THE EFFECTS OF ALCOHOL ON GENE EXPRESSION IN RAT BRAIN TISSUE

by

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Halil Rıdvan ÖZ
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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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M. S. Thesis – Genetics and Bioengineering
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ABSTRACT

Alcohol is a type of addiction material for human and affects molecular mechanism of human brain by chancing expression of some genes in brain. Our aim is to detect some genes' expressions alteration. In this study, ethanol is used for addiction of rats in order to understand molecular effects of alcohol addiction on human brain. Three groups of rats are used, first group is become alcohol addicted by using ethanol, second one is alcohol deficiency group and the last one is negative control group. There are 6 rats (Long Evans) in each group and totally 18 rats. Addiction process continues totally 22 days. At the end of process, all rats are killed and extracted their brains. Three parts of brain is used for this study, frontal cortex, striatum, and hippocampus. For genetic analysis, RNA extraction, cDNA synthesis and Real Time PCR protocols are applied for each group members' sample. We hypothesized that there can be difference of gene expression level between each group. If we detect this difference, it can provide knowledge about effect of alcohol on genetic background of brain. If the genes which we determine for this analysis are homolog in human, it can be clue for understanding how alcohol addiction alters brain molecular mechanism in human.

Keywords: Alcohol, addiction, brain, gene expression, RT PCR

ALKOLÜN SIÇAN BEYİN DOKUSUNDAKİ GEN EKSPRESYONU ÜZERİNE ETKİSİ

Nilüfer SEVİNÇ

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ÖZ

Alkol bağımlılık yapan bir maddedir ve beyindeki bazı genlerin ekspresyonunu değiştirerek insan beynindeki moleküler mekanizmayı etkiler. Amacımız bazı genlerin ekspresyonlarının değişimini belirlemektir. Bu çalışmada alkol bağımlılığının insan beyni üzerindeki moleküler etkilerini anlamak için etanol kullanılarak sıçanlar bağımlı yapılmıştır. Üç grup sıçan kullanılmıştır: Birinci grup etanol kullanılarak bağımlı hale getirilmiştir, ikinci grup alkol yoksunluk grubudur, sonuncu ise negatif kontrol grubudur. Her grupta 6 sıçan (Long Evans) vardır ve toplam 18 sıçan kullanılmıştır. Bağımlılık protokolü toplamda 22 gün sürmüştür. Protokol sonunda, bütün sıçanlar öldürülmüş ve beyinleri çıkarılmıştır. Bu çalışma için üç beyin bölgesi kullanılmıştır, frontal korteks, striatum ve hipokampüs. Bundan sonra gen ekspresyon analizleri için, RNA izolasyonu, cDNA sentezi ve Real Time PCR protokolleri her grup üyesine ait örnek için uygulanmıştır. Hipotezimiz gruplar arasındaki gen ekspresyon farklılığını saptamaktır. Eğer bu farklılığı saptarsak, bu alkolün beynin genetik altyapısı üzerindeki etkisi konusunda bilgi sağlayabilir. Eğer sıçanlara ait bu genlerden bazılarının insanda homologu varsa, bu alkol bağımlılığının insan beyninin moleküler mekanizmasını nasıl değiştirdiğini anlamamız için bir ipucu sağlayabilir.

Anahtar Kelimeler: Alkol, bağımlılık, beyin, gen ekspresyonu, RT PCR

DEDICATION

To my grandfather Zeki Sevinç (died),

my father Nimet Sevinç, my mother Yurdise Sevinç,

my brother Ertuğrul Sevinç, my grandmother Hayati Sevinç,

and

my aunt Şenay Zengin...

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SYMBOL/ABREVIATION

AGMA : Agmatinase

Alc : Alcohol addicted group

AMPA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ARDEC : Arginine decarboxylase

ARG : Arginase

AV : Average

BDNF : Brain-derived neutrophic factor

C : Frontal cortex

CNS : Central nervous system

Cont : Control group

Ct : Cycle threshold

DLS : Dorsolateral striatum

DMS : Dorsomedial striatum

F : Forward Primer

GABA : Gamma-aminobutyric acid

GADPH : Glyceraldehyde-3-phosphate dehydrogenase

H : Hippocampus

LTP : Long-term potentiation

NMDA receptors: *N*-Methyl-D-aspartic acid or *N*-Methyl-D-aspartate receptors

NOS : Nitric oxide synthase

R : Reverse Primer

S : Striatum

SAT1 : Spermidine/spermine N (1)-acetyltransferase (SPD/SPM acetyltransferase)

SNP : Single-nucleotide polymorphism

Wth : Withdrawal group

CHAPTER 1

INTRODUCTION

1.1 ADDICTION

Addiction is a brain disease characterized by craving for a drug, loss of control over consumption, development of tolerance and dependence, while simultaneously the repertoire of social functioning not related to intake behaviour declines dramatically (Keifer, 2011). In other words, the addictions are associated with maladaptive and destructive behaviours that are persistent, compulsive, and uncontrolled use of a drug or an activity. This is not basic issue that are preventable by law or depend on individual choice (Merikangas and Risch, 2003). For this reason, addiction affect the world in a negative way by changing life of society.

Addiction require use: a choice that is itself modulated not only by environment, but also by genes. To understand the factors that compel some individuals to consume excessively, addiction research has focused on the identification of brain mechanisms that support reinforcing actions of addictive compounds and the progression of changes in neural function induced by chronic consumption (Keifer, 2011). Namely, addictive agents induce adaptive chances in brain function; these changes are bases for tolerance and for the establishment of craving, withdrawal and affective disturbance, which persist long after consumption ceases (Roberts and Koob, 1997). This self-maintaining and progressive neurobiology of addictions makes them chronic and relapsing disorder, however, today partly prevented and treated using untargeted and only partially effective methods (Goldman et al., 2005). New scientific researches showes genetic background of addiction. Environmental and genetic factors contribute to individual differences in vulnerability to initiating use of addictive agents and in vulnerability to the shift from use to addiction. Cross- sectional studies on large twin samples also indicate that a mixture of environmental and genetic influences are shared between

diseases and provide a link between the normal range of behavioral variation and psychopathology (Kendler et al., 2003).

The addictions are interrelated to other psychiatric diseases by common neurobiological pathways, including those that modulate reward, behavioral control, and the anxiety or stress response. Furthermore, the addictions encompass non-substance related behaviors that are widespread and that might access the same neurobiological pathways that modulate impulsive, compulsive behavior and mood (Reuter et al., 2005). These diverse behaviors, including binge eating, compulsive gambling and playing video games, resemble the substance addictions in their clinical course and harmful effect. For instance, the hedonic properties of food can stimulate feeding behavior even when energy requirements have been met, contributing to weight gain and obesity. Similarly, the hedonic effects of drugs of abuse can motivate their excessive intake, culminating in addiction. Common brain substrates regulate the hedonic properties of palatable food and addictive drugs, and recent reports suggest that excessive consumption of food or drugs of abuse induces similar neuroadaptive responses in brain reward circuitries. In other words, obesity and drug addiction may share common molecular, cellular and systems-level mechanisms (Kenny, 2011).

Our future understanding of addictions will be enhanced by the identification of genes that have a role in altered substance specific vulnerabilities such as variation in drug metabolism or drug receptors and a role in shared vulnerabilities such as variation in reward or stress resiliency. In addition, identifying gene-environment interactions is a crucial issue in the study of addictions, which by definition depend on exposure to an addictive agent and are strongly modulated by other environmental factors. The story of genes in addictions and other complex behavioral diseases seems to be one of incremental progress as the functional significance of sequence variations is discovered and then related both to intermediate phenotypes and to the complex diseases that are emergent from an intermediate neurobiology (Goldman et al., 2005).

As a summary, the origins of addiction vulnerability are complex; the underlying genetic factors need to be identified to solve the causes of these pervasive and relatively intractable disorders. For this, the establishment of widely accepted definitions has created a unifying framework for research and for the clinical treatment of disorders that frequently share neurobiological and clinical course (Hasin, 2003).

Different technologies have revealed a variety of genes and pathways underlying addiction; however, each individual technology can be biased and incomplete. So, publications between 1976 and 2006 linking genes and chromosome regions to addiction by single-gene strategies, microarray, proteomics, or genetic studies are integrated. This study identifies human addiction-related genes and developed KARG (http://karg.cbi.pku.edu.cn), the first molecular database for addiction-related genes with extensive annotations and a friendly Web interface (Li C-Y, 2008).

1.2 ALCOHOLISM

Alcohol is one of common type of addiction material shown in figure 1.1 that affect people social, economic and familial lives and alcoholism is one of prevalent type of addiction (Vega et al., 2002).

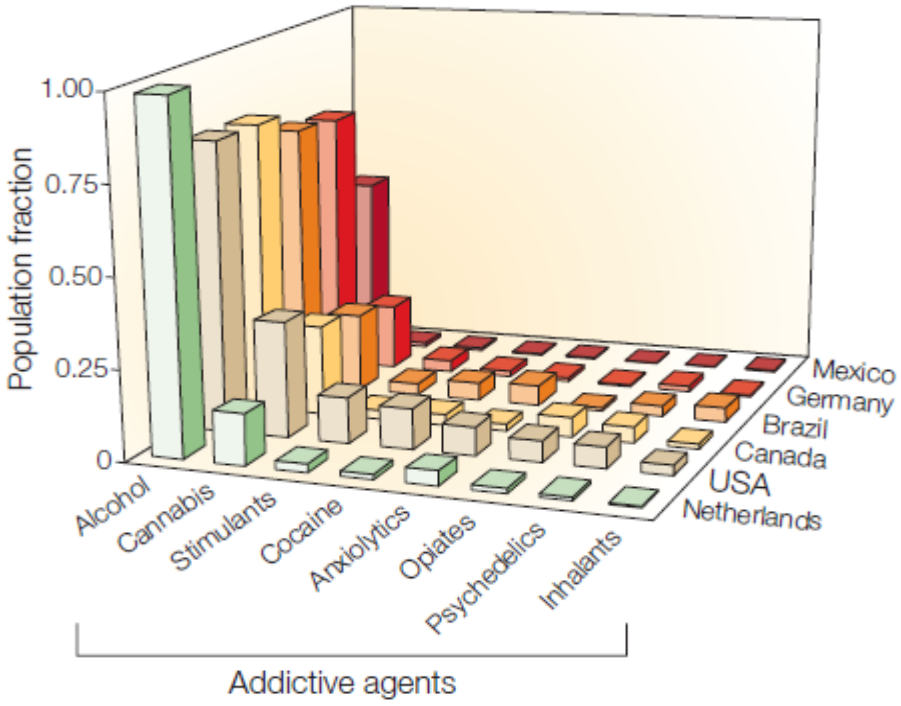


Figure 1.1 Lifetime prevalence of substance use in 6 countries: Alcohol use is defined as having consumed 12 or more drinks in at least 1 year. Other drug use is defined as reporting use of the drug more than five times ever (Goldman et al., 2005).

Normally, the brain is protected from drugs and foreign substances by a filter system which only allows water to pass through. Because the molecular structure of alcohol is similar to water, it is able to pass through the barrier or filter. Since the brain controls critical body functions like perception, speech and judgment, alcohol's effect on

the brain can be very dangerous. Depending on the amount of alcohol in the bloodstream, the longevity of the effects vary. As more and more alcohol is consumed, the effects intensify exponentially.

Alcohol seductive characteristic arise from that in very small amounts, alcohol can help a person feel more relaxed or less anxious, because it temporarily blunts the effects of stress hormones. It typically leaves you feeling worse than ever. For this reason, people prefer more amount, but more alcohol causes greater changes in the brain, namely it depresses the brain and nervous system. Alcohol is a depressant namely it slows the function of central nervous system by mimicking either the brain's natural sedating chemicals or blocking the brain's natural ability to produce stimulating chemicals. By the way, it prevents some of the messages trying to get to the brain. This alters a person's perceptions, emotions, movement, vision, and hearing. Alcohol use can also cause mood changes and loss of inhibitions as well as violent or self-destructive behavior (Russell, 2011).

If we examine the effect of alcohol on people who are different age groups, our first group is pregnancy period of women. The moderate consumption of ethanol by pregnant women can have significant consequences on the developing nervous system of the fetus. Consumption of ethanol during pregnancy at levels considered to be in the moderate range can generate fetal alcohol effects (behavioral, cognitive anomalies) in the offspring. A number of factors–including gestational period, the periodicity of the mother's drinking, genetic factors, etc.–play important roles in determining the effect of ethanol on the developing central nervous system (Eckardt et al., 1998).

Alcoholism affect teenager more than adults, namely young brain is more sensitive to the effect of alcohol, because it can cause alterations in the structure and function of the developing brain, which continues to mature into a person's mid 20s. In adolescence, brain development is characterized by dramatic changes to the brain's structure, neuron connectivity, and physiology (Tapert et al., 2004). These changes in the brain affect everything from emerging sexuality to emotionality and judgment.

Whole adolescent brain parts not mature at the same time, for instance the limbic areas mature earlier than frontal lobes of the brain. If the limbic areas' and frontal lobes' functions are thought, the frontal lobes are responsible for executive functions, namely recognize consequences of your actions and to choose between good and bad actions such as judgement, reasoning, problem-solving and impulse control, however the limbic

areas regulate memories associated with emotions and reward response. These are associated with an adolescent's lowered sensitivity to risk. That is to say, differences in maturation among parts of the brain can result in impulsive decisions or actions and a disregard for consequences (Brown et al., 2008).

As a conclusion, young people who abuse alcohol have poorer memory, more depression, anxiety or self-harm and more likelihood of excess drinking later in life due to effect of alcohol on brain function. Because, the adolescent brain (< 20 years) is more sensitive to the effects of alcohol and the effects of alcohol excess cause memory impairment, behavioral/judgement impairment, shortly alcohol binge drinking damages the brain.

In order to understand effects of alcoholism, studies about alcohol must be molecular level. Because, alcohol dependence is not a basic habit that cannot limit only alcohol drinking behavior. New scientific researches show genetic aspects of alcoholism. Alcoholism affects molecular mechanism of human brain by chancing expression of some genes in brain (Crabbe and Philips, 1998). In order to understand genetic background of alcoholism, influence of alcohol on brain molecular mechanism must be detected specifically. Many researches about genetic aspects of alcohol addiction are applied based on SNP analysis in human. But generally animals especially rats are chosen as a model organism, because animal models allow researchers to use methods that would be unethical with human subjects. Both human and animal studies indicate that genetic factors play a role in the development of alcoholism, leading researchers to focus on identifying genes associated with alcoholism or a predisposition to alcoholism (Tabakoff and Hoffman, 2000).

Genetic mapping of quantitative trait loci (QTLs) has been used to identify chromosomal locations of genes influencing responses related to alcohol QLTs marks a new period of synthesis in pharmacogenetic research, in which networks of drug related behaviors their underlying pharmacological, physiological and biochemical mechanisms and particular genomic regions of interest are being identified (Crabbe et al., 1994).

Genetic models of alcohol related behaviors include inbred strains, recombinant inbred strains, and transgenic/knock-out mice are obtained in order to make easier alcohol intake in rats (Gora - Maslak et al., 1991; Wehner and Bowers, 1995). However, genetically engineered mice were not used for this research, in order to understand rats become dependent or not, based on expression analysis.

In research on alcohol addiction, rats were chosen as model organisms for determining alterations of gene expression based on alcohol intake in brain, because animal models allow us to use methods that would be unethical with human subjects. Methodology of alcohol intake was applied in rats by using ethanol in liquid diet (Uzbay and Kayır; 2008). All procedures in this study are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (USA).

1.3 ALCOHOL RELATED BRAIN PARTS

Alcohol is a central nervous system depressant that slows the function of central nervous system and also it can appear to be a stimulant, since it depresses the part of the brain that controls inhibitions. Central nervous system include the brain and the spinal cord, and its function about making the person think, speak, move, namely when a person thinks of something he wants his body to do, CNS sends a signal to that part of the body.

When effect of alcohol on brain is thought deeply, at the neurochemical level, the moderate consumption of ethanol selectively affects the function of GABA, glutamatergic, serotonergic, dopaminergic, cholinergic, and opioid neuronal systems. Ethanol can affect these systems directly or the interactions between and among these systems become important in the expression of ethanol's actions. The behavioral consequences of ethanol's actions on brain neurochemistry, and the neurochemical effects, based on dose and time-related, themselves, can change significantly even on the rising and falling phases of the blood ethanol curve. The behavioral effects of moderate ethanol intake can perceive as reinforcing through either positive (e.g., pleasurable, activating) or negative (e.g., anxiolysis, stress reduction) reinforcement mechanisms in human or animals. Genetic factors and gender play an important role in the metabolism and behavioral actions of ethanol, and doses of ethanol producing pleasurable feelings, activation, and reduction of anxiety in some humans or animals can have aversive, sedative, or no effect in others (Eckardt et al., 1998).

In this study, detection of gene expression differences on rat brain tissue depend on alcohol intake, provide knowledge about influence of alcohol on brain molecular mechanism. Actually, effect of alteration in gene expression based on alcohol in brain,

shows genetic background of alcoholism. The main purpose of this research has already understood this background of brain. However, some parts of brain are especially affected by addiction agents than other parts; such as they activate dopaminergic neurons in mesolimbic system (Pierce and Kumaresan, 2006) that included frontal cortex, nucleus accumbens, ventral tegmental area, hippocampus, striatum are shown in figure 1.2. For example, Cholinergic interneuron's, GABAergic parvalbumin expressing interneuron's, GABAergic somatostatin expressing interneuron's in striatum are some types of neurons that are related to dopamine receptor system and reward mechanism. These systems are also related to addiction

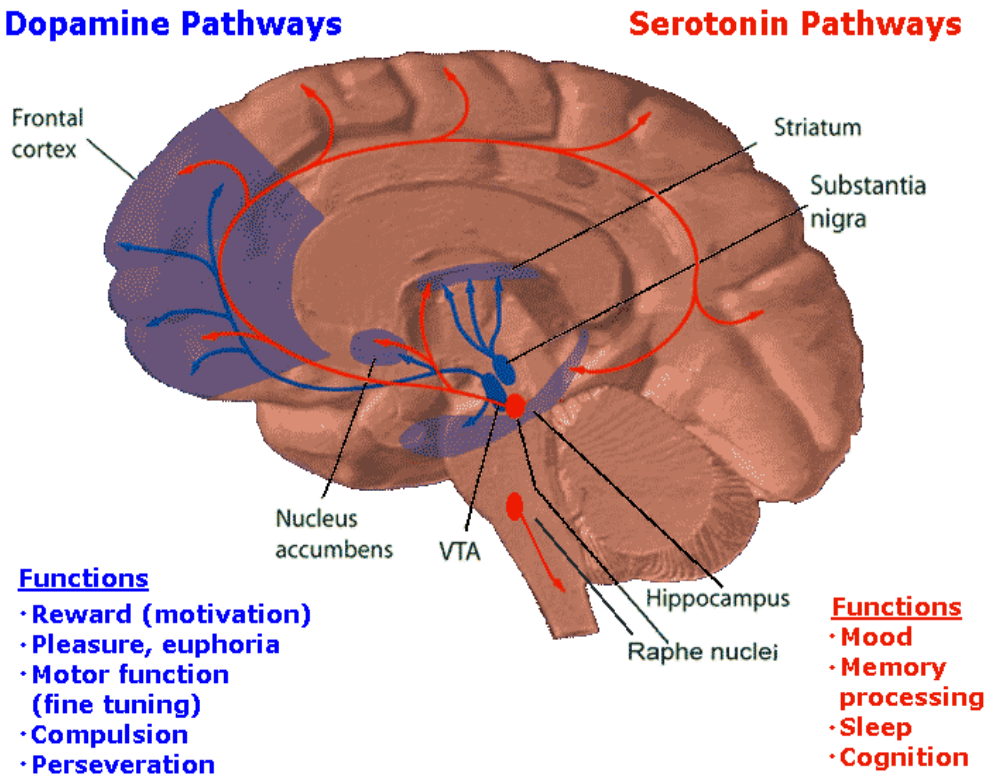


Figure 1.2 Mesolimbic pathway: Include dopamine and serotonin pathways, and also their functions are shown. (http://en.wikipedia.org/wiki/File:Dopamineseratonin.png)

Alterations in expression of genes that control signal transduction neurotransmitter synthesis, receptor abundance and activity changes in brain circuitry (Neuron connections) in limbic system included hippocampus, striatum, frontal cortex etc. changes in behavior, dependence, tolerance (Cruz et al., 2008).

1.3.1 Hippocampus

Although alcohol acts as a general CNS depressant, but it also affects specific areas of the brain to a more extent degree than others such as hippocampus. The hippocampus like as amygdala shown in figure 1.3 is the part of the brain where memories are made. Memory impairment due to alcohol has been linked to disruption of hippocampal function (White, 2003). For example, after just one or two drinks, if alcohol reaches the hippocampus, a person may have trouble remembering something he just learned, such as a name or a phone number. In addition, drinking a lot of alcohol quickly can cause a blackout, namely not being able to remember entire events, such as what he did last night.

In other words, when alcohol damages the hippocampus, a person may find it hard to learn and to hold on to knowledge, because of alcohol in particular affecting gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) neurotransmission which negatively impacts long-term potentiation (LTP) at molecular level. Alcohol severely disrupts the ability of neurons to establish long–lasting, heightened responsiveness to signals from other cells. This heightened responsiveness is known as long–term potentiation (LTP). The molecular basis of LTP is associated with learning and memory. So, LTP used as a model for studying the neurobiology underlying the effects of drugs, including alcohol, on memory. One of the key requirements for the establishment of LTP in the hippocampus is that a type of signal receptor known as the NMDA receptor becomes activated. Activation of the NMDA receptor allows calcium to enter the cell, which sets off a chain of events leading to long–lasting changes in the cell's structure or function, or both. Alcohol interferes with the activation of the NMDA receptor, thereby preventing the influx of calcium and the changes that follow. This is believed to be the primary mechanism underlying the effects of alcohol on LTP, though other transmitter systems probably are also involved (White, 2003).

Particularly, damage to hippocampal CA1 cells adversely affects memory formation, and this disruption has been linked to dose-dependent levels of alcohol consumption. At higher doses, alcohol significantly inhibits neuronal activity in the CA1 and CA3 pyramidal cell layers of the hippocampus (Hiller at al., 2004; Ryabinin, 1998).

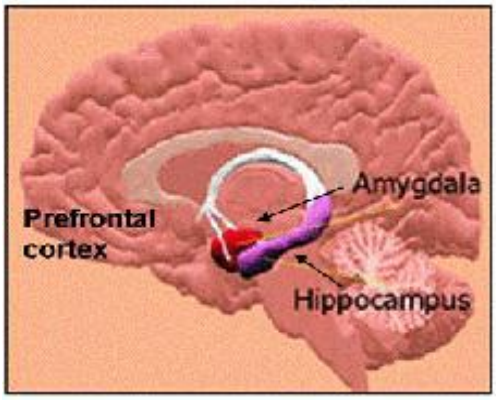


Figure 1.3 Image of hippocampus: Memory center of brain with amygdala are shown. (http://www.humanstress.ca/stress/effects-of-stress-on-memory/stress-hormones-and-memory/page-2.html)

Alcoholism affect not only behavioral and molecular aspect of human, but also it can cause anatomic, morphologic and physiologic alteration in human brain. Namely, alcohol influence hippocampal volume. Smaller hippocampal volumes have been reported in the brains of alcoholic patients than in those of healthy subjects. Since, in chronic alcoholism, the reduction of hippocampal volume is proportional to the reduction of the brain volume (Agartz et al., 1999).

1.3.2 Frontal Lobes – Prefrontal Cortex

The frontal lobe included prefrontal cortex and its parts shown in figure 1.4 is involved in functions such as creative thinking, forming ideas, planning of future actions, decision making, artistic expression, using self-control, aspects of emotional behaviour, as well as spatial working memory, language and motor control.

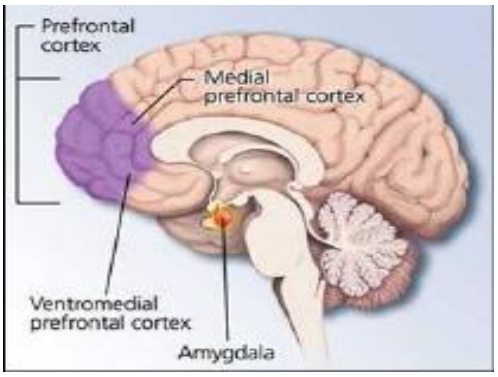


Figure 1.4 Image of frontal lobes included prefrontal cortex and also its regions. (http://www.mcaay.org.au/component/attachments/download/19.html)

The importance of the frontal lobes derives from rich connections, both afferent and efferent, with almost all other parts of the central nervous system. Frontal connections with cortical sensory areas, providing information from the external milieu, occur either by direct cortical–cortical afferents or via the thalamus. The occipital, parietal, and temporal sensory association cortices connect to both the anterior temporal and inferior parietal areas; in turn, each of these has direct afferent connections to the frontal cortex. The prefrontal cortex receives projections from olfactory sensation; it is thus the only cortical area interacting with all four sensory modalities. The prefrontal cortex occupies the anterior portion of the frontal lobes, so like as frontal lobes's characteristics, prefrontal cortex functions are about planning complex cognitive behaviors, moderating correct social behaviour, personality expression, decision making, emotional regulation, planning and organisation. The prefrontal cortex is the single largest brain region in human beings, having been estimated to constitute 29% of the total cortex. As a summary, the frontal lobe also has well-developed connections with limbic and subcortical areas that provide monitoring of the internal milieu (Moselhy et al., 2001).

When alcohol reaches the frontal lobe, loss of reason and inhibitions occur, namely this effect of alcohol cause that a person may hard to control her/his emotions and urges and acts without thinking.This results in the careless, reckless behavior that intoxicated people exhibit. In a social setting this is particularly dangerous because of the loss of self-restraint. Intoxicated people often find themselves doing and saying things they normally would not. Having sex with a stranger, stripping in public and driving while drunk are a few examples of how alcohol affects the frontal lobe. After the effects of alcohol wear off, many people are very surprised to learn what they did while intoxicated. But, the consequences of their actions remain. In addition, ıf person continue to drink alcohol over a long period of time, this can damage the frontal lobes forever, so abnormal behaviors become characteristics of this person (Hiller et al., 2004). Specifically, when the prefrontal cortex is damaged or its activity is decreased, behavior can change dramatically and people can lose much of their inhibition and ability to weigh the consequences of their actions (Woodward J. And Lovinger D., 2008).

As known, the prefrontal cortex region of the brain is involved in decision making. New rodent findings show that prefrontal cortex neuron N-methyl-D-aspartic

acid (NMDA) receptors are especially sensitive to concentrations of alcohol achieved during drinking. This suggests that alcohol's alteration of NMDA receptor function may inhibit normal prefrontal cortex function. For this reason, researches believed that abnormal function in the prefrontal cortex region of the brain contributes to the impulsive behavior and lack of control over drinking that characterize alcohol dependence, but how this occurred was unknown. In order to understand this unknown molecular mechanism of prefrontal cortex, researchers study on ion channels that control the activity of prefrontal cortex neurons. It is hypothesized that alcohol may affect one or more of these ion channel gates, leading to alterations in the function of the prefrontal cortex and that this may contribute to an individual's inability to control their drinking. In addition, this may help to explain why many alcoholics appear to lose control over their drinking despite serious adverse consequences that can arise, such as loss of job, family or even health (Woodward and Lovinger, 2008). Professor Woodward and his team study on NMDA, gamma-aminobutyric acid (GABA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the absence and presence of alcohol. The major finding was that alcohol, at concentrations that are associated with mild to moderate intoxication significantly inhibited the function of the NMDA receptor ion channel without affecting AMPA or GABA ion channels and results provide an explanation for how alcohol affects the ability of the prefrontal cortex to carry out its normal duties, when NMDA receptors are inhibited, as with alcohol, the ability of the neuron to carry out its task is affected, thereby reducing the ability of an individual to control their behavior and possibly leading them to engage in actions that are not beneficial. In other words, the normal risk/benefit assessment that this brain region engages in is disrupted (Abernathy et at., 2010).

The pre frontal cortex continues to develop until early 20s by synaptic pruning and myelination. By the way, improved brain function cause increased efficiency of “the network” and increased speed of neuronal transmission. Young brain is more sensitive to the effect of alcohol on brain structural and functional development and adaption as the prefrontal cortex is stil developing, less sensitive to cues that serve to moderate alcohol intake (Spear, 2002; Spear, 2004). Adolescents who abuse alcohol have smaller prefrontal cortices, smaller prefrontal white matter volumes, white matter structural irregularities. Also, teenagers who abuse alcohol have smaller hippocampal volumes, more sensitive hippocampus to alcohol neurotoxic effects (Agartz, 1999).

1.3.3 Striatum

The striatum is a subcortical part of the forebrain. The striatum, in turn, gets input from the cerebral cortex. According to anatomical subdivisions striatum two regions have been identified, dorsal and ventral striatum. In primates (including humans), the striatum is divided by a white matter tract called the internal capsule into two sectors called the caudate nucleus (medially) and putamen (laterally) are shown in figure 1.5.

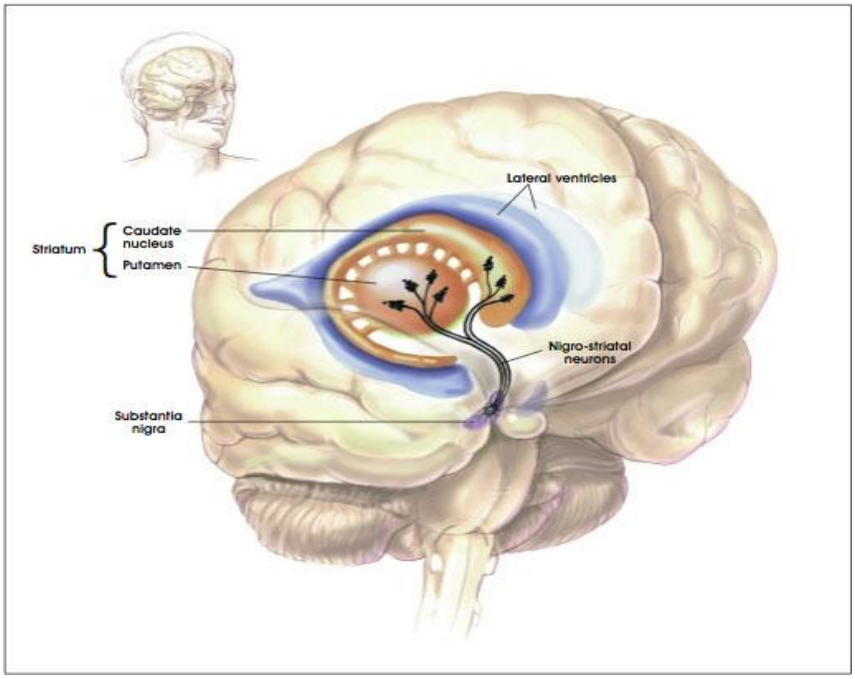


Figure 1.5 Image of striatum and its components, also functional related parts in brain. (http://brainmind.com/BasalGanglia.html)

Although there is an extensive literature linking the cognitive control of executive functions specifically to the prefrontal cortex, more recent studies suggest that these functions depend on reward-related circuitry linking prefrontal, premotor, and sensorimotor cortices with the striatum. Reduced neuronal activity as well as dysfunctional connectivity between the ventral striatum and the dorsolateral prefrontal cortex is associated with alcohol craving and impairment of new learning processes in abstinent alcoholics (Chen et al., 2011). Also, corticostriatal network controls functionally heterogeneous decision processes involving (1) actions that are more flexible or goal directed, sensitive to rewarding feedback, and mediated by discrete regions of association cortices particularly medial, orbitomedial, premotor, and anterior cingulate cortices together with their targets in caudate/dorsomedial striatum and (2)

actions that are stimulus bound, relatively automatic or habitual, and mediated by sensorimotor cortices and dorsolateral striatum/putamen (Balleine et al., 2007).

The striatum is heterogeneous in terms of neurons. It is composed of these neuronal cell types. The most ratio of neurons in striatum includes “medium spiny neurons” (96%). The remaining are “Deieters’ neurons (2%) and cholinergic interneurons (1%). In spite of the small ratio, cholinergic interneurons are tonically active in primates and their functions are about reward-related events. Other neuronal cell types in striatum are GABAergic interneurons and they express dopamine receptors, so they are important for reward mechanism.

Metabotropic dopamine receptors are present both on spiny neurons and on cortical axon terminals. Second messenger cascades triggered by activation of these dopamine receptors can modulate pre- and postsynaptic function, both in the short term and in the long term. The striatum is best known for its role in the planning and modulation of movement pathways but is also potentially involved in a variety of other cognitive processes involving executive function, such as working memory (Voytek and Knight, 2010). In humans the striatum is activated by stimuli associated with reward, but also by aversive, novel, unexpected or intense stimuli, and cues associated with such events. The ventral tegmental dopaminergic neurons that innervate portions of the striatum have long been accepted to be the site of rewarding feeling.

Dopamine release in ventral striatum is a common element of drug reward, but alcohol has an unusually complex pharmacology, and humans vary greatly in their alcohol responses. This variation is related to genetic susceptibility for alcoholism, which contributes more than half of alcoholism risk. Development of novel, mechanism-based pharmacotherapies will require an improved understanding of the neurobiology that underlies addictive properties of alcohol. Compared to other addictive drugs, alcohol has a complex pharmacology. Sedative, ataxic and anxiolytic alcohol effects are primarily mediated through GABA and glutamate signaling. Alterations of dopaminergic, glutamatergic, and GABAergic signaling within different regions of the striatum by alcohol is critical for alcohol craving, consumption, dependence, and withdrawal in humans and animal models. Within the DLS of mice and nonhuman primates withdrawn from alcohol after chronic exposure, glutamatergic transmission in striatal projection neurons is increased, while GABAergic transmission is decreased. Glutamatergic transmission in DMS projection neurons is also increased in ethanol

withdrawn rats (Chen et al., 2011). In contrast, rewarding properties of alcohol such as euphoria and psychomotor stimulation are thought to involve endogenous opioids and mesolimbic dopamine (DA). In response to alcohol, m-opioid receptor (OPRM1) activation in the ventral tegmental area suppresses the activity of inhibitory GABAergic interneurons, resulting in disinhibition of mesolimbic dopamine neurons and mesolimbic dopamine release from their terminals in the ventral striatum. Accordingly, m-opioid receptor blockade is a treatment for alcohol dependence.

Humans vary substantially in their alcohol responses, and this variability is related to genetic susceptibility for alcohol use disorders, which accounts for more than half the disease risk in this condition. Striatal mesolimbic dopamin release is a common element of drug reward, and alcohol-induced mesolimbic dopamine release has been shown both in rodents and in humans. There is, however, marked individual variation in alcohol induced behavioral responses thought to be related to mesolimbic dopamine activation, such as psychomotor stimulation. Functional variation in opioid genes may contribute to this variation by modulating alcohol-induced mesolimbic dopamine release (Ramchandani et al., 2011).

A decrease in dopamine type 2 receptors (D2) and mesolimbic dopamine transmission predisposes animals to consume alcohol. Studies in rodents have reported a decrease in D2 receptor density in the caudate–putamen and nucleus accumbens of alcohol-preferring rats compared with non-alcohol-preferring rats (McBride et al., 1993). Furthermore, lower dopamine concentrations in the mesolimbic terminals have also been measured in alcohol-preferring compared with alcohol-nonpreferring rodents (Murphy et al., 1982). These studies suggest that a deficit in mesolimbic dopamine function, either presynaptic (low dopamine levels) or postsynaptic (low D2 receptor density), may be associated with alcohol dependence (Martinez et al., 2005).

In addition, according to another research about dopamine receptor, in abstinent alcoholic patients, a low availability of dopamine D2/3 receptors in the ventral striatum and adjacent putamen was associated with a high level of craving for alcohol. Alcohol craving may also depend on presynaptic dysfunction of striatal dopamine production, which may contribute to the risk of relapse. Rat studies indicated that acute and chronic alcohol intake stimulates dopamine release in the ventral and dorsal striatum. Chronic alcohol intake, however, reduced the availability of striatal dopamine D2/3 receptors, which may represent a compensatory down-regulation that ensures homeostasis of

central dopaminergic neurotransmission. Based on this study report, reduced availability of dopamine D2/3 receptors in the ventral striatum and adjacent putamen of abstinent alcoholic subjects, which was associated with a high level of craving for alcohol and an increase in brain activation elicited by alcohol associated (as opposed to control) cues. Because low striatal dopamine D2/3 receptor availability was inversely correlated with alcohol craving in abstinent alcoholic subjects (Heinz et al., 2005).

1.4 TARGET MOLECULAR PATHWAY

In this research, genes of agmatinase, arginine decarboxylase, arginase, SAT1 enzymes are our target. As known, enzymes are key factors of several molecular mechanisms in cells and this role of proteins is thought in nervous system, they act as a role on signal transduction in neurons and affect molecular mechanisms of cells in brain. Our collaborators (Uzbay T. and Kayır H.) researches about agmatine pathway includes arginase, arginine decarboxylase, nitric oxide synthase, ornithine decarboxylase, agmatinase, diamine oxidase and SAT1 enzymes shown in figure 1.6 are referred for this study. However, we examine expression of arginase, arginine decarboxylase, agmatinase and SAT1 for this study. Also, BDNF used as a positive control of alcohol dependence and GADPH for housekeeping gene.

Our aim is to apply expression analysis of agmatinase, arginine decarboxylase, arginase, SAT1, BDNF genes against to alcohol consumption in rats. Expression analysis result are compared between withdrawal, alcohol intake and control groups and we will conclude that alteration of gene expression based on alcohol is able to detected, and which genes are really related to alcohol abuse, and this conclusion may become a clue for understanding how alcohol addiction alter brain molecular mechanism. In other words, according to genetic analysis results, we can comment on genetic background of alcohol on this pathway in brain. If results of this study will be supported literature survey, they can be used for improvement about diagnosis and treatment of alcoholism.

1.4.1 Polyamine/Agmatine Pathway

Recent evidence suggests that agmatine, which is an intermediate in polyamine biosynthesis, might be an important neurotransmitter in mammals. Namely, Agmatine is a cationic amine formed by decarboxylation of arginine by the enzyme arginine decarboxylase (Uzbay et al., 2000). It is a biologically active substance that is synthesized in the brain, stored in synaptic vesicles in regionally selective neurons, accumulated by uptake, released by depolarization, and inactivated by agmatinase. In addition, it is contained axon terminals and interacts with cell specific receptors, namely binds to a 2- adenoceptors and imidazoline binding sites, and is widely distributed in rat tissue, such as serum, viscera and brain including astrocytes. Also, it blocks NMDA receptor channels and other ligand-gated cationic channels. Furthermore, agmatine inhibits nitric oxide synthase (NOS) in rats, and induces the release of some peptide hormones. That is to say, it elicits biological actions within the central nervous system. As a result of its ability to inhibit both hyperalgesia and tolerance to, and withdrawal from, morphine, and its neuroprotective activity, agmatine has potential as a treatment of chronic pain, addictive states and brain injury (Reis and Regunathan, 2000).

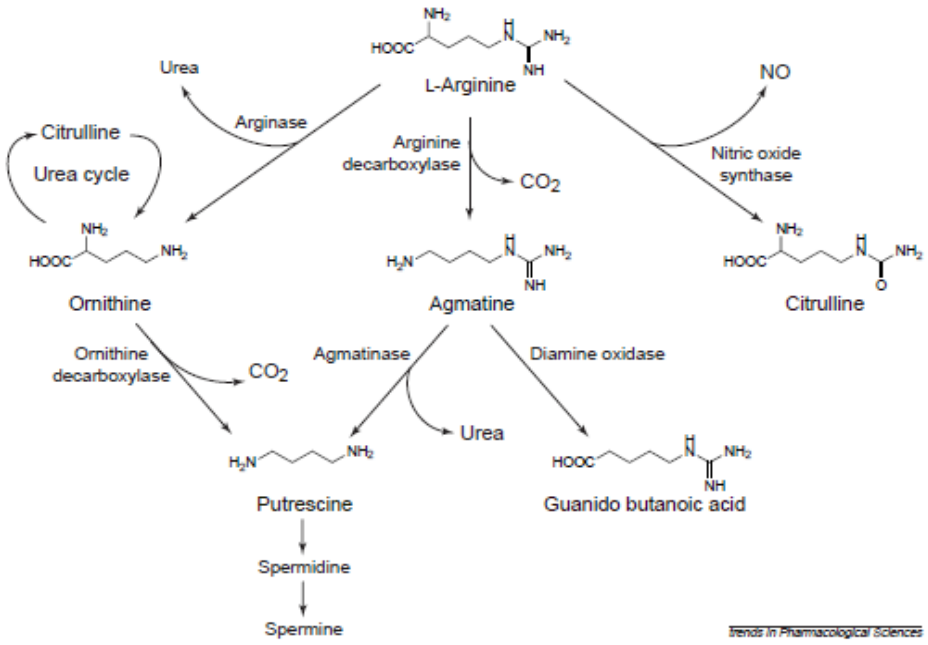


Figure 1.6 Metabolic pathways of agmatine and related compounds. Agmatine is produced from L-arginine by the action of arginine decarboxylase. Agmatine can then be metabolized to produce either spermine during polyamine biosynthesis, or guanido butanoic acid (Reis and Regunathan, 2000).

Effects of agmatine, which is an endogenous polyamine metabolite formed by decarboxylation of L-arginine, have been investigated on the ethanol withdrawal syndrome in rats and it is demonstrated that agmatine, has some inhibitory effects on the withdrawal syndrome in ethanol-dependent rats. In other words, agmatine seems to be a new and a potent pharmacologically active agent on mechanisms involved in development of ethanol physical dependence in rats, and it may have therapeutic potential in the treatment of ethanol-type dependence (Uzbay et al., 2000).

If the effect of alcohol on agmatine molecular mechanism in brain is examined deeply, the beneficial effects of agmatine on ethanol dependence may be explained by four mechanisms: α_2-adrenergic receptor activation, imidazoline receptor activation, NMDA (*N*-methyl-D-aspartate) receptor blockage and NOS (nitric oxide synthase) inhibition or NO-NMDA cascade inhibition. Drugs like clonidine, which bind to α_2-adrenergic receptors, have prominent inhibitory effects on the signs of ethanol withdrawal in rats and humans, and agmatine binds to these receptors as well. Although agmatine has not been revealed to have agonistic activity at α_2-adrenergic receptors, these data imply that agmatine may possibly inhibit ethanol withdrawal via an effect similar to clonidine. However, agmatine has agonistic activity at imidazoline receptors. This property may be responsible for the beneficial effects of agmatine on ethanol withdrawal, as this property is shared with clonidine, another effective inhibitor of ethanol withdrawal syndrome. A third explanation of the effects of agmatine on ethanol withdrawal may be a central inhibition of NOS by agmatine. NOS inhibitors produce a marked attenuation of the signs of ethanol withdrawal syndrome in rats (Uzbay and Oglesby, 2001; Uzbay, 2012). Some studies demonstrated that agmatine inhibited enzyme NOS in brain (Galea et al., 1996; Uzbay, 2012). A further possibility may be an interaction with central excitatory amino acidergic mechanisms through NO. Activation of NMDA subtype of excitatory amino acid receptors causes an influx of Ca2+ into neurons, leading to calmodulin-dependent activation of NOS. Thus, the activation of NMDA receptors may be accompanied by the generation of NO. The role and significance of glutamatergic stimulation in the development of physical dependence to ethanol is well known, and NMDA receptor blockers attenuate the severity of ethanol withdrawal syndrome in rats. NMDA receptor activation relies upon NO as a significant neuronal messenger. Thus, NOS inhibition in the glutamate system could also be responsible for the inhibitory effect of agmatine on ethanol withdrawal signs.

Furthermore, agmatine is also a selective blocker of NMDA receptor channels in rats, and its beneficial effects on ethanol withdrawal may be associated with a direct interaction with NMDA-type glutamatergic receptors (Uzbay, 2012). Although the above explanations are reasonable, more direct evidence from experimental studies is necessary to understand the mechanism of the effect of agmatine on ethanol dependence. As a summary, all of these knowledge from Uzbay (2012); according to this, agmatine is a novel transmitter in brain, and it involves action mechanisms affecting several neurobiological processes such as neuroprotective effects in the CNS. Respectable studies also indicate that agmatine has beneficial effects on the withdrawal syndromes of several abused drugs, such as morphine and ethanol. All of the data imply that agmatine and the polyamine system may represent a new and effective target for understanding the action mechanisms of some CNS disorders and for the development of new pharmacological strategies in the treatment of these disorders.

In recent years, evidence has emerged implicating dysregulation of the polyamine system as an important factor in suicide and other psychiatric disorders. Alterations of spermidine/spermine N1-acetyltransferase (SAT1), the rate-limiting enzyme in polyamine catabolism, have been one of the most robust findings implicating this system in the neurobiology of suicide (Fiori and Turecki, 2011).

1.4.2 Brain-derived neurotrophic factor (BDNF)

BDNF belongs to the nerve growth factor (NGF) family of neurotrophic factors. BDNF and its receptor TrkB are widely distributed throughout the brain, and the BDNF/TrkB pathway plays an important role in neuronal proliferation, differentiation and survival, as well as synaptic plasticity. More recently, BDNF has been implicated in psychiatric disorders such as depression, anxiety and also drug addiction.

Human studies have linked BDNF to alcohol addiction. For example, the region of chromosome 11 containing the BDNF gene has been implicated as a susceptibility locus for addiction to multiple drugs of abuse, including alcohol (Uhl et al., 2001; Jeanblanc et al., 2009), and a single nucleotide polymorphism in the BDNF gene has been linked with an earlier onset of alcoholism (Matsushita et al., 2004; Jeanblanc et al., 2009). These evidence that suggests a role for BDNF in regulating behavioral responses to alcohol in rodents.

Specifically, a reduction in BDNF expression or inhibition of the BDNF receptor TrkB increases ethanol consumption and preference (Jeanblanc et al., 2006). Moreover, according to Jeanblanc and his team (2009) observation both acute systemic administration of ethanol and voluntary ethanol intake increase BDNF expression in the dorsal striatum of mice and they showed that this increase in BDNF level triggers the expression of downstream effectors, including the dopamineD3 receptor and preprodynorphin, and that inhibition of the dopamineD3 receptor or of the dynorphin receptor, the κ opioid receptor, blocks the BDNF-mediated decrease in ethanol consumption. Together, these studies suggest that BDNF may act as an endogenous negative regulator of ethanol intake. However, the localization of this regulatory effect remains unknown.

As a conclusion, ethanol treatment increases BDNF expression specifically in the dorsal striatum. In addition, whether BDNF within the dorsal striatum regulates ethanol self-administration in Long–Evans rats and also a greater increase in BDNF expression after ethanol self-administration in the dorsolateral striatum than in the dorsomedial striatum. The dorsal striatum has been implicated in the control of goal-directed behaviors and in the formation of habit. Specifically, the dorsomedial striatum plays a role in response–outcome learning, whereas the dorsolateral striatum regulates stimulus–response, or habit learning. In addition, the lateral and medial parts of the dorsal striatum have distinct anatomical inputs and outputs. Therefore, Jeanblanc and his team (2009) interested in determining whether and where BDNF within the subregions of dorsal striatum controls the level of ethanol self-administration. According to their findings, it is demonstrated that the BDNF pathway within the dorsolateral striatum controls the level of ethanol self-administration. This results suggest that an endogenous signaling pathway within the same brain region that mediates drug-taking behavior also plays a critical role in gating the level of ethanol intake (Jeanblanc et al., 2009).

1.4.3 Housekeeping Gene – GADPH

In molecular biology researches, quantitative gene expression data are often normalized to the expression levels by housekeeping genes, because an inherent assumption in the use of housekeeping genes is that expression of the genes remains

constant in the cells or tissues under investigation (Barber et al., 2005). In this study, GADPH used as a housekeeping gene.

Housekeeping gene involved in basic functions needed for the sustenance or maintanence of the cell. Housekeeping genes are constitutively expressed at a relatively constant level (they are always turned on), because they should always be present and should always have the same levels. If the constant is not consistent, it suggests that there may be a problem with the sample or the process being used, since The proteins produced by a housekeeping gene vary, but are involved in some way in processes necessary to the survival of a cell. hence, they are essential to a cell and always present under any conditions. It is assumed that their expression is unaffected by experimental conditions, namely they are expressed in all cells of an organism under normal and pathophysiological conditions (Eisenberg and Levanon, 2003; Butte et al., 2001). The proteins they code are generally involved in the basic functions, some may be involved in sustaining cell function, while others may be involved in cell maintenance such as metabolism, cell signaling, gene expression etc. necessary for the sustenance or maintenance of the cell. Housekeeping genes tend to produce proteins at steady rates, and errors in their expression can lead to cell death. Examples of housekeeping genes include actin, GAPDH and ubiquitin. GADPH act as a role on carbohydrate metabolism in cell (Eisenberg and Levanon, 2003).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes used in comparisons of gene expression data (Barber et al., 2005). GAPDH catalyses the conversion of glyceraldehyde 3-phosphate as the name indicates. This is the 6th step of the breakdown of glucose (glycolysis), and thus serves to break down glucose for energy and carbon molecules, an important pathway of energy and carbon molecule supply located in the cytosol of eukaryotic cells. Glyceraldehyde 3-phosphate is converted to D-glycerate 1,3-bisphosphate in two coupled steps. The first is favourable and allows the second unfavourable step to occur.

In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes, including transcription activation, initiation of apoptosis, and endoplasmic reticulum to golgi vesicle shuttling.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

This study include main two parts are animal studies and molecular application. For this reason, materials that were used in these parts were explained under two subtitles for materials.

2.1.1 Animal Studies

2.1.1.1 Animals and Laboratory

18 adult male Long Evans rats (342-506g weight at the beginning of the experiment) separated three groups (Each group has n=6): Alcohol addicted, withdrawal, and negative control groups.

For chronic ethanol exposure, rats were housed individually in a quiet and temperature- and humidity- controlled room (22+-3°C and 60+-5%, respectively) in which a 12 h light/dark cycle was maintained (7:00-19:00 h light). This laboratory conditions were provided by our collaborators at Faculty of Medicine Department of Medical Pharmacology, Psychopharmacology Research Unit, Gülhane Military Medical Academy, Ankara.

2.1.1.2 Liquid Diet

Ethanol was given in the modified liquid diet (composition : cow milk 925 ml, 25-75ml ethanol (96.5% ethyl alcohol), vitamin A 5000 IU and sucrose 17g) to first 12 rats.

In addition, liquid diet was freshly prepared daily and presented at the same time of the day (09:30h).

2.1.2 Molecular Application

2.1.2.1 Primers

Primers of five target and one housekeeping genes was used for this study and nucleotide chain of forward and reverse primers of these genes are shown in Table 2.1.

Table 2.1 Forward (F) and reverse (R) primers of target genes and their nucleotid lines.

Primer of Gene	Nucleotide Chain
BDNF - F	AGTCTCCAGGACAGCAAAGC
BDNF - R	GTCGTCAGACCTCTCGAACC
SAT1 - F	TCTACCACTGCCTGGTTGC
SAT1 - R	CTGCAGCGACACTTCATAGC
GAPDH - F	TCATGAAGTGTGACGTTGACATCCGT
GAPDH - R	CCTAGAAGCATTTGCGGTGCAGGATG
ARDEC - F	TCAAGTATGCTGCCAAGCAC
ARDEC - R	AAACTCACACCCACCACCTC
ARG - F	TTTCCTGGATCAAACCTTGC
ARG - R	GTCAAAGCTCAGGTGGATCG
AGMA - F	AAGTGTGGATGAGGGACTGC
AGMA - R	ATCTGCTGCCTGATCTCTGC

2.1.2.2 Homogenization

MagNA Lyser Green Beads ROCHE kit and its instrument was used for homogenization of rat brain tissue samples. Protocol is applied by essential 2.0 ml sample tubes filled with ceramic beads shown in Figure 1.1 for homogenizing solid cellular sample material using the MagNA Lyser Instrument shown in Figure 2.2.

Figure 2.1 MagNA Lyser sample tube: 2 ml screw-cap tube prefilled with 1.4 mm ceramic beads used for homogenization of tissue samples.
(http://www.roche-applied-science.com/proddata/gpip/3_8_7_1_2_1.html)

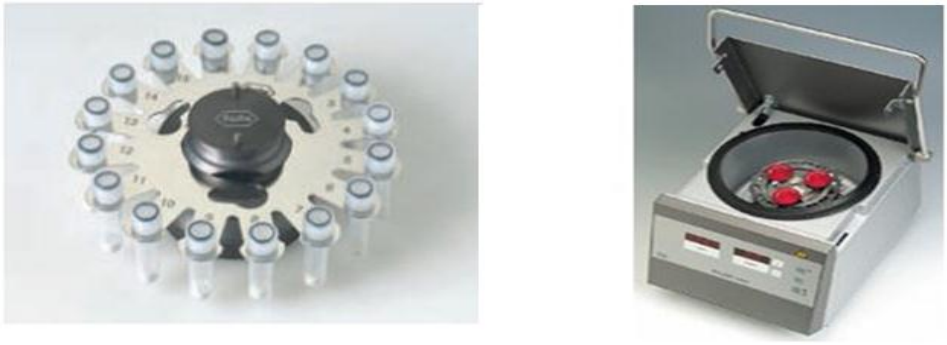


Figure 2.2 MagNA lyser instrument: MagNA lyser sample tubes are placed in its instrument for homogenization of tissue samples. (http://www.roche-applied-science.com/sis/geneexpression/index.jsp?id=gene_071050; http://www.roche-applied-science.com/proddata/gpip/3_8_7_1_1_1.html)

2.1.2.3 RNA Isolation

High Pure RNA Isolation (Roche) kit was used for RNA isolation and its included chemicals' contents and functions are shown in Table 2.2.

Table 2.2 Contents of Roche RNA isolation kit.

Chemicals/Materials	Content/Function
Lysis/Binding Buffer	4.5 M guanidine-HCl, 50 mM Tris-HCl, 30% Triton X-100 (w/v), pH 6.6 (25°C).
DNase I, recombinant, lyophilizate	10 KU lyophilized DNase I. Resuspend in 0.55 ml Elution Buffer.
DNase Incubation Buffer	1 M NaCl, 20 mM Tris-HCl and 10 mM $MnCl_2$, pH 7.0 (25°C).
Wash Buffer I	33 ml (add 20 ml absolute ethanol before first use) 5 M guanidine hydrocloride and 20 mM Tris-HCl, pH 6.6 (25°C); final concentrations after addition of 20 ml absolute ethanol.
Wash Buffer II	10 ml (add 20 ml absolute ethanol before first use) 20 M NaCl, 2 mM Tris-HCl, pH 7.5 (25°C); final concentrations after addition of 20 ml absolute ethanol.
Elution Buffer	30 ml Water, PCR Grade
High Pure Filter Tubes	One bag with 50 polypropylene tubes with two layers of glass fiber fleece, for uptake of up to 700 µl sample volume
Collection Tubes	One bag with 50 polypropylene tubes (2 ml)

2.1.2.4 Spectrophotometric Measurement

In order to measure amount and purity of isolated RNA, Nanodrop-Thermo device shown in Figure 2.3 and its connected computer system, shown in Figure 2.4, was used.

Figure 2.3 Thermo nanodrop device: Usage for spectrophotometric measurement of isolated RNA quantity and purity.
(http://technologyinscience.blogspot.com/2012/05/nuclecacidquantificationdnarna.html)

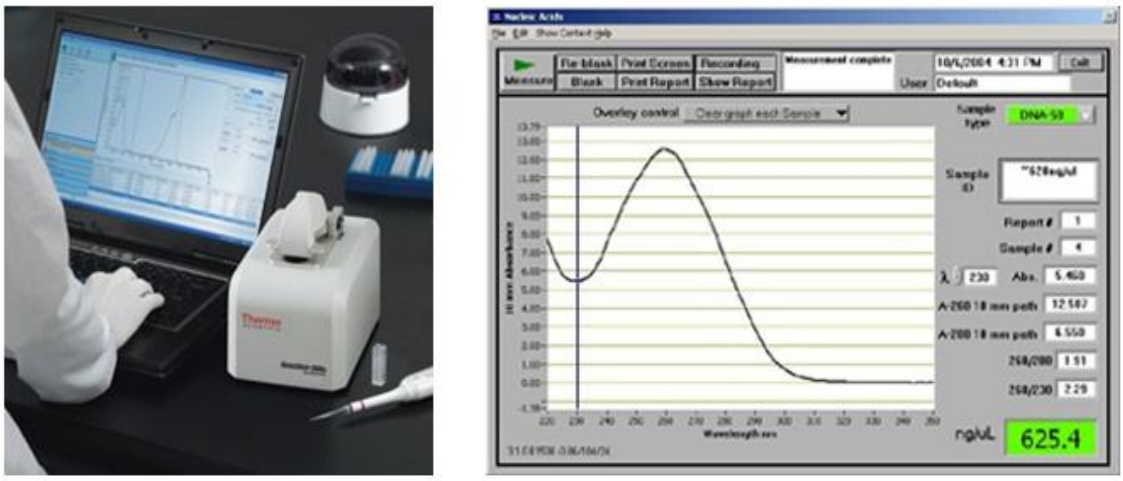


Figure 2.4 Nanodrop device connected with computer.
(http://www.dddmag.com/products/2010/05/spectrophotometer;
http://www.nuigalway.ie/surgery/research/laboratory_facilities.html)

2.1.2.5 Agarose Gel Electrophoresis

After RNA isolation and cDNA synthesis agarose gel electrophoresis was applied by its equipments such as combs, tray, and tank shown in Figure 2.5 and also power supply is shown in Figure 2.6. In addition, in order to prepare agarose gel, chemicals were used. Agarose gel chemicals and their contens are shown in Table 2.3.

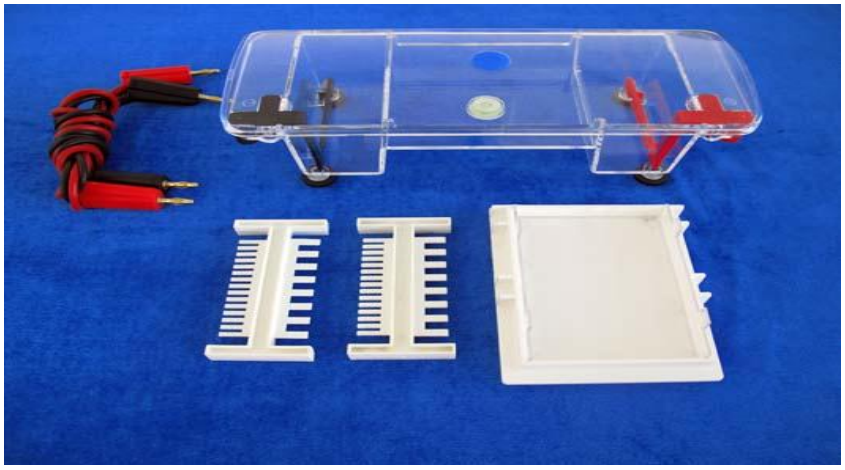


Figure 2.5 Agarose gel electrophoresis equipments: Comb, tray, tank and cablos. (http://www.bio-equip.cn/enshow1equip.asp?equipid=8114&division=502)

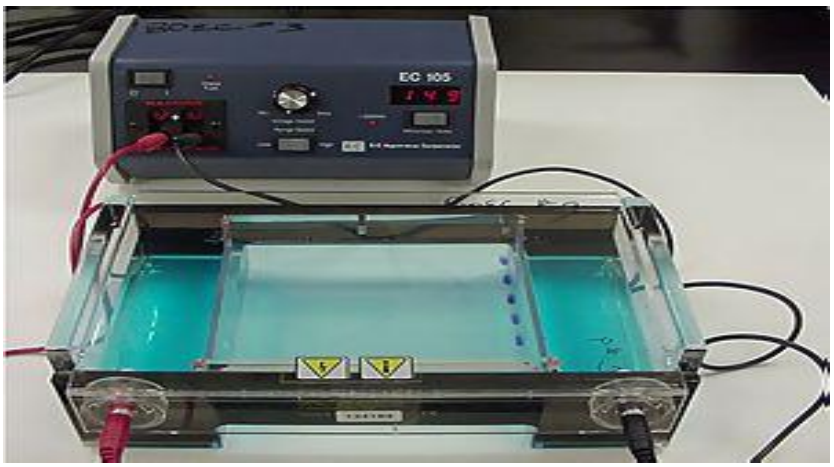


Figure 2.6 Power supply and tank of agarose gel electrophoresis. (http://www.biotech.iastate.edu/ppt_presentations/html/Fingerprntng/StudentInstruction-gel/07.html)

Table 2.3 Agarose gel electrophoresis chemicals and their contents/functions.

Chemical	Content/Function
Agarose (1/1.5%)	Prona, Sigma, St. Louis, USA 0.4 gr / 40 ml 1X TAE (For RNA isolation) 2.25 gr / 150ml 1X TAE (For cDNA synthesis)
50X/1X TAE Buffer	242 g Tris-Base 57.1 ml Glacial Acetic acid 100 ml 500 mM EDTA (pH:8.0) 40 Mm Tris, 20 mM acetic acid, 1 mM EDTA (1X TAE)
6X Loading Dye	0.5 mM Tris-HCl (pH 7.6), %0.03 Bromophenol Blue (BPB), % 0.03 xylene cyanol FF, %60 glycerol, 1 mM EDTA
DNA Ladder	GeneRuler™ 100 bp DNA Ladder, Fermentas 100 µl (0.5 µg/µl) 100 bp sized DNA fragments in 10mM Tris-HCl (pH 7.6), 1mM EDTA.
DNA Stain	SafeView (Invitrogen) / Ethidium Bromide

2.1.2.6 cDNA Synthesis

cDNA was synthesized from isolated RNAs of each samples by "RevertAid™ First Strand cDNA Synthesis" kit (Fermantas) according to kit protocol detailed explained in methods part. However, cDNA synthesis kit contents are shown in Table 2.4. In this table, chemicals included in kit and their contents, in addition their quantities based on number of reaction, namely reaction times, are shown. In other words, two kinds of kit according to reaction times are 20 and 100 rxns, so each one included required amount these numbers. #K 1621 and #K 1622 are represent the code number of kits. For this study, #K 1622 was used.

Table 2.4 Components of the cDNA kit and included amounts of chemicals.

RevertAid™ First Strand cDNA Synthesis Kit	**20 rxns #K 1621**	**100 rxns #K 1622**
RevertAid™ M-MuLV Reverse Transcriptase (200 u/µl)	25 µl	120 µl
RiboLockTM RNase Inhibitor (20 u/µl)	25 µl	120 µl
5X Reaction Buffer 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl^2$, 50 mM DDT	150 µl	500 µl
10mM dNTP Mix	50 µl	250 µl
Oligo$(dT)_{18}$ Primer 100 µM, 0.5 µg/µl (15 A_{260} u/ml)	25 µl	120 µl
Random Hexamer Primer 100 µM, 0.2 µg/µl (6 A_{260} u/ml)	25 µl	120 µl
Forward GADPH Primer, 10 µM 5' – CAAGGTCATCCATGACAACTTTG – 3'	20µl	20 µl
Reverse GADPH Primer, 10 µM 5' – GTCCACCACCCTGTTGCTGTAG – 3'	20µl	20 µl
Control GADPH RNA 1.3 kb 3'-poly(A) tailed RNA transkript, 0.05 µg/µl	20µl	20 µl
Water, nuclease-free	2x1.25 µl	2x1.25 µl

2.1.2.7 Real Time PCR

"SYBR Premix Ex Taq II (Tli RNaseH Plus)" (TaKaRa) kit was used for Real Time PCR. The product code of kit is RR82A and this for 200 reactions. Firstly, the features of kit is explained, after in Table 2.5 components of kit and amounts are shown. Lastly, in Figure 2.7, devices that were used for RT PCR are shown. Two kinds of devices were used according to number of samples. The first one is **"**Rotor-Gene 6000 (Corbett)" for 36 samples (from Fatih University Biology Department Labaratuary), the second one is "Stratagene MX3000p" for 96 samples by using 96 well plate (from Istanbul University DETAE Labaratuary).

i. The features of kit as follows:

- RNase H included in reaction mix reduces inhibition from mRNA/cDNA hybrids in the qPCR reaction.
- Eliminates need for RNase H digestion step when using low RNase H RTs.
- High sensitivity detects as few as 100 copies.
- Accurate quantitation for excellent standard curves for many qPCR instruments.
- Compatible with all qPCR instruments.
- Includes SYBR Green I for intercalator-based real time PCR (qPCR).

ii. Kit components

Table 2.5 Components and their amounts of the RT PCR TaKaRa kit.

Chemicals	Amount
SYBR *Premix Ex Taq* II (Tli RNaseH Plus) (2X conc.)[1]	1 ml × 5
ROX Reference Dye (50X conc.)[2]	200 µl
ROX Reference Dye II (50X conc.)[2]	200 µl

1: Contains *TaKaRa Ex Taq* HS, dNTP Mixture, Mg^{2+}, Tli RNaseH and SYBR Green I
2: This component is to be used for analyses using a device that corrects fluorescent signals between wells such as the real-time PCR device by Applied Biosystems.

iii. Devices for RT PCR

Figure 2.7 RT PCR devices: First is **"**Rotor-Gene 6000 (Corbett)" for x36 samples and second is "Stratagene MX3000p" for x96 well plate. (http://www.sci.muni.cz/botany/systemgr/index.php?show=molekular&res=1600; http://vet.osu.edu/idmel/facilities)

2.2 METHODS

This study include main two parts are animal studies and molecular application. For this reason, methods that were applied were explained under two subtitles for methods.

2.2.1 Animal Studies

Figure 2.8 Long Evans rat: Used for animal studies. (bioquicknews.com)

2.2.1.1 Chronic Exposure to Ethanol

All procedures in this study are in accordance with the "Guide for the Care and Use of Laboratory Animals" as adopted by the National Institutes of Health (USA)

At the beginning of study, the modified liquid diet without ethanol for 7 days were given all the rats. Then, liquid diet with 2.4% ethanol was administered for 3 days, after that the ethanol concentration was increased to 4.8% for the following 4 days and finally to 7.2% for 14 days to first 12 rats. Until this time, there are only two groups, first 12 rats are alcohol intake group, the last 6 are negative control group. At 21st day, alcohol intake group divided into two (n=6) groups (alcohol and wihdrawal) according to their total weights, namely 18 rats divided into three groups shown in Table 2.6.

Table 2.6 Rat order number and its group.

Groups	Number of rats
Alcohol addicted	3,4,6,7,11,12
Withdrawal	1,2,5,8,9,10
Negative control	13,14,15,16,17,18

At the end of the exposure to 7.2% ethanol containing liquid diet (21st day); for withdrawal (third) group, diet with ethanol was withdrawn and replaced with isocaloric ethanol-free diet. Ethanol-dependent rats (second group) continued to same ethanol containing (7.2%) liquid diet. Negative control group was fed liquid diet without ethanol during whole procedure.

In addition, the weight of the rats was recorded every day, and daily ethanol intake was measured and expressed as g per kg per day. By using these recorded data, alcohol consumption tables were formed and one sample part is shown in Table 2.7, also whole detailed one is in Appendix. Control rats (n = 6) were pair fed with an isocaloric liquid diet containing sucrose as a caloric substitute to ethanol.

Table 2.7 Sample of recorded data for alcohol exposure.

17.09.2011 (%2,4)			**18.09.2011 (%2,4)**		
Weight	**ml**	**g/kg**	**Weight**	**ml**	**g/kg**
481	114	4,607	485	50	2,004
515	150	5,662	533	138	5,033
492	120	4,741	502	110	4,26
483	134	5,393	493	104	4,101
504	140	5,4	516	132	4,973
469	98	4,062	469	100	4,145
390	105	5,234	400	120	5,832
421	116	5,356	434	98	4,39
396	105	5,155	403	102	4,92
373	120	6,254	382	94	4,784
482	122	4,92	493	134	5,284
353	110	6,058	360	110	5,94
453	150	6,437	450	110	4,752
424	146	6,694	430	138	6,239
414	135	6,339	422	110	5,067
394	130	6,414	406	106	5,075
520	140	5,234	532	120	4,385
426	95	4,335	433	140	6,285
	AV:	**5,461**		**AV:**	**4,859**

2.2.1.2 Surgery

At 22nd day, all rats were killed, and their brains are extracted. For this study, only three parts of brain were used: frontal cortex, striatum, hipocampus.

While separating brain parts 30% sucrose/PBS solution was used to provide the composition of cells against cell distruption due to osmotic pressure . Also, all laboratory equipments were sterilized by ethanol during experiment.

Each brain tissue sample was put in DNase- RNase free 1.5ml eppendorf tubes that marked according to group number and brain parts and the tubes were placed into liquid nitrogen. At last, all sample tubes were collected from liquid nitrogen and they were stored in -80 C until genetic experiment.

2.2.2 Molecular Application

2.2.2.1 Primer Design

Table 2.8 Primer characteristics.

Product size	200-300 bp
Length	20 bp
Tm	60^{o}C
GC cont	40-60
PolyX	3
GC Clamp	2 3’

According to reference primer characteristics shown in Table 2.8, Primer3 program was used for primer design (http://frodo.wi.mit.edu/). Also, NCBI was used (eg : Fasta format) and GEO profiles was used for provide knowledge about all expression studies of gene which encode enzyme and ensamble.org was used for representation of intron-exon details.

2.2.2.2 Homogenization

The rotor was placed on the MagNA Lyser Cooling Block and 54 of MagNA Lyser Green tubes into the rotor. The samples (18x3) and lysis buffer were added to MagNA Lyser Green Beads. The rotor was placed into the instrument and it was secured with the retention plate. Screws were tightened. All the samples were homogenized according to MagNA Lyser Operator's Manual. Centrifuge the sample to pellet the cell debris, supernatant was used for RNA isolation. All procedure is summarized step by step as image in Figure 2.9.

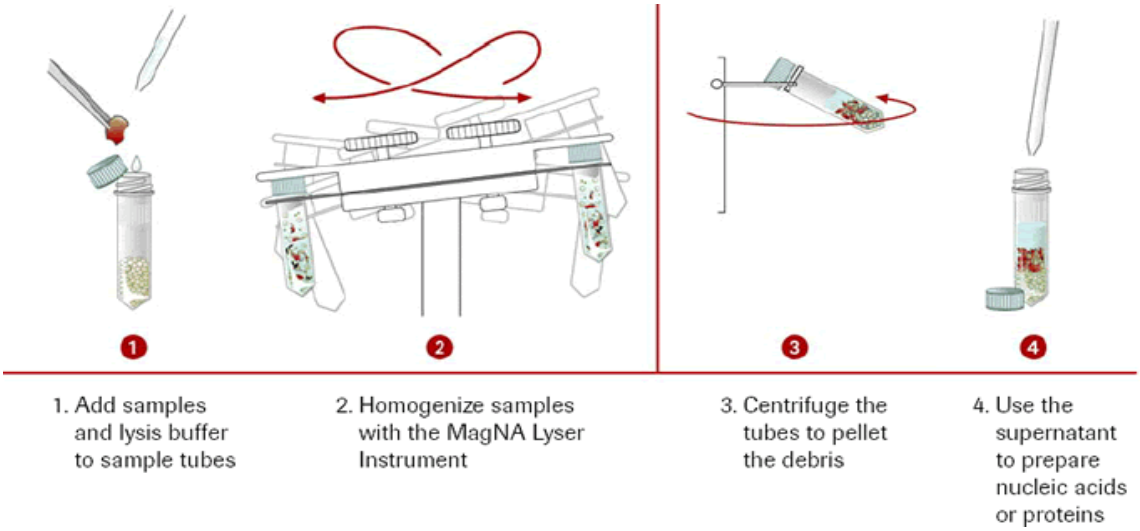


Figure 2.9 Summary of tissue sample homogenization protocol: MagNA Lyser system step by step as image**.**
(http://www.roche-applied science.com/sis/automated/lyser/index.jsp?&id=ml_01010)

2.2.2.3 RNA Isolation

200 µl sample was taken for each sample and put into 1.5 ml microtubes. 400 µl Lysis/Binding buffer was added on each sample tubes and vortex for 15s. High Pure Filter tubes were inserted in collection tubes. Each sample were transfered to High Pure Filter tube, and centrifugation was applied all sample tubes 15 s 8000xg. After centrifugation, each filter tube was removed from collection tube, flowthrough liquid in collection tubes were discarded after that, filter tubes and used collection tubes were combined again. For each sample tubes, 90 µl DNase incubation buffer and 10 µl DNase I were mixed and added on upper reservior of the filter tube and incubated for 15 min at +15 to +25 C. After incubation, 500 µl Wash Buffer I was added to upper

reservior of filter tubes for each sample and centrifugation was applied 15s at 8000xg. Each flowthrough was discarded and each filter tube was combined with used collection tubes. 500 µl Wash Buffer II was added to upper reservior of filter tubes for each sample and centrifugation was applied 15s at 8000xg. Each flowthrough was discarded and each filter tube was combined with used collection tubes again. 200 µl Wash Buffer II was added to upper reservior of filter tubes for each sample and centrifugation was applied for 2 min at maximum speed approximately 13000xg in order to remove any residual Wash Buffer. Extra centrifugation was applied to ensure removal of residual Wash Buffer. The collection tubes were discarded and the filter tubes were inserted into a clean, sterile 1.5 ml microcentrifuge tube.

In order to elute the RNA, 75 µl Elution Buffer was added to upper reservior of each sample filter tube and centrifugation was applied the sample tubes for 1 min at 8000xg. Finally, we provide eluted RNA of each sample in the microcentrifuge tubes. 2 µl RNA sample was used for measurement of spectrophotometer values of each sample RNA by nanodrop device. After spectrohotometric analysis, sample tubes were stored -80^{o}C.

2.2.2.4 Agarose Gel Electrophoresis

It was applied for control of provided RNAs. 50X TAE stock solution was diluted 50:1 with distilled water to make a 1X working solution.

1% agarose gel was prepared. 0.4 g agarose was dissolved in 40 ml 1X TAE buffer by heating. After dissolving, gel was cooled. For visualization under UV, 2 µl ethidium bromide was added and mixed.

Comb was placed on tray of agarose gel and agarose gel was poured on tray of agarose gel electrophoresis tank and waiting to become solidity by cooling. Also, tank was filled up by 1X TAE. After gel solidity, comb was discarded from gel. 10 µl samples were mixed 2 µl loading dye and this was loaded in wells on agarose gel (marker was not used). Electrophoresis was applied at 85V for 40 min, and the agarose gel was visualized under UV.

2.2.2.5 cDNA Synthesis

11 microliter template RNA and 1 µl oligo(dT) primer were mixed into a sterile, nuclease-free tube on ice. Centrifugation was applied all sample tubes at 5000xg. After centrifugation, reagents were added amounts according to Table 2.9 on each sample tubes, total volume become 20 µl. After that, mixing and centrifugation were applied. Incubation for 60 min at 42 C and termination the reaction by heating at 70 C for 5 min. Storage at -20 C.

Table 2.9 Amount of reagents for cDNA synthesis.

Reagents	**Amounts**
5x Reaction Buffer	4 µl
RiboLock RNase inhibitor	1 µl
10mM dNTP Mix	2 µl
RevertAid M-MuLV Reverse Transcriptase	1 µl

2.2.2.6 Control PCR

In order to control cDNA synthesis application, sample cDNAs were multiplied by control PCR. 2 µl cDNA of each sample were put in PCR tubes and after following reagents were added on each sample cDNA tubes that amounts according to Table 2.10.

Table 2.10 Amounts of reagents for control PCR.

Reagents	**Amounts**
PCR Master Mix	12.5 µl
Forward GADPH Primer	0.5 µl
Reverse GADPH Primer	0.5 µl
Water, nuclease-free	9.5 µl
Final volume	25 µl (for each 54 sample)

All procedure was applied on ice and after praparation of PCR tubes, centrifugation was also applied. PCR was performed according to Table 2.11:

Table 2.11 PCR profile for control PCR application.

Step	**Temperature, ^{o}C**	**Time**	**Number of Cycles**
Initial denaturation	94	3 min	1
Denaturation	94	30s	35
Annealing	58	30s	
Extension	72	45s	

2.2.2.7 Agarose Gel Electrophoresis

After control PCR, agarose gel electrophoresis was applied for visualization and control for next parts of study. 1.5% Agarose gel was prepared. 2.25 g agarose was dissolved in 150 ml 1X TAE buffer by heating. After dissolving, gel was cooled. For visualization under UV, 10 µl SafeView was added and mixed

Comb was placed on tray of agarose gel and agarose gel was poured on tray of agarose gel electrophoresis tank and waiting to become solidity by cooling. Also, tank was filled up by 1X TAE. After gel solidity, comb was discarded from gel. 10 µl samples were mixed 2 µl loading dye and this was loaded in wells on agarose gel. Also, marker (2 µl marker + 2 loading dye + 8 µl distilled water) was used as a parameter. Electrophorese at 85V for 40min and visualization under UV.

2.2.2.8 Real-Time PCR

After cDNA synthesis RT PCR was applied in two steps according to Table 2.12. Firstly, RT PCR was applied triple for housekeeping gene GADPH. After that, RT PCR repeated twice for all target genes by all samples. Also, optimization for BDNF repeated according to results of melting graphes. All RT PCR application except optimization were applied according to PCR profile shown in Table 2.12.

Table 2.12 PCR profile of RT PCR application.

Cycle	Cycle point
Hold@ 95°C, 0 min 40 secs	
Cycling (35 repeats)	Step 1 @ 95°C, hold 8 secs
	Step 2 @ 63°C, hold 15 secs
	Step 3 @ 72°C, hold 15 secs, acquiring to Cycling A (Green)
Melt (70-95°C), hold secs on the 1st step, hold 3 secs on next steps, Melt A (Green)	

i.Real Time PCR for GADPH

Before RT PCR, all samples were diluted ratio of 1:50. For this, 2 µl cDNA and 98 µl double distilled water were mixed for each sample (54) and 2 µl sample was taken from here for RT PCR. Following reagents were added according to amounts shown in Table 2.13 for each sample on PCR tubes and centrifugation. RT PCR was applied according to PCR profile shown in Table 2.12

Table 2.13 Amounts of reagents for GADPH RT PCR.

Reagents	Amounts
PCR Mix	10 µl
Primer (F + R)	0.8 µl
dd H_2O	7.2 µl
Final volume	20 µl

ii. RT PCR for Agmatinase, Arginine Decarboxylase, Arginase, SAT1, BDNF

For each gene, 1 µl cDNA was taken and added to 96 well plate for each sample (5x54) and following reagents were added amounts shown in Table 2.14 in 96 well plate for each sample and centrifugation applied. RT PCR was applied according to PCR profile shown in Table 2.12. Because of sample number, 96 well plate was used for Stratagene MX3000p RT PCR device.

Table 2.14 Amounts of reagents for target genes RT PCR.

Reagents	**Amounts**
PCR mix	7.5 µl
Primer (F and R)	0.6 µl
dd H_2O	5.9 µl
Total volume	15 µl

CHAPTER 3

RESULTS

For gene expression analysis, there are basic three steps, the first step is RNA isolation and others are cDNA synthesis and PCR/RT PCR. Results include four basic parts; RNA isolation, cDNA synthesis, RT PCR and analysis parts. The first one is RNA isolation, its results contain spectrophotometric measurement and agarose gel images are explained below. Also, cDNA results includes only agarose gel images. Third part mainly contain Ct values of housekeeping and target genes. The last part is analysis that includes expression, statistical analysis and graphes.

3.1 RNA ISOLATION

First results are obtained in RNA isolation part. Results of RNA isolation includes spectrophotometric measurement values for determining amount and purity of isolated RNAs. Secondly, agarose gel images for visualization to control only presence or absence of all sample RNAs.

3.1.1 Spectrophotometric Measurement

After RNA isolation application, spectrophotometric measurement was applied. By this way, amount and purity of isolated RNAs were determined and shown in Table 3.1. In Table 3.1, concentration represents amount of isolated RNAs from tissue samples and 260/280 represents purity of RNAs.

Table 3.1 Spectrophotometric results of sample* RNAs.

#	**Sample ID**	**RNA Conc. (**ng/µl)	**260/280**
1	13C	721,7	1,98
2	18C	332,6	2,1
3	16H	433,8	2,11
4	15S	231,5	2,11
5	13H	449,2	2,1
6	18S	286,2	2,1
7	17S	350,8	2,11
8	14S	67,9	1,98
9	17C	480,8	2,11
10	15C	315,2	2,12
11	16S	273,1	2,11
12	14C	230,4	2,07
13	14H	414,9	2,09
14	13S	141,6	2,11
15	15H	447,7	2,1
16	17H	479,9	2,09
17	18H	175,1	2,09
18	10C	360,4	2,07
20	8S	312	2,11
21	7C	114,5	1,86
22	11C	396,3	2,1
23	9S	146,8	2,02
24	10S	270,8	2,09
25	12S	244	2,1
26	10H	249,4	2,08
27	9H	220,2	2,07
28	11S	253,2	2,08
29	11H	312,9	2,09
30	7H	395,3	2,09
31	12H	448,1	2,1

*C: Frontal cortex, S: Striatum, H: Hippocampus.

Table 3.1 Continue spectrophotometric results of sample* RNAs.

#	**Sample ID**	**RNA Conc. (**ng/µl)	**260/280**
32	8H	375,9	2,09
33	9C	122	2
34	16C	2,1	1,58
35	12C	307,1	2,08
36	6H	18,9	2,05
37	5S	333,7	2,07
38	5H	229,6	1,98
39	4C	230,3	1,77
40	8C	379,2	2,1
41	2C	38,1	2,13
42	6C	36,1	1,99
43	5C	110,8	2,24
44	3S	87,8	2,06
45	1C	101,9	1,83
46	2S	71,9	2,09
47	4S	29,2	2,25
48	6S	106,5	2,05
49	3C	101,4	2,07
50	3H	228,9	2,12
51	4H	298,2	2,08
52	2H	31,5	1,5
53	1H	144,4	2,1
54	1S	97,4	2,06
55	7S	392	2,09

*C: Frontal cortex, S: Striatum, H: Hippocampus.

3.1.2 Agarose Gel Electrophoresis Results (RNA Isolation)

After RNA isolation, agarose gel electrophoresis was applied for qualitative evaluation of RNA isolation results. By the way, isolated RNAs of all samples can be visualized. Images of agarose gel results are shown in Figure 3.1.

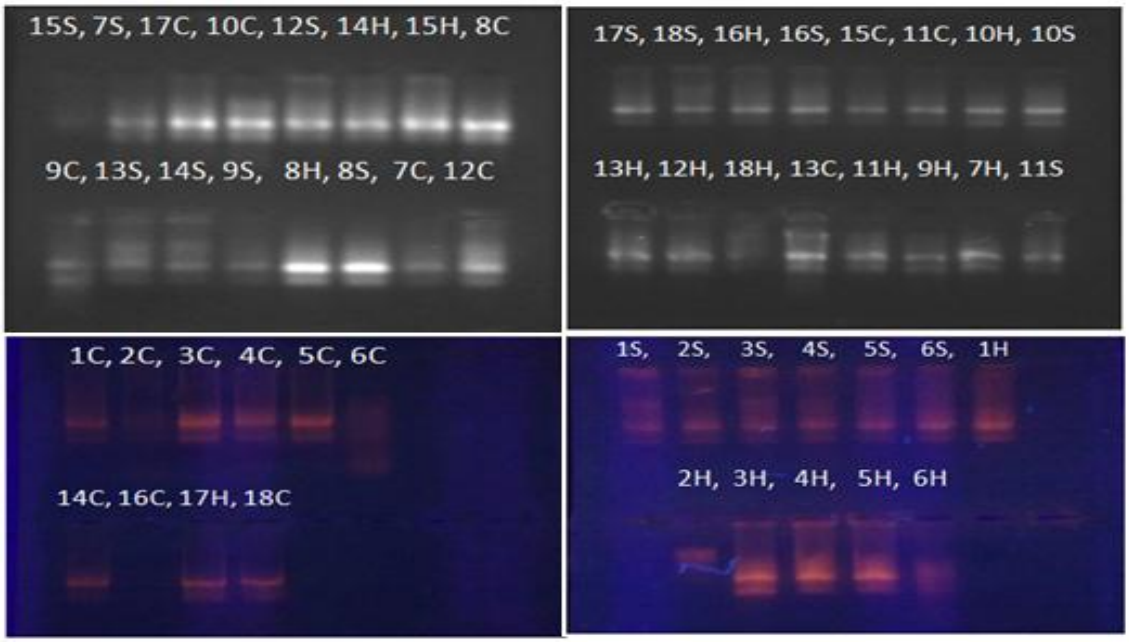


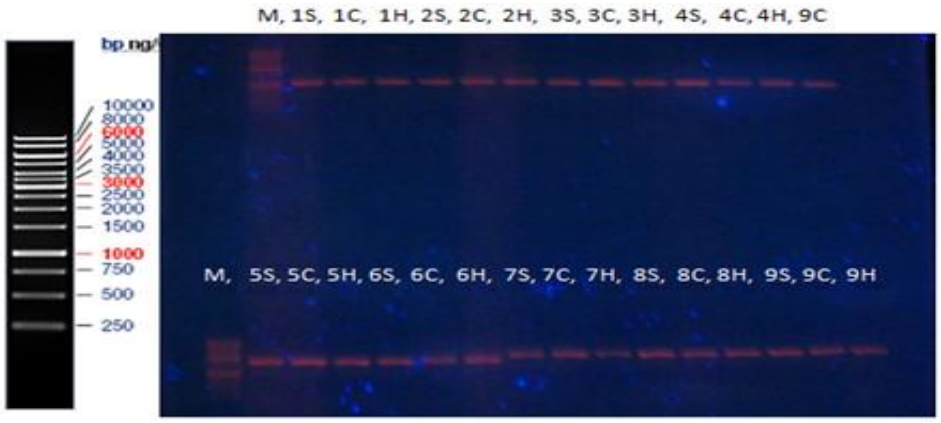
Figure 3.1 Images of 1 % agarose gel for RNA isolation: Content: 0.4 g agarose was dissolved in 40 ml 1X TAE buffer and 2 µl ethidium bromide; 10 µl samples* were mixed 2 µl loading dye.
(*C: Frontal cortex, S: Striatum, H: Hippocampus)

3.2 cDNA SYNTHESIS

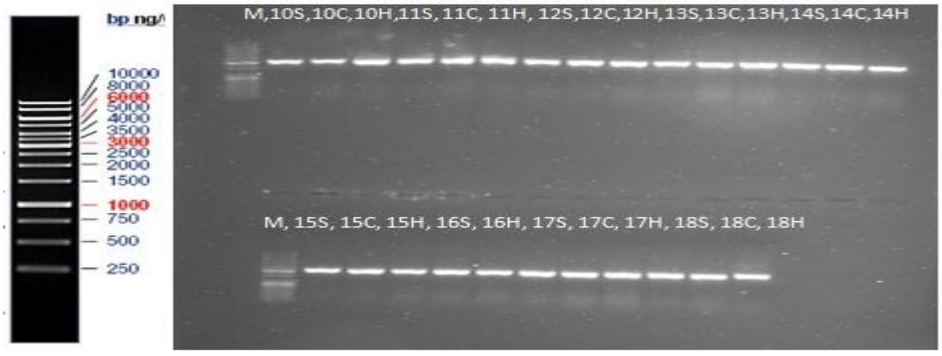
cDNA results includes only agarose gel images for all samples' cDNAs.

3.2.1 Agarose Gel Electrophoresis

After cDNA synthesis, agarose gel electrophoresis was applied for visualization and control presence of all samples cDNAs. Two images of agarose gel results are shown as (a) and (b) in Figure 3.2.



(a)



(b)

Figure 3.2 (a) Image of 1.5 % agarose gel for cDNA synthesis results of samples* between 1 and 9 for three brain parts: Content: 2.25 g agarose was dissolved in 150 ml 1X TAE buffer and 10 µl SafeView. (b) Image of 1.5 % agarose gel for cDNA synthesis results of samples between 10 and 18 for three brain parts, gel content was same as (a).
(*C: Frontal cortex, S: Striatum, H: Hippocampus; M:Marker)

3.3 REAL-TIME PCR

RT PCR results are divided into two sections for housekeeping and target genes.

3.3.1 RT PCR Results for GADPH

After cDNA synthesis, firstly RT PCR was applied for housekeeping gene GADPH, after that for target genes. Melting and quantitation reports of GADPH are shown below. Melting report includes melting graph and Tm values Table 3.2

3.3.1.1 Melting Report

By RT PCR, two main results were obtained, melting and quantitation results. Melting report for GADPH contains melting graph, shown in Figure 3.3 and Tm values according to each sample's peak on graph, shown in Table 3.2.

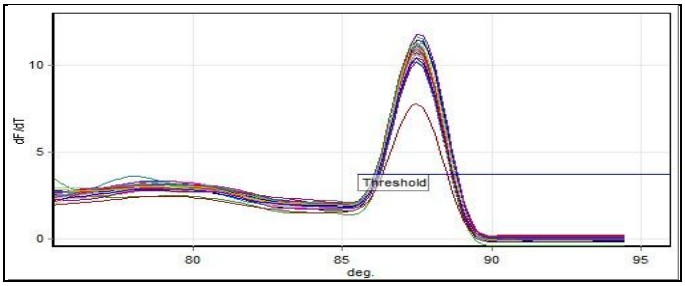


Figure 3.3 Melting graph of GADPH.

Table 3.2 Tm* values of GADPH** are grouped based on brain parts***.

Sample	Tm (°C)	Sample	Tm (°C)	Sample	Tm (°C)
1S	87,48	1H	87,48	1C	87,28
2S	87,48	2H	87,48	2C	87,28
3S	87,48	3H	87,48	3C	87,28
4S	87,48	4H	87,48	4C	87,16
5S	87,48	5H	87,60	5C	87,16
6S	87,48	6H	87,60	6C	87,16
7S	87,48	7H	87,60	7C	87,16
8S	87,48	8H	87,60	8C	87,28
9S	87,48	9H	87,60	9C	87,28
10S	87,48	10H	87,60	10C	87,28
11S	87,48	11H	87,48	11C	87,34
12S	87,48	12H	87,48	12C	87,46
13S	87,48	13H	87,48	13C	87,58
14S	87,48	14H	87,48	14C	87,58
15S	87,48	15H	87,48	15C	87,76
16S	87,48	16H	87,48	16C	****
17S	87,48	17H	87,48	17C	87,76
18S	87,48	18H	87,48	18C	87,88

*Tm: Melting temperature; **GADPH: Glyceraldehyde 3-phophate dehydrogenase; ***C: Frontal cortex, S: Striatum, H: Hippocampus **** While RNA isolation RNA of 16C could not be obtained due to not enough homogenization, so its place on tables is empty from now on.

3.3.1.2 Quantitation Report

By RT PCR, secondly quantitation results of GADPH were obtained; include quantitation graph, shown in Figure 3.4 and GADPH Ct values of each sample according to brain parts, shown in Table 3.3.

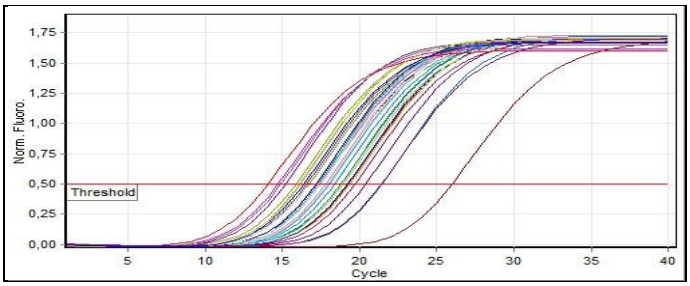


Figure 3.4 Quantitation graph of GADPH.

Table 3.3 Ct values of GADPH* grouped based on brain parts**.

Sample	Ct	Sample	Ct	Sample	Ct
1S	19,29	1H	17,83	1C	19,05
2S	19,41	2H	26,03	2C	20,8
3S	19,24	3H	16,76	3C	16,74
4S	20,44	4H	15,89	4C	18,49
5S	18	5H	17,36	5C	18
6S	18,41	6H	21,48	6C	25,95
7S	18,8	7H	17,22	7C	19,22
8S	17,18	8H	15,22	8C	18,38
9S	18,81	9H	19,72	9C	26,46
10S	14,74	10H	17,43	10C	17,41
11S	17,83	11H	16,74	11C	16,78
12S	18,03	12H	16,16	12C	17,48
13S	17,47	13H	14,93	13C	18,01
14S	18,96	14H	16,58	14C	18,49
15S	17,49	15H	14,08	15C	17,67
16S	17,41	16H	16,2	16C	***
17S	17,45	17H	16,4	17C	17,32
18S	17,44	18H	21,44	18C	15,53

*GADPH: Glyceraldehyde 3-phophate dehydrogenase **C: Frontal cortex, S: Striatum, H: Hippocampus *** While RNA isolation RNA of 16C could not be obtained due to not enough homogenization, so its place on tables is empty.

3.3.2 RT PCR Results of Target Genes

Target genes of this study are Agmatinase (AGMA), Arginase (ARG), Arginine Decarboxylase (ARDEC), Spermidine/spermine N1-acetyltransferase (SAT1) and Brain Derived Neutrophic Factor (BDNF). Here, their Ct values' tables shown in Table 3.4, 3.5 and 3.6 formed according to brain regions hippocampus, striatum, and front. cortex.

Table 3.4 Hippocampus (H) Ct values of target genes*.

Sample	SAT1	ARG	ARDEC	AGMA	BDNF
1H	33,69	27,58	22,93	27,91	25,18
2H	34,9	27,89	28,7	30,64	32,66
3H	31,98	28,1	23,34	28,33	24,51
4H	33,32	27,80	21,96	26,93	23,6
5H	20,36	23,77	21,27	26,31	23,65
6H	22,98	27,02	25,56	26,1	27,04
7H	17,39	20,88	19,36	25,38	21,92
8H	17,81	20,85	19,18	25,38	22,36
9H	19,82	23,02	23,01	27,14	25,09
10H	18,08	21,85	20,49	25,45	22,46
11H	17,91	21,81	20,74	26,36	22,77
12H	17,14	21,48	19,79	26,29	23,31
13H	18	21,40	20,33	25,63	22,67
14H	17,92	21,31	20,11	25,04	22,88
15H	17,52	20,98	17,52	25,33	22,52
16H	18,70	21,92	20,02	24,77	22,84
17H	18,44	21,29	20,76	25,48	21,97
18H	23,44	23,92	28,78	27,21	29,85

*ARG: Arginase, AGMA: Agmatinase, ARDEC: Arginine Decarboxylase, SAT1: Spermidine/spermine N(1)-acetyltransferase, BDNF: Brain Derived Neutrophic Factor.

Table 3.5 Striatum (S) Ct values of target genes*.

Sample	SAT1	ARG	ARDEC	AGMA	BDNF
1S	33,79	27,43	23,34	24,99	28,76
2S	32,92	27,82	22,89	26,01	31,96
3S	34,73	27,09	22,93	25,32	31,99
4S	34,56	27,70	22,95	25,19	31,25
5S	32,63	25,93	21,47	23,96	27,32
6S	31,8	27,24	21,64	23,96	28,46
7S	32,85	25,44	21,64	24,46	32,73
8S	20,10	22,54	20,48	21,98	24,86
9S	29,73	21,85	17,40	20,15	22,49
10S	27,79	20,08	16,36	19,1	23,51
11S	25,49	20,84	17,12	19,68	26,35
12S	27,7	20,03	16,91	18,96	26,81
13S	28,33	16,82	17,65	20,78	23,88
14S	28,38	20,87	17,24	20,33	24,39
15S	**	18,74	16,33	19,25	25,15
16S	15,85	19,18	15,83	19,24	25,64
17S	30,79	27,89	21,58	23,81	29,87
18S	34,24	27,25	22,26	24,63	28,34

*ARG: Arginase, AGMA: Agmatinase, ARDEC: Arginine Decarboxylase, SAT1: Spermidine/spermine N(1)-acetyltransferase, BDNF: Brain Derived Neutrophic Factor. **For each gene, RT PCR was double applied for all samples, but 15 S result was not obtained, so its place on each table is empty from now on.

Table 3.6 Frontal cortex (C) Ct values of target genes*.

Sample	SAT1	ARG	ARDEC	AGMA	BDNF
1C	20,27	23,67	22,71	27,79	25,7
2C	23,97	25,63	23,82	28,32	27,37
3C	19,23	22,37	20,44	25,51	23,87
4C	19,41	23,52	21,64	25,1	25,51
5C	19	22,61	20,69	26,55	24,69
6C	24,31	27,83	25,81	28,61	29,39
7C	19,78	23,38	20,52	26,13	23,75
8C	17,39	20,77	18,66	23,84	22,11
9C	25,98	29,23	28,64	30,52	32,85
10C	17,78	19,36	17,53	22,49	19,79
11C	17,52	20,21	19,08	23,73	22,12
12C	17,54	19,52	17,76	24,01	22,61
13C	16,31	22,51	17,61	22,82	20,84
14C	16,14	22,52	16,25	22,12	17,79
15C	18,11	22,47	18,01	22,12	21,43
17C	15,58	22,43	18,32	22,43	20,79
18C	19,21	22,12	20,8	26,29	24,43

*ARG: Arginase, AGMA: Agmatinase, ARDEC: Arginine Decarboxylase, SAT1: Spermidine/spermine N(1)-acetyltransferase, BDNF: Brain Derived Neutrophic Factor.
While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on tables from now on.

3.4 ANALYSIS

The last part of this chapter is analysis of results. By using quantitative results of experiment firstly expression analysis was applied by using Ct values of GADPH and target genes, according to expression analysis results namely by using Δcts, graphes are drawn and t-Test (Two samples for averages) is applied for statistical analysis.

3.4.1 Expression analysis

For expression analysis Ct values of housekeeping and target gene were used, namely subtraction GADPH from target genes' Ct values ($\Delta ct = Ct_{target} - Ct_{housekeeping}$), Δct values for each gene were obtained and in first section Table 3.7, 3.8, and 3.9 shows Δcts according to brain parts, and second part, Δcts grouped according to target genes.

3.4.1.1 Analysis for Brain Parts

Table 3.7 Δct values of target genes* on striatum (S).

Sample	Δct-SAT1	Δct-ARG	Δct-ARDEC	Δct-AGMA	Δct-BDNF
1S	14,51	8,14	4,05	5,7	9,47
2S	13,51	8,41	3,48	6,6	12,55
3S	15,49	7,85	3,69	6,08	12,75
4S	14,12	7,26	2,51	4,75	10,81
5S	14,63	7,93	3,47	5,96	9,32
6S	13,39	8,83	3,23	5,55	10,05
7S	14,05	6,64	2,84	5,66	13,93
8S	2,92	5,36	3,30	4,8	7,68
9S	10,92	3,04	-1,41	1,34	3,68
10S	13,05	5,34	1,62	4,36	8,77
11S	7,66	3,01	-0,71	1,85	8,52
12S	9,67	2	-1,12	0,93	8,78
13S	10,86	-0,64	0,18	3,31	6,41
14S	9,42	1,91	-1,72	1,37	5,43
15S	**	1,25	-1,16	1,76	7,66
16S	-1,56	1,77	-1,57	1,83	8,23
17S	13,34	10,44	4,13	6,36	12,42
18S	16,80	9,81	4,82	7,19	10,90

*ARG: Arginase, AGMA: Agmatinase, ARDEC: Arginine Decarboxylase, SAT1: Spermidine/spermine N(1)-acetyltransferase, BDNF: Brain Derived Neutrophic Factor **Although RT PCR was double applied for all samples, but 15 S result was not obtained

Table 3.8 Δct values of target genes* on hippocampus (H).

Sample	Δct-SAT1	Δct-ARG	Δct-ARDEC	Δct-AGMA	Δct-BDNF
1H	15,86	9,75	5,11	10,08	7,35
2H	8,87	1,86	2,67	4,61	6,63
3H	15,22	11,34	6,58	11,57	7,75
4H	17,43	11,91	6,07	11,04	7,71
5H	3,01	6,41	3,91	8,94	6,29
6H	1,51	5,54	4,08	4,62	5,56
7H	0,17	3,66	2,14	8,16	4,7
8H	2,59	5,63	3,96	10,16	7,14
9H	0,1	3,31	3,28	7,42	5,37
10H	0,65	4,42	3,06	8,02	5,03
11H	1,17	5,07	4	9,62	6,03
12H	0,98	5,32	3,63	10,13	7,15
13H	3,07	6,47	5,41	10,70	7,74
14H	1,34	4,73	3,53	8,46	6,3
15H	3,44	6,9	3,44	11,25	8,44
16H	2,51	5,72	3,82	8,57	6,64
17H	2,04	5,52	4,36	9,08	5,57
18H	2,01	2,48	7,34	5,77	8,41

*ARG: Arginase, AGMA: Agmatinase, ARDEC: Arginine Decarboxylase, SAT1: Spermidine/spermine N(1)-acetyltransferase, BDNF: Brain Derived Neutrophic Factor.

Table 3.9 Δct values of target genes* on frontal cortex (C).

Sample	Δct-SAT1	Δct-ARG	Δct-ARDEC	Δct-AGMA	Δct-BDNF
1C	1,22	4,62	3,65	8,74	6,65
2C	3,17	4,83	3,02	7,52	6,57
3C	2,49	5,63	3,71	8,76	7,13
4C	0,92	5,03	3,15	6,61	7,02
5C	1	4,61	2,69	8,55	6,69
6C	-1,63	1,88	-0,14	2,65	3,44
7C	0,56	4,16	1,3	6,91	4,53
8C	-0,98	2,39	0,28	5,46	3,73
9C	-0,47	2,77	2,18	4,06	6,39
10C	0,37	1,95	0,12	5,08	2,38
11C	0,74	3,43	2,3	6,95	5,34
12C	0,06	2,04	0,28	6,53	5,13
13C	-1,71	4,5	-0,39	4,81	2,83
14C	-2,34	4,03	-2,23	3,63	-0,7
15C	0,44	4,8	0,34	4,45	3,76
17C	-1,74	5,11	1,01	5,11	3,47
18C	3,68	6,59	5,27	10,76	8,91

*ARG: Arginase, AGMA: Agmatinase, ARDEC: Arginine Decarboxylase, SAT1: Spermidine/spermine N(1)-acetyltransferase, BDNF: Brain Derived Neutrophic Factor.
While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on tables.

3.4.1.2 Analysis for Target Genes

For each target gene, Δct was calculated by using $\Delta ct = Ct_{target} - Ct_{housekeeping}$ formula and Δct values were grouped based on experiment groups; alcohol, withdrawal and control against brain regions for each target gene. The first one is SAT1 gene, Table 3.10 shows comparing Δct values of SAT1 between groups according to brain parts. Table 3.10 style was repeated one by one for each gene ARG, ARDEC, AGMA and

BDNF. Namely, Table 3.11 is same as Table 3.10, however for ARG gene; Table 3.12 is for ARDEC, Table 3.13 is for AGMA and Table 3.14 is for BDNF.

Table 3.10 Comparing SAT1* Δct values of brain parts** according to groups***.

	SAT1	**H**	**C**	**S**
Cont	13	3,07	-1,71	10,86
	14	1,34	-2,34	9,42
	15	3,44	0,44	****
	16	2,51	*****	-1,56
	17	2,04	-1,74	13,34
	18	2,01	3,68	16,81
	Average	**2,41**	**-0,33**	**9,77**
Alc	3	15,22	2,49	15,49
	4	17,43	0,92	14,12
	6	1,51	-1,63	13,39
	7	0,17	0,56	14,05
	11	1,17	0,74	7,66
	12	0,98	0,06	9,67
	Average	**6,08**	**0,52**	**12,39**
Wth	1	15,86	1,22	14,51
	2	8,87	3,17	13,51
	5	3,01	1	14,63
	8	2,59	-0,98	2,92
	9	0,1	-0,47	10,92
	10	0,65	0,37	13,05
	Average	**5,18**	**0,72**	**11,59**

* SAT1: Spermidine/spermine N(1)-acetyltransferase; **C: Frontal cortex, S: Striatum, H: Hippocampus;***Alc: Alcohol, Cont: Control, Wth: Withdrawal;**** For each gene, RT PCR was double applied for all samples, but 15 S result was not obtained, so its place empty on each table.***** While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on table.

Table 3.11 Comparing ARG* Δct values of brain parts** according to groups***.

	ARG	H	C	S
Cont	13	6,47	4,5	-0,64
	14	4,73	4,03	1,91
	15	6,9	4,8	1,25
	16	5,72	****	1,77
	17	5,52	5,11	10,44
	18	2,48	6,59	9,81
	Average	**5,31**	**5,01**	**4,09**
Alc	3	11,34	5,63	7,85
	4	11,91	5,03	7,26
	6	5,54	1,88	8,83
	7	3,66	4,16	6,64
	11	5,07	3,43	3,01
	12	5,32	2,04	2
	Average	**7,14**	**3,69**	**5,93**
Wth	1	9,75	4,62	8,14
	2	1,86	4,83	8,41
	5	6,41	4,61	7,93
	8	5,63	2,39	5,36
	9	3,31	2,77	3,04
	10	4,42	1,95	5,34
	Average	**5,23**	**3,53**	**6,37**

*ARG: Arginase **C: Frontal cortex, S: Striatum, H: Hippocampus; ***Alc: Alcohol, Cont: Control, Wth: Withdrawal; **** While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on table.

Table 3.12 Comparing ARDEC* Δct values of brain parts** according to groups***.

	ARDEC	H	C	S
Cont	13	5,41	-0,39	0,18
	14	3,53	-2,23	-1,72
	15	3,44	0,34	-1,16
	16	3,82	****	-1,57
	17	4,36	1,01	4,13
	18	7,34	5,27	4,82
	Average	**4,65**	**0,79**	**0,78**
Alc	3	6,58	3,71	3,69
	4	6,07	3,15	2,51
	6	4,08	-0,14	3,23
	7	2,14	1,3	2,84
	11	4	2,3	-0,71
	12	3,63	0,28	-1,12
	Average	**4,42**	**1,76**	**1,74**
Wth	1	5,11	3,65	4,05
	2	2,67	3,02	3,48
	5	3,91	2,69	3,47
	8	3,96	0,28	3,31
	9	3,28	2,18	-1,41
	10	3,06	0,12	1,62
	Average	**3,67**	**1,99**	**2,42**

*ARDEC: Arginine Decarboxylase **C: Frontal cortex, S: Striatum, H: Hippocampus; ***Alc: Alcohol, Cont: Control, Wth: Withdrawal; **** While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on table.

Table 3.13 Comparing AGMA* Δct values of brain parts** according to groups***.

	AGMA	H	C	S
Cont	13	10,71	4,81	3,31
	14	8,46	3,63	1,37
	15	11,25	4,45	1,76
	16	8,57	****	1,83
	17	9,08	5,11	6,36
	18	5,77	10,76	7,19
	Average	**8,97**	**5,75**	**3,64**
Alc	3	11,57	8,76	6,08
	4	11,04	6,61	4,75
	6	4,62	2,65	5,55
	7	8,16	6,91	5,66
	11	9,62	6,95	1,85
	12	10,13	6,53	0,93
	Average	**9,19**	**6,41**	**4,14**
Wth	1	10,08	8,74	5,7
	2	4,61	7,52	6,6
	5	8,94	8,55	5,96
	8	10,16	5,46	4,8
	9	7,42	4,06	1,34
	10	8,02	5,08	4,36
	Average	**8,21**	**6,57**	**4,79**

*AGMA: Agmatinase **C: Frontal cortex, S: Striatum, H: Hippocampus; ***Alc: Alcohol, Cont: Control, Wth: Withdrawal; **** While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on table.

Table 3.14 Comparing BDNF* Δct values of brain parts** according to groups***.

	BDNF	**H**	**C**	**S**
Cont	13	7,74	2,83	6,41
	14	6,3	-0,7	5,43
	15	8,44	3,76	7,66
	16	6,64	****	8,235
	17	5,57	3,47	12,42
	18	8,41	8,91	10,91
	Average	**7,18**	**3,65**	**8,51**
Alc	3	7,75	7,13	12,75
	4	7,71	7,02	10,81
	6	5,56	3,44	10,05
	7	4,7	4,53	13,93
	11	6,03	5,34	8,52
	12	7,15	5,13	8,78
	Average	**6,48**	**5,43**	**10,81**
Wth	1	7,35	6,65	9,47
	2	6,63	6,57	12,55
	5	6,29	6,69	9,32
	8	7,14	3,73	7,68
	9	5,37	6,39	3,68
	10	5,03	2,38	8,77
	Average	**6,3**	**5,4**	**8,58**

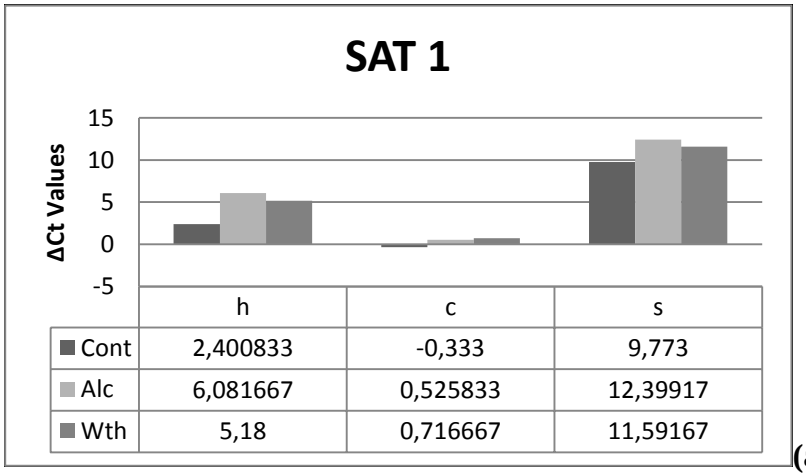
*BDNF: Brain-Derived Neutrophic Factor; **C: Frontal cortex, S: Striatum, H: Hippocampus; ***Alc: Alcohol, Cont: Control, Wth: Withdrawal; **** While RNA isolation, 16C RNA could not be obtained due to not enough homogenization, so it is absent on table.

3.4.1.3 Charts

Second part of analysis is graphes, includes three subtitle; column, pie and line charts. All kinds of these charts were drawn according to Δct values by using tables in expression analysis part.

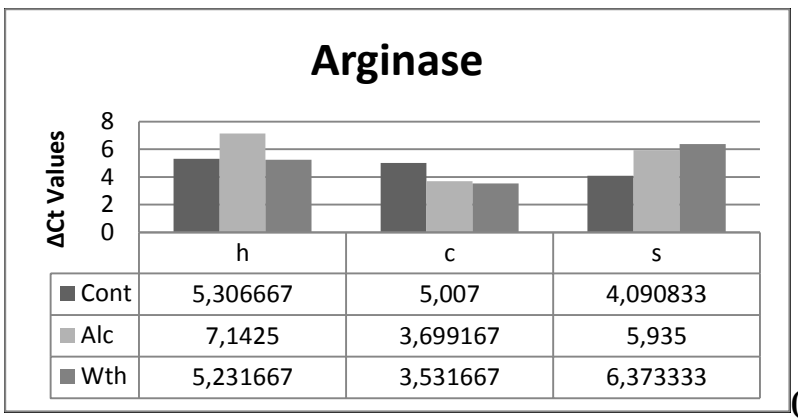
i. Column Chart

Column chart for each gene was drawn by using average Δct values on Table 3.10 for SAT1, Table 3.11 for Arginase, Table 3.12 for Arginine decarboxylase, Table 3.13 for Agmatinase, and Table 3.14 for BDNF in expression analysis part. The purpose for using average Δct values on these tables to represent on column chart is comparing average Δct values between groups in brain parts for target genes shown in one by one Figure 3.5



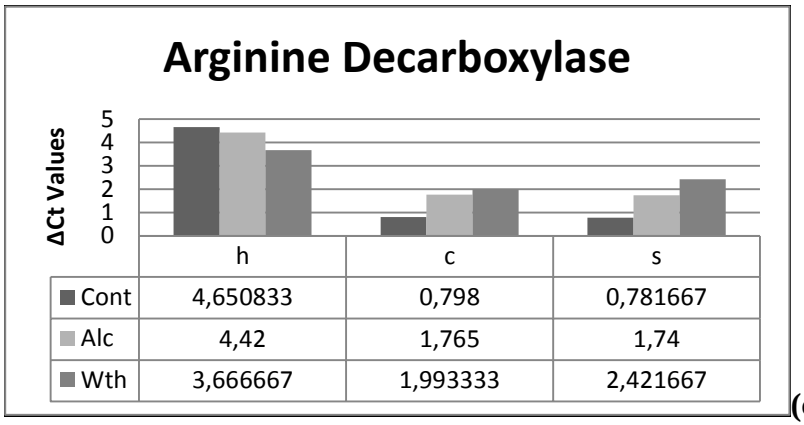
	h	c	s
Cont	2,400833	-0,333	9,773
Alc	6,081667	0,525833	12,39917
Wth	5,18	0,716667	11,59167

(a)



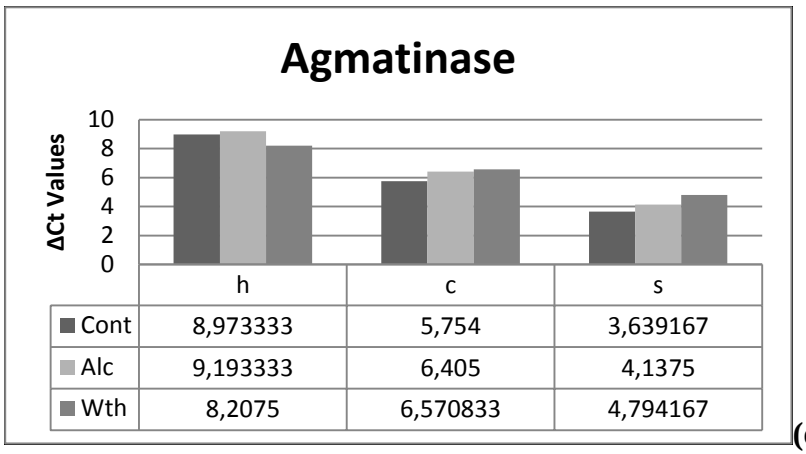
	h	c	s
Cont	5,306667	5,007	4,090833
Alc	7,1425	3,699167	5,935
Wth	5,231667	3,531667	6,373333

(b)



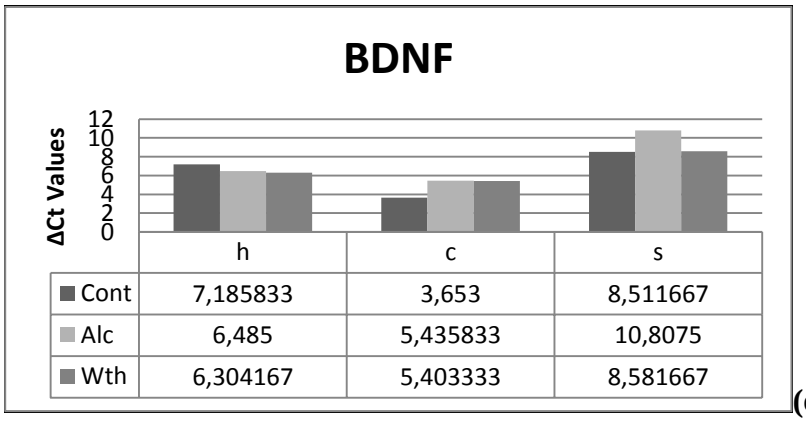
	h	c	s
Cont	4,650833	0,798	0,781667
Alc	4,42	1,765	1,74
Wth	3,666667	1,993333	2,421667

(c)



	h	c	s
Cont	8,973333	5,754	3,639167
Alc	9,193333	6,405	4,1375
Wth	8,2075	6,570833	4,794167

(d)



	h	c	s
Cont	7,185833	3,653	8,511667
Alc	6,485	5,435833	10,8075
Wth	6,304167	5,403333	8,581667

(e)

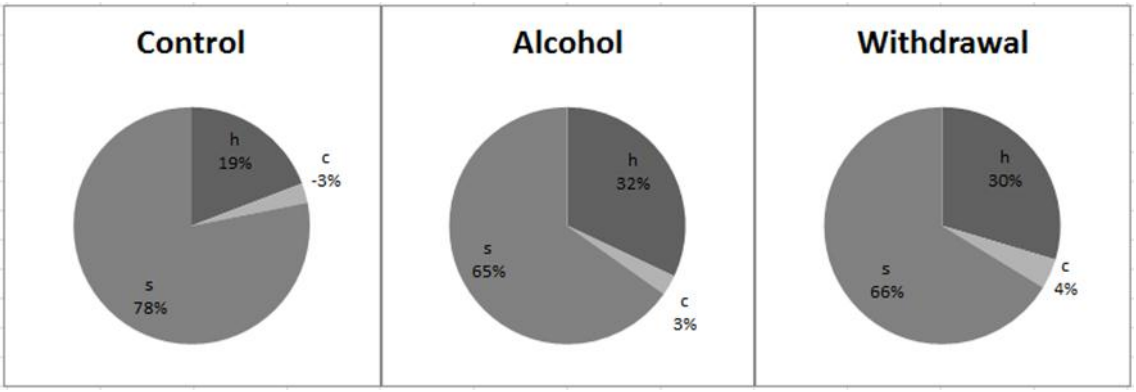
Figure 3.5 Comparing average Δct values by column chart: Representation of comparing average Δct values between groups* in brain parts** for target gene*** (a) SAT1 (b) ARG (c) ARDEC (d) AGMA (e) BDNF by column chart.

*Cont: Control, Alc: Alcohol, Wth: Withdrawal; ** h: Hippocampus, s: Striatum, c: Frontal cortex; *** SAT1: Spermidine/spermine N(1)-acetyltransferase (SPD/SPM acetyltransferase), Arginase, Arginine decarboxylase, Agmatinase, BDNF: Brain-Derived Neutrophic Factor.

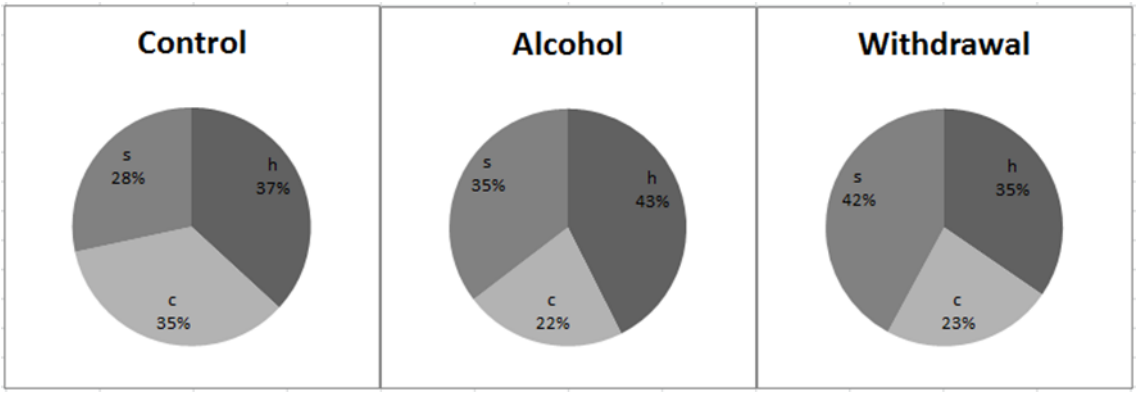
ii. Pie Chart

Pie chart for each gene was drawn by using average Δct values on tables of target genes in expression analysis part like as column chart. The purpose of pie chart is to represent comparing Δct values of brain parts in each group themselves and between groups for each target gene one by one shown in Figure 3.6.

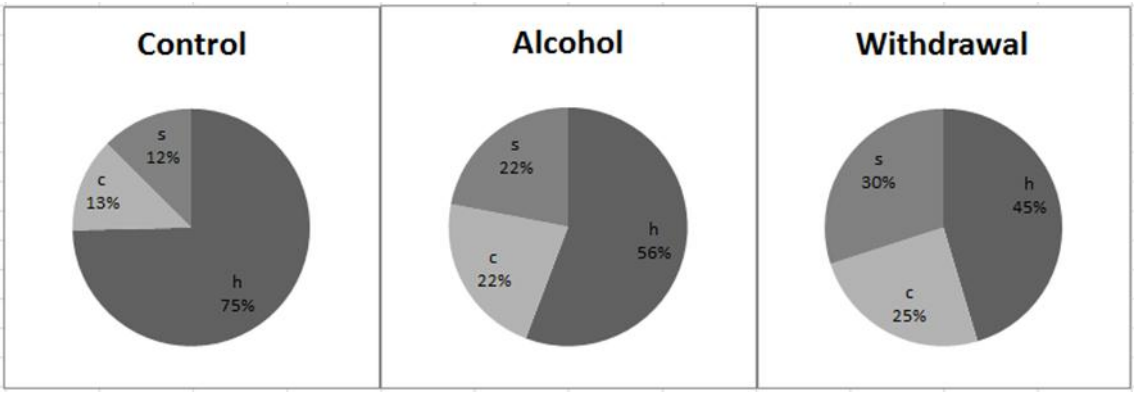
(a) SAT1



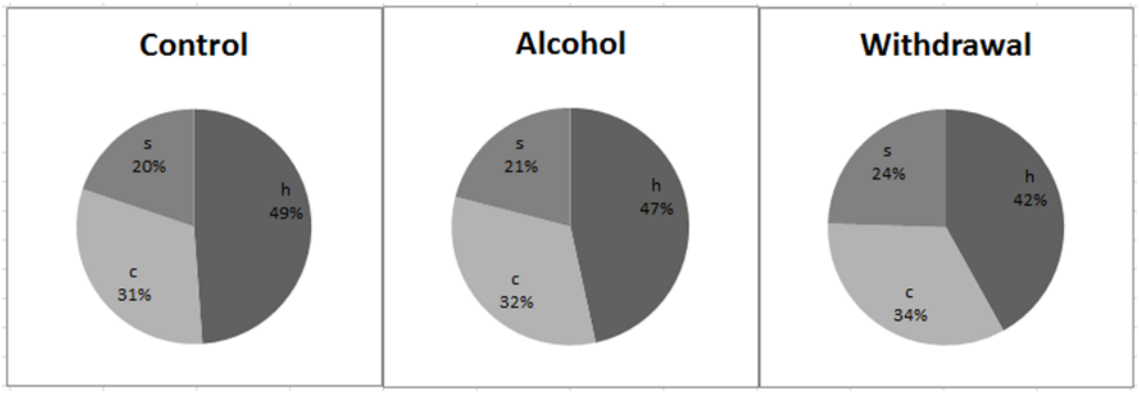
(b) Arginase



(c) Arginine decarboxylase



(d) Agmatinase



(e) BDNF

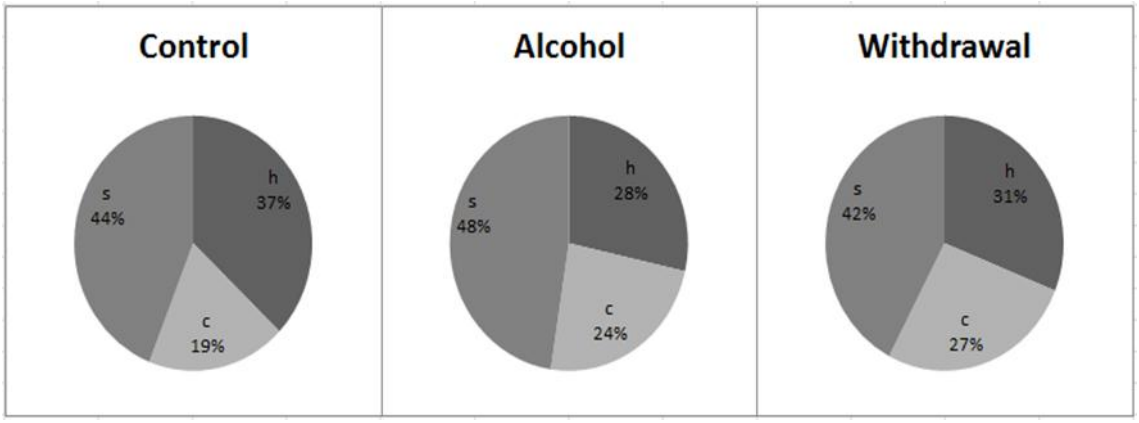


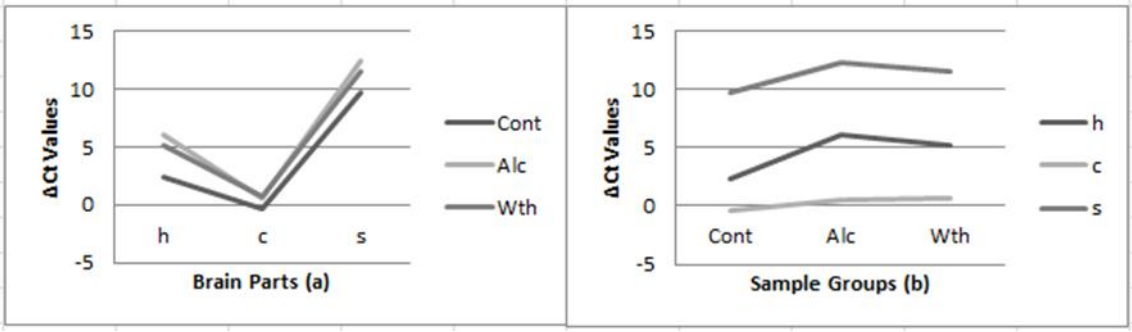
Figure 3.6 Comparing average Δct values by pie chart: Representation of comparing average Δct values of brain parts* in each group themselves and between groups** for each target gene***(a) SAT1 (b) ARG (c) ARDEC (d) AGMA (e) BDNF by pie chart.

*h: Hippocampus, s: Striatum, c: Frontal cortex; **Cont: Control, Alc: Alcohol, Wth: Withdrawal; *** SAT1: Spermidine/spermine N(1)-acetyltransferase (SPD/SPM acetyltransferase), Arginase, Arginine decarboxylase, Agmatinase, BDNF: Brain-Derived Neutrophic Factor.

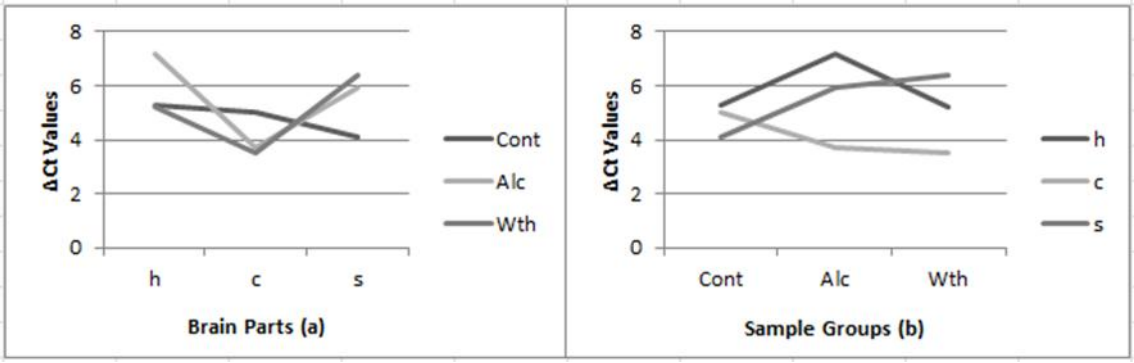
iii. Line Chart

The last type of graph is line chart that was drawn for each target gene by using average Δct values on tables of target genes in expression analysis part like as previous charts. For each target gene, two types of line charts are shown in Figure 3.7 that represent (a) Average Δct of groups comparing according to brain regions. (b) Average Δct of brain regions comparing according to groups.

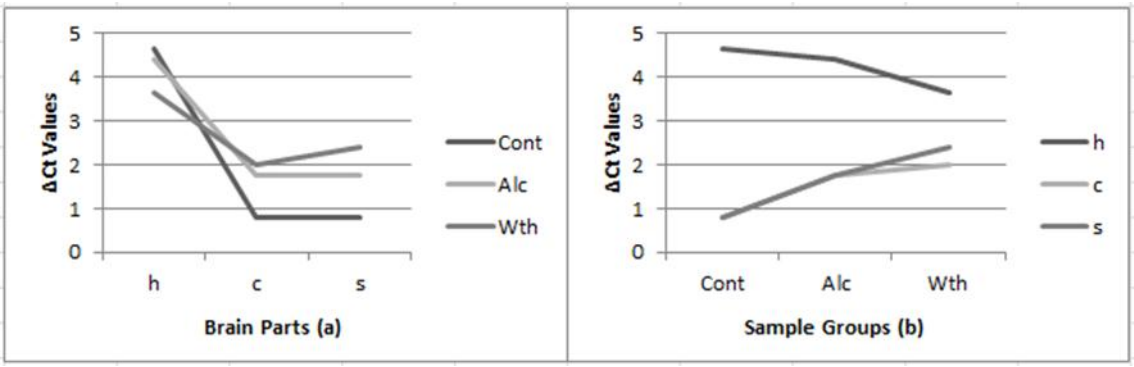
(1) SAT1



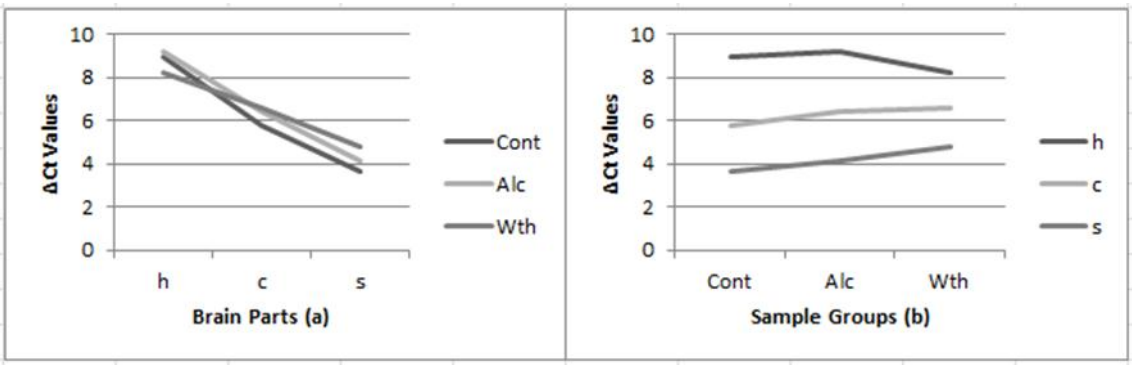
(2) Arginase



(3) Arginine decarboxylase



(4) Agmatinase



(5) BDNF

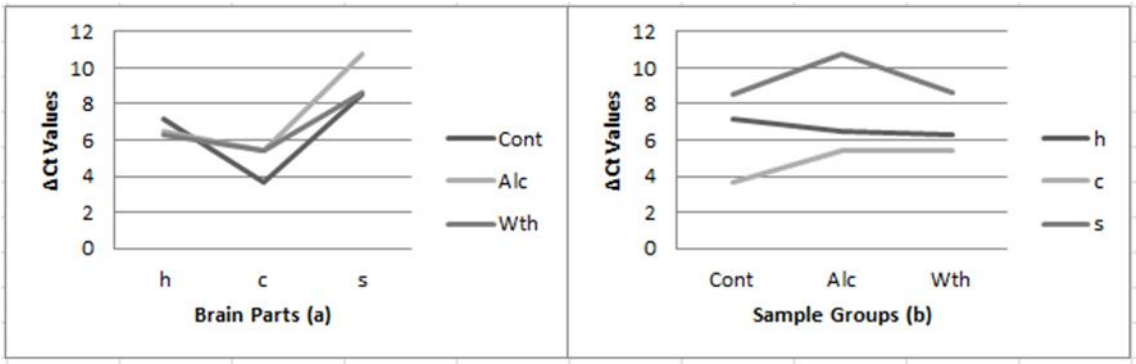


Figure 3.7 Comparing average Δct values by line chart: For each target gene* (1) SAT1 (2) ARG (3) ARDEC (4) AGMA (5) BDNF one by one (a) Average Δct of groups** comparing according to brain regions***. (b) Average Δct of brain regions*** comparing according to groups**.

*SAT1: Spermidine/spermine N(1)-acetyltransferase (SPD/SPM acetyltransferase), Arginase, Arginine decarboxylase, Agmatinase, BDNF: Brain-Derived Neutrophic Factor; **Cont: Control, Alc: Alcohol, Wth: Withdrawal; ***h: Hippocampus, s: Striatum, c: Frontal cortex.

3.4.2 Statistical Analysis

By using expression analysis results, namely Δct values of target genes; statistical analysis was applied by t-Test (Two samples for averages). Results of t-Test were P values for comparison between groups of each gene in brain regions. These P values were grouped according to three brain parts shown in Table 3.15 for target gene statistical analysis. Also, an extra t-Test was applied for comparison of brain parts and Table 3.16 shows P values for comparison between brain parts in each group of each target gene.

3.4.2.1 Analysis for Target Genes

The aim is to compare each gene's alcohol, withdrawal and control groups Δct values by statistical analysis. P values shows expression difference significance between groups, in other words smaller value than 0.05 of P value represent expression difference significance. In Table 3.15 P values were grouped into three brain parts.

Table 3.15 P Values of striatum, hippocampus and frontal cortex.

Striatum	**Alc/Cont**	**Wth/Cont**	**Alc/Wth**
SAT1	0,28	0,164	0,498
ARG	0,285	0,219	0,274
ARDEC	0,327	0,217	0,103
AGMA	0,401	0,261	0,19
BDNF	0,127	0,488	**0,068**

Hpcampus	**Alc/Cont**	**Wth/Cont**	**Alc/Wth**
SAT1	0,161	0,154	0,3
ARG	0,141	0,471	0,16
ARDEC	0,405	0,11	0,164
AGMA	0,446	0,23	0,273
BDNF	0,167	0,11	0,395

F.Cortex	**Alc/Cont**	**Wth/Cont**	**Alc/Wth**
SAT1	0,374	0,326	0,457
ARG	0,31	0,423	0,442
ARDEC	0,248	**0,023**	0,359
AGMA	0,286	0,314	0,488
BDNF	0,199	**0,034**	0,355

SAT1: Spermidine/spermine N(1)-acetyltransferase (SPD/SPM acetyltransferase), ARG:Arginase, ARDEC: Arginine decarboxylase, AGMA: Agmatinase, BDNF: Brain-Derived Neutrophic Factor; Cont: Control, Alc: Alcohol, Wth: Withdrawal; **

3.4.2.2 Analysis for Brain Parts

When expression analysis results were examined, average Δct differences between brain regions were more noticed than groups' differences. For this reason, an extra t-Test was applied for comparison of brain parts and Table 3.16 shows P values for comparison between brain parts in each group of each target gene.

Table 3.16 Comparison of P values between brain parts.

BDNF	**H-C**	**S-C**	**AGMA**	H-S	
Cont	0,061		**Cont**	0,011	
Alc		0,001	**Alc**	0,01	
Wth		0,032	**Wth**	0,017	
ARG	**H-C**	**S-C**	**SAT1**	**S-C**	**S-H**
Alc	0,013		**Cont**	0,015	0,061
Wth		0,0014	**Alc**	0,0001	0,034
			Wth	0,00037	0,024
ARDEC	**H-C**	**H-S**			
Alc	0,0015	0,0151			
Cont	0,0008	0,0031			

SAT1: Spermidine/spermine N(1)-acetyltransferase (SPD/SPM acetyltransferase), Arginase, Arginine decarboxylase, Agmatinase, BDNF: Brain-Derived Neutrophic Factor; Cont: Control, Alc: Alcohol, Wth: Withdrawal; H: Hippocampus, S: Striatum, C: Frontal cortex.

CHAPTER 4

DISCUSSION

Addiction is a brain disease characterized by maladaptive and destructive behaviours that are persistent, compulsive, and uncontrolled use of a drug or an activity. (Keifer, 2011). People take addictive drugs to elevate mood, but with repeated use these drugs produce serious unwanted effects, which can include tolerance to some drug effects, sensitization to others, and an adapted state - dependence - which sets the stage for withdrawal symptoms when drug use stops. The most serious consequence of repetitive drug taking, namely, is addiction: a persistent state in which compulsive drug use escapes control, even when serious negative consequences ensue. Researches about addiction and addictive drugs aims to progress in understanding the molecular and cellular mechanisms of tolerance, dependence and withdrawal, because until nowadays, we understand little of the neural substrates of compulsive drug use and its remarkable persistence (Hyman and Malenka, 2001). Alcohol is one of common type of addiction material is that affect people social, economic and familial lives and alcoholism is one of prevalent type of addiction (Vega et al., 2002).

Alcohol dependence become a danger especially teenagers, because young brain is more sensitive to the effect of alcohol that's reason, it can cause alterations in the structure and function of the developing brain, which continues to mature into a person's mid 20s. In adolescence, brain development is characterized by dramatic changes to the brain's structure, neuron connectivity, and physiology (Tapert et al., 2004). These changes in the brain affect everything from emerging sexuality to emotionality and judgment.

Vulnerability to alcoholism not only about environmental factors, but also it is related genetic mechanism of brain. In order to understand effects of alcoholism, studies about alcohol must be molecular level; because, alcohol dependence is not a basic habit

that cannot limit only alcohol drinking behavior. New scientific researches show molecular pathways of alcoholism on brain. Alcoholism affects molecular mechanism of human brain by chancing expression of some genes in brain (Crabbe and Philips, 1998). In order to understand genetic background of alcoholism, influence of alcohol on brain molecular mechanism must be detected specifically. Aim of this research is detection of gene expression alteration in agmatine pathway depend on alcohol intake on specific parts of rat brain.

Both human and animal studies indicate that genetic factors play a role in the development of alcoholism, leading researchers to focus on identifying genes associated with alcoholism or a predisposition to alcoholism (Tabakoff and Hoffman, 2000). However, because of this is an expression study, rats were chosen as model organisms for determining alterations of gene expression based on alcohol intake in brain, because animal models allow us to use methods that would be unethical with human subjects. For this reason, rat brain tissue was used for gene expression analysis, and methodology of animal studies, alcohol intake was applied in rats by using ethanol in liquid diet (Uzbay and Kayır; 2008).

Gene expression studies are related with proteins, enzymes; for brain studies are neurotransmitters. All these ones acts as a role on brain molecular mechanism such as signal transduction pathway. Alcohol and other addictive agents cause alterations in expression of genes that control signal transduction, neurotransmitter synthesis, receptor abundance and activity changes in brain circuitry (Neuron connections) in mesolimbic system changes in behavior, dependence, tolerance (Cruz et al., 2008) by activating dopaminergic neurons (Pierce and Kumaresan, 2006). This represents some parts of brain are more sensitive against addictive drugs including alcohol than other parts. So, for this study molecular application part, not whole brain; only tissue of selected brain regions of rat were used, namely some of mesolimbic system components; striatum, frontal cortex and hippocampus. Namely, our purpose is to detection expression differences between alcohol, withdrawal and control groups for each target gene in this brain regions. Target genes Agmatinase, Arginine decarboxylase, Arginase, SAT1 enzymes' coding genes that are included in agmatine pathway and also BDNF was used as positive control, and GADPH as housekeeping gene. For molecular application, RNA

isolated from rat brain tissues of striatum, frontal cortex and hippocampus. cDNA synthesized from this RNA was used quantitative analysis of Real Time PCR.

Cells in all organisms regulate gene expression and turnover of gene transcripts (mRNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation. In order to detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase. But in our study, we isolated RNA not mRNA, and synthesized cDNA from this RNA was used quantitative analysis of Real Time PCR.

Real-time PCR is a high standard biotechnological tool for molecular applications. Because it provides fast, accurate and sensitive DNA analysis at various genetic and epigenetic levels. Development of PCR technologies based on gene expression studies and fluorophores permits measurement of DNA amplification during PCR in real time, namely, the amplified product is measured at each PCR cycle. The data thus generated can be analysed by computer software to calculate relative gene expression in several samples. Real-time PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. Real-time PCR can be used to quantify nucleic acids by two common methods: relative quantification and absolute quantification. Relative quantification method compares the gene expression of one sample to that of another sample: drug-treated samples to an untreated control, for example, using a reference gene for normalization, that is our method for this study. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards based on a standard curve, which is prepared from samples of known template concentration. The concentration of any unknown sample can then be determined by simple interpolation of its PCR signal (Cq) into this standard curve (Dhanasekaran et al, 2010). The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes (Housekeeping genes).

In detailed explanation of DNA quantification by real-time PCR relies on plotting fluorescence against the number of cycles on a logarithmic scale. The number of cycles at which the fluorescence exceeds the threshold is called the cycle threshold, Ct. During the exponential amplification phase, the sequence of the DNA target doubles every cycle. For example, a DNA sample whose Ct precedes that of another sample by 3 cycles contained $2^3 = 8$ times more template. However, the efficiency of amplification is often variable among primers and templates. Therefore, the efficiency of a primer-template combination is assessed with serial dilutions of DNA template. In order to obtain more significant results for this study, samples diluted several times before RT PCR, especially repeated for housekeeping gene.

In this study, for quantification of gene-specific mRNA expression level, determined Ct values by quantitative RT PCR were used for calculation of Δct. Ct of housekeeping gene was subtracted from gene of interest Ct and provided Δct for each target gene. Another method for RT PCR quantitative analysis that was not applied for this study is the C_t for an RNA or DNA from the gene of interest is divided by C_t of RNA/DNA from a housekeeping gene in the same sample to normalize for variation in the amount and quality of RNA between different samples. This normalization procedure is commonly called the $\Delta\Delta C_t$-method and permits comparison of expression of a gene of interest among different samples (Schefe et al., 2006). However, for such comparison, expression of the normalizing reference gene needs to be very similar across all the samples. Choosing a reference gene fulfilling this criterion is therefore of high importance, and often challenging, because only very few genes show equal levels of expression across a range of different conditions or tissues (Nailis et al., 2006). This reference gene is housekeeping.

Housekeeping gene involved in basic functions needed for the sustenance or maintanence of the cell. So, housekeeping genes are constitutively expressed at a relatively constant level, because they should always be present and should always have the same levels. If the constant is not consistent, it suggests that there may be a problem with the sample or the process being used, since the proteins produced by a housekeeping gene vary, but are involved in some way in processes such as metabolism, cell signaling, gene expression etc. necessary to the survival of a cell, for this reason errors in their expression can lead to cell death. Hence, they are essential to a cell and always present under any conditions. It is assumed that their expression is unaffected by

experimental conditions, namely they are expressed in all cells of an organism under normal and pathophysiological conditions (Eisenberg and Levanon, 2003; Butte et al., 2001). So, housekeeping genes are used as reference for molecular studies. The most common used ones are actin, GAPDH and ubiquitin. In our study, GADPH was used as housekeeping gene. GADPH act as a role on carbohydrate metabolism in cell (Eisenberg and Levanon, 2003).

For quantitative expression analysis of each target gene by RT PCR in this study, Δct values show expression difference between GADPH and target gene. GADPH expresses relatively constant level cells, but target genes expresses differently. In other words, according to housekeeping gene, if sample has more amount cDNA, it reaches threshold earlier, namely for this condition Δct value become less; however if sample has less amount cDNA, it reaches threshold late, so Δct value become more. As a result, less Δct means more expression, more Δct means less expression level. Housekeeping gene provide a reference for expression analysis. For expression analysis each gene Δct values were grouped as alcohol, withdrawal, control groups and prefrontal cortex, hippocampus, striatum.

For statistical analysis, for each brain parts, t-Test between alcohol-control, withdrawal-control, alcohol-withdrawal groups applied for each gene, and P values of each were obtained. P values were become tables for each brain parts. t-Test provide to confirm qualitative results to quantitative and significance of this results. If P value is smaller than 0.05, this result shows significant expression difference (Yuan et al., 2006) between alcohol, withdrawal and control groups. According to our P table (Table 3.15), only three significant result were obtained: At prefrontal cortex, between withdrawal and control groups of ARDEC and BDNF gene and approximately significant value (P = 0,068) at striatum, between alcohol and withdrawal for BDNF.

In this study, BDNF was used as positive control; because according to literatures more recently, BDNF has been implicated in psychiatric disorders such as depression, anxiety and also drug addiction. Specifically, BDNF may act as an endogenous negative regulator of ethanol intake. A reduction in BDNF expression or inhibition of the BDNF receptor increases ethanol consumption and preference (Jeanblanc et al., 2006). In other words, ethanol treatment increases BDNF expression specifically in the dorsal striatum. In addition, whether BDNF within the dorsal striatum regulates ethanol self-

administration in Long–Evans rats. The dorsal striatum has been implicated in the control of goal-directed behaviors and in the formation of habit, as we think this role of striatum, it is important for addiction process, namely uncontrolled using of addictive drug become a habit by the time. Specifically, dorsolateral striatum regulates stimulus–response, or habit learning. Findings of Jeanblanc (2009) study confirmed this, according to Jeanblanc (2009) study, a greater increase in BDNF expression after ethanol self-administration in the dorsolateral striatum than in the dorsomedial striatum and it is demonstrated that the BDNF pathway within the dorsolateral striatum controls the level of ethanol self-administration. This results suggest that an endogenous signaling pathway within the same brain region that mediates drug-taking behavior also plays a critical role in gating the level of ethanol intake (Jeanblanc et al., 2009).

If we examine our results of BDNF, this can support the literature findings. Firstly, at column graph of BDNF for striatum (Figure 3.5), BDNF expression in striatum decrease with alcohol intake (Δct is inverse proportion to expression level of gene). Namely, ethanol consumption increase affect reduction of BDNF expression level in striatum according to our findings shown in figure 3. 5. At withdrawal, alcohol withdrawn in liquid diet, namely any ethanol included as control groups liquid diet, so BDNF expression level increase, and approximately same as control groups expression level again. Secondly, at pie graph (Figure 3.6), parallel result same as column graph; by alcohol intake, expression level decrease and by withdrawn alcohol, expression level increase without ethanol included diet. Thirdly, at line graph, in both graph represent parallel results same as other charts. At statistical analysis, according to t-Test results, comparing alcohol and withdrawal groups at striatum expression levels, a significant P value was obtained. This shows, expression difference between alcohol and withdrawal groups is not only qualitative, but also quantitative and significant.

All target genes of enzymes for this research, are included in agmatine pathway. In this pathway, agmatine is a cationic amine, which is an intermediate in polyamine biosynthesis produced from decarboxylation of L-arginine by the action of arginine decarboxylase enzyme and then metabolized to produce either spermine during polyamine biosynthesis, or guanido butanoic acid, is might be a novel important neurotransmitter that is biologically active substance in brains of mammals, also its inactivation is by Agmatinase (Uzbay et al., 2000). It involves action mechanisms

affecting several neurobiological processes such as neuroprotective effects in the CNS. Respectable studies also indicate that agmatine has potential as a treatment of chronic pain, addictive states and brain injury (Reis and Regunathan, 2000) and also has beneficial effects on the withdrawal syndromes of several abused drugs, such as morphine and ethanol.

Specifically, effects of agmatine, have been investigated on the ethanol withdrawal syndrome in rats and it is demonstrated that agmatine, has some inhibitory effects on the withdrawal syndrome in ethanol-dependent rats. For example, clonidine drugs which bind to α_2-adrenergic receptors, have prominent inhibitory effects on the signs of ethanol withdrawal in rats and humans, and agmatine binds to these receptors as well. Although agmatine not been have agonistic activity, these data imply that agmatine may possibly inhibit ethanol withdrawal via an effect similar to clonidine. This is beneficial effects of agmatine on ethanol withdrawal, as this property is shared with clonidine, another effective inhibitor of ethanol withdrawal syndrome. (Uzbay, 2012). All of the data imply that agmatine seems to be a new and a potent pharmacologically active agent on mechanisms involved in development of ethanol physical dependence in rats, and it may have therapeutic potential in the treatment of ethanol-type dependence (Uzbay et al., 2000). For this study, we aimed to understand that agmatine and the polyamine system affect on mechanism of alcohol addiction.

If we examine agmatine pathway enzymes' results in detailed, firstly on figure 3.5 at column graph for comparison groups between brain regions, for SAT1 enzyme gene, in all brain regions, average Δct values change parallel between groups, namely firstly alcohol intake cause to decrease SAT1 expression, but after with withdrawn of alcohol affect on increase its expression, also SAT1 most expressed in frontal cortex and the least one in striatum. For Arginase, there is not parallel results between brain areas like SAT1, also Δct between groups and brain parts close to each other. However, for Arginine decarboxylase and Agmatinase (their effect on agmatine directly, synthesize and inhibition enzymes) results are parallel, namely for both of them, the least expressed on hippocampus. In addition, for both of them, ethanol exposure decrease their expression in striatum and frontal cortex. This shows us that, agmatine inhibit ethanol withdrawal (Uzbay, 2012), so expression of synthesized enzyme Arginine decarboxylase must increase, so agmatine increase, and inhibitor enzyme must be

decrease according to literature knowledge, at withdrawal, agmatine must be increase for action of withdrawal inhibition. However, Arginine decarboxylase decrease with alcohol intake and Agmatinase has same result, this is contradiction. Secondly at pie graph on figure 3.6 for comparison groups with their brain parts' results. For SAT1, remarkable difference seen between brain parts and this parallel all groups. Namely, SAT1 most expressed in frontal cortex and the least one in striatum. For arginase, its expression decrease by alcohol intake in striatum. For Arginine decarboxylase, expression increase by alcohol intake in hippocampus, this is expected result, because arginine decarboxylase synthesize arginine, so at withdrawal inhibition, agmatine increase. But for other brain parts, expression decrease. For agmatinase, namely inhibitor of agmatine, result for striatum and frontal cortex are consistent based on literature, it must be decrease however decreasing percentages are tiny amounts. Thirdly at line chart on figure 3.7, two graphes for each gene shows average Δct of groups comparing according to brain regions and average Δct of brain regions comparing according to groups. For SAT1, both graphes represent paralel results between groups, the expected result is detect difference between groups. However, at arginase line charts, this expected difference between groups remarkably seen, at first one in spite of same Δct for control and withdrawal in hippocampus, increase in striatum and frontal cortex for control group, but for withdrawal increase in frontal cortex however decrease for striatum, in the second chart alcohol consumption cause to decrease arginase expression for striatum and hippocampus, but increase in frontal cortex. For arginine decarboxylase paralel result in first chart but in second one, ethanol consumption decrease Arginine decarboxylase expression in striatum and frontal cortex, but increase in hippocampus. For agmatinase, remarkable result seen in first chart, Agmatinase expression most in striatum, least in hippocampus, there is no remarkable difference between groups.

I applied an extra t-Test between brain parts results, because I noticed that significant differences at Δct average values between brain parts for each alcohol, withdrawal and control groups and I chose only these groups (not all groups) values for t-Test. My chosen values did not mislead to me, all ones' P values were less than 0.05. According to Table 3.16, for example BDNF expression between striatum and prefrontal cortex for alcohol groups reflects BDNF expresses prefrontal cortex more than striatum for alcohol group.

In addition, if comparison of Δct average values for each gene on Table 3.10, 3.11, 3.12, 3.13, 3.14 are examined deeply, some Δct values between h, c, and s for alcohol, withdrawal and control groups increase or decrease parallel. For instance, ratio or difference between alcohol and withdrawal Δct values of striatum, hippocampus, and prefrontal cortex approximately near. This means that e.g SAT1 approximately double expresses hippocampus than striatum.

According to our results, firstly we comment on according to P values comparison between alcohol-control, withdrawal-control, alcohol-withdrawal results, at striatum BDNF expresses withdrawal tissue more than alcohol; at prefrontal cortex BDNF expresses control tissue more than withdrawal; and also at prefrontal cortex ARDEC expresses control tissue more than withdrawal.

If we obtained more significant variation, this provide us some hints about genetic background of alcohol addiction, and if these rat genes are homolog with human, this will be a important clue for understanding how alcohol alter agmatine pathway on brain specifically. Maybe this can make easier improvement about alcoholism diagnosis and treatment. However, after t-Test, we obtained significant P values between brain parts. Dependence affect mesolimbic pathway on brain; striatum, prefrontal cortex, and hippocampus are some parts of mesolimbic system, namely alcohol influences all of them, but according to our results, we can comment on that more affective area. According to examination of P values, the most affective area depend on alcohol intake was prefrontal cortex.

CHAPTER 5

CONCLUSION

Addiction is not only behavioral problem in society; it is also a common psychiatric disorder. Because addictive agents affect brain molecular mechanisms such as signal transduction system by enzymes and neurotransmitters. For this reason, genetic analyses are applied in researches for diagnosis and treatment of addiction.

Alcohol is one of prevalent type of addictive material in world. Same as other drugs of abuse, genetic background of brain can be varied depend on alcohol intake. This study aim is to determine differences on molecular mechanism of brain depend on alcohol. Our selected pathway enzymes are related to variations because of alcoholism on brain neuronal pathway. One of these enzymes' gene alteration detection may even give us a clue about addiction genetics.

In order to provide concrete results about expression differences quantitative Real Time PCR was applied. For gene expression analysis, brain tissue is needed, so rats were used as a model organism of human. Three parameters are compared each other in this study, Δct express selected genes' expression difference between alcohol intake, withdrawal, and control tissue samples. For statistical analysis, t-Test was applied and according to P values, specific differences shows genetic mechanism depend on alcohol intake between control and other groups. Actually expected result is to detect a demonstrative difference between control and alcohol or withdrawal group samples by selected genes. But only, BDNF and ARDEC give a signal about expected results. Besides, an extra t-Test was applied for Δct differences between brain specific parts for each gene. General result of this, we comment on that prefrontal cortex is most affective area depend on alcohol intake according to our statistical results

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APPENDIX

Four groups of tables from animal studies are shown in this part. These are detailed information, so placed in appendix A.

During animal studies, for each rat, their weights and liquid diet consumptions were recorded. Table A.1 represents each day ethanol consumption, weight and ratio both of them for each animal. Also, percentage values in each day title shows ethanol ratio in liquid diet. Numbers of each animal placed in the right part of the table A.1. Totally, there are five tables included in table A.1 throughout whole animal studies except last two days, because of recording was forgotten.

Table A.1 Alcohol consumption tables.

16.09.2011 (%2,4)			**17.09.2011 (%2,4)**			**18.09.2011 (%2,4)**			**19.09.2011 (%4,8)**			
weight	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	
461	114	4,807	481	114	4,607	485	50	2,004	460	130	10,99	1
506	112	4,303	515	150	5,662	533	138	5,033	543	130	9,308	2
476	110	4,492	492	120	4,741	502	110	4,26	512	90	6,834	3
468	110	4,569	483	134	5,393	493	104	4,101	498	104	8,12	4
488	130	5,179	504	140	5,4	516	132	4,973	515	120	9,059	5
460	100	4,226	469	98	4,062	469	100	4,145	484	76	6,105	6
380	82	4,195	390	105	5,234	400	120	5,832	414	92	8,64	7
409	92	4,373	421	116	5,356	434	98	4,39	431	85	7,668	8
384	90	4,556	396	105	5,155	403	102	4,92	407	92	8,789	9
358	114	6,19	373	120	6,254	382	94	4,784	378	120	12,34	10
470	130	5,377	482	122	4,92	493	134	5,284	495	88	6,912	11
342	111	6,309	353	110	6,058	360	110	5,94	366	140	14,87	12
429	142	6,435	453	150	6,437	450	110	4,752	460	50	4,226	13
401	138	6,69	424	146	6,694	430	138	6,239	435	120	10,73	14
393	128	6,332	414	135	6,339	422	110	5,067	421	50	4,618	15
380	110	5,627	394	130	6,414	406	106	5,075	401	100	9,696	16
511	124	4,717	520	140	5,234	532	120	4,385	543	128	9,165	17
410	80	3,793	426	95	4,335	433	140	6,285	430	100	9,042	18
	AV:	**5,121**		**AV:**	**5,461**		**AV:**	**4,859**		**AV:**	**8,728**	

Continue to alcohol consumption table A.1

20.09.2011			21.09.2011			22.09.2011			23.09.2011 (%7,2)			
weight	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	
486	114	9,12	491	112	8,869	483	90	7,245	484	90	10,845	1
547	140	9,951	552	144	10,14	544	106	7,576	540	86	9,288	2
513	106	8,034	516	110	8,288	519	90	6,742	516	88	9,946	3
505	100	7,699	505	116	8,931	507	102	7,822	502	88	10,223	4
522	114	8,491	526	120	8,87	521	104	7,761	518	80	9,0069	5
479	100	8,117	475	72	5,893	483	86	6,923	473	60	7,3979	6
402	86	8,318	412	100	9,437	416	110	10,28	410	81	11,522	7
444	90	7,881	443	100	8,777	450	90	7,776	449	64	8,3129	8
411	90	8,514	412	82	7,738	420	100	9,257	404	50	7,2178	9
383	90	9,136	386	108	10,88	393	115	11,38	378	57	8,7943	10
500	128	9,953	510	132	10,06	508	140	10,71	519	118	13,26	11
370	90	9,457	376	106	10,96	378	90	9,257	375	75	11,664	12
450	50	4,32	454	150	12,85	462	140	11,78	466	140	17,521	13
450	140	12,1	450	140	12,1	449	110	9,525	449	130	16,886	14
421	90	8,312	430	130	11,75	429	110	9,969	434	132	17,738	15
414	80	7,513	412	110	10,38	417	140	13,05	416	110	15,421	16
539	108	7,79	553	130	9,14	556	116	8,112	553	140	14,765	17
437	90	8,007	440	100	8,836	444	92	8,056	450	123	15,941	18
	AV:	**8,484**		**AV:**	**9,661**		**AV:**	**9,068**		**AV:**	**11,986**	

24.09.2011			25.09.2011			26.09.2011			27.09.2011			
weight	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	
486	140	16,8	485	85	10,22	503	110	12,75	495	122	14,37	1
535	110	11,99	546	115	12,28	543	122	13,1	547	146	15,57	2
517	90	10,15	516	84	9,494	527	104	11,51	522	110	12,29	3
495	70	8,247	490	56	6,665	500	65	7,582	480	65	7,898	4
514	85	9,644	512	76	8,657	514	66	7,489	506	90	10,37	5
470	50	6,204	476	68	8,331	476	50	6,126	462	60	7,574	6
404	50	7,218	398	50	7,327	400	50	7,29	387	45	6,781	7
442	60	7,917	441	70	9,257	444	50	6,568	431	60	8,119	8
404	55	7,94	403	60	8,683	407	74	10,6	391	40	5,966	9
375	50	7,776	372	40	6,271	370	35	5,517	363	40	6,426	10
516	114	12,88	521	132	14,78	525	120	13,33	512	110	12,53	11
372	140	21,95	374	80	12,47	371	60	9,432	364	60	9,613	12
474	115	14,15	476	140	17,15	477	140	17,12	477	90	11	13
446	112	14,65	452	110	14,19	446	110	14,38	447	130	16,96	14
433	110	14,82	437	80	10,68	437	138	18,42	434	140	18,81	15
416	105	14,72	415	110	15,46	416	130	18,23	413	110	15,53	16
544	118	12,65	543	120	12,89	540	110	11,88	540	120	12,96	17
546	110	11,75	463	134	16,88	455	130	16,66	448	100	13,02	18
	AV:	**11,75**		**AV:**	**11,2**		**AV:**	**11,55**		**AV:**	**11,43**	

Continue to alcohol consumption table A.1

28.09.2011			29.09.2011			30.09.2011			01.10.2011			
weight	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	
500	90	6,998	494	101	7,949	492	100	11,85	491	106	12,59	1
544	126	9,005	548	110	7,804	533	96	10,5	538	100	10,84	2
513	80	6,063	514	86	6,505	509	90	10,31	514	95	10,78	3
483	40	3,22	483	60	4,83	467	50	6,244	476	108	13,23	4
504	55	4,243	510	82	6,251	501	77	8,963	498	66	7,729	5
461	30	2,53	459	50	4,235	448	56	7,29	447	38	4,958	6
395	56	5,512	389	58	5,797	379	50	7,694	380	50	7,674	7
442	66	5,806	437	70	6,228	424	54	7,428	427	56	7,649	8
400	50	4,86	397	58	5,68	391	66	9,844	396	68	10,01	9
365	34	3,622	369	50	5,268	358	60	9,774	361	50	8,078	10
518	120	9,007	520	120	8,972	515	120	13,59	519	104	11,69	11
372	60	6,271	367	64	6,78	364	105	16,82	360	64	10,37	12
473	74	6,083	463	74	6,214	459	84	10,67	464	94	11,81	13
451	90	7,759	446	92	8,02	450	110	14,26	452	120	15,48	14
438	140	12,43	438	130	11,54	437	105	14,01	433	130	17,51	15
415	88	8,244	410	86	8,155	404	115	16,6	405	90	12,96	16
542	95	6,815	543	114	8,163	530	110	12,1	530	70	7,703	17
443	90	7,899	443	90	7,899	444	105	13,79	440	90	11,93	18
	AV:	**6,465**		**AV:**	**7,016**		**AV:**	**11,21**		**AV:**	**10,72**	

02.10.2011			03.10.2011			04.10.2011			05.10.2011			
weight	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	**weight**	**ml**	**g/kg**	
496	98	11,52	486	86	10,32	484	100	12,05	477	70	8,558	1
533	90	9,848	531	90	9,885	533	120	13,13	534	112	12,23	2
514	80	9,077	518	92	10,36	510	84	9,606	514	90	10,21	3
477	50	6,113	472	68	8,402	459	57	7,242	457	64	8,167	4
498	58	6,792	494	64	7,556	486	64	7,68	478	50	6,1	5
450	40	5,184	446	64	8,369	440	83	11	435	68	9,117	6
373	28	4,378	367	46	7,31	368	60	9,509	370	72	11,35	7
430	56	7,595	414	50	7,043	418	70	9,767	414	58	8,17	8
395	50	7,382	391	64	9,546	386	70	10,58	390	86	12,86	9
363	40	6,426	356	50	8,191	356	50	8,191	357	70	11,44	10
527	85	9,406	519	114	12,81	522	108	12,07	517	116	13,09	11
360	64	10,37	362	74	11,92	357	80	13,07	358	110	17,92	12
456	60	7,674	444	40	5,254	443	54	7,109	441	63	8,331	13
450	102	13,22	450	110	14,26	445	108	14,15	439	108	14,35	14
434	136	18,28	443	120	15,8	433	114	15,35	434	130	17,47	15
410	110	15,65	406	120	17,24	395	70	10,34	397	90	13,22	16
527	90	9,96	522	110	12,29	520	100	11,22	516	72	8,138	17
440	100	13,25	450	130	16,85	448	110	14,32	450	120	15,55	18
	AV:	**9,562**		**AV:**	**10,74**		**AV:**	**10,91**		**AV:**	**11,46**	

In alcohol consumption tables, there are not only weight and liquid diet consumption values, but also ratio both of them are placed. Namely, for each animal weight proportion to liquid diet consumption according to formula =(3000*(ml*0,01944))/kg were calculated and placed in table A.1. In addidion, these ratio values were grouped based on experiment groups alcohol (Alc), withdrawal (Wth) and control (Cont) for easily comparison between groups are shown in table A.2. However, because of totally 22 days of experiment, table continue to second page. (Also, last two days are absent because weight walue recording was forgotten.

Table A.2 Ethanol exposure perceptions comparison between groups.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Day**
								Alc
4,49	4,74	4,26	6,83	8,03	8,29	6,74	9,95	**3**
4,57	5,39	4,10	8,12	7,70	8,93	7,82	10,22	**4**
4,23	4,06	4,14	6,11	8,12	5,89	6,92	7,40	**6**
4,19	5,23	5,83	8,64	8,32	9,44	10,28	11,52	**7**
5,38	4,92	5,28	6,91	9,95	10,06	10,71	13,26	**11**
6,31	6,06	5,94	14,87	9,46	10,96	9,26	11,66	**12**
4,86	**5,07**	**4,93**	**8,58**	**8,60**	**8,93**	**8,62**	**10,67**	**AV**
								Wth
4,81	4,61	2,00	10,99	9,12	8,87	7,24	10,84	**1**
4,30	5,66	5,03	9,31	9,95	10,14	7,58	9,29	**2**
5,18	5,40	4,97	9,06	8,49	8,87	7,76	9,01	**5**
4,37	5,36	4,39	7,67	7,88	8,78	7,78	8,31	**8**
4,56	5,15	4,92	8,79	8,51	7,74	9,26	7,22	**9**
6,19	6,25	4,78	12,34	9,14	10,88	11,38	8,79	**10**
4,90	**5,41**	**4,35**	**9,69**	**8,85**	**9,21**	**8,50**	**8,91**	**AV**
								Cont
6,43	6,44	4,75	4,23	4,32	12,85	11,78	17,52	**13**
6,69	6,69	6,24	10,73	12,10	12,10	9,53	16,89	**14**
6,33	6,34	5,07	4,62	8,31	11,75	9,97	17,74	**15**
5,63	6,41	5,08	9,70	7,51	10,38	13,05	15,42	**16**
4,72	5,23	4,38	9,17	7,79	9,14	8,11	14,76	**17**
3,79	4,34	6,29	9,04	8,01	8,84	8,06	15,94	**18**
5,60	**5,91**	**5,30**	**7,91**	**8,01**	**10,84**	**10,08**	**16,38**	**AV**

9	10	11	12	13	14	15	16	Day
								Alc
10,15	9,49	11,51	12,29	6,06	6,51	10,31	10,78	3
8,25	6,67	7,58	7,90	3,22	4,83	6,24	13,23	4
6,20	8,33	6,13	7,57	2,53	4,24	7,29	4,96	6
7,22	7,33	7,29	6,78	5,51	5,80	7,69	7,67	7
12,88	14,78	13,33	12,53	9,01	8,97	13,59	11,69	11
21,95	12,47	9,43	9,61	6,27	6,78	16,82	10,37	12
11,11	**9,84**	**9,21**	**9,45**	**5,43**	**6,19**	**10,33**	**9,78**	AV
								Wth
16,80	10,22	12,75	14,37	7,00	7,95	11,85	12,59	1
11,99	12,28	13,10	15,57	9,01	7,80	10,50	10,84	2
9,64	8,66	7,49	10,37	4,24	6,25	8,96	7,73	5
7,92	9,26	6,57	8,12	5,81	6,23	7,43	7,65	8
7,94	8,68	10,60	5,97	4,86	5,68	9,84	10,01	9
7,78	6,27	5,52	6,43	3,62	5,27	9,77	8,08	10
10,34	**9,23**	**9,34**	**10,14**	**5,76**	**6,53**	**9,73**	**9,48**	AV
								Cont
14,15	17,15	17,12	11,00	6,08	6,21	10,67	11,81	13
14,65	14,19	14,38	16,96	7,76	8,02	14,26	15,48	14
14,82	10,68	18,42	18,81	12,43	11,54	14,01	17,51	15
14,72	15,46	18,23	15,53	8,24	8,16	16,60	12,96	16
12,65	12,89	11,88	12,96	6,81	8,16	12,10	7,70	17
11,75	16,88	16,66	13,02	7,90	7,90	13,79	11,93	18
13,79	**14,54**	**16,11**	**14,71**	**8,20**	**8,33**	**13,57**	**12,90**	AV

17	18	19	20	Day
				Alc
9,08	10,36	9,61	10,21	3
6,11	8,40	7,24	8,17	4
5,18	8,37	11,00	9,12	6
4,38	7,31	9,51	11,35	7
9,41	12,81	12,07	13,09	11
10,37	11,92	13,07	17,92	12
7,42	**9,86**	**10,42**	**11,64**	AV
				Wth
11,52	10,32	12,05	8,56	1
9,85	9,88	13,13	12,23	2
6,79	7,56	7,68	6,10	5
7,60	7,04	9,77	8,17	8
7,38	9,55	10,58	12,86	9
6,43	8,19	8,19	11,44	10
8,26	**8,76**	**10,23**	**9,89**	AV
				Cont
7,67	5,25	7,11	8,33	13
13,22	14,26	14,15	14,35	14
18,28	15,80	15,35	17,47	15
15,65	17,24	10,34	13,22	16
9,96	12,29	11,22	8,14	17
13,25	16,85	14,32	15,55	18
13,00	**13,61**	**12,08**	**12,84**	AV

Here third group of tables are shown in table A.3. By using only average values of each group alcohol (Alc), withdrawal (Wth) and control (Cont) for each day, table A.3 is formed. Also, according to table A.3, graph was drown and it is shown in figure A.1.

Table A.3 Ethanol exposure perception average values of groups.

Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Alc	4,86	5,07	4,93	8,58	8,60	8,93	8,62	10,67	11,11	9,84
Wth	4,90	5,41	4,35	9,69	8,85	9,21	8,50	8,91	10,34	9,23
Cont	5,60	5,91	5,30	7,91	8,01	10,84	10,08	16,38	13,79	14,54
AV	5,12	5,46	4,86	8,73	8,48	9,66	9,07	11,99	11,75	11,20

Days	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Alc	9.21	9,45	5,43	6,19	10,33	9,78	7,42	9,86	10,42	11,64
Wth	9.34	10,14	5,76	6,53	9,73	9,48	8,26	8,76	10,23	9,89
Cont	16.11	14,71	8,20	8,33	13,57	12,90	13,00	13,61	12,08	12,84
AV	11.55	11,43	6,46	7,02	11,21	10,72	9,56	10,74	10,91	11,46

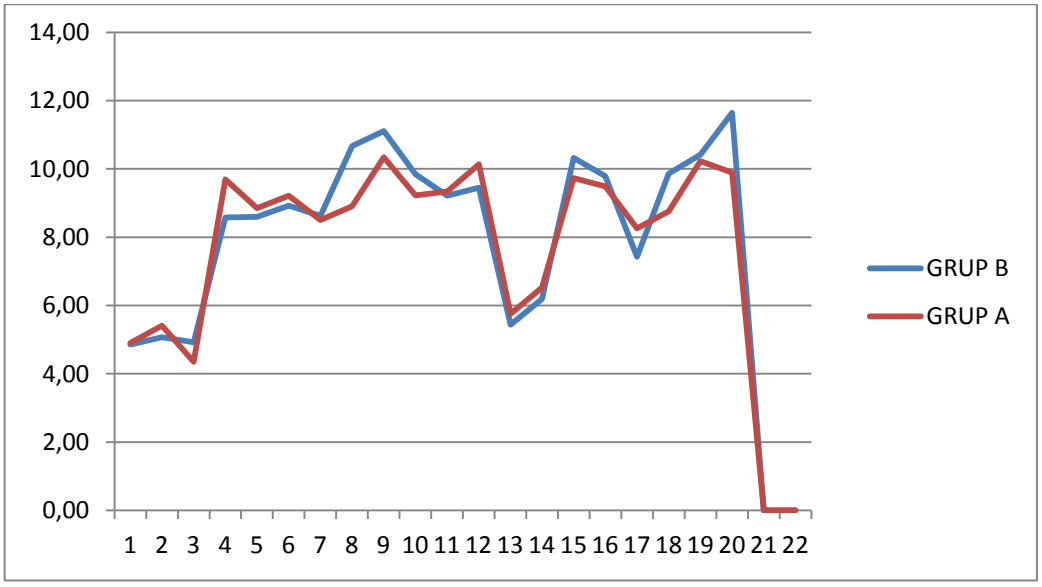

Figure A.1 Comparison graph, according to average perception values of alcohol and withdrawal groups shown in table A.3. Group A represents withdrawal, group B represents alcohol groups.

At nineteenth day (4.10.2011), recorded data of weight, consumption and ethanol exposure ratio, according to average values closeness, groups of animal were determined for withdrawal procedure, namely which animal was included in which group, and table A.4 shows this determining groups.

Table A.4 Determining groups of animals according to average weight, consumption and their ratios.

		Weight	**Consmption**	**(g/kg)**
Wth	**1**	484	100	12,05
	2	533	120	13,13
	5	486	64	7,68
	8	418	70	9,77
	9	386	70	10,58
	10	356	50	8,19
	AV	**443,83**	**79,00**	**10,23**
Alc	**3**	510	84	9,61
	4	459	57	7,24
	6	440	83	11,00
	7	368	60	9,51
	11	522	108	12,07
	12	357	80	13,07
	AV	**442,67**	**78,67**	**10,42**