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The Effect of Dietary Ganglioside on Lipid Composition of Synaptosomal Plasma Membrane and Myelin Fractions of Developing Rat Brain

by

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ABSTRACT

Gangliosides are sialic acid-containing glycosphingolipids, concentrated in the central nervous system. Gangliosides are involved in the growth, development, and function of neurons. Human milk is a dietary source of gangliosides for the developing infant. However, infants fed formula receive little to no nutritional ganglioside. Recent studies have shown that dietary ganglioside affects total ganglioside content of whole brain tissue in weanling rats. This research examines if differences in dietary ganglioside affect the lipid composition of synaptosomal plasma membrane and myelin fractions of developing rat brain.

Weanling rats were fed nutritionally complete diets varying only in lipid composition. The protein, ganglioside, phospholipid, sphingolipid, and cholesterol composition of synaptosomal membrane and myelin brain fractions was analyzed. Dietary treatment increased the relative proportion of GM_4 and lysophosphatidylcholine in synaptosomal plasma membrane, as well as myelin GQ_{1b} and glucosylceramide. Alterations in the lipid composition of synaptosomal membrane and myelin indicate changes in brain function.

This thesis is dedicated to the love of science and the pursuit of truth.

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ABBREVIATIONS

ANOVA	Analysis of variance
ANSA	Anilino naphthalene sulfonic acid
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatased
BSSL	Bile salt-stimulated lipase
°C	Celsius (degrees)
C18	Eighteen carbons
Cer	Ceramide
CNS	Central nervous system
CoA	Coenzyme A
d	Day
DHA	Docosahexaenoic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
ER	Endoplasmic reticulum
F	Control diet fat
g	Gram
GalCer	Galactosylceramide
GlcCer	Glucosylceramide
GD_{1a}	Ganglioside (disialic acid; "a" isomer)

GD_{1b}	Ganglioside (disialic acid; "b" isomer)
GD_3	Ganglioside (diasialic acid)
GM_1	Ganglioside (monosialic acid)
GM_2	Ganglioside (monosialic acid)
GM_3	Ganglioside (monosialic acid)
GM_4	Ganglioside (monosialic acid)
GT_{1a}	Ganglioside (trisialic acid; "a" isomer)
GT_{1b}	Ganglioside (trisialic acid; "b" isomer)
GQ_{1b}	Ganglioside (quadrasialic acid; "b" isomer)
HCl	Hydrochloric acid
HDL	High density lipoprotein
HOM	Homogenate (whole brain)
HPTLC	High performance thin-layer chromatography
hr	Hour
in.	Inch
IUPAC	International Union of Physics and Applied Chemistry
K^+	Potassium
KCl	Potassium chloride
kg	Kilogram (10^3 gram)
Km	Michaelis constant
LacCer	Lactosylceramide
LDL	Low density lipoprotein
LPC	Lysophosphatidylcholine

M	Molar concentration
mg	Milligram (10^{-3} gram)
$MgCl_2$	Magnesium chloride
min	Minute
mL	Milliliter (10^{-3} liter)
mmol	Millimole (10^{-3} mole)
mM	Millimolar concentration (10^{-3} molar)
MTC	Mitochondria
MYE	Myelin
n	Population size
N	Nomal concentration
Na^+	Sodium
NaCl	Sodium chloride
NANA	N-acetylneuraminic acid
Neu5Ac	N-acetylneuraminic acid
NeuAc	Neuraminic acid
NGF	Nerve growth factor
nm	Nanometer (10^{-9} meter)
nmol	Nanomole (10^{-9} mole)
p	Significance level
P	Phosphorus
P1	First pellet
P2	Second pellet

PC	Phosphatidylcholine
PDGF	Platlet-derived growth factor
PE	Phosphatidylethanolamine
pH	Measure of acidity: $-\log[H^+]$
Pi	Inorganic phosphorus
PI	Phosphatidylinositol
PKC	Protein kinase C
PL	Phospholipid
PO_4	Phosphate
PMSF	Phenylmethylsulphonyl fluoride
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
rpm	Rotations per minute
s	Second
S1	First supernatant
S2	Second supernatant
SD	Standard deviation
SDH	Succinate dehydrogenase
SM	Sphingomyelin
SPM	Synaptosomal plasma membrane
TLC	Thin-layer chromatography
UDP	Uridine diphosphate
UTP	Uridine triposphate

UV	Ultraviolet
µg	Microgram (10^{-6} gram)
µL	Microliter (10^{-6} liter)
µmol	Micromole (10^{-6} mole)
µM	Micromolar concentration (10^{-6} molar)
VLDL	Very low density lipoprotein
Vmax	Maximal velocity
vol	Volume
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
X	Times or magnification

CHAPTER 1. LITERATURE REVIEW

A. INTRODUCTION

Following birth, the human infant relies upon ingestion of nutrients for continued growth and development. During this critical stage, infant nutrition is normally supplied by human milk or infant formula. Human milk has naturally evolved as a food source to meet infant biological requirements, but is not always available for every newborn. Knowing this, it is essential that infant formulas, fed as alternatives to mother's milk, contain all the components necessary for optimal development. Infant formulas currently on the market do not contain all of the nutrients found in human milk.

Gangliosides are one type of nutrient present in mother's milk, but lacking in an artificial infant diet. This chapter discusses the nature and importance of gangliosides in the human body, and particularly, the brain. It is evident that many gaps remain in our understanding of the functional roles of gangliosides, especially those of dietary origin. This thesis is one of the first to address the unexplored area of dietary ganglioside, and examines the relationship between nutritional ganglioside and the lipid composition of important brain fractions.

B. GANGLIOSIDES

i) History

Gangliosides were first discovered by Professor Ernst Klenk in the late 1930's, isolated from the brain of a Niemann-Pick patient (1). These novel substances were later recovered from a Tay-Sachs patient, and then from normal brain (1). Professor Klenk coined the term *ganglioside* to reflect the glycosidic nature of the molecules and their apparent localization in the ganglion cells of the brain (1). Since then, researchers have made great strides in determining the molecular structures, physiological distributions, metabolism and functions of individual ganglioside species.

ii) Structure

Gangliosides are acidic members of a large family of glycosphingolipids (Table 1.1A & B). All glycosphingolipids contain a hydrophobic ceramide moiety that acts as a membrane anchor, and an extracellular-oriented glycan chain (2). The ceramide portion consists of a long-chain amino alcohol sphingoid base, usually D-erythro-sphingosine, which is acylated at the terminal amino group with a fatty acid (2). The carbohydrate moiety is linked at the primary hydroxyl group of the sphingol in a glycosidic beta linkage (3). The term glycosphingolipid encompasses all compounds containing at least one monosaccharide and a sphingoid. (4). In gangliosides, the first sugar moiety attached to ceramide is glucose, so gangliosides can also be termed glucosphingolipids, except for GM_4, which is a galactosphingolipid (3, 5).

A ganglioside is a sialoglycosphingolipid, meaning that it carries one or more sialic acid residues on the glycan chain (4). Sialic acids are a family of 9-carbon monosaccharide, neuraminic acid derivatives (6). There are at least 43 variations of sialic acid, in which isomerization is made possible due to substitution at various positions (6). The most common sialic acid in mammalian tissues, however, is N-acetylneuraminic acid (NANA). N-glycolylneuraminic acid is also common, and found substantially in porcine tissue. N-glycolylneuraminic acid is generally not detected in humans, but has been found in some cancers (6).

Table 1.1A. Classification of glycosphingolipids based on properties (4, 7)

Glycosphingolipid classes	Number of molecules
Neutral glycosphingolipids	214
Mono-, oligoglycosylsphingoids	
Mono-, oligoglycosylceramides	
Acid glycosphingolipids	323
Sialoglycosylsphingolipids (gangliosides)	
Sulfoglycosylsphigolipids (sulfatides)	
Alkaline glycosphingolipids	14
Amphoteric glycosphingolipids	27

Table 1.1B. Classification of glycosphingolipids based on carbohydrate structure (7)

Glycan chain series	Structure
Gala (Ga)	Galα1-4(6)Galβ1-Cer
Globo (Gb)	Galα1-4Galβ1-4Glcβ1-Cer
Isoglobo (iGb)	Galα1-3Galβ1-4Glcβ1-Cer
Lacto (Lc)	GlcNAcβ1-3Galβ1-4Glcβ1-Cer
Neolacto (nLc)	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
Ganglio (Gg)	GalNAcβ1-4Galβ1-4Glcβ1-Cer
Isoganglio (iGg)	GalNAcβ1-3Galβ1-4Glcβ1-Cer
Lactoganglio (LcGg)	GalNAcβ1-4(GlcNAcβ1-3)Galβ1-4Glcβ1-Cer
Mollu (Ml)	Manα1-3Manβ1-4Glcβ1-Cer
Muco (Mc)	Galβ1-4Galβ1-4Glcβ1-Cer
Arthro (Ar)	GlcNAcβ1-3Manβ1-4Glcβ1-Cer
Spirometo (Sp)	Galβ1-4Glcβ1-3Galβ1-Cer
Gangliosides	
a-series	(NeuAcα2-3)Galβ1-4Glcβ-Cer
b-series	(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ-Cer
c-series	(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ-Cer
α-series	(NeuAcα2-6)GalNAcβ1-4Galβ1-4Glcβ-Cer
β-series	(NeuAcα2-8NeuAcα2-6)GalNAcβ1-4Galβ1-4Glcβ-Cer

In addition to variation in the number and composition of sialic acid and hexose monomers in the glycan head-group, gangliosides also vary in fatty acid composition. The acyl residue of ceramide is usually a saturated fatty acid (8). Glycosphingolipid fatty acids tend to be longer and more saturated than in glycerolipids (3). In fact, sphingolipids account for a substantial portion of the body's long chain fatty acids (9). Tissue sphingolipids have more 20-24 carbon fatty acids, whereas circulating sphingolipids contain mostly 16:0 and 18:0 (10). Gangliosides of normal tissues, except neuroblastoma cells, contain almost no alpha-hydroxy fatty acids (3). The general ganglioside structure is outlined (Figure 1.1).

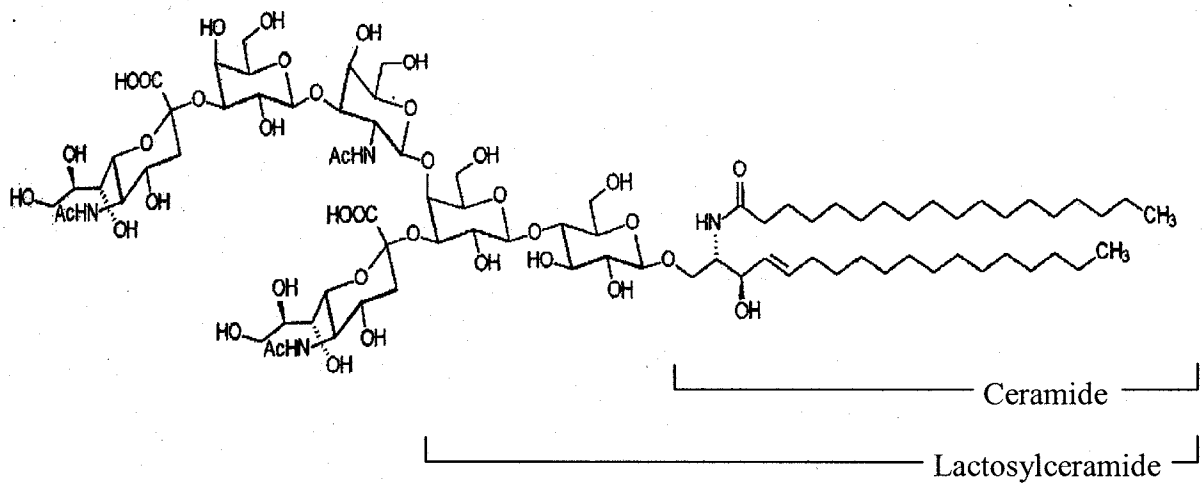

Figure 1.1. Structure of ganglioside GD_{1a} (2)

iii) Nomenclature

The Nomenclature Commission of the International Union of Biochemistry has dictated a standardized procedure for naming gangliosides (Table 1.2; 4). Roman numerals I-IV indicate the position of the sugar residue with respect to ceramide. Arabic numeral superscripts indicate the position of the bond between the first and second sugar residue.

However, the nominal method developed by Dr. Lars Svennerholm is most commonly published because it is simpler and easy to remember (Table 1.2). The letter G is common to all gangliosides, and is followed by a Latin prefix M (mono-), D (di-), T (tri-), etc., which refers to the number of sialic acid residues. An Arabic numeral subscript corresponds to the number of sugar residues in the oligosaccharide moiety (1 = 4 monomers; 2 = 3 monomers, etc.). A letter subindex (a, b, c) refers to the biosynthetic pathway (8).

Table 1.2. Ganglioside nomenclature (8)

Svennerholm method	IUB method
GM_4	I^3NeuAc-GalCer
GM_4	I^3NeuAc-GlcCer
GM_3	II^3NeuAc-LacCer
GM_3	II^3NeuGc-LacCer
GM_2	II^3NeuAc-$GgOse_3$Cer
GM_1	II^3NeuAc-$GgOse_4$Cer
GD_3	$II^3(NeuAc)_2$-LacCer
GD_3	$II^3(NeuGc)_2$-LacCer
GD_3	II^3(NeuAc, NeuGc)-LacCer
GD_2	$II^3(NeuAc)_2$-$GgOse_3$Cer
GD_{1a}	II^3NeuAc, IV^3NeuAc-$GgOse_4$Cer
GD_{1b}	$II^3(NeuAc)_2$Cer
GT_{1a}	$IV^3(NeuAc)_2$, II^3NeuAc-$GgOse_4$Cer
GT_{1b}	IV^3NeuAc, $II^3(NeuAc)_2$-$GgOse_4$Cer
GT_{1c}	$II^3(NeuAc)_3$-$GgOse_4$Cer
GQ_{1b}	$IV^3(NeuAc)_2$, $II^3(NeuAc)_2$-$GgOse_4$Cer
GQ_{1c}	IV^3NeuAc, $II^3(NeuAc)_3$-$GgOse_4$Cer
GP_{1b}	$IV^3(NeuAc)_3$, $II^3(NeuAc)_2$-$GgOse_4$Cer
GP_{1c}	$IV^3(NeuAc)_2$, $II^3(NeuAc)_3$-$GgOse_4$Cer
LM_1	IV^3NeuAc-$nLcOse_4$Cer
LD_1	$IV^3(NeuAc)_2$-$nLcOse_4$Cer

IUB, International Union of Biochemistry

iv) Localization and distribution

Gangliosides and other glycosphingolipids are present in eukaryotic cells. They form specific patterns in cellular membranes that change with growth, differentiation, viral transformation and oncogenesis (2). Gangliosides are widely distributed in most vertebrate tissues. Their composition varies among species, gender, individuals, tissue and cell type (8). Gangliosides are especially abundant in neural tissues, where they are highest in grey matter and in the central nervous system (8). The ganglioside concentration of neural membrane lipids is 5- 10%, however, since they are almost exclusively localized in the extracellular monolayer, the concentration is 10-20% of that

fraction (5, 11). Gangliosides orient themselves in the lipid bilayer, with the hydrophilic portion exposed to the extracellular environment (8). Extraneural tissues and fluids with substantial ganglioside content include: skeletal and smooth muscle, liver, pancreas, spleen, kidney, placenta, thymocytes, lymphocytes, plasma, amniotic fluid and milk (3, 8, 12). Sialic acid is independently present in many human body fluids, such as saliva, mucins, gastric juice, serum, urine, tears and milk. The term *sialos* is from the Greek, meaning saliva. Sialic acid increases in pregnant women over the course of gestation, and is higher in the saliva of breast-fed infants (6). Mammalian CNS has highest concentration of sialic acid (65% = ganglioside, 32% = glycoprotein, 3% = free) (6). In other body tissues, the proportion of sialoglycoprotein exceeds that of ganglioside (5). Table 1.3 demonstrates the differences in ganglioside and sialic acid content between human milk and bovine milk used in infant formulas, as well as the ganglioside content of human brain.

Table 1.3. Ganglioside content of milk and brain (6, 8)

Measure	Bovine milk	Human Milk
Range of total ganglioside (mg of LBSA/kg)	7.5(c) - 1.4(m)	11.93 (c) - 0.82 (m)
Predominant species (relative %)	**GD3**, GM3, GT3 (80-90 %)	GD3 (c), **GM3** (m) (95%)
Presence of complex gangliosides	?	GD1a, GD1b, GT1b, GQ1b
Total sialic acid (mmol/L)	0.48	3.72 (c) - 1.48 (m)
Neu5Ac % of sialic acid	73*	100
		Human Brain
Total ganglioside (mg/g fresh weight)		125
Predominant species (relative %)		GM1, GD1a, GD1b, GT1b (80-90 %)
Total sialic acid (µg/g fresh weight)		890 (cerebral cortex)
Neu5Ac % of sialic acid		100

LBSA = lipid-bound sialic acid, (c) = colostrum, (m) = mature
Major ganglioside species is underlined. * From bovine milk-based infant formula.

v) Biosynthesis

Gangliosides are synthesized in all tissues of the body, via the pathways illustrated (Figure 1.2). The assembly process begins in the endoplasmic reticulum (ER), progresses through the Golgi complex, and is finalized in the plasma membrane. The rate-limiting step for glycosphingolipid production is the condensation of serine and palmitoyl-CoA to 3-dehydrosphinganine in the endoplasmic reticulum (13, 14). 3-dehydrosphinganine reductase produces D-erythrosphinganine, which is converted to D-erythro-dihydroceramide, and finally ceramide, by acylation on the ER cytosolic surface (14). The stepwise addition of glucose and galactose, on the cytosolic and lumenal Golgi surfaces, results in lactosylceramide (15, 16). Galactosylceramide is formed separately in the ER lumen (16). In the terminal Golgi, GM_3 is the first ganglioside synthesized, through sialylation of lactosylceramide (17). GM_3 can be sialylated to GD_3, the first of the b-series gangliosides, or modified by the addition of N-acetylgalactosamine to GM_2, the first of the a-series (17). More complex gangliosides are likewise synthesized in the *trans* Golgi network (15). Gangliosides are transported to the plasma membrane, where they may be further modified by membrane-bound sialidase or sialyltransferase, or recycled (18, 19, 20, 21). Exogenous gangliosides are also taken up by the cell and inserted into the plasma membrane, although most are degraded via the endocytic pathway (18, 22).

N-acetylneuraminic acid is formed in the cytosol via the phosphorylation of glucose to fructose-6-phosphate (6). Glycosamine-6-phosphate synthase catalyzes the transformation to glucosamine-6-phosphate. Acetyl CoA is added to form N-acetylglucosamine-6-phosphate, which is altered by N-acetylglucosamine-6-phosphate

mutase into N-acetylglucosamine-1-phosphate. From this substrate and UTP, UDP-N-acetylglucosamine is created. Sialic acid synthesis is limited by the key enzyme UDP-N-acetylglucosamine-2-epimerase, needed to form the intermediate product, N-acetylmannosamine. There is evidence that this enzyme is deficient in the livers of young animals such as rats, mice and guinea pigs (6, 23, 24). Therefore, the human infant liver may also have limited capacity for sialic acid synthesis during early postnatal life. N-Acetylmannosamine is phosphorylated to N-Acetylmannosamine-6-phosphate, from which N-Acetylneuraminic acid-9-phophate is synthesized by the enzyme N-acetylneuraminate-9-phosphate synthase. Finally, N-acetylneuraminic acid results from the action of N-acetylneuraminate-9-phosphatephatase (6).

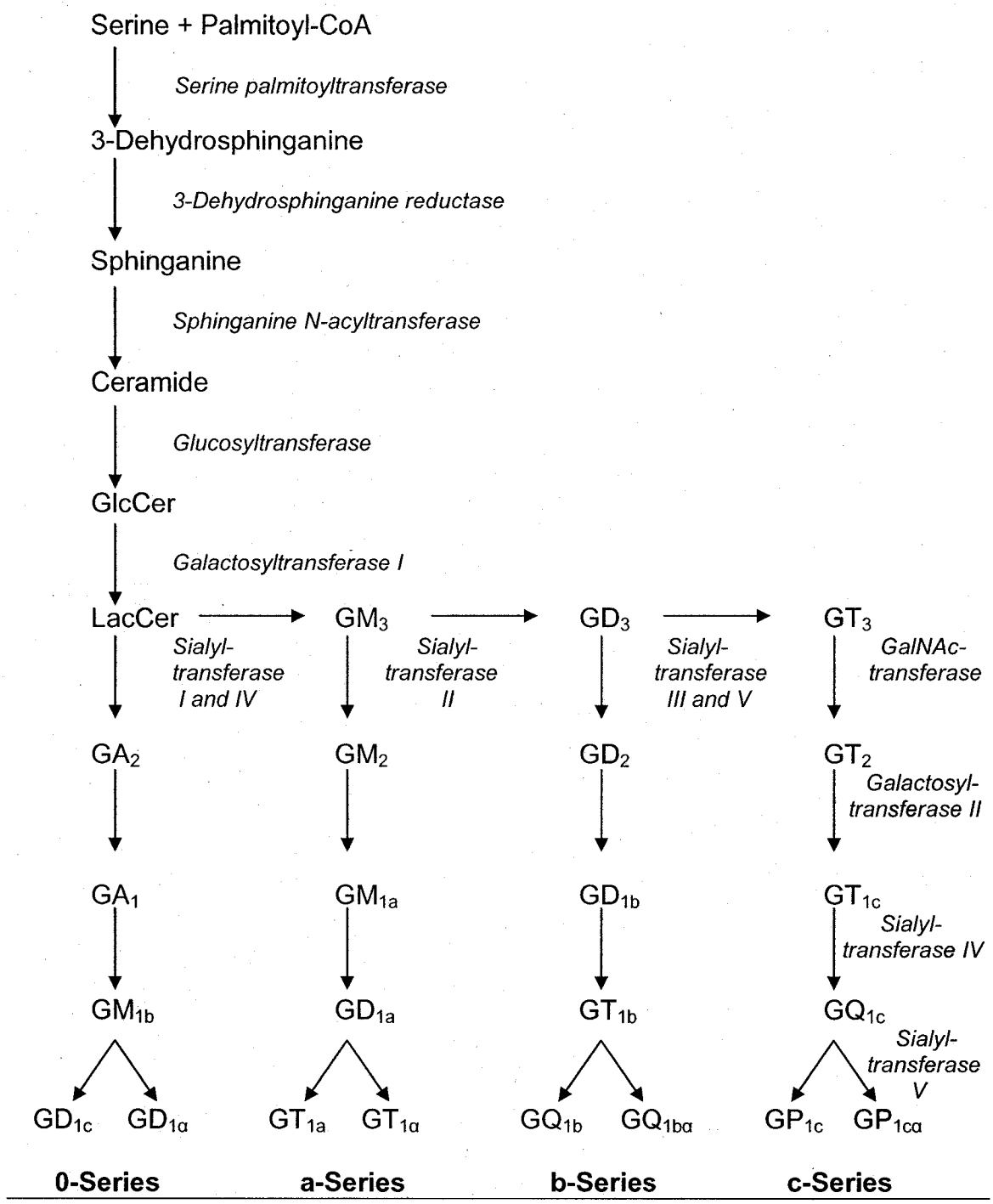

Figure 1.2. Ganglioside biosynthesis pathways (2, 14)

vi) Function

Despite considerable progress in determining the chemical, physical and metabolic properties of gangliosides, little is known about their biological roles. The

high concentration of gangliosides in synaptic membrane naturally leads to speculation about their involvement in synaptic function. It is difficult to ignore how changes in brain ganglioside profiles coincide with neurodevelopmental events. Indeed, deficiencies in brain ganglioside or sialic acid, while not usually lethal, are related to mental retardation and poor learning behaviour (6). In fact, sialic acid may have implications for evolutionary development and intellectual capacity (6). Ganglioside knockout studies lead to multiple abnormalities, including: calcium dysregulation, axonal degeneration, myelination defects, and impaired motor coordination (25). Gangliosidoses, storage diseases that accumulate gangliosides in nervous tissue, are characterized by neuronal morphological abnormalities, most notable being extensive neurite proliferation, culminating in the increased length and number of cell processes (11). The postulated roles for gangliosides fall under the following broad categories: membrane shape and stability; cell recognition and adhesion; biosignal transduction and modulation of membrane proteins; calcium trafficking, signaling and homeostasis; immunological function; pathogen binding; development and mature cellular function; neurotrophic and neuroprotective effects (2, 3, 6, 11, 12, 25). In most, if not all cases, ganglioside function is achieved through some interaction with calcium ions.

Ganglioside function is undoubtedly related to structure. The possession of both hydrophobic and hydrophilic domains with associated negative charge likely facilitates binding to a variety of membrane proteins, whose functions are thereby modified (25). Gangliosides form aggregates with membrane-bound proteins, like ion channels, which may be a method of controlling ion flow across the membrane (6). These associations probably occur in microdomains, which provide the environment for coalescence of

individual glycosphingolipids or clusters with a variety of signal transducing systems (25).

Most of the biological functions of gangliosides are attributed to their oligosaccharide portions (26). The fatty acid in the ceramide tail may also be important, but this is, as yet, unexamined. The presence of gangliosides modifies membrane organization, as well as a host of physical properties. Ganglioside-calcium interactions affect membrane fluidity, surface potential, condensation and surface pressure (6). GD_3 has been shown to mediate membrane permeability to ions and metabolites (11). Moreover, gangliosides affect membrane excitability and electrical conductance (6, 12). These properties of gangliosides seem well suited for functional importance in the central nervous system.

Essential changes in membrane organization occur through ganglioside interaction (12). Gangliosides are critical factors in the retention and organization of fibronectin into the extracellular matrix (27). The principal ganglioside for this role is GT_{1b}, followed by GD_{1a}, GM_1, GM_2, and GM_3 (27). Integrins may exist as a functional complex with GD_2, and possibly other ganglioside species (3). An enrichment of gangliosides in the detergent-insoluble matrix binding sites of the kidney indicates the importance of gangliosides for maintaining a strong matrix in harsh environments (3, 28). Therefore, gangliosides provide cell membranes with high chemical and mechanical stability, while protecting against degradation and uncontrolled membrane fusion (2). They also control cell-cell interactions through recognition and binding, facilitating cell aggregation when required.

Cell-to-cell adhesion may involve carbohydrate-to-carbohydrate interaction on the surface of counterpart cells (25). Cell aggregation is hindered by repulsive effects of negatively charged sialic acids in plasma membranes. In contrast, the binding of positive calcium ions may facilitate cell adhesion by breaking up the negative electric fields (6). Exogenous gangliosides in culture have been shown to inhibit cell adhesion and spreading ability through modulation of fibronectin, vitronectin, laminin, and collagen (3, 12).

The role of gangliosides in cell adhesion seems minor in comparison to their extensive associations with specific membrane proteins, of which the conformations, and hence, activities are thereby altered (25). Gangliosides interact with growth factor receptors, protein kinases, phosphatases, ion transporters, and other regulatory machinery, potentially including cytosolic proteins (8, 25). Gangliosides are capable of modulating the functional properties of integral neuronal membrane proteins like Na^+-K^+ ATPase and activating adenylate cyclase and phosphodiesterases (29, 30). In the renal epithelium, gangliosides modulate apical membrane sodium channels (3).

Gangliosides are recognized as signal biotransducers for regulation of cell growth (27). Exogenous gangliosides alter growth properties of a variety of cells, either suppressing normal growth or inducing profound trophic events like neuritogenesis. As calcium mobilization appears to be an early event in the cellular response to mitogens, gangliosides may exert their effects through modulating plasma membrane calcium channels (27). Cell growth is also dependent on growth factors and insulin. GD_3 inhibits epidermal growth factor (EGF) receptor kinase, whereas platlet-derived growth factor (PDGF) is influenced by GM_1, GD_{1a}, GD_{1b} and GT_{1b}. GM_1 has a synergistic enhancing

effect on nerve growth factor (NGF). Neuritogenic differentiation of most neurons is induced by NGF. GQ_{1b} mimics NGF activity in types of neuroblastoma cells, stimulating neurite outgrowth (3, 12).

Gangliosides and other sphingolipids help regulate cell proliferation and differentiation. Exogenous gangliosides will arrest cell growth *in vitro* through extension of the G1 phase (3). Cellular differentiation, induced by agents like retinoic acid and butyrate, is associated with increased cellular synthesis of GD_3 (3). Metabolites of sphingolipids are potent physiological mediators of mitogenesis. Ceramide has antimitogenic effects, leading to cell differentiation, cell cycle arrest, senescence and apoptosis (2). Sphingosine-1-phosphate has the opposite effects (9). Ganglioside will protect against apoptosis through blockade of protein kinase C translocation from the cytosol to the plasma membrane for activation of PI3-kinase. Nuclear GM_1 is similarly suggested for a role in homeostatic mechanisms and protection against apoptosis (25). Accelerated growth of liver and spleen in Gaucher's disease may be due to accumulation of glucosylceramide (3). It is important to realize that many of the studies reporting ganglioside effects on cell growth or membrane-bound enzymes, have been based on the use of high concentrations of exogenous gangliosides (10-100μM), and little consideration has been given to whether the gangliosides were inserted into the plasma membrane in a physiologically relevant manner (27).

Gangliosides are involved in immunoregulation in a variety of capacities. Gangliosides have essential regulatory roles in T-cell function, leukocyte differentiation, and the differentiation of different lymphocyte subpopulations (8, 12). In cancer, PKC enhances signal transduction in oncocytes and activated platelets or endothelial cells

during inflammation. PKC activity is highly susceptible to polysialogangliosides, glycosphingolipid and sphingosine derivatives (12). Gangliosides also play a role in immunosupression by tumour cells (11).

Gangliosides have been identified as receptors for certain bacterial toxins and viruses. GM_1 is the natural membrane receptor for cholera toxin. *E. coli* heat-labile enterotoxin, tetanus toxin and Sendai virus also bind to gangliosides (27). However, it is a glycoprotein that is the natural receptor for Sendai virus (11). Virus resistant cells become susceptible to infection once gangliosides are incorporated into the plasma membrane (11). Since harmful bacteria, viruses and pathogens use cell surface carbohydrates as sites for recognition and binding to the target host, oligosaccharide sequences in human milk can act as decoys in the intestinal lumen. Both free and bound sialylated oligosaccharides in human milk prevent binding of rotavirus, cholera toxin and *E. coli* associated with neonatal meningitis and sepsis (6). Milk gangliosides have an inhibitory effect on *E. coli* adhesion to intestine, and may be a component for host defense over all stages of lactation. This suggests the importance of breast-feeding during the first few months before the immune system is mature and stable colonic microflora has been established (6).

Gangliosides may play a role in a host of neurodevelopmental processes in mammalian brain (31). The presence of GM_1 is important during membrane growth (32). Feline neurons with GM_1 gangliosidosis show extensive proliferation of neurites. Adding GM_1 to cultured neuroblastoma cells also results in an increase in length and number of cell processes (11). Gangliosides are required in areas of membrane extension for axonal and dendritic growth. Exogenous gangliosides incorporated into the neuronal membrane

increase neuritogenesis and synaptogenesis, whereas lack of sufficient gangliosides related to perinatal ethanol exposure, is associated with a loss of neurites (6). Evidence shows that gangliosides, especially GM_1, enhance calcium flux, resulting in the generation of new axons (25). However, exogenous gangliosides often lead to dendritogenesis, so the entry pathway is critical in determining the signaling mechanism (25).

Gangliosides are involved in synaptic transmission and potentiation, although the exact mechanisms are unclear. Gangliosides form clusters in synaptic membrane around calcium pumps supplying ions for the cell. Calcium binds to sialic acid, and calcium-ganglioside interactions may modulate neuronal functions for short-term transmission, and long-term adaptations, including the storage of information (6). Sialic acid on gangliosides may act as an extracellular storage mechanism for calcium, which is essential for synaptic transmission. This is thought to activate second messenger pathways to induce potentiation (6). Neurotransmitter release is affected by exogenous gangliosides (25). Endogenous ganglioside function in synaptic transmission is likely based on the formation of calcium-ganglioside complexes, and the subsequent dissociation of calcium (33). Sialic acid is also postulated to be an actual receptor for neurotransmitter in the CNS (6).

Gangliosides are associated with cholinergic function (34). Treatment with gangliosides greatly influences the regeneration and reinnervation process of cholinergic and adrenergic fibre (35). Combining GM_1 with nerve grafts in animal models of neurological damage attenuates behavioural deficits and normalizes choline-acetyltransferase activity (36, 37, 38). Whether the mechanism is related to

neuroprotection or enhanced neuronal recovery is still under investigation. The impaired electrical property of neurons is implicated in a number of psychological disorders. Schizophrenic patients show decreased sialic acid in cerebrospinal fluid glycoprotein, while lithium treatment in rats changed the sialic acid composition of synaptosomes, perhaps improving membrane electric properties (6).

The chemical, histologic and behavioural connection in neuroscience features prominently in ganglioside literature. Cell recognition, contact and position functions are necessary for information handling and storage by the nervous system (39). Synaptic connections, possibly corresponding to memory, lost due to devascularizing lesions or aging, may be protected or replaced by gangliosides (6). Intraperitoneal NANA injection in rat pups increased cerebellar ganglioside and glycoprotein sialic acid and increased maze test scores (6). These effects were not accompanied by change in brain weight, cell size, cell number, DNA, RNA or protein content, and were persistent into adulthood. Scientists question whether certain complex gangliosides, like GD_{1b} or GQ_1, may be more important for intelligence (6).

vii) Implications in health and disease

Upon recent examination, glycosphingolipids, including dietary sources, are emerging as important regulators of some health and disease processes. The abnormal expression, degradation, or distribution of gangliosides impacts health in such pathologies as gangliosidoses, peripheral neuropathies, autoimmune disorders and secretory diarrhea. Gangliosides also possess therapeutic potential in cancer and neurological disease or trauma.

As shown in Table 1.4, gangliosidoses are a subclass of lysosomal storage diseases in which a deficiency in any one of the enzymes involved in the degradation pathway results in the accumulation of undigested gangliosides or their intermediates (40). In the nervous system, such disorders lead to neurodegeneration, mental retardation, dementia, motor dysfunction, sensory deficits, increased startle response and seizures (40). These symptoms are related to changes in neural connectivity and accompanied by apoptosis, demyelination and gliosis (40). The injured neurons alter their microenvironment by releasing inflammatory cues (40). Although gangliosidoses are primarily discovered in infants, a milder, later onset may occur in adolescents and adults.

Table 1.4. Gangliosidoses and other glycosphingolipidoses (40).

Disease	Enzyme deficiency	Storage product
Gangliosidoses		
GM1 gangliosidosis	β-galactosidase	GM1
Tay Sachs diseases	β-hexosaminidase A	GM2
Sandhoff diseases	β-hexosaminidase A & B GM2 activator protein	GM2
Niemann-Pick diseases	Sphingomyelinase	GM2, GM3*
Non-ganglioside glycosphingolipidoses		
Gaucher disease	β-glucocerebrosidase	GlcCer
Krabbe disease	Galactosylceraminidase	GalCer
Fabry disease	β-galacosidase A	
Faber disease	Ceramidase	
Wolman's disease	Acid lipase	
Austin's disease	Multiple sulfatases	
Metachromatic leukodystrophy	Arylsulfatase A	

* Storage of gangliosides is secondary to sphingomyelin or cholesterol and mucopolysaccharides (Niemann-Pick type A/B), or dermatan or heparan sulfate (Niemann-Pick type C).

Anti-ganglioside anti-bodies are responsible for the peripheral neuropathy in Guillain-Barré syndrome (GM_1, GM_2, GQ_{1b}), Miller-Fisher syndrome (GQ_{1b}), multifocal motor neuropathy (GM_1) and chronic idiopathic ataxic neuropathy (GM_3, GD_{1b}, GD_3, GQ_{1b}) (41). Often the disease will be triggered by an immune response to a pathogen containing ganglioside-like epitopes, as in *Campylobacter jejuni* or cytomegalovirus infections preceding Guillain-Barré syndrome (41). The role of antibodies in neuropathy is not fully understood, however direct neuronal damage, ion channel dysfunction, inhibition of remyelination, axonal degeneration and blockage of conduction are plausible mediators (41). It is not too far-fetched to consider that other autoimmune disorders may result from abnormal glycosylation of plasma membrane gangliosides or other oligosaccharide-containing molecules, changing self-recognition patterns to mimic those of infectious species. In some cases, administering exogenous gangliosides may improve symptoms, as in diabetic neuropathy (11).

Massive secretory diarrhea results from bacterial toxins that are endocytosed by the intestinal epithelium. Cholera toxin and *E. coli* heat-labile toxin type I have subunits with high affinity for GM_1 binding. Signaling pathways in the endoplasmic reticulum stimulate chloride secretion, which is the fundamental event responsible for secretory diarrhea (41).

Gangliosides integrate a multitude of cellular responses by changing the functional integrity of intracellular membranes, actively mediating apoptotic programs (40). The endoplasmic reticulum is the primary store of intracellular calcium, especially in neurons. Since cytosolic calcium concentration is tightly controlled around 0.1 μM, any additional calcium is a potent signal. Interplay between the endoplasmic reticulum

and mitochondria during stress response can result in the dissipation of mitochondrial transmembrane potential, DNA fragmentation, the release of reactive oxygen species, and ultimately, apoptosis (40, 41). Gangliosides GM_1, GM_3 and GD_3, in particular, are potent mediators of cell death (40, 41). GD_3 is found only in trace amounts in the adult human brain, but it is expressed in high levels in activated microglia and reactive astrocytes (40). It is also increased in the brain in Creutzfeldt-Jakob disease and multiple sclerosis, and along with GM_3 in pronounced dysfunction of the blood-brain barrier (40). Shedding of gangliosides in neuroinflammatory conditions is thought to be directly responsible for the induction of apoptosis in oligodendrocytes, the cells responsible for myelination in the central nervous system (40).

Tumor cells also synthesize and shed a large amount of gangliosides into their microenvironment, which regulate cell functions through immunosupression and apoptosis (41). Tumor formation and metastasis is enhanced in this microenvironment absent of immune response. In the presence of certain gangliosides or their metabolites, however, tumor cell death is very possible. Altered ganglioside profiles may be a mechanism by which cancerous cells evade regulation of their cycles and stimulation of apoptotic signals (42). Dietary sphingolipid degradation in the lower intestine is potentially responsible for protection against colon cancer. Sphingolipid metabolites, ceramide and sphingosine, are highly bioactive compounds that affect cell regulatory pathways, and appear to reduce the appearance of aberrant colonic crypts (9, 43). Understanding the behaviour of oncocytes and the signaling pathways of certain gangliosides and their metabolites may be useful in the production of diagnostic markers and therapeutic agents for cancer (41).

Neurosurgeons are investigating the ability of certain gangliosides to enable neuroregeneration or reinnervation. GM_1 and its internal ester, AGF2, have been shown to enhance neurite outgrowth and promote regeneration of damaged peripheral and central nerves, while GM_1 antibodies and other gangliosides may prevent regeneration (36). Neuroregeneration following surgery may be facilitated by adding exogenous GM_1, or by creating GM_1 through the action of sialidase on existing complex ganglioside species. GM_1 shows synergistic action with nerve growth factor on the survival and development of grafted cells (37, 44). Exogenous GM_1 attenuates behavioural deficits in experimentally brain-damaged animals (36, 37, 44). Primarily, gangliosides appear to exert their effects without limiting the extent of neuronal damage or initial impairments, but through accelerating the rate of recovery (36). GM_1 may also improve memory deficits related to senility, suggesting that this ganglioside is involved in neuronal plasticity, survival or transmission (38, 45).

C. BRAIN FRACTIONS

i) Synaptosomal plasma membrane

Synaptosomal plasma membrane (SPM) is the plasma membrane component of isolated pre- and postsynaptic nerve terminals, also called synaptosomes. The SPM fraction of the brain is obtained by homogenization and fractionation of nerve tissue, followed with synaptosomal lysis to empty subcellular organelles and constituents from the isolated vesicles (46).

The synaptosomal plasma membrane is a functional component of the synapse, and thus an important regulator of neurotransmission, learning, and memory. This

membrane is relatively enriched in gangliosides as well as the transporter, Na^+-K^+ ATPase, which is commonly used as a biochemical marker of SPM (25, 46, 47). Synaptosomal plasma membrane is also characterized by a higher lipid/protein ratio than most other membranes (about 1.5 mg/1.0 mg) (46). Cholesterol, which is a critical component of all brain membranes, functions to regulate SPM fluidity (48). Dietary treatments, including ethanol and polyunsaturated fatty acid (PUFA), have been shown to alter SPM lipid composition and associated enzyme activity (19, 49, 50).

ii) Myelin

Myelin in the central nervous system is formed through outgrowth of oligodendrocytes that wrap around neuronal axons (51). This multilaminal sheath of plasma membrane functions as an electrically insulating layer, thereby increasing the speed at which impulses propagate along the nerve fiber. Whereas impulses move continuously as waves in unmyelinated fibers, they propagate by saltation in myelinated fibers. The myelin sheath also helps prevent escape of electrical current from the axon. Myelinated axons are white in appearance and are often referred to as the brain's "white matter".

Myelination begins in rat brain between 7 and 10 days of postnatal age and continues well into adulthood (52, 53). Increase in brain weight after 100 days is almost exclusively due to continued myelination (53). The maximum rate of myelin deposition: 3.5 mg /brain/day, occurs around 20 days postnatal (52, 53). The timing of myelinogenesis in young rats is sensitive to dietary lipid (52). As the myelin matures, it becomes increasingly dehydrated and enriched in lipid content (53).

Dry myelin is generally composed of 70-80 % lipid and 20 % protein, although the exact chemical composition will vary (53). Important myelin proteins such as proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte protein (MOP) can be used as biochemical markers for myelin (52, 54). Myelin and oligodendrocytes commonly possess high concentrations of glycosphingolipids, particularly galactolipid (55, 56). The primary lipid species is galactosylceramide (56). Sphingomyelin and cholesterol are also important lipids for endowing myelin its strength and intermediate fluid properties (56). Myelin lipid is 78 % phospholipid by weight, and 16-24 % sphingolipid (53). Gangliosides are an intrinsic component of myelin (57, 58). Interestingly, the ganglioside composition of myelin differs substantially from the composition of originating oligodendrocytes, which show greater ganglioside complexity and increased GD3 content (59). Myelin ganglioside consists primarily of GM1, GD1b, and also GM4 in mammalian and avian CNS (58, 59, 60, 61). This divergence may arise through alteration of myelin gangliosides by *in situ* myelin-associated neuraminidase (59).

D. GANGLIOSIDES IN THE BRAIN

The brain has an exceedingly high concentration, molecular diversity and complexity of gangliosides compared to extraneural organs and tissues (62). Ganglioside concentration is three times higher in cerebral grey matter compared to white matter, and grey matter has fifteen times the ganglioside content of large visceral organs, such as liver, lung and spleen, and five hundred times the content of intestinal mucosa (6). The

major brain gangliosides of higher vertebrates, accounting for 80-90 % of total ganglioside, are: GM1, GD1a, GD1b, GT1b (6, 8). White matter may also contain GM4, which is particularly abundant in primate and avian brains (6, 8). In fact, oligodendroglia are independently higher in GM1, GM3 and GD3, as well as GM4, compared to other brain cell types, while GD3 is an important component of spinal cord fluid (62). Astroglia contain twice the ganglioside concentration of neurons, although this is largely explained by the increased ratio of plasma membrane to cytoplasmic volume (62). Gangliosides are distributed over a large part of the neuronal surface, and are generally concentrated in clusters on synaptic membranes in the vicinity of membrane-bound calcium pumps (6).

The ganglioside profile of neural tissue changes significantly during brain development. The concentration increases approximately three-fold from the tenth gestational week to five years of age (6). During this period, GM1 and GD1a increase 12-15 times (6). GT1b is the major ganglioside during the third to fifth gestational month, but its concentration drops rapidly to term and slowly increases again up to 50 years (6).

All body tissues, including the brain, have the ability to synthesize gangliosides and other sphingolipids. The brain will also take up sphingolipids intact from circulating plasma. This action is regulated by the blood brain barrier, which is characterized by tight junctions, high metabolic capacity, low pinocytic vesicular traffic, and efficient efflux mechanisms (63). Evidence shows that the brain is highly selective of the lipids taken up from circulation (64). And despite dynamic turnover, brain lipids are relatively resistant to change in composition (65, 66). It seems likely that the consistent patterns

observed in brain lipids are functionally important, and perhaps even critical during vulnerable periods in infant brain development.

It is unknown at this time if dietary sources of ganglioside will enhance brain ganglioside content, or if they are necessary to supplement *de novo* synthesis during early growth and development conditions. Recent studies have revealed a potential deficiency in the key enzyme UDP-N-acetylglucosamine-2-epimerase in the livers of young animals (6, 23, 24). Such a deficiency could limit an infant's capacity for synthesizing sialic acid. Coincidentally, this is the period at which the gut is most permeable to dietary sialic acid, and perhaps also a period at which the blood brain barrier is functionally immature (6).

E. DIETARY GANGLIOSIDE

i) Introduction

The metabolism of gangliosides and other dietary sphingolipids is poorly understood. Sphingolipids, although minor constituents of foods with negligible caloric value, are highly bioactive. They are comparable in that manner to other nutrients such as cholesterol and tocopherols. Consumption in the United States is estimated to be on the order of 0.3-0.4 g/d (9). The sphingolipid content of food varies considerably. Dairy products, eggs, and soybeans are the richest sources per kilogram, while the contribution of meat, including fish, is also significant. Mammalian tissues and milk contain ceramide, sphingomyelin, cerebrosides, gangliosides and sulfatides. Plants, fungi and yeast have mainly cerebrosides and phosphoinositides (9). Sphingomyelin is the most prevalent, and most studied dietary sphingolipid. Therefore, most of the information in this section is based upon the kinetics of sphingomyelin.

Sphingolipids are nonessential nutrients, as they are synthesized *de novo* in most, if not all tissues. However, in some metabolic conditions, or during early infant growth, certain sphingolipids or their components (i.e. sialic acid) may be conditionally essential. Sphingolipid synthesis may be subject to some degree of feedback regulation, influenced by the consumption of dietary sphingolipid (9). Sphingolipid, cholesterol and omega-3 PUFA metabolism are shown to be interactive (9). Through interference with cholesterol metabolism and absorption, and by the action of sphingolipid metabolites, dietary sphingolipids have important postulated roles in colon cancer, atherosclerosis and immunity (9).

ii) Digestion

Dietary sphingolipids are mixed with sphingolipids from bile and sloughed mucosal cells in the intestinal lumen. The course of digestion shows a time-dependent decrease in sphingolipid, a transient appearance of ceramide, and an increasing replacement of sphingolipid and ceramide with fatty acid products (67). The initial step is the action of alkaline sphingomyelinase (sphingomyelin) or lactase-phlorizin hydrolase (glycosylceramides) to cleave the head group (i.e. phosphocholine) from the ceramide portion (68). Intestinal glucoceramidase also cleaves the oligosaccharide groups from glycosylceramides and glycosylsphingosines, with the exception of GM1 and asialo GM1 (69). Dietary ceramide is further degraded to sphingoid base and fatty acid by the action of neutral ceramidase, and to a minor extent by bile salt-stimulated lipase (BSSL) from the pancreas or from human milk (10, 67, 68). Another ceramidase present in the gut is acid lysosomal ceramidase, which occurs generally in all tissues (10). Sphingoid bases

are first phosphorylated and then converted to long-chain aldehydes or ethanolamine phosphate (10). Sphingolipid metabolites are extremely bioactive, which has implications in disease processes like colon cancer, or in the modulation of gut physiology. Sphingosine and ceramide generally inhibit growth and induce apoptosis, while sphingosine-1-phosphate, readily synthesized through the action of abundant intestinal sphingosine kinase, is a potent mitogen and inhibitor of apoptosis (9). Sphingolipid hydrolase activity at the brush border membrane may additionally influence sterol absorption and lipoprotein secretion of intestinal tissue (68).

Alkaline sphingomyelinase and neutral ceramidase have similar topology. They are located in the brush border membrane of the small intestine, and exert their activity in the lumen. Pancreatic and biliary secretions also contribute neutral and alkaline sphingomyelinases respectively (9). Peak sphingolipid enzyme activity occurs in the distal jejunum, where most of the lipid digestion is complete but bile salt concentration is still high (68). However, the course of sphingolipid digestion is quite extended and incomplete (10, 68, 70). No enzyme activity is present in the stomach, while some activity may be present in the human duodenum. The activity increases along the length of the small intestine to a maximum in the distal jejunum, and then declines steadily throughout the ileum and colon (10). Further degradation of ceramide by intestinal or bacterial lipases probably occurs in the colon. This is suggested by the slow and extended time course of absorption (1-10 hr), and by the remaining potential activity of enzymes against incompletely digested sphingolipids as they travel through the colon (70). BSSL exerts its ceramidase activity mostly in the proximal small intestine (67, 68). Both sphingomyelinase and ceramidase activities are influenced by bile salts (10, 68).

Despite the substantial sphingomyelinase, glucoceramidase and ceramidase activity of rat small and large intestine, sphingolipid digestion capacity is limited (9, 10).

Gangliosides, glycoproteins and some free oligosaccharides have sialic acid moieties. Sialic acid content in the diet is highest in fish eggs and milk (6). Cleavage of the sialic acid portion of gangliosides during digestion occurs via sialidase activity in the intestinal mucosa (6). This activity is especially high during the suckling period. The terminal position of sialic acid may permit hydrolysis even if the remainder of the compound resists digestion. There is also some speculation that autohydrolysis is possible due to the pKa range of the acid (6). However, sialic acid is not released in incubation when exposed to pancreatic and mucosal enzyme mixtures (6). Sialidases of bacterial origin cleave sialic acid in the colon from fermenting human milk oligosaccharides, although it is unknown whether sialic acid can be absorbed across the colon (6).

iii) Absorption

Free sphingoid bases and fatty acids are easily taken up by intestinal mucosa (10). Within the enterocyte, these components are further degraded to fatty acids, or are reincorporated into intestinal sphingolipids (9). Ceramide, however, is less readily absorbed. Long chain ceramides, due to their molecular structure and low solubility in bile salt micelles, are expected to show decreased absorption, if they are absorbed at all (68, 70). Very little dietary ceramide appears intact in the chyle (68).

Rat enterocytes are highly permeable to sialic acid; about 90 % is absorbed in pups (6). Sialic acid is not absorbed as well in older animals, once brain growth is

completed (6). Following uptake into tissue, sialic acid is cleaved by Neu5Ac lyase to N-acetylmannosamine and pyruvic acid. N-acetylmannosamine is used for resynthesis of sialic acid-containing compounds.

iv) Circulation

Some components of dietary sphingolipids are transported from the mucosa to the systemic circulation (9, 70). Chylomicrons may be involved in lymphatic transport of sphingolipid metabolites, whereas small amounts of free sphingoid bases are also present in the blood, associated with albumin and blood cells (9). Sphingolipids are integral components of lipoprotein membranes. They are highest in LDL, followed by VLDL and then HDL (9).

Sialic acid is transported to extraintestinal tissues. Dietary sialic acid appears in the brain after 6 hr (6). Intraperitoneal injection of sialic acid has been shown to increase brain ganglioside content (71). Since it is not a hydrophobic molecule, NANA most likely occurs freely in the plasma serum.

v) The suckling infant

Human milk is a major source of dietary sphingolipid. Milk from different species varies in ganglioside content and composition. Human milk has increased amounts of ganglioside compared to infant formulas made from bovine milk (8). The profile of human milk ganglioside changes over the course of lactation. GD3 is highest in the colostrum, and even higher in preterm milk relative to other ganglioside species (8). As lactation matures, the proportion of GD3 declines and is replaced by increasing

GM3 as the most prominent ganglioside (8). This is likely linked to maturation of the mammary gland, as a similar pattern is observed in other developing tissues. Human milk ganglioside is almost exclusively associated with the membrane fraction of fat globules, which is derived mainly from the apical plasma membrane of mammary apocrine secretory cells (8). The third week of lactation in humans shows an increase in total lipids, including gangliosides (8).

Sialic acid is one of the most variable fractions in human milk (6). In milk, sialic acid is incorporated into gangliosides, but is also a component of mucins, other glycoproteins and free oligosaccharides (6). Glycolipid sialic acid is present at 0.016 mM in colostrum, and 0.006 mM in mature milk (6). Almost 50 % of human milk oligosaccharides are sialylated (6). Sialic acid in milk is protective against intestinal pathogens, and ganglioside-supplemented infant formula positively modifies the intestinal ecology of preterm infants (8). All tissues have the capability to synthesize this molecule, but one of the key enzymes in the liver is low at birth, potentially increasing the importance of dietary sialic acid (6).

It appears that infants are born ready to digest sphingolipids. Sphingolipid enzyme activity, such as sphingomyelinase, is expressed pre-natally, and is present in human fetus meconium as early as 26 weeks gestation (10, 68). The presence of sphingolipid may even encourage maturation of the infant small intestine (10). Although most ceramidase activity in adults in not attributed to bile salt-stimulated lipase, BSSL is responsible for most of the ceramidase activity in milk (10, 68).

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CHAPTER 2. RESEARCH PLAN

A. RATIONALE

This research highlights the important relationship between diet and structure and function of brain cell membranes during development. Previous studies have established that nutritionally complete diets, differing only in fatty acid composition, affect synaptosomal plasma membrane composition and function (1, 2, 3, 4). Synaptosomal membrane is the site of neurotransmitter uptake and release, and the fundamental synaptic component for the processes of learning and memory. Myelin is also essential in the central nervous system in order to maintain proper electrical function. Disorders of myelination during brain development are usually lethal. It is unknown, at this time, whether the lipid composition of nutritionally complete diets affects myelin composition and function.

While gangliosides represent a substantial component of membrane-bound lipids in the central nervous system, their role in nutrition has received little attention. Gangliosides are involved in the growth, development, function, and recovery of neurons. Rapid increases in the concentrations of specific brain gangliosides coincide with major periods of neural cell proliferation and differentiation. Moreover, gangliosides are particularly concentrated in synaptic membrane.

Recent experiments have shown that neurological, and other, tissues are also responsive to the presence of dietary gangliosides (5). Human milk is a source of dietary gangliosides for the developing infant. However, infant formulas fed as acceptable

alternatives to mother's milk do not currently include significant amounts of ganglioside. Thus, infants fed formula do not receive similar amounts or types of gangliosides in their diets compared to breast-fed infants, if they receive any dietary ganglioside at all.

The purpose of this research is to help advance contemporary understanding of interactions between dietary ganglioside and neurological development, using the rat as a model. The relationship between gangliosides in the diet and chemical features of synaptosomal membrane and myelin has never been studied. The knowledge gained from this research may have important implications for both optimal and abnormal brain function, as well as for understanding the role of diet in infant health and development.

B. OBJECTIVES

This research was conducted to determine if dietary ganglioside alters the lipid profiles of synaptosomal membrane and myelin fractions of developing rat brain. The objectives of this research were to determine:

1. if changing dietary ganglioside alters the ganglioside content of synaptosomal membrane or myelin fractions of developing rat brain.
2. if changing dietary ganglioside alters the phospholipid, sphingolipid or cholesterol content of synaptosomal membrane or myelin fractions of developing rat brain.

C. HYPOTHESES

1) The addition of gangliosides GD_3, or GD_3 and GM_3, to the diet of weanling rats will increase total ganglioside content and alter ganglioside composition in synaptosomal membrane and myelin brain fractions.

2) The addition of gangliosides GD_3, or GD_3 and GM_3, to the diet of weanling rats will change the phospholipid, sphingolipid and cholesterol contents in synaptosomal membrane and myelin brain fractions.

The hypotheses posed are tested as described in Chapter 3, by feeding weanling rats nutritionally complete diets differing in ganglioside content. Synaptosomal plasma membrane and myelin fractions are isolated from the developing brains. Lipids are extracted from these fractions, separated by thin layer chromatography, and analyzed by spectrophotometry. The ganglioside, phospholipid, sphingolipid, and cholesterol contents are compared among dietary treatment groups.

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CHAPTER 3. THE EFFECT OF DIETARY GANGLIOSIDE ON LIPID COMPOSITION OF SYNAPTOSOMAL PLASMA MEMBRANE AND MYELIN FRACTIONS OF DEVELOPING RAT BRAIN

A. INTRODUCTION

Following birth, the human infant relies upon ingestion of nutrients for continued growth and development. During this critical stage, infant nutrition is normally supplied by human milk or infant formula. Human milk has naturally evolved as a food source to meet infant biological requirements, however, it is not always possible for every newborn to receive human milk. Knowing this, it is essential for infant formulas, fed as alternatives to mother's milk, to contain all the components necessary for optimal development. Infant formulas currently on the market do not contain all of the nutrients found in human milk.

While it is agreed that all components of human milk are there for a reason, the functions and importance of many remain elusive. Only recently have long chain polyunsaturated fatty acids (PUFAs) been revealed as vital nutrients for infant health, and added to most commercial infant formulas (1). Dietary PUFAs have since received a great deal of attention, while gangliosides, another type of lipid found in human milk, have been largely ignored in nutrition research.

Gangliosides are glycosphingolipids containing one or more sialic acid residues. They are composed of a hydrophobic ceramide base and a hydrophilic oligosaccharide chain. Gangliosides are present in almost every human cell type and tissue, but are

especially abundant in the grey matter of the central nervous system, where they account for 5 - 10 % of neural membrane lipids (2). Gangliosides are involved in many fundamental processes, including: stabilizing membranes, cell recognition and adhesion, biosignal transduction, immune response, neurotrophism and neuroprotection (2, 3, 4, 5, 6, 7).

Human milk is a dietary source of gangliosides for the developing infant. Human colostrum is rich in GD_3, but this ganglioside is replaced in prominence by GM_3 as lactation matures (8). Together, GD_3 and GM_3 account for 50-60 % of the lipid-bound sialic acid in human milk (8). Formula-fed infants do not receive the same quantities or types of gangliosides in their diet. If the formula is plant-based, an infant will not acquire any dietary ganglioside at all. If dietary ganglioside is important for developing brain, formula-fed infants are at a disadvantage. A recent experiment demonstrated that feeding ganglioside to weanling rats increased the ganglioside content of whole brain tissue (9).

This paper investigates whether differences in dietary ganglioside affect the lipid composition of synaptosomal plasma membrane and myelin fractions of developing rat brain. Changes in the lipid composition of these brain fractions may indicate an important change in their functional abilities to regulate neurotransmission and electrical conductance in brain. It is hypothesized that: 1) the addition of gangliosides GD_3, or GD_3 and GM_3, to the diet of weanling rats will increase total ganglioside content and alter ganglioside composition in synaptosomal plasma membrane and myelin brain fractions; and 2) the addition of gangliosides GD_3, or GD_3 and GM_3, to the diet of weanling rats will change the phospholipid, sphingolipid and cholesterol contents in synaptosomal membrane and myelin brain fractions.

The relationship between dietary ganglioside and the chemical features of synaptosomal plasma membrane and myelin has not been studied. This research may have important implications for infant nutrition, as well as for advancing understanding of the role of diet in optimal and abnormal brain function.

B. MATERIALS AND METHODS

i) Diets

Four dietary treatments, varying only in lipid composition: 1) control diet (F); 2) GD_3 ganglioside diet (F + GD_3); 3) GD_3/GM_3 ganglioside diet (F + GD_3/GM_3); and 4) ganglioside and long chain PUFA diet (F + GD_3/GM_3 + PUFA) were fed. Composition of the basal diet fed is illustrated (Table 3.1). The control diet fat (F) was a blend of oils, which reflected the fatty acid composition of a commercial infant formula. Dietary fatty acids were composed of approximately 31 % saturated, 48 % monounsaturated and 21 % polyunsaturated fatty acids, with an 18:2n-6 to 18:3n-3 ratio of 6.0. Ganglioside mixtures were combined with the fat blend at 0.1 % of fat. For the diet containing PUFA, arachidonic acid and docosohexaenoic acid (DHA) comprised 1.0 % and 0.5 % of the combined fat blend, respectively. In the GD_3 ganglioside diet, GD_3 was the predominant ganglioside species, accounting for 92 % of the total ganglioside. The remainder consisted of GM_3 and GD_{1a}. In the GD_3/GM_3 ganglioside diets, GD_3 and GM_3 gangliosides were present in approximately equal ratio. Together, GD_3 and GM_3 accounted for 99 % of the ganglioside mixture. All diets required for the entire feeding

period were made in one day and stored at – 20 °C. Diet in the rat cages was refreshed every 2-3 days.

Table 3.1. Basal diet composition

	Ingredients	Amount (g/kg)
Basal diet		*800.00*
	Casein	270.00
	Corn starch	200.00
	Dextrose	207.65
	Non-nutritive Cellulose	50.00
	Vitamin mixture[a]	10.00
	Mineral mixture[b]	50.85
	Choline chloride	2.75
	Inositol	6.25
	L-Methionine	2.50
Fat blend		*200.00*
	Corn oil	42.00
	Coconut oil	92.00
	Canola oil	46.00
	Oleic acid oil	20.00

* The basal diet composition was adjusted, as required, to compensate for ingredients contained within the ganglioside powder.
[a] A.O.A.C (40055) Harlan Teklad, Madison, WI, USA
[b] B-T (170750) Harlan Teklad, Madison, WI, USA

ii) Animals

Weanling, male Sprague-Dawley rats (n = 72) were used for this experiment. Pups were reared in two successive cohorts, differing in age by one week. All animals were kept on a 12 hr light/dark cycle, at a constant temperature of 23 °C, and regulated humidity. Pregnant female rats were obtained from Charles River Canada at 21 days gestation. The dams were housed one per cage with access to standard rat chow and tap water *ad libitum.* Litters were culled to 11 pups, saving the males when possible. Male

pups were weaned at 18-19 days postpartum and housed three per cage. Males from the same litter were divided among the treatment groups, so that each treatment contained a similar mixture of pups from each of the litters. Each cage supported *ad libitum* access to one of four modified diets and tap water. The pups were fed for a period of two weeks prior to harvesting of the brain tissue. Body weight and food intake were recorded daily upon weaning for 3 days and every 2-3 days following. Total food consumption for each cage was divided by the number of animals in the cage. This experiment was approved by the University of Alberta, Agriculture, Forestry and Home Economics Faculty Animal Policy and Welfare Committee.

iii) Extraction of brain tissue

Following anesthetizing with carbon dioxide, rats were euthanized by cervical dislocation and the whole brain tissue was excised. Three rats from each dietary treatment were killed each day over a period of three days. Whole brains extracted from the rats were kept on ice in 10 % (w/v) sucrose buffer with enzyme inhibitor cocktail (Table 3.2). To increase the volume of brain tissue available for biochemical analysis, three rat brains from the same treatment group were pooled to comprise one sample.

Table 3.2. Enzyme inhibitor cocktail composition

Ingredient	EDTA	Pepstatin A	Aprotinin	Leupeptin	Trypsin Inhibitor	PMSF
Amount	1 mM	5µg/mL	5µg/mL	5µg/mL	5µg/mL	1 mM
pH (7.0-7.4)						

* Chemicals from Sigma, St. Louis, MO, USA.

iv) Isolation of brain fractions

Synaptosomal plasma membrane, myelin and mitochondrial fractions were isolated from the fresh brain samples by a procedure developed by Cruz and Gurd (1978) and standardized in this laboratory (10, 11, 12). All methods were performed at 0-4 °C. Brain samples were sliced by razor blade into small fractions and homogenized (12 strokes) in a glass-glass homogenizer in 18 mL (6 vol) of 10 % sucrose buffer with enzyme inhibitor cocktail. A 200 μL aliquot of brain homogenate was saved on ice for use as a comparative standard. The remaining homogenate was centrifuged for 10 min at 3 000 g (JA-20 rotor, J2-21 centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA). The supernatant was saved, while the pellet was washed in 18 mL of 10 % sucrose buffer (5 strokes) and centrifuged again. The two supernatants were pooled and centrifuged for 20 min at 10 500 g. The pellet was collected and resuspended in 25 mL of 10 % sucrose buffer for washing. The resuspension was centrifuged at 3 000 g for 10 min and the resulting supernatant was centrifuged at 10 500 rpm for 20 min. This washed pellet was resuspended in 24 mL (8 vol) of 5mM Tris-HCl (pH 8.1) and let stand for 30 min on ice to lyse the synaptosomes. Three homogenizer strokes were applied to ensure release of all synaptosomal contents. The mixture was ultracentrifuged (SW-27 rotor, L8-70M ultracentrifuge, Beckman Instruments Inc., Palo Alto, CA, USA) for 25 min at 24 000 rpm. The pellet was completely resuspended in 8.5 mL of 35 % sucrose solution, also containing enzyme inhibitor cocktail. A discontinuous sucrose gradient (8.5 mL of 29 % (w/v) sucrose, 7.8 mL of 24 % (w/v) sucrose, 2.0 mL of 10 % sucrose buffer) was constructed on top of the suspension. The gradient was ultracentrifuged for 110 min at 96 000 g. The myelin (MYE) fraction was collected at the 24-29 % sucrose interface; the

synaptosomal plasma membrane (SPM) fraction was collected at the 29-35 % sucrose interface, and a mitochondrial (MTC) fraction as the pellet. All fractions were pelleted and resuspended in 1.0 mL of 10 % sucrose buffer for immediate protein content and/or purity analysis. The saved homogenate (HOM) fraction was mechanically homogenized (3 s; PTA 7 probe: ¼ in., Kinematica Polytron PT 10 35, Brinkmann Instruments (Canada) Ltd., Rexdale, ON) and made to 1.0 mL with 10 % sucrose buffer.

v) Protein content and purity of brain fractions

Protein content

Total protein content was measured using a BCA™ kit (Pierce, Rockford, IL, USA). Bovine serum albumin (Sigma, St. Louis, MO, USA) was used as a protein standard. SPM, HOM and MTC samples were diluted 4 times. MYE was measured without dilution. Sample and standard aliquots of 10 µL were incubated in a covered microplate with 200 µL of working reagent for 30 min at 37 °C. Optical density was measured at 562 nm (Spectramax 384 Plus Microplate Spectrophotometer, Molecular Devices, Sunnyvale, CA, USA).

Electron microscopy

A preliminary screening of SPM and MYE fraction purity was performed by electron microscopy. Sample aliquots were suspended in 1 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer, rinsed three times in 0.1 M cacodylate buffer, and fixed in 1:3 (v/v) osmium tetraoxide/0.2 M cacodylate buffer for 1 hr. Samples were dehydrated in increasing concentrations of ethanol, soaked three times in propaleneoxide for 10 min,

and embedded in resin. Technical assistance from Dr. Ming Chen of the University of Alberta Medicine/Dentistry EM Unit is gratefully acknowledged.

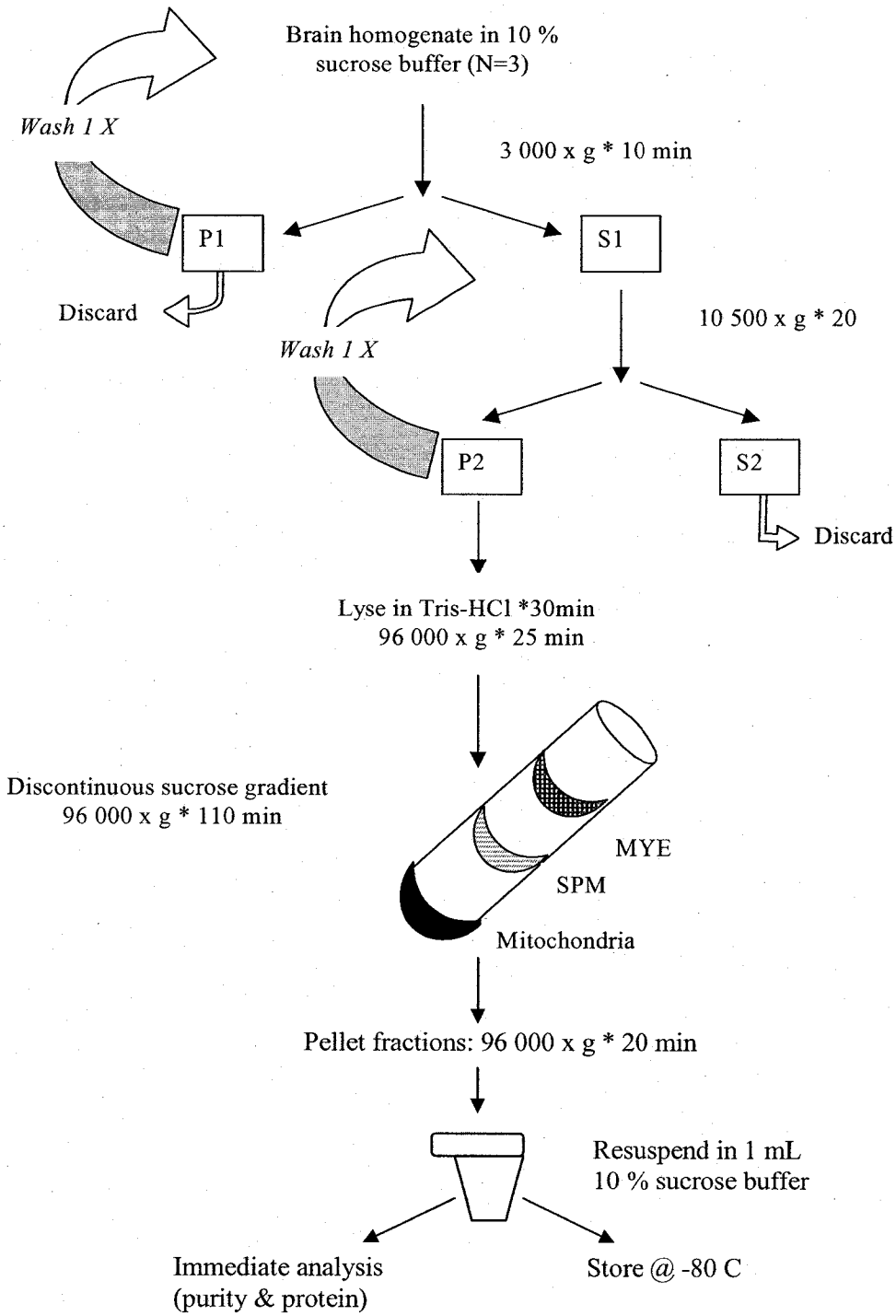

Figure 3.1. Procedure for isolating brain fractions

Enzymatic analyses

Synaptic plasma membrane purity was also determined enzymatically. Na^+-K^+ ATPase assay was used to compare enzyme activity between SPM and HOM fractions (13, 14). Two 50 µL aliquots of diluted SPM and HOM sample were incubated at 37 °C for 15 min in 950 µL of freshly prepared reaction buffer containing 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 30 mM histidine and 3 mM ATP, pH 7.4, in the presence or absence of 10 mM ouabain, a specific inhibitor of Na^+-K^+ ATPase (15). The reaction was terminated with addition of 500 µL of 10 % (w/v) trichloroacetic acid. Following microcentrifugation at 2100 x g for 10 min (Eppendorf Centrifuge 5415C, Brinkmann Instruments (Canada) Ltd., Mississauga, ON), 100 µL of supernatant was removed for microdetermination of inorganic phosphorous (16), using K_2HPO_4 as a standard. Sample and standard aliquots (100 µL) were adjusted to 4 mL with distilled water and incubated at 37 °C for 1.5-2 hr with 4 mL of freshly prepared reagent C. Optical density was read at 820 nm (Spectramax 384 Plus). Na^+-K^+ ATPase activity was expressed as µmol Pi/mg protein/hr.

Mitochondrial contamination of the synaptosomal plasma membrane fraction was determined by succinate dehydrogenase assay in comparison to the mitochondrial fraction (17). SPM and MTC samples were diluted 4 times in 10 % sucrose buffer, with a total volume of 200 µL. Sample aliquots (10 µL) were mixed with 990 µL of reagent in glass-stoppered tubes. The tubes were gently shaken in a water bath at 37 °C for 15 min (Dubnoff Metabolic Shaking Incubator, Precision Scientific, Chicago, IL, USA). The reaction was terminated with 1 mL of 10 % (w/v) trichloroacetic acid. Formazan product was extracted with 4 mL of ethyl acetate. Samples were vortexed (Genie 2™, Allied

Fisher Scientific, Bohemia, NY, USA) to ensure complete colour extraction immediately prior to spectrophotometry at 490 nm (Spectramax 384 Plus). Succinate dehydrogenase activity was expressed as nmol product/mg protein/min. Remaining samples were snap frozen in liquid nitrogen and stored at –80 °C.

vi) Lipid extraction

Myelin and SPM lipids were extracted using the Folch method in 30 mL 2:1 (v/v) chloroform/methanol (18). Test tubes were shaken at low speed for 3 hr (Dubnoff Metabolic Shaking Incubator). Next, 6 mL of 0.025 % (w/v) calcium chloride solution was gently mixed in by inverting the tubes 5 times. The polar and non-polar phases were allowed to separate overnight at 4 °C. Gangliosides were derived from the upper (polar) phase, while phospholipids, sphingolipids, and cholesterol were derived from the lower (non-polar phase). For extracting gangliosides, the upper phase was removed and the lower phase was washed with Folch theoretical upper phase solution, chloroform/methanol/water (3:48:47, v/v/v), and centrifuged for 5 min at 1000 rpm (Sorvall SS-3 Automatic Superspeed Centrifuge, Ivan Sorvall Inc., Norwalk, CT, USA). The two upper phase solutions were pooled and filtered through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA), prewashed with 5 mL of methanol and 10 mL of 2:1 (v/v) chloroform/methanol (19). The cartridges were washed with 7 mL of distilled water to remove salts and water-soluble contaminants. The gangliosides were eluted with 5 mL of methanol and 10 mL of 2:1 (v/v) chloroform/methanol. Gangliosides, as well as the non-polar phase, were dried under nitrogen gas, redissolved in a known volume of 2:1 (v/v) chloroform/methanol, and stored at 4 °C until analysis.

vii) Ganglioside analysis

Gangliosides were measured as ganglioside-bound NANA (20). Individual gangliosides were separated from an aliquot of ganglioside sample by silica gel high performance thin-layer chromatography (HPTLC; Whatman Inc, Clifton, NJ, USA) in chloroform/methanol/0.25 % calcium chloride solvent (55:45:10, v/v/v). After development, dry TLC plates were sprayed with 0.1 % (w/v) anilino naphthalene sulfonic acid (ANSA) and exposed to UV light to visualize the lipid bands. Ganglioside bands were scraped into test tubes. A standard curve was prepared fresh for each analysis using NANA (Sigma, MO, USA). All test tubes were made up to 500 μL with distilled water and sonicated (5 s; Sonic 300 Disembrator, Artek Systems Corp., Farmingdale, NY, USA). All tubes were reacted with 500 μL of resorcinol-HCl, using Teflon-lined screw caps, at 160 °C for 9 min (Fisher Isotemp® Oven 255G, Allied Fisher Scientific, Bohemia, NY, USA). The purple-blue colour produced was extracted by adding 500 μL of butylacetate/butanol (85:15, v/v), with centrifugation for 10 min at 350 x g to precipitate the silica particles (Sorvall SS-3). The optical density was determined by spectrophotometry (Hewlett Packard, 8452A) at 580 nm. Ganglioside amount was expressed as μg NANA/mg protein. Total ganglioside amount was measured as described above, without TLC separation. Total ganglioside was also determined via addition of individual ganglioside amounts.

viii) Phospholipid analysis

Individual phospholipids were separated on H-grade TLC plates (Whatman Inc, Clifton, NJ, USA) in chloroform/methanol/2-propanol/0.25 % potassium

chloride/triethylamine solvent (30:9:25:6:18, v/v/v/v/v). Dry TLC plates were sprayed with 0.1 % ANSA to visualize the lipid bands under UV light. The phospholipid bands were scraped into test tubes. A phosphorous standard curve was prepared for each analysis using various concentrations of phosphorous standard (1.5 mM KH_2PO_4 in 8.5 % (w/v) sucrose solution), dried on a heating block. Perchloric acid (1 mL) was added to all tubes. In order to digest the phospholipid, the test tubes were heated in blocks for 3 hr at 210 °C, capped with marbles. To cooled tubes, 6 mL of colour-producing reagent was added (Table 3.3). Tubes were capped and reacted in boiling water for 7-10 min. The blue colour was extracted by adding 2 mL of 85:15 (v/v) butylacetate/butanol. The optical density was determined at 790 nm (Spectramax 384 Plus). Phospholipid amount was expressed as μg Pi/mg protein. Total phospholipid was determined via addition of individual phospholipid amounts.

Table 3.3. Composition of colour-producing reagent for phospholipid analysis

Product	Amount
Colour-producing reagent	
5 % ammonium molybdate	0.4 mL
Reducing reagent	0.4 mL
Distilled water	8.0 mL
Reducing reagent	
Sodium bisulfite	2.500 g
Sodium sulfite	0.500 g
4-amino-3-hydroxy-naphthalene-1-sulfonic acid	0.042 g
Distilled water	250 mL

ix) Sphingolipid analysis

Lipid aliquots required preparation before sphingolipid analysis. Lipid aliquots were methylated by incubation in 2 mL of 3 N hydrochloric acid in methanol at 80 °C for

20 hr (Fisher Isotemp® Oven). Following incubation, 1.5 mL of distilled water and 4.0 mL of chloroform were added. The non-polar phase was extracted for lipid analysis and dried under nitrogen gas. Lipid samples were shaken slowly at room temperature for 1 hr in 1 mL of chloroform and 3 mL of 0.2 N sodium hydroxide in methanol (Dubnoff Metabolic Shaking Incubator). An additional 3 mL of chloroform and 3 mL of 1 M sodium chloride were combined with the solution. Samples were centrifuged for 10 min at 350 x g (Sorvall SS-3). The nonpolar phase was extracted, dried, and redissolved in 200 uL of 2:1 (v/v) chloroform/methanol. Lipid samples were separated on G-grade TLC plates (Whatman Inc, Clifton, NJ, USA) in two consecutive solvent systems: 1) 60:35:8 (v/v/v) chloroform/methanol/distilled water, run to one-third of the plate, dried, and 2) 90:2:8 (v/v/v) chloroform/methanol/ammonium hydroxide, run to the top of the plate. The plates were sprayed with 0.1 % ANSA to visualize the lipid bands under UV light. The sphingolipid bands were scraped into test tubes. A standard curve was prepared for each analysis using various concentrations of d-sphingosine (Sigma, St. Louis, MO, USA), dried under nitrogen gas. All test tubes were incubated for 1 hr at 45 °C in a dark oven (Fisher Isotemp® Oven) with 1 mL of 4 % (w/v) aqueous sodium bicarbonate and 1 mL of 1 % (w/v) aqueous trinitrobenzene sulfuric acid (TNBS). Following incubation, 1 mL of 1 N methanolic HCl was added. Sphingolipids were extracted by the addition of 2 mL of hexane to all samples. Samples were washed with another 2 mL of hexane, and the pooled hexane fraction was dried under nitrogen gas. The dried fractions were redissolved in 2 mL of ethanol. Optical density was measured at 340 nm (Spectramax 384 Plus). Sphingolipid amount was expressed as μg sphingosine/mg protein.

x) Cholesterol analysis

Total cholesterol amount was determined by cholesterol test kit (Infinity™, Thermo Electron Corp., Melbourne, AUS). Cholesterol sample and standard aliquots were dried under nitrogen gas and redissolved in equal volumes of ethanol. Cholesterol solutions were reacted with 400 mL of cholesterol reagent for 5 min at 37 °C. The product was microcentrifuged for 10 min at 16 000 x g (Eppendorf 5415C) to remove any contaminating particles from the solution. Colour intensity was read at 520 nm (Spectramax 384 Plus).

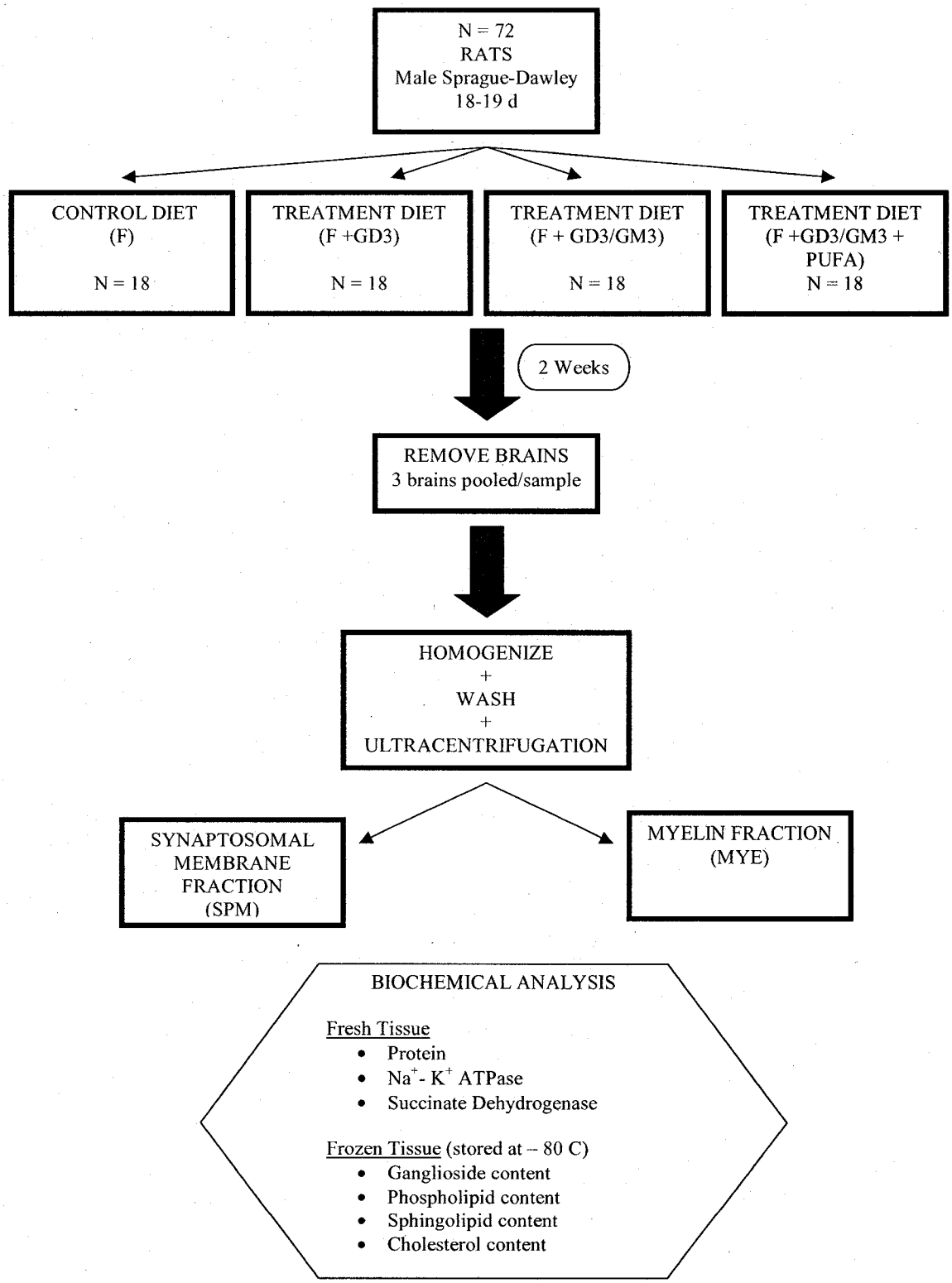

Figure 3.2. Summary of experimental methodology

xi) Statistical analysis

The values shown are means ± standard deviation (SD). For statistical analysis, the data were blocked for the procedure day (1-3) to eliminate the possible confounding effect of time on diet treatment results. Significant differences between the control group and treatment groups were determined by one-way analysis of variance (ANOVA) with SAS (SAS Institute Inc, Version 8.2, NC, USA). Duncan multiple range test was used to determine significant effects of dietary treatment or day, at a significance level of $p<0.05$.

C. RESULTS

i) Animal growth and tissue

Upon weaning, rats weighed 39.4 ± 1.9 g. There was no significant difference in the mean body weight of rats among treatment groups. Upon euthanasia, the rats weighed 136.4 ± 6.7 g. There was no significant difference in the mean body weight of rats among treatment groups. However, there was a consistent trend for reduced body weight in the control group compared to the GD_3 ganglioside group and the ganglioside and PUFA group. The difference was, on average, 10.8 g, but diminished as the age of the rats increased. Rats were euthanized over a period of three days. Rats gained approximately 9.5 g each procedure day. Body weights ranged from 126.5 ± 8.0 g on Day 1 to 145.6 ± 5.7 g on Day 3 of euthanasia. There was no difference in the mean food intake between treatment groups throughout the feeding period. The brain tissue mass did not change with diet or time. Each brain weighed approximately 1.75 g, while each pooled brain sample weighed 5.4 ± 0.1 g.

ii) Protein content and purity of brain fractions

Protein content

There was no difference in the protein content of brain fractions among dietary groups (Figure 3.3). Whole brain homogenate had the most protein (5.0 ± 0.5 mg/mL), followed by SPM (1.4 ± 0.4 mg/mL) and then myelin (0.5 ± 0.1 mg/mL). All brain fractions, except myelin, were diluted 4 times for protein content analysis.

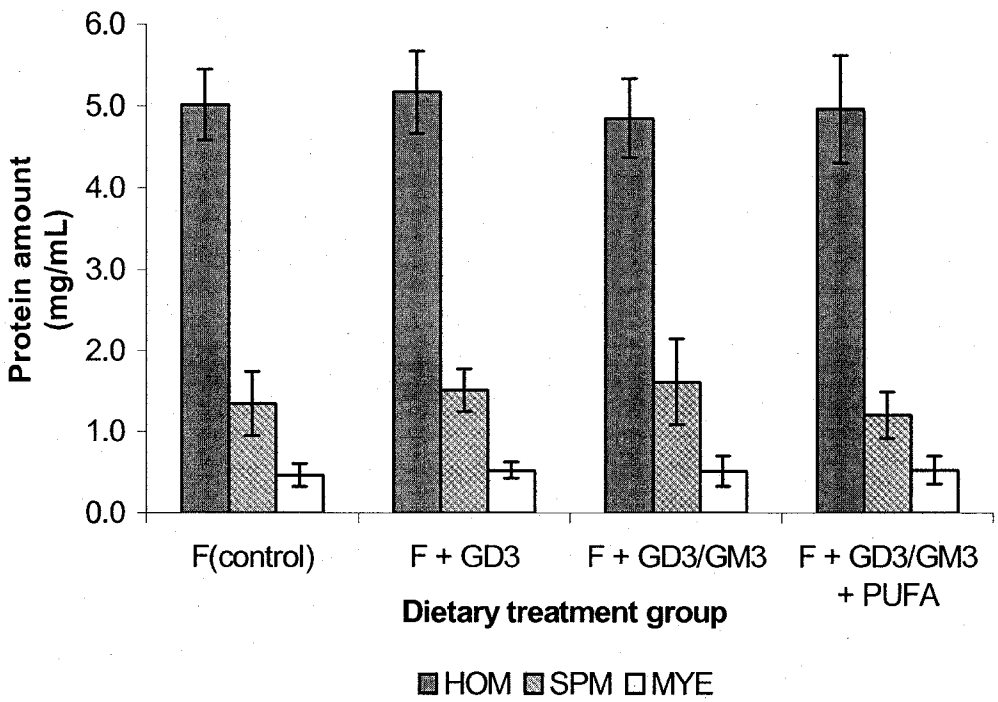

Figure 3.3. Protein content (mg/mL) of homogenate, synaptosomal plasma membrane and myelin for each dietary treatment group.

Electron microscopy

Electron micrographs of myelin and synaptosomal plasma membrane are shown in Figure 3.4.

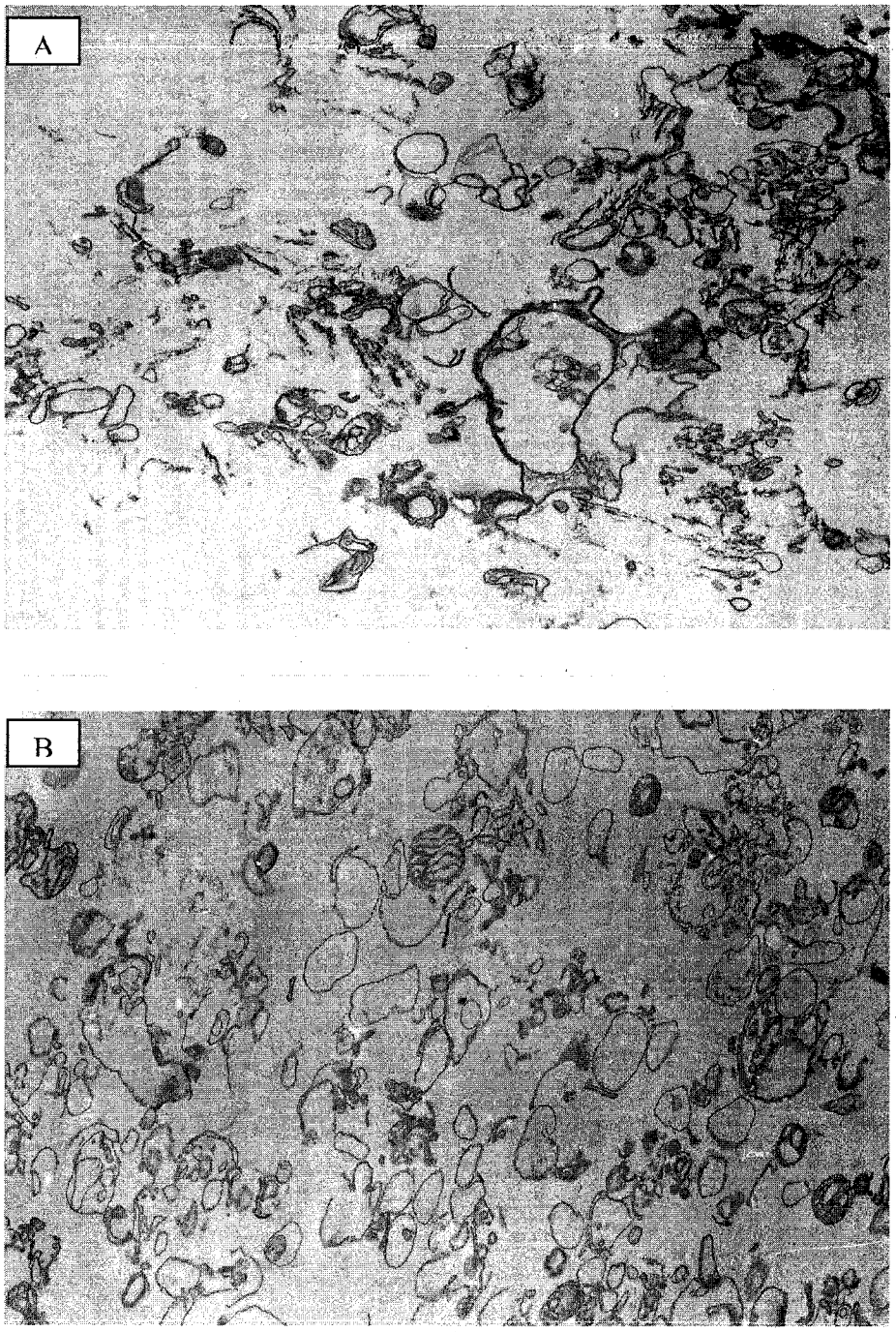

Figure 3.4. EM photographs of A. MYE (3600X) and B. SPM (3600X).

Enzymatic analyses

Na^+-K^+ ATPase is a marker for synaptosomal plasma membrane. Therefore, enzyme activity should be greater in the SPM fraction, compared to whole brain homogenate. Na^+-K^+ ATPase activity was 30.7 ± 11.5 μmol Pi/mg protein/hr in the SPM fraction and 5.2 ± 1.9 μmol Pi/mg protein/hr in the homogenate. The SPM/HOM ratio of Na^+-K^+ ATPase activity was 6.5 ± 3.4. Variation in the ganglioside and PUFA diet group was considerably greater due to a few high values from the SPM fraction. Na^+-K^+ ATPase activity may be an indication of SPM function. There was a trend evident in the SPM/HOM ratios between dietary treatments: F + GD_3/GM_3 diet < F + GD_3 diet < F diet < F +GD_3/GM_3 +PUFA ($p = 0.12$).

Succinate dehydrogenase (SDH) is a marker for mitochondrial contamination. Therefore, enzyme activity should be negligible in the SPM fraction compared to the mitochondrial fraction. Succinate dehydrogenase activity was 0.09 ± 0.05 nmol/mg protein/min in SPM and 0.53 ± 0.1 nmol/mg protein/min in mitochondria. The SPM/MTC ratio of succinate dehydrogenase activity was 8.8 ± 6.7. There was no difference in the enzyme activity or SPM purity among dietary treatments. There was a nonsignificant trend ($p=0.08$) for decreased mitochondrial SDH activity over time: Day 1> Day 2 > Day 3.

Table 3.4. Activities of Na^+-K^+ ATPase (μmol Pi/mg protein/hr) and succinate dehydrogenase (nmol/mg protein/min) in synaptosomal plasma membrane relative to whole brain homogenate and mitochondria, respectively, as a measure of sample purity.

	Na^+-K^+ ATPase (μmol Pi/mg protein/hr)		
Diet	SPM	HOM	SPM/HOM ratio
F (control)	33.0 ± 7.3	5.3 ± 1.6	6.7 ± 1.7
F + GD_3	29.6 ± 4.7	5.5 ± 2.1	5.6 ± 1.5
F + GD_3/GM_3	23.1 ± 4.2	4.8 ± 1.8	5.7 ± 2.7
F + GD_3/GM_3 + PUFA	37.6 ± 19.5	5.4 ± 2.2	8.0 ± 6.0
Mean	30.7 ± 11.5	5.2 ± 1.9	6.5 ± 3.4
	Succinate dehydrogenase (nmol product/mg protein/hr)		
Diet	SPM	MTC	SPM/MTC ratio
F (control)	0.1 ± 0.1	0.5 ± 0.1	10.5 ± 10.4
F + GD_3	0.1 ± 0.0	0.5 ± 0.1	7.1 ± 2.5
F + GD_3/GM_3	0.1 ± 0.0	0.5 ± 0.1	9.2 ± 5.4
F + GD_3/GM_3 + PUFA	0.1 ± 0.1	0.5 ± 0.1	8.3 ± 7.4
Mean	0.1 ± 0.0	0.5 ± 0.1	8.8 ± 6.7

ii) Total ganglioside content of synaptosomal plasma membrane and myelin

Dietary treatment did not change total ganglioside content in synaptosomal plasma membrane or myelin (Figure 3.5). The total ganglioside amount in SPM was 19.7 ± 3.0 μg NANA/mg protein. Myelin has a significantly higher lipid to protein ratio compared to SPM. The total ganglioside content of myelin was 48.5 ± 14.1 μg NANA/mg protein. There was a significant difference in myelin total ganglioside due to procedure day ($p = 0.02$), with Day 3 showing the greatest ganglioside content, and Day 2 the least.

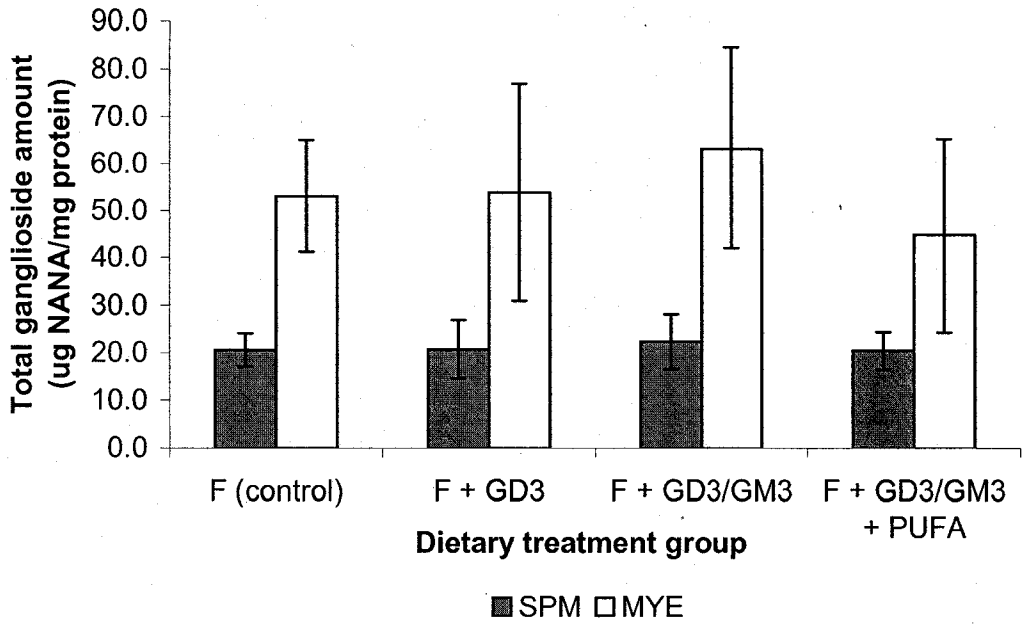

Figure 3.5. Total ganglioside content (μg NANA/mg protein) of synaptosomal plasma membrane and myelin brain fractions for each dietary treatment group.

iii) Individual ganglioside composition of synaptosomal plasma membrane and myelin

Individual gangliosides were measured and expressed as relative percentages of total ganglioside-bound sialic acid (Table 3.5). Gangliosides present in both brain fractions included: GQ_{1b}, GT_{1b}, GD_{1b}, GT_{1a}, GD_{1a}, GD_3, GM_1, GM_2, GM_3, and GM_4. There were significant differences in the relative amounts of certain gangliosides depending on dietary treatment. In synaptosomal plasma membrane, GM_4 was higher in the ganglioside and PUFA diet compared to all other diets ($p = 0.003$). In myelin, the complex ganglioside GQ_{1b} was higher in the GD_3 diet compared to the control diet ($p = 0.009$). The procedure day produced significant differences for GD_{1b}, GM_3 and GM_4 relative amounts in synaptosomal plasma membrane ($p = 0.01$-0.02), with Day 2 or Day 3 usually showing the greatest percentage. In myelin, procedure day also affected GQ_{1b} relative percentage ($p = 0.003$).

Table 3.5. Relative percentages of individual ganglioside-bound sialic acid (%) in synaptosomal plasma membrane and myelin brain fractions for each dietary treatment group.

Ganglioside	Brain fraction	Dietary treatment				
		F (control)	F + GD_3	F + GD_3/GM_3	F + GD_3/GM_3 + PUFA	All diets
GQ_{1b}	SPM	16.5 ± 5.0	17.6 ± 5.4	17.4 ± 4.1	16.4 ± 3.0	16.9 ± 3.6
	MYE	11.9 ± 1.6	15.7 ± 1.4*	12.5 ± 3.9	12.5 ± 2.0	12.5 ± 2.9
GT_{1b}	SPM	12.0 ± 3.6	14.3 ± 2.7	13.6 ± 2.9	12.4 ± 1.5	13.0 ± 2.2
	MYE	11.5 ± 3.7	11.9 ± 3.3	10.2 ± 3.2	10.9 ± 2.1	10.5 ± 2.6
GD_{1b} + GT_{1a}	SPM	7.1 ± 3.1	7.5 ± 1.7	6.9 ± 1.8	6.3 ± 1.6	6.6 ± 1.7
	MYE	5.9 ± 1.8	5.4 ± 1.0	4.3 ± 2.5	5.4 ± 2.3	4.8 ± 2.4
GD_{1a}	SPM	21.1 ± 7.0	22.0 ± 6.7	21.8 ± 4.8	20.1 ± 3.2	21.3 ± 4.0
	MYE	19.6 ± 6.0	21.3 ± 4.9	23.0 ± 3.3	17.9 ± 2.0	20.4 ± 2.7
GD_3	SPM	9.5 ± 4.0	8.8 ± 3.3	11.6 ± 8.0	10.2 ± 2.8	10.9 ± 5.4
	MYE	22.4 ± 12.3	13.0 ± 3.3	17.4 ± 10.3	17.2 ± 4.4	17.3 ± 7.4
GM_1	SPM	10.9 ± 3.1	9.7 ± 2.3	12.5 ± 2.0	11.3 ± 1.7	11.9 ± 1.9
	MYE	9.8 ± 3.6	13.1 ± 2.2	12.7 ± 2.5	11.4 ± 1.3	12.0 ± 1.9
GM_2	SPM	12.7 ± 20.4	10.7 ± 16.7	6.2 ± 3.6	5.8 ± 2.5	6.0 ± 3.0
	MYE	4.3 ± 2.3	4.6 ± 2.2	4.0 ± 3.1	5.0 ± 2.1	4.5 ± 2.6
GM_3	SPM	5.0 ± 1.4	5.5 ± 4.2	5.3 ± 2.2	7.8 ± 2.2	6.5 ± 2.2
	MYE	8.3 ± 3.8	6.2 ± 2.4	5.8 ± 1.2	8.7 ± 3.5	7.3 ± 2.3
GM_4	SPM	5.1 ± 1.2	5.5 ± 2.7	5.9 ± 2.3	9.6 ± 2.9*	7.8 ± 2.6
	MYE	6.3 ± 2.9	8.8 ± 1.6	10.1 ± 6.6	10.9 ± 4.1	10.5 ± 5.4
GD/GM ratio	SPM	1.4 ± 0.7	1.5 ± 0.9	1.5 ± 0.5	1.1 ± 0.2	1.3 ± 0.6
	MYE	1.8 ± 0.6	1.2 ± 0.2	1.5 ± 0.7	1.2 ± 0.4	1.4 ± 0.6

* $p < 0.05$

In SPM, GD_{1a}, GQ_{1b}, GT_{1b}, GM_1, and GD_3 were the most abundant gangliosides (> 70 % lipid-bound NANA). In myelin, GD_{1a} and GD_3 were most abundant. The myelin brain fraction had greater proportions of GD_3, GM_3 and GM_4, compared to synaptosomal plasma membrane, at the expense of GM_2 and the complex gangliosides. There was no difference in the ratio of disialogangliosides to monosialogangliosides among dietary groups. The ratio was also comparable between SPM and MYE fractions (SPM: 1.3 ± 0.6; MYE: 1.4 ± 0.6).

iv) Phospholipid profile of synaptosomal plasma membrane and myelin

There was no significant difference in total phospholipid content of SPM or myelin among dietary treatment groups (Figure 3.6). Total phospholipid content of synaptosomal plasma membrane was 66.7 ± 23.9 μg P/mg protein. Total phospholipid content of myelin was 91.6 ± 24.2 μg P/mg protein. There was no significant difference in total phospholipid content due to procedure day.

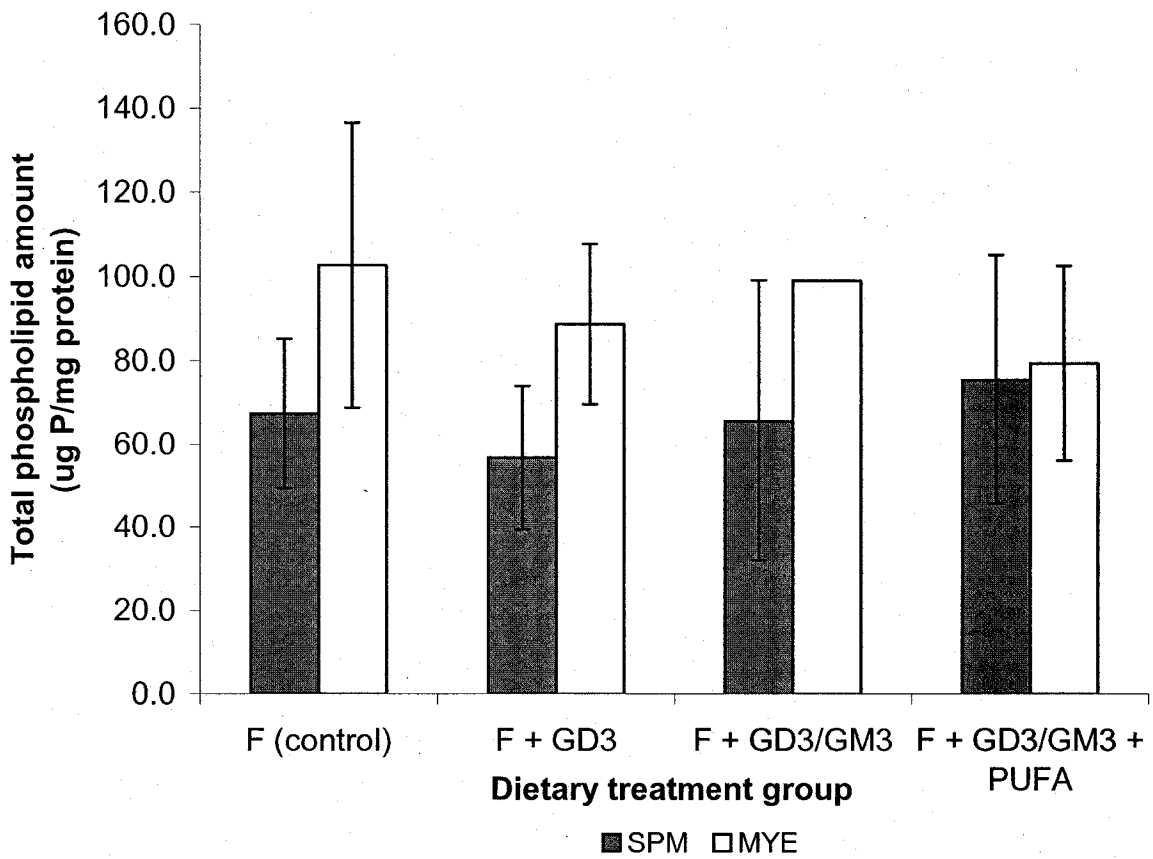

Figure 3.6. Total phospholipid content (μg P/mg protein) of synaptosomal plasma membrane and myelin brain fractions for each dietary treatment group.

Individual phospholipids were measured, and expressed as relative percentages of total phospholipid amount (Table 3.6). Phospholipids present in both brain fractions

included: lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). Dietary treatment did produce significant changes in the relative amounts of one phospholipid species. In synaptosomal plasma membrane, LPC was relatively increased in the ganglioside and PUFA diet compared to all other treatment groups ($p = 0.02$). In myelin, there was no change in individual phospholipid proportions among dietary treatment groups. Procedure day produced significant differences in the relative percentage of LPC in synaptosomal plasma membrane ($p = 0.0001$), as well as in the relative percentages of SM and PE in myelin ($p = 0.02$; $p = 0.03$). Day 1 showed the greatest amounts most frequently. There was no difference in the PC/PE ratios among diet groups, and the ratios were similar between SPM and myelin (SPM: 1.7 ± 0.3; MYE: 1.3 ± 0.6). The most abundant phospholipid species in both brain fractions were PC and PE.

Table 3.6. Relative percentages of individual phospholipids (%) in synaptosomal plasma membrane and myelin brain fractions for each dietary treatment group.

Phospholipid	Brain fraction	Dietary treatment				
		F (control)	F + GD_3	F + GD_3/GM_3	F + GD_3/GM_3 + PUFA	All diets
LPC	SPM	3.2 ± 2.3	2.5 ± 1.4	3.9 ± 1.4	5.4 ± 3.2*	3.8 ± 2.1
	MYE	4.2 ± 1.1	2.5 ± 1.6	3.3 ± 1.5	4.2 ± 1.2	3.5 ± 1.4
SM	SPM	8.0± 2.0	8.2 ± 3.2	6.6 ± 1.1	8.5± 2.6	7.8 ± 2.2
	MYE	6.1 ± 1.2	5.6 ± 1.4	6.6 ± 1.7	7.3 ± 3.4	6.4 ± 1.9
PC	SPM	45.2 ± 3.7	45.2± 2.9	44.8 ± 3.8	44.6 ± 6.1	44.9 ± 4.1
	MYE	37.7± 4.9	40.2 ± 4.9	41.1 ± 1.4	37.5 ± 7.9	39.1 ± 4.7
PS	SPM	10.4 ± 4.0	10.8 ± 2.2	9.7± 3.0	9.6 ± 3.0	10.1 ± 3.1
	MYE	12.6 ± 2.4	13.5 ± 3.6	14.1 ± 2.4	15.6 ± 5.4	14.0 ± 3.4
PI	SPM	6.6 ± 1.4	6.1 ± 1.7	6.0 ± 1.9	5.1 ± 1.7	5.9 ± 1.7
	MYE	6.2 ± 2.4	5.9 ± 1.3	5.9 ± 1.7	6.6 ± 1.5	6.2 ± 1.7
PE	SPM	26.6 ± 4.8	27.1 ± 2.8	29.0 ± 3.5	26.9 ± 5.7	27.4 ± 4.2
	MYE	33.2 ± 2.9	32.2 ± 4.2	29.1 ± 7.7	28.8 ± 8.6	30.8 ± 5.8
PC/PE ratio	SPM	1.7 ± 0.4	1.7 ± 0.1	1.6 ± 0.3	1.7 ± 0.5	1.7 ± 0.3
	MYE	1.2 ± 0.2	1.2 ± 0.4	1.5 ± 0.5	1.5 ± 1.0	1.3 ± 0.6

* $p < 0.05$

v) Sphingolipid profile of synaptosomal plasma membrane and myelin

The sphingolipid profile, other than gangliosides, was analyzed. The amounts of individual sphingolipids were determined as relative percentages of total sphingolipid amount (Table 3.7). Sphingolipids present in both brain fractions included: sphingomyelin (SM), lactosylceramide (LacCer), galactosylceramide (GalCer), glucosylceramide (GlcCer), and ceramide (Cer). There were no significant differences in the SPM sphingolipid profiles among diet groups. However, in myelin, glucosylceramide was present in greater proportion in the ganglioside and PUFA diet compared to all other treatment groups ($p = 0.02$). Procedure day produced significant differences in the relative amounts of galactosylceramide, glucosylceramide, and ceramide from myelin,

however, there was no trend evident for which day showed the highest values. In synaptosomal plasma membrane, SM, GalCer and Cer were present in the highest proportions. In myelin, GalCer was the most prevalent sphingolipid.

Table 3.7. Relative percentages of individual sphingolipids (%) in synaptosomal plasma membrane and myelin brain fractions for each dietary treatment group.

Sphingolipid	Brain fraction	Dietary treatment				
		F (control)	F + GD_3	F + GD_3/GM_3	F + GD_3/GM_3 + PUFA	All diets
SM	SPM	27.6 ± 6.6	27.0 ± 10.8	25.9 ± 6.0	21.7 ± 2.4	25.7 ± 7.1
	MYE	23.2 ± 11.1	19.0 ± 3.0	18.5 ± 5.4	17.9 ± 4.9	19.7 ± 6.7
LacCer	SPM	15.5± 2.4	14.7 ± 4.7	14.9 ± 3.7	18.1± 2.8	15.7 ± 3.6
	MYE	16.5 ± 2.4	16.2 ± 2.0	16.0 ± 6.4	15.5 ± 3.4	16.0 ± 3.7
GalCer	SPM	21.5 ± 3.5	19.7± 6.2	21.9 ± 4.5	20.5 ± 1.9	20.9 ± 4.2
	MYE	30.3± 8.2	27.3± 4.2	30.2 ± 7.4	26.1 ± 4.6	28.5 ± 6.2
GlcCer	SPM	16.3 ± 2.2	15.0 ± 4.1	18.6± 3.2	17.2 ± 3.1	16.8 ± 3.3
	MYE	15.9 ± 3.1	17.3 ± 2.6	16.5 ± 6.6	24.2 ± 7.5*	18.5 ± 6.0
Cer	SPM	19.1 ± 4.9	23.7 ± 6.5	18.7 ± 2.4	22.6 ± 2.7	20.9 ± 4.8
	MYE	15.2 ± 2.9	19.0 ± 4.9	17.0 ± 3.5	18.1 ± 1.7	17.3 ± 3.5

* $p < 0.05$

vi) Cholesterol content of synaptosomal plasma membrane and myelin

There was no difference in cholesterol content among dietary treatment groups for SPM or myelin brain fractions (Figure 3.7). Synaptosomal plasma membrane contained 62.4 ± 12.6 μg/mg protein, while myelin contained 57.3 ± 4.8 μg/mg protein. For myelin, procedure Day 3 yielded a significantly greater amount of cholesterol ($p = 0.05$).

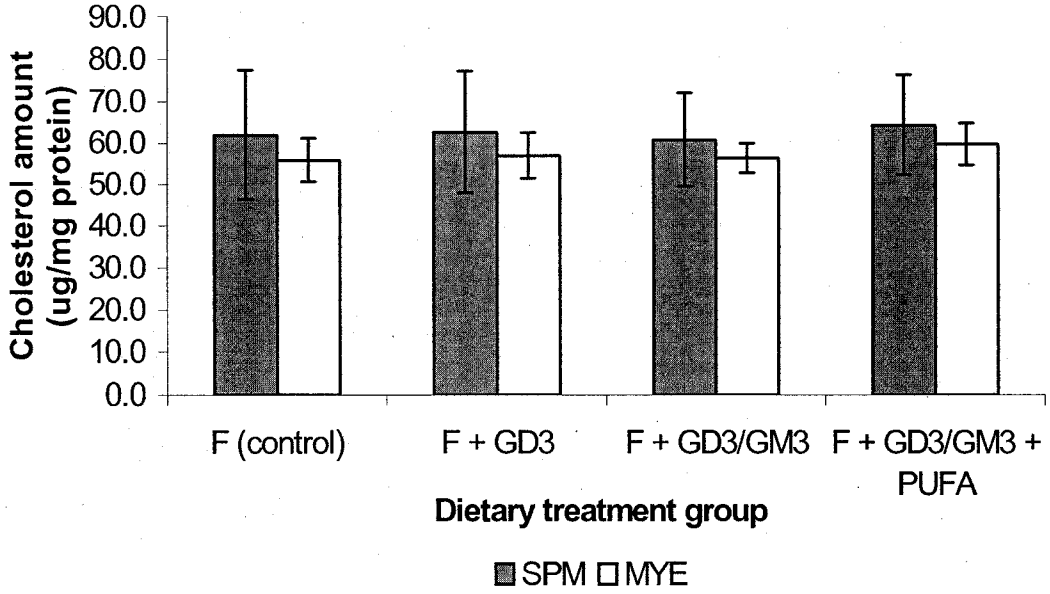

Figure 3.7. Total cholesterol content (μg/mg protein) of synaptosomal plasma membrane and myelin brain fractions for each dietary treatment group.

The approximate ratio of ganglioside/phospholipid/cholesterol (w/w/w) content in the brain fractions was 0.3:1:1 for synaptosomal plasma membrane and 1:2:1 for myelin.

D. DISCUSSION

This is the first experiment to examine the relationship between dietary gangliosides and the biochemical features of synaptosomal plasma membrane and myelin. Understanding this relationship is key to understanding the role of diet in infant neurological development. The objective of this experiment was to determine if changing the ganglioside content of nutritionally complete diets alters the ganglioside, phospholipid, sphingolipid or cholesterol content of synaptosomal plasma membrane or myelin fractions of developing rat brain.

Animal growth and tissue

Rats weighed 136.4 ± 6.7 g at 33 d postnatal age. Each brain weighed approximately 1.75 g. In comparison, average body and brain weights of Sprague-Dawley rats at 30 d were reported to be 68 g and 1.4 g respectively (21). Brain weights averaging 1.6 g were found for 35 d old Sprague-Dawley rats (22). The increased body and brain weights observed in this experiment can be explained by the higher fat diet containing "F" fat blend fed to these animals, as opposed to standard rat chow fed in the other experiments. Dietary treatment containing ganglioside and/or PUFA had no significant effect on body and brain weights compared to the control diet.

Gangliosides

Gangliosides present in both brain fractions included: GQ_{1b}, GT_{1b}, GD_{1b}, GT_{1a}, GD_{1a}, GD_3, GM_1, GM_2, GM_3, and GM_4. Gangliosides GD_{1a}, GQ_{1b}, GT_{1b}, GM_1, and G_{D3} represented the highest proportion of lipid-bound NANA (>70 %) in SPM, while in myelin, GD_{1a} and GD_3 were predominant. Other studies reporting the ganglioside compositions of neurological tissues have concluded that the major brain gangliosides of higher vertebrates are GM_1, GD_{1a}, GD_{1b} and GT_{1b}, which account for 80-90 % of total ganglioside-NANA content (23). Park *et al.* (2005) fed a diet similar to the one used in this experiment, and found GD_{1a}, GT_{1b}, GD_{1b}, GQ_{1b}, and GM_1 to be the major brain ganglioside species in 33 day old rats (9). This experiment differs from published reports on the relative importance of GD_{1b} and GD_3. However, none of the other studies measured the ganglioside composition of SPM specifically. Myelin gangliosides are predominately GM_1, GD_{1b} and GM_4 (24). GM_1 was reported to represent over 60 % of myelin total ganglioside-NANA in adult rats, and approximately 38 % in 31 d old rats

(25, 26). In this experiment, GD_{1a} was most abundant and there were elevated proportions of GD_3 and the complex gangliosides (24). This altered ganglioside profile is consistent with that of less mature myelin, which has somewhat lower GM_1 content, or even oligodendroglia, which are characterized by increased GD_{1a} compared to GD_{1b}, increased GD_3, and increased ganglioside complexity (24, 26, 27). This may be explained by the relatively young age of the rats studied, although by 33 days, the rate of myelination is slowing. The high dietary fat content may also be a large contributing factor, delaying myelin maturation in the rat brain, or altering the myelin ganglioside profile. Contamination with spinal cord myelin, or other neurological membranes of higher GD_3 or GD_{1a} content, is also a plausible explanation for the discrepancy. Ganglioside GM_4 is an important ganglioside for primate myelin, but may not be a component of SPM (23, 28). However, Ueno *et al.* (1978) reported GM_4 to represent 1.5 % of human cerebral grey matter ganglioside-bound sialic acid and 26.6 % of cerebral myelin ganglioside-NANA (24). The presence of GM_4 in SPM (7.8 ± 2.6 %) and MYE (10.5 ± 5.4 %) in this experiment may be explained as a result of altered dietary fat, contamination by other neurological material, or more sensitive analysis. Park *et* al. (2005) did not measure GM_4 in the whole brain tissue of rats fed similar diets (9). Other CNS tissues containing significant amounts of GM_4 are human spinal cord and cord myelin. The presence of GM_4 in other neurological cells or subcellular compartments of any species has not been clearly examined.

The total ganglioside amount in SPM was 19.7 ± 3.0 μg NANA/mg protein. Myelin has a significantly higher lipid to protein ratio compared to SPM. The total ganglioside content of myelin was 48.5 ± 14.1 μg NANA/mg protein. Curatolo (1987)

also reported the protein content of myelin to be relatively low (20 %) (2). The myelin brain fraction had greater proportions of GD_3, GM_3 and GM_4, compared to synaptosomal plasma membrane, at the expense of GM_2 and the complex gangliosides. This is consistent with other published findings (29). The disialoganglioside/monosialoganglioside ratio was similar in myelin compared to SPM (SPM: 1.3 ± 0.6; MYE: 1.4 ± 0.6). This is in agreement with Ueno *et al.* (1978), whose data show ratios of 0.4 for both (24). The higher ratio obtained in this experiment is due to the lower relative percentages of GM_1 detected.

Total ganglioside amount in SPM or myelin was not changed by diet. Park *et al.* (2005) reported a small increase, approximately 10 %, in ganglioside content of whole brain tissue from rats fed diets including similar amounts of ganglioside (9). This divergence may be a result of conservation tactics within SPM and myelin to maintain homogenous ganglioside amounts. Gangliosides are known to have specific bioactive roles within the nervous system, thus gangliosides within these CNS components may be too important for dietary dependence. Changes in whole brain ganglioside content may instead be a reflection of changes in the ganglioside content of other brain fractions. For instance, whole brain homogenate contains the blood circulating in brain microvessels. Since the ganglioside content of blood is affected by dietary intake, this fraction may partially explain the increase in ganglioside found in whole brain tissue (9). Other brain components that may increase their ganglioside contents are astrocytes, microglia and endothelial cells. It is also possible that the membranes of internal organelles are more sensitive to changes in ganglioside than cellular plasma membranes, or that the short time period (2 weeks) was not long enough to allow for turnover of gangliosides within the

fractions examined. The findings by Park *et al.* (2005) were reported as mg NANA/g wet tissue. Therefore, it is conceivable that any increases in ganglioside content in this experiment were matched by similar, nonsignificant increases in protein content. Alternatively, high variation in the results of this experiment, due to increased complexity of the methodology, may be masking any smaller dietary effect. The age of the animals, although consistent with Park *et al.* (2005), may be an important barrier to registering change in ganglioside content. It has been shown that the intestines of young animals are extremely efficient in sphingolipid digestion and the absorption of NANA. Intestinal sialidase activity is increased during the suckling period (7). In young rats, over 90 % of intraperitoneally injected NANA was absorbed, and 3 - 4 % reached the brain within 6 hr (30). However, in animals older than 30 days, brain biochemistry was unaffected. Brain development occurs more rapidly at younger ages. The brain is likely most sensitive to dietary ganglioside during the critical stages of brain cell differentiation, synaptogenesis, myelination, and even growth. The capability of young mammals to synthesize gangliosides has not been examined in the absence of mother's milk. Potential deficiencies in ganglioside metabolic enzymes may limit the young animal's brain capacity for ganglioside synthesis, making dietary contribution conditionally essential. The animals used in this experiment were 33 days old. At this age, myelination was nearly complete and the brains had almost reached adult size.

There were significant differences in the relative amounts of certain gangliosides in each brain fraction, depending on dietary treatment. In synaptosomal plasma membrane, GM_4 was higher in the ganglioside and PUFA diet compared to all other diets ($p = 0.003$). In myelin, the complex ganglioside GQ_{1b} was higher in the GD_3 diet

compared to the control diet ($p = 0.009$). The presence of dietary long chain polyunsaturated fatty acids appears to be responsible for the difference in relative GM_4 composition. Although, it is also possible that the effect depends upon combined action of ganglioside and PUFA. Alternatively, GM_4 may not be an important ganglioside of SPM. This significant difference may have been amplified by unrelated changes in the relatively minor amounts of GM_4 detected in each dietary treatment group. Nevertheless, the biological importance of this effect remains unknown. GM_4 is the least complex of the detected gangliosides, and its function has not been studied. Interestingly, GM_4 is unique in its classification as a galacto-ganglioside, and its presence in myelin is restricted to central nervous tissue of a select few species, indicative of a relatively late evolutionary appearance (24, 31, 32). It has also been suggested that GM_4 complexes with MBP in myelin, thereby receiving protection from neuraminidase activity (27). The increased relative amount of GQ_{1b} in myelin is also mysterious. Although GQ_{1b} is a relatively minor component of myelin, it is very complex, and implicated in important functions related to intelligence (7) and the stimulation of neurite outgrowth (4). The possible advantages or disadvantages of increased GQ_{1b} content in myelin are open for speculation. It is interesting that the diet containing GD_3 alone is responsible for this effect. The GD_3 content of the F + GD_3 diet was three times the amount of the other diets containing GD_3. As GD_3 is an important ganglioside for developing tissues, the presence of surplus GD_3 may be a substrate for maturing brain cells to synthesize more complex ganglioside species, such as GQ_{1b}. Ganglioside GD_3 is a precursor for GQ_{1b} synthesis in the b-pathway. Alternatively, this significant difference may also have been amplified by random changes in the relatively minor amounts of GQ_{1b} detected in each diet group.

Park *et al.* (2005) did not detect changes in ganglioside composition in the whole brain tissue of developing rats fed ganglioside (9). However, they also did not measure GM_4. Increases in the GQ_{1b} content of myelin, as shown in this experiment, may be offset by other changes in different brain fractions, resulting in zero net change in whole brain tissue.

Phospholipids, sphingolipids and cholesterol

Phospholipids present in both synaptosomal plasma membrane and myelin included: LPC, SM, PC, PS, PI and PE. The same phospholipids were measured in synaptosomal plasma membrane by Magruder *et al.* (1984) (33). Total phospholipid content of synaptosomal plasma membrane was 66.7 ± 23.9 μg P/mg protein. Magruder *et* al. (1984) measured 19.5 ng P/mg protein in SPM (33). Total phospholipid content of myelin was 91.6 ± 24.2 μg P/mg protein. The most important phospholipids were PC and PE in both fractions. This is consistent with other findings indicating the abundance of PC and PE in neurological tissues (12, 34). The PC/PE ratio in this experiment was 1.2 for myelin. These results agree with the value of 1.4 in published literature (21). The ratio of 1.7 obtained for synaptosomal plasma membrane cannot be compared.

Asialo-sphingolipids present in both brain fractions included: SM, LacCer, GalCer, GlcCer, and Cer. These results are in agreement with published findings showing the presence of GalCer, GlcCer, and LacCer in all cells and SM in brain tissue (35, 36). In synaptosomal plasma membrane, SM, GalCer and Cer were present in the highest proportions. In myelin, GalCer was the most prevalent sphingolipid. This is in agreement with Curatolo (1987), who also found GalCer to be the primary glycolipid of

myelin at 24 % (2). Abe and Norton (1978) also reported GalCer and SM to be in highest relative proportion in both calf myelin and grey matter (36).

Synaptosomal plasma membrane contained 62.4 ± 12.6 μg cholesterol/mg protein, while myelin contained 57.3 ± 4.8 μg cholesterol/mg protein. Curatolo (1987) also commented on the relative importance of cholesterol in myelin (28 %) (2). This experiment is in agreement that myelin contains relatively high proportions of cholesterol and relatively low proportions of protein.

The approximate ratio of ganglioside-NANA/phospholipid-P/cholesterol (w/w/w) content in the brain fractions was 0.3:1:1 for synaptosomal plasma membrane and 1:2:1 for myelin. The phospholipid/cholesterol ratio of SPM is in agreement with Shapiro and Barchi (1981), who reported the normal ratio to be 0.3:0.4 mol/mol (37). The molar ratio of myelin phospholipid to cholesterol was also reported to be approximately 1:1 although some variation was apparent depending on animal age (21). The difference observed in this experiment is likely explained by the dissimilar diet. Of course, the molar amount of ganglioside depends upon the mono-, di-, and polysialoganglioside composition. Svennerholm (1964) reported that lecithins represent approximately 50 % of brain lipids, while Norton and Poduslo (1971) found that phospholipids represented 71 % of neuronal and glial cell lipids (38, 39). This experiment does not contradict such a possibility for either SPM or MYE.

There were no significant differences in total phospholipid or cholesterol content of SPM or myelin influenced by dietary treatment, nor were there any differences in the phospholipid profiles of myelin or sphingolipid profiles of SPM among treatment groups. However, in the diet also containing PUFA, there was a significant increase in the

relative phospholipid content of synaptosomal plasma membrane LPC ($p = 0.02$), as well as a significant increase in the relative sphingolipid content of myelin GlcCer ($p = 0.02$), over all other diet groups. The biological importance of these differences is unknown, and may be attributed to the presence of dietary PUFA alone, or in conjunction with ganglioside. Neither LPC, nor GlcCer, are major lipid components of synaptosomal plasma membrane or myelin. Equivalent research has never been conducted, so these results cannot be compared. However, LPC is normally low in SPM, and increases may be attributed to cleavage of PC by phospholipase A_2 (33). In a study by Park *et al.* (2005), dietary ganglioside and PUFA were both found to alter retinal phospholipid composition with no change, or a decrease in total phospholipid content. In general, they found increased relative proportions of PC and PS, and decreases in PI and PE (40). It is unknown why these changes were important for retina, but not for synaptosomal plasma membrane or myelin.

For most lipid analyses there were significant differences attributed to procedure day. However, there was no consistent trend for which procedure day produced which effect, so differences cannot be attributed to the increased age or mass of the animals. It is evident, only, that conducting this analytical experiment in successive groups will increase variation in the results, which can amount to statistically significant differences.

Na^+-K^+ ATPase activity

Sodium-potassium adenosinetriphosphatase showed a nonsignificant trend ($p = 0.12$) for increased activity in the diet group also containing PUFA. This enzyme may indicate a functional change in SPM, as it has a critical role in maintaining the ion gradients required for nerve impulses and, possibly, indirectly modulating synaptic action

(41). Increased Na^+-K^+ ATPase activity has been shown in animals injected with GM_1, GD_{1a}, GD_{1b} and GT_{1b} (28, 42), as well as in animals fed DHA (43). However, changes in PUFA content can affect SPM fraction density, thereby influencing results in this experiment. In order to determine if diet containing ganglioside and/or PUFA truly increases Na^+-K^+ ATPase activity, it is necessary to measure the enzyme kinetics Km and Vmax.

Conclusions

In conclusion, nutritionally complete diets differing in ganglioside content produce significant differences in the ganglioside profiles of SPM and myelin, but not total ganglioside amount. This is in partial agreement with Hypothesis 1. However, the difference produced in SPM may be attributed to dietary PUFA, rather than ganglioside. Dietary treatment with ganglioside also produced significant differences in the phospholipid profiles of SPM, and in the sphingolipid profiles of myelin. However, both of these differences may be attributed to dietary PUFA, rather than ganglioside. Total phospholipid and cholesterol contents were unaffected by diet. These results are in partial agreement with Hypothesis 2. Further experimentation is warranted to determine if dietary ganglioside can affect Na^+-K^+ ATPase activity in SPM, and if animal age influences the response of neurological lipids to dietary ganglioside.

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CHAPTER 4. THESIS SUMMARY AND CONCLUSIONS

The hypotheses tested in this thesis have been addressed as follows:

Hypothesis 1.

The addition of gangliosides GD_3, or GD_3 and GM_3, to the diet of weanling rats will increase total ganglioside content and alter ganglioside composition in synaptosomal plasma membrane and myelin brain fractions.

Dietary ganglioside did not affect the total ganglioside content of synaptosomal plasma membrane or myelin fractions of developing rat brains. However, dietary ganglioside (GD_3 and GM_3) combined with dietary PUFA increased the relative percentage of GM_4 in synaptosomal plasma membrane. The relative percentage of GQ_{1b} in myelin also increased when ganglioside GD_3 was fed in the diet.

Hypothesis 2.

The addition of gangliosides GD_3, or GD_3 and GM_3, to the diet of weanling rats will change the phospholipid, sphingolipid and cholesterol contents in synaptosomal plasma membrane and myelin brain fractions.

Dietary ganglioside did not affect the total phospholipid or cholesterol contents of synaptosomal plasma membrane or myelin fractions of developing rat brain. However, dietary ganglioside (GD_3 and GM_3) combined with dietary PUFA increased the relative percentage of lysophosphatidylcholine in synaptosomal plasma membrane and glucosylceramide in myelin.

Taken together, these results suggest that dietary PUFA may be a more important factor for influencing lipid composition of synaptosomal plasma membrane and myelin, but that increased dietary GD_3 also has the potential to alter ganglioside composition of myelin. Since the lipid composition of synaptosomal plasma membrane was not modified by the presence of dietary ganglioside alone, it is probable that synaptosomal plasma membrane has regulated mechanisms controlling ganglioside and other lipid content that are insensitive to the levels of circulating ganglioside. The functional importance increased GM_4 and lysophosphatidylcholine in synaptosomal plasma membrane or GQ_{1b} and glucosylceramide in myelin remains unknown. Important factors potentially influencing the results of this study include: animal age, the use of sequential experimentation periods, duration of feeding, and type or amount of ganglioside fed. It is important to note that the sensitivity of rat brains to particular gangliosides found in human milk may also differ substantially from the sensitivity of developing human brains.

The ganglioside content of neurological tissues is an important indication of developmental stage, functional capability, and perhaps even intelligence. Since mother's milk is a rich source of ganglioside while infant formulas currently available contain little to no ganglioside, the importance of dietary ganglioside needs to be examined in human clinical trials. This is the first research to examine the relationship between dietary ganglioside and the lipid composition of synaptosomal plasma membrane and myelin. The knowledge gained from this thesis will be helpful in future studies examining the effects of dietary ganglioside on neuroanatomy and brain function.