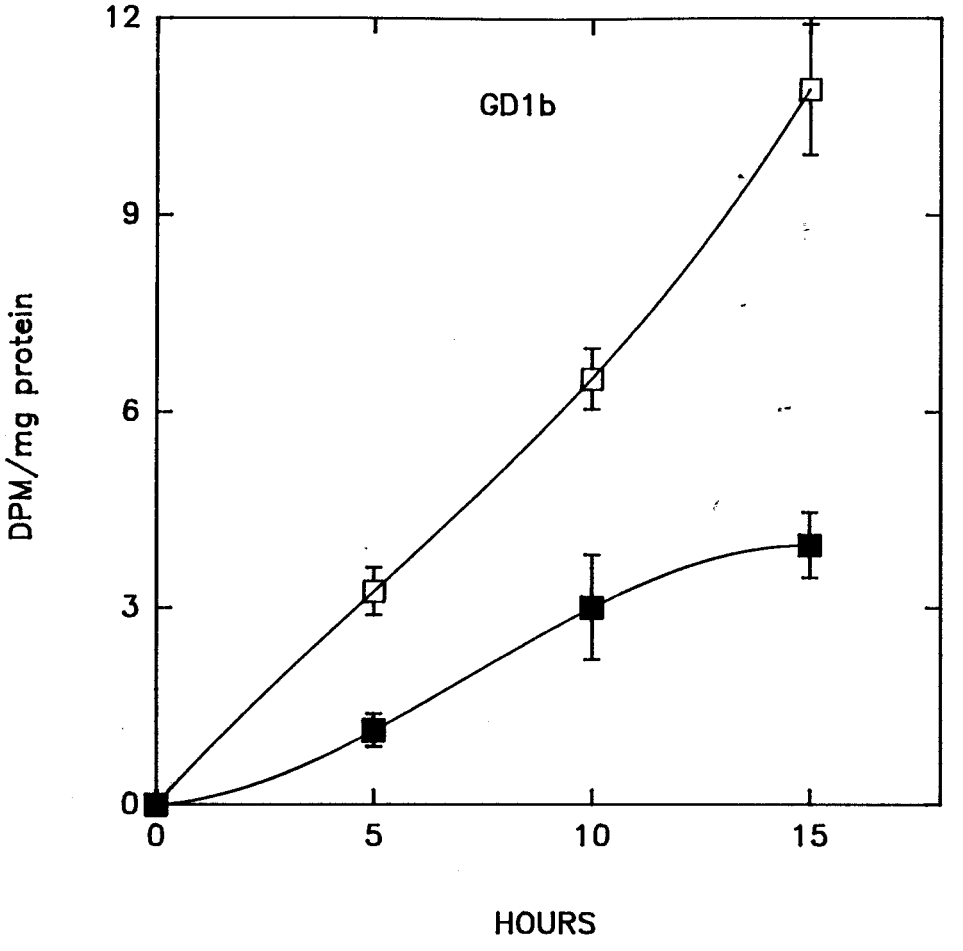


Figure 33

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GD1b band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, $^{**}p<.01$ for all time points when compared to control.

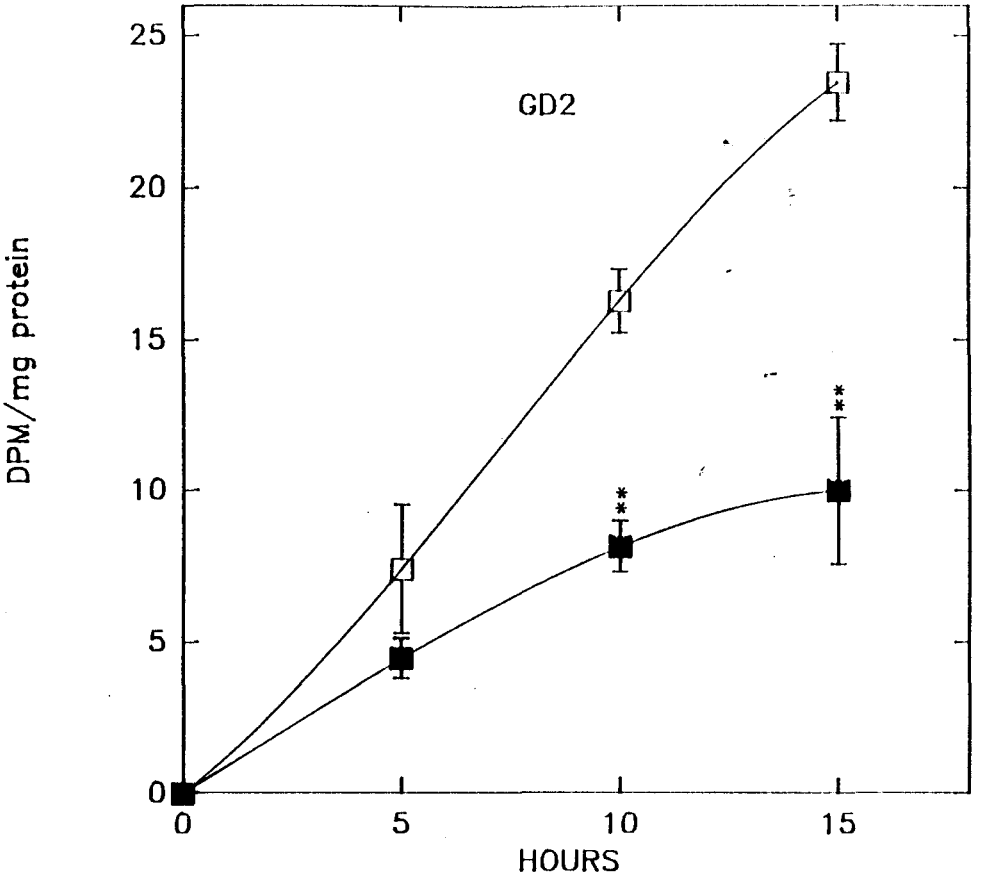


Figure 34

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GD2 band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, **p<.01 for all time points except 5 hour (not significant) when compared to control.

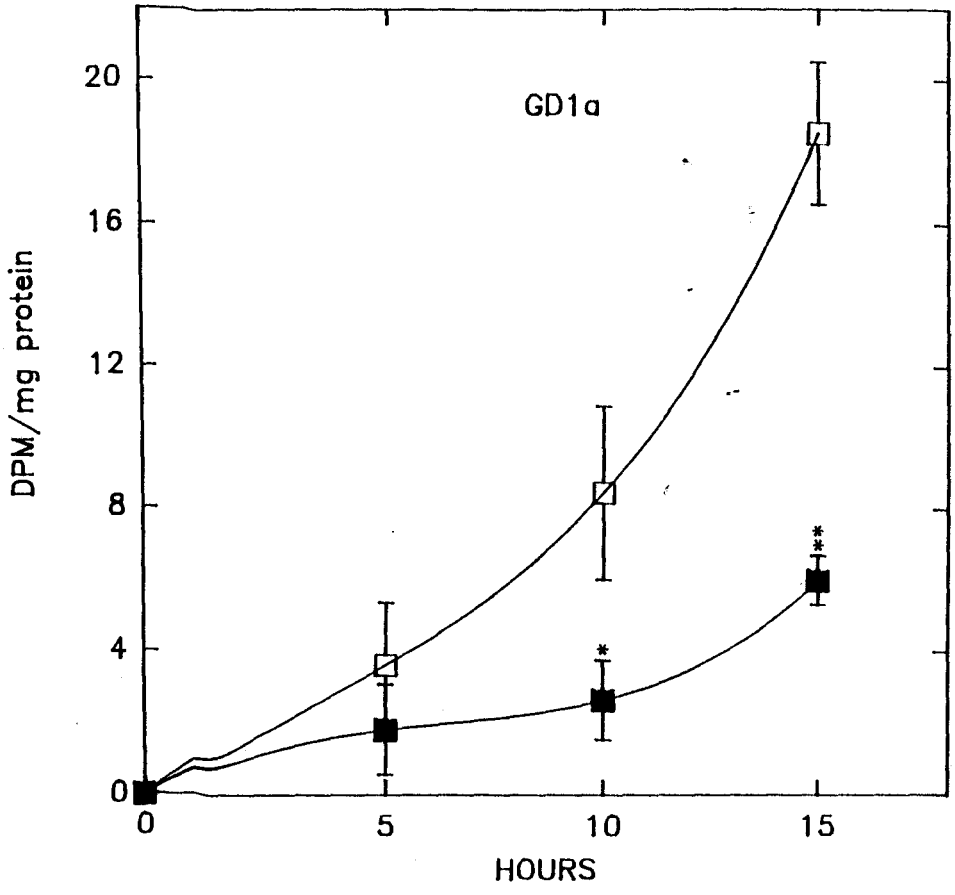


Figure 35

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GD1a band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, *p<.05, **p<.01, 5 hour time point was not significant when compared to control.

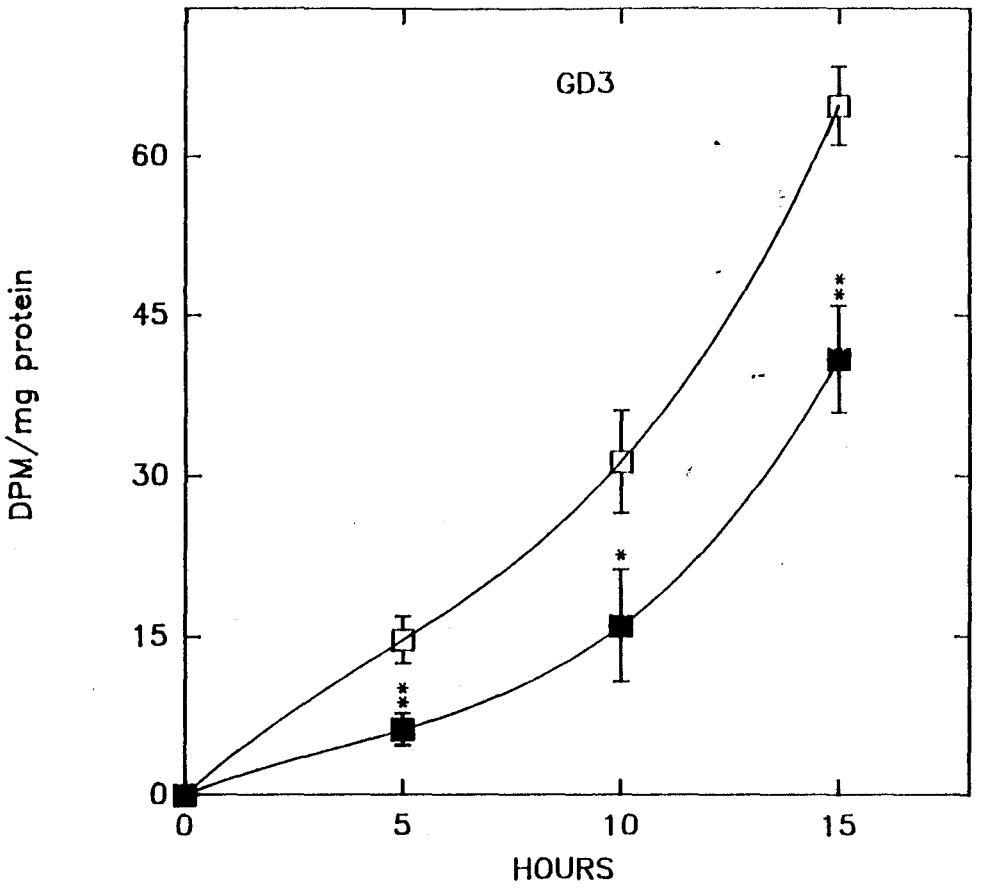


Figure 36

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 µM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GD3 band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 µM) treated cells. (n=3, ± SD, *p<.05, **p<.01)

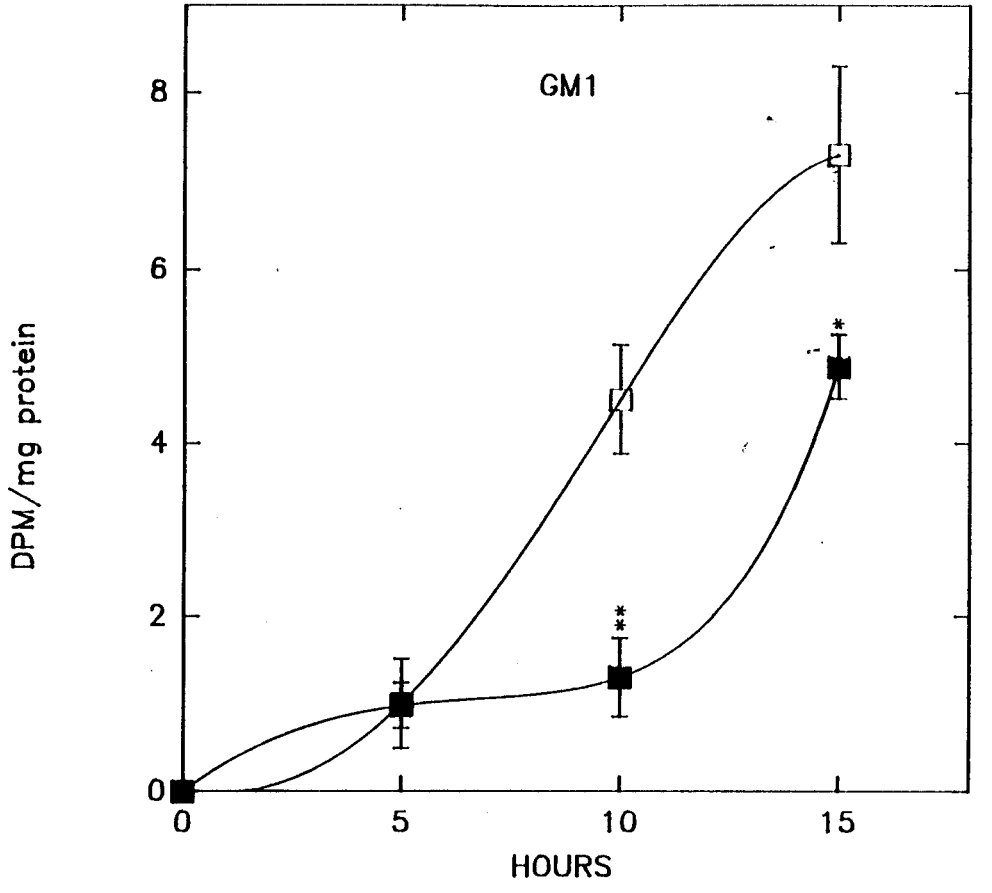


Figure 37

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 µM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GM1 band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 µM) treated cells. (n=3, ± SD, *p<.05, **p<.01, 5 hour time point was not significant when compared to control.

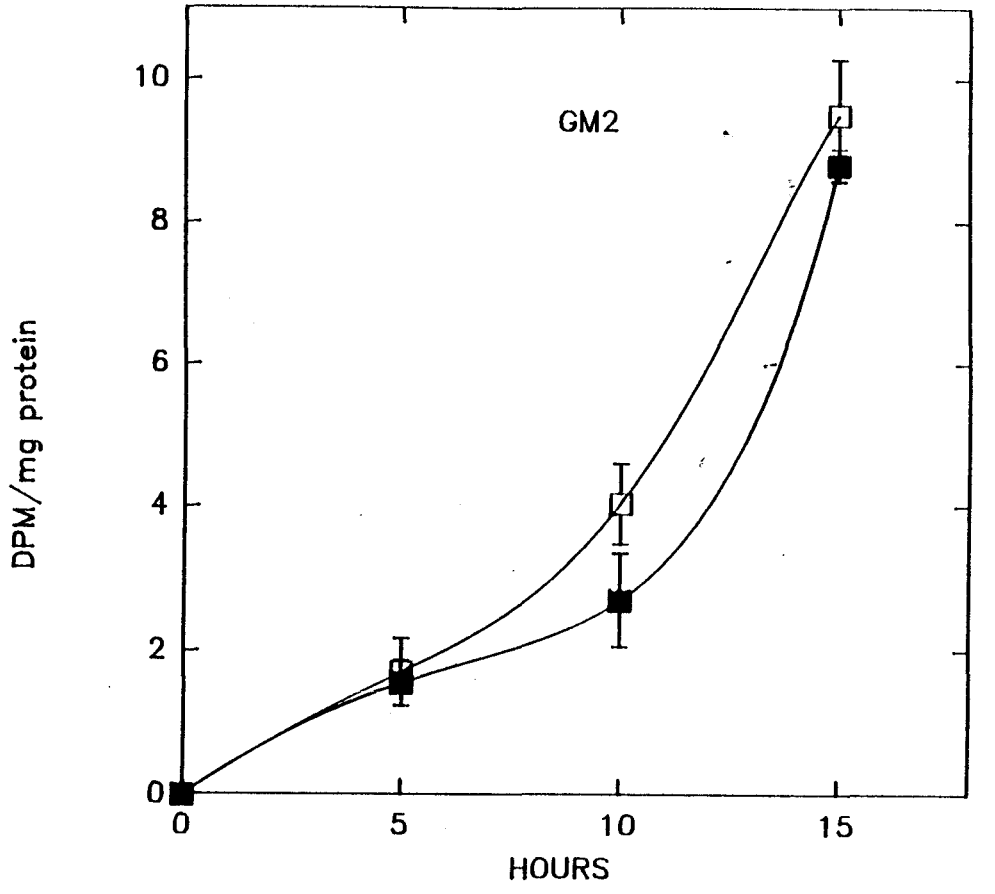


Figure 38

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 µM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GM2 band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 µM) treated cells. (n=3, ± SD, All time points were not significant when compared to control).

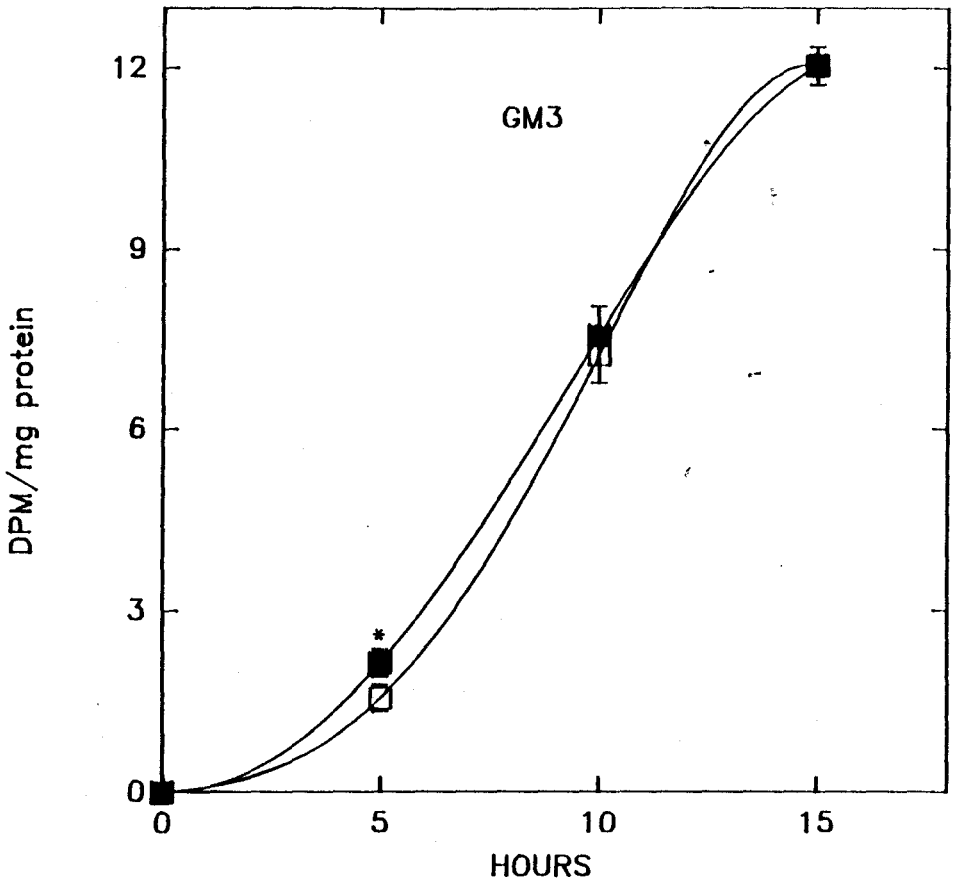


Figure 39

Primary neurons were labeled with [^{14}C]-N-acetylmannosamine and treated with or without 0.1 μM monensin for the times indicated. Gangliosides were isolated and then separated by HPTLC. The GM3 band was scraped and the amount of radioactivity was determined. Open squares - control Filled squares - monensin (0.1 μM) treated cells. (n=3, ± SD, All time points were not significant except for the 5 hour time point (*$p<.05$) when compared to control.

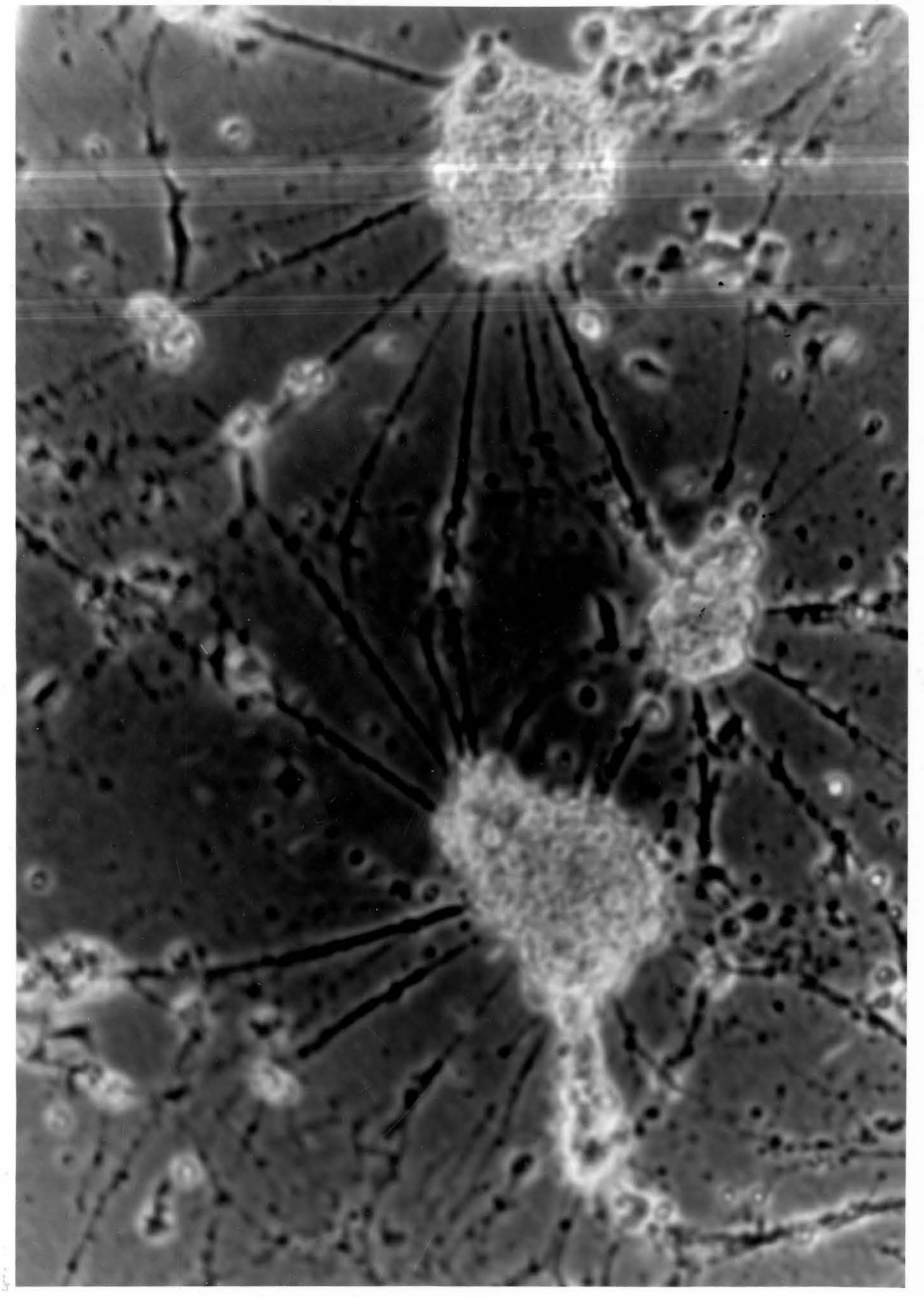


Figure 40

6 day primary neurons were exposed to 1.0 µM colchicine for 16 hours. The photograph of colchicine treated neurons is presented. (Magnification 100 X)

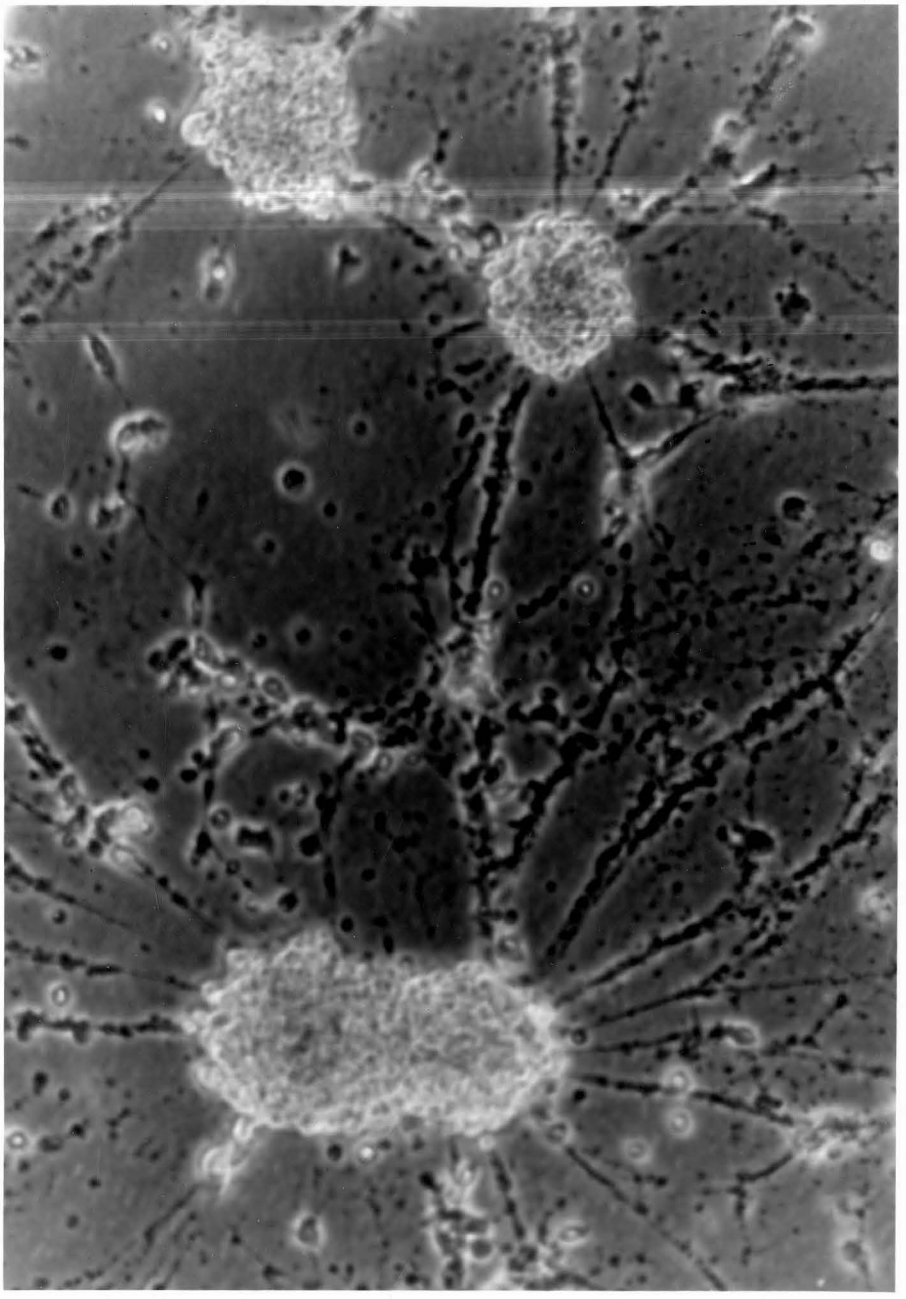


Figure 41

6 day primary neurons were exposed to 1.0 µM vinblastine for 16 hours. The photograph of vinblastine treated neurons is presented. (Magnification 100 X)

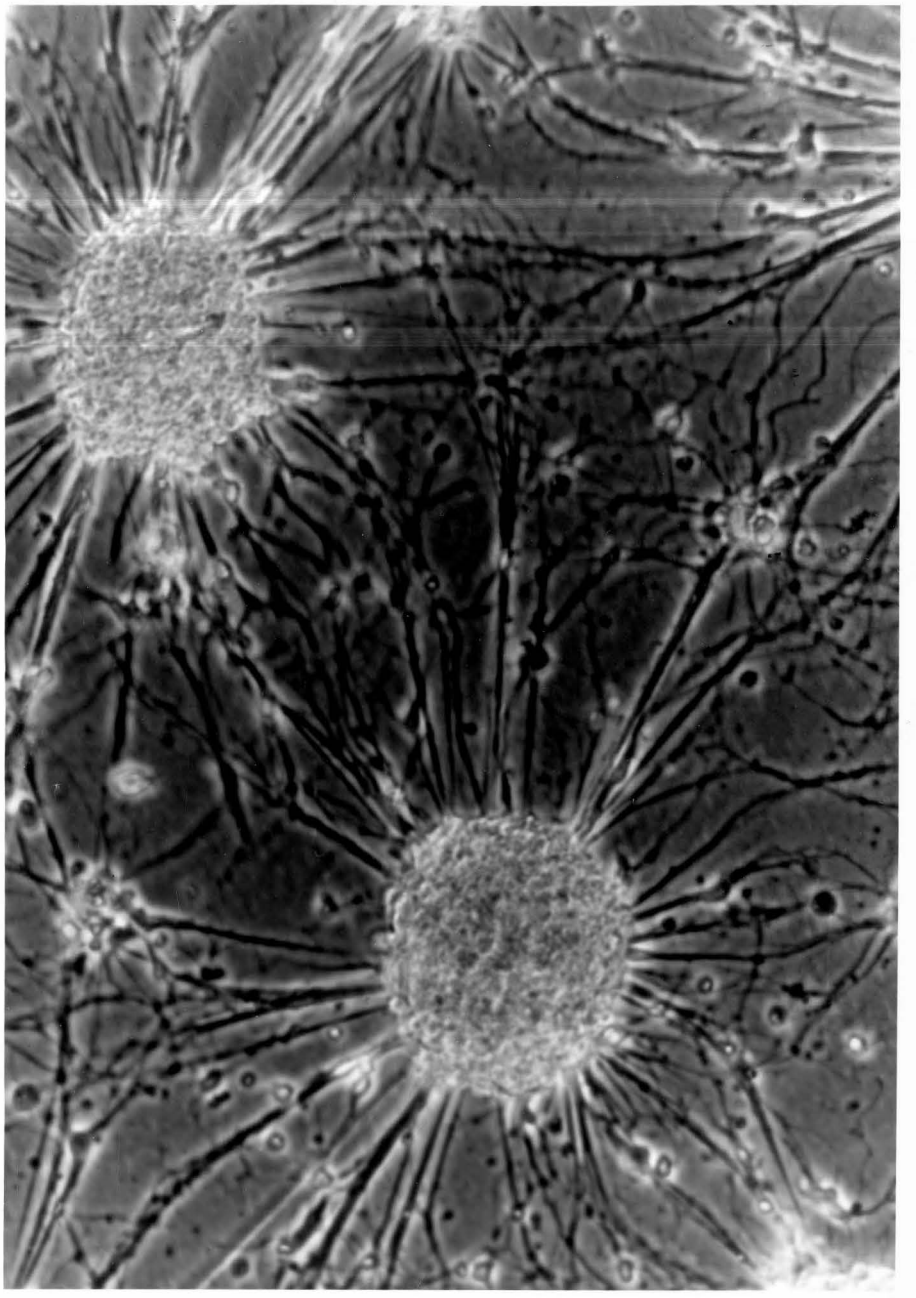


Figure 42

6 day primary neurons were exposed to 1.0 µM cytochalasin D for 16 hours. The photograph of cytochalasin D treated neurons is presented. (Magnification 100 X)

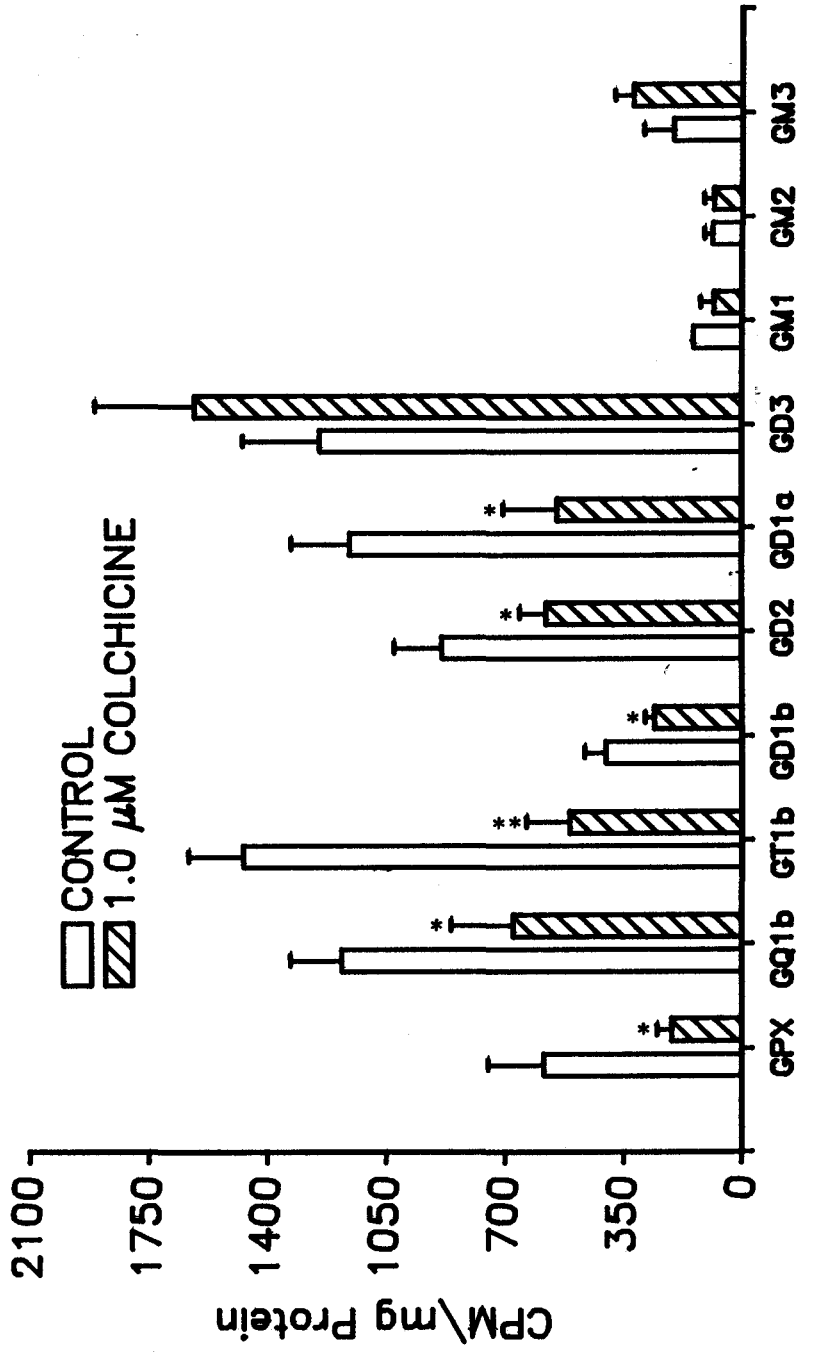


Figure 43

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 1.0 μM colchicine for 15 hours. Gangliosides were isolated and then separated by HPTLC. Individual ganglioside bands were scraped and the amount of radioactivity in each band was determined. (n=3, ± SD, *p<.05, **p<.01, remaining time points were not significant when compared to control.

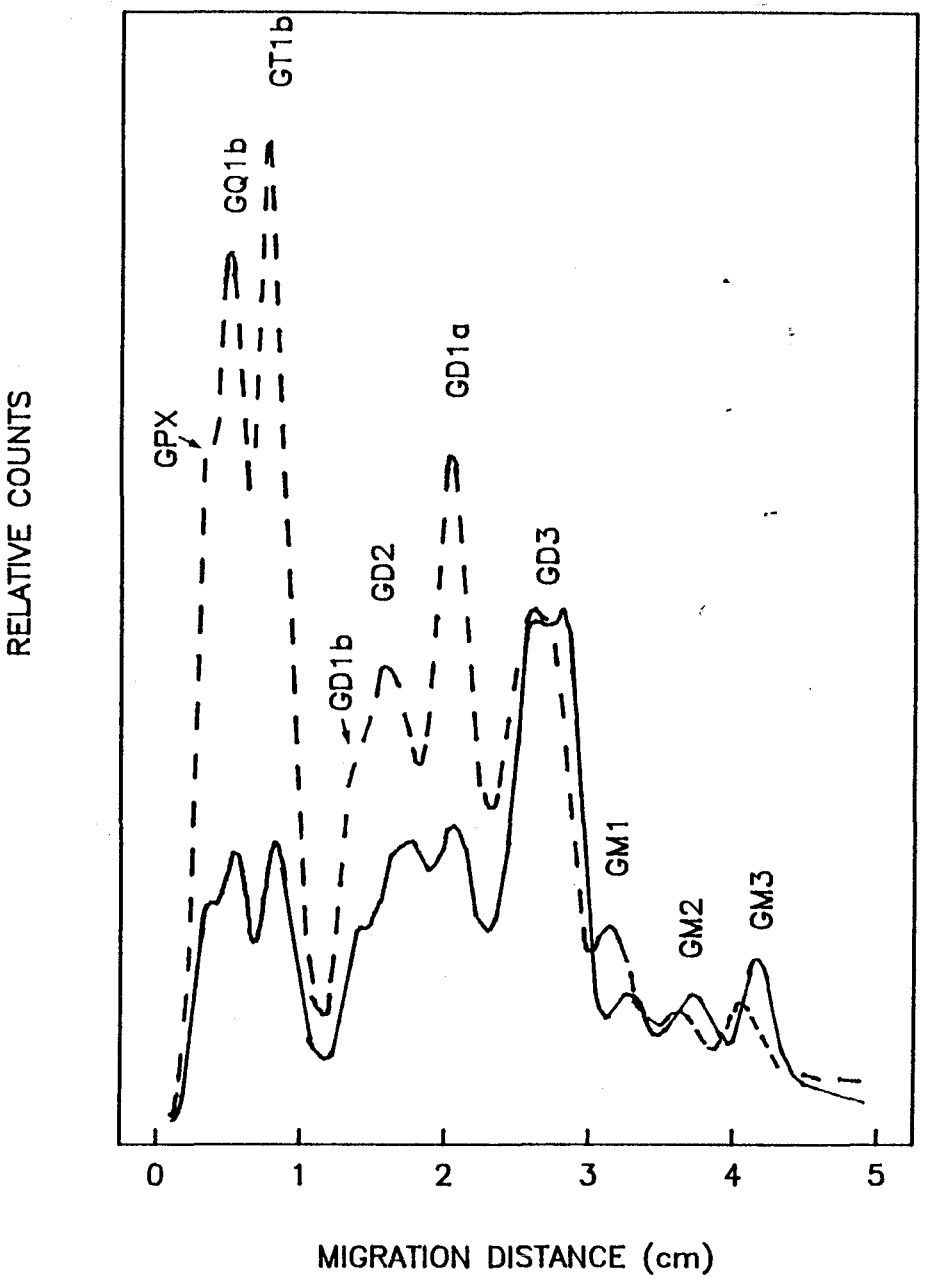


Figure 44

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 1.0 μM colchicine for 15 hours. Gangliosides were isolated and then separated by HPTLC. The HPTLC plate was placed under a radioactive TLC scanner for 10 mins. The RTLC scan comparing control (dotted line) to colchicine treated (solid line) is presented.

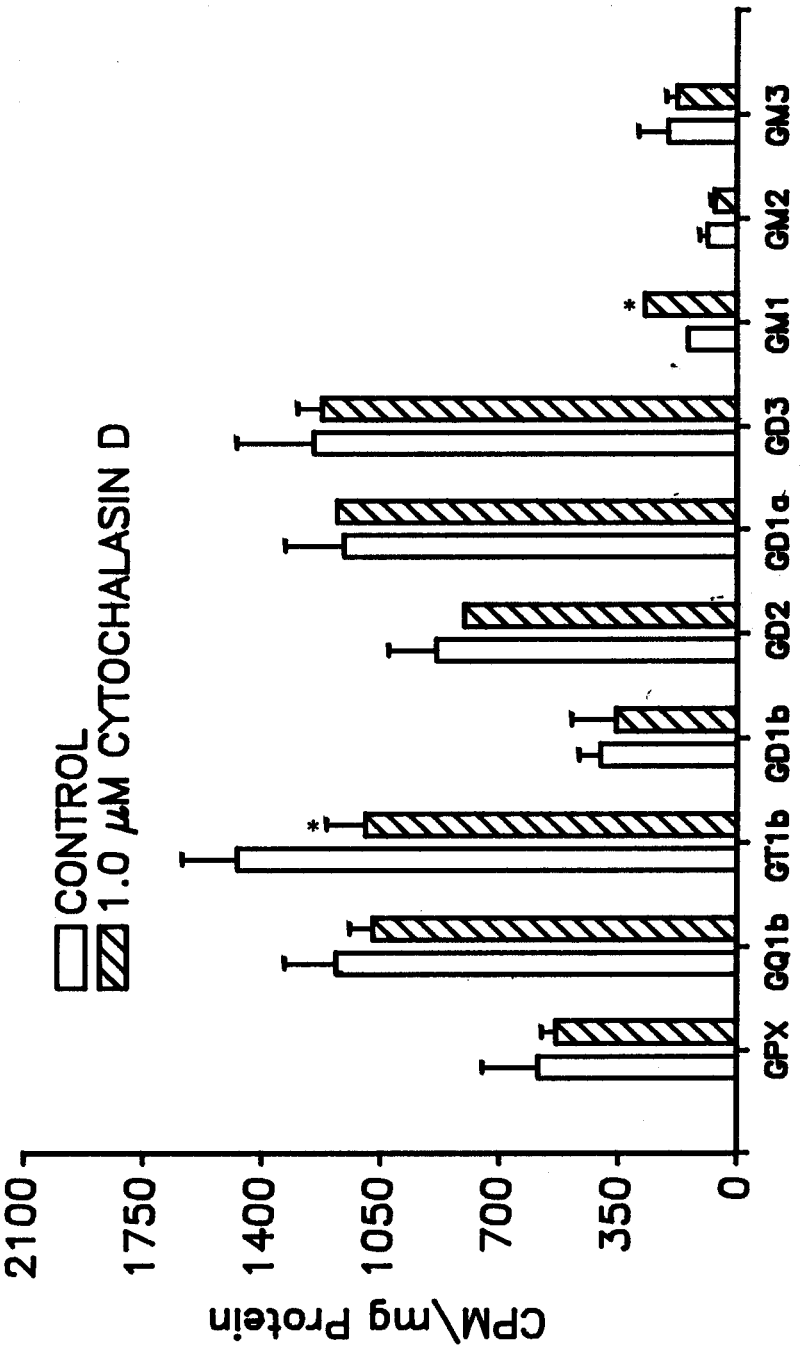


Figure 45

Primary neurons were labeled with [^{3}H]- galactose and treated with or without 1.0 μM cytochalasin D for 15 hours. Gangliosides were isolated and then were separated by HPTLC. Individual ganglioside bands were scraped and the amount of radioactivity in each band was determined. (n=3, ± SD, *p<.05, remaining time points were not significant when compared to control.

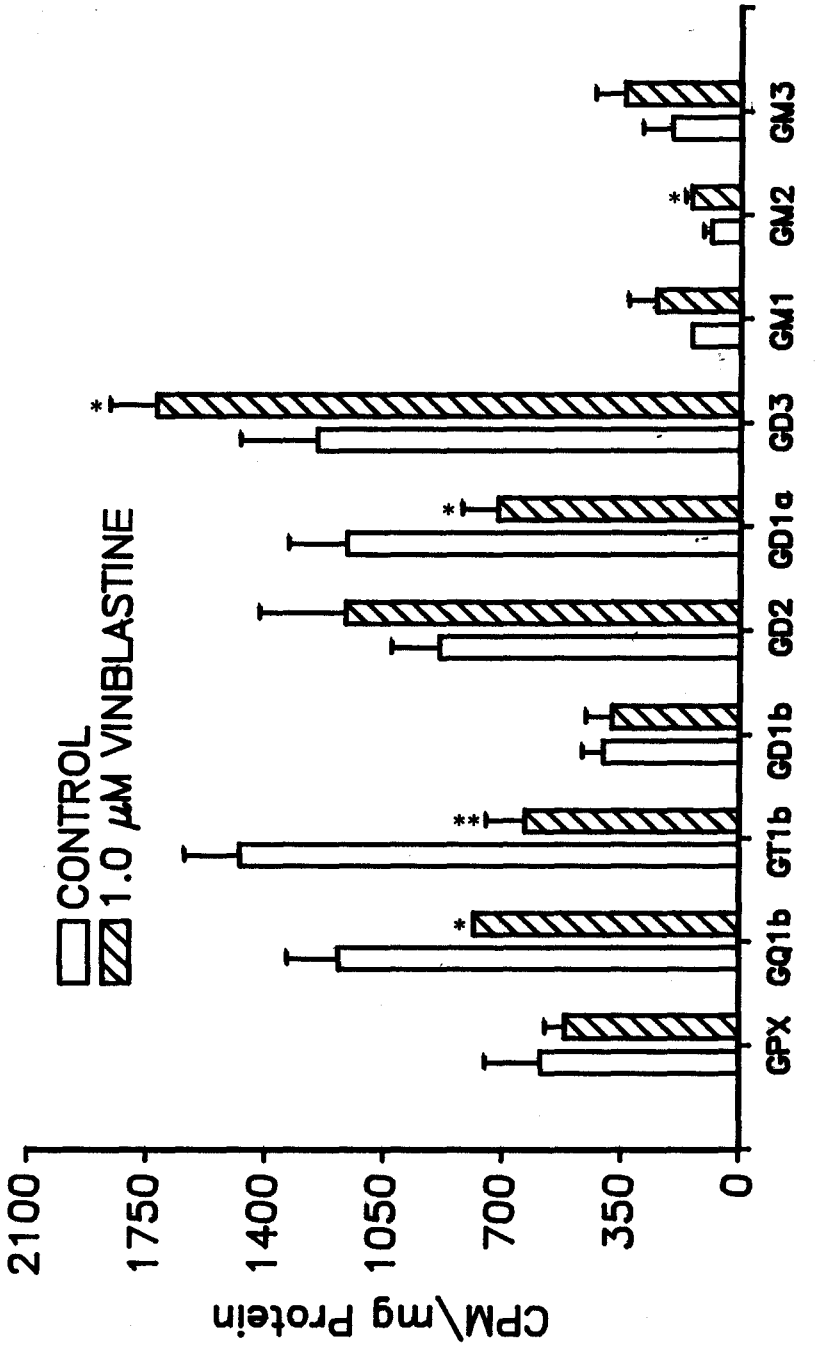


Figure 46

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 1.0 μM vinblastine for 15 hours. Gangliosides were isolated and then were separated by HPTLC. Individual bands were scraped and the amount of radioactivity in each band was determined. (n=3, ± SD, *p<.05, **p<.01, remaining time points were not significant when compared to control.

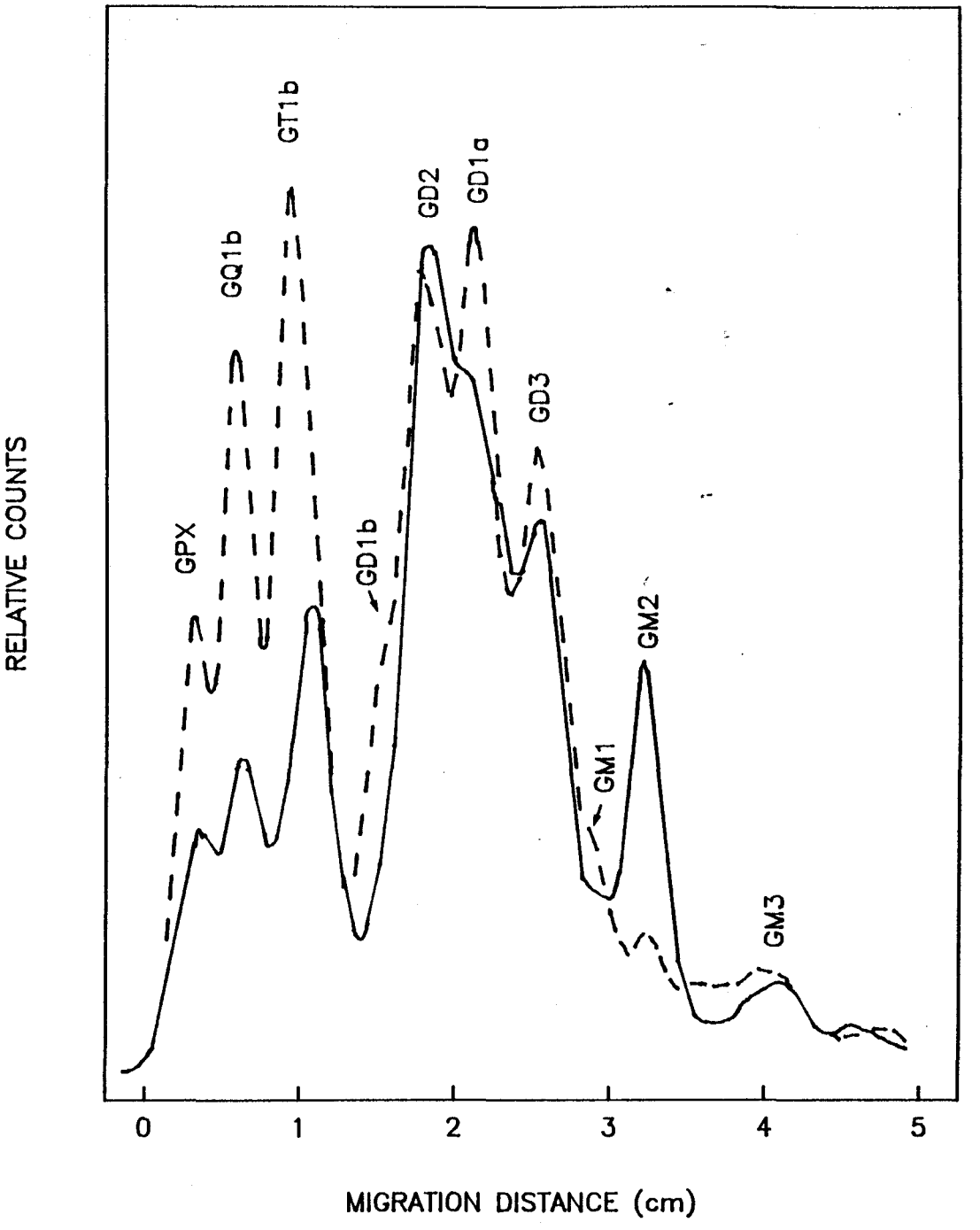


Figure 47a

Primary neurons were labeled with [^{3}H]- galactose and treated with or without cobalt (0.5 mM $CoCl_2$-$6H_2O$) for 18 hours. Gangliosides were isolated and then separated by HPTLC. The HPTLC plate was placed under a radioactive TLC scanner for 10 mins. The RTLC scan of control (dotted line) was compared to cobalt treated (solid line) neurons.

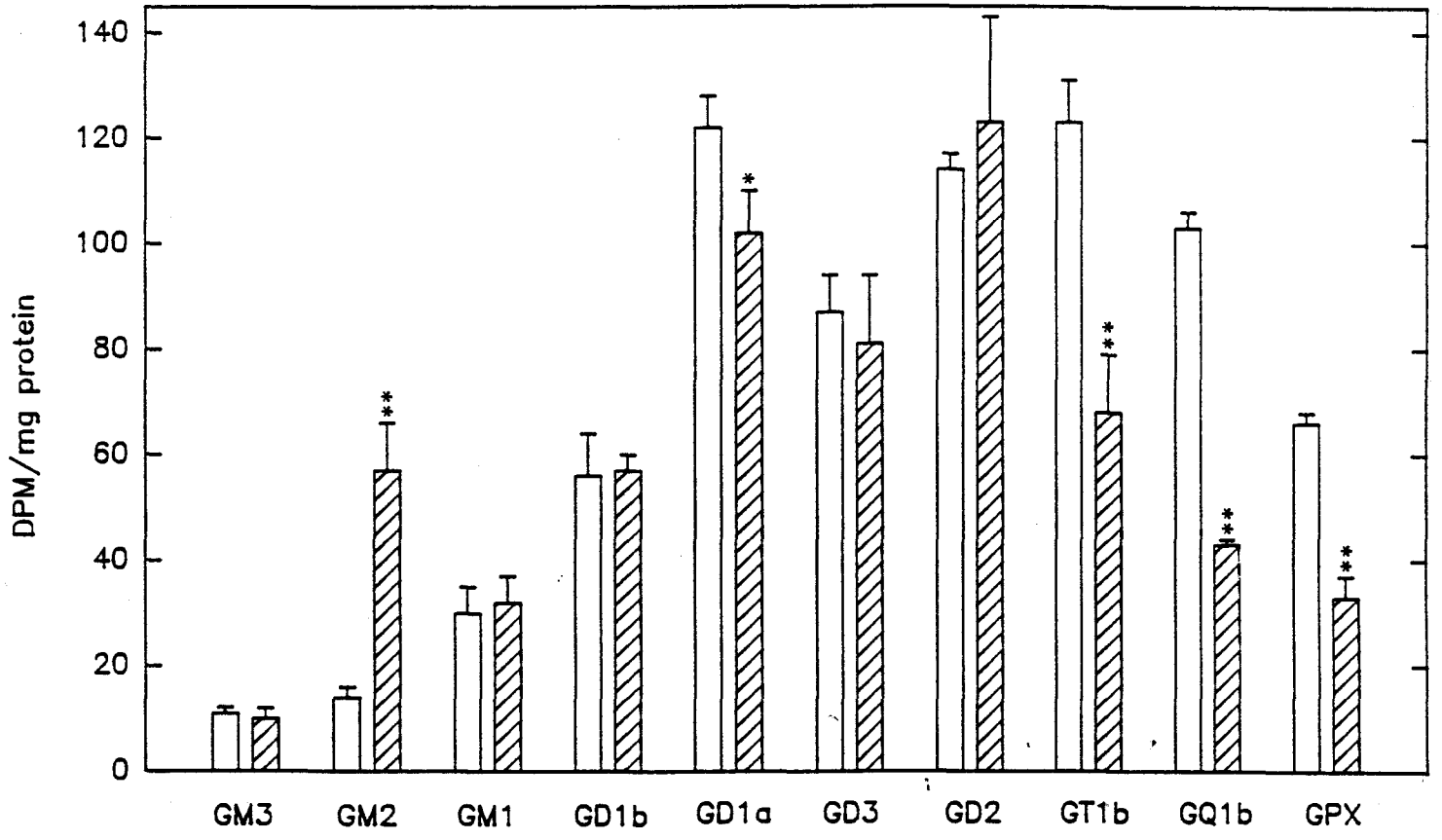


Figure 47b

Figure 47b Primary neurons were labeled with [^{3}H]- galactose and treated with or without cobalt (0.5 mM $CoCl_2$-$6H_2O$) for 18 hours. Gangliosides were isolated and then separated by HPTLC. Individual ganglioside bands were scraped and the amount of radioactivity in each band was determined. (control-open bars, cobalt-hatched bars)(n=3, ± SD, *p<.05, **p<.01, remaining time points were not significant when compared to control).

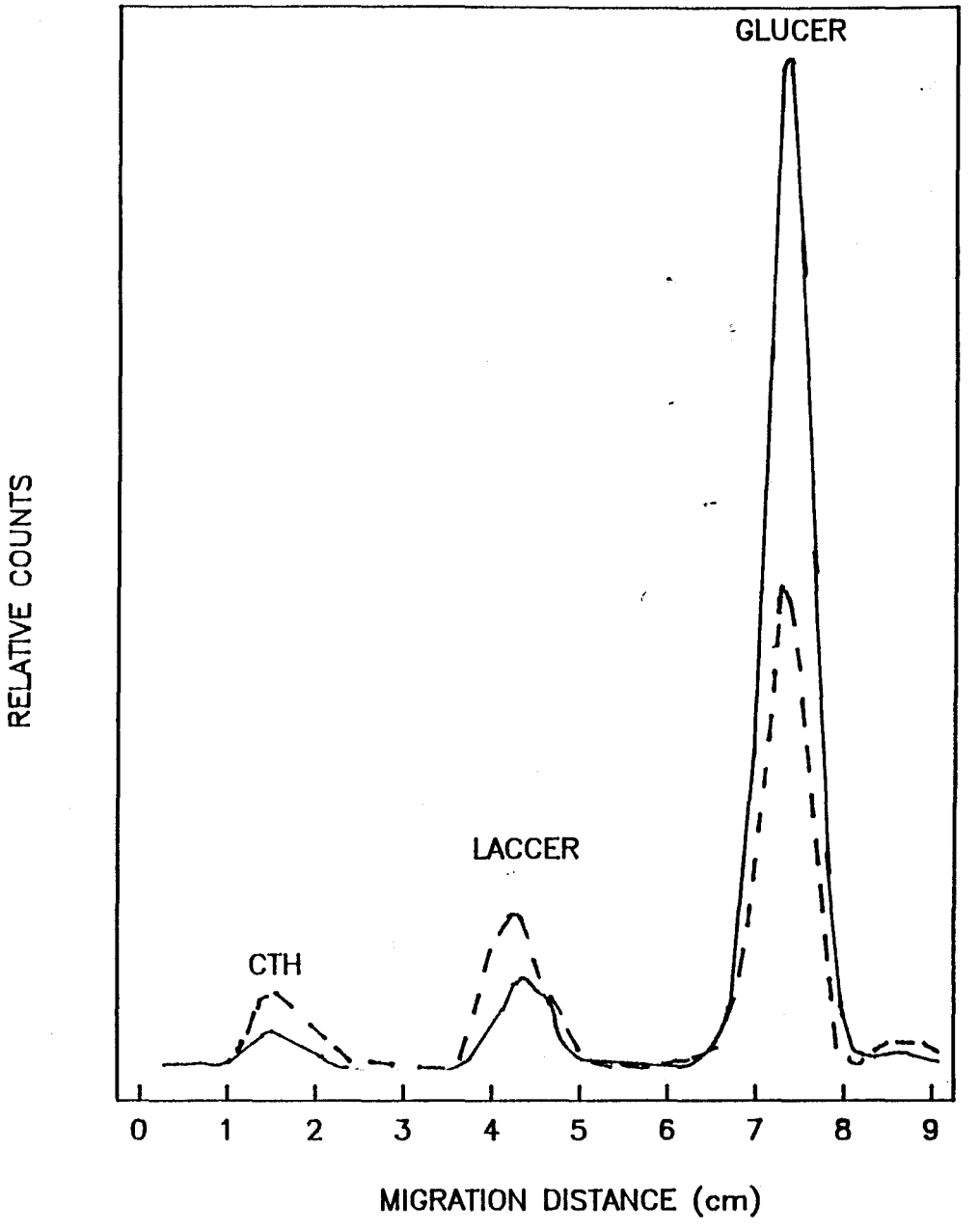


Figure 48

The neutral glycolipids of chick primary neurons were labeled with [^{3}H]-galactose and treated with or without cobalt (0.5 mM $CoCl_2$-$6H_2O$) for 18 hours. Neutral glycolipids were isolated and then separated by HPTLC. The HPTLC plate was placed under the radioactive TLC scanner for 10 mins. The RTLC scan of control (dotted line) was compared to cobalt treated (solid line) neurons.

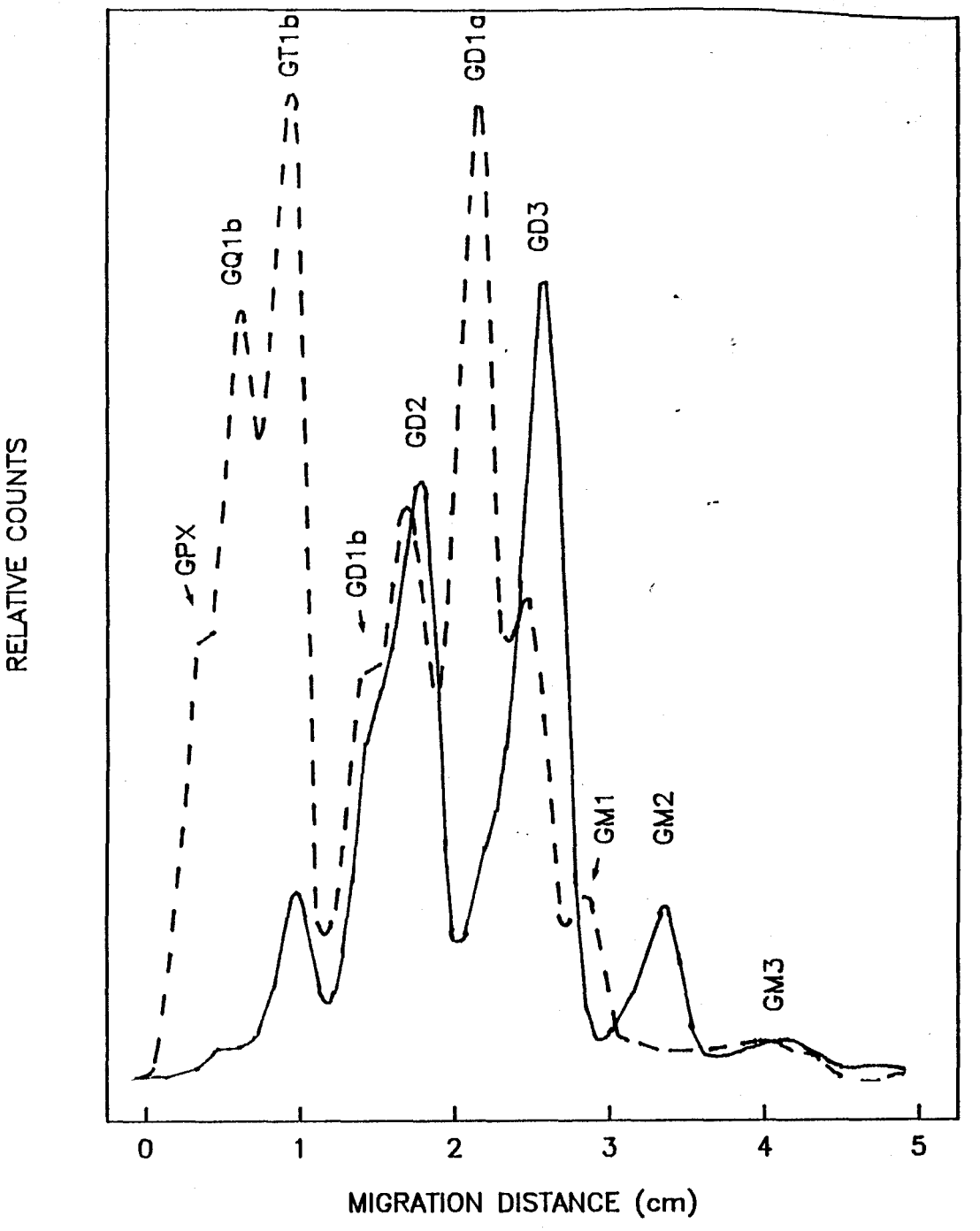


Figure 49a

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 1.0 μM A23187 for 15 hours. Gangliosides were isolated and then separated by HPTLC. The HPTLC plate was placed under a radioactive TLC scanner for 10 mins. The RTLC scan of control (dotted line) was compared to A23187 treated (solid line) neurons.

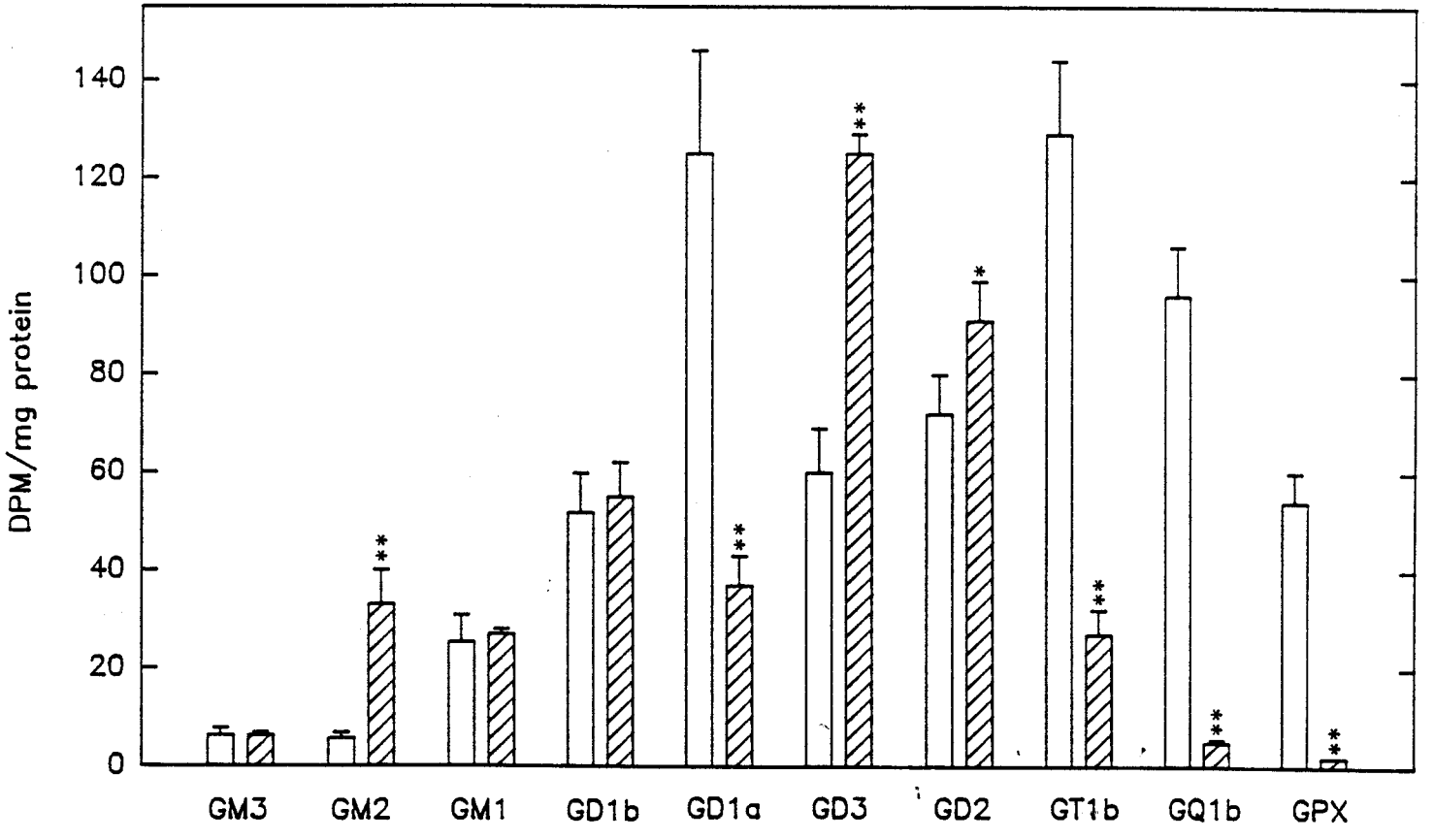


Figure 49b Primary neurons were labeled with [^{3}H]-galactose and treated with or without 1.0 µM A23187 for 15 hours. Gangliosides were isolated and then separated by HPTLC. Individual gangliosides were separated by scraping and the amount of radioactivity in each band was determined. (control-open bars, A23187-hatched bars)(n=3, ± SD, *p<.05, **p<.01, remaining time points were not significant when compared to control).

Figure 49b

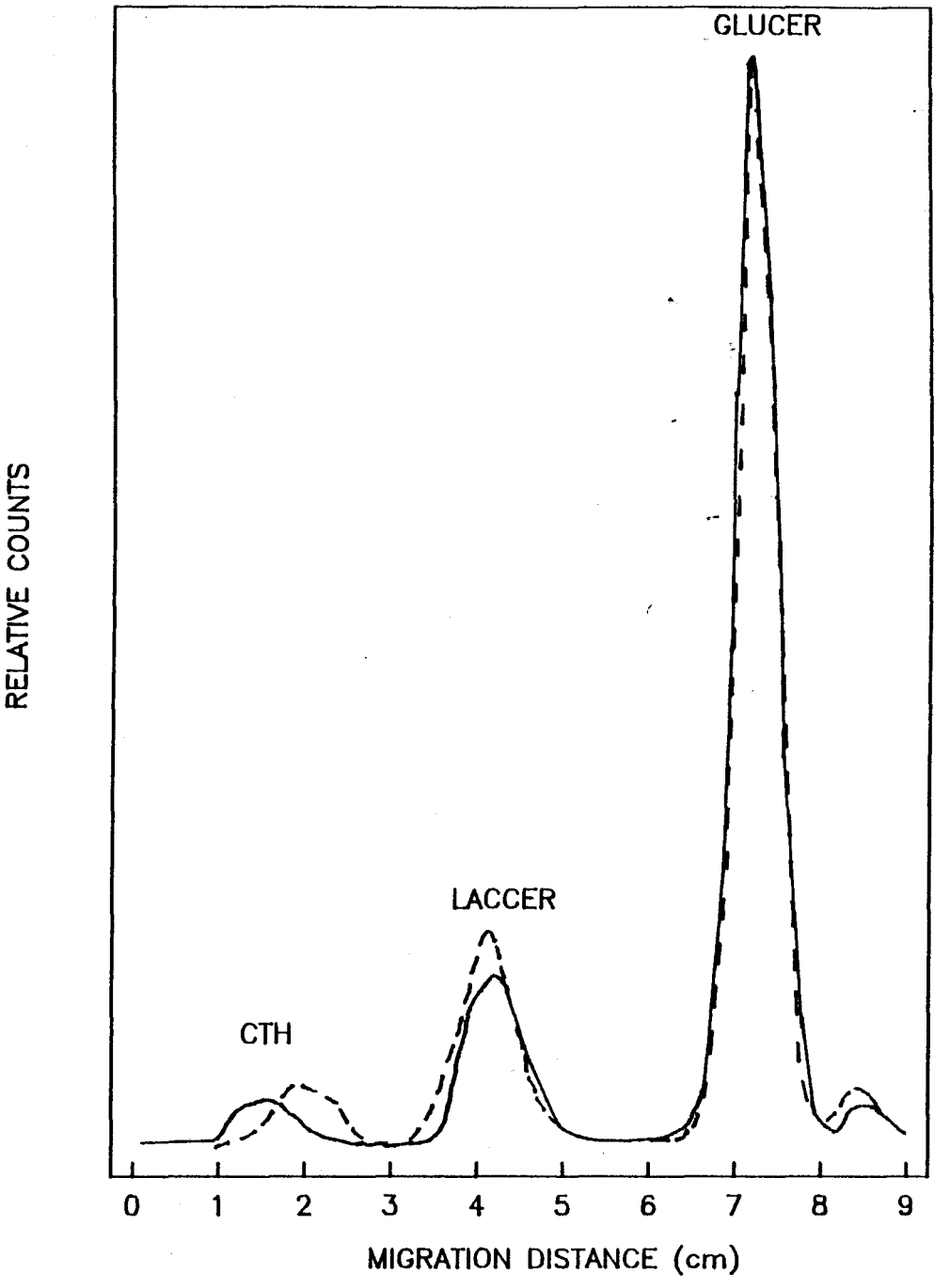


Figure 50

Primary neurons were labeled with [^{3}H]-galactose and treated with or without 1.0 μM A23187 for 15 hours. Neutral glycolipids were isolated and then separated by HPTLC. The HPTLC plate was placed under a radioactive TLC scanner for 10 mins. The RTLC scan of control (dotted line) was compared to A23187 treated (solid line) neurons.

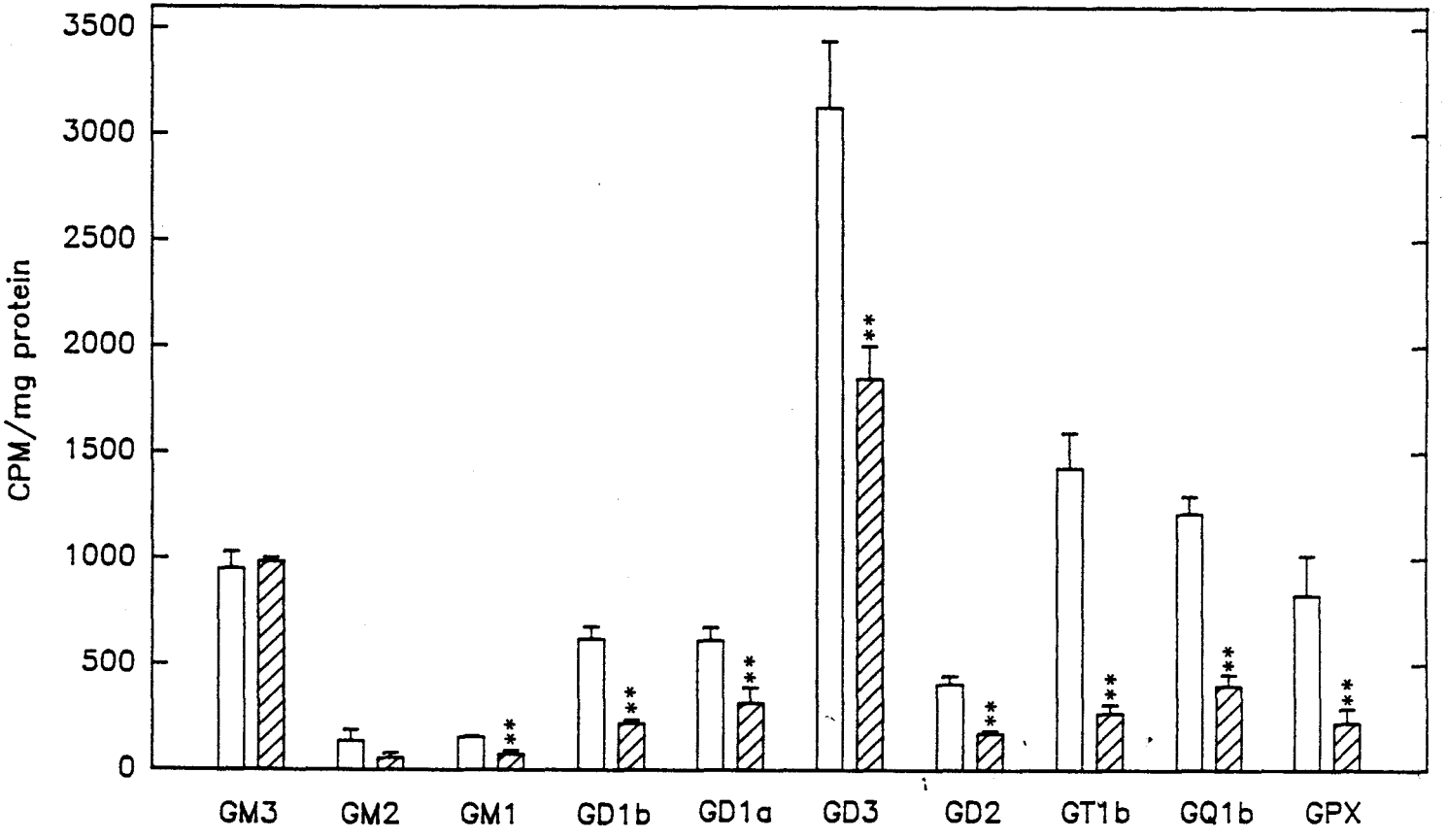


Figure 51 Primary neurons were labeled with [^{3}H]-galactose and treated with or without 0.1 µM nigericin for 18 hours. The gangliosides were isolated and then separated by HPTLC. Individual gangliosides were scraped and the amount of radioactivity in each band was determined. (Control - open bars, nigericin - hatched bars)(n=3, ± SD, **p<.01, remaining time points were not significant when compared to control).

Figure 51

CHAPTER V

DISCUSSION

Gangliosides are major constituents of the nervous system and are purported to play a wide variety of roles in neuronal functioning (Wiegandt, H. 1982). The biochemical metabolism and chemical characterization of gangliosides has been worked out in detail from in vitro studies. Addition of activated sugar substrates to ceramide is catalyzed by highly specific glycosyltransferases which add the substrates sequentially (Figure 2). Most of these glycosyltransferases are thought to be located at the luminal surface of the Golgi complex (see review). Studies have revealed that the Golgi complex is organized and compartmentalized. Some proteins which exit the endoplasmic reticulum, via vesicles, enter the cis face of the Golgi complex where they pass through the medial cisternae and then finally the trans cisternae in a sequential manner. Proteins exiting the Golgi complex are sorted and transported to there ultimate destination. As proteins pass through the Golgi complex they are acted upon by various enzyme activities which modify the protein structure. Evidence indicates that some of these enzyme activities are associated only with the cis, medial, or trans compartments of the Golgi complex which allows for an ordered processing. This same ordered processing may be important for metabolism

of gangliosides which are thought to pass through the Golgi complex. Specific glycosyltransferases may reside in a distinct compartment of the Golgi complex. Certain cisternae may also contain the postulated multiglycosyltransferase complexes which are thought to be comprised of two or more transferases. The transport of several classes of proteins through the Golgi complex can be inhibited by monensin, a monovalent cationophore. Monensin specifically disrupts Golgi processing events somewhere between the cis and trans cisternae. This disruption of processing may be a direct result of transport inhibition caused by the dilation of the cisternae or, as some speculate, may involve a decrease in transport of activated sugar substrates from the cytoplasm to the lumen of the Golgi. Recent evidence indicates that monensin does not inhibit activated sugar transport (Capasso, 1984). In one study there was an accumulation of activated sugars in the Golgi lumen of thymocyte cells that are treated with monensin (Verbert, et. al 1987).

Monensin has been used in C6 and N18 cells (Miller-Prodraza and Fishman, 1984), fibroblasts (Saito, et. al. 1985) and, in this study, on primary neurons to perturb glycosphingolipid processing. There are several similarities and several distinct differences between primary neurons and cell lines when monensin is used to disrupt labeling of gangliosides. Primary neurons contain several higher gangliosides (GPX, GQ1b, GT1b, GD1b, and GD2) which are not

found in the other cell lines studied and so can not be directly compared. The labeling of GPX, GQ1b, GT1b, GD1b, and GD2 is inhibited significantly when primary neurons are treated with monensin (Figure 16). Labeling of GD3 is only moderately inhibited by monensin in primary neurons (Figure 23) but is significantly inhibited in fibroblasts. GD1a labeling was significantly inhibited by monensin in C6 cells, N18 cells, fibroblasts, and in primary neurons (Figure 22). Labeling of the monosialogangliosides GM1 and GM2, was inhibited by monensin in N18 cells, fibroblasts, and primary neurons (Figures 24 and 25). Labeling of GM3 was reduced in C6 cells, N18 cells, and fibroblasts after monensin treatment but was unchanged in primary neurons (Figure 16) indicating a distinct difference between transformed cell lines and primary cells. There were also distinct differences in the labeling changes induced by monensin between neutral glycolipids in cell lines and primary neurons. Glucosylceramide labeling was greatly increased in C6 cells, N18 cells, and fibroblasts after treatment with monensin but the labeling of primary neurons was not significantly changed (Figure 28). The labeling of lactosylceramide was greatly increased in both primary neurons (Figure 27) and fibroblasts after monensin treatment but was actually reduced in both C6 cells and N18 cells. These striking differences seen between cell lines and primary neurons may suggest altered ganglioside metabolism in transformed cells. The inability of monensin to have any

inhibitory effect on the rate (Figure 26) or the specific activity of labeling in GM3, suggests that GM3 is synthesized in an extra-Golgi compartment. Since there is little change in GM3 labeling after monensin treatment this suggests a high turnover rate. The location of GM3 in an extra-Golgi compartment may be an important control point because ganglioside biosynthesis bifurcates at GM3 to form the "a" series gangliosides and the "b" series gangliosides.

Nigericin was also examined for its effect on ganglioside anabolism in primary neurons. The results with nigericin confirm the results seen with monensin (Figure 51). Labeling of GM3 was unaffected by nigericin after 18 hours treatment. GD3 was inhibited to a lesser degree by nigericin then the other gangliosides. Nigericin also increased the label incorporation into lactosylceramide but had no effect on incorporation into glucosylceramide (Table 4).

These studies on primary neurons were also carried out using [^{14}C]- N-acetyl-mannosamine in order to specifically label the gangliosides and to determine if there were any significant differences when compared to [^{3}H]-galactose labeling. The inhibition of ganglioside labeling ([^{14}C]-N-acetyl-mannosamine) caused by monensin was qualitatively similar to the inhibition of labeling seen with [^{3}H]-galactose except for GM2 (Figures 30 to 39). The labeling of ganglioside GM2 by [^{14}C]-N-acetyl-mannosamine was unaffected by monensin treatment (Figure 38) but was inhibited when [3H]-

galactose was used as a label (Figure 26).

Cytoskeletal inhibitors provided further evidence for the compartmentalization of ganglioside anabolism. The higher gangliosides GPX, GQ1b, GT1b, GD1b, GD1a, and GD2 had decreased incorporation of label when treated with colchicine (Figure 43). There was no change in the labeling of GM3 and GD3 after colchicine treatment. Colchicine also had no effect on the label incorporation into glucosylceramide but had a small inhibitory effect on labeling of lactosylceramide (Table 6).

Cytochalasin D (1.0 μM) which inhibits polymerization of actin had little effect on the labeling of gangliosides (Figure 45) or on neutral glycolipids (Table 6). Although cytochalasin D can interfere with microtubule contraction this data would suggest that it has little effect on the processing events in the Golgi.

Vinblastine which crystallizes free tubulin caused a small increase in labeling of GD3 (Figure 46) and had no effect on the labeling of GM3 in primary neurons. The labeling of GT1b, GQ1b, and GD1a was inhibited by vinblastine. This data with cytoskeletal inhibitors supports the idea outlined above that GM3 is regulated differently intracellularly than the other gangliosides. That GM3 is unique among gangliosides is supported by several other studies. HeLa cells contain mostly GM3 and GD3 and changes in the morphology of these cells is closely associated with GM3 synthesis (Fishman and Brady, 1976). Rapid differentiation of epithelial cells

is also closely associated with GM3 synthesis (Hakomori, 1981). Epidermal growth factor dependent phosphorylation of the EGF receptor appears to be regulated by GM3 (Hanai, 1988) and may regulate cell growth. The growth of 3T3 cells, KB cells, and A431 cells was inhibited by GM3 (for review see Usuki, 1988). Usuki (1988) speculates that the removal of sialic acid from GM3 at the membrane surface may regulate cell growth. The only anabolic defect in ganglioside biosynthesis discovered to date was in a patient who possessed only GM3 and GD3 in the brain (Kanfer, 1983). GM3 is the major ganglioside found in many mammalian tissues. Changes in GM3 levels and in GM3 synthetase are associated directly with oncogenic transformation (Hakamori, 1981). The data in this paper supports the hypothesis that GM3 is an important ganglioside and shows that the synthesis of GM3 is very closely regulated.

The cytoskeletal inhibitors had little effect on the labeling of the neutral glycolipids. This supports the idea that neutral glycolipids are synthesized in the endoplasmic reticulum or in a pre-Golgi compartment and that there is a separation of the initial glycosphingolipid glycosylation site from that of higher glycosylations. Treatment of primary neurons with cobalt and A23187 also supports the hypothesis of distinct glycosylation sites

Cobalt caused a significant reduction in labeling of the higher gangliosides (GPX, GQ1b, and GT1b) in primary neurons (Figure 47b). The labeling of GM3 and GD3 remained

unchanged after cobalt treatment which again suggests the close regulation of these gangliosides. This data also suggests that higher glycosylation sites are distinct from intermediate glycosylation sites. One significant difference between cobalt treatment and monensin treatment was the enhancement of label into GM2 after cobalt treatment. Both GD3 and GM2 are the first step in the "b" series and in the "a" series after GM3 branches. There may be a distinct regulatory component which affects which pathway ganglioside anabolism will follow. Cobalt, which is thought to specifically inhibit transport between the endoplasmic reticulum and the cis face of the Golgi, caused a large increase in the incorporation of label into glucosylceramide but a reduction of label into lactosylceramide. This data suggests that cobalt interferes at another earlier step of ganglioside anabolism than does monensin. This provides further evidence for a distinct compartmentalization of glycosylation sites.

When primary neurons are treated with the calcium ionophore A23187 there is an large inhibition of label into GPX, GQ1b, GT1b, and GD1a (Figure 49a and 49b). A23187 had no effect on the label incorporation into GM1, GM3, and GD1b. The incorporation of label into GD3 and GM3 was greatly increased by treatment with A23187. The increase in calcium levels and the corresponding increase in labeling of GD3 and GM2 after treatment with A23187 suggest a role for calcium in regulating which pathway GM3 will follow. This data also

supports the idea of separate compartmentalization of neutral glycolipid and ganglioside anabolism. There was no apparent effect of A23187 on the incorporation of label into lactosylceramide or glucosylceramide (Table 8).

SUMMARY

The transport pathways of gangliosides in chick primary neuronal cultures were examined by perturbing these pathways with monensin, colchicine, vinblastine, cytochalasin D, cobalt, and A23187. Results from this study confirm that there is a distinct compartmentalization of earlier glycosylation sites from later glycosylation sites in the ordered processing of gangliosides. Cobalt inhibits the earliest step in the biosynthetic pathway of gangliosides because it caused a significant increase in labeling of glucosylceramide (Table 7) while the other inhibitors had no effect. The synthesis of glucosylceramide may reside in the endoplasmic reticulum because cobalt is known to interfere with transport between the endoplasmic reticulum and the Golgi. There was a significant enhancement of label into lactosylceramide (Table 4) after monensin and nigericin treatment but the other inhibitors had no effect. Lactosylceramide synthesis may be in an extra-Golgi compartment. When the Golgi is disrupted by monensin treatment the transport from this extra- Golgi compartment to the Golgi may be inhibited.

There was no significant change in the labeling of GM3 for any of the inhibitors studied. Synthesis of GM3 appears to be very closely regulated and might be synthesized in a rapid anabolic extra-Golgi compartment. There is no change in the labeling of GM3 yet there is an increase in the labeling

of lactosylceramide. If monensin is stimulating lactosylceramide synthesis you might think that GM3 would be inhibited but this is not the case. One might speculate that two pathways may exist at the dynamic border between the endoplasmic reticulum and the Golgi. One pathway for ganglioside biosynthesis might destine lactosylceramide to be delivered to the Golgi while the second pathway might be destined for the plasma membrane. Lactosylceramide may be required for ectoenzyme synthesis of gangliosides on the plasma membrane (Dreyfus et. al). Since there are two pathways for lactosylceramide to take it may be only one pathway that is significantly affected by monensin. The incorporation of label into higher gangliosides (GPX, GQ1b, GT1b, GD1b ,GD1a, and GD2) are inhibited by monensin yet there is no accumulation of label into GM3. This lack of change in GM3 caused by all the inhibitors studied may result from a high turnover of this ganglioside. Such a high turnover might suggest an important control point in ganglioside biosynthesis. It is also possible that GM3 synthesis is regulated at the plasma membrane surface and therefore these intracellular inhibitors would have little effect. The fact that GM3 remains in transformed cells while the higher gangliosides are lost also suggests that GM3 is important for the cell.

Transport between this extra-Golgi compartment and the cis face of the Golgi may involve calcium regulation because the calcium ionophore A23187 causes an enhancement of

label into GD3 and GM2 which are both the next step in ganglioside synthesis after GM3. The gangliosides in the pathways following GM2 and GD3 have greatly diminished labeling after treatment with A23187. This could have important implications in the control of ganglioside anabolism. The cell might control ganglioside anabolism by altering calcium concentration in the local area of ganglioside biosynthesis. This study reveals for the first time a compartmentalization of key ganglioside biosynthetic pathways and an ordered processing of ganglioside metabolism in the primary neuron.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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