Regulation of n-3 PUFA biosynthesis in proliferating and differentiated PC12 pheochromocytoma cells

Institute of Brain Chemistry (IBCHN)

Faculty of Life Sciences,

London Metropolitan University

A thesis Submitted
for
the Degree of Doctor of Philosophy (Ph.D.)

Presented by

Ora Msika M.Phil (Hons)

Directors of Studies: Kebreab Ghebremeskel

Michael Crawford

Ephraim Yavin

Author's declaration

I attest that the work presented in this thesis entitled "Regulation of n-3 PUFA biosynthesis in proliferating and differentiated PC12 pheocromocytoma cells" has not been submitted in support of any qualification in this or other educational institutions in the UK or elsewhere.

Ora Msika

Date August 2012

Table of contents

Acknowledgments

First of all, I would like to especially thank Pr Ephraim Yavin for his invaluable supervision, support and guidance along the project. I wish to express special gratitude to Dr Annette Brand for sharing her scientific knowledge. I would like to express my gratitude to Pr Kebreab Ghebremeskel for his guidance during the writing of this thesis.

Then, Pr Michael Crawford, Dr Allain Amador Bueno, Dr Rosana Cabello-Moruno, Dr Yoeju Min, Dr Kot Nyuar, Dr Yiqun Wang and all the PhD students from IBCHN Ahmed Daak, Amanda Hallott, Marita Neville and especially Katia Mariniello for her friendship, help and answering my questions.

I would like to thank every member of all the other research institutes (IHRP, MRU), in particular Dr Ephraim Ansa-Addo for his friendship, help, support and enthusiasm and Samireh Jorfi (CMIRC).

I am very grateful to Pr Menachem Segal for giving me the opportunity to be a member of his research group at the Weizmann Institute of Science. I also would like to thank all my colleagues from the Weizmann Institute of Science.

I wish to thank Dr Ana Juknat for her kind help in mastering several molecular biology techniques and for her scientific suggestions and advice. I am very grateful for the time she dedicated to this thesis.

Special thanks to Dr Martin-Paul Agbaga from the University of Oklahoma, *HSC,* who assisted me with molecular biology studies.

Finally, I would like to thank my family for their support as well as the Institute of Brain Chemistry and Human Nutrition (IBCHN) and the London Metropolitan University, for the privilege and opportunity to undertake my studies in such an institution. I would like to acknowledge the Gulton Foundation (USA), Mother and Child Foundation (UK) and Letten Foundation (Norway) who supported this work. This work was supported by grants from the Gulton Foundation (USA), Mother and Child Foundation (UK) and Letten Foundation (Norway).

Original publication and conference contributions

This thesis is based on the following publication and published abstracts. In addition some unpublished data are presented.

Original publication

Msika O, Brand A, Crawford MA, Yavin E (2012). NGF blocks polyunsaturated fatty acid biosynthesis in n-3 fatty acid-supplemented PC12 cells. Biochim Biophys Acta 1821: 1022-1030.

Conference contributions

(I) Msika O, Brand A, Crawford MA, Yavin E (2010). PC12 pheochromocytoma cells posses the ability to synthesize omega-3 polyunsaturated fatty acids from alpha-linolenic acid. Presented at the 9th conference of the International Society for the study of fatty acids and lipids (ISSFAL) in Maastricht, Holland (28V-02VI-2010).

(II) Msika O, Brand A, Crawford MA, Yavin E (2010). PC12 pheochromocytoma cells posses the ability to synthesize omega-3 polyunsaturated fatty acids from alpha-linolenic acid. Presented at the ISN (International Society for Neurochemistry) International School in Catania, Sicily, Italy (17-21V-2010).

Abstract

Regulation of polyunsaturated fatty acid (PUFA) biosynthesis in proliferating and NGF-differentiated PC12 pheochromocytoma cells deficient in n-3 docosahexaenoic acid (DHA, 22:6n-3) was studied. A dose- and time dependent increase in eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and DHA in phosphatidylethanolamine (EPG) and phosphatidylserine (SPG) glycerophospholipids (GPL) *via* the elongation/desaturation pathway following alpha-linolenic acid (ALA, 18:3n-3) supplements was observed.That was accompanied by a marked reduction of eicosatrienoic acid (Mead acid, 20:3n-9), an index of PUFA deficiency. EPA supplements were similarly converted to 22:5n-3 and 22:6n-3. On the other hand, supplements of linoleic acid (LA, 18:2n-6) were not effectively converted into higher n-6 PUFA intermediates nor did they impair elongation/desaturation of ALA. A marked decrease in the newly synthesized 22:5n-3 and 22:6n-3 following ALA or EPA supplements was observed after nerve growth factor (NGF)-induced differentiation. NGF also inhibited the last step in 22:5n-6 formation from LA.

NGF alters differently the expression of several genes involved in the FA metabolism: Elovl5, Elovl2, Fads2 and Ppara genes encoding, respectively, for ELOVL5 elongase, ELOVL2 elongase, Delta6 desaturase (D6D) and the nuclear transcription factor PPARα. LA up-regulates the expression of the above genes, whereas ALA specifically up-regulates the mRNA levels of Fads2 and Elovl5.

The results found in this thesis emphasise the importance of overcoming n-3 PUFA deficiency, and raise the possibility that growth factor regulation of the last step in PUFA biosynthesis may constitute an important feature of neuronal phenotype acquisition.

Index

List of tables

List of figures

Abbreviations

Aβ	Amyloid β
ARA	Arachidonic acid (20:4n-6)
AD	Alzheimer's disease
AKT	Protein kinase B
ATP	Adenosine triphosphate
ALA	Alpha-linolenic acid (18:3n-3)
ATRA	All-*trans*-retinoic acid
bFGF	Basic fibroblast growth factor
BHT	Butylated hydroxytoluene
cDNA	complementary DNA
CO_2	Carbon dioxide
COX	Cyclooxygenase
CPG	Choline phosphoglyceride
Ct	Cycle threshold
D5D	Δ 5 desaturase
D6D	Δ 6 desaturase
DDCt	Delta-Delta-Ct
ddH_2O	double deionised water
DHA	Docosahexaenoic acid (22:6n-3)
DHGLA	Dihomo-γ-linolenic acid
DMSO	Dimethyl sulfoxide minimum
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid (22:5n-3)
n-6 DPA	Docosapentaenoic acid (22:5n-6)
DTA	Docosatetraenoic acid (22:4n-6)
EDTA	Ethylenediamine tetraacetic acid
ELOVL	Elongation of very -long-chain fatty acid
EPA	Eicosapentaenoic acid (20:5n-3)
EPG	Ethanolamine phosphoglyceride
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
FA	Fatty acid
Fads1	Δ5 desaturase
Fads2	Δ6 desaturase
FAME	Fatty acid methyl esters
FBS	Foetal bovine serum
GC	Gas chromatography
GLA	γ-linolenic acid
Gln	L-glutamine
GPCR	G-protein coupled receptor

GPL	Glycerophospholipid
GM	Growth medium
HIP	Hexane/isopropanol
HS	Horse serum
IPG	Inositol phosphoglyceride
LA	Linoleic acid (18:2n-6)
LCPUFA	Long chain polyunsaturated fatty acid
LOX	Lipoxygenases
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEFA	Non-esterified FA
NGF	Nerve Growth Factor
OA	Oleic acid (18:1n-9)
PBS	Phosphate buffered saline
PC12 cells	Rat adrenal pheochromocytoma cells
PCR	Polymerase chain reaction
Pen/Str	Penicillin/Streptomycin
PLC	Phospholipase C
PLL	Poly-L-lysine
PPARs	Peroxisome proliferators activated receptors
PPARα	Proliferator-activated receptor α
PPRE	Peroxisome proliferators response elements
PUFA	Polyunsaturated fatty acid
QRT-PCR	Quantitative Real-time PCR
RA	Retinoic acid
RNA	Ribonucleic acid
RT	Reverse transcription
RXR	Retinoic acid receptor
SCD	Stearoyl CoA desaturase
SDA	Stearidonic acid
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
SM	Sphingomyelin
SPG	Serine phosphoglyceride
SPM	Sphingomyelin
SREBP	Sterol Regulatory Element-Binding Protein
SREs	Sterol response element
TAE	Tris-acetate-EDTA
TLC	Thin Layer Chromatography
v/v	Volume per volume
w/v	Weight per volume

Chapter 1

General Introduction

1.1 Cell membrane

1.1.1 Structure

The accepted membrane model, "the fluid mosaic model", describing the plasma membrane structure as a fluid lipid bilayer, was presented in 1972 by Singer and Nicolson (Singer and Nicolson, 1972). According to this model, the biological membrane is formed by combining two lipidic layers, within which various proteins are embedded. These lipidic layers are composed of several lipids. Specifically, phospholipids are amphipathic lipids arranged in the bilayer with their hydrophobic moieties (fatty acids (FAs)) in the center of the membrane and the hydrophilic head groups point toward the water that surrounds both sides of the bilayer (the extracellular side and the cytosol). Membrane proteins can be associated within the lipid bilayer in various ways:

-Peripheral membrane proteins are bound to only one side of the membrane.

-Transmembrane proteins span the membrane and interact with the hydrophobic tails of the lipid molecules in the interior of the bilayer through their hydrophobic regions. Furthermore, the carbohydrates are covalently linked to lipids (glycolipids) or proteins (glycoproteins).

Thus, the membrane is a fluid structure with a "mosaic" of various proteins embedded in a lipid bilayer (Figure 1.1).

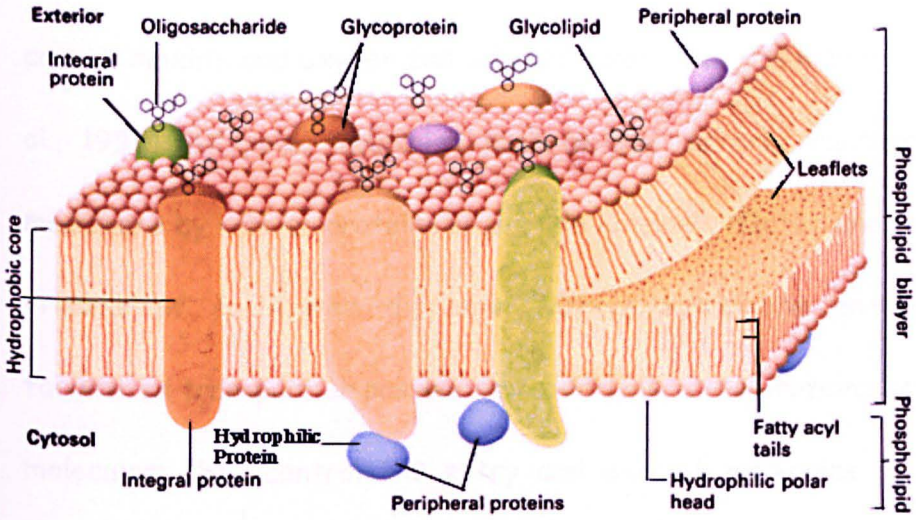


Figure 1.1 The "mosaic fluid model" (Singer-Nicolson 1972): adopted from http://personal.cicbiomagune.com/ireviakine/cellmembrane.html.

1.1.2 Cell membrane functions

The cell membrane acts as a physical boundary between the internal and the external environments of the cell and maintains their respective compositions. Moreover, it has two main functions: cellular transport and cellular signalling.

1.1.2.1 Cellular transport

The cell membrane consists of a semi-permeable lipid bilayer. As described in section 1.1.1, the membrane is composed of proteins and lipids. Lipids provide permeability for the membrane whereas proteins provide selectivity. The membrane is permeable to some molecules, depending on their size, polarity and charge (Walter and Gutknecht, 1986). The molecules move following a gradient concentration (from higher to lower concentrations); this mechanism does not

require energy and is called diffusion. Only small non-polar molecules such as carbon dioxide and oxygen can diffuse across the cell membrane (Boxerman et al., 1995). All polar molecules such as glucose are transported across the membrane by carrier proteins or protein channels (Qutub and Hunt, 2005). This process of facilitated diffusion without expending energy is known as facilitated transport or passive transport. Here the proteins act as a carrier of molecules; they control the entry and exit of molecules in the cell. These carrier proteins bind molecules at one side of the membrane, then undergo conformational changes and release them on the other side of the membrane (Lehnart and Marks, 2007).

In contrast, larger and polar molecules can be transported against the gradient concentration through the membrane by transporter proteins or pumps. This mechanism requires an expenditure of energy; it is called active transport (Glynn, 1968). Both transporters and pumps present two active sites: one binds the molecule to be transported and the other binds a molecule of adenosine triphosphate (ATP) that provides energy for the transport (Glynn, 1968). Transporters are classified into three types: uniporters, symporters and antiporters. Uniporters transport one solute across the membrane. One solute is transported simultaneously or sequentially with another in the same direction by symporters, or alternatively, in the opposite direction by antiporters (Lehnart et al., 2007). The Na^+/K^+ pump is a good example of an antiporter; it exchanges Na^+ (out of the cells) against K^+ (into the cells) while expending ATP

(Skou, 1965). The mechanism by which solutes are transported across the plasma membrane has been the focus of numerous studies, but few of these have examined the transport of FAs into cells.

FAs enter cells through transporters or by diffusion and are transported to intracellular compartments. A question under debate is the transport mechanism of FAs into all cells and all membranes. Two models of FA transport have been proposed. The first mechanism of FA transport across cell membranes is protein-mediated (Hui, 1997). Several protein transporters are involved in this process, such as plasma membrane fatty acid binding protein (FABPpm) (Stremmel, 1985), fatty acid translocase (FAT) (Abumrad, 1993) and fatty acid transport protein (FATP) (Schaffer, 1994). Very few studies have examined the transport of FAs across the blood brain barrier into brain cells. Edmond (2001) proposed a model where lipoproteins from the plasma bind to receptors on the luminal surface of the endothelial cell. Then, FAs are released from these lipoproteins by lipolysis inside the cell. The second mechanism is the diffusion of FAs across cell membranes. FA diffusion has been described in several cell types such as adipocytes (Kamp et al., 2003), HepG2 cells (Guo et al. 2006), HEK cells (Meshulam et al., 2006), and myocytes (Wu et al., 2000). A model for FA diffusion into brain cells has been proposed by Halmiton and Brunaldi (2007). First, FA-albumin complex binds to the luminal surface of the endothelial cell. Second, FAs are ionized and diffuse across the lipid bilayer of the blood brain barrier and plasma membrane of neural cells by reversible flip

flop. In cytoplasm of endothelial cells and neural cells, FAs diffuse unbound or bound to FABP in cytosol. The brain selectively takes up certain FAs such as PUFAs (LA, ALA, ARA, EPA or DHA). Whether the brain takes up saturated FAs has been the subject of controversy (Noronha, 1990; Freed, 1994; Edmond, 1998).

1.1.2.2 Cell signalling

All cells have protein receptors on their plasma membrane, which receive information from their environment. Membrane receptors recognise and transmit a variety of extracellular stimuli to the cell. The cell, in turn, responds to these external stimuli through a set of intracellular mechanisms (chemical, ionic and electrical events), known as cell signalling. Receptors are often large oligomeric transmembrane proteins, with two domains: (1) one facing the exterior environment, the ligand-binding domain, with high affinity for specific substrates, and (2) an effector domain located on the cytosolic side (Massague et al., 1980; Huber et al., 2004). Specific ligands (substrates) bind to the receptor. This event causes conformational changes in the receptor, consequently transmitting the signal to the effector domain (Donner and Yonkers, 1983). As a result of these conformational changes, the effector site is exposed to intracellular signalling proteins or molecules, the signal is propagated through the cytoplasm (signalling transduction cascades), and physiological responses are elicited (Berg et al., 2002). Many cellular functions

and responses are affected when the membrane lipid composition is altered. Receptors are sensitive to the lipid environment within which they interact. Modifications in the lipid composition involve conformational changes in the receptors (Spector and Yorek, 1985). Lipid rafts and caveolae contain a variety of different protein receptors, and are thus considered signal transduction centers.

1.1.3 Rafts and caveolae

The plasma membranes contain specialized microdomains involved in physiological processes associated with the cell surface. These domains are ordered regions enriched by the association of certain lipids, cholesterols and proteins. They are classified as lipid rafts (Hooper, 1999), which are subdivided into the following three types: (Brown and London 2000; Simons and Ikonen 1997).

- Outside rafts: sphingomyelin and (glyco) sphingolipid-enriched membranes (GEM) that are localized in the exoplasmic leaflet of the plasma membrane
- Inside rafts: phosphoglycerides such as phosphatidylethanolamine, phosphatidylserine or polyphosphoinositol (*e.g.*, PIP2)-rich rafts that are found in the cytoplasmic leaflet of plasma membrane glycerolipids
- Caveolae: sphingolipid-enriched flask-shaped plasma membrane invaginations

Microdomains are very dynamic; they can form and disperse constantly (Simons and Ikonen 1997), allowing lateral and rotational movement of lipids and

proteins within the membrane. This biophysical property is attributed to the distribution of the asymmetric rafts in the lipid bilayer. In the cytoplasmic leaflet, rafts are enriched in phosphoglycerides, which usually contain unsaturated acyl chains, whereas in the endoplasmic leaflet the sphingolipids contain long, saturated acyl chains (Brown and London, 2000). Because of their kinked acyl chains, the unsaturated acyl chains of phosphoglycerides tend to be loosely packed with cholesterol molecules and therefore form a liquid-disordered membrane that enables rapid lateral and rotational lipid movement (Edidin, 2003). In contrast, sphingolipids and cholesterol are tightly packed, and as a consequence, have very little mobility (liquid-ordered phase). The rigid properties in the liquid-ordered phase are attributed to Van der Waals interactions between the saturated acyl chains of sphingolipids, the hydrogen bonding between neighbouring sphingolipids and the bonding between sphingolipid amide and the 3-OH group of cholesterol. Therefore, the cholesterol content in the plasma membrane plays an essential role in ensuring the stability of rafts. In particular, in neurons, cholesterol stabilizes lipid rafts, and depletion of cholesterol/sphingolipid leads to a gradual loss of synapses and spines, which is a main characteristic of neurodegenerative diseases (Hering et al., 2003). Ceramides can self-aggregate, resulting in the formation of ceramide-based microdomains or rafts (Zhang et al., 2009). These structures play several functions during signal transduction (Grassme et al., 2001). Moreover, they play important roles in regulating apoptosis, cell

differentiation, transformation and proliferation (Kawamura et al., 2009; Jana et al., 2010). In addition, specific proteins are attached to lipid rafts such as glycosylphosphatidylinositol (GPI) anchored-proteins (Brown and Rose, 1992); this emphasises the role of lipid rafts in the participation of signalling transduction (Figure 1.2). In contrast, caveolae contain a unique protein, caveolin, which exhibits a high affinity for cholesterol (Rothberg et al., 1992) (Figure 1.2). Caveolae play a role both in cell signalling (Li et al., 1996) and in endocytosis and transcytosis events (Parton and Simons, 2007). They are found in most cell types, especially endothelial cells, but not in neuronal cells, red blood cells, platelets and lymphocytes (Fra et al., 1995; Gorodinsky and Harris, 1995).

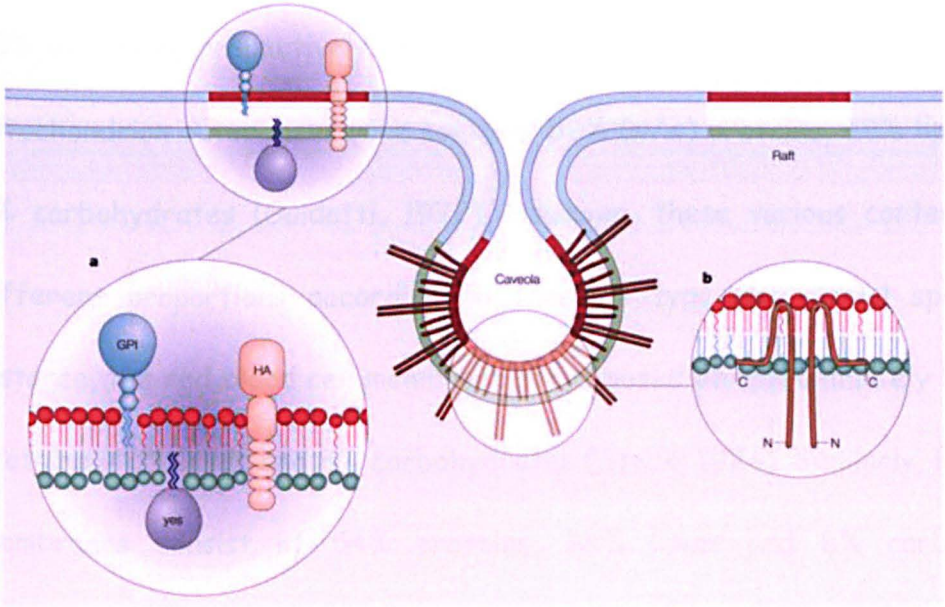


Figure 1.2 Model of the organization lipid rafts and caveolae in the plasma membrane: adopted from Simons and Ikonen (1997).

a) In the exoplasmic leaflet, lipid rafts enriched with sphingomyelin and glycosphingolipids (red) contain proteins attached to the leaflet by their acyl tails from -glycosylphosphatidylinositol (GPI) anchors (i.e., the Src-family kinase Yes).

In the cytoplasmic leaflet, lipid rafts are enriched with glycerolipids (green).

Specific proteins can be attached to the lipid bilayer through their transmembrane domains, such as the influenza virus protein haemagglutinin (HA).

b) Caveolae are formed by self-associating caveolin molecules that form a hairpin loop in the membrane.

1.2 Membrane lipids

All biological membranes, including organelle sub-cellular membranes, are mostly composed of lipids, proteins and some carbohydrates. In addition to acting as a permeability barrier for cells and organelles, membrane lipids play an important role along with proteins in regulating cellular activities such as cell adhesion, motility and signalling. In general, lipids and proteins are found at a ratio of

30% and 70%, respectively. As an example of a sub-cellular membrane, the mitochondrion outer membrane contains 52% (w/w) proteins, 48% lipids and 2-4% carbohydrates (Guidotti, 1972). However, these various contents are in different proportions according to the cell type and animal species. For instance, the red blood cell membrane is composed of approximately 52% (w/w) proteins, 40% lipids and 8% carbohydrates (Steck, 1974). Similarly, hepatocyte membranes consist of 54% proteins, 36% lipids and 8% carbohydrates (Guidotti, 1972). In contrast, the corresponding proportions in myelin sheaths are 20% (w/w), 75% and 5%, respectively (Guidotti, 1972).

There are three major classes of membrane lipids: phospholipids, sphingo- and glycosphingolipids, and cholesterol; the former is the most abundant in the cell membrane (Berg et al., 2002). The proportions of these lipids vary among different cell types. The neuron membrane is composed of 41% phospholipids, 22% cholesterol and 28% glycolipids (Sastry, 1985). The proportions of phospholipids, cholesterol and glycolipids in myelin sheaths are 41%, 22% and 28%, respectively (Bruyn and Garcin, 1969). In comparison, the human erythrocyte membrane lipid composition represents 61% phospholipids, 22% cholesterol and 11% glycolipids (Keller et al., 1998).

1.2.1 Cholesterol

Cholesterol is the main non-polar lipid in cell membranes. Structurally, cholesterol consists of a steroid ring, a single polar hydroxyl group and a

hydrocarbon chain (Figure 1.3). In the membrane, cholesterol is oriented between phospholipids molecules with its polar end (hydroxyl group) towards the outside surface of the monolayer and with its hydrophobic end projecting into the interior of the membrane. Hydrophobic interactions between phospholipids and cholesterol contribute to the maintenance of membrane fluidity and permeability. In addition, cholesterol is tightly packed, thus maintaining membrane microdomains and lipid raft dynamics, and it plays a major role in cell signalling (Simons and Ikonen, 1997). Cholesterol has a variety of important functions. It is the precursor of steroid hormones (cortisol, cortisone and aldosterone), sex hormones (progesterone, estrogen and testosterone) and vitamin D (Payne and Hales, 2004). In addition, its hydroxyl group can be esterified with a long FA chain, producing cholesterol esters. Cholesterol esters are stored in the cytoplasm of the cell and contribute to the pool of FAs.

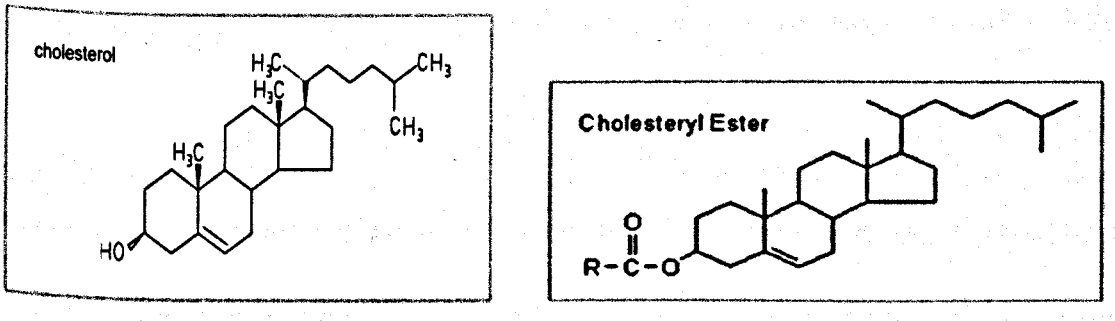


Figure 1.3: Cholesterol (a) Cholesteryl ester (b): chemical structures

1.2.2 Phospholipids

Phospholipids (PLs) serve primarily as structural elements of the membrane and are formed biosynthetically from four molecular components:

- a hydrophobic tail containing one or two long chain fatty acids (LCFAs),
- a phosphoric acid, polar head group bearing a negative charge at neutral pH,
- a complex amino alcohol (ethanolamine, choline, serine or inositol),
- and a backbone, consisting of glycerol or sphingosine.

PLs constitute the major class of polar lipids owing to the presence of polar head groups. PLs with glycerol backbones are known as glycerophospholipids or phosphoglycerides.

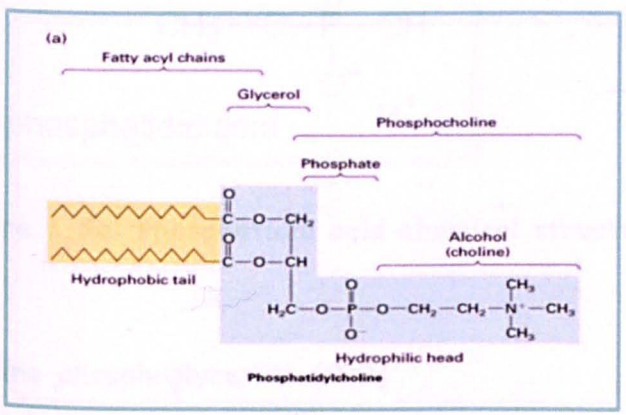


Figure 1.4: Phosphoglyceride chemical structure

1.2.2.1 Phosphoglycerides

The glycerol moiety at position 1 (*C*-1 or *sn-1*) and 2 (*C*-2 or *sn-2*) binds to two FAs. In general, at *C*-1 the FA can be saturated (*C*16 or *C*18) and at *C*-2 unsaturated (*C*18 to *C*20), whereas in position 3 (*C*-3 or *sn-3*) the glycerol binds to phosphoric acid through a phosphate ester bond. The complex amino alcohol

is linked to the phosphate group through a second phosphate ester bond (Figure 1.4). On the basis of the amino alcohol group, phosphoglycerides can be classified into choline, ethanolamine, serine and inositol phosphoglycerides.

Phosphatidic acid

Phosphatidic acid or 1,2-diacyl-*sn*-glycerol-3-phosphate is a PL without an amino alcohol group. It is the biosynthetic metabolic precursor of most phosphoglycerides and is found in trace amounts under normal circumstances (Figure 1.5a). Based on the type of amino alcohol complexes, PLs can be further divided into several subclasses.

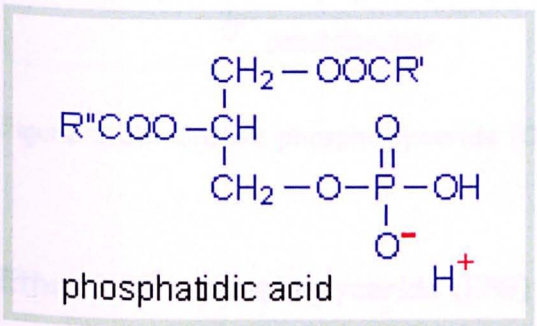


Figure 1.5a: Phosphatidic acid chemical structure

Choline phosphoglyceride (CPG)

Phosphatidylcholine or 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine possesses a choline group as its complex amino alcohol group (Figure 1.5b). CPG is usually the major PL in the cell plasma membrane and is located on the outer leaflet of the cell membrane (Bretscher, 1972; Verkleija et al., 1973). CPG can be found in different proportions, depending on the tissue type. For example, in human grey matter, CPG represents 39% of the total GPL content and 48% in human

skeletal muscle (Hawthorne and Ansell, 1982). Generally, in animal cells such as liver (Wood and Harlow, 1969) and brain grey matter cells (Yabuuchi and O'Brien 1968), CPG tends to contain lower proportions of arachidonic acid (ARA) and docosahexaenoic acid (DHA) and more of the C18 unsaturated fatty acids than does ethanolamine phosphoglyceride (EPG). The polyunsaturated components are concentrated in position *sn*-2 with saturated fatty acids most abundant in position *sn*-1 (Wood and Harlow, 1969).

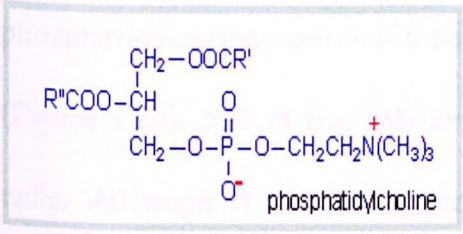


Figure 1.5b: Choline phosphoglyceride (CPG) chemical structure

Ethanolamine phosphoglyceride (EPG)

Ethanolamine phosphoglyceride or 1,2-diacyl-*sn*-glycerol-3-phosphorylethanolamine contains an ethanolamine group as its complex amino alcohol group (Figure 1.5c). EPG is the second most abundant phospholipid class in animal cells. Although it is found in all living cells, it is particularly abundant in nervous tissue such as brain white matter and the spinal cord. EPG is located in the internal monolayer of the plasma membrane (Bruce, 1974; Hawthrone and Ansell, 1982). In general, it contains higher proportions of ARA and DHA than CPG. These PUFAs are mainly linked to position *sn*-2 whereas saturated FAs are most abundant at position *sn*-1 (Wood and Harlow, 1969).

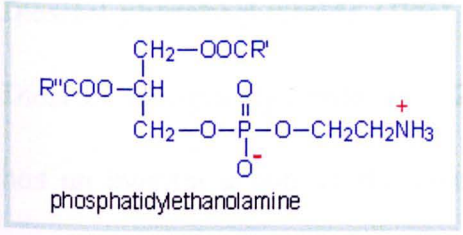


Figure 1.5c: Ethanolamine phosphoglyceride (EPG) chemical structure

Serine phosphoglyceride (SPG)

Serine phosphoglyceride or 1,2-diacyl-*sn*-glycero-3-phospho-L-serine or phosphatidylserine contains a serine group as its complex amino alcohol group (Figure 1.5d). SPG is the only amino acid-containing phosphoglyceride in animal cells. Although it comprises less than 10% of the total phospholipids, the greatest concentration appears in myelin found in brain tissue. In animal cells, the FA composition of SPG varies from tissue to tissue. In human plasma, 1-stearoyl-2-oleoyl and 1-stearoyl-2-arachidonoyl species predominate, but in brain and many other tissues 1-stearoyl-2-docosahexaenoyl species are very abundant (Wood and Harlow, 1969). SPG is located entirely on the inner side of the plasma membrane and is involved in cell signalling transduction events such as in the protein kinase C pathway (Bolsover et al., 2003).

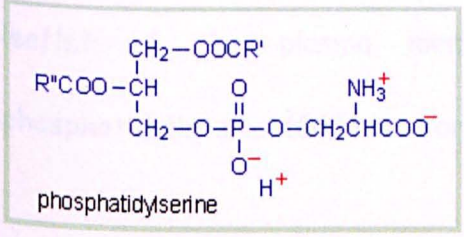


Figure 1.5d: Serine phosphoglyceride (SPG) chemical structure

Inositol phosphoglyceride (IPG)

Inositol phosphoglyceride or 1,2-diacyl-*sn*-glycero-3-phospho-1-D-myo-inositol has an inositol group as its complex amino alcohol group (Figure 1.5e). IPG is especially abundant in brain tissue, where it can comprise 10% of the phospholipid content. In animal tissues, the characteristic feature is a high content of stearic acid and ARA. All the stearic acids are linked to position *sn*-1 and all the ARAs are linked to position *sn*-2. About 78% of the total lipid may consist of a single molecular species *sn*-1-stearoyl-*sn*-2-arachidonoyl-glycero-phosphorylinositol (Wood and Harlow, 1969; Thompson and MacDonald, 1975; 1976).

IPG is located entirely on the inner monolayer of the plasma membrane and is involved in cell signalling. IPG can be hydrolysed by phospholipase C (PLC) into two second messengers, namely, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which are intermediates in the IP3/DAG signalling pathway (Fukami et al., 2010). In addition, IPG is the primary source of the arachidonate required for eicosanoid biosynthesis (including prostaglandins) in animals (Smith, 1992; Zhou and Nilsson, 2001; Carlson and Levitan, 1990). IPG is considered to be an anchor that can link a variety of proteins to the external leaflet of the plasma membrane via a glycosyl bridge (glycosyl-phosphatidylinositol (GPI)-anchored proteins).

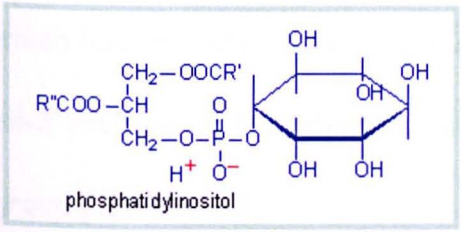


Figure 1.5e: Inositol phosphoglyceride (IPG) chemical structure

Ether lipids

Ether lipids are lipids in which one of the carbon atoms on the glycerol backbone is attached to an alkyl chain by a vinyl ether bond rather than by ester linkage. Plasmalogens are a type of ether lipids, they ressemble to glycerophospholipids except that the fatty acyl chains at *sn-1* position of the glycerol is attached to the glycerol backbone by a vinyl ether bond (Figure 1.5f). Three major classes of plasmalogens have been identified: choline, ethanolamine and serine plasmalogens. Usually, the highest proportion of the plasmalogen form is found in the EPG class, with smaller amounts in CPG, and usually little or none in other phospholipids such as IPG. This is the case in brain tissue, and particularly in myelin, where plasmalogens are found in high proportion in the EPG class and in lesser amounts in CPG, with little or no presence in other glycerophospholipids. In contrast, cardiac tissue plasmalogens are found to be enriched in CPG species.

Another type of ether lipid, platelet-activating factor (PAF) synthesized by neurons in injured brain (Lindsberg et al., 1991), is an active mediator in

ischemic brain damage (Nishida et al., 1996). It is an ether glycerophospholipid which has an acetyl group at the *sn-2* position instead of a fatty acyl chain. The *sn-3* position of the glycerol backbone is attached to a phosphocholine head group just like CPG.

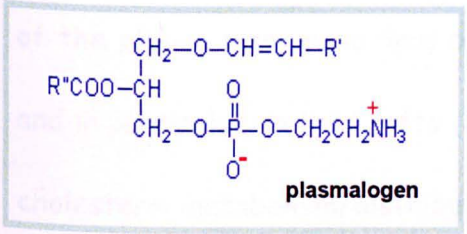


Figure 1.5f: plasmalogen (pEPG) chemical structure.

1.2.2.2 Sphingolipids

Sphingolipids are composed of a polar head group and two non-polar tails, with a long-chain amino alcohol, and a sphingosine backbone instead of glycerol. The sphingosine is linked on one side to two FAs through an amide bond and on the other side to a phosphate group. The phosphate group can be esterified to a hydroxyl group, OH (ceramides; simplest sphingolipid form) (Figure 1.6a), or to an ethanolamine, serine or choline group (sphingomyelins) (Figure 1.6b). Ceramides can be glycosylated with one or more oligosaccharide residues (cerebrosides and gangliosides), so they can be placed in the glycosphingolipid family (Figure 1.6c). Cerebrosides are the principal glycosphingolipid in brain tissue, and they are found in all nervous tissues, amounting to 2% of the dry weight of grey matter and 12% of white matter (Wherret and Cumings, 1963).

Sphingomyelins serve as a precursor for ceramides, long-chain bases and sphingosine-1-phosphate, as part of the 'sphingomyelin cycle', as well as many other important sphingolipids. Sphingomyelins account for about 10% of animal cellular lipids and are found in particularly large amounts in the myelin sheath (O'Brien and Sampson, 1995). Sphingomyelins are localised in the outer leaflet of the plasma membrane lipid bilayer (Van Echten-Deckert and Herget, 2006) and in particular, in lipid rafts (Ayuyan and Cohen, 2008), where they influence cholesterol metabolism/distribution (Slotte and Bierman, 1998).

In general, all sphingolipids are located on the outer leaflet of the plasma membranes and are particularly abundant in neural tissue.

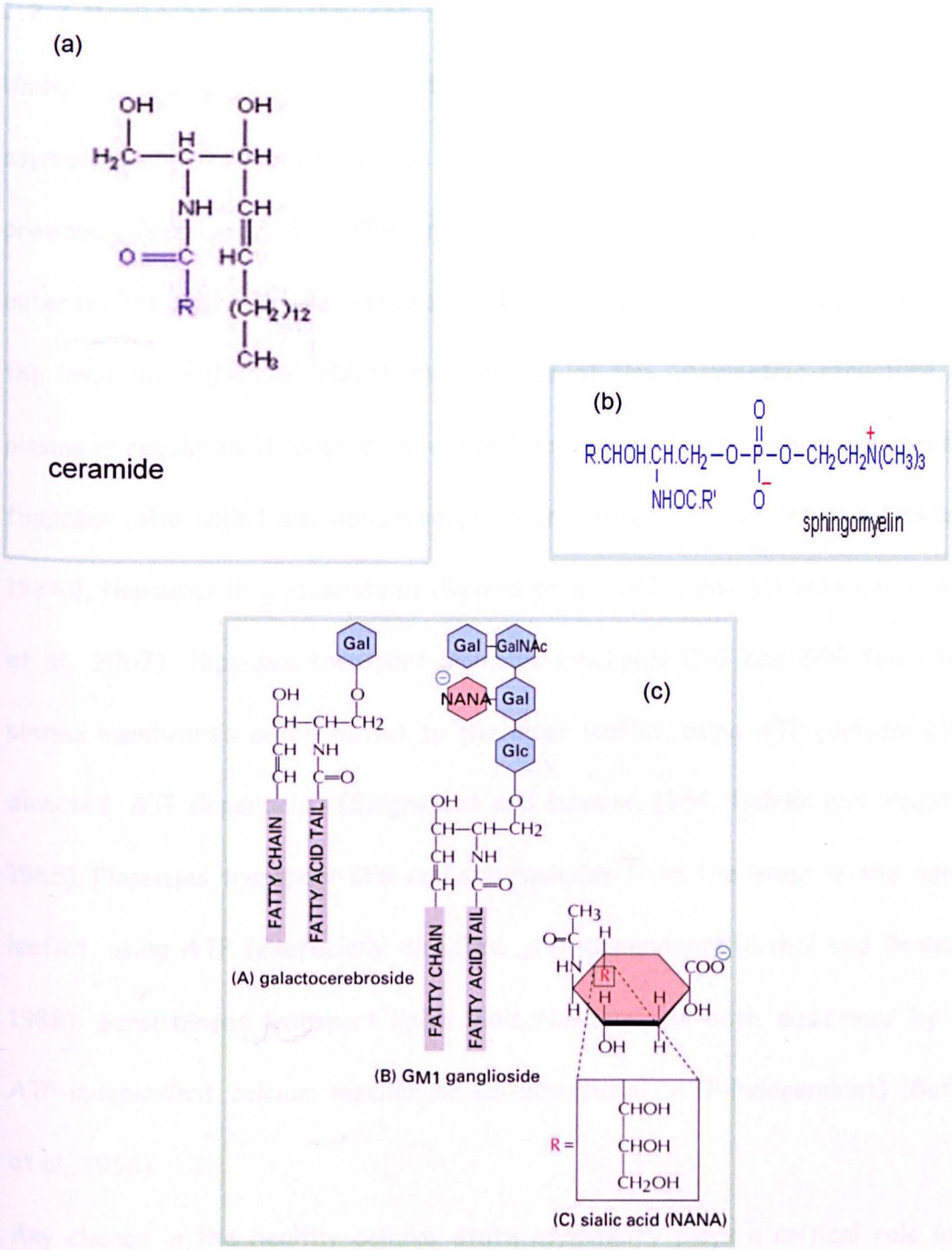


Figure 1.6: Ceramide (a), sphingomyelin (b) and glycosphingolipid chemical structures.

1.2.3 Membrane asymmetry and its regulation

Under normal physiological conditions, cell membrane phospholipids are asymmetrically distributed across the bilayer (Bretscher, 1972). As stated previously (section 1.2.2.1), CPG and sphingolipids are mainly localised on the outer leaflet of the plasma membrane, whereas SPG, EPG and IPG are located in the inner layer (Daleke, 2003). Maintenance of the asymmetric structure of plasma is regulated through a convoluted balance of transmembrane enzymes: flippases (also called aminophospholipid translocase) (Seigneuret and Devaux, 1984)), floppases (P-glycoprotein) (Bevers et al., 1999) and scramblases (Sahu et al., 2007). Flippases transport aminophospholipids EPG and SPG from the plasma membrane's outer leaflet to the inner leaflet, using ATP (cytofacially-directed, ATP dependent) (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985). Floppases transport CPG and sphingolipids from the inner to the outer leaflet, using ATP (exofacially directed, ATP-dependent) (Bitbol and Devaux, 1988). Scramblases transport lipids indiscriminately in both directions by an ATP-independent calcium mechanism (bi-directional, ATP-independent) (Buton et al., 1996).

Any change in the healthy cellular state asymmetry plays a critical role in a variety of biological processes. A loss of asymmetry in phospholipids is associated with many pathological phenomena. For instance, the translocation of SPG to the outer leaflet is a marker of cell apoptosis and leads to the activation of the phagocytosis by phagocytes (Fadok et al., 2000). In addition,

exposure of EPG to the cell surface is a signal that activates cytokinesis (Emoto et al., 1996).

1.3 Fatty acid components of plasma membrane phospholipids

Fatty acids (FAs) constitute the building blocks of two types of lipids: lipid storage fats (Triacylglycerols (TAGs)) and structural phospholipids.

FAs are carboxylic acids with long hydrocarbon chains, whose length varies from 4 to 30 carbon atoms. However, commonly the FAs found in plasma membrane have 16 to 24 carbons; mostly they have 16 and 18 carbons. This is formed by the biosynthesis of FAs, where C2-unities are combined to form chains of various lengths. The chemical and biochemical properties such as melting point of FAs are mostly dependent upon the chain length of the FAs, and the degree of unsaturation (double bond content). The melting point is an indicator of the fluidity/rigidity; the higher the melting point is, the greater is the rigidity of the structure.

1.3.1 Fatty acid nomenclature

Three systems of nomenclature are used for the designation of FAs: the trivial name, the systematic name, and the shorthand notation.

The trivial name is derived from the name of the main source of the FA. For example, palmitic acid is a principal constituent from palm oil, and oleic acid is obtained mainly from olive oil.

The systematic designation follows the rules set by International Union of Pure and Applied Chemistry (IUPAC) for the Nomenclature of Organic Compounds. The number of carbons is counted from the carboxyl acid end and double bonds are annotated following the cis/trans or E/Z notation.

The shorthand nomenclature consists of the number of carbon atoms, a colon, the number of double bonds and their position. The position of the double bond is indicated in two different ways. In the Δ^x representation, Δ indicates the double bond, and x gives the position of the double bond counting from the carboxyl acid end. Each double bond is preceded by *cis* and *trans*. In the n-x, ω-x or omega-x representation, x gives the position of the double bond counting from the terminal methyl carbon (designated as *n* or ω) toward the carbonyl carbon.

Depending on the degree of unsaturation, FAs can be either saturated (contain no carbon-carbon double bonds in the acyl chain), monounsaturated (one double bond) or polyunsaturated (multiple double bonds).

1.3.2 Saturated fatty acids

Saturated fatty acids contain no carbon-carbon double bonds (Figure 1.7). Saturated components make up 10-40% of the total fatty acids in most natural lipids. Long-chain saturated FAs (12:0 upwards) have relatively high melting points owing to the absence of unsaturation. Such saturated FAs also increase the rigidity of membranes. In biological systems, the most common saturated

fatty acids are 16 and 18 carbon fatty acids (palmitic acid C16:0, stearic acid C18:0).

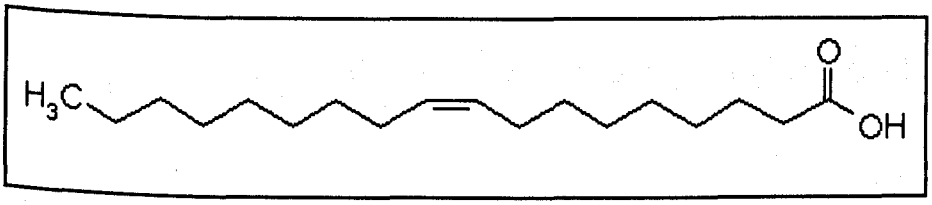


Figure 1.7: Saturated fatty acid

1.3.3 Monounsaturated fatty acids

Monounsaturated fatty acids are monoenoic components; they contain one carbon-carbon double bond in their acyl chain. In most animal cell membranes, the double bond is of the *cis-* or *Z*-configuration, although some FAs with *trans-* or *E*-double bonds are known. The configuration isomerism has an important effect on the FA physical and chemical properties. The *cis-* configuration results in a non-straight structure and enhances membrane fluidity. In contrast, *trans*-FAs are straight chains with physical properties similar to saturated FAs and consequently increase membrane rigidity. In animal tissues, Oleic acid, 18:1(n-9), is by far the most abundant monoenoic FA (Figure 1.8). It is the biosynthetic precursor of the (n-9) family FAs.

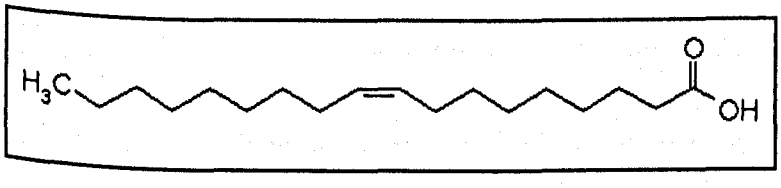


Figure 1.8: Monounsaturated fatty acids: Oleic acid, 18:1(n-9)

1.3.4 Polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) have two or more double bonds. Depending on the number of carbon atoms, from the last double bond to the terminal methyl group, the PUFAs can be divided into three major families (omega 3, 6 and 9 or n-3, n-6 and n-9 respectively). The double bonds can be uninterrupted (allenic), conjugated or interrupted by single (one-methylene-interrupted) or several methylenes (poly-methylene-interrupted). Allenic FAs are found in some plant seeds and in insects (Mikolajczak et al., 1967; Lie Ken Jie et al., 2003). One-methylene-interrupted FAs are found in most animal cells. In contrast, poly-methylene-interrupted FAs are found in plants (Berdeaux and Wolff, 1996) and insects (Christie et al., 1988). Conjugated FAs are mostly present in rumen microorganisms (Bauman et al., 1999; Wallace et al., 2007) and therefore are found in high amounts in meat and diary products and in individuals who consume these kinds of food (Chin et al., 1992). The presence of several double bonds decreases the melting point and confers extremely flexible molecules.

1.3.4.1 The n-9 FA family

The family of PUFAs, with a double bond between the C-9 and the C-10 carbon from the carboxyl end, are termed omega 9 or n-9 FAs. The precursor for this n-9 FA family is Oleic acid, OA, 18:1n-9. OA is the most abundant monounsaturated FA in animal tissues; it is synthesised from a saturated FA, stearic acid (C18:0) by a Δ9 desaturase. Δ9 desaturase, also called stearoyl CoA

desaturase (SCD), inserts the first cis-double bond between C-9 and C-10 from the carboxyl end of the fatty acyl chain (Mahfouz et al., 1980; Corl et al., 2001). OA is first desaturated by a Δ6 desaturase (Δ6) into 18:2n-9, then 18:2n-9 is elongated into 20:2n-9, and finally 20:2n-9 is desaturated by a Δ5 desaturase (Δ5) into a very important product, named mead acid (20:3n-9). Mead acid is a marker of essential fatty acid deficiency; it accumulates under conditions of n-6 or n-3 fatty acid deficiency. This unsaturation biosynthetic pathway takes place in the endoplasmic reticulum (ER) (Figure 1.10).

1.3.4.2 The n-3 and n-6 FA family

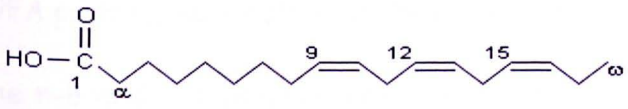
The family of PUFAs, with a double bond between the third (C-3) and the fourth (C-4) carbon from the methyl end of the acyl chain, are termed omega 3 or n-3 fatty acids, and those with a double bond between C-6 and C-7 are termed omega 6 or n-6 fatty acids.

The precursor of the n-3 family is alpha-linolenic acid (ALA, 18:3n-3), and the n-6 family is linoleic acid (LA, 18:2n-6). LA is synthesised from OA by a Δ12 desaturase, whereas ALA is synthesised from LA by a Δ15 desaturase. Mammalians' metabolism cannot introduce a C=C-bond beyond C-9 in the FA chain, because of the absence of the Δ12 and Δ15 desaturases. For the same reason, the body cannot convert an n-3 to an n-6 FA, or vice-versa. ALA and LA are referred to as essential fatty acids (EFA) because they cannot be synthesised in a mammal's body and their absence in the body causes clinical

abnormalities (see section 1.3.7). Thus, ALA and LA have to be obtained from external sources via the diet. ALA is found in leafy vegetables and certain vegetable oils such as flaxseed oil; LA is found in seeds and their oils (Belitz et al., 2004). However, mammals are able to synthesise long-chain polyunsaturated fatty acid (LCPUFAs) from these precursor PUFAs. The main LCPUFAs of the n-3 family are Eicosapentaenoic acid (EPA, 20:5n-3) and DHA (22:6n-3), synthesised from ALA, and ARA (20:4n-6), from the n-6 family, which originates from LA.

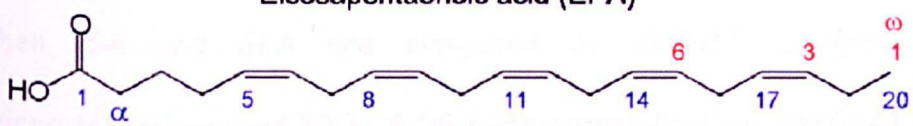
Alternatively, EPA and DHA can also be obtained from marine species, such as fish (salmon, tuna and trout) and algae, whereas ARA is mostly present in animal products, such as red meat and dairy products (Insel et al., 2012).

Alpha-linolenic acid (ALA)

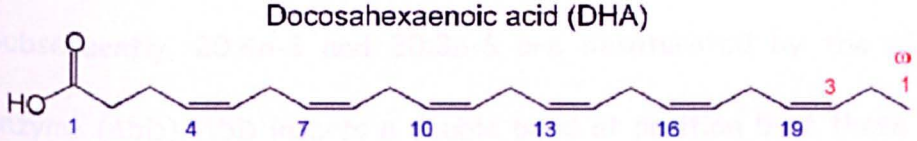


Linoleic acid (LA)

Eicosapentaenoic acid (EPA)



Docosahexaenoic acid (DHA)



Arachidonic acid (AA)

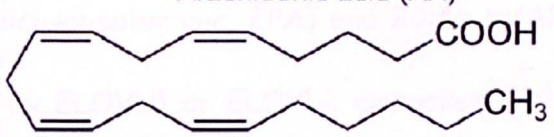


Figure 1.9: Chemical structures of ALA, LA, DHA, EPA and ARA.

1.3.5 Biosynthesis of long-chain n-6 and n-3 polyunsaturated fatty acids (LCPUFAs)

According to the pathway elucidated by Sprecher et al. (1986, 1992), the synthesis of n-3 and n-6 PUFA occurs in two compartments: the endoplasmic reticulum (ER) and the peroxisome.

The n-3 and n-6 PUFA are synthesised from alpha-linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) acids by a series of elongation and desaturation steps

(Hornstra et al., 1995) which occur in the ER. The final step of this n-6 and n-3 PUFA pathway takes place in the peroxisomal compartment.

The n-6 and n-3 families compete for the same enzymes for forming double bonds (desaturases) and for lengthening the carbon chain (elongases).

First, alpha-linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) are desaturated by a Δ6 desaturase (D6D) enzyme at position 6 from the carboxyl end to form 18:4n-3 (stearidonic, SA) and 18:3n-6 (gamma linolenic, GLA) FAs, respectively. Then, SA and GLA are elongated by ELOVL5 elongase in 20:4n-3 (eicosatetraenoic) and 20:3n-6 (di-homo-gamma-linolenic, DHGLA), respectively. Subsequently, 20:4n-3 and 20:3n-6 are desaturated by the Δ5 desaturase enzyme (Δ5D), Δ5D inserts a double bond at position 5 of these FAs to yield 20:5n-3 (eicosapentaenoic, EPA) and 20:4n-6 (ARA). 20:5n-3 and 20:4n-6 are elongated by ELOVL5 or ELOVL2 elongases, which gives rise to 22:5n-3 (n-3 docosapentaenoic, DPA) and 22:4n-6 (adrenic). Consequently, 22:5n-3 and 22:4n-6 are elongated by ELOVL2 elongase in 24:5n-3 and 24:4n-6. Then, 24:5n-3 and 24:4n-6 undergo a second Δ6 desaturation to 24:6n-3 and 24:5n-6 in ER.The latter two FAs are translocated to the peroxisomes where they undergo one cycle of beta-oxidation to form 22:6n-3 (DHA) and 22:5n-6 (n-6 docosapentaenoic, DPA) (Figure 1.10). This peroxisomal beta-oxidation reaction involves 3 enzymes: straight-chain acyl-CoA oxidase (SCOX), dehydrogenase-bifunctional protein (DBP) and 3-ketoacyl CoA thiolase. Finally, DHA and n-6 DPA are transported back to the ER for incorporation into phosphoglycerides,

whereas an excess of these two products remains in peroxisomes and is degraded. The same organelle (peroxisome) is involved in both synthesis and degradation.

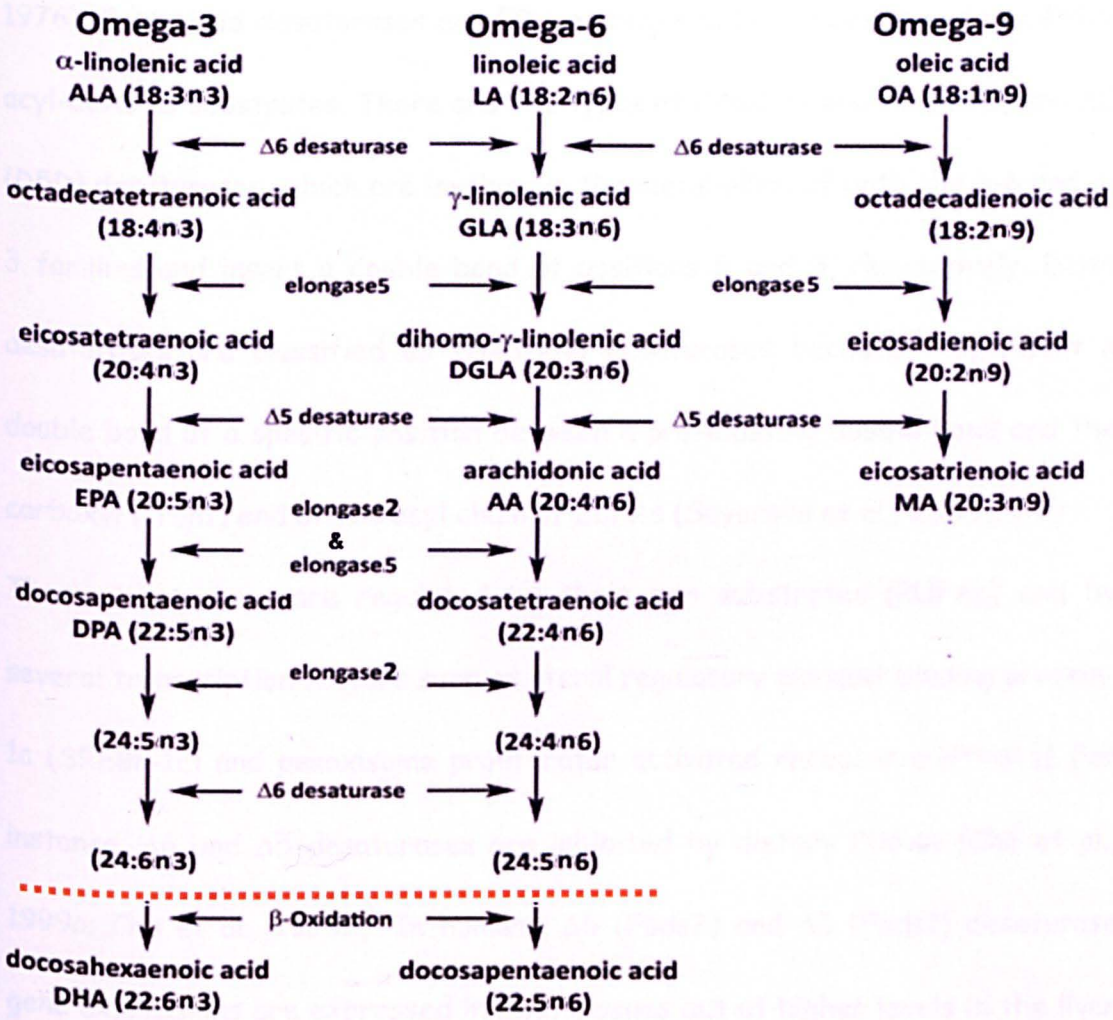


Figure 1.10: Pathway of metabolism and synthesis of omega-3, omega-6, and omega-9 PUFAs (adapted and modified from Le et al., 2009).

1.3.6 The desaturase process

The desaturase reaction involves few components: an oxygen molecule, a cofactor (NAD(P)H), an electron transport system (cytochrome b5 reductase and cytochrome b5 (electron donor)) and a fatty acid desaturase (Enoch et al., 1976). Fatty acid desaturases are ER membrane-bound enzymes and use fatty acyl-CoAs as substrates. There are two types of desaturases: Δ6 (D6D) and Δ5 (D5D) desaturases, which are involved in the metabolism of both the n-6 and n-3 families and insert a double bond at positions 6 and 5, respectively. Delta desaturases are classified as front-end desaturases because they insert a double bond at a specific position between a pre-existing double bond and the carboxyl (front) end of the acyl chain of LCFAs (Sayanova et al., 2003).

These desaturases are regulated by their own substrates (PUFAs) and by several transcription factors such as sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator activated receptor-*α* (PPAR*α*). For instance, Δ6 and Δ5 desaturases are inhibited by dietary PUFAs (Cho et al., 1999a; Cho et al., 1999b). In humans, Δ6 (Fads2) and Δ5 (Fads1) desaturase gene expressions are expressed in most tissues but at higher levels in the liver (Cho et al., 1999a; Cho et al., 1999b).

1.3.7 Elongation process

The elongation cycle includes 4 steps: The first step begins with condensation by an elongase (ELOVL) of a fatty acyl-CoA to a malonyl-CoA molecule (the

donor of two carbon units). The second step consists of a reduction reaction where the elongase interacts with 3-keto acyl-CoA reductase. The third step is a dehydratation reaction involving a dehydratase. Finally, the fourth step is a reduction reaction implicating *trans*-2, 3-enoyl-CoA reductase.

Seven fatty acid elongases (ELOVLs) subtypes (ELOVL1-7) have been identified in the mouse, rat and human. ELOVL1, ELOVL3, ELOVL6 and ELOVL7 elongate saturated and monounsaturated fatty acids. On the other hand, ELOVL2, ELOVL4 and ELOVL5 are specific for PUFAs synthesis, whereas ELOVL2 and ELOVL5 are involved in n-6 and n-3 PUFA biosynthesis. ELOVL2 uses C20-22 PUFAs as substrates (ARA, EPA, adrenic and n-3DPA). Substrates of ELOVL5 consist of 18 and 20 carbons polyunsaturated fatty acyl-coA (18:4n-3, 18:3n-6, ARA and EPA) (Figure 1.10).

Elovl1, Elovl5 and Elovl6 gene expression is ubiquitous. Elovl5 gene expression is highest in the liver, testis and adrenal glands. However, Elovl2, Elovl3, Elovl4, and Elovl7 gene expression is tissue-specific. Elovl2 gene expression is highest in the testis and liver and is significant in the kidney, brain, lung and white adipose tissue. The Elovl transcript expressions have been shown to be regulated by the diet in rat liver (Wang et al., 2005). Moreover, the Elovl2 and Elovl5 expressions are also regulated at the transcriptional level by several transcription factors such as sterol regulatory element binding protein-1a (SREBP-1a) and PPAR*α*.

1.3.8 Essentiality of fatty acids

In 1929, Burr and Burr discovered the essentiality of two FAs, LA and ALA. These FAs were classified as essentials because of their ability to ameliorate some classic symptoms of essential fatty acid deficiency, such as dermatitis, kidney lesion, infertility, premature death and growth retardation (Burr and Burr, 1929).

Experimental works carried out in rats fed on a fat-free diet showed that the rats generated these clinical abnormalities (stated above) and that these impairments were reversed by supplementing their diets with LA and ALA (Burr and Burr, 1929). Moreover, dietary ALA deficiency has been associated with retinal anomalies, but also with alterations in learning and memory performances (Bourre et al., 1989). Feeding rats sunflower oil (low in ALA) led to anomalies in their electroretinogram, behavioural tests, learning and memory and resulted in premature death (Bourre et al., 1989). Okuyama reported higher learning ability and retinal function in rats fed perilla oil (53% n-3) compared with those fed safflower oil (containing 75% LA) and also with those fed soybean oil (53% n-6 and 4% n-3). These general behavioral patterns were observed in rats for two generations (Okuyama, 1992). Following an n-3-deficient diet, rats performed poorer in olfactory-based behavioral tasks and spatial tasks compared with adequately fed animals (Salem et al., 2001).

Nevertheless, some of the clinical signs of LA and ALA deficiency are due to the absence of their respective metabolites. LA is the precursor of the longer

chain PUFA ARA, whereas ALA is the precursor of EPA, and DHA. ARA, EPA and DHA are present in all biological membranes. In particular, DHA and ARA are especially prominent in neural and retinal tissues (Singh, 2005). These FAs are required for proper differentiation and functioning of brain cells (Bourre et al., 1993). Deprivation of ALA in the diet dramatically alters the FA composition of various organs including the brain. ALA deficiency was associated with reduced amounts of DHA, which was compensated by an increase of n-6DPA in brain cells and organelles of the nervous system: neurons, astrocytes, oligodendrocytes, myelin, nerve endings, endoplasmic reticulum and retina (Bourre et al., 1993). Bourre et al. also demonstrated that by switching from an n-3-deficient diet to an n-3-containing diet, the speed of recovery from these abnormalities is very slow for brain cells compared with other organs (Bourre et al., 1993). Changes in brain membrane PUFA composition are directly correlated with changes in cognitive functions. N-3 deficiency influences neurotransmitter systems in rats, particularly the dopamine systems of the frontal cortex, which is associated with learning ability (Zimmer et al., 2000).

In humans, n-3 FAs play a therapeutic role in neurological disorders; they may reduce cognitive decline and the risk of dementia including the Alzheimer type (Conquer et al., 2000).

1.4 Lipids and the nervous system

Nervous tissue has the highest lipid concentration immediately after adipose tissue. The dry weight of the adult human brain is composed of 50-60% of lipids including 22% of lipids in the cerebral cortex and 24% in the white matter, mostly structural lipid in the form of phospholipids (Sastry, 1985). The major PLs in the adult rat brain are CPG 35.5%, EPG 34.1%, SPG 12.2%, SM 5.7% and IPG 3.1% (Sastry, 1985). The brain is the organ that contains the greatest quantities of DHA, an important amount of ARA and very little LA and ALA in their glycerophospholipids (Tinoco et al., 1982; Sastry, 1985). An average of one in three FAs in the nervous system is polyunsaturated. In humans, DHA is more concentrated in phospholipids from the grey matter (cortex) than from the white matter. DHA is highly enriched in neural membranes constituting approximately 30-40% of the PLs in the grey matter of the cerebral cortex and the photoreceptor cells in the retina (Innis et al., 1991; Lauritzen et al., 2001).

The brain is composed of several types of cells: neurons mostly in the grey matter (1/4 of the brain weight), glial cells (astrocytes and oligodendrocytes) mostly in the white matter and endothelial cells that are associated with the cerebral capillaries. Glial cells, mainly astrocytes, play important roles in the support and segregation of neurons, the regulation of neuronal communication, neurosecretion, synaptic plasticity and activity modulation. Membrane lipid composition appears in different proportions according to the brain cell type.

DHA and ARA are the major LCPUFAs and exhibit great specificity according to the structure, as illustrated in Table 1.1. Among subcellular fractions of brain tissue, the highest concentrations of DHA are found in the synaptosomal plasma membranes (Breckenridge et al., 1972) and synaptic vesicles (Breckenridge et al., 1973). DHA preferentially accumulates in synaptosomes, astrocytes and myelin (Bourre et al., 1992; Breckenridge et al., 1973).

In grey matter, the ARA to DHA ratio is nearly 1:2, whereas the myelin fraction has almost a 3:1 ratio. The retina contains a very high level of DHA in the rod outer segment of the photoreceptor membrane. The FA compositions of the same lipid class from the retina (Anderson, 1970) are similar to those of the same class from grey matter of the brain (O'Brien and Sampson, 1965). This is consistent with the fact that the retina is derived from grey matter (Jampel, 1966).

Species	Fraction	Lipid	ARA (% of total FA)	DHA (% of total FA)	Ref.
Human	Grey matter	EPG	13.8	24.3	O'Brien and Sampson (1965)
		SPG	1.6	36.6	
		CPG	4.1	3.1	
Human	White matter	EPG	6.4	3.4	
		SPG	2.0	5.6	
		CPG	1.3	0.1	
Human	Retina	EPG	13.4	22.2	Anderson (1970)
		SPG	5.0	18.5	
		CPG	4.1	4.6	
Rat	Synaptosomal plasma membrane	EPG	14.6	32.4	Breckenridge et al. (1972)
		SPG	2.0	34.1	
		CPG	4.3	2.7	
		IPG	36.9	4.7	
Rat	Synaptic vesicles	EPG	16.7	30.6	Breckenridge et al. (1973)
		SPG	2.8	37.0	
		CPG	6.0	6.9	
		IPG	33.0	2.6	
Rat	Neurons	TLE	10.3	8.3	Bourre et al.
Rat	Synaptosomes	TLE	12.2	12.5	

Rat	Oligodendroglia	TLE	9.3	5.1	(1984)
Rat	Myelin	TLE	8.6	1.4	
Rat	Astrocytes	TLE	10.3	12.1	

Table 1.1 DHA and ARA compositions in different brain lipid classes in selected mammalian tissues. ARA and DHA are expressed as the % of total FA content; EPG, ethanolamine phosphoglyceride; SPG, serine phosphoglyceride; CPG, choline phosphoglyceride; IPG, inositol phosphoglyceride and TLE, total lipid extract from GC data.

Prevalence and role of EPA in brain cells

In contrast to the predominant n-3 PUFA in brain, DHA; EPA is a relatively minor FA in the brain. EPA is found in trace amounts in the cortex (Makrides et al., 1994) and it accounts for approximately 0.2% of total FAs in EPG (Philbrick et al., 1987). In the brain, EPA plays a major role as a precursor for the biosynthesis of hydroxy FA derivatives known as eicosanoids. In particular, Yerram et al. (1989) showed that brain microvessel endothelial cells were able to convert EPA to prostaglandin PGE3, PG13. Several *in vitro* and *in vivo* studies have shown that EPA supplements have beneficial effects on cell survival through the modulation of the neurotrophin receptor expression. EPA has protective effects against neurotoxicity-induced by lipopolysaccharide in rat hippocampal cells (Lonergan et al., 2004) and against neurotoxicity-induced by staurosporine in SH-SY5Y cells (Kou et al., 2008).

1.4.1 Biosynthesis of DHA and ARA in the nervous system

Accretion of LCPUFAs, especially DHA and ARA, in the brain, takes place predominantly during prenatal life and early ageing. LCPUFAs are readily

accumulated in the brain during brain development, between the last trimester of gestation up to two years of age after birth (Clandinin et al., 1980; Martinez, 1992). The developing foetus and the newborn have a limited capability to synthesise DHA and ARA and rely mostly on the mother's supply. These two PUFAs are maternally supplied to the foetus *via* the placenta (Crawford et al., 1976; Innis, 2005) and to the newborn *via* maternal milk (Crawford et al., 1981; Innis, 2004).

During a lifetime, there are several sources of DHA and ARA; they can arise through the blood stream and are derived from the diet, from their endogenous biosynthesis in the liver (Scott and Bazan, 1989) and partially, from local biosynthesis in the brain (Moore et al., 1991). Work with primary cultures of astrocytes and neurons suggested that the main site in the brain for the biosynthesis of n-3 and n-6 LCPUFAs is the astrocyte cell (Moore et al., 1991). Neuronal cultures were able to elongate and desaturate ALA (18:3n-3) into 20:3n-3, 22:5n-3, but only a very small amount of DHA (22:6n-3) was made. In contrast, pure cultures of primary astrocytes actively elongated and desaturated 18:3n-3 into higher n-3 intermediary metabolites, 20:5n-3, 22:5n-3 as well as into 22:6n-3.

Similar patterns were observed for the n-6 PUFA family metabolism. Neuronal cultures produced large amounts of 20:2n-6 from LA (18:2n-6) but no ARA (20:4n-6), and adrenic acid (n-6DTA, 22:4n-6) was synthesised from direct

supplementation with 20:4n-6. In contrast, astrocytes produced 20:2n-6, 20:3n-6, ARA, n-6DTA and n-6DPA from their n-6 precursor.

Further studies have suggested that endothelial cells and astrocytes co-operate in the elongation and desaturation of n-3 and n-6 precursors in the brain and in the transfer of their final products, DHA and ARA, to neurons. The work of Moore et al. (1990) on cultured murine cerebromicrovascular endothelium incubated with n-3 and n-6 precursors showed an elongation/desaturation in EPA and ARA, respectively. Astrocytes subsequently completed the conversion of EPA to DHA and of ARA to n-6DPA; both intermediary metabolites ended up in the neuronal lipid cache.

In concluding from these *in vitro* studies on PUFA synthesis, neurons do not have the ability to form LCPUFAs from their essential FA precursors. The abundance of LCPUFAs (especially ARA and DHA) in neuronal tissue is supplied by cerebrovascular endothelium and astrocytes (Moore, 2001).

1.4.2 Role of LCPUFAs in neural membrane

Ontogenetic events such as neurogenesis (the generation of nerve cells) and synaptogenesis (the formation of synapses) in the developing brain, along with nerve regeneration and stem cell differentiation in the mature brain, are all biological processes that depend upon essential lipid components as well as on highly specific membrane proteins for their proper conclusion (Lauritzen, 2001;

Alessandri, 2004). Notable among these essential lipids are the ubiquitous LCPUFAs DHA and ARA, which are integral components of neuronal membranes. Both of them are presumed to play important roles in providing unique membrane biophysical properties supporting cellular signalling including regulation of gene expression and generation of cell membrane lipid mediators (eicosanoids). DHA, in particular, facilitates neuronal functions and its inadequate levels are strongly associated with impaired vision, memory losses and learning disabilities.

1.4.2.1 DHA and ARA effects on membrane physical properties

The plasma membrane is assimilated as a dynamic structure, whose physical properties are highly dependent on its lipid composition. The organization of membranes in microdomains (e.g., rafts and caveolae) and its physical properties (e.g., fluidity) are highly dependent on the lipid content and interactions (Day et al., 2009).

Membrane fluidity is affected by the lipid composition of the membrane, specifically, the ratio between cholesterol and unsaturated FAs. Cholesterol reduces the membrane fluidity index, and conversely, unsaturation (PUFAs) increases it. N-3 and n-6 PUFAs alter the FA unsaturation index and the rigidity of the membrane. Dietary PUFAs, in particular, DHA and ARA, when incorporated, can alter membrane fluidity. Upon an increase in the DHA and ARA or an increase in the overall double bond contents in the membrane, the

physical properties including the fluidity of the membranes rise (Stillwell and Wassall, 2003). The membrane flexibility differs between DHA and ARA because of their different degrees of conformational flexibility in membranes (Feller et al., 2002).

1.4.2.2 DHA and ARA contributions on neurogenesis

Both n-3 and n-6 LCPUFAs play crucial roles in neuronal growth and in the development of synaptic processes for neuronal cell circuitry.

In the developing embryonic rat brain, neurogenesis is inhibited or delayed (Bertrand et al., 2006) and migration of immature neuronal cells to their final position, where they establish permanent synaptic relationships, is delayed, following maternal n-3 dietary deficiency (Yavin et al., 2009). PUFAs (ARA and DHA) have been shown to be involved in neuronal arborisation, which is the main characteristic of neurogenesis and synaptogenesis. N-3 fatty acid deprivation during development resulted in marked decreases in neurite growth and synaptogenesis (Cao et al., 2009).

In vitro studies showed that DHA stimulates neurite outgrowth induced by NGF in PC12 cells (Ikemoto et al., 1997; Dagai et al., 2009), whereas ARA suppresses this process. DHA also promotes neuronal differentiation and arborization in hippocampal neurons from rat primary cultures, whereas ARA and n-6DPA did not have any effect (Calderon and Kim, 2004). DHA also increased spontaneous synaptic activity in mouse hippocampal neurons (Cao et

al., 2009). Tixier-Vidal et al. (1986) reported that ARA and DHA simultaneously permitted the full synaptogenesis of cultured mouse foetal hypothalamic cells. ARA and DHA regulate the formation of synaptic vesicles in this system.

1.4.2.3 DHA influences the size of the neuron

DHA affects additional neuronal morphological changes. Loss of DHA decreases the cell body size of rat hippocampus neurons (Ahmad et al., 2002a), and neurons in the hippocampus of the human DHA-deficient brain had significantly smaller cell bodies (Ahmad et al., 2002b). Thus, a DHA deficiency in the brain induces a reduction in neuron size.

Dietary DHA deficiency decreases the NGF content in the rat hippocampus neurons, so consequently, DHA promotes NGF expression in the brain (Ikemoto et al., 2000).

1.4.2.4 DHA interaction with membrane proteins and its involvement in signalling transduction cascades

Enrichment of membranes with PUFAs, in particular DHA, affects the membrane's biophysical properties, and subsequently the membrane receptors' organization and protein functions.

DHA may interact in specific sites of membrane proteins where it modulates the activity of channel and transport proteins.

Using patch clamp techniques, Poling et al. (1995, 1996) demonstrated that membrane DHA modulates the activity of certain ionic channels such as

voltage-gated K^+ channels by interacting with the external domain of the K^+ channel. DHA also modulates Na^+/ K^+-ATPase (Huang et al., 1986), which are the neurotransmitter and amino acid transporters (Poling et al., 1995). DHA promotes optimal function of G-proteins coupled receptor families (GPCRs) in neuronal and retinal tissues such as rhodopsin (Litman et al., 2001). GPCRs are usually found in membranes rich in PUFAs, specifically in DHA-rich domains (Brown, 1994). Absorption of light leads to the formation of a photoactivated rhodopsin meta-II state (MII) in equilibrium with an inactive rhodopsin meta-I state (MI). The conformational transition from a non-active to active rhodopsin (MI-MII equilibrium) is thought to be favored by lipids with DHA chains (Mitchell et al., 2003).

Studies carried out with Nuclear Magnetic Resonance (NMR) revealed that the DHA-acyl chains bind to rhodopsin (Soubias et al., 2006) to stabilize it and enhance its kinetics (Grossfield et al., 2006).

DHA can alter the activity of cellular enzymes. It has been shown to enhance the diacylglycerol-dependent activation of protein kinase C (PKC) when it is incorporated into EPG rather than CPG (Chen and Murakami, 1994; Giorgione et al., 1995). Furthermore, PKC plays a role in neural differentiation and plasticity. Activation of PKC enhances neurite outgrowth in several cell lines such as PC12 cells and in primary neurons (Hundle et al., 1995). DHA also plays a role in the phosphatidylinositol-3 kinase/ Akt (PIK3/Akt) signalling cascades involved in neuronal survival (Akbar et al., 2005). DHA promotes the PIK3/Akt pathway,

which is involved in preventing apoptotic cell death induced by staurosporine in Neuro 2A cells (Akbar et al., 2002).

1.4.2.5 N-3 and n-6 PUFAs modulate gene expression

N-3 and n-6 PUFAs were reported to act as regulators of gene expression (Deckelbaum et al., 2006).

FAs are delivered to cells either as lipoprotein complexes or as non-esterified FAs (NEFAs). Non-esterified FAs enter cells through transporters or by diffusion and are transported to intracellular compartments for metabolism, or to the nucleus, where they interact with transcription factors (Bordoni et al., 2006). PUFAs, in particular, DHA and ARA, physically bind and activate nuclear transcription factors; thereafter, the complex of PUFA-transcription factors binds cis-regulatory elements of target genes and regulates their gene expression. FAs have been shown to up-regulate or down-regulate the transcription of genes by altering the rate of gene transcription (Jump et al., 1993; Landschulz et al., 1994; Tang et al., 2003) or the transcripts' stability (Sessler et al., 1996). Several transcription factors interacting with PUFAs have been identified.

The first family of nuclear transcription factors are the peroxisome proliferator activated receptors (PPARs), which consist of four isoforms: PPARs, PPARα, PPAR β/δ and PPAR γ. The PPARs are activated by unsaturated PUFAs (ALA, LA and ARA; Kliewer et al.; Krey et al., 1997) and by eicosanoids

(Yu et al., 1995), often at micromolar concentrations (Gottlicher et al., 1992). Upon activation with a specific ligand, the PPARs heterodimerize with the retinoid X receptor (RXR), which itself, is itself activated by 9-*cis* retinoic acid (Bardot et al., 1993; Desvergne and Wahli, 1999). Also, DHA (Fan et al., 2003; Lengqvist et al., 2004), n-6DPA and ARA (Lengqvist et al., 2004) have been shown to bind with a low affinity and activate RXR. The activated PPAR-RXR complex binds to specific DNA sequences (here, peroxisome proliferators response elements (PPREs)) on target genes. A number of genes that respond to PPAR activation have been reported: genes involved in β-oxidation, such as carnitine palmitoyltransferase I (Brandt et al., 1998), and genes involved in lipid metabolism, such as acyl-CoA synthetase (Schoonjans et al., 1995), and the FA transporter protein (FATP) (Frohnert et al., 1999).

In addition to PPARs, another family of nuclear receptors that has been shown to be activated by PUFAs are the liver X receptors (LXR). *In vitro* studies have shown that PUFAs can bind LXRα, and inhibit the LXRα activity in hepatoma (Ou et al., 2001) and in HEK 293 cells (Yoshikawa et al., 2002). However, further research is needed to clarify whether PUFAs play a role in modulating LXRα activity *in vivo* (Jump et al., 2005).

LXRs regulate those genes involved in FA and cholesterol metabolism (Repa et al.,2000), including SREBP-1c, lipoprotein lipase, fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and stearyl CoA desaturase-1 (SCD1). The activated ligand-LXRα complex binds a DNA sequence (LXRE) located in the

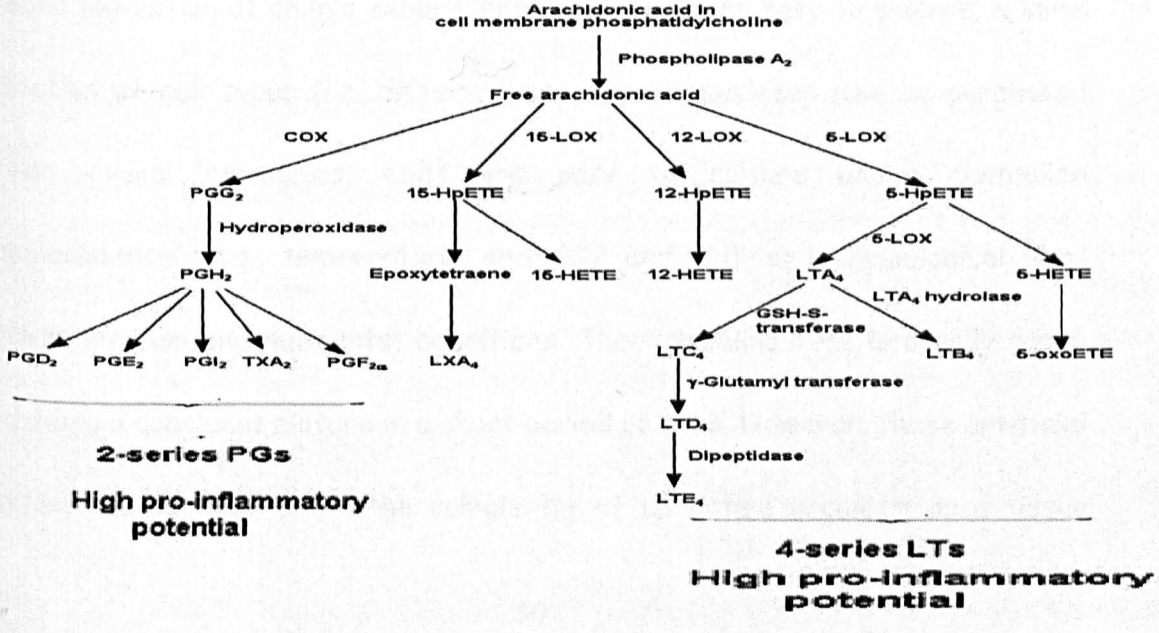
promoter of sterol regulatory element binding protein-1c (SREBP-1c). SREBP-1c is the predominant SREBP isoform in human and rodent livers. SREBP-1c transcription is suppressed by PUFAs as a result of their ability to inhibit LXRα activity (as stated before) (Kim et al., 1999). Consequently, transcription of SREBP-1c target genes containing a sterol response element (SREs) is decreased. SREBP-1c plays a central role in controlling the transcription of genes involved in FA and triglyceride synthesis (Brown and Goldstein, 1977) such as ACC, FAS, glycerol phosphate acyl transferase and SCD1.

1.4.2.6 Second messenger derivatives of n-3 and n-6 metabolites

In response to extracellular stimuli (such as stress, endotoxins, hormones, and cytokines), cells are able to generate second messenger derivatives of n-3 and n-6 metabolites (Zucali et al., 1986; Fu, 1990). Initially, arachidonoyl and docosahexaenoyl chains are released from the membrane choline glycerophospholipids *via* the action of phospholipase A_2 (PLA_2) (Clark et al., 1990) or from the membrane phosphatidylinositol-4,5-biphosphate *via* the actions of phospholipase C (PLC) and a diacylglycerol lipase (DAG) (Bell et al., 1979). Then, two families of enzymes, namely, cyclooxygenase (COX; COX1 and COX2) (Seibert et al., 1995) and lipoxygenase (LOX; 5-LOX, 12-LOX or 15-LOX) (Izumi et al., 1991; Jakobson et al., 1992), catalyze the FA oxygenation (Tassoni et al., 2008). FAs are metabolised into a variety of oxygenated derivatives known as eicosanoids (prostaglandins (PGs), thromboxanes (TXs),

leukotrienes (LTs), hydroperoxyeicosatetraenoic (HPETEs), hydroxyeicosatetraenoic (HETEs), hydroxyeicosapentaenoic acids (HEPEs) and lipoxins (LXs)) (Figure 1.11). COX catalyzes the biosynthesis of PGs and TXs, while LOX produces LTs, HPETEs, HETEs, HEPEs and LXs (Schmitz and Ecker, 2008). Oxygenation of ARA gives origin to 2-PGs, 2-TXs, 5-HETEs, 5- HPETEs, and 4-LTs; DHGLA to 1-PGs; whilst EPA leads to the production of 3- PGs, TXs, 5-LTs, and HEPEs (Schmitz and Ecker, 2008). In addition, *via* the sequential action of 5-LOX, EPA generates the E-series of resolvins, whilst DHA is the precursor of docosanoids (hydroxy-DHA (HDHAs), neuroprotectins and D-series resolvins) (Kohli and Levy, 2009). Eicosanoids are biosynthesized by several different cell types. For instance, leukotrienes are mainly synthesized by immune cells such as leukocytes, macrophages, and mast cells (Funk, 2001). The eicosanoids are biologically active substances affecting every tissue of the body. They are involved in a wide range of functions, such as mediation of allergic and inflammatory reactions (pro-inflammatory), renal function, as well as vaso- and broncho-constriction (Allen, I. C. et al., 2006; Yu et al., 2009; Xu et al., 2010). The docosanoids are a potent group of anti-inflammatory and neuroprotectants found in brain tissue under stress conditions (Mukherjee et al., 2004; Bazan, 2005). Most recently, DHA has been found to serve as a precursor of docosanoids that include neuroprotectins, docosatrienes, resolvins of the D series (resolvin D1 or RvD1) (Serhan, 2005), a potent group of anti-inflammatory, anti-apoptotic and neuroprotective compounds.

Under certain stress conditions, two DHA-oxygenation pathways give rise to two DHA-derived messengers: On the one hand, to 10,17S-docosatriene (neuroprotectin D1, NPD1), and on the other hand, to the synthesis of resolvin-type messengers (17R-series resolvins, 17R-DHA) (Marcheselli et al., 2003). NPD1 has been shown to possess some protective actions in neural and retinal systems under several stress conditions. NPD1 protects human retinal pigment epithelial cells (RPE) against cell injury-induced oxidative stress (Bazan 2005; Mukherjee, 2004), and human brain cells during brain ischemic stroke, presumably by augmenting neuroprotective signalling cascades (Marcheselli, 2003). A beneficial role of NPD1 in Alzheimer's disease has been also recently indicated (Bazan, 2009). Experimental studies with human brain cells, exposed to amyloid beta-42 peptide (Aβ-42), have shown that NPD1 down-regulates pro-inflammatory events and induces anti-apoptotic and neuroprotective gene expression that represses Aβ-42-induced neurotoxicity. To conclude, NPD1 promotes cell survival during adverse events such as stroke, neurotrauma and neurodegenerative diseases.



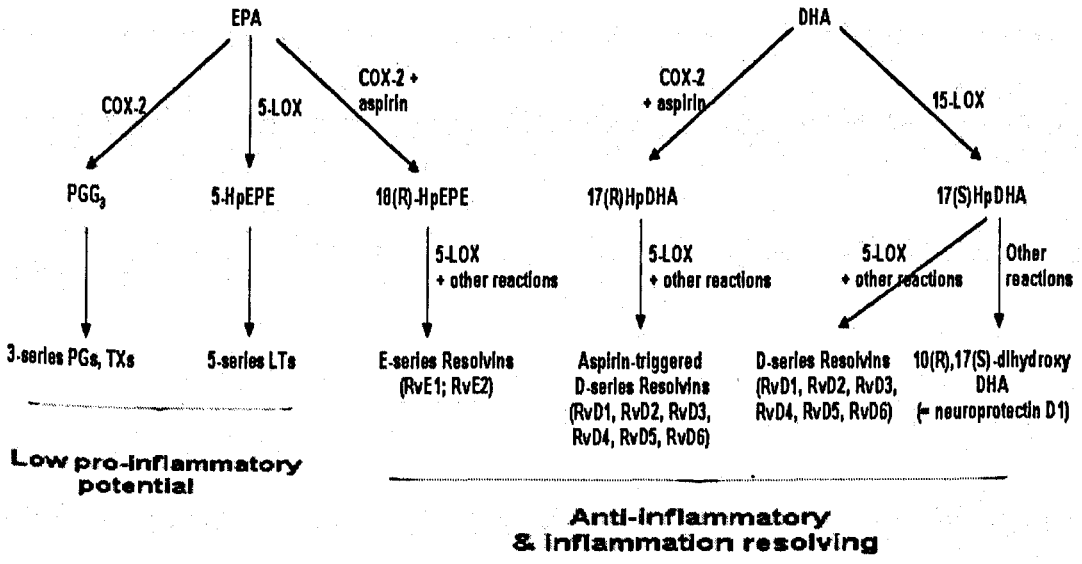


Figure 1.11: Eicosanoids synthesis pathway from ARA, EPA and DHA (adapted and modified from http://www.gustrength.com/health:omega-3-fatty-acids-and-inflammation).

1.5 PC12 cells as a model in neurobiology

In order to study brain lipid dynamics in health and disease, several animal model systems have been employed over the past decades. From the point of view of the reductionist, an *in vitro* cell culture system using pure brain cell populations would be advantageous over a structure, an organ or an entire animal. The advantages of using *in vitro* cultured cells are financial and ethical (public opposition of animal experiments). Cell lines are easy to acquire; a large selection of cell types (i.e. different species, tissues) can now be purchased from several companies. Cells are easy to culture under controlled physiochemical (e.g., temperature, and CO2 and pH), and physiological (e.g., growth medium and nutrients) conditions. Their doubling time is usually rapid, providing a confluent culture in a short period of time. However, these artificial systems do not represent the complexity of an entire organism or a tissue

(Freshney, 2010). Over the years, many cells resembling neurons, astrocytes, oligodendrocytes and microglia have been adapted for short-lived growth (primary) or perpetual growth (transformed). For many years, the PC12 cell line has been considered a suitable model for studying the molecular mechanisms underlying both neuronal differentiation and survival, owing to their ability to differentiate into sympathetic-like neurons and to extend neurite outgrowth (Gollapudi and Oblinger 1999a, b). The PC12 cell line, established in 1976 (Greene and Tischler, 1976), is a clonal cell line derived from pheochromocytoma cells of the rat adrenal medulla. Pheochromocytoma cells were first described in the 1970s, when Warren and Chute as well as others reported the *in vitro* induction and partial characterisation of a transplantable rat pheochromocytoma, which expressed differentiated properties similar to adrenal chromaffin cells (Warren and Chute, 1972; Delellis et al., 1973).

An earlier report showed that tumour-derived chromaffin cells extend long, branching neuronal-like phenotypes after treatment with nerve growth factor (NGF) protein (Greene and Tischler, 1976). Like chromaffin cells, PC12 cells also differentiate into a neuronal-like phenotype upon treatment with NGF, including synthesis and storage of dopamine (Westerink and Ewing, 2008). Furthermore, the PC12 cell line was considered a suitable model for exploring the nervous system, since it permits rapid studies of the cellular and molecular mechanisms present in neuronal cells, which can then be confirmed using cultured primary neurons.

PC12 cells have been extensively employed in neurobiology and neurochemical investigations (such as the role of growth factors associated with receptor activation and down-stream events), in cellular physiology and in the pathological mechanisms governing stress and cell death.

Use of PC12 cells in cellular physiology: Calcium (Ca^{2+}) signals

Ca^{2+} transport in undifferentiated and differentiated PC12 cells displays properties similar to the Ca^{2+} transport of sympathetic neurons (Duman et al., 2008). Therefore, PC12 cells serve as a useful tool to investigate the cellular physiology associated with the synaptic activity. Early work conducted in PC12 cells by Jia et al. (1999) illustrated the effect of neurotrophins on calcium uptake. NGF treatment of PC12 cells increased the calcium currents, as recorded by patch clamp techniques. Another study carried out in PC12 cells by Ghosh and coworkers (1994) suggested that Ca^{2+} can regulate gene expression (e.g., through the calcium response element (CaRE)) by multiple signalling pathways. Agell et al. (2002) showed that Ca^{2+} can modulate the survival of PC12 cells through the Ras/Raf/mitogen-activated protein kinase kinase /extracellular signal-regulated kinase (Ras/Raf/MEK/ERK) pathway. Stimulation with neurotrophins or growth factors (as described next), increases intracellular Ca^{2+}, which greatly activates the Ras/Raf/MEK/ERK pathway associated with cell survival.

Use of PC12 cells in cell death studies

Differentiated PC12 cells, as a model for neural cells, have many applications in human neurodegenerative diseases such as Alzheimer's disease (AD) (el-Agnaf and Irvine, 2002). AD is characterised by deposits of amyloid beta-peptide (Aβ) in the brain or amyloidogenesis. Amyloidogenesis is caused by a non-Aβ component (NAC) and Aβ peptides. PC12 cells have been used to investigate the neurotoxicity of peptides related to AD. It has been shown that PC12 cells are particularly sensitive to NAC and Aβ peptides (Bodles et al., 2001). Some of the mechanisms involved in neurotoxic cell death have been investigated. One early response to Aβ, studied in PC12 cells, is the inhibition of cellular redox activities and cell death (Shearman et al., 1994). A recent report showed that PC12 cells, transfected with a mutated amyloid precursor protein (*app*) gene, became more sensitive to apoptosis induced by reduction of trophic factors, such as serum reduction (Leutz et al., 2002).

Moreover, treatment of PC12 cells with NGF, neurotrophins or cytokines stimulated APP expression in all constructs (Ge and Lahiri, 2002), suggesting that different growth factors up-regulate the expression of the *app* gene.

1.5.1 Proliferation and differentiation of PC12 cells

Under regular culture conditions, PC12 cells grow, divide and possess properties analogous to those of immature rat adrenal chromaffin cells. After exposure to peptide growth factors such as nerve growth factor (NGF) and to a lesser

extent to acidic fibroblast growth factor (aFGF), or basic fibroblast growth factor (bFGF), PC12 cells cease to replicate, extend long nerve-like processes and express a number of markers reminiscent of differentiated neurons (Togari et al., 1983; Wagner and D'Amore, 1986; Rydel and Greene, 1987). PC12 cells have also been exposed to retinoic acid (RA). RA did not induce any morphological differentiation (manifested by the lack of neurites) but like neurotrophins, affected cell proliferation and induced several neural gene transcripts (Cosgaya et al., 1996, 1998; Matsuoka et al., 1989). Similar to sympathetic neurons, PC12 cells exposed to NGF could generate action potentials (Dichter et al., 1977; Green and Tischler, 1976) and have been useful in elucidating the mode of action of NGF in particular, and other growth factors in general (Fujita et al., 1989).

1.5.2 NGF and neurotrophin effects on PC12 cells

The nerve growth factor belongs to a large family of growth factors, known as the neurotrophin family. The neurotrophin family comprises five members: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and neurotrophin-5 (NT-5). The neurotrophic factors act on neurons of either the peripheral or central nervous systems by inducing neurite growth and specific genes related to neural differentiation. These neurotrophic substances are also involved in cell survival.

NGF signalling pathway mechanism

NGF binds to specific cell surface receptors, after which it initiates a variety of cellular responses involving a number of signal-transducing proteins that undergo activation by phosphorylation. Interestingly, NGF has the ability to activate two different receptors that stimulate neuronal cell survival and differentiation. NGF and neurotrophins (BDNF, NT-3, and NT-4/5) bind with low affinity to Low-Affinity Nerve Growth Factor Receptor (LNGFR) or a member of the tumour necrosis factor receptor 1 (p75), which is widely distributed on both neuronal and non-neuronal cells. NGF and neurotrophins (NT-3, NT-4/5) bind with high affinity to a tyrosine kinase receptor (TrkA). These receptors are often present on the same neuronal cell and can generate synergic or antagonist effects on neurons when challenged with various neurotrophins. Activation of TrkA by NGF mediates the differentiation and survival of neurons, whereas activation of p75 by NGF mediates neuronal apoptotic cell death. The fate of the neuron depends on the ratio of p75 to TrkA receptors present on the cell (Davies et al., 1993; Barrett and Bartlett, 1994; Lee et al., 1994).

Activation of TrkA receptors by NGF induces the phosphorylation of downstream signalling molecules *via* a Ras-dependent pathway leading to activation of the ERK or of the mitogen-activated protein kinases (MAPK) (Kaplan and Stephens, 1994; Greene and Kaplan, 1995) and also *via* other enzymes, such as PI-3K (Yao and Cooper, 1995). The phosphorylated MAPK is

then translocated in the nucleus where MAPK activates specific transcription factors that consequently induce the expression of specific genes involved in differentiation and survival (Figure 1.12).

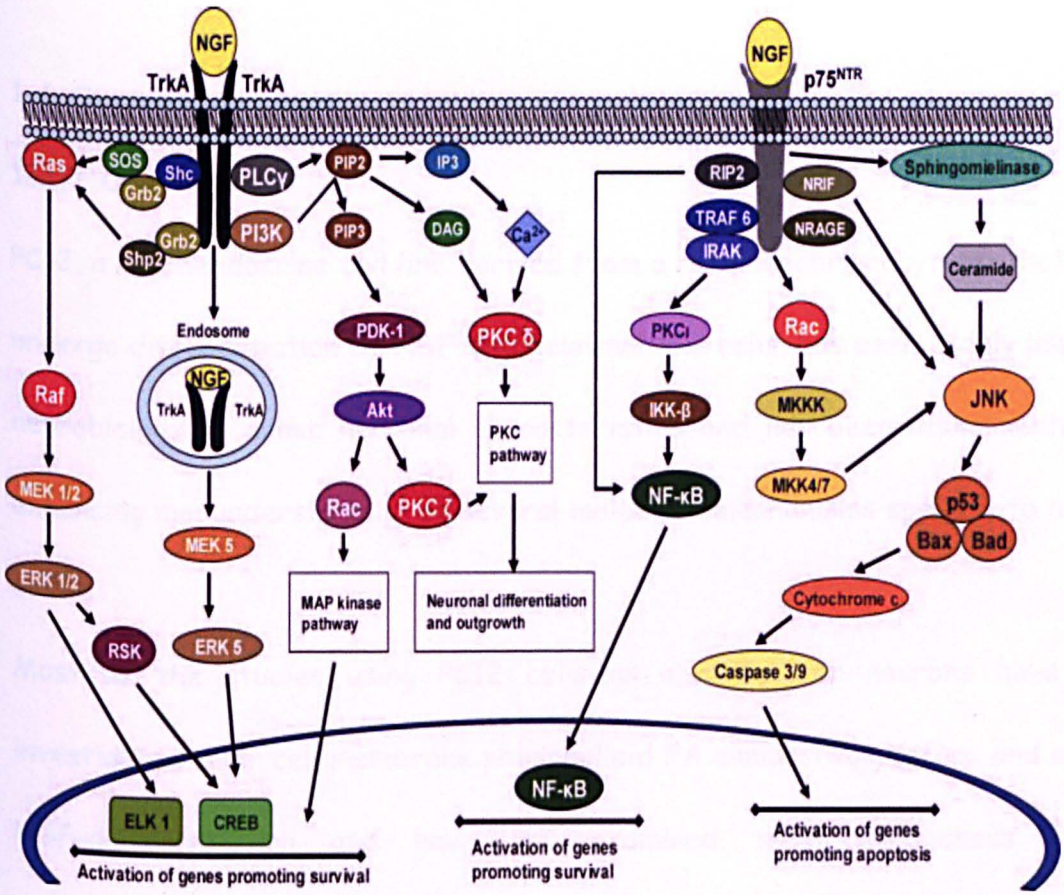


Figure 1.12: NGF signalling pathways in PC12 cells and neurons (adapted from Niewiadomska et al., 2011).

Molecular signalling pathways involved in PC12 cell differentiation and survival have been extensively studied *in vitro*. For instance, the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/ ERK pathway controls apoptosis, proliferation or differentiation in many cell types including PC12 cells. High activation of the Ras/Raf/MEK/ERK pathway is associated with neuronal and

PC12 cell differentiation (Vaudry et al., 2002). In contrast, low activation of this pathway is associated with cell proliferation (Agell et al., 2002; Vaudry et al., 2002).

1.6 Overview and aims

1.6.1 Overview

PC12, a neuroendocrine cell line derived from a rat pheochromocytoma that can undergo differentiation by NGF into neuronal-like cells, has been widely used in neurobiology to mimic neuronal characteristics and has been instrumental in enhancing our understanding of several molecular mechanisms specific to nerve cells.

Most of the studies using PC12 cells as a model for neurons have not investigated their cell membrane phospholipid FA composition before and after NGF-differentiation and have not examined their endogenous PUFA biosynthetic pathway and the relative effect of NGF-differentiation. Moreover, regulation of PUFA biosynthesis in neural cells by neural growth factors and dietary FAs has, to our knowledge, never been studied before and it is of major importance in understanding the features of neuronal phenotype acquisition.

1.6.2 Aims

The specific aims of this research programme are as follows:

1. Profiling PUFA and other FA compositions found in EPG, CPG and SPG PLs before and after PC12 cell differentiation.

2. Profiling PUFA and other FA content of EPG, CPG and SPG PLs in PC12 cells before and following essential fatty acid precursors (i.e., LA and ALA) as well as other long-chain n-3 and n-6 family supplementations.

3. Examining the kinetics of the PUFA elongation/desaturation pathway in producing endogenous intermediates under competitive and non-competitive conditions.

4. Examining the kinetics of the PUFA elongation/desaturation pathway in producing endogenous intermediates before and after differentiation with NGF.

5. Examining the expression of several gene transcripts associated with the PUFA elongation/desaturation pathway after NGF and FA supplements.

Chapter 2
Materials and Methods

2.1 Technical devices

Please refer to Appendix 1.

2.2 Materials

2.2.1 Chemicals

Please refer to Appendix 2.

2.2.2 Buffers

2.2.2.1 50X TAE

242 g Tris hydroxymethylaminoethane
57.1 ml Glacial Acetic Acid
100 ml 0.5 M Ethylenediamine tetraacetic acid (EDTA)
Bring final volume to 1 L with ddH_2O

2.2.2.2 0.5 M EDTA

146.125 g EDTA
1000 ml ddH_2O
Add 10 M Sodium hydroxide (NaOH), until the pH reaches 8.

2.2.2.3 1X TAE

20 ml from 50X TAE
980 ml ddH_2O

2.2.2.4 Agarose 1%

1 g Agarose
100 ml 1X TAE
2.5 µl of ethidium bromide (EtBr)

2.2.2.5 6X DNA/RNA loading buffer

30% Glycerol
1X TAE
Bromophenol blue

2.2.2.6 1X loading buffer

6X loading buffer
1X TAE

2.3 Cell culture

All experiments were carried out with a rat adrenal pheochromocytoma cell line, PC12 cells. The PC12 cell line was obtained from Dr G Guroff, NIH who sub-cloned it from the original cell line generated by Dr D Schubert (Salk Institute, San Diego).

2.3.1 Cell growth medium

The growth medium consists of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 2 mM L-glutamine (Gln, PAA), 100 units/µg penicillin/streptomycin (Pen/Str, PAA), 10% horse serum (HS, Sigma-Aldrich), and 5% foetal bovine serum (FBS, PAA) and stored at 4°C as detailled by Schonfeld et al., 2007.

2.3.2 Maintaining cell lines

PC12 cells were seeded in 25 cm^2 flasks (Nunc) pre-coated with poly-L-lysine (PLL, 50 µg/ml, Sigma-Aldrich). PC12 cells were maintained in 80% growth medium and 20% conditioned medium at 37°C in a humidified atmosphere of 5% CO_2 (Incubator Heraeus CO_2-Auto-Zero, Thermo Electron Corporation). Conditioned medium consists of spent media harvested from cultured cells and

filtered through a 0.22 µm membrane (VWR). PC12 cells were split, depending on confluence every 3 to 5 days. Briefly, cells were incubated with 0.05% (v/v) trypsin/EDTA (PAA) for 5 min at 37°C with 5% CO_2; the cells were detached and growth medium was added to inactivate the trypsin. The trypsin solution was removed by centrifugation (Eppendorf microfuge) at 1000 rpm for 3 min. Cell pellets were resupended in the appropriate growth medium-conditioned medium volume and seeded at the desired concentration into new culture flasks.

2.3.3 Freezing of cells

PC12 cells grown to almost 80% confluence were first trypsinised to bring them into suspension. Suspended cells were washed with growth medium and the trypsin solution was removed by centrifugation at 1000 rpm for 3 min. Cells were resuspended in the freeze mix (90% FBS (v/v) + 10% dimethyl sulfoxide minimum, DMSO (v/v), Sigma-Aldrich) and transferred into cryo-vials (Fisher Scientific). The cryo-vials were frozen at -80°C in special cryo- boxes (Nalgene), which ensure a temperature decrease of 1°C per minute. The frozen cryo-vials were transferred to liquid nitrogen cell storage tanks (Arpege 70) after 24 hours for long-term storage.

2.3.4 Thawing of cells

To defrost cells, cryo-vials were removed from liquid nitrogen and immediately thawed in a water bath at 37°C (Grant). The cell mixture was transferred to a

15 ml centrifuge tube (Fisher Scientific) containing 5 ml of fresh growth medium. Cells were centrifuged at 1000 rpm for 5 min. To remove the DMSO, the medium was discarded and the sedimented cells were resuspended in fresh growth medium. The cells were then placed into 25 cm^2 flasks (flasks of the same size as had been used prior to freezing) and incubated at 37°C with 5% CO_2.

2.4 Polyunsaturated fatty acid (PUFA) supplementations

2.4.1 FA-foetal bovine serum solution

FAs were dissolved in 100% ethanol (Hayman) at a concentration of 100 mM. Then, this FA solution was diluted in FBS to achieve a final concentration of 10 mM. The FA mixture was incubated at 37°C for 30 minutes in order to allow the binding of FAs to the FBS.

2.4.2 Medium for FA treatments

The medium for FA treatments consists of DMEM supplemented with 2 mM L-glutamine and 100 units/μg penicillin/streptomycin.

2.4.3 PUFA supplement procedure

For preparing PUFA supplements, PC12 cells were seeded in 25 cm^2 flasks pre-coated with PLL (50 μg/ml) and allowed to divide for 24 hours. The growth medium was changed after 1 day to DMEM containing 1% FBS and either

supplemented or not with different concentrations of various types of FA (Sigma-Aldrich), such as alpha-linolenic acid (ALA, 18:3n-3); linoleic acid (LA, 18:2n-6); eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) or arachidonic acid (ARA, 20:4n-6) in the absence or presence of nerve growth factor (NGF, 100 ng/ml, Alomone Labs) or all-*trans*-retinoic acid (ATRA, 1 or 10 μM, Sigma-Aldrich) for designated time periods, at 37°C in a humidified atmosphere of 5% CO_2.

2.5 Lipid analysis

2.5.1 Lipid extraction

Briefly, at the end of the incubation, cellular lipids were extracted and subjected to FA analysis using combined thin layer chromatography (TLC, LAMAG)/gas chromatography (GC, HRGC MEGA Series 2-Fisons, Instruments, Italy). Medium was removed and the cell monolayer was washed twice with Dulbecco's phosphate buffered saline (PBS, Sigma-Aldrich). In order to collect plasma membrane total lipids, PBS was removed, flasks were placed on ice and cells were washed and then subjected to 3 ml of lipid extraction solvent, hexane/isopropanol (3/2 by vol., Fischer Scientific) containing 0.05% butylated hydroxy toluene (BHT, Sigma-Aldrich), as described previously by Hara and Radin, 1978.

The organic layer was collected after 10 min in an Eppendorf microfuge and cells were rinsed again with a similar solvent mixture. The combined lipid

extract was dried under a stream of nitrogen (Techne Dri-Block DB3), and then redissolved in 1.8 ml of chloroform/methanol (2/1 by vol.) containing 0.05% BHT. The lipid extract was subjected to a washing procedure of Folch's extraction (Folch et al., 1957). The lipid extract in 1.8 ml of chloroform/methanol (2/1 by vol.) was washed with 0.6 ml of water by vortexing, and centrifugation at 4000 rpm for approximately 1 min at 4°C.

After centrifugation, a clear separation of lipid and non-lipid components appears. The aqueous phase (upper layer) is removed, and the organic phase (bottom layer) that contains the lipids is transferred into another clean tube.

2.5.2 Thin layer chromatography

The separation is based on the principle that different components of total lipids have diverse affinities to stationary and mobile phases.

The total cell lipid extract in chloroform/methanol (2/1 by vol.) was applied onto 10x20 cm pre-activated silica gel G plates (MERCK) heated at 100°C for 1 hour. The thin layer of silica gel is the stationary phase. The mobile phase is a mixture of solvents chloroform/methanol/40% methylamine (65/35/15, v/v/v by vol.) containing 0.01% BHT. Plates were placed in the equilibrated tank for about 20 minutes until the solvent reached 1 cm from the top of the plate. Plates were removed from the tank, dried and glycerophospholipid bands were detected by spraying them with a solution of 2',7'-dichlorofluorescein (0.1%

w/v) (Sigma-Aldrich) in methanol and they were visualised under ultraviolet light (533 nm) (LAMAG).

The order of glycerophospholipid fraction separations from the origin is shown in the photograph below (Figure 2.1) and consists (starting from the origin) of inositol phosphoglycerides (IPG), serine phosphoglycerides (SPG), sphingomyelin (SM), choline phosphoglycerides (CPG) and ethanolamine phosphoglycerides (EPG).

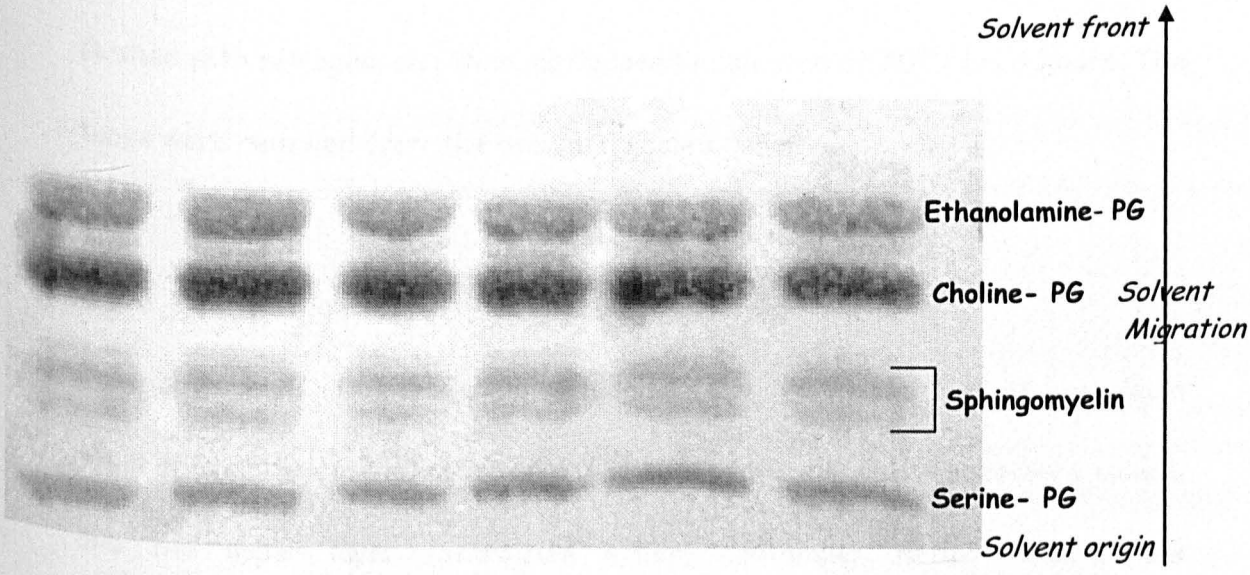


Figure 2.1: A representative image of a typical one-dimentional TLC analysis of PC12 cell glycerophospholipids: Total lipid extract of PC12 cells were loaded onto the TLC plate. GLycerophospholipids fractions were separated by chloroform/methanol/methylamine (65:30:15, v/v/v), stained with solution of 2',7'-dichlorofluorescein (0.1% w/v) in methanol and visualised under UV light (533 nm).

PL quantification as detailed previously by Dittmer and Lester (1935)

Alternatively, in order to quantify the amounts of each GPL after TLC separation, the TLC plate was stained with ammonium molybdate solution (Sigma) instead of 2',7'- dichlorofluorescein. Molybdate stains only the lipid-

containing phosphorus (Pi). The plate was photographed immediately thereafter; the color intensity was recorded and measured by densitometry.

2.5.3 Fatty acid methyl ester (FAME) procedure

2.5.3.1 Trans-methylation of fatty acids

Glycerophospholipid fractions were scraped from TLC plates and transferred directly into methylating tubes (Corning) with 1 ml of freshly prepared methylating reagent (15% acetyl chloride in dry methanol v/v). Tubes were flushed with nitrogen, and then methylated in an oven at 70°C for 3 hours. The tubes were removed from the oven and cooled down.

2.5.3.2 Extraction of fatty acid methyl esters

To each tube, 1 ml of 5% sodium chloride (w/v, VWR) and 1 ml of petroleum ether (Fisher Scientific) containing 0.01 % BHT were added. Tubes were mixed, and the upper layer (petroleum ether) containing methyl esters, was transferred into a new tube containing 0.5 ml of 2% potassium bicarbonate (w/v) to neutralise the acidity. 1 ml of petroleum ether was again added to the original tube and mixed vigorously. FAMEs in 1ml of petroleum were transferred to the tube containing 2% potassium bicarbonate. FAMEs in 2 ml of petroleum ether were removed from the tube containing 2% potassium bicarbonate (Fisher Scientific) and dried with 100 mg granular sodium sulphate (Acros Organics). The FAMEs in petroleum ether were eventually transferred to a 3.5

ml vial and dried down at 37°C under a stream of nitrogen. The resulting FAMEs were redissolved in 0.5 ml of heptane (Fisher Scientific) containing 0.01% BHT and stored at -20°C until analysis.

2.5.4 Gas chromatography

FAMEs were separated by a gas chromatograph (GC, HRGC MEGA 2 Series, Fisons Instruments, Italy) fitted with a capillary column (BPX70 column, SGE, forte, 0.25 μm diameter) connected to a flame ionisation detector. Hydrogen was used as a carrier gas at flow rate of 1 ml/minute. The injector, oven, and detector temperatures were set at 250, 200, and 280 °C, respectively. The FAMEs were identified by comparing their retention times with those of a mixture of individual FA standards (Appendix 1 and 2). Peak areas were calculated using data acquisition software from the EZChrom chromatography data system (Agilent EZ Chrom Elite chromatography Data System version 3.2, Scientific Software, Inc., Pleasanton, CA). The percentage area of a particular FAME was calculated as shown below:

Percentage area of a particular FAME = (Area of a particular FAME /Total area of FAMEs) x 100.

2.5.5 Data analysis

My protocol was validated with C15:0 and C17:0 standards. The molar concentration of each individual FA has been determined by injecting C15:0 and

C17:0 standards. The values were similar to the percentage of FA. In the thesis, the values will be referred according to the percentage of FA. Data are presented as an average of %FA ± standard error of the mean (SEM) of triplicate or more experiments.

2.6 Gene expression

2.6.1 RNA extraction

Total RNA was extracted from non-treated and treated PC12 cell samples following the protocol of the RNeasy purification kit (Qiagen) according to the manufacturer's instructions, as described previously by Butovsky et al. (2005). Medium was removed and the cell monolayer was washed twice with PBS followed by lysis with 1 ml of RLT buffer. The resulting lysate was transferred to a QIAshredder spin column attached to a 2ml collection tube that was subjected to serial centrifugations (2 times for 2 min at 10,000 rpm). Subsequently, 1 ml of 70% ethanol per 1ml RLT buffer was added to each cell homogenate. The samples were vigorously mixed, transferred to RNeasy spin columns in a 2ml collection tube and centrifuged 3 times for 15 s at 10,000 rpm. The flow-through was discarded and the RNA samples were adsorbed on the silica gel membrane of the RNeasy spin column. Unwanted cellular contents such as proteins and DNA were washed away by consecutive buffers and centrifugations. 700 µl of buffer RW1 was added to the column, the column was spinned, and the flow-through was discarded. 500 µl of buffer RPE was added

to the column, the column was spinned as before, and the flow-through was discarded. The RNeasy spin column was briefly air dried in order to remove all traces of ethanol. 30 µl RNase-free water (Sigma) was added onto the centre of the silica membrane and the RNA was eluted from the columns.

The quantity and purity of total RNA extracted from each sample were assessed by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The relative amounts of RNA were determined by measuring the absorbance at 260 nm. The RNA purity was provided by measuring the optical density ratio at 260/280 nm (it indicates contaminants such as proteins) and 260/230 nm (it shows contaminants such as chaotropic salts). Additionally, the integrity of the RNA was checked by separating the RNA on an ethidium bromide (EtBr, Sigma-Aldrich) /gel electrophoresis as described in section 2.3.8. A representative image of the gel is shown in Appendix 3.

2.6.2 Conversion of RNA into cDNA

Total RNA (2 µg) was converted to single-stranded cDNA following the protocol of the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) using a reaction volume of 20 µl, as indicated in the manufacturer's protocol. 1ul oligo-dT primer (Invitrogen) was added to the RNA samples (in a volume to reach 2ug) and the volume was adjusted to 10 µl with Nuclease-free water. RNA

samples were denaturated at 75°C for 5 min and subsequently cooled on ice for 1 min. Then, 10 ul of the following Master Mix was added to each RNA sample.

Reverse transcription (RT) Master Mix composition:

5X Reaction Buffer	4 µl
10mM dNTP Mix	2 µl
Nuclease-free water	2.5 µl
RiboLock™ RNase Inhibitor	0.5 µl
RevertAid™ M-MuLV Reverse Transcriptase	1 µl

Each sample (20 µl) was incubated at 42°C for 1h followed by 75°C for 10 min.

Summary of the thermal cycle conditions:

	Temperature (°C)	Time (min)
	RNA	
Step 1	75	5
Step 2	ICE	1
	RNA + Master Mix	
Step 3	42	60

Step 4	75	10
Step 5	4	∞

To verify complete removal of genomic DNA, the RNA samples were subjected to mock reverse transcription (in the absence of reverse transcriptase), and were then run in a quantitative Real-time polymerase chain reaction (qRT-PCR).

2.6.3 Real-time quantitative polymerase chain reaction (qRT-PCR)

Synthesised cDNA was diluted 80 times in nuclease-free water. The diluted cDNA was mixed with ABsolute Blue QPCR SYBR Green ROX Mix (Thermo Scientific) and four sets of gene-specific forward and reverse primers (Integrated DNA Technologies) in 0.1 ml strip tubes (Gene Target Solutions). The samples were subjected to qRT-PCR using the Rotor-Gene 6000 (Corbett Research). For each set of primers a no template control (the total mix without cDNA) was run. In order to obtain the dilution curves, 5 different concentrations of cDNA were tested and PCR amplification efficiencies were calculated for each primer reaction.

The primers used for qRT-PCR are listed below (Wang et al., 2006 and Tu et al., 2010):

Beta Actin fwd, 5'-ACT ATT GGC AAC GAG CGG TT-3'

Beta Actin rev, 5'-TGT CAG CAA TGC CTG GGT ACA-3'

Elovl2 fwd, 5'-TTT GGC TGT CTC ATC TTC CA-3'

Elovl2 rev, 5'-GGG AAA CCG TTC TTC ACT TC-3'

Elovl5 fwd, 5'-TAC CAC CAT GCC ACT ATG CT-3'

Elovl5 rev, 5'-GAC GTG GAT GAA GCT GTT GA-3'

Fads2 fwd, 5'-TGT CCA CAA GTT TGT CAT TGG-3'

Fads2 rev, 5'-ACA CGT GCA GGC TCT TTA TG-3'

Ppara fwd, 5'-CCT GTG AAC ACG ATC TGA AAG-3'

Ppara rev, 5'-ACA AAA GGC GGA TTG TTG -3'

The reactions were set in a volume of 15 μl as follows:

3 μl cDNA (diluted 1/80)

7.5 μl 2X ABsolute Blue QPCR SYBR Green ROX Mix

0.75 μl of 10 μM Forward+Reverse Primers

3.75 μl of Nuclease-free water

Thermal cycling conditions were set as follows:

	Temperature (°C)	Time	
Step 1	95	15 min	
Step 2	94	15 s	45 cycles
	60	30 s	
	72	30 s	
Step 3	4	∞	

All reactions were performed in triplicate.

PCR products were detected on an ethidium bromide/gel electrophoresis apparatus (as described in section 2.3.8) and were shown to have the expected amplicon size. A representative image of the gel is shown in Appendix 4.

2.6.4 Data analysis

Fluorescence was measured at the end of each PCR cycle. An amplification curve (fluorescence versus the number of cycles) was generated and a threshold cycle value, C_t was calculated from the exponential phase (C_t is defined as the number of cycles required for the fluorescent signal to exceed the threshold or background level). The relative amounts or expressions of

genes were calculated using the Rotor-Gene 6 software. This software allows to generate a standard curve for each gene (C_t versus the log DNA concentration) and the calculation of each gene concentration present in each unknown sample. For each sample, the ratio of the target gene concentration to the housekeeping gene concentration was calculated. Beta actin was used as an internal standard (housekeeping gene), and all the results were normalized to the expression levels of the beta actin gene.

2.6.5 RNA/DNA gel electrophoresis

Gel Casting

The gel was prepared by dissolving 1.5 g of agarose in 100 ml of 1X TAE in a microwave. Ethidium bromide (10 mg/ml) was added to the melted agarose.

The gel was poured into a gel cassette to a depth of 0.5 cm and a comb was inserted in order to create wells where the samples were loaded.

The gel solidified and the comb was removed. The gel was placed in the electrophoresis apparatus and was completely covered with 1X TAE.

Sample loading on the gel

Briefly, 10 μl of DNA or 5 μl of RNA samples were mixed with 2 or 1 μl of 6X loading buffer. Each mixture and a DNA-ladder marker were loaded in each well. The gel was run at 80 V, until the bromophenol blue reached the end of the gel. Bands corresponding to RNA or DNA (PCR products) were visualised

under UV light (UVP Bioimaging Systems, UK). PCR products were identified by their size compared to the DNA-ladder marker.

2.7 Data analysis

Statistical analysis for lipid analysis and molecular biology data were performed by the unpaired t-test for repeated measures (average of 3 or more independent experiments) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). $P<0.05$ was considered statistically significant.

Chapter 3

Metabolic conversion of ALA and reversal of n-3 PUFA-deficient status in PC12 cells

3.1 Introduction

Much work has been conducted on neural-derived clonal cell lines to examine aspects of PUFA biosynthesis and its consequences on cell function (Murphy and Horrocks, 1993; Langelier et al., 2005; Martin et al., 2006; Kan et al.; 2007). PC12, a neuroendocrine cell line derived from a pheochromocytoma of rat adrenal medulla (Greene and Tischler, 1976), has been extensively utilized as a model for a variety of neuronal functions. PC12 cells are easily cultured and can be differentiated into a neuronal phenotype using nerve growth factor (NGF). In the presence of NGF, the proliferating cells stop dividing and develop properties related to neurons by promoting neurite outgrowth and inducing a number of neuronal markers (Vaudry et al., 2002). Thus, PC12 cells have often been used as an *in vitro* system for neural cells in order to study biophysical, biochemical, physiological (Williams and McGee, 1981) and neurotoxicological (Mundy et al., 2010) responses. Specifically, they have been used to study calcium homeostasis, neurotransmitter synthesis and exocytosis (Westerink and Ewing, 2008; Williams and McGee, 1981) as well as neuronal receptor functions (Sak and Illes, 2005). These cells have also been used to study aspects of PUFA biosynthesis (Marszalek et al., 2005), as well as the effects of DHA supplements on cell function (Williams et al., 1982; Dagai et al., 2009; Ikemoto et al., 1997; Kawashima et al., 2010). Since these events might involve cell membrane properties, we reasoned that it is necessary to assess the lipid composition of these cultured cells in comparison with that of the normal brain.

Only a few and mostly early studies have attempted to analyse the membrane lipid content of primary neurons in cultured cells from the brain (Yavin and Menkes, 1974; Bourre et al., 1983; Moore et al., 1991); usually the population from a dissociated culture brain is heterogeneous (neurons and other non-neuronal cells) and is also short-lived.

We have little information until now about the lipid composition of PC12 cells. The FA composition of total phospholipids (Ikemoto et al., 1997; Williams and McGee, 1981) and individual phospholipids (Traynor et al., 1982) has been studied briefly in PC12 cells. It was originally found that they contain only limited amounts of DHA (Williams and McGee, 1982), in contrast to neuronal cells, which possess a well-diversified lipid profile enriched in DHA and ARA (Murphy et al., 1993).

One obvious reason for this discrepancy is the inadequate level of nutritional components, particularly essential fatty acids, during long-term propagation *in vitro*. A second reason could be the lack of external signals associated with cell differentiation. Both circumstances may lead to altered membrane lipid profiles and impaired terminal differentiation (Chung et al., 2010).

Therefore, the mere characterisation of the FA phospholipid's composition in PC12 cells should first provide information on basal differences and similarities between this cell line and the original nervous tissue.

Furthermore, the addition of PUFAs may supply an indication of the utilisation rates of these FAs for the building up of "neuron-like" membrane phospholipids in view of the possible deficiencies noted above.

Objectives

The objectives of this series of experiments employing lipidomic techniques were as follows:

- To analyse the baseline fatty acid (FA) composition of ethanolamine, choline and serine phosphoglycerides in PC12 cells
- To analyse the effects of PUFA supplements on FA levels in PC12 cells
- To establish a lipid enrichment combination to approximate the FA profiles of cellular membranes from the PC12 cell line to the values observed in neurons

3.2 PC12 cells are deficient in n-3 PUFA

3.2.1 Procedures

To investigate the FA composition, PC12 cells were seeded in 25 cm^2 flasks and incubated at 37°C under 5% CO_2 humidified conditions for 24 h. After one day, the growth medium (15% serum) was removed,the cells were rinsed in DMEM and the cells were incubated for three days in DMEM supplemented with 1% foetal bovine serum (FBS) in the absence of any additional FA. After incubation, medium was removed and cellular lipids were extracted with hexane/isopropanol (3/2 by vol.) and subsequently subjected to TLC for major

GPL separation. FAMEs in different GPLs were analysed by GC as described in section 2.5.

3.2.2 Results

Table 3.1 depicts the typical profile of an FA composition determined in the three major classes of GPLs, namely, ethanolamine phosphoglycerides (EPGs), choline phosphoglycerides (CPGs) and serine phosphoglycerides (SPGs) in proliferating (no NGF) cells. These three lipid classes constituted the bulk of GPLs (78.5%) as established by determining the total lipid phosphorus (Pi) content. The relative distributions of the GPL classes were measured by densitometry, as described in Materials and Methods (section 2.5.2); they reached values of 26.3%±1.21; 43.3%±2.65; 8.9%±0.35 and 10.1%±0.4 for the EPG, CPG, SPG and IPG classes, respectively.

Substantial differences in the FA composition have been noted among the GPL classes: within the saturated FA species, palmitate (C16) and stearate (C18) were the major components; they appeared in variable amounts depending on the polar head group. The variability in monounsaturated FAs was also distinct, with oleic acid (18:1n-9, OA) being the major FA in all three GPL species.

The first and most notable fact is that the total PUFA content in EPG is 23.51%, which is more than 4-fold higher than that of CPG (5.41%) and nearly 1.4-fold greater than SPG, a relatively minor cellular GPL (9% of the total lipid Pi). Of the total FAME, profoundly low levels (4.44%±0.17; 0.97%±0.04 and

5.81%±0.63) of n-3 PUFA in the EPG, CPG and SPG classes, respectively, were noted. Of these, DHA (22:6n-3) comprised the major part, accounting for 3.08%±0.07, 0.22%±0.01 and 3.39%±0.55 of the total FAME, respectively. Within the n-6 family, ARA (20:4n-6) was fairly high in EPG (14.89%±0.2) but was rather low in CPG (1.55%±0.07) and SPG (3.4%%±0.17).

The low levels of DHA in SPG, a GPL important for signal transduction during neuronal development, are particularly conspicuous. Overall, these values are by far lower when compared with neurons and glia cells isolated from the CNS (Kawashima and al., 2010; Bourre et al., 1984). The overall deficiency of n-3 PUFA in the EPG of the PC12 cells is slightly compensated by an increase in Mead acid (20:3n-9) (5.96±0.42%), the final elongation product of OA (18:1n-9) and a typical marker for n-3 deficiency.

The levels of 20:3n-9 are an indicator that the PC12 cells grew under conditions characterized by a marked PUFA deficiency (Chung et al., 2010). We reasoned that this deficiency may be in part related to the lack of ALA in contrast to exceedingly high levels of LA (>40-fold) in the growth medium (see the appendix 5), or to impaired biosynthesis of n-3 PUFAs.

Table 3.1: Major FAME in EPGs, CPGs and SPGs of proliferating PC12 cells. PC12 cells were grown for 3 days in growth medium. After 3 days, the FAME components in different GPLs was determined. The values, expressed as the % FAMEs, are averages ± SEM of triplicates of triplicate cultures of three independent experiments.

	Phospholipid classes		
	EPG	CPG	SPG
% lipid phosphorus	26.3%±1.21	43.3%±2.65	8.9%±0.35
	% mean FA ± SEM	% mean FA ± SEM	% mean FA ± SEM
FA	Saturated fatty acids		
16:0	8.0 ± 0.32	16.43 ± 4.1	4.17 ± 0.16
18:0	13.07 ± 0.28	8.74 ± 1.68	36.83 ± 0.66
20:0	0.61 ± 0.03	0.49 ± 0.06	1.05 ± 0.07
22:0	0.25 ± 0.02	0.26 ± 0.02	1.28 ± 0.03
24:0	0.22 ± 0.02	0.23 ± 0.01	0.83 ± 0.07
	Plasmalogen species		
16:0 DMA	2.77 ± 0.07	0.27 ± 0.03	<0.1
18:0 DMA	5.38 ± 0.38	0.55 ± 0.03	0.13±0.05
18:1 DMA	1.68 ± 0.09	0.02 ± 0.002	<0.1
	Monounsaturated fatty acids (and Mead acid)		
16:1n-7	1.47 ± 0.21	5.39 ± 0.45	0.87 ±0.07
18:1n-7	7.51 ± 0.61	14.96 ± 0.57	8.62 ± 0.17
18:1n-9	23.96 ± 0.49	38.02 ± 0.46	22.52 ± 0.63
20:1n-9	2.69 ± 0.10	3.02 ± 0.12	2.63 ± 0.12
20:3n-9	5.96 ± 0.42	0.94 ±0.06	1.58 ± 0.41
22:1n-9	0.53 ± 0.07	0.3 ±0.001	<0.1
24:1n-9	0.16±0.01	0.18 ±0.003	0.46 ±0.01
	n-6 and n-3 PUFA s		
18:2n-6	2.63 ± 0.57	2.78 ± 0.11	2.57 ± 0.07
20:2n-6	0.03 ± 0.01	0.09 ± 0.01	0.06 ± 0.01
20:3n-6	0.99 ± 0.03	0.57 ± 0.003	4.49 ± 0.06
20:4n-6	14.89± 0.2	1.55 ± 0.07	3.4 ± 0.17
22:4n-6	0.99 ± 0.03	0.07 ± 0.01	1.03 ± 0.17
22:5n-6	0.56 ± 0.03	0.04 ± 0.002	0.63 ± 0.11
Σ n-3 PUFA	4.44%±0.17	0.97%±0.04	5.81%±0.63
20:5n-3	0.5 ± 0.08	0.68 ± 0.04	1.16 ± 0.14
22:5n-3	0.86 ± 0.07	0.07 ± 0.01	1.26 ± 0.1
22:6n-3	3.08 ± 0.07	0.22 ± 0.01	3.39 ± 0.55

Hence, it appears that a typical proliferating PC12 cell has lower levels of endogenous n-3 PUFA species in its membrane, which renders it unsuitable for studying many neuronal characteristics that require a proper PUFA profile. Therefore, modifying the membrane composition by supplementing the culture medium with PUFAs appears to be essential when studying physiological and pharmacological responses of PC12 cells.

3.3 The effects of FA supplementations on FA levels in PC12 cells

3.3.1 Procedures

PC12 cells were grown for 1 day in growth medium containing 10% HS and 5% FBS supplements. After one day, the growth medium was removed and after being rinsed in DMEM, 10 μM of LA, ALA, ARA or DHA in serum-deprived (1% FBS) medium was added (as described in section 2.4.3). After 3 days, the medium was removed and cellular lipids were extracted with hexane/isopropanol (3/2 by vol) and subsequently subjected to one dimentional TLC for major GPL separation followed by GC analysis of the individual FAME in different GPL classes as indicated in Materials and Methods (section 2.5.2).

3.3.2 Results

As shown in Table 3.2, there were little or no changes in the mass GPL composition in the presence of the FA supplements. Following ALA or DHA supplements, however, an overall increase of 2.9- and 2.4-fold, respectively,

was noted in n-3 PUFA of the EPG class. At this time, n-6 PUFA slightly increased (1.14- and 1.18-fold, respectively), whereas that of MUFA was reduced by about 30% compared with non-supplemented cells. A similar trend in n-3 PUFA elevation was noted in CPG and SPG after ALA or direct DHA supplementation, in contrast with a decrease in MUFA content. Table 3.2 also shows that the n-6 PUFA profile of all three GPL species increased after 3 days of supplement with LA (1.6-, 2.7- and 1.3-fold for EPG, CPG and SPG, respectively). This was accompanied by a reduced MUFA level. The amount of n-6 PUFA in the three major GPL species also increased after 3 days of administering ARA (1.4-, 1.8- and 1.3-fold for EPG, CPG and SPG, respectively). Also notable in Table 3.2 is an overall decrease (between 20 and 40%) in n-9 PUFA (Mead acid) following these FA supplements. Thus, it appears that given appropriate FAs, PC12 cells can reshuffle the FA profile towards an unsaturated status. To further examine in detail the dynamic changes in the n-6 PUFA and n-3 PUFA cellular profile, ALA and LA were added to cells at increasing concentrations.

Table 3.2: Changes in the FAME composition regarding EPGs, CPGs and SPGs in PC12 cells after various supplements. PC12 cells were treated for 72 hours with 10 µM of LA, ALA, ARA or DHA. The entire (98.5%) FAME profile of either the EPG, CPG or SPG classes, expressed as the % FAMEs, are averages ± SEM of triplicates. The experiment was repeated three times. * ($p<0.05$) indicates a significant difference for non-supplemented cells. NS indicates no significant difference compared with non-supplemented cells.

Suppl.	Control	ALA	DHA	LA	ARA
%lipid phosphorus	24.7±1.6 ^{ns}	26.2±0.9 ^{ns}	23.9±2.1 ^{ns}	25.3±1.8 ^{ns}	27.2±2.1 ^{ns}
	%FA ± SEM	%FA ± SEM	%FA ± SEM	%FA ± SEM	%FA ± SEM
	EPG				
	Saturated fatty acids				
16:0	8.0 ± 0.32	9.69 ± 1.74	7.86 ± 1.71	8.95 ± 1.58	9.53 ± 1.87
18:0	13.07 ± 0.28	14.24 ± 0.76	17.47 ± 2.91	14.50 ± 1.08	14.90 ± 1.51
20:0	0.61 ± 0.03	0.47 ± 0.03	0.60 ± 0.07	0.50 ± 0.05	0.52 ± 0.04
22:0	0.25 ± 0.02	0.22 ± 0.02	0.33 ± 0.02	0.30 ± 0.03	0.31 ± 0.02
SAFA	22.16 ± 0.79	25.21±1.31 ^{ns}	26.63±3.0 *	25.28±0.83 ^{ns}	28.58±1.74 *
	Plasmalogen species				
16:0 DMA	2.77 ± 0.07	2.75 ± 0.36	2.11 ± 0.44	2.23 ± 0.18	2.58 ± 0.50
18:0 DMA	5.38 ± 0.38	4.67 ± 0.55	4.46 ± 0.69	4.15 ± 0.32	4.17 ± 0.72
18:1 DMA	1.68 ± 0.09	1.26 ± 0.13	1.38 ± 0.27	1.18 ± 0.09	1.26 ± 0.22
DMA	9.83±0.38	8.5±0.75 ^{ns}	8.58±1.51 ^{ns}	7.84±0.87 *	7.59±1.33 *
	Monounsaturated fatty acids				
16:1n-7	1.47 ± 0.21	0.37 ± 0.05	0.28 ± 0.07	0.44 ± 0.18	0.39 ± 0.07
18:1n-7	7.51 ± 0.61	3.62 ± 0.32	3.56 ± 0.28	3.74 ± 0.28	3.91 ± 0.19
18:1n-9	23.96 ± 0.49	19.61 ± 1.38	18.41 ± 1.61	20.19 ± 1.22	20.57± 1.74
20:1n-9	2.69 ± 0.10	1.72 ± 0.25	1.9 ± 0.22	1.83 ± 0.25	2.16 ± 0.19
MUFA	35.63±0.35	25.33±1.14 *	24.15±1.87 *	26.2±0.78 *	25.19±1.31 *
n-9 PUFA	5.96 ± 0.42	2.35 ± 0.25 *	2.8±0.21 *	2.3±0.39 *	3.35±0.19 *
	n-6 and n-3 PUFAs				
18:2n-6	2.63 ± 0.57	3.9±0.63	2.77±0.87	6±0.74	4.67±1.35
20:3n-6	0.99 ± 0.03	1.48 ± 0.14	1.26 ± 0.17	1.61 ± 0.33	1.29 ± 0.14
20:4n-6	14.89 ± 0.2	15.01 ± 2.21	16.41 ± 0.26	19.32 ± 1.37	18.4 ± 2.12
22:4n-6	0.99 ± 0.03	1.22 ± 0.24	1.63 ± 0.12	2.43 ± 0.39	1.87 ± 0.47
22:5n-6	0.56 ± 0.03	0.64 ± 0.14	1.06 ± 0.02	1.81 ± 0.46	1.08 ± 0.29
n-6 PUFA	19.07 ± 0.55	22.26±0.67 ^{ns}	23.12±0.77 *	31.18±0.66 *	26.39±1.61 *
20:5n-3	0.5 ± 0.08	0.76 ± 0.19	0.04 ± 0.01	0.02 ± 0.01	0.08 ± 0.01
22:5n-3	0.86 ± 0.07	3.97 ± 0.29	0.85 ± 0.13	0.88 ± 0.11	1.10 ± 0.12
22:6n-3	3.08 ± 0.07	8.11 ± 1.02	9.61 ± 0.1	3.84 ± 0.36	3.94 ± 0.56
n-3 PUFA	4.44 ± 0.17	12.84±0.66 *	10.86±0.07 *	5.17±0.38 ^{ns}	5.38±0.53 ^{ns}
	CPG				
	Saturated fatty acids				
16:0	16.43 ± 4.1	24.35 ± 0.94	25.06 ± 2.06	22.85 ± 0.86	26.50 ± 0.83
18:0	8.74 ± 1.68	8.17 ± 1.13	9.72 ± 0.13	8.26 ± 0.94	9.16 ± 0.16
20:0	0.49 ± 0.06	0.54 ± 0.07	0.60 ± 0.06	0.53 ± 0.07	0.60 ± 0.01
22:0	0.26 ± 0.02	0.32 ± 0.06	0.35 ± 0.06	0.32 ± 0.04	0.34 ± 0.02
SAFA	25.17 ± 2.34	33.03±1.06 *	36.1±2.06 *	31.24±1.58 *	37.02±1.02 *
	Plasmalogen species				
16:0 DMA	0.27 ± 0.03	0.26 ± 0.03	0.22 ± 0.02	0.18 ± 0.02	0.24 ± 0.10
18:0 DMA	0.55 ± 0.03	0.32 ± 0.1	0.06 ± 0.00	0.22 ± 0.08	0.09 ± 0.06

18:1 DMA	0.02 ± 0.002	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.06 ± 0.02
DMA	0.84±0.02	0.7±0.10*	0.34±0.01*	0.47±0.06*	0.41±0.18*
		Monounsaturated fatty acids			
16:1n-7	5.39 ± 0.45	4.64 ± 1.41	0.63 ± 0.18	3.61 ± 1.44	0.91 ± 0.09
18:1n-7	14.96 ± 0.57	13.18 ±1.23	9.77 ± 0.14	13.83 ± 1.68	9.35 ± 0.02
18:1n-9	38.02 ± 0.46	35.21 ± 0.9	36.07 ± 0.40	32.56 ± 1.32	35.3 ± 0.59
20:1n-9	3.02 ± 0.12	2.8 ± 0.19	2.91 ± 0.35	2.6 ± 0.23	2.89 ± 0.04
MUFA	61.39 ± 0.4	55.84±1.66ns	48.74±0.89*	52.61±1.94ns	48.46±0.73*
n-9 PUFA	0.94 ±0.06	0.58±0.05*	0.73±0.046*	0.58±0.05*	0.7±0.09*
		n-6 and n-3 PUFAs			
18:2n-6	2.78 ± 0.11	2.41±0.04	3.33±0.34	6.64±0.47	4.08±0.26
20:3n-6	0.57 ± 0.003	0.68 ± 0.11	1.14 ± 0.1	1.05 ± 0.1	1.01 ± 0.02
20:4n-6	1.55 ± 0.07	2.01 ± 0.24	3.39±0.08	4.1 ± 0.11	3.83 ± 0.12
22:4n-6	0.07 ± 0.01	0.07 ± 0.01	0.16 ± 0.02	0.23 ± 0.02	0.18 ± 0.02
22:5n-6	0.04 ± 0.002	0.04 ± 0.01	0.08 ± 0.02	0.16 ± 0.00	0.14 ± 0.02
n-6 PUFA	4.44 ± 0.08	5.22±0.08ns	8.1±0.46*	12.18±0.14*	9.23±0.14*
20:5n-3	0.68 ± 0.04	0.8 ± 0.23	0.03±0.01	0.43 ± 0.14	0.02 ± 0.01
22:5n-3	0.07 ± 0.01	0.49 ± 0.04	0.17 ± 0.02	0.12 ± 0.02	0.14 ± 0.00
22:6n-3	0.22 ± 0.01	0.73 ± 0.01	1.22 ± 0.18	0.4 ± 0.06	0.58 ± 0.00
n-3 PUFA	0.97 ± 0.04	2.1±0.29*	1.43±0.17*	0.95±0.07ns	0.75±0.01*
		SPG			
		Saturated fatty acids			
16:0	4.17 ± 0.16	9.47 ± 2.68	14.01 ± 0.83	13.09 ± 0.8	16.94 ± 1.05
18:0	36.83 ± 0.66	36.91 ± 2.01	32.66 ± 2.14	23.59 ± 4.22	31.76 ± 1.71
20:0	1.05 ± 0.07	0.92 ± 0.02	0.98 ± 0.15	1.13 ± 0.35	0.93 ± 0.11
22:0	1.28 ± 0.03	1.00 ± 0.12	0.91 ± 0.22	0.86 ± 0.27	0.84 ± 0.11
SAFA	41.14±0.46	47.64±1.62ns	49.08±1.68*	38.67±6.91ns	51.03±0.98*
		Plasmalogen species			
16:0 DMA		0.02 ± 0.00	0.01 ± 0.01	0.05 ± 0.02	0.01 ± 0.00
18:0 DMA	0.13±0.05	0.08 ± 0.01	0.15 ± 0.04	0.09 ± 0.02	0.12 ± 0.01
18:1 DMA		0.04 ± 0.03	0.07 ± 0.04	0.05 ± 0.02	0.05 ± 0.02
DMA	0.13±0.05	0.15±0.04*	0.23±0.11*	0.19±0.05*	0.18±0.04*
		Monounsaturated fatty acids			
16:1n-7	0.87 ±0.07	0.39 ± 0.18	0.06 ± 0.03	0.08 ± 0.04	0.11 ± 0.03
18:1n-7	8.62 ± 0.17	5.41 ± 0.94	3.26 ± 0.47	2.52 ± 0.32	3.25 ± 0.09
18:1n-9	22.52 ± 0.63	15.4 ± 0.72	14.42 ± 0.75	29.12 ± 6.7	14.96 ± 0.8
20:1n-9	2.63 ± 0.12	1.7 ± 0.16	1.16 ± 0.00	0.87 ± 0.04	1.3 ± 0.08
MUFA	34.64± 0.25	23.38±2.38*	18.89±1.26*	20.11±0.0*	19.62±0.66*
n-9 PUFA	1.58±0.41	0.49±0.09*	0.79±0.00*	0.21±0.18*	1.01±0.06*
		n-6 and n-3 PUFAs			
18:2n-6	2.57 ± 0.07	2.05 ± 0.18	1.9 ± 0.15	6.55 ± 0.88	2.21 ± 0.34
20:3n-6	4.49 ± 0.06	3.74 ± 0.27	3.46 ± 0.03	2.41 ± 1.05	4.12 ± 0.1
20:4n-6	3.4 ± 0.17	2.51 ± 0.34	3.52 ± 0.36	3.43 ± 0.54	5.37 ± 0.07
22:4n-6	1.03 ± 0.17	0.82 ± 0.07	0.88 ± 0.02	0.93 ± 0.1	1.63 ± 0.31
22:5n-6	0.63 ± 0.11	0.4 ± 0.07	0.6 ± 0.04	0.8 ± 0.00	1.22 ± 0.31
n-6 PUFA	11.50±0.56	9.53±0.19*	10.37±0.46*	14.58±1.11*	14.56±0.07*
20:5n-3	1.16 ± 0.14	0.7 ± 0.22	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.01
22:5n-3	1.26 ± 0.1	6.31±1.15	0.71 ± 0.15	0.63±0.04	0.14 ± 0.00
22:6n-3	3.39 ± 0.55	7 ± 0.33	6.33 ± 0.44	1.08 ± 0.76	0.58 ± 0.00
n-3 PUFA	5.81±0.63	14.06±1.6*	7.08±0.61*	1.72±0.74*	5.76±0.7ns

3.4 Concentration-dependent conversion and esterification of n-3 ALA and n-6 LA metabolites into cellular GPLs

3.4.1 Procedures

After investigating the FA profile of PC12 cells in the absence of NGF, experiments were set up to examine the potential of the elongation/desaturation pathway of ALA (18:3n-3) and LA (18:2n-6) in proliferating PC12 cells. Briefly, cells were seeded in 25 cm^2 flasks and incubated at 37°C under 5% CO_2 humidified conditions. After one day, the growth medium (15% serum) was removed, and the cells were incubated for three days in 1% FBS medium containing different n-3 ALA and n-6 LA concentrations. Cellular lipids were extracted with hexane/isopropanol (3/2 by vol) and subsequently subjected to TLC for major GPL separation followed by GC analysis of the FA in different GPLs as described in Materials and Methods (section 2.5)

3.4.2 Results

The percentage of the major n-3 PUFA intermediates, eicosapentaenoic (EPA, 20:5n-3), docosapentaenoic (DPA, 22:5n-3), and docosahexaenoic (DHA, 22:6n-3) synthesised from several ALA concentrations (up to 80 μM) in the main GPLs: EPG (panel a), CPG (panel b) and SPG (panel c) are shown in Figure 3.1. These three lipid classes constituted the bulk of GPLs (78.5%, see Table 3.2), as established by determining the total lipid phosphorus content.

Supplements of ALA resulted in a concentration-dependent increase in EPA (between 0.58±0.02 basal and 9.27±0.40 at 80 μM; $p<0.05$), and elevated DPA levels (between 0.69±0.03 basal and 13.75±0.51 at 80 μM; $p<0.05$) in the EPG species (panel a). In contrast, DHA content reached a maximum level at 40 μM (10.47±0.17; $p<0.05$), followed by a decline in spite of an increase in ALA concentration in the medium. In the presence of 40 μM ALA, a robust conversion and incorporation of EPA and DPA up to 4.93%±0.3 ($p<0.05$) and 9.42%±0.2 ($p<0.05$), respectively, from negligible levels was seen in EPG. At this concentration, DHA was increased approximately 3-fold from its basal levels (from 3 to 10%; $p<0.05$). Furthermore, after 3 days in the presence of ALA, PC12 cells converted and esterified n-3 higher intermediates of ALA such as EPA, DPA and DHA into EPG (panel a).

A similar analysis was carried out in CPG (panel b), which usually contains a lower proportion of PUFAs, as shown in Table 3.1; nearly identical elongation/desaturation and esterification took place over a three-day period for CPG species, which were comparable to EPG. Notable, however, are the much lower levels of PUFAs in CPG than in EPG. As anticipated, DHA levels in CPG were a little over 1%.

A similar incorporation pattern was seen in the SPG fraction (panel c); PC12 cells were able to elongate/desaturate ALA in a concentration-dependent fashion. The levels of EPA remained constant and low (between 1.16±0.14 basal and 1.37±0.09 at 80 μM; $p<0.05$) after treatment with increasing concentrations

of ALA. Moreover, the content of DPA increased in an ALA concentration-dependent manner and DHA reached a maximum (8.23±0.25; $p<0.05$) when it was saturated with 40 μM of ALA. Interestingly, increasing the ALA concentration to 80 μM caused an increase in DPA (in EPG 13.75±0.51; $p<0.05$/ in SPG 14.18±0.47; $p<0.05$) rather than in the DHA levels (in EPG, 8.6±0.19; $p<0.05$/ in SPG, 5.63±0.33; $p<0.05$) in both GPLs, as previously shown in neuroblastoma cells (Langelier et al., 2005) and in primary astrocyte cultures (Innis et al., 2002). Overall, the data indicate that an increase ($p<0.05$) in n-3 PUFA metabolites took place, in agreement with a possible reversal of the deficiency status.

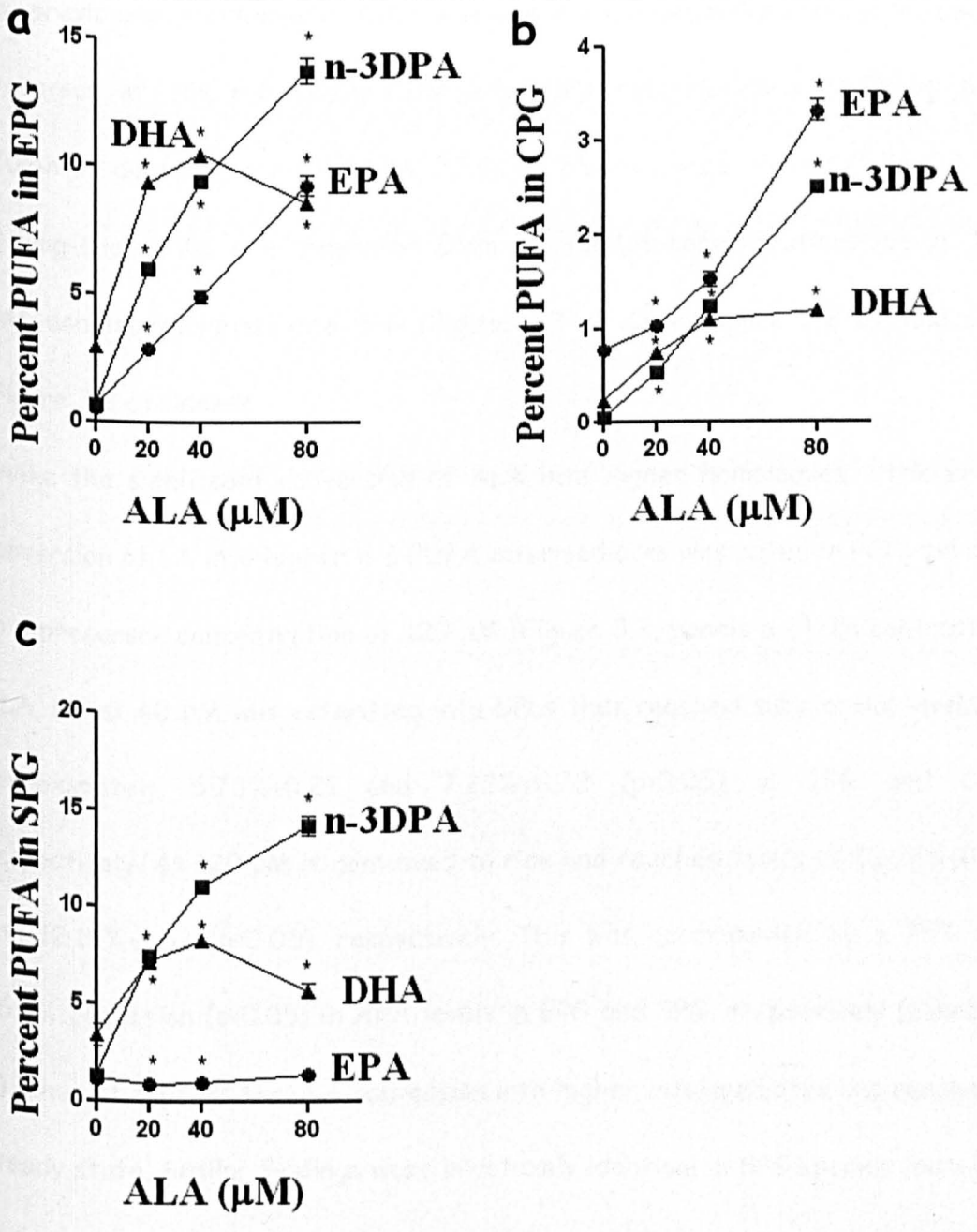


Figure 3.1: Dose-dependent conversion and esterification of ALA metabolites into cellular GPLs. PC12 cells were incubated with various concentrations of ALA (0-80 μM). After 3 days, the FAME components in different GPLs was determined. FAME, expressed as the % of FA in either EPG, CPG and SPG, are averages ± SEM of triplicates. The experiment was repeated three times. * ($p<0.05$) indicates a significant difference for FA non-supplemented cells. The values are SEM of triplicate experiments.

As previously mentioned, PC12 cells were also supplemented with LA, a precursor of the n-6 family. The n-6 PUFA intermediates, including ARA (20:4n-6), docosatetraenoic (DTA, 22:4n-6), and docosapentaenoic (DPA, 22:5n-6) long-chain FAs, are converted from various LA concentrations (up to 120 μM), and incorporated into EPG (Figure 3.2 a), CPG (Figure 3.2 b), and SPG (Figure 3.2 c) classes.

Unlike the significant conversion of ALA into higher homologues, little or no conversion of LA into higher n-6 PUFA intermediates was noted in PC12 cells up to a precursor concentration of 120 μM (Figure 3.2, panels a-c). In contrast to ALA, LA at 40 μM was esterified into GPLs that reached substantial levels of approximately 5.73%±0.21 and 7.22%±0.72 ($p<0.05$) in EPG and CPG, respectively. At 120 μM it continued to rise and reached levels of 10.57%±0.48 and 12.15%±1.58 ($p<0.05$), respectively. This was accompanied by a 75% and 46.4% reduction ($p<0.05$) in ARA levels in EPG and CPG, respectively (panels a, b). Thus, it appears that LA conversion into higher intermediates has reached a steady state. Similar findings were practically identical in SPG species (panel c).

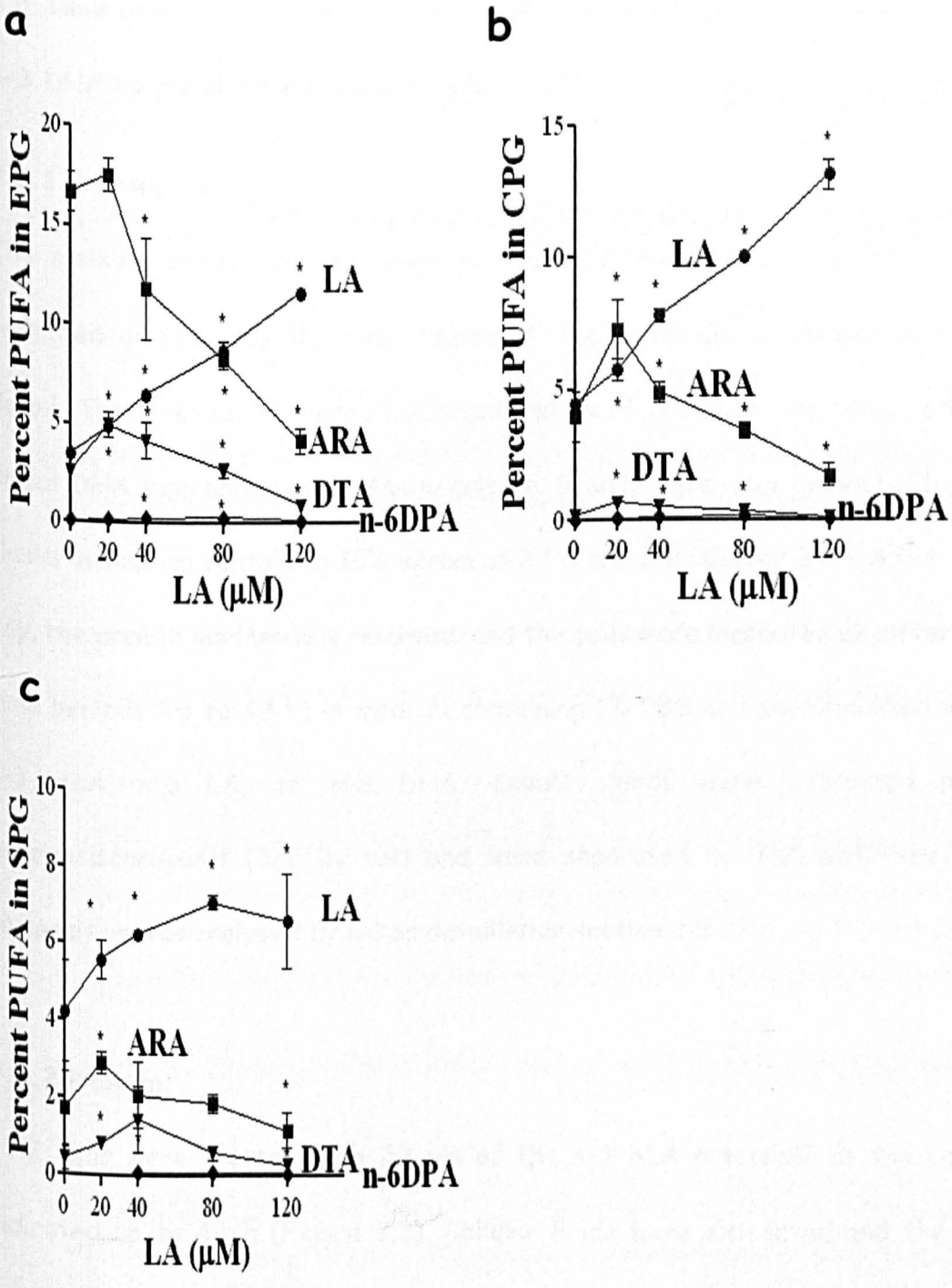


Figure 3.2: Dose-dependent conversion and esterification of LA metabolites into cellular GPLs. The experimental conditions were identical to those in Figure 3.1. The values are SEM of triplicate experiments. FAME, expressed as the % of FA in either EPG, CPG and SPG, are averages ± SEM of triplicates. The experiment was repeated three times. * ($p<0.05$) indicates a significant difference for FA non-supplemented cells.

3.5 Time course of conversion and esterification of n-6 LA, n-3 ALA and n-3 DHA metabolites into cellular GPLs

3.5.1 Procedures

The striking differences between ALA and LA handling by the PC12 cells prompted us to study the time course of the metabolic conversion in more detail. The kinetics of elongation/desaturation of n-3 ALA was compared to direct DHA supplement over time in culture. Briefly, cells were grown in 25 cm^2 flasks in medium containing 15% serum at 37°C and 5% CO_2 for 24 h. After one day, the growth medium was removed, and the cells were incubated at different time periods (up to 48 h) in medium containing 1% FBS and supplemented with n-3 ALA, n-6 LA, or n-3 DHA. Cellular lipids were extracted with hexane/isopropanol (3/2 by vol) and were separated by TLC and their FA composition was analysed by GC as detailled in section 2.5.

3.5.2 Results

PC12 cells were treated with 20 μM of the n-3 ALA precursor at the times indicated up to 48 h (Figure 3.3). Cellular lipids were extracted and the FA profile was analysed according to the method described before. The percentage of ALA incorporated and the n-3 PUFA intermediates EPA, DPA and DHA biosynthesised from ALA in a time-dependent manner are shown in EPG (panel a) and SPG (panel b). A gradual rise in the levels of 20:5n-3 and 22:5n-3 corresponding to a decrease in the precursor (18:3n-3) was observed in EPG

species (panel a), after 6 h and 12 h incubations. Notably, 6 h following 20 μM ALA addition, 20:5n-3 (from 1.02% to 2.14%; $p<0.05$) and 22:5n-3 (from 2.39% to 5.14%; $p<0.05$) increased both by approximately 2-fold in EPG (panel a). Although at early times, 22:6n-3 remained unchanged, it was nearly 2.5-fold higher after 48 h (from 4.27% to 9.65%; $p<0.05$). At 6 h, some ALA (approximately 4.0±0.3) was directly esterified into EPG; by 48 h, however, it leveled off completely. At 48 h, a clear conversion of 20:5n-3 into 22:5n-3 and 22:6n-3 was observed, thus, indicating that the PC12 cells were able to synthesise n-3 intermediates from their precursor, ALA, in a time-dependent manner. Notably, by 48 h DHA and DPA together comprised about 20% of the FAME in EPG. Since SPG is more enriched with ALA-derived n-3 metabolites, we examined the changes as a function of time. In SPG species (panel b), a remarkable rise in 22:5n-3 and 22:6n-3 levels was also observed. Indeed, after 2 days, the percentage of 22:5n-3 (from 0.88% to 5.98%; $p<0.05$) and 22:6n-3 (from 1.49% to 4.75%; $p<0.05$) in SPG was at least 3-fold higher than in non-supplemented cells (panel b). These data further confirm the previous results shown in Figure 3.1, which indicated effective endogenous elongation/desaturation reactions in PC12 cells.

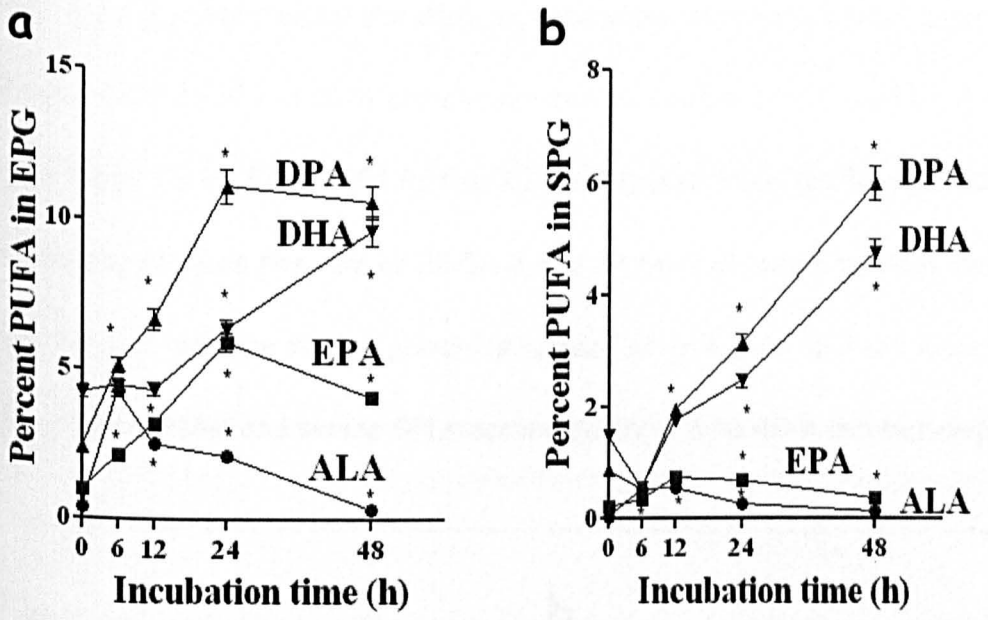


Figure 3.3: Time course of conversion and esterification of ALA metabolites into cellular GPLs. PC12 cells were incubated for designated time periods (up to 48 h), in the presence of 20 μM ALA. The conditions were identical to those in Figure 3.1. The values, expressed as the % FAMEs, are averages ± SEM of triplicates of three independent experiments. * ($p<0.05$) indicates the significant difference for non-supplemented cells (time 0).

The kinetics of directly incorporating DHA (20 μM) into GPLs was followed-up over a period of 48 h. Figure 3.4 shows the levels of esterified DHA and other n-3 ALA metabolites (EPA and DPA) in EPG (panel a) and SPG (panel b) cellular GPLs. Notably, a striking increase in DHA was attained by 6 hours, and by 12 hours, uptakes nearly reached the saturation level. As expected, no changes in the levels of 20:5n-3 and 22:5n-3 contents were observed over the course of time. A 48-h direct supplement of DHA raised the amount of the esterified

product in EPG to 19.7%±2.1 (panel a), which is similar to the sum of 22:5n-3 and 22:6n-3 generated by the ALA-supplemented cells (Figure 3.3 panel a). Similar kinetics of n-3 DHA enrichment can be seen in SPG (panel b). A 4-5-fold rise (from 1.5 to 8; $p<0.05$) in the 22:6n-3 levels from preformed 22:6n-3 is nearly the same as the sum of 22:5n-3 and 22:6n-3 attained by ALA conversion. The results indicate that a powerful uptake of n-3 DHA and its incorporation into ethanolamine and serine GPLs occurs during a 6 to 48-h incubation period.

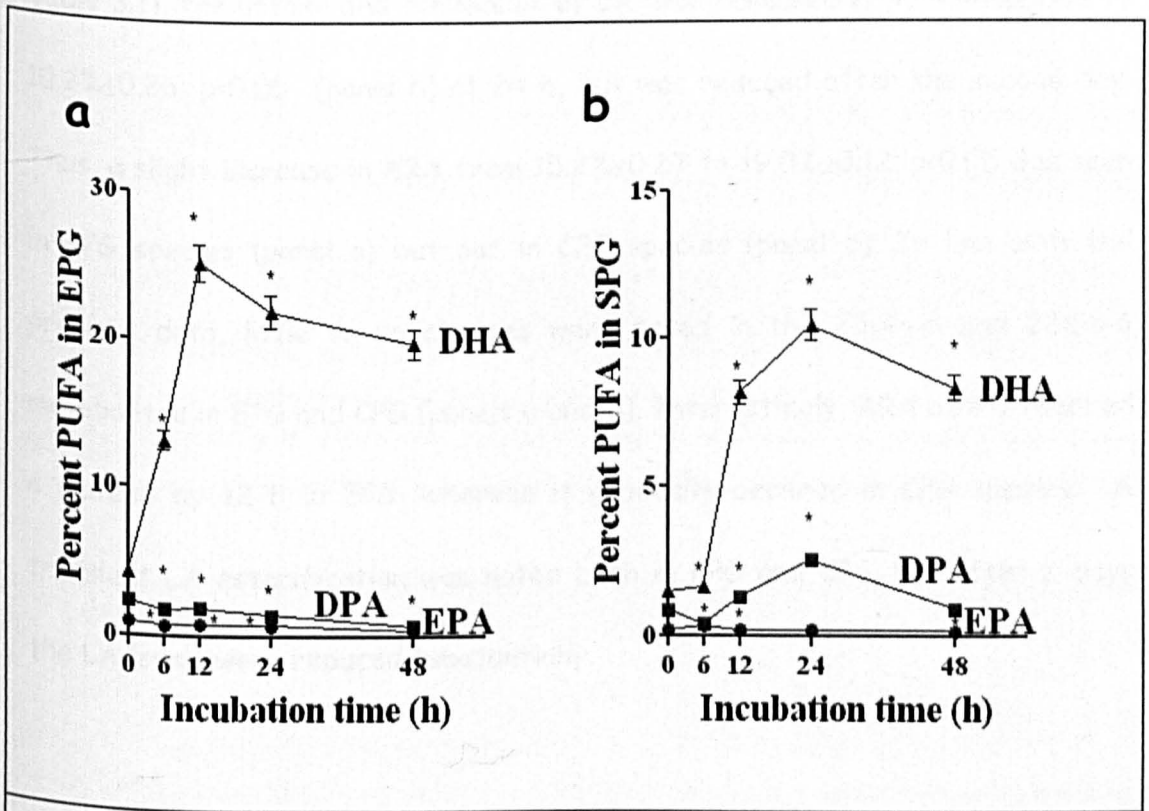


Figure 3.4: Time course of esterification of DHA into cellular GPLs. PC12 cells were incubated for designated time periods (up to 48 h), in the presence of 20 μM DHA. The experimental conditions were identical to those in Figure 3.1. The values, expressed as the % FAMEs, are averages ± SEM of triplicates of three independent experiments. * ($p<0.05$) indicates a significant difference for non-supplemented cells (time 0).

The kinetics of LA (20 μM) directly incorporated into GPLs was followed-up over a period of 48 h. The time-dependent course for LA and the subsequent higher n-6 PUFA intermediates (ARA, DTA and n-6DPA) are shown in EPG (panel a), and CPG (panel b) GPLs (Figure 3.5). In comparison with ALA, the metabolic conversion of LA was by far less active (panels a and b). Uptake and conversion of LA into EPG (panel a) was moderately increased from 3.41%±0.17 to 6.65%±0.23; $p<0.05$ at 24 h. However, into CPG, the major cellular GPL (see Table 3.1), the uptake and conversion of LA was increased from 4.44%±0.04 to 10.2%±0.26; $p<0.05$ (panel b) at 24 h, but was reduced after the second day. Thus, a slight increase in ARA from 15.2%±0.27 to 19.0%±0.12; $p<0.05$ was seen in EPG species (panel a) but not in CPG species (panel b). In line with the previous data, little or no changes were noted in the 22:4n-6 and 22:5n-6 metabolites in EPG and CPG (panels a and b). Interestingly, ARA nearly reached a plateau by 12 h in EPG, whereas it gradually declined in CPG species. A transient LA esterification was noted both in EPG and CPG, but after 2 days the LA levels were reduced substantially.

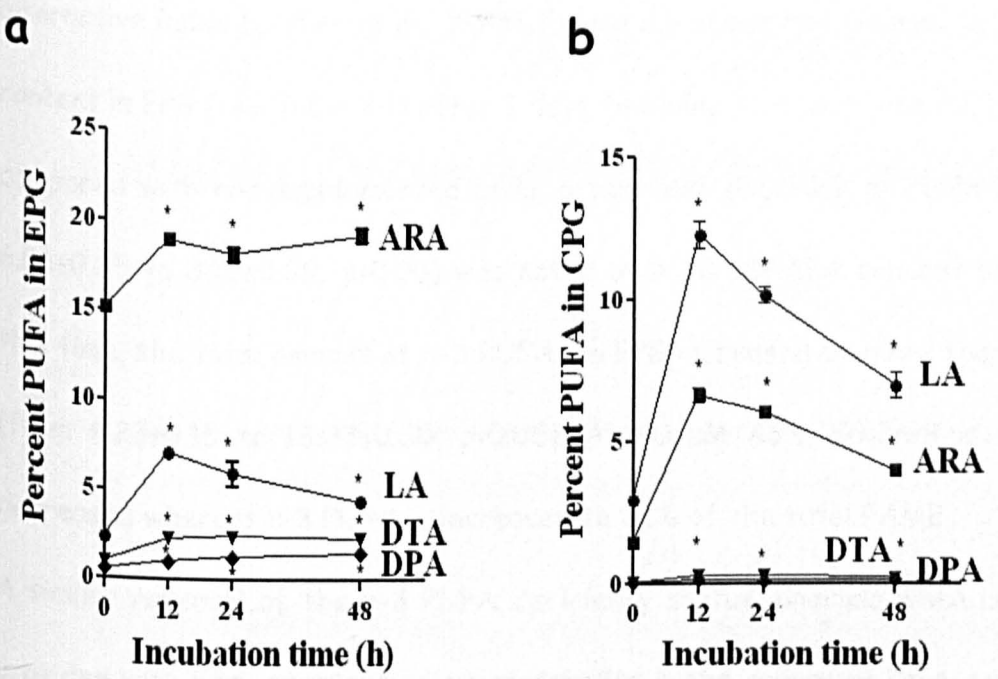


Figure 3.5: Time course of conversion and esterification of LA metabolites into cellular GPLs. PC12 cells were incubated for designated time periods (up to 48 h), in the presence of 20 μM LA. The experimental conditions were identical to those in Figure 3.1. The values, expressed as the % FAMEs, are averages ± SEM of triplicates of three independent experiments. * ($p<0.05$) indicates a significant difference for non-supplemented cells (time 0).

3.6 Recovery from n-3 deficient status in PC12 cells following ALA or EPA supplements

The decrease in 20:3n-9 following PUFA supplements (Table 3.2) warranted particular attention since it has been strongly associated with n-6 PUFA deficiency (Lerner et al., 1995). On the other hand, the well-established index of n-3 PUFA deficiency (22:5n-6/22:6n-3) is not suitable in the present study, mainly because PC12 cells synthesise little if any 22:5n-6.

Therefore, we used Mead acid, the n-9 PUFA higher metabolite, as an alternative index (Lerner et al., 1995). Figure 3.6 shows the changes in 20:3n-9 content in EPG (see Table 3.1) after 3 days following ALA supplements. Notably, compared with non-supplemented cells, a two-fold decrease in 20:3n-9 (from 6.06±0.25 to 3.18±0.09; $p<0.05$) was noted at a 20 μM ALA concentration. At this time, the total amount of n-3 PUFAs in EPG increased by more than 4-fold (from 4.23±0.15 to 18.23±0.30; $p<0.05$). At 40 μM ALA, 20:3n-9 is further decreased whereas n-3 PUFA is increased to 25% of the total FAME.

A similar reversal of the n-3 PUFA deficiency status emerges when cells are provided with EPA, an intermediary metabolite in the course of DHA synthesis. At 10 μM EPA, 20:3n-9 is reduced by approximately 40% and at 40 μM EPA is nearly absent. At the latter concentration, the amount of n-3 PUFAs attained is at a level of nearly 38%. This strongly suggests that the culture-imposed deficiency status can be easily reverted by treating cells with suitable n-3 FA precursors.

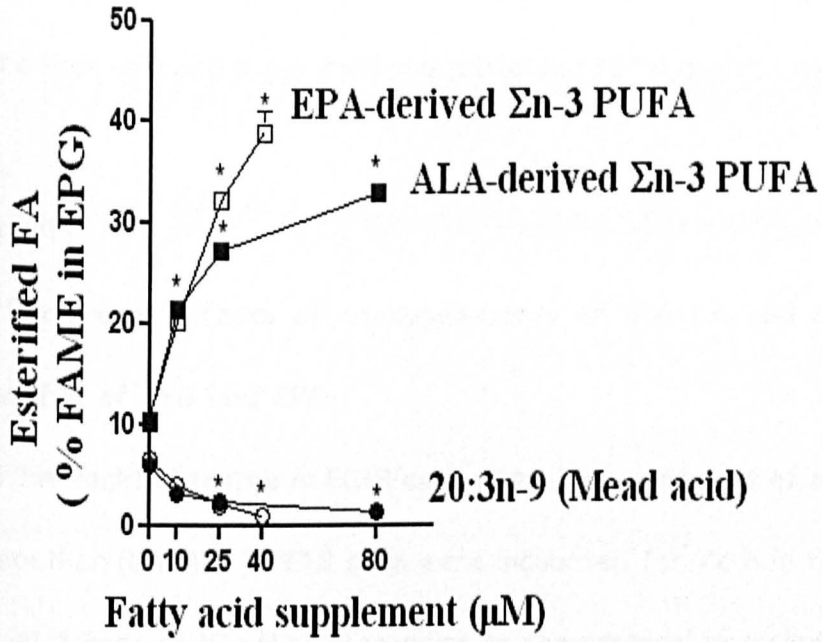


Figure 3.6: Recovery from n-3-deficient status in PC12 cells following ALA or EPA supplements. PC12 cells were incubated for 72 h with the indicated concentrations of ALA (closed symbols) or EPA (open symbols). The experimental conditions were similar to those detailed in Figure 3.1. FAME±SEM of 20:3n-9 (circles) and the sum of 20:5n-3; 22:5n-3 and 22:6n-3 (squares), expressed as the percent of EPG, was obtained from triplicate cultures. *p<0.05 indicates a significant difference for FA non-supplemented cells.

3.7 Effects of n-6 and n-3 PUFA co-supplements on the FA profile of cellular GPLs

3.7.1 Procedures

A principal aim of this study was to design a FA cocktail in order to build up a proper molecular repertoire of n-3 and n-6 FAs for PC12 cells similar to that of neuronal cells.

To this end, cells were seeded in 25 cm^2 flasks and incubated in a humidified atmosphere of 37°C and 5% CO_2 for 24 h. The following day, the growth medium was removed and replaced with DMEM containing 1% FBS in the

presence of different n-3 and n-6 PUFA combinations for 48 h or 72 h. Cellular lipids were then extracted, purified and subjected to FA analysis by GC.

3.7.2 Results

3.7.2.1 Reciprocal effects of co-supplements of n-6 LA and n-3 ALA on the FA profile of EPG and CPG

The first FA cocktail tested in PC12 cells was a co-supplement of n-3 ALA and n-6 LA together (LA+ALA). PC12 cells were incubated for 72 h in the presence of 10 μM ALA and/ or 10 μM LA according to the protocol described in section 3.7. The n-3 PUFA profile of EPG (panel a) and CPG (panel b) as well as the n-6 PUFA profile of EPG (panel a') and CPG (panel b') are depicted in Figure 3.7. In EPG species (panel a), after co-supplementing with 10 μM ALA and 10 μM LA, the EPA, DPA and DHA levels rose by approximately 8-(from 0.04 to 0.29; $p<0.05$), 4-(from 1 to 4; $p<0.05$) and 2-fold (from 9.68 to 3.75; $p<0.05$), respectively, as compared with the controls (Con). However, there was no change in the n-6 FA profile in the EPG species (panel a'). In the CPG species (panel b), the EPA, DPA and DHA levels were elevated by 5-(from 0.02 to 0.09; $p<0.05$), 4-(from 0.55 to 0.12; $p<0.05$) and 3-fold (from 1.30 to 0.47; $p<0.05$), respectively, as compared with Con. In addition, there was a small increase (>1.4-fold) in ARA (4.42±0.22) compared with Con (3.16±0.16; $p<0.05$) and in DTA (>1.8-fold; 0.25±0.01 compared with Con (0.14±0.01; $p<0.05$) percentages in the n-6 FA profile (panel b').

The two essential FAs, ALA and LA at equimolar concentrations, compete for the elongation/desaturation pathway. Figure 3.7 clearly shows that LA did not prevent a conversion of ALA into 20:5n-3, nor did it affect the elongation /desaturation into the 22:5n-3 and 22:6n-3 metabolites.

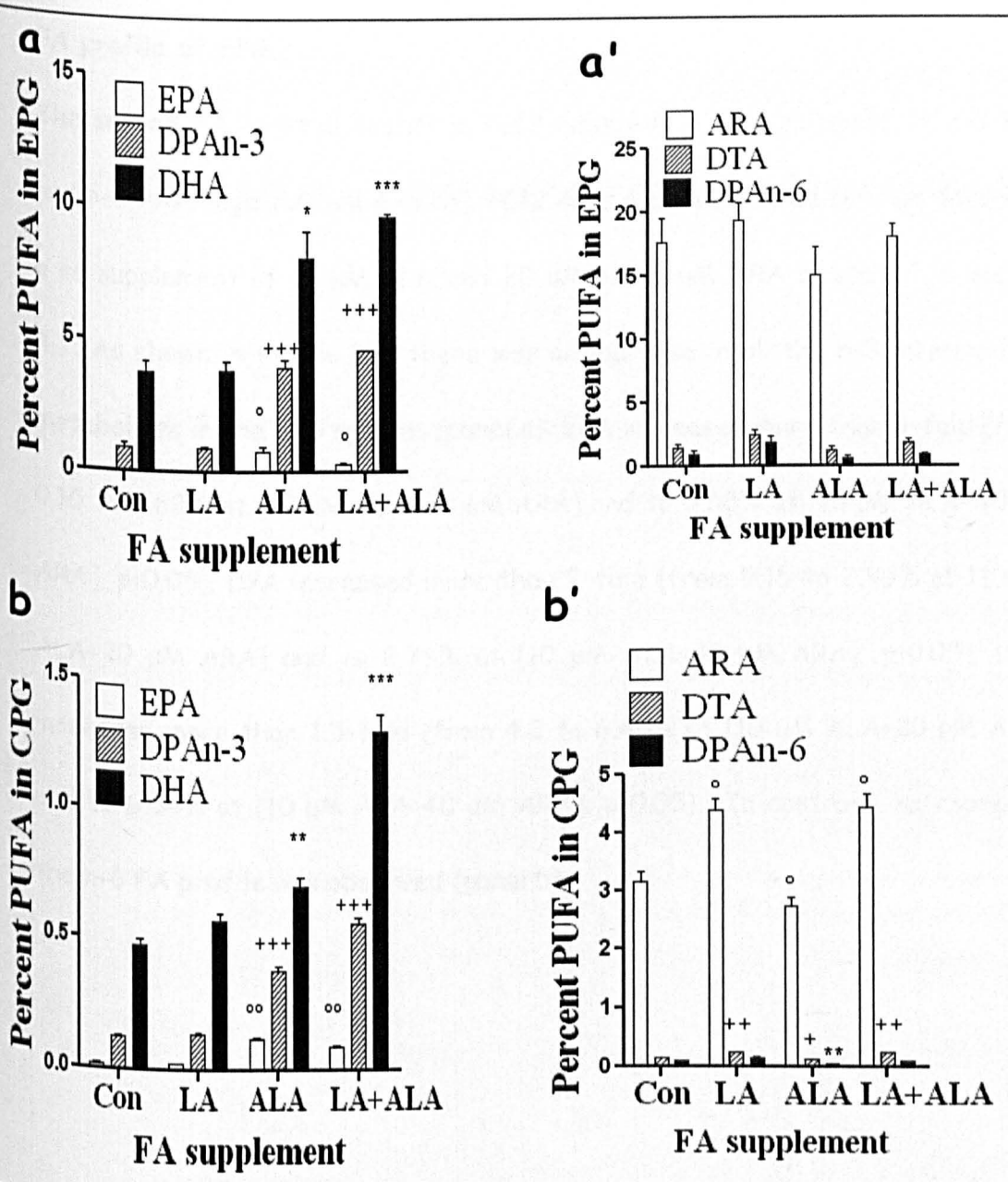


Figure 3.7: Effects of LA co-supplements on ALA metabolic conversion and esterification in EPG and CPG. PC12 cells were incubated in the presence of 10 µM ALA or/and 10 µM LA. The experimental conditions were similar to those detailed in Figure 3.1. The values, expressed as the % FAMEs, are averages ± SEM of triplicates of three independent experiments. ° ($p<0.05$), °° ($p<0.005$), °°° ($p<0.001$) indicate a significance difference for control untreated cells (Con) for n-3 EPA or the n-6 ARA percent. + ($p<0.05$), ++ ($p<0.005$), +++ ($p<0.001$) indicate a significance difference for control untreated cells (Con) for n-3 DPA or n-6 DTA percent.* ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significance difference for control untreated cells (Con) for n-3 DHA or n-6 DPA percent.

3.7.2.2 Reciprocal effects of n-6 ARA and n-3 ALA co-supplements on the FA profile of EPG

The second FA cocktail tested in PC12 cells was a co-supplement of n-3 ALA and n-6 ARA together (ALA+ARA). PC12 cells were incubated for two days with a co-supplement of 10 μM ALA and 20 μM or 40 μM ARA as stated in section 3.7. As shown in Figure 3.8, there was an increase in all the n-3 intermediary metabolites in the EPG species (panel a): EPA increased more than 3-fold (from 0.15 to 0.62% at [10 μM ALA+20 μM ARA] and to 0.50% at [10 μM ALA+40 μM ARA]; $p<0.05$). DPA increased more than 2-fold (from 0.15 to 2.99% at [10 μM ALA+20 μM ARA] and to 2.78% at [10 μM ALA+40 μM ARA]; $p<0.05$). DHA increased more than 1.5-fold (from 4.3 to 6.46% at [10 μM ALA+20 μM ARA] and to 6.04% at [10 μM ALA+40 μM ARA]; $p<0.05$). In contrast, no change in the n-6 FA profile was observed (panel b).

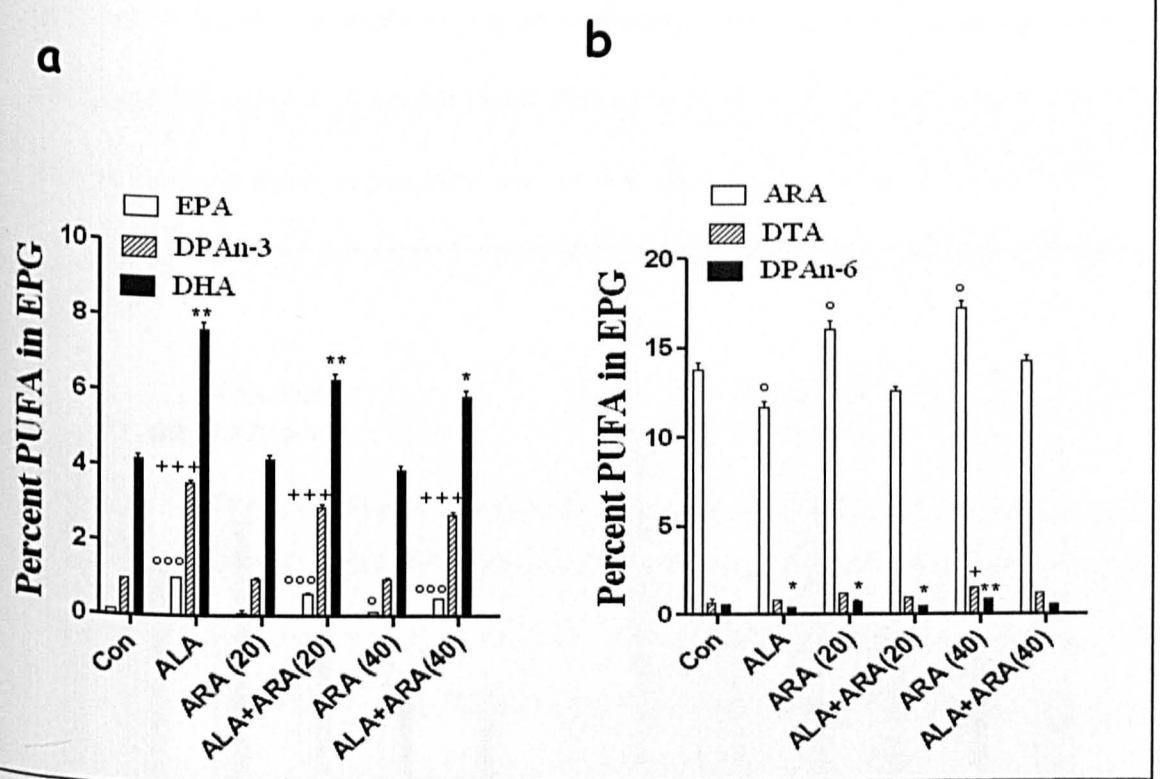


Figure 3.8: Effects of ARA co-supplements on ALA metabolic conversion and esterification in EPG. PC12 cells were incubated for 48 h in the presence of 10 µM ALA or/and 20 or 40 µM ARA. The experimental conditions were similar to those detailed in Figure 3.1. The values, expressed as the % FAMEs, are averages ± SEM of triplicates of three independent experiments. ° ($p<0.05$), °° ($p<0.005$), °°° ($p<0.001$) indicate a significance difference for control untreated cells (Con) for n-3 EPA or n-6 ARA percent. + ($p<0.05$), ++ ($p<0.005$), +++ ($p<0.001$) indicate a significance difference for control untreated cells (Con) for n-3 DPA or n-6 DTA percent.* ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significance difference for control untreated cells (Con) for n-3 DHA or n-6 DPA percent.

3.7.2.3 Reciprocal effects of n-6 ARA and n-3 DHA co-supplements on the FA profile of EPG and CPG

PC12 cells were incubated for two days with a co-supplement of 10 µM or 20 µM DHA and 20 µM ARA (ARA+DHA). Cellular lipids were extracted and the content of FA was analysed (see section 3.7). A combination of both DHA (up to 20 µM) and ARA (20 µM) (Figure 3.9) caused a small rise in ARA levels (>1.3-

fold) and a 3-fold increase in the percentage of DHA in the EPG (panel a) and CPG (panel b) species. A proportional decrease in ARA/DHA or ARA/n-3 ratio in a DHA concentration-dependent manner was observed in these two major GPLs.

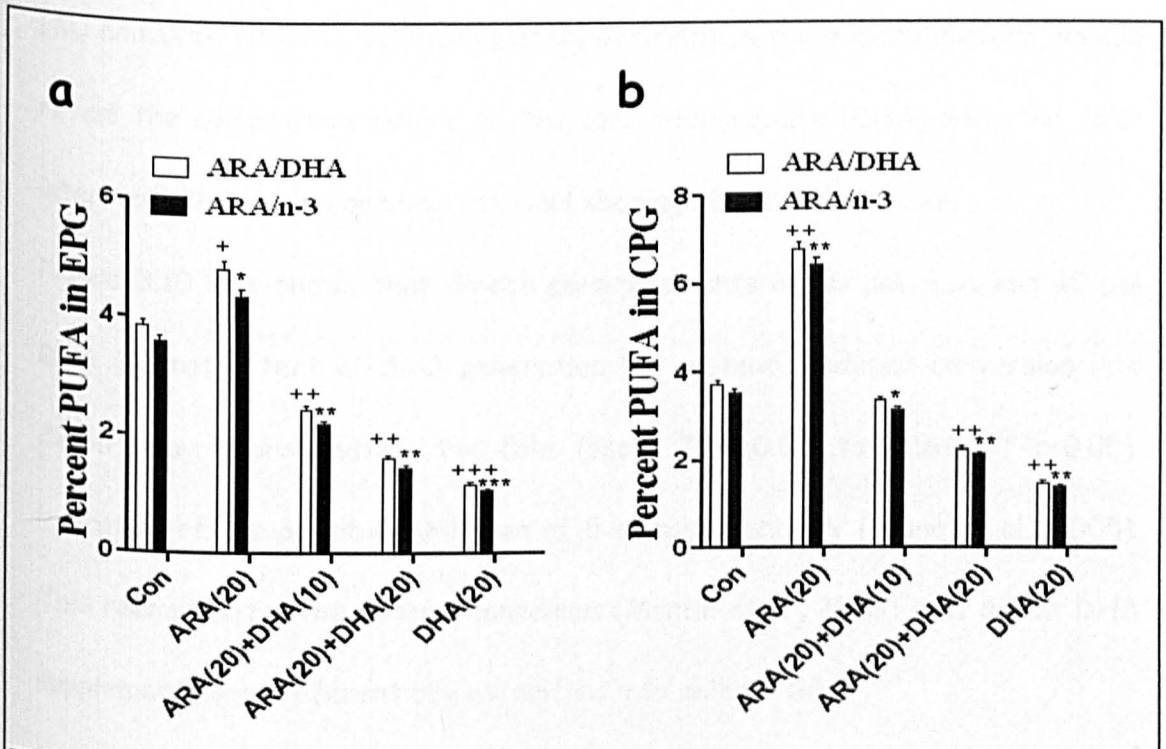


Figure 3.9: Effect of the DHA and ARA supplements on the ARA and DHA profiles of EPG and CPG lipids. PC12 cells were incubated for 48 h in the presence of 10 or 20 μM DHA or/and 20 μM ARA. The experimental conditions were similar to those detailed in Figure 3.1. The values, expressed as the ARA/DHA and ARA/n-3 ratio in EPG, were obtained from triplicate cultures of three independent experiments. + ($p<0.05$), ++ ($p<0.005$), +++ ($p<0.001$) indicate a significance difference for control untreated cells (Con) for ARA/DHA percent.* ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significance difference for control untreated cells (Con) for the ARA/n-3 percent.

3.7.4 Reciprocal effects of co-supplements of n-3 ALA and n-3 DHA on the FA profile of EPG

In previous experiments, supplements with LA or ARA did not result in excessive accumulation of ARA during the formation of their higher

intermediates (22:4n-6 and 22:5n-6). In contrast, the presence of ALA in the medium prompted PC12 cells to generate most of the n-3 intermediary metabolites including EPA, DPA and DHA. Therefore, a combination of ALA (10 μM) and DHA (10 μM), both completely deficient in the growth medium, should reveal the competitive nature of the last intermediate (DHA) with the first intermediate (ALA) regarding the final shaping of the GPL species.

Figure 3.10 also shows that direct co-supplements of 10 μM ALA and 10 μM DHA did not affect 20:5n-3 generation but instead, reduced conversion into 22:5n-3 by approximately two-fold (from 2.06±0.05 to 0.9±0.17; $p<0.05$), indicative of the possible inhibition of 5 elongase activity (Wang et al., 2005). This reemphasizes the general consensus (Martin et al., 2006) that direct DHA supplements are preferentially esterified into cellular GPL.

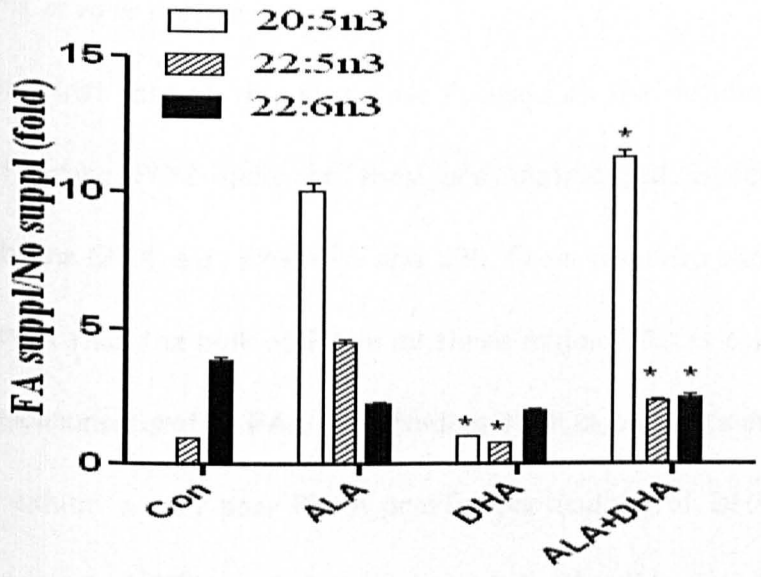


Figure 3.10: Effects of DHA co-supplements on ALA metabolic conversion and esterification in EPG. PC12 cells were incubated for 48 h in the presence of 10 μM ALA or/and 10 μM DHA. The experimental conditions were similar to those detailed in Figure 3.1. The values are expressed as the relative changes in the major n-3 FAME profile of EPG after single or mixtures of FA supplements. Comparisons were made to non-supplemented cultures. The values are SEM of triplicate cultures of three independent experiments.* ($p<0.05$) indicates a significant difference for ALA-supplemented cells.

3.8 Discussion

The ample use of PC12 in neurobiology and neuropathology research (Vaudry et al., 2002; Suggate et al., 2009; Malagelada et al., 2010; Ravni et al., 2006; Nakamura et al., 2001) justified a revisit of these cells in order to examine their metabolic capacity to generate synthesized n-3 or n-6 PUFA metabolites from added precursors. The lack of information regarding their membrane lipid composition and the extensive use of these cells as models for neurons in

neurobiology and neuropathology research prompted us to evaluate the validity of this *in vitro* system.

In the first part of this study, we focused on the detailed FA composition of proliferating PC12 cells as they are distributed among the three major membrane GPLs, e.g., EPG, CPG and SPG. From the data shown in Table 3.1, it is apparent that the bulk of FA in all three major GPLs is composed of saturated and monounsaturated FAs. The finding that in comparison with neurons, PC12 cells exhibit a very poor PUFA profile, particularly of DHA but also ARA, was less expected. This stands in contrast to the FA composition reported for various cells and brain structures isolated from the rodent brain (Bourre et al., 1984). For example, in neurons and synaptosomal membranes the amount of ARA is 10.3% and 12.2%, respectively, whereas that of DHA is 8.3% and 12.5%, respectively (see Table 1.1). Thus, compared with neurons, the levels of ARA and DHA in PC12 cells were down by 1.5- and 5-fold ($p<0.05$), respectively (see Appendix 6 and Table 1.1). In analogy to animal studies, this constitutes a marked deficiency that is not uncommon under culture conditions. For example, oligodendroglia-like, OLN93 cells (Brand et al., 2010), septal-derived SN56 cells and hippocampal-derived HT22 cells (Martin et al., 2006) are all poor in membrane PUFA content. The most feasible explanation is the lack of sufficient amounts of essential FAs in the synthetically defined media or the serum additives given to these cells, a condition that may alter their cellular and functional properties.

Given the marked PUFA deficiency in PC12 cells, we have explored ways to enhance the content of both the n-3 and n-6 PUFA families to attain a proper molecular repertoire that resembles that of neuronal cells. Addition of FA supplements (ALA, DHA, LA or ARA) did not affect the amount of individual GPL under these conditions (Table 3.2). However, supplementing PC12 cells with FAs reshuffles the FA profile towards a specific unsaturated status (Table 3.2). To this end, cultured cells were supplemented with a number of FA cocktails (a combination of FAs) at different concentrations for various periods of time. In addition, ALA and LA precursors were studied when competing with their respective final products DHA and ARA, in order to evaluate the metabolic regulation of the endogenous elongation/desaturation pathway. The latter is common to the n-3 and n-6 PUFA families (Anrtholm and Mohrhauer, 1963; Mohrhauer and Holman, 1963). It consists of mutual serial enzyme-catalysed elongation/desaturation reactions that occur in the endoplasmic reticulum (ER) and which culminate in beta oxidation in the peroxisome (Sprecher et al., 1986; Sprecher et al., 1992) (see Figure 1.2). The general consensus is that both the n-3 and n-6 PUFA families compete for the same desaturase and elongase enzymes; however, many aspects of this physiological regulation to attain a certain profile of GPL molecular species remain unclear. Very few studies have addressed the question of how the FA metabolism is regulated in PC12 cells (Ikemoto et al., 1997; Traynor et al., 1982), although these cells have been widely used in a variety of studies in neurobiology and in

human neuropathology research (Vaudry et al., 2002; Marszalek et al., 2005; Suggate et al., 2009; Vaisid et al., 2008; Malagelada et al., 2010). The kinetics of ALA conversion into higher intermediates indicates that PC12 cells possess an active elongation/desaturation pathway. Thus, increasing the ALA concentration in the culture medium results in a nearly linear rise in EPA and DPA levels into all major GPLs. EPA and DPA are usually absent from normal neurons. The accumulation of these metabolites in PC12 cells appears to be characteristic of transformation. We show that the conversion of ALA into DHA was high and reached a maximum with the 40 μM ALA supplement (Figure 3.1). The results show that the ALA products are selectively distributed; most of the products are esterified to the aminoethanol GPLs (Figure 3.1). A similar distribution pattern was observed in human SH-SY5Y neuroblastoma cells. Similar to PC12 cells, the SH-SY5Y cells effectively converted ALA into EPA, DPA and DHA in a concentration-dependent manner (Langelier et al., 2005).

From the time course of ALA and EPA accumulation in EPG, a clear precursor-product relationship is evident. Thus, the maximum incorporation of the precursor ALA into EPG was seen at 6 h, followed by a sharp decline by 48 h (Figure 3.3). EPA, on the other hand, gradually increased but unlike ALA, it leveled off by 48 h, suggesting a slower turnover. Interestingly, by 48 h, DPA and DHA also approached a plateau (approx. 11% and 10%, respectively), suggesting a maximum capacity (approximately 20% of the 22 carbon PUFA) for incorporation into EPG, independent of ALA availability. The latter was

ineffective even at 80 μM (Figure 3.1). Given the fact that direct supplements of DHA (Figure 3.4) raised the amount of DHA in EPG to approximately the same level (20%), we think that the 22-carbon long-chain PUFAs are tightly regulated by the cellular biosynthetic machinery. We also studied the conversion of LA into higher members of the n-6 family as serum supplements, which are routinely added to most synthetic cell culture media (~1/40 for the ALA/LA ratio (appendix 5)).

Notably, LA supplements added to the medium of proliferating cells were rapidly incorporated without conversion (Figure 3.2) in all three major GPLs, namely, the EPG, CPG and SPG classes, in a concentration-dependent manner. ARA, the first major intermediate of LA conversion, was only marginally elevated under these conditions. Furthermore, only a small conversion into n-6 DTA and n-6 DPA (20 μM LA) was evident. This finding is striking because the parallel conversion of ALA was far higher (Figure 3.1 vs 3.2). This strongly suggests that although the two precursors share the same metabolic pathway, the physiological needs of n-6 and n-3 families for cell growth may be different. Based on the data, we concluded that 20 μM LA is an adequate concentration to sustain the metabolic needs of new membranes in proliferating PC12 cells.

The finding of a severe PUFA deficiency in cultured PC12 cells prompted us to study the optimal conditions for attaining a proper lipid neuronal-like phenotype. This objective needs to be considered in light of the endogenous

conversion of the ALA and LA precursors by the elongation/desaturation metabolic pathway as well as by the competition that may arise as a result of direct final products added to the culture medium. Therefore, in addition to studying the metabolic conversion of each precursor in isolation, the influence of the reciprocal competition between ALA and LA and between ARA and DHA, on the ultimate assembly of the GPL molecular species also warranted a thorough investigation. When the simultaneous supplement of the n-3 and n-6 precursors (ALA+LA) was applied, all n-3 PUFA intermediates were elevated, whereas those of n-6 remained unchanged (Figure 3.7). This is in agreement with the notion that under conditions of n-3 deficiency, the metabolic machinery for PUFA elongation/desaturation in PC12 cells showed a preference for the n-3 precursor in order to meet the cell membrane's needs. In other words, there was a clear lack of competition between the two precursors on the same enzymes. In this context, note that rat liver microsomes failed to convert LA into 18:3n-6 after being exposed to increasing ALA concentrations, suggesting that Δ6 desaturase may exhibit a higher affinity for ALA (Brenner et al., 1969). These are obviously in vitro studies that lack the complexity and functional compartmentalisation of the intact cell or the cell-cell interaction in tissues and therefore may not be relevant to PC12 cells. At this time, we do not have any molecular clues to account for this intriguing observation except to suggest that the preferential build-up of GPL with the proper PUFA composition in the endoplasmic reticulum could be the rate-limiting and

regulatory step. This is based on a preliminary data analysis of the molecular species, a topic that is currently under investigation.

Additional competition studies were carried out by us in PC12 cells using dual DHA and ALA supplements. Under these conditions, the combination did not appear synergistic, presumably because the EPG profile is already enriched with long-chain 22-hydrocarbons (i.e., DPA, Figure 3.9). On the other hand, when the two final products of the elongation/desaturation pathway, ARA and DHA, were added together to the culture medium, preferential uptake and incorporation of the latter was noted already at 10 μM (Figure 3.8). Similar findings have been reported in bone marrow-derived mesenchymal stem cells (Kan et al., 2007) as well as in SN56 and HT22 cells (Martin et al., 2006). One explanation for this phenomenon may be related to the preferential activation of DHA by the Acyl CoA synthetase long-chain (Acsl) isoform (Marszalek et al., 2004; Marszalek et al., 2005). Although this mechanism may account for the first step in free PUFA activation, the regulatory basis for the actual partition into GPLs or other lipid acceptors, depending on the cell lineage or the cell's physiological status, still remains unknown. Nevertheless, the present results suggest that a combination of ALA and DHA should serve as a most suitable molecular repertoire for n-3 PUFA-deficient cells (Figure 3.9) to match levels comparable to those observed in nerve cells.

Conclusions

1. PC12 cells are deficient in n-3 metabolites and this deficiency can be recovered by the supplementation of n-3 PUFAs.
2. ALA is taken up, converted into DHA and esterified into phospholipids, particularly EPG and SPG, in a process where EPA and DPA are also formed. We find this is in marked contrast to neuronal cells which show little or no presence of EPA and DPA.
3. The ALA-converted DHA reaches a level near that obtained after direct supplements of DHA.
4. LA is marginally converted to ARA or its higher n-6 PUFA metabolites indicating a plateau most likely attributable to the high levels of LA present in the medium.
5. LA supplementations do not inhibit ALA conversion to higher n-3 PUFAs suggesting a lack of competition for the same elongases and desaturases.

Chapter 4

The effects of growth factors on PUFA biosynthesis

4.1 Introduction

The pheochromocytoma clonal cell line, PC12, has been widely used for investigating neuronal differentiation and for studying nerve growth factor (NGF) actions. PC12 cells originate from chromaffin cells and in response to several factors; they differentiate into sympathetic neuron-like cells. The most common factor is NGF (Greene et al., 1976; Fujita et al., 1989), but the acidic fibroblast growth factor (aFGF) (Rydel and Greene, 1987), the basic fibroblast growth factor (bFGF) (Rydel and Greene, 1987; Togari et al., 1985) and retinoic acid (RA) (Scheibe et al., 1991) are also well known. A great deal of our understanding about the mode of action of growth factors and their signaling cascades has been achieved with NGF.

AFGF and bFGF are peptide hormones abundant in the nervous tissue (Lobb and Fett, 1984; Thomas et al., 1984; Gospodarowicz et al., 1984) and play important roles during differentiation. BFGF, for example, induces neurite outgrowth in cultured dissociated hippocampal and cerebral cortical neurons (Walicke et al., 1986; Morisson et al., 1986).

The vitamin A metabolite, all-*trans* retinoic acid (ATRA), plays a crucial role in the development of the peripheral nervous system (PNS) and the central nervous system (CNS). RA has been shown to stimulate neurite outgrowth of sympathetic and dorsal root ganglia (DRG) neurons (Haskell et al., 1987) and dissociated neurons from the neural tube (Corcoran et al., 2000) derived from chick embryo explants. RA promotes neurite extensions of several types of

primary neurons in the cortex (Ved and Pieringer, 1993), retina (Mey and Rombach, 1999) and olfactory system (Whitesides et al., 1998). It also affects transformed cultured cells such as neuroblastoma (Petroni et al., 1996a; 1996b; Sidell et al., 1983; 1984; 1986; Shea et al, 1985) and teratocarcinoma (Jones-Villeneuve et al, 1983). In principle, it is believed that ATRA inhibits cell division, and subsequently this inhibition results in cell differentiation into a neuronal phenotype. RA induces neurite expansion in subclones of PC12 cells and the expression of neural markers in the parental PC12 cell line (Scheibe et al., 1991).

Since these events presumably involve alterations in the cell membrane, we have focused our attention to changes in membrane phospholipids after exposure to various differentiation factors including NGF, bFGF and RA. In the following experiments, changes in the FA composition and FA biosynthesis in individual phospholipids were assessed after adding these agents.

Changes in the overall phospholipid composition and FA content of individual phospholipids in PC12 cells after a three-day or seven-day exposure to NGF have been reported in the past (Traynor et al., 1982; Ikemoto et al., 1997). However, there have been no studies regarding the impact of NGF, bFGF or RA on n-3 PUFA or n-6 PUFA biosynthesis in PC12 cells. In other cell lines, such as neuroblastoma cells, the effect of differentiation toward a neuronal phenotype by several agents (dimethylsulfoxide (DMSO), 5'-bromodeoxyuridine and dibutyryl cAMP, RA) on the FA composition has been studied. Gulaya et al.

(1989) measured the changes in phospholipid classes and the changes in FA content in glycerophospholipids of neuroblastoma Cl300 Nl8 cells following differentiation by different agents (DMSO, 5'-bromodeoxyuridine or dibutyryl cAMP). In N1E-115 cells, the effect of DMSO-induced differentiation on the phospholipid composition and on the FA content of glycerophospholipids was also assessed (Murphy and Horrocks, 1993). In SK-N-BE cells, the effect of RA-induced differentiation on the percentage of the levels of major FAs and on the conversion of LA and EPA has been also studied (Petroni et al., 1996a, b).

Objectives

The objectives of this study were to investigate in PC12 cells the following:

- The optimal conditions for morphological changes (typical of neuronal differentiation) after challenging cells with NGF, bFGF or ATRA
- The FA composition before and after differentiation
- The appearance of individual metabolites generated from linoleic acid (18:2n-6) and linolenic acid (18:3n-3) precursors and the target phospholipids for their incorporation

4.2 Effect of NGF, bFGF and ATRA on PC12 cell proliferation

4.2.1 Procedures

For morphological observations, PC12 cells were seeded in 25 cm^2 flasks and incubated at 37°C under 5% CO_2 humidified conditions for 24 h. After one day, the growth medium (15% serum) was removed, and the cells were incubated for 16 hours or three days in DMEM supplemented with 1% foetal bovine serum (FBS) with or without additional effectors: NGF (100 ng/ml), bFGF (50 ng/ml) or ATRA (1 μM or 10 μM). Images were taken with a Nikon phase contrast microscope.

4.2.2 Results

The morphological changes of PC12 cells after exposure to a number of agents are illustrated in Figure 4.1.

Proliferating PC12 cells grown in serum-supplemented (15%) medium and incubated in serum-deprived (1% FBS) medium appear small and round and, as described by Green and Tischler (1976), they resemble chromaffin-like cells.

Sixteen hours following NGF addition, cell flattening and the formation of short cytoplasmic extensions (neurites or processes) were observed under microscopy. Three days after adding NGF, extended and more robust neurites were noted.

Morphological responses to bFGF treatment are very similar to those seen after NGF at the beginning of the process (a 16-hour treatment). After 16 h,

with the addition of bFGF, extended robust neurites are observed in PC12 cells. However, after 72 h with bFGF, the neurite outgrowth ceased. These observations are in concordance with those of Togari et al. (1985), who reported that 50 ng/ml pituitary bFGF elicited unstable neurite expansion by PC12 cells, which after three days, had deteriorated. Thus, bFGF appears to initiate transient neurite outgrowth.

Three days after adding RA to serum-deprived (1%) medium, no neurite processes were noted. Thus, RA did not induce neurite outgrowth in PC12 cells. This observation is in line with studies undertaken by Scheibe and co-workers (1991), who showed that the parental PC12 cell line did not elaborate neurites in response to RA, in contrast to a subclone mutant deficient in cAMP-dependent protein kinase A (PKA).

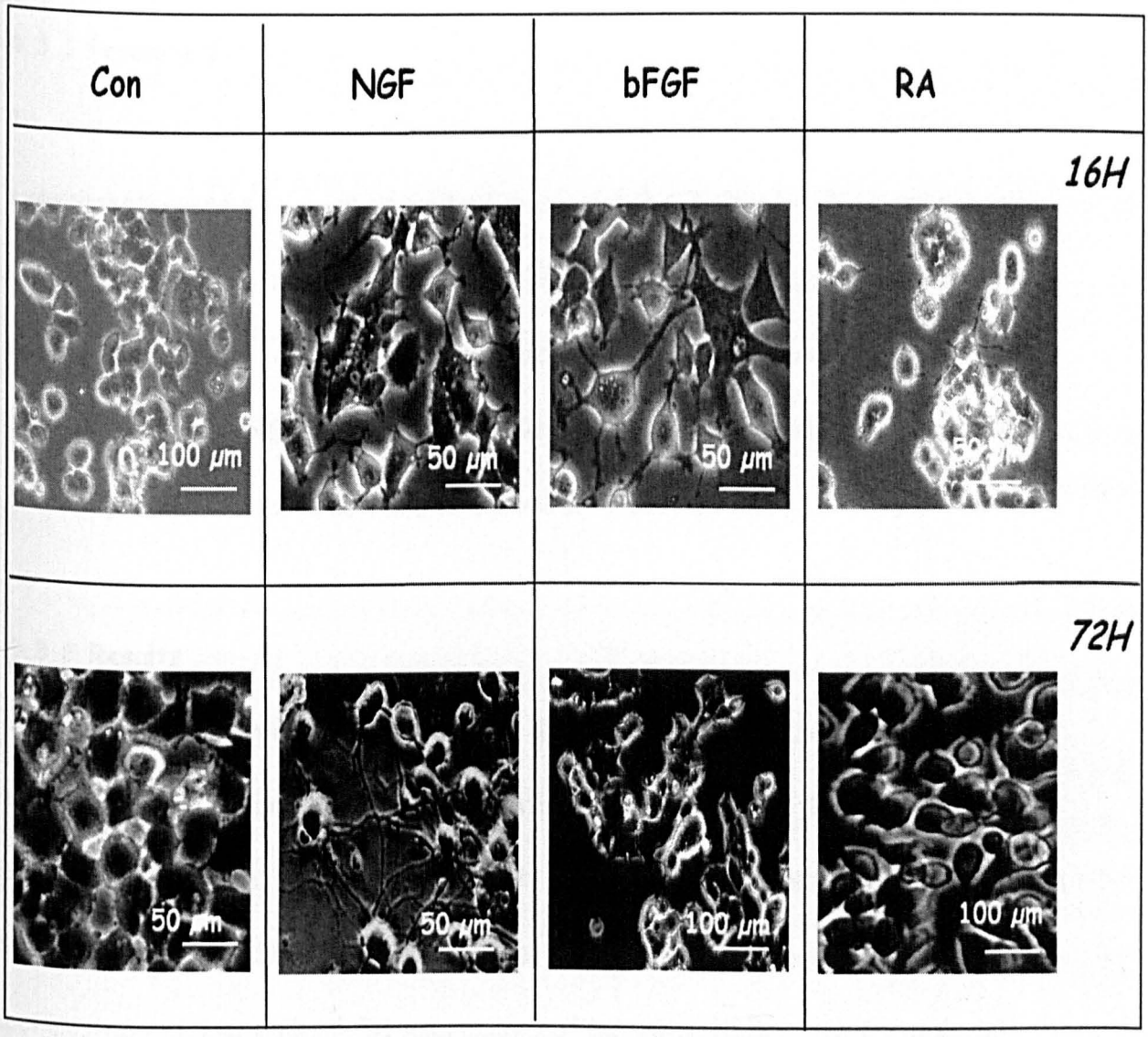


Figure 4.1 Microscopic images of proliferating, NGF-differentiated, bFGF-differentiated and RA-differentiated PC12 cells. 16 hours: Proliferating cells in serum-deprived medium (1%). After NGF (100 ng/ml) or bFGF (50 ng/ml) short extensions were noted. After ATRA (1 μM or 10 μM), no morphological differentiation was observed. Three days after adding NGF, extended and more robust neurites were noted. After adding bFGF, the processes regressed. After ATRA, no morphological differentiation was observed. Scale bar = 100 μm, Scale bar = 50 μm.

4.3 NGF does not alter the FA profile

4.3.1 Procedures

To investigate the FA composition of differentiated (+NGF) PC12 cells, experiments were set up as described in section 2.4.3. Briefly, PC12 cells were incubated in DMEM supplemented with 1% FBS in the presence of NGF (100 ng/ml) and in the absence of any additional FA for three days. After differentiation by NGF, the EPG, CPG and SPG were extracted from PC12 cells and analysed for FA composition, as described in section 2.5.

4.3.2 Results

Table 4.1 depicts typical profiles of FAs determined in the three major classes of GPLs (EPG, CPG and SPG) in differentiated (+NGF) cells. This table clearly shows that despite the fact that PC12, after 3 days, exhibited a neuronal morphology after NGF addition, little or no changes took place in the composition of the bulk of FA especially PUFAs (ARA, 15% in -NGF (table 3.1) to 12% in +NGF; DHA, 3% in -NGF (table 3.1) to 2% in +NGF in EPG).

Table 4.1: Major FAME in EPG, CPG and SPG of NGF-differentiated PC12 cells. PC12 cells were grown for three days in DMEM containing 1% FBS and 100 ng/mL NGF. After incubation, the FAME components in different GPLs was determined. The values, expressed as the % FAMEs, are averages ± SEM of triplicate cultures of three independent experiments. Control data is found in (-NGF; table 3.1).

	+NGF (100 ng/ml)		
	Phospholipid classes		
	EPG	CPG	SPG
	% mean FA ± SEM	% mean FA ± SEM	% mean FA ± SEM
FA	Saturated fatty acids		
16:0	8.68 ±0.8	24.23±0.73	13.06±1.47
18:0	11.51 ±0.61	5.98±0.28	27.95±2.87
20:0	0.38 ±0.04	0.24±0.01	0.63±0.07
22:0	0.24 ±0.02	0.16±0.009	0.39±0.13
24:0	0.15±0.05	0.34±0.006	0.42±0.14
	Plasmalogen species		
16:0 DMA	3.03±0.43	0.16±0.02	<0.1
18:0 DMA	4.03±0.35	0.53±0.04	0.21±0.04
18:1 DMA	1.57±0.18	0.03±0.003	0.06±0.03
	Monounsaturated fatty acids (and Mead acid)		
16:1n-7	1.39±0.08	<0.1	0.16±0.01
18:1n-7	8.3±0.84	14.73±0.9	6.7±0.82
18:1n-9	29.6±1.09	39.5±0.16	24.1±3.15
20:1n-9	3.18±0.32	3.35±0.05	2.22±0.42
20:3n-9	4.7±0.28	0.61±0.04	3.02±1.27
22:1n-9	0.22±0.04	<0.1	<0.1
24:1n-9	0.21±0.02	0.46±0.01	0.44±0.03
	N-6 and n-3 PUFAs		
18:2n-6	3.9±0.79	3.59±0.14	7.02±2.2
20:2n-6	0.03±0.009	0.16±0.003	0.06±0.02
20:3n-6	0.97±0.05	0.58±0.05	3.65±0.33
20:4n-6	12.04±0.54	1.1±0.04	3.17±0.19
22:4n-6	0.67±0.07	0.05±0.009	0.78±0.09
22:5n-6	0.34±0.03	0.05±0.006	0.33±0.1
20:5n-3	0.68±0.05	0.85±0.02	0.82±0.03
22:5n-3	0.66±0.11	0.09±0.01	1.03±0.17
22:6n-3	2.07±0.16	0.2±0.01	3.08±0.58

4.4 Effect of NGF-induced cell differentiation on n-3 and n-6 PUFA metabolism

4.4.1 Procedures

To investigate the effect of NGF addition on n-3 PUFA and n-6 PUFA metabolism, cells were grown in 25cm^2 flasks, incubated at 37°C and 5% CO_2. After one day, the growth medium was removed and replaced with DMEM containing 1% FBS in the presence of NGF (100 ng/ml) and ALA, EPA, DHA or LA for short or long incubation periods as described in section 2.4.3. Cellular lipids were extracted with hexane/isopropanol (3/2 by vol) and subjected to FA analysis using combined one-dimensional TLC/ GC as detailed in section 2.5.

4.4.2 Results

4.4.2.1 Effect of NGF-induced cell differentiation on conversion and esterification of n-3 ALA metabolites into cellular EPG and SPG

For time-course studies, NGF-challenged PC12 cells were subjected to the n-3 ALA precursor (20 μM) for designated time periods (up to 48 h). Cellular lipids were extracted and FA content was profiled following the protocol described in section 4.3. In NGF-differentiated PC12 cells, the percentage of ALA incorporated and the n-3 PUFA intermediates EPA, DPA and DHA biosynthesized from ALA in a time-dependent manner are shown in EPG (Figure 4.2 panel a), (SPG) (Figure 4.2 panel b). In EPG species (panel a), apart from 20:5n-3, a drastic decline in 22:5n-3 and 22:6n-3 was noted within the first 12

h after adding NGF. After 24 h, the levels of 22:5n-3 and 22:6n-3 were reduced by approximately 38% and 46% ($p<0.05$) compared with NGF-untreated cells (see Figure 3.3), respectively, whereas conversion into 20:5n-3 was not affected. By 48 h, the reduction in DHA approached 70% ($p<0.05$).

In contrast, NGF caused only a small reduction (18%) in the level of 22:6n-3 in SPG species (panel b), at 24h. Interestingly, ALA levels in SPG of NGF-treated cells remained relatively high compared with NGF untreated cells. These data suggest that the elongation/desaturation from 20:5n-3 to 22:6n-3 is robustly impaired as a result of adding NGF.

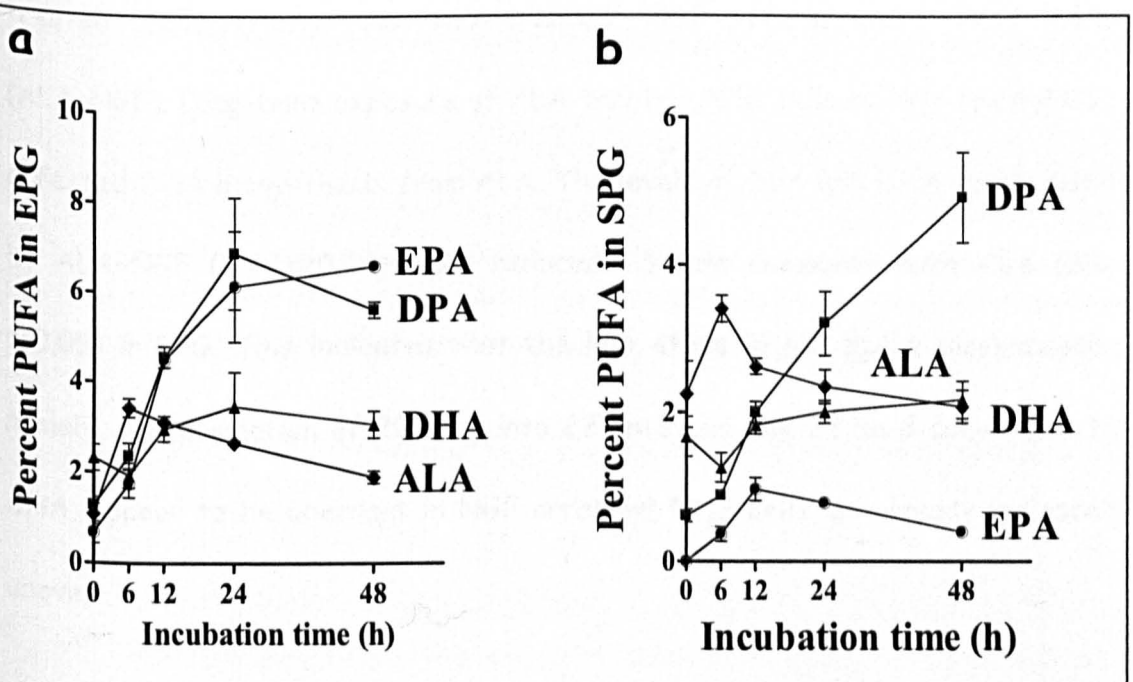


Figure 4.2: Time course of conversion and esterification of ALA metabolites into cellular GPLs after treatment with NGF. PC12 cells were incubated for designated time periods (up to 48 h), in the presence of 20 μM ALA and 100 ng/ml NGF. After 3 days, the FAME components in different GPLs was determined. The values, expressed as the % of FAMEs, are averages ± SEM of triplicate cultures of three independent experiments. * ($p<0.05$) indicates a significant difference for non-supplemented cells (time 0).

4.4.2.2 Effect of NGF-induced cell differentiation on the conversion and esterification of ALA metabolites into cellular EPG after prolonged incubation

In order to confirm the results obtained from the time course studies, PC12 cells were subjected to >4 days incubation with or without NGF (100 ng/ml) and ALA (20 μM). The n-3 PUFA profile in EPG was determined as described previously (see section 4.3).

Figure 4.3 illustrates the percentages of EPA, DPA and DHA in EPG species in non-supplemented (non suppl.), NGF-differentiated PC12 cells (NGF), ALA-treated cells (ALA) and ALA-treated NGF-differentiated PC12 cells (ALA+NGF). Long-term exposure of ALA-treated PC12 cells to NGF (ALA+NGF) affected DHA biosynthesis from ALA. The levels of DPA and DHA synthesised by ALA+NGF (5%; $p<0.05$) were reduced 1.5-fold compared with ALA (8%; $p<0.05$) in EPG. This indicates that the last steps in n-3 PUFA biosynthesis, namely, the elongation of 20:5n-3 into 22:5n-3 and the 22:5n-3 conversion to DHA, appear to be aberrant in NGF-arrested PC12 cells, as already indicated above.

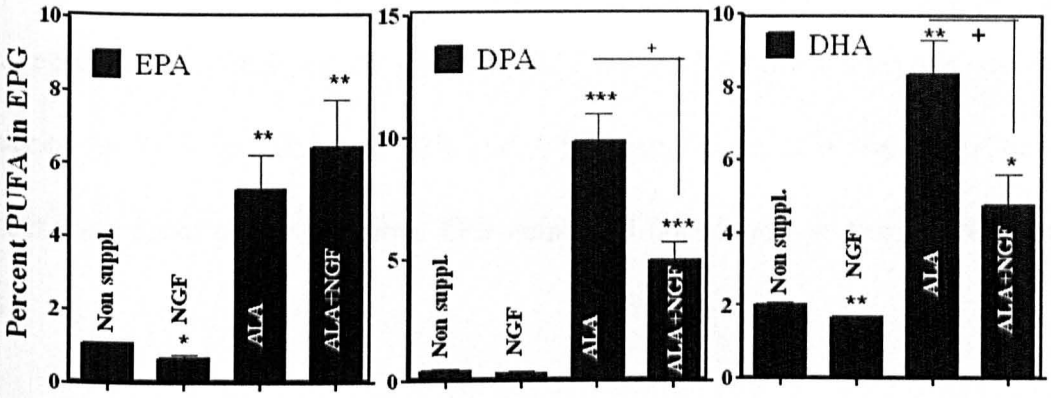


Figure 4.3: Effect of long-term NGF-induced cell differentiation on the conversion and esterification of ALA metabolites into EPG. PC12 cells grown under similar conditions as described in Figure 4.2 were supplemented for >4 days with 20 μM ALA in the absence or presence of 100 ng/ml NGF in 1% FBS. After 3 days, cellular lipids were extracted and the FA profile from EPG was determined. The figure, which depicts the % of FAMEs in the major n-3 series, are averages ± SEM, which were obtained from triplicate cultures of three independent experiments. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significant difference for control untreated cells (Non suppl.). + ($p<0.05$), ++ ($p<0.005$), +++ ($p<0.001$) indicate a significant difference between non-NGF and NGF-differentiated cells.

4.4.2.3 Effect of NGF-induced cell differentiation on the conversion and esterification of EPA metabolites into cellular EPG after prolonged incubation

From the previous experiments, we concluded that NGF appears to block the conversion step from EPA to DHA when cells were fed with ALA. Therefore, we decided to evaluate the generality of this phenomenon by directly adding the EPA precursor to the PC12 cells.

To further examine this issue, increasing supplements of EPA (10-40 μM) were given to PC12 cells in the presence or absence of NGF (100 ng/ml) for 3 days, and cellular lipids were extracted as described in section 4.3. As shown in

Figure 4.4, there was a general decline in the amount of EPA higher metabolites incorporated into EPG. At 25 μM EPA, 22:5n-3 and 22:6n-3 biosynthesis was reduced by 24% ($p<0.05$) and 42% ($p<0.05$), respectively. On the other hand, direct incorporation of EPA into EPG remained unchanged in the presence of NGF.

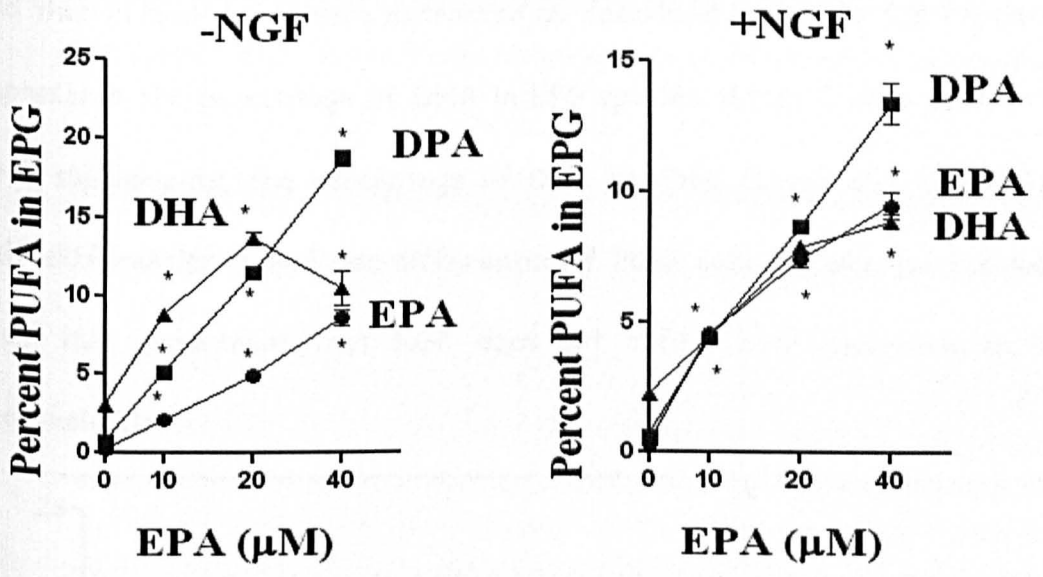


Figure 4.4: Effect of NGF on the conversion and esterification of EPA into cellular EPG. PC12 cells grown under similar conditions as described in Figure 4.2 were supplemented for 3 days with n-3 EPA (0 - 40 μM) in the absence or presence of 100 ng/ml NGF in 1% FBS. After incubation, the FAME components in EPG was determined. * ($p<0.05$) indicates a significance difference for control untreated cells (Non-suppl.). The figure, which depicts the % of FAMEs of the major n-3 series, are averages ± SEM, which were obtained from triplicate cultures of three independent experiments. * ($p<0.05$) indicates a significant difference for non-supplemented cells (time 0).

4.4.4.4 Effect of NGF-induced cell differentiation on esterification of DHA into cellular EPG after prolonged incubation

From the data above, it is clear that NGF affects the n-3 elongation/desaturation pathway; however, NGF could also be interfering with DHA esterification into phospholipids. To rule out this possibility, PC12 cells were incubated without or with NGF (100 ng/ml) and DHA (20 μM) for 2 days, and then cellular lipids were extracted as described in section 4.3. Figure 4.5 represents the percentage of DHA in EPG species. After 2 days with direct DHA supplements, the percentage of DHA reached almost 20% ($p<0.001$) in NGF-differentiated and non-differentiated PC12 cells. It can be concluded from this experiment that NGF does not affect DHA incorporation into phospholipids.

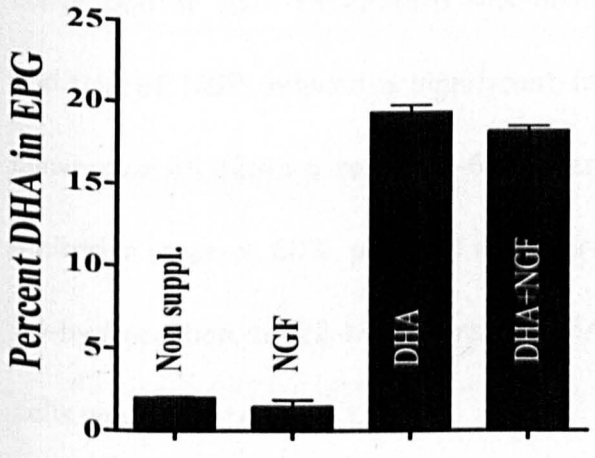


Figure 4.5: NGF does not affect DHA incorporation. PC12 cells were supplemented for 2 days with 20 μM DHA in the absence or presence of 100 ng/ml NGF in 1% FBS. After incubation, the FAME components in EPG was determined. The medium was removed and cellular GPLs were separated and processed as described in Materials and Methods. The values, expressed as the % of FAMEs, are averages ± SEM, which were obtained from triplicate cultures of three independent experiments. *** ($p<0.001$) indicates a significance difference for control untreated cells (Non suppl.).

4.4.4.5 Effect of NGF-induced cell differentiation on the conversion and esterification of LA metabolites into cellular EPG after prolonged incubation

It has been long established that n-3 PUFA and n-6 PUFA share the same enzymes for PUFA biosynthesis (Brenner and Peluffo, 1966). Therefore, the common metabolic pathway for n-3 PUFA and n-6 PUFA elongation/desaturation prompted us to study the biosynthesis of n-6 PUFA from the LA precursor. Therefore, PC12 cells were incubated with or without NGF (100 ng/ml) and LA (20 μM) for 3 days, and then cellular lipids were extracted as described in section 4.3.

As illustrated in Figure 4.6, while typically, the conversion of n-6 metabolites is by far smaller than that of n-3 metabolites, clearly, adding NGF strongly inhibited the 20- to 22-hydrocarbon-long FA elongation. In contrast, the conversion of ARA to 22:4n-6 was doubled in the presence of LA, and the addition of NGF induced a significant (approx. 33%; $p<0.001$) reduction. The conversion of 22:4n-6 to 22:5n-6 via beta-oxidation induced an even stronger inhibition (approx. 60%, $p<0.001$) in the presence of NGF. Reduced elongation of 20-hydrocarbon to 22-hydrocarbon-LCFAs by NGF was also evident in PC12 cells supplemented with LA.

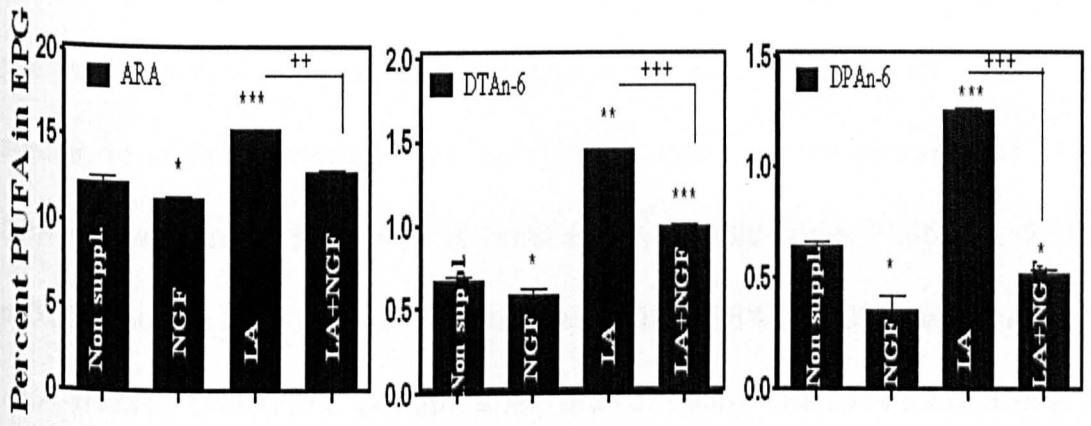


Figure 4.6: Effect of NGF-induced cell differentiation on the conversion and esterification of LA metabolites into EPG. PC12 cells were supplemented for 3 days with 20 μM LA in the absence or presence of 100 ng/ml NGF in 1% FBS. After incubation, the FAME components in EPG was determined. The figure, which depicts the % of FAMEs in the major n-6 series, are averages ± SEM, which were obtained from triplicate cultures of three independent experiments. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significant difference for control untreated cells (Non suppl.). + ($p<0.05$), ++ ($p<0.005$), +++ ($p<0.001$) indicate a significant difference between non-NGF and NGF-differentiated cells.

4.5 Effect of bFGF-induced cell differentiation on the conversion and esterification of n-3 ALA metabolites into cellular EPG

The effect of another differentiation factor, basic fibroblast growth factor (bFGF), on n-3 biosynthesis was studied. As already noted in Figure 4.1, bFGF differentiates PC12 cells morphologically, manifested by a transient outgrowth of neurites (after 72h, neurite outgrowth ceased).

Therefore, bFGF (50 ng/ml) was added along with 20 μM ALA to cells for 3 days, and cellular lipids were extracted as described in section 4.3.

The levels of each n-3 intermediary metabolite biosynthesised from an n-3 precursor in EPG in PC12 cells differentiated with bFGF (ALA+ bFGF) are similar to undifferentiated PC12 cells (ALA). Again, in the presence of NGF, DPA biosynthesised from ALA is reduced by 1.7-fold (from 11.35% to 7.11%; $p<0.05$) and the DHA level is approximately half (4.28%; $p<0.05$), compared with ALA-treated cells (7.5%; $p<0.05$). BFGF did not inhibit the DHA biosynthesis in PC12 cells as compared with NGF (Figure 4.7).

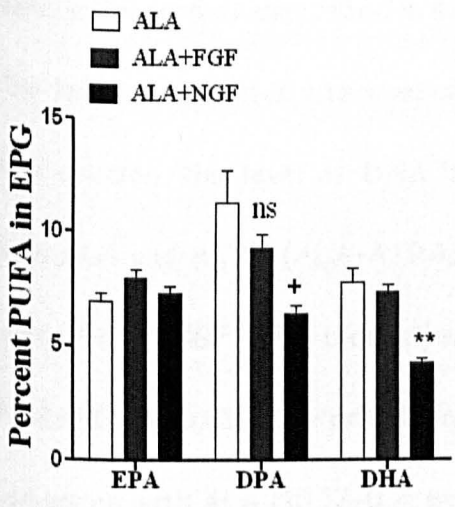


Figure 4.7: bFGF does not inhibit ALA conversion into DHA in EPG fatty acid profiles. PC12 cells were pre-incubated in DMEM containing 1% FBS medium in the presence of 50 ng/ml bFGF (ALA+FGF) or 100 ng/ml NGF for 12 h and for 3 days with 20μM ALA and NGF. After incubation, the FAME components in EPG was determined. + ($p<0.05$) indicates a significance difference for ALA-treated cells (ALA) for the percentage of DPA. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significance difference for ALA-treated cells (ALA) for the percentage of DHA.

4.6 Retinoic acid (ATRA) slightly enhances ALA conversion to DHA into cellular EPG

Unlike NGF, ATRA, a compound known to induce neuronal differentiation, has been shown in PC12 cells to enhance the expression of several neural genes without affecting neurite outgrowth. Therefore, the effect of RA, a naturally occurring metabolite of vitamin A, on n-3 FA elongation/desaturation pathway was investigated. ATRA at different concentrations (1 or 10 μM) or 100 ng/ml NGF was added with 20 μM ALA to the medium for 3 days, and cellular lipids were extracted as described in section 4.3.

The level of each n-3 intermediary metabolite in EPG is shown in Figure 4.8. In EPG species, the level of DHA biosynthesised is higher in PC12 cells treated with ALA and ATRA (ALA+ATRA) than in ALA-treated PC12 cells. Again, in the presence of NGF, DPA biosynthesised from ALA is reduced by 1.5-fold (from 9.07±0.18 to 6.31±0.18; $p<0.05$) and the DHA level is half (4.82±0.13; $p<0.05$), compared with ALA (10.17±0.2; $p<0.05$), or with ALA+ATRA-treated cells. ATRA did not inhibit the DHA biosynthesis in PC12 cells as compared with NGF; it slightly enhanced it.

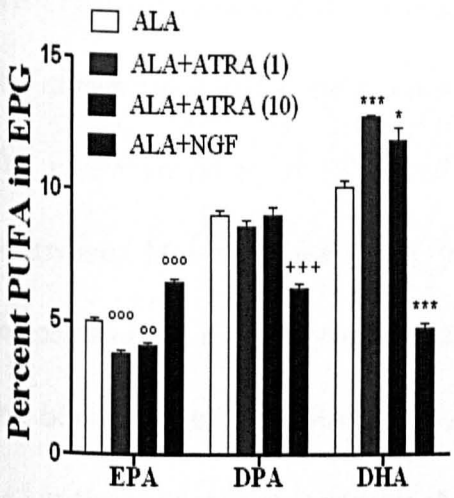


Figure 4.8: Retinoic acid (ATRA) does not inhibit of ALA conversion into DHA in EPG fatty acid profiles. PC12 cells was incubated in the presence of 20 μM ALA, or a combination of both ALA and 1 μM or 10 μM ATRA (ALA+ATRA), or a combination of both ALA and 100 ng/ml NGF (ALA+NGF) for 72 h. After 3 days, the FAME components in different GPLs was determined. °° ($p<0.005$), °°° ($p<0.001$) indicate a significance difference for ALA- treated cells (ALA) for the percentage of EPA. +++ ($p<0.001$) indicates a significance difference for ALA-treated cells (ALA) for the percentage of DPA. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) indicate a significance difference for ALA-treated cells (ALA) for the percentage of DHA.

4.7 Discussion

From the morphological point of view, both NGF and bFGF stimulate neurite outgrowth in PC12 cells. However, there is a notable dissimilarity in the ways PC12 cells respond to these different signalling molecules. The neurites that are formed in response to NGF are stable in comparison with those induced by bFGF. As in the case of the latter, the neurites started to retract within three days. This observation is in agreement with a previous report (Togari et al. (1985)), although Rydel and Greene (1987) found no evidence of deterioration.

With respect to adding RA, we also did not notice any neurite expansion; as was also shown by Scheibe and coworkers (1991).

The characteristics of PC12 cells differentiated by these agents have been described. However, we have little information until now about the lipid composition of differentiated PC12 cells. In these original studies, we tackle the biosynthesis of PUFA from various essential FA precursors (i.e. ALA and LA) in the presence or absence of NGF.

Clearly, despite the fact that NGF induces morphological changes in PC12 cells (i.e. membrane reshuffling) after a three-day exposure with NGF, the overall detailed FA profile remains unchanged (Table 4.1). A similar observation was reported by Traynor and coworkers (1982). They reported no significant modification of the total phospholipid composition of the cells and in the FA content of individual phospholipids, after NGF treatment for 3 days. In contrast, Ikemoto and coworkers (1997) showed that NGF treatment increased EPG synthesis and the sum of n-3 PUFAs and consequently decreased the n-6/n-3 ratio of the phospholipid acyl chains. For other cell lines, there are reports indicating that the differentiation process is associated with modifications of the lipid metabolism. For instance, differentiated Cl300 N18 neuroblastoma cells exhibited elevated EPG and SPG levels, an increase in the saturated FA content in IPG, EPG and CPG and also a decrease in the unsaturated FA content compared with undifferentiated cells (Gulaya et al.,

1989). In a similar study, N1E-115 neuroblastoma cell differentiation induced by DMSO decreased DHA proportions (Murphy and Horrocks, 1993).

In chapter three, PC12 cells were shown to have the ability to synthesise all the n-3 or n-6 (to a lesser extent) metabolites from their precursor. In this chapter, we examined whether various differentiation factors such as NGF, bFGF or RA could influence this ability. First of all, the consequences of ALA and LA metabolism in NGF-treated, n-3 PUFA-deficient PC12 cells were investigated.

Supplements of ALA were effectively converted to 20:5n-3 by NGF-treated cells. Conversion of the precursor to EPA is time-dependent, similarly to the results obtained for proliferating cells (Figures 4.1 and 3.3). In marked contrast to the latter, NGF-treated cells were unable to elongate and desaturate 20-hydrocarbon-long chains (EPA) to 22-hydrocarbon-long chain metabolites (DPA and DHA). In particular, conversion of ALA to 22:6n-3, as manifested by its levels in both EPG and SPG, was strongly reduced. Incubations of longer duration (4.74±0.86; $p<0.05$), however, were manifested by a slight increase (compared to 3.06±0.29; $p<0.05$ at 48h), in the production of DHA after adding ALA, yet the levels were still below those attained with ALA (8.32±0.95; $p<0.05$) (Figure 4.2 and Figure 4.3). This striking observation strongly suggests that NGF blocked the formation of DPA and DHA but not that of EPA. The possible site of NGF action is illustrated below in the metabolic chart of long-chain FA elongation/desaturation.

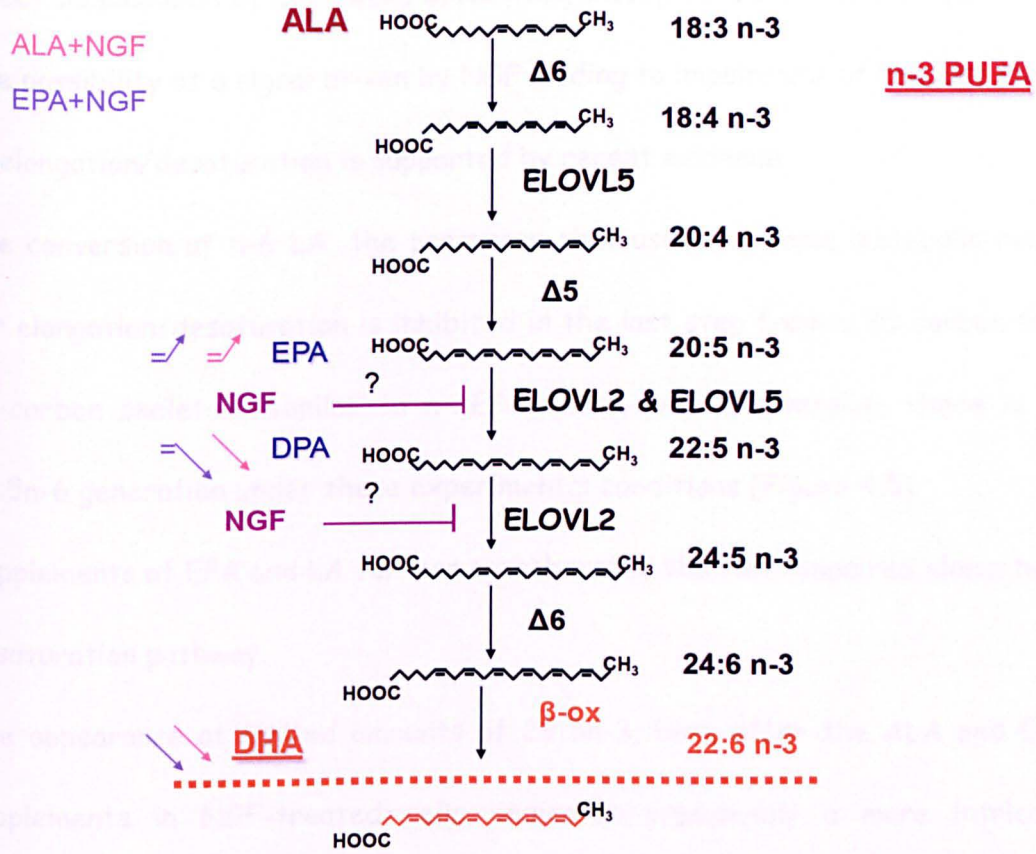


Figure 4.9: Schematic illustration of the results obtained for the n-3 PUFA metabolism in proliferating and NGF-differentiated PC12 cells. Δ6 and Δ5 represent desaturase enzymes; ELOVL2 and ELOVL5 elongase activities and β-ox peroxisomal β oxidation. Abbreviations: ALA, α-linolenic acid; EPA, eicosapentaenoic; DHA, docosahexaenoic acid; NGF, nerve growth factor.

A more refined experiment to determine the possible site of inhibition was performed using EPA supplements (Figure 4.3). Notably, EPA was little converted to DPA but not to DHA even after 3 days of incubation. Under these conditions the peroxisomal decarboxylation of 24-carbon to 22-carbon DHA appears impaired. Another possibility would be that esterification of DHA into

GPLs is inhibited by NGF. However, this does not seem to be the case because direct supplements of DHA were effectively incorporated into EPG (Figure 4.4). The possibility of a signal driven by NGF leading to impairment of the last steps of elongation/desaturation is supported by recent evidence.

The conversion of n-6 LA, the precursor that uses the same metabolic route for elongation/desaturation is inhibited in the last step from a 20-carbon to a 22-carbon skeleton. Similar to n-3EPA and n-3ALA conversion, there is no 22:5n-6 generation under these experimental conditions (Figure 4.5).

Supplements of EPA and LA further corroborated the NGF-impaired elongation desaturation pathway.

The appearance of limited amounts of 22:5n-3, both after the ALA and EPA supplements in NGF-treated cells, indicates presumably a more intricate metabolic regulation. One possible explanation might be that the residual activity of ELOVL5, the enzyme present in rodents, which is responsible for the 18- hydrocarbon to 20-hydrocarbon chain length elongation, may also enable the elongation of the 20-hydrocarbon skeleton (Leonard et al., 2004). From our studies, it is tempting to hypothesise that NGF may have activated an intrinsic cellular signal to shut off the PUFA elongation/desaturation pathway as part of a phenotypical expression of neuronal characteristics. As such, it is possible that NGF may affect local signals such as estrogen receptor activation (Merot et al., 2009) to regulate PUFA metabolism, a hypothesis that remains to be examined.

To further examine whether NGF inhibition is a general phenomenon that might be shared by other compounds inducing PC12 cell differentiation, we examined the impact of another growth factor, bFGF, on PUFA conversion by PC12 cells. BFGF induced a neuronal phenotype in PC12 cells, confirmed by the occurrence of neurite outgrowth (Figure 4.1). BFGF has been examined in the presence of ALA supplements. In bFGF-differentiated cells, ALA conversion into DPA and DHA levels in EPG were similar to the ones in undifferentiated cells. Compared with NGF-differentiated PC12 cells, bFGF did not inhibit the conversion pathway (Figure 4.6).

ATRA, another compound inducing PC12 cell differentiation, has been examined in the presence of ALA supplements. ATRA did not induce neurite extension (Figure 4.1). It was reported to increase the low-affinity receptor (p75) expression in PC12 cells (Scheibe and Wagner, 1992, Cosgaya et al., 1996) similarly to NGF (Miller et al., 1991). Like NGF, RA affected cell proliferation and induced several gene transcripts. ALA conversion into DPA and DHA, in the presence of ATRA (1-10 μM), slightly enhanced DHA levels in EPG rather than inhibited them (Figure 4.7). In that respect, ATRA cannot mimic the action of NGF on an ALA elongation/desaturation enzymatic cascade. Similar to our findings, ATRA increased the elongation/desaturation of n-6LA and induced neurite outgrowth in a human neuroblastoma (NB) cell line, whereas the conversion of EPA was not affected (Petroni et al., 1996a, b).

In conclusion, the fact that NGF plays a regulatory role in PUFA biosynthesis is a unique property for NGF and this can be assessed in a wider context to help resolve some controversial findings regarding how neuronal and glial cells contribute to the endogenous capacity of the brain in synthesising its own PUFA constituents.

In such a scenario, we hypothesise that all neuroblasts and stem cells in the subventricular zone in the developing nervous system have full capacity to synthesise PUFA from essential FA precursors. Upon exposure to growth factors or neuromodulator-induced terminal neural differentiation, neurons may cease to produce their own DHA and consequently become highly dependent on outside sources such as astrocytes. The latter seems to retain this capacity throughout life.

Conclusions

1. NGF and bFGF induce a morphological differentiation of PC12 cells as observed by the induction of neurite outgrowths. No such behavior occurred with ATRA treatment.
2. The addition of NGF causes a striking inhibition of the last steps in elongation/desaturation of ALA and LA suggesting a possible interference with the last stages of the 22-hydrocarbon long chain biosynthesis.

3. BFGF and ATRA did not induce any inhibition in the elongation/desaturation of ALA.

Chapter 5

The effects of long-chain n-3 and n-6 supplements and NGF on gene expression

5.1 Introduction

In the previous chapters, we provided novel evidence that proliferating PC12 cells can convert more efficiently ALA into DHA than LA into ARA metabolites. On the other hand, we found that NGF-differentiated cells were robustly inhibited ($p<0.05$) in their ability to convert either precursor to its corresponding metabolite DHA and ARA, respectively.

We reasoned that the cause of this inhibition is an interference with the last stages of the 22-hydrocarbon-long chain intermediate biosynthesis of the n-3 and n-6 PUFA families. This biosynthetic pathway consists of several key catalytic reactions including two elongases (ELOVL5 and ELOVL2), a desaturase (delta6 desaturase) and peroxisomal beta oxidation catalytic activity (PPARα, Peroxisome proliferator-activated receptor alpha). Finding such a disparity in the conversion of PUFAs in response to different signals may be caused by differential regulation of specific genes that encode for the enzymes indicated above and/or their affinities to substrates. Therefore, in the first stage, we set out to examine in detail the effects of FA supplements on the expression of several of the above transcripts in proliferating or NGF-differentiated cells using quantitative real-time PCR (qRT-PCR) analysis.

The impact of FA supplements on elongase and desaturase gene expressions has been thoroughly evaluated previously in animals fed with different PUFA-enriched diets. *In vivo* studies have indicated that both elongase and

desaturase expression are under tight physiological regulation. Wang and co-workers (Wang et al., 2005; 2006), for example, have shown that the hepatic Elovl5 expression level was reduced by 50% to 75% in mice fed with a high-fat diet (60% of calories as fat). However, the abundance of Elovl2 and delta6 desaturase (Fads2) mRNA did not change in mice fed with a high-fat diet compared with mice fed with a low fat diet (10% of calories as fat). These results indicated that Elovl5 expression is regulated by an n-3 PUFA-enriched diet and a high fat diet, in contrast to Elovl2, which was not physiologically controlled (Wang et al., 2005; 2006). In a mouse model, other laboratories showed that desaturases were affected by the diet; high PUFA diets decreased hepatic delta6 and delta5 desaturase mRNA expression levels and correspondingly, their enzymatic activities (Nakamura et al., 2000).

It is well established that several PUFAs appear as regulators of the gene expression of key steps in FA metabolism via transcriptional factors and peroxisome proliferator-activated receptors (PPARs) (Jump et al., 2005). The latter are members of the nuclear hormone receptor superfamily. Regarding this stage, three isoforms of PPARs (PPARα, PPARγ and PPARβ) have been identified. PPARs possess in their structure two domains: a ligand-binding domain and a DNA-binding domain. PPARs are activated by binding to specific ligands such as FA (polyunsaturated, conjugated and branched), eicosanoids and the synthetic ligand (4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid

(WY14643). Upon activation, PPARs heterodimerise with an activated retinoid X receptor (RXR), which has been previously activated by binding to its ligand, 9-cis retinoic acid (vitamin A). The activated PPAR-RXR complex can bind to specific DNA sequences (peroxisomal proliferator response elements, PPREs) on the promoter of target genes and as such can regulate their transcriptional expression. PPARα has been shown to regulate a wide variety of genes encoding those proteins involved in FA transport (Wierzbicki et al., 2009; Martin et al., 1997; Fourcade et al., 2001), FA binding proteins (Wierzbicki et al., 2009), FA-acyl-CoA synthesis (Martin et al., 1997), FA uptake and β-oxidation (Ribet et al., 2010) and peroxisomal fatty acid β-oxidation enzymes, including FA acyl-CoA oxidase (SCOX) and dehydrogenase-bifunctional protein (DBP) (Reddy et al., 1986). Furthermore, PPARα also plays a crucial role in FA elongation and desaturation. Wang et al (2005; 2006) reported that PPARα regulates elongases and desaturases. Studies have been carried out in wild-type mice and PPARα-deficient mice, both fed with a diet containing the PPARα ligand, WY14643. In wild-type mice, WY14643 induces hepatic Elovl5 elongase and delta6 desaturase expression; however, in PPARα-deficient mice WY14643 had no effect (Wang et al., 2005; 2006).

The role of n-3PUFA, n-6PUFA and their corresponding eicosanoid metabolites, functioning as ligands and activators of PPARα, has been previously examined. Kliewer et al. (1994), using mammalian cells co-transfected with a PPARα

expression vector containing the luciferase reporter gene, showed that PPARα was highly activated by WY14643 and to a lesser extent by LA. A few years later the same group (Kliewer et al., 1997) demonstrated that saturated (palmitic acid), monounsaturated (OA) FAs, PUFAs (LA, ALA, and ARA) and eicosanoids (cyclooxygenase and lipoxygenase metabolites) act as ligands for PPARα. Other studies, using a similar approach, also demonstrated that the activation of PPARα was strongest in response to PUFAs followed by monounsaturated and saturated FA treatments (Keller et al., 1993). They also showed that there was no difference in the activation of PPARα between the n-6 PUFAs (ARA and LA) and n-3 PUFAs (DHA, EPA and ALA) treatments.

Objectives

The objectives of this series of experiments employing molecular biology techniques were to investigate the following:

•The basal levels of Elovl5, Elovl2 and Fads2 gene transcripts encoding for ELOVL5 elongase, ELOVL2 elongase and Delta6 desaturase (D6D), respectively, and Ppara encoding for the nuclear transcription factor PPARα in proliferating PC12 cells.

• The levels of Elovl5, Elovl2, Fads2 and Ppara gene expressions following n-3 ALA and n-6 LA supplements.

• The levels of Elovl5, Elovl2, Fads2 and Ppara gene expression following NGF-induced cell differentiation.

• The levels of Elovl5, Elovl2, Fads2 and Ppara gene expressions following a combination of PUFA and NGF.

5.2 The effect of NGF and n-3 ALA on the mRNA expression of PUFA pathway genes

5.2.1 Procedures

In order to determine whether the differentiation growth factor NGF or n-3 PUFA supplements induced changes in the expression of Elovl5, Elovl2, Fads2 and Ppara genes, we performed quantitative real-time PCR (qRT-PCR) analysis. PC12 cells were exposed for either 72 h or 96 h to NGF (100 ng/ml) and to n-3ALA (20 μM), and the RNA of the cells was extracted, converted to cDNA and then qRT-PCR was performed according to the protocol described in Materials and Methods (section 2.6). While at 6 and 24 h, the mRNA levels greatly fluctuated, but at 72 and 96 h, the values were stable and this enabled us to assess the mRNA abundance following exposure to NGF and n-3 PUFA. The expression level of each target gene in treated and control cells was normalised to the housekeeping gene (beta actin), whereas the expression of genes in treated cells was reported relative to control cells (considered as one).

5.2.2 Results

Figure 5.1 illustrates the effects of n-3 ALA supplements on mRNA levels in PC12 cells in the absence or presence of NGF. After 3 days following NGF supplements, PC12 cells exhibited a substantial elevation in the levels of Elovl5 (Fig 5.1a), Elovl2 (Fig 5.1b), Fads2 (Fig 5.1c) and Ppara (Fig 5.1d) compared with non-supplemented (CON) cells. In NGF-differentiated cells, the gene expression level of Elovl5 was up-regulated by 3.10 ± 0.39-fold (3.10 ± 0.39 in NGF versus 1 in CON; $p<0.005$), of Elovl2 by 1.73 ± 0.19-fold ($p<0.05$), of Fads2 by 2.66 ± 0.18-fold ($p<0.001$), and also with the transcription factor Ppara by 2.29 ± 0.30-fold ($p<0.001$).

ALA supplement alone did not significantly increase the expression in two out of four transcripts, Elovl2 and Ppara (panels b and d). Interestingly, a combination of the two supplements (NGF and ALA) was not additive, but in contrast, Elovl5 and Elovl2 gene expressions were suppressed in comparison with the stimulation attained by NGF. NGF-differentiated cells with modified lipid content exhibited an up-regulation of 1.43 ± 0.22-fold ($p<0.05$) and 1.84 ± 0.39-fold ($p<0.005$) in Elovl2 and Ppara mRNA levels compared with 1.73 ± 0.19-fold ($p<0.05$) and 2.29 ± 0.30-fold ($p<0.001$), respectively in NGF-differentiated cells.

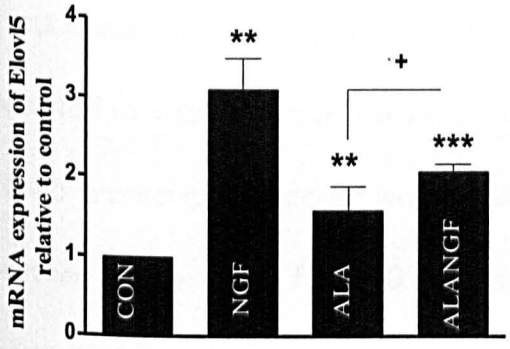


Figure 5.1a: Fatty acid elongase5, Elovl5

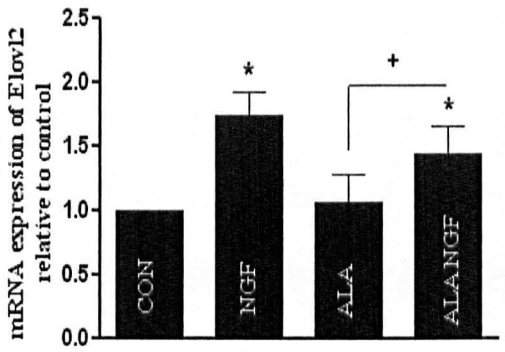


Figure 5.1b: Fatty acid elongase2, Elovl2

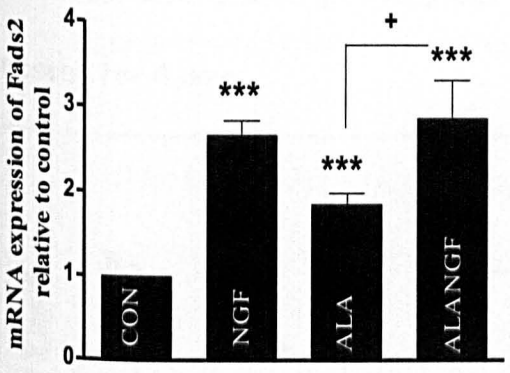


Figure 5.1c: Fatty acid desaturase 6, Fads2

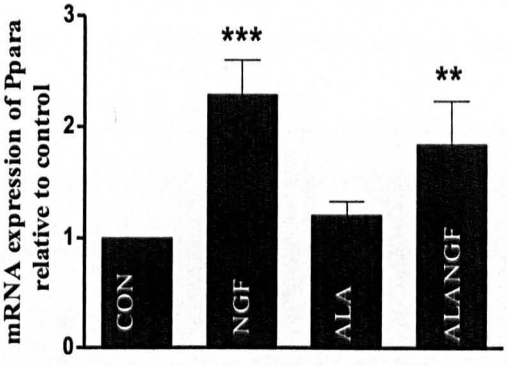


Figure 5.1d: Peroxisome proliferator-activated receptor alpha, Ppara

Figure 5.1: Transcripts of Elovl5, Elovl2, Fads2 and Ppara expression after ALA and NGF treatments. PC12 cells were subjected to 20 μM ALA for 3 days in the presence or absence of 100 ng/ml NGF or a combination of the two. Cells were harvested and qRT-PCR analysis was performed. Values are average ± SEM of triplicates. The experiment was repeated three times on separate occasions. ** ($p<0.005$), *** ($p<0.001$) indicate a significant difference for control untreated cells (con). + ($p<0.05$) indicates a significant difference between non-NGF and NGF-differentiated cells.

Incubating for a longer duration (96 h) in the presence of NGF (Figure 5.2) resulted in a sustained increase (3.56 ± 0.51; $p<0.001$) in the expression of the Elovl5 transcript compared with a 72 h incubation. In the presence of ALA, no further change (1.55 fold ± 0.39; Ns compared with 1.58 ± 0.29-fold $p<0.005$) was noted. Interestingly, a relative but significant increase (3.05 ± 0.67; $p<0.05$ vs. 2.06 ± 0.10 $p<0.001$), but nevertheless still below the 72 h value (3.10 ± 0.39; $p<0.05$ vs. 3.56 ± 0.51; $p<0.001$) was observed when both NGF and ALA were present for 4 days.

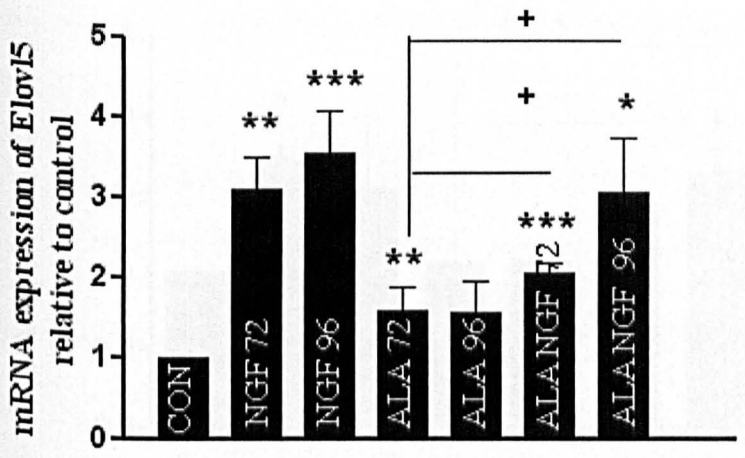


Figure 5.2 Transcripts of Elovl5 expression after 72h or 96h ALA and NGF treatments. PC12 cells were subjected to 20 μM ALA for 72h or 96h in the presence or absence of 100 ng/ml NGF or a combination of the two. Cells were harvested and qRT-PCR analysis was performed. Values are average ± SEM of triplicates. The experiment was repeated three times on separate occasions. ** ($p<0.005$), *** ($p<0.001$) indicate a significant difference for control untreated cells (con). + ($p<0.05$) indicates a significant difference between non-NGF and NGF-differentiated cells.

The Elovl2 mRNA level also was quantified in cells treated for 96 h (Figure 5.3) and the values were practically identical to those seen after 72h incubation. NGF alone sustained the levels between 1.53 and 1.73-fold, whereas ALA alone did not at all change the expression of Elovl2 mRNA. A major difference with Elovl5 expression was, however, that ALA was unable to suppress the NGF-induced expression of the Elovl2 transcript (1.65 ± 0.14-fold; $p<0.05$ vs. 1.43 ± 0.22; $p<0.05$).

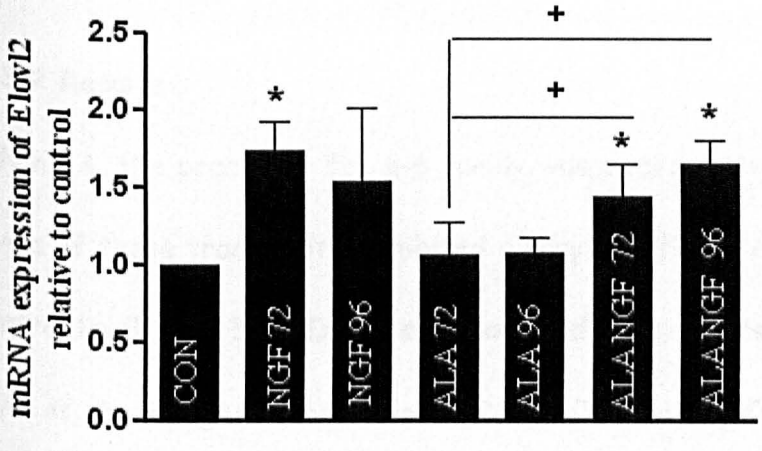


Figure 5.3: Transcripts of Elovl2 after 72h or 96h ALA and NGF treatments. PC12 cells were subjected to 20 μM ALA for 72h or 96h in the presence or absence of 100 ng/ml NGF or a combination of the two. Cells were harvested and qRT-PCR analysis was performed. Values are average ± SEM of triplicates. The experiment was repeated three times on separate occasions. ** ($p<0.005$), *** ($p<0.001$) indicate a significant difference for control untreated cells (con). + ($p<0.05$) indicates a significant difference between non-NGF and NGF-differentiated cells.

5.3 The effect of NGF and n-6 LA on mRNA expression of PUFA pathway genes

5.3.1 Procedures

PC12 cells were incubated with or without 20 μM of n-6 LA in the absence or presence of 100 ng/ml NGF for 72 h as described in section 2.4.3. The expression of these genes was assessed by qRT-PCR as described in section 2.6. The expression level of each target gene in treated and control cells was normalised to the housekeeping gene (beta actin), whereas the expression of genes in treated cells is reported relative to control cells (considered as one).

5.3.2 Results

When LA, the precursor for n-6 family was added instead of ALA, the mRNA levels of these transcripts exhibited a very significant enhancement above the control levels (Fig 5.4). In LA-supplemented cells, the relative gene expression level of the elongases rose (Elovl5, 2.31 ± 0.15-fold; $p<0.005$ and Elovl2, 1.71 ± 0.71-fold; $p<0.05$). LA also elevated the desaturase Fads2 and the transcription factor Ppara mRNA levels by 2.51 ± 0.41-fold; $p<0.005$ and 1.82 ± 0.08-fold; $p<0.005$.

Most interesting, however, in NGF-differentiated cells, three out of the four gene transcripts (Figure 5.4, panels a, b and d) did not change after LA supplements, suggesting that a different regulation mechanism exists for the

two FA precursors. There was no significant difference in the Elovl5 mRNA levels between the NGF and LA+NGF treatments (3.10 ± 0.39-fold; $p<0.005$ and 3.11 ± 0.25; $p<0.001$, respectively). Also, no statistically significant difference in Elovl2 mRNA levels, compared with LA-supplemented undifferentiated (1.71 ± 0.71-fold; $p<0.05$) and differentiated (1.95 ± 0.10-fold; $p<0.05$) cells (Figure 5.6) was observed. Moreover, LA did not significantly change the mRNA level of the NGF-up-regulated Ppara (2.29 ± 0.30-fold; $p<0.001$), as shown in LA+NGF-treated cells (2.56 ± 0.31-fold; $p<0.005$).

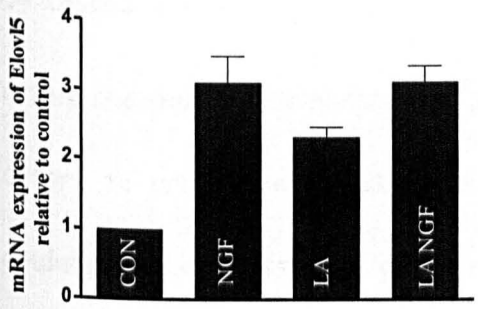


Figure 5.4a: Fatty acid elongase5, Elovl5

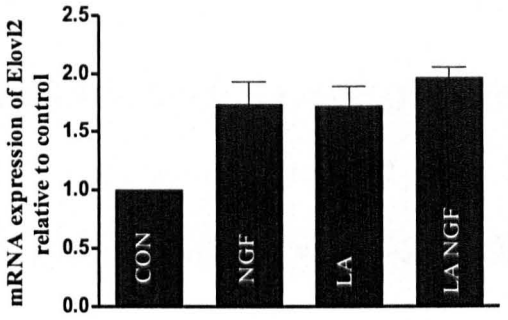


Figure 5.4b: Fatty acid elongase2, Elovl2

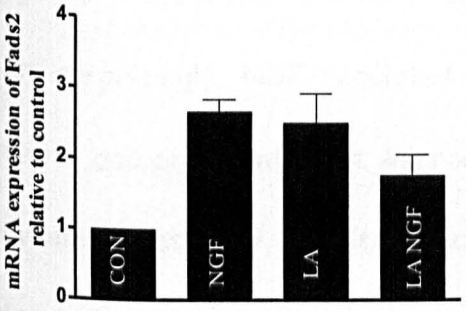


Figure 5.4c: Fatty acid desaturase 6, Fads2

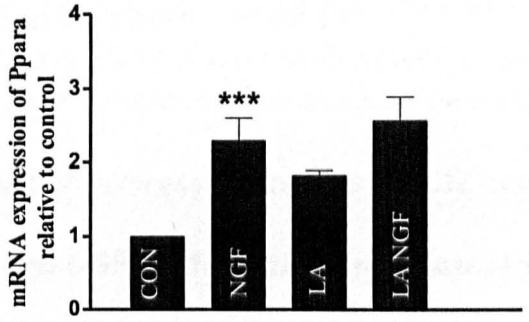


Figure 5.4d: Peroxisome proliferator-activated receptor alpha,Ppara

Figure 5.4: Transcripts of Elovl5, Elovl2, Fads2 and Ppara expression after n-6LA and NGF treatments. PC12 cells were subjected to 20 μM LA for 3 days in the presence or absence of 100 ng/ml NGF or a combination of the two. Cells were harvested and qRT-PCR analysis was performed. Values are average ± SEM of triplicates. The experiment was repeated three times on separate occasions. ** ($p<0.005$), *** ($p<0.001$) indicate a significant difference for control untreated cells (con). + ($p<0.05$) indicates a significant difference between non-NGF and NGF-differentiated cells.

5.4 Discussion

In this chapter, we demonstrated that NGF, one of the most powerful growth factors to arrest cell division and induce differentiation in PC12 cells, up-regulates the expression of gene transcripts associated with PUFA metabolism. To the best of our knowledge, there have been no reports concerning the expression of PUFA-associated genes in PC12 cells or in neuronal cells all together. This is the first time such studies have been carried out.

Not surprisingly, NGF regulates many gene transcripts involved in neuronal growth and differentiation. Microarray mRNA expression analysis in PC12 cells revealed that NGF and its precursor pro-NGF differentially modulate the expression of genes involved in several cellular mechanisms (D'Onofrio et al., 2011). Treatment for 1h or 4h with NGF modulates the expression of genes in the cell cycle, as well as DNA repair, intracellular trafficking/ synaptic activity, ionic transport and transcription factors. These sets of genes (except the transcription factor set) are common with NGF and pro-NGF treatments. However, whereas the transcription factor family is specific for the NGF treatment dataset, the lipid metabolism genes are specific for the pro-NGF set (D'Onofrio et al., 2011).

Works by others carried out in rat sympathetic neurons confirmed the regulation of genes by NGF. It was found that after 5 days NGF elevated up to 7-fold the mRNA levels of the p75 low-affinity NGF (LNGF) receptor and

tyrosine hydroxylase (TH) and up to 4-fold Ta1 α-tubulin (Ta1) mRNA (Ma et al., 1992). Treatment with NGF and also with FGF and EGF up-regulated NGF I-A mRNA in PC12 cells, a transcription factor that is homologous to the TFIIIA transcription factor (Cho et al., 1989).

Regulation of genes associated with PUFA metabolism has been studied mainly in cells of non-neuronal origin. For instance, PPARα was found to be expressed in rat primary hepatocytes (Pawar et al., 2003) and in Hek293 cells (Pawar et al., 2003), Elovl5 was found in primary hepatocytes (Wang et al., 2005), whereas Fads2 was expressed in the human promyelocytic cell line HL60 (Slagsvold et al., 2007) and in HepG2 cells (Tang et al., 2003). Tissue-specific expression of these FA elongases, desaturases and PPARα has been revealed by Northern analysis in rat and human species. In the adult rat, it was shown that PPARα was widely expressed in tissues with high lipid catabolism such as the liver, kidney, heart and adrenal gland (Kliewer et al., 1994). Elovl5, on the other hand, was detected in a broad array of rat tissues such as the liver, lung, brain, brown adipose tissue, skin and heart (Wang et al., 2005). In contrast, Elovl2 was only expressed in rat liver, lung, brain and kidney. Human delta 6 desaturase mRNA was found to be distributed in equal proportions in the liver, lung and heart but it is present in higher levels in the brain (Cho et al., 1999b).

A second novel finding documented in these studies pertains to the impact of FA supplementations on gene expression. Importantly, we showed that

significant differences exist between n-6 LA and n-3 ALA supplements with respect to the expression of the PUFA elongation/desaturation genes. The dietary effects on the mRNA expression levels for the elongase, desaturase and Ppara activities have been demonstrated in several *in vivo* and *vitro* studies. The emerging data reveal strong nutritional regulation, giving rise to the relatively novel field of nutrigenomics. Feeding rats with fish oil (diet enriched in n-3 PUFA (EPA,DPA and DHA) decreased the expression of delta5, delta6 desaturases and Elovl5 elongase mRNA expression by more than 50%, however, with no significant effect on Elovl2 expression (Wang et al., 2005). Nakamura et al. (2004) corroborated these studies by showing the suppression of all FA desaturase (delta5, delta6 and delta9 desaturases) expression in rats fed with a fish oil diet. These results were confirmed in n-3 PUFA-deficient rats, which displayed an up-regulation of the hepatic Fads1, Fads2, Elovl2 and Elovl5 compared with controls fed on an adequate n-3 PUFA diet (Igarashi et al., 2007). It is not clear as to whether this up-regulation is due to other FA components that may stimulate these genes in the deficient diet. Interestingly, the mRNA expression of all these enzymes in brain tissue was not affected by dietary conditions, i.e. the mRNA levels did not differ between adequate and deficient n-3 PUFA supplements (Igarashi et al., 2007). Nevertheless, Elovl5, Elovl2, delta5 and delta6 expression levels were influenced by different dietary conditions. For example, feeding rats with a diet enriched in corn oil (high in

LA) suppressed the hepatic abundance of Fads2 mRNA (Cho et al., 1999b). Mice fed on a high glucose diet after an intake of LA exhibited a decrease in the hepatic abundance of delta6 desaturase mRNA in comparison with mice fed on a high glucose diet without LA (Nakamura et al., 2000). More recently (Tu et al., 2010), rats were fed with different diets containing different ALA compositions and several parameters including phospholipid FA composition and the gene expression of Fads1 (delta5 desaturase), Fads2, Elovl2, Elovl5 and Ppara were analyzed in the liver. No significant difference in mRNA levels of all these genes involved in PUFA metabolism was observed when the ALA dietary concentration was elevated. However, the levels of EPA and DPA in phospholipids rose in proportion to ALA. Therefore, this recent study suggests that n-3 PUFA biosynthesis is strongly governed by the substrate concentration rather than by the levels of the transcripts (Tu et al., 2010).

Other studies have shown that delta6 and delta5 desaturase genes may be regulated by a PPARa-dependent and -independent mechanism. Matsuzaka et al. (2002) showed that delta6 and delta5 desaturase gene expression is regulated *via* another transcription factor, a sterol regulatory element binding protein-1c (SREBP-1c). In transgenic mice over-expressing SREBP-1c, the suppression of hepatic delta5 desaturase and delta6 desaturase expressions by fish oil was abolished. FA desaturase genes are regulated by both PPARa and SREBP-1c (Nakamura and Nara, 2002) and fish oil activates PPARa (Jump et al., 2004).

The nutritional impact in the intact animal is far more complex than in cells maintained under relatively well-controlled in vitro conditions. Therefore, taking conclusions from one model to another should be exercised with great caution. Equally prominent are the differences in whole animals in relation to tissue, organ, age, sex and species. Tissue culture also has its drawbacks, mainly related to unforeseen conditions such as n-3 PUFA deficiency, as detailed in this work or an accumulation of toxic products in the medium.

In this respect some caution needs to be exercised regarding the choice of time for the expression studies by qRT-PCR. We indicated that at 72 h, a greater stability of the mRNA levels as opposed to shorter time periods (6 h and 24 h) was found. Thus, at early times, the levels of transcripts were inconsistent and were subject to great fluctuations. This may not be surprising since mRNA stability is known to be influenced by cell growth rates, substratum, as well as cycle and environmental factors such as hormones, growth factors and ions (Ross, 1995), which adds up to tissue culture drawbacks. Therefore, we cannot rule out the possibility that some of the transcripts may be transiently expressed at the earlier times. Moreover, it has been stated in many studies that the gene expression program is time-dependent. Studies by Werner et al. (2005) showed that the number of genes up-regulated in response to a stimulus increased with time. After mouse embryonic fibroblasts were stimulated for one hour with tumor necrosis factor

(TNF), microarray gene expression profiling revealed 176 up-regulated genes, but after 8-hour stimulation, 687 genes were up-regulated. Genes are expressed at different times and their expression can be transient. This phenomenon has been observed during the process of cell differentiation and it is called the "temporal control of the gene expression programme" (Verdeil et al., 2006).

The third and presumably the principal finding arising from these experiments is that a combination of NGF and ALA decreased the expression of the Elovl2 and Elovl5 transcripts. In other words, ALA supplements effectively suppressed NGF-stimulated, Elovl2 and Elovl5 gene expression. This is in accordance with the lipidomic data (Chapter 4, Figure 4.2), indicating reduced DHA levels in the NGF-differentiated, ALA-supplemented cells. This finding strongly suggests that NGF and ALA play a complex regulatory role in gene expression. Notable in this case is the impact on Elovl2, which is the principal elongase to catalyse 22 carbon-long synthesis from the 20 carbon-long (20:4n-6 and 20:5n-3) intermediate (see the scheme, Figure 1.14 Chapter 1).

Conclusions

In summary, the present data indicate two presumably distinct but mutually related routes of signals for stimulation of gene expression. The first route,

which is regulated by NGF, results in an increase in the PUFA-associated genes. This route, however, is co-regulated by the availability of the ALA precursor to induce down-regulation of gene expression (particularly Elovl2 and Elovl5), and its outcome is the robust suppression of the final step in the generation of 22-carbon chain products. The second route is regulated by n-6 LA, which also results in an increase in the PUFA-associated genes. Although these genes appear to be up-regulated, they do not necessarily lead to enhanced production of n-6 DTA and n-6 DPA (Chapter 3, Figure 3.2) nor do they stimulate n-3 DPA and n-3 DHA (Chapter 3, Figure 3.7) in ALA-supplemented and NGF-untreated cells. Importantly, NGF supplement neither co-regulates LA-induced gene expression when added together, nor enhances the production of n-6 DTA and n-6 DPA (Chapter 4, Figure 4.6)

The present data suggest that the last step in n-3 PUFA biosynthesis is probably regulated by the nature of the FA precursor rather than by the immediate levels of the transcripts.

Chapter 6
General discussion

PC12 pheochromocytoma cells, a widely used *in vitro* model for NGF-induced neuronal differentiation, have been instrumental in investigating many human neuropathologies including Alzheimer disease (AD) (Vaisid et al., 2008), Parkinson disease (Lipman et al., 2006; Abu-Raya et al., 2000) and bipolar disorders (Detera-Wadleigh, 2001).

The abundant neurite outgrowth and other biochemical characteristics that distinguish NGF-induced cell differentiation in these cells highlight the importance of examining membrane lipid composition and regulation of PUFA metabolism in both proliferating and differentiated cells. The general discussion that follows addresses new findings pertaining to PUFA metabolism and evaluates their validity or lack thereof in the context of previous studies using these cells as models in neurobiology and neuropathology research.

To this end, the data suggest that PC12 cells possess an active elongation and desaturation pathway that converts ALA into all its upstream metabolites, particularly DHA. Increasing the ALA concentrations in the culture medium of PC12 cells results in a nearly linear rise in EPA and DPA levels. This biosynthetic capability also characterises many transformed cells (Langelier et al., 2005) and primary astrocytes (Williard et al., 2001) but not neuronal cells (Moore et al., 1991). The principal retailored GPLs following ALA conversion by PC12 cells are the EPG and SPG GPL classes. That is evident already at low precursor concentrations (Figure 3.1) and is dependent on time of exposure (Figure 3.3).

Interestingly, LA supplements are also preferentially incorporated into these two aforementioned aminoethanol GPLs. However, the majority of the retailored FAs including CPG (Figures 3.2 and 3.5) consist of esterified LA itself with little or no upstream metabolites (Figure 3.2). Furthermore, ALA conversion is not affected by the presence of LA (Figure 3.7), therefore reaffirming previous observations that ALA exhibits a higher selectivity than does LA in using the elongation and desaturation enzymes for DHA biosynthesis (Alessandri et al., 2008). Thus, in spite of sharing similar enzymes for their conversion into upstream metabolites, the biosynthesis of the two essential FAs is independently regulated by presumably additional cellular mechanisms (Innis and Dyer, 2002). The present study shows that supplements of preformed DHA to cells are by far more effectively incorporated into the aminoethanol GPL species than are DHA generated from ALA conversion, over the same time period (Figures 3.3 and 3.4), which is in accordance with observations made in other neural cell lines (Langelier et al., 2005). Nevertheless, the sum of the esterified n-3 DPA and DHA residing in the aminoethanol GPLs from ALA conversion, compared with that arising from preformed n-3 DHA, is very similar, suggesting that a nearly steady-state occupancy of the *sn-2* position exists for these GPLs. This steady-state occupancy is also evident by the lack of ARA enrichment either via LA conversion or by direct ARA supplements. The rigorous maintenance of the

PUFA content under conditions where FA precursors are not rate limiting is presumably driven by intracellular regulatory mechanisms underlying GPL biosynthesis (Innis and Dyer, 2002).

The second topic posed in this study addresses the issue of inadequate levels of DHA, a genuine n-3 PUFA deficiency. In whole animals, such a deficiency is usually resolved by effective elongation-desaturation of LA that gives rise to docosapentaenoic acid (DPA, 22:5n-6) via a mechanism of reciprocal replacement (Bernoud et al., 1998). The molecular basis for this reciprocal substitution and its possible effects on membrane function are not entirely clear.

For PC12 cells propagated in ALA-deficient growth medium (~1/40 for the ALA/LA ratio), the inability of LA to serve as substrate to produce 22:5n-6 [see Figures 3.1, 3.2 and 4.6] is puzzling. Indeed, a small fraction of 22:4n6 is converted to 22:5n-6 (Figure 4.6). Also, 22:5n-3 and 22:6n-3 are generated, rather substantially, from the ALA precursor via similar enzymes. The most favourable explanation is that the reciprocal replacement (i.e. replacement of 22:6n-3 with 22:5n-6) is subject to stringent regulation, it includes any intracellular cellular regulatory mechanism responsible for GPL biosynthesis in the endoplasmic reticulum (ER). Whereas at the ER level a certain repertoire of FA is attained (Vance and Vance, 2004), it is the local acylation-deacylation modifications at specific membrane domains that determine the ultimate FA

molecular profile (Brenner and Peluffo, 1966). With PC12 cells and most transformed cell lines, this reciprocal rearrangement does not appear to be a prerequisite for growth. Interestingly, OA (18:1n-9) appears to serve in the reciprocal substitution because of its abundance in the serum-containing culture medium (40 μM), and it is more effective than LA in replenishing ALA as a substrate for elongation and desaturation.

This important point, which was discovered in this study, has helped us to utilise the levels of Mead acid (20:3n-9) as a surrogate index of n-3 PUFA deficiency even though it was originally reported as an indicator of n-6 PUFA deficiency (Lerner et al., 1995). Indeed, when n-3 FA supplements such as ALA or EPA were added, the Mead acid index of deficiency was drastically reduced (Figure 3.6). This correlation strongly indicates that 20:3n-9 can be used as a substitute in either n-6 or n-3 PUFA deficiencies. This deficiency is almost an inherent metabolic property characterising transformed cell lines and warrants a cautious approach particularly in neuro-functional attributes of these cells. Such caution should also be applied to many neural-derived cell lines including SN56 and HT22 cells originating from the septum and hippocampus, respectively (Martin et al., 2006), N1E 115 mouse neuroblastoma (Murphy and Horrocks, 1993), or rat oligodendroglia (Brand et al., 2010) and mesenchymal cells (Kan et al., 2007), all of which appear to be deficient in n-3 PUFA.

A third issue pursued in this study addresses the ultimate metabolic fate of ALA and LA in PC12 cells after treatment with NGF. Within the time frame studied (72 h), NGF supplements promoted neurite outgrowth in cells growing in regular ALA-inadequate medium (Table 4.1). ALA addition at micromolar concentrations was effectively converted to 20:5n-3 by NGF-treated cells. In contrast, NGF-treated cells were unable to elongate and desaturate 20-long chain hydrocarbons to 22-long chain hydrocarbon metabolites. In particular, the conversion of ALA to 22:6n-3, as manifested by its levels in both EPG and SPG, was strongly reduced. Supplements of EPA (Figure 4.4) and LA (Figure 4.6) further corroborated the findings that NGF impaired the elongation desaturation pathway. This work further examines whether NGF inhibition is a general phenomenon that might be shared by other compounds that induce PC12 cell differentiation, such as basic fibroblast growth factor (bFGF) or retinoic acid (RA). It allows the conclusion that bFGF or RA did not impair the conversion pathway in comparison with NGF-differentiated PC12 cells. The fact that NGF played a regulatory role in PUFA biosynthesis appears to be unique to NGF. This adds to the growing list of the biological effects of this particular growth factor, which is essential to the differentiation of certain populations of neuronal cells. Are there other growth factors, in addition to those mentioned before (e.g., BDNF) that are essential for PUFA metabolism in other

neuronal cell populations (in the cortex, in the hippocampus, Purkinje cells in the cerebellum)? This open question remains to be resolved.

At this time, the role of NGF in PUFA metabolism can only speculated. It has been previously established that the production of DHA in mammalian cells is regulated by ELOVL2, (Holman, 1998; Vance and Vance, 2004) and is responsible for the 20 to 22 carbon elongation. Nevertheless, it is possible that a limited amount of DHA may be generated by the second cytosolic enzyme, ELOVL5, from the elongation desaturation pathway. Although the main substrate for this enzyme is 18:4n-3, it has been suggested that it may catalyse, at least in rodents, the elongation of 20 carbons to 22 carbons (Leonard et al., 2004). This enzyme could be largely responsible for the limited amounts of 22:5n-3 formed after either ALA or EPA (Figure 4.4) supplements.

With respect to the large inhibition of DHA biosynthesis, the current studies led us to propose that NGF may activate an intrinsic cellular signal (Lands, 2000) that shuts off the PUFA elongation/desaturation pathway during the last step, as part of phenotypic expression of neuronal characteristics. Under these circumstances, the contribution of neuronal and glial cells to the endogenous capacity of the brain to synthesise its own PUFA constituents may be an epigenetically associated phenomenon.

It is well known that in the course of neurogenesis, immature neuroblasts cease dividing and after migration and strong apoptotic elimination, assume a finite

position in the neuronal network (Rakic, 2006). It is hypothesised that at some point during this sequel, normal neurons appear to lose their capability to produce DHA and ARA by themselves. They begin to rely on glia and possibly other non-neural cells for continuous provision through effective transport systems of these *de novo* synthesised PUFAs, as has been demonstrated in studies carried out by Moore (2001). It is now established that at some crucial points in ontogeny certain growth signals are generated that determine the course of differentiation from the pluripotent nature of the dividing germinal cells. This thesis proposes that NGF may constitute such a signal for PC12 cells or for neurons *in vivo* (Levi-Montalcini, 1987; Tessler, 2004), whereas neurotrophic agents such as BDNF are essential growth factors that appear to determine neuronal fate (Mattson et al., 2004). In this scenario, during the course of differentiation, neurons may cease to produce PUFA by themselves and relay DHA and ARA supplies provided directly *via* the diet or by neighbouring astrocytes. This is in accordance with previous studies whereby primary rat foetal cerebral cells were able to elongate and desaturate both ALA and LA to DHA and ARA when a small population of glia cells was present in the mixture (Yavin and Menkes, 1974). When purer preparations of dissociated rat cortical and cerebellar neuronal cultures were incubated with ALA, DHA was not formed (Moore et al., 1991). In contrast, pure astrocytic cultures effectively elongated and desaturated ALA to DHA and LA to ARA (Moore et

al., 1991). Overall, neurotrophic-induced differentiation may give rise to one of the most fundamental molecular switches in the process of cell lineage partition. At this time, this possibility remains to be rigorously studied in view of a controversial finding suggesting that hippocampal neurons are able to produce DHA under culture conditions (Kaduce et al., 2008).

It is suggested that all neuroblasts and stem cells in the subventricular zone in the developing nervous system have full capacity to synthesise PUFA from essential FA precursors. Upon the introduction of growth factors or neuromodulator-induced terminal differentiation, neurons may cease to produce their own DHA and become highly dependent on outside sources such as astrocytes. The latter seems to retain this capacity throughout life.

In conclusion, this work demonstrated that undifferentiated PC12 cells possess the capability to convert essential FA into higher intermediates and also established the necessity to provide ALA or DHA metabolites in future studies employing cells of transformed or stem cell origin. Unraveling the requirements of n-3 PUFA constituents for membrane synthesis and the temporal code details of their regulation should shed light on the conditions needed for proper neuronal function or aberrations during disease and aging.

A fourth objective pursued in this study has been to evaluate the transcriptional changes of several key genes involved in FA metabolism (FA elongases Elovl5 and Elovl2), desaturases (Fads2) and peroxisome proliferator-

activated receptor a (Ppara) after NGF-induced differentiation and after ALA or LA supplements in PC12 cells. The rationale for these studies was based on previous works carried out in rat sympathetic neurons or PC12 cells, where it was shown that NGF up-regulated the mRNA level of several genes such as the transcription factor, NGF I-A, p75 low-affinity NGF (LNGF) receptor, tyrosine hydroxylase (TH) and Ta1 a-tubulin (Ta1) (Cho et al., 1989; Ma et al., 1992). In the present studies, PC12 cells supplemented with NGF for 3-4 days significantly up-regulated several key gene transcripts including Elovl5, Elovl2, Fads2 and Ppara.

A wide range of studies have reported that mRNA expressions of FA desaturase (Wang et al., 2005; Nakamura et al., 2004; Igarashi et al., 2007) and elongase (Wang et al., 2005; Igarashi et al., 2007) are regulated by PUFA supplemented or PUFA-deficient diets. In this current study, ALA supplements did not alter the levels of Elovl2 and Ppara but up-regulated the expression of Elovl5 and Fads2 in proliferating PC12 cells. In contrast, supplements of LA up-regulated the expression of all (Elovl5, Elovl2, Fads2 and Ppara) gene transcripts. This is a surprising observation given the fact that this supplement of exogenous LA is on top of the already high basal levels (0.15 mM concentration of basal LA in the growth medium) (see appendix 5). The impact of FA supplements on gene expressions has been previously reported and the emerging picture is not conclusive. For instance, exposure to LA in HepG2 cells

up-regulated the mRNA levels of Fads2. In contrast, exposure to ALA (at the same concentration) did not affect Fads2 mRNA levels (Harnack et al., 2009). Similarly, ALA but not LA up-regulated gene expression of Ppara in the same system (Harnack et al., 2009). Treatment of foam cells with an n-3 ligand such as EPA increased PPARα mRNA expression, although an n-6 ligand such as LA had no significant effect on PPARα mRNA levels (Reza et al., 2009).

In dietary studies, feeding rats with an n-3 PUFA-deficient diet up-regulated Fads2, Elovl2 and Elovl5 mRNA expressions (Igarashi et al., 2007), whereas a diet enriched in LA down-regulated Fads2 mRNA levels (Cho et al., 1999b).

In qRT-PCR experiments, higher transcript up-regulation was attained by LA compared with ALA (Figures 5.1-5.4) when both were added at the same concentration. Furthermore, it appears that the enhanced levels of these transcripts did not reflect a straightforward correlation with the obtained lipidomic data. As documented by the latter approach, PC12 cells synthesised more effectively all the n-3 intermediates from ALA and EPA rather than corresponding n-6 intermediates from the LA precursor. For example, after 72 h incubation with ALA, a higher percentage of n-3 PUFA metabolites such as EPA, DPA and DHA were found in the cells. In contrast, incubation with LA under the same conditions showed little incorporation of the precursor or its higher metabolites into the cells. Thus, the regulation of PUFA biosynthesis is differential and is clearly dependent on the substrate source, i.e., of n-3 or n-6

origin. Gregory and co-workers (2011) reported that the activity of elongase and desaturase enzymes is also dependent on the substrate source. The Elovl2 activity was at least 2.0-fold higher with the n-3 substrates EPA and DPA, respectively, compared with their n-6 homologs, ARA and 22:4n-6. Notable in this context, Fads2 exhibited Δ6 desaturase activity with ALA and LA, and with 24:5n-3 (Gregory et al., 2011). However, substrate competition studies revealed a higher affinity of Δ6 desaturase for ALA (Brenner et al., 1969).

It is still unclear whether the changes in the FA composition of cell lipids are directly attributable to the increased mRNA expression of genes encoding for proteins related to lipid metabolism by FAs or are due to the increased demand for substrate availability and/or cellular needs. Song He et al. (2002) hypothesised that up-regulation of Δ6 desaturase gene transcription might be a compensatory response to an increased demand in PUFAs caused by modulation of Ppara-inducing FA oxidation.

From lipidomic data, the biosynthesis pathway of ALA or LA was working properly in proliferating cells (ALA or LA) and was inhibited in NGF-differentiated cells (ALA+NGF or LA+NGF). In NGF-differentiated cells, the last steps in the n-3 and n-6 biosynthesis pathway, the formation of 22-long-chain hydrocarbon metabolites was inhibited, although both ELOVL2 and ELOVL5 could catalyse n-3 EPA to n-3 DPA or n-6 ARA to 22:4n-6. It still

remains uncertain whether one or both elongases normally contribute to EPA or ARA elongation. ELOVL2 and ELOVL5 each have at least two n-3/n-6 substrates, suggesting n-3 or n-6 PUFA competition between substrates. SDA (18:4n-3)/18:3n-6 and n-3EPA/ARA are both ELOVL5 substrates. ELOVL2 has n-3EPA/n-6ARA and n-3DPA/22:4n-6 as substrates. Moreover, ELOVL2 during the step of elongation of n-3DPA to 24:5n-3 is limited by substrate availability and is considered as a control point in the biosynthetic pathway (Gregory et al., 2011). The blockade during this step by NGF raises the possibility of competitive inhibition between the substrate (EPA or ARA) and the growth factor (NGF). The inability to synthesise DHA from ALA or n-6DPA from LA in NGF-differentiated cells could be due to an absolute block by the growth factor at the delta-6-desaturase, the rate-limiting enzyme of the pathway (Sprecher et al., 1981) or during the peroxisomal step.

It was expected that by PCR assay, a down-regulation rather than an up-regulation of the genes involved in the PUFA pathway in NGF-differentiated cells would be identified. However, an up-regulation in the mRNA expression does not always mean a translation to an active protein that will act on a substrate. The literature has reported poor correlations between the level of mRNA and the level of protein. For instance, quantitative studies of mRNA and protein expressions within the same lung adenocarcinomas showed that only 17% of the proteins exhibited a correlation between their mRNA and protein

levels (Chen et al., 2002). Other works using pulse labelling with radioactive nucleosides or amino acids supported the lack of a correlation between protein and mRNA half-lives/turnover (Schwanhausser et al., 2011). There are two main reasons for the absence of a correlation between mRNA and protein levels reported in the literature. First, there are post-transcriptional mechanisms involved in turning mRNA into protein. Second, there are post-translational mechanisms affecting the proteins' half-lives as the result of cells controlling the protein synthesis and degradation (Greenbaum et al., 2003). The cellular abundance of a given protein depends not only on the transcriptional process of its gene—it also depends on the balance between post-transcriptional and post-translational mechanisms (Schwanhausser et al., 2011).

In NGF-differentiated cells, it is reasonable to assume that the enzyme activities of the PUFA pathway taking place in the ER are fully operant, at least until the step of DPA synthesis, whereas the final synthesis of 24:5n-3, 24:6n-3 and/or the peroxisomal enzyme activities would be inhibited. From this point of view, NGF might be a potent inhibitor at the level of ELOVL2 and ELOVL5. Further experiments should be carried out to quantify the protein's expression and its relative activities.

Concluding remarks and future work

This work has focused on the intriguing regulation of PUFA biosynthesis in proliferating and NGF-differentiated PC12 cells.

The PC12 cell FA profile has been characterised, it has been found to be extremely deficient in n-3 metabolites, which were compensated by a high level of Mead acid (20:3n-9). Therefore, care should be exercised in interpreting data in which these cells are used in studying signal transduction in neurobiology or in neuropathology.

PC12 cells can take up ALA and convert it to higher intermediates, all of which, are esterified in EPG and SPG phosphoglycerides.

PC12 cells can take up LA and incorporate it directly without conversion primarily into EPG. LA is marginally converted to ARA or its higher intermediates, indicating a plateau most likely attributed to the already sufficient levels of LA in the medium. LA supplements do not inhibit ALA conversion to n-3 PUFA metabolites, suggesting a lack of competition for the same elongation/desaturation enzymes and tight cellular regulation for preferential ALA conversion.

In expanding the current work, one can foresee the following objectives and questions that remain to be unravelled:

1. What is the molecular mechanism driving the preferential utilisation of ALA over LA?

2. Do PC12 cells share the same enzymes for the ALA and LA conversion pathway and if so, how are they regulated?

3. A detailed kinetic analysis of FA molecular species of membrane phospholipids by Q-TOF and stable isotope incorporation is needed. Stable isotopes such as ALA, EPA, ARA and LA could be applied in order to follow-up the intermediary steps of PUFA biosynthesis.

4. The mechanism by which intrinsic signals such as growth factors and dietary lipid molecules interact to regulate specific gene transcripts in transformed or differentiated cells needs to be investigated. We have shown by qRT-PCR analysis that LA but not ALA regulates the gene expression of several transcripts (delta6 desaturase, Elovl5 and Elovl2 elongases and Ppara). On the other hand, ALA, and not LA, seems to down-regulate the gene expression of the Elovl5 and Elovl2 elongases. Therefore, the combination of NGF and ALA causes a striking inhibition of the last steps in the elongation/desaturation of ALA, suggesting a possible specific interference with ELOVL2 elongase.

In order to confirm the results of qRT-PCR, analysis can be done by correlating the gene levels (Elovl5, Elovl2, Fads2) with the protein levels (ELOVL5, ELOVL2, delta6 desaturase) by using Western blot techniques.

To use RNA interference (RNAi) in order to silence Fads1 (encoding for delta5 desaturase) or Elovl2 and to look for the changes at the protein levels (Western blot) and at the FA profile (TLC/GC).

5. Evaluation of neural-related receptors' binding efficiency and the outcome of electrical and/or synaptic activity in n-3-deficient and n-3-enriched cells is needed.

In summary, these novel results suggest that phospholipid biosynthesis in proliferating cells, after assuming a neuronal phenotype (NGF induction), may be suppressed by the presence of dietary FA (n-3 ALA in particular), which changes the course of metabolism in mature neuronal cells.

Bibliography

Abu-Raya, S., Tabakman, R., Blaugrund, E., Trembovler, V., & Lazarovici, P. (2002). Neuroprotective and neurotoxic effects of monoamine oxidase-B inhibitors and derived metabolites under ischemia in PC12 cells. *European Journal of Pharmacology, 434*(3), 109-116.

Abumrad N. A., El-Maghrabi M. R., Amri E. Z., Lopez E., and Grimaldi P. A. (1993) Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation: homology with human CD36. *J. Biol. Chem.* 268, 17,665-17,668.

Agell, N., Bachs, O., Rocamora, N., & Villalonga, P. (2002). Modulation of the Ras/Raf/MEK/ERK pathway by Ca^{2+}, and calmodulin. *Cellular Signalling, 14*(8), 649-654.

Ahmad, A., Murthy, M., Greiner, R. S., Moriguchi, T., & Salem Jr, N. (2002a). A decrease in cell size accompanies a loss of docosahexaenoate in the rat hippocampus. *Nutritional Neuroscience, 5*(2), 103.

Ahmad, A., Moriguchi, T., & Salem, N. (2002b). Decrease in neuron size in docosahexaenoic acid-deficient brain. *Pediatric Neurology, 26*(3), 210-218.

Akbar, M., Calderon, F., Wen, Z., & Kim, H. Y. (2005). Docosahexaenoic acid: A positive modulator of akt signaling in neuronal survival. *Science's STKE, 102*(31), 10858.

Akbar, M., & Kim, H. Y. (2002). Protective effects of docosahexaenoic acid in staurosporine-induced apoptosis: Involvement of phosphatidylinositol-3 kinase pathway. *Journal of Neurochemistry, 82*(3), 655-665.

Alessandri, J. M., Extier, A., Langelier, B., Perruchot, M. H., Heberden, C., Guesnet, P., et al. (2008). Estradiol favors the formation of eicosapentaenoic acid (20: 5n-3) and n-3 docosapentaenoic acid (22: 5n-3) from alpha-linolenic acid (18: 3n-3) in SH-SY5Y neuroblastoma cells. *Lipids, 43*(1), 19-28.

Alessandri, J. M., Guesnet, P., Vancassel, S., Astorg, P., Denis, I., Langelier, B., et al. (2004). Polyunsaturated fatty acids in the central nervous system: Evolution of concepts and nutritional implications throughout life. *Reproduction Nutrition Development, 44*(6), 509-538.

Allen, I. C., Hartney, J. M., Coffman, T. M., Penn, R. B., Wess, J. and Koller, B.H.

(2006). Thromboxane A2 induces airway constriction through an M3 muscarinic acetylcholine receptor-dependent mechanism. American Journal of Physiology Lung Cellular and Molecular Physiology 290(3):L526-33.

AN, R. T. H. O. L. M., & MOHRHAUER, H. (1963). A hypothesis involving competitive inhibitions in the metabolism of polyunsaturated fatty acids. *Acta Chem.Scand, 17*, 1.

Anderson, R. E. (1970). Lipids of ocular tissues: IV. A comparison of the phospholipids from the retina of six mammalian species. *Experimental Eye Research, 10*(2), 339-344.

Ayuyan, A. G., & Cohen, F. S. (2008). Raft composition at physiological temperature and pH in the absence of detergents. *Biophysical Journal, 94*(7), 2654-2666.

Bardot, O., Aldridge, T., Latruffe, N., & Green, S. (1993). PPAR-RXR heterodimer activates a peroxisome proliferator response element upstream of the bifunctional enzyme gene. *Biochemical and Biophysical Research Communications, 192*(1), 37-45.

Barrett, G. L., & Bartlett, P. F. (1994). The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proceedings of the National Academy of Sciences, 91*(14), 6501.

Bauman, D., Baumgard, L., Corl, B., & Griinari, J. (1999). Biosynthesis of conjugated linoleic acid in ruminants. *Proc. Am. Soc. Anim. Sci, , 3.* pp. 1-15.

Bazan, N. G. (2005). Neuroprotectin D1 (NPD1): A DHA-Derived mediator that protects brain and retina against cell Injury-Induced oxidative stress. *Brain Pathology, 15*(2), 159-166.

Bazan, N. G. (2009). Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and alzheimer's disease. *Journal of Lipid Research, 50*(Supplement), S400-S405.

Belitz, H., Grosch, W., & Schieberle, P. (2004). *Food chemistry* Springer Berlin etc.

Bell, R., Kennerly, D. A., Stanford, N., & Majerus, P. W. (1979). Diglyceride lipase: A pathway for arachidonate release from human platelets. *Proceedings of the National Academy of Sciences, 76*(7), 3238.

Berdeaux, O., & Wolff, R. L. (1996). Gas-liquid chromatography-mass spectrometry of the 4, 4-dimethyloxazoline derivatives of Δ5-unsaturated polymethylene-interrupted fatty acids from conifer seed oils. *Journal of the American Oil Chemists' Society, 73*(10), 1323-1326.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). Signal-transduction pathways: An introduction to information metabolism.

Bernoud, N., Fenart, L., Benistant, C., Pageaux, J., Dehouck, M., Moliere, P., et al. (1998). Astrocytes are mainly responsible for the polyunsaturated fatty acid enrichment in blood-brain barrier endothelial cells in vitro. *Journal of Lipid Research, 39*(9), 1816-1824.

Bertrand, P. C., O'Kusky, J. R., & Innis, S. M. (2006). Maternal dietary (n-3) fatty acid deficiency alters neurogenesis in the embryonic rat brain. *The Journal of Nutrition, 136*(6), 1570-1575.

Bevers, E. M., Comfurius, P., Dekkers, D. W. C., & Zwaal, R. F. A. (1999). Lipid translocation across the plasma membrane of mammalian cells. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1439*(3), 317-330.

Bitbol, M., & Devaux, P. F. (1988). Measurement of outward translocation of phospholipids across human erythrocyte membrane. *Proceedings of the National Academy of Sciences, 85*(18), 6783.

Bodles, A. M., Guthrie, D. J. S., Greer, B., & Irvine, G. B. (2001). Identification of the region of non-Aβ component (NAC) of alzheimer's disease amyloid responsible for its aggregation and toxicity. *Journal of Neurochemistry, 78*(2), 384-395.

Bolsover, S. R., Gomez-Fernandez, J. C., & Corbalan-Garcia, S. (2003). Role of the Ca2 /phosphatidylserine binding region of the C2 domain in the translocation of protein kinase Ca to the plasma membrane. *Journal of Biological Chemistry, 278*(12), 10282-10290.

Bordoni, A., Di Nunzio, M., Danesi, F., & Biagi, P. (2006). Polyunsaturated fatty acids: From diet to binding to PPARs and other nuclear receptors. *Genes & Nutrition, 1*(2), 95-106.

Bourre, J., Pascal, G., Durand, G., Masson, M., Dumont, O., & Piciotti, M. (1984). Alterations in the fatty acid composition of rat brain cells (neurons, astrocytes, and oligodendrocytes) and of subcellular fractions (myelin and synaptosomes) induced by a diet devoid of n-3 fatty acids. *Journal of Neurochemistry, 43*(2), 342-348.

Bourre, J., Bonneil, M., Chaudiere, J., Clement, M., Dumont, O., Durand, G., Lafont H., Nalbone G., Pascal G. & Piciotti M. (1992). Structural and functional importance of dietary polyunsaturated fatty acids in the nervous system. *Advances in Experimental Medicine and Biology, 318*, 211.

Bourre, J., Bonneil, M., Clement, M., Dumont, O., Durand, G., Lafont, H., Nalbone G. & Piciotti M. (1993). Function of dietary polyunsaturated fatty acids in the nervous system. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 48*(1), 5-15.

Bourre, J., Faivre, A., Dumont, O., Nouvelot, A., Loudes, C., Puymirat, J. &Tixier-Vidal A. (1983). Effect of polyunsaturated fatty acids on fetal mouse brain cells in culture in a chemically defined medium. *Journal of Neurochemistry, 41*(5), 1234-1242.

Bourre, J., Francois, M., & Youyou, Dumont A. M., Piciotti M. , Pascal G. & Durand G. (1989). The effects of dietary alpha-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. *J.Nutr, 119*, 1880-1892.

Boxerman, J. L., Bandettini, P. A., Kwong, K. K., Baker, J. R., Davis, T. L., Rosen, B. R. &Weisskoff RM. (1995). The intravascular contribution to fmri signal change: Monte carlo modeling and diffusion-weighted studies in vivo. *Magnetic Resonance in Medicine, 34*(1), 4-10.

Brand, A., Bauer, N. G., Hallott, A., Goldbaum, O., Ghebremeskel, K., Reifen, R. & Richter-Landsberg C. (2010). Membrane lipid modification by polyunsaturated fatty acids sensitizes oligodendroglial OLN-93 cells against oxidative stress and promotes up-regulation of heme oxygenase-1 (HSP32). *Journal of Neurochemistry, 113*(2), 465-476.

Brand, A., & Yavin, E. (2001). Early ethanolamine phospholipid translocation marks stress-induced apoptotic cell death in oligodendroglial cells. *Journal of Neurochemistry, 78*(6), 1208-1218.

Brandt, J. M., Djouadi, F., & Kelly, D. P. (1998). Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor α. *Journal of Biological Chemistry, 273*(37), 23786-23792.

Breckenridge, W., Gombos, G., & Morgan, I. (1972). The lipid composition of adult rat brain synaptosomal plasma membranes. *Biochimica Et Biophysica Acta (BBA)-Biomembranes, 266*(3), 695-707.

Breckenridge, W., Morgan, I., Zanetta, J., & Vincendon, G. (1973). Adult rat brain synaptic vesicles II. lipid composition. *Biochimica Et Biophysica Acta (BBA)-General Subjects, 320*(3), 681-686.

Brenner, R. R., & Peluffo, R. O. (1966). Effect of saturated and unsaturated fatty acids on the desaturation in vitro of palmitic, stearic, oleic, linoleic, and linolenic acids. *Journal of Biological Chemistry, 241*(22), 5213-5219.

Brenner, R. R., & Peluffo, R. O. (1969). Regulation of unsaturated fatty acids biosynthesis 1. effect of unsaturated fatty acid of 18 carbons on the microsomal desaturation of linoleic acid into [gamma]-linolenic acid. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 176*(3), 471-479.

Bretscher, M. S. (1972). Asymmetrical lipid bilayer structure for biological membranes. *Nature, 236*(61), 11-12.

Brown, D. A., & London, E. (2000). Structure and function of sphingolipid-and cholesterol-rich membrane rafts. *Journal of Biological Chemistry, 275*(23), 17221-17224.

Brown, D. A., & Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell, 68*(3), 533-544.

Brown, E. R., & Subbaiah, P. V. (1994). Differential effects of eicosapentaenoic acid and docosahexaenoic acid on human skin fibroblasts. *Lipids, 29*(12), 825-829.

Brown, M. F. (1994). Modulation of rhodopsin function by properties of the membrane bilayer. *Chemistry and Physics of Lipids, 73*(1-2), 159-180.

Brown, M. S., & Goldstein, J. L. (1997). The SREBP pathway: Regulation review of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell, 89*, 331-340.

Bruce, Å. (1974). Skeletal muscle lipids. II. changes in phospholipid composition in man from fetal to middle age. *Journal of Lipid Research, 15*(2), 103-108.

Bruyn, G. W., & Garcin, R. (1969). *Handbook of clinical neurology* North-Holland Pub. Co.

Burr, G. O., & Burr, M. M. (1929). A new deficiency disease produced by the rigid exclusion of fat from the diet. *Journal of Biological Chemistry, 82*(2), 345-367.

Buton, X., Morrot, G., Fellmann, P., & Seigneuret, M. (1996). Ultrafast glycerophospholipid-selective transbilayer motion mediated by a protein in the endoplasmic reticulum membrane. *Journal of Biological Chemistry, 271*(12), 6651-6657.

Butovsky, E., Juknat, A., Goncharov, I., Elbaz, J., Eilam, R., Zangen, A. & Vogel Z. (2005). In vivo up-regulation of brain-derived neurotrophic factor in specific brain areas by chronic exposure to Δ9-tetrahydrocannabinol. *Journal of Neurochemistry, 93*(4), 802-811.

Calderon, F., & Kim, H. Y. (2004). Docosahexaenoic acid promotes neurite growth in hippocampal neurons. *Journal of Neurochemistry, 90*(4), 979-988.

Cao, D., Kevala, K., Kim, J., Moon, H. S., Jun, S. B., Lovinger, D. & Kim HY. (2009). Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. *Journal of Neurochemistry, 111*(2), 510-521.

Carlson RO, Levitan IB. Regulation of intracellular free arachidonic acid in Aplysia nervous system. J Membr Biol. 1990 Jul;116(3):249-60.

Cassimeris, L., Lingappa, V. R., & Plopper, G. (2011). *Lewin's cells* Jones & Bartlett Learning.

Chen, G., Gharib, T. G., Huang, C. C., Taylor, J. M. G., Misek, D. E., Kardia, S. L. R., et al. (2002). Discordant protein and mRNA expression in lung adenocarcinomas. *Molecular & Cellular Proteomics, 1*(4), 304-313.

Chen, S. G., & Murakami, K. (1994). Effects of cis-fatty acid on protein kinase C activation and protein phosphorylation in the hippocampus. *PDA Journal of Pharmaceutical Science and Technology, 48*(2), 71-75.

Chin, S., Liu, W., Storkson, J., Ha, Y., & Pariza, M. (1992). Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *Journal of Food Composition and Analysis, 5*(3), 185-197.

Cho, H. P., Nakamura, M., & Clarke, S. D. (1999). Cloning, expression, and fatty acid regulation of the human Δ-5 desaturase. *Journal of Biological Chemistry, 274*(52), 37335-37339.

Cho, H. P., Nakamura, M. T., & Clarke, S. D. (1999). Cloning, expression, and nutritional regulation of the mammalian Δ-6 desaturase. *Journal of Biological Chemistry, 274*(1), 471-477.

Cho, K., Skarnes, W., Minsk, B., Palmieri, S., Jackson-Grusby, L., & Wagner, J. (1989). Nerve growth factor regulates gene expression by several distinct mechanisms. *Molecular and Cellular Biology, 9*(1), 135-143.

Christie, W., Brechany, E. Y., & Stefanov, K. (1988). Silver ion high-performance liquid chromatography and gas chromatography—mass spectrometry in the analysis of complex fatty acid mixtures: Application to marine invertebrates. *Chemistry and Physics of Lipids, 46*(2), 127-135.

Chung, J., Kubota, H., Ozaki, Y., Uda, S., & Kuroda, S. (2010). Timing-dependent actions of NGF required for cell differentiation. *PLoS One, 5*(2), 9011.

Clandinin, M., Chappell, J., Leong, S., Heim, T., Swyer, P., & Chance, G. (1980). Intrauterine fatty acid accretion rates in human brain: Implications for fatty acid requirements. *Early Human Development, 4*(2), 121-129.

Clark, J. D., Milona, N., & Knopf, J. L. (1990). Purification of a 110-kilodalton cytosolic phospholipase A2 from the human monocytic cell line U937. *Proceedings of the National Academy of Sciences, 87*(19), 7708.

Conquer, J. A., Tierney, M. C., Zecevic, J., Bettger, W. J., & Fisher, R. H. (2000). Fatty acid analysis of blood plasma of patients with alzheimer's

disease, other types of dementia, and cognitive impairment. *Lipids, 35*(12), 1305-1312.

Corcoran, J., Shroot, B., Pizzey, J., & Maden, M. (2000). The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *Journal of Cell Science, 113*(14), 2567-2574.

Corl, B., Baumgard, L., Dwyer, D., Griinari, J., Philips, B., & Bauman, D. (2000). The role of delta-9-desaturase in the production of cis-9, trans-11 CLA and other delta-9 desaturated fatty acids in milk fat. *Journal of Dairy Science, 83*(^ s1)

Cosgaya, J. M., Garcia-Villalba, P., Perona, R., & Aranda, A. (1996). Comparison of the effects of retinoic acid and nerve growth factor on PC12 cell proliferation, differentiation, and gene expression. *Journal of Neurochemistry, 66*(1), 89-98.

Crawford, M., Hassam, A., & Stevens, P. A. (1981). Essential fatty acid requirements in pregnancy and lactation with special reference to brain development. *Progress in Lipid Research, 20*, 31-40.

Crawford, M., Williams, G., Hassam, A., & Whitehouse, W. (1976). Essential fatty acids and fetal brain growth. *The Lancet, 307*(7957), 452-453.

Dagai, L., Peri-Naor, R., & Birk, R. (2009). Docosahexaenoic acid significantly stimulates immediate early response genes and neurite outgrowth. *Neurochemical Research, 34*(5), 867-875.

Daleke, D. L. (2003). Regulation of transbilayer plasma membrane phospholipid asymmetry. *Journal of Lipid Research, 44*(2), 233-242.

Daleke, D. L., & Huestis, W. H. (1985). Incorporation and translocation of aminophospholipids in human erythrocytes. *Biochemistry, 24*(20), 5406-5416.

Davies, A. M., Lee, K. F., & Jaenisch, R. (1993). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron, 11*(4), 565.

Day, C. A., & Kenworthy, A. K. (2009). Tracking microdomain dynamics in cell membranes. *Biochimica Et Biophysica Acta (BBA)-Biomembranes, 1788*(1), 245-253.

Deckelbaum, R. J., Worgall, T. S., & Seo, T. (2006). n-3 fatty acids and gene expression. *The American Journal of Clinical Nutrition, 83*(6), S1520-1525S.

Delellis, R. A., Merk, F. B., Deckers, P., Warren, S., & Balogh, K. (1973). Ultrastructure and in vitro growth characteristics of a transplantable rat pheochromocytoma. *Cancer, 32*(1), 227-235.

Desvergne, B., & Wahli, W. (1999). Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocrine Reviews, 20*, 649-688.

Detera-Wadleigb, S. D. (2001). Lithium-related genetics of bipolar disorder. *Annals of Medicine, 33*(4), 272-285.

Dichter, M. A., & Tischler, A. S. (1977). Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. *Nature, 268*, 501-504.

Dittmer, J. and Lester, R. (1964). A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. Journal of lipid research, 15, 126-7.

Donner, D., & Yonkers, K. (1983). Hormone-induced conformational changes in the hepatic insulin receptor. *Journal of Biological Chemistry, 258*(15), 9413-9418.

D'Onofrio, M., Paoletti, F., Arisi, I., Brandi, R., Malerba, F., Fasulo, L. & Cattaneo A. (2011). NGF and proNGF regulate functionally distinct mRNAs in PC12 cells: An early gene expression profiling. *PloS One, 6*(6), 20839.

Duman, J. G., Chen, L., & Hille, B. (2008). Calcium transport mechanisms of PC12 cells. *The Journal of General Physiology, 131*(4), 307-323.

Edidin, M. (2003). The state of lipid rafts: From model membranes to cells. *Annual Review of Biophysics and Biomolecular Structure, 32*(1), 257-283.

Edmond J., Higa T. A., Korsak R. A., Bergner E. A., and Lee W.-N. P. (1998) Fatty acid transport and utilization for the developing brain. *J.*

Neurochem. 70, 1227-1234.

Edmond, J. (2001). Essential polyunsaturated fatty acids and the barrier to the brain: The components of a model for transport. Journal of Molecular Neuroscience, 16, 181-193, discussion 215-121.

El-Agnaf, O., & Irvine, G. B. (2000). Review: Formation and properties of amyloid-like fibrils derived from [alpha]-synuclein and related proteins. *Journal of Structural Biology, 130*(2-3), 300-309.

Emoto, K., Kobayashi, T., Yamaji, A., Aizawa, H., Yahara, I., Inoue, K. & Umeda M. (1996). Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis. *Proceedings of the National Academy of Sciences, 93*(23), 12867.

Enoch, H., Catala, A., & Strittmatter, P. (1976). Mechanism of rat liver microsomal stearyl-CoA desaturase. studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid. *Journal of Biological Chemistry, 251*(16), 5095-5103.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A. B., & Henson, P. M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature, 405*(6782), 85-90.

Fan, Y. Y., Spencer, T. E., Wang, N., Moyer, M. P., & Chapkin, R. S. (2003). Chemopreventive n-3 fatty acids activate RXRa in colonocytes. *Carcinogenesis, 24*(9), 1541-1548.

Feller, S. E., Brown, C. A., Nizza, D. T., & Gawrisch, K. (2002). Nuclear overhauser enhancement spectroscopy cross-relaxation rates and ethanol distribution across membranes. *Biophysical Journal, 82*(3), 1396-1404.

Folch, J., Lees, M. and Stanley, G. H. (1957). A simple method for the isolation and purification of total lipid from animal tissues.Journal of Biological Chemistry.

Fourcade, S., Savary, S., Albet, S., Gauthe, D., Gondcaille, C., Pineau, T., Bellenger J, Bentejac M, Holzinger A, Berger J & Bugaut M. (2001). Fibrate induction of the adrenoleukodystrophy-related gene (ABCD2). *European Journal of Biochemistry, 268*(12), 3490-3500.

Fra, A. M., Williamson, E., Simons, K., & Parton, R. G. (1995). De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proceedings of the National Academy of Sciences, 92*(19), 8655.

Freed L. M., Wakabayashi S., Bell J. M., and Rapoport S. I. (1994) Effect of inhibition of beta-oxidation on incorporation of [U-14C]palmitate and [1-14C] arachidonate into brain lipids. *Brain Res.* 645, 41-48.

Freshney, R. I. (2010). *Culture of animal cells: A manual of basic technique and specialized applications* Wiley-Blackwell.

Frohnert, B. I., Hui, T. Y., & Bernlohr, D. A. (1999). Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *Journal of Biological Chemistry, 274*(7), 3970-3977.

Fu, J. Y., Masferrer, J., Seibert, K., Raz, A., & Needleman, P. (1990). The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *Journal of Biological Chemistry, 265*(28), 16737-16740.

Fujita, K., Lazarovici, P., & Guroff, G. (1989). Regulation of the differentiation of PC12 pheochromocytoma cells. *Environmental Health Perspectives, 80*, 127.

Fukami, K., Inanobe, S., Kanemaru, K., & Nakamura, Y. (2010). Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *Progress in Lipid Research, 49*(4), 429-437.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. Science **294**(5548): 1871-5.

GE, Y. W., & Lahiri, D. (2002). Regulation of promoter activity of the APP gene by cytokines and growth factors. *Annals of the New York Academy of Sciences, 973*(1), 463-467.

Ghosh, A., Ginty, D. D., Bading, H., & Greenberg, M. E. (1994). Calcium regulation of gene expression in neuronal cells. *Journal of Neurobiology, 25*(3), 294-303.

Giorgione, J., Epand, R. M., Buda, C., & Farkas, T. (1995). Role of phospholipids containing docosahexaenoyl chains in modulating the activity of protein kinase C. *Proceedings of the National Academy of Sciences, 92*(21), 9767.

Glynn, I. (1968). Membrane adenosine triphosphatase and cation transport. *British Medical Bulletin, 24*(2), 165-169.

Gollapudi, L., & Oblinger, M. M. (1999). Estrogen and NGF synergistically protect terminally differentiated, ERα-transfected PC12 cells from apoptosis. *Journal of Neuroscience Research, 56*(5), 471-481.

Gollapudi, L., & Oblinger, M. M. (1999). Stable transfection of PC12 cells with estrogen receptor (ERα): Protective effects of estrogen on cell survival after serum deprivation. *Journal of Neuroscience Research, 56*(1), 99-108.

Gorodinsky, A., & Harris, D. A. (1995). Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. *The Journal of Cell Biology, 129*(3), 619-627.

Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A., & Böhlent, P. (1984). Isolation of brain fibroblast growth factor by heparin-sepharose affinity chromatography: Identity with pituitary fibroblast growth factor. *Proceedings of the National Academy of Sciences, 81*(22), 6963.

Göttlicher, M., Widmark, E., Li, Q., & Gustafsson, J. A. (1992). Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proceedings of the National Academy of Sciences, 89*(10), 4653.

Grassmé, H., Jekle, A., Riehle, A., Schwarz, H., Berger, J., Sandhoff, K., Kolesnick R & Gulbins E. (2001). CD95 signaling via ceramide-rich membrane rafts. *Journal of Biological Chemistry, 276*(23), 20589-20596.

Grassmé, H., Schwarz, H., & Gulbins, E. (2001). Molecular mechanisms of ceramide-mediated CD95 clustering. *Biochemical and Biophysical Research Communications, 284*(4), 1016-1030.

Greenbaum, D., Colangelo, C., Williams, K., & Gerstein, M. (2003). Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol, 4*(9), 117.

Greene, L. A., & Kaplan, D. R. (1995). Early events in neurotrophin signalling via trk and p75 receptors. *Current Opinion in Neurobiology, 5*(5), 579-587.

Greene, L. A., & Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the National Academy of Sciences, 73*(7), 2424.

Gregory, M. K., Gibson, R. A., Cook-Johnson, R. J., Cleland, L. G., & James, M. J. (2011). Elongase reactions as control points in long-chain polyunsaturated fatty acid synthesis. *PloS One, 6*(12), e29662.

Grossfield, A., Feller, S. E., & Pitman, M. C. (2006). A role for direct interactions in the modulation of rhodopsin by ω-3 polyunsaturated lipids. *Proceedings of the National Academy of Sciences of the United States of America, 103*(13), 4888-4893.

Guidotti, G. (1972). The composition of biological membranes. *Archives of Internal Medicine, 129*(2), 194.

Gulaya, N., Volkov, G., Klimashevsky, V., Govseeva, N., & Melnik, A. (1989). Changes in lipid composition of neuroblastoma C1300 N18 cell during differentiation. *Neuroscience, 30*(1), 153-164.

Guo, W., Huang, N., Cai, J., Xie, W., & Hamilton, J. A. (2006). Fatty acid transport and metabolism in HepG2 cells. American Journal of Physiology: Gastrointestinal and Liver Physiology, 290, G528-G534.

Hamilton JA, Brunaldi K. A model for fatty acid transport into brain. J Mol Neurosci. 2007 Sep; 33(1):12-7.

Hara, A. and Radin, S. (1978). Lipid extraction of tissues with a low-toxicity solvent. Analytical biochemistry 90, 420-426.

Harnack, K., Andersen, G., & Somoza, V. (2009). Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids. *Nutrition & Metabolism, 6*(1), 8.

Haskell, B. E., Stach, R. W., Werrbach-Perez, K., & Perez-Polo, J. R. (1987). Effect of retinoic acid on nerve growth factor receptors. *Cell and Tissue Research, 247*(1), 67-73.

Hawthorne, J. N., & Ansell, G. B. (1982). *Phospholipids* Elsevier Science.

Hering H, Lin CC, Sheng M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. J Neurosci. 2003 Apr 15;23(8):3262-71.

Holman, R. T. (1998). The slow discovery of the importance of ω3 essential fatty acids in human health. *The Journal of Nutrition, 128*(2), 427S-433S.

Hooper, N. M. (1999). Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). *Molecular Membrane Biology, 16*(2), 145-156.

Huang, W. H., Kakar, S. S., & Askari, A. (1986). Activation of (Na++K+)-ATPase by long-chain fatty acids and fatty acyl coenzymes A. *Biochemistry International, 12*(4), 521-528.

Huber, T., Botelho, A. V., Beyer, K., & Brown, M. F. (2004). Membrane model for the G-protein-coupled receptor rhodopsin: Hydrophobic interface and dynamical structure. *Biophysical Journal, 86*(4), 2078-2100.

Hui T. Y. and Bernlohr D. A. (1997) Fatty acid transporters in animal cells. *Frontiers Biosci.* **2**, 222-231.

Hundle, B., McMahon, T., Dadgar, J., & Messing, R. O. (1995). Overexpression of-protein kinase C enhances nerve growth factor-induced phosphorylation of mitogen-activated protein kinases and neurite outgrowth. *Journal of Biological Chemistry, 270*(50), 30134-30140.

Igarashi, M., Ma, K., Chang, L., Bell, J. M., & Rapoport, S. I. (2007). Dietary n-3 PUFA deprivation for 15 weeks upregulates elongase and desaturase expression in rat liver but not brain. *Journal of Lipid Research, 48*(11), 2463-2470.

Ikemoto, A., Kobayashi, T., Watanabe, S., & Okuyama, H. (1997). Membrane fatty acid modifications of PC12 cells by arachidonate or docosahexaenoate affect neurite outgrowth but not norepinephrine release. *Neurochemical Research, 22*(6), 671-678.

Ikemoto, A., Nitta, A., Furukawa, S., Ohishi, M., Nakamura, A., Fujii, Y., & Okuyama H. (2000). Dietary n-3 fatty acid deficiency decreases nerve growth factor content in rat hippocampus. *Neuroscience Letters, 285*(2), 99-102.

Innis, S. M. (2004). Polyunsaturated fatty acids in human milk. *An Essential Role in Infant Development.Adv Exp Med Biol, 554*, 27-43.

Innis, S. M., & Dyer, R. A. (2002). Brain astrocyte synthesis of docosahexaenoic acid from n-3 fatty acids is limited at the elongation of docosapentaenoic acid. *Journal of Lipid Research, 43*(9), 1529-1536.

Innis, S. (2005). Essential fatty acid transfer and fetal development. *Placenta, 26*, S70-S75.

Innis, S. M. (1991). Essential fatty acids in growth and development. *Progress in Lipid Research, 30*(1), 39-103.

Insel, P., Ross, D., McMahon, K., & Bernstein, M. (2012). *Discovering nutrition-book only* Jones & Bartlett Publishers.

Izumi, T., Rådmark, O., Jörnvall, H., & Samuelsson, B. (1991). Purification of two forms of arachidonate 15-lipoxygenase from human leukocytes. *European Journal of Biochemistry, 202*(3), 1231-1238.

Jakobsson, P. J., Steinhilber, D., Odlander, B., Rådmark, O., Claesson, H. E., & Samuelsson, B. (1992). On the expression and regulation of 5-lipoxygenase in human lymphocytes. *Proceedings of the National Academy of Sciences, 89*(8), 3521.

Jampel, R. S. (1966). Neurology of the visual system. *Archives of Ophthalmology, 76*(1), 155.

Jana, A., Hogan, E. L., & Pahan, K. (2009). Ceramide and neurodegeneration: Susceptibility of neurons and oligodendrocytes to cell damage and death. *Journal of the Neurological Sciences, 278*(1-2), 5-15.

Jia, M., Li, M., Liu, X. W., Jiang, H., Nelson, P. G., & Guroff, G. (1999). Voltage-sensitive calcium currents are acutely increased by nerve growth factor in PC12 cells. *Journal of Neurophysiology, 82*(6), 2847-2852.

Jia, M., Li, M., Liu, X. W., Jiang, H., Nelson, P. G., & Guroff, G. (1999). Voltage-sensitive calcium currents are acutely increased by nerve growth factor in PC12 cells. *Journal of Neurophysiology, 82*(6), 2847-2852.

Jones-Villeneuve, E., Rudnicki, M. A., Harris, J. F., & McBurney, M. (1983). Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Molecular and Cellular Biology, 3*(12), 2271-2279.

Jump, D. B. (2004). Fatty acid regulation of gene transcription. *Critical Reviews in Clinical Laboratory Sciences, 41*(1), 41-78.

Jump, D. B., Botolin, D., Wang, Y., Xu, J., Christian, B., & Demeure, O. (2005). Fatty acid regulation of hepatic gene transcription. *The Journal of Nutrition, 135*(11), 2503-2506.

Jump, D. B., Clarke, S. D., MacDougald, O., & Thelen, A. (1993). Polyunsaturated fatty acids inhibit S14 gene transcription in rat liver and cultured hepatocytes. *Proceedings of the National Academy of Sciences, 90*(18), 8454.

Kaduce, T. L., Chen, Y., Hell, J. W., & Spector, A. A. (2008). Docosahexaenoic acid synthesis from n-3 fatty acid precursors in rat hippocampal neurons. *Journal of Neurochemistry, 105*(4), 1525-1535.

Kamp, F., Guo, W., Souto, R., Pilch, P. F., Corkey, B. E., & Hamilton, J. A. (2003). Rapid flip-flop of oleic acid across the plasma membrane of adipocytes. Journal of Biological Chemistry, 278, 7988-7995.

Kan, I., Melamed, E., Offen, D., & Green, P. (2007). Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation. *Journal of Lipid Research, 48*(3), 513-517.

Kaplan, D. R., & Stephens, R. M. (1994). Neurotrophin signal transduction by the trk receptor. *Journal of Neurobiology, 25*(11), 1404-1417.

Kawamura, H., Tatei, K., Nonaka, T., Obinata, H., Hattori, T., Ogawa, A., et al. (2009). Ceramide induces myogenic differentiation and apoptosis in drosophila schneider cells. *Journal of Radiation Research, 50*(2), 161-169.

Kawashima, A., Harada, T., Kami, H., Yano, T., Imada, K., & Mizuguchi, K. (2010). Effects of eicosapentaenoic acid on synaptic plasticity, fatty acid profile and phosphoinositide 3-kinase signaling in rat hippocampus and differentiated PC12 cells. *The Journal of Nutritional Biochemistry, 21*(4), 268-277.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., & Wahli, W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proceedings of the National Academy of Sciences, 90*(6), 2160.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., & Wahli, W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proceedings of the National Academy of Sciences, 90*(6), 2160.

Keller, S., Pitcher III, W., Huestis, W., & McConnell, H. (1998). Red blood cell lipids form immiscible liquids. *Physical Review Letters, 81*(22), 5019-5022.

Kim, H. J., Takahashi, M., & Ezaki, O. (1999). Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver A POSSIBLE MECHANISM FOR DOWN-REGULATION OF LIPOGENIC ENZYME mRNAs. *Journal of Biological Chemistry, 274*(36), 25892-25898.

Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand P, Wahli W, Willson TM, Lenhard JM & Lehmann JM. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proceedings of the National Academy of Sciences, 94*(9), 4318.

Kliewer, S., Forman, B., Blumberg, B., Ong, E., Borgmeyer, U., Mangelsdorf, D., e Umesono K. & Evans RM. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proceedings of the National Academy of Sciences, 91*(15), 7355.

Kliewer, S., Forman, B., Blumberg, B., Ong, E., Borgmeyer, U., Mangelsdorf, D., Umesono K. & Evans RM. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proceedings of the National Academy of Sciences, 91*(15), 7355.

Kohli, P., & Levy, B. D. (2009). Resolvins and protectins: Mediating solutions to inflammation. *British Journal of Pharmacology, 158*(4), 960-971.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M. G. & Wahli W. (1997). Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Molecular Endocrinology, 11*(6), 779-791.

Lands, W. (2000). Stories about acyl chains. *Biochim.Biophys.Acta, 1483*(1), 1-14.

Landschulz, K., Jump, D., MacDougald, O., & Lane, M. (1994). Transcriptional control of the stearoyl-CoA desaturase-1 gene by polyunsaturated fatty Acids1. *Biochemical and Biophysical Research Communications, 200*(2), 763-768.

Langelier, B., Alessandri, J. M., Perruchot, M. H., Guesnet, P., & Lavialle, M. (2005). Changes of the transcriptional and fatty acid profiles in response to n- 3 fatty acids in SH-SY5Y neuroblastoma cells. *Lipids, 40*(7), 719-728.

Lauritzen, L., Hansen, H., Jørgensen, M., & Michaelsen, K. (2001). The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Progress in Lipid Research, 40*(1-2), 1-94.

Lee, K. F., Davies, A. M., & Jaenisch, R. (1994). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development, 120*(4), 1027-1033.

L., G., P. and Lingappa, V. R. London, Jones and Barlett LLC: 232-290.

Le HD, Meisel JA, de Meijer VE, Gura KM, Puder M. (2009). The essentiality of arachidonic acid and docosahexaenoic acid. Prostaglandins Leukot Essent Fatty Acids, 81(2-3):165-70.

Lengqvist, J., de Urquiza, A. M., Bergman, A. C., Willson, T. M., Sjövall, J., Perlmann, T., & Griffiths WJ. (2004). Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor a ligand-binding domain. *Molecular & Cellular Proteomics, 3*(7), 692-703.

Leonard, A. E., Pereira, S. L., Sprecher, H., & Huang, Y. S. (2004). Elongation of long-chain fatty acids. *Progress in Lipid Research, 43*(1), 36.

Lerner, R., Lindström, P., Berg, A., Johansson, E., Rosendahl, K., & Palmblad, J. (1995). Development and characterization of essential fatty acid deficiency in human endothelial cells in culture. *Proceedings of the National Academy of Sciences, 92*(4), 1147.

Leutz, S., Steiner, B., Marques, C. A., Haass, C., Müller, W. E., & Eckert, A. (2002). Reduction of trophic support enhances apoptosis in PC12 cells expressing Alzheimer's APP mutation and sensitizes cells to staurosporine-induced cell death. *Journal of Molecular Neuroscience, 18*(3), 189-201.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science, 237*(4819), 1154-1162.

Li, S., Couet, J., & Lisanti, M. P. (1996). Src tyrosine kinases, Ga subunits, and H-ras share a common membrane-anchored scaffolding protein, caveolin. *Journal of Biological Chemistry, 271*(46), 29182-29190.

Lie Ken Jie, M., Lau, M., Lam, C., Alam, M., Metzger, J., & Biermann, U. (2003). Novel halo-oxo-allenic fatty ester derivatives from epoxidized methyl santalbate (methyl trans-11-octadecen-9-ynoate). *Chemistry and Physics of Lipids, 125*(2), 93-101.

Lindsberg PJ, Hallenbeck JM, Feuerstein G. Platelet-activating factor in stroke and brain injury. Ann Neurol. 1991 Aug;30(2):117-29.

Lipman, T., Tabakman, R., & Lazarovici, P. (2006). Neuroprotective effects of the stable nitroxide compound tempol on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in the nerve growth factor-differentiated model of pheochromocytoma PC12 cells. *European Journal of Pharmacology, 549*(1-3), 50-57.

Litman, B. J., Niu, S. L., Polozova, A., & Mitchell, D. C. (2001). The role of docosahexaenoic acid containing phospholipids in modulating G protein-coupled signaling pathways. *Journal of Molecular Neuroscience, 16*(2), 237-242.

Lobb, R. R., & Fett, J. W. (1984). Purification of two distinct growth factors from bovine neural tissue by heparin affinity chromatography. *Biochemistry, 23*(26), 6295-6299.

Ma, Y., Campenot, R., & Miller, F. (1992). Concentration-dependent regulation of neuronal gene expression by nerve growth factor. *The Journal of Cell Biology, 117*(1), 135-141.

Mahfouz, M., Johnson, S., & Holman, R. (1980). The effect of isomerictrans-18: 1 acids on the desaturation of palmitic, linoleic and eicosa-8, 11, 14-trienoic acids by rat liver microsomes. *Lipids, 15*(2), 100-107.

Malagelada, C., Jin, Z. H., Jackson-Lewis, V., Przedborski, S., & Greene, L. A. (2010). Rapamycin protects against neuron death in in vitro andIn vivo models of parkinson's disease. *The Journal of Neuroscience, 30*(3), 1166-1175.

Marcheselli, V. L., Hong, S., Lukiw, W. J., Tian, X. H., Gronert, K., Musto, A., Hardy M, Gimenez JM, Chiang N, Serhan CN & Bazan NG. (2003). Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *Journal of Biological Chemistry, 278*(44), 43807-43817.

Marszalek, J. R., Kitidis, C., Dararutana, A., & Lodish, H. F. (2004). Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth. *Journal of Biological Chemistry, 279*(23), 23882-23891.

Marszalek, J. R., Kitidis, C., DiRusso, C. C., & Lodish, H. F. (2005). Long-chain acyl-CoA synthetase 6 preferentially promotes DHA metabolism. *Journal of Biological Chemistry, 280*(11), 10817-10826.

Martin, G., Schoonjans, K., Lefebvre, A. M., Staels, B., & Auwerx, J. (1997). Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARα and PPARγ activators. *Journal of Biological Chemistry, 272*(45), 28210.

Martín, V., Almansa, E., Fabelo, N., & Díaz, M. (2006). Selective polyunsaturated fatty acids enrichment in phospholipids from neuronal-derived cell lines. *Journal of Neuroscience Methods, 153*(2), 230-238.

Martinez, M. (1992). Tissue levels of polyunsaturated fatty acids during early human development. *The Journal of Pediatrics, 120*(4), S129-S138.

Massague, J., Pilch, P. F., & Czech, M. P. (1980). Electrophoretic resolution of three major insulin receptor structures with unique subunit stoichiometries. *Proceedings of the National Academy of Sciences, 77*(12), 7137.

Matsuzaka, T., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Yoshikawa, T., Hasty, A. H., Tamura, Y., Osuga, J., Okazaki, H., Iizuka, Y., Takahashi, A., Sone, H., Gotoda, T., Ishibashi, S. and Yamada, N. (2002). "Dual regulation of mouse Delta(5)- and Delta(6)-desaturase gene expression by SREBP-1 and PPARalpha." Journal of Lipid Research 43(1): 107-14.

Matsuoka, I., Mizuno, N., & Kurihara, K. (1989). Cholinergic differentiation of clonal rat pheochromocytoma cells (PC12) induced by retinoic acid: Increase of choline acetyltransferase activity and decrease of tyrosine hydroxylase activity. *Brain Research, 502*(1), 53-60.

Mattson, M. P., Maudsley, S., & Martin, B. (2004). BDNF and 5-HT: A dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. *Trends in Neurosciences, 27*(10), 589-594.

Mérot, Y., Ferrière, F., Gailhouste, L., Huet, G., Percevault, F., Saligaut, C. & Flouriot G. (2009). Different outcomes of unliganded and liganded estrogen receptor-α on neurite outgrowth in PC12 cells. *Endocrinology, 150*(1), 200-211.

Meshulam, T., Simard, J. R., Wharton, J., Hamilton, J. A., & Pilch, P. F. (2006). Role of caveolin-1 and cholesterol in transmembrane fatty acid movement. Biochemistry, 45, 2882-2893.

Mey, J., & Rombach, N. (1999). Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells in vitro. *Neuroreport, 10*(17), 3573.

.

Mey, J., & Rombach, N. (1999). Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells in vitro. *Neuroreport, 10*(17), 3573.

Meyer, A., Cirpus, P., Ott, C., Schlecker, R., Zähringer, U., & Heinz, E. (2003). Biosynthesis of docosahexaenoic acid in euglena gracilis: Biochemical and molecular evidence for the involvement of a Δ4-fatty acyl group desaturase. *Biochemistry, 42*(32), 9779-9788.

Mikolajczak, K., Rogers, M. F., & Smith, C. (1967). An octadecatrienoic acid from lamium purpureum L. seed oil containing 5, 6-allenic and trans-16-olefinic unsaturation. *Biochemical Journal, 105*(3), 1245.

Miller, F. D., Mathew, T. C., & Toma, J. G. (1991). Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. *The Journal of Cell Biology, 112*(2), 303-312.

Mitchell, D. C., Niu, S. L., & Litman, B. J. (2003). Enhancement of G protein-coupled signaling by DHA phospholipids. *LIPIDS-CHICAGO THEN CHAMPAIGN-AMERICAN OIL CHEMISTS SOCIETY, 38*(4), 437-444.

Mohrhauer, H., & Holman, R. T. (1963). Effect of linolenic acid upon the metabolism of linoleic acid. *The Journal of Nutrition, 81*(1), 67-74.

Moore, S. A. (2001). Polyunsaturated fatty acid synthesis and release by brain-derived cells in vitro. *Journal of Molecular Neuroscience, 16*(2), 195-200.

Moore, S. A., Yoder, E., Murphy, S., Dutton, G. R., & Spector, A. A. (1991). Astrocytes, not neurons, produce docosahexaenoic acid (22: 6n-3) and arachidonic acid (20: 4?-6). *Journal of Neurochemistry, 56*(2), 518-524.

Moore, S. A., Yoder, E., & Spector, A. A. (1990). Role of the Blood-Brain barrier in the formation of Long-Chain ω-3 and ω-6 fatty acids from essential fatty acid precursors. *Journal of Neurochemistry, 55*(2), 391-402.

Morrison, R. S., Sharma, A., De Vellis, J., & Bradshaw, R. A. (1986). Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. *Proceedings of the National Academy of Sciences, 83*(19), 7537.

Msika, O., Brand, A., Crawford, M. A., & Yavin, E. (2012). NGF blocks polyunsaturated fatty acids biosynthesis in n-3 fatty acid-supplemented PC12 cells. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,*

Mukherjee, P. K., Marcheselli, V. L., Serhan, C. N., & Bazan, N. G. (2004). Neuroprotectin D1: A docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America, 101*(22), 8491.

Mundy, W. R., Radio, N. M., & Freudenrich, T. M. (2010). Neuronal models for evaluation of proliferation in vitro using high content screening. *Toxicology, 270*(2-3), 121-130.

Murphy, E. J., & Horrocks, L. A. (1993). Effects of differentiation on the phospholipid and phospholipid fatty acid composition of N1E-115 neuroblastoma cells. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1167*(2), 131-136.

Nakamura, M. T., Cho, H. P., & Clarke, S. D. (2000). Regulation of hepatic delta-6 desaturase expression and its role in the polyunsaturated fatty acid inhibition of fatty acid synthase gene expression in mice. *The Journal of Nutrition, 130*(6), 1561-1565.

Nakamura, M. T., & Nara, T. Y. (2004). Structure, function, and dietary regulation of Δ6, Δ5, and Δ9 desaturases. *Nutrition, 24*

Nakamura, M., & Nara, T. (2002). Gene regulation of mammalian desaturases. *Biochemical Society Transactions, 30*(6), 1076-1079.

Nakamura, S., Takahashi, T., Yamashita, H., & Kawakami, H. (2001). Nicotinic acetylcholine receptors and neurodegenerative disease. *Alcohol, 24*(2), 79-81.

Noronha J. G., Bell J. M., and Rapoport S. I. (1990) Quantitative brain autoradiography of [9,10-3H] palmitic acid incorporation into brain lipids. *J. Neurosci. Res.* 26, 196-208.

Niewiadomska G, Mietelska-Porowska A, Mazurkiewicz M (2011). The cholinergic system, nerve growth factor and the cytoskeleton. Behav Brain Res. ,221(2):515-26.

Nishida, K. and Markey, S.P. Platelet-activating factor in brain regions after transient ischemia in gerbils Stroke. 1996 Mar;27(3):514-8.

O'Brien, J. S., & Sampson, E. L. (1965). Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin. *Journal of Lipid Research, 6*(4), 545-551.

Okuyama, H. (1992). Minimum requirements of n-3 and n-6 essential fatty acids for the function of the central nervous system and for the prevention of chronic disease. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, NY), , 200.* (2) pp. 174-176.

Ou, J., Tu, H., Shan, B., Luk, A., DeBose-Boyd, R. A., Bashmakov, Y. & Goldstein JL, Brown MS. (2001). Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proceedings of the National Academy of Sciences, 98*(11), 6027.

Parton, R. G., & Simons, K. (2007). The multiple faces of caveolae. *Nature Reviews Molecular Cell Biology, 8*(3), 185-194.

Pawar, A., & Jump, D. B. (2003). Unsaturated fatty acid regulation of peroxisome proliferator-activated receptor a activity in rat primary hepatoctes. *Journal of Biological Chemistry, 278*(38), 35931-35939.

Pawar, A., Botolin, D., Mangelsdorf, D. J. and Jump, D. B. (2003). The role of liver X receptor-alpha in the fatty acid regulation of hepatic gene expression. The Journal of biological chemistry 278(42): 40736-43.

Payne, A. H., & Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews, 25*(6), 947-970.

Petroni, A., Blasevich, M., La Spada, P., Papini, N., & Galli, C. (1996). Arachidonic acid synthesis and lipid metabolism in retinoic acid-differentiated neuroblastoma cells. *Journal of Lipid Mediators and Cell Signalling, 14*(1-3), 39-44.

Petroni, A., Blasevich, M., Papini, N., La Spada, P., & Galli, C. (1996). Changes in arachidonic acid levels and formation and in lipid synthesis in the human neuroblastoma SK-N-BE during retinoic Acid-Induced differentiation. *Journal of Neurochemistry, 67*(2), 549-556.

Poling, J. S., Karanian, J. W., Salem Jr, N., & Vicini, S. (1995). Time-and voltage-dependent block of delayed rectifier potassium channels by docosahexaenoic acid. *Molecular Pharmacology, 47*(2), 381-390.

Poling, J., Vicini, S., Rogawski, M., & Salem Jr, N. (1996). Docosahexaenoic acid block of neuronal voltage-gated K channels: Subunit selective antagonism by zinc. *Neuropharmacology, 35*(7), 969-982.

Qiu, X., Hong, H., & MacKenzie, S. L. (2001). Identification of a Δ4 fatty acid desaturase fromThraustochytrium sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression inSaccharomyces cerevisiae and brassica juncea. *Journal of Biological Chemistry, 276*(34), 31561-31566.

Qutub, A. A., & Hunt, C. A. (2005). Glucose transport to the brain: A systems model. *Brain Research Reviews, 49*(3), 595-617.

Rakic, P. (2006). A century of progress in corticoneurogenesis: From silver impregnation to genetic engineering. *Cerebral Cortex, 16*(suppl 1), i3-i17.

Ravni, A., Bourgault, S., Lebon, A., Chan, P., Galas, L., Fournier, A. & Vaudry H, Gonzalez B, Eiden LE, Vaudry D. (2006). The neurotrophic effects of PACAP in PC12 cells: Control by multiple transduction pathways. *Journal of Neurochemistry, 98*(2), 321-329.

Reddy, J. K., Goel, S. K., Nemali, M. R., Carrino, J. J., Laffler, T. G., Reddy, M. K., & S J Sperbeck, T Osumi, T Hashimoto, N D Lalwani (1986). Transcription regulation of peroxisomal fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase in rat liver by peroxisome proliferators. *Proceedings of the National Academy of Sciences, 83*(6), 1747.

Repa, J. J., & Mangelsdorf, D. J. (2000). The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annual Review of Cell and Developmental Biology, 16*(1), 459-481.

Reza, J. Z., & Doosti, M. (2009). Modulation peroxisome proliferators activated receptor alpha (PPAR α) and acyl coenzyme A: Cholesterol Acyltransferase1 (ACAT1) gene expression by fatty acids in foam cell. *Lipids in Health and Disease, 8*(1), 38.

Ribet, C., Montastier, E., Valle, C., Bezaire, V., Mazzucotelli, A., Mairal, A., Viguerie N & Langin D. (2010). Peroxisome proliferator-activated receptor-α control of lipid and glucose metabolism in human white adipocytes. *Endocrinology, 151*(1), 123-133.

Ross, J. (1995). mRNA stability in mammalian cells. *Microbiological Reviews, 59*(3), 423-450.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., & Anderson, R. G. W. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell, 68*(4), 673-682.

Rydel, R. E., & Greene, L. A. (1987). Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. *The Journal of Neuroscience, 7*(11), 3639-3653.

Sahu, S. K., Gummadi, S. N., Manoj, N., & Aradhyam, G. K. (2007). Phospholipid scramblases: An overview. *Archives of Biochemistry and Biophysics, 462*(1), 103-114.

Sak, K., & Illes, P. (2005). Neuronal and glial cell lines as model systems for studying P2Y receptor pharmacology. *Neurochemistry International, 47*(6), 401-412.

Salem, N., Litman, B., Kim, H. Y., & Gawrisch, K. (2001). Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids, 36*(9), 945-959.

Sastry, P. (1985). Lipids of nervous tissue: Composition and metabolism. *Progress in Lipid Research, 24*(2), 69.

Sayanova, O. V., Beaudoin, F., Michaelson, L. V., Shewry, P. R., & Napier, J. A. (2003). Identification of primula fatty acid [delta] 6-desaturases with n-3 substrate preferences1. *FEBS Letters, 542*(1-3), 100-104.

Schaffer J. E. and Lodish H. F. (1994) Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell* 79, 427-436.

Scheibe, R. J., Ginty, D. D., & Wagner, J. A. (1991). Retinoic acid stimulates the differentiation of PC12 cells that are deficient in cAMP-dependent protein kinase. *The Journal of Cell Biology, 113*(5), 1173-1182.

Schmitz, G. and Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. Progress in lipid research 47(2): 147-55.

Schoonjans, K., Watanabe, M., Suzuki, H., Mahfoudi, A., Krey, G., Wahli, W., Grimaldi P, Staels B, Yamamoto T & Auwerx J. (1995). Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. *Journal of Biological Chemistry, 270*(33), 19269-19276.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen W & Selbach M. (2011). Global quantification of mammalian gene expression control. *Nature, 473*(7347), 337-342.

Scott, B. L., & Bazan, N. G. (1989). Membrane docosahexaenoate is supplied to the developing brain and retina by the liver. *Proceedings of the National Academy of Sciences, 86*(8), 2903.

Seibert, K., Masferrer, J., Zhang, Y., Leahy, K., Hauser, S., Gierse, J., Koboldt C, Anderson G, Bremer M & Gregory S. (1995). Expression and selective

inhibition of constitutive and inducible forms of cyclooxygenase. *Advances in Prostaglandin, Thromboxane, and Leukotriene Research, 23*, 125-127.

Seigneuret, M., & Devaux, P. F. (1984). ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: Relation to shape changes. *Proceedings of the National Academy of Sciences, 81*(12), 3751.

Serhan, C. N. (2005). Novel eicosanoid and docosanoid mediators: Resolvins, docosatrienes, and neuroprotectins. *Current Opinion in Clinical Nutrition & Metabolic Care, 8*(2), 115.

Sessler, A. M., Kaur, N., Palta, J. P., & Ntambi, J. M. (1996). Regulation of stearoyl-CoA desaturase 1 mRNA stability by polyunsaturated fatty acids in 3T3-L1 adipocytes. *Journal of Biological Chemistry, 271*(47), 29854.

Shea, T. B., Fischer, I., & Sapirstein, V. S. (1985). Effect of retinoic acid on growth and morphological differentiation of mouse NB2a neuroblastoma cells in culture. *Developmental Brain Research, 21*(2), 307-314.

Shearman, M. S., Ragan, C. I., & Iversen, L. L. (1994). Inhibition of PC12 cell redox activity is a specific, early indicator of the mechanism of beta-amyloid-mediated cell death. *Proceedings of the National Academy of Sciences, 91*(4), 1470.

Sidell, N., Altman, A., Haussler, M. R., & Seeger, R. C. (1983). Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Experimental Cell Research, 148*(1), 21-30.

Sidell, N., Lucas, C. A., & Kreutzberg, G. W. (1984). Regulation of acetylcholinesterase activity by retinoic acid in a human neuroblastoma cell line. *Experimental Cell Research, 155*(1), 305-309.

Sidell, N., Sarafian, T., Kelly, M., Tsuchida, T., & Haussler, M. (1986). Retinoic acid-induced differentiation of human neuroblastoma: A cell variant system showing two distinct responses. *Pathobiology, 54*(5-6), 287-300.

Simons, K., & Ikonen, E. (1997). Functional rafts in cell membranes. *Nature, 387*(6633), 569-572.

Singer, S., & Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Landmark Papers in Cell Biology*, 296-307.

Singh, M. (2005). Essential fatty acids, DHA and human brain. *Indian Journal of Pediatrics, 72*(3), 239-242.

Skou, J. C. (1965). Enzymatic basis for active transport of na and K across cell membrane. *Physiological Reviews, 45*(3), 596-618.

Slagsvold, J., Thorstensen, K., Kvitland, M., Mack, M., & Bjerve, K. (2007). Regulation of desaturase expression in HL60 cells. *Scandinavian Journal of Clinical & Laboratory Investigation, 67*(6), 632-642.

Slotte, J. P., & Bierman, E. L. (1988). Depletion of plasma-membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular cholesterol pools in cultured fibroblasts. *Biochemical Journal, 250*(3), 653.

Smith WL. Prostanoid biosynthesis and mechanisms of action. Am J Physiol. 1992 Aug;263(2 Pt 2):F181-91.

Song He, W., Nara, T. Y., & Nakamura, M. T. (2002). Delayed induction of [delta]-6 and [delta]-5 desaturases by a peroxisome proliferator. *Biochemical and Biophysical Research Communications, 299*(5), 832-838.

Soubias, O., Teague, W. E., & Gawrisch, K. (2006). Evidence for specificity in lipid-rhodopsin interactions. *Journal of Biological Chemistry, 281*(44), 33233-33241.

Spector, A. A., & Yorek, M. A. (1985). Membrane lipid composition and cellular function. *Journal of Lipid Research, 26*(9), 1015-1035.

Sprecher, H. (1986). The metabolism of (n-3) and (n-6) fatty acids and their oxygenation by platelet cyclooxygenase and lipoxygenase. *Progress in Lipid Research, 25*, 19-28.

Sprecher, H. (1992). Interconversions between 20-and 22-carbon n-3 and n-6 fatty acids via 4-desaturase independent pathways. *Essential Fatty Acids and Eicosanoids: Invited Papers from the Third International Congress*, pp. 18-22.

Sprecher, H. (1996). New advances in fatty-acid biosynthesis. *Nutrition, 12*(1), S5-S7.

Sprecher, H., Luthria, D. L., Mohammed, B., & Baykousheva, S. P. (1995). Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of Lipid Research, 36*(12), 2471-2477.

Steck, T. L. (1974). The organization of proteins in the human red blood cell membrane. *The Journal of Cell Biology, 62*(1), 1-19.

Stillwell, W., & Wassall, S. R. (2003). Docosahexaenoic acid: Membrane properties of a unique fatty acid. *Chemistry and Physics of Lipids, 126*(1), 1-27.

Stremmel W., Strohmeyer G., Borchard F., Shaul K., and Berk P. D. (1985) Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membrane. *Proc. Natl. Acad. Sci. USA* **82**, 4-8.

Suggate, E. L., Ahmed, Z., Read, M. L., Eaton-Charnock, K., Douglas, M. R., Gonzalez, A. M., et al. (2009). Optimisation of siRNA-mediated RhoA silencing in neuronal cultures. *Molecular and Cellular Neuroscience, 40*(4), 451-462.

Tang, C., Cho, H. P., Nakamura, M. T., & Clarke, S. D. (2003). Regulation of human Δ-6 desaturase gene transcription. *Journal of Lipid Research, 44*(4), 686-695.

Tassoni, D., Kaur, G., Weisinger, R. S., & Sinclair, A. (2011). The role of eicosanoids in the brain. *Asia Pacific Journal of Clinical Nutrition, 17*(S1), 220-228.

Tessler, A. (2004). Neurotrophic effects on dorsal root regeneration into the spinal cord. *Progress in Brain Research, 143*, 147-154.

Thomas, K. A., Rios-Candelore, M., & Fitzpatrick, S. (1984). Purification and characterization of acidic fibroblast growth factor from bovine brain. *Proceedings of the National Academy of Sciences, 81*(2), 357.

Thompson, W., & MacDonald, G. (1975). Isolation and characterization of cytidine diphosphate diglyceride from beef liver. *Journal of Biological Chemistry, 250*(17), 6779.

Thompson, W., & Macdonald, G. (1976). Cytidine diphosphate diglyceride of bovine brain. *European Journal of Biochemistry, 65*(1), 107-111.

Tinoco, J. (1982). Dietary requirements and functions of alpha-linolenic acid in animals. *Progress in Lipid Research, 21*(1), 1.

Tixier-Vidal, A., Picart, R., Loudes, C., & Bauman, A. F. (1986). Effects of polyunsaturated fatty acids and hormones on synaptogenesis in serum-free medium cultures of mouse fetal hypothalamic cells. *Neuroscience, 17*(1), 115-132.

Togari, A., Baker, D., Dickens, G., & Guroff, G. (1983). The neurite-promoting effect of fibroblast growth factor on PC12 cells. *Biochemical and Biophysical Research Communications, 114*(3), 1189-1193.

Togari, A., Dickens, G., Kuzuya, H., & Guroff, G. (1985). The effect of fibroblast growth factor on PC12 cells. *The Journal of Neuroscience, 5*(2), 307-316.

Tonon, T., Harvey, D., Larson, T. R., & Graham, I. A. (2003). Identification of a very long chain polyunsaturated fatty acid Δ4-desaturase from the microalga< i> pavlova lutheri</i>. *FEBS Letters, 553*(3), 440-444.

Traynor, A. E., Schubert, D., & Allen, W. R. (1982). Alterations of lipid metabolism in response to nerve growth factor. *Journal of Neurochemistry, 39*(6), 1677-1683.

Tu, W. C., Cook-Johnson, R. J., James, M. J., Muhlhausler, B. S., & Gibson, R. A. (2010). Omega-3 long chain fatty acid synthesis is regulated more by substrate levels than gene expression. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 83*(2), 61-68.

Vaisid, T., Kosower, N. S., Elkind, E., & Barnoy, S. (2008). Amyloid β peptide toxicity in differentiated PC12 cells: Calpain-calpastatin, caspase, and membrane damage. *Journal of Neuroscience Research, 86*(10), 2314-2325.

van Echten-Deckert, G., & Herget, T. (2006). Sphingolipid metabolism in neural cells. *Biochimica Et Biophysica Acta (BBA)-Biomembranes, 1758*(12), 1978-1994.

Vance, J. E., & Vance, D. E. (2004). Phospholipid biosynthesis in mammalian cells. *Biochemistry and Cell Biology, 82*(1), 113-128.

Vaudry, D., Stork, P., Lazarovici, P., & Eiden, L. (2002). Signaling pathways for PC12 cell differentiation: Making the right connections. *Science's STKE, 296*(5573), 1648.

Ved, H., & Pieringer, R. (1993). Regulation of neuronal differentiation by retinoic acid alone and in cooperation with thyroid hormone or hydrocortisone. *Developmental Neuroscience, 15*(1), 49-53.

Verdeil, G., Chaix, J., Schmitt-Verhulst, A. M., & Auphan-Anezin, N. (2006). Temporal cross-talk between TCR and STAT signals for CD8 T cell effector differentiation. *European Journal of Immunology, 36*(12), 3090-3100.

Verkleij, A., Zwaal, R., Roelofsen, B., Comfurius, P., Kastelijn, D., & Van Deenen, L. (1973). The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochimica Et Biophysica Acta (BBA)-Biomembranes, 323*(2), 178-193.

Voss, A., Reinhart, M., Sankarappa, S., & Sprecher, H. (1991). The metabolism of 7, 10, 13, 16, 19-docosapentaenoic acid to 4, 7, 10, 13, 16, 19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. *Journal of Biological Chemistry, 266*(30), 19995-20000.

Wagner, J. A., & D'Amore, P. A. (1986). Neurite outgrowth induced by an endothelial cell mitogen isolated from retina. *The Journal of Cell Biology, 103*(4), 1363-1367.

Walicke, P., Cowan, W., Ueno, N., Baird, A., & Guillemin, R. (1986). Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proceedings of the National Academy of Sciences, 83*(9), 3012.

Wallace, R. J., McKain, N., Shingfield, K. J., & Devillard, E. (2007). Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *Journal of Lipid Research, 48*(10), 2247-2254.

Walter, A., & Gutknecht, J. (1986). Permeability of small nonelectrolytes through lipid bilayer membranes. *Journal of Membrane Biology, 90*(3), 207-217.

Wang, Y., Botolin, D., Christian, B., Busik, J., Xu, J., & Jump, D. B. (2005). Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. *Journal of Lipid Research, 46*(4), 706-715.

Wang, Y., Botolin, D., Xu, J., Christian, B., Mitchell, E., Jayaprakasam, B., et al. (2006). Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *Journal of Lipid Research, 47*(9), 2028-2041.

Warren, S. Chute, Rn. (1972) . Pheochromocytoma. *Cancer, 29: 327-331*

Werner, S. L., Barken, D., & Hoffmann, A. (2005). Stimulus specificity of gene expression programs determined by temporal control of IKK activity. *Science's STKE, 309*(5742), 1857.

Westerink, R., & Ewing, A. G. (2008). The PC12 cell as model for neurosecretion. *Acta Physiologica, 192*(2), 273-285.

Wherrett, J., & Cumings, J. (1963). Detection and resolution of gangliosides in lipid extracts by thin-layer chromatography. *Biochemical Journal, 86*(2), 378.

Whitesides, J., Hall, M., Anchan, R., & LaMantia, A. S. (1998). Retinoid signaling distinguishes a subpopulation of olfactory receptor neurons in the developing and adult mouse. *The Journal of Comparative Neurology, 394*(4), 445-461.

Wierzbicki, M., Chabowski, A., Zendzian-Piotrowska, M., & Gorski, J. (2009). Differential effects of in vivo PPAR alpha and gamma activation on fatty acid transport proteins expression and lipid content in rat liver. *J Physiol Pharmacol, 60*, 99-106.

Williams, T. P., & McGee Jr, R. (1982). The effects of membrane fatty acid modification of clonal pheochromocytoma cells on depolarization-dependent exocytosis. *Journal of Biological Chemistry, 257*(7), 3491-3500.

Williard, D. E., Harmon, S. D., Kaduce, T. L., Preuss, M., Moore, S. A., Robbins, M. E. C., et al. (2001). Docosahexaenoic acid synthesis from n-3 polyunsaturated fatty acids in differentiated rat brain astrocytes. *Journal of Lipid Research, 42*(9), 1368-1376.

Wolfe, L., & Horrocks, L. (1994). Eicosanoids. *Basic Neurochemistry*, 475-490.

Wood, R., & Harlow, R. (1969). Structural analyses of rat liver phosphoglycerides. *Archives of Biochemistry and Biophysics, 135*, 272-281.

Wu, M. L., Chan, C. C., & Su, M. J. (2000). Possible mechanism(s) of arachidonic

acid-induced intracellular acidosis in rat cardiac myocytes. Circulation Research, 86, E55-E62.

Xu, Z. Z., Zhang, L., Liu, T., Park, J. Y., Berta, T., Yang, R., Serhan, C. N. and Ji, R. R. (2010). Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. Nature medicine **16**(5): 592-7, 1p following 597.

Yabuuchi, H., & O'Brien, J. S. (1968). Positional distribution of fatty acids in glycerophosphatides of bovine gray matter. *Journal of Lipid Research, 9*(1), 65-67.

Yao, R., & Cooper, G. M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science, 267*(5206), 2003-2006.

Yavin, E., Himovichi, E., & Eilam, R. (2009). Delayed cell migration in the developing rat brain following maternal omega 3 alpha linolenic acid dietary deficiency. *Neuroscience, 162*(4), 1011-1022.

Yavin, E., & Menkes, J. H. (1974). Polyenoic acid metabolism in cultured dissociated brain cells. *Journal of Lipid Research, 15*(2), 152-157.

Yoshikawa, T., Shimano, H., Yahagi, N., Ide, T., Amemiya-Kudo, M., Matsuzaka, T., et al. (2002). Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *Journal of Biological Chemistry, 277*(3), 1705-1711.

Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., et al. (1995). Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *Journal of Biological Chemistry, 270*(41), 23975-23983.

Yu, Y., Lucitt, M. B., Stubbe, J., Cheng, Y., Friis, U. G., Hansen, P. B., Jensen, B. L., Smyth, E. M. and FitzGerald, G. A. (2009). Prostaglandin F2 alpha elevates blood pressure and promotes atherosclerosis. Proceedings of the National Academy of Sciences of the United States of America **106**(19): 7985-90.

Zhang, Y., Li, X., Becker, K. A., & Gulbins, E. (2009). Ceramide-enriched membrane domains--structure and function. *Biochimica Et Biophysica Acta (BBA)-Biomembranes, 1788*(1), 178-183.

Zhou L, Nilsson A. Sources of eicosanoid precursor fatty acid pools in tissues. J Lipid Res. 2001 Oct;42(10):1521-42.

Zimmer, L., Delion-Vancassel, S., Durand, G., Guilloteau, D., Bodard, S., Besnard, J. C., et al. (2000). Modification of dopamine neurotransmission in the nucleus accumbens of rats deficient in n-3 polyunsaturated fatty acids. *Journal of Lipid Research, 41*(1), 32-40.

Zucali, J. R., Dinarello, C. A., Oblon, D. J., Gross, M. A., Anderson, L., & Weiner, R. S. (1986). Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E2. *Journal of Clinical Investigation, 77*(6), 1857.

Appendices

Appendix 1

Technical devices	Suppliers
Capillary column, BPX70	SGE, forte, 0.25 μm diameter
Cell culture flasks (25cm^2)	Nunc
Centrifuge 5804R	Eppendorf
Centrifuge 5810R	Eppendorf
15 ml Centrifuge tubes	Fisher Scientific
50 ml Centrifuge tubes	Fisher Scientific
Cryo-vials 1.8 ml	Fisher Scientific
Cryo-boxes or Cryo-box	Nalgene
Electrophoresis	Amersham pharmacia biotech
Filter bottle 150 ml	Fisher Scientific
Filter bottle 500 ml	Fisher Scientific
Filter tips 10 μl	Fisher Scientific
Filter tips 200 μl	Fisher Scientific
Filter tips 1000 μl	Fisher Scientific
Fluorescence microscope (1X81)	Olympus Corporation,Germany
FLUOstar Omega plate reader	BMG Labtech,UK
Gas chromatography	HRGC MEGA Series 2-Fisons, Instruments, Italy
GC vials	Klnesis
GC vials caps	Klnesis
3.5 ml glass vial	Fisher Scientific
Incubator Heraeus CO_2-Auto-Zero	Thermo Electron Corporation
Liquid nitrogen cell storage tank	Arpege 70
Methylated tubes	Corning
Microplate (48-well)	Nunc
Microplate (96-well)	Nunc
Microcentrifuge 5417R	Eppendorf
Microcentrifuge tube 1.5 ml	Fisher Scientific
Microcentrifuge tube 2 ml	Fisher Scientific
Microcentrifuge tube 0.5 ml	Fisher Scientific
NanoDrop ND-1000 spectrophotometer	NanoDrop Technologies
Nikon Inverted Microscope, TS100	Nikon Eclipse, Japan
Sample concentrator N_2 gas reservoir	Techne Dri -Block DB3
PCR tubes with attached cap 0.2 ml	Fisher Scientific
pH-Meter 766 Calimatic	Jenway
Rotor-Gene 6000	Corbett Research
Spectrometer	Lambda 35 UV/VIS

Sterile syringe filter with 0.2 μm cellulose	Spectrometer PerkinElmer
0.1 ml strip tubes and caps	VWR
Test tubes	Gene Target Solutions
Thermocycler	Fisherbrand
Glass Developing Tank for Thin Layer Chromatography	Biometra T300
TLC spray cabinet II	LAMAG
½ TLC silica gel 60 (10x20 cm)	LAMAG
UVP Chemiluminescence	MERCK
UV lamp	UVP Bioimaging Systems, UK
Water-bath	LAMAG
	Grant

Appendix 2

Chemicals	Suppliers
ABsolute Blue QPCR SYBR Green ROX Mix	Thermo Scientific
Absolute Ethanol	Hayman
Acetic acid glacial	Fisher scientific
Acetyl chloride, 98%	Acros Organics
Agarose, for routine use	Sigma-Aldrich
All-*trans*-retinoic acid	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
Buffer M-MuLV RT	Fermentas
Buffer RPE	Quiagen
Buffer RW1	Quiagen
Butylated hydroxytoluene	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Chloroform	Fisher scientific
Desoxynucleotide Mix (dNTP)	Sigma-Aldrich
2',7'-dichlorofluorescein	Sigma-Aldrich
D(+)-glucose	Sigma-Aldrich
Dimethyl sulfoxide minimum 99.5% *GC*	Sigma-Aldrich
Dry Methanol	Fisher scientific
Dulbecco's modified Eagle's medium	Sigma-Aldrich
Dulbecco's phosphate buffered saline (10x)	Sigma-Aldrich
Ethidium bromide solution	Sigma-Aldrich
Ethylenediamine tetraacetic acid, disodium salt dehydrate	Fisher Scientific
Foetal bovine serum gold	PAA
Free Fatty Acid	Sigma-Aldrich
Heat inactivated,sterile,filtered horse serum	Sigma-Aldrich
GC individual FA standard	Sigma-Aldrich
Glycerol (Glycerin)	Sigma-Aldrich
Hexane	Fisher Scientific
Heptane	Fisher Scientific
Isopropanol	Fisher Scientific
L-glutamine	PAA
Methanol	Fisher Scientific
Methylamine	Acros Organics
Molybdenum blue spray reagent, 1.3%,	Sigma-Aldrich
Nerve growth factor	Alomone Labs, Jerusalem, IL
Oligo-dT primer	Invitrogen
PCR primers	Integrated DNA Technologies

Penicillin/Streptomycin	PAA
Poly-L-lysine	Sigma-Aldrich
Potassium chloride	BDH
Potassium hydrogen carbonate	Fisher Scientific
Potassium phosphate	Sigma-Aldrich
Petroleum ether	Fisher Scientific
QIAshredder spin column	Qiagen
Quick-Load 100 bp DNA ladder	BioLabs
RevertAid™ First Strand cDNA Synthesis Kit	Fermentas
RevertAid™ M-MuLV Reverse Transcriptase	Fermentas
RiboLock™ RNase Inhibitor	Fermentas
RNase-free water	Sigma-Aldrich
RNAase Zap	Spyglass
RNeasy purification kit	Qiagen
RNeasy spin column	Qiagen
Sodium chloride	VWR
Sodium hydroxide pellets	BDH
Sodium sulphate	Acros Organics
Tris (hydroxymethyl)aminomethane, ultrapure grade	Sigma-Aldrich
Trypsin/EDTA (1X)	PAA

Appendix 3

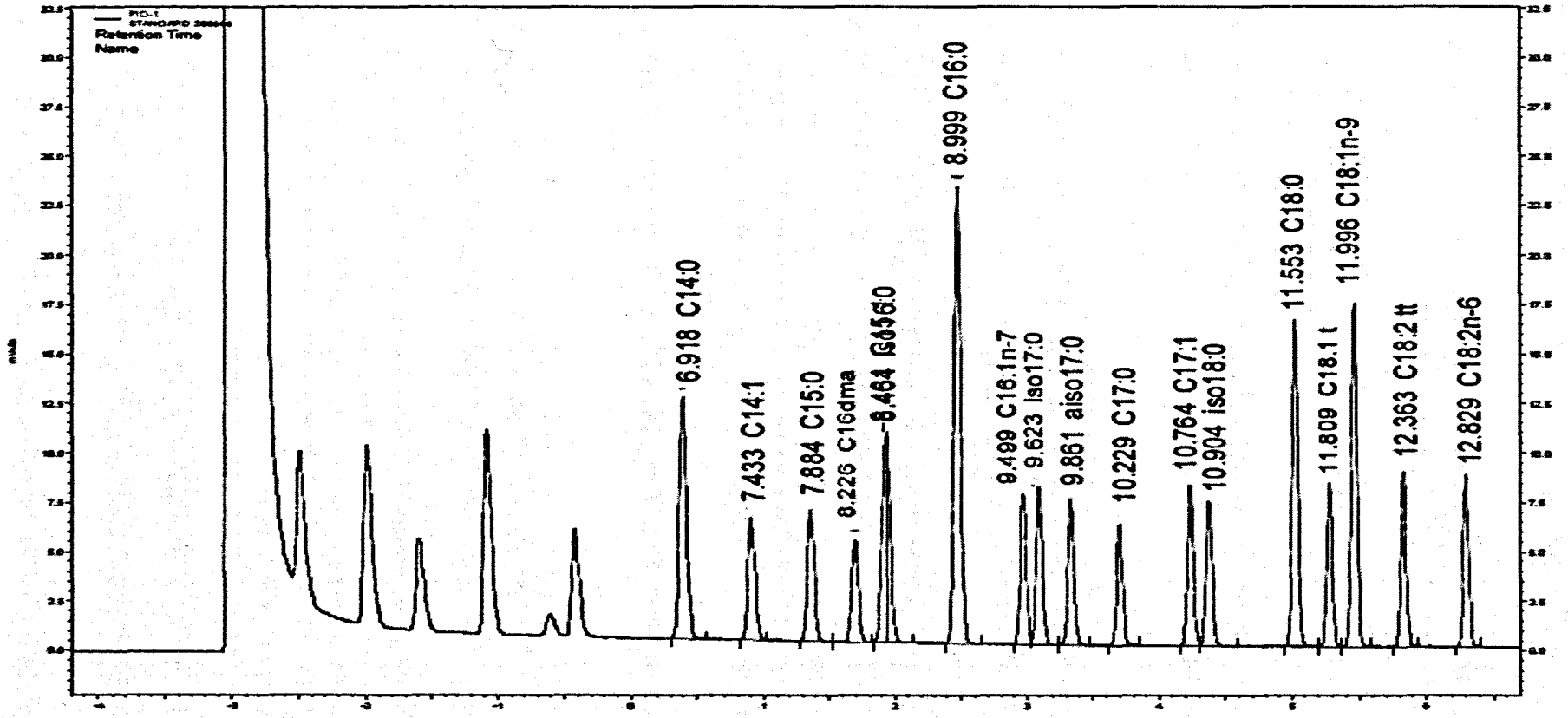
Appendix 3: Lipid Standards: Fatty Acid Methyl Ester (FAME) mixture (100 mg ampoule contains a mixture of individual FAMEs; the weight percentages of each component are indicated).

Trivial Name	Systematic name	Abbreviation	Weight (%)
Butyric acid Methyl Ester	Butanoic acid Methyl Ester	4:0	4
Caproic acid Methyl Ester	Hexanoic acid Methyl Ester	6:0	4
Caprylic acid Methyl Ester	Octanoic acid Methyl Ester	8:0	4
Capric acid Methyl Ester	Decanoic acid Methyl Ester	10:0	2
Lauric acid Methyl Ester	Dodecanoic acid Methyl Ester	12:0	4
	Tridecanoic acid Methyl Ester	13:0	2
Myristic Acid Methyl Ester	Tetradecanoic acid Methyl Ester	14:0	4
Myristoleic acid Methyl Ester	Tetradecenoic acid Methyl Ester	14:1	2
	Pentadecanoic acid Methyl Ester	15:0	2
	Cis-10-Pentadecenoic acid Methyl Ester	15:1	2
Palmitic acid Methyl Ester	Hexadecanoic acid Methyl Ester	16:0	6
Palmitoleic acid Methyl Ester	Hexadecenoic acid Methyl Ester	16:1	2
Margaric acid	Heptadecanoic acid Methyl Ester	17:0	2
	Cis-10-Heptadecenoic acid Methyl Ester	17:1	4
Stearic acid Methyl Ester	Octadecanoic acid Methyl Ester	18:0	4
Oleic acid Methyl Ester	Octadecenoic acid Methyl Ester	18:1	4
Elaidic acid Methyl Ester		18:1	2
Linoleic acid Methyl Ester	Octadecadienoic acid Methyl Ester	18:2	2
Linolelaidic acid		18:2	2
Linoleic acid Methyl Ester	Octadecadienoic acid	18:2	2
Linolelaidic acid Methyl Ester		18:2	2
α- linolenic acid Methyl Ester		18:3	2
γ- linolenic acid Methyl Ester		18:3	2
Arachidic acid Methyl Ester	Eicosanoic acid Methyl Ester	20:0	4
Gadoleic acid Methyl Ester	Cis-11-eicosenoic acid Methyl Ester	20:1	2
	Cis-11,14-eicosadienoic acid Methyl Ester	20:2	2
	Cis-11,14,17-eicosatrienoic acid Methyl Ester	20:3	2
Arachidonic acid Methyl Ester	Eicosatetraenoic acid Methyl Ester	20:4	2
Timnodonic acid Methyl Ester	Cis-5,8,11,14,17-eicosapentaenoic acid Methyl Ester	20:5	2

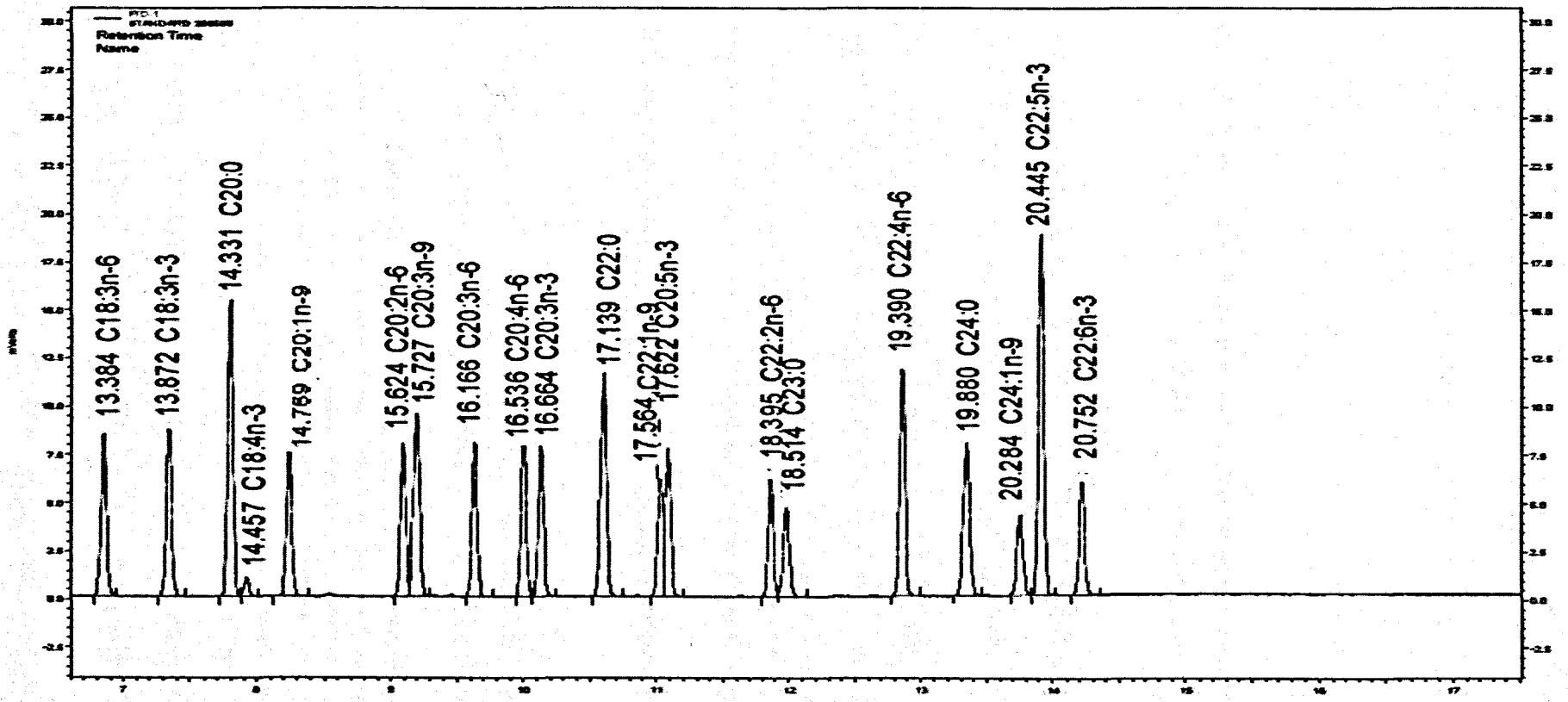
Heneicosanoic acid Methyl Ester		21:0	2
Behenic acid Methyl Ester	Docosanoic acid Methyl Ester	22:0	4
Erucic acid Methyl Ester	Docosenoic acid Methyl Ester	22:1	2
Brassic acid Methyl Ester	Cis-13,16-docosadienoic acid Methyl Ester	22:2	2
Docosahexaenoic acid Methyl Ester	Cis-4,7,10,13,16,19-docosahexaenoic acid Methyl Ester	22:6	2
Tricosanoic acid Methyl Ester		23:0	2
Lignoceric acid Methyl Ester	Tetracosanoic acid Methyl Ester	24:0	4
Nervonic acid Methyl Ester	cis-tetracosenoic acid Methyl Ester	24:1	2

Appendix 4

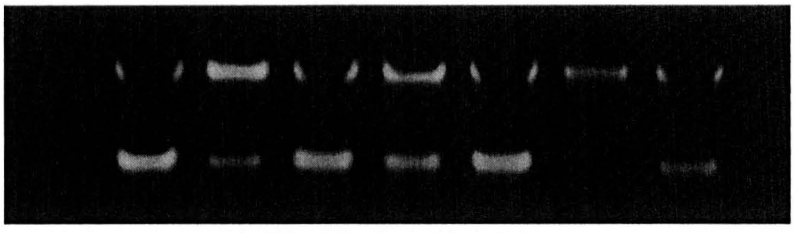
Appendix 4: A chromatogram of a fatty acid methyl ester standard mix



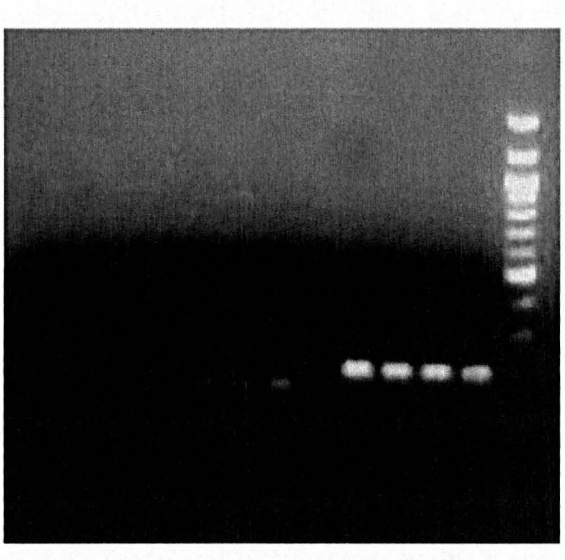
Continued from previous page



Appendices 5 & 6



Appendix 5: Quality of total RNA extracted from PC12 cells. Total RNA samples were resolved in 1% agarose gel and visualised with ethidium bromide.



Appendix 6: Validation results of qRT-PCR. PCR products were resolved in 1% agarose gel and visualised with ethidium bromide.
Ba (beta actin), Elovl (Elongation of very long-chain fatty acids)

Appendix 7

Appendix 7: The fatty acid compositions of DMEM medium, foetal bovine serum (FBS), horse serum (HS) and growth medium (GM; 5%FBS and 10% HS) used in this study were as follows:

	DMEM (μM)	FBS (μM)	HS (μM)	GM (μM)
FA	Saturated fatty acids			
16:0 palmitic	6.18	68.62	285.22	64.35
18:0 stearic	3.25	32.99	338.15	62.8
	Monounaturated fatty acids			
18:1n-9 oleic	0.6	53.31	182.56	39.66
	n-6 and n-3 PUFAs			
18:2n-6 LA	0.12	21.73	767.91	154.89
18:3n-3 ALA		1.91	21.87	4.22
20:4n-6		12.91	12.25	3.50
20:5n-3	0.06	3.48		0.35
22:6n-3		8.19		0.29

Appendix 8

Appendix 8: Fatty acid profile of proliferating PC12 cells' total lipid extract (TLE). PC12 cells were grown for three days in serum under semi-starvation (1% FBS) conditions and lipids were extracted and processed for GC as described in Materials and Methods. Data are reported as mean percentage of total FA± SEM.

FA	Mean % ± SEM
Saturated fatty acids	
16:0	17.54 ± 1.97
18:0	13.57 ± 1.40
20:0	0.68 ± 0.07
22:0	0.45 ± 0.072
24:0	0.33 ± 0.15
Plasmalogen species	
16:0 DMA	1.43 ± 0.34
18:0 DMA	1.33 ± 0.6
18:1 DMA	0.38 ± 0.24
Monounsaturated fatty acids	
16:1n-7	0.93 ± 0.23
18:1n-7	7.3 ± 0.51
18:1n-9	30.5 ± 0.83
20:1n-9	2.64 ± 0.35
20:3n-9	2.42 ± 0.29
22:1n-9	0.47± 0.15
24:1n-9	0.58 ± 0.16
N-6 and n-3 PUFAs	
18:2n-6	6.42 ± 0.42
20:2n-6	0.22 ± 0.09
20:3n-6	1.18 ± 0.27
20:4n-6	6.22 ± 0.75
22:4n-6	0.36 ± 0.03
22:5n-6	0.33 ± 0.20
20:5n-3	0.22 ± 0.11
22:5n-3	0.34 ± 0.046
22:6n-3	1.2 ± 0.17