# INTRODUCTION

Second to the adipose tissue, brain is the most lipid concentrated organ in the body. More concretely, brain is very rich in two long chain polyunsaturated fatty acids (LcPUFA), arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), derivatives of linoleic acid (LA, 18:2n-6) and α-linolenic acid (LNA, 18:3n-3), respectively. All of them play vital roles in the organism but, only LA and LNA are considered as essential since they cannot be synthesized, being the diet the only source. However, the synthesis rate of AA and DHA from their parent fatty acids is too slow to cover body requirements, especially in stages with high lipid metabolism like development, and is for this reason why the two fatty acids must be present in the diet as well. Hence, an appropriated dietary lipid composition is important first, for a proper development and later on, for the maintenance of the organism functions.

A large number of PUFA-deficient dietary studies were carried out the last decades, all of them pointed out to the necessity of PUFAs for a proper development of the central nervous system. In addition, while PUFA-deficient diets have been implicated with impairment or completely lost of cognitive functions, PUFA-enriched diets have been related with an improvement of the learning capacity. Thus, it is clear that PUFAs are somehow involved in memory and learning processes although the exact mechanism how they act is still poorly understood.

The hypothesis more studied is the influence that dietary lipids, as future components of membrane phospholipids, might have on the membrane fluidity. This biophysical property is determined by different factors, like temperature, cholesterol level and, phospholipid composition. Since the body temperature in mammals is constant, alterations in membrane lipid composition might be considered as the responsible of changes in the fluidity and, consequently in whole cell function. The first hints of the active role played by the lipids in the cell functioning, were provided by experiments showing that the activity of a number of transmembrane and membrane-

associated enzymes was modified by the membrane fluidity. Importantly, among these enzymes, are those implicated in signal transduction.

However, processes like behavioral changes, learning, loss of memory and, aging are too complex to be explained by a simple fluidity-dependent mechanism. Thus, more elements are needed for a better comprehension of the role that PUFAs are playing in such processes. Two relatively recent discoveries might help to shed some light on this topic: the lipid rafts and the fatty acid interaction with the peroxisome proliferator activated receptors (PPAR).

Lipid rafts are membrane subdomains with a specific lipid and protein composition. PUFAs have been shown to change lipid raft composition and therefore, the activity of the proteins embedded in them may also be altered. On the other hand, PPAR are ligand-dependent nuclear factors that act as transcription factors binding directly to some specific sequences in the DNA chain. Interestingly, fatty acids and some lipid derivates were found to be ligands of these receptors. Therefore it is likely to link diet with gene expression patterns. All this information open a wide range of possible mechanisms through which the effects and the need for PUFAs could be better understood.

The present thesis attempts to provide new data that highlight the importance of dietary polyunsaturated fatty acids in mammalian brain metabolism and activity, and that might serve to establish a new mechanism to explain the fundamental role that PUFAs display in the brain.

# AIMS OF THE STUDIES

Compared to liver or adipose tissue, brain lipid metabolism is still poorly understood. It has been well established that the main source of brain polyunsaturated fatty acids is the diet, and this would imply a key role of the dietary lipid composition in the final lipid composition of the brain. However, such importance has usually been restricted to certain periods when the lipid metabolism is very high, as it happens during the brain and nervous system development. During these stages there is a continuous membrane formation for the newly formed cells, consequently a high concentration of available PUFAs is required. One of the consequences of a poor supply of them is a minor learning capacity of the animal.

PUFAs have been also involved in the aging process. Losses of PUFAs have been reported in aged animals and in a certain of neurodegenerative diseases. That loss is usually accompanied by an impairment of the cognitive functions.

This linkage established between dietary PUFAs and learning, aroused the interest to understand the underlying mechanism. Work focused on the n-3 and/or n-6 deficient diets highlighted the importance of the dietary lipid composition. Interestingly not too much work has been done using PUFA-sufficient diets.

Based on all these facts, we aimed to carry on experiments on the role of dietary lipids in the composition of the neuronal membranes of PUFA-sufficient animals. Four different diets with a particular lipid composition were designed. The effectiveness to exert changes and the nature of these changes were determined analyzing the phospholipid composition by HPLC and GC.

Owing to the relationship existing between the metabolic rate and the age, the latter was an important variable to be taken in consideration. For this reason, three groups of rats differing in age were used in our studies. The first group consisted in rats

fed from conception and they were fed till reaching adulthood (6-week-old). The other two groups were 3-month and 2-year-old rat. Later on some measurements of the learning capacities were performed.

Finally, dietary lipids have been involved in alterations of gene expression in liver and adipose tissue. It was intriguing to see whether they would have similar effects in brain.

In this view the objectives were as follows:

1. To investigate the capacity of neuronal membranes to accumulate PUFAs and the relation of this accumulation with the dietary lipid composition.
2. To determine the importance of starting time and duration of feeding period.
3. To examine the changes in the membrane at the molecular level using gas chromatography, to analyze changes in total fatty acids and HPLC for changes in molecular species composition.
4. To reveal the relationship between age and phospholipid turnover in the neuronal membranes.
5. To investigate whether it is possible to restore PUFAs levels lost during aging process and to correlate changes in membrane composition with improvement of learning abilities.
6. To compare the phospholipid composition in forebrain, cerebellum and hippocampus and their response to diet.
7. To confirm the involvement of dietary lipids in brain gene expression and to reveal the importance of the exact lipid composition of the diet.
8. With the synthesis of the results obtained from the experiments, to find generalities regarding the role of polyunsaturated fatty acids in brain function and metabolism.

# 1.THEORETICAL BACKGROUND.

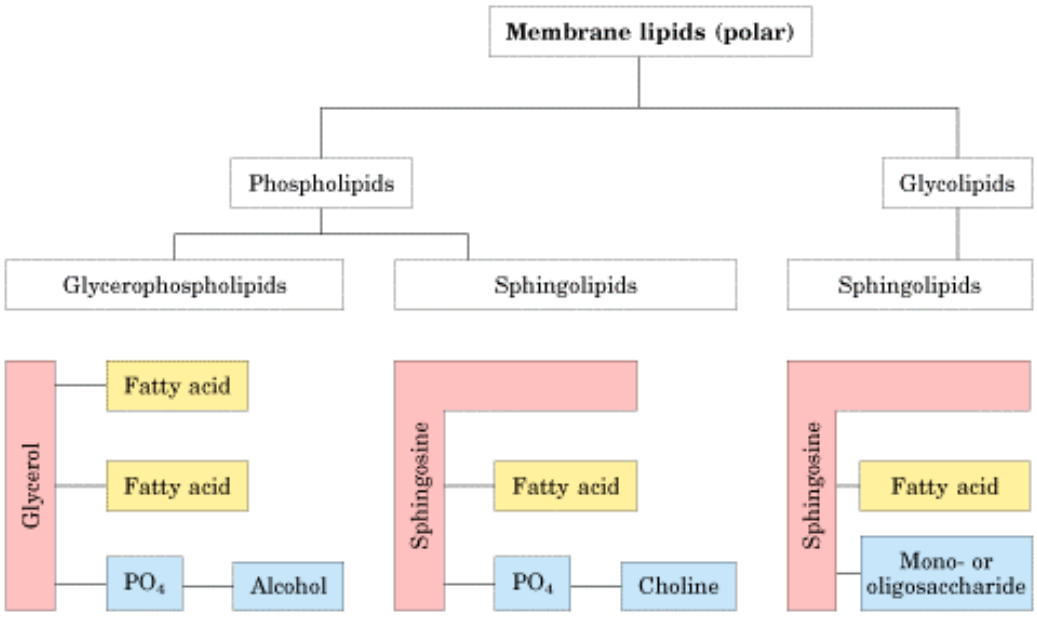
## 1.1. Classification and function of lipids in mammals.

### 1.1.1. Membrane lipids.

Among the tissues of the body, nervous tissue is one of the richest in lipids. In brain, lipids account for about 10% of the fresh weight and half of the dry matter. Apart from their quantitative importance, the lipids of the nervous tissue show a great deal of structural diversity although the biological significance of such diversity in lipids is, however, not clear (188).

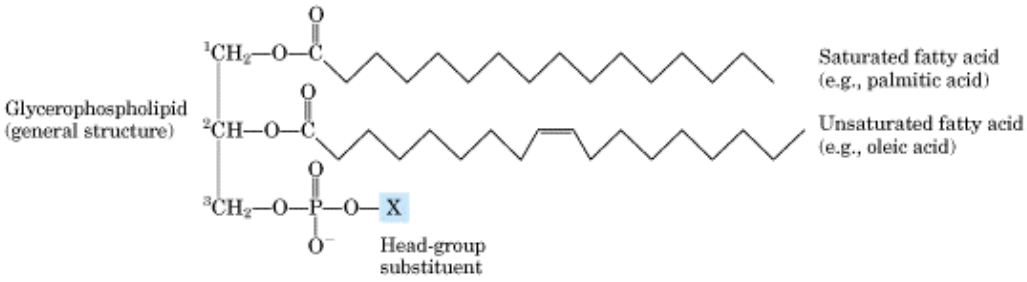
Brain is characterized by a high content of neutral lipids: cholesterol, glycolipids and phospholipids (1), which are the three major kinds of membrane lipids. The largest component in neutral lipids is cholesterol, making up 30% of total lipids. The glycolipids posses a sphingosine backbone where the amino group is acylated by a fatty acid, and the primary hydroxyl group is linked to one or more sugars residues. Finally, the phospholipids can be derived from glycerol, a three-carbon alcohol, or sphingosine, a more complex alcohol (fig. 1.1.).

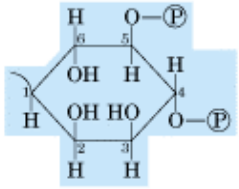
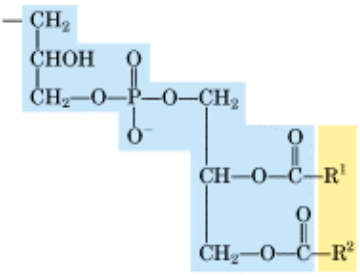
Figure 1.1. Classification of membrane lipids.



Phospholipids derived from glycerol are called phosphoglycerides. They consist of a glycerol backbone, two fatty acid chains esterified at C-1 and C-2 through the carboxyl group, and a phosphorylated alcohol, esterified at the C-3. The simplest is the phosphatidic acid, but in the majority of phosphoglycerides, the phosphate group is esterified to a hydrophilic group, like, ethanolamine, choline, serine or inositol. Phosphoglycerides are the major phospholipid components ubiquitously found in cell membranes and they are usually considered as the building blocks of all the membranes.

Figure.1.2. Structure and classification according to polar head of the glycerophospholipids.

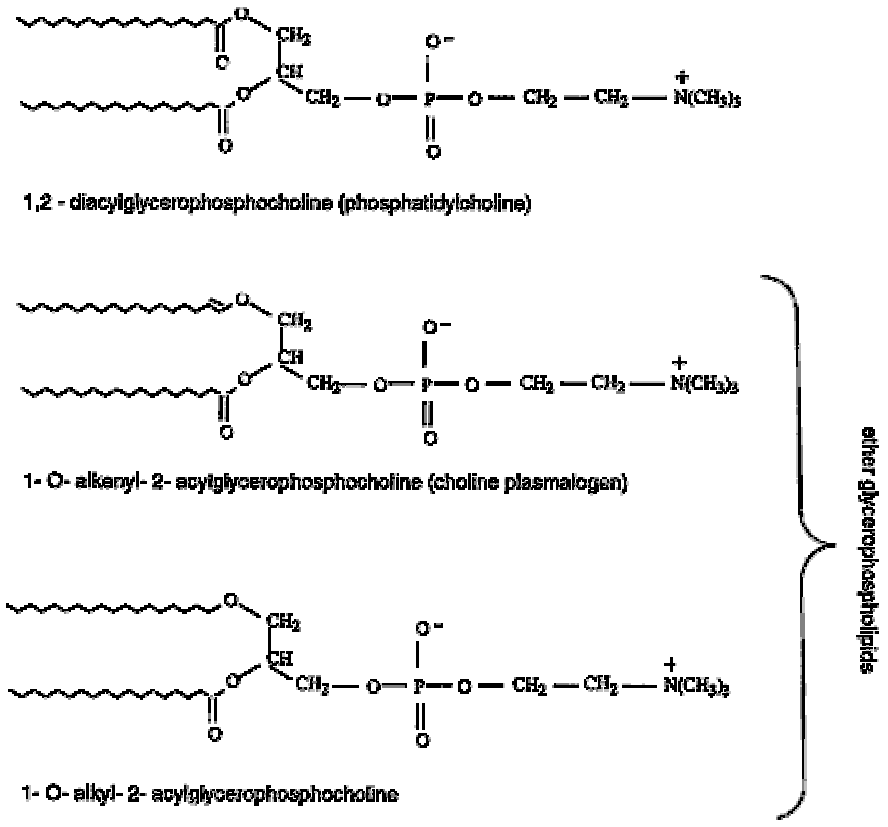



| Name of glycerophospholipid | Name of X | Formula of X | Net charge (at pH 7) |
|---|---|---|---|
| Phosphatidic acid | — | — H | −1 |
| Phosphatidylethanolamine | Ethanolamine | — $CH_2$—$CH_2$—$\overset{+}{N}H_3$ | 0 |
| Phosphatidylcholine | Choline | — $CH_2$—$CH_2$—$\overset{+}{N}(CH_3)_3$ | 0 |
| Phosphatidylserine | Serine | — $CH_2$—$CH$(—$\overset{+}{N}H_3$)—$COO^-$ | −1 |
| Phosphatidylglycerol | Glycerol | — $CH_2$—$CH$(—OH)—$CH_2$—OH | −1 |
| Phosphatidylinositol 4,5-bisphosphate | *myo*-Inositol 4,5-bisphosphate |  | −4 |
| Cardiolipin | Phosphatidyl-glycerol |  | −2 |

The nature of the headgroup is an important feature for the interaction with proteins within the membrane. The acidic headgroups phosphatidylserine and phosphatidylinositol may give rise to electrostatic interactions with positively charged residues. Furthermore, the size of the headgroups is of importance, i.e. bulky headgroups leave less space for proteins to insert (218).

Previous classification of phosphoglycerides was established according to nature of the hydrophilic moiety. The hydroxyl at *sn*-2 position is always linked to the fatty acid through an ester linkage fatty acid, but in *sn*-1 there are three possibilities. This lends an additional criteria to classify the phosphoglycerides according to the linkage between the fatty acid and the hydroxyl at the *sn*-1 position (fig. 1.3). Therefore, three phosphoglycerides subclasses can be distinguished: diacyl-, alkyl-, and alkenyl-phosphoglycerides.

Figure1.3. Classification of phosphoglycerides according to the hydrophobic part.



Despite the extensive repertoire of membrane lipids, all of them possess a critical common structural theme: membrane lipids are amphipathic molecules, containing both a hydrophilic and a hydrophobic moiety. For this reason, the favored structure for most phospholipids and glycolipids in aqueous media is lipid bilayer.

### 1.1.2. Classification and function of fatty acids.

The percentage of free fatty acids in the organism is very low, usually they are forming part of phospholipids and glycolipids. In most animal cells, phospholipids are predominantly of a mixed-chain variety, meaning that the two acyl chains are structurally different. Typically in *sn*-1 position the fatty acid is saturated and contains an even number of carbon atoms ranging from 14 to 22, predominantly 16 and 18 carbons. Whereas at the *sn*-2 position contains 1-6 *cis* double bonds (Δ-bonds) and may have 16-22 carbons (123). This variation in acyl chain and in headgroup composition results in the presence of hundreds of different lipid species.

Fatty acids carry out many functions that are necessary for normal physiological function and good health. Saturated fatty acids are involved in energy production, energy storage, lipid transport, the synthesis of phospholipids and sphingolipids needed for membrane synthesis, and the covalent modification of many regulatory proteins. Monounsaturated fatty acids also are involved in many of these processes and play key role in maintaining optimal fluidity of the membrane lipid bilayer. Although these are clearly vital physiological processes, the term “essential” is not applied to them. The designation is reserved for those polyunsaturated fatty acids (PUFAs) that are required for good health and, in addition, cannot be completely synthesized in the body. As it is presently used, essentiality implies that the fatty acid not only performs a vital function, but it is also is required dietary nutrient (200).

There are four families of polyunsaturated fatty acids derived from the parent fatty acids: linoleic acid ($C_{18:2}$n-6), α-linolenic acid ($C_{18:3}$n-3), oleic acid ($C_{18:1}$n-9), and palmitoleic ($C_{16:1}$n-7). The two families derived from the n-6 and n-3 polyunsaturated

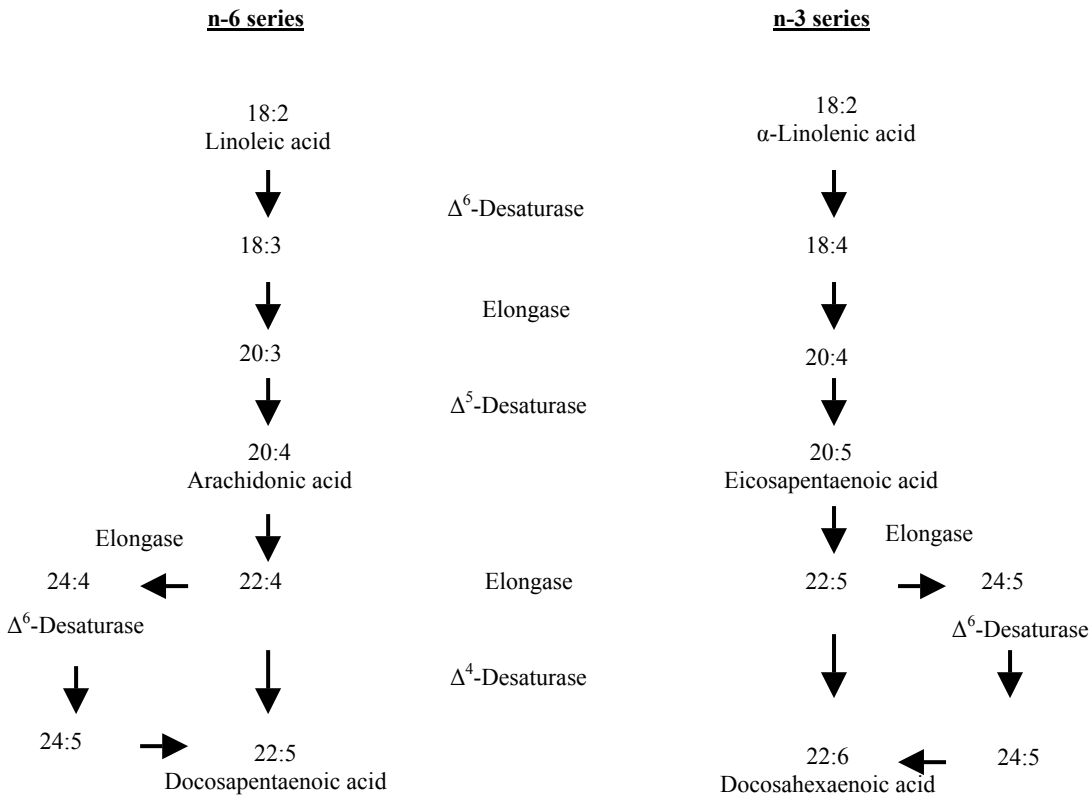
fatty acids, linoleic and α-linolenic acids, are the essential fatty acids (EFAs). The two families of essential fatty acids have distinct nutritional and metabolic effects and they should be differentiated (table 1.1.). Linoleic and α-linolenic acids are the parent fatty acids for the direct precursors of various classes of eicosanoids, dihomo-γ-linolenic, arachidonic, and eicosapentaenoic acids. The other two families derived from palmitoleic and oleic acids are considered to be non-essential fatty acids, because humans can synthesize them from saturated fatty acid precursors (67).

Table 1.1. Biochemical functions of the n-6 and n-3 fatty acids.

| Class | Fatty acid | Function |
|---|---|---|
| n-6 | arachidonic | Eicosanoids synthesis |
| | | Component of the inositol phospholipids |
| n-6 | linoleic | Synthesis of lipids that form the epidermal permeability barrier |
| n-3 | eicosapentaenoic | Eicosanoids synthesis |
| | | Structural analog and competitor of arachidonic acid |
| n-3 | docosahexaenoic | Structure of membrane lipid domains |
| | | Modulation of integral membrane proteins |
| | | Metabolism of phosphatidylethanolamine, ethanolamine plasmalogens, and phosphatidylserine |
| | | Formation of free radicals |
| | | Regulation of gene expression. |

Interconversion among the four families of unsaturated fatty acids does not occur in humans. However, the enzymes for the desaturation and elongation reactions by which the parent fatty acids are converted to their derivative fatty acids are shared by all four families (69) (fig. 1.4). The rates of desaturation and elongation differ among the families, and the rank of the families by order of decreasing rates is n-3 > n-6 > n-9 > n-7 (69).

Figure 1.4. Metabolic pathways of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3).




For each fatty acid, two alternative pathways are shown, involving a Δ6-desautrase plus a set of retro-conversion or a $\Delta^4$-desaturation. It is not clear with of these pathways that are correct, but most experimental data point to the one involving retroconversion (126).

## 1.1.3. Phosphoglycerides in biomembranes.

Phospholipids form the building blocks of all biomembranes. The exact composition in phosphoglycerides determines the fluidity of the membrane, one of the most important property if not the most, since a large number of membrane functions depend on it. Membranes at physiological conditions are in the liquid crystalline phase $L_\alpha$ also called, fluid lamellar phase. The regulation of acyl chain composition is important to keep the bilayer in this phase (151). On one hand, the length of the acyl chain has to match with the hydrophobic length of the transmembrane segment that needs to accommodate (113). On the other hand, an increase in the unsaturation leads to an increase in fluidity in general terms (93).

One of the characteristics of the biomembranes is the asymmetric distribution of phospholipids between the two leaflets (table 1.2). Most kind of phospholipid, as well as cholesterol, are generally present in both membrane leaflet, although they are often more abundant in one or the other. For instance, in plasma membranes almost all the sphingomyelin and phosphatidylcholine, both of which have a positively charged head group, are found in the exoplasmic leaflet. In contrast, lipids with neutral or negative polar head groups, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), are preferentially located in the cytosolic leaflet.

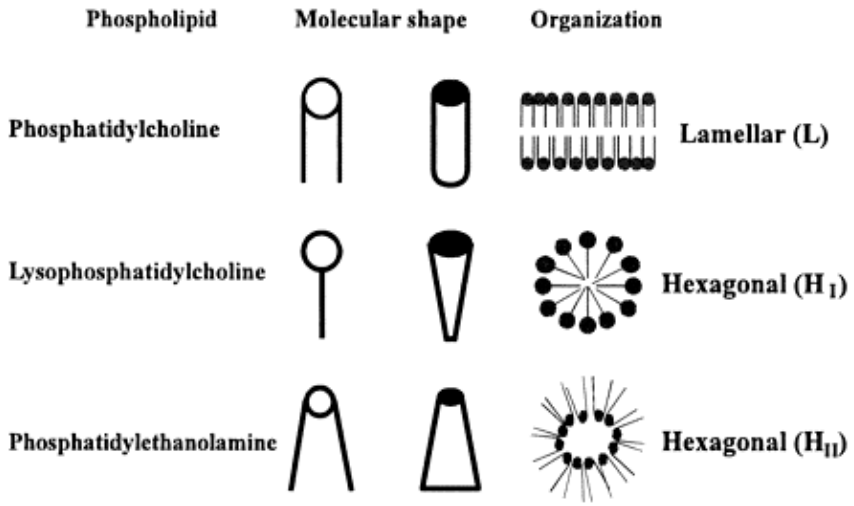
Table 1.2. Asymmetrical distribution of phospholipids in the membrane between the inner and the outer leaflet.

| Membrane phospholipid | Percent of total membrane phospholipid | Distribution in membrane |
|---|---|---|
| | | Inner monolayer 100 — 0 — Outer monolayer 100 |
| Phosphatidylethanolamine | 30 | |
| Phosphatidylcholine | 27 | |
| Sphingomyelin | 23 | |
| Phosphatidylserine | 15 | |
| Phosphatidylinositol | 5 | |
| Phosphatidylinositol 4-phosphate | | |
| Phosphatidylinositol 4,5-bisphosphate | | |
| Phosphatidic acid | | |

Interestingly, the combination of acyl chains and headgroup size determines the shape of the lipid (218). Phosphatidylcholine with saturated or single unsaturated tails, present a cylinder-shape, since head group and tail has similar cross-sectional areas. Lysophospholipids, with larger head group than tail cross-sectional areas, constitute inverted cones; whereas molecules such as PS or PE with smaller head group than tail cross-sectional areas, constitute lipids with a roughly cone shape (156). Depending on

the shape of the lipid the supramolecular organization, or packing, is different, originating the widespread bilayer (or lamellar, L) structure, or and the non-lamellar (tubular micelles) $H_I$ and $H_{II}$ phases (63).

Figure 1.5. Lipid shape and supramolecular organization (polymorphism).




The role of PUFAs in determining the fluidity of the membrane was studied in model systems and it was shown that polyunsaturation loosens chain packing and decreases the strength of cohesive interactions in the membranes. As a consequence, bending stiffness is decreased (175) and water permeability increased in comparison with membranes of saturated and monounsaturated lipids (165). Moreover these studies suggested that polyunsaturated chains, when paired with saturated ones, cause changes of membrane lateral organization and interfacial properties. This would provide the key to understand the effect of the high content of PUFAs in biomembranes (93).

The Singer-Nicholson fluid mosaic concept proposes that the bilayer functions as a neutral two-dimensional solvent, having little influence on membrane protein function. But biophysicist found that lipids exist in several phases in model lipid bilayer, including gel, liquid-ordered and liquid-disordered states, in order of increasing fluidity. In the gel state lipids are semi-frozen, whereas at the other extreme, the liquid-disordered state, the whole lipid bilayer is fluid, as proposed by the Singer-Nicholson model. In the liquid-ordered phase, phospholipids with saturated hydrocarbon chains

pack tightly with cholesterol but nevertheless remain mobile in the plane of the membrane (193). This suggested that the coexistence of different phases or microdomains within the membrane might be possible.

Further investigations confirmed the hypothesis of the presence of domains, i.e., zones in the membrane where the concentration of the components differs from that of surrounding membrane environment. Currently, it is generally accepted that these domains, or rafts, exist within the plane of the membranes (146). They consist of dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the bilayer. The preponderance of saturated hydrocarbon chains in cell sphingolipids allows for cholesterol to be tightly intercalated, similar to the organization of the liquid-ordered state in model membranes. The inner leaflet is probably rich in phospholipids with saturated fatty acids and cholesterol, but its characterization is still incomplete. The membrane surrounding lipid rafts is more fluid, as it consists mostly of phospholipids with unsaturated and therefore kinked, fatty acyl chains and cholesterol (193).

Rafts are thought to facilitate signal transduction and to regulate interactions among the lipid and protein components of signal pathways (27, 197): 1. cell signaling is sensitive to the cellular concentration of cholesterol (173) and, 2. lipid rafts can include or exclude proteins to variable extents. Furthermore, there are a number of protein modifications that increase the affinity of them for rafts. Some examples are the glycosylphosphatidylinositiol (GPI)-anchored proteins, doubly acylated proteins, such as Src-family kinase or the α-subunits of heterotrimeric G proteins, cholesterol-linked and palmitoylated proteins such as transmembrane proteins (193).

Recent studies showed that lipids rafts are also sensitive to modification by PUFAs being modified both the phospholipid component and the proteins associated with rafts, and altering therefore, the signal transduction pathways (107).

### 1.1.4. Studies on dietary lipids.

Dietary n-3 PUFAs have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels (152), cardiovascular (127) and immune function (97), insulin action (44), and neuronal development and visual function (186). Ingestion of n-3 PUFAs will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoids synthesis, and signaling as well as the regulation of gene expression (57). However, cell-specific lipid metabolism and the expression of fatty acid-regulated factors likely play an important role in determining how cells respond to changes in PUFAs composition (107).

In healthy humans the desaturation of the parent n-6 and n-3 PUFAs, LA and LNA acids, to the derivative fatty acids seems to proceed slowly and ineffectively, whereas the steps of elongation are in general more rapid. A large excess of a fatty acid from one family in the diet may inhibit the desaturation of smaller amounts of a fatty acid from another family, as the enzymes of desaturation and elongation are shared among all families. n-3 PUFAs especially may exert a substantial inhibitory effect on the desaturation of n-6 PUFAs. Thus, complex feedback mechanisms may regulate the metabolism of essential fatty acids (69).

Among the symptoms of EFA deficiency are fatigue, dermatological problems, immune problems, weakness, gastrointestinal disorders, heart and circulatory problems, growth retardation, and sterility. In addition to these symptom conditions, a lack of dietary EFA has been implicated in the development or aggravation of breast cancer, prostate cancer, rheumatoid arthritis, asthma, preeclampsia, depression, schizophrenia and attention deficit and hyperactivity disorders (ADHD) (243).

### 1.1.5. Lipids and gene expression.

The understanding of tissue-specific effects of fatty acids relays on the knowledge of how these substances and their derivatives flux and signal in the organism. This includes how they are taken up, assimilated, transported in the blood,

distributed to cells and then either utilized as membrane constituents or metabolized to downstream products, some of them being potent biological mediators (91).

Initially, the effects of fatty acids were thought to be mediated via changes in cellular membrane composition or via effects on signaling cascades (10). The latter have been extensively studied recently, opening a new field of transcriptional regulation of gene activity via fatty acids (50). The effects of fatty acids on gene expression have received considerable attention because this represents a direct route for fatty acids to regulate gene function (106). n-3 PUFAs have rapid effects on gene expression: changes in mRNAs encoding several lipogenic enzymes can be detected within hours of feeding animals diets enriched in n-3 PUFAs (26, 190). Moreover, these effects are sustained as long as the n-3 PUFAs remains in the diet. In these case, the fatty acid acts like a hormone to control the activity or abundance of key transcription factors (107).

The discovery of soluble nuclear receptors for fatty acids allowed to link the gene regulation directly with the fatty acids. Indeed, it is now established that as members of the steroid/thyroid hormone receptors family, the peroxisome proliferator-activated receptors (PPARs) mediate the effect of fatty acid on gene expression and cell fate (128).

To date, three PPAR isoforms have been cloned: α, β and γ, with tissue-selective expression (208), ligand-specific activation and ability to heterodimerize (144) with retinoid X receptors (RXR) for which three isoforms: α, β, γ haven also been isolated. The PPAR/RXR heterodimers interact in target genes with PPAR response elements (PPREs). It was demonstrated that long-chain FA, whether saturated or not, certain prostaglandins and other eicosanoids were PPAR activators (51, 112). These compounds were then proposed as natural ligands for all three PPARs. By analogy, the PPERs present in the promoter of regulated genes were defined as FA response elements. Among FA, PUFAs were show to be the most potent. Later on it was

determined that these FAs and eicosanoids could indeed directly bind to PPARs (73, 120).

Thus, for a particular cell type, a potentially complex level of transcriptional control may be exerted on a PPRE-containing gene according to: a) levels of each PPAR and RXR isoforms, b) the lipid and retinoid ligands encountered by the cell and in consequence, c) the levels of different PPAR-ligand:RXR-ligand heterodimers combinations that compete for binding the PPRE (48).

## 1.2. The brain

### 1.2.1. Brain lipid composition

The total fatty acid composition indicates a large content of polyunsaturates in brain. In human brain, n-6 PUFAs account for 17.1% of the total fatty acids, and n-3 PUFAs account for 9.7% AA and its elongation product, adrenic acid make up 90% of the n-6 in the brain and DHA accounts for 95% of the n-3 PUFAs (144).

At least six categories of PUFAs effects on brain functions have been noted and discussed elsewhere (243), namely: a) modifications of membrane fluidity, b) modifications of the activity of membrane bound enzymes; c) modifications of the number and affinity of receptors; d) modifications of the function of ion channels; e) modifications of the production and activity of neurotransmitters; and f) signal transduction and neuronal growth factors.

Ethanolamine phosphoglycerides (PE) are quantitatively the major phospho-lipids in the nervous tissues and the predominant form is the ethanolamine plasmalogen (1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphoryl ethanolamine). It accounts for 50-60% of the PE in the whole brain of many species. Cellular functions of plasmalogens are poorly understood, but they have been implicated in membrane fusion, production of eicosanoids and they seems to have antioxidative properties (67).

Although nervous tissues are capable of synthesizing fatty acids *in situ*, they also take up fatty acids from the circulating blood (188). One of the major factors in regulating the activity of fatty acids in the brain is the blood-brain-barrier (BBB). In fact, the BBB is one of the possible sites for elongation and desaturation of linoleic acid (n-6) and α-linolenic (n-3), a vital step in the production of longer fatty acids that controls numerous brain functions, and it is more extensive for n-3 than for n-6 FA (246). In addition, essential fatty acids can modify the BBB's function by influencing the rate of binding to certain proteins and consequently its rate of penetration into the brain (246).

### 1.2.2. Neuronal activity.

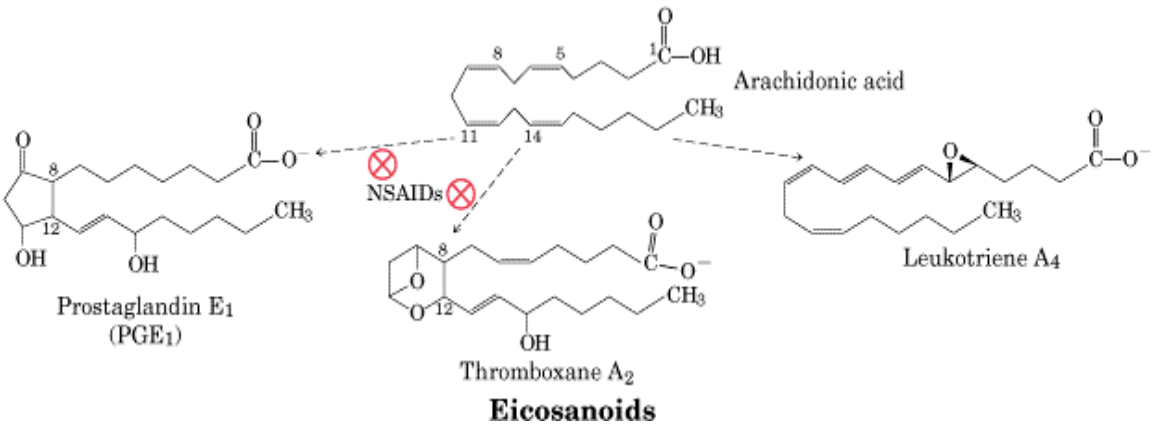
The physical state of the neuronal membrane is critical in the control of transfer of neuronal information, which depends on the exchange of ions between the inner and outer walls of the membrane. Although the physical state of the membrane needs to be at some "optimal" value, i.e., neither too rigid nor too fluid in order for the exchange between the cell and outside to be at maximum efficiency, membrane lipids and fatty acids are able to change the index of membrane fluidity (246).

A dominating concept in understanding the effects of EFAs activity concerns the state of the neuronal membrane. EFAs may exert a direct influence on neuronal activity through several different mechanisms that can influence and modify behavior. (246). The modification of the index of membrane fluidity in peripheral membranes may be influenced by such factors as dietary fatty acids in particular PUFAs, alcohol or aging (158, 246). It is noteworthy that the effect of PUFAs is not restricted to absolute levels of fatty acids alone, rather it appears that the relative amounts of ω-3 and ω-6 fatty acids in the cell membranes is the responsible for affecting cellular function (247). In addition to PUFAs, cholesterol has been shown to exert a major role in determining the membrane fluidity index (246).

As it was previously mentioned, lipids and EFAs can modify the activity of membrane-bound enzymes, neurotransmitter receptors and ion channels. For instance, fatty acid-binding proteins (FABP), phospholipase A2, protein kinase C, acetyltransferase, and membrane-associated iron enzymes are sensitive to fatty acids. Cholinergic receptors, nicotinic receptors, muscarinic receptors, and N-methyl-D-aspartate receptors are some of the neurotransmitter receptors affected by EFAs (243).

Furthermore, glycerophospholipids are the main reservoirs of PUFAs, including DHA and AA. The exact function of these PUFA is not clear although AA is the main substrate for synthesis for various eicosanoids mediators (fig. 1.6.) and DHA appears to exert a role in membrane fluidity and long-term potentiation, a process that is necessary for memory function (130).

Figure. 1.6. Structure the three families of eicosanoids derived from the arachidonic acid.




### 1.2.3. Brain development.

Body development is one of the stages of life characterized by high metabolic requirements. Among dietary constituents, lipids play an important role not only because of their high-energy value, but also because they are sources of fat-soluble vitamins and EFAs contained in the fat of natural food. Furthermore, lipids play both

structural and functional roles, and these roles are particularly relevant in the brain (187).

The total fatty acid content increases four to five-fold in brain during the period of brain maturation due to the rapid accretion of lipids (188). The bulk of weight increase of rat brain occurs postnatally, with the growth spurt paralleling myelinogenesis (4), whereas neuronal proliferation and differentiation are nearly complete by birth. Thus, neuronal membrane phospholipid formation and fatty acid accretion should be substantial at this period. The failure to acquire sufficient and appropriate PUFAs at such critical periods in fetal development may have harmful and irreversible consequences for postnatal growth and function (82). Dietary PUFAs determine to a great extent membrane level of these fatty acids and are particularly important for ensuring harmonious cerebral development (22).

One important source of PUFAs during the postnatal stage is the maternal milk (103, 116). Numerous studies have associated alterations in dietary fats of maternal diet during pregnancy or during weaning period with alterations in fatty acid composition of cell membranes and organelles in brain and learning and behavioral deficits in rats and mice (36, 67, 158, 188).

Maintenance of high DHA levels is also necessary for an optimal brain development and function (158). In animal studies, reduction in brain DHA leads to a variety of behavioral and visual deficits. These animals appeared to be recovered from their DHA losses when switched to an n-3 fatty acid adequate diet and they also recovered from deficits in spatial task performance, however changes in electroretinograms and mean arterial blood pressure did not appear to be reversible (158).

### 1.2.4. Importance of PUFAs supply during nervous system development.

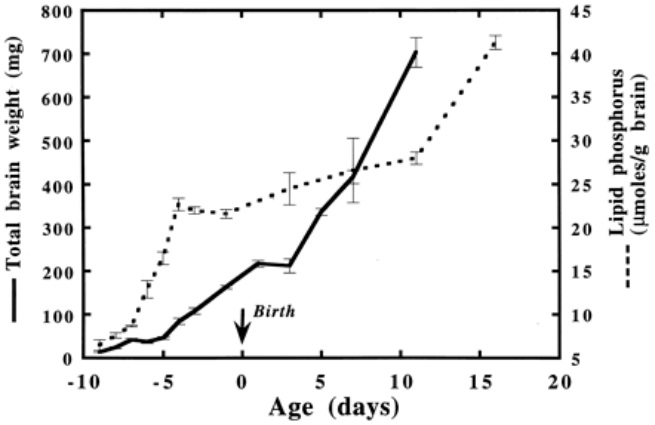
In adult vertebrates, the formation of nervous system and brain development at early stages is remarkably similar. The cells, regions, and various structures of the brain do not develop uniformly, as in other tissues and organs (52, 53).

In rats the development events occur at about 18 and 20 days of gestation, respectively (fig. 1.7). Known exceptions that continue to proliferate postnatally include some neurons of the hippocampus and the cerebellum (41). It is during prenatal and early postnatal periods when long chain PUFAs, especially DHA, are actively accumulated in high amounts in the brain, where they are involved in neurogenesis and synaptogenesis (40, 83, 145, 194).

Green et al. (83) examined the fatty acid content of the fetal rat brain at two time points during its development in late pregnancy (82) and concluded that the accretion rate of DHA at this developmental stage is the most rapid. Then, they extended the time periods of lipid analysis, both to middle pregnancy and to the first 2 weeks of postnatal life. They demonstrated that the major accumulation of all the FAs occurred between embryonic days (E) E14-E17, but the only fatty acid whose steep accretion continued up to birth was DHA (fig. 1.8).

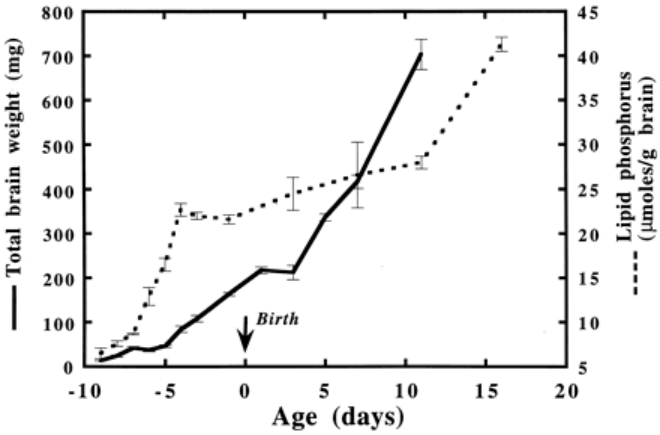
The developing fetal brain can synthesize saturated and monounsaturated fatty acids (45, 150). High rates of lipogenesis in fetal liver may act as a source of fatty acids for the fetus (198). Regarding PUFAs, the placenta determines initially the amount of these fatty acids available to the fetus by controlling the passage of AA and DHA, but then the fetus (by its own metabolism) starts to synthesize its own AA and DHA (47). However, while *in utero*, it is clear that the fetus relies on the mother for its supply of many fatty acids, particularly the essential fatty acids (41).

Figure 1.7. Total brain weight and total membrane lipids during development in rats




Total brain weight expressed in mg (continuous line) and total membrane lipids expressed as μmoles lipid phosphorus per gram brain (dotted line) during development in rats. At days -9 and -8 (E12 and E13) whole heads, rather than brains, were used. Values are means ± SEM of 4 –6 samples (83).

Figure 1.8. Accumulation of the major PUFAs in the rat brain during development




Docosahexaenoic acid, 22:6n-3: continuous line; Arachidonic acid, 20:4n–6: dotted line. At days -9 and -8 (E12 and E13) whole heads, rather than brains, were used. Values are μg FA per gram tissue, mean ± SEM of 4 –6 samples (83).

In humans, during the third trimester of human development, n-3 and n-6 fatty acids accrue in fetal tissues as an essential component of structural lipids, and rapid synthesis of brain tissue occurs. It is apparently critical that the developing fetus obtains the correct types and amounts of fatty acids to ensure complete and proper development of the brain. Timing of the availability of these fatty acids is also an important factor. Collectively, this quantitative information indicates that large amounts of DHA and AA are required during development of neural tissue when cellular differentiation and active synaptogenesis are taking place (41).

Despite the accumulating evidences that both term and preterm infants are able to convert LA to AA and LNA to DHA (49, 185, 205), is still controversial whether the capacity of these metabolic systems proceeds at sufficient rat to meet tissue demands for long chain PUFAs (126). Several randomized, controlled trials, where the key variable is addition of DHA, or DHA and AA to infant formula, indicate that the supplement supports improved performance in visual acuity tasks, (14, 32, 140, 213), visual recognition memory (34), learning a means-end problem –solving task (232) and higher Bayley mental developmental index (15, 33) or developmental quotient scores (3). However other trials have shown no benefit in neural function for long-chain polyunsaturated supplements (99, 9, 104, 141, 189). Although AA and DHA are always present in human milk (103, 116) the issue whether their addition to infant formula is necessary to promote optimal function is still undecided in North America (153)

### 1.2.5. Role of lipids in the cognitive processes.

Animal models of n-3 deficiency can be used to help to define the nature and extent of the functional deficits in the nervous system that are associated with this dietary treatment. In these studies, a vegetable oil low in LNA such as safflower oil is given to rats for two generations to induce a significant decline in levels of brain and retinal DHA (211). In this manner, the variable to be studied may be magnified and behavioral consequences may be more easily discerned. The results of such studies indicated that rats on a diet low in n-3 fats performed more poorly in brightness discrimination (238) and shock avoidance tasks (19, 214). Differences in exploratory behavior (62) and elevated plus-maze performance (159) were observed as well. Some researchers reported poorer performance in water maze task (46, 75, 102, 159, 225), whereas others have found no differences in spatial task performance (223, 224).

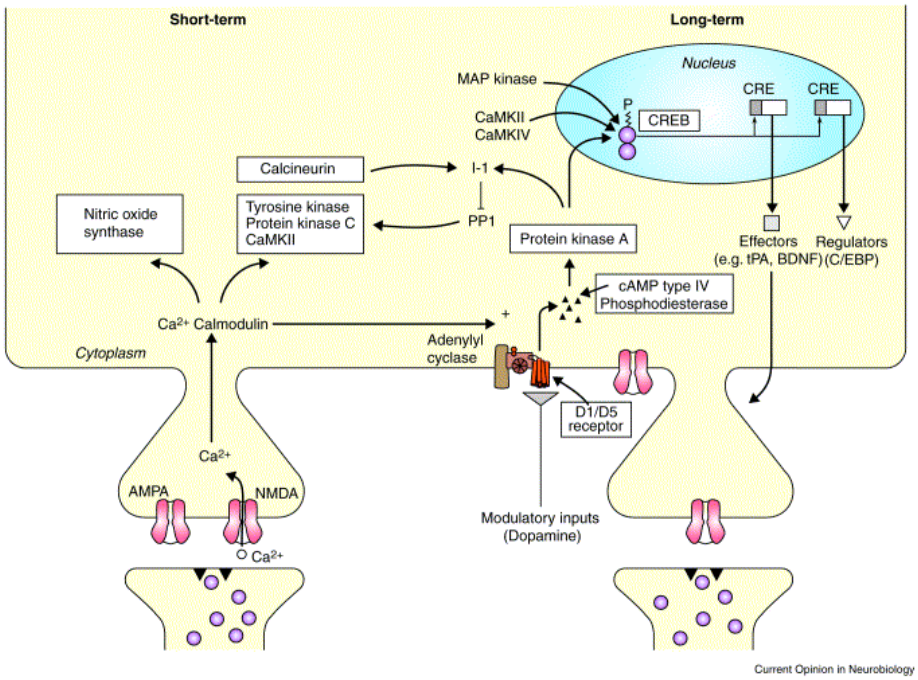
Animals can learn because of changes in the brain which allow new information to be acquired, stored and later recalled. At the cellular level, changes probably occur at synapse. This idea is formalized in the Hebb's rule (1945), which states that learning

and memory are based on modifications of synaptic strength among neurons that are simultaneously active, implying that enhanced synaptic coincidence detection could lead to better learning and memory.

Long-term potentiation (LTP) of synaptic transmission in the hippocampus is the primary experimental model for investigating the synaptic basis of learning and memory in vertebrates. LTP is induced by brief, repeated stimulation of defined neural pathways in the hippocampus. The best understood form of LTP is the one involving N-methyl-D-aspartate (NMDA) receptor complex activation. This subtype of glutamate receptor endows LTP with Hebbian characteristics, and allows electrical events at the postsynaptic membrane to be transduced into chemical signals with, in turn, are thought to active both pre- and postsynaptic mechanisms to generate a persistent increase in synaptic strength (17, 18).

On a molecular level, studies of LTP in hippocampal area CA1 have focused on the NMDA receptor and intracellular signaling events downstream of $Ca^{2+}$ influx through the NMDA receptor (2). In the fig. 10 are depicted the molecular events underlying the early and late phases of LTP. Stimulation of NMDA-type glutamate receptors, as a result of postsynaptic depolarization through AMPA receptors and the binding of glutamate, allows $Ca^{2+}$ to enter the postsynaptic neuron. Among the immediate effects of $Ca^{2+}$ are the activation of CaMKIII, PKC and calcineurin. Long-lasting LTP occurs when adenylyl cyclase is activated by $Ca^{2+}$ or by modulatory inputs, which stimulated adenylyl cyclase through G-protein-coupled receptors. This leads to increases in cAMP levels, which activate PKA, which translocates into the nucleus where it phosphorylates CREB. Other protein kinases, such as CaMKII, CaMKIV and MAP kinase, also regulate gene expression, and it is now understood that there is extensive crosstalk among these different kinase pathways (230). Experiments examining the molecular basis of memory have found many of these signaling molecules to be important for acquisition and consolidation.

Figure 1.9. Molecular events that underlie the early and late phases of LTP (2).




BBNF, brain-derived neurotrophic factor; C/EBP, CCAAT enhancer binding protein; CRE, cAMP response element; I-1, protein phosphatase inhibitor-1 ; PP1, protein phosphatase-1; tPA, tissue plasminogen activator.

### 1.2.6. Changes in brain lipid composition during aging and neurodegenerative diseases.

The aging in brain is associated with many biochemical, physiological and behavioral deficiencies including reduction of long-term potentiation (LTP), learning and memory loss, sleep disturbance, pain threshold alteration, and disturbed thermoregulation (148).

The ability of the brain to create new synapses is diminished during this period for reasons that are not understood. Concurrently, there are major biochemical changes in the brain that affect the neuronal membrane, the “site of action” for many essential functions: conduction of neuronal information along the axon, regulation of membrane bound enzymes, control of the ionic channels structure and activity, and maintenance of various types of receptors (247).

Changes in the neuronal membrane due to aging, involve a great increase of cholesterol levels whereas PUFAs' levels decreases (6). This age-related change in membrane composition, which decreases membrane fluidity (11, 64), might be predicted to affect the membrane associated functions (149). On the other hand, the underlying cause of the age-related impairment in LTP is not known, although it seems to be related to defects in glutamate release, and with a decrease in depolarization-induced transmitter release, both have been linked with a decrease in membrane fluidity (148).

Alzheimer's disease (AD) is the most widespread progressive neourodegenerative disease, major symptoms include short- and long-term memory loss, impairments of language and speech, decline of abstract reasoning and visual-spatial perceptual changes (43).

AD brains are characterized in part by the presence of cerebrovascular amyloid deposits and senile plaques. The principal proteinaceous component of these plaques is β-amyloid an amphiphilic peptide that forms aggregates in aqueous solutions or below physiological pH. The mechanism behind Aβ toxicity has been debated and several mechanisms have been suggested, including perturbations in membrane fluidity (119), free radical production and lipid peroxidation (29), formation of ion channels (133), changes in lipid metabolism (117), and increased phospholipase activity (196).

Changes in phospholipid composition have been observed in AD brains, showing decreased levels of AA and DHA levels in PC, PE (172) and PI (201). The loss of phospholipids may be related to the loss of synapses in AD (229). Another changes observed are: 1)an elevated activity of lipolytic enzymes (65) and in concentrations of phospholipid degradation metabolites (169) and 2) an increase of prostaglandins and lipid peroxides levels (221), suggesting that lipid peroxidation along with alterations in membrane integrity plays an important role in pathophysiology of AD (66). Even

though still is not clear whether all these changes occur prior to or post-disease onset (43), lipid metabolism appears to play an important role in this disease.

Other neurodegenerative diseases have been related to the changes in lipid metabolism. Comparison of FA levels between individuals with AD, cognitively impaired nondemented (CIND) and other kind of dementia (OD) to normal elderly individuals clearly showed differences in the fatty acid composition of total PL. The decreased levels of EPA, DHA and total n-3 levels in the plasma PL of patients with AD, OD and CIND suggested that the dietary intake of the n-3 fatty acids is lower than that of normal subjects. The possibility of an increased breakdown, either before of after disease onset, cannot be ruled out (43).

Finally, Horrobin (95) proposed a hypothesis relating disorders in lipid metabolism and schizophrenia. Noting that prostaglandins are derived from membrane EFAs, he hypothesized that defective phospholipase or cyclo-oxygenase function could lead to prostaglandins deficiency that might also be sensitive to dietary variations in EFA precursor availability. Recent modifications of this hypothesis have involved EFAs depletion, a defective uptake of EFAs into membrane phospholipids (95) or a defective conversion of EFAs into long-chain polyunsaturated analogs (139).

The importance of dietary lipids in all these disorders was highlighted by recent studies suggesting that diets rich in PUFAs are inversely correlated with the development of dementia, and in particular Alzheimer's disease, (81, 109). The treated patients presented improvements in mood, cooperation, appetite, sleep, and short-term memory (244). Also improvements in serum fatty acid and learning tests were observed in elderly with mild to moderate dementia after a diet supplemented with DHA (210).

### 1.2.7. Gene expression in brain.

PPARs are distributed in a wide range of organs with tissue specificity for each other, except PPARβ, which seems ubiquitously expressed. Molecular biological and

histological studies explored which PPAR subtype is present in the central nervous system and they showed a distinct expression pattern for the three subtypes of PPAR depending on type of cell, region of the brain (118) and developmental stage (24).

Various potential PPAR and RXR ligands were found in the CNS and they appeared to play profound roles in both the normal and disease state (48). During development, various milk diet-derived long chain FA, such as DHA, cross the blood-brain barrier with ease (145) and, according to the type of FA, may then be selectively, catabolised for fuel (8), conserved for important structural and functional roles (20, 56, 160) or anabolised to generate small quantities of certain very long chain FA peculiar to the CNS (171). retinoids have also been demonstrated to enter the CNS (166) and they exert potent RXR-mediated effects of CNS development (94, 191). Finally, cytotoxic long-chain FAs accumulate in peroxisomal disorders such as Zellweger's syndrome (179).

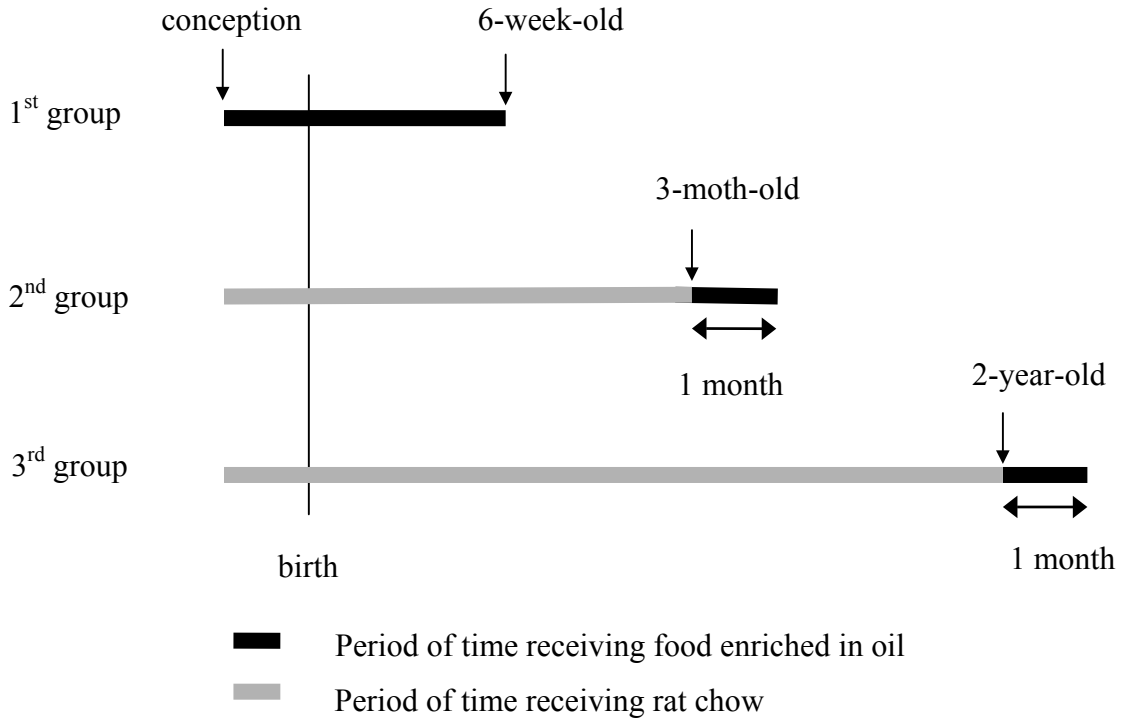
# 2. MATERIALS AND METHODS

## 2.1. Experimental Animals.

Three groups of Wistar rats of different ages were kept on normal rat chow (control groups) and rat chow supplemented with diverse dietary oils for different time periods. The first group was consisted of rats fed from conception till they became 6-week-old. The second and third group were 3-month and 2-year-old respectively when they started to receive food and the feeding period was for a month (figure 2.1)

Once the established feeding period finished, the animals were sacrificed by decapitation. The cerebellum and hippocampus were removed from the whole brain. For simplicity this part will be referred as forebrain, although strictly hippocampus is also included in it. The three regions were snap-frozen in liquid nitrogen and kept until processing at –80ºC.

Figure 2.1. Design of the experiments.




## 2.2. Diets.

One of the aims of this work was to determine the effect of dietary PUFAs on the neuronal membrane lipid composition. For this reason, each one of the diets was enriched in one particular fatty acid or had a particular ratio of LA(n-6) to LNA(n-3). The fatty acid composition of each diet is given in the table 2.1.

- **Diet 1**: rat chow enriched with fish oil, rich in DHA, a ω-3 fatty acid.
- **Diet 2**: rat chow enriched with perilla oil, rich in LNA, a ω-3 fatty acid, precursor of the DHA.
- **Diet 3**: rat chow enriched with a mixture of perilla and soybean oil where the ratio LNA (ω-3)/LA (ω-6) was 4.7:1.
- **Diet 4**: rat chow enriched with a mixture of perilla and soybean oil where the ratio LNA (ω-3)/LA (ω-6) was 2.9:1.

Table 2.1. Fatty acid composition of diets

| Fatty acid, wt % | Control | Fish oil | Perilla oil | LA/LNA 4.7:1 | LA/LNA 2.9:1 |
|---|---|---|---|---|---|
| 16:0 | 13.6 | 7.5 | 8.9 | 11.8 | 15.28 |
| 16:1n-7 | 0.8 | 1.3 | TR | | 1.10 |
| 18:0 | 3.1 | 2.2 | 2.5 | 3.0 | 5.44 |
| 18:1n-9 | 17.0 | 16.2 | 19.5 | 24.5 | 23.18 |
| 18:1n-7 | - | 1.5 | 1.2 | | 1.53 |
| 18:2n-6 | 51.0 | 23.0 | 28.0 | 49.4 | 37.54 |
| 18:3n-3 | 6.2 | 2.5 | 39.4 | 10.4 | 12.97 |
| 20:1n-9 | - | 1.3 | - | - | - |
| 20:4n-6 | 0.1 | - | - | - | - |
| 20:5n-3 | 0.7 | 12.0 | - | - | - |
| 22:5n-3 | 0.2 | 2.6 | - | - | - |
| 22:6n-3 | 1.2 | 26.9 | - | - | 0.77 |
| ratio LA/LNA | 8.2 | 9.2 | 0.7 | 4.7 | 2.9 |
| ratio n-6/n-3 | 6.2 | 0.5 | 0.7 | 4.7 | 2.3 |

Only the most common fatty acids are listed. TR, traces

## 2.3. Analytical techniques.

### 2.3.1. Extraction of lipids

Lipids of brain, cerebellum and hippocampus tissues were extracted according to the method described by Folch et al. (71). The samples were homogenized in chloroform/methanol (2:1 v/v) with an Ultra Turrax blender, and left in the refrigerator for at least 4 hours. The mixture was filtered to remove the rest of tissues. To facilitate the complete separation of the organic and aqueous phases a certain volume of KCl 0.2 M was added, approximately the 20% of the total volume. It was left in the refrigerator for at least one more hour. The aqueous phase was removed using a water pump. The lower organic phase was filtered using a silicone treated filter paper and dried by rotatory evaporation at 35º C. The extracted lipids were dissolved in benzene containing butylated hydroxytoluene (0.1%), to avoid fatty acid oxidation, and stored at –20ºC until assayed.

### 2.3.2. Separation of phospholipid classes

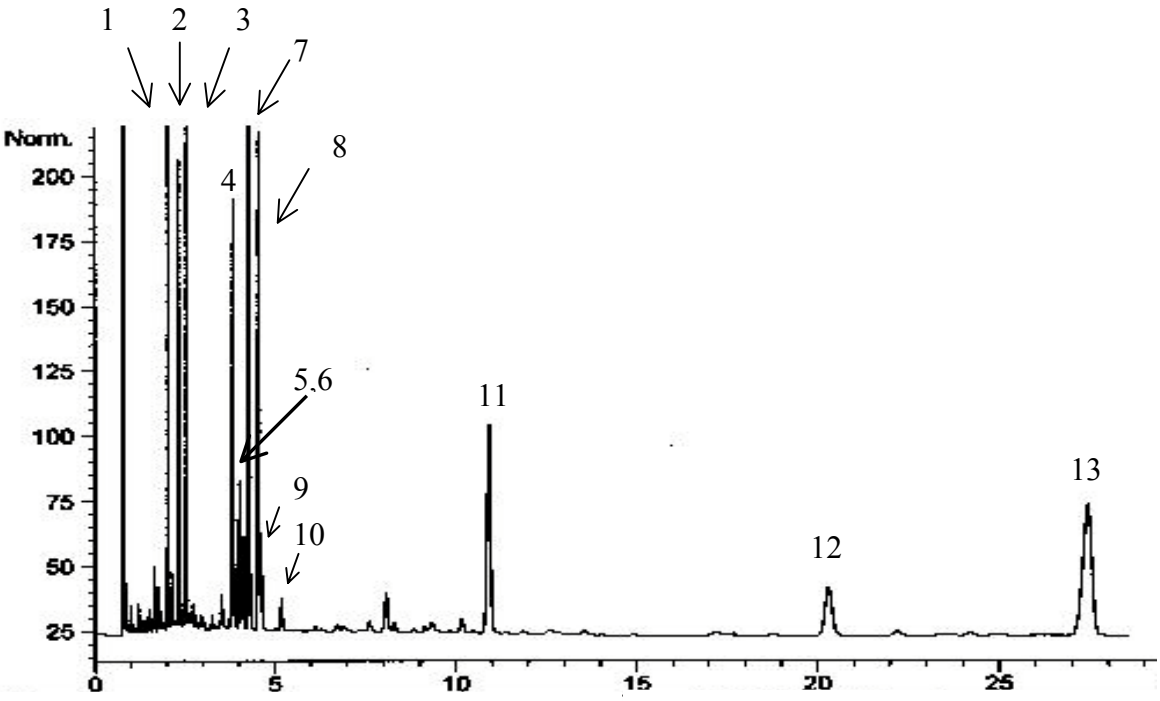
Polar phospholipids were separated into subclasses by thin layer chromatography on Silicagel G plates using the following solvent system: methylacetate/ propan-2-ol/ chloroform/ methanol/ 0.25% (w/v) aqueous KCl (25:25:25:10:9) (220). Phospholipid subclasses were visualized by 254 nm UV light after spraying with a 0.5% 1-anilino, 8-naphthalene sulphonic acid in 50% methanol. The spots corresponding to phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidyl-inositol were scratched off and eluted from the silica gel with chloroform/methanol 2:1 (v/v), dried by rotatory evaporation at 35ºC, and finally under nitrogen. The phospholipid residue was redissolved in benzene with BHT and stored at –20º C.

### 2.3.3. Fatty acid analysis

*Preparation of ester derivatives*. Lipids were transmethylated with absolute methanol containing 5% HCl at 80ºC under inert atmosphere for 3 hours. After cooling the tubes, 3 ml of distilled water and 1 of n-hexane were added and vortexed for one

minute. The upper layer was transferred to a test tube and evaporated to dryness under nitrogen. The residue was redissolved in 10 µl of n-hexane. To inject to the gas chromatograph 1 µl of n-hexane, 2 µl of air and 1 µl of sample had to be taken in this order using a Hamilton syringe.

Figure.2.2. Example of a gas chromatogram of a phosphatidylethanolamine.




Order of elution of the different fatty acids:

| Position | Fatty acid | Position | Fatty acid | Position | Fatty acid |
|---|---|---|---|---|---|
| 1 | | 6 | DMA 18:0 | 11 | 20:4n-6 |
| 2 | 16:0 | 7 | 18:0 | 12 | 22:5n-6 |
| 3 | 16:1n-9 | 8 | 18:1n-9 | 13 | 22:6n-3 |
| 4 | DMA 18:0 | 9 | 18:1n-7 | | |
| 5 | DMA 18:0 | 10 | 18:2n-6 | | |

Separation of fatty acid ester derivatives. Fatty acid methyl esters were separated on a 30 m long FFAP column (0.32 mm i.d., Supelco, Bellefonte, PA) using a Hewlett Packard gas chromatograph, Model 8890. Peaks were identified by using authentic standards from Sigma (St. Louis, MO).

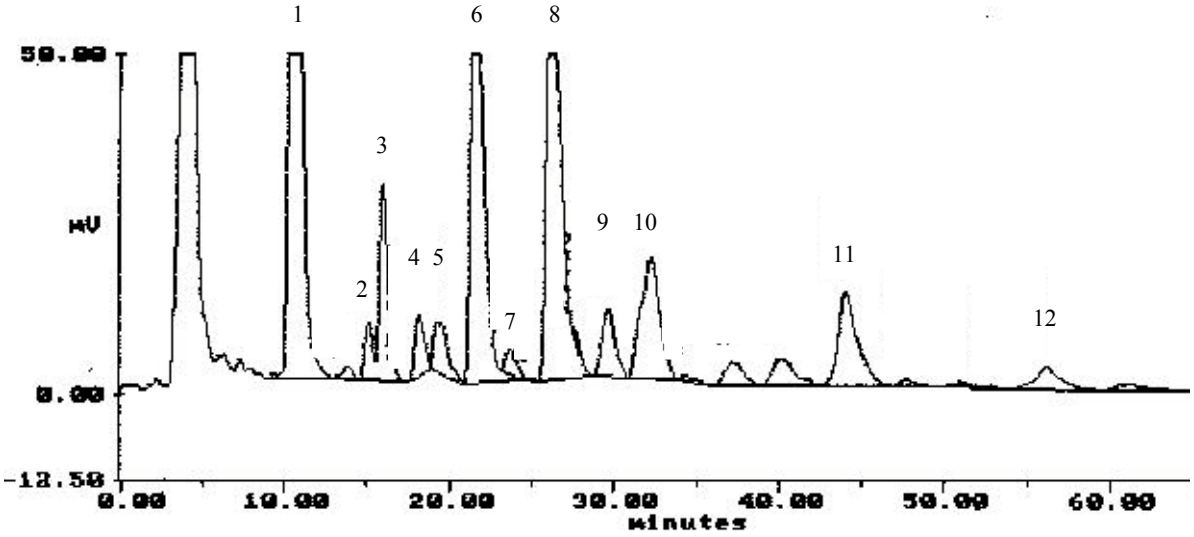
### 2.3.4. Separation of molecular species of phosphatidylcholines and ethanolamines

*Preparation of derivatives*. Approximately 1 mg of the phospholipids was evaporated to dryness under nitrogen in a test tube. The residue was resuspended in 1 ml diisopropylether, 1 ml of 30 mM Tris-borated buffer (pH 7.5). Then 20 µl phospholipase C of *Bacillus cereus* was added. The reaction was allowed to proceed at room temperature overnight with stirring. In order to stop the reaction 2 ml of hexane/isopropylether (1:1 v/v) were added. The mixture was vortexed and centrifuged for 2 minutes at 2000 r.p.m. The upper phase was separated and transferred in new test tubes and evaporated at room temperature in a centrifugal evaporator for 30 minutes. The phospholipids were mixed with 10 µl of N,N-diisopropylethylamine and 200 µl of 9-anthroyl chloride solution in a sealed test tube and heated at 60ºC for 0.5 hour. After cooling the tubes in ice, the reaction was stopped by adding 3 ml of 100 mM $NH_4OH$ solution. The reaction product was extracted with 3.5 ml of chloroform and washed for three times with 3 ml of 100 mM $NH_4OH$ solution. The lower phase was collected into new test tubes and evaporated to dryness.

*Separation of derivatives into subclasses*. The anthronyl-diarylglycerol derivatives were separated into diacyl, alkylacyl, and alkenylacyl subclasses by TLC. The TLC plates were developed with hexane /toluene / diisopropylether (10.0:9.0:0.8 v/v/v). The three subclasses were easily visualized under UV light. As an internal standard derivatised 12:0/12:0 was applied. Then the spot corresponding to each subclass was scratched off and extracted with 2ml of hexane/diisopropylether. After 10 minutes the silica gel was discarded and the solvent evaporated. The final product was dissolved in acetonitrile and kept at –20º C until analysis

*Separation and quantification of the molecular species*. The molecular species composition of isolated diacyl, alkylacyl and alkenyl phosphatidylcholines and phosphatidylethanolamines was determined according to Takamura et al. (206). The diarylglycerides obtained by digestion with phospholipase C from *Bacillus cereus* (Sigma) were converted to their anthroyl derivatives according to Takamura et al. (207). Each glycerophospholipid subclass, was segregated into individual molecular species on a Supelcolsil LC-18 column (particle size 5µm, 25 x 2.1mm, Supelco, Bellefonte, PA) using acetonitrile:propane-2-ol, 75:25 (v/v) as solvents with a flow rate of 0.2 ml/min on a Merck-Hitachi L6220 pump equipped with a fluorescent detector (Model F 1050).

Figure 2.3. HPLC tracing of a diacylphosphatidylethanolamine




| Order of elution | Molecular specie | Order of elution | Molecular specie |
|---|---|---|---|
| 1 | 12:0/12:0 (internal standard) | 7 | 16:0/18:2 |
| 2 | 18:1/22:6 | 8 | 18:0/20:4 |
| 3 | 16:0/22:6 | 9 | 18:1/18:1 |
| 4 | 18:1/20:4 | 10 | 16:0/18:1 |
| 5 | 16:0/20:4 | 11 | 18:0/18:1 |
| 6 | 18:0/22:6 | 12 | 18:0/18:0 |

The excitation and emission wavelengths were 360 and 460 nm, respectively. Peaks were determined using authentic diacylglycerol standards (16:0/16:0, 22:6/22:6, 20:4/20:4, 16:0/22:6, 18:0/22:6, 18:0/20:4, 18:0/22:6, 16:0/18:2, 16:0/18:1, 18:0/18:1) from Avanti Polar Lipids Inc (Birmingham, AL) and by comparison of their relative retention time with published data (Bell and Dick, 1991) derivatised.

## 2.3. Gene expression analysis

Gene expression was studied by DNA microarray techniques. Rat brain, liver, and ganglion cDNA libraries were constructed in TriplEx cloning vector according to the manual of the SMART cDNA construction kit (CLONTECH).

For gene expression analysis, total RNA was purified from each group. Total RNA was amplified by using an antisense RNA amplification protocol. The RNA was purified, concentrated, and measured spectorscopilcally. Three micrograms this RNA was labeled with Cy3-labeled or Cy-5-labeled dCTP. After hydrolysis of the RNA, labeled cDNA was purified and hybridized.

Relative quantitative reverse transcription-PCR was performed to validate the gene expression changes observed with microarrays. (114)

## 2.4 Behavioral tests

Behavioral tests were done by the Behavioral testing of spatial learning took place in a Morris water maze consisting of a circular black polyethylene tub with a slightly sloping wall (diameter at to 140 cm, at bottom 130 cm, depth 40 cm) and was filled with clear tap water at a temperature of approximately 23 ºC. A black polyethylene cylinder (diameter 10 cm), submerged 1.5 cm below the surface of the water, served as escape platform.

During each of 7 daily sessions the animals received 4 trials. A trial was started by placing the rat into the pool, facing the wall of the tank. Each of the 4 starting positions (arbitrarily assigned north, east, south and west) was used once in a series of 4 trials while their order was randomised. The escape platform stayed at the same quadrant in the middle between the centre of the pool and the wall of the quadrant. A trial was terminated as soon as the rat climbed onto the escape platform and the next trial was started. Rats that did not find the platform within 90 s were direct with hand to the platform in the water and allowed to stay there for 30 s. After completion of the daily session the rats were dried and returned to their home cages.

Scoring behaviour of the rats in the water maze the escape latency reaching the hidden platform was registered at each trial. Averaged escape latency of the 4 trials at each daily session was calculated which served for statistical analysis of the acquisition of spatial learning.

# 3. RESULTS

## A. Study of PUFA metabolism in rat brain

### A.1. Effects of the dietary lipids on the phospholipid fatty acid and molecular species composition in forebrain membranes.

The effect of dietary lipids on the organism and more concretely on the nervous system is a widely studied topic due to its relation to the human health. Although there is no standard procedure to analyze these effects, in the majority of the experiments carried out, the feeding period is maintained at least for two generations. The main reason is because there is the general thought that brain has a slow lipid metabolism and therefore, a rather long time is needed to deplete and to increase DHA level in brain phospholipids (21, 5).

On the other hand, it has been well established that during the last stage of the embryogenesis and in the early postnatal period, there is a large acquisition of long chain polyunsaturated fatty acids (80, 81) and this is critical for an adequate development of the nervous system (184). Most neuronal cells cease dividing and proliferating after weaning in the rat; therefore, the nutritional status during gestation and lactation is considered crucial for the development of brain and neuronal functions.

For this reason, the first step for a better understanding in the brain lipid metabolism was to analyze whether changes in the diet during embryogenesis and early postnatal periods would have any effect on the final composition of the brain membrane.

#### A.1.1. Results in *rats fed from conception until* adulthood.

The use of four different diets in this study had principally two aims:

1. To establish the importance of the DHA supply in the diet for its adequate accumulation during nervous system development. For this purpose, two groups of rats

were fed either with fish oil, containing a 26.9% of DHA, or with perilla oil, containing 39.4% of LNA (DHA precursor).

2. To analyze the importance of a particular ratio n-6 to n-3 fatty acids for the accumulation of DHA in brain membranes. For this, two different diets were designed: one with a ratio LA (n-6) to LNA (n-3) = 4.7 to 1 and, in the second one was 2.9 to 1.

The changes were analyzed at both fatty acid and molecular species level and the results are given as a percentage of total fatty acid and total molecular species respectively. In all the tables values represent means ± S.D., n = 3-6. The statistical significance of the mean differences was determined by one-way ANOVA. Values marked with the superindex: a, b, c and d, have the following meaning: a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.005$ and, d: $P < 0.001$ compared to values obtained in control rats.

The results dealing are dealing almost exclusively with the fatty acid and molecular species composition of the phosphatidylethanolamine. This phosphoglyceride is the richest in PUFA and it proved to be the most active metabolically.

Table 1.1. shows the fatty acid content in forebrain in rats fed from conception. The first remarkable fact is the very low presence or even total absence of the precursor fatty acids, LA and LNA. The largest percentage of these two fatty acids has been oxidized to obtain energy. Eventually, some percentage of LA and LNA can be metabolised to AA and DHA respectively.

On the other hand, the results show an accumulation of DHA (12.3%) in the fish oil diet while in the perilla oil diet there is a slight decrease (5.1%). These fact agree with the idea that although the brain has the necessary machinery to synthesize long chain polyunsaturated fatty acids from their precursors, that process is relatively slowly and therefore the largest percentage of accumulated DHA might come from dietary

sources. In contrast the level on AA is lower in the fish oil diet than in the perilla oil diet.

Table 3.1. Fatty acid composition of PE in forebrain of rats fed from conception.

| Fatty acid | Control | Fish oil | Perilla | LA/LNA 4.7:1 | LA/LNA 2.9:1 |
|---|---|---|---|---|---|
| DMA 16:0 | 5.11 ±0.51 | 4.00±0.93 | 4.71±0.86 | 3.53±0.47 | 0.96 ±0.12 |
| 16:0 | 8.31 ±1.53 | 6.34±2.2 | 8.28±2.6 | 4.49±0.54 | 6.84±1.06 |
| 16:1 n-7 | 0.68 ±0.21 | 1.08±0.36 | ND | 1.46 | ND |
| DMA 18:0 | 14.51 ±2.00 | 10.54±2.19 | 14.04±2.16 | 10.35±0.74 | 12.69±1.19 |
| 18:0 | 17.05 ±2.00 | 19.00±2.9 | 17.13±1.45 | 15.00±1.65 | 17.55±2.54 |
| 18:1n-9 | 12.18±1.49 | 12.75±2.56 | 12.23±1.19 | 11.43±0.83 | 11.19±2.29 |
| 18:2n-6 | 0.80±0.02 | 0.71±0.32 | 0.88±0.34 | 0.52±0.03 | 0.70±1.22 |
| 18:3n-3 | 1.90±0.17 | 0.42±0.22 | ND | ND | ND |
| 20:5n-3 | ND | 0.10 | ND | ND | ND |
| **20:4n-6** | **9.64±1.57** | **9.07±3.46** | **10.1±1.48** | **11.33±0.63** [a] | **11.33±2.31** [a] |
| **22:6n-3** | **19.73±2.00** | **22.16±2.86** [c] | **18.39±2.92** | **20.92±1.34** [a] | **19.95±2.97** |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$; DMA: dimethyl acetal; ND. not determined:

Finally, it is noteworthy the effect the diet LA/LNA 4.7:1 has on the fatty acid composition. Although the accumulation of DHA is not as high as in fish oil diet, the interesting fact is that level of AA not only has been maintained but presents a increase compared to the control. Similar changes are observed at this level in the LA/LNA 2.9:1 diet.

Table 3.2. shows the changes in molecular species composition in ethanolamine phosphoglycerides. In the tables are listed only the levels of the AA and DHA-containing species. The main reason is because they are in the focus of this study and in addition, these species showed the higher susceptibility to the different diets. Of the three subclasses, diacyl-, alkyl-, and alkenyl- phosphoglycerides, the discussion will be focused on the changes in the diacyl and alkenyl-PE, yet they represent the 10-15 and 75-80% of the total phosphatidylethanolamine in brain, respectively.

Table 3.2. Molecular species composition of PE in forebrain of rats fed from conception.

| | Control | Perilla | Fish oil | LA/LNA 4.7:1 | LA/LNA 2.9:1 |
|---|---|---|---|---|---|
| **diacyl-PE** | | | | | |
| 18:1/22:6 | 1.93±0.46 | 1.46±0.70 | 2.57±0.63 | 2.62±0.80 | 2.49±0.09 |
| 16:0/22:6 | 6.56±0.29 | 5.30±2.13 | 7.54±3.24 | 12.31±2.10 | 8.33±0.38 |
| **18:0/22:6** | **29.68±2.09** | **31.88±2.85** | **34.22±0.50** [c] | **36.31±4.09** [d] | **28.56±1.28** |
| 18:1/20:4 | 2.67±0.06 | 2.02±0.88 | 3.07±1.97 | 3.40±0.87 | 3.58±0.44 |
| 16:0/20:4 | 2.15±0.63 | 1.54±0.92 | 2.04±0.41 | 2.92±1.35 | 2.18±0.22 |
| **18:0/20:4** | **25.16±1.44** | **23.40±2.50** | **19.92±0.50** [d] | **22.15±2.12** [a] | **23.56±2.60** |
| **alkyl-PE** | | | | | |
| 18:1/22:6 | 3.63±1.02 | 2.80±1.56 | 4.80±0.86 | 3.15±0.56 | 3.53±1.04 |
| 16:0/22:6 | 9.36±2.09 | 6.92±1.46 | 6.72±1.83 | 9.57±0.72 | 9.41±1.35 |
| 18:0/22:6 | 12.59±1.29 | 11.22±2.34 | 11.02±1.84 | 13.70±1.06 | 14.37±1.23 |
| 18:1/20:4 | 4.89±0.44 | 6.45±2.36 | 5.27±3.55 | 5.23±1.27 | 4.15±0.51 |
| 16:0/20:4 | 5.88±1.12 | 5.17±1.21 | 2.26±0.53 | 6.05±0.32 | 5.14±0.45 |
| 18:0/20:4 | 4.98±0.36 | 4.91±0.87 | 2.60±0.52 | 5.47±0.48 | 6.32±0.66 |
| **alkenyl-PE** | | | | | |
| 18:1/22:6 | 3.70±0.58 | 3.620.96 | 5.50±0.68 | 5.06±0.79 | 4.52±0.06 |
| 16:0/22:6 | 8.92±0.80 | 9.821.35 | 12.23±1.65 | 14.08±2.56 | 11.04±1.33 |
| **18:0/22:6** | **20.23±2.48** | **20.71±1.50** | **22.16±1.85**[a] | **23.36±1.83** [b] | **21.47±1.14** |
| 18:1/20:4 | 4.64±0.35 | 4.50±1.00 | 4.07±0.78 | 5.89±1.02 | 4.43±0.19 |
| 16:0/20:4 | 5.08±0.89 | 4.84±0.97 | 4.02±0.92 | 6.39±1.23 | 4.67±1.20 |
| **18:0/20:4** | **10.08±0.73** | **7.38±2.81** | **6.55±0.21** [d] | **8.66±0.62** | **8.96±1.71** |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

In outline, the changes observed at the fatty acid level are reflected at the molecular species level. The single but remarkable exception to this is the diet LA/LNA 2.9:1, presenting no changes at this level comparing to the control diet.

Comparing perilla and fish oil diets it is obvious that the accumulation of 18:0/22:6 is only achieved when already formed DHA is given in the diet. As a counterpart, there was a significant decrease in AA-containing species.

A very interesting change came out from the rats fed with LA/LNA 4.7:1, comparing to the fish oil diet there is a higher accumulation in diacyl 18:0/22:6 (15.3 in fish oil vs. 22.3% in LA/LNA 4.7:1) whereas the decrease in 18:0/20:4 was less pronounced (20.8% in fish oil vs. 12% in LA/LNA 4.7:1). 16:0/22:6 was the third molecular specie more affected by the diet, showing the highest accumulation in the case of the LA/LNA 4.7:1 diet.

Alkenyl-PE shows exactly the same tendency as the diacyl-PE, although the changes are less extent.

In brain a very low percentage of alkyl-PC can be found while alkenyl-PC is almost absent. Hence, only diacyl phosphatidylcholine molecular species are listed in the table 3.3. All together, molecular species containing either DHA or AA represent roughly the 20% the rest are species containing saturated and monounsaturated fatty acids. For these reasons, from now on the data regarding phosphatidylcholine molecular species will be omitted

Table. 3.3. Molecular species composition of diacyl -PC in forebrain of rats fed from conception.

| | **Control** | **Fish oil** | **Perilla** | **LA/LNA4.7:1** | **LA/LNA 2.9:1** |
|---|---|---|---|---|---|
| 18:1/22:6 | 0.48±0.17 | 0.89±0.29 | 0.34±0.07 | 0.81±0.22 | 0.56±0.21 |
| 16:0/22:6 | 2.54±1.09 | 4.44±1.84 | 2.00±0.22 | 7.12±5.63 | 3.35±1.18 |
| 18:1/20:4 | 0.94±0.27 | 1.16±0.56 | 1.10±0.49 | 1.81±1.03 | 1.36±0.32 |
| 16:0/20:4 | 3.41±1.49 | 3.73±1.82 | 3.52±0.75 | 4.73±1.09 | 4.71±0.06 |
| 18:0/22:6 | 4.11±1.69 | 8.13±4.43 | 3.35±1.12 | 5.47±2.61 | 2.81±0.75 |
| 16:0/18:2 | 1.99±0.62 | 2.69±1.64 | 2.67±0.32 | 4.30±1.58 | 2.33±0.68 |
| 18:0/20:4 | 6.14±1.68 | 5.25±1.16 | 5.35±0.10 | 6.21±1.06 | 5.94±0.64 |
| 18:1/18:1 | 3.69±0.35 | 3.85±0.50 | 3.36±0.31 | 2.59±0.82 | 3.00±0.31 |
| 16:0/18:1 | 31.07±4.71 | 30.22±1.99 | 31.60±0.62 | 30.53±2.15 | 35.06±2.75 |
| 16:0/16:0 | 10.43±2.76 | 11.53±0.81 | 13.83±1.24 | 13.63±2.30 | 15.14±1.15 |
| 18:1/18:0 | 20.49±3.58 | 17.92±2.15 | 18.85±1.16 | 15.79±1.49 | 14.98±2.50 |
| 18:0/16:0 | 4.36±0.78 | 4.04±0.79 | 5.08±0.56 | 3.34±1.82 | 5.25±1.52 |

**A.1.2. Results in *3-month-old* rats fed for one month with PUFA-enriched diet.**

Once the results showed clearly that diet could influence in a significant manner the membrane composition, the next step was to examine whether there would be similar changes in animals with nervous system already developed. At this stage, lipid metabolism has declined till basal level and, it is generally though that is rather difficult to alter DHA levels. For this purpose, 3-month-old rat were fed during one month with fish oil and LA/LNA=4.7:1 diets, yet these were the two diets exerting the highest influence on membrane composition.

Table. 3.4. Fatty acid composition of PE in forebrain of rat 3-month-old rats.

| **Fatty acid** | **Control** | **Fish oil** | **LA/LNA 4.7:1** |
|---|---|---|---|
| DMA 16:0 | 1.61±0.42 | 1.39±0.46 | 1.52±0.79 |
| 16:0 | 4.55±0.96 | 5.65±1.20 | 7.14±1.53 |
| 16:1 n-7 | 0.95±0.32 | 0.71±0.16 | 1.37±0.30 |
| DMA 18:0 | 12.38±0.95 | 13.58±1.46 | 11.67±2.83 |
| 18:0 | 14.11±1.74 | 14.80±1.17 | 14.88±0.95 |
| 18:1n-9 | 13.73±1.13 | 13.11±0.95 | 12.90±0.84 |
| 18:1n-7 | 2.59±0.48 | 2.40±0.24 | 2.12±1.24 |
| 18:2n-6 | 0.14±0.31 | 0.60±0.05 | 1.94±1.81 |
| **20:4n-6** | **9.62±0.76** | **8.71±0.36** | **9.35±2.05** |
| 22:4n-6 | 6.13±0.44 | 5.14+0.20 | 5.09±2.62 |
| **22:6n-3** | **15.73±0.69** | **19.67±0.63**[b] | **19.61±1.49**[b] |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

The analysis ethanolamine phosphoglycerides at fatty acid and molecular species composition levels showed that, one month of PUFA- enriched diet was enough to alter significantly the PE composition in brain membrane. This fact would indicate that, although brain development has already finished, there is still an active lipid metabolism responsible for that DHA accumulation. Interestingly, the DHA accumulation PE was almost identical for both diets (25%), whereas AA level decrease was higher in rats fed with fish oil than in the rats fed with LA/LNA=4.7:1.

Table 3.5 Molecular species composition of PE in forebrain of rat 3-month-old rat.

| | Control | Fish oil | LA/LNA4.7:1 |
|---|---|---|---|
| **Diacyl-PE** | | | |
| 18:1/22:6 | 2.01±0.03 | 1.99±0.20 | 1.62±0.18 |
| 16:0/22:6 | 6.65±0.24 | 6.38±0.62 | 5.89±0.72 |
| **18:0/22:6** | **27.74±1.33** | **32.29±1.10** [d] | **29.52±1.32** [a] |
| 18:1/20:4 | 3.88±0.29 | 2.02±0.42 | 4.05±2.27 |
| 16:0/20:4 | 2.57±0.18 | 1.73±0.19 | 2.66±0.32 |
| **18:0/20:4** | **20.11±0.39** | **19.00±1.36** | **22.52±1.63** [d] |
| **Alkyl-PE** | | | |
| 18:1/22:6 | 3.85±0.77 | 4.65±1.42 | 5.27±0.28 |
| 16:0/22:6 | 7.04±1.31 | 6.97±1.96 | 9.71±1.94 |
| 18:0/22:6 | 11.50±0.61 | 11.70±2.33 | 13.42±1.95 |
| 18:1/20:4 | 5.15±0.74 | 6.31±2.19 | 6.11±1.45 |
| 16:0/20:4 | 4.99±0.09 | 3.86±0.59 | 5.33±0.04 |
| 18:0/20:4 | 5.37±1.80 | 5.13±0.65 | 5.98±1.47 |
| **Alkenyl-PE** | | | |
| 18:1/22:6 | 2.96±0.70 | 4.18±0.56 | 3.30±0.37 |
| 16:0/22:6 | 6.29±1.40 | 7.55±0.97 | 7.04±1.22 |
| **18:0/22:6** | **15.39±0.46** | **17.83±1.12** [c] | **19.23±1.05** [c] |
| 18:1/20:4 | 4.98±0.92 | 4.56±0.32 | 4.32±0.25 |
| 16:0/20:4 | 3.18±0.55 | 2.84±0.37 | 2.99±0.46 |
| **18:0/20:4** | **7.48±0.81** | **7.05±0.63** | **7.78±1.09** |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

The data in table 3.5. corroborates the fatty acid results showing significant alterations, either in diacyl-PE or alkenyl-PE, in the most abundant molecular species, 18:0/22:6 and 18:0/20:4. However, it should be noticed that not all changes are of the same magnitude as in the rats fed from conception. This difference is more obvious in the rats fed with LA/LNA 4.7:1 diet: while in the youngest rats, 18:0/22:6 increased by 22.3% in the 3-month-old rats it increased by 6.4%. The effect on the AA-containing species was the opposite as the observed in the rats fed from conception and, diacyl-18:0/20:4 level increased by 12%.

In the alkenyl subclass, the diet LA/LNA=4.7:1 had a higher effect on the 18:0/22:6 level than the fish oil. It is remarkable that the accumulation of this molecular specie was 10% higher in this group than in the rats fed from conception. There were not significant changes in the 18:0/20:4 levels.

### A.1.3. Results in *2-year-old* rats fed for one month with PUFA-enriched diet.

It is well known that during aging a large number of changes occur in the brain, some of them related with lipids: their metabolism is slowed down and there is a loss of DHA content. Despite these facts and due to the positive results obtained with the 3-month-old rats, it was decided to carry out an identical experiment using 2-year-old rats. Surprisingly, the same alterations as in the younger animals were observed.

Table 3.6. Fatty acid composition in PE in forebrain of 2-year-old rat.

| Fatty acid | Control | Fish oil | LA/LNA 4.7:1 |
|---|---|---|---|
| DMA 16:0 | 5.33±0.38 | 4.65±0.55 | 3.84±0.37 |
| 16:0 | 5.75±1.56 | 5.52±0.13 | 8.22±2.32 |
| 16:1 n-7 | 0.73±0.29 | 0.63±0.15 | 1.09±0.61 |
| DMA 18:0 | 18.12±0.35 | 18.18±1.14 | 13.17±1.18 |
| 18:0 | 13.81±0.74 | 13.83±0.81 | 15.93±1.46 |
| 18:1n-9 | 13.22±0.76 | 13.72±0.56 | 12.26±1.29 |
| 18:1n-7 | 5.44±0.53 | 5.59±1.28 | 2.41±0.28 |
| 18:2n-6 | 0.83±0.22 | 0.61±0.2 | 0.90±0.19 |
| **20:4n-6** | **10.92±1.05** | **9.32±0.57** | **8.98±1.30** |
| 22:4n-6 | 5.6±0.38 | 5.05±0.35 | 4.72±0.67 |
| **22:6n-3** | **15.97±0.56** | **17.21±0.54**[a] | **18.51±1.26**[b] |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

In the table 3.6., it can observed that effectively, there was an accumulation of DHA in the phosphatidylethanolamine. Despite this increase in DHA, it should be mentioned that when DHA levels of old rats are compared to levels of young rats, there is a general decrease. In young rats the level was around 20-22%, whereas in aged rats

is about 16-18.5% of the total fatty acid content. Interestingly, AA levels appeared to be maintained in all three groups around.

Table 3.7. Molecular species of PE in forebrain of 2-year-old rats.

| | Control | Fish oil | LA/LNA 4.7:1 |
|---|---|---|---|
| **Diacyl-PE** | | | |
| 18:1/22:6 | 1.67±0.30 | 1.84±0.28 | 1.60±0.75 |
| 16:0/22:6 | 6.13±1.05 | 6.92±1.08 | 5.66±1.77 |
| **18:0/22:6** | **25.13±1.38** | **29.62±1.49** [d] | **25.67±1.44** |
| 18:1/20:4 | 3.91±1.46 | 2.74±0.33 | 2.89±0.98 |
| 16:0/20:4 | 2.96±0.86 | 2.19±0.34 | 2.28±0.45 |
| **18:0/20:4** | **22.15±1.11** | **20.45±1.19** | **21.28±1.78** |
| **Alkyl-PE** | | | |
| 18:1/22:6 | 3.83±1.28 | 6.97±1.28 | 4.79±0.58 |
| 16:0/22:6 | 9.79±1.70 | 9.37±3.00 | 11.30±1.36 |
| 18:0/22:6 | 9.14±1.39 | 13.53±1.09 | 16.04±2.09 |
| 18:1/20:4 | 3.85±1.63 | 4.82±1.98 | 4.91±1.03 |
| 16:0/20:4 | 5.27±1.53 | 4.51±1.38 | 5.93±0.42 |
| 18:0/20:4 | 8.09±1.93 | 5.6±1.02 | 7.96±0.95 |
| **Alkenyl-PE** | | | |
| 18:1/22:6 | 4.05±0.62 | 4.42±0.53 | 4.99±1.14 |
| 16:0/22:6 | 7.13±1.45 | 7.29±1.17 | 8.03±1.61 |
| **18:0/22:6** | **13.59±1.09** | **14.37[x]±0.90** [d] | **17.79±1.99** [d] |
| 18:1/20:4 | 6.45±1.40 | 4.81±0.65 | 6.60±1.24 |
| 16:0/20:4 | 4.42±0.75 | 2.96±0.90 | 4.18+0.91 |
| **18:0/20:4** | **7.92±0.60** | **6.57±0.81** [b] | **8.01±0.95** |

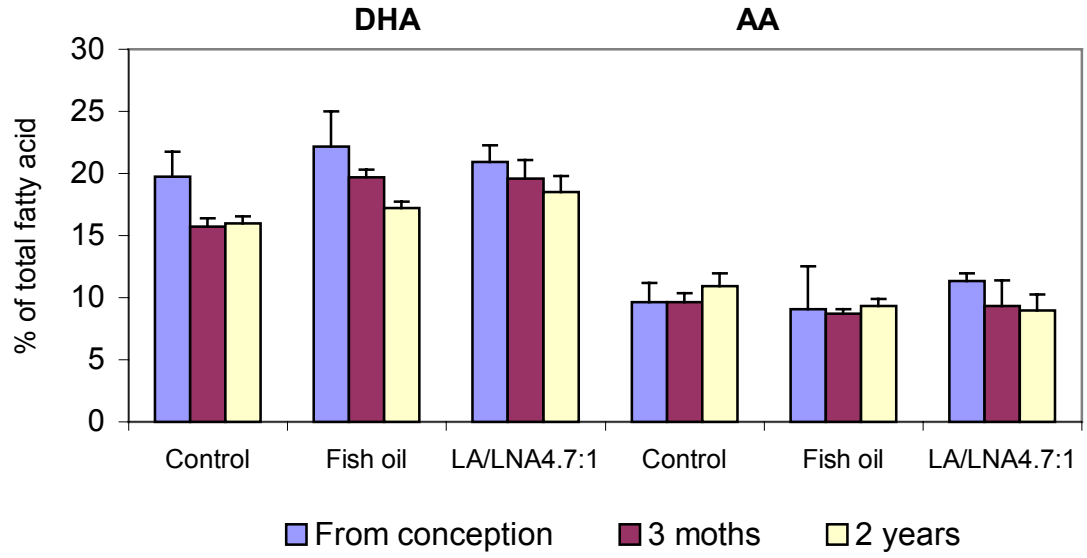
a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

PE molecular species were also affected, although only fish oil diet had an important effect on the composition increasing the diacyl-18:0/22:6 by 18% and decreasing diacyl-18:0/20:4 by 7.7%. Interestingly, the effect of the diet LA/LNA 4.7:1 on these diacyl species was very low but it affected dramatically the alkenyl subclass: 18:0/22:6 was increased by 31% and the 18:0/20:4 is decreased by 11%.

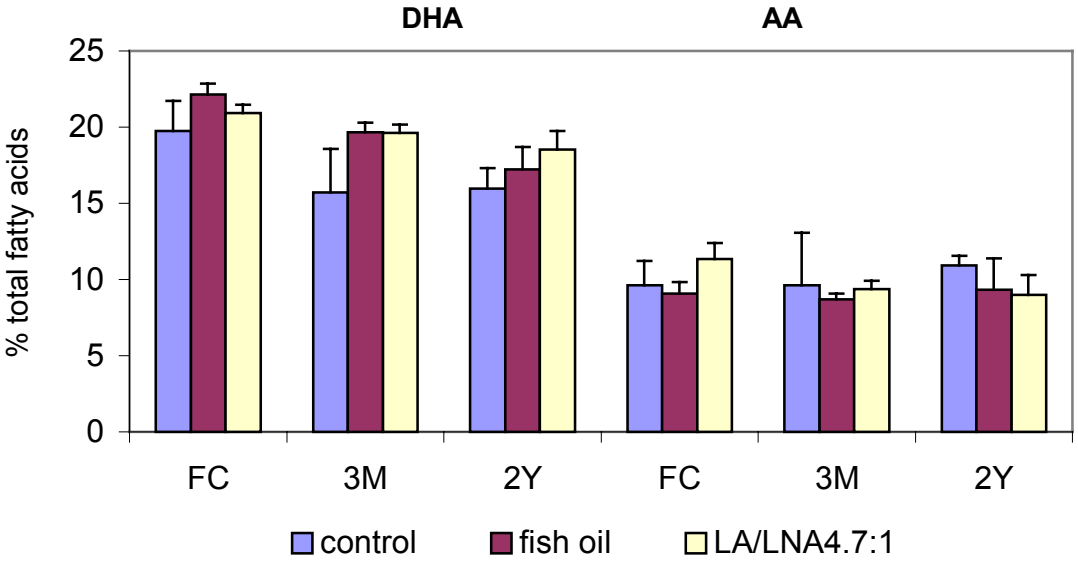
At this stage some tendencies may be established relating diet, age and level of fatty acid or molecular. Next, some sets of figures are shown, relating in a different manner these three variables. In the first set (fig. 3.1), AA and DHA levels are represented in relation to age (a) and diet (b).

Figure. 3.1. DHA and AA levels in PE of forebrain in relation to diet and age.

a) Effect of the age:




b) Effect of the diet

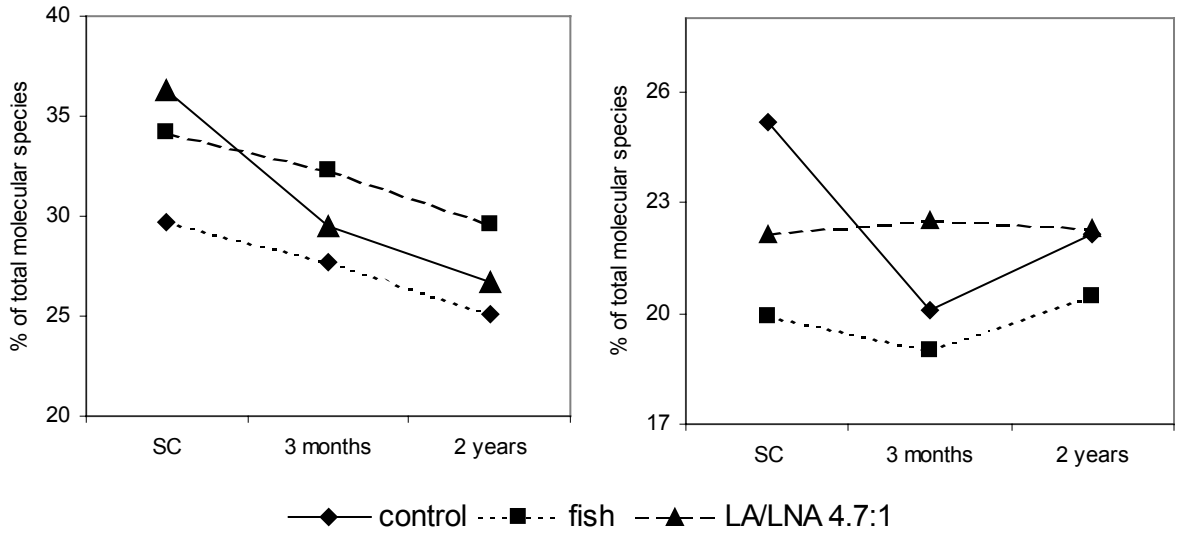



FC: rats fed from conception; 3M: 3-month-old; 2Y: 2-year-old

The age-dependency of DHA level is very well reflected in the figure 3.1.a). In the three diets there is a decrease in its level as the age increase. It is also clear from fig. 3.1.b) that DHA level is influenced by the dietary lipids. Despite the differences in age, the three groups are able to accumulate DHA. Regarding to AA levels, they do not show clearly an age-dependency. The values remain within a relative narrow range. Only in the 2-year-old rats there no beneficial effect of the diet on the AA level.

In figures 3.2. a) and b) are represented the levels of the two more abundant PE molecular species in the diacyl subclass, 18:0/22:6- and 18:0/20:4-PE. It can be observed that, in the case of the 18:0/22:6-PE there is a clear relation between the level of this molecular specie and the age, the level declines as the age increases.

Figure 3.2 Level of a) 18:0/22:6- and b) 18:0/20:4-PE in the diacyl subclass in relation to age and diet in rat forebrain.



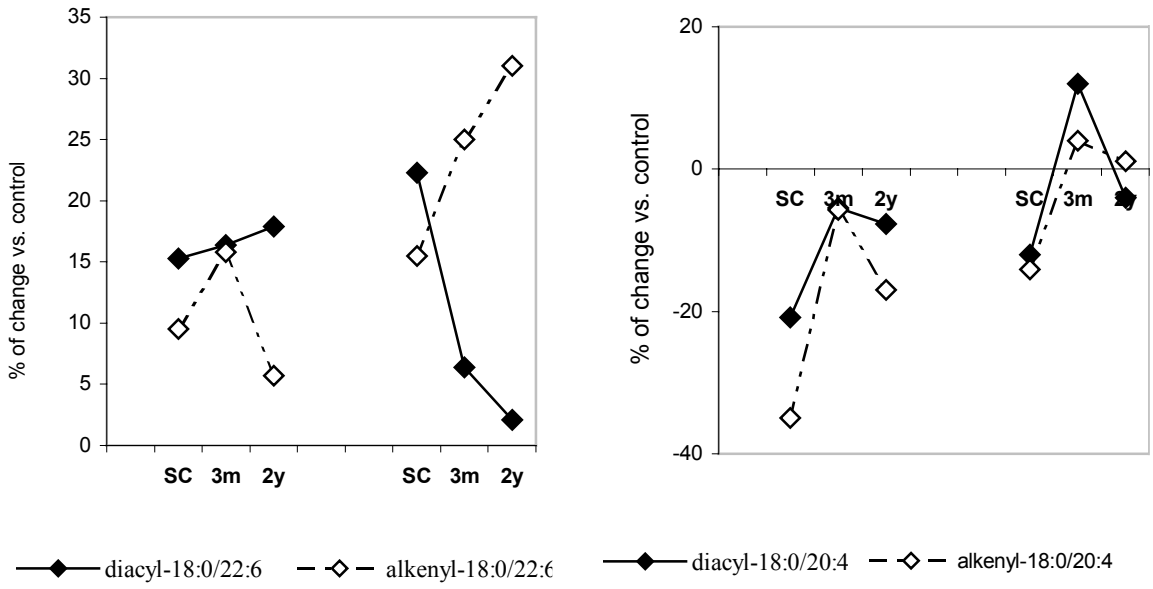

SC: rats fed from conception; 3 months: 3-month-old rats; 2 years: 2-year-old rat

However for 18:0/20:4-PE, this tendency is not observed. Instead, it seems that age does not affect its level when rats are fed with PUFA-enriched diets, yet only in control rat some pronounced changes could be observed. It is tentative to speculate, that the responsible for this maintenance of the 18:0/20:4 level is the PUFA-enriched diet.

Finally, the increase or decrease in 18:0/22:6-PE and 18:0/20:4-PE versus the control was calculated for both subclasses (fig. 3.3.). Thus, the fish oil diet seems to affect the alkyl-18:0/22:6 level in a similar way. In the three groups of animals, the increase is around a 16%. The alkenyl-18:0/22:6 shows a considerable increase in young rats, but it is very low in the old rats.

Fig. 3.3. Changes in a) 18:0/22:6-PE and b) 18:0/20:4-PE levels in rat forebrain

a) DHA containing species b) AA containing species




SC = rats fed from conception; 3 m = 3-month-old rats 2y: 2-year-old rats

In the case of rats fed with LA/LNA 4.7:1, percentage of change in alkyl-18:0/22:6 is increasing with the age of the animal, while in the alkenyl is decreasing. This would suggest some reorganization in the molecular species within the PE.

Finally it is quite obvious that AA containing molecular species does not follow any age-dependent tendency neither in fish oil diet nor in the LA/LNA=4.7:1 diet, but curiously enough the changes followed the same pattern in both diets.

## A.2. Effects of the dietary lipids on the phospholipid fatty acid and molecular species composition in hippocampus and cerebellum membranes.

Hippocampus and cerebellum are two distinguishable parts of the brain with different functions. Cerebellum is involved in the coordination of voluntary motor movement, muscle tone, balance and equilibrium. On the other hand, hippocampus has been involved in the process of memory, being important for the formation of the Long Term Potentiation (LTP), one form of the synaptic plasticity. Taking into account these differences, it was considered interesting to analyze both hippocampus and cerebellum membranes lipid composition.

It is clear from the results obtained in the forebrain's analysis that the most affected subclasses are the diacyl- and alkenyl-PE. Within the six molecular species listed previously, the major changes occurred in the 18:0/22:6 and 18:20:4. For these reasons, the tables presented in this chapter, will deal only with the changes produced on these two molecular species in the diacyl and alkenyl subclass. In addition, the three groups differing in age will be disused simultaneously.

### A.2.1. Results of hippocampus membranes analysis.

Table 3.1. collects the changes produced by the dietary lipids in the hippocampus membrane. In the younger group, the changes in composition of the hippocampus membrane maintain the general tendency observed in the forebrain membranes. Fish oil diet provoked the major changes in the molecular species composition. In fact, the increase in 18:0/22:6 level, is the highest observed in this

study: 43.6% in diacyl-PE and a 33.3% in alkenyl-PE. The decrease in AA containing species is remarkable as well: 18.3% in diacyl PE and 35% in alkenyl-PE.

Table 3.8. Molecular species composition of alkyl and alkenyl-PE in hippocampus of rats fed from conception, 3-month and 2-year-old.

| | Control | Fish oil | Perilla oil | LA/LNA 4.7:1 | LA/LNA 2.9:1 |
|---|---|---|---|---|---|
| **From conception** | | | | | |
| **Diacyl-PE** | | | | | |
| 18:0/22:6 | 25.54 ±0.41 | 36.68 ±2.63 [d] | 27.07 ±1.43 | 29.80 ±0.96 [b] | 28.24 ±1.93 |
| 18:0/20:4 | 26.91 ±1.38 | 21.98 ±2.05 [c] | 26.17 ±1.37 | 24.57 ±2.31 | 27.10 ±0.87 |
| **Alkenyl- PE** | | | | | |
| 18:0/22:6 | 21.08 ±3.53 | 28.10 ±0.19 [d] | 22.44 ±0.76 | 20.64 ±0.53 | 21.42 ±0.84 |
| 18:0/20:4 | 10.09 ±1.21 | 8.29 ±1.08 [a] | 10.16 ±0.10 | 10.45 ±1.03 | 9.44 ±0.22 |
| **3-month- old** | | | | | |
| **Diacyl- PE** | | | | | |
| 18:0/22:6 | 23.82 ±1.02 | 29.83 ±0.72 [b] | | 22.55 ±2.77 | |
| 18:0/20:4 | 23.56 ±1.27 | 21.64 ±1.02 | | 23.64 ±3.48 | |
| **Alkenyl- PE** | | | | | |
| 18:0/22:6 | 17.42 ±1.10 | 21.60 ±0.72 [d] | | 19.56 ±0.43 [a] | |
| 18:0/20:4 | 8.59 ±0.53 | 7.59 ±0.06 [a] | | 8.57 ±0.68 | |
| **2-year- old** | | | | | |
| **Diacyl- PE** | | | | | |
| 18:0/22:6 | 20.96 ±1.84 | 22.77 ±1.49 [a] | | 24.63 ±0.09 [b] | |
| 18:0/20:4 | 26.17 ±0.71 | 23.41 ±1.19 [a] | | 29.29 ±1.60 [b] | |
| **Alkenyl- PE** | | | | | |
| 18:0/22:6 | 17.53 ±0.68 | 19.82 ±0.90 [a] | | 20.75 ±1.53 [b] | |
| 18:0/20:4 | 6.04 ±0.33 | 7.95 ±0.81 | | 9.18 ±0.53 [d] | |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

LA/LNA 4.7:1 diet produced an increase in the diacyl-18:0/22:6 but in less degree than in the brain, whereas the alkenyl subclass was nearly unaffected. This diet decreased the level of the 18:0/20:4 in both subclasses. Finally, LNA-enriched diet (perilla diet) and LA/LNA=2.9:1 diet, had only marginal effects on the diacyl-18:0/22:6 level, being the increase similar in both diets (6 and 10%).

In 3-moth-old rats, fish oil diet affected in nearly exact way both subclasses, increasing DHA-containing species by 25% in and a decreasing AA-containing species by 10%. LA/LNA 4.7:1 had very little influences, the most remarkable change was produced in the alkenyl-18:0/22:6 increased by 12%.

Besides the high increase in the diacyl-18:0/22:6 in the youngest group, the most striking finding was the increase in old rats of the alkenyl-18:0/20:4: it increased by 32% in fish oil fed rats and by 52% in the case of LA/LNA 4.7:1 diet.

**A.2.2. Results of cerebellum membranes analysis.**

The table 3.9. summarizes the results obtained in the analysis of cerebellum phospholipids. In rats fed from conception, once more the fish oil diet is the one producing more changes in the diacyl species, although these changes are smaller than the produced in brain or hippocampus. Regarding the alkenyl species, while the increase in 18:0/22:6 was similar in both diets (25%), the decrease in 18:0/20:4 is quite different: by 30% in fish oil diet decreased but only by 2.4% in LA/LNA 4.7:1 diet.

The most interesting results may be the obtained in the aged rats. In the 3-month-old rats, fish oil diet provoked profound changes on the diacyl species, increasing the 18:0/22:6 by 26% and decreasing the 18:0/20:4 by 22%. Effects of LA/LNA 4.7:1 diet were similar but in less degree, both decrease and increase were close to 9%. In the alkenyl subclass, changes were different there was a slight increase due to the fish oil diet in 18:0/22:6 level, and a decrease in both diets by 15% in 18:0/20:4 level.

Finally, the most unexpected results were obtained in the 2-year-old rats. Not only there was not the usual increase in DHA- and AA-containing species, but the decrease in them was considerable.

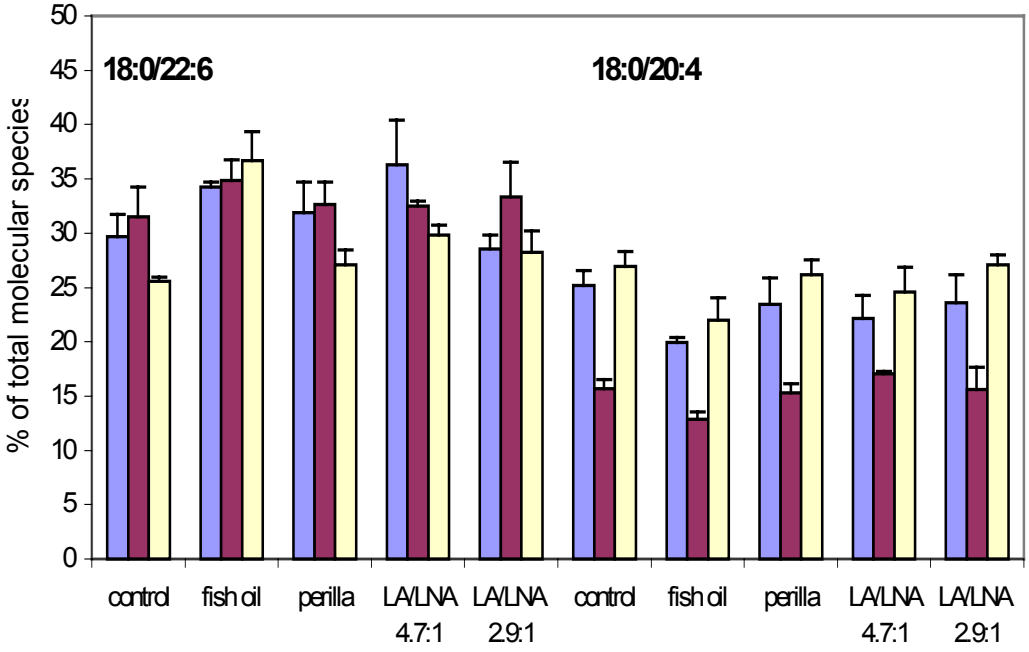
Table 3.9. Molecular species composition of alkyl and alkenyl-PE in cerebellum of rats fed from conception, 3-month and 2-year-old.

| | Control | Fish oil | Perilla | LA/LNA4.7:1 | LA/LNA2.9:1 |
|---|---|---|---|---|---|
| **From conception** | | | | | |
| **Diacyl-PE** | | | | | |
| 18:0/22:6 | 31.53±2.72 | 34.83±1.94 [a] | 32.63±2.06 | 32.49±0.42 | 33.32±3.19 |
| 18:0/20:4 | 15.70±0.85 | 12.84±0.67 [d] | 15.32±0.81 | 17.05±0.23 | 15.63[a]±2.05 |
| **Alkenyl-PE** | | | | | |
| 18:0/22:6 | 19.74±1.62 | 24.54[d]±2.50 | 22.16[a]±2.59 | 24.41[c]±0.89 | 22.32±0.21 |
| 18:0/20:4 | 7.51±0.62 | 5.25[d]±0.20 | 5.98[d]±0.46 | 7.33±0.14 | 6.50±0.17 |
| **3-moth-old** | | | | | |
| **Diacyl-PE** | | | | | |
| 18:0/22:6 | 31.48±0.93 | 39.84±1.30 [c] | | 34.50±2.30 [a] | |
| 18:0/20:4 | 15.90±0.58 | 12.43±0.29 [d] | | 14.59±0.72 [a] | |
| **Alkenyl-PE** | | | | | |
| 18:0/22:6 | 21.14±1.91 | 22.20±1.14 | | 18.75±0.87 [a] | |
| 18:0/20:4 | 6.69±0.50 | 5.71[a]±0.17 | | 5.61±0.48 [b] | |
| **2-year-old** | | | | | |
| **Diacyl-PE** | | | | | |
| 18:0/22:6 | 29.93±0.73 | 26.91±2.43 | | 25.47[b]±1.20 | |
| 18:0/20:4 | 13.99±0.46 | 13.10±1.54 | | 14.10±1.17 | |
| **Alkenyl-PE** | | | | | |
| 18:0/22:6 | 19.94±2.27 | 16.44±0.23 [c] | | 15.21±0.65 [c] | |
| 18:0/20:4 | 4.54±0.30 | 4.26±0.26 | | 3.46±0.18 [b] | |

Next, some figures summarize all the results obtained in the analysis of the molecular species composition of the three brain regions for each one of the three groups differing in age. In each set, first figure correspond to the diacyl subclass and the second to the alkenyl subclass.

Figure 3.4. Levels of 18:0/22:6 and 18:0/20:4 in diacyl and alkenyl -PE subclasses in forebrain, cerebellum and hippocampus **of rats fed from conception.**

a) Changes in the diacyl-PE molecular species.




b) Changes in the diacyl-PE molecular species.

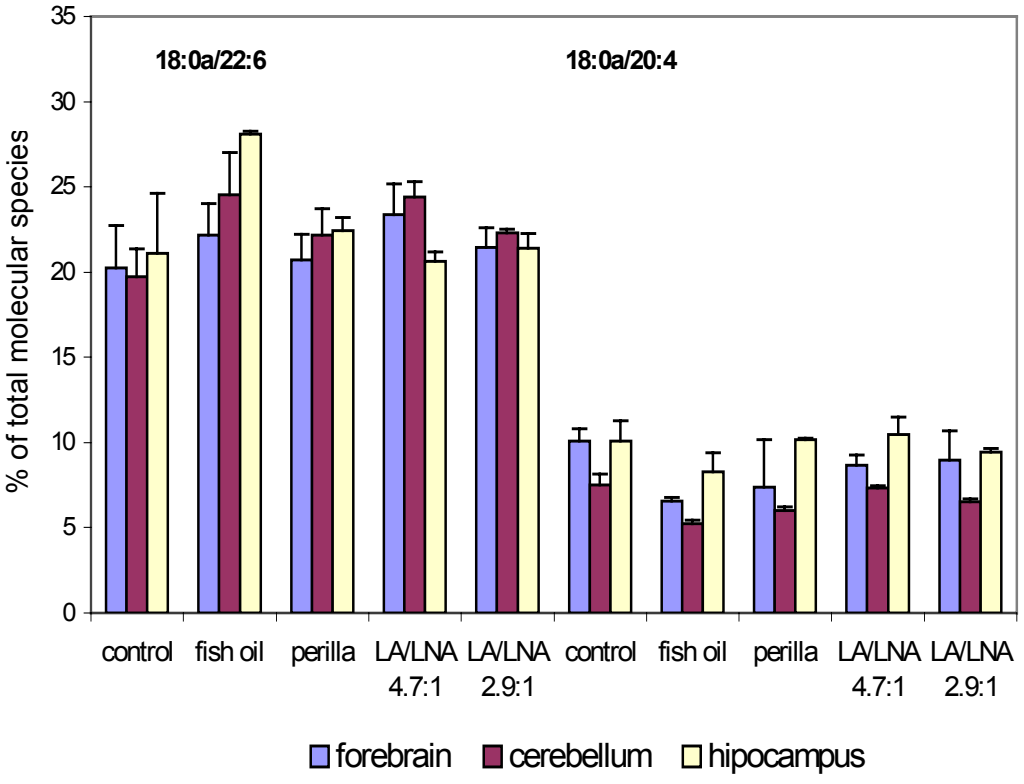
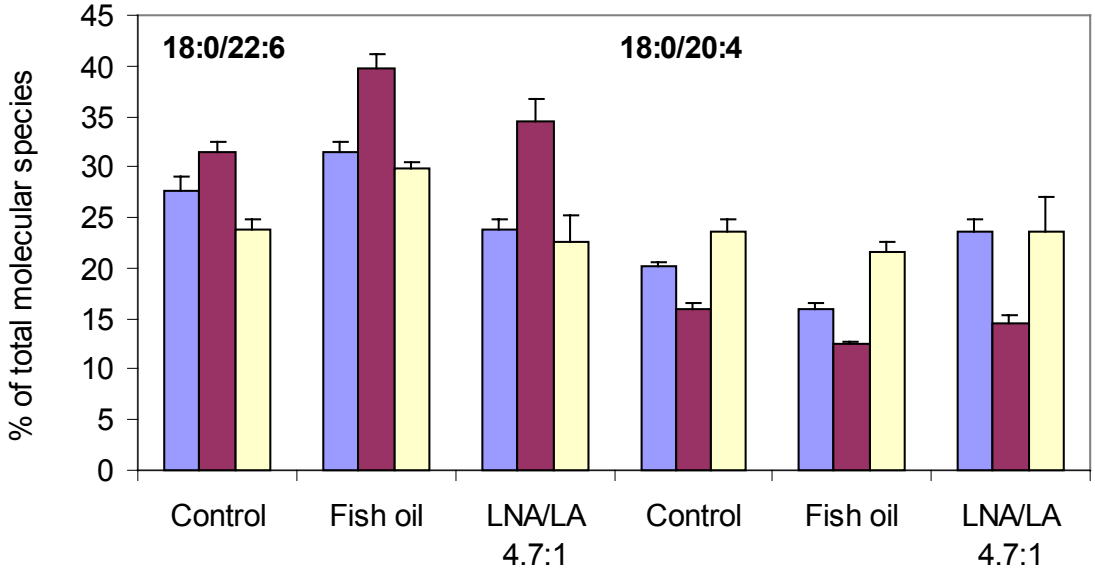



Figure 3.5. Levels of 18:0/22:6 and 18:0/20:4 in diacyl and alkenyl -PE subclasses in

forebrain, cerebellum and hippocampus of **3-month-old** rat.

a) Changes in the diacyl-PE molecular species.




b) Changes in the alkenyl-PE molecular species.

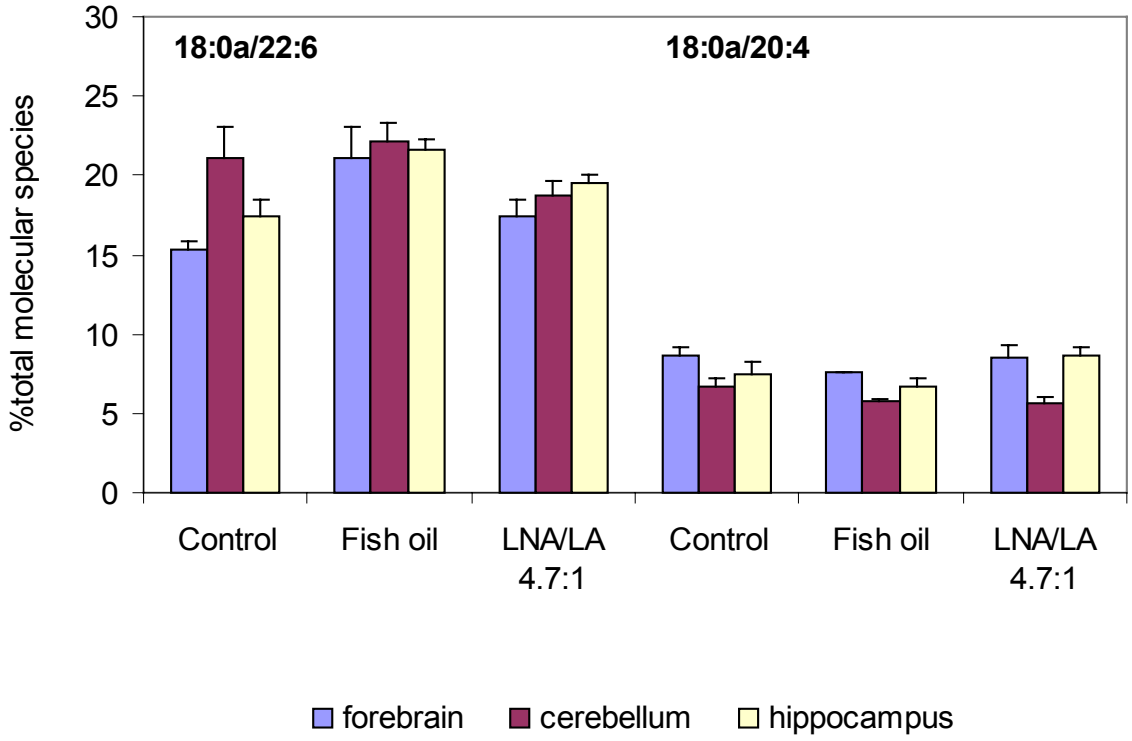
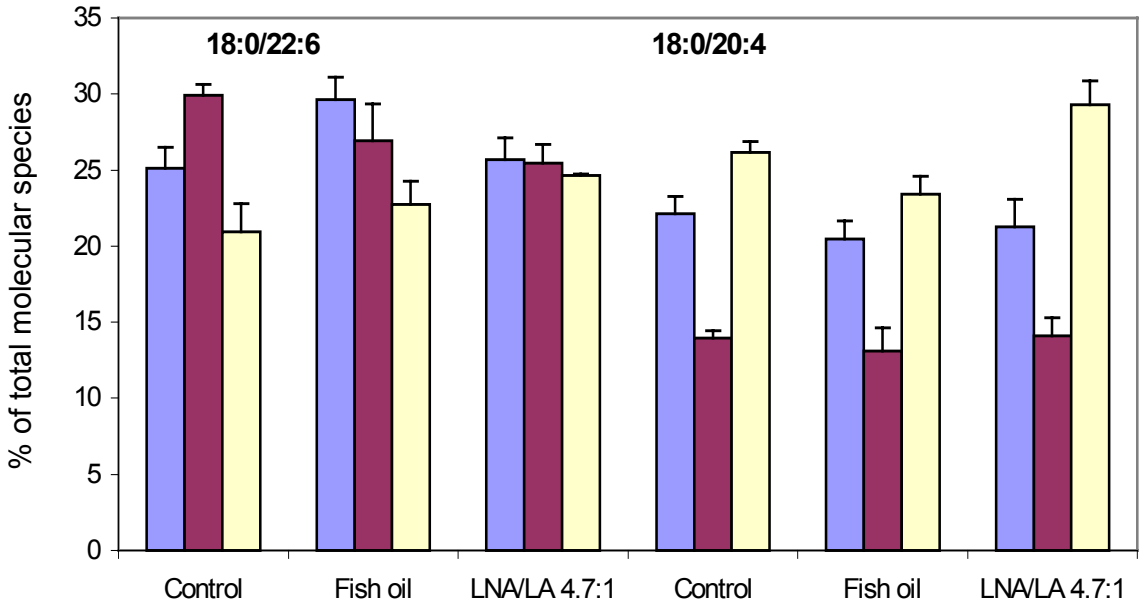
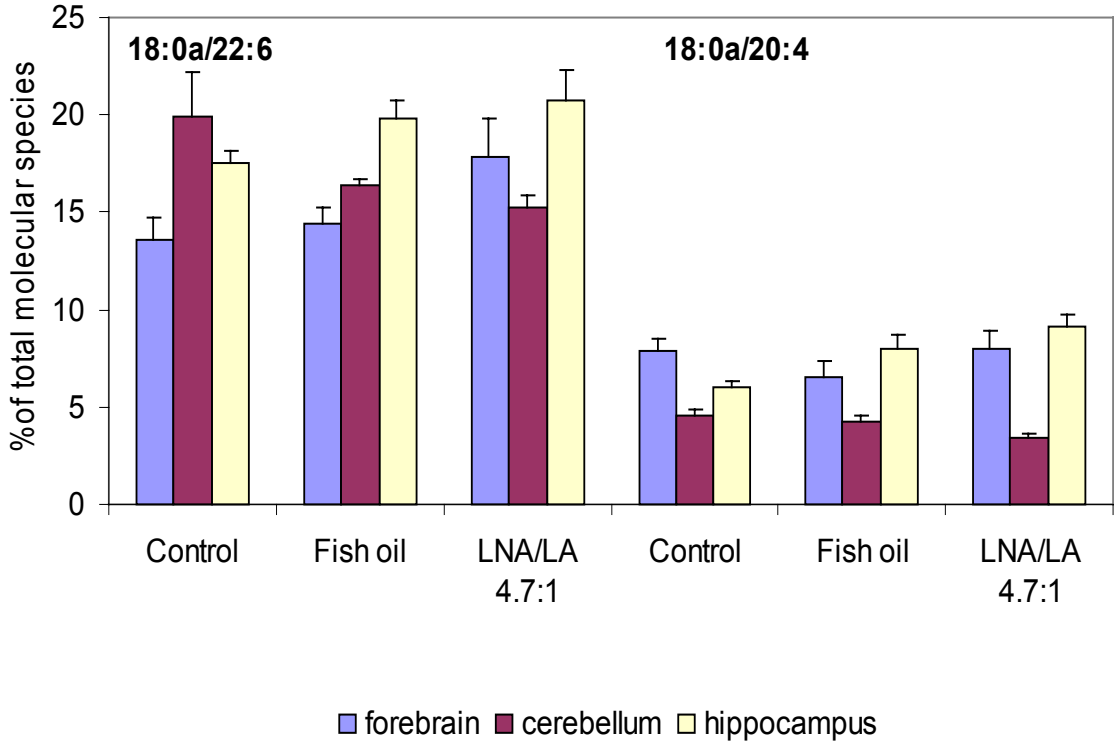



Figure 3.6. Levels of 18:0/22:6 and 18:0/20:4 in diacyl and alkenyl -PE subclasses in forebrain, cerebellum and hippocampus of **2-year-old** rat.

a) Changes in the diacyl-PE molecular species.




b) Changes in alkenyl-PE molecular species.




These figures show clearly the differences in molecular species composition existing between the three brain regions. Cerebellum appears to be very rich in diacyl-

18:0/22:6, specially in the adult animals. Hippocampus and brain show, in general, a similar molecular species composition, although hippocampus contains an amount slightly higher of diacyl 18:0/20:4.

Following these observations in lipid composition it was calculated the ratio 18:0/22/6 to 18:0/20:4 in each region and for each one of the 11 different groups analysed in this study. It was surprising that for a particular brain region, this ratio was kept in a relatively narrow interval. The average for each region is represented in table 3.10.

Table.3.10. Average of the ratio 18:0/22:6 to 18:0/20:4 in diacyl and alkenyl-PE subclasses in brain, cerebellum and hippocampus of the 11 different group analyzed.

| | **Brain*** | **Cerebellum** | **Hippocampus** |
|---|---|---|---|
| Diacyl-PE | 1.39 ± 0.21 | 2.22 ± 0.41[d] | 1.08 ± 0.25[a] |
| Alkenyl-PE | 2.41 ± 0.04 | 3.72 ± 0.63[d] | 2.44 ± 0.04 |

a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.001$

(*significance was calculated comparing to brain's ratio)

It is remarkable the fact that for the diacyl-PE, each region presents a particular ratio, maintained in all groups, despite the existing differences in age and diet. For the alkenyl-PE, the ratio is the same for brain and hippocampus, but they differ considerably from cerebellum's ratio.

## B. EFFECTS OF DIETARY LIPIDS ON GENE EXPRESSION.

One of the major functions of DHA is related to its involvement in cognitive processes. Once its level had been reduced, the animal models underperformed in learning test and other mental functions (19, 84, 125), which could be reversed by supplying DHA or other polyunsaturated fatty acid, such a linolenic acid (20). Recent evidence attributed a similar function to a proper mixture of n-6 to n-3 octadeca-polyenoid fatty acids (214, 240, 245).

The exact mode of action of PUFA-containing phospholipids on cognitive functions is still not known. Since polyunsaturated fatty acid content is known to affect membrane biophysical properties, it was proposed that the beneficial effect on mental functions of these fatty acids occurred at this level (245). However, it is evident that storing and processing of incoming information is a more complex process and it cannot be a simple function of membrane physicochemical properties (114). One of the plausible mechanisms could involve the gene expression machinery.

It was previously shown by Kitajka et al. (114) that fish oil and perilla oil induced changes on the gene expression in the brain. The expression levels of 102 cDNAs, representing 3.4% of the total DNA elements on the array, were significantly altered. The changes ranged from –5 fold to + 7 fold. It was interesting to observe that the two kinds of oils exerted almost the same effect on the gene expression profile. Only one gene, coding for a membrane protein with unknown function, exhibited specific up-regulation in response to fish oil feeding. Again, only one gene was specifically down-regulated in perilla oil-fed rat brain. Altogether expression of 55 genes increased whereas the expression of 47 genes was decreased by fish oil or perilla oil feeding. Among the overexpressed genes there were membrane, cytoskeletal, regulatory, receptor, ion channel and synaptic proteins, and genes related to lipid and energy metabolism, neurotransmission, signal transduction, endocytosis and synaptic vesicle recycling (114).

Based on these observations it was interesting to analyze the gene expression in brain of rats fed with the oil mixture, LA/LNA 4.7:1. Of the 55 genes found

overexpressed after feeding with perilla oil (rich in LNA) only 20 of these genes responded with an increased expression when the LA/LNA 4.7:1 combination was fed to the rats (12). These genes are listed in the table 3.11. where for comparison, data obtained for perilla oil are also given (12, 114).

In table 3.12 the list of down-regulated genes by LA/LNA 4.7:1 is compared to the results obtained by feeding perilla oil to rats (12). Four were found down-regulated by the LA/LNA 4.7:1 diet. Two of them (Syntaxin binding protein 2 gene and NIPSNAP2 gene) are coding for proteins which are related to vesicular transport, 2 are related to regulatory proteins/kinases (a non-receptor tyrosine kinase type 2 and STK-1 serine/threonine kinase), an other to membrane protein (transthyretrin), to lipid metabolism (dihydrolipoamide branched. chain transacylase), to ion channels (E1-E2 ATPase) and 6 to unknown functions.

The degree of down-regulation by LA:LNA (4.7:1) mixture was different from that obtained for perilla oil. The extent of down-regulation in case of four genes (dihydrolipoamide branched chain transacylase E2, non-receptor tyrosine kinase (tyk 2) and syntaxin binding protein MUNC 18-2, BAC 10818 containing the Ercc-4 gene), 8 of them responded with an increased down regulation (such as STK-1 (serine/threonine kinase)) or U1 small nuclear ribonucleoprotein homologue), while transthyretrin was down regulated to a lower extent (-2.54 vs. –4.76) (12).

Table 3.11. Effects of perilla oil, and LA/LNA 4.7:1 diet on rat brain gene expression and genes with altered gene expression (upregulated genes)

| Gene product | Perilla oil Fold-Δ | LA: LNA 4.7:1 Fold-Δ |
|---|---|---|
| **Lipid metabolism** | | |
| Serine palmitoyl transferase subunit II | 3.34 | 4.92 |
| Farnesyl pyrophosphate syn. (testis) | 3.94 | 6.51 |
| **Mithocondrial enzymes** | | |
| Ubiquinol-cytochrome c red. (6.4kDa) | 1.54 | 2.31 |
| NADH dehydrog./ cyt. c oxidase subunit I | 3.73 | 5.39 |
| Cytochrome c oxidase subunit II (Cox2) | 3.84 | 7.01 |
| Cytochrome b mRNA | 3.56 | 5.04 |
| **Synaptic proteins** | | |
| Synuclein. alpha | 1.85 | 2.71 |
| **Cell adhesion. synaptic traffic** | | |
| D-cadherin precursor | 1.90 | 2.64 |
| **Membrane proteins** | | |
| Sec24 prot (Sec24A isoform) | 5.52 | 6.55 |
| Signal transduction | | |
| Calmodulin (RCM3) | 1.54 | 2.42 |
| Endocytosis. synaptic vesicle recycling | | |
| Clathrin-ass. adaptor prot homolog (p47B) | 2.76 | 3.47 |
| **Ceramid-activated prot. phosphatase** | | |
| Prot. phosphatase 2A | 1.62 | 2.81 |
| **Others** | | |
| Ubiquitin-prot. ligase Nedd4-2 | 5.56 | 9.83 |
| Elongation factor 1-alpha | 4.76 | 7.79 |
| Beta-globin | 4.47 | 6.41 |
| Ribosomal protein L7a | 5.60 | 8.40 |
| **Unknown** | | |
| M.musculus adult male cerebellum cDNA | 4.85 | 8.34 |
| M.musculus adult small intestine cDNA | 4.64 | 5.95 |

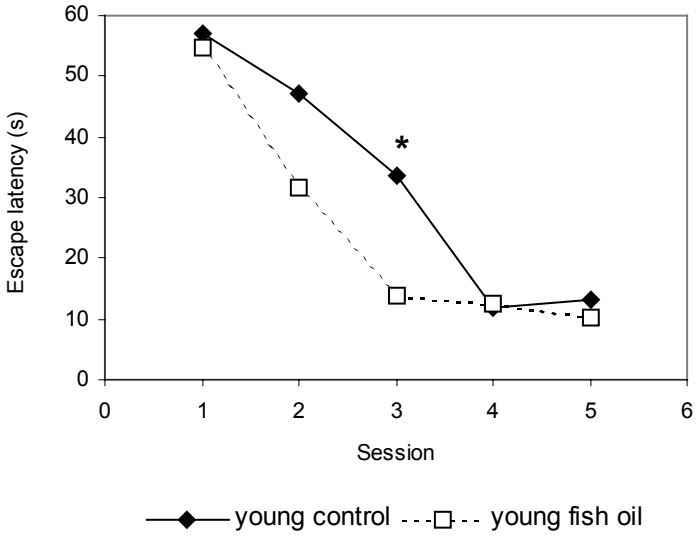
Table 3.12. Effects of perilla oil and LA/LNA 4.7:1 diet on rat brain gene expression and genes with altered gene expression (downregulated genes)

| Gene product | Perilla oil Fold-Δ | LA: LNA 4.7:1 Fold-Δ |
|---|---|---|
| **Lipid metabolism** | | |
| Dihydrolipoamide branched chain transacylae E2 | -3.89 | -3.84 |
| **Regulatory proteins. kinases. phosphorylases** | | |
| Non-receptor tyrosine kinase(tyk2) | -2.42 | -2.38 |
| STK-1 (serine/threonine kinase) | -1.59 | -2.87 |
| **Membrane protein** | | |
| Syntaxin binding prot MUNC 18-2. Stxbp2 | -1.98 | -2.17 |
| Transthyretin | -4.76 | -2.54 |
| **Receptor. ion channels** | | |
| Putative E1-E2 ATPase | -4.03 | -5.1 |
| **Vesicular transport** | | |
| NIPSNAP2 prot | -1.59 | -2.27 |
| **Others** | | |
| RNA binding-protein AB0169092 | -1.96 | -2.26 |
| BAC 10818 containing the Ercc-4 gene | -2.20 | -2.27 |
| U1 small nuclear ribonucleoprotein homolog | -2.62 | -8.13 |
| **Unknown** | | |
| HSPC016 mRNA (*H.sapiens*) | -2.35 | -2.9 |
| Clone PLACE 1004814 | -2.30 | -3.4 |
| Clone ct7-326b16 | -2.44 | -3.48 |

## C. EFFECTS OF A DHA-ENRICHED DIET ON THE COGNITIVE ABILITIES.

DHA has been repeatedly related with improvement in the cognitive function (184, 152), and it resulted interesting to investigate if one month of feeding with fish oil in adult rats was enough to evoke that benefit. Spatial learning test were performed to 3-month and 2-year-old rats after a feeding period of one month. The results were compared with the performance of two control groups (3-month and 2-year-old) receiving normal rat chow in their diet.

Figure. 3.7. Morris water maze test performed by 3-month-pld fed either with normal rat chow or with rat chow enriched in fish oil

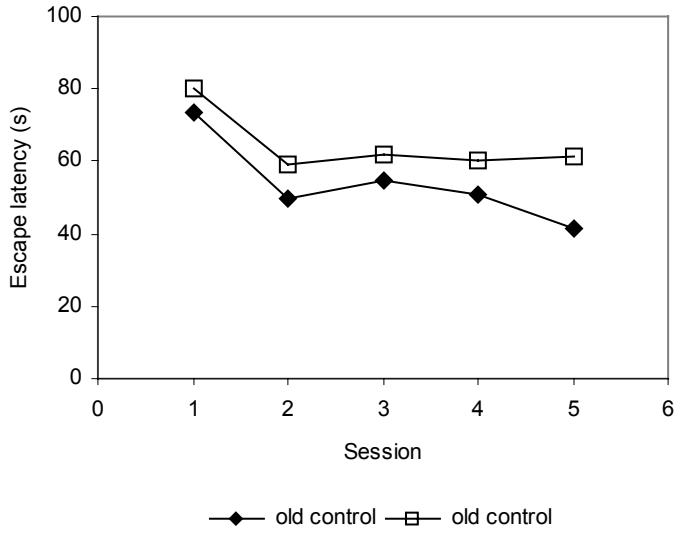



In Fig. 3.7.it is represented the results of the Water Morris test for the four groups of rats. The figure shows that young rats on fish oil tended to perform better in Morris water maze test than the control ones the difference reached the $p<0.05$ significance level at the 3rd session ($t=2.24$, $df=17$, $p<0.05$).

In contrast, the old rats kept on fish oil did not show improvement in their spatial memory (Fig. 3.8) indicating that elevated level of DHA might not have been sufficient

to evoke the known beneficial effect of n-3 polyunsaturated fatty acids on learning. Performance was also not different with the old rats if the main latencies per each session were computed (data not shown).

Figure. 3.8. Morris water maze test performed by 2-year-old fed either with normal rat chow or with rat chow enriched in fish oil




Furthermore, evaluating the daily improvement during the test young rats showed a highly significant learning effect throughout the course of 5 daily sessions ($F_{[4,68]}$=49.03, $p<0.0001$) while aged rats just reached significance to improve their daily memory performance ($F_{[4,56]}$=3.88, $p<0.05$, ANOVA with repeated measures).

# 4. DISCUSSION.

Generally, studies concerning the beneficial effects of n-3 or/and n-6 acids essential fatty acids on animals involve a two-generation diet regimen in which the mother is raised on an n-3/n-6 deficient diet. Afterward, the offspring is fed for a certain time with a diet rich in the fatty acids and the effects are discussed. Such treatment has generally been found to be necessary to induce a marked decline in brain DHA (186).

To our knowledge, not too many studies have been carried out regarding EFA supplementation in sufficient animals. For this reason, it was considered important to study effects that EFA supplementation might have on healthy animals. Hence, the principal difference between most of the works found in the literature and this thesis is that EFA sufficient animals stood in the center of our attention.

The main reason of focusing this study on the brain lipids is that despite intensive research, there is still some controversy on how dietary lipids affect the brain's function and metabolism.

On the other hand, analyzes were performed on total membrane phospholipids, and the possible differences that might exist in the molecular species composition of each subcellular membrane were not taken into account. Nevertheless it was considered, that changes in the phospholipid levels of the whole cell reflect the changes taking place in each particular cellular membrane. Further efforts will focus on determining the changes that might exist in separated membranes, especially in synaptosomes.

Finally the main bulk of work in lipid analysis was expressed in terms of changes in fatty acid composition. Here, however, both fatty acids and molecular species composition has been analyzed. The degree of information is higher and might give some new differences that could be shadowed if only fatty acid analysis was done.

**Importance of PUFAs in brain.**

It is well accepted that profound n-3 PUFAs deficiency acts on both brain composition and brain functions such as neurotransmission and behavior.

In 1976, Lamptey and Walker (125) showed that n-3 fatty acid deficient rats had inferior learning ability. Later, several studies showed that in severely n-3 fatty acid-depleted rodents the performance in a learning test was reduced (20, 75, 84, 153, 163, 215, 226, 240). Finally, studies focused on the depletion of a particular PUFA. Reduction in brain DHA lead to a variety of behavioral and visual deficits (76, 84, 153, 225). Meanwhile it turned out that LNA deficient diets impaired performance in a variety of learning task (74, 153, 222, 238) and altered several sensory processes such as olfaction (85) and audition (23). Results of human infant studies suggest, that a feeding formula containing low n-3 PUFAs leads to suboptimal cognitive and visual function (15, 35, 42, 79, 101, 141, 232).

The recovery from DHA losses was possible in animals when the n-3 deficient diet was switched to and n-3 fatty acid adequate diet (42, 158). These animals recovered from deficits concerning their spatial task performance, as well (155). Despite the efforts taken to understand how EFAs exert their beneficial effect on the brain, the underlying mechanism is still unclear.

During learning or memory storage, different signal transduction pathways are involved. A large number of proteins are implicated, some of them are integral proteins while others are membrane-associated ones. It would be reasonable to suggest that an increase in the efficacy of the signal transduction would lead to an enhancement of the cognitive abilities. Two different mechanisms could be proposed: 1. either to increase the activity of the involved proteins or 2. to increase the number of them. While the first one is directly related to the biophysical and biochemical properties of the membrane, the second would implicate changes in gene expression.

In this work it has been demonstrated, that dietary lipids act by both of the proposed mechanisms. Although different, these two mechanisms do not exclude each other. In fact, it is perfectly reasonable that the combination of the two effects might explain the beneficial effects of the EFAs of the brain.

**The brain responds differently to PUFA-sufficient diets than to PUFA-deficient diets.**

Brain lipid metabolism shows differences when compared to the rest of organs. It has been known for a long time, that when an adult mammal consumes a diet low in DHA and its n-3 precursors, the DHA content of its nervous system is much less altered, than that of other organs, i.e., DHA is said to be tenaciously retained once neural development has completed (182, 183). However, when n-3 deficiency occurs during neuronal development, levels of brain and retinal DHA decline significantly (161, 164, 182, 183, 211).

Once depleted, the brain recovers its DHA rather slowly (251). A recent study in rats provided the time courses of DHA recovery when the rats were repleted with a diet containing both α-linolenate and DHA (158). The half-time of repletion of DHA levels, was 2.9 weeks for the brain while only 0.3 and 0.5 weeks for the liver and plasma, respectively. Moreover the recovery seemed not to be homogenous throughout the different brain regions (36), and in addition, this biochemical recovery was not always correlated with functional recovery as assessed by behavioral tests (36, 37, 98).

Therefore, if the ability to recover from the EFA deficiency depends on the developmental stage when it happens in, it is reasonable to think that the beneficial effects of the EFA will also present the same time-dependence. Hence, maximal benefits were expected when the diet was given during prenatal and early postnatal periods, the time of the intensive accumulation of long chain PUFAs, especially DHA in the brain (40, 83, 194).

It was shown that feeding rats from conception and for only one generation it was enough to alter significantly both the fatty acid and molecular species composition of PE (Table 3.1, 3.2). This would suggest that brain lipid metabolism responds differently according to its EFA status. When there is a deficiency, the brain tries to maintain the EFA levels through different mechanisms, like increasing the brain's synthesis DHA, enhancing the recruitment of DHA from the plasma, or synthesis of DHA from docosapentaenoic acid and LNA (167). In fact, the conversion of LNA to DHA increases during essential fatty acid deficiency (61). Williard et al. (234) showed that astrocytes were able to synthesize DHA. So it seems, that the brain has different mechanisms to maintain the PUFAs' levels. Although PUFA-depletion is very difficult to achieve in the brain, the accumulation of PUFA's turned to be less restricted.

The highest accumulation of DHA was observed when the main source of PUFAs was the DHA-enriched diet (fish oil diet) (table 3.1). This result is consistent with other works found in the literature (186, 266). This increase in DHA levels was accompanied by a decrease in AA levels. This is a consequence of the competition shown between the two families of fatty acids for the elongation-desaturation enzymatic machinery (69) (fig. 1.4). Dietary studies on rats and other animals have shown that 18:3n-3 is a strong suppressor of n-6 acid metabolism, whereas 10 times as much 18:2n-6 is required to give an equal suppression on n-3 metabolism (126). Wainwright et al. (266) made the same observations in their experiments.

Diets containing a high percentage of LNA (perilla diet and LA/LNA=2.9 diet) lead to a small or no accumulation of DHA (table 3.1 and 3.2). This is consistent with our knowledge about the fate of dietary LNA in the body. LNA is involved in three different metabolic pathways: β-oxidation, carbon recycling and synthesis of DHA. Studies in humans and animals reveal that a major catabolic route of metabolism of LNA is β-oxidation. Another major route in rats and primates involves carbon recycling into *de novo* lipogenesis in brain and other tissues in pregnant, fetal, and infant animals (25). A third metabolic pathway of LNA, often thought of as the main pathway, is being

the precursor of EPA, DPE (22:5n-3) and DHA. However, diets rich in LNA do not necessarily lead to high tissue DHA levels. Sinclair et al. (195) addressed this issue and showed that the tissues where the most LNA accumulated were muscle, skin, and adipose tissue (78). They argue that several reasons could be responsible for the relatively poor efficiency of the conversion of LNA to longer chain PUFAs. First, as noted above, a substantial proportion of LNA is either diverted β-oxidation or found distributed throughout all major tissue lipid pools. Second, LA is a competitive inhibitor of the metabolism of LNA to 18:4n-3, which is the precursor of long-chain n-3 PUFAs (fig 1.4.). Furthermore, diets rich in LA decrease the expression level of the hepatic Δ-6 desaturase (38).

Finally, the diet with the ratio LA/LNA=4.7:1 showed the highest accumulation of DHA-containing molecular species in the forebrain of young rats (table 3.2.). The AA-containing species decreased but to a lesser degree than in the case of fish oil diet. This lower decrease can be explained simply by the increased level of LA in the diet, the precursor of the AA. However there is no definitive explanation for the large increase in DHA containing species. Similar observation was made by Jumpsen et al. (108). They fed rats from conception until 6 weeks of age, with diets differing in the n-6/n-3 ratio and analyzed the changes in fatty acid composition in PE and PC in neuronal cells. In PE of rats, the highest content of DHA was achieved with the LA/LNA ratio of 4.1. (108).

It is well known that Δ-6 and Δ-3 desaturases are influenced by the n-3 and n-6 content of the diet, so it can be speculated that a ratio of LA/LNA close to 4 is the optimal one to convert the LA in AA and LNA to DHA. However, other possibilities cannot be ruled out. Dietary PUFAs also affect the permeability of the blood-brain barrier, the gene expression in liver and adipose tissues and finally the activity of the lipid binding proteins.

It should be mentioned that our rats were sacrificed when they were six weeks old, so only four weeks after weaning. Maternal milk contains a small percentage of PUFAs (103, 116) and this amount is influenced by the mother's diet (77, 248). Therefore mother's milk is an additional source of PUFAs, which is not included in the adult rat's diet.

Despite this difference, a significant accumulation of DHA is observed in the three month-old (table 3.4., 3.5.) and the two year-old rat brain (table 3.6., 3.7.) after being fed for a month with DHA-rich diet. A similar tendency is obtained with the LA/LNA=4.7 diet, although changes are less pronounced. In aged rats, levels of DHA decreased whereas AA was maintained between certain levels (fig 3.1. a). However these lower levels have been shown to change in response to dietary PUFAs. So age appeared to be irrelevant concerning the accumulation of DHA (fig 3.3. b).

In the case of three month-old rats it could be argued whether the development has been completed or not. In accordance with Sinclair's data (194), the brain has reached only approximately 65% of its final weight at this age. However, the accumulation of C22 species has risen up till 90%. Therefore it could be assumed, that in these rats the major part of the increase in DHA level is due to dietary effects rather than to the development accumulation.

**Alterations of architecture and biophysical properties of the membrane due to changes in the molecular species composition.**

Glycerophospholipids have a clear structural role in neural membranes but they are also the main reservoirs of PUFAs, including DHA. Each phospholipid class (PE, PC, PS and PI) has a distinct and unique molecular profile, and the number of different combinations is extremely high. The role of such diversity in the cell is still far from being understood, although evidences are pointing to a specific function for each different molecule. Both *de novo* biosynthetic and remodeling pathways appear to

contribute to the unique molecular species assembly in these phospholipid classes. (158).

Recently, Salem et al. (186) demonstrated the phenomenal degree of specificity that the membrane has regarding its composition. They showed that substitution of DHA (22:6n-3) for EPA (22:5n-6), a common fatty acid occurring in n-3 deficient animals, is not enough to recover the learning abilities, despite the high similarity between these two molecules. It is clear from these experiments that subtle changes in membrane composition might lead to considerable functional changes.

Changes in the ethanolamine phosphoglycerides were the main aim of this study, since quantitatively they are the major phospholipids in the nervous tissues (188).

Levels of DHA and AA in forebrain PE are clearly affected when fish oil and LA/LNA=4.7:1 diet were given from conception (table 3.1.). DHA and AA containing phosphoglycerides have been proposed to play a special role in biomembranes including those of neurons. This is due to their favorable biophysical properties, like high motility and motional freedom (134, 184, 186). The results of NMR spectroscopy and X-ray diffraction studies suggested that PUFAs chains prefer flexible, looped and helical structures with rapid transitions among a large number of conformers. This provides increased flexibility to receptor-rich neural membranes that contain high concentrations of DHA. The alteration of elasticity of neural membranes according to the number of double bonds per fatty acid is one possible role for lipid polyunsaturation (186).

In addition, it is widely recognized that phospholipid acyl chain unsaturation plays a major role in determining many important bilayer properties, including phase transition temperature (110, 162), bilayer thickness (212), area per molecule (93), and acyl packing (202). Lundbaek et al. (136) and de Kruijff (54) pointed out the importance of molecular geometry of phospholipids and packing properties of membranes in stabilizing intrinsic proteins in their functional state.

Regarding the molecular species composition, our attention was focused on two principal subclasses, the diacyl and the alkenyl-PE, since both together account for approximately the 90% of the total PE (data not shown). Fodor et al. (70) showed in model experiments how diacyl phosphatidylethanolamines tended to increase membrane packing (70). On the other hand ethanolamine phosphoglyceride molecules are conic shaped and are prone to form so called non-bilayer phases (fig. 1.5). This tendency is greater in the case of the alkenyl subclass due to the presence of the vinyl ether linkage.

Using HPLC techniques at least 24 different molecular species can be separated. DHA and AA containing species represent a 70 and 55 % of the total diacyl-PE and alkenyl-PE, respectively. Furthermore, 18:0/22:6 and 18:0/20:4 are the ones present in the highest concentration, together they account for approximately more than a 50% of the total diacyl PE and a 30% of the alkenyl-PE species. Therefore it can be assumed that changes in the levels of these two particular molecular species will be the most important both qualitatively and quantitatively. Within the two subclasses, 18:0/22:6 and 18:0/20:4 are the two molecular species responding most sensitively to dietary changes. Applegate and Glomset (7) published a molecular model for *sn*-1 stearic acid *sn*-2 docosahexaenoic acid containing phospholipids, where the molecule adapted a rod like shape. On the other hand, Zabelinskii et al. (252) calculated that the surface area of 1-monounsaturated, 2-polyunsaturated molecular species is roughly 20 % greater than that of the corresponding sn-1 saturated, sn-2 polyunsaturated species.

Taking all these studies into consideration, it is reasonable to assume, that in the animals fed with fish oil and LA/LNA 4.7:1 diets, the changes observed in PE both in the total amount of DHA and AA, will certainly alter the biophysical properties of the neural membranes.

**Importance of ratio n-6/n-3.**

Despite the beneficial effects demonstrated for the DHA, diets containing very high levels of long-chain (n-3) FA and extremes in the n-6/n-3 ratio have been shown to affect brain development. Weisinger et al. (228) showed, that in newborn guinea pigs fed with diets containing large amounts of fish oil (n-6/n-3 ratio 2.54), which in turn resulted in high levels of DHA in the retina, had electroretinograms that resemble those of animals fed with n-3 FA-restricted diets. Studies carried out by Wainwright showed how diets with high contents of n-3 (ratio of n-6/n-3 was 0.3) had a negative effect on nervous system development (226).

DHA is considered to be a molecule highly susceptible to lipid peroxidation (92), and therefore the ingestion of DHA rich diets has been related with an enhancement of liver and kidney lipid peroxidation (121). However, the role of PUFAs in the cell oxidative stress is still unclear since the peroxidation of PUFAs is thought to be influenced by the microenvironment in which they exist (239). Accordingly, it appears that the location of PUFAs, that is the distribution in different lipid classes as well as in different tissues, should be taken into account when lipid peroxide formation in tissues is investigated (122).

Besides its role as structural component, AA is well characterized as a precursor of bioactive mediators, such as eicosanoids, leukotrienes, and prostaglandins, which are involved in sleep regulation (216), febrile response (217), and pain perception (157), as well as in inflammatory responses. Free AA is known to activate protein kinases and ion channels, inhibit neurotransmitter uptake, and enhance synaptic transmission (111). AA and platelet-activating factor, derived from AA-containing phospholipids are suggested to function as retrograde messengers in long-term potentiation of synapses in the hippocampus CA1 region (233), and to be involved in the migration of neurons in the cerebral cortex (87).

Finally, recent studies proposed that not only DHA, but also n-6 PUFAs might be involved in learning and cognitive performance (98, 153). Ikemoto et al. (98) provided evidence, that increased n-6 PUFAs level in the brain under n-3 fatty acid deficiency is another critical factor affecting the reversibility of learning performance in the brightness-discrimination test.

All these facts show the importance of an appropriate level of AA in optimal cell functioning. Therefore it seems, that it is the ratio of n-3 and n-6 fatty acids which plays a critical role, and not the absolute level of a particular PUFA.

Yehuda et al. (240) already pointed out the importance of the ratio of the two EFAs. This was demonstrated in a study in which the administration of a narrow range ratio of 4:1 α-linolenic to linolenic acids exerted beneficial effects on learning (240), pain threshold and thermoregulation (240). The role of EFAs in various neurological diseases has been recently discussed (244), and studies show that such a ratio is effective in reversing symptoms observed in animal models of learning deficit (241), epilepsy (241), and multiple sclerosis (244). The effects of such a ratio on brain appears as an increase in the percentage of PUFAs and in a reduction in the level of cholesterol in the neuronal membrane. These two effects lead to improved membrane structure and behavioral function (246). In addition, a study showed that it also exerts beneficial effect in rats given a diluted dose of the experimental allergic encephalomyelitis (EAE) toxin (244). Nevertheless, little is known about the molecular basis of the benefits and so far, no studies analyzing the alterations in the molecular species composition have been made.

In this study the three different ratios of LA/LNA, 0.7 (perilla oil diet), 2.9 (mixture of perilla-soybean oil), 4.7 (mixture of perilla-soybean oil) were compared to the control, (ratio LA/LNA 8.2). It was clearly shown how 4.7 is the ratio exerting a major effect on brain. Interestingly, the main difference between the fish oil and the LA/LNA 4.7:1 diets was their effect on the 18:0/20:4-PE. While in the case of animals

fed with fish oil, there was always a decrease (from 5.5 up to 35%) in both subclasses, this effect was less pronounced during the LA/LNA 4.7:1 diet, when in some cases, even some accumulation was observed. A possible mechanism might be suggested if some very recent advances in raft studies were taken into consideration.

Experimental evidence is available proving that rafts play a very important role in the regulation of cell signaling. These domains appear to facilitate interactions among the lipid and protein components of signal pathways, thereby regulating these processes (27, 197). It has been demonstrated that lipid rafts are sensitive to modification by PUFAs. This data were obtained studying T-cells, (129) but similar situations might occur in neuronal membranes as well.

On the other hand, although rafts are usually associated with cholesterol, sphingomyelin and saturated fatty acids, recent studies showed that these structures are enriched in arachidonic acid and plasmenylethanolamine (170). It is known, that arachidonic acid-containing plasmenylethanolamines are the major source of released arachidonic acid in different cell types (86, 72, 174). Hence, changes in AA containing species observed in this work could alter lipid raft composition and consequently, alter signal transduction. This, added to the increase in 18:0/22:6 observed in this diet as well, might be one of the explanations of the beneficial effects on learning observed by Yehuda et al. (240) in their study with the LA/LNA 4:1 diet.

Once the relation between dietary PUFAs and rafts was established, some hypothesis could be created to explain the beneficial effect of the PUFAs on learning abilities.

Induction of LTP in hippocampus requires NMDA receptor activation (148), which in turn, needs the participation of the AMPA receptors (fig. 10). Suzuki et al. (203), demonstrated the differential distribution of the NMDA and AMPA-type glutamate receptor subunits in the distinct compartments of the postsynaptic region: the

dendritic raft and the PSD. About half of the AMPA-type was found in the dendritic raft fraction, whereas NMDA receptors were mostly localized to the PSD fraction. Dentritic rafts were also enriched in molecules involved in the processing of extracellular signals, for example, components of the ras/MAPK pathway were also present. They suggested that for efficient signaling, interactions between the dendritic raft and PSD is essential. (203).

PKC is one of the protein kinases involved in the long-term potentiation (figure 10) and may participate in the regulation of certain brain functions, including memory (143). It has been shown how n-6 PUFAs increases the activity of this enzyme in several target organs, including the colon (39), the mammary gland (90) and the skin (177). Mice exposed to high n-6 exhibited shortened immobility in the swimming test, greater aggressiveness in the resident-intruder paradigm, higher locomotor activity in an open field and increased PKC activity, although it remained unclear whether all these effects were due to changes in the PKC activity (176).

Delion et al. (56) proposed that impaired behavioral responses observed in n-3 deficient animals could involve monoaminergic neurotransmission processes. Selective depletion of monoamines has been shown to reduce LTP in rat hippocampus (135)(fig. 10). Other observed alterations were a reduction in the vesicular monoamine transporter binding sites (115) and a decrease in the dopamine-stimulated release that probably resulted from a significantly diminished number of storages vesicles in dopaminergic terminals (253, 254).

Owing to the greater propensity of alkenyl-PE to adopt a hexagonal phase it has been suggested that alkenyl-PE may be involved in membrane fusion, a process occurring during synaptic transmission (80). Vesicles containing alkenyl-PE undergo fusion six times more rapidly than those containing diacyl-PE (227). In fact, alkenyl-PE is abundant in several subcellular membranes that undergo rapid membrane fusion, just like synaptic vesicles (227).

Finally, Black et al. (16) showed that learning is responsible for synapse formation in the cerebellar cortex. Later, Yoshida reported that dietary n-3 fatty acid deficiency and a learning task synergistically induced ultrastructural changes in synapses in the hippocampus (249) and some functional changes in the brain microsomal membrane surfaces (250). It is known that large amounts of DHA and AA are required during development of neural tissue when cellular differentiation and active synaptogenesis are taking place (41). Therefore, this PUFA requirement for synaptogenesis could be extended to the whole life period.

But the influence of PUFAs is not restricted to the signal pathways involved in LTP formation. The inner leaflet, the richest in PE and therefore the most affected by dietary lipids, establishes a large number of interactions with different membrane-associated proteins, like the G- proteins. Escriba *et al.*. (63) demonstrated the importance of propensity of diacyl phosphatidylethanolamines to form non-bilayer phases for G-proteinfunction. Litman et al. (134) suggested a role of DHA containing phospholipids in G protein-coupled signaling pathways. Furthermore, DHA could stimulate diacyglycerol kinase activity by about 2 fold (219). Vaidyananthan et al. (219) concluded that DHA, through its stimulatory effect on DG kinase may regulate the signaling events in growth-related situations in the brain, such as synaptogenesis.

**Aging and changes in the membrane.**

Aging has long been known to have detrimental effects upon the function of the nervous system, but the precise molecular mechanisms involved have not been elucidated. Multiple signaling pathways involved in inflammation, antioxidant defenses, developmental gene regulation, and cellular signaling have been implicated as playing a role in the age-related deterioration (59, 60, 89, 105, 137, 149, 199).

As part of the aging process, a decrease in the expression and function of the NMDA receptor (138, 199, 231), in NMDA receptor binding densities and in NMDA-stimulated release of transmitters (138) has been observed. Finally LTP is also

negatively altered in aged rodents (138). All these changes have been correlated with poor performance in reference memory tasks.

But, aging has also been associated with modifications in the lipid composition of brain cell membranes in rats (30) and in humans (204). A decrease in the levels of the major phospholipid classes, PE and PC, was observed, whereas the levels of minor phospholipid classes, PI and PS were markedly increased in frontal cortex of 24-month-old rats. Other investigators have found a moderate decrease in total phospholipid levels in locations such as hippocampus, frontal white matter, frontal gray matter, and pons in human brains (168).

Although the restoration of membrane PUFAs concentrations in aged brain has been reported to be down-regulated with age, McGahon et al. (149) showed that it is possible. They showed how a DHA enriched diet could reverse the age-related decreases in membrane DHA and partially reversed the age related decrease in AA concentration. This was shown in 22-moth-old rats fed for 8 weeks with DHA-enriched diet. In this work, with a similar diet, the levels of DHA and DHA-containing species were raised up to the level of the rats fed from conception (fig. 3.1 a) and b)). This would suggest that lipid metabolism is not as slow in aged animals as it is generally thought.

Therefore, taking into account the relation that might exist between PUFAs and the learning processes, one could expect that once DHA levels are restored, the cognitive abilities would follow the same tendency.

Some studies support this idea, for example Yehuda et al. (247) showed an improvement in Morris water maze performance in old rats (22-24 months) following pretreatment with the diet LA/LNA 4:1 (247). McGahon et al. (149) showed that age-related decreases in LTP and glutamate release were reversed in 22-month-old rats which were fed on a n-3 diet for eight weeks (149).

In this work no improvement in Morris' water maze performance was observed in old rats after one month feeding with DHA-enriched diet, despite the changes in the molecular species. This result is in completely agreement with Lim et al. (2001), who suggested that it might take some time after the incorporation of DHA, to observe an improvement in learning ability to occur. In their experiment with mice, the improvement in maze-learning ability was evident one month after the start of the feeding trial, whereas the increased DHA levels in brain were apparent after feeding for just 2 weeks (131).

From these results it can be concluded that an elevated level of DHA or DHA containing phosphatidylethanolamines alone cannot be made responsible for its observed beneficial effect on mental activities. Changes in gene expression could be one of the candidates to account for the improvement in cognitive abilities.

**Forebrain, hippocampus and cerebellum respond differently to dietary PUFAs.**

Recent studies showed that the PUFAs- composition of cerebral membranes was not homogenous throughout the brain and that it was altered differently in response to n-3 PUFAs deficiency according to cerebral region (37, 55).

Carrie et al. (37) studied the effects of n-3 PUFAs deficiency and phospholipid supplementation on eleven different brain regions of mice. They showed that saturated fatty acids levels were higher in the hippocampus than in the midbrain whereas the cerebellum was rich in MUFA. The region with the highest DHA level was the frontal cortex.

The effect of n-3 PUFAs deficiency on the fatty acid composition of alkenyl and diacyl phosphatidylethanolamines in the cortex, striatum, and cerebellum were studied by Favrelière et al. (67). They did not find changes in the proportion of plasmalogen in the trhee brain regions. However, they did find marked changes in FA content of alkenyl- and diacyl-PE.

Jumpsen et al. (108) studied the effect of diets varying in n-6 and n-3 PUFAs content on brain fatty acid composition in neonatal rats. The types of responses to the different diets depended on the examined phosphoglycerides, cell types and brain regions.

All these studies examined differences it total fatty acids in different phosphoglycerides. In this work the molecular species of phosphatidylethanolamine for both regions: the cerebellum and the hippocampus were analyzed. The results are summarized in the figs. 3.4, 3.5 and 3.6. , and it is clear that each region has a particular molecular composition. The brain and the hippocampus present similar distribution in molecular species, although the hippocampus is slightly richer in 18:0/20:4. The cerebellum is particularly rich in 18:0/22:6 in both subclasses. The three regions had parallel responses to the diets, independent of the age of the rats. The only remarkable differences were observed in the hippocampus of the rats fed with fish oil from conception. The increase in diacyl- and alkenyl-18:0/22:6 was much larger compared to the other diets. It is also noteworthy, that in old rats the parallelism was not so clearly observed in diacyl and alkenyl 18:0/22:6, but it was perfectly maintained in the case of 18:0/20:4.

All these data would suggest that the different regions of the brain respond similarly to dietary lipids. This does not necessarily contradict the data of the literature, since most of them refer to a deficient status. In fact, these results would reinforce the hypothesis that the brain responds differently according to its PUFA status, being more permissive to accumulation of PUFAs than to depletion.

Despite the tolerance that the brain would show to the accumulation, the observed changes could be somehow restricted to the maintenance of a particular ratio among different molecular species. At least this is what one could suggest from the data shown in table 3.10., where the ratios of 18:0/22:6 to 18:0/20:4 are compared. Surprisingly this ratio was maintained in each region within a narrow range despite the

different experimental conditions. Neither age nor diet seems to affect this ratio considerably. Taking all these facts into account, it could be reasonable to hypothesize, that in addition to a particular phospholipid composition, the ratio of different molecular species would play a critical role in the proper maintenance of brain functions.

In fact, the idea that the brain tries to maintain a particular phospholipid composition was already suggested by Burdge et al. (28). Brain phospholipids of guinea pig (28), rat (96), neonatal monkey (132) and adult human brain (40) presented a high similarity, suggesting an evolutionary convergence and conservation of molecular species composition. Burdge et al. (28) suggested that optimal membrane phospholipid molecular species composition is of critical importance to the brain function and is maintained largely independent of diet (28). The results obtained in this work would suggest that the brain tries to maintain the proper ratio between the different molecular species, rather than a particular lipid composition.

All these changes in lipid composition, observed in the different brain regions, will certainly have some implications at the neurotransmission level. In fact, Itokazu et al. (100) showed, that intracerebroventricular administration of DHA influences hippocampal neurotransmission and synaptic plasticity in anesthetized rats. They suggested, that DHA possibly serves as an intrinsic modulator of hippocampal neurotransmission and synaptic plasticity

Delion et al. (55) have shown that chronic dietary α-linolenic acid deficiency alters dopaminergic and serotoninergic neurotransmission in rats, but these changes were only observed in the frontal cortex, which seems to be more sensitive to n-3 deficiency than the striatum. On the other hand, they reported that the proportions of different phospholipid classes in the frontal cortex, striatum, and cerebellum were not modified by α-linoleic acid deficiency. Carrie et al. (36) reported a nearly 30% decrease of synaptical vesicles in the terminals of the hippocampus CA1 region in LNA

deficient animals, compared to controls. These animals showed learning impairments as well (249).

**Gene expression and PUFAs.**

The first clear hint that gene expression can be related with learning processes came when Tang et al. (209) showed that the overexpression of the subunit 2B of the NMDA receptor in the forebrains of transgenic mice leaded to enhanced activation of the receptor, and therefore facilitated synaptic potentiation (209). These mice exhibited superior ability in learning and memory in various behavioral tasks, showing that NR2B is critical in gating the age-dependent threshold for plasticity and memory formation.

A further step was taken, when Routtenberg et al. (181) showed, that overexpression of the growth-associated GAP-43 protein results in enhanced learning and synaptic plasticity. This molecule has been implicated in input-dependent alterations of synaptic morphology (13), and was previously implicated in memory storage processes (31, 192). It was also shown that it binds to actin (88) and fodrin (178), and such protein-protein interactions may affect morphological changes in the neuron (181).

On the other hand, Kitajka et al. (114) showed how fish oil and perilla oil diets accounted for changes in brain gene expression. This work was extended analyzing the effects of the LA/LNA 4.7:1 diet on gene expression. The most striking result was that comparing perilla and LA/LNA 4.7:1, 20 genes showed a higher overexpression in the latter (table 3.11), suggesting that the exact lipid composition plays an important role in the regulation of the gene expression.

Mechanisms relating dietary lipids, gene expression and PPARs have already been intensely studied. However, these works focused mainly on adipose tissue and liver, little has been done in brain.

The X-ray crystal structure of the ligand-binding domain (LBD) of the three PPARs was determined (236). The LBD contains a cavity large enough to dock a variety of hydrophobic ligands with low affinity, in accordance with the promiscuous binding properties of the PPARs for eicosanoids and PUFAs (58).

Xu et al. (235) showed that α-linolenic acid bound to PPARβ, one of the three PPAR subtypes described so far, with the highest affinity, followed by linoleic acid. Arachidonic acid also showed this preference for PPARβ, although its binding was not as strong as LNA's and LA's. According to the docking model fatty acids shorter than 14 or longer than 20 carbon atoms would not fit into the binding site of PPAR and would be exposed to solvent. Activation of PPARs by DHA would likely require its prior conversion back to EPA, a process that requires peroxisomal β-oxidation (107).

These two facts could possible explain the difference of the expression patterns observed during LA/LNA=2.9:1 diet and the other diets (LNA diet and DHA diet).

However, DHA does bind the RXR receptors (147). Recent experiments using cell lines showed that DHA is a potent activator of RXR. The reported effect was highly specific to DHA in comparison to other $C_{22}$, $C_{20}$ and $C_{18}$ unsaturated FA (147). Given the fact that RXR responsiveness to DHA is affected by heterodimerization and that PPAR and RXR are common partners, it is possible that DHA deficiency alters cellular pathways under the transcriptional control of both RXR and PPAR or other heterodimerization partners.

In turn, the expression of PPAR's genes has been shown, in a whole-body experimental model tested to be moderately sensitive to long-term n-3 FA deficiency. (180). The dietary intervention in the study is highly specific to the n-3 FA series, preserving the total amount of fat as well as the amount of n-6 FAs. It was suggested that the dietary intake of n-3 FA could primarily involve modulation of the activity rather than amount of PPAR. In addition, other transcriptional factors may be involved

in the response to n-3 deficiency. This supports the idea that PUFA-enriched diets may exert their beneficial actions in part by interacting with the PPAR.

It is interesting to point out, that the expression of transcription factors shows regional and developmental stage dependence. Braissant's et al. (24) showed that in contrast to the specific distribution of PAARα and γ, PPAR**β** is expressed ubiquitously and very early during embryogenesis, with a peak of expression in the developing nervous system on embryonic stage E13.5. Furthermore, the expression levels are higher than in the adulthood. Its ubiquitous expression suggests a specific role. One possibility could be a role in membrane lipid synthesis and turnover. Another possibility would be a function of PPARβ at the onset of differentiation processes. Indeed, they showed that the peak of ubiquitous expression of PPARβ during embryogenesis correlates with the period of greatest cell differentiation activity, particularly in CNS (24).

Cullingford et al. (48) demonstrated that during brain development, the levels of PPAR mRNAs are different for each isotype. In different brain regions of 11-day-old rats, the order of abundance for PPAR mRNAs is β>α>>>γ. It is possible to speculate, that similar age-dependent expression of PPARs could exist in older animals too. This would be one of the possible explanation for the delay in the improvement of learning abilities in the older rats despite the restored DHA levels (fig. 3.7.)

In addition to nuclear receptors, PUFAs and their metabolites bind to proteins that are members of the family of intracellular lipid-binding proteins (iLBPs), which include cellular retinoic acid-binding proteins (CRABPs) and fatty acid binding proteins (FABPs). It has traditionally been believed, that the general function of iLBPs is to solubilize and protect their ligands in aqueous spaces and to facilitate transport across the cytosol. However, it seems that iLBPs have diverse and specific roles in regulating the metabolism and activities of their ligands.

The brain lipid-binding protein (BLBP) is a brain-specific member of the FABP family, expressed at high levels in the developing central nervous system (68, 124). Several lines of evidence suggest that it plays an important role in neuron/glia interactions during central nervous system development. Xu et al. (237) reported, that the binding specificity of BLBP presents a particular pattern, and suggested that DHA could be the physiological ligand for BLBP, since the affinity of this interaction is the highest yet reported for a FAPB/ligand interaction. These pieces of evidence strongly support, that the role of BLBP in the developing nervous system may be closely related to the essential requirement for DHA in CNS development in vivo (237). Very interesting results came out from Tan et al. (2002), relating FABPs and PPARs. They showed that FABPs act in concert with PPARs and, importantly, that they do so with strict selectivity for both receptor subtypes and ligands (Tan et al. 2002).

However PPARs might not be the only way of action of the PUFAs on gene expression. Several recent observations suggest, that the dogma stating that FA regulation of gene transcription is a simple PPAR-mediated process, should be revised. In many instances the effects of PPAR ligands are disconnected from those of FAs. Indeed, there is no single mechanism for FA regulation of gene transcription. The FA action on genes may be either indirect or direct. Depending on the cell-specific context and the target gene, FAs can take very different routes to alter transcription. Several transcription factors, different from PPARs, are likely candidates (58).

# CONCLUSIONS

In the last 20 years, the role of polyunsaturated fatty acids in the organism has changed dramatically. In the field of nutrition, they have been recognized as essential for the human being and their benefits on learning, mental disorders, cardiovascular diseases have been clearly established. At the cellular level, PUFAs may exert their role as free fatty acids, being second messenger in the signal transduction pathways, precursors of other active biomolecules, or they may become part of the phospholipids, being the building blocks of the membranes and regulating their biophysical properties.

Although big efforts have been taken to unravel the mechanisms used by PUFAs to exert their function, recent advances relating PUFAs in raft signalling and in gene expression regulation have demonstrated that these mechanisms are rather complex.

The main aim of this thesis was to improve our knowledge on lipid metabolism in brain. For this purpose, different feeding experiments were carried out on rats, and either diet or age were considered as variables. Regional differences within the brain were analyzed as well.

From the brain phospholipid analyzes it can be concluded that:

1. The significant changes observed in the levels of DHA and AA-containing molecular species in rats fed from conception indicates that the brain has a faster response to diet in PUFA-sufficient conditions.

2. The presence of DHA or an adequate ratio of n-6 to n-3 fatty acids in the diet is necessary to obtain a significant accumulation of DHA-containing species.

3. The accumulation of DHA is possible in aged animals, indicating that losses in PUFAs characteristic of the aging process can be reversed by an adequate diet.

4. Forebrain, cerebellum and hippocampus have a characteristic lipid composition and the three regions responded similarly to the diets.

5. It's the ratio of the molecular species, and not their absolute level, that the brain tries to maintain.

Although aged animals accumulated DHA up to the levels of the younger rats, they did not perform better in the learning test. Based on this observation it can be proposed that:

1. DHA does not only regulate the biophysical properties of the membrane, but it also participates in another mechanism, that would account for the improvement in learning. This mechanism would need a longer time to be affected by the changes in DHA levels. One of the possible mechanisms used by the DHA could be the regulation of the gene expression.

The analysis of brain gene expression in animals fed with LA/LNA 4.7:1 diet showed differences compared to the previous experiments, when rats where fed with fish and perilla oil (114). 20 genes showed a higher overexpression and some of them are involved in lipid metabolism, energy household and neurotransmission. Therefore it can be concluded that:

1. Regulation of the gene expression in the brain is sensitive to alteration in the dietary lipid composition.

**On the basis of the presented results we propose that alteration in membrane architecture and function, due to the accumulation of DHA-containing molecular species, coupled with alterations in gene expression profiles may contribute to the observed beneficial impact of n-3 type polyunsaturated fatty acids on cognitive functions.**

# ÖSSZEFOGLALÁS

A biológiai membránok fontos szerepet játszanak a celluláris folyamatok szabályozásában. Ezek a szerkezetek határolják a sejteket a külvilág felé, de ezek közvetítik a külvilág ingereit/üzeneteit a sejtek számára, hogy az ezekre adaptiv válaszokat adhassanak. A membránok egyik fontos alkotóelemei a fehérjék mellett a lipidek, pontosabban a foszfolipidek. A foszfolipidek zsírsav és fejcsoport összetétele szigorúan van szabályozva.

A központi idegrendszer lipidekben az egyik leggazdagabb szerv, és ezeknek a lipideknek döntő tömege membránokhoz kötődik. A foszfolipidek egyik fő komponensei a zsírsavak, melyeknek egy része az állati szervezetek számára esszenciális, vi. ezeket a szervezetek maguk nem képesek előállítani ezeket, ill. precursoraikat a táplálékkal kell felvegyék. Ezek a zsírsavak szerkezetileg vagy a linolsav (n-6), vagy pedig a linolénsav (n-3) családba tartoznak. A központi idegrendszer membránjainak domináló politelítetlen zsírsava az arachidonsav (20:4n-6, AA) és a docosahexaensav (22:6n-3, DHA). Elfogadott tény, hogy a központi idegrendszer működéséhez speciális foszfolipid strukturákat igényel, és ezt az igényt elsősorban a DHA tartalmú molekula specieszek elégítik ki. Amennyiben, valamilyen okból kifolyólag a neuronok membránjainak DHA szintje csökken, az súlyos következményekkel jár. Ismeretes, hogy bizonyos neurodegenerativ betegségekben (pl. Azheimer kór,) szenvedő egyének, vagy idős korban az agyi DHA szint alacsonyabb a normálisnál és ezekben az esetekben a mentalis funkciók is sérültek. Mesterségesen esszenciális zsírsavhiányossá tett patkányok tanulási képessége és memóriája is károsodik, mely DHA adagolással helyreállítható.

A legfontosabb DHA raktárok a központi idegrendszerben az etanolamin és szerin foszfoglyceridek. Az előbbiek (diacyl, alkylacyl, alkenylacyl foszfastidyletanolaminok) a neurális membránokban, míg az utóbbi a retinában és a

látásban játszik nélkülözhetetlen szerepet. Noha ezek a tények mAr régen ismeretesek, a DHA dinamikáját a központi idegrendszerben sok intenziv kutatás ellenére sem ismerjük kielégítően. A legtöbb kutatást esszenciális zsírsavhiányos állatokon végezték, melyeknek során vizsgálták az eredeti DHA szint viszaálitásának körülményeit. Ezek a kisérletek egyértelműen bizonyították a DHA nélkülözhetetlenségét a kognitív funkciókban. A hibája ennek a megközelítésnek az, hogy esszenciális zsírsavhányos állapot humán vonatkozásban rettentően ritka, de a DHA hiány természetesen patológiás körülmények között (Alzheimer kór Zelweger kór stb.) ismert. Felfogásunk szerint egészen más egy DHA hiányos állapot következményeit vizsgálni, ami csupán a kérdés egy adott aspektusára ad választ, mint azt kutatni, hogy egy normális állapotban a DHA milyen szerepet játszik a membránok összetételének és a kapcsolt funkciók fenntartásában.

A fent elmondottalból kiindulva vizsgálatainkat 3 különböző korcsoportba tartozó patkányokon végeztük: 1./ a fogantatástól 2 hónapos korig, 2./ 2 hónapos állatok, 3./ 2 éves állatok, melyeket a következő diéták valamelyikével etettünk (5% zsírtartalom): halolaj (tuna, 25% DHA), perilla olaj (60% LNA), LA/LNA= 4.7:1, ill 2.9:1 arányú elegye. Az 1. csoporba tartozó patkányok a fogantatástól iverérettségükig halolajat kaptak, míg minden más patkány csak 1 hónapig kapott a fenti kompozíciók valamelyikéből. A kisérlet végén az állatokat leöltük, az agyukból 3 régiót:, nagyagy, kisagy, hippocampus, különítettünk el, melyeket folyékony nitrogénben fagyasztottunk le és a felhasználásig –70 C-on tároltuk. Minden mintának megvizsgáltuk a zsírsavösszetételét (GLC), és a fontosabb foszfolipidek molekuláris öszetételét (HPLC). A diszertációban azonban csak a foszfatidyletanolaminokra szorítkozunk, minthogy ez a csoport válaszolt a diétás hatásokra, a foszfatdylkolinok zsírsav és molekuláris összetétele jóformán változatlan maradt minden esetben. Ezen túlmenően egyes mintákon megnéztük a diéta hatását a génexpresszióra, valamint az idős patkányok és a hozzátartozó fiatal állatok esetében tanulási tesztet (Morris water maze) is végeztünk. Úgy gondoltuk, hogy ezzel a megközelítéssel jól körül tudjuk járni ezt a meglehetősen komplex problémát. Feltételezésünk szerint ui. az agyi folyamatok, mint pl. tanulás,

sokkal bonyolultabb háttérel birnak, mint egyszerű membránlipid összetétel, noha az ingervezetésben ill. az ioncsatornák működésében ez sem elhanyagolható momentum.

A munka egyik legfontosabb megfigyelése az, hogy az alkalmazott diéták majdnem mindegyikének hatására növekedett a nagyagyban a foszfatidyletanolaminok DHA tartalma továbbá a diacyl 18:0/22:6 speciesz aránya. Kivételt perilla olaj képezett, valamint a LA/LNA=2:1 keveréke., de ez a takarmányozási idő és az életkor függvénye is volt. Fontos megfigyelésnek tartjuk, hogy 2 éves patkányok esetében 1 hónapos halolaj ill LA/:NA=47:1 keverékének fogyasztása elegendőnek mutatkozott az előrehaladott kor miatt csökkent DHA szint és a halolaj esetében a diacyl és alkenylacyl 18:0/22:6 foszfatidyletanolamin szintjének restaurálására. Ennek a megfigyelésnek humánbiológiai implikációi is lehetnek: elképzelhető, hogy előrehaladott korban az embereknél is pótolható a DHA veszteség. A másik fontos üzenete ezeknek a kutatásoknak, hogy a DHA tartalmú foszfatidyletanolaminok szintje dietás kontrol alatt áll a nagyagyban még egészséges állapotban is. Azt is fontosnak tartjuk, hogy a perilla olaj tartalmú dieta (magas LNA) szintén e specieszek szintjének növekedést eredményezte, mely arra utal, hogy egy bizonyos C-18n-6/n-3 arány esetében vagy a máj, vagy maga a központi idegrendszer jelentős mennyiségű DHAt képez a linolénsabból. Mindezeket a változásokat az AA tartalmú specieszek megfelelő csökkenése kisérte.

A hippocampus és kisagy hasonló tendenciájú válaszokat adott Érdekes volt megfigyelni, hogy pl. a hippocampus az 1. állatcsoportban sokkal erőteljesebb választ adott a halolajra, mint a 2 ill. 3 csoportban. Úgy gondoljuk, hogy mindezek az egyes agyi régiók szövettani és funkcionális különbségeire vezethetők vissza.

Minthogy az n-3 zsírsavak kognitiv funkciókkal kapcsolatos vonatkozásai már korábban ismeretesek voltak érdekesnek láttunk néhány tanulási tesztet végezni olyan idős és fiatal patkányokon, melyek csak 1 hónapig kaptak halolajat. Az láttuk, hogy a 2 csoportba tartozó patkányok tanulási képessége javult a kontrolhoz képest, míg a 3 csoportba tartozóké nem, annak ellenére, hogy a DHA tartalmú specieszek aránya ugyanezen idő alatt elérte a faiatal állatokban mért értékeket. A diploma munka megírásának ideje alatt ezt a kisérletet megsmételtük olyan 2 éves ptkányokkal, melyek 3 hónapig kaptak halolajat, immár pozitiv eredménnyel. Nilvánvaló, hogy a DHA tartalmú specieszek arányának egyszerű növekedése nem jár a mentális képességek javulásával. Az azonban biztos, hogy a DHA ill. a diacyl és alkenylacyl 18:0/22:6 speciesz ezekben a folyamatokban nélkülözhetetlen szerepet játszik.

Viszont előző eredményeiinkre alapozva feltéteteztük, hogy a neuronok genetikai apparátusa szerepet játszhat ezekben a folyamatokban. Ebben a disszertációban a LA/LNA=4.7:1 arányú keverékének hatását mutatjuk be a génexpesszióra. Azért választottuk ezt a kompoziciót, mert mások kutatásai szerint a patkányok tanulási készségre igen előnyös hatással van. 20 gén expresszióját növelte ez a kompozició, többek között számos mitochodriális genét, a synucleinét, számos membrán proteint, melyek a jelátvitelben játszanak szerepet. A téma szempontjából fontos lehet a synuclein szerepe, melyről mások már kimutatták, hogy ez a fehérje akkor képződik az énekes madarak agyában, amikor azok énekelni tanulnak. Kb. 20 gén supresszálódott a dieta hatására. Feltehetőleg van valami egyensúly a kétféle géncsoport között.

Természetesen ezek a kisérletek nem adnak támpontot a DHA génexpresszióra gyakorolt hatásának mechanizmusára nézve, de felvetődhet az a lehetőség, hogy a folyamatban a PPAR/RXR receptorok, valamint bLBP játszhatnak szerepet. Ez a rendszer bonyolult módokon kapcsolatba léphet a DNA megfelelő sequenciájával.

Lehetséges azonban, hogy ebben a szabad DHA játszik szerepet, minthogy a sokkal nagyobb 18:0/22: nem tud a fehérje hydrofob gödrébe beleférni.

Úgy gondoljuk, hogy ezek a kutatások rávilágitottak a DHA ill. a DHA tartalmú foszfolipidek dinamikájára az agyban és egyes esetekben talán új utakat nyitottak az ilyen tipusú kutatások számára. Azt mindenféleképpen reméljük, hogy hozzásegítettek az agyi lipdanyagcsere egyes vonatkozásainak jobb megismeréséhez.

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