INVESTIGATION OF THE POTENTIAL CORRELATION BETWEEN THE COGNITIVE PERFORMANCE AND LEVELS OF BRAIN FATTY ACIDS IN YOUNG AND AGED MICE

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submitted by **BERRAK YETİMLER** in partial fulfillment of the requirements for the degree of **Master of Science in Biological Sciences Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Musa Doğan
Head of Department, **Biological Sciences** _____

Assoc. Prof. Dr. Ewa Jakubowska Dogru
Supervisor, **Biological Sciences Dept., METU** _____

Examining Committee Members:

Assoc. Prof. Dr. Turgay Çelik
Medical Pharmacology Dept., GMMA _____

Assoc. Prof. Dr. Ewa Jakubowska Doğru
Biological Sciences Dept., METU _____

Prof. Dr. Orhan Adalı
Biological Sciences Dept., METU _____

Assist. Prof. Dr. Hakan Kayır
Medical Pharmacology Dept., GMMA _____

Assist. Prof. Dr. Tülin Yanık
Biological Sciences Dept., METU _____

Date: 11.02.2011

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Berrak Yetimler

Signature :

ABSTRACT

INVESTIGATION OF THE POTENTIAL CORRELATION BETWEEN THE COGNITIVE PERFORMANCE AND LEVELS OF BRAIN FATTY ACIDS IN YOUNG AND AGED MICE

Yetimler, Berrak

M. Sc., Department of Biological Sciences

Supervisor: Assoc. Prof. Dr. Ewa Jakubowska Doğru

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The aim of the present study was to elucidate the possible relationship between the levels of various brain fatty acids and learning indices in aged and young mice classified as “good” or “poor” learners basing on their performance in a spatial learning task, the Morris Water Maze. The levels of several fatty acids including palmitic, stearic, oleic, linoleic, arachidonic acid (AA) and docosahexaenoic acid (DHA) were measured using gas chromatography separately in samples from four different brain areas: hippocampus, cortex, striatum and hypothalamus. The level of oleic acid in the cerebral cortex was significantly higher in young-good learners as compared to young-poor learners and higher in young-poor learners than in old-poor learners, with no significant difference in the concentration of this acid between old-good and old-poor learners.

The most consistent correlation between animals’ learning capacity and brain fatty acid’ level was found for the arachidonic acid in the hippocampal region: AA level was significantly lower in young-good learners as compared to both young-poor learners” and old-good learners” with young-good learners showing significantly better performance than the two other groups. Interestingly, except hypothalamus, no significant between-group differences were recorded for the remaining fatty acids including DHA, in none of the four brain regions examined.

Keywords: Aging, Spatial Learning and Memory, Fatty Acid, MWM, Gas Chromatography

ÖZ

GENÇ VE YAŞLI FARELERDE BEYİNDEKİ YAĞ ASİDİ SEVİYELERİ İLE MEKANSAL ÖĞRENME ARASINDAKİ İLİŞKİNİN İNCELENMESİ

Yetimler, Berrak

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Mevcut çalışmada, bir mekansal öğrenme testi olan Morris Su Labirenti'ndeki performanslarına göre "iyi" ve "kötü" öğrenenler olaral ayrılan genç ve yaşlı dişi farelerin öğrenme performansları ile beyindeki yağ asidi seviyeleri arasındaki olası ilişkinin aydınlatılması amaçlanmıştır. Palmitik, stearik, oleik, linoleik, araşidonik asit (AA) ve dokozahekzaenoik asid (DHA) yağ asitlerinin seviyesi gaz kromatografisi (GC) ile ölçülmüştür. Bu değerlendirilme dört farklı beyin bölgesinden alınan örneklerle bağımsız olarak yapılmıştır. Bu bölgeler; hipokampus, korteks, striatum ve hipotalamustur. Serebral kortekstеki oleik asid seviyesi genç-iyi öğrenen grupta genç-kötü öğrenen gruba göre anlamlı derecede yüksek bulunmuştur. Ayrıca genç-kötü öğrenenlerde yaşlı-kötü öğrenen gruba göre oleik seviyesi yine anlamlı derecede yüksek bulunmuştur ancak yaşlı-kötü ve yaşlı-iyi öğrenen gruplar arasında anlamlı bir fark bulunamamıştır.

Öğrenme performansları ve yağ asidi seviyeleri arasındaki en anlamlı korelasyon sadece genç farelerin hipokampus bölgesindeki araşidonik asit seviyeleri için bulunmuştur: AA seviyesi hem genç-kötü öğrenen gruba, hem de yaşlı-iyi öğrenen gruba göre daha iyi performans sergileyen genç-iyi öğrenen grupta anlamlı derecede daha düşük bulunmuştur. Çalışmanın ilginç bulgularından biri, hipotalamus dışında DHA dahil olmak üzere diğer yağ asidi seviyeleri için gruplar arasında herhangi bir anlamlı ilişki tespit edilememiş olmasıdır.

Anahtar kelimeler: Yaşlanma, Mekansal Öğrenme, Yağ asidi, MWM, Gaz Kromatografisi

To My Family

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CHAPTER 1

INTRODUCTION

1.1. Aging

Biological aging is defined as the gradual accumulation of changes which are responsible for the sequential alterations that seen with the advancing age and the related progressive increases of the probability of diseases and eventually death. These variations can arise from disease, environment, immune dysfunction, and to an inborn process; the aging process (Ashok, 1999).

Another well-accepted definition is the decline of the cell's ability of maintaining homeostasis. At the time this goes beyond a certain level, the survival capacity of the organism is under jeopardy (Gershon, 2000).

Aging is universal among multicellular organisms. It is evolutionarily conserved among species and affected by both genetic and environmental factors. The principle mechanisms of aging can cause detrimental effects on the cell and organ functions and these changes can promote diseases can kill the organism such as cancers, cardiovascular diseases and neurodegenerative diseases (Cutler, 2006). However, the progression or the rate of aging varies widely between different species, in different individuals within a species, in organs and tissues within a single organism, in cells types in a tissue and even sub-cellular compartments and macromolecules within a cell. (Rattan and Clark, 2005) Thus, it is hard to make a single definition of such a complex and diverse phenomenon and elucidate all of the parameters involved in aging process. The longevity of a given species is a consequence of various factors including frailty (i.e., the intrinsic vulnerability to death) and senescence (i.e., the rate of change in frailty by time) (Galvin, 2005)(de Magalhaes, 2004).

Today, there is a remarkable increase in the human life expectancy in many countries around the world (Muhs, 2006). Average life expectancy has increased from 65.6 years for men and 71.1 for women in 1950, to 74.8 for men and 80.1 for women in 2003 (Hoyert *et al*., 2005). The health care conditions are improving, thus the ratio of older population in the overall population is estimated to double over the next fifty years. However, at the same time the incidence of neurodegenerative diseases that linked to the aging process like stroke and dementias can increase (Haga, 2009). Thus the question of aging and age-related diseases has become a substantial field of study for social, medical and economical concerns in the pursuit of creating better life standards.

Today, there is a great number of diverse experimental data on different perspectives of the complex process of aging for years, however, the fundamental causes of physiological attrition that is seen with advanced age in an organism has not yet been fully understood (Thakur, 1993).

1.1.1. The Characteristics of Aging

The general characteristics of aging can be defined as increased mortality with advancing age after maturation, modifications in biochemical composition in tissues with age, progressive impairment in physiological ability with age, impaired ability to react adaptively to environmental stimuli with age and enhanced susceptibility and vulnerability to diseases (Troen, 2003).

1.1.2. Aging Theories

Aging phenomena are very complex and there are many different hypotheses attempting to explain them. Numerous papers have been published so far indicating the very complex nature of the process (Troen, 2003).

Aging theories can generally be classified into two categories: stochastic and developmental-genetic theories (Table 1). It should be clearly understood that these categories are not totally exclusive. Actually, there is probably a spectrum from birth to senescence that shows a declining effect of active genetic influences

and an increasing impact of stochastic events. This would correspond to the change in importance from general to species-specific genes (Troen, 2003).

Table 1. Theories of aging (Modified from Troen, 2003).

AGING THEORIES	
Stochastic Theories	**Developmental-Genetic Theories**
- Somatic Mutation and DNA Repair Theories	- Longevity Genes Theory
- Error-Catastrophe Theory	- Accelerated Aging Syndromes
- Protein Modification Theory	- Neuroendocrine Theory
- Free Radical (Oxidative Stress) / Mitochondrial DNA Theories	- Immunologic Theory
	- Cellular Senescence / Telomere Theory
	- Cell Death Theory

1.1.2.1. Stochastic Theories

Stochastic theories propose that random extrinsic factors promote collective damages at different levels of the organism and these damages eventually lead aging. DNA damage or impairment in repair mechanisms, detriments in cell organelles and tissues by oxygen free radicals, cross-linking of proteins and impaired removing mechanisms for removing damaged molecules can be given as an example of these damages. These effects can accumulate and lead to the progression of aging process and senescence (Rajawat *et al,* 2009).

1.1.2.1.1. Somatic Mutation and DNA Repair Theories

The “somatic mutation theory” (Dorman *et al.,* 1997) concentrate on intrinsic factors. This theory suggests that mutations in DNA of the somatic tissue are the fundamental driving force of aging process. These mutations then can generate non-functional proteins/enzymes that cannot function properly. The gradual addition of mutations can induce structural impairments and gradually the death of the living. The theory got its name “somatic” since in particular, mutations in somatic tissues can be detrimental (Merker, 2001).

A different pattern of this theory is called the DNA Repair Theory. The repairing capability of DNA for UV or radiation induced damages in cell cultures taken from divergent species shows a certain relationship with the maximum lifespan potential (Hart *et. al.,* 1974). There are a number of papers showing that the total DNA repairing ability is not showing any variation with the age of the organism. However, there is some evidence suggesting that the repair mechanism at specific regions of DNA might be essential in different varieties of fully differentiated cells of the organism (Hanawalt *et. al.,* 1992).

1.1.2.1.2. Error-Catastrophe Theory

This theory suggests that the errors that randomly occur in the synthesis may be finally seen in the proteins that involve in the DNA synthesis or the other template biomolecules (Orgel, 1963).

Normally if there is an error in a protein, these missynthesized proteins will be eliminated by the natural turnover and restored by the normal protein molecules. However if the defective proteins function as a part of the protein translation system, they may cause impairments in the products they synthesized. This may cause propagation of the error and lead to the cumulation of the error having proteins. This situation would cause an “error-catastrophe” which is not appropriate for vital functioning (Troen, 2003). Among many papers about structural changes of proteins in the aging process, there is still no direct support for protein missynthesis related with age. These varied proteins that appear in the aged cells of the organism rather go under post-translational mechanisms like oxidation and glycation (Kristal *et. al.,* 1992, Levine *et. al.,* 1996). However, aggregation of the error containing proteins with aging deteriorates the normal cellular turnover systems and induces reduced clearance. In theory, this might additionally induce the impairment in the gene expression regulation to a degree that is not acceptable for normal functioning. The increment in altered proteins may result from decreased clearance in older cells (Gracy *et. al.,* 1985).

1.1.2.1.3. Protein Modification Theory

There are some variations correlated with age at the steady state levels of proteins (Troen, 2003). Also, some alterations that lead functional changes arise. Reduced specific activity in different enzymes, alterations in heat stability and enhanced carbonyl content of proteins are coupled to the aging process (Levine *et al.*, 1996).The cause of the variations may be attributed to direct oxidization of the amino acid residues, metal-catalyzed oxidation and modification by lipid oxidation products and glycation. It is suggested that the addition of the variated proteins can deteriorate cellular and eventually organ functions. (Bjorksten, 1974 and Kohn, 1978)

The glycation/cross-linking hypothesis is derived from the knowledge on proteins, DNA and other structural molecules can form cross-links to each other with advancing age (Cerami, 1985). Glycation is the fundamental way of the cross-linking. Proteins that go under post-translational modifications contain sugar moieties. These glycated molecules can be diminished by a complex reaction series (Maillard reaction) to produce many different end-products that called advanced glycation end products (AGEs). In case the two sticky ends of AGEs attach to neighbouring proteins, permanent, impairing cross links occur (Rajawat, 2009). Impaired proteins are generally broken down by proteases; but if there are cross-linkages in proteins, proteases are inhibited, thus impaired proteins accumulate (Bonnefont-Rousselot *et al.,* 2000). There are a number of reports that support the implication of glycation/cross-linking in the aging process. These AGEs enhance with the advancing age and are involved in skin disintegration, diabetes, eye disorders and amyloid accumulation. Also various extracellular matrix proteins show enhanced cross-linking in aging. A normal extracellular matrix is important for diffusion of essential molecules and thus is essential for normal organ function.

Collagen cross linking has been proved to be engaged to some age-related changes in skin to some degree (Reiser *et al.*, 1987, Robins, 2007). Cross-linking of the proteins in the lenses of the eyes (crystalline) has an essential role in age-related cataract development (Kumar *et al.,* 2007). Cross linking of proteins (elastins) in arterial walls may possibly have a function in the improved hardness

of the vascular walls seen with the advancing age and it can be taken account as a cause of at least some forms of atherosclerosis, such as diabetic angiopathy (Peppa and Vlassara, 2005), also like the age-related reduction in kidney function. Additionally, there are some evidence that glycation also plays a role in the formation of beta-amyloid which accumulates together in Alzheimer's patients brains.

1.1.2.1.4. Free Radical (Oxidative Stress) / Mitochondrial DNA

From the time it was first suggested in 1956, this theory is still known widely and there is still debate on the theory up today (Weinert and Timiras, 2003). Harman first noticed the resemblance between reactions after ionizing radiation and aging and he developed the "oxidative damage" theory of aging (Harman, 1956). This theory proposes that the accumulation of the free radicals with time leads to gradual damage to the cell functions, tissues, organs and systems causing the death of the organism. Also it indicates that free radicals are the main agents of aging at a molecular level. After twenty years, he discovered that mitochondria is the main source for the production of reactive oxygen species and it greatly supports to oxidative damage (Harman, 1981). Free radicals or reactive oxygen species (ROS) are ions or very tiny molecules that in an unstable distribution since they carry an unpaired electron in the outer shell of the atom. Since they have an unstable configuration, free radicals are highly in reaction with neighbouring molecules taking up an electron to balance themselves at the same time turning the attacked molecule into a free radical so that the first free radical attack produces an endless dynamic cycle. There are two major free radicals: Superoxide radical (O_2^-) and hydroxyl radical (OH^-) (Fridovich, 1989; Sohal and Weindruch, 1996).

Molecules that have antioxidant activity like carotenoids, flavonoids, glutathione, uric acid, vitamins A, C and E, and enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase are major free radical scavengers that keep the cells away from oxidative damage attack. The magnitude of the oxidative damage results from the interaction of pro-oxidant and antioxidant forces in the cells. The delicate balance between the two factors forms the eventual pattern of oxidative damage in aging. The theory proposes that ROS formation is natural in

biological systems. In fact, ROS are normally by-products of the aerobic metabolism of cells. Accumulated free radicals which cannot be effectively reduced by the antioxidants can lead to detrimental results, like cross linking of several biomolecules, DNA-strand breaks, lipid oxidation and aggregation of alkyl radicals and aldehydes.

The major support to this theory is obtained from the life span experiments with flies and worms. Transgenic mice that express SOD enzyme with improved lifespan suggest that free radical scavenging enzymes are decent enough for retarding aging in Drosophila (Tower, 2000). Also it was reported that flies that have elevated lifespan have increased SOD levels and augmented resistance against oxidative stress (Arking *et al.,* 2000).

Mutated worms with increased lifespan have also resistance against oxidative stress at the same time exhibiting an elevation in SOD levels and catalase activity. Long-lived mutant worms are also resistant to oxidative stress and show an age-dependent increase in SOD and catalase activity (Larsen, 1993). The increased life span by the activity of synthetic small molecules which can act like catalase and/or SOD reflects that antioxidants can retard the aging process in *C. elegans* (Melov, 2000). Evidently, ROS injuries show an inverse correlation with the life span in small organisms like worms (Weinert and Timiras, 2003). Some experiments with mammals reveal that dietary antioxidants can diminish the accumulation of oxidated molecules in mice; however they are not capable of increasing life span (Mehlhorn, 2003). SOD mutation possessing rodents are very ill and die very early but there is not data about them such as they age fast. In rodents, omnipresent over synthesis of SOD enzyme doesn't seem to increase life span suggesting that SOD does not border the life span (Huang *et al.,* 2000). Ionizing radiation forms free radicals; however the unexpected fact is that subjection to chronic radiation results in elevation of the rodent longevity (Caratero *et al.,* 1998). That impact of chronic radiation may be clarified in case it causes the stable activation of the cellular defence mechanisms.

Mitochondrial DNA (mtDNA) is particularly vulnerable to chronic reactive oxygen species damage because the addition of somatic mutations coupled to aging causes deleterious effects on the normal functioning. These functional

impairments include impaired mitochondria respiration, augmented energy output and even further ROS production (Rajawat, 2009). There is a dilemma about mitochondria considering that the organelle is at the same time the essential energy and ROS generator. ROS seems to have effects on the regulation of cell signaling, differential gene expression, cell replication, specialization and apoptosis (Sen and Packer, 1996; Suzuki *et al.*, 1997). The free radical generation in the heart, kidney and liver in a variety of mammals exhibit a negative correlation with longevity (Sohal *et al.,* 1989). A number of papers published so far both make verifying (Balaban *et al.,* 2005; Muller *et al.,* 2007; Finkel and Holbrook, 2000) and disclaiming conclusions about this theory. Substantial information has been brought to light and the theory of oxidative damage in aging has now both supporting (Balaban *et al.,* 2005; Muller *et al.,* 2007; Finkel and Holbrook, 2000) and contradicting evidence (Raha and Robinson, 2000; Gurber *et al.,* 2008; Vijg and Campisi, 2008).

There are some characteristic features of mtDNA which make it vulnerable to damaging mutations (Rajawat, 2009). mtDNA is transmitted maternally. It replicates in the course of lifespan in proliferating and also in nonproliferating post-mitotic cells. Also, mtDNA does not bear introns and does not possess any effective DNA repair mechanism; elevating the probability for exposing random mutations that impossible to repair. mtDNA exposes to mutations much more than nuclear DNA (Ozawa, 1997). The congregation of mutated mtDNA is abundantly observed in aging cells but at the same time, total substitution of the normal mitochondria with the damaged one is reported (Khrapko *et al.,* 1999). Contemporary papers propose that peroxisomes implicate in the coordination of free radicals and ROS generation (Schrader and Fahimi, 2006). Peroxisomes involve in the fatty acid pathways and responsible for fatty acid generation,, oxidisation, and elimination of peroxides. Enhanced production of peroxisomal ROS and variations in fatty acid oxidation activity is the two main involment of peroxisomes in aging. Alterations in fatty acid oxidation can affect the lipid content of the membranes. There are some researchers imply that membranes are fundamental for the normal cellular functioning and detritions in the structure of membranes is the key event leading aging (Zs-Nagy, 1994). Various changes in the membrane structure with age is represented. Lipid peroxidation (Matsuo *et al*., 1993), and the cumulation of the lipid dolichol (Dolfi *et al*., 2003) that can heavily

disrupt the transmembrane signaling can be given as an example to these changes.

1.1.2.2. Developmental-Genetic Theories

These theories propose that the aging is an element of the genetically programmed and controlled perpetuation of the development process and maturation. That concept can be attractive but, the effects of aging are very different from strictly controlled and rigorous processes of development. Besides, evolution processes for the optimization of reproduction; so the gene expression in advanced age possibly doesn't have a major function in the evolution of the species. These theories are strengthened by the fact that the maximum life span is very specific for each species.

1.1.2.2.1. Longevity Genes Theory

The theory considers aging as a part of the natural biological processes that are essential for life. (Cutler, 1992) In long term, these processes may cause negative effects in organism. The suggestion that all organisms have the same causes for aging and they have common mechanisms of governing aging rate is the unique feature of this hypothesis. The mechanisms and reasons underlying aging can be far complicated; nevertheless, there can be less varied main processes that control the aging rate and life span. The genes that control these few key processes are named 'longevity determinant genes'.

The discovery on longevity genes found in lower eukaryotes such as yeast and *Caenorhabditis elegans* (*C. elegans)* and the accelerated aging syndromes present in humans have supported the reliability in genetic theories of aging (Turker and Martin, 1999). Yet the genetic and stochastic theories of aging shouldn't be considered as independent mechanisms.

Abundant reports on aging indicate that that maximum life span (MLSP) is genetically regulated in various species however; the degree of heritability seems to be smaller than 35% (Scott *et al.,* 2002). Although the effect seems to be

relatively insignificant, genetic mutations can still effectively alter the senescence process.

Various genes affect both the mean and maximum lifespan of yeast (Foll *et. al.,* 1999). These genes express some products that function in differently, such as regulation of stress response, evaluation of the nutritional status, enhancing metabolic capacity, and silencing the genes which contributes to aging process. The studies with *C. elegans* mutants with increased lifespan have elucidated various genes that may be relevant in the aging process (Mendenhall *et al.,* 2006).

A strain of *Drosophila melanogaster* which shows around 35% increase in mean lifespan and enhanced resistance to different types of stress such as shortage of nutrition, high temperature, and dietary paraquat (an insecticide which is a free-radical producer (Lin *et al.,* 1998). The same conclusion cannot be derived from the mammal studies as clear as studies with the other organisms. However, it is reported that in mice and humans that have elevated longevity, immune loci is involved (Foll *et. al.,* 1999). Also, a mutation was observed in the gene for the signaling molecule p66 (shc) in mice that effectively increases the resistance against oxidative stress. That mutation has the powerful effect of enhancing the life span by 30% (Walker and Benzer, 2004). A well-known concept about aging that it promotes a differential gene expression patterns in muscle and brain parallel to inflammatory and oxidative stress, and diminished expression of both metabolic and biosynthetic genes.

1.1.2.2.2. Accelerated Aging Syndromes

There are some human genetic diseases that display some features of accelerated aging although none of them mimic the exact phenotype of normal aging. These diseases can be useful for studying the genetic mechanisms of aging. Werner's syndrome ("adult" progeria), Down's syndrome and Hutchinson Gilford syndrome (the classic early onset progeria developed in children), can be given as example (Turker and Martin, 1999).

Recently, many different mouse models were built up which show some characteristics of aging features observed in people (Kuro-o, 2001). For instance,

klotho mouse is affected from a malfunction in a single gene which encodes a membrane protein. It performs several essential age-related phenotypes that can also be observable in human elder populations. These phenotypes are reductions in lifespan, diminished physical activity, premature thymic involution, skin disorders, arteriosclerosis, osteoporosis, emphysema, and lipodystrophy. Also some lines of senescence-accelerated mice (SAM) which show alterable aging phenotypes parallel to multigenic effects. However, none of the mouse models exhibits all of the phenotypes related to human aging. Nevertheless, the animal models are essential as a model study for elucidating the potential events and changes at molecular level of aging.

1.1.2.2.2. The Neuroendocrine Theory

The theory suggests that aging is connected to alterations in both in neural and endocrine functions. These functions are essential for controlling and monitoring the transmission with and reactiveness of all of the organs and systems to the outer environment; tuning the physiological reactions to environmental stimuli; and sustaining an optimum level in order to control reproduction and survival.

An essential feature about the theory is the view about the hypothalamo-pituitary-adrenal (HPA) axis as a key regulator, the “pacemaker” that expresses both the first beginning and ending of life stages. The main function of the HPA axis is to orchestrate the physiological arrangements required for the protection and continuum of the intrinsic “homeostasis” in the presence of the sequiental alterations in the neighborhood of the organism.

Currently, this theory has gathered some positive evidence from the research of an ancestral insulin pathway which is responsible for the supervision of stress reactions and life span of nematode C*. elegans* (Kawano *et al.,* 2000).

Mutations in various genes that involved in this pathway contribute to resistance to several external stress factors and extension in life span (Udelsman *et al.,* 1993). Some of the same genes are preserved and present in humans. In C. *elegans*, these genes compose a primal neuroendocrine system in which the insulin/IGF-I peptide evaluates and collects the information from external stress determinants.

The final combined responses exhibit a crucial role in following and controlling the metabolic and reproductive condition of the organism to organize suitable energy arrangements and finally increase in the longevity (Tatar *et al.,* 2003). So it can be inferred that this primal neuroendocrine system present in lower organisms has the ability to both control the destination and magnitude of the reactions and additionally prevention of the stress responses to be out of balance. The important studies with nematodes suggest deeper investigation of the organization of the programming of various influences that interact to orchestrate the maximum life span.

A number of researches conducted with humans show progressive reductions in the abundance of the peripheral hormones in the normal existence of trophic hormones (Mobbs, 1996). These outcomes may both result from the elevation in the response to the peripheral hormones by HPA or the unbalanced reduced expression of the stimulating hormone for the synthesis. Nevertheless, the fact that lower organisms that share common aging characteristics doesn't involve intricate neuroendocrine pathways like in higher vertabrates, should be taken into account. The alterations observed in the neuroendocrine pathways can be resulted from the essential changes happening in all cells of the body and may be a resulting indication of the general characteristics of aging and its effects.

1.1.2.2.3. Immunologic Theory

This theory is mainly aroused from the two findings that the normal abilities of the immune system is reduced with increasing age (proved by a diminished response of T cells to mitogens and reduced durability against infectious diseases); and elevations in autoimmune activities with age; like elevated levels of serum autoantibodies (Walford, 1974). The tendency in aging is the elevations in the ratio of memory T cells coupled to increased synthesis of a multi drug resistance protein; p-glycoprotein. (Miller, 1996). Additionaly, humoral immune system activity is also diminished with advancing age, as reported by reduced antibody generation and an unequal loss in the capacity to produce IgG and IgA (immunoglobulin G and A) antibodies with high affinity. Also variations in the longevity of different mice lines are correlated to specific alleles in the major histocompatibility gene complex (MHC) (Yunis and Salazar, 1993). Additionally,

these genes have influences on the regulation of multifunctional oxidases (P-450 system), DNA repair functions, and enzymes involved in free radical elimination. It is suggested that mouse and human histocompatibility genes can be correlated to life span working with distinct mechanisms, in mice with vulnerability to lymphomas and vulnerability to infectious diseases in humans. Some reports indicate that cytokine gene polymorphisms can influence histocompatibility genes effecting longevity (Caruso *et al.,* 2001).

The same commentary opposed to the neuroendocrine theory can be applied to the immune theory of aging. Lower vertabrates do not have advanced immune systems, yet they show the features of aging as well. Also it is challenging to discriminate the essential alterations that occur in many kinds of cells and tissues and the consequential results driven by the altered immune system with accompanying age.

1.1.2.2.4. Cellular Senescence / Telomere Theory

The cellular senescence theory of aging was first proposed at the time cell senescence was defined as an event that borders the multitude of cell divisions in a human cell culture (Hayflick, 1965). Limitation in replication capacity is observed following characteristic times of cell division process and finally causes impairments of cell function and disruption in cell homeostasis (Campisi, 2003).

The differences between cellular senescence and stress-induced senescence (SIS) should be clarified. Replicative senescence is a different type of cellular senescence that finally causes the pruning of telomeres which are specific compartments consisted of a repeating DNA sequence and it can be found at the end of every linear chromosome. In every cell division, a tiny fraction of DNA is lost at each chromosome end causing in telomere shortening, changed telomere structure, and finally replicative senescence (Blackburn, 2000). Activation of the telomerase enzyme can elongate telomeres again, prohibits replicative senescence and immortalize primary human cell cultures (Bodnar *et al.,* 1998). On the other hand; SIS results from reactions to miscellaneous stressor factors; covering but not limited to DNA damage, changes in the heterochromatin structure, and amplified mitogenic signals resulted from oncogene activation

(Campisi, 2003). Characteristically immortalized cells like stem cells, germ cells, and T lymphocytes produces telomerase enzyme and they can both preserve their telomere length or impede telomere shortenings (Wright *et al.,* 1996).

The obligation of existence of proper telomerase activity for maintaining human cell immortality and the knowledge that telomeres are shrinked with age made the scientists develop the concept that telomere length regulates cell replicative life span of the organism and involved in the aging process. Some studies on rodents have raised little reliability of this concept. Gene targeting experiments conducted with mice manifested that telomerase-deficient mice do not age fast; in fact, distinct aging phenotypes are not exhibited for several generations (Blasco *et. al.,* 1997). That result indicated that telomere detritions are not contributing to aging of mice but resemblance between aging and the late-generation telomerase-deficient mice phenotype implies that some kinds of cellular senescence may promote aging.

Unlike the mice studies, it can be said that in humans, as proved by some reports, telomere pruning may promote aging. The mutation responsible for dyskeratosis congenita disease (DKC), affects an enzyme which is required for metabolism of the telomerase RNA subunit (hTR) (Mitchell *et al*., 1999). A rare dominant autosomal form of DKC derived from a direct mutation on the hTR gene, giving credits to the suggestion that DKC is developed through telomerase dysfunction. Also supportingly, some DKC patients develop an increased incidence of carcinomas, proposing the notion that telomere shortening may involve in the development of cancer that is more widespread with advancing age (Vulliamy *et al.*, 2001). The relative involvement of replicative senescence and SIS in the aging process is not clearly elucidated and still controversial up to this date.

1.1.2.2.5. Cell Death Theory

Two different types of cell death; necrosis and apoptosis are identified. Massive cell damage which is mostly followed by inflammation may cause necrosis of the cell. Necrosis is mainly an accidental process and involves aggregation of chromatin, swelling of the organelles, and eventually breaking down of membrane and cell. Oppositely, apoptosis is driven genetically, an active, “suicide” process as

a reaction to environmental or internal stimuli, generally not requires critical external injury of the cell (Lockshin and Zakeri, 1991). Apoptosis is essential for the organism to maintain homeostasis. “Programmed cell death” and “apoptosis” terms generally used as having the same meaning, however, they are different terms indeed. Programmed cell death is a part of the development of the organism, on the other hand, apoptosis is a type of cell death (Campisi, 2003). Programmed cell death required elevated levels of lysosomal enzyme and it seldomly causes the laddering of DNA which is featured in apoptosis.

Cell death is a typical feature in many of the neurodegenerative diseases frequently observed in aging (Warner *et al.,* 1997). Selective loss of neurones is observed in Alzheimer’s disease (hippocampus and cortex), Parkinson’s disease (substantia nigra), Huntington’s disease (striatum), and amyotrophic lateral sclerosis (motor neurons). β-amyloid protein is cytotoxic to normal culture of neuronal cells, which sequentially undergo apoptosis process. Research continues to reveal the prospective contribution of apoptosis in aging and aging related diseases (Ellis *et al.,* 1991).

1.2. The Aging Brain

In the regular aging process, cognitive abilities seems to diminish more slowly compared to physical ability nevertheless it occurs at varies extent in aging individuals.

The alteration that observed with age in the capacity of remembering distinct types of information is called ‘age-associated memory impairment’ (AAMI) (Crook *et al*., 1986). Alterations in memory in aging are changeable among individuals and not every type of memory are affected in a same degree. In humans, first memory deficits may be noticed by the fifth decade of life (Albert *et al*., 1987).

During aging, different anatomical, histological, cellular, and subcellular alterations occur in the human brain. The wide variety of changes, mostly masked by compensating reactions, makes the identification of salient decays due to age a difficult task.

1.2.1. Cognitive performance in aging

Gradual impairments in cognitive function, loss of memory, reduced capability to learn new information or gather new abilities are widespread elements of the aging process. The magnitude of the damage is very alterable and it may result from several inducements covering intrinsic and genetic factors or intrinsic factors of the "natural senescence" of the cell.

Variations in regional brain volumes and unity of the white matter with the progressing age are related to cognitive capacity. On the other hand, the collection of the reports about that topic indicates that the extent of the mentioned relationship is not very effective (Raz, 2000; Gunning-Dixon and Raz, 2000). Structure-function connections are involved in some fields of cognitive aging of the brain. Better ability on some executive tasks (e.g., Wisconsin Card Sorting Test) is coupled to larger prefrontal cortices, and less WMH (White matter hyperintensities) in MRI scans (Raz *et al*., 1998; Gunning- Dixon and Raz, 2003). Larger hippocampal volumes and higher amounts of a neuronal metabolic marker (NAA) were detected in younger adults that have better spatial memory performance than the older ones (Driscoll *et al*., 2003).

Also there are some data about specific negative correlations between cortical volume in some of the prefrontal regions and performance on working memory tasks. In healthy elderly, the correlation between simultaneously measured regional volumes and cognitive performance is not fully clarified yet. Significant extent of the individual variations may arise from determinants different than aging such as vascular disorders (Salat *et al.*, 2002; Van Petten *et al*., 2004).

The question about to which level the impairment can be evaluated as a result of ‘normal’ or nonpathological aging and to what extent is the result of pathological neurodegeneration such as in Alzheimer’s disease has been addressed by several neuroscientists. One of the recent studies has focused on the topic and also evaluated the possible contribution of apolipoprotein E4 (ApoE4) which is a genetical risk factor for Alzheimer’s disease (Mayeux *et al*., 2001). In the experiments, memory is impaired by time in a big proportion of healthy old individuals however, no significant loss of visuospatial/cognitive function or

language, which is reduced in Alzheimer's disease, was recorded. On the other hand, the presence of an ApoE4 allele causes a sharper reducement, which shows ApoE is a crucial factor for the normal brain functioning as well as its specific function in Alzheimer's disease pathogenesis. The result underlies the notion that genetical influences may have effects in the non-pathological functional reduction in the brain and other genes that affecting the cognitive decline observed with progressive aging will be probably identified. Environment can also be a contributor because poor education has been revealed to be a possible cause in accelerated memory decline and dementia (Schmand *et al.*, 1997).

Another model of cognitive aging proposes that age-related alterations in the cognitive functions with age can be attributed to regional and early age-related disintegration in the frontal lobes of the brain. Since the frontal lobes are considered as substrates that are most closely related to executive functioning, alterations in their structures may be a possible contributor to impairments in executive functioning. Executive losses sequentially make the conclusion towards to the well-known changes in a variety of cognitive processes, covering memory (Phillips and Henry, 2008). Today, there is a still controversy on this discussion about the significance of more fundamental alterations in processing speed versus specific impairments in executive control as contributor agents in cognitive aging.

One of the most effected cognitive function with aging; episodic memory is covering the subjective experiences and past events (Mahwah, 2000). The impairments in episodic memory that observed in some elderly may either result from defects that occur at the time of the first storage of the information (encoding), the maintaining of the information (storage), and also the recovery of the stored information (retrieval). Behavioral studies which influenced attention during these three phases indicates that encoding deficits may have a major role episodic memory impairments in elderly (Anderson *et al*., 2000).

Non-twin experiments demonstrate that cognitive decline coupled to aging is more prevalent in women compared to men (Read *et al*., 2006). However, there is still argument about whether this finding is an artifact due to sex differences in longevity (Bachman *et al*., 1992).

1.2.2. Anatomical changes in the brain associated with non-pathological aging

A significant decrease in brain weight is a widely documented sign of aging. In neurologically normal elderly individuals, it has been estimated that this decrease begins at the age of 60 years and proceeds at a rate of 2 to 3 grams per year. Considering the average brain weights of a young adult men (1400 g) and women (1250 g), by the age of 80, an overall reduction of about 3 to 5% occurres. (Bertoni-Freddari *et al*., 2006) The volume loss is observed with elevations in the ventricular volume and other cerebrospinal fluid (CSF) spaces. The most effected brain structres by aging are the hippocampus and frontal lobes. Over the age range of 30–90 years, the volume losses are 14% in the cerebral cortex, 35% in the hippocampus and 26% cerebral white matter, with partially bigger loss of white matter when exceeding the age 50 (Esiri *et al.*, 1997; Jernigan *et al*., 2001).

There are many reports focused on neuronal numbers in the aging brain so far. Yet a debate is going on this topic. This may be dependent to different technical problems like the shrinkage of neuronal cell bodies making the cells very hard to be counted within a particular class of neurones.(Esiri *et al*., 1997). Modern stereological methods have revealed a 10% loss of neurons in the human cerebral cortex (Pakkenberg and Gundersen, 1997). Also neurones in some other brain regions including those in the hippocampus, and amygdala were reported to be lost with age. Some other neurones show shrinkage absence of loss and still other brain structres are likely to be isolated from shrinkage and neuronal loss. The nucleus basalis can be given as an example. It is an area which is associated to Alzheimer's disease that the neurons exhibit some disease-related signs of shrinkage and loss (Chiu *et al*., 1984).

Recently, the development of more accurate counting techniques raised more reliable estimations and led to the conclusion that normal aging does not involve a significant neuronal numeric decrease through neuron death. Unbiased stereological analyses have shown that, across the overall lifespan, the loss of neurons is around 10%, but this cannot be evaluated as a specific time-related significant impairment since sex has been indicated to be more important than age in modulating the total neuron number in a given brain region, for example, the

neocortex (Pakkenberg and Gundersen, 1997). The marked heterogeneity of the whole brain and even of specific brain regions makes it difficult to interpret any decrease of neuron number from a functional standpoint, thus only those studies conducted in localized CNS zones with clearly demonstrated functions and connections have provided useful information in this manner. Because of their documented direct implication in memory; enthorinal cortex and hippocampus have been investigated in different animal models and in human beings. Thus even if the lack of significant neuron loss cannot be shown as the main cause of a functional decline, the neuronal circuits on which the specific function, for example, memory, relies may undergo a progressive damage leading to impaired brain performances.

Brain shrinkage associated with aging can be related to the overall effects of stress that controlled by the secretion of corticosteroids. Elevated glucocorticoid levels are connected to synaptic losses in the hippocampus, hippocampal atrophy, and cognitive decline with age (Sapolsky *et al*., 1987; McEwen, 2002), and stress hormones released in an acute or chronic manner are damaging for the memory performance at all ages (Lupien *et al*., 2005). On the other hand, the promotion of glucocorticoids to brain aging is connected to the physiological and cellular environment and some of the mentioned effects can be reversed (Lee *et al*., 2000; Nicolle *et al*., 2001). Yet some early evidence points towards a link between corticosteroid secretion and hypothalamic-pituitary-adrenal (HPA) axis responses with age-related shrinkage in some brain regions (Sapolsky *et al*., 1987; Lupien *et al*., 1998; Magri *et al*., 2000; Wolf *et al*., 2002). In addition, reduction in cortisol levels can cause in reversing back of the observed hippocampal atrophy (Starkman *et al.*, 1999).

In summary, at present, the well documented brain shrinkage due to age does not seem to be resulted from the numeric loss of neurons that has been demonstrated to be far less significant than previously thought and it commonly occurs in localized CNS regions. Atrophy of neurons and of their contacts is reported as the main causative event responsible for the age-related reduction in brain volume, and this provides a rationale for interventional strategies to repair dysfunctional neuronal networks.

1.2.2.1. Dendritic Changes

An age-related reduction in the number of dendrites and dendritic spines has been reported in normal aging process, resulting in an overall decrease of dendritic complexity. The regressive changes occurring at the dendrite tree affect mainly frontal and temporal cortex and the limbic system in the human brain. The first step in the age-related alterations of dendrites is the loss of spines followed by changes in shape and size of basilar dendrites and then of the branches of the apical shaft (Scheibel *et al.*, 1975). Dendrites are receptor membranes of the neurons, and their spines amplify this function and have been reported to isolate increases of synaptic calcium transport utilized for information storage (Koch *et al*., 1992). As a result, the age-related loss of dendrites and dendritic spines isolates neurons and leads to impairment in cell-to-cell communication. However, because of its plasticity, the old CNS is still capable of compensating to some extent for the age-related loss of neurons and dendritic retraction by increasing the dendritic growth. In parahippocampal pyramidal cells of cognitively normal individuals the continual growth of dendrites was reported to occur well into the eighth decade of life, whereas in pathological brain aging, for example, AD, this compensating reaction has not been found (Buell and Coleman, 1979).

1.2.2.2. Glia Alterations

Glial cells and neurons interact intensively; thus alterations in glial support occurring with advancing age may be one of the reason of impaired neural functions.

An improved characterization of primary glial changes occurring in the aging CNS has been achieved by the use of recently developed immunohistochemical and silver impregnation methods that helped to reveal new aspects of time-related glial degeneration. As an example, paired helical filaments (PHF), long believed to occur only in neurons were also demonstrated in astrocytes from Alzheimer's Diseases patients (Nakano *et al*., 1992). In addition, the existence of abnormal argyrophilic structures immunoreactive with anti-tau antibodies has been demonstrated in glial cells which support the notion that not only neural but also glial cytoskeletal abnormalities are related to the specific pathological conditions.

With specific reference to physiological aging, reactive gliosis, probably occurring as a consequence of neuronal damage, is the most widespread of all glial changes. Also, it was found that in the eighth decade there is an age-associated elevation in the amount of cerebral cortical astrocytes immunoreactive for glial fibrillary acid protein (GFAP) (Hansen *et al*., 1987). This reaction is directly connected with the essential function of astrocytes that play important roles both in nutritional supply, via glucose transfer to neurons, and in the metabolism of glutamic acid, used as neurotransmitter.

Over age 60, in the human brain, microglia also show changes such as altered morphology, the increased number of reactive microglia expressing the immunomodulatory cytokine interleukin-1, which is connected to elevated tissue levels of interleukin-1 mRNA (Mrak *et al*., 1995), an alteration implicated in AD pathogenesis.

In summary, on the basis of these data, in addition to the age-related neuronal changes, glial degenerative phenomena also must seriously be taken into account when studying brain aging.

1.2.2.3. Vascular Changes

Age-dependent damage of blood vessels may cause a significant reduction of blood supply to the brain. Since atherosclerosis and amyloid angiopathy are reported to represent the most important changes responsible for the age-related progressive decay in blood cerebral perfusion, they were thoroughly investigated in the brain of both humans and experimental animal models. The results have provided detailed information on mechanisms, determinants, and causative events involved in brain aging.

1.2.2.3.1. Atherosclerosis

Atheroma is the term used to define the caseous material, containing high amounts of lipids, found in plaque-like thickenings of the interior portion of the vessel wall. As a consequence, perivascular demyelinating lesions affecting axons and the cerebral white matter are formed.

1.2.2.3.2. Amyloid Angiopathy

Amyloid deposition in the wall of cerebral blood vessels is a well-known change found both in physiological and pathological aging of the human brain. Amyloid fibrils are deposited extracellularly and derived from soluble circulating proteins that have undergone partial proteolysis and polymerization finally resulting in insoluble aggregates. Different components take part in the final composition of amyloid deposits and include, in addition to the straight, nonbranching protein fibrils (diameter: 7-10 nm), a serum glycoprotein (P-component) showing a structural homology with c-reactive protein (an acute phase reactant) and sulphated proteoglycans. The favorite sites for amyloid deposition are the outer surface of the endothelial basement membrane of the capillaries, the basement membrane of pericytes, and the basement membrane of the smooth muscle cells of veins and arteries. Amyloid angiopathy is particularly severe in temporal and occipital regions, increasing in these brain regions the risk of a spontaneous intracerebral mass hemorrhage (Haan *et al*., 1994).

More than 30 types of amyloidoses are classified, with respect to their location and distribution in the body (Bertoni-Freddari *et al*., 2006). With specific reference to brain aging and age-related neurodegenerative diseases, the most common and important one is βA4 amyloidosis. The amyloid precursor protein (APP), a protein spanning the cellular membrane, is secreted by nerve and other types of cells. In the brain, nerve cells and their dendrites are the main sources of APP. APP is then transported along the perivascular spaces to the subarachnoid space and may deposit in external regions of the blood vessel walls. Although βA4 amyloid angiopathy may occur without evident clinical signs, it is almost frequently combined with the presence of amyloid deposits, as senile plaques in the cerebral parenchyma (hippocampus, frontal cortex, and amygdala) of AD patients.

1.2.3. Changes in gene and protein expression in the aging brain

There are several studies examining gene expression patterns in brain tissue during the life span (Aenlle et al.,2009; Blalock *et al*., 2003; Erraji-Benchekroun *et al*., 2005; Prolla, 2002; Terao *et al*., 2002). Monitoring the gene expression in

aging mouse brain have revealed that approximately 2% of the 6347 genes examined performed either elevations or reductions in expression of up to 2.1-fold in magnitude (Lee *et al.*, 2000). An early proteome analysis study revealed that a similar proportion of proteins are altered during the time of aging (Tsugita *et al*., 2000). There were increases in genes implicated in inflammation, stress responses and reductions in genes for trophic activities, protein metabolism and DNA repair. Some changes were reported to be ameliorated by caloric restriction (Tsugita *et al*., 2000). Different research conducted on hypothalamus and cerebral cortex of aged mice showed resembling alterations to some degree, in addition there were elevations in protease levels possibly essential in the modification of the amyloid precursor protein (APP) and reduction in protein phosphatase 2A levels which is a tau phosphatase (Jiang *et al.*, 2001). It must be added these conclusion are only tentative and showing that alterations in gene expression with age may lead to Alzheimer-type neurodegeneration and more analysis is required for revealing the pathogenesis of Alzheimer's disease at a molecular level.

The aging of the brain is definitely influenced by the genetic factors. This conclusion can be assumed from the partition of the ApoE allotype to age-related cognitive impairments and research on SAM mice. Inbred lines of mice may be accepted as a credible model for studying the genetic influences on the aging brain. Current reports have revealed that gene loci which affect the hippocampus structure can be determined (Lu *et al*., 2001).

1.2.4. Age-related Alterations of the Synaptic Structural Dynamics

The synaptic contact zones are functionally differentiated areas of the neuronal membrane. The synaptic terminal regions show dynamic changes responsible for continuous remodelling to optimize signal transduction and information processing. Synaptic plasticity is the commonly used term defining functionally controlled adaptive responses of the synaptic contact zones, including the various different morphological, biochemical, molecular, and genetic alterations observed at synapses as a result of external stimulation. (Bertoni-Freddari *et al*., 2006)

1.2.4.1. Synaptic Numeric Density

The synapses play different roles as interaction zones among neurons and the synapse number reflects the magnitude of neural inputs towards to the neurons of a CNS region. The magnitude of the postsynaptic potential is affected by the density of the synapses in a given tissue area or volume (Nv), and Nv may essentially modify the processing of information. (Desmond and Levy, 1986)

In the adult CNS which is totally differentiated, the synaptic pattern can still be altered in the activity-dependent manner by the growth of novel connections. reduction in the number of synaptic contacts has been indicated to happen with age, however, the amount and the significancy of the age-related decrease of Nv is directly connected to the specific vulnerability to aging of the CNS region that under investigation and on counting method.

A reduction of synaptic numbers in the aging process was revealed by different authors in varying CNS areas studied from different animal species and people, giving positive evidence about the changes is a omnipresent characteristic of the aging brain.

1.2.4.2. Synaptic Average Size

It is presumable that the synaptic contact area size can affect the quantitiy of transmitters released, in addition the extent of the transportation of many substances that function in information processing. So it can be inferred that increase or decrease of the synaptic size may cause functional alterations.

In the brain of aged subjects, both the laboratory animals and the humans, an elevation in the synapse size in different regions of the CNS has been found. Interestingly, similar changes have been also reported in the CNS of Alzheimer's Disease patients (Bertoni-Freddari *et al*., 1990; DeKosky and Scheff, 1990). Such synaptic changes were reported both in brain areas known to be very sensitive to aging and age-related pathologies (e.g., the hippocampus) and in regions documented to be less sensitive to time-related damage (e.g., the cerebellar

cortex), suggesting that this modification in the composition of the synaptic population is a general characteristics of the CNS aging.

1.2.4.3. Synaptic Volume Density

The cumulation of the areas of the synaptic contact zones that present in a defined tissue volume (usually 1 μm^3) responsible for the response capacities of the neural circuits of a given defined CNS area. The term that is used for defining the parameter is surface density (Sv), and just like other morphological characteristics of the synaptic junctional areas, it may be affected from alterations as a result of a number of modulating actions like electric stimulation, behavioral conditioning, and trophic factors. The Sv value may be affected by the density and the lenght of the stimulus and also to the level of plasticity of the specific CNS area which is analyzed (Desmond and Levy, 1986) Memory and learning capabilities additionally depends on the sufficiency of Sv values, because an improved network of contact gives a microanatomical structural basis of the essential functions.

In people, Sv has been demonstrated to be significantly reduced in the old hippocampus, however, a non-significant age related decrease of Sv was noted the cerebellar cortex. More reduction of Sv was observed only in the hippocampus in the AD patients, likely in relation with persisting presence of senile plaques in the CNS zone, which is a characteristic feature of the disease (Bertoni-Freddari et al, 1990, 1996). The Sv is reduced in physiological aging and also in AD. It may result from the numeric loss of contacts (Nv) but also due to enlargement of the average synaptic size (S).

1.2.5. Neurotransmitter Systems

Neurotransmitters play a central role in neuron-to-neuron information processing and in transferring information from neurons to target cells. The major neurotransmitters in the CNS implemented in “hot lines” of signaling are amino acids: the glutamate, gamma aminobutyric acid (GABA) and glycine while classical modulatory neurotransmitters include acetylcholine, the catecholamines (norepinephrine, epinephrine, and dopamine), and serotonine. In addition, neurotransmission may be accomplished and/or modulated by several peptides

(e.g., enkephalin, substance P, and cholecystokinin), gases (nitric oxide and carbon dioxide), and metals, acting as neuromodulators (zinc).

During aging, the levels and activity of neurotransmitters and related enzymes decline in many brain regions, and the corresponding receptors may or may not respond to these changes by increasing their number and/or affinity. In details, with reference to human beings, it has been found that brain acetylcholine transferase levels as well as muscarinic binding decrease in aging (Perry, 1980). The levels of striatal dopamine uptake sites, dopamine and dopamine transporters show an age-associated decline (Kish et al., 1992) as do serotonin binding sites, α2 and β1 adrenoceptors, and cortical GABAergic innervations (Allen et al., 1983; Kalaria et al., 1989). Alterations due to age in tissue levels of glutamate and aspartate were also noticed in different brain areas (Banay-Schwartz *et al.*, 1992). Studies in different discrete zones of the CNS of laboratory animals (rats) have shown that while the concentration of serotonin is constant throughout the lifespan, dopamine and norepinephrine progressively decease starting from adulthood. Thus, in the aging rat brain, the ratio of serotonin to catecholamines progressively increases, but the functional manifestation of this imbalance may be due to the impairment of only one of these three neurotransmitters.

1.2.6. Energy Metabolism: The Critical Role of Mitochondrial Function Decay

The human brain, representing less than 2% of the body weight, receives about 16 to 17% of the cardiac output and under resting conditions uses about 20% of the total oxygen consumption. The energy demand of the nervous tissue is much higher as compared with other tissues. Most of this energy is supporting the maintenance of steep transmembrane ionic gradiends on which the nerve cells excitability depends. A lot of energy is also used for neurotransmitter turnover.

1.2.6.1. Impairment of Mitochondrial Structural Dynamics in Aging

Mitochondrial morphology is known to be non-homogenous and it can show a great variability among different cells in the organ level or among the cellular sub regions in the cell. For instance, in neurons the greatest density of mitochondria is found in dendrites. (Bertoni-Freddari *et al*., 1993; Bereiter-Hahn and Voth, 1994;

Bertoni-Freddari *et al*., 2001). Mitochondria are very dynamic structures easily undergoing “remodeling” in order to meet the actual energy demands of the cells.

According to a number of studies, mitochondria numbers show a reduction in elderly. In contrast, mitochondria size shows an elevation maintaining the cumulative mitochondrial volume density (i.e., the mitochondrial fraction/μm³ of tissue) stable over the entire course of the life of the organism. A notable elevation in the percent of the enlarged organelles was revealed at the end of synapses of older animals. Those organelles that are oversized, like mega mitochondria (MM), were reported to be a risk factor for the homeostasis and life of the cell in physiologically challenging circumtances (Bertoni-Freddari *et al*., 1993; Solmi, 1994; Walter *et al*., 1999).

1.2.6.2. Cytochemistry Of Succinic Dehydrogenase (SDH) Activity In The Aging Brain

Recently, the notion that the reactive oxygen species (ROS) formation when the normal physiological process of cellular respiration is not managed perfectly in old cells has been supported, and their production may account for the notable harm to mitochondrial DNA (mtDNA) and the membranes of the cell leading to the changes in the morphology of mitochondria and to a gradual deterioration of functions of the mitochondria. The notion is particularly supported by the observations in old neurons that the building up of the mtDNA mutations has been resulted both from ROS damage at the time of cellular respiration and the replication number of mtDNA.

Studies conducted with aged rats demonstrated that the volume density (the fraction of organelles/ μm³ of cytoplasm or neuropil) of mitochondria having SDH is notably reduced in perykaria of big neurons, like the cerebellar Purkinje cells and hippocampal CA1 pyramidal cells. In all kinds of these cells, this reduction is resulted from to a noticable loss of numbers of the SDH-positive organelles, as their average volume is not showing any notable alteration. Purkinje and CA1 cells with their large soma, rich dendritic arbor, lengthy projections, and high energy demands are especially vulnerable to any decrease in the amount of ATP and

thus, are valuable models to elucidate the cellular bioenergetic metabolism with age in neoruns. These data support the idea that, in aging, mitochondria of neurons may not be capable to supply the energy requerments for the regular processes at the time ATP need is increased.

1.2.6.3. Cytochemistry Of Cytochrome Oxidase Activity In The Aging Brain

The population of mitochondria is not consisted of organelles that having equal yield in functioning, in contrast, each mitochondrion shows its own characteristics. That characteristic in the metabolism of mitochondria is defined as mitochondrial metabolic competence (MMC) (Bertoni-Freddari *et al*., 2003, 2005), and it is a subject of study of age related alterations by preferential cytochemistry of cytochrome oxidase (COX) activity determined in each organelle by computerized image analyzers and morphometric programs. COX, which is an integral transmembrane protein of the inner membrane of mitochondria, it is involved in the electron transport chain as the final enzyme and provides a credible endogenous marker of metabolism in neurons (Wong-Riley, 1989).

A current investigation conducted on mitochondria of synapses in the cerebellum of both adult and aged rats made the comparisons of the age-related MMC vs. size of individual mitochondrion. Aged animals shows a notable reduction in the MMC of small and medium-sized organelles by 31.6 and 26.4% in turn, but in samples from adult and aged old rats the proportion of oversized mitochondria seemed to have the same capability in order to supply ATP. Considering all of the changes that occur with age in mitochondrial ultrastructure and also SDH and COX functioning, it appears that the reduction of MMC may have a preliminary and essential role in the formation and progression of synaptic disorders linked to physiological and pathological aging of the brain.

1.2.7. Cellular changes

The characteristic signs of Alzheimer's disease can also be observed in old people with normal cognitive functioning but in significantly lower amounts compared to the observed numbers in the patients (Anderton, 1997). Nevertheless, the incidence of AD is affected from some genetical determinants like ApoE4 and

other genes which are not determined yet (Myers *et* al., 2000). So it is possible that the risk of developing disease is a result from the interaction of the cumulative effects of both genetic and environmental elements. It is perhaps more likely that the combination of genetic and environmental factors regardless of the age of the organism.

1.2.7.1. Accumulation of age pigments

The accumulation of age pigments, or lipofuscin, is a distinctive feature of nerve cells (both neurons and glia) in aging. Since lipofuscin forms during adult life and accumulates with age, it has been suggested that it represents the morphological outcome of "wear and tear" cellular function. Lipofuscin is a by-product of lipid peroxidation and originates from secondary lysosomes that transform into lipopigment granules. In neurons, the membranes undergo a continuous turnover with the involvement of lysosomal degradation of proteins and lipids. The interaction between the products of the peroxidation of polyunsaturated fatty acids and other biological molecules leads to the formation of these pigments, which are difficult to digest, and accumulate as residual bodies within the cytoplasm. Any type of biological molecule may become a constituent of lipofuscin, and this finds a well-grounded support in the documented ultrastructural heterogeneity of neuronal pigments reported in the aging human brain (Boellaard and Schlote, 1986). Lipofuscin contains peroxidised protein and lipids and may manifest the elevated failure of cells to reduce the peroxidation-induced cell disruption products.

Lipofuscin accumulation has been proposed to be a function of cellular metabolic activity rather than of chronological age and, in this context, repetitive metabolic accidents may lead to the aggregation of a nonfunctional by-product of cellular metabolism. This assumption finds support in the fact that caloric restricted animals (i.e., that have undergone a reduction in metabolic rate) show less lipofuscin accumulation associated with a longer lifespan than age-matched normally fed littermates (Moore *et al*., 1995).

1.2.7.2. Neurofibrillary Tangles and Neuropil Threads

Neurofibrillary tangles and senile plaque formation has been known as the histopathological distinctive signs of Alzheimer's disease (Anderton, 1997). On the other hand, these signs can also be detected in the brain of healthy people. However, in the example of Hirano bodies and granulovacuolar changes which are the most characteristic sign of AD, the amount of all of the four signs is much higher and much more prevalent. In the regular aging process, the quantity of tangles that resides the cell body of affected neurones is lower compared to the patients and only present in hippocampus, amygdala and entorhinal cortex. On the other hand, studies with sections from both cognitevely healthy and disintegrated people from middle age to late age it was demonstrated that at the time there is a minimum neurofibrillary tangles presence, they are located in the transentorhinal region. Brains in which elevated neurofibrillary alterations occur, the entorhinal cortex can be given as an example also more detrition may be occured in the hippocampus, connected to cognitive impairment (Braak and Braak, 1991). Neurofibrillary tangles are ultrastructres consisted of paired helical filaments (PHF) and irregular straight filaments, including microtubule associated protein tau and ubiquitin. Examination of biopsy samples of AD patients has manifested that in neurones heavily damaged by PHF, the regular cytoskeleton of microtubules and neurofilaments of the neuron is completely destroyed (Flament-Durand and Couck, 1979; Gray *et al*., 1987). So, some of the neuronal loss is may result from the absence of a properly functioning cytoskeleton. Formation of neurofibrillary tangles inside the neurons' processes changes the morphology of neural fibers. These abnormal fibers are called neuropil threads. In normal aging they are only seen in the entorhinal cortex, hippocampus and amygdala generally, however, they are much common in AD patients (Braak *et al*., 1986).

1.2.7.3. Senile plaques

The zones of grey matter up to 200 μm in diameter composed of a extracellular core of amyloid located in the centre enveloped by the swollen extraordinary neurites are defined as senile plaques. They can be also called neuritic or amyloid plaques. The core in the center includes various proteins however; the essential protein is a tiny peptide consisted of 39–43 amino acids that called amyloid β-

peptide (Aβ) which accumulate on fibrils. A smaller fraction of neuritic plaques can also be observed in the brains of cognitively normal elderly but in AD, they heavily increase in number. Patients suffer from Down's syndrome manifest all of the characteristic alterations observed in AD by the age of fourty. However in young Down's syndrome patients the first symptom related to Alzheimer's disease pattern is the presence of 'diffuse deposits' of Aβ (Mann and Esiri, 1989). After that, the neurofibrillary tangles and neuritic plaques emerge. This information has provided a reliable contribution to the notion suggesting that amyloid deposition is the driving factor for the remaining alterations in the brain of Alzheimer's patients. There are some reports suggesting a relation between the decline in the cognitive functions and the cumulative brain Aβ load, however; 'diffuse Aβ deposits' are demonstrated in high levels lacking any neuritic interaction in various brain regions of healthy people (Dickson, 1996).

1.2.7.4. Granulovacuolar degeneration

Vacuoles filled with basophilic granules are located in the cell bodies in mostly pyramidal cells of the hippocampus. The degree of the degeneration shows an elevation with age and is intensely present in Alzheimer's disease (Xu *et al*., 1992). Still there is not so much data about the composition of granulovacuoles and other granules that interact with antibodies to cytoskeleton and proteins present in the tangles (Stadelmann *et al*., 1999; Dickson *et al*., 1993; Kahn *et al*., 1985).

1.2.7.5. Hirano bodies

Hirano bodies are structures with rode shape sizing up to 30 μm long and 8 μm wide and they are present in or neighboring to hippocampal pyramidal cells. Their numbers show an elevation with aging and commonly found in higher numbers in AD. They are ultrastructres which are present as paracrystalline arrays consisted of 60–100 nm long filaments. As seen in the PHF/straight filaments and granulovacuolar degeneration, Hirano bodies is also likely to be constituted of cytoskeletal proteins and also other proteins present in tangles but especially the proteins associated with microfilaments such as tropomyosin, α-actinin and vinculin (Schwab *et al*., 2000; Lee *et al*., 1999).

1.2.7.6. Congophilic angiopathy or cerebral amyloid angiopathy

This is an alteration that involves the extracellular accumulation of Aβ in the cerebral blood vessels' walls. The levels of it shows an elevation in aging and a great abundance in AD. Massive accumulation of Aβ which may have genetic background results in fatal haemorrhage (Wattendorff *et al*., 1995).

1.2.7.7. Infarcts and Leucoaraiosis

The gradual cumulation of many small infarcts is a different contributor to the occurrence of dementia. However, they are also present in the brains of healthy old people in a lesser degree. The name leucoaraiosis corresponds to the attenuation in the white matter that commonly occurs. Leucoaraiosis is a term used to describe thining white matter usually taking place near the ventricles and can be monitored by CT scanning (Nencini, *et al*., 1993). These alterations are usually widespread and abundantly present in the aged brains and it is also found in AD. The reason for the white matter alterations is unclear but may result from partial ischemia due to infarcts formation.

1.2.8. Calcium dyshomeostasis and synaptic communication in aged hippocampus

Calcium does have an essential role in mitochondrial fission which is concerned to be a fundamental mechanism of brain aging (Bossy-Wetzel *et al*., 2003; Lenaz *et al*., 2002), Also it is possible that direct regulation of the Ca^{+2} channels by free radicals can be present in old organisms (Annunziato *et al*., 2002). This notion was first developed for Alzheimer's disease, however, it can be both applied to pathological and normal aging (Khachaturian, 1984).

Reports about the impaired Ca^{+2} regulation at the CA1 neurons in old rodents gave rise to the suggestion of "Ca^{+2} hypothesis of brain aging and dementia" (Landfield and Pitler, 1984; Thibault and Landfield, 1996). Mechanisms that operate in the aging of the brain and/or related to varied synaptic function and impairments in cognitive functions contain Ca^{+2} uptake and release with the endoplasmic

reticulum and mitochondria, plasma-membrane-associated influx and elimination of the intracellular Ca^{+2}, and Ca^{+2} buffering composition and power.

Recent reports indicate that Ca^{+2}-dependent processes modify Ca^{+2} signaling pathways and damages the synaptic function dependent to advancing age, parallel to the Ca^{+2} hypothesis of the aging brain and dementia. The synaptic plasticity can be reduced with age, however, increased Ca^{+2} signals in aged neurons may lead to greater K^{+} efflux, which can be a contributing factor in the elevations in synchronized burst firing in hippocampus (Patrylo *et al*., 1994). Yet more research is needed to be done in order to reveal the relationship between brain Ca^{+2} homeostasis and general excitability in brain aging.

1.3. Aging brain and plasticity

In the adult mammalian brain, the areas which are most susceptible to aging are at the same time shoving great plasticity. Adult neurogenesis in primates, is only observed in the association cortices (prefrontal, inferior temporal and posterior parietal) and in the hippocampus but not in the primary sensory areas (Gould *et al*., 1999). Factors that induce plasticity like growthassociated protein (GAP-43) are found very few in the brainstem, tectum and tegmentum but found abundantly in the associative areas of the neocortex, in the dentate gyrus (molecular layer), in the neostriatum, and in the amygdala (Neve *et al*., 1988; Benowitz *et al*., 1989).

Opposite to the notion suggesting that general damage to the brain happens in the normal aging, data obtained from different studies shows that thereis a general tendency in protection of normal functioning in the aged brain and generally the alterations that do occur are proportionally districted to distinct sub regions of the hippocampus (Barnes, 1994; Rosenzweig and Barnes, 2003). In addition, counterbalancing activities may occur in the aged brain. The pramary cells in the hippocampus do not show any alterations with age in all species. The majorty of the physical characteristics of hippocampal neurons does not show any alterations with age; such as resting membrane potential, input resistance, the magnitude of the action potential, time constant, and EPSP rise time and half-width. The elevated afterhyperpolarizing potential in aged CA1 pyramidal neurons of hippocampus is an exception (Landfield and Pitler, 1984).

Despite there are various distinct age related alterations were identified that may cause reduction in the excitability of aged neurons, electrical contacts with gap junctions can be more abundant in old hippocampus neurons and may cause a general reduction of the threshold for action potential discharge which is a mechanism showing the potential for elevated excitability of old neurons.

1.4. Fatty Acids

Fatty acids are aliphatic carboxylic acid with different hydrocarbon lengths at one end of the chain which is coupled to terminal carboxyl (-COOH) group. The general formula is $R\text{-}(CH_2)_n\text{-}COOH$. Fatty acids can be saturated, monounsaturated or polyunsaturated resulting from the double bonds numbers. The fatty acids have varying chain lengths from 4 to 28 carbon atoms. Long-chain fatty acids (LCFA) are composed of aliphatic tails with 16 or more carbons. These type of fatty acids involve the polyunsaturated fatty acids (PUFAs) that include two or more double bonds in the hydrocarbon chain.

In plants, animals, and microorganism, fatty acids are frequently in an unbranched structure and the fatty acids even having between 12 and 22 carbon atoms react with glycerol to generate lipids. In the living organisms, lipids serve as an energy store, constitute an important building material for the cell membranes, and are required for the healthy skin, cholesterol metabolism, and prostaglandin production.

The palmitic and stearic acids are the most abundant saturated fatty acids that are found in animals and plants. Stearic acid is a saturated derivative of monoansaturated oleic acid. Stearic acid (18:0) has the highest molecular weight among saturated fatty acids and commonly found in fats and oils. It constitutes about 12–14%, while palmitic acid constitutes about 25–28% of the total animal fat. Palmitic acid is the first fatty acid that is generated in the lipogenesis process from which production of longer fatty acids is possible. Retinyl palmitate serves as an antioxidant.

Fifty to sixty percent of the total plant and animal fat contains unsaturated fatty acids (UFAs). Unsaturated fatty acits are mono- or polyunsaturated (PUFAs). There are two main families of PUFAs – the omega-3 and the omega-6 families. There are different systems of nomenclature for naming the fatty acids, however, fatty acids are generally called by their trivial names, e.g. “linoleic acid (LA)”. In delta-x naming system, every double bond is indicated by Δ^x, representing where the double bond is located on the xth carbon–carbon bond starting counting from the carboxylic acid end. Every double bond have by a cis- or trans- prefix, showing the conformation of the molecule around the bond. The n-x nomenclature of fatty acids makes the classification by their physiological features. A double bond is located on the xth carbon–carbon bond, counting from the terminal methyl carbon (designated as n or ω) toward the carbonyl carbon.

Humans are not able to synthesize double bonds at position 6 or lower, therefore omega-3 (n-3) and omega-6 (n-6) PUFAs are essential fatty acids for the normal functionin of the body and required to be taken from the diet. Linoleic acid (LA) (C18:2, cis,cis Δ^9,Δ^{12}, n-6), which is the main element of the omega-3 family, is found very common in nature, and it can be found in the seeds of many plants. Alpha-linolenic acid (ALA) (C18:3, cis,cis,cis Δ^9, Δ^{12}, Δ^{15}, n-3) which is the main element of omega-3 family is not common as LA and imainly found in soya bean, rapeseed and flaxseed oil. Some studies showed that linoleic acid performs dynamic reducing effect of serum cholesterol levels.

Removing the omega-6 double bond from linoleic acid produces oleic acid. Linoleic acid is found in the lipid fraction of cell membrane and is used in the biosynthesis of arachidonic acid (AA). For most mammals, however, arachidonic acid is among the essential fatty acids. Arachidonic acid is a polyunsaturated omega-6 fatty acid found in the phospholipids (especially phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositides) of the cell membranes. Arachidonic acid is freed from a phospholipid molecule by phospholipase A2 (PLA_2) enzyme that splits the fatty acid, but can also be generated from DAG by diacylglycerol lipase. Arachidonic acid is cumulated fast during the brain development in the last trimester of gestation. An impairment in the AA levels can cause a decrease in the growth of infants. Arachidonic acid has also a role in the suppression of the growth of human lung tumour A549 cells.

ALA is the precursor to the synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and at the same time reported to contribute to the generation of various eicosanoids, and in the lowering the blood pressure and also reduction of blood triglyceride levels.
The long-chain n-3 PUFAs, like EPA and DHA can be capable for the protection and curing the diseases lile hypertension, arthritis, inflammatory and autoimmune diseases and also cancer. But, the most characteristic effect of the two PUFAs is the reduction in plasma triglyceride levels and causing a decrease in chance for developing fatal coronary heart diseases controls by different ways, such as the decrease of triglyceride synthesis and chylomicron synthesis from intestines and in addition to inhibition of fatty acid synthesis and triglyceride formation in the liver .

Docosahexaenoic acid, can be received with a diet especially when the diet is rich in the seafood, but it can also be obtained from the intrinsic production from α-linolenic acid. DHA is the most common omega-3 fatty acid which is present in brain and retina. DHA levels stands for 40% of the polyunsaturated fatty acids (PUFAs) in the brain and 60% of the PUFAs in the retina. Fifty percent of the weight of a neuron membrane weight is consisted of DHA (Meharban, 2005). DHA can be found in three phospholipid structres: phosphatidylethanolamine, ethanolamine plasmalogens, and phosphatidylserine (PS). PS controls apoptosis, and reduced DHA levels causes a decreae in neural cell PS and elevation of the neuronal death (Serhan *et al*., 2004).

DHA was reported to modulate the carrier-mediated transport of choline, glycine, and taurine, the function of delayed rectifier potassium channels, and the response of rhodopsin contained in the synaptic vesicles and also having other important functions (Spector, 1999). DHA is metabolized to form the docosanoids, which include several families of potent hormones.

1.5. The role of fatty acids in neural functions

According to classical fluid mosaic membrane model, the cell membrane is a lipid bilayer incorporating a mosaic of proteins having a wide range of functions including transport and communication with other cells and the extracellular

medium. The latter function is of particular importance for neurons generating and transmitting signals within complex neural networks. The membrane is a very dynamic structure wherein the content, localization, and functional properties of the component macromolecules (lipids and proteins) are changing in activity-dependent manner. These changes in the composition of membrane lipids and proteins account for the adult neuroplasticity responsible for remodeling neural networks according to behavioral experience of the animal (learning and memory function).

The lipid and protein composition of the membrane affects the lipid-protein interaction and thus the membrane functional properties such as conductance of ion channels, clustering or dispersal of membrane receptors, their sensitivity to signaling molecules, activity of G-proteins and other enzymes involved in the intracellular signal transduction pathways, production of secondary messenger molecules, etc. Membrane phospholipids contain a variety of fatty acids of which mono- and polyunsaturated fatty acids constitute a substantial fraction. On the other hand, it is known that PUFAs are one of the main targets for the reactive oxygen species whose production increases with aging, and that increased peroxidation of membrane lipids leads to deterioration of neurons' functions and behavioral deficits.

The omega-3 DHA and omega-6 AA are the most common fatty acids in the brain (Crawford and Sinclair, 1971). In the cell membrane, there is about 12-15% more DHA than the other fatty acids. In recent clinical studies, it has been demonstrated that DHA shows protection effects on the brain and its dietary supplementation contributes to the amelioration of cognitive deficits in elderly subjects (Issa *et al*., 2006; Lukiw *et al*., 2005; Yurko-Mauroa, 2010) It has been also reported that DHA facilitates neurogenesis (Kawakita *et. al*., 2006), increases synaptic spine density in hippocampus (Sakamoto *et al*., 2007), and synaptic functions (Darios and Davletov, 2006; Wurtman *et al*., 2006). Another polyunsaturated fatty acid found in high amounts in the plasmalemma in neurons, the arachidonic acid, is involved in cellular signaling as a lipid retrograde second messenger implemented in the regulation of signaling enzymes, such as PLC-γ, PLC-δ and PKC-α, -β and -γ isoforms. Among other things, AA helps to maintain hippocampal cell membrane fluidity. Arachidonic acid is also known as a key inflammatory intermediate being

a substrate for the production of prostaglandins. However, under normal metabolic conditions, the increased levels of arachidonic acid in the body tissues are unlikely to trigger or increase inflammation since AA is metabolized to both pro-inflammatory and anti-inflammatory molecules such as tumor-necrosis factor-beta (Harris *et al*., 2009, Ferrucci *et al*., 2006). It was also reported that AA may protect the brain from oxidative stress by activating perioxisomal proliferator-activated receptor-y (Wang *et al*., 2006). It also activates syntaxin-3 (STX-3), a protein involved in the growth and repair of neurons (Darios, *et al*., 2006). Arachidonic acid supplementation showed some positive effects in human amnesiac patients, reversing the cognitive dysfunction resulted from either organic brain damages or age (Kotani *et al*., 2006).

In view of all this, it is hypothesized that a cognitive status of young but especially elderly subjects may be related to the brain levels of essential fatty acids.

1.6. The Aim of the Study

The aim of the present study was to elucidate the possible relationship between the levels of various brain fatty acids and learning/memory indices in aged and young mice classified as “good” or “poor” learners basing on their performance in a spatial learning task, the Morris Water Maze.

The levels of several fatty acids including palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acid were measured separately in samples from four different brain areas: hippocampus, cortex, striatum and hypothalamus. All these brain regions were shown to be implemented in different forms of learning and memory including episodic memory (hippocampus) and procedural memory (striatum).

CHAPTER 2

MATERIALS AND METHODS

2.1. Subjects

In the current study, 17 2-3 months old (young) and 46 22-23 months (old) female Swiss Webster (SW) mice were chosen as a subject. Mice were acquired from the Department of Medical Pharmacology of Gülhane Military Medical Academy (GMMA). During the experiments, animals were kept in the animal house in the Department of Biological Sciences at METU. In the animal house, the temperature was kept at 22°C ± 1 and light-dark cycle (12h light/12 h dark) was controlled. The animals had accessed freely to water and food (laboratory chow) during the study.

2.2. Apparatus

2.2.1. Morris Water Maze

Morris Water Maze (MWM, Morris, 1984) is a system that widely used for testing the place learning and long-term spatial memory (an equivalent of the human declarative memory) in small rodents such as rats and mice. (Figure 1)

Figure 1. Morris Water Maze apparatus.

In the present study, it was a circular tank, 60 cm high and 100 cm in diameter. The water depth was kept at 45 cm deep, and the temperature of water was automatically kept constant at 22 °C (±1) with a heater. A non-toxic dye was mixed into to water to give it an untransparent look. A clear square Plexiglas platform (10×10 cm) which can be moved preferably was placed at the middle of one of the quarters of the tank. When the platform is placed, the water level was set to cover 1cm top of the platform. So the mice could readily get onto the platform for avoiding the contact with water. With two abstract lines that intersect at the middle of the maze, four quarters referred as NW, NE, SW and SE were obtained in the software. The tank was divided into four quadrants (NE, NW, SE, and SW) by two imaginary perpendicular lines crossing in the centre of the tank. The room where the experiment took place was decorated with some clues outside the maze. The coordination of the clues was never changed till the end of the experiments. The ceiling lamps in the room provided light indirectly for the maze (Dursun, 2006).

2.2.2. Gas Chromatography (GC)

Fatty acid methyl ester (FAME) analyses were carried out in a Shimadzu gas chromatography system connected to a flame ionization detector. The gas chromatography studies were performed at the Medical Pharmacology department in Gülhane Millitary Medical Academy (GMMA).

The technique of chromatography is a very popular procedure among the contemporary chemical assays. The word chromatography is the combination of two Greek words; which are “chromos” that means color and “graphy” that denotes to write. Currently, chromatographic breakdown can be performed on samples both with and without color. Among the earliest developed chromatographic methods, gas chromatography (GC) is very popular yet and very reliable today. GC is commonly preferred method, because it is intensely selective, sensitive, giving precise results, performing with high resolution and also it can be worked with samples with greatly varied concentrations. GC remains as a credible method of study with growing range of application in various fields.

GC technique is fast, highly capable to give peaks. In addition, it can be equipped with different specific detectors such as mass spectrometer (MS) providing even more specific results. With various detectors' combination with GC, its selective power is amplified. Thus generally it is preferred as a technique for the study of easily vaporizing mixed elements because of the above mentioned advantages of the method (Figure 2).

Figure 2. Gas Chromatograph with an autosampler.

A mobile flowing phase, a column that includes the stationary phase, injection part, detector and a computer system are the elements of the GC system. The samples go through the column and with their characteristic preference among the stabile phase in the column and the flowing carrier gas that causes the separation.

The mobile flowing phase is a gas, which can be hydrogen, helium or nitrogen. It provides a mobile context in which sample can move across. The flow of the gas can be controlled.

Injector is responsible for the introduction of the sample solution to the GC system. The injector temperature can be adjusted in order to have a proper partitioning.

The column is the core of the GC system. It is the part of the system where the main partitioning takes place. The characteristic composition and structure of the column constitutes the immobile phase and it is a major influence for the sample partition. Column properties can affect many features of the process like time of

the analysis. The common immobile phases which are coated onto the inner side of the column have high molecular weights such as polysiloxane, polyethylene glycol, or polyester polymers. Columns sizes are highly variable; their length varies between 15 to 60 meters and their diameter can be from 0.25 to 0.32 millimeters. The column for the study must be chosen considering the chemical characteristics of the sample.

Oven: The oven contains the column. The oven temperature is adjustable in a great range starting from cryogenic temperatures to 300°C by the software. Oven is a protective field from all of the extreme temperatures. The oven provides a stable and precise temperature for the column. In addition, the oven temperature can be adjusted as a program with specific rate and time for the increases and the decreases that are desired. By having the programming, specific adjustments can be achieved that fits best to the feature of the sample and broadly separated peaks can be obtained in the analysis.

The detector is located at the end of the column. The compounds are vaporized and separated across the column and then enter to the detector. After the interaction, a signal is produced and a peak representing the characteristics and the concentration is obtained from each substance. There are a variety of detectors that can be used dependent on the sample type and the purpose of the analysis. The sensitivity can range from 10^{-15} to 10^{-6} gram of a single component. Depending on the aim of the analysis and the detector type, introduction of an internal or external standard of the desired compound may be necessary in order to get the exact position and retention time of the specific peak of the compound that will be analyzed. Detectors that are commonly used are; thermal conductivity detector (TC), mass spectrometer (GC-MS), flame ionization detector (FID) and electron capture detector (ECD).

Thermal conductivity detector is principally based on the electrical current obtained from a wire in the mobile phase flow. The detection is achieved by the changes in the voltage derived from the change in the compounds' molecular volume.

The flame ionization detector (FID) includes a tiny flame that uses hydrogen or oxygen. The substances get through the flame causes ion production and a current occurs which can be measurable by the detector (Figure 3.). It is known as

a very sensitive detector especially for the analysis of molecules that have C-H bonds but not for the other substances.

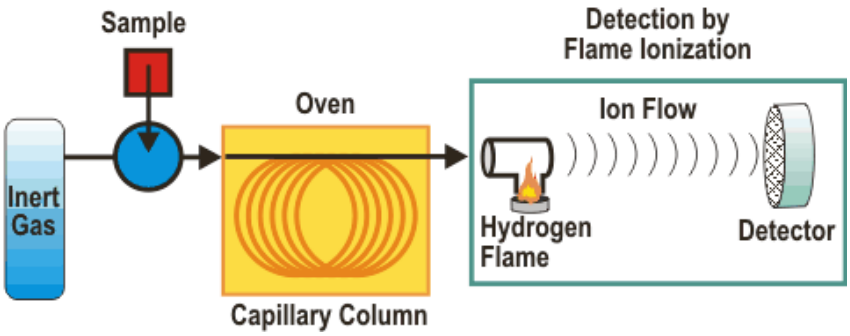

Figure 3. A schematic representation of a FID detector.

Electron capture detector (ECD) uses a radioactive source for the separation of the compounds. The ions are produced from the source causes an ion current which can be measured by the detector. It is a suitably used with the FID detector. Argon is used as the mobile phase.

Mass spectrometer (MS) can be a valuable tool with specific separation of analyts when coupled to a gas chromatograph. MS provides the identification of each molecule analyzed and also gives information about the concentration. The ions are detected regarded to their molecular weight and give a peak with a characteristic mass to charge ratio. The spectra obtained from the analysis are often compared to data from previously defined spectra in the “library” of thousands of substances. The coincidence makes reliable identification possible without using an internal standard.

In gas chromatography, the sample vapour passes through the column and separates into its components - a separation that is governed by the distribution between the mobile and stationary phase. The degree of separation between the sample and the stationary phase is determined by flow rate, the nature of the stationary phase, the surface area exposed to the carrier gas, and the column temperature. GC uses temperature changes to improve separations. Then the gas escapes from the column entering to the detector. A signal is derived from the detector giving a peak specific for each compound of the sample. Every peak can be observed at their specific retention time. The separation efficiency is called as

resolution. As the individual components emerge from the column, the detector obtains a signal and relays the message to the data.

GC is not available for the identification of the components unless coupled to a MS detector. Nevertheless, in routine analysis, the standard is used for making a calibration curve for the components to be analyzed. So with the knowledge of the retention time of a known substance, the analysis can yield a great specificity (Figure 4).

Figure 4. A typical chromatogram showing specific peaks of the fatty acid in the study

Today, the studies on fatty acids and their derivatives can easily be conducted with the current methods in gas chromatography. There is a great variety both in the column types and detector types. An appropriate combination must be chosen in order to yield results with both high resolution and specificity. The most common derivatization of fatty acids for the GC analysis and the FID detector is fatty acid methyl esters (FAME) formation (Shantha and Napolitano, 1992).

2.3. Experimental Procedure

2.3.1. Handling

All of the animals were handled and weighted for consecutive seven days prior to the experiment, in order to get the animals used to the experimenter.

2.3.2. Shaping Training

Shaping training was executed to make animals get used to the new environment, learn to swim and escape onto the platform. Since the new environment and contacting with water can be a very stressful factor interfering with the mnemonic performance of the animal. During a day, the platform was centered in a random quarter 30 cm away from the pool's edge. Around the tank, a dark curtain was placed in order to avoid the interference of other spatial clues and/or the movement of the experimenter.

Each mouse was introduced to the maze four times per session from varying points: starting from a very close point the platform, and then from gradually increasing distance for reaching the platform. Every time the subjects swam in order to escape from the water until the time they escape to the platform. When an animal couldn't find the platforms location in 60 seconds, it was then gently directed and put onto the platform by the experimenter.

2.3.3. MWM Acquisition Training and Probe Trial Tests

During place learning, platform has been moved to a new position and curtains were removed. The platform position was kept constant throughout the training. The place learning was carried out for seven days with one session in each day and four trials per session. Each mouse introduced to the water while facing the pool borders from one of the four starting points that named as N, S, E and W). The releasing point was determined in a random way while avoiding the repetition of the pattern in consecutive days and each releasing point was used for one time in every session. Each trial ended when the subject found the platform and the subject was kept on the platform for 15 seconds. The subjects were given 60 seconds per session to find the platforms location. Afterwards, the mouse was taken to the home cage for approximately 5 minutes in their inter-trial interval.

Software for the video tracking (EthoVision System by Noldus Information Technology, Holland) was employed to track and record the animals movement in the maze. The movements of the subjects were recorded with a camera was placed onto the ceiling just above the tank and was coupled to a microprocessor.

The following parameters were recorded by The Noldus EthoVision video-tracking system:

1) Swim path trajectory of the subject,
2) Escape latency to the platform: Time, in seconds, between the starting point and reaching the escape platform,
3) Distance swum: The distance that the subject swum, in centimetres, from the starting point to the escape platform,
4) The average swimming velocity of the subject.

2.3.4. Probe trial

After the 7 days of place learning, animals were subjected to a 45 second probe trial, a place preference test used to additionally measure the strength of the place memory in mice.
In the course of the probe trial, the platform was removed from the tank and thus unavailable to the animals. At the software, an abstract annulus (annulus 40) which is 40 cm in diameter was placed around the circumference where the platform was used to be located. The time spent of subjects in the platform quadrant and the annulus 40 in order to find the platform was recorded.

2.3.5. Sample Preparation for Gas Chromatography Measurements

2.3.5.1. Decapitation, Brain Removal and Dissection.

On the completion of behavioral studies, mice were anesthesized with the overdose of ketamine and xylasine mixture and mice were decapitated. Brains were removed and hippocampus, hypothalamus, striatum and cortex were disected. All the tissue samples were immediately frozen in liquid nitrogen and stored at – 80°C until analysis by gas chromatography (GC).

2.3.5.2. Tissue Homogenization and Lipid Extraction

Each tissue sample was dissected into small pieces with a razor blade. Homogenization was done using a homogenizator. 2 ml n-hexane (GC grade)

containing butylated-hydroxy toluene (BHT) (80mg/lt) was used as solvent. After centrifugation in 4000 rpm, the upper phase (supernatant) was taken.

2.3.5.3. Fatty acid methyl ester (FAME) formation

Gas chromatography is a common process for the study of long chain fatty acids that have more than 12 carbon atoms in many fields of study. But the carboxylated contents cannot be analzed by GC easily because their structure is highly polar. In order to get a clear result, derivatization of the fatty acids is required and it ensures more volatile and nonpolar compounds.

In order to achieve that, a very commonly used derived type of fatty acids; fatty acid methyl esters (FAMEs) were obtained by methylation. For methylation, methanolic HCl (%5 v/v) was added to the solution. Then samples were incubated in hot water bath at 75°C for one hour. After one hour, tubes were centrifugated in 4000 rpm. The clear upper phase were taken into vials and used for GC analysis.

2.3.6. Standarts

All the standarts used; Arachidonic acid, Linoleic Acid, Oleic Acid, Stearic Acid, Palmitic Acid and DHA methyl esters were purchased from Sigma & Aldrich and were GC grade.

For standardization, 0.1 grams for solid standards and 3-4 drops for liquid standards were dissolved in 5ml n-hexane each. Then 20µl of each standard solution was diluted in 1ml n-hexane and taken into GC vials. Measurements for each of the standard samples were carried out by the same method as for the experimental samples.

2.3.7. Calibration

Evaluation of data for FAME studies were carried out with “% area” calculation. In order to do that, each of the standart fatty acids’ retention time was introduced to the software from standardization measurements. Undesired and irrelevant peaks were eliminated before calibration.

2.3.8. FAME Analysis

As mentioned earlier, the FAME measurements of the samples were carried out in Shimadzu gas chromatography equipped with a flame ionization detector (FID). The working method parameters were as described below:

DB-23 was selected as a column for the analysis of FAME's. The column temperature was set to 90°C at the beginning, and with 5 °C rate, the final temperature was held constant at 240 °C. Total analysis time was 52 minutes. The detector temperature was set to 250°C. Helium was used as a carrier gas. Gas flow was 30ml/min. Hydrogen flow was 40 ml/min and air flow was 400 ml/min. Injection volume was 1 µl.

Each analysis resulted in the separation of the fatty acids as a function of their hydrocarbon chain length, in other word; their molecular weight. Every fatty acid has a specific retention time dependent on the analysis method and can be identified by its specific peak.

2.4. Data Analysis

In the behavioral experiments, from all data, group means ± standard error of mean (SEM) were calculated. The data were analyzed with treatment as independent factor, and sessions or trials as repeated measures. Tukey test was used for Post Hoc analysis of the data. In the GC studies, the results were expressed as means ± standard error of mean (SEM). A "p" value which is lesser than or equal to 0.05 was considered as statistically significant.

In order to evaluate the data, old and young groups were divided into 3 groups according to their escape latency performances on MWM test. These groups are: Good learners (escape latency is shorter than group average – 3SEM in at least 3 days of last 4 training days), poor learners (in which escape latency is longer than group average + 3SEM in at least 3 days of last 4 training days), and intermediate group (the others).

In both of the behavioral and GC studies, the statistical package SPSS 15.0 for windows was used to compare the results with ANOVA.

All of the steps in the current study were conducted according to the rules in the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA) (Institute of Laboratory Animal Sources Commission on Life Sciences, National Research Council, 1996).

CHAPTER 3

RESULTS

3.1. Results of Behavioral Tests

3.1.1. Learning Tests

3.1.1.1. Classical MWM Training

Figure 5 shows the course of spatial learning in the MWM throughout the 7 consecutive training days, 4 trials per day, in the groups of young (2-3 months old, n=14) and old (22-23 months old, n=31) Swiss Webster (SW) mice. As seen from this figure, in both groups, the escape latency to reach the platform was decreasing over the first 3 days of training to reach the asymptotic level of about 33 seconds in old and 25 seconds in young subjects.). The two-way (group x day) ANOVA for repeated measures yielded a significant day effect ($F_{(6:258)}$ = 11.36, p≤ 0.001),with group effect and day x group interaction insignificant.. As seen from Fig. 5, there was no difference between the groups at the beginning of the training, however, faster decrement in escape latencies was observed in the group of young animals as compared to the old subjects. Repeated measure ANOVA performed for the last 4 days of training revealed insignificant day effect, insignificant group x day interaction and significant group effect ($F_{(1:43)}$ = 4.34, p≤ 0.043)

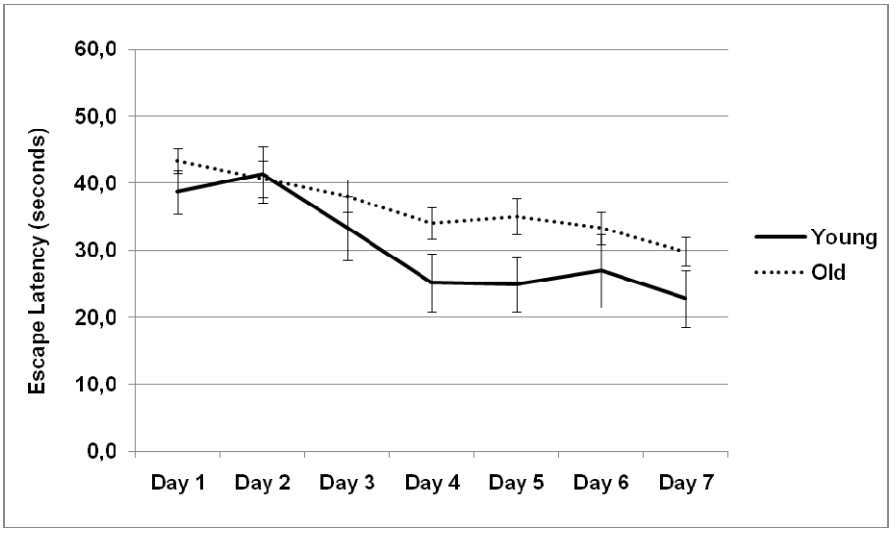

Figure 5. Mean escape latency (± SEM) in the water maze calculated for each age group and each training day separately. Error bars denote SEM.

However, as seen in Fig. 6, there was a great difference in the swimm velocities between old and young subjects with old animals swimmining significantly slower . In this situation, escape latency is not a credible measure of animals' learning capabilities. Therefore, in this study, the animals learning capacity was evaluated basing on the swim distance to reach the platform.

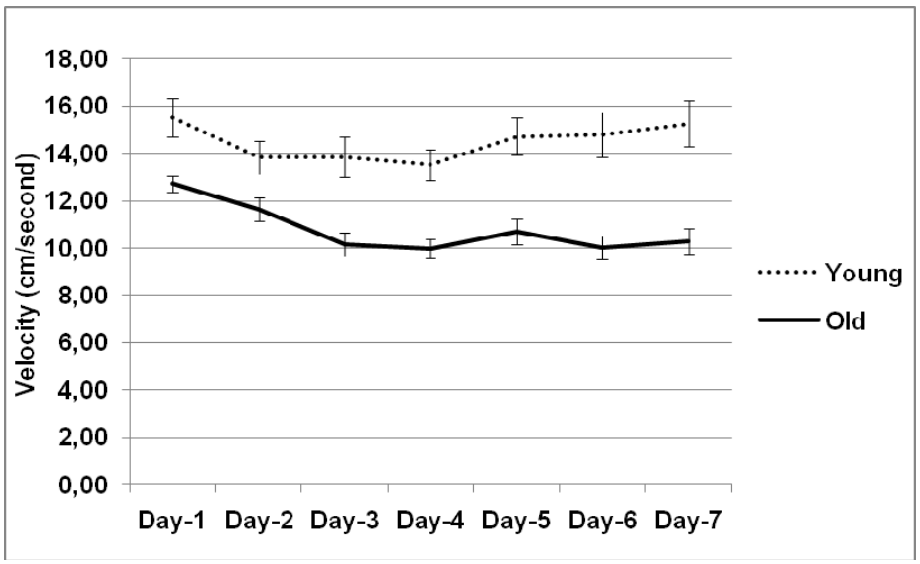

Figure 6. Mean swimming velocities (± SEM) calculated for each group and each day of training separately. Error bars denote SEM.

As seen from Fig. 7, in both age groups, swim distance shortened steadily throughout the first 3 days of training to reach a similar asymptotic level on the following 4 days. The two-way (group x day) ANOVA for repeated measures yielded day effect significant ($F_{(6:258)}$ = 20,26, p≤ 0.001), with the main group effect and day x group interaction insignificant.

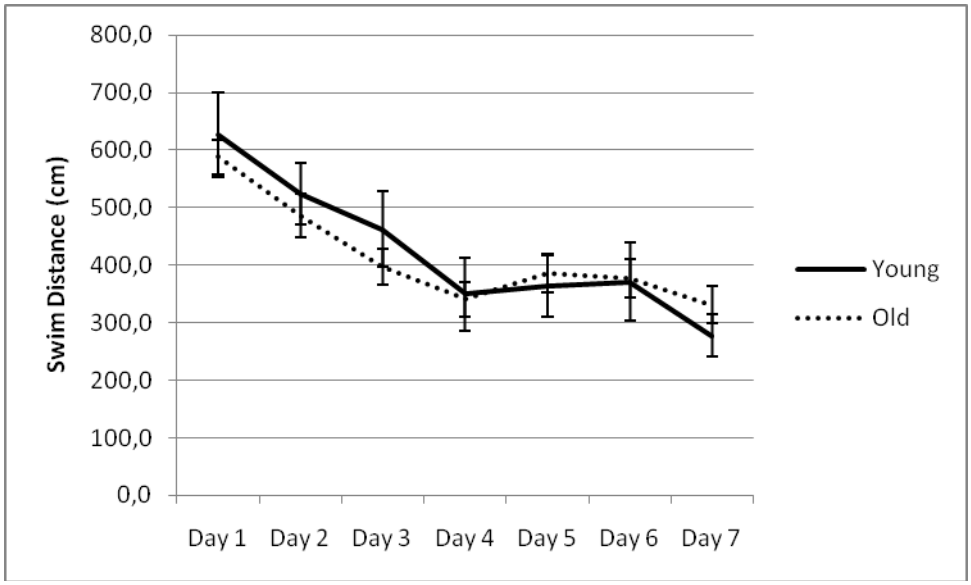

Figure 7. Mean swim distance (± SEM) in the water maze calculated for each of the seven training days and each age group independently. Error bars denote SEM.

Basing on their performance during the last 4 days of the training, both aged and young mice were divided into three subgroups: the “good learners” with swim distance ≤ group mean – 3SEM, the “poor learners” with swim distance ≥ group mean + 3SEM, and the intermediate group .

According to this classification; in young group of 14 mice, 3 good learners, 3 poor learners and 8 intermediate learners were determined. In aged group of 31 old mice, 7 good learners, 8 poor learners and 16 intermediate learners were determined.

Figure 8 demonstrate the learning curves for the young group treated as a whole and the old –good and old-poor learners independently.

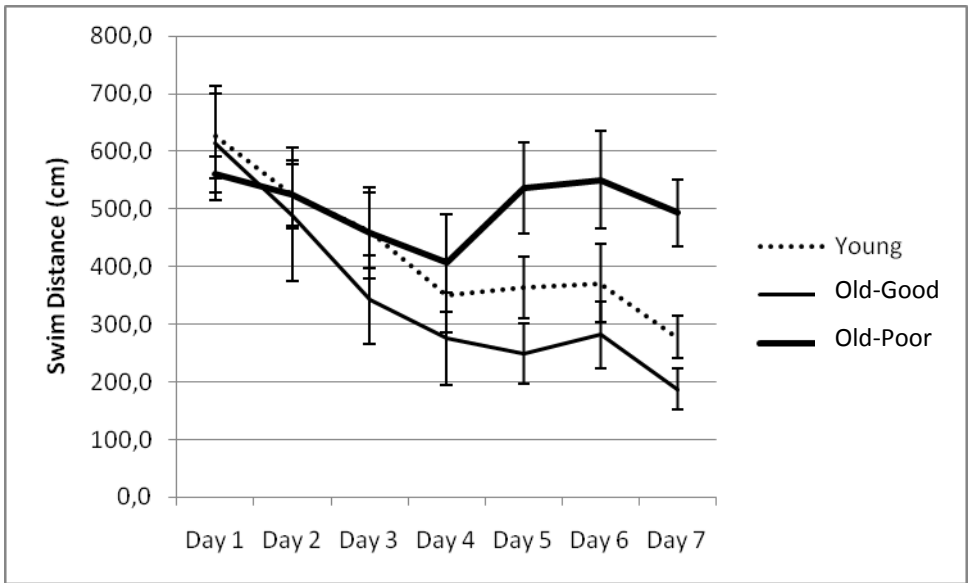

Figure 8. Mean swim distance (± SEM) in the water maze calculated for each training day independently in young mice, old-poor, and old-good learners group. Error bars denote SEM.

As seen from this graphic, towards the end of the training, the old good-learners performed better than the average young mice, while the old poor-learners demonstrated substantially worse perfromance as compared with the two remaining groups. The two-way (group x day) ANOVA for repeated measures yielded a significant day effect ($F_{(6:156)}$ = 12,02, p≤ 0.001), significant day x group interaction ($F_{(12:156)}$ = 2,05, p≤ 0.023), but found the main group effect insignificant. However, repeated measures ANOVA performed for the last 4 days of training revealed both the day effect and the group effect significant ($F_{(3,78)}$ = 2,49, p ≤ 0.066, and $F_{(2,26)}$ = 4,05, p ≤ 0.029, respectively). The LSD post-hoc comparisons confirmed significantly worse performance in old poor-learners as compared to both, young mice and old good-learners (p≤ 0.05 and p≤ 0.01, respectively).

Figure 9 demonstrates learning curves for the groups of young and old good and poor learners. The two-way (group x day) repeated measures ANOVA yielded a significant day effect ($F_{6,114)}$ =,10,826, p≤ 0.001), significant day x group interaction (($F_{18,114)}$ =,1,804, p≤ 0.033), and significant main group effect ($F_{(3:19)}$ = 4,935, p≤ 0.011). The LSD post-hoc comparisons confirmed significantly worse performance

in both old and young poor-learners as compared to both old (p≤ 0,003 and 0,0001, respectively) and young good-learners (p≤ 0.001 and p≤ 0.003, respectively), with no significant difference between young versus old good learners or young versus old poor learners. Repeated measures ANOVA performed for the last 4 days of training revealed the day effect and the day x group interaction insignificant which indicates that within the last 4 days of training, all the groups achieved the asymptotic performance level. However, the main group effect remained significant ($F_{(3.19))}$ = 9,114, p≤ 0.001).

The LSD post-hoc comparisons confirmed significantly worse performance in both old and young poor-learners as compared to both, old (p≤ 0.003 and 0.006, respectively) and young good-learners (p≤0.0001 and p≤0.001, respectively), with no significant difference between young versus old good learners or young versus old poor learners. Despite of the lack of an overall significant difference between young and old good learners, as seen from the Fig.9 young good learners achieved the asymptotic performance level on 4th day of training while the old good learners achieved similar performance level only on the 7th day of training.

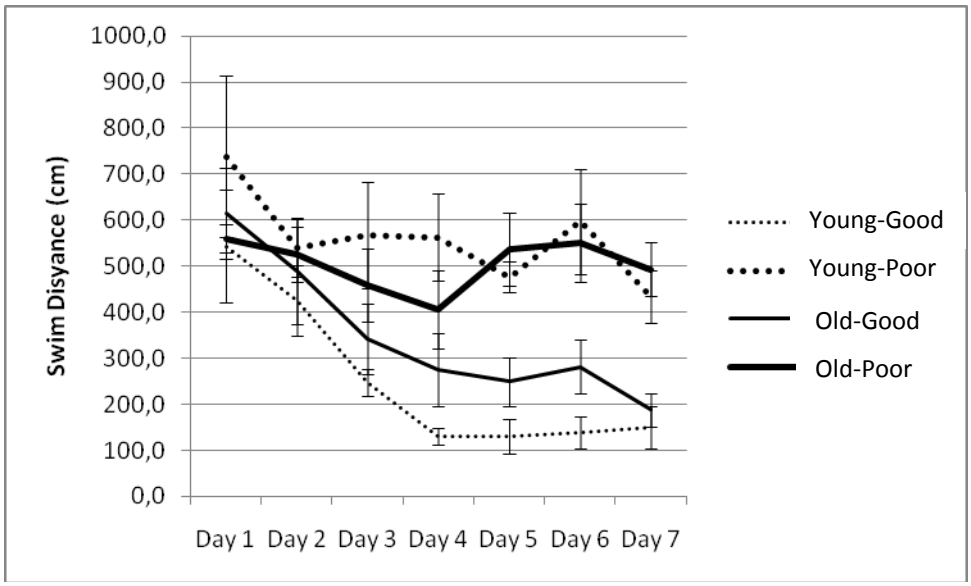

Figure 9. Mean swim distance (± SEM) in the water maze calculated for each training day and each group independently. Error bars denote SEM.

Due to the additional animal losses prior to decapitation, biochemical assays were carried out on 12 young mice: 4 good, 4 poor, and 4 intermediate-learners, and 15 old mice out of which 6 belonged to good, 4 to poor and 5 to the intermediate-learners. Figure 10 presents learning curves for the groups of young and old good- and poor-learners used in biochemical assays. The results of this analysis are similar to the results presented in Fig.9. The two-way (group x day) repeated measures ANOVA yielded a significant day effect ($F_{(6:84)}$ = 9,15, p≤ 0.001), and significant main group effect ($F_{(3:14)}$ = 5,92, p≤ 0.008). The LSD post-hoc comparisons confirmed significantly worse performance in both old and young poor-learners as compared to both, old (p≤ 0.042 and 0.025, respectively) and young good-learners (p≤ 0.008 and p≤ 0.005, respectively), with no significant difference between young versus old good-learners or young versus old poor-learners. However, the young good-learners achieved the asymptotic level of performance earlier (the 4th training day) than the old good-learners (the 7th training day).

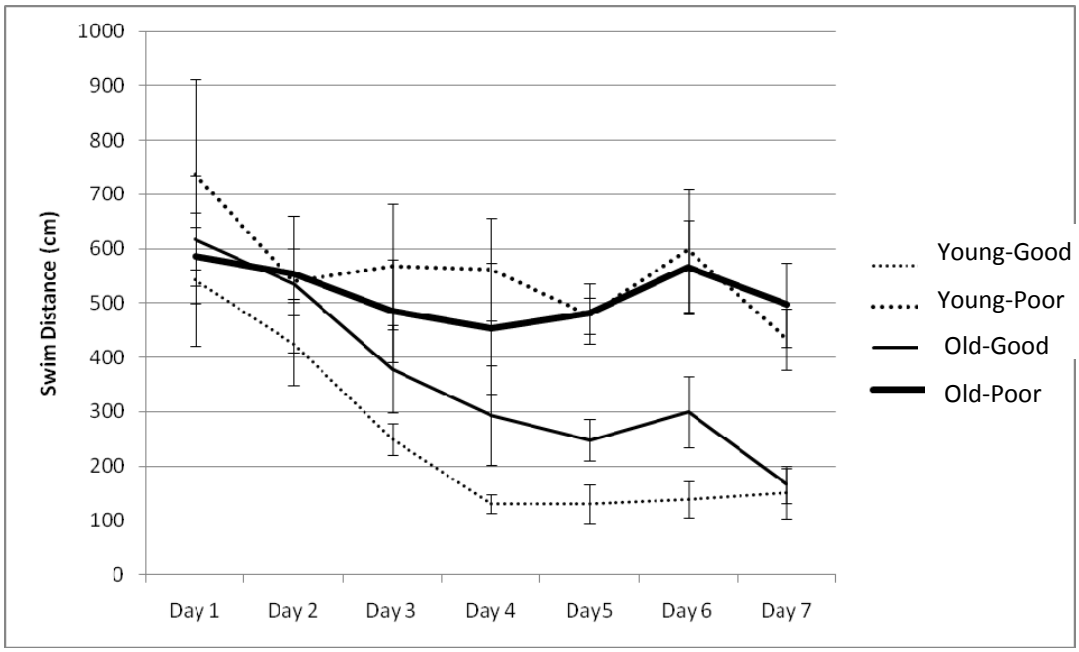

Figure 10. Mean swim distance (± SEM) in the water maze calculated for each training day for the subgroups of young and old good- and poor-learners consısting of animals used in biochemical assays. Error bars denote SEM.

3.2. Gas Chromatography Studies

Gas chromatography (GC) analysis was made for 6 different fatty acids: palmitic acid, stearic acid, oleic acid, linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in four distinct brain regions; cortex, hippocampus, striatum and hypothalamus belonging to to young versus old mice and to young good- and young poor-learners as well as old good- and old poor-learners. The levels of examined fatty acid methyl esters (FAMEs) were determined by the area under the peaks and given as "% area".

As seen from Fig. 11-13, in the cortex, hippocampus, and striatum, no significant difference was found in the levels of six examined fatty acids between young and old mice groups including good, intermediate and poor learners.

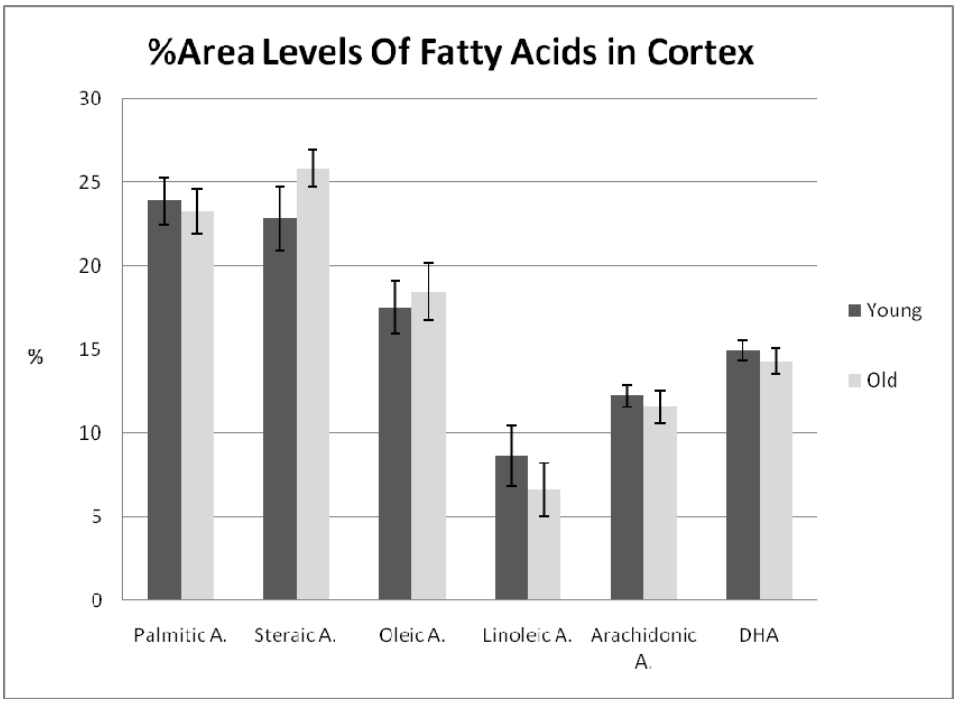

Figure 11. The comparision of levels of six fatty acids in the cortex of young and old mice. Error bars denote ± SEM

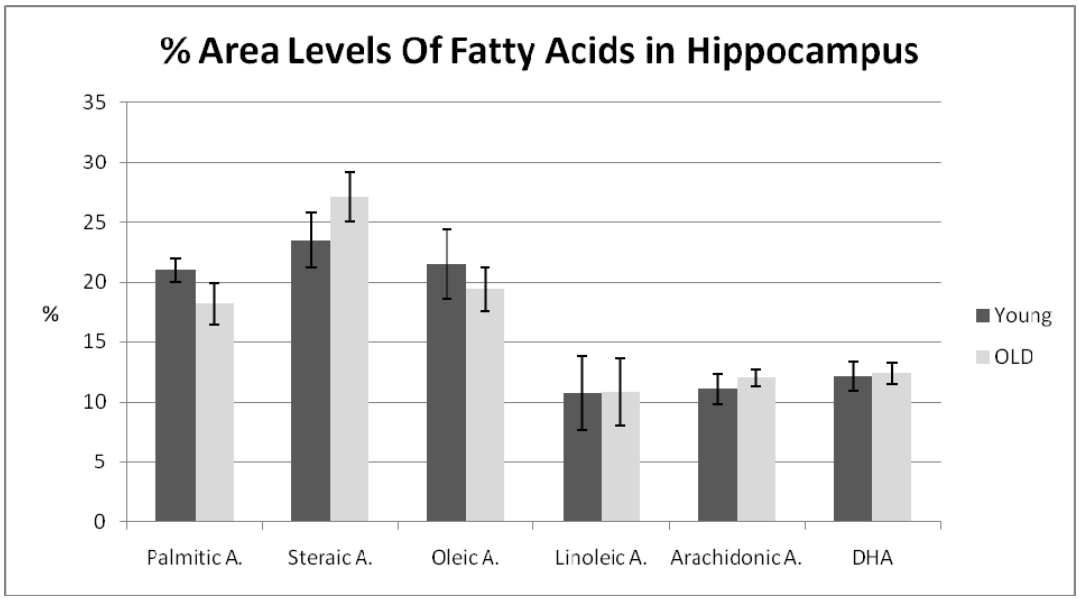

Figure 12. The comparision of levels of six fatty acids in the hippocampus of young and old mice. Error bars denote ± SEM.

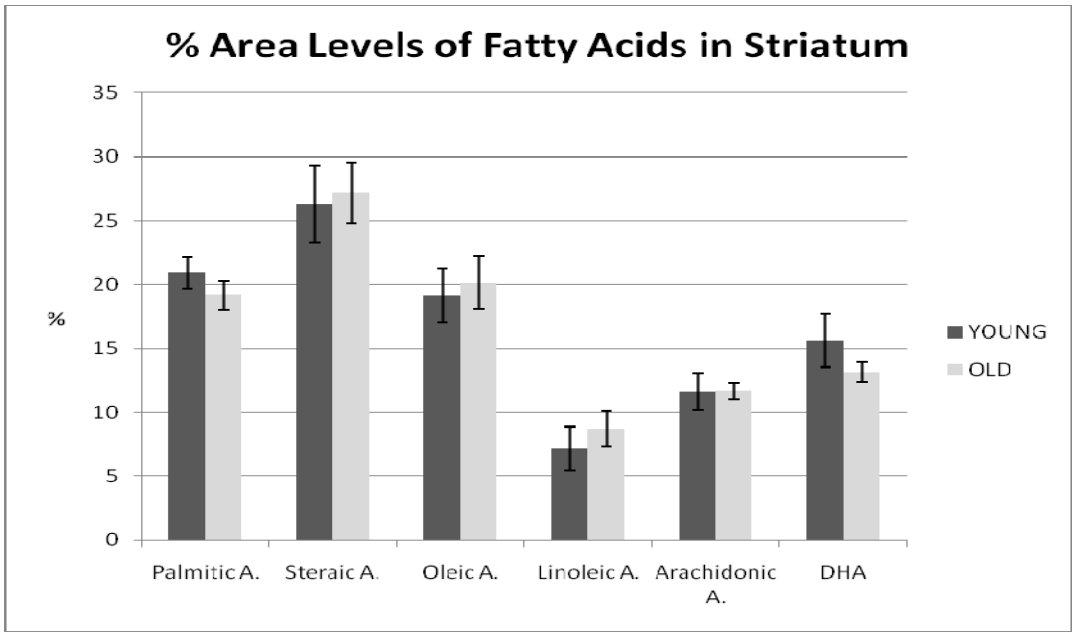

Figure 13. The comparision of levels of six fatty acids in the striatum of young and old mice.. Error bars denote ± SEM.

As it is shown in Fig.14., in the hypothalamus of the old mice, the level of oleic acid was significantly higher ($p \leq 0.018$) while the level of DHA was significantly lower ($p \leq 0.016$) as compared to the same regions in the young mice.

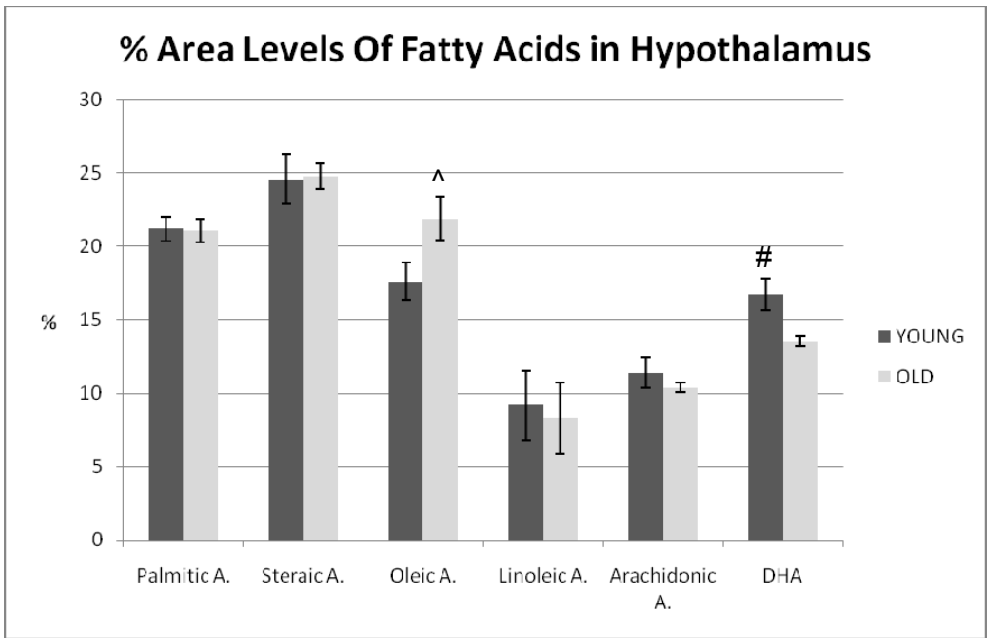

Figure 14. The comparision of levels of six fatty acids in the hypothalamus of young and old mice. Error bars denote ± SEM. Asterisks denote the level of significance at p≤ 0.018 ^ and at p≤ 0.016 #.

When the levels of fatty acids in four brain regions including cortex, hippocampus, striatum and hypothalamus were compared between mice groups classified according to their performance in the MWM as young poor- and good learners and old poor- and good-learners, the main group effect was found significant (($F_{(3:15)}$ = 3,23, p≤ 0.05) for arachidonic acid (AA) in the hippocampus only.

The post hoc LSD test confirmed significantly higher level of AA in young poor-learners as compared to young good-learners (p≤ 0,014) and significantly higher level of AA in old good-learners as compared to young good-learners (p≤ 0.032). In the same brain region, the level of linoleic acid was significantly (p≤ 0.034) higher in young good-learners as compared to old good learners and significantly (p ≤ 0.025) lower levels of stearic acid was found in young good learners as compared old good learners (see Figure 15).

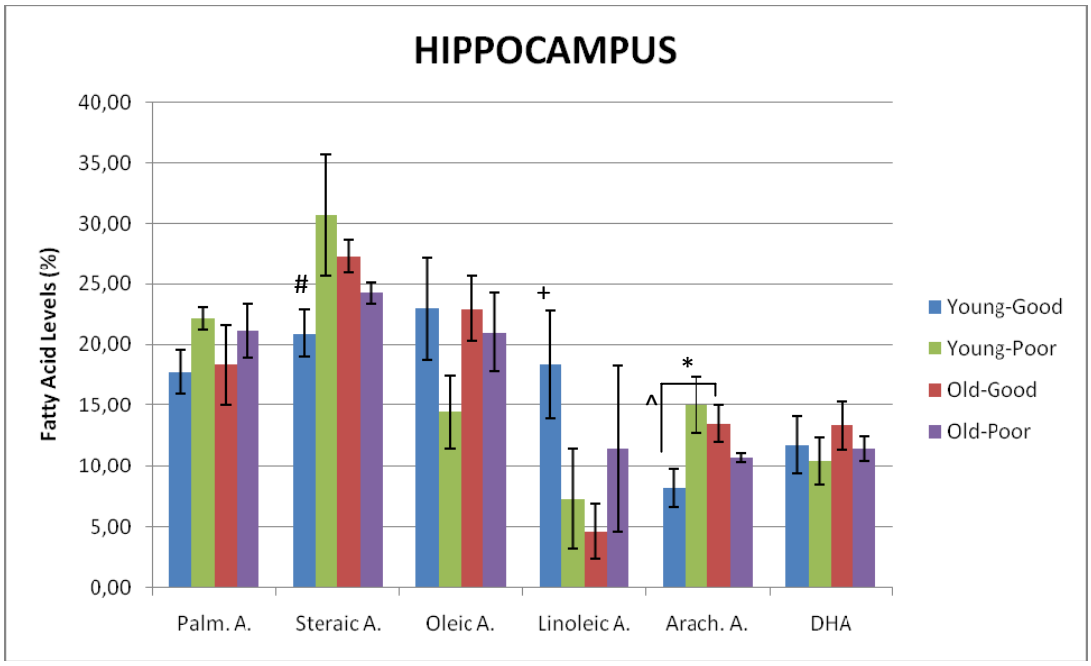

Figure 15. The comparision of levels of six fatty acids in the hippocampus of young and old good- and poor-learners. Error bars denote ± SEM. Asterisks denote the level of significance at p≤ 0.014* , p≤ 0.032^, p≤ 0.034+ and p≤ 0.025#.

Pearson's correlation analysis confirmed a significant (p≤ 0.0146) negative correlation between hippocampal levels of arachidonic acid and animals performance in the spatial memory task but only in young subjects (Fig. 16 A). No significant correlation was found for the same two measures in the group of old mice (Fig.16 B).

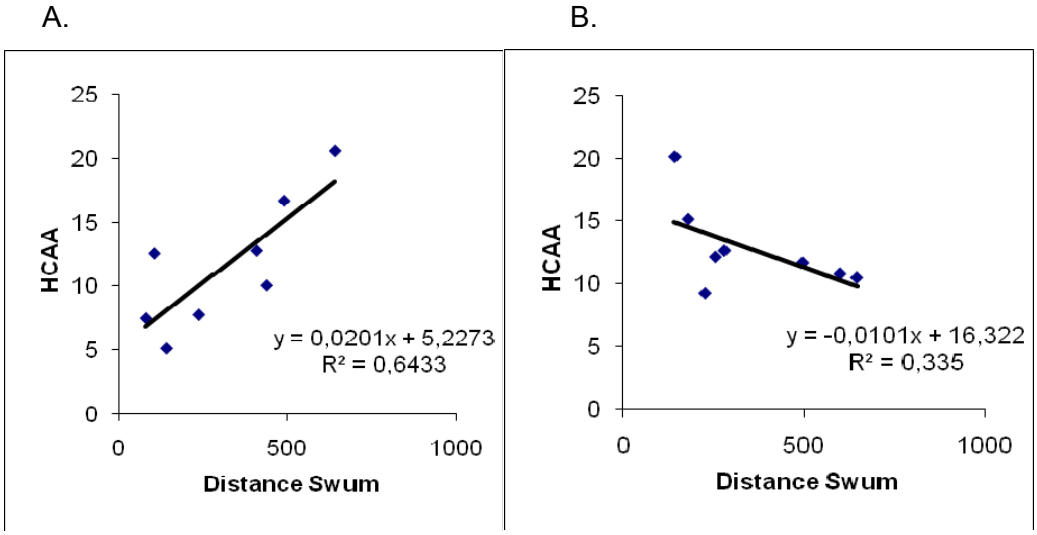

Figure 16. Pearson's correlation between the mean swim distance (cm) calculated for the last 3 days of training in the MWM and the hippocampus AA levels in the group of young mice.

In the cortex (Fig. 17), the level of oleic acid was found significantly higher in poor-young learners compared to the good-young learners ($p \leq 0.047$).

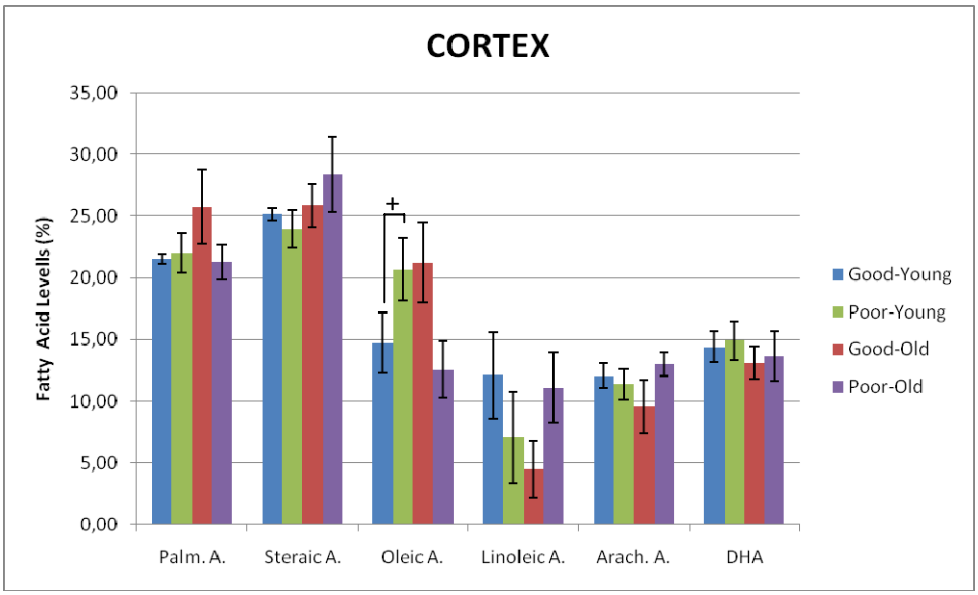

Figure 17. The comparision of levels of six fatty acids in the cortex of young and old good and poor learners. Error bars denote ± SEM. Asterisk denotes the level of significance at $p \leq 0.047+$.

The opposite was true for the old mice but the difference was only marginally significant ($p \leq 0{,}084$).

In striatum, no significant between-group differences were found both between groups and within groups (Figure 18).

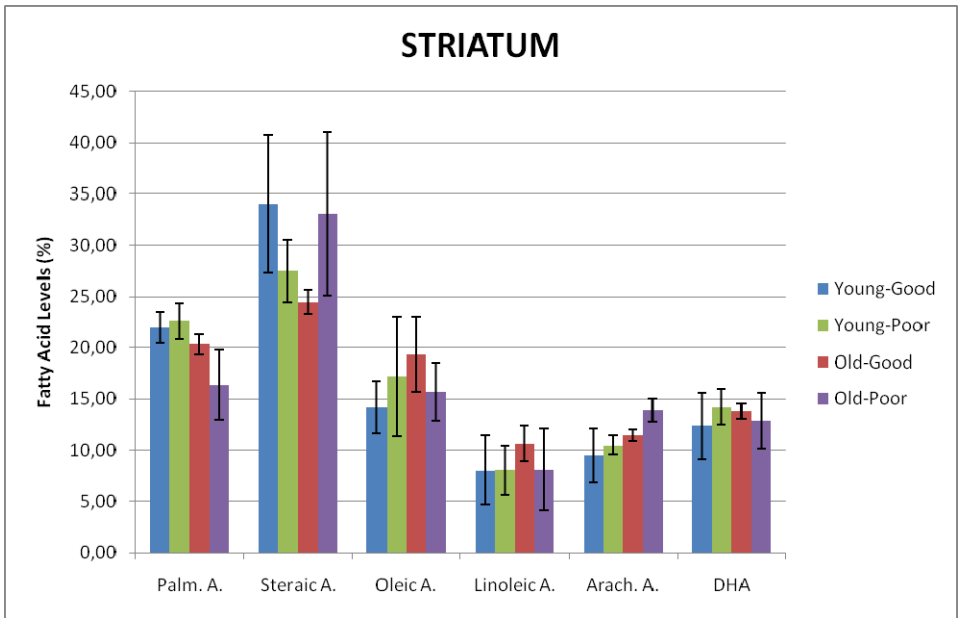

Figure 18. The comparision of levels of six fatty acids in striatum of young and old good and poor learners. Error bars denote ± SEM.

In hypothalamus (Figure 19), DHA level in young good-learners was found significantly ($p \leq 0{,}041$) higher than DHA level in old poor-learners but not than that in young poor-learners.

Also the stearic acid level was significantly lower $p \leq 0{,}048$ in young good-learners than in young poor-learners while no difference was noted in the stearic acid level between old good and poor learners.

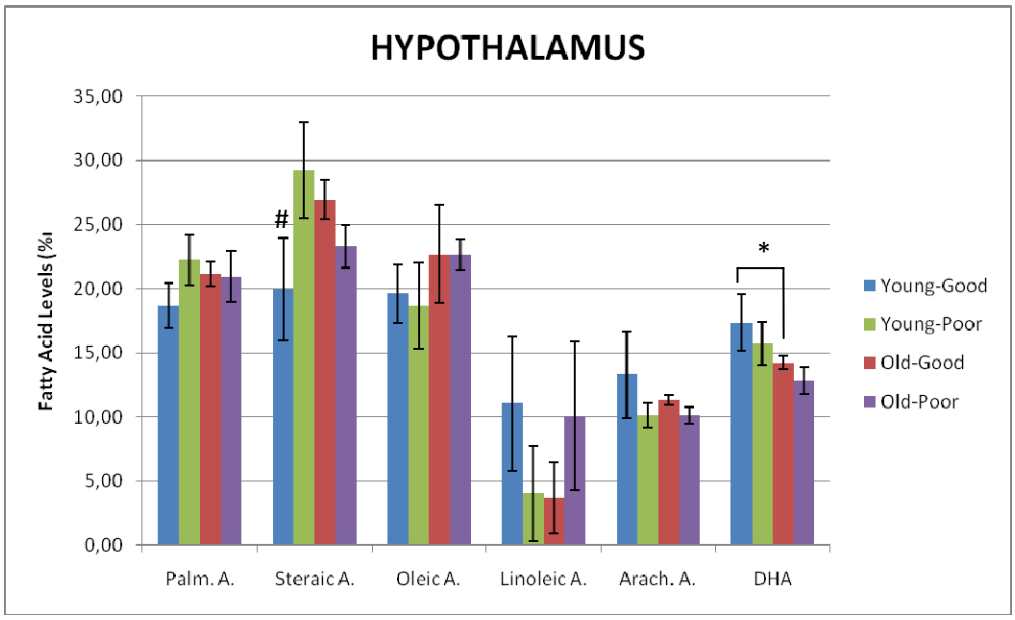

Figure 19. The comparision of levels of six fatty acids in hypothalamus young and old good and poor learners. Error bars denote ± SEM. Asterisks denote the level of significance at p =0,041* and at p= 0,048#

CHAPTER 4

DISCUSSION

In the present study, the levels of palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acid were measured in four different brain areas: hippocampus, cortex, striatum and hypothalamus of young and aged female mice classified as "good" or "poor" learners basing on their performance in a spatial learning task, the Morris Water Maze.

The results of behavioural testing in the spatial reference memory task , the MWM, confirmed a well known general fact that the learning capacity is declining with aging. However, a great individual variation in learning scores between aged subjects was observed. Some of the aged mice demonstrated significant deficits in the applied learning task while some other mice from the same age group performed as well as the young subjects. These results remain in line with earlier reports by other authors showing that chronological aging is not always parallel to biological aging (Barnes, 1988, Baxter and Gallagher, 1996, Gallagher *et al*., 2003, Gallagher and Pelleymounter, 1988, Geinisman *et al*., 1995, Rapp and Amaral, 1995).

According to the results obtained in the present study, there are no regional differences in the relative concentrations of different fatty acids in the rat brain. In all examined brain samples, the saturated fatty acids, palmitic and stearic, showed the highest concentrations, with intermediate levels of monounsaturated oleic acid, and the relatively lower levels of polyunsaturated acids, especially linoleic acid. The consistency of these results is at the same time proving the accuracy of the performed measurements.

Between-group differences were specific to the type of fatty acid and the brain region examined. In the cerebral cortex, a significant difference was found only in the concentration of oleic acid between young poor and young good learners with higher levels in young-poor learners. However, a significantly lower level of oleic acid was shown in old poor learners as compared to young poor learners despite of the lack of a significant difference between the two latter groups in the place learning task. These results are arguing against the relevance of the fluctuations in the cortical oleic acid concentration for the learning process. In the cerebral cortex, the concentration of stearic acid was significantly higher in old poor learners as compared to young poor learners. The latter difference also cannot be related to the animal's learning skills since there was no significant difference in the task performance between these two groups.

In the hypothalamus, opposite to the cortex, the oleic acid concentration in young-poor learners was significantly lower than that in both, old poor and old good learners. Alike in the cerebral cortex, these fluctuations in oleic acid levels did not correlated with animals' performance in the place learning task.

In the striatum, no significant between-group differences were noted for any of the fatty acids studied.

In contrast, in hippocampus, the structure known as the most important for spatial learning and memory, a significant difference ($p \leq 0.01$) in AA concentration was found between young poor and young good learners, with higher AA levels in poor learners. Also a significantly higher AA level was recorded in old good learners as compared to young good learners which correlated with the significantly lower memory scores in the old good learners as compared to young good learners. The Pearson's correlation analysis confirmed the significant negative correlation between learning scores and hippocampal levels of AA in young but not in old subjects. The potential adverse effect of relatively higher AA concentration in mouse hippocampus of young mice is contradictory to the earlier reports by other authors related to protective antioxidant effects of AA (Wang et al., 2006), activation of syntaxin-3, a protein postulated to be involved in the growth and repair of neurons (Darios *et al*., 2006), and beneficial effects of AA supplementation in human amnesic patients, (Kotani *et al*., 2006). It is, however,

consistent with some contradictory findings such as a recent clinical report suggesting that disturbed metabolism of AA and its increased levels may be associated with neurological disorders such as Alzheimer's disease (Rapoport, 2008). Sanchez-Mejia and Mucke, (2010) reported also that AA and specific isoforms of phospholipase A(2) (PLA2) appear to be critical mediators in beta-amyloid (Aβ)-induced pathogenesis, leading to learning, memory, and behavioral impairments in mouse models of AD. The negative correlation between hippocampal levels of AA and learning scores may be accounted by the AA action as a retrograde neurotransmitter at glutamatergic synapse where it is inhibiting glutamate release from the presynaptic terminals (Herrero *et al*., 1992).

In the current study, among old animals, the significant difference in place learning between poor and good learners was not paralled by the significant difference in the hippocampal AA concentration. On the other hand, despite of the lack of a significant difference in the animal performance between young and old poor learners, the level of AA was significantly higher in young poor learners as compared to old poor learners. These results suggest that AA levels in the hippocampus may have impact on learning capacity in young subjects but not so much in the old ones. The cognitive status of the elderly subjects must be then determined by some other factors.

The unexpected finding of the present study was the absence of significant differences between both young and old subjects as well as between good and poor learners in the brain levels of DHA. The only significant difference was found between overall old and young hypothalamus DHA levels as higher levels in young group. But this finding doesn't correlate with the learning skills in MWM test. This data suggests the lack of the relation between aging and animal's cognitive status and the brain levels of DHA. The latter finding is contradictory to several reports from clinical and animal studies about amelioration of cognitive deficits in AD patients and reduction of amyloid plagues and tau tangles in mouse model of AD after dietary DHA supplementation (Issa *et al*., 2006, Lukiw *et al*., Yurko-Mauroa, 2010). It is also inconsistent with earlier reported beneficial effects of DHA promoting neurogenesis (Kawakita *et al*., 2006, increasing synaptic functions (Sakamoto *et al*., 2007, Darios *et al*., 2006, Wurtman *et al*., 2006). This last finding is, however, in line with the recently published clinical studies showing that

the supplementation with DHA compared with placebo had no effect on the rate of cognitive and functional decline in patients with mild to moderate Alzheimer disease (Quinn *et al*., 2010). Interestingly, it has been also reported that when DHA, commonly known to have beneficial effects on cognitive functions, was administered to AD patients in combination with arachidonic acid, plaque formation was greater than without the arachidonic acid.

CHAPTER 5

CONCLUSION

1. The results of behavioural testing in the spatial reference memory task confirmed that the learning capacity is declining with age, However, a great individual variation was revealed in learning scores between aged subjects suggesting that biological aging is not always parallel to chronological aging.

2. The relative levels of palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acids in the four examined brain structures including cerebral cortex, striatum, hypothalamus, and hippocampus were very similar.

3. Except the hypothalamus, no significant relation has been found between the brain levels of DHA omega-3 acid and the animal's age or cognitive status. This finding remains in line with a recent discussion about the valuability of DHA supplementation as an effective protective treatment against aging and dementia. Present study contributes to this debate.

4. The only significant correlation between learning performance and the brain fatty acid levels was found for arachidonic acid in the young mice hippocampus (the structure known to be critical for spatial learning and memory). Arachidonic acid levels were inversely correlated with the learning performance in young but not in old mice suggesting that in mice, the hippocampal levels of AA may affect spatial learning (an equivalent of episodic learning in human) only in young subjects and possibly other mechanisms are implicated in old mice brain.

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