EFFECTS OF CHRONIC ETHANOL CONSUMPTION ON MEMORY AND MOLECULAR CHANGES IN THE HIPPOCAMPUS OF YOUNG ADULT WISTAR RATS

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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.

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ABSTRACT

EFFECTS OF CHRONIC ETHANOL CONSUMPTION ON MEMORY AND MOLECULAR CHANGES IN HIPPOCAMPUS OF YOUNG ADULT WISTAR RATS

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The aim of the present study was to examine retention of spatial reference memory after 6 (Experiment I) and 15days (Experiment II) of binge-like drinking and during alcohol withdrawal in young adult Wistar rats. Prior to alcohol treatment, rats received Morris Water Maze (MWM) training. Afterwards, rats were intragastrically administered ethanol at the dose increasing from 4.5g-to-12g/kg. Intubation control groups (n=7 and n=10, respectively) received infusions of a sucrose solution without ethanol. Subsequently, all subjects were given a single probe trial in the MWM to test memory retention. In both experiments, there were three alcohol groups: A0 group (n=7) tested 4h after the last alcohol administration for acute effects of ethanol; A24 group (n=7) tested 24h after alcohol cessation, when acute ethanol effects disappear but withdrawal symptoms does not develop yet; A72 group (n=7) tested 72h after the last ethanol infusion for withdrawal effects. Finally, potential molecular changes in hippocampus were examined using Fourier Transform Infra-Red (FT-IR) spectroscopy. The blood alcohol concentration was 605.67±36mg/dl.

In Experiment I, due to the low overall level of performance in the memory retention task the behavioral effects of ethanol could not be evaluated and no significant between–group differences were observed in Experiment II. In Experiment I, no significant changes in the molecular make-up of the hippocampus were noted. Conversely, in Experiment II, significant changes in protein, lipid, and nucleic acid profiles related to ethanol intake and withdrawal were found. They are linked to both development of tolerance to ethanol and adverse withdrawal effects.

Key words: Wistar rat, chronic ethanol abuse in adulthood, spatial learning and memory, MWM, FT-IR spectroscopy

ÖZ

GENÇ ERİŞKİN WİSTAR SIÇANLARDA ETANOL ALIMININ HAFIZA ÜZERİNDEKİ ETKİSİNİN VE HİPOKAMPÜSTE OLUŞAN MOLEKÜLER DEĞİŞİKLİKLERİN İNCELENMESİ

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Bu çalışmanın amacı, 6 (Deney I) ve 15 günlük (Deney II) aşırı alkol tüketiminden sonra ve tüketimin sonlandırılması sırasında oluşan uzun süreli mekansal bellekteki etkilerinin genç erişkin sıçanlarda incelenmesidir. Sıçanlara alkol verilmeden önce Morris su tankı eğitimi uygulandı. Daha sonra sıçanlara intragastrik yolla 4.5g'dan başlayarak 12g/kg'a kadar artan dozda etanol verildi. İntübasyon kontrol grubuna (n=7 ve n=10, sırasıyla) etanol içermeyen sükroz solüsyonu verildi. Akabinde, bütün sıçanlara Morris su tankında hatırlama seviyelerini ölçen test denemesi uygulandı. Her iki deneyde de 3 tane alkol grubu vardı: A0 (n=7) grubu alkolün akut etkilerini ölçmek için alkolün kesilmesinde 4 saat sonra, A24 grubu (n=7), alkolün kesilmesindn 24 saat sonra, yani akut etkilerin kaybolduğu ama yoksunluk semptomlarının hala gözlemlenmediği bir zamanda, ve A72 grubu (n=7) son alkol verilmesinde 72 saat sonra, fiziksel yoksunluk sendromunun başladığı düşünülen sürede test edildiler. Son olarak, hipokampüste alkol kullanımına bağlı oluşabilecek moleküler değişimler Fouier Dönüşüm Kızılötesi Spektroskopisi (FTIR) kullanılarak incelendi. Alkolün kesilmesinde sonra kandaki alkol seviyesi 605,67±36 mg/dl olarak belirlendi.

Deney I de, test denemesinde sıçanların genel performansları çok düşük olduğundan, etonolün davranış üzerindeki etkileri konusunda değerlendirme yapılmadı. Deney II de ise test denemesinde gruplar arasında hiçbir fark gözlemlenmedi. Deney I de, sıçanların hipokampüslerinde önemli düzeyde moleküler bir fark görülmedi. Buna karşılık, Deney

II de, alkol alımına ve alkol yoksunluğuna bağlı olarak, protein, lipid ve nükleik asit profillerinde önemli değişimler bulundu. Bu değişimlerin hem etanole karşı tolerans gelişmesinden hem de alkol yoksunluğunun zararlı etkilerinden kaynaklandığı düşünülmektedir.

Anahtar Kelimeler: Wistar sıçan, erişkin yaşta kronik alkol tüketimi, mekansal öğrenme ve bellek, MWM, FT-IR Spektroskopisi.

To My Mother

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LIST OF ABBREVIATIONS

A	Alcohol Group
AA	Arachidonic Acid
ACh	Acetyl Choline
AChE	Acetylcholinesterase
ADH	Alcohol Dehydrogenase
AgNORs	Argyrophilic Nucleolar Organizing Regions
AWS	Alcohol Withdrawal Syndrome
BAC	Blood Alcohol Concentration
BDNF	Brain Derived Neurotrophic Factor
BFCS	Basal Forebrain Cholinergic System
CA	Cornu Ammonis
cAMP	cyclic Adenosine-Mono-Phosphate
ChAT	Choline Acetyl Transferase
Cl	Clor
CNS	Central Nervous System
CRE	cAMP Response Element
CREP	cAMP Response Element Binding Protein
CT	Computerized Tomography
DA	Dopamine
DG	Dentate Gyrus

DT	Delirium Tremens
E	East
EDC	Ethanol Derive Calories
FAE	Fetal Alcohol Effects
FAS	Fetal Alcohol Syndrome
FTIR	Fourier Transform Infra-Red
GABA	Gamma-Amino-Butyric-Acid
GD	Gestation Day
GFAP	Glial Fibrillary Acidic Protein
IC	Intubation Control
KBr	Potasium Bromide
LTD	Long Term Depression
LTP	Long Term Potentiation
MRI	Magnetic Resonance Imagining
MS	Medial Septum
MWM	Morris Water Maze
N	North
NAc	Nucleus Accumbens
NbM	Nucleus basalis of Mynert Complex
NE	North-East
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate

NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPC	Neuronal Progenitor Cells
NT 3/4	Neurotrophin 3/4
PC	Phosphotidylcholine
PD	Postnatal Day
PE	Phosphotidlyethanolamine
PKA	Phospho Kinase A
PKC	Phospho Kinase C
PLA_2	Phospholipase A_2
PS	Phosphotidylserine
RBCF	Regional Cerebral Blood Flow
ROS	Reactive Oxygen Species
S	South
SP	Spingomyelin
SW	South-West
Trk	High Affinity Tyrosine Kinase Family Receptor
VDB	Ventral Diagonal Band
VTA	Ventral Tegmental Area
W	West

CHAPTER 1

INTRODUCTION

1.1. Chemical Properties of Ethanol

For thousands of years, ethanol has been the most widely abused drug in the world. Today alcohol is known as a potent teratogen but it seems to have serious adverse effects also in the postnatal life after a chronic abuse.

Ethanol is a simple molecule that easily dissolves in water, and can be almost completely absorbed into the bloodstream after oral ingestion. Consequently, the rate of alcohol entrance into the body tissues depends upon the blood supply to the tissues. Therefore, the alcohol concentration in the highly vascularized organs (i.e. central nervous system (CNS)) rapidly comes into equilibrium with that in the systemic arterial blood.

The complexity and the multitude of the ethanol effects in living organisms paradoxically rely on the simplicity of its chemical structure. The hydroxyl group provides a dipole that favors the formation of hydrogen bonds (or the breakage of preexisting ones) with electron acceptor or electron donor groups of proteins or polar head groups of membrane phospholipids (Barry and Gawrish, 1994). The formation of hydrogen bonds makes ethanol soluble in water in all proportions. Via hydrogen bonds ethanol can also modify the organization of water molecules in the extracellular matrix (Yurttas *et al.*, 1992), thereby altering the solvation of ligands or ions that interact with receptor proteins (Fadda and Rossetti, 1998).

1.2. Animal Models in Alcohol Studies

Today, both in the developed and developing countries, alcoholism is still a serious problem having a negative influence on the human health and countries' economy. Therefore, a lot of research is carried out regarding the ethanol's effects on the biological systems, the potential prevention strategies, and the therapeutic methods. Due to the legal and ethical constraints on research with humans, most of this research has been done using animal models. Among different animal species, rodents and particularly rats have been most widely used in these studies. It is mainly because of the

ease of handling, short gestation period and relatively low cost to purchase, housing and feeding (Keane and Leonard, 1989). The mechanisms of alcohol metabolism were shown to be similar in humans and rats, with the exception that rats as small endotherms have faster metabolic rate than man has and, therefore, metabolize alcohol more quickly.

There are different methods of alcohol administration to experimental animals such as subcutaneous/intraperitoneal injections, inhalation, liquid diet, and intraoral/intragastric infusion (gavage, intubation). No method is ideal because each has its advantages and disadvantages. The most commonly used methods are liquid diet and intragastric gavage. Alcohol containing liquid diet serves as the animal's sole source of nutrition. Alcohol is added to this diet either at a low concentration usually equivalent ~18% ethanol derived calories (EDC) or at a higher concentration usually equivalent ~35% EDC. These alcohol concentrations result in daily alcohol intake of ~12 and ~18 g/kg/day respectively. This method generally includes two control groups. The first is pair-fed to either 18% or 35% alcohol group and receives a similar liquid diet with a carbohydrate i.e. sucrose, substituted for the alcohol (Berman and Hannigan, 2000; Driscoll *et al.,* 1990). This procedure equates the total daily caloric intake across groups and therefore, serves as a control for reduced caloric intake that is typical in the alcohol treated animals and it might result in malnutrition. The second control group has continuous access to standard laboratory chow and water. If the alcohol group differs from both control groups, and the two control groups do not differ from each other, the effect may be attributed to alcohol intake per se. Alcohol administration with a liquid diet is more natural (Uzbay and Kayaalp, 1995), however, a basic disadvantage of this procedure is that there is a great individual variation in the consumption of alcohol-containing solutions, and thus variation in the blood alcohol concentrations across the subjects. Additionally, the peak blood alcohol concentration (BAC) obtained with this method is relatively low.

To ensure equal ethanol intake by all the experimental animals, and obtain a high peak BAC direct intraoral or intragastric intubation (gavage method) is applied. This method is sometimes referred to as "binge-like drinking". Using a gavage method, alcohol can be delivered in doses varying between 2 and 12 g/kg/day. In this procedure, alcohol is mixed with a vehicle and administered directly to the stomach via a feeding needle. To

increase the portion of the day with elevated BAC, the absolute daily dose may be divided into two or three administrations. This method also includes two controls. One control is pair-fed to alcohol group and receives the same volume of fluid as the alcohol group via intubation, except that carbohydrate is substituted isocalorically for alcohol. The other control group has continuous access to standard laboratory chow and water (Berman and Hannigan, 2000; Driscoll *et al.,* 1990).

1.3. Ethanol Teratogenity

As mentioned earlier, ethyl alcohol is rapidly absorbed from the stomach and gastrointestinal tract following ingestion, and is evenly distributed throughout the fluids and tissues in the body. It also readily crosses the placental barrier producing approximately equal maternal and fetal BAC (Waltman and Iniquez, 1972). The embryo and fetus are dependent on the maternal liver to metabolize alcohol because the fetus does not have the hepatic alcohol dehydrogenase (ADH), the major metabolizing enzyme for alcohol. Therefore, the elimination of alcohol from the fetus is through a passive diffusion of alcohol across placenta and then maternal elimination. In addition, the rate of alcohol elimination from amniotic fluid is approximately half that from maternal blood, resulting in relatively high alcohol concentrations in amniotic fluid when alcohol levels are low or eliminated from maternal blood. Thus, amniotic fluid may act as a reservoir for alcohol, and the fetus can be actually exposed to it for a longer period than predicted based on maternal alcohol concentration (Brien *et al.* 1983)

In the 1970s, it was recognized that *in utero* ethanol exposure of the human fetus could result in a neurodevelopmental syndrome called fetal alcohol syndrome (FAS) or in less severe form of impairment referred to as fetal alcohol effects (FAE) (Jones *et al*. 1973). Both conditions seem to be related to alcohol-induced cell deletions in the developing brain and result in the reduced brain mass at birth. Cells in the CNS show higher sensitivity to alcohol and therefore, experience more rapid cell death (apoptosis) than other cells in the developing embryo. In experiments on animal models of FAS/FAE, ethanol was shown to induce a massive wave of apoptosis (Goodlett *et al.,* 2005; Ikonomidou *et al*., 2000; Light *et al*., 2002).

In experiments using animal models of FAS, it has been demonstrated that prenatal or early postnatal (neonatal) exposure to alcohol leads to microencephaly with significant

growth deficits in the cerebrum including basal forebrain, cerebellum, and brain stem of rats of either sex. Morphological, neurochemical, and electrophysiological studies suggest that among brain structures the cerebellum and hippocampal formation are most vulnerable to the teratogenic consequences of perinatal (pre- and neonatal) exposure to alcohol (Bonthius and West, 1990**;** Goodlett *et al*., 1997; Livy *et al.,* 2003; Mihalick *et al.*, 2001; Miki *et al*., 2003). In humans, quantitative magnetic resonance imaging studies have documented that certain structural anomalies can be detected in FAS subjects, including corpus callosum anomalies, reductions in the anterior cerebellar vermis and basal ganglia (nucleus caudatus), and narrowing of gray matter density in certain regions of association cortex in parietal, temporal and frontal lobes (Archibald *et al.,* 2001; Riley *et al*., 2004).

The neuroteratogenic effects of alcohol would depend on the amount and duration of prenatal alcohol exposure, but more than that on the timing of the exposure relative to the developmental stage of the cells and tissues involved (Goodlet *et al.,* 2005). The critical periods of alcohol exposure overlap with periods of greatest development and/or maturation of organ systems. For humans, the major brain growth spurt occurs during the third trimester of gestation and growth then continues for about two years postnatally (West, 1987). In contrast, the major brain growth spurt in the rat occurs during the first 10-14 days of postnatal life, the equivalent of the human third trimester (West *et al.,* 1989).

Another factor that is determining the adverse effects of ethanol insult is the peak BAC. Peak BAC appears more critical than the alcohol daily dose in determining the degree of severity of brain damage and behavioral deficits. There is a handful of data suggesting that patterns of alcohol consumption which producing high BAC, such as binge drinking, may be especially harmful to the brain of the developing fetus (West *et al.,* 1989). Peak BAC above 425 mg/dl was shown as lethal, while BAC threshold for producing microencephaly was between 140 and 197 mg/dl with female rats more susceptible to adverse alcohol effects than male (Pierce and West, 1986).

Alcohol exposure during brain development may produce neuron alteration in multiple ways, including inhibition of protein synthesis, alterations in lipid solubility, and thus disruption of membrane integrity and/or disruption of cytoskeletal elements. Other putative mechanisms through which chronic prenatal alcohol may show its adverse

effects on the developing nervous system are: disrupted cellular energetic: altered energy metabolism (Fattoretti *et al.*, 2003; Snyder *et al.,* 1992) leading to oxidative stress and activation of the mitochondrial pathway of apoptosis (Cartwright *et al*., 1998; Ikonomidou *et al*., 2000; Light *et al.,* 2002; Zhang *et al*., 1998); suppression of protein and DNA synthesis (Shibley and Pennington, 1997); altered regulation of gene expression and reduced retinoic acid signaling (mainly due to the competitive interaction of ethanol with ADH, an enzyme critical also for synthesis of retinoic acid) (Deltour *et al*., 1996; Peng *et al.,* 2004); disruption of midline serotonergic neural development and thus serotonin signaling (Whitaker-Azmitia *et al.,* 1996) (both retinoic acid and serotonin signaling are important for normal neuronal differentiation and maturation in the developing brain); disruption of cell-to-cell interactions: inhibition of L1 cell adhesion molecule (L1 CAM) function (Charness *et al.,* 1994; Ramanathan *et al*., 1996; Wilkemeyer and Charness, 1998). Prenatal exposure to alcohol was also reported to interact with neurotransmitter systems and to interfere with growth-factor signaling or other cell-signaling pathways (Bonthius *et al*., 2004; Zhang *et al.,* 1998). Decreased sensitivity of the adult rat (PN 70-90) hippocampus (CA1 area) to NMDA (Morriset *et al.,* 1989), and alterations in the expression of hippocampal $GABA_A$ receptor and its pharmacological properties (Iqbal *et al.,* 2004) were observed after perinatal alcohol exposure. Alterations in receptor functions may affect signal transmission in the hippocampus and contribute to hippocampal-related behavioral deficits described in fetal alcohol rats. It has been also reported that chronic but even acute (single intragastric alcohol infusion on the GD 15) prenatal administration of alcohol led to decreased expression and decreased brain levels of neurotrophins such as NGF and BDNF (Angelucci *et al.,* 1997; Climent *et al.,* 2002; Tapia-Arancibia *et al.,* 2001). Chronic alcohol intake during gestation and/or lactation was also shown to decrease expression of p75, low affinity NGF receptor (Seabold *et al.,* 1998), and increase the ratio of truncated to full-length brain-derived neurotrophic factor's (BDNF), TrkB receptors in the developing cerebral cortex (Climent *et al.,* 2002). These changes are accompanied by reduction in neurotrophin-activated extra- and intracellular signal transduction pathways leading to increased loss and/or dysfunction of cholinergic neurons, the neurons known to be dependent on neurotrophin support. Reduction in the number of cholinergic neurons in the basal forebrain gives rise to the cholinergic deafferentation of the hippocampus and cortical mantle.

‘‘Secondary’’ sources of damage during prenatal alcohol exposure are altered placental functions or other intrauterine factors (Randall *et al.,* 1989), hypoxia/ischemia (Savoy-Moore *et al.*, 1989), acetaldehyde formation (Sreenathan *et al*., 1982).

In line with morphological data indicating towards great cell losses in cerebellum, basal ganglia, hippocampus, and some associative cortices, perinatal alcohol intoxication is most affecting motor and cognitive functions. Both in humans and in rodents, one of the most characteristic effects of perinatal alcohol intoxication is locomotor hyperactivity. Locomotor hyperactivity has been consistently reported in children and in preweaning and juvenile rats (Abel, 1982; Mattson *et al*., 2001; Tran *et al.,* 2000). It was also shown that preweaning rats prenatally exposed to alcohol were worse than the control in their performance on rotating drum, and fell off an inclined plane at a less steep angle, what suggested alcohol-induced decrease in muscle strength and sensorimotor coordination (Abel and Dintcheff, 1978). Cognitive deficits including attention and learning impairments were also frequently reported in both humans and rodents after perinatal exposure to ethanol. It has been postulated that such deficits may be observed even in the absence of full-blown fetal alcohol syndrome (Girard *et al*., 2000). Cognitive deficits have been demonstrated especially in spatial tasks sensitive to hippocampal damage such as spatial navigation in the Morris Water Maze (MWM) (Girard *et al.,* 2000; Hamilton *et al*., 2003; Johnson and Goodlett, 2002), food-rewarded spatial navigation in the radial arm maze (Neese *et al.,* 2004; Reyes *et al.,* 1989) and place acquisition as well as conditional alternation in T-maze (Lee and Rabe, 1999; Nagahara and Handa, 1997).

1.4. Effects of Chronic Exposure to Ethyl Alcohol in Adult Subjects on Brain Morphology, Physiology, and Behavior

1.4.1. Morphological Studies

Neuropathological studies as well as neuroimaging observations such as computerized tomography (CT) or magnetic resonance imaging (MRI) in human alcoholics have shown reduction in the brains’ weight and volume related to a decrease in the brain gray but especially white matter volume (Harper and Kril, 1985; Pfefferbaum *et al*., 1992; Shear *et al*., 1994; Wilkinson, 1982). Decreased volume of the brain tissue was associated with the increase in the size of the ventricles. In the cerebral cortex, a patchy

loss of cortical neurons and a widening of the sulci was reported (Cala *et al.*, 1978; De la Monte, 1988; Harper *et al.,* 1985; Jernigan *et al.,* 1991). According to the report by Hunter *et al*. (1989), the frontal lobes appear to be more seriously affected than other cortical regions due to reduction of regional cerebral blood flow (RBCF) in the frontal lobe and periventricular regions of alcoholics. It was also postulated that the mammillary bodies of the hypothalamus, the medial dorsal thalamic nucleus, and the nerve fibers connecting these two structures are the main diencephalic structures damaged (Fadda and Rossetti, 1998; Harper and Matsumoto, 2005).

In animal studies, chronic alcohol intake was reported to produce a serious damage to the hippocampus and the basal forebrain cholinergic system (BFCS), structures known to be involved in learning and memory (Connor *et al.,* 1991; Dunnet *et al.,* 1987). In rodents, chronic ethanol consumption resulted in a decreased number of the hippocampal CA1 and CA3 pyramidal neurons, mossy fiber-CA3 synapses, dentate gyrus granule cells and local circuit interneurons (Bengoechea and Gonzalo, 1991; Beracochea *et al*., 1987; Cadete-Leite *et al.,* 1989 a, b; Walker *et al.,* 1980).

Franke *et al* (1997) reported a significant loss of the total number of hippocampal pyramidal and dentate gyrus granule cells after 36-week ethanol treatment (10% v/v by liquid diet) in Wistar rats. Regional differences in the vulnerability to the neurotoxic effects of chronic ethanol intake were found: CA3 > CA1 + CA2 > > CA4 > DG. Similar loss of hippocampal pyramidal and dentate gyrus granule cells was observed in laboratory rats maintained on ethanol-containing diets for 5 months followed by a 2-month alcohol-free period (Walker *et al*., 1980). Lukoyanov *et al*. (1999) also reported 18% cell loss in CA1 and 19% cell loss in CA3 hippocampal regions in the rats consuming alcohol at the average dose of 7.5 g/kg/day between 2 and 15 months of age. Arendt *et al.* (1988) has observed adverse morphological changes in the target areas of the BFCS: neocortex and hippocampus, after much shorter period of adult ethanol intoxication (12 weeks on 20% v/v alcohol containing liquid diet). Cortical and hippocampal degeneration is associated with the damage to the cholinergic structures of the basal forebrain observed upon the chronic exposure to ethanol in adult rodents. The loss of neurons in BFCS seems to be more pronounced in the medial septum and diagonal band nuclei than in the nucleus basalis (Arendt *et al*., 1988). The nucleus basalis innervates the neocortex, whereas the cholinergic septohippocampal pathways

terminate in various dendritic segments of the hippocampal formation and modulate hippocampal activity (Mesulam *et al.*, 1983). Neurodegeneration of these cholinergic pathways is therefore expected to alter the function of the innervated structures.

Degenerative changes in the basal forebrain were shown to be parallel by the concomitant reduction of presynaptic cholinergic markers (synthesis, content, and release of acetylcholine) in the neocortex and hippocampus (Arendt *et al.,* 1988). In the latter study, the number of acetylcholinesterase (AChE)-positive neurons in the basal nucleus of Meynert complex (NbM, Ch1 to Ch4) was 83 % of control values. Activity of choline acetyltransferase (ChAT) and AChE in the basal forebrain was simultaneously reduced to 74 % and 81 % respectively, and content of acetylcholine (ACh) to 56% of control value. In another study (Miller and Rieck, 1993), chronic exposure to dietary ethanol (6.7 % v/v alcohol containing liquid diet), lasting 42 day (6 weeks) produced marked changes in the cortical plexus of AChE-positive fibers. The AChE-positive plexus in ethanol-treated rats was reduced in all cortical layers, in comparison to age-matched pair-fed control and chow-fed rats. The most marked reduction was evident in layers II/III, IV, and VIa. In this study, no detectable ethanol-induced change in the density of cresyl violet-stained neurons either in the horizontal limb of the diagonal band of Broca or in the nucleus basalis was reported. However, the density of AChE-positive neurons in the nucleus basalis was significantly lower in ethanol-fed rats than in controls. Thus, it appears that a mere 6 weeks of ethanol exposure is sufficient to alter the cholinergic innervations of the cerebral cortex. Alcohol-induced loss of the cells in hippocampal formation was shown to be aggravated during withdrawal from alcohol (Paula-Barbosa *et al*., 1993).

Neuronal degeneration in selected cerebral cortical regions involved in memory and olfaction was also observed after repetitive ethanol intoxication through intragastric delivery 3 times daily for 4 days ("binge-like drinking") in adult rats (Collins *et al*., 1996). In these studies, neuronal damage was visualized with the de Olmos cupric silver technique for degenerating neurons and processes (argyrophilia), and was quantitated by total counts and densities of argyrophilic cells/fields. Argyrophilia was noted only in ethanol-intoxicated rats with mean blood ethanol levels for days 2 to 4 above 300 mg/dl. However, it increased substantially between 350 and 550 mg/dl. In highly intoxicated rats, argyrophilia was most extensive among hippocampal dentate gyrus granule cells,

pyramidal neurons in layer 3 of the entorhinal cortex, and olfactory nerve terminals in the olfactory bulb. Degenerating pyramidal neurons were also consistently seen in the insular cortex and olfactory cortical regions, such as the piriform and perirhinal cortices. There were few argyrophilic neurons in the CA regions of the hippocampus and none in the cerebellum, regions generally shown to have cell loss in long-term ethanol feeding models, but degenerating mossy fibers in the CA2 region were observed.

There are also some reports that binge-like administration of ethanol at the dose of 5 g/kg to adult rats reduced hippocampal neurogenesis by inhibiting both neural progenitor cells (NPC) proliferation and cell survival (He *et al*., 2005; Nixon and Crews, 2002). However, in addition to reports about ethanol-induced neural degeneration and suppressed adult neurogenesis, there are few contradictory reports postulating increased neurogenesis in the adult brain in response to ethanol administered at moderate concentrations (6g/kg/day) (Aberg *et al*., 2005; Miller, 1995).

1.4.2. Physiological Studies

Ingested alcohol is absorbed into the bloodstream from the stomach and intestines. All blood from the stomach and intestines first goes through the liver before circulating around the whole body. Therefore, the highest concentration of alcohol is in the blood flowing through the liver. Thus, in heavy drinkers, liver more than other organs is exposed to alcohol intoxication. This leads with time to three types of liver pathological conditions: fatty liver, hepatitis, and cirrhosis. The liver cells can metabolize only a certain amount of alcohol per hour. Therefore, under alcohol abuse, when more alcohol is ingested than the liver can deal with, the level of alcohol in the bloodstream rises and affects other organs and organ systems. As mentioned earlier, one of the most affected systems is central nervous system where alcohol leads to nervous tissue degeneration and activity abnormalities.

In vivo research supports the notion that ethanol's effects on the brain's cellular activity are region-specific. **Acute** ethanol administration was shown to suppress cellular activity in the medial septum (Givens, 1996), but did not alter cellular activity in the lateral septum (Givens and Breese, 1990), and produced an increased cellular activity in the ventral tegmental area (Gessa *et al.,* 1985). Peripheral or intrasystemic acute administration of alcohol was also reported to suppress the spontaneous activity of

hippocampal pyramidal cells, disrupt the hippocampal theta rhythm (Givens, 1995), and alter the activity of hippocampal place-cells (Alexandrov *et al*., 1993; Matthews *et al*., 1996; White *et al.,* 2000). Acute ethanol inhibited the induction of long-term potentiation (LTP) both in hippocampal slices (Blitzer *et al*., 1990; Morrissett and Swartzwelder, 1993) and in freely behaving animals (Givens and McMahon, 1995). The potency of ethanol in depressing LTP correlated well with its potency in inhibiting the response to N-methyl-D-aspartate, an agonist at the glutamate NMDA receptors. Alterations in excitatory amino acid receptors have been reported following acute administration to ethanol administered in a pharmacologically relevant dose (Crews *et al*. 1996). The NMDA receptor, which regulates an ion channel permeable to calcium and sodium, is believed to play an important role in memory, learning, and the generation of seizures (Finn and Crabbe, 1997). In addition to antagonizing activity at NMDA receptors, ethanol reduces the overall level of glutamate released at synapses within the hippocampus. Decreased levels of glutamate in hippocampal synapses likely contribute to reduced levels of activity in hippocampal pyramidal and granule cells. On the other hand, it is known that ethanol potentiates the effects of gamma-amino-butyric-acid (GABA, the major inhibitory neurotransmitter in the CNS) at some subtypes of the $GABA_A$ receptor (Grobin *et al.,* 1998). Thus, ethanol may disrupt hippocampal function also by potentiating GABA-mediated inhibition in hippocampal microcircuits (White *et al.,* 2000).

Chronic alcohol consumption was also reported to affect hippocampal physiology and reduce hippocampal LTP. As reported by Peris *et al.* (1997), decrease in the magnitude of hippocampal LTP observed after chronic alcohol treatment (liquid diet delivered for 28-42 weeks) lasted as long as 7 months after ethanol withdrawal. Chronic alcohol intoxication was shown not only to produce suppression of hippocampal LTP but also to reduce long-term depression (LTD) in the CA3-CA1 Schaffer collateral pathway (Thinschmidt *et al*., 2003).

1.4.3. Behavioral Studies

In human subjects, acute ethanol is known to have anxiolytic, sedative, hypnotic, anticonvulsant, and motor incoordinating effects. It also impairs attention concentration and memory (Dougherty *et al.,* 2000). At high concentrations, it acts as an anesthetic and respiratory depressant. Mounting evidence suggests that cognitive abilities mediated

by the hippocampus might be particularly sensitive to the effects of moderate to high doses of ethanol. Early speculation that altered activity in the hippocampus might give rise to memory impairments followed from the observation that intoxicated subjects perform poorly on tasks that are sensitive to hippocampal system damage (Ryback, 1971). In humans, ethanol disrupts performance on a variety of short-term memory tasks from verbal list learning (Acheson *et al*., 1998; Lister *et al.,* 1991; Miller *et al*., 1978) to pattern recognition (nonspatial task) and spatial learning (Bowden and Carter, 1993; Parker *et al.,* 1976; Stokes, 1991; Uecker and Nadel, 1996; Weissenborn and Duka, 2003).

In animal studies, in addition to the spatial (Gibson, 1985; Melchior *et al*., 1993; White *et al*., 1997) and nonspatial (Givens, 1996; Givens and McMahon, 1997) working memory deficiencies, moderate doses of ethanol disrupt the acquisition and performance of spatial reference memory tasks (Markwiese *et al*., 1998; Matthews *et al.,* 1995; White *et al.,* 1998), while sparing the acquisition and performance of nonspatial reference memory tasks (Devenport *et al.,* 1989; Markwiese *et al*., 1998; Matthews *et al.,* 1995; White *et al*., 1998). In addition, ethanol produces a shift in bias from the use of spatial information to the use of nonspatial information to solve learning and memory tasks (White *et al.,* 2000). Due to its specific effect on spatial navigation, acute ethanol administration was shown by some authors as a valuable non-invasive method of producing reversible hippocampal dysfunction to be applied in the studies on the role of hippocampus in learning and memory (Matthews *et al.,* 1999).

Cognitive-processing deficits are one of the main symptoms of chronic alcohol abuse in humans. Therefore, most of the animal studies deals with effects of chronic ethanol intoxication on learning and memory. As mentioned earlier, hippocampal LTP that is considered a cellular correlate of spatial memory was shown to be reduced by both acute and chronic alcohol abuse. It is no surprising then, that chronic alcohol consumption does not impair or impairs less the acquisition of non-spatial memory tasks and yet produces deficits in reference and working spatial memory, the type of memory dependent on the integrity of the BFCS and hippocampus. In most of these studies, animals were subjected to a prolonged ethanol containing liquid diet and not submitted to withdrawal prior to testing. In one of those studies (Franke *et al*., 1997), after 36 weeks of ethanol treatment (10% v/v in liquid diet) parallel to the cell loss

within the hippocampal formation, a significant behavioral impairment in the acquisition of a complex elevated labyrinth was observed in alcohol-treated Wistar rats. In another study (Arendt, 1994), impaired acquisition of radial maze spatial task due to increase in both within-trial working and long-term reference memory errors was reported in rats after alcohol administration lasting 28 weeks (20% v/v ethanol in drinking water). Similar results were reported by Hodges *et al*. (1991), too.

However, not all results are consistent. Santin and colleagues (2000) reported that chronic intoxication by alcohol, beginning soon after weaning (PD 21) and lasting over 4 months, did not produce an impairment in the reference memory task (place learning in the MWM), and only deficits in alcohol-treated animals occurred in spatial working memory task when platform location was valid only for one trial consisting of acquisition (sample) and retention (test) swims applied 15 sec. or 5 min. apart. Rats showed greater performance deficit when instead of one, four trials per day were carried out. On the acquisition trials when the platform position was varied, alcohol-treated animals swam a greater distance before locating the escape platform in the pool in spite of being familiar with the environment. This seemingly manifested an impaired ability to use spatial strategies required to explore a known environment in which there is a reward. In the working memory task with four daily trials, a deficit observed in the performance of the alcohol-treated animals during both sample and test swim was even greater suggesting strong influence of earlier trials upon the following trial (a proactive interference). In the studies by Pereira *et al*. (1998), after oral administration of 20% v/v alcohol solution for 6 months, male Fisher rats were either trained de novo or retrained in the radial maze task. The only significant difference between control and alcohol pre-treated groups was observed in the memory retention task (retraining) carried out one year after the original training. Interestingly, this memory deficiency occurred without concomitant decrease in the cortical cholinergic parameters (in vitro AChE activity and stimulated ACh release). Conversely, Lukoyanov *et al*. (1999), after 13 months alcohol intoxication (20% v/v alcohol containing liquid diet), did not observe in rats any deficits either in place learning or spatial working memory in the MWM despite 18-19% loss of hippocampal neurons. Interestingly, the same author, in the more recent publication, did report impairment in both the acquisition and retention of place learning in the Morris water maze after much shorter (6 months) exposure to alcohol. There was, however, a procedural difference between these two studies: in the latter study, rats before being

tested were withdrawn from alcohol for at least one month (Lukoyanov *et al*., 2003). These results may confirm the notion that cognitive deficits related to chronic exposure to alcohol in the adulthood are aggravated during the withdrawal period. In the studies by Blokland *et al*. (1993), no effect of chronic alcohol treatment (20% aqueous solution) was found in rats tested in three different tasks: the Morris spatial navigation task, a cone-field task, and a temporal discrimination task. Also in a radial arm maze task that depended upon extra-maze cues (visuo-spatial) or intra-maze cues (various odors), rats fed alcohol for 28 weeks prior to testing and during the period of behavioral testing consistently performed the same or better than the control subjects (Steigerwald and Miller, 1997). No significant differences between control and alcohol pretreated adult rats were also noted after repeated binge like alcohol administration where rats were exposed to alcohol (5.0 g/kg intraperitoneally) or isovolumetric saline at 48 hr intervals over 20 days, and then tested twenty days later on delayed (5 and 60 min) non-matching-to-position task in the 8-arm radial maze (White *et al*., 2000). In this study, only animals treated with ethanol during adolescence exhibited some working memory impairments and only when tested under an ethanol challenge (1.5 g/kg intraperitoneally). Garcia-Moreno *et al*. (2002) observed behavioral impairment in non-spatial working memory task (a spontaneous delayed non-matching-to-sample test) only after long 60 min delay.

1.4.4. Molecular Correlates of the Morphological, Physiological, and Behavioral Deficits Induced By Chronic Ethanol Abuse

Despite of widespread abuse of ethanol and the presence of vast data about ethanol-induced brain damage and behavioral deficits, molecular mechanism underlying deteriorating effects of alcohol intoxication on the nervous system and behavior remain elusive.

One of the postulated effects of ethanol in the brain tissue is a direct, specific interaction with neuronal membrane lipids and proteins thereby altering their function. This would be followed by compensatory changes in single brain structures or in a particular neurotransmitter system or even in membrane receptors/ion channels alone.

Several molecular correlates of fetal alcohol syndrome described earlier may also be encountered during adult alcohol insult. Among them is distorted cellular energy

metabolism leading to oxidative stress and followed by changes in DNA, protein and lipid structures, altered regulation of gene expression and protein synthesis, disruption of membrane integrity and disruption of cytoskeletal elements, interference with neurotransmitter systems and growth factor signaling, and eventually apoptotic cell death.

1.4.4.1. Oxidative Stress and Free Radicals (Reactive Oxygen Species) Production

Oxidative stress is attractive as a possible mechanism for the alcohol-induced brain damage for many reasons. Among all internal organs, the brain has the highest energy utilization and thus, processes large amounts of O_2 in a relatively small mass. It also has a very high content of substrates available for oxidation (i.e., polyunsaturated fatty acids and catecholamine) in conjunction with low antioxidant activities (Halliwell, 2006). In addition, certain regions of the CNS, such as the hippocampus and cerebellum, may be particularly sensitive to oxidative stress because of their especially low, relatively to other brain regions, endogenous levels of Vitamin E, an important biochemical antioxidant (Wilson, 1997). Such a depressed defense system may be adequate under normal circumstances. However, in pro-oxidative conditions, such as during alcohol exposure, these low antioxidant defenses can predispose the brain to oxidative damage.

Two mechanisms are known by which alcohol may produce oxidative stress: First, enhanced production of free radicals (acetaldehyde, a byproduct of alcohol metabolism, additionally alcohol stimulates the activity of enzymes such as cytochrome P450s, which contribute to the production of reactive oxygen species (ROS)); Second, alcohol consumption suppresses activity of antioxidants that are necessary for free radical elimination. Therefore, the combination of increased free radical production and decreased free radical elimination can cause toxic levels of free radical exposure, leading to mitochondrial dysfunction, cell damage, and cell death.

Some experimental data are supporting the notion that alcohol-induced oxidative stress to great extent may be responsible for the adverse structural and functional changes occurring in the CNS under both fetal and postnatal alcohol intoxication. Alcohol was reported to induce the generation of ROS such as superoxide, hydrogen peroxide, and hydroxyl anions in cultured neural crest cells (Davis *et al*., 1990). An increase in ROS

has been detected in the cortices of rats exposed to alcohol acutely on either postnatal day 7 or 21 (Heaton *et al*., 2003).

Alcohol-induced damage to cellular lipids (lipid peroxidation) even after acute alcohol exposure has been observed in several tissues such as rat liver homogenates (Di Luzio and Hartman, 1967); cerebellum (Rouach *et al*., 1987; Uysal *et al*., 1986); maternal and fetal hepatic tissue (Chen *et al*., 2000; Henderson *et al*., 1995); and fetal brain (Henderson *et al*., 1999). In addition, a diet high in saturated fats (which are more resistant to peroxidation) was found to alleviate hyperactivity, a common behavioral outcome of fetal alcohol exposure (Abel and Reddy, 1997), suggesting that lipid peroxidation may play an important role in the neuropathology of FAS after the observations (Montoliu *et al.*, 1994; Nordmann *et al.*, 1990).

Alcohol-induced oxidative damage to nucleic acids has been evidenced by increased levels of 8-OHdG, an oxidatively altered base, with the detection of molecular techniques in mouse and rat mitochondrial DNA (Cahill *et al.*, 1997; Wieland and Lauterburg, 1995). In addition, DNA fragmentation and nuclear DNA strand breaks — characteristics of oxidative DNA damage— have also been observed in cultured rat hepatocytes (Ishii *et al*., 1996), and in hippocampal and cerebellar tissue from rats administered alcohol chronically (Renis *et al*., 1996).

Finally, increased protein carbonyl formation, one of the most general and commonly used indicators of oxidative protein damage, has been observed in the blood of alcoholic patients (Mutlu-Turkoglu *et al*., 2000), in the liver (Abraham *et al*., 2002; Rouach *et al.,* 1997), and in the intestinal mucosa of adult male rats following alcohol exposure (Altomare *et al*., 1998).

Oxidative stress induced by alcohol due to increased activity of mitochondrial oxidative enzymes and reduced antioxidative defenses may be potentiated by alcohol–induced oxygen deficiency (hypoxia) in tissues. Ethanol causes hypoxia by increasing oxygen consumption. In the brain, hypoxia usually leads to neural membrane depolarization and increased release of excitatory amino acid neurotransmitters, mainly glutamate. Elevated glutamate acting on NMDA receptors by increased cellular calcium loading can lead to so called amino acid excitotoxicity. Hypoxia-related overstimulation of NMDA glutamatergic receptors and increased cellular calcium loading results in:

(1) Increased activity of nitric oxide synthase (NOS) and thus increased formation of a nitric oxide (NO) which itself belongs to the ROS and contributes to the increased production of free radicals, and in addition, acting as a retrograde neurotransmitter which enhances glutamate release from the presynaptic neurons establishing a neurotoxic feed-forward cycle (Stamler *et al*., 1992; Uzbay and Oglesby, 2001);

(2) The calcium-dependent activation of phospholipase A_2 (PLA_2) and release of arachidonic acid (AA) which also leads to the generation of ROS (Dumuis *et al.*, 1988). AA alike NO was also shown to increase glutamate release (Williams, 1989) and reduce glutamate uptake (Volterra *et al*., 1994);

(3) Increased calcium uptake into mitochondria causing the production of ROS that interferes with the function of mitochondria and other plasma membranes (Harper and Matsumoto, 2005).

The resemblance of the argyrophilic distribution observed upon severe, repetitive ("binge-like") ethanol intoxication in adult rats to the regional neuropathology that occurs in experimental seizures confirms that the ethanol-induced degeneration may have an excitotoxic basis (Collins *et al*., 1996).

1.4.4.2. Ethanol Effects on the Plasma Membrane Lipids and Proteins

In adult brain, alcohol exerts its pharmacological effects by altering the physiochemical properties of cellular plasma membranes (Hunt, 1975). Adverse effects of chronic alcohol administration, tolerance and physical dependence development, as well as withdrawal syndrome appear to be at least partially associated with ethanol-induced maladaptive changes within neural membranes (Hunt, 1975). As it is known, plasma membranes are made by phospholipid bilayer and embedded in it proteins. The aliphatic moiety of ethanol molecule provides a lipophilic group that can interact with non-polar domains of macromolecules. However, in contrary to what is generally believed, ethanol has low solubility in lipids: it localizes in the polar head group region and very little within the lipids of the neuronal membrane (Barry and Gawrish, 1994). Nevertheless, alcohol readily penetrates cell membranes and alters the fatty acid interaction of the lipid layers, thereby increasing membrane “fluidity” and permeability. It has been hypothesized that during acute alcohol intoxication, increased fluidization of cellular membranes is responsible for impaired neural information processing (Chin and

Goldstein, 1977). In addition, one of the mechanisms postulated to explain the ethanol's depressant effects on neural activity was disordering the lipid bilayer of the plasma membranes (Chin and Goldstein, 1977; Goldstein, 1984). By increasing the rate and range of motion of lipid molecules, ethanol was believed to indirectly disrupt the function of membrane-bound proteins and decrease the excitability of the cell (Peoples *et al*., 1996).

After prolonged consumption, however, alcohol was shown to increase the cholesterol/phospholipid ratio in membranes, thereby altering the lipid layers to increase membrane rigidity (Buck and Haris, 1991; Deitrich *et al.,* 1989). In a study done by Rottenberg, Waring, and Rubin (1981), in animals chronically fed with alcohol, brain synaptosomal membranes became resistant to the fluidizing effects of alcohol and showed a reduction in alcohol binding.

However, alcohol was postulated to affect the structure of plasma membranes not only by increasing fluidity of lipid layers. It was shown that alcohol promotes phospholipases-mediated release of fatty acids from complex lipids, and thus, alters the membrane lipid composition of various cells and organelles (Rubin and Rottenberg, 1982). These released fatty acids may be the primary source for enzymatic synthesis of fatty acid ethyl esters and prostaglandins found after alcohol exposure (Hungund *et al.,* 1988). In addition, ethanol-induced changes in the levels of membrane phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) have been reported in a number of systems. According to a research done by Miller *et al*. (1997), ethanol-induced increases in levels of PE and PS and ethanol-induced decreases in the levels of PC and sphingomyelin (SP) were observed in the brains of chick embryos with a single dose of ethanol administration.

Another effect of both acute and chronic alcohol on membrane lipids is its effect on the membrane gangliosides, one of the major lipid components of neural membranes. Exogenous gangliosides have neuroprotective actions against a variety of neural insults, including those induced by alcohol exposure (Hungund and Mahadik, 1993; Mahadik and Karpiak, 1988). Particularly, sialic acid groups are considered to play an important role in the extracellular Ca^{++} and perhaps other cation binding and in the transport across synaptic membranes (Rahmann *et al.,* 1991). It was reported by some authors that both acute and chronic alcohol treatment might affect content, composition, and/or

distribution of brain gangliosides (Klemm and Foster, 1986; Vrbaski *et al*., 1984). Alcohol was postulated to interact with the anchoring sialo-compounds in the lipid bilayer, thereby distorting the orientation of these compounds in the extracellular space, making sialic acid more susceptible to destruction by endogenous neuraminidase. Alcohol-induced change in the surface properties of gangliosides may be more important than its actions in the lipid bilayer (Hungund and Mahadik, 1993).

The type of lipid compositional changes or altered lipid arrangement, however, may depend on type of alcohol, the route of alcohol administration, the animal species used, and the length and duration of exposure to alcohol.

As far as the ethanol effect on the membrane proteins is concerned, ethanol can upset the natural thermal balance that maintains membrane architecture and can alter membrane microdomains that determine protein–membrane and protein–ligand interactions (Wang *et al*., 1993). Recent works, however, point to a specificity of action of ethanol directly on membrane proteins producing conformational changes that alter their function (Eyring *et al*., 1973; Franks and Lieb, 1994; Li *et al*., 1994; Lovinger, 1997). For instance, alcohols could directly interact with proteins such as neurotransmitter-gated ion channels to alter their function in at least three general ways. First, alcohols could interact with the agonist-binding site to act as agonists or competitive antagonists. Second, alcohols could bind to a modulatory site on the receptor and act as allosteric modulators, thereby making agonist binding, or channel opening more or less favorable. Third, alcohols could bind to a site within the ion channel lumen and physically occlude the channel, thus acting as open-channel blockers (Peoples *et al*., 1996).

1.4.4.3. Interference with Neurotransmitter Systems

1.4.4.3.1. Glutamate

As mentioned earlier, ethanol, when administered acutely in a pharmacologically relevant dose, selectively and potently inhibits the function glutamatergic NMDA receptors. On the NMDA receptor, ethanol directly interacts with an allosteric site that is independent of the recognition site for the agonist glutamate or glycine, and reduces agonist efficacy by modulating the kinetics of the channel gating (Wright *et al*., 1996). Direct inhibition of NMDA receptor by ethanol may counteract earlier described

ethanol's hypoxic effects on glutamatergic neurotransmission. However, chronic exposure to ethanol causes adaptive up-regulation in sensitivity of NMDA receptors that can result in an increased vulnerability for glutamate-induced cytotoxic response especially upon the alcohol withdrawal. Increased 'sensitization' of neuronal cells to excitotoxic insults is considered one of the most important factors in the mechanism underlying ethanol dependence, withdrawal symptoms and ethanol-induced brain damage.

1.4.4.3.2. GABA

Alcohol-GABA interactions are also involved significantly and directly in the central effects of alcohol. In particular, alcohol activates the $GABA_A$ receptor-coupled Cl^- channel, thereby increasing Cl^- conductance and postsynaptic inhibition by means of a transient decrease in the postsynaptic membrane potential. As mentioned before, acute ethanol administration potentiates GABA-mediated inhibition both *in vitro* and *in vivo*, in several brain regions such as cortex, substantia nigra pars reticulata, medial septum, and according to recent reports hippocampus, too (Givens and Breese 1990; Givens and McMahon 1997; Matthews *et al*. 1995).

1.4.4.3.3. Dopamine

It has been shown that low to moderate doses of ethanol activate the dopaminergic pathways of the brain, which are strongly linked to reward and addiction, while high doses of ethanol can produce anesthetic and toxic effects and suppress dopaminergic activity (Budygin *et al*., 2005). Alterations in brain dopaminergic system are related to ethanol-induced physical dependence and withdrawal (Uzbay *et al*., 1998; Weiss *et al*., 1996). Ethanol increases the firing rate of DA neurons in VTA (Gessa *et al*., 1985) through what has been shown recently to be direct excitatory cellular activation (Brodie *et al*., 1999). Ethanol, like most drugs of abuse, elevates extracellular DA concentrations in the NAc (Di Chiara and Imperato, 1988). Over the course of chronic ethanol exposure, adaptations develop in mesolimbic DA function to counter sustained stimulation of this system by ethanol. If this is the case, an altered sensitivity of dopamine receptors during chronic treatment with ethanol may be responsible for the resultant decreased sensitivity to the effects of ethanol that accompanies the development of tolerance (Hoffman and Tabakoff, 1977). Although ethanol acutely activates mesolimbic DA neurotransmission, withdrawal from chronic ethanol leads to

substantial decrements in VTA DA neuron activity (Shen and Chiodo, 1993) and extracellular NAc DA levels (Rossetti *et al*., 1992; Weiss *et al.,* 1996). This suggests that chronic ethanol exposure causes mesolimbic DA hypofunction, a condition significant for maintenance of addiction by promoting ethanol intake to compensate for its decreased efficacy on DA release and by motivating resumption of drinking during withdrawal to reverse DA deficits.

1.4.4.4. Second Messenger Systems, Gene Expression Regulation, and New Protein Synthesis

Chronic ethanol administration was reported to alter PKC and cAMP-PKA signaling in neuronal cells (Diamond and Gordon, 1997). It was reported that ethanol promotes activation and translocation of the PKA catalytic subunit (Calpha) into the nucleus in cell lines and primary neuronal cultures. PKA Calpha translocation to the nucleus is followed by cAMP Response Element binding protein (CREB) phosphorylation and cAMP Response Element (CRE)-mediated gene expression (Asyyed *et al*., 2006). However, at the transcriptional level, ethanol was shown to diminish experience-dependent c-fos expression in hippocampal neurons (Ryabinin, 1998). In addition evaluation of transcriptional neuronal activity by measuring the argyrophilic nucleolar organizer regions (AgNORs) in the dentate gyrus, CA3, and CA1 hippocampal areas from adult male rats receiving chronic administration of ethanol and after withdrawal showed that chronic intake of alcohol decreases protein synthesis in hippocampal neurons with most affected CA3 region (Garcia-Moreno *et al*., 2001).

1.4.4.5. Reduction of Neurotrophic Support

Trophic factors are produced by a variety of cells; in the nervous system by both neurons and glial cells. Both during embryonic development and the postnatal life, neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), or neurotrophins-3/4 (NT-3/4) play a vital role in neuronal survival and maturation, and are important for the regulation of naturally occurring apoptotic cell death. Members of classical neurotrophin family are closely related peptide factors, that are evolutionary very conservative. NGF, BDNF, and NT-3/4 are encoded by three distinct genes identified in all higher vertebrates, including teleost fishes.

Recent studies suggest that chronic exposure to ethanol can reduce the availability of BDNF and alter its receptor (TrKB) function (Climent *et al.,* 2002). It has been also reported that long-term postnatal alcohol consumption causes a reduction in the level of ChAT, decrease in NGF levels, and a reduction in the distribution of NGF-receptors in the basal forebrain (Miller and Mooney, 2004). It has been found that the highest levels of NGF mRNA are present in hippocampus, amygdala, olfactory bulb, and cerebral cortex, the areas innervated by the BFCS. The highest levels of NGF itself were found in the BFC structures such as medial septal nucleus and nucleus basalis magnocellularis (Mufson *et al.,* 1994). In the CNS, the highest sensitivity to NGF (highest density of NGF receptors) was also shown by the cholinergic neurons of the basal forebrain for which NGF seems to act as a target-derived neurotrophic factor. Brain distribution of BDNF overlaps largely with NGF distribution.

Angelucci *et al*. (1997) reported that, in rats, a single intragastric administration of ethanol on the 15th day of gestation affected density of NGF receptors (low-affinity p75, and high-affinity tyrosine kinase A (TrkA) receptors)**,** decreased the NGF level, and reduced the numbers of cholinergic neurons expressing p75 in the BFCS. These changes were still observed at PD 60. These results suggest that one cause of the deleterious effects induced by ethanol consumption might be the low availability of neurotrophins. Heaton and co-workers (2000) showed that in a transgenic mice's CNS overexpressing NGF under the control of the glial fibrillary acidic protein (GFAP) promoter, effects of ethanol neurotoxicity in the developing cerebellum were ameliorated**.** Lukoyanov *et al.* (2003) and Cadete-Leite *et al.* (2003) demonstrated that intra-cerebro-ventricular NGF administration protected against loss of cholinergic neurons in the MS/VDB, prevented decrease in the fiber density within the septohippocampal system, and ameliorated memory deficits in rats withdrawn from prolonged (6 months) alcohol intake.

1.5. Development of Ethanol Tolerance

Tolerance that develops with a chronic use of a drug is considered as a neuroadaptive process, which attempts to reduce the acute effects of the substance and to maintain homeostasis. As a result of adaptation, in spite of permanent presence of a drug, an organism functions almost normally, compensating for drug effects.

In pharmacological terms, tolerance can be defined as a shift to the right of the dose response curve so that higher doses of the drug are required to produce the same effects. The development of tolerance is a complex event that may involve different mechanisms. Pharmacokinetic or metabolic tolerance is related to enzymatic induction due to which a drug is eliminated more rapidly from the organism so the drug acts for a shorter time therefore its total effect is weaker (Kalant *et al*., 1971). On the other hand, pharmacodynamic tolerance is related with rapid desensitization due to receptor phosphorylation and decrease receptor affinity to ligand and/or changed receptor coupling with G protein and 2nd messenger-generating enzymes, changed Ca^{++} distribution, receptor internalization, or down-regulation of receptor synthesis (Kalant *et al.*, 1971; Tabakoff *et al*., 1982). Alcohol alike benzodiazepines, barbiturates, or opiates, belongs to drugs that quickly develop tolerance.

In humans, repeated administration of the lower ethanol doses is sufficient for the development of tolerance to the behavioral effects of ethanol such as motor impairment, hypothermia, hypnosis. Continuous intoxication or high doses administered at short intervals are required for cellular tolerance to develop. Tolerance after high doses of ethanol develops at slow rate and disappears faster after interruption of the treatment. Conversely, the tolerance after low doses can develop even within a single administration (acute tolerance) and lasts for weeks after discontinuing ethanol administration (Pohorecky and Roberts, 1992; Poulos and Cappell, 1991).

There are probably multiple mechanisms of ethanol tolerance development. Increased rigidity of the membrane observed after chronic alcohol exposure appears to be an adaptation mechanism to counteract the fluidizing effect of alcohol, and is presumed to be one of the factors underlying the development of tolerance (Deitrich *et al*., 1989).

GABA and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, and the neurons using these neurotransmitters constitute >80% of all neurons in the brain. Therefore, the neuroadaptive changes that occur in these systems have primary relevance to the neurochemical and behavioral effects associated with ethanol abuse. As mentioned earlier, alcohol was reported to act through a dual mechanism increasing $GABA_A$ and reducing NMDA receptor functions. Naturally occurring neuroadaptation to chronic ethanol intake responsible for ethanol tolerance development results in damping $GABA_A$ receptor function, and up-regulation of NMDA

receptors (Iorio *et al*., 1992). Alcohol-dependent decrease in the sensitivity of $GABA_A$ receptor-mediated responses was reported in cerebral cortex (Sanna *et al*. 1993), nucleus accumbens (Szmigielski *et al*. 1992), and medial septal nucleus (Criswell *et al.* 1993). Suppression of $GABA_A$ receptor functions may arise from conformational changes in receptor structure, alterations in $GABA_A$ receptor subunit composition, and/or internalization of the receptor complex (Morrow, 1995). The most likely mechanism is alcohol-induced conformational change in $GABA_A$ receptor structure due to phosphorylation or dephosphorylation of these receptors. On the other hand, up-regulation of NMDA receptor functions seems to occur through the increase in the number of these receptors (Nutt, 1999).

1.6. Development of Ethanol Dependence

Substance dependence is characterized by compulsive, uncontrolled craving for a substance and attempts to get it at all costs and despite of obvious health and life-threatening consequences.

Physical dependence to a drug of abuse such as alcohol develops because of adaptive changes evoked by chronic use of a substance and aiming the maintenance of homeostasis. Due to physical dependence, substance (i.e. alcohol) withdrawal produces so called withdrawal or abstinence symptoms that are highly unpleasant. Therefore, substance craving (seeking) starts mainly in order to avoid disagreeable effects of the drug absence. Strong physical dependence is produced by alcohol, opiates, anxiolytic drugs, barbiturates.

However, substance dependence (or drug addiction) is also related to a disturbance of the reward system responsible for goal-oriented arousal mediating cortical responses with emotional quality such as curiosity or pleasure. The pleasure centers in the brain are connected with the ascending dopaminergic, noradrenergic and serotonergic projections of mesencephalic nuclei innervating cortex (prefrontal lobe) and the forebrain emotional (limbic) structures (i.e. shell of nucleus accumbens). Many addictive substances directly or indirectly stimulate release of neurotransmitters or mimic their action at the receptor level in the reward system. Drugs, which increase stimulatory neurotransmitter actions, increase the mood and cause very potent psychical dependence.

1.7. Withdrawal Syndrome and Its Effects on Brain Functions and Behavior

In human alcoholics, there are three clinical stages of alcohol withdrawal syndrome (AWS): minor; major, and severe referred to as delirium tremens (DT) (McMicken, 1990). According to Fadda and Rossetti (1998), minor withdrawal usually occurs 24-48 hr after cessation and manifest as mild autonomic hyperactivity, nausea, anorexia, tremor, tachycardia, hyperreflexia, anxiety, and insomnia. Major withdrawal symptoms usually occur after 3 days of abstinence with more profound hyperactivity, disorientation, diaphoresis, fever, seizures, and hallucinations. Severe withdrawal usually occurs after 5 days of abstinence and is characterized by gross tremor, profound confusion, extreme agitation, fever, incontinence, mydriasis, seizure, and frightening, visual or auditory hallucinations. Seizures generally consist of one or two grand mal attacks and may develop into status epilepticus. Their incidence is reported to be from 5% to 15% of subjects undergoing withdrawal. Hallucinations that may occur during major AWS can be visual, auditory, tactile, and olfactory. The incidence is similar to that of seizures. Frightening visual or persecutory auditory hallucinations are associated more often with severe withdrawal and may occur without DT (alcoholic hallucinosis).

Symptoms of alcohol withdrawal in animals include tremors and other motor dysfunction as well as autonomic overactivity. One of the most commonly studied symptoms is convulsions. Susceptibility to chemically induced convulsions and audiogenic seizures (i.e., seizures elicited by sound stimuli) is also increased during alcohol withdrawal. Additional measures of withdrawal include increased anxiety and increased change in behavioral reactivity to stimuli (Finn and Crabbe, 1997)

Interestingly withdrawal symptoms are more readily produced by drugs that inhibit neuronal activity: opiates increase K^+ currents while barbiturates, anxiolytics and alcohol are $GABA_A$ receptor agonists increasing Cl^- influx to the neuron.

Following repeated administration of alcohol, the brain attempts to restore normal functioning through adaptations such as tolerance and physical dependence that reduce alcohol's initial perturbing effects. New biological status requires, however, the continued presence of alcohol. When a person terminates a prolonged drinking session, the adaptations that developed to offset alcohol's initial inhibitory actions are unopposed, resulting in a rebound hyperexcitability, or withdrawal syndrome. It is

postulated that the neurochemical changes that occur during alcohol withdrawal (i.e., reduced function of inhibitory neurotransmission and increased activity of excitatory neurotransmission) not only contribute to the withdrawal syndrome, but also may cause long-term changes in brain excitability by a kindling-like process (Glue and Nutt, 1990). Drug withdrawal is critical for manifestation of drug dependence and the expression of the withdrawal syndrome is generally considered the major causal factor for the onset and development of the neuropathological alterations. Alcohol withdrawal disturbs new adaptive balance established between the brain inhibitory and excitatory neurotransmission resulting in increased stimulation of up-regulated glutamatergic NMDA receptors, elevated NO synthesis and glutamate release, and thus increased glutamate concentrations in the brain, increased cystolic calcium loading (through NMDA receptor ionophores but also via up-regulated voltage-sensitive calcium channels) and eventually amino acid excitotoxicity that may lead to apoptotic cell deletions (Gonzales *et al.*, 1996; Nutt, 1999). Paula-Barbosa *et al*. (1993) demonstrated that alcohol-induced loss of the cells in hippocampal formation was aggravated during withdrawal from alcohol. Parallel to apoptotic cell loss, seizure-induced increase in neural progenitor cells proliferation resulting in aberrant neurogenesis was also observed (Parent *et al*., 1997).

Electrophysiological, neurochemical, and behavioral evidence indicate that at pharmacological relevant doses ethanol activates the mesolimbic dopaminergic system (Fadda *et al*., 1991; Gessa *et al*., 1985; Weiss *et al.*, 1993). Increased activation of $GABA_A$ receptors in the VTA was postulated to mediate ethanol-induced increase of dopamine release in the NAc, one of the brain reward centers responsible for hedonic experiences. Conversely, withdrawal from chronic ethanol treatment and thus decrease in $GABA_A$ receptor simulation was reported to be associated with a profound decrease in DA release (Rossetti *et al.*, 1992; Weiss *et al*., 1996). The latter effect is linked to a reduction in neuronal activity of VTA dopaminergic neurons (Diana *et al*., 1993) and results in increased reward threshold (Koob, 2003).

All these withdrawal-associated changes in the brain functions and morphology can be resulted with adverse effects on behavior and cognitive functions.

1.8. The Summary of Ethanol Effects

Ethyl alcohol is one of the most common drugs of abuse in human population and is known as a potent teratogen. In human, prenatal exposure to higher doses of alcohol was shown to trigger a massive wave of apoptotic neurodegeneration in many different regions of the developing brain and result in Fetal Alcohol Syndrome (FAS) or Fetal Alcohol Effects (FAE). FAS and FAE are characterized by structural and behavioral anomalies such as facial dysmorphogenesis, and motor and/or cognitive deficits.

Alike chronic perinatal alcohol intoxication, long-term alcohol intake in the adulthood, was also reported to have highly adverse effects on both the brain morphology and behavior. In chronic ethanol feeding models significant cell losses were reported in cerebellum, hippocampus, and neocortex. Parallel to morphological damage, chronic ethanol consumption by adult subjects was shown by several authors to result in the impairment of sensorimotor functions as well as memory and learning with more severe deficits in spatial (hippocampus-dependent) than nonspatial memory tasks. However, not all the experimental results were consistent. Generally, the adverse effects related to chronic exposure to alcohol in the adulthood were reported to aggravate during the withdrawal period.

Despite of widespread abuse of ethanol and increased knowledge about related brain damage and behavioral deficits, still it is little known about molecular mechanisms underlying ethanol neurotoxicity due to chronic ethanol intake. Many different mechanisms have been proposed and most of them seem to converge on production of free radicals and oxidative stress as a main cause of ethanol damaging effects.

1.9. Aim of the Study

The aim of the present study was to revisit the issue of the chronic adult alcohol insult on the retention of spatial reference memory in rats and if possible, to correlate the behavioral output with the protein, nucleic acids, and lipid profiles in the rat hippocampus, a brain structure critical for memory formation. There is a vast body of data related to ethanol-induced molecular changes in the peripheral tissues and in the brain. However, most of these data were obtained using classical assay techniques such as mass spectrometry, liquid/gas chromatography, gel electrophoresis, etc. In the present study, molecular characteristics of the brain tissue in control and ethanol-exposed

animals were examined using Fourier Transform Infra-Red (FT-IR) spectroscopy, a new technique characterized by high sensitivity in detecting changes in the functional groups belonging to tissue components. Using this method information about the lipid conformation and the protein secondary structure can be obtained simultaneously with a single experiment.

In the present study, alcohol was delivered chronically by intragastric intubation (binge-like drinking). Retention of spatial memory was examined at different times (4, 24, and 72 h) after the last alcohol administration to be able to differentiate between the acute and chronic alcohol intoxication and the withdrawal effect.

This study may contribute to better understanding of the molecular mechanisms of alcohol neurotoxicity.

CHAPTER 2

MATERIALS AND METHODS

2.1. Subjects

Large number of 3.5 - 4 months old, naive, male Wistar rats, obtained from the Hıfzısıhha Serum-Production Facility (Ankara), were used in the present study. Throughout the experiments, rats were kept in the animal house, in the Department of Biological Sciences at METU, with controlled temperature (22 ± 1°C), under 12 h/12 h light/dark cycle (lights on at 07:00 a.m., lights off at 07:00 p.m.), and with free access to water and food (laboratory chow). Tests were carried out in the light phase of the light/dark cycle.

2.2. Apparatus

2.2.1. Morris Water Maze

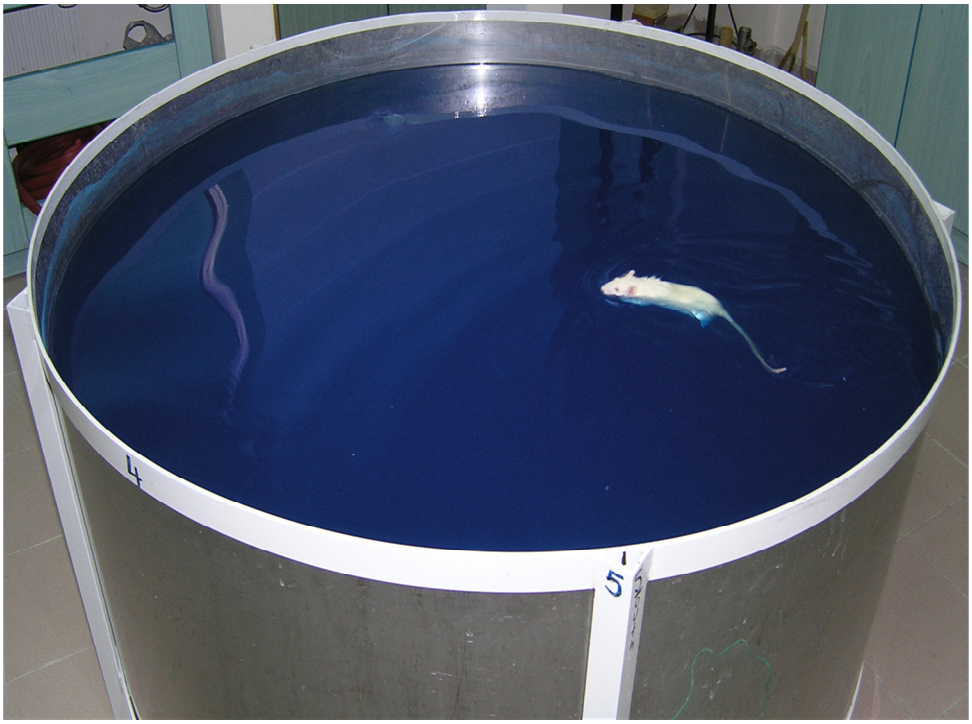

Figure 1. Morris Water Maze Apparatus

Morris Water Maze (MWM) is commonly used to test spatial learning and memory in rodents. It is a circular tank, 60 cm high and 150 cm in diameter. It was filled to the depth of 45 cm with water maintained at 23 °C (±1) by an automatic heater. Nontoxic blue watercolor paint was added to make water opaque. Computerized video tracking system (EthoVision System by Noldus Information Technology, Holland) was used to track the animal in the pool and to record data. The Noldus EthoVision video-tracking system was automatically recording the following measures:

1. Swim path trajectory,
2. Escape latency: the time, in seconds, between the start location and escape platform,
3. Swim distance (path length): the distance swum, in centimeters, from the start location to the escape platform,
4. Mean swim velocity.

On the computer screen, the pool was divided into four quadrants by two imaginary perpendicular lines crossing in the centre of the pool. The quadrants were marked by the four compass points as North-East (NE), North-West (NW), South-East (SE), and South-West (SW). A movable platform (11 ×11) made of transparent Plexiglas and thus invisible to the animals, was located in the centre of one of the quadrants. The top of the platform was 2 cm below the surface of the water such that the animal could climb on it in order to escape from the water. A camera was mounted to the ceiling above the pool and was connected to a microprocessor. Experimental room was furnished with several extra-maze cues immobile throughout the entire experimental period. Indirect illumination was provided by diffused light coming from the sides of the room.

2.2.2. FTIR spectrometer

Infrared spectra were obtained using a Perkin-Elmer SpectrumOne FTIR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) equipped with a MIR TGS detector.

2.3. Experimental Procedure

2.3.1. Experimental Design

Two different experimental designs were used to determine the alcohol effects on spatial memory retention and the molecular make-up of the hippocampus as assessed by FT-IR spectroscopy.

Experiment I

I. Stage: Six sessions of place learning in the Morris Water Maze (MWM)

II. Stage: Six days of alcohol/isocaloric solution administration by intragastric intubation (binge-like drinking)

III. Stage: Memory retention test (probe trial) in the MWM, Group A0 (n=7) was tested 2 h after the last ethanol administration, Group A24 (n=7) tested 24 h after ethanol withdrawal, and Group A72 (n=7) tested 72 h after ethanol withdrawal. Isocaloric control group (IC (n=7)) was subjected to the memory retention test 72 h after last intubation. This experimental protocol was adopted from Celik *et al.* (2005).

IV. Stage: Decapitation of the animals, three hrs after the completion of the probe trial Removal of the hippocampi and storage at -80°C for the spectroscopic examination.

V. Stage: FT-IR spectroscopic analyses of the brain tissue.

Experiment II

Experiment II differed from the Experiment I in: (1) controlling the acquired place preference at the end of MWM acquisition training by application of a probe trial followed by two retraining sessions, and (2) in longer, lasting for 15 days, binge-like ethanol treatment. In the Experiment II, the group size was as follows: A0 (n=7), A24 (n=7), A72 (n=7), and IC (n=10).

2.3.2. Behavioral Tests

2.3.2.1. Handling

For five consecutive days prior to the beginning of experiments, all rats were daily weighed and handled each for 30 s, to get used the animals to the experimenter.

2.3.2.2. MWM Acquisition Training and Probe Trial Tests

In the MWM, the rats use hippocampus-dependent long-term spatial memory to learn the position of a hidden platform in reference to the stable throughout the experiment visuo-spatial distal cues belonging to the room. During place learning in the MWM, rats were given six sessions and each session had four trials. Inter-trial intervals lasted

approximately 5 min. Each rat was released into the water facing the pool wall at one of the four starting points (N, S, E, W) that were used in a pseudorandom order such that each position was used once during the session. Every trial lasted maximum 60 s or until the animal found the platform where it remained 15 s and then was put to the waiting cage for the inter-trial interval. On the first experimental session, if the animal did not find platform within the 60 s, the experimenter guided it gently to the platform. On the completion of 6 sessions of MWM training, the animals were randomly divided into 4 groups: three alcohol (A0, A24, and A72) groups and one control (IC) group. There were no significant differences in swim latency between the groups.

In the Experiment II, the acquisition training was the same except that on the following day after the training completion, animals were tested for the place preference in 60 s probe trial carried out without the platform. On the day after the probe trial, all animals were subjected to two retraining sessions, 4 trials each, to restore the place habit.

2.3.2.3. Probe Trial: A Memory Retention Test

Probe trial was also used as a memory retention test after the alcohol treatment. Retention of place memory was evaluated in three individual groups of ethanol-administered, ethanol-withdrawn (24^{th} and 72^{nd} h of withdrawal) and control rats. The probe trial is used to assess the strength of the acquired response and, indirectly, to assess degree of learning. On a 60 s lasting probe trial, the platform was removed from the pool. On the computer screen, an imaginary 40 cm diameter annulus (annulus 40) was drawn around the place where originally platform was located. The total time an animal spent in: (a) platform quadrant (NE); (b) the opposite quadrant (SW); and (c) the annulus 40 were recorded.

2.3.3. Alcohol Administration

Adapting Majchrowicz protocol (1975), the behavioral intoxication states of ethanol-treated rats were rated on a scale of 1 to 5 after each treatment. The rating was used to determine the largest dose of ethanol that could be tolerated.

The alcohol was delivered to the rats by the intragastric intubation method using stainless curved feeding needle directly into stomach of the rat (Needle, Curved, 18ga, 3 in, Stoelting Co. USA).

Figure 2. The moment of intragastric intubation

Ethyl alcohol (99.8 % v/v, Merck) was used in this study. The alcohol was prepared as a 25 % (v/v) solution mixed with distilled water (Experiment I) or 50% light PINAR Milk (Experiment II). In Experiment I, animals in IC group received the same volume of fluid with sucrose, which substituted isocalorically for ethanol. In Experiment II, where high alcohol dose was administered over a longer period, alcohol solution was prepared on the milk basis. In this experiment, IC group used to receive the same volume of milk as alcohol groups but without ethanol. In Experiments I, the total daily dose of ethanol was stepwise increased from initial 4.5 g/kg/day to the final 12 g/kg/day within the first four days of alcohol administration (see Table 1).

Table 1. Dose and time table of alcohol administration in Experiment I

Time	Dose in one intubation	Total daily dose
1st day	1,5 g/kg	4,5 g/kg/day
2nd and 3rd days	2,0 g/kg	6,0 g/kg/day
4th day	3,0 g/kg	9,0 g/kg/day
5th and 6th days	4,0 g/kg	12,0 g/kg/day

In the Experiment II, the total daily dose of ethanol was stepwise increased from the initial 6g/kg/day to 12 g/kg/day within the first thirteen days of alcohol administration.

In both Experiments I and II, the total daily dose of alcohol was divided into three equal doses delivered by intragastric intubation 3hr (Experiment I) or 4 hr (Experiment II) apart. The alcohol administration protocol was strictly timed such that the rats received the alcohol at the same time each day. In Experiment I, alcohol and isocaloric solution were given to animals at 10:00 a.m., 01:00 p.m., and 04:00 p.m. After the last dose, 50% milk and water mixture was prepared and 50 cc of this mixture was given to each animal as a protection of stomach mucosa. In Experiment II, three equal doses of ethanol solution were given to animals 4 h apart, at 10:00 a.m., 02:00 p.m., and 06:00 p.m. Throughout the experiments, all animals had ad libitum access to laboratory chow and water.

2.3.4. Blood Alcohol Concentration (BAC)

The BAC were measured in a separate groups of rats (n=3) receiving the same ethanol treatment as other animals but not subjected to behavioral tests. Blood samples (3–4 ml) were taken by intracardiac puncture under the ether anesthesia on the last day of ethanol administration, 3 hr after the last intragastric intubation (Abel, 1978; Tran *et al.,* 2000). Samples were collected into tubes containing EDTA and centrifuged (1000 rpm for 10 min.) at room temperature. The supernatants were separated and alcohol level was determined by the Biolabo alcohol assay. Levels were expressed as mg/dl.

2.3.5. Decapitation

In both Experiments I and II, approximately three hours after the completion of a probe trial, animals were decapitated by a guillotine, brains were removed and the hippocampi were dissected and stored at - 80^0C until the FT-IR studies.

2.3.6. FTIR Spectroscopic Measurements

2.3.6.1. FTIR spectroscopy

Spectroscopy is defined as the study of the interaction of electromagnetic radiation with matter. Spectroscopic techniques involve irradiation of a sample with some form of electromagnetic radiation, measurement of the scattering, absorption, or emission in terms of some measured parameters, and the interpretation of these measured parameters to give useful information.

The term “infrared” covers the range of the electromagnetic spectrum between 0.78 and 1000 μm. In the context of infrared spectroscopy, wavelength is measured in “wavenumber”. The infrared spectrum can be divided into three regions according to wavenumber: the *far infrared* (400-20 cm^{-1}), the *mid infrared* (4000-400 cm^{-1}) and the *near infrared* (14285- 4000 cm^{-1}). Most infrared applications employ the mid-infrared region, but the near and far infrared regions can also provide information about certain materials.

The atoms in a molecule are constantly oscillating around average positions. Bond lengths and bond angles are continuously changing due to this vibration. The vibrational levels and hence, infrared spectra are generated by the characteristic twisting, bending, rotating and vibrational motions of atoms in a molecule. As shown in Figure 3 vibrations can either involve a change in bond length (*stretching*) or bond angle (*bending*).

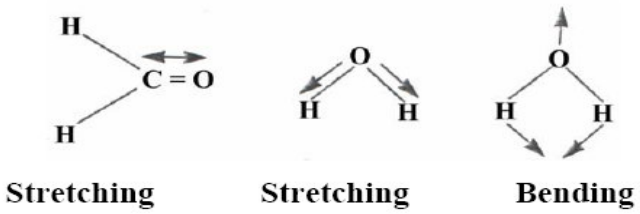

Figure 3. Types of normal vibration in a linear and non-linear triatomic molecule. Atomic displacements are represented by arrows (in plane of page) (Arrondo *et al*., 1993).

The value of infrared spectrum analysis comes from the fact that frequencies and intensities are sensitive to local structure, orientation, physical state, conformation, temperature, pressure and concentration (McDonald, 1986).

Fourier transform infrared (FT-IR) spectroscopy is a new technique that monitors different functional groups by measuring the vibrations of molecules due to electromagnetic radiation at infrared region (10^3-10^5 nm). This technique is mostly used in different scientific areas to provide quantitative and qualitative information about the sample.

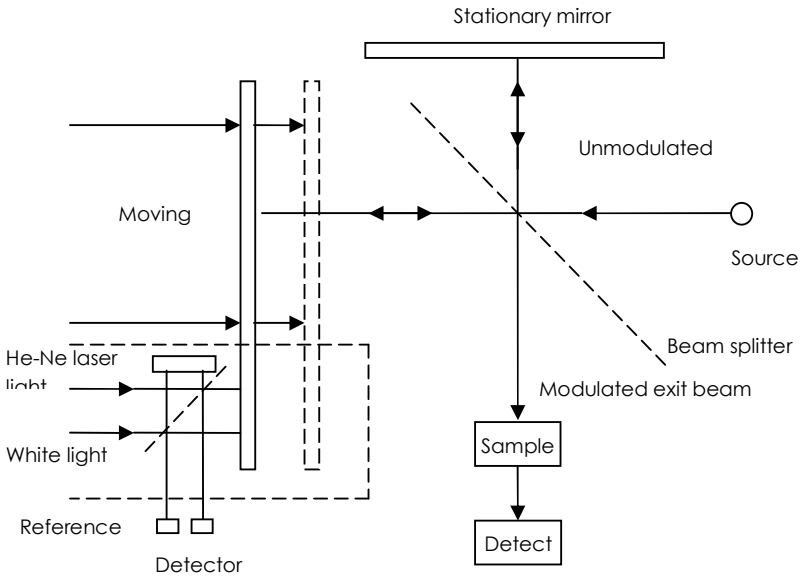

Figure 4. Instrumentation of FT-IR spectrometer

In biological research, the FT-IR technique gains more importance because it can investigate the biological systems at molecular level without giving any harm to their structure **(**Haris and Severcan; 1999; Jackson *et al.* 1997; Liu *et al.* 2002; Melin *et al.*, 2000; Mourant *et al*. 2003**).** Moreover, it is known that FT-IR is used in different areas like determination of secondary structure of proteins, interaction of biological macromolecules with other molecules, identification and diagnosis of pathologic conditions like cancer and diabetes in tissue level, systematics of living things (Boyar and Severcan, 1997; Fukuyama *et al*., 1999; Li *et al*., 2002; Severcan *et al.,* 2000; Toyran *et al*., 2004).

FT-IR spectroscopy provides a precise measurement method, which requires no external calibration. It is a rapid and sensitive technique. The instruments are relatively easy to use and data processing is simple with the computer software, which are user-friendly (Manoharan *et al.*, 1993; Rigas *et al.*, 1990). Moreover, system permits permanent data storage, manipulation of data and quantitative calculations (Garip *et al.*, 2007; Gorgulu *et al.,* 2007; Yono *et al.*, 1996). Since a computer is already used to obtain the Fourier transform, it is easy to perform many scans to improve the signal-to-noise ratio (noise adds up as the square root of the number of scans, whereas signal adds linearly). Highly improved signal to noise ratio is achieved by the averaging of numbers of scans per

sample. Frequency and bandwidth values can be determined routinely with uncertainties of better than ± 0.05 cm^{-1}.

The system can be applied to the analysis of any kind of material and is not limited to the physical state of the sample. Samples may be aqueous solutions, viscous liquids, suspensions, inhomogeneous solids or powders, single crystals, detergent micelles, etc. It is a valuable technique due to its high sensitivity in detecting changes in the functional groups belonging to tissue components, such as lipids, proteins and nucleic acids (Cakmak et al., 2006; Kneipp *et al*. 2000; Severcan *et al*., 2000; Toyran *et al*., 2004). The shift in the peak positions, bandwidths, and intensities of the bands all give valuable structural and functional information, which may have diagnostic value (Dogan *et al*., 2007; Severcan *et al*. 2000; Toyran *et al*., 2006; Yono *et al*. 1996). Moreover, information about the lipid conformation and the protein secondary structure can be obtained simultaneously with a single experiment.

With developments in FT-IR instrumentation, it is now possible to obtain high quality spectra from aqueous protein solutions (Arrondo and Goñi, 1999; Haris and Severcan, 1999; Surewicz *et al*., 1993). FT-IR spectroscopy technique requires only small amounts of sample (10 μg), and the size of the sample is not important (Haris and Severcan, 1999). Digital subtraction (that is, point-by-point subtraction of the separate spectra by a computer) can be used to produce good difference spectra. This method has great advantages in obtaining infrared spectra in aqueous solutions (Campbell and Dwek, 1984). The overlapping H_2O absorption bands can be digitally subtracted from the spectrum of the protein solution. In addition, the broad infrared bands in the spectra of the proteins can be analyzed in detail by using second derivative and deconvolution procedures. These procedures can be utilized to reveal the overlapping components within the broad absorption bands (Arrondo and Goñi, 1999; Surewicz *et al*., 1993).

2.3.6.2. Sample Preparation for FT-IR Studies

The hippocampus samples were dried overnight in a LABCONCO freeze drier (Labconco FreeZone®, 6 liter Benchtop Freeze Dry System Model 77520) in order to remove the water content. The samples then were ground for 2 minutes in agate mortar containing liquid nitrogen to obtain powder. Then, small quantities of the samples (0.001 grams) were mixed with potassium bromide (KBr) at a 1/150 ratio to produce a

homogenous powder. KBr is most commonly used alkali halide disk serving as a beam condensing system. The mixture was dried again in the freeze drier for 4 hours to remove all traces of remaining water. In this procedure, the water solution of sample and halide is frozen and a strong vacuum is applied to frozen solid. The mixture was then compressed for 6 min under a pressure of ~100kg/cm^2 (1300psi) in an evacuated die to obtain a thin KBr disk. KBr disk or pellet is transparent to IR light in the spectral region of interest so an impeded spectrum of the compound is obtained. This sinters the mixture and produces a clear transparent disk (Stuart, 1997).

2.3.6.3. Spectroscopic Measurements

The dilution with KBr or some other reagent is necessary to obtain better quality FT-IR spectra. Although the used KBr is always infrared spectroscopic grade, there is a possibility that it may give some small absorption bands interfering with sample spectra. To overcome these problems the spectrum of air and KBr transparent disk was recorded together as background and subtracted automatically by using appropriate software (SpectrumOne software, (Perkin-Elmer)). Figure 5 shows the FT-IR spectrum of 100% pure KBr pellet.

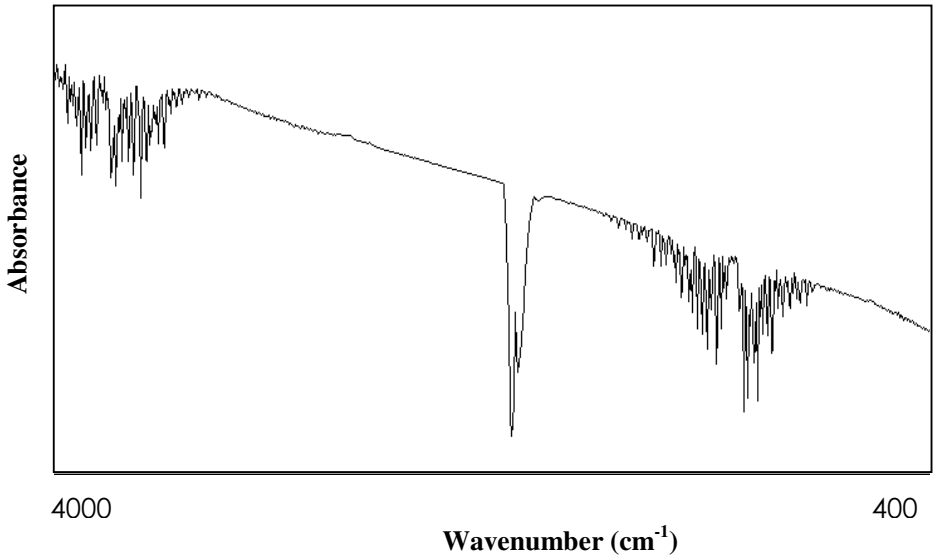

Figure 5. The spectrum of 100 % pure KBr Pellet

The FT-IR spectrum was recorded in the 4000-400 cm^{-1} region at room temperature. Each interferogram was collected with 100 scans per sample at 4 cm^{-1} resolution. Each

sample was scanned under the same conditions with three different pellets, all of which gave identical spectra. The average spectra of these three replicates were used in detailed data analysis and statistical analysis. Collections of spectra and data manipulations were carried out using SpectrumOne software. The band positions were measured using the second derivative of the spectra. Using the same software, the spectra were first smoothed with nineteen-point Savitsky-Golay smooth function to remove the noise after the averages of three replicates of the same samples were taken. After that, the spectra were baseline corrected. The spectra were normalized with respect to specific bands for visual demonstration. The purpose of the normalization is to remove differences in peak heights between the spectra acquired under different conditions. It allows a point-to-point comparison to be made (Smith and Jackson, 1999). The shifting of the frequencies was examined before the normalization process. Band areas were calculated from smoothed and baseline corrected spectra using SpectrumOne software. The bandwidth values of specific bands were calculated as the width at 0.80 x height of the signal in terms of cm^{-1}.

2.4. Data Analyses

In the behavior tests, from all measures 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. The statistical package SPSS 10.0 for windows was used to compare the results with ANOVA.

In the FT-IR studies, the results were expressed as means ± standard deviation (SD). The data were analyzed statistically using non-parametric Mann–Whitney U test with the Minitab statistical Software Release 13.0 program. A ‘p’ value less than or equal to 0.05 was considered as statistically significant. The degree of significance was denoted as less than or equal to $p<0.05$*, $p<0.01$**.

All procedures in the present study were performed in accordance with the rules in the Guide for the Care and Use of Laboratory Animals adopted by National Institutes of Health (USA) (Institute of Laboratory Animal Sources Commission on Life Sciences, National Research Council, 1996).

CHAPTER 3

RESULTS

3.1. Blood Alcohol Concentration

In the rats subjected to binge drinking, the average blood alcohol concentration estimated at the end of the alcohol treatment (12 g/kg/day), 3h after the third intubation, was 605,67 ± 36 mg/dl. The range was 569 mg/dl - 641 mg/dl.

3.2. Results of Behavioral Tests

3.2.1. Learning Tests

3.2.1.1. Classical MWM Training

In both Experiments I and II, two-way repeated measures ANOVA (group x day) performed for escape latency yielded a significant day effect ($F_{(5:120)} = 62.70$ $p < 0.001$, and $F_{(5:135)} = 36.12$ $p < 0.001$, respectively) showing a general decrease in overall latency throughout the training period. No between-group differences were revealed ($F_{(15:120)} = 0.87$ $p=0.60$, $F_{(15:135)} = 0.93$ $p= 0.53$, respectively). On the fourth day of the acquisition training, all rat groups reached the asymptotic performance level with mean escape latency oscillating around 10s. (Figure 6 and Figure 7, respectively).

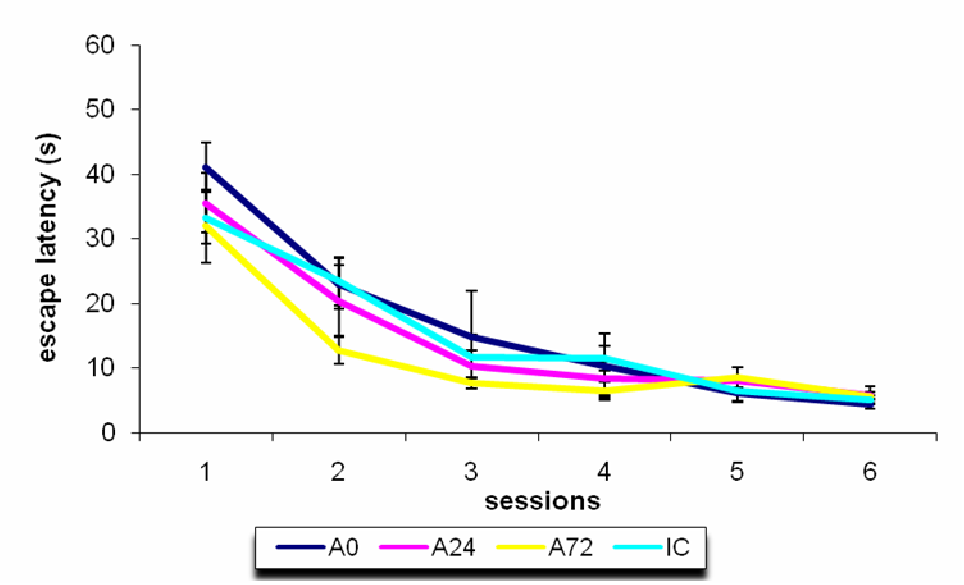

Figure 6. Mean escape latency ± SEM for the Experiment I to locate invisible platform in the water maze calculated for each training day and each treatment group independently

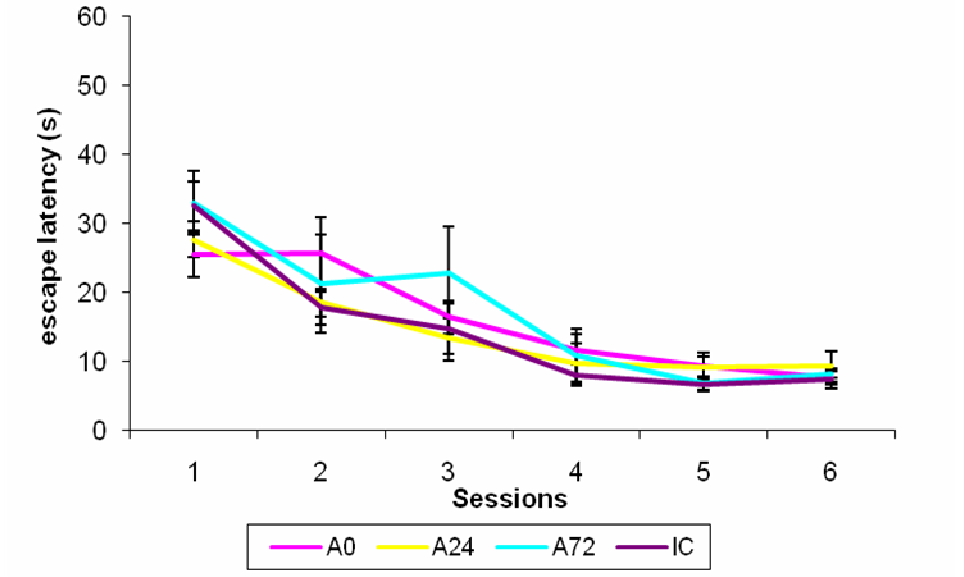

Figure 7. Mean escape latency ± SEM for the Experiment II to locate invisible platform in the water maze calculated for each training day and each treatment group independently.

In both Experiments, no between-group differences were also found in the swim distance measure.

3.2.1.2. Probe Trial at the End of the MWM Training (Experiment II)

This test was done only in the Experiment II to measure the levels of learning of the rats. As seen from Figure 8, in all the groups, the average percent time spent in the platform quadrant was around 50%. No significant between-group differences were revealed in this measure.

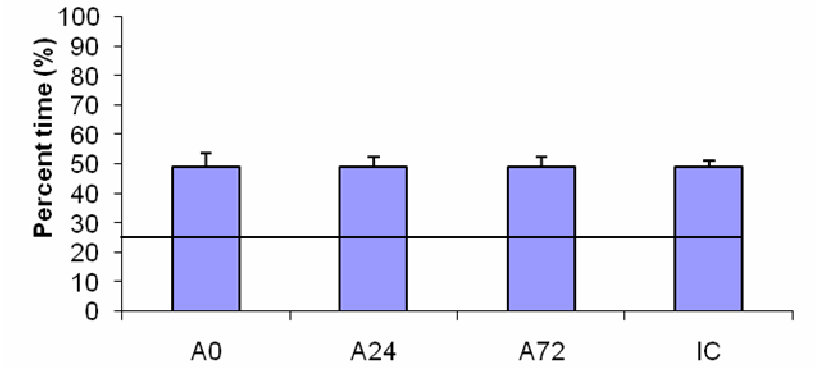

Figure 8. Mean percentage of time (± SEM) spent in the platform quadrant on the 60-s probe trial carried out on the completion of MWM training, in the Experiment II, in each treatment group independently. Line at 25% represents chance level.

3.2.1.3 Retraining

This retraining procedure was introduced in the Experiment II after the probe trial (carried out without escape platform) to refresh the rats memory about the initial platform position. In this task, in all groups, the time of reaching the hidden escape platform was under the 10 s. No significant between-group differences were noted.

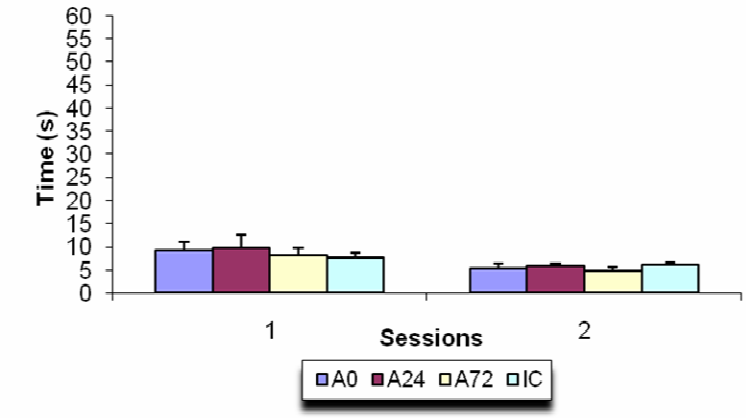

Figure 9. Mean escape latency ± SEM for the Experiment II to locate invisible platform in the water maze calculated for each retraining session and each treatment group independently.

3.2.2. Probe Trials Applied After the Completion of Ethanol Treatment: Memory Retention Test

As mentioned earlier, animal's performance in the water maze on the one day 60-s probe trial (memory retention test) was assessed by the percentage of time spent in the platform quadrant (Figure 10 and Figure 11); the ratio of time spent in the platform quadrant to time spent in the opposite quadrant (Figure 12 and Figure 13); and time in annulus 40 (Figure 14 and Figure 15).

3.2.2.1 Percent time in the platform quadrant

In the Experiment I, one-way ANOVA performed on the percent time in the platform quadrant yielded significant group effect ($F_{(3; 27)} = 6.37$, $p=0.002$). Subsequent post hoc comparisons using the LSD test (SPSS statistical package) confirmed significantly better performance in A24 group as compared with both A0 and A72 alcohol groups ($p<0.05$). A24 group was marginally better also from IC group ($p = 0.077$). However, the performance in control and two alcohol groups (A0 and A72) remained at the chance level (Figure 10).

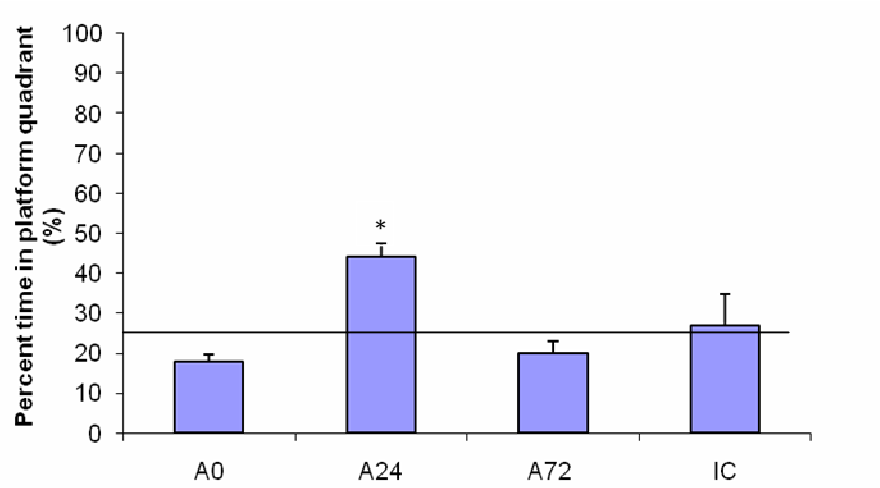

Figure 10. Mean percentage time spent in the platform quadrant on the 60-s probe trials in each treatment group independently in the Experiment I. Error bars denote SEM. Line at 25% represents chance level. Asterisk indicates significant difference at $p \leq 0.05$.

In contrast to the Experiment I, in the Experiment II, the probe trial performance as assessed by the percent time spent in the platform quadrant was in all groups above the chance level (Figure 11) but no significant between-group differences were recorded.

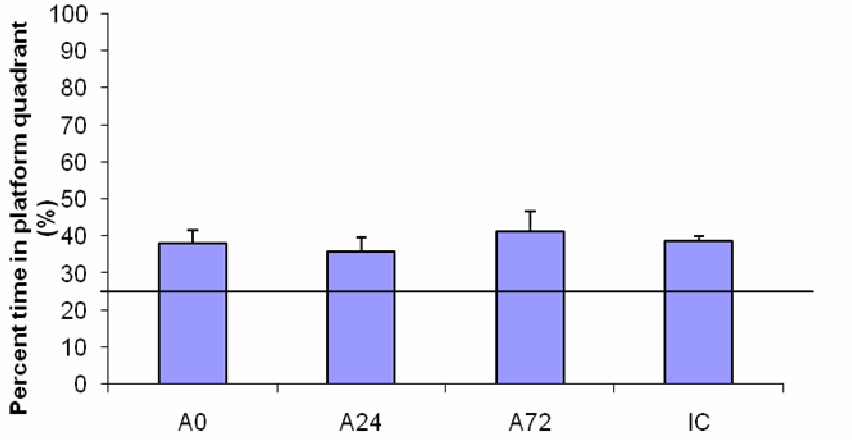

Figure 11. Mean percentage time spent in the platform quadrant on the 60-s probe trials in each treatment group independently in the Experiment II. Error bars denote SEM. Line at 25% represents chance level.

3.2.2.2. NE/SW ratio

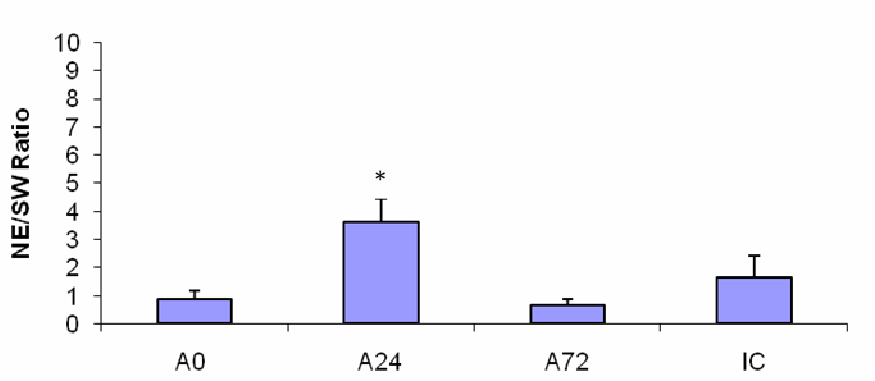

Figure 12. Ratio of the total time spent in the platform quadrant (NE) to the total time spent in the opposite quadrant (SW) for each treatment group independently for the Experiment I. Asterisk indicates significant difference at $p \leq 0.05$

As seen from the Figure 12, in the Experiment I, the best index of performance was shown by A24 group. One-way ANOVA performed on NE/SW ratios yielded significant group effect ($F_{(3;\ 27)} = 5.25$, $p=0.006$). Subsequent post hoc comparisons using the LSD test (SPSS statistical package) confirmed significantly better performance in A24 group as compared with all other groups ($p < 0.05$).

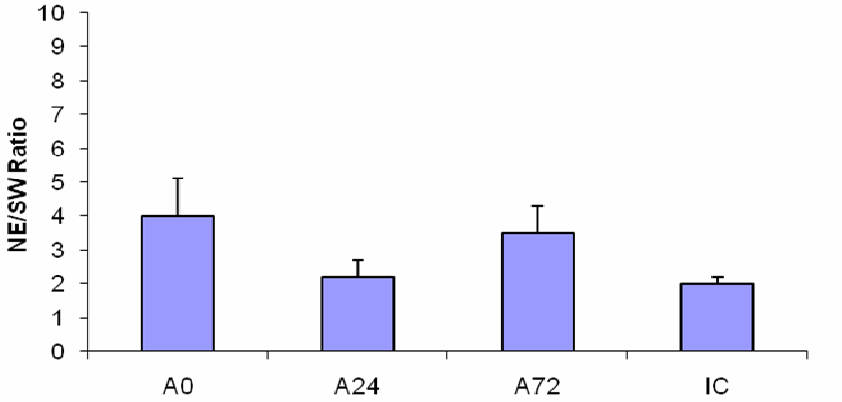

Figure 13. Ratio of the total time spent in the platform quadrant (NE) to the total time spent in the opposite quadrant (SW) for each treatment group independently for the Experiment II**.**

In contrast to the Experiment I, in the Experiment II, also on this learning measure no significant between-group differences were revealed ($F_{(3; 30)} = 2.05$, p=0.13).

3.2.2.3. Time spent in the annulus 40

This measure is used because sometimes, during the probe trial, two rats may spent the same time and swim the same distance in the platform quadrant but when their swim trajectories are compared one of the rats appear to swim at much shorter distance to the previous platform location thus, showing better performance than the other animal.

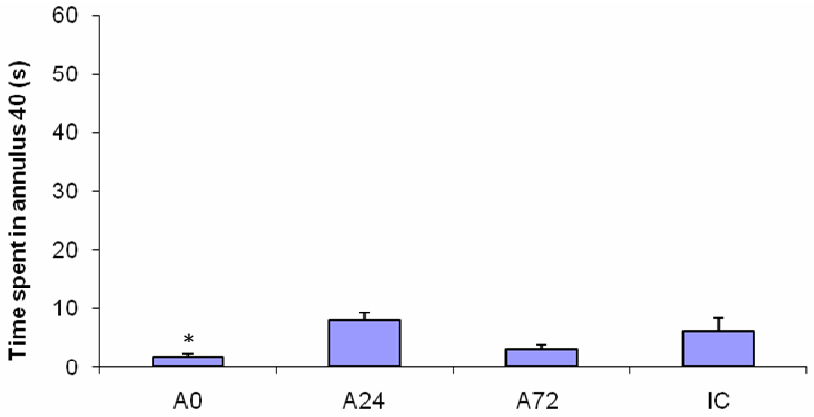

Figure 14. Time in annulus 40 ± SEM calculated for each treatment group independently for the Experiment I. Asterisk indicates significant difference at $p \leq 0.05$

In Experiment I, also on this measure A24 group performed the experiment to be better than the remaining groups. One-way ANOVA confirmed a significant overall group effect ($F_{(3; 27)} = 4.28$, $p = 0.015$). Here too, the performance of A24 group did not significantly differ from that of control animals but was significantly better than in A0 and A72 groups ($p<0.05$). There was no significant difference between A0 and A72 groups, but the difference between A0 versus IC group was yielded significant (p=0.032).

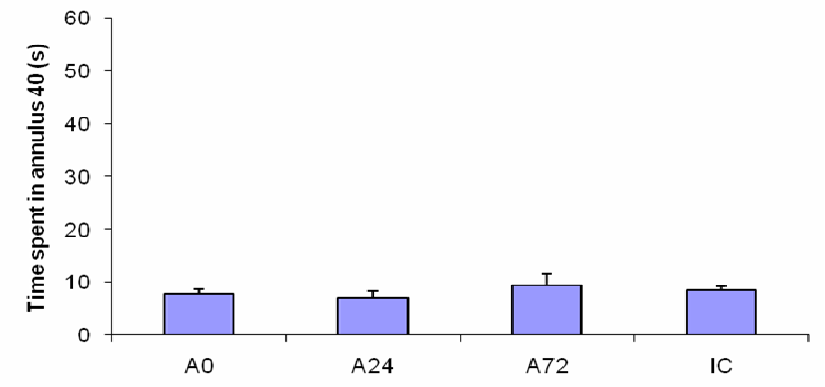

Figure 15. Time in annulus 40 ± SEM calculated for each treatment group independently for the Experiment II.

However, no significant between-group differences were observed in the time spent in annulus 40 in the Experiment II.

3.3. FT-IR Studies

3.3.1. General Band Assignment of Hippocampus

Since the positions and intensities of many of the infrared absorption bands can be correlated with the presence of specific groups of atoms in the system studied (Steele, 1971), it is possible to assign specific wavelength molecular absorption bands to specific vibrational modes of particular functional groups. According to these specific groups, the absorption bands of a representative infrared spectrum obtained from untreated rat hippocampus in the 4000-400 cm^{-1} wavenumber range was demonstrated in the Figure 16. The main absorption bands have been labeled in this figure and are defined in detail in Table 2 according to the literature (Banyay *et al*., 2003; Cakmak *et al*., 2006; Jackson *et al*., 1998; Jamin *et al*., 1998; Lyman *et al*., 1999; Melin *et al*., 2000; Rigas *et al*., 1990; Takahashi *et al*., 1991; Toyran *et al*., 2006; Wong *et al*., 1991).

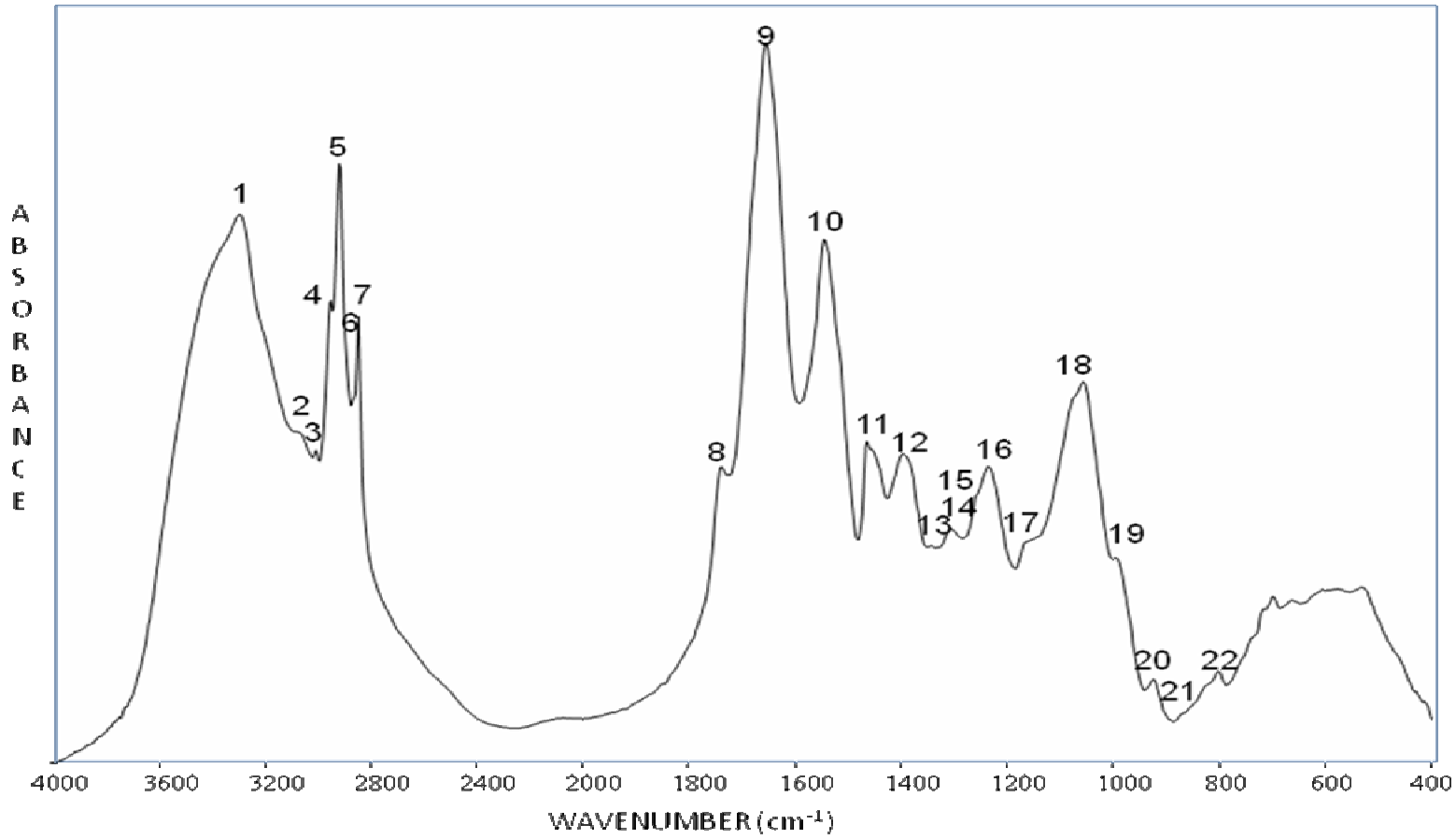

Figure 16. The representative FT-IR spectrum of control group in the 4000-400 cm^{-1} region. The spectra were normalized with respect to the Amide A mode at around 3360 cm^{-1}. (Absorbance in arbitrary units).

Table 2. General band assignment of brain tissue (hippocampus)

Peak No	Wavenumber (cm^{-1})	Definition of the spectral assignment
1	3304	O-H stretching (Amide A), hydrogen-bonded intermolecular OH groups of proteins and glycogen
2	3066	C-H and N-H stretching (Amide B) of protein
3	3014	Olefinic=CH stretching vibration: unsaturated lipids, cholesterol esters
4	2961	CH_3 asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
5	2921	CH_2 asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
6	2872	CH_3 symmetric stretch: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids
7	2851	CH_2 symmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
8	1745	(ester) Carbonyl C=O stretch: lipids
9	1658	Amide I (protein C=O stretching)
10	1549	Amide II (protein N-H bend, C-N stretch)
11	1468	CH_2 Bending: mainly lipids, with the little contribution from proteins
12	1402	COO^- symmetric stretch: fatty acids
13	1344	Amide III vibrations of collagen
14	1311	CH_2 twisting and bending (protein, lipid), nucleic acids
15	1262	PO^-_2 asymmetric stretch, non-hydrogen-bonded: mainly nucleic acids with the little contribution from phospholipids
16	1236	PO^-_2 asymmetric stretch, fully hydrogen-bonded: mainly nucleic acids with the little contribution from phospholipids
17	1172	CO-O-C asymmetric stretching: glycogen and nucleic acids
18	1083	PO^-_2 symmetric stretch: nucleic acids and phospholipids C-O stretch: glycogen, polysaccharides, glycolipids
19	970-995	$C\text{-}N^+\text{-}C$ stretch: nucleic acids, ribose-phosphate main chain vibrations of RNA
20	925	z-type DNA
21	876	Vibrations in N-type sugars in nucleic acid backbone
22	801	Vibrations in N-type sugars in nucleic acid backbone

3.3.2. Comparison the Spectra of Alcohol and Control Hippocampi

FTIR spectral data were collected over the frequency range of 4000-400 cm^{-1}. As seen from Figure 16 and the band assignment given in Table 2, it is a complex spectrum that

contains several bands belonging to lipids, proteins, and nucleic acids. Therefore, for more detailed analysis, the investigations were performed in three different regions: The first range was 3800-3030 cm^{-1}, the second range was 3030-2800 cm^{-1}, and the third range was 1800-400 cm^{-1}. All control spectra overlapped. All alcohol spectra, which were different from control spectra, also overlapped. For this reason, for the following discussions only one control and alcohol spectrum was chosen as a representative spectrum.

Figures 17 and 18 show the infrared spectra of control and alcohol groups in 3800-3030 cm^{-1} region and in 3030-2800 cm^{-1} region, respectively.

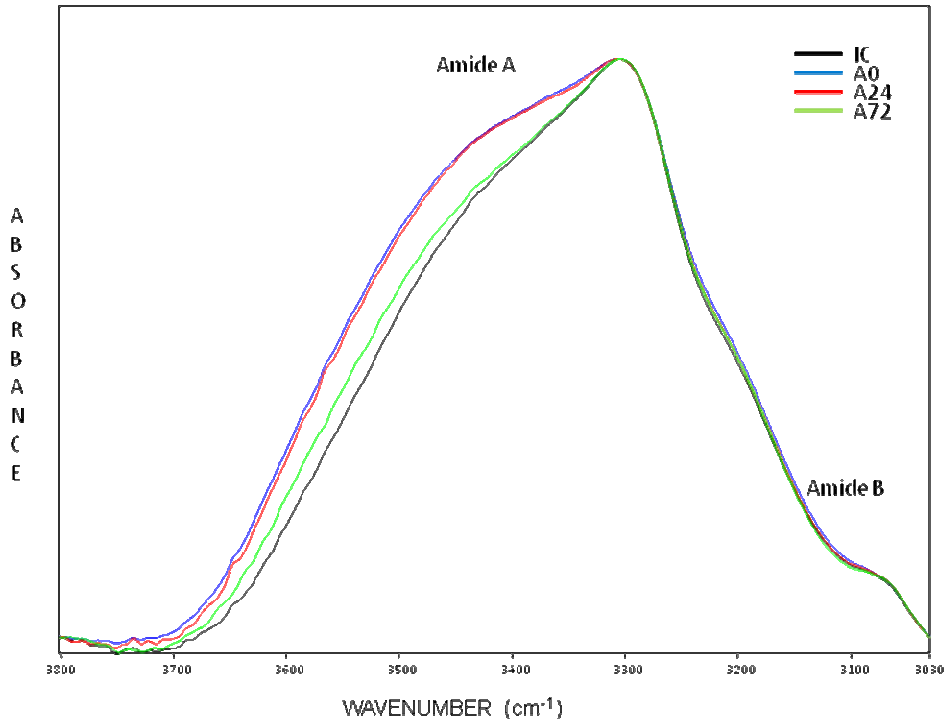

Figure 17. The representative infrared spectra of control and alcohol groups in the 3800-3030 cm^{-1} region. The spectra were normalized with respect to the Amide A mode at 3360 cm^{-1} (Absorbance in arbitrary units). (IC for intubation control group, A0 for ethanol acute effects group, A24 for ethanol chronic effects group, and A72 for ethanol withdrawal effects group).

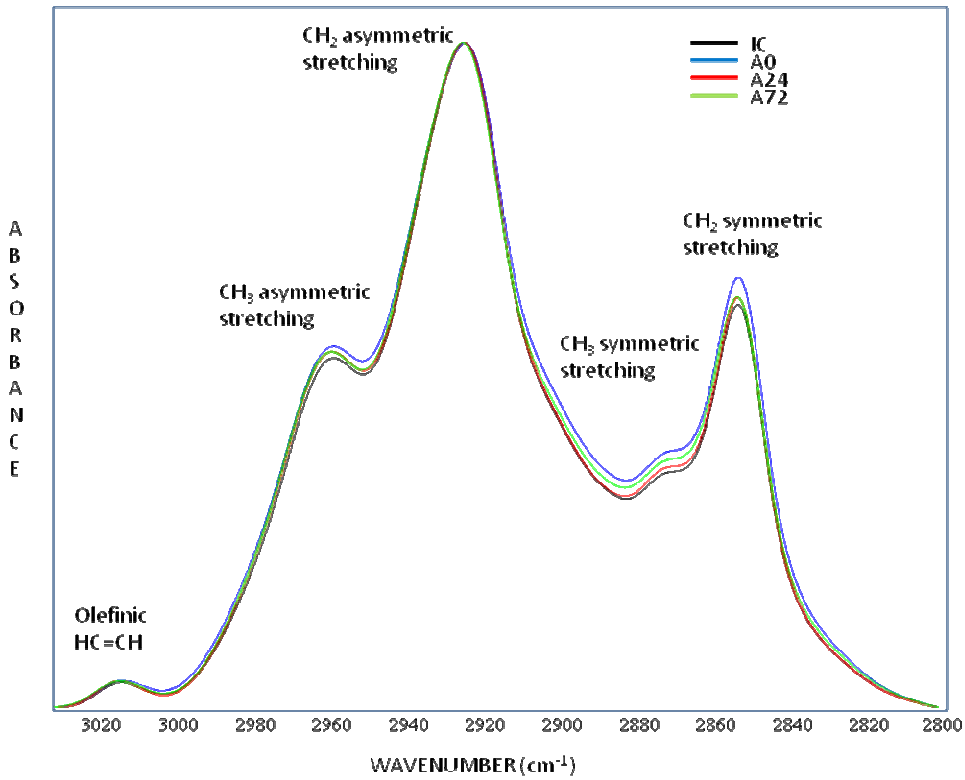

Figure 18. The representative infrared spectra of control and alcohol groups in the 3030-2800 cm^{-1} region. The spectra were normalized with respect to the CH_2 asymmetric stretching mode at 2921 cm^{-1} (Absorbance in arbitrary units). (IC for intubation control group, A0 for ethanol acute effects group, A24 for ethanol chronic effects group, and A72 for ethanol withdrawal effects group).

Figure 19 demonstrates the infrared spectra of control and alcohol groups in 1800-400 cm^{-1} region.

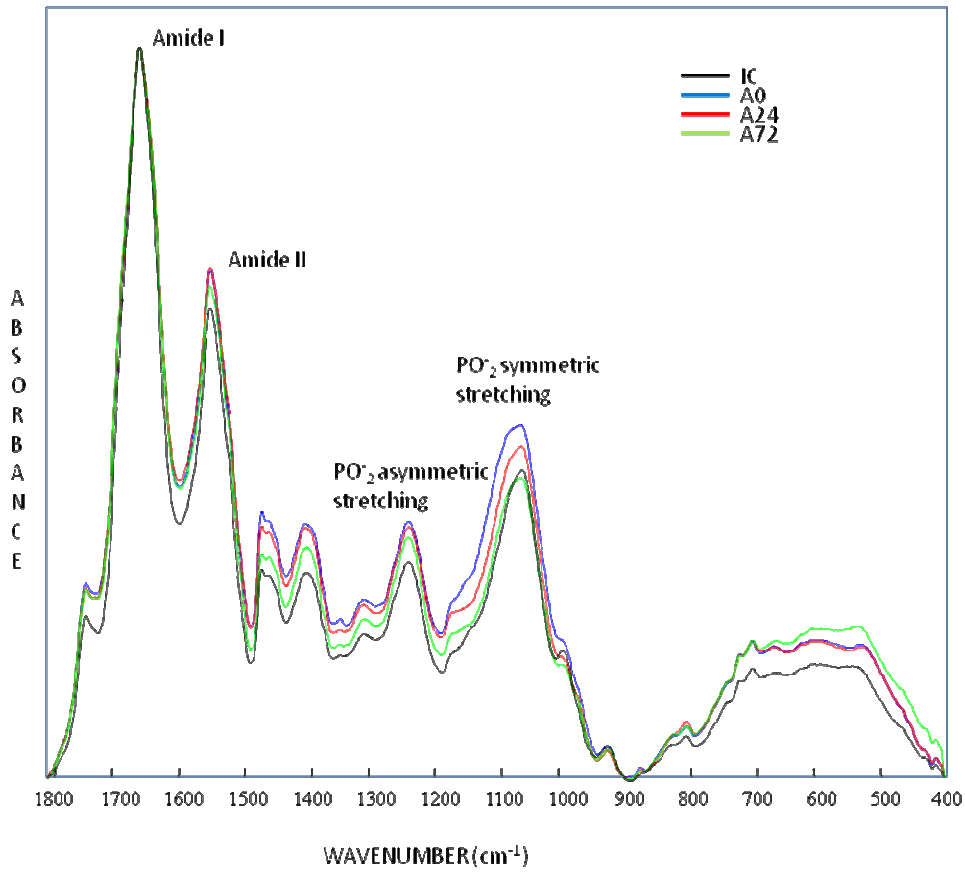

Figure 19. The representative infrared spectra of control and alcohol groups in the 1800-400 cm^{-1} region. The spectra were normalized with respect to the Amide I mode at 1658 cm^{-1} (Absorbance in arbitrary units). (IC for intubation control group, A0 for ethanol acute effects group, A24 for ethanol chronic effects group, and A72 for ethanol withdrawal effects group).

As it could be seen from the figures, the spectra obtained from control and alcohol-pretreated groups exhibit noticeable differences in area and frequency values in the analyzed regions. The spectral differences between the control and alcohol groups will be later discussed in more details.

3.3.3. Numerical Comparisons of the Bands of Control and Alcohol Groups Spectra

To determine the possible differences in the spectra between the individuals of the same group, the group means and standard deviations for band areas and the band frequencies

were analyzed in alcohol groups with respect to control group. Statistical analysis was done by a non-parametrical Mann-Whitney U-test. The results of this analysis are presented in Table 3 for band frequencies and in Table 4 for band areas. In the tables, only significant differences are shown.

Table 3. Numerical summary of the detailed differences in the band frequencies of control and alcohol group spectra. The values represent the mean ± SD for each sample. The degree of significance is denoted as p<0.05*, p<0.01**.

	BAND FREQUENCY			
Band No	**IC (n=8)**	**A0 (n=7)**	**A24 (n=7)**	**A72 (n=7)**
1	3303,61 ± 3,81	3297,81 ± 1,16↓*	3299,24 ± 3,75	3304,60 ± 1,00
2	3065,57 ± 2,26	3063,85 ± 0,23↓*	3063,56 ± 1,15*	3066,37 ± 1,03
4	2960,69 ± 0,22	2960,18 ± 0,12↓**	2960,43 ± 0,12*	2960,46 ± 0,17
5	2920,87 ± 0,38	2921,24 ± 0,11↑	2921,01 ± 0,53	2921,39 ±0,20**
7	2851,15 ± 0,13	2851,31 ± 0,06↑*	2851,25 ± 0,21	2851,33 ± 0,08*
11	1468,16 ± 0,06	1468,07 ± 0,05↓**	1468,11 ± 0,06	168,08 ± 0,03*
12	1402,13 ± 0,49	1401,59 ± 0,11↓*	1401,70 ± 0,25	1402,18 ± 0,37
15	1262,20 ± 0,26	1261,83 ± 0,17↓*	1261,98 ± 0,42	1262,25 ± 0,71
18	1082,54 ± 0,97	1084,26 ± 1,18↑*	1083,65 ± 0,66*	1084,10 ± 1,24*
21	876,05 ± 2,79	874,00 ± 0,43↓	873,63 ± 0,69*	875,09 ± 1,41

Table 4. Numerical summary of the detailed differences in the band areas of control and alcohol groups spectra. The values represent the mean ± SD for each sample. The degree of significance is denoted as p<0.05*, p<0.01**.

	BAND AREA			
Band No	**IC (n=8)**	**A0 (n=7)**	**A24 (n=7)**	**A72 (n=7)**
1	64,04 ± 12,27	91,02 ±9,00↑**	86,51 ± 19,99*	77,36 ± 12,06
2	7,80 ± 1,38	9,12 ± 0,92↑	7,96 ± 1,16	9,00 ± 1,38
3	1,96 ± 0,33	2,52 ± 0,23↑**	2,54 ± 0,34*	3,06 ± 0,44**
4	5,50 ± 0,98	7,38 ± 0,67↑**	6,65 ± 1,02	6,80 ± 0,97
5	9,65 ± 1,92	13,61±1,35↑**	11,77 ± 2,01	11,68 ± 1,73
6	1,44 ± 0,26	1,94 ± 0,21↑**	1,62 ± 0,23	1,76 ± 0,25
7	3,57 ± 0,65	4,68 ± 0,49↑**	4,67 ± 0,66*	3,94 ± 0,56
8	2,40 ± 0,28	3,52 ± 0,20↑**	2,36 ± 0,33	3,51 ± 0,46**
9	18,18 ± 4,29	29,22 ± 2,56↑**	24,89 ± 5,92	24,01 ± 4,25*
10	11,36 ± 2,46	18,53 ± 1,57↑**	15,63 ± 3,21*	14,67 ± 2,51*
11	3,53 ± 0,63	5,42 ± 0,54↑**	4,68 ± 0,75**	4,32 ± 0,66*
12	4,68 ± 0,85	6,92 ± 0,68↑**	6,13 ± 0,94*	5,54 ± 0,86
13	0,73 ± 0,14	1,09 ± 0,12↑**	0,85 ± 0,12	0,73 ± 0,11
14	2,16 ± 0,39	3,22 ± 0,30↑**	2,80 ± 0,43*	2,75 ± 0,42*
16	5,97 ± 1,04	8,10 ± 0,64↑**	7,77 ± 1,24*	7,27 ± 1,05*
17	1,63 ± 0,29	2,35 ± 0,25↑**	2,72 ± 0,36**	2,48 ± 0,34**
18	10,12 ± 2,16	14,26 ± 2,23↑**	13,83 ± 2,03**	11,21 ± 1,67
20	0,31 ± 0,12	0,27 ± 0,14↓	0,33 ± 0,05	0,25 ± 0,09
21	0,02 ± 0,01	0,05 ± 0,01↑**	0,04 ± 0,02	0,04 ± 0,01*
22	0,39 ± 0,06	0,52 ± 0,03↑**	0,52 ± 0,14*	0,44 ± 0,04

Table 5. Numerical summary of the detailed differences in the bandwidth of control and alcohol groups spectra. The values represent the mean ± standard deviation for each sample. The degree of significance is denoted as p<0.05*, p<0.01**.

	BANDWIDTH			
Band Name	**IC (n=8)**	**A0 (n=7)**	**A24 (n=7)**	**A72 (n=7)**
CH_2 asymmetric stretching	12,39 ± 0,18	12,21 ± 0,10↓*	12,29 ± 0,29	12,06 ± 0,16**

3.3.4. Detailed Spectral Analysis

3.3.4.1. Comparison of Control and Alcohol Spectra in 3800-3030 cm^{-1} Region

The bands centered at 3304 cm^{-1} (Amide A) and 3066 cm^{-1} (Amide B) correspond to the OH and/or the NH stretching mode, the NH and/or the CH vibrations, respectively. The Amide A band contains strong absorptions arising from OH stretching modes of proteins and polysaccharides (Cakmak *et al*., 2006; Melin *et al*., 2000).

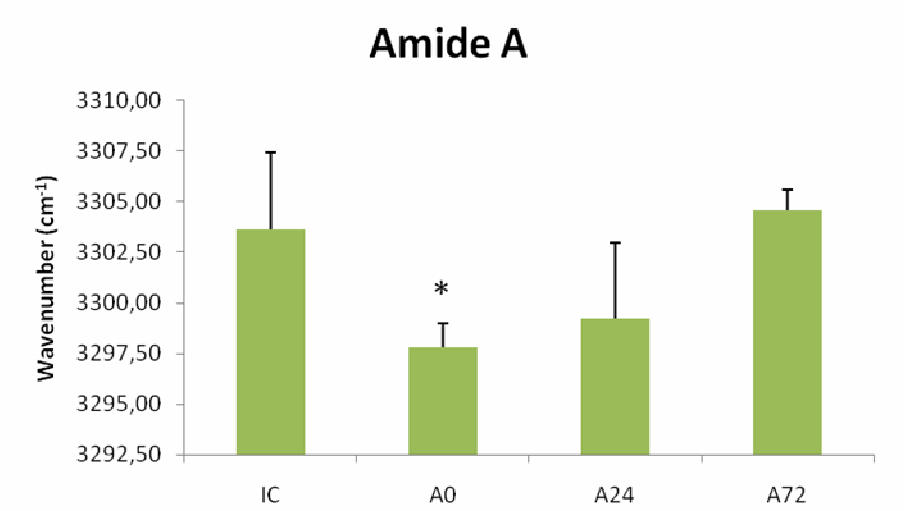

Figure 20. The changes in the band frequency at the Amide A region for control and alcohol group spectra. Error bars denote the standard deviation. Asterisks denote the level of significance at p<0.05* and p<0.01**.

As seen from Figure 20, in A0 and A24 groups with respect to the control group, the frequency of Amide A shifted to lower values. This shift was significant in A0 group only ($p<0.05$*). Moreover, there was an increase in the band area of Amide A for all alcohol groups, but it reached significance in only in A0 ($p<0.01$**) A24 groups ($p<0.05$*) when compared with the control group (Table 4).

The band centered at 3066 cm^{-1}, the Amide B, results from the C-H and N-H stretching of the proteins.

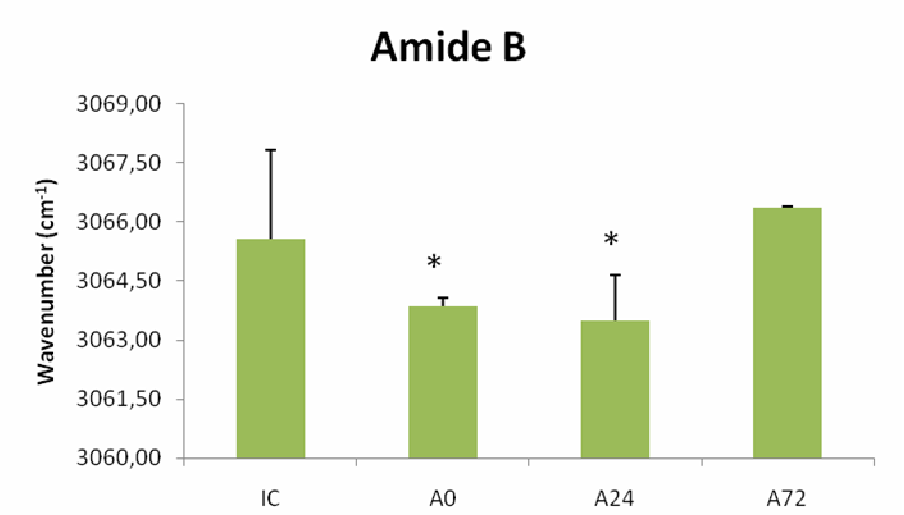

Figure 21. The changes in the band frequency at the Amide B region of control and alcohol group spectra. Error bars denote standard deviation. Asterisks denote the level of significance at $p<0.05$.

Figure 21 shows that there was a significant decrease in band frequency in A0 and A24 groups ($p<0.05$*), but not in A72 group. An increase in band areas in all groups was yielded insignificant.

3.3.4.2. Comparison of Control and Alcohol Spectra in 3030-2800 cm^{-1} Region

In this region, the band centered at 3014 cm^{-1} monitors stretching mode of the H-C=C-H vibrations (olefinic band). The region between 3000-2800 cm^{-1} represents the bands in the C-H region. These bands are centered at 2961 cm^{-1} , 2921 cm^{-1} , 2872 cm^{-1} , 2851 cm^{-1} , and they monitor the CH_3 and the CH_2 asymmetrical, and the CH_3 and the CH_2

symmetrical vibrations, respectively (Cakmak *et al*., 2006; Chang and Tanaka, 2002; Melin *et al*., 2000; Severcan *et al*., 2003).

The olefinic band which is located at 3014 cm^{-1} and is due to CH stretching mode of the HC=CH groups can be used as a measure of unsaturation in the phospholipid acyl chains (Liu *et al*., 2002; Melin *et al*., 2000; Severcan *et al*., 2005b; Takahaski *et al*., 1991). In the frequency of the olefinic band, there was an insignificant decrease in all alcohol groups. However, the band areas in all alcohol groups significantly increased ($p<0.01$** in A0 and A72 groups and $p<0.05$* in A24 group).

In the CH_3 asymmetric stretching and CH_2 asymmetric and symmetric stretching bands which originate from lipids (Mendelson and Mantsch, 1986; Severcan *et al.,* 2000), there were significant changes. Conversely, in the CH_3 symmetric stretching band, which originates from proteins, insignificant changes in band frequencies were noted. In the CH_3 asymmetric stretching band, there was a significant decrease in band frequency in A0 group ($p<0.01$**) and A24 group ($p<0.05$*) (Figure 22). On the other hand, as seen from Figure 23 and Figure 24, in the lipid bands in CH region, there were shifts to higher values. This shift was significant in A72 group ($p<0.01$**) in CH_2 asymmetric stretching and in A0 an A72 group ($p<0.05$**) in CH_2 symmetric stretching band. Also, in A0 ($p<0.05$*) and A72 group ($p<0.01$**), a significant decrease in the bandwidth of the CH_2 asymmetric stretching band was observed (Table 5). In all groups, in all C-H region, there was a general increase in the band areas but this change appeared to be significant only in A0 group ($p<0.01$**). A significant increase was also observed in A24 group but only in CH_2 symmetric stretching band ($p<0.05$*).

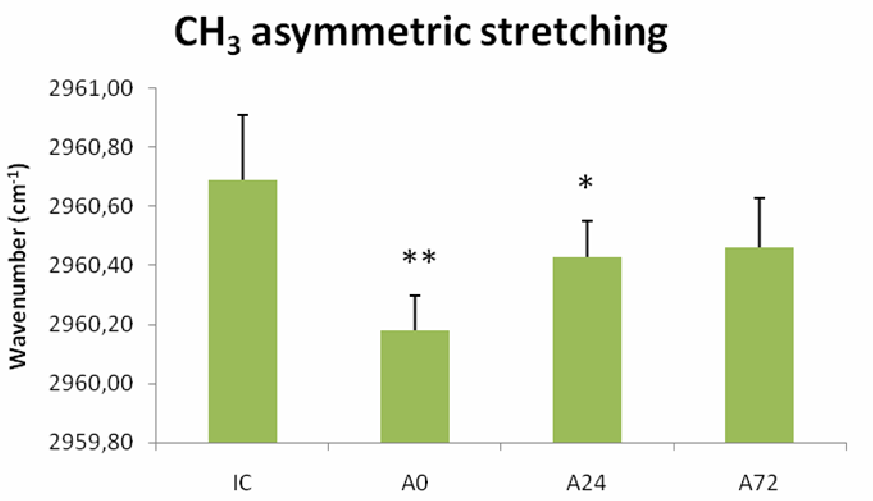

Figure 22. The changes in the band frequency at the CH_3 asymmetric stretching mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisks denote the level of significance at $p<0.05$ and $p<0.01$.

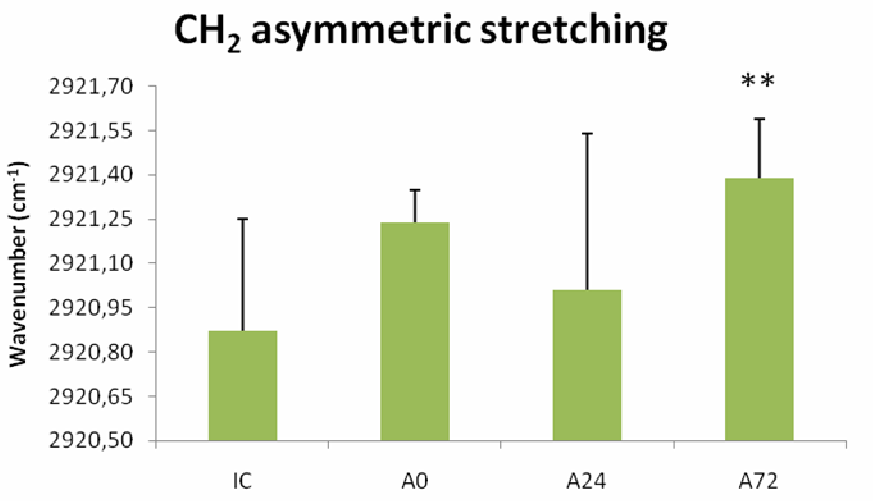

Figure 23. The changes in the band frequency at the CH_2 asymmetric stretching mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisks denote the level of significance at $p<0.01$.

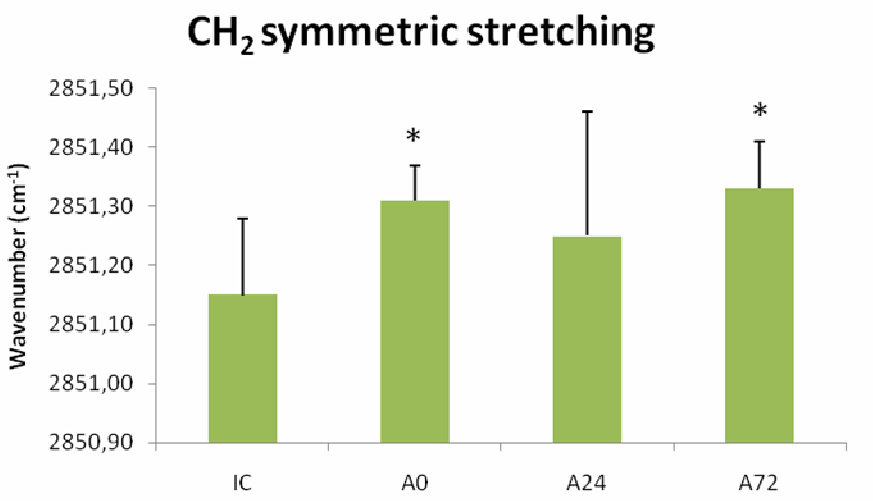

Figure 24. The changes in the band frequency at the CH_2 symmetric stretching mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisks denote the level of significance at $p<0.05$.

3.3.4.3. Comparison of Control and Alcohol Spectra in 1800-400 cm^{-1} Region

The 1800-400 cm^{-1} band region is considered as a fingerprint region for different tissues due to having bands originating from the interfacial and head-group modes of the membrane lipids and from the protein and nucleic acid vibrational modes (Mendelsohn and Mantsch, 1986).

The band centered at 1745 cm^{-1} is mainly assigned to the C=O ester stretching vibration in phospholipids (Melin *et al*., 2000; Severcan *et al*., 2003). In this study, in all alcohol groups, there were insignificant changes in the band frequencies. On the other hand, there was a significant increase in band area in A0 and A72 groups ($p<0.01$**).

The Amide I and Amide II vibrations of structural proteins were centered at 1658 and 1549 cm^{-1}, respectively. The Amide I correspond to the C=O stretching and to the C-N stretching (60 %) vibrational modes weakly coupled with the N-H bending (40%) of the polypeptide and protein backbone and Amide II is assigned to the N-H bending (60%) and the C-N stretching (40%) modes of protein (Haris and Severcan, 1999; Melin *et al*., 2000; Takahaski *et al*., 1991; Wong *et al*., 1991).

In Amide I and in Amide II band frequency, there were insignificant changes in all alcohol groups. However, in all groups, there was a significant increase in band area in Amide II band ($p<0.05$* in A24 and A72 groups). This increase was most pronounced in the A0 group ($p<0.01$**). In Amide I band, there was also observed a significant increase in band area in all groups except A24 group.

The band which is labeled as Amide III located at 1344 cm^{-1} falls into the region of C-N and the C-C stretching and the N-H bending vibrations of collagen (Camacho *et al.,* 2001; Gough *et al*., 2003). As it can be seen from the Table 3, in all alcohol groups, changes in band frequency were insignificant and the only significant change in the band area was seen as an increase in the A0 group ($p<0.01$**).

The band at 1468 cm^{-1} is assigned to the CH_2 bending mode of protein and lipids (Manoharan *et al.*, 1993). In the present study, there was a significant decrease in A0 and A72 groups ($p<0.01$** and $p<0.05$*, respectively) in band frequency with respect to control group. However, there was a significant increase in band area in all alcohol groups as compared to control ($p<0.01$** in A0 and A24, $p<0.05$* in A72 group).

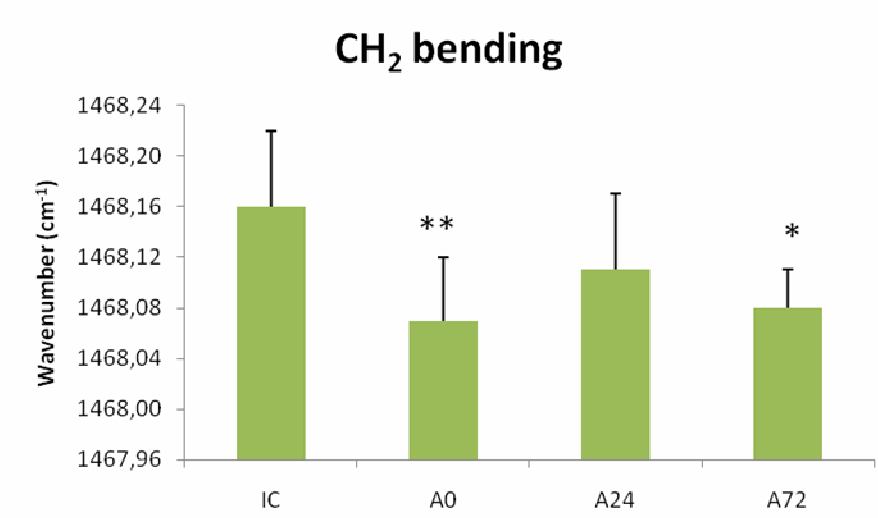

Figure 25. The changes in the band frequency at the CH_2 bending mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisks denote the level of significance at $p<0.05$ and $p<0.01$.

A significant decrease in the band frequency of 1402 cm^{-1} which is due to the COO- symmetric stretching vibration of amino acid side chains and fatty acids (Cakmak *et al*.,

2006; Jackson *et al*., 1998) was observed only in A0 group ($p<0.05$*) (Figure 26). In band areas, there was found a significant increase in A0 ($p<0.01$**) and in A24 ($p<0.05$*) groups.

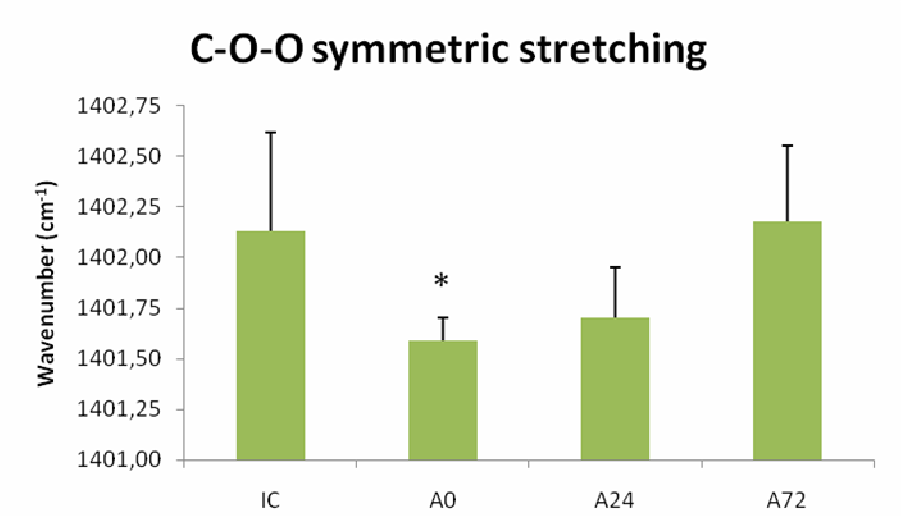

Figure 26. The changes in the band frequency at the C-O-O symmetric stretching mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisk denotes the level of significance at $p<0.05$.

In the 1280-900 cm^{-1} frequency range, several macromolecules (i.e. polysaccharides and phosphate carrying compounds such as phospholipids and nucleic acids give absorption bands (Melin *et al*., 2000). The relatively strong bands at 1262, 1236 and 1083 cm^{-1} are mainly due to the asymmetric and symmetric stretching modes of phosphodiester groups, the P=O bond present in the phosphate moieties (PO^-_2) of nucleic acids backbone structures and phospholipids (Wong *et al*., 1991).

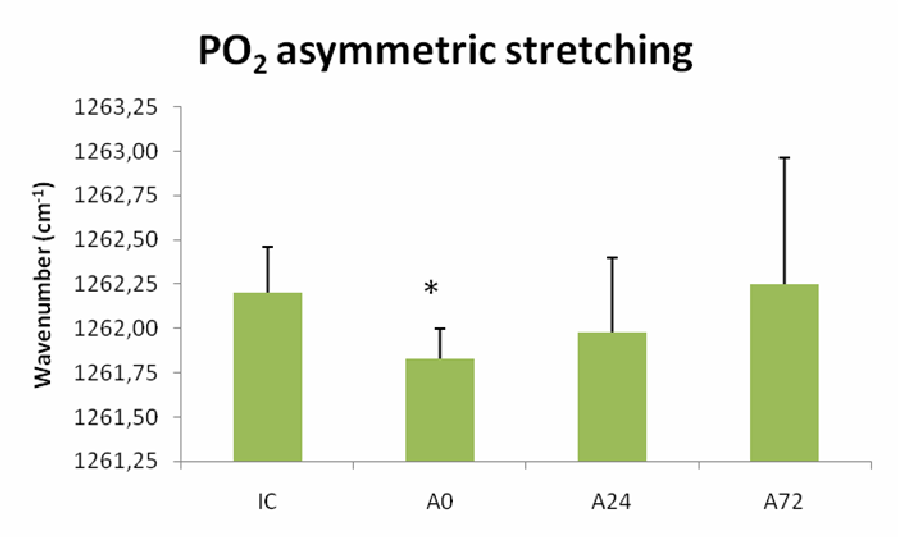

Figure 27. The changes in the frequency at the PO_2 asymmetric stretching mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisk denotes the significance at $p<0.01$.

Figure 27 shows that in the frequencies of the band which was centered at 1262 cm^{-1} and there was a significant shift to lower values in A0 group ($p<0.05$*). In the band, which was centered at 1236 cm^{-1}, there were no significant changes in band frequency. However, in the band areas of this band there was a significant increase in all alcohol groups ($p<0.05$*), but this increase was most pronounced in the A0 group ($p<0.01$**).

The weak band centered at 1172 cm^{-1} is due to asymmetric stretching mode of the CO-O-C groups present in glycogen and nucleic acids (Cakmak *et al*., 2006; Rigas *et al*., 1990). The decreases in band frequencies in all alcohol groups as compared to the controls were insignificant. On the other hand, in alcohol groups, there was a significant increase in band areas ($p<0.01$**).

The PO_2 symmetric stretching band is generally located at 1083 cm^{-1} (Banyay *et al*., 2003; Lyman *et al.*, 1999). In this band frequency, there was a significant shift to higher values in all alcohol groups ($p<0.05$*) (Figure 28). In the band areas, a significant increase was observed also in the A0 and A24 groups ($p<0.01$**).

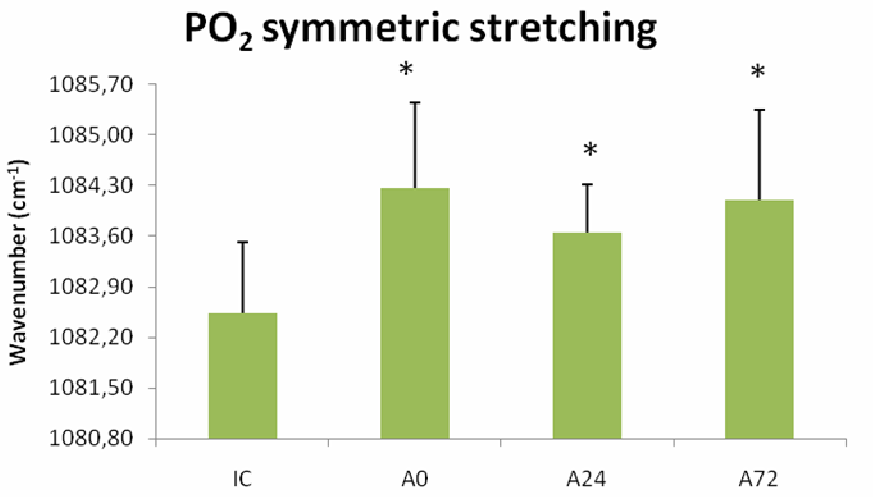

Figure 28. The changes in the band frequency at the PO_2 symmetric stretching mode of control and alcohol group spectra. Error bars denote the standard deviation. Asterisks denote the level of significance at $p<0.05$.

The spectral region between 995-970 cm^{-1} is assigned to the ribose-phosphate main chain vibrations of RNA (Banyay *et al.*, 2003). In all alcohol groups, there were insignificant changes in the band frequencies.

The bands centered at 876 cm^{-1} and 801 cm^{-1} both originate from the vibrations of N-type sugars in the sugar phosphate backbone of nucleic acids (Banyay *et al.,* 2003; Taillander and Liquier, 1992). The 801 cm^{-1} band is also coupled with furanose-phosphodiester chain vibrations (Banyay *et al.,* 2003). In all alcohol groups, there was a shift to lower values in the band, which was centered at 876 cm^{-1}. The shift was significant in A24 group ($p<0.05$*) (Figure 29). However, there were insignificant shifts in frequency for the band, which was centered at 801 cm^{-1} for all alcohol groups. In A0 group, there was a significant increase in the band area for both bands ($p<0.01$**). However, a significant increase in the band area was observed in A24 only at 801 cm^{-1} and in A72 only at 876 cm^{-1}.

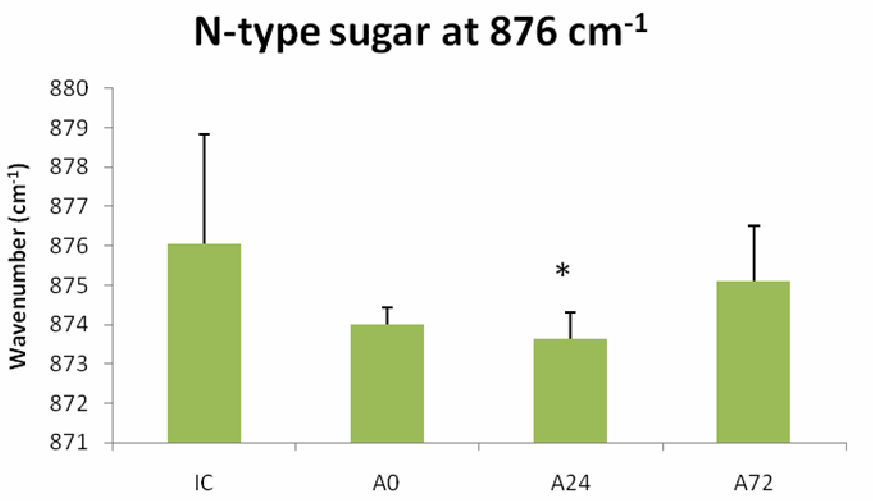

Figure 29. The changes in the band frequency at the N-type sugar at 876 cm^{-1} band frequency of control and alcohol group spectra. Error bars denote the standard deviation. Asterisk denotes the level of significance at $p<0.05$.

Table 6. Numerical summary of the detailed differences in the lipid-to-protein ratio, lipid ester-to-protein ratio, and nucleic acid-to-protein ratio of control and alcohol group spectra. The values represent the mean ± SD for each sample. The level of significance is denoted as $p<0.05$*, $p<0.01$**.

Ratio	IC (n=8)	A0 (n=7)	A24 (n=7)	A72 (n=7)
Lipid / Protein [Band (4+5+7)/Band 9]	1,03±0,06	0,88±0,04↓**	0,95±0,10	0,94±0,04*
Lipid Ester/ Protein [Band 8/ Band 9]	0,14±0,03	0,12±0,01↓*	0,10±0,01**	0,15±0,01
Nucleic Acid/Protein [Band (20+21+22)/Band 9]	0,04±0,01	0,03±0,00↓**	0,04±0,00	0,03±0,00*

The amount of proteins and lipids in the membranes is an important factor affecting the membrane structure and dynamics (Szalontai *et al.*, 2000). From the FTIR spectrum, a precise lipid-to-protein ratio can be derived by calculating the ratio of the areas of the bands arising from lipids and proteins. As seen from Table 6, the ratio of the area of the

CH_2 and CH_3 asymmetric stretching band and CH_2 symmetric stretching band to the area of Amide I the which gives lipid-to-protein ratio was lower in both A0 ($p<0.01$**) and A72 ($p<0.05$*) groups. Similarly, a decrease was observed in the ratio of lipid ester to protein which was calculated from the ratio of area of the C=O ester stretching to the area of Amide I.

The DNA to protein ratio that was calculated by the ratio of the area of PO_2 symmetric stretching band and N-type sugars to the area of Amide I band was given in Table 6. As seen from the Table, there was also a decrease in this ratio in both A0 ($p<0.01$**) and A72 groups ($p<0.05$*).

CHAPTER 4

DISCUSSION

As mentioned earlier, there are many experimental studies examining effects of chronic alcohol intoxication on learning, with learning tasks carried out both under the continuing alcohol challenge, and after the alcohol withdrawal. On the other hand, there are quite few studies investigating effect of chronic alcohol administration and alcohol withdrawal on memory retention. Therefore, the present study examined the effect of chronic alcohol intake and withdrawal on the retention/retrieval of the memory task. In the present study, the data from behavioral tests suggest that with the applied experimental protocol, rats exposed to binge-like drinking over 6 to 15 days, with high daily ethanol dose (up to 12 g/kg/day) resulting in high BAC of an average 605.67 ± 36 mg/dl were not impaired in the retention of place memory as assessed by the probe-trial in the MWM carried out at different delays after the cessation of ethanol administration (4, 24 and 72 hr). In Experiment I, performance in A24 group was significantly better than in A0 group in which adverse acute effects of alcohol could be expected, and in A72 group in which negative effects of alcohol withdrawal could be already expected. However, in this experiment, performance in the control group on a probe trial carried out 9 days after the completion of the initial acquisition training was at the chance level indicating towards the poor task acquisition by the animals. In this situation, any between-group differences can hardly be linked to the ethanol effects. Therefore, the experiment was repeated using the same training procedure but controlling the strength of acquired place preference by introducing a probe trial on the completion of the training, and applying two retraining sessions prior to alcohol administration. In Experiment II, despite of longer lasting alcohol administration no significant between-group differences in the performance on the probe trial were observed. Performance in all groups was above the chance level.

The lack of an adverse effect of binge like alcohol intake on memory retention and/or retrieval in the present study is in line with the results of some earlier studies investigating impact of adult chronic ethanol administration on cognitive functions. According to the report by White and co-workers (2000), no significant differences between control and alcohol pretreated adult rats were noted after repeated binge like

alcohol administration where rats were exposed to alcohol (5.0 g/kg intraperitoneally) or isovolumetric saline at 48 hr intervals over 20 days, and then tested twenty days later on delayed (5 and 60 min) non-matching-to position task in the 8-arm radial maze, a task testing spatial working memory. There are also reports about the absence of significant effects of a long term ethanol administration by the liquid diet (usually 20% v/v alcohol solution) where the BACs are much lower than those obtained after binge like intake but treatment lasts much longer (4 -13 months). Lack of the effects of a liquid ethanol diet was reported on place learning or spatial working memory in the MWM and in the radial arm maze task depended upon extra-maze visuo-spatial cues (Blokland *et al*., 1993; Lukoyanov *et al*., 1999; Pereira *et al*., 1998; Santin *et al*., 2000; Steigerwald and Miller, 1997). In the study by Pereira and co-workers (1998), the only significant difference between control and alcohol pre-treated groups was observed in the memory retention task (as evaluated by the course of retraining) carried out one year after the original learning, thus when the task's demands were highly increased.

In many other studies, however, an adverse effect of chronic ethanol intake in adult subjects on their performance in hippocampus-dependent cognitive tasks was demonstrated, with working spatial memory usually more affected than the reference spatial memory (Arendt, 1994; Franke *et al*., 1997; Hodges *et al.,* 1991; White *et al.,* 1997). Mild deficits in spatial working memory were reported even after relatively short intoxication with ethanol - 26 days of ethanol containing liquid diet followed by 17 days withdrawal period (Santucci *et al*., 2004). The discrepancy between the experimental results related to the adult chronic ethanol intake on cognitive functions may suggest that the effects of chronic ethanol consumption in the adulthood are affected by some other factors such as rats' strain and age, task complexity, method and duration of ethanol administration, overall ethanol dose etc. It has been also proposed that a potential damaging effect of repeated ethanol administration on the brain may be ameliorated by some kind of compensatory process. It has been, for instance postulated that with ethanol administered at a moderate dose even over a relatively long period of time, synaptic reorganization in the hippocampal formation of alcohol-fed rats may compensate for functional deficits related to neuronal loss. In line with this notion, it was reported that parallel to a progressive loss of both hippocampal granule and CA3 pyramidal cells even after 6 to 12 months of ethanol containing liquid diet there was observed maintenance of the relative number of mossy fibers (MF)-CA3 synapses and

the increased proportion of the MF plasmalemma occupied by synapses (Cadete-Leite *et al.,* 1989a; Lukoyanov *et al*., 2000). The latter findings suggest the formation of new contacts and can be interpreted as a compensation process that, however, "broke down" in rats maintained on alcohol diet for 18 months. Another possible compensatory mechanism may be increased adult neurogenesis observed by some authors in response to moderate ethanol intoxication (Aberg *et al*., 2005; Miller, 1995). Adult neurogenesis occurs in two regions of the brain: the forebrain subventricular zone (SVZ) and hippocampal dentate gyrus (DG). Hippocampal neurogenesis is postulated to have a contribution to learning and memory formation (Shors *et al.,* 2001). In a normal brain, aberrant neurogenesis may have adverse effects on brain circuitry but it may have beneficial effects in the brain under the alcohol insult.

The lack of an acute effect of alcohol on animal performance on the probe trial in the MWM (A0 group) remains in line with a finding by Boulouard and co-workers (2002) that initial impairment of spatial memory by an acute ethanol administration is reversed in chronic ethanol-consuming animals what may suggest that chronic ethanol consumption did induce tolerance to the spatial memory deficits induced by acute ethanol intake. On the other hand, the lack of any adverse behavioral effect on a probe trial in A72 group, may suggest that under the applied experimental protocol, in the strain of rats used in the present study alcohol withdrawal symptoms have not developed yet. It was actually confirmed by lack of hyperactivity to environmental stimuli and tremor or seizure incidents.

In addition to behavioral data, FTIR spectroscopy was used to investigate the effects of ethanol on hippocampus molecular makeup. In infrared spectroscopic analysis, the concentration of related functional groups is determined by the area of the bands in absorption spectra (Freifelder, 1982; Toyran and Severcan, 2003; Toyran *et al*., 2004) and the frequency changes are related with the changes in the structure of macromolecules (Arrondo and Goni, 1999; Haris and Severcan, 1999).

In Experiment I, in parallel with the lack of behavioral deficits in alcohol-exposed rats, FTIR spectroscopic method applied to evaluate the presumed deteriorating effects of repeated ethanol intake on the molecular make-up of the hippocampal formation also did not show any apparent between-group differences in protein, lipid, and nucleic acid profiles.

In the Experiment II, wherein alcohol was administered over the longer time period, despite of the lack of behavioral effects of repeated ethanol administration, in alcohol groups, some significant changes were observed in the protein, lipid and nucleic acids profiles in the tissue samples taken from the hippocampus. As seen from Table 3 and Table 4, there were significant changes in the band frequency and band area values for all bands in the FTIR spectrum.

In the dried samples, Amide A band located at 3304 cm^{-1} is assigned to absorptions arising from the proteins and glycogen content rather than water. In this study, there was observed an increase in the area of this band suggesting that repeated alcohol administration might trigger an increase in gene expression and protein synthesis or decrease in protein degradation. This increase in the protein content declined with alcohol withdrawal. The increase in protein content can be related to adaptive up-regulation of some receptors-ion channel complexes such as g-amino butyric acid type-A ($GABA_A$), NMDA, and serotonin that has been reported by some authors as a result of chronic alcohol intoxication (Lovinger, 1993; Matsumoto *et al.,* 2001; Nutt, 1999). The increase in the NMDA receptor expression upon repeated alcohol administration is thought to compensate for diminished glutamate neurotransmission due to blockage of these receptors by ethanol (Nutt, 1999; Lovinger, 1993). At the same time, a significant decrease in the Amide A band frequency was observed which may suggest a decreased contribution from glycogen to the OH absorption. The glycogen is degraded to pyruvate, which is the substrate for glutamate production (Gibbs *et al*, 2006). The decrease in the glycogen can be caused by an increase in the production of glutamate that also can be considered as a compensatory process against ethanol related suppression of glutamate neurotransmission (Reynolds and Brien, 1994).

The increase in protein content in the hippocampal tissue upon the chronic alcohol administration was confirmed by an increase in the area of Amide I (1658 cm^{-1}) and Amide II (1549 cm^{-1}) bands. In the present study, an increase in these band areas was significant in all alcohol groups.

As mentioned earlier, the change in the frequency of Amide I band is indicative about protein conformation changes, and as such, it is very useful for determination of protein secondary structure (Cakmak *et al.*, 2006; Haris and Severcan, 1999; Lyman *et al.*, 1999; Melin *et al.,* 2000; Takahashi *et al*., 1991; Wong *et al.*, 1991). Amide I band

(1658 cm^{-1}) is assigned to α-helix structures of proteins and the shift to lower frequency values (1639 cm^{-1}) indicates an increase in the content of random coil structure (Melin *et al.*, 2000; Dousseau and Pezolet, 1990). In this study, in all alcohol groups, there were insignificant shifts in the frequency of Amide I and Amide II bands indicating lack of changes in the secondary structure of the proteins and the content of the random coil structure with the chronic consumption of ethanol. On the other hand, however, the significant shifts to lower values in A0 and A24 groups for both Amide A and Amide B could argue for some changes in protein structure occurring upon the ethanol administration

The change in the area of COO symmetric stretching band (1402 cm^{-1}) indicates a change in the content of amino acids and fatty acids (Cakmak *et al.*, 2006). In the present study, an increase in the area of this band was observed in all alcohol groups. This indicates that the content of amino acids and fatty acids increased due to ethanol administration. Alcohol-related increase in the content of amino acids may be an indication of an increase in protein turnover while an increase in the fatty acid content may result from the fact that alcohol promotes phospholipases-mediated release of fatty acids from complex lipids, and thus, alters the membrane lipid composition of various cells and organelles (Rubin and Rottenberg, 1982). The change was most profound in the A0, and it decreased when the alcohol was withdrawn. There was also observed a significant shift to lower values in band frequency in A0 group suggesting changes in the amino acids and fatty acids structures.

The area of the band located at 1345 cm^{-1} indicates the content of collagen (Camacho *et al.,* 2001; Gough *et al.,* 2003). The 1365-1300 cm^{-1} spectral region contains bands assigned to plane-bending hydroxyl modes in saccharides (Cael *et al*., 1973). There is no collagen in the brain except the blood capillaries. Therefore, there might have been a contribution from saccharide molecules to this band. The change in the band area indicating increase in saccharides content was most pronounced in A0 group.

As previously mentioned, the C-H region (3050-2800 cm^{-1}) is populated by absorptions arising from the C-H stretching, both asymmetric and symmetric, vibrations of olefinic (HC=CH), -methyl ($-CH_3$) and methylene ($-CH_2$) groups, most of which originate from lipid structures (Kneipp *et al*., 2000). Increases in the band areas in this region (except CH_3 symmetrical stretching band) indicate an increase in the lipid content (Cakmak *et*

al., 2006; Severcan *et al*., 2000; Severcan *et al*., 2003). In the present study, the band areas of this region were significantly elevated in A0 group and this increase declined in A72 group. These results suggest that there was an increase in the lipid content with alcohol administration, however, upon alcohol withdrawal, the lipid content declines approaching the control values. These increases in lipid content were further supported by an increase in the area of the CH_2 bending band (1468 cm^{-1}) also originating from lipids (Severcan *et al*., 2000; Severcan *et al*., 2003). The information about accumulation of lipids in the tissues can be also derived from the area of the C=O ester stretching band originating from the vibration in triglycerides (Cakmak *et al.*, 2006; Nara *et al.*, 2002). However, in the present study, in C=O ester stretching band, a significant increase in the band area was observed only in acute alcohol group (A0) and in alcohol withdrawal group (A72). Increase in the C=O ester stretching band area can result from the ethanol metabolism in the brain which is terminated by the formation of the nonoxidative end-product, fatty acid ethyl esters (Bora and Lange, 2003). The presence of fatty acid ethyl ester synthase activity at the neuronal membrane might alter the fluidity of membranes by promoting an alteration in fatty acid composition (Chin and Goldstein, 1977). Fatty acid ethyl ester can impair mitochondrial function, which might also be the reason for the changes in protein content, and thus, cause damage to the brain (Laposata and Lange, 1986). The C=O ester stretching band gives valuable information not only about the lipid accumulation but also about the interfacial region of lipid assemblies (Kneipp *et al,* 2000; Takahashi *et al*, 1991). The frequency of C=O ester carbonyl band in the interfacial region of membrane lipids is an important monitor of the ability of lipids to interact with each other and with proteins via hydrogen bonds because of their proton-accepting ability (Wong *et al.,* 1991; Severcan *et al.,* 2005a). However, in this experiment, there were no significant changes in this band frequency suggesting no significant changes in the hydrogen bonding ability of lipids.

As seen from the Table 5, a decrease was observed in the lipid ester to protein ratio as well as in the lipid to protein ratio, which indicated that the protein increase was more profound than the increase in the lipid ester and lipid content.

The area of olefinic band can be used as a measure of lipid peroxidation (Liu *et al.*, 2002; Severcan *et al.*, 2005b; Takahashi *et al.*, 1991). In the present study, a significant increase in this band area was observed which indicates an increase in lipid peroxidation

and its end-products upon ethanol intoxication. Alcohol-related increase in lipid peroxidation was reported in fetal and adult brain tissue also by other authors (Henderson *et al.,* 1999; Montoluni *et al*., 1994; Nordman *et al*., 1990; Rouach *et al.,* 1987; Uysal *et al*., 1986). On the other hand, the frequency shift of the olefinic band to the lower values implies an increase in order of the system (Bizeau *et al.*, 2000; Severcan *et al.*, 2003). In the present study, however, in all alcohol groups, the olefinic band frequency shifts to lower values remained insignificant which means that the order of the system did not change significantly.

The shift to lower frequencies of the CH_3 asymmetric stretching band can be used for the detection of an increase in the order in the deep interior part of the lipid chains (Severcan *et al*., 2000). In the current study, the CH_3 asymmetric stretching band shifted to lower values in all alcohol groups but it was significant in A0 ($p<0.01$**) and in A24 groups ($p<0.05$*) which suggests an increase in the order of the deep interior upon ethanol administration. This result is consistent with previous finding where an increase in bilayer order was reported due to ethanol treatment (Lewis *et al*., 1989; Beaugé *et al*., 1988). This ethanol effect is transient and disappears with alcohol withdrawal.

The frequency shifts in both CH_2 asymmetric and symmetric stretching bands indicate alterations in the composition and structure of cellular membrane systems. The frequencies of these CH_2 asymmetric and symmetric bands are conformation-sensitive and respond to changes in the trans-gauche ratio in acyl chains (Mendelsohn and Mantsch, 1986; Severcan, 1997). In the current study, in A0 and A72 groups, a significant shift to the higher values in the band frequencies for both bands was observed. This suggested that acute ethanol administration but also ethanol withdrawal lead to an increase in disordering of the membrane lipids. These results are consistent with the previous studies, which suggest that ethanol disorders the membrane (Sun and Sun, 1985; Goldstein and Chin, 1981). The disordering effect became even more pronounced in the alcohol withdrawal group. The disordering of the membrane unsaturated lipids upon alcohol withdrawal can result from the increase in lipid peroxidation. This would be in line with earlier findings related with alcohol effects on lipid bilayers (Bae *et al.,* 2005).

Membrane dynamics is detected by monitoring the bandwidth of the C-H stretching bands (Severcan *et al*., 2003; Severcan *et al*., 2005b). The increase in the bandwidth corresponds to an increase in membrane fluidity (Severcan *et al*., 2005b). In the present study, a decrease in the lipid dynamics (membrane fluidity) was observed by monitoring the decrease in the bandwidth of the CH_2 asymmetric stretching band. This decrease was significant in A0 and A72 groups. It was reported earlier that acute alcohol causes an increase in membrane fluidity (Chin and Goldstein, 1977, Goldstein, 1984), whereas chronic alcohol administration tends to increase membrane rigidity, which may be due to an increase in the cholesterol/lipid ratio (Deitrich, 1989, Buck and Haris, 1991) as in our case.

The 1300-1000 cm^{-1} spectral range is commonly coupled with the infrared bands of the stretching modes of the P=O bond present in the PO^-_2 moieties of phospholipids and nucleic acids and with distinct ring vibrations of carbohydrates (C-O, C-C and C-O-C) such as the sugars in the backbone of DNA and RNA (Mendelsohn and Mantsch, 1986). The phosphate-stretching vibrations are believed to provide valuable information about the head-groups of the phospholipids in the polar-nonpolar interface of membranous structures (Mendelsohn and Mantsch, 1986). Furthermore, these infrared bands can also monitor alterations in the quantity, conformational state, and the degree and position of phosphorylation of the nucleic acids in DNA and RNA (Kneipp *et al*, 2000). These vibrations are mainly the PO_2 asymmetric and symmetric stretching bands located at 1236 cm^{-1} and 1083 cm^{-1} respectively, originating from the phosphate-stretching vibrations of nucleic acids and phospholipids head groups (Lyman *et al.,* 1999; Banyay *et al.*, 2003). As mentioned before, the frequency of the PO_2 asymmetric stretching mode is between 1220 cm^{-1} and 1260 cm^{-1} and shows the degree of hydrogen bonding (Rigas *et al.*, 1990; Wong *et al.*, 1991). It is known that as the hydrogen bonding increases, the frequency values shift to lower value in PO_2 asymmetric stretching vibration. In the absorption spectra of the hippocampus, in the region of the PO_2 asymmetric stretching band, two bands were observed which were located at 1262 cm^{-1} and 1236 cm^{-1}. The band located at 1262 cm^{-1} is due to non-hydrogen-bonded PO^-_2 groups and the band located at 1236 cm^{-1} is due to hydrogen-bonded PO^-_2 groups. In our spectra, the band located at 1262 cm^{-1} was shifted to lower values significantly in A0 group only ($p<0.05$*). The changes can result from a decrease in non-hydrogen bonding

and an increase in the hydrogen bonding of PO^{-}_{2} in this group due to ethanol high capacity of forming hydrogen bonding.

The band area of the band at 1172 cm−1 is assigned to the content in glycogen and nucleic acids (Rigas *et al.,* 1990). There were significant increases in the band areas in all three alcohol groups as compared to control. This may result from the increases in nucleic acid concentration, which in turn, can be a result of an increased gene expression due to ethanol administration and withdrawal**.**

Moreover, there was a significant increase in the area of the PO_2 symmetric stretching band. Hence, this result indicates an increase in the content of nucleic acids and phospholipids in the membrane structures. In the present study, there were significant shifts to higher values in band frequency of the PO_2 symmetric stretching band, which suggest a change in DNA and RNA conformation, possibly related to the changes of the hydrogen bonding in the DNA and RNA backbones (Dovbeshko *et al*, 2000).

The spectral region between 995-970 cm^{-1} is assigned to the ribose-phosphate main chain vibrations of RNA (Banyay *et al.*, 2003). The band centered at 970 cm^{-1} is generally assigned to symmetric stretching mode of dianionic phosphate monoester of nucleic acids (Ci *et al.*, 1999; Cakmak *et al.*, 2003) and ribose-phosphate main chain vibrations of the RNA backbone (Banyay *et al.*, 2003). In the present study, no significant changes were observed between the groups in these bands.

The vibrations of N-type sugars in the sugar phosphate backbone of nucleic acids are centered at 876 cm^{-1} and 801 cm^{-1} (Banyay *et al.*, 2003). The 801 cm^{-1} band is also coupled with furanose-phosphodiester chain vibrations (Banyay *et al.*, 2003). There were no significant changes in the band frequencies for the band centered at 801 cm^{-1}. However, there was a significant shift to lower values in A24 group of the band located at 876 cm^{-1} that suggest the structural changes in the backbone of the nucleic acids with chronic consumption of ethanol. On the other hand, there was an increase in the band areas for both bands, so, an increase in nucleic acids content was observed in the spectra from alcohol groups. As seen from Table 6, there was a significant decrease in the ratio of nucleic acid to the protein content which suggesting that the increase in the protein content was more profound compared to the nucleic acids content.

To sum up, there are considerable changes in proteins, lipids, and nucleic acid profiles in the hippocampal structure upon the ethanol intoxication and withdrawal. In **A0 group**, there was a significant increase in the protein band areas. The increase in the protein content can result from the increase in synthesis of some receptors i.e. NMDA, to compensate the damping of these receptors by ethanol. Parallel to this, a significant decrease in the glycogen content, the precursor of the glutamate synthesis, was observed. Moreover, in A0 group, a significant increase in the lipid content was observed. As seen from the bands of the CH region, the ethanol consumption increased the lipid disordering and decreased in the lipid dynamics (membrane fluidity). At the same time, in A0 group, a significant increase in amino acid, fatty acid, and nucleic acids content was noted. An increase in amino acid content may indicate towards an increase in the protein turnover leading to the increase in the protein synthesis. On the other hand, an increase in the fatty acid content may be due to alcohol–induced release of fatty acids from complex lipids. Increased content of nucleic acids may be related to an increase in gene expression and protein synthesis. In addition to the variations in the content of the basic macromolecules, changes in their structure were also observed. In **A24 group**, a significant increase in both the protein and lipid content was noted, however, this increase was less pronounced than in the A0 group. Similar to A0 group, there was also observed an increase in lipid disordering. Some structural changes in the protein and backbone of DNA and RNA were also present. The increase in the nucleic acids content was as significant as that in A0 group. Generally, the changes in proteins, lipids and nucleic acids content and/or structure recorded in A24 group were similar but less profound than these observed in A0 group. Molecular changes in A0 group in addition to the chronic effects of alcohol administration could be potentiated by the acute ethanol effects whereas the changes in A24 resulted from only by the chronic effects of the alcohol. In **A72 group**, there was still a significant increase in both lipid and protein content observed; however, this increase was not as profound as in A0 and A24 groups. In this group, the changes in the protein structure were insignificant. However, in A72 group, decrease in the membrane fluidity and lipid disordering was more profound than in A0 and A24 groups and can be related to withdrawal-induced oxidative stress and increased lipid peroxidation. These findings are consistent with some earlier reports by other authors about an increased oxidative stress during the alcohol withdrawal and it's aggravation of alcohol-induced behavioral deficits.

CHAPTER 5

CONCLUSION

In the present study, the retention of spatial reference memory after six (Experiment I) and 15 days (Experiment II) of binge-like drinking and during alcohol withdrawal was examined in the young adult Wistar rats. In both experiments, there were three alcohol groups tested at different times after the alcohol withdrawal: A0 group tested 4 h after the last alcohol administration for the acute effects of ethanol; A24 group tested 24 h after alcohol cessation, when acute ethanol effects disappear but withdrawal symptoms does not develop yet; and A72 group tested 72 h after the last ethanol infusion, at the time when the beginning of physical withdrawal syndrome may be expected.

In Experiment I, both A0 and A72 groups, but not A24 group, showed significantly worse performance on the probe trial than the control animals what could confirm adverse effect of acute alcohol intoxication and alcohol withdrawal on behavior. However, in this experiment, the overall level of performance after delay was low (at or slightly above the chance level). When, in the Experiment II, on the completion MWM training and prior to the ethanol administration, the level of learning (strength of place preference) was tested and found satisfactory in all animal groups, despite longer alcohol intoxication resulting in high BAC (605 mg/dl) no significant between–group differences were observed.

In the Experiment I, six days of moderate-to-high dose alcohol intoxication did not produce any significant changes in the molecular make-up of the hippocampus as assessed by FT-IR technique.

In the Experiment II, despite of the lack of significant behavioral deficit there were observed significant changes in protein, lipid, and nucleic acid profiles related to ethanol intake and withdrawal. In general, in all groups, there was observed ethanol-induced increase in the total content of proteins, lipids, as well as amino, fatty, and nucleic acids, which can be considered as a compensatory process, related to the development of alcohol tolerance. This increases diminished along with the alcohol withdrawal.

In all alcohol groups, increased lipid disordering and decrease in membrane fluidity were noted which may have adverse effects on the cell functions. In A72 group, the increase in lipid disordering and the decrease in membrane fluidity were most profound because of aggravated effects of ethanol withdrawal due to increased oxidative stress.

As a conclusion, instead of not observing any significant differences in the behavioral output, the significant changes were noted at the molecular level upon ethanol administration, which can disrupt the cellular functions.

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