DETERMINATION OF HYPOTHALAMIC NEUROPEPTIDE LEVELS
INVOLVED IN APPETITE REGULATION IN ATYPICAL
ANTIPSYCHOTIC DRUG, RISPERIDONE TREATMENT

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ABSTRACT

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Although the use of atypical antipsychotic drugs is successful in the treatment of schizophrenia, they cause complications in the long term use that is mainly weight gain. In this study, circulating levels of hypothalamic neuropeptides/hormones, which are related to appetite regulation; neuropeptide Y (NPY), alpha melanocyte stimulating hormone (α-MSH), cocaine and amphethamine regulated transcript (CART) and plus leptin in male schizophrenic patients who were treated with an atypical antipsychotic drug, risperidone, which is a serotonin antagonist, for 4 weeks was investigated. Based on the hypothesis that the risperidone treatment might alter the circulating levels of those neuropeptides through the serotonergic antagonism, it results in the weight gain. Leptin plasma levels were increased in the risperidone treated patients accompanying by weight gain vs controls and

NPY, α-MSH, CART levels were decreased in the patients before the treatment but they were not changed after treatment. To determine alterations of those candidate genes mRNA expression levels, male Wistar rats were orally administered with risperidone for 4-weeks. Rat studies show that the mRNA expression and plasma levels of POMC, AgRP, and NPY were decreased but CART mRNA levels were increased while their plasma levels were decreased unexpectedly. In conclusion, the serotonergic antagonism of risperidone on POMC neurons may cause increase in appetite; and hence, increased weight gain and leptin levels, even in a short term trial.

Keywords: Schizophrenia, Risperidone, Hypothalamic neuropeptides, Obesity

ÖZ

ATİPİK ANTİPSİKOTİK RİSPERİDON TEDAVİSİNDE İŞTAH KONTROLÜNDE ROL ALAN HİPOTALAMİK NÖROPEPTİTLERİN SEVİYELERİNİN İNCELENMESİ

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Atipik antipsikotikler, şizofreni tedavisinde başarı göstermelerine rağmen, uzun dönem kullanımlarında, en önemlisi kilo alımı olmak üzere, çeşitli yan etkiler göstermektedir. Bu çalışmada, iştah düzenlenmesinde rol alan, hipotalamik nöropeptiler/horomonların (nöropeptit Y (NPY), alfa melanosit stimule eden hormon (α-MSH) ve kokain ve amfetamin ile regüle edilen transkript (CART)) ve leptinin, serotonerjik antagonist olan atipik antipsikotik risperidon ile 4 hafta tedavi edilen erkek şizofren hastalardaki plazma seviyeleri incelendi. Risperidonun, serotonerjik antagonizm ile bu nöropeptilerin plazma seviyelerini değiştirebileceği hipotezine bağlı olarak, ilacın kilo alımına neden olabileceği düşünülmektedir. Risperidon tedavisi gören hastalarda kontrole göre plazma leptin seviyeleri artmış ve kilo aldıkları gözlemlenmiştir. Bununla birlikte, NPY, α-MSH ve CART seviyeleri tedaviden önce düşük olup, ilaç tedavisiyle değişiklik göstermemiştir. Bu aday

genlerin mRNA ekspresyon seviyelerinin belirlenmesi için erkek Wistar sıçanlara 4 hafta oral yoldan risperidon verilmiştir. Sıçanlarla yapılan çalışma, POMC, AgRP ve NPY mRNA ekspresyonlarının ve plazma seviyelerini azaldığını, fakat beklenmedik bir şekilde CART mRNA seviyelerinin düştüğünü, plazma seviyelerinin ise arttığını göstermiştir. Sonuç olarak, risperidonun kısa dönem kullanımda dahi POMC nöronları üzerindeki serotonerjik antagonizmi ile iştahta artışa, bu nedenle de kilo alımına ve leptin seviyelerinde artışa neden olduğu düşünülmektedir.

Anahtar kelimeler: Şizofreni, Risperidon, Hipotalamik nöropeptitler, Obezite

To my present and future family…

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"The only reason for time is so that everything doesn't happen at once."

Albert Einstein

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LIST OF ABBREVIATIONS

Δ**Ct**	Difference of the Ct values
5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	Serotonin
5-HT$_1$ - 5-HT$_7$	Serotonin receptors
5-HTP	5-hydroxytryptophan
AC	Adenylate cyclase
ACTH	adenocorticotrophic hormone
AgRP	Agouti-related peptide
AMPK	Adenosine monophosphate kinase
BBB	Blood Brain Barrier
BMI	Body Mass Index
bp	base pair
cAMP	cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated transcript
cDNA	complementary deoxyribonucleic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
Ct	Threshold cycle
D$_1$-D$_4$	Dopamine receptors
DEPC	Diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
E	Efficiency of qPCR
EDTA	ethylenedaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	extrapyramidal side effects
ERK	Extracellular signal regulated kinase
FFA	Free fatty-acid
FOXO1	Forkhead Box Protein O1
FP	forward primer
GAPDH	Glyceraldeyhde-3-phosphate dehyrdogenase
GIRK	G-protein coupled inwardly-rectifying potassium channel
GPCR	G-protein coupled receptor
HPLC	High Pressure Liquid Chromatography

IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
LSD	Lysergic acid diethylamide
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MC-R	melanocortin receptor
M-Mul-V	Moloney Murine Leukemia Virus
MRI	Magnetic Resonance Imaging
mRNA	messenger ribonucleic acid
NPY	Neuropeptide Y
O.D.	Optical Density
Ob-R	Leptin receptor
PBS	phosphate-buffered saline
PC	prohormone convertase
PI3-K	phosphatidylinositol-3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
qRT-PCR	quantitative real time polymerase chain reaction
R^2	coefficient of determination in qPCR
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse primer
rpm	revolutions per minute
SERT	Serotonin reuptake protein
SH2	Src homology 2
SOCS	suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-EDTA buffer
TRH	thyroid stimulating hormone-releasing hormone
WHO	World Health Organization
Y_1R-Y_6R	NPY receptors
α-MSH	alpha melanocyte stimulating hormone

CHAPTER 1

INTRODUCTION

1.1 Schizophrenia

Schizophrenia is a chronic, psychotic disorder that causes perturbations in cognition, hallucinations, delusions, disorganized speech and catatonic or bizarre behavior (DSM IV, 1994). Its lifetime prevalence is 1% and generally seen in young adulthood ages (Andreasen, 2000). The exact causes of schizophrenia are not known clearly. However, it is generally thought that the genetic basis is very significant risk factor as well as the birth injuries or viral infection during pregnancy. Psychosocial effects do not seem to be a "cause" for schizophrenia (Frith, 1992). The symptoms such as hallucinations, delusions are called "positive symptoms" since they are abnormally happening. Also, a schizophrenic patient shows "negative symptoms" of the illness such as the reduction of spontaneous behavior, and those symptoms are the ones that a normal person has but a schizophrenic patient loses it (Andreasen, 1985).

1.1.1 Anatomical abnormalities in brains of schizophrenic patients

The behavioral abnormalities might be the results of neurodevelopmental deficits and brain anatomical and physiological changes. By the use of Magnetic Resonance Imaging (MRI) technology, the best studied and visualized difference in schizophrenic patients is lateral ventricular enlargement, however, it is a non specific disturbance since this can be also

seen in other psychiatric conditions (Shenton *et al*., 1997). There is no statistically significant difference in the volume of the gray matter in brain of schizophrenic patients in comparison to the healthy individuals (Pearlson, 1997). According to the MRI studies, there are abnormalities in hippocampus, amygdala, and parahippocampal cortex of the patients when compared to controls (Shenton *et al*., 1997). While some researchers found thalamic abnormalities in schizophrenic patients (Andreasen *et al*., 1994), some could not observe any difference between control and patient groups (Portas *et al*., 1998) due to the fact that since thalamus has a heterogeneous structure in terms of anatomy, it is difficult to visualize it by MRI technology (Pearlson, 1999). When compared to healthy individuals, the most important change in schizophrenic patients' brain anatomy is that volumes of some parts of the brain regions differ as seen in a number of MRI studies (Daniel *et al*., 1995). When the gender difference is considered, the prevalence of the schizophrenia does not differ between men and women significantly, however, the differences might be seen in the age of onset or the treatment response (Goldstein, 1995). When the genetic basis of the schizophrenia is studied, it was shown that if a person has a schizophrenic first-degree relative, the unaffected person might be carrying pathologic genes (Cannon *et al*., 1994). Although the exact mechanism of the involvement of genes in the pathological condition of the disease is not known clearly (Wickham, 1997), it is suggested that the candidate genes might be responsible for the cortical migration or development (Ross and Pearlson, 1996).

1.1.2 Biochemical abnormalities in brains of schizophrenic patients

Disturbances in the neurotransmitter systems were identified in the brains of the schizophrenic patients. The increased dopamine transmission, which is a cathecolamine neurotransmitter, in the central nervous system (CNS) was found to be correlated with the positive symptoms of the schizophrenia (Van

Rossum, 1967). Also, gamma aminobutyric acid (GABA) pathway, an inhibitory neurotransmitter, was found to be disrupted in the schizophrenic patients (Wassef *et al*., 2003). Moreover, the alterations in histaminergic, adrenergic and noradrenergic pathways were found to be related to the schizophrenia pathophysiology (Rauscher, *et al*., 1980; Kemali, *et al*., 1982).

1.1.2.1 Serotonergic pathway and abnormalities in schizophrenia

Serotonin, 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter that is synthesized from tryptophan by two enzymes which are L-tryptophan-5-monooxygenase and 5-hydroxytryptophan decarboxylase (Fig.1.1) (Costa and Meek, 1974).
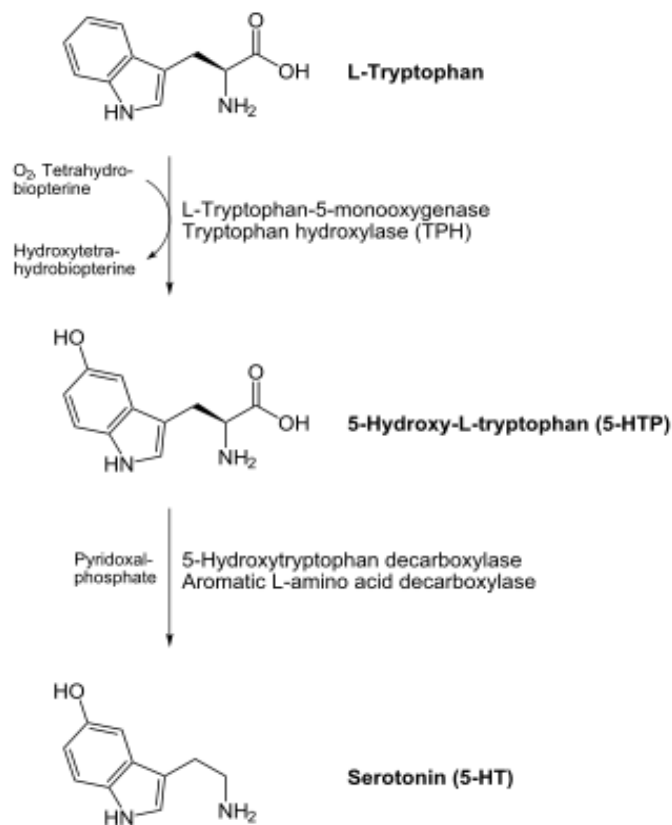
Figure 1. 1 Serotonin biosynthesis pathway

Serotonin is synthesized in CNS and it is generally associated with mood, anxiety, cognition and homeostatic activity in the brain. The cell bodies of serotonin neurons are mainly found in raphe nuclei and they send their axon projections to almost all brain regions, including cortex, thalamus, hypothalamus, striatum and amygdala to exert their physiological functions (Lam *et al*., 2010; Charnay and Léger, 2010).

After the biosynthesis of serotonin, it is sent to the vesicles to be packed. In case of the depolarization of the neuron, the calcium ion concentration increases and the vesicles containing serotonin fuse with cell membrane for the release of serotonin to the synaptic cleft. Serotonin reaches to postsynaptic neuron and via its receptors, and it initiates the downstream pathways in the neuron. The excess serotonin is taken back to the neuron by serotonin reuptake protein (SERT) and that serotonin is again packed in vesicles. Free serotonin in the cytoplasm is metabolized by the enzyme monoamine oxidase and it is converted to its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) (Fig.1.2) (Nichols and Nichols, 2008).
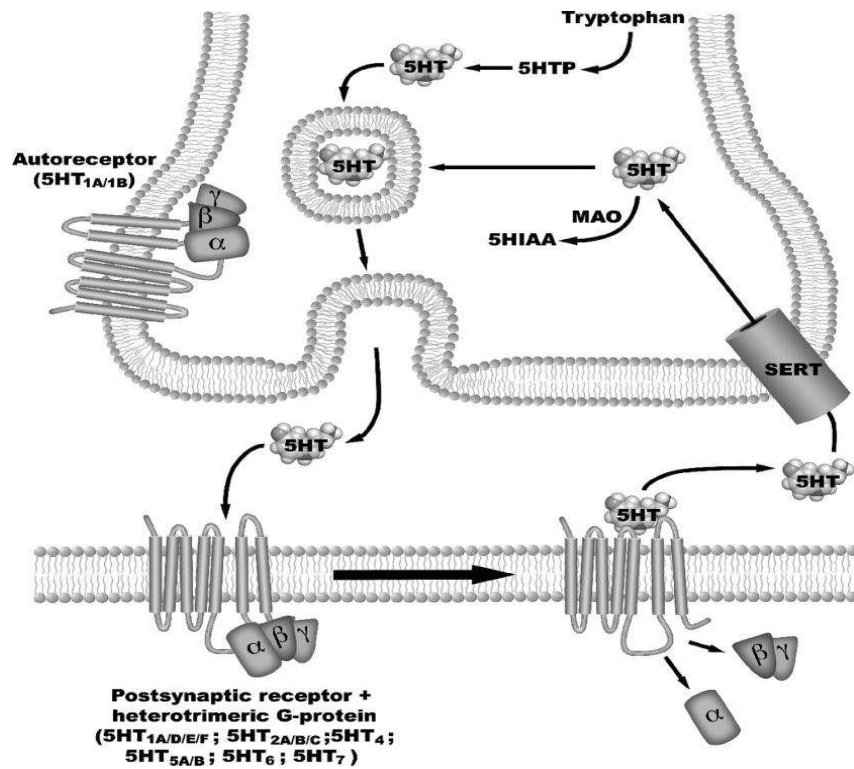
Figure 1. 2 The serotonergic synapse (Nichols and Nichols, 2008)

Serotonin receptors, which are in seven groups, namely 5-HT$_1$, 5-HT$_2$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, 5-HT$_7$, belong to G-protein coupled receptor (GPCR) family with 13 genes encoding for those receptors. Only the 5-HT$_3$ receptor is a ligand-gated ion channel type receptor (Nichols and Nichols, 2008). Some of those receptor groups include more than one member, such as 5-HT$_1$ has 1A, 1B, 1D, 1E and 1F subgroups, while 5-HT$_2$ has 2A, 2B and 2C subgroups. 5-HT$_1$ receptors, by coupling to G$_i$, inhibits the adenylate cyclase (AC) activity and results with the neuron hyperpolarization by downstream pathways. The 5-HT$_2$ receptor family couples to G$_q$, activating phospholipase C (PLC) pathway and depolarization of the neuron (Fig.1.3) (Lam *et al.*, 2010).
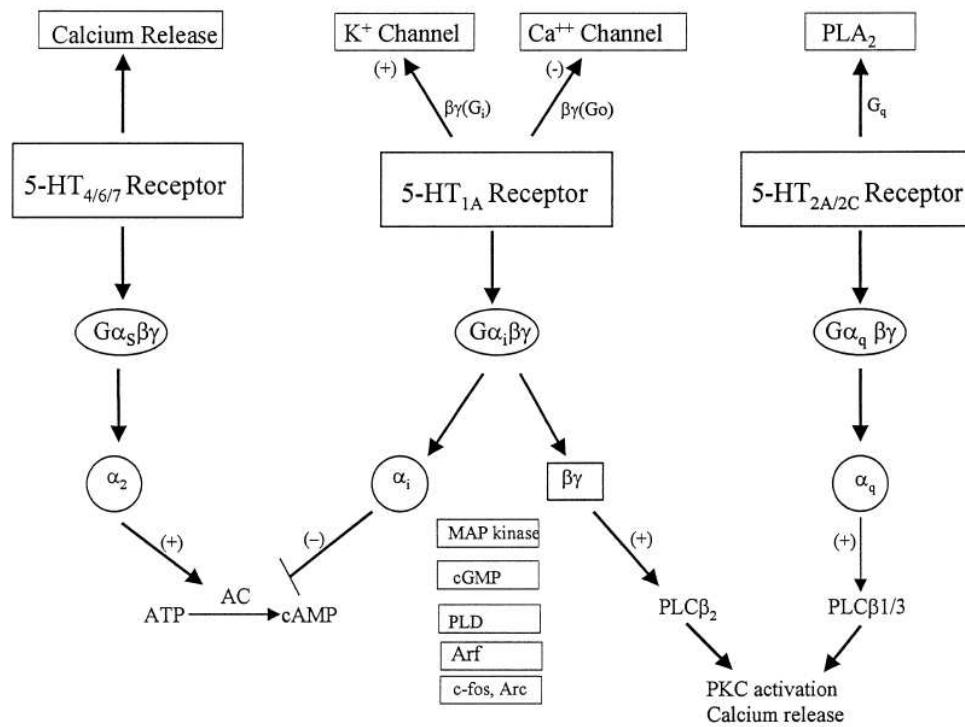
Figure 1. 3 Serotonin receptors' downstream pathways (Aghajanian and Sanders-Bush, 2002).

In 1950s, it was shown that the hallucinogenic drug lysergic acid diethylamide (LSD) resembles serotonin in terms of its structure. LSD caused the inhibition of the serotonin neuron firing in the raphe nucleus. It was hypothesized that the schizophrenia might be associated with a dysfunction in serotonergic system (Gaddum, 1954; Wooley and Shaw, 1954). LSD, by direct agonism of presynaptic 5-HT$_{1A}$, inhibits the serotonergic cells in raphe nuclei, but it is more potent agonist of 5-HT$_2$ receptors. This agonism results with positive symptoms of schizophrenia which is suggesting the increased 5-HT$_2$ functions might be related to schizophrenic episodes (Abi-Dargham *et al*., 1997; Aghajanian, 1994). The serotonin metabolite 5-HIAA was investigated in the cerebrospinal fluid (CSF) of schizophrenic patients and there were, increase, decrease or no change in 5-HIAA level, which could not give explanation about the serotonin in schizophrenia (Ashcroft *et al*., 1966; Wode-Helgodt *et al*., 1977; Rimon *et al*. 1971). Furthermore, serotonin concentration in

hypothalamus and hippocampus of post-mortem schizophrenic patients decreases (Winblad *et al.*, 1979), while in the subcortical areas it increases (Farley *et al.*, 1980). Also, the density of 5-HT transporters is found to be decreased in the frontal cortex of schizophrenic patients (Laruelle *et al.*, 1993). $5-HT_{2C}$ receptor expression was also shown to decrease significantly in the postmortem schizophrenic patients' brains (Castensson *et al.*, 2003).

1.2 Treatment of schizophrenia

1.2.1 Typical and atypical antipsychotics

The treatment of schizophrenia could be possible after the discovery of antipsychotic drugs in 1950's. The first antipsychotic drug to be discovered and applied to the patients was chlorpromazine which was synthesized and tested by Charpentier and Courvoisier in 1950, in France, however, its mechanism of action was not known (Stip, 2002). After dopamine was discovered as a central neurotransmitter, it was found that the major mechansim of action of antipsychotic drugs was thorugh dopaminergic antagonism (Carlsson, 1963). After this discovery, also, serotonergic mechanism of action of antipsychotics was also proposed (Meltzer, 1989;99(Suppl)). Those antipsychotics discovered firstly were named as "typical antipsychotics" and it was shown that they cause extrapyramidal side effects (EPS), leading to involuntary movements such as parkinsonism, on patients (Leucht, 1999). It is suggested that the reason for the EPS is that the one of the main pathways of dopaminergic system in brain which is between the substantia nigra and striatum and called as nigrostriatal pathway is blocked (Taylor, 2006). Besides the EPS, chlorpromazine has also other side effects such as increase in plasma creatine kinase activity and increase in plasma prolactin levels (Meltzer, 2004). Apart from chlorpromazine, the typical antipsychotics that were used were reserpine, fluphenazine, prochloroperazine, and thioredazine, which were followed by the

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involvement of butyrophenones (Domino, 1999). In 1960s, German scientists studied on the antipsychotic efficacy and EPS linkage and finally they discovered clozapine, the first 'atypical antipsychotic', with minimum EPS and capable of treating both positive and negative symptoms of schizophrenia (Hippius, 1996). Since the mechanism of action of typical antipsychotics is through dopamine receptor blockage, after the discovery of atypical antipsychotics, the serotonin/dopamine antagonism theory was postulated and it was suggested that if a drug's affinity for serotonin receptor is higher than that of dopamine receptor, it leads to less EPS and more drug efficacy (Meltzer *et al.*, 1989) After the succes of clozapine, the discovery of new atypical antipsychotics continued and the second one to be discovered and approved was risperidone in 1994 (Marder & Meibach, 1994).

1.2.2 Risperidone

Risperidone belongs to benzisoxazole derivatives antipsychotic family and its chemical name is 3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1- piperidinyl]ethyl] -6,7,8,9-tetrahydro-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one, with the chemical formula $C_{23}H_7FN_4O_2$, the molecular weight is 410.49 (Risperdal Prescribing Information, 2008).
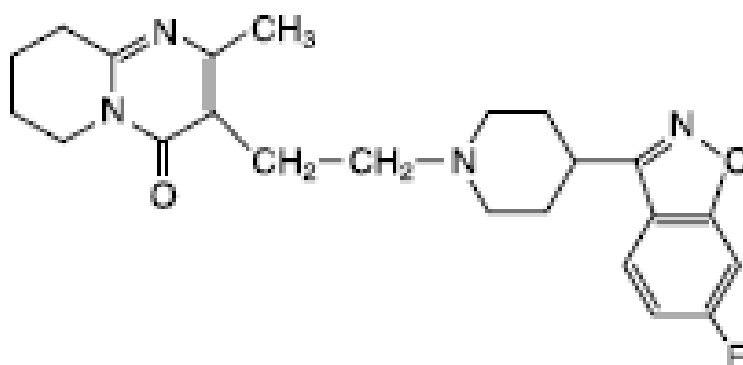
Figure 1. 4 The chemical structure of risperidone

Risperidone is metabolized through hydroxylation reaction by cytochrome P450 2D6 (CYP 2D6) enzyme in the liver. It is converted to 9-hydroxyrisperidone and together with risperidone, they exert the pharmacological action (Risperdal Prescribing Information, 2008).

Risperidone is a potent antagonist for 5-HT_{2A} and dopamine-2 receptor (D_2) (Gardner *et al.*, 2005). It has effects on both positive and negative symptoms of schizophrenia and also it shows low incidence of EPS (Davis and Janicak, 1996).

Table 1. 1 Relative dopamine and serotonin receptor affinities of risperidone (Miyamoto *et al*., 2005)

Receptor	Risperidone affinity
D_1	+
D_2	+ + +
D_3	+ +
D_4	-
5-HT_{1A}	-
5-HT_{1D}	+
5-HT_{2A}	+ + + +
5-HT_{2C}	+ +
5-HT_6	-
5-HT_7	+ + +

The atypical antipsychotics, besides treating the schizophrenic symptoms, have an important side effect of weight gain (Müller *et al*., 2004). Ten weeks risperidone treatment of the schizophrenic patients revealed that risperidone causes two kilograms of weight gain, suggesting its moderate weight gain

effect (Allison *et al*., 1999). The clinicians in Gülhane School of Medicine (GATA) reported that the patients generally do not want to continue the treatment due that the long term use of risperidone causes a significant increase in the body weight and leads to obesity.

1.3 Obesity

Obesity is becoming an important worldwide epidemic day by day. With its increasing prevalence in last decades, obesity is a major health problem accompanied by economical, social and psychological problems worldwide. According to the definition of World Health Organization (WHO), obesity is "abnormal or excessive fat accumulation that may impair health". Body Mass Index (BMI) is the index that is used for the measurement of overweight state and obesity. It is calculated by the division of body weight in kilograms to the square of the height in meters (kg/m^2).

Table 1. 2 Classification of BMI according to WHO.

	BMI cut-off points
Underweight	**< 18.50**
Severe thinness	< 16.00
Moderate thinness	16.00 – 16.99
Mild thinness	17.00 – 18.49
Normal range	**18.50 – 24.99**
Overweight	**≥ 25.00**
Pre-obese	25.00 – 29.99
Obese	**≥ 30.00**
Obese class I	30.00 – 34.99
Obese class II	35.00 – 39.99
Obese class III	≥ 40.00

According to the data collected by Turkish Statistical Association, 50.5% of men and 49.4% of women above the age 15 are obese or overweight in Turkey (www.tuik.gov.tr, 2011). The Fig.1.5 shows the worldwide obese percentage of adults according to the countries, survey conducted by WHO (http://apps.who.int/bmi/index.jsp, 2009). Moreover, WHO estimates that by the year 2015, approximately 2.3 billion people will be overweight and over 700 million of them will be obese.

BMI adults % obese (> =30.0), Most recent

≥ 50.0	
40.0	- 50.0
30.0	- 40.0
20.0	- 30.0
10.0	- 20.0
5.0	- 10.0
0.0	- 5.0
no data	
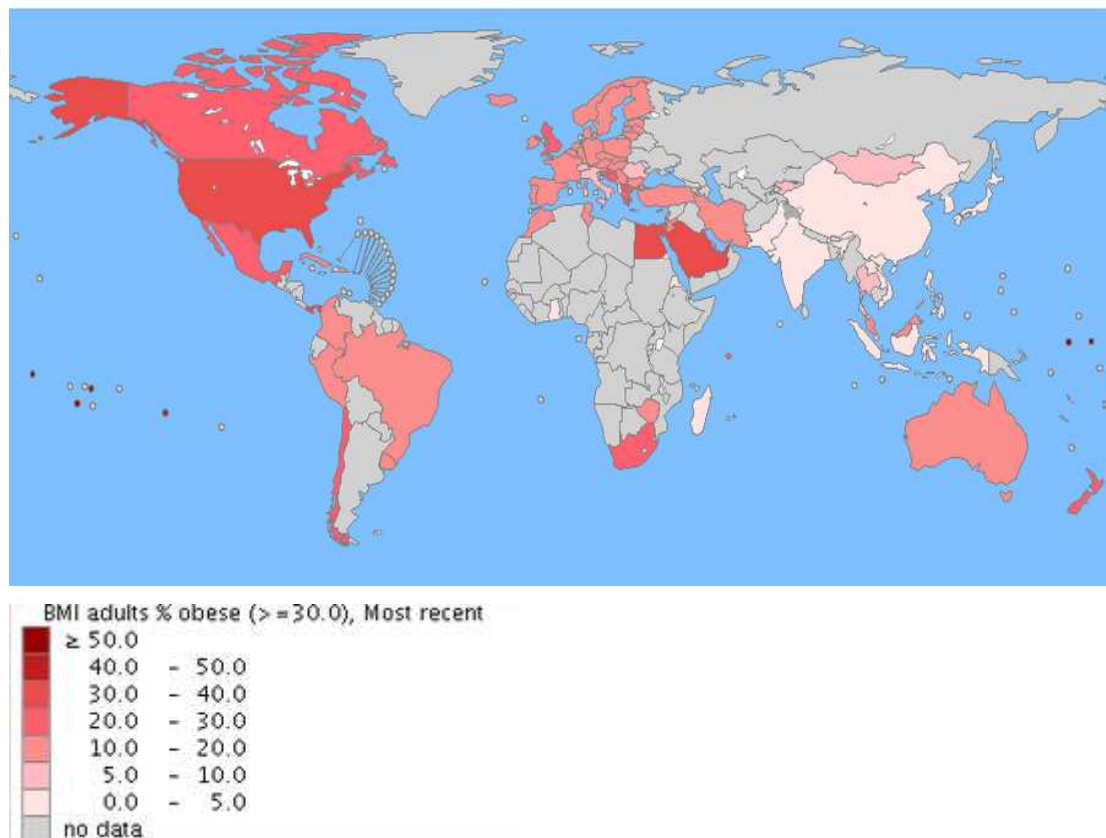
Figure 1. 5 Percentage of obese adults worldwide (http://apps.who.int/bmi/index.jsp, 2009).

A number of researches have been carried out for the etiology of obesity and it was found that the genetic factors' contribution to the disease is 30-40% while the environmental factors' contribution is 60-70% (Pi-Sunyer and Xavier, 2002). Moreover, there is an interaction between genetic and environmental

factors such that some people genetically predispose to obesity that only under certain environmental factors. Among environmental factors, sedentary life styles, high-fat diets, eating behaviors, and drugs' side effects commonly lead to obesity (Stunkard, 1988).

Obesity is the second cause of mortality in the U.S. after smoking. Obesity is associated with other disorders such as type-2 diabetes, hyperlipidemia, cardiovascular diseases, collectively resulting with metabolic disorder (Hurt *et al.*, 2011). The most important consequences of obesity could be listed as insulin resistance, type-2 diabetes, and the increased levels of free fatty acids (FFAs). Insulin, after secretion from the β-cells in Langerhans islands in pancreas upon the increase of glucose in blood, binds to its receptors on the target cell. When insulin binds to its receptor, it causes autophosphorylation of tyrosine leading to downstream pathways. As a result of this intracellular downstream pathway, glucose uptake via glucose transporters increases. However, in obese people, the ability of insulin receptor to autophosphorylate the tyrosine decreases and this reduction causes insulin resistance (Caro *et al.*, 1989). Moreover, obese people have some other defects in the glucose metabolism, such as decreased glucose transport, and dysfunctions in some important enzymes in glucose metabolism pathways. Those abnormalities, however, may normalize after the weight loss of the obese person (Friedman *et al.*, 1992; Segal *et al.*, 1991). Another important consequence of obesity is type 2 diabetes which has both genetic and environmental background. It is shown that BMI and waist circumference are associated with the risk of type-2 diabetes. Neither all obese people have type-2 diabetes nor are all diabetic patients obese (Timpson *et al.*, 2009). Apart from the genetic background, environmental factors, one of the most important of which is obesity, have effects on type-2 diabetes progress. This type of diabetes is seen due to insulin resistance and β-cell impairment of pancreas Langerhans islands. The β-cell impairment leads to disruption of the insulin secretion, and consequently

hyperglycemia is seen in those patients. Moreover, it was shown that type-2 diabetic patients have 40% less β-cells when compared to healthy individuals (Feinglos and Bethel, 2008).

When the fat mass in obese people is elevated, lipolysis and FFA mobilization increases and as a consequence FFA oxidation is increased in muscles and the liver. As a result, muscles start to use FFA as an alternative energy source and gluconeogenesis increases in the liver. Those metabolic abnormalities in glucose metabolism lead to hyperglycemia and impaired glucose tolerance (Fig.1.6) (Jensen *et al.*, 1989).
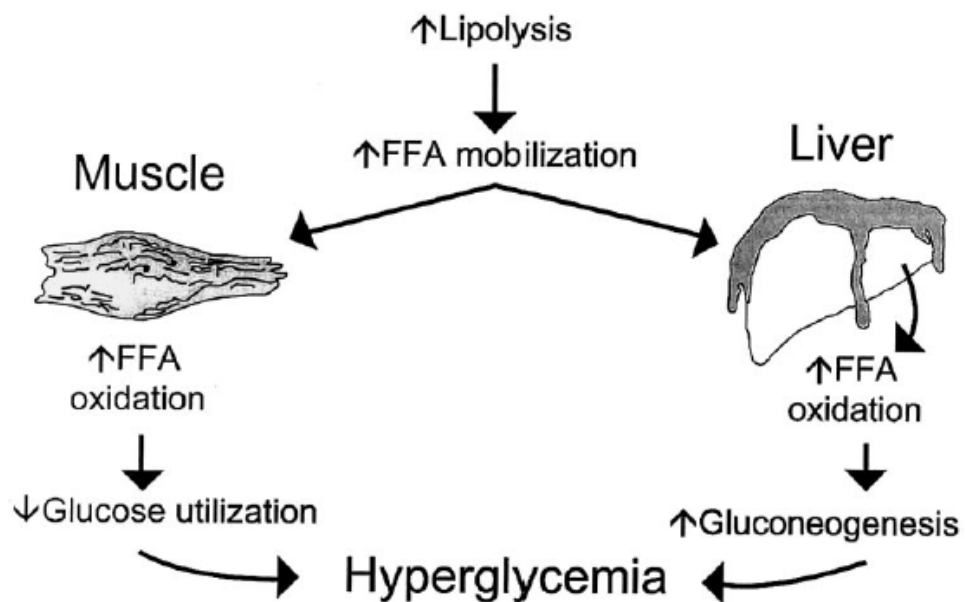
Figure 1. 6 Effect of lipolysis on glucose metabolism (Pi-Sunyer and Xavier, 2002).

1.4 Central and peripheral control of eating

Body weight is controlled by the interaction between the brain and peripheral signals. There are a number of gastrointestinal hormones in the periphery that have roles in the body weight regulation. Those hormones, cholecystokinin

(CCK), glucagon-like peptide 1 (GLP-1), ghrelin, adiponectin, peptide YY (PYY), control the energy balance in parallel to the control of eating by CNS (Havel, 2001).

The hypothalamus is the primary CNS part for the regulation of energy homeostasis (Rohner-Jeanrenaud *et al.*, 1996). The arcuate nucleus (ARC) of hypothalamus is the most important part for the food intake control and the site where synthesis and secretion of orexigenic and anorexigenic neuropeptides take place. (Funahashi *et al.*, 2000).
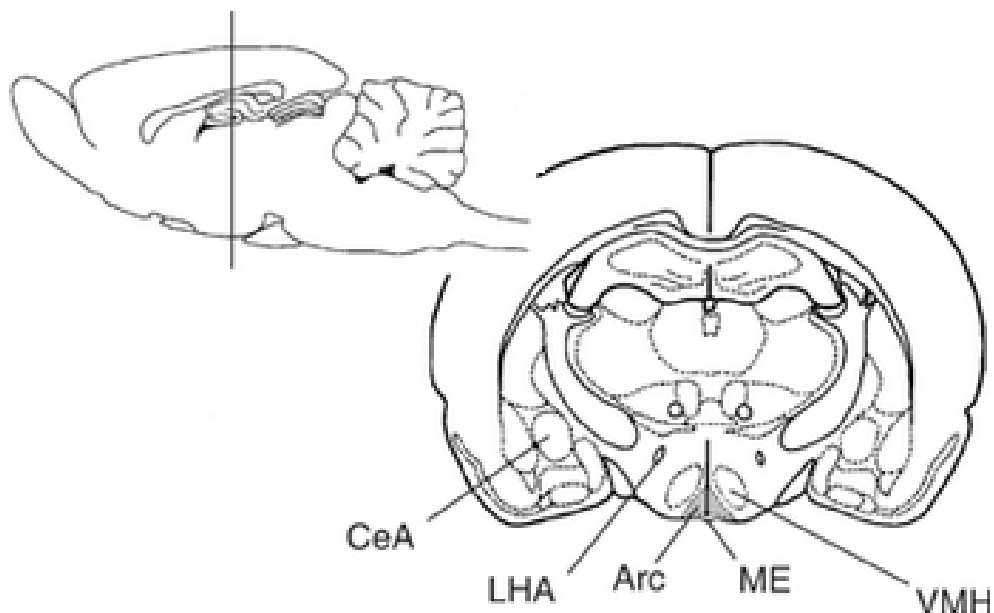
Figure 1. 7 Arcuate nucleus (ARC) of hypothalamus, in the schematic diagram of the coronal section of the rat brain (CeA: Central nucleus of amygdala; LHA: Lateral hypothalamic area; ME: Medial eminence; VMH: Ventromedial nucleus of the hypothalamus) (Kishi and Elmquist, 2005)

1.4.1 Leptin

Leptin is a 16 kDa protein hormone which is synthesized in and secreted from the white adipose tissue and signals to the hypothalamus (Margetic *et al.*,

2002). After cloning of mice and human leptin (ob) protein, which is the time of its discovery, the mice lacking the *ob* gene and obese, it was deduced that replacement of leptin caused a decrease in the food intake and obesity symptoms in the mice (Zhang *et al*., 1994; Halaas *et al*., 1995). The *ob* gene is located on the chromosome 7q31.3 and is 650 kb with 3 exons and 2 introns with coding regions on exon 2 and 3. There are a number of regulatory elements on the promoter site of *ob* gene (Fig.1.8) but the tissue specific expression regulation still remains unknown in white adipose tissue (Gong *et al*., 1996). Apart from the white adipose tissue, leptin is also secreted from various kinds of tissues such as stomach, intestine, placenta, and testis (Considine and Caro, 1999).
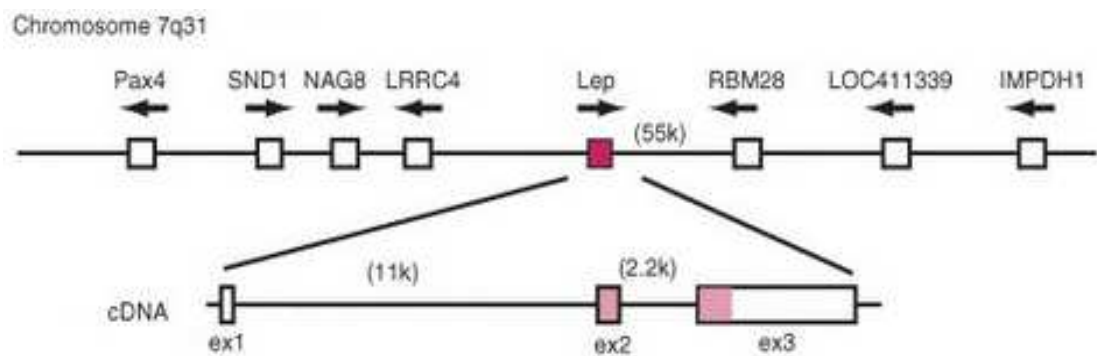
Figure 1. 8 Leptin gene and its transcript in human (Kurokawa *et al*., 2005)

The most important physiological role of leptin is the control of food intake via CNS. To exert its function, leptin binds to its receptors (Ob-R). There are six splice variants of the leptin receptor gene identified, Ob-Ra, Rb, Rc, Rd, Re, Rf, all of which share the same extra-cellular ligand binding domain which consists of 816 amino acids. However, their intracellular C-terminus domains differ between variants (Tartaglia *et al*., 1995). Figure 1.9 shows the isoforms of leptin receptor. Leptin receptor family is a member of class I cytokine receptors. All isoforms contain a cytokine receptor homologous domain in the extracellular part. Also, all share two conserved disulfide bonds in the N-

terminus. Among the identified isomers, only the Ob-Rb has the necessary intracellular domain for the activation of the JAK-STAT (Janus activated kinase-signal transducers and activators of transcription) signal transduction pathway. Moreover, Ob-Re does not have any intracellular or transmembrane domains, and circulates as a soluble receptor (Tartaglia, 1997).
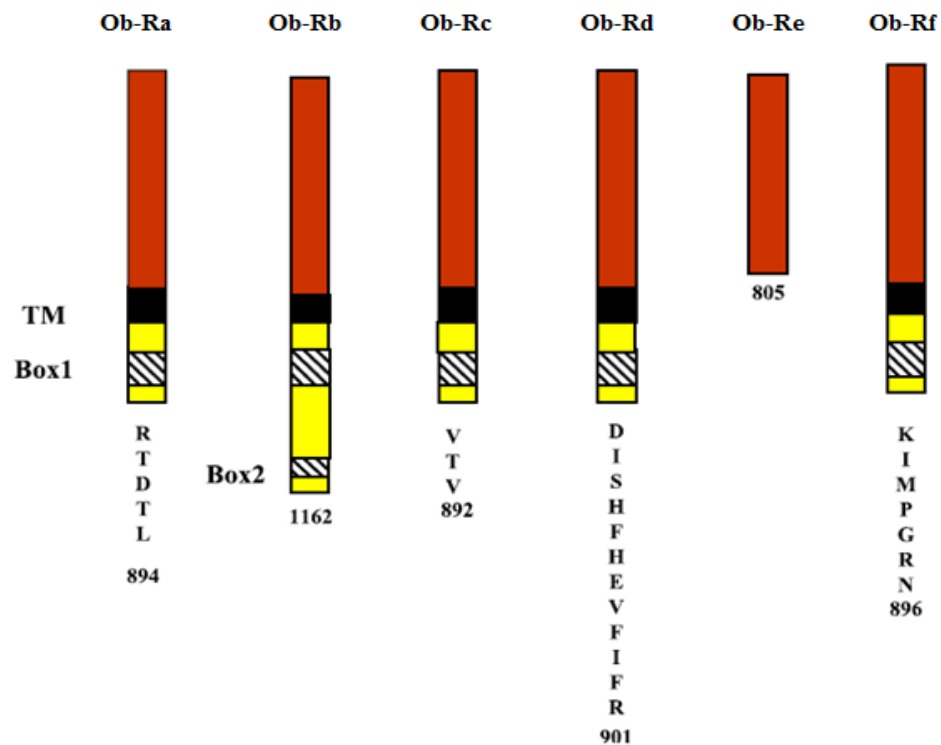
Figure 1. 9 Leptin receptor isoforms. The domain structures of the alternatively spliced variants. (Ahima and Osei, 2004).

Once the leptin binds to its receptor the main downstream mechanism is activated by the JAK-STAT pathway. Ob-Rb has three conserved tyrosine residues which are identified as Y985, Y1077 and Y1138 in mice. Y1138 is the part which activates signal transducer and activator of transcription 3 (STAT3) with its SH2 (Src homology-2) domain and this activation results with homodimerization and translocation of STAT3 which binds to specific promoter elements of target genes (Bates *et al*., 2003; Muraoka *et al*., 2003). Ob-Rb activates the targets such as insulin receptor substrate-1 (IRS-1),

mitogen activated protein kinase (MAPK), extracellular signal regulated kinase (ERK), Akt, adenosine monophosphate kinase (AMPK) and phosphatidylinositol-3-kinase (PI-3) which are also activated by insulin signaling. Therefore, leptin and insulin function together to control the body weight. (Niswender and Schwartz, 2003; Porte *et al*., 2002).
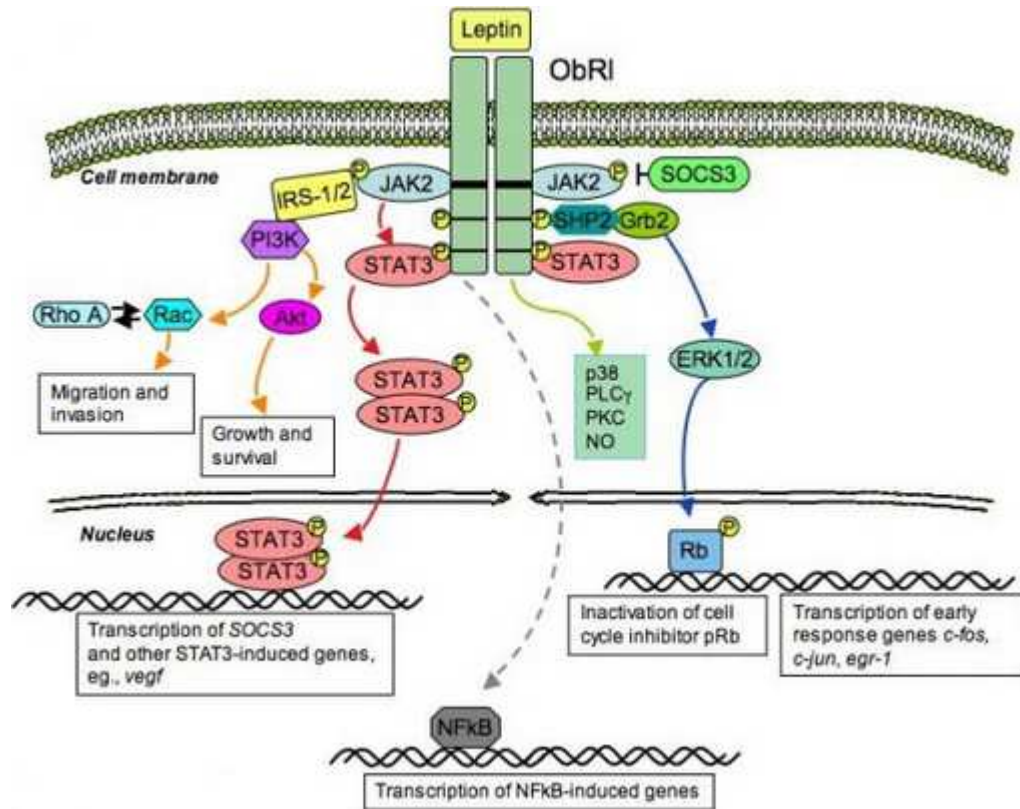
Figure 1. 10 Genereal signal transduction pathways triggerred by leptin receptor (Garofalo and Surmacz, 2006)

Ob-Rb expression is high in the hypothalamus and it was shown that Ob-Rb defected mice showed no STAT3 activation in the brain which is indicating that hypothalamus is the main part for the leptin action (Ghilardi *et al*., 1996; Fei *et al*., 1997).

Leptin is circulating in the bloodstream both as bound to plasma proteins and in its free form (Sinha *et al.*, 1996). The way of transmission of leptin to the brain is suggested by crossing the blood brain barrier (BBB) via receptor mediated mechanism (Pardridge, 1986). There is a high level of expression of Ob-Ra, the short form of leptin receptor isomers, in microvessels of brain providing the internalization of leptin (Bjorbaek *et al.*, 1998; Golden *et al.*, 1997). Moreover, leptin and Ob-Ra is found in cerebrospinal fluid (CSF) but it was shown that leptin concentration in CSF is approximately 100 fold less than the plasma leptin concentrations which indicate CSF is not the primary leptin carrier site (Tartaglia *et al.*, 1995). Leptin receptors in the brain associated with the food intake regulation are mainly found in the arcuate nucleus of hypothalamus, where leptin controls the expression and synthesis of orexigenic and anorexigenic peptides, which increase and decrease the food intake, respectively (Dyer *et al.*, 1997).

One important mechanism in obesity is the leptin resistance pathway. Leptin resistance can be defined as the decreased response to the leptin and indicated by the increased plasma leptin levels in obesity. In people with leptin resistance, appetite regulation cannot be achieved properly and food intake cannot be inhibited as the fat mass increases even if secreted leptin amount also increases as result of it. Mechanisms involved in development of leptin resistance are mainly leptin receptor signaling and leptin transport, yet the exact mechanism is not known (Houseknecht *et al.*, 1998). Elevated leptin levels impair leptin receptor signaling thus the expected response corresponding to those leptin levels are not generated. In the case of presence of high leptin levels in the plasma, the transport of leptin through BBB is impaired so that leptin cannot reach its target neurons to generate a response. In addition, defects in cellular trafficking of leptin receptors and targeted signaling pathways may also contribute to development of leptin resistance.

Moreover, insulin resistance is mostly seen together with the leptin resistance in obesity (Margetic *et al*., 2002).

1.4.2 Hypothalamic neuropeptides that regulate food intake

The synthesis and secretion of the orexigenic (Neuropeptide Y and Agouti-related peptide) neuropeptides and the anorexigenic (Pro-opiomelanocortin and cocaine and amphetamine regulated transcript) take place in the ARC of the hypothalamus.

1.4.2.1 Orexigenic peptides

Mainly two orexigenic neuropeptides exist in the ARC which are neuropeptide Y (NPY) and agouti related peptide (AgRP).

1.4.2.1.1 Neuropeptide Y (NPY)

NPY is a neuropeptide having 36 amino acids and found in the mammalian nervous system (Larhammar *et al.*, 1987). NPY is abundantly found in the different parts of the brain, peripheral sympathetic nerves, and adrenal medulla, and it is localization is with cathecolamines, serving as neurohormone, neurotransmitter, and neuromodulator in the nervous system, immune system, and vascular system via its different types of receptors (Zukowska *et al.*, 2003). NPY, being one of the most abundant peptides in the brain, reaches its highest concentrations in the arcuate nucleus of the hypothalamus, brainstem, and anterior pituitary. In those sites, it is responsible for two important physiological functions, which are regulation of food intake and stress response (Hirsch and Zukowska, 2012). The stress response generated by NPY differs in the central and peripheral parts. In periphery, NPY causes an increase in the stress response, while, in the brain, it helps the body to cope with the stress. It

was shown that NPY injection to the paraventricular nucleus (PVN) resulted in the increasing levels of adenocorticotrophin hormone (ACTH), which triggers the release of cortisols from the adrenal glands, important in the stress response (Crowley, 2004).

NPY gene expression in the ARC of hypothalamus is mainly regulated by leptin receptors found on the NPY neurons. The proposed mechanism for the NPY gene expression repressed by the leptin binding to its receptor is shown in Fig.1.11. When leptin binds to its receptor, it activates the JAK-STAT pathway and STAT3 is phosphorylated. When STAT3 is activated via phosphorylation, it activates a transcription inhibitor factor, SOCS3 (suppressor of cytokine signaling 3). The induced SOCS3 antagonizes the STAT3 activation on the leptin receptor and inhibits the transcription of *NPY* via histone deacetylases (HDAC). In the reverse condition, one of the HDAC inhibitors, trichostatin A, inhibits the repression effect of SOCS3 which eventually cause *NPY* expression (Higuchi *et al.*, 2005).
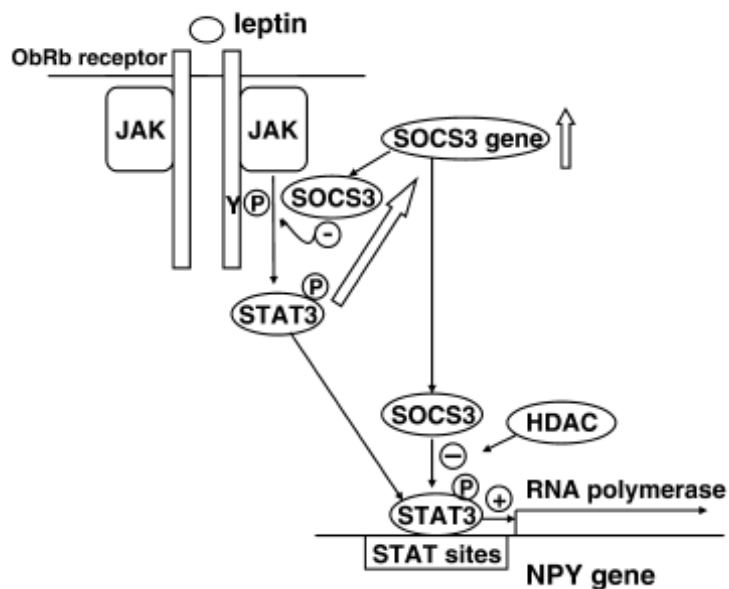
Figure 1. 11 Proposed mechanism of how leptin controls the NPY gene expression (Higuchi, *et al*., 2005).

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The receptors of NPY belong to the GPCR family. Five different NPY receptors are identified in the mammals, which are Y_1R, Y_2R, Y_4R, Y_5R and Y_6R (Michel *et al.*, 1998). The distribution of those receptors are mainly confined to the hypothalamic area in the brain, however, there are also NPY receptors in the periphery (Fetissov *et al.*, 2004). The downstream pathway activated via NPY receptors are generally the inhibition of the adenylate cyclase pathway and reduction in cAMP levels. Moreover, in some cell types, the response might be through the potassium and calcium channels (Merten and Beck-Sickinger, 2006; Herzog *et al.*, 1992). Among those receptors, the ones important in the feeding behavior and stress responses are Y_1R, Y_2R, and Y_5R.

Y_1R is mainly found in the cerebral cortex, thalamus, and amygdala in the CNS (Cabrele and Beck-Sickinger, 2000). Moreover, it was shown that Y_1R is also found in the adipose tissue and in the vascular smooth muscle cells (Lindner *et al.*, 2008; Castan *et al.*, 1993). The Y_1R expression is controlled by three promoters and which one of those will be activated depends on the tissue type (Ball *et al.*, 1995). Glucocorticoids were shown to have a stimulatory effect on the Y_1R in the hypothalamic region (Bournat and Allen, 2001). Furthermore, although the NPY expression is upregulated during hunger periods, the expression of Y_1R mRNA was shown to be decreased in the ARC (Cheng *et al.*, 1998). Also, obese rats were shown to have decreased Y_1R mRNA expression in the hypothalamus (Beck *et.al*, 2001). On the other hand, the role of NPY in the stress responses was found to be related to Y_1R, and NPY exerts its function via activation of the Y_1R (Eva *et al.*, 2006). It is important that the Y_1R expression is highly upregulated in the dentate gyrus and the medial amygdala of the schizophrenic patients (Kishi *et al.*, 2005). Moreover, the activation of the Y_1R inhibits the behavioral response to the dopaminergic stimulation in schizophrenic patients (Kask and Harro, 2000). The downstream pathways stimulated upon the binding of the agonists to Y_1R are initiated by

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the internalization of the receptor via clatrin coated pits (Gicquiaux *et al.*, 2002).

The Y_2R is found in different brain regions such as amygdala, hypothalamus, thalamus, hippocampus and brainstem of the rat brain (Gustafson *et al.*, 1997). It was shown that when the Y_2R is knock-down in mice, it causes an increase in the NPY levels in the arcuate nucleus of the hypothalamus (Sainsbury *et al.*, 2002). This observation might be explained by the presence of Y_2R in the NPY neurons to down-regulate its expression in the presynaptic manner (King *et al.*, 2000). Except for the arcuate nucleus, Y_2R was found to be expressed in different parts of hypothalamus. High levels of expression were shown to be in the rostral hypothalamus, and the Y_2R in that region was thought to have roles in the reproductive behavior (Swanson, 1987). Moreover, Y_2R was found to be strongly associated with feeding behavior. NPY injection to the PVN of the hypothalamus was found to be decreasing the food intake (Leibowitz and Alexander, 1991). Related to the other important role of NPY, which is stress response, the Y_2R knockout mice displayed diminished anxiety behaviors, which might explain the role of Y_2R in stress responses of NPY (Redrobe *et al.*, 2003). Furthermore, mice having deficiency in the Y_2R signaling showed schizophrenia-related behaviors (Karl *et al.*, 2010).

The Y_5R localization throughout the brain is distributed in different brain areas, and the density of Y_5R in the hypothalamus is too low (Dumont *et al.*, 1998). The studies showed that Y_5R, not by itself, but with the Y_1R, is modulating the feeding behavior (Duhault *et al.*, 2000). To support this idea, it was revealed that Y_5R knockout mice do not show any changes in the feeding behavior or weight gain, which might be explaining that Y_5R cannot control the food intake itself or the lack of Y_5R might be compensated for in terms of the feeding behavior (Kanatani *et al.*, 2000). Because of the colocalization of Y_5R with the stress related neurotransmitters, such as corticotrophin releasing factor (CRF)

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and GABA, it was shown that Y_5R involves in the stress-related behaviors (Walker *et al.*, 2009). However, no direct involvement of the Y_5R in the schizophrenia is described in the literature.

There are numerous studies showing the relation between the NPY levels and schizophrenia. Alterations of the NPY levels were detected in the temporal cortex and the CSF of the schizophrenic patients, but no significant change in the hypothalamic tissue was observed (Frederiksen *et al.*, 1991; Widerlov *et al.*, 1988). Moreover, the NPY mRNA levels of the schizophrenic patients in the prefrontal cortex were shown to significantly decrease when compared to healthy individuals (Kuromitsu *et al.*, 2001). The antipsychotic treatment, on the other hand, was shown differences in terms of the typical and atypical antipsychotic treatments. Obuchowicz (1996) demonstrated that the treatment of rats with typical antipsychotics, but not the atypical ones, resulted an increase in the hypothalamic NPY levels (Obuchowicz, 1996).

1.4.2.1.2 Agouti-related Peptide (AgRP)

Human AgRP is a 132 aminoacid protein while the rodent AgRP encodes 131 aminoacid protein (Brown *et al.*, 2001). There are a number of physiological roles of AgRP, one of which is the increasing the food intake and decreasing the energy expenditure (Stutz *et al.*, 2005). Moreover, AgRP has neuroendocrine functions on the regulation of the hypthalamo-pituiatry axis, such as increasing the ACTH levels or suppressing the thyroid stimulating hormone-releasing hormone (TRH) and decreasing the thyroid hormone levels in the periphery (Dandona *et al.*, 2004; Fekete *et al.*, 2002).

AgRP was shown to be an antagonist of the melanocortin receptors (MCRs), and it is also an inverse agonist of the MC4R (melanocortin receptor 4), and this effect is suggested to be the role of AgRP in the feeding control

mechanism (Nijenhuis *et al.*, 2001). In the CNS, AgRP mRNA was found to be colocalized with the NPY mRNA at a percentage of 95% (Broberger *et al.*, 1998). It was suggested that AgRP has a modulatory role in the feeding behavior, not required for the stimulation of feeding because it was shown that AgRP levels is higher in pregnancy and lactation when the body needs higher energy (Rossi *et al.*, 1998; Rocha *et al.*, 2003). Moreover, AgRP injection to the PVN of the rats resulted with an increased food intake, which suggests the localization of AgRP in the PVN and its effect on feeding (Wirth *et al.*, 2000).

The cellular synthesis pathway of the AgRP is the same with the NPY expression, in which leptin binding to its receptor, activated the JAK/STAT pathway to inhibit the expression of NPY, together with AgRP. This mechanism also works with the phosphatidylinositol 3-OH-kinase signaling pathway (Morrison *et al.*, 2005). After the expression of AgRP, it is stored in the secretory granules intracellularly, and its release mechanism is regulated secretory pathway. Its posttranslational modification is done by the prohormone convertases (PCs), specifically PC1/3 cleaves the prohormone and provides the formation of full-length bioactive AgRP (Creemers *et al.*, 2006). The important part of AgRP is its 83-132 C terminal fragment since this fragment was shown to have the role in melanocortin receptors' antagonism, so the regulation of the food intake (Rossi *et al.*, 1998).

In stress conditions, AgRP expression was shown to decrease significantly in rats while NPY expression significantly increases. Therefore, it was suggested that overeating during the stressful periods might be related to the disruption of NPY/AgRP mechanism (Kas *et al.*, 2005). Furthermore, although there is not much evidence for the relationship of AgRP and schizophrenia, AgRP was suggested to have alterations in schizophrenic patients (Bernstein *et al.*, 2010).

1.4.2.2 The ARC anorexigenic peptides

Two ARC neuropeptides have anorexigenic roles which are namely Pro-opiomelanocortin (POMC) and Cocaine and Amphetamine Regulated Transcript (CART).

1.4.2.2.1 Pro-opiomelanocortin (POMC)

POMC is a prohormone that is synthesized in the brain, pituitary gland, skin, and the immune system of humans (Yeo *et al.*, 2000). The expression in the brain was found to be in the ARC of the hypothalamus and the nucleus tractus solitarius (Young *et al.*, 1998). The expression control of POMC by leptin is induced by JAK/STAT pathway, so that binding of STAT3 to POMC promoter, histone acetylases are recruited and POMC mRNA expression increases (Kim *et al.*, 2006). However, when STAT3 gene is deleted, POMC mRNA expression slightly decreased, suggesting an additional mechanism in the POMC expression regulation (Xu *et al.*, 2007). The other mechanism in the POMC gene expression was found to be phospatidylinositol 3-kinase (PI_3K) pathway. Once inositol-3-phosphate (PI_3) is activated, it phosphorylates the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Plum *et al.*, 2006). PI_3K phosphorylates the forkhead box protein O1 (FOXO1) and once FOXO1 reaches to nucleus, it increases the expression of POMC (Fig.1.12) (Belgardt *et al.*, 2008).
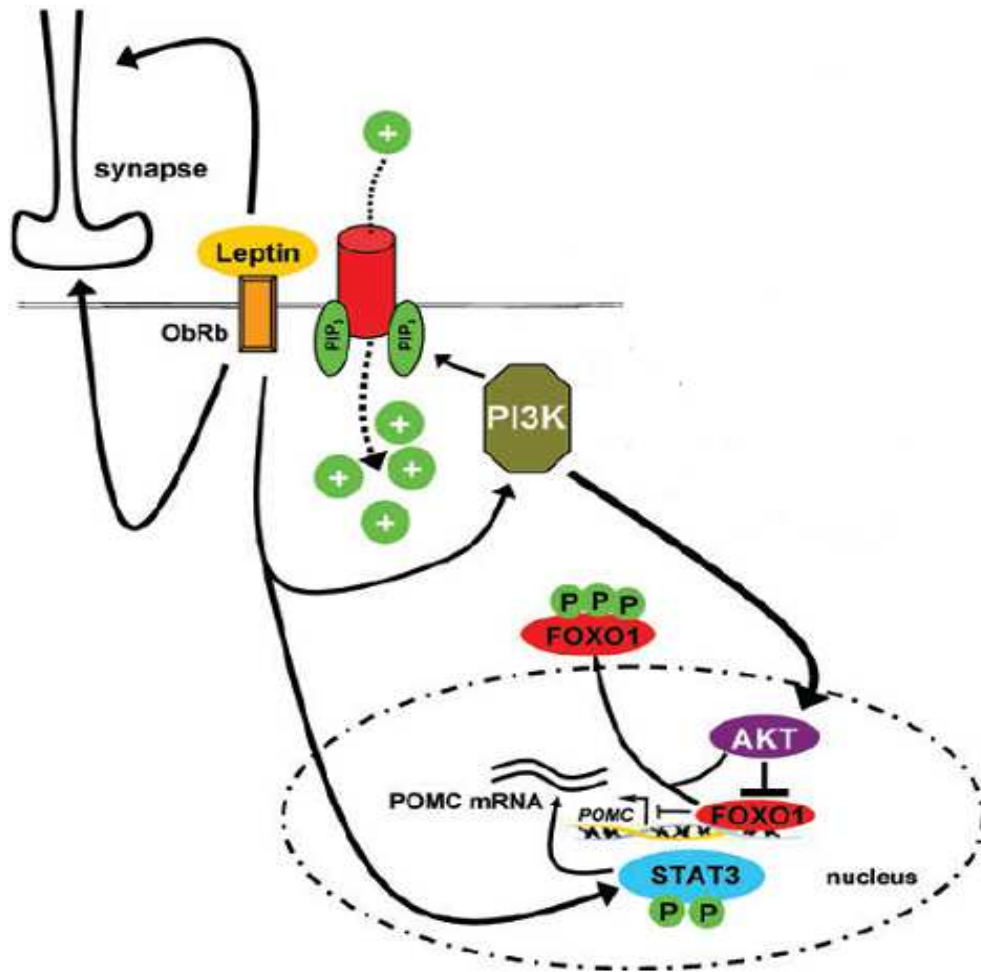
Figure 1. 12 The expression control of POMC by leptin (Belgardt *et. al*, 2009).

After the expression of POMC as a pro-hormone, it is post-translationally cleaved by PCs to form ACTH, β-endorphin, α-, β-, and γ-melanocyte stimulating hormones (MSH) (Fig.1.13) (Castro and Morrison, 1997).
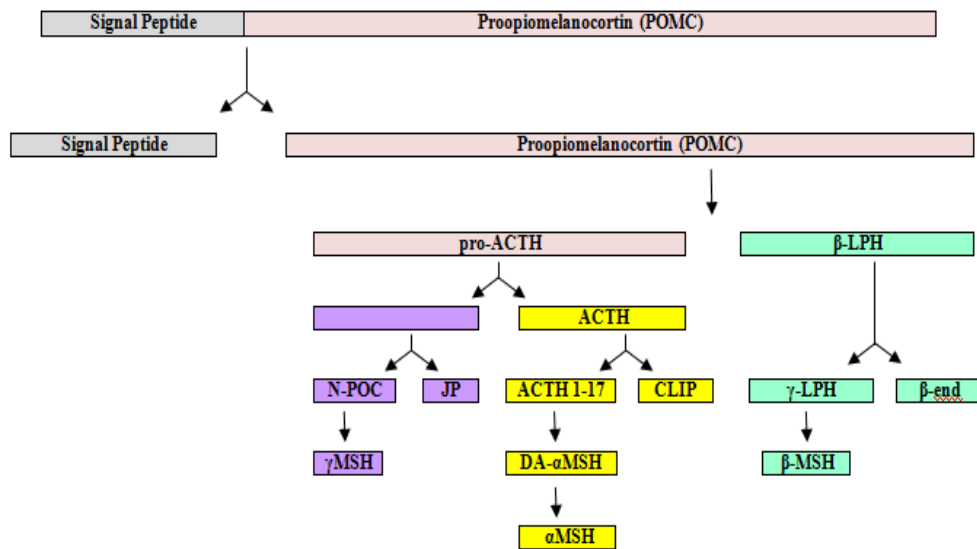
Figure 1. 13 The post-translational cleavage of POMC (modified from Pritchard *et al.*, 2002).

The POMC-derived anorexigenic peptide α-MSH exerts its functions on MCRs. More specifically, α-MSH binds to MC3R and MC4R for the appetite regulation (Pritchard *et al.*, 2002). MC4R is highly expressed in the brain, especially in the parts related to feeding mechanisms, such as PVN, dorsomedial hypothalamus (DMH), and LHA (Mountjoy *et al.*, 1994). The MC4R knockout rats showed hyperphagic behavior, together with obesity, but their growth, reproduction or thyroid functions were normal, which is indicating the significance of MC4R in the feeding control mechanism (Vaisse *et al.*, 1998). On the other hand, MC3R expression was shown to be in the POMC neurons suggesting that MC3R might have a feedback role in the ARC of the hypothalamus (Jegou *et al.*, 2000). Moreover, MC3R knockout mice showed obesity but not hyperphagic behavior (Butler *et al.*, 2000). The central administration of α-MSH resulted with a severe reduction in the food intake due to strong MC4R agonism (Fan *et al.*, 1997).

Another control mechanism on the POMC neurons is the serotonergic receptors. It was shown that 5-HT$_{2C}$ receptor agonists activate the α-MSH expressing neurons (Heisler *et al.*, 2002). Moreover, when serotonin activity increasing agents were administered to the CNS, decrease of the food intake and the weight loss was seen (Simansky, 1996). Serotonin administration was found to increase the POMC mRNA expression in the anterior pituitary of rats (Kageyama *et al.*, 1998). The serotonin binding to its 5-HT$_{2C}$ receptor activates the GPCR subunits, and activated α-subunit increases the PLC activation, resulting with the formation of diacylglycerol (DAG) and IP$_3$ from the membrane lipid PIP$_2$. Moreover, this process results with the activation of G-protein coupled inwardly-rectifying potassium channels (GIRKs) and the potassium is released from the POMC neuron. This is suggested to be the mechanism how 5-HT$_{2C}$ receptor, activates the POMC neuron (Figure 1.14) (Qiu et al., 2007)
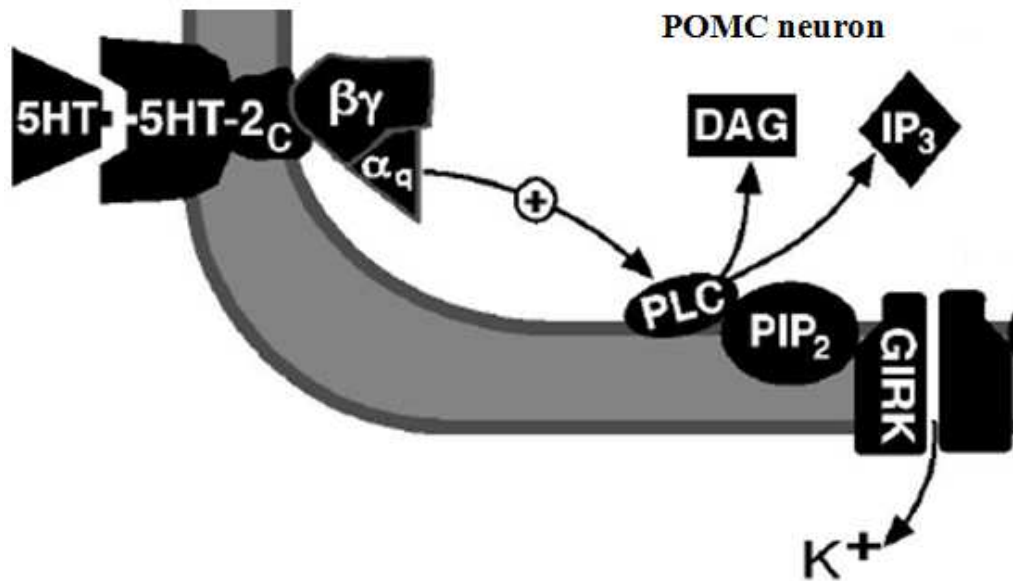
Figure 1. 14 The downstream pathway how serotonin activates POMC neuron (Qiu *et al.*, 2007).

POMC-derived hormones, especially ACTH, have significant roles in stress response. The stress conditions in humans was shown to result in a significant increase in the plasma ACTH levels (Meyerhoff *et al.*, 1988). Moreover, the POMC mRNA expression was increased during the stress condition of rats in different hypothalamic region (Baubet *et al.*, 1994). The stimulation of POMC-derived hormones α-MSH and β-endorphin, inhibit the central stress system responses (Charmandari *et al.*, 2005).

1.4.2.2.2 Cocaine and amphetamine regulated transcript (CART)

CART expression was found in the highest concentrations in the hypothalamus (Kuhar and Vechia, 1999). CART expression was also detected to be in different tissues, such as pituitary, retinal ganglion cells, olfactory bulb, spinal cord, cortical neurons, and medulla of the adrenal glands (Couceyro *et al.*, 1997). CART neurons were colocalized with POMC neurons in the ARC of the hypothalamus (Elias *et al.*, 1998). Two important roles of CART are the regulation of feeding behavior as an anorexigenic peptide and the regulation of the stress responses (Vrang *et al.*, 1999; Smith *et al.*, 2004).

The human and rat CART peptides differ in terms of their length and form. The rat pro-CART, after the cleavage of the 27 amino acid leader sequence, consists of long and short forms, with 102 and 89 amino acids, respectively (Kristensen *et al.*, 1998). On the other hand, pro-CART is just found as its short form in humans (Douglass and Daoud, 1996). The bioactive CART is formed by the cleavage of pro-CART with PCs, specifically PC2 and PC1/3 work together to form the resulting bioactive CART peptide (Dey *et al.*, 2003). By the alternative splicing of the CART forms, a number of biologically active CART peptide is formed in different tissues (Dylag *et al.*, 2006).

CART mRNA expression, similar to the POMC expression, is activated by the leptin receptor, JAK/STAT mechanism. The mouse and human CART promoter sites are conserved and suggests that leptin affects the CART mRNA expression same in both species (Dominguez *et al.*, 2002).

Due to the different biologically active forms of CART and its different physiological roles in the body, it was suggested that there might be more than one CART receptor (Vicentic *et al.*, 2006). The CART receptor has not cloned yet but there is litle information about the CART receptors on neuron-like cells, and hence, its downstream pathways remain to be elucidated (Lin *et al.*, 2011; Cheung and Mao, 2012).

The wide distribution of the CART in the body was shown and there is a remarkable CART expression in the hypothalamo-pitutary axis, which is shown as the role of CART in the stress responses (Koylu *et al.*, 2006). It was shown that when CART is administered to the hypothalamic cell cultures, it causes the stimulation of CRF and introcerebroventricular CART administration results with increase in the ACTH and cortisol levels, which are triggering the stress responses (Stanley *et al.*, 2001).

1.4.3 The interaction between anorexigenic and orexigenic peptides in the arcuate nucleus of the hypothalamus

While NPY and AgRP exert their function on feeding mechanism via their neuronal projections to PVN and LH, POMC and CART project their axons to medial hypothalamus (Konturek *et al.*, 2005), there is a strong feedback mechanism between those neuropeptides in the ARC.
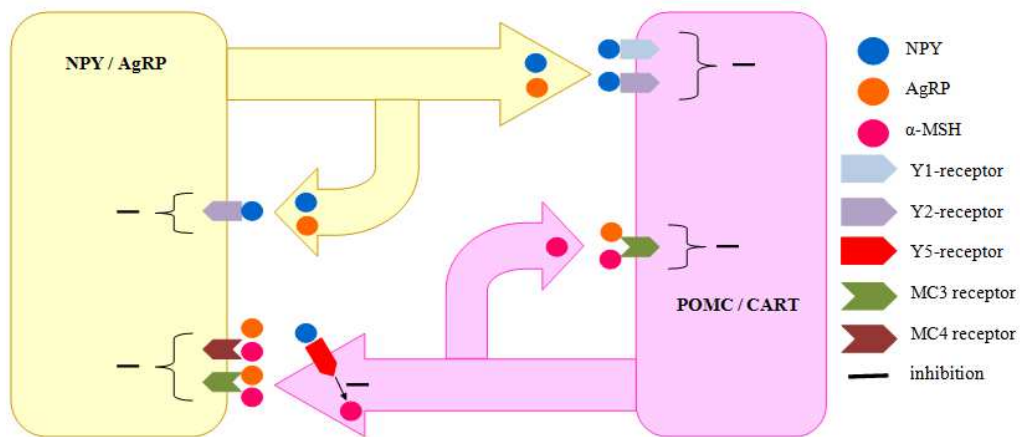
Figure 1. 15 The interactions between the orexigenic and anorexigenic peptides in arcuate nucleus of hypothalamus (modified from Mercer *et al.*, 2011).

As described in the Fig.1.15, the synthesis and secretion of NPY and AgRP have inhibitory roles on POMC/CART neurons, by binding of NPY to Y1 and Y2 receptors, and binding of AgRP to the MC3R. Moreover, when NPY binds to its Y5 receptor on the presynaptic cleft of POMC/CART neuron, it causes the inhibition of α-MSH release to the synaptic cleft and prevents the interaction with melanocortin receptors on NPY/AgRP neuron. Furthermore, NPY binds to Y2 receptor on the NPY/AgRP neuron to downregulate its and AgRP's expression. On the other hand, α-MSH secreted from POMC neurons, binds to MC3 and MC4 receptors to inhibit the expression of NPY and AgRP. Also, α-MSH has a feedback role, by binding to MC3 receptor on the POMC/CART neuron, it inhibits the synthesis of POMC and CART as a negative feedback regulator (Mercer *et al.*, 2011).

1.5 The studies about the weight gain side effect of risperidone on rats

Besides the human studies, there are a number of studies showing the effects of antipsychotics on the rats. However, risperidone, as an antipsychotic causing moderate weight gain, was used in a limited number of studies with different routes of administration and similar physiological measurements. Risperidone

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injection to the rats in a dose dependent manner, its lowest dose administered (0.005 mg/kg), caused an increase in the food intake, significant body weight gain, and an increase in the leptin gene expression in the adipose tissue. However, the same study showed that the highest concentration (0.5 mg/kg) caused a reduction in the body weight of the rats. These differences in the results were explained as the stress condition caused by the daily injection (Ota *et al.*, 2002). Subcutaneous administration of risperidone to both male and female rats caused a significant increase in the body weight in the female rats, while caused no significant change in the male rats. This was explained as not the sex difference but the route of administration (Baptista *et al.*, 2002). When risperidone is administered by intraperitoneally, it caused a significant increase in the food intake and the body weight of female rats (Fell *et al.*, 2004). Risperidone administration in the peanut butter to the mice resulted with an increased food intake and decreased activity, leading to a significant increase in the body weight (Cope *et al.*, 2009). Twenty-one days of risperidone administration to rats caused no change in the leptin receptor genes and the peptides having role in the appetite regulation in the hypothalamus (Ota *et al.*, 2005). Overall, these studies indicate that the most important side effect of risperidone is the weight gain, revealed in both rat and human studies.

1.6 The aim of the study

For some time, the weight gain side effect of risperidone was ignored since it treats the schizophrenic symptoms. Due to the weight gain effect of risperidone, the patients generally do not want to continue the treatment, which affects the consistency of the treatment. In the long term use, risperidone causes a significant increase in the body weight and, consequently, leads to obesity. This situation results in depression and social incompatibility of the patients, even if they are recoverd from the symptoms of schizophrenia.

In this study, we aimed to explore the alterations of the appetite regulating neurohormones, POMC, CART, NPY, and AgRP in both expression levels and in periphery, together with the adipocyte derived hormone leptin's peripheral levels after 4-weeks of risperidone treatment. We hypothesized that, risperidone, via the serotonergic antagonism, might have causing alterations in the expression levels and plasma levels of those candidate orexigenic and anorexigenic peptides, which have roles in appetite regulation, synthesized and secreted from the ARC of the hypothalamus.

Firstly, the peripheral changes of those hormones were detected in male schizophrenic patients who were treated with risperidone for 4-weeks. After that, in order to explain those changes further, those candidate genes' (POMC, CART, NPY, AgRP) expressions and peripheral levels were determined in the hypothalamus and plasma samples; respectively, of the male Wistar-rats who were administered risperidone for 4-weeks.

CHAPTER 2

MATERIALS AND METHODS

2.1 Patients and treatment

Random twenty-four male schizophrenic patients who applied to Gülhane School of Medicine (GATA) were included in this study. Patients having Axis I diagnosis (except for a psychotic disorder) or an extra medical disorder, or using psychoactive substances and drugs affecting the CNS, were not included in the study. The diagnosis of first episodes of schizophrenia was confirmed by means of the Structured Clinical Interview Form for DSM-IV (SCID) (Spitzer *et al.*, 1992). After the diagnosis, the twenty-four patients were hospitalized. However, seven of them had to be excluded from the study due that two of them were decided to be treated with a different drug during the hospitalization and five of them were diagnosed as bipolar disorder instead of schizophrenia during the six months of follow up. Thus, the study group consisted of seventeen male schizophrenic patients. As soon as the patients were hospitalized their weight and height were measured, and their whole blood samples were collected into tubes containing EDTA to prepare the plasma samples. Those values of the patients immediately after the hospitalization and before the risperidone treatment were referred as "before treatment" throughout this study. Moreover, the control group was including seventeen healthy individuals with no endocrinological or psychological health problems. The control and the patient group had similar sociodemogrophic features, were in the same age interval (20-30 years old) and the same gender. The control

groups' weight and height were also measured and their whole blood samples were collected for the plasma preparation.

Immediately after the hospitalization and the measurements, patients were started to be administered risperidone at a dose of 2-4 mg/day, which was the dose decided by the clinicians. The dose change was applied according to the response of the patients and the final dose was 4-8 mg/day, with a mean value of 4.6±1.2 mg/day. The duration of the risperidone treatment was four weeks. At the end of the fourth week, the weight and the height of the patients were again measured and their blood samples were again collected to determine the values after the treatment, which was referred as "after treatment" throughout this study. All the measurements and the collection of the blood samples were done early in the morning before breakfast.

During the hospitalization period, the patients were fed with the standard hospital food with 2500 kcal/day but they were allowed to extra food intake by using the hospital cafeteria.

All the procedures were approved by the GATA Human Research Ethics Committee for the ethics and patients rights, and the consent letters were obtained from patients or their legal guardians.

2.2 Plasma sample preparation

4 ml of blood samples of both patient and control groups were collected twice at the beginning and at the end of the four-week period in GATA by nurses. Blood samples were in EDTA-preserved blood tubes and their plasma samples were prepared by 3.000x g centrifugation for 10 minutes. Plasma samples were collected into serum tubes and kept at -80^0C until use in experiments.

2.3 Rat Studies

2.3.1 Animals

Four weeks old 25 male Wistar rats were used for this study. The age of the rats were decided as they have intact hypothalamic orexigenic and anorexigenic neurons in the hypothalamus (Garcia *et al*., 2010). Fifteen of them were for the risperidone administration group and 10 of them were in the vehicle group. The vehicle group was the control group of the study to ignore the effect of the sucrose in the drug administration. Rats were housed three of them per cage under a 12-hour light/dark cycle (lights on at 7 a.m.), in a room maintained at a temperature of $22^0C \pm 2°C$ and a humidity of 60% with free access to water and 200 g routine food *ad libitum* daily. Each day meals were weighed to determine food consumption. The rats used in this study were healthy rats, not schizophrenic rats. Despite the existence of some schizophrenia model rats, since schizophrenia is a complex disorder as mentioned in introduction part, and there are a number of disturbances in the brain neurotransmitter system, the clinicians in GATA do not think that any of the models exactly reflects the schizophrenia.

Also, in this study male rats were used due to the hormonal complexity and the menstrual cycle's effects on the hypothalamic systems (Carey *et al*., 1995). In addition to this knowledge, since the human study group was consisting of men, for the correlation of the results, we studied with the male rats.

2.3.2 Preparation and administration of risperidone

Risperdal® (risperidone) (Janssen Pharmaceuticals, Inc., USA) 1mg/ml oral solution was used for the drug administration which was administered in 10% sucrose solution. Since the half life of risperidone is less than 12 hours in rats

(Van Beijsterveldt *et al*., 1994), it was administered for two times, one is in the morning (09:00 a.m.) and the second one is in the evening (6:00 p.m.); therefore 2mg/kg rat/day risperidone dose was used (Aravagiri *et al*., 1998). The average weight of the risperidone group was 160.71 grams; therefore each rat received 0.32 mg / day. For each rat, 0.16 ml of Risperdal® was mixed with 1,84 ml of 10% sucrose solution to a final volume of 2 ml solution. The pH of this solution was adjusted to approximately 8.00 by addition of 1M of NaOH corresponding to the physiological pH. Each rat had received the drug orally with the help of syringes as shown in Fig.2.1. The dose was fixed and according to their weights the needed volume was calculated and administered. The route of the drug administration in this study was chosen as the least stressful one, which is the oral administration with syringe, not by gavage or injection (Schleimer *et al.*, 2005).

The vehicle group which included 10 rats was fed with 2 ml of 10% sucrose solution twice daily at the same time points with the risperidone group under the same conditions. Two of the rats in the risperidone group were excluded from the study since they were injured by their cage mates and sacrificed. Another one is excluded due to an inflammation on its eye. Moreover, two of them were excluded during the RNA isolation step due to experimental errors. Experiments and statistical analyses were done with 10 rats in the risperidone group and 10 rats in vehicle group.

Each rat was weighed once a week and both their weights and food consumptions are represented in the Figure 3.6 and Figure 3.7, respectively.

Figure 2. 1 The administration way of the sucrose solution and risperidone solutions.

2.3.3 Removal of hypothalamus

First of all, all equipments, such as scissors, foreceps, pipette tips, microcentrifuge tubes, and etc., to be used in the procedures and the subsequent parts were treated with DEPC (diethyl pyrocarbonate). DEPC (1ml, Sigma Aldrich, Germany) was added to 1 lt of distilled water and all equipments were kept in this solution overnight under the hood. Then, excess DEPC solution was removed and all equipment was autoclaved.

Rats were sacrificed by giotin after 10 seconds of CO_2 treatment. Following the decapitation, rats' hypothalamus region was removed (Fig.2.2) and put into microcentrifuge tubes with 400 µl of RNA*later* solution (Qiagen, Germany). Tissues were kept at 4^0C overnight in the RNA*later* solution, and they were removed from the solution by the foreceps and passed into a new microcentrifuge tubes to be stored at -80^0C until RNA isolation process.

Figure 2. 2 The hypothalamic part (in the red circle) in the removed brain region of the rat

2.3.4 Collection of blood sample and plasma preparation

Immediately after the rats were sacrificed, their trunk blood was collected into EDTA-preserved tubes and their plasma samples were prepared by 3.000 x g centrifugation for 10 minutes. Plasma samples were collected into serum tubes and kept at -80^{0}C until use.

2.3.5 Total RNA Isolation

For the determination of the expression changes in the hypothalamic tissue, first of all, the total RNA were isolated. The RNA isolation protocol was a modification of Chomczynski, 1993. Tissues were removed from -80^{0}C and put onto ice. Before thawing, 1 ml of phosphate buffered saline (PBS) (Sigma Aldirch, Germany) was added onto the tissues. The preparation of the 1x PBS solution was putting one tablet of the PBS into 200ml of distilled water. The solution was containing 0.01M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, with the pH 7.4 at room temperature. For the partial removal of RNA*later* solution inside the tissue; tissues were centrifuged at 12.000 x g for 40 seconds. Tissues were removed from PBS solution and put

into new microcentrifuge tubes and 1 ml of TRI reagent (Sigma Aldrich, Germany) was added to each tissue. Tissues were homogenized with the glass-teflon homogenizer system on ice and after homogenization; they were incubated at room temperature for 10 minutes. After the incubation, the samples were centrifuged at 12.000 x g for 10 minutes at 4^0C. After the centrifugation, the supernatant was taken to another microcentrifuge tube and the pellet containing the cell debris was discarded. Two hundred µl of chloroform (Sigma Aldrich, Germany) was added to each sample and vortexed for 30 seconds. The samples were incubated for 3 minutes at room temperature and they were centrifuged at 12.000 x g for 15 minutes at 4^0C. At the end of the centrifugation, three layers were observed. The lower layer contained proteins; the interphase was DNA and the upper aqueous phase was total RNA. In order to isolate the total RNA free from protein and DNA contamination, the upper aqueous phase was taken to a new microcentrifuge tube and 200µl of chloroform was added and vortexed for 30 seconds. The samples were then incubated for 7 minutes at room temperature and centrifuged at 12.000 x g for 15 minutes at 4^0C. After the centrifugation, the upper aqueous phase taken to another microcentrifuge tube and 500µl of isopropanol (Sigma Aldrich, Germany) and 2 µl of glycogen was added to the sample. The samples were incubated at -20^0C for 40 minutes and then centrifuged at 12.000 x g for 25 minutes at 4^0C. The supernatant was removed and 1ml of 75% ethanol was added to wash the pellet. The samples were centrifuged at 7.500 x g for 5 minutes at 4^0C and after the centrifugation the ethanol was removed by air-drying. 30µl of nuclease free water was added to the pellet to dissolve total RNA. Then, the samples were incubated at 65^0C for 10 minutes to remove the RNA*later* stucked protein or DNA clusters that will interfere with the subsequent procedures. Finally, the concentrations of the RNA samples were measured by using the NanoDrop 2000 (Thermo Scientific, US). By dropping 2 µl of the samples to the NanoDrop, the concentration measurement, A_{260}/A_{280}

and A_{260}/A_{230} measurements were done by the software programme of NanoDrop 2000.

2.3.6 DNase Treatment of the samples

For the removal of DNA contaminaton, if any, in the total RNA samples, deoxyribonuclease (DNase) treatment procedure was applied. DNase treatment was done by using the Ambion DNA-*free*^{TM} DNAse Treatment Kit (Invitrogen, Germany). The experiment was done according to manufacturer's instructions. 0.1 volume of the RNA solution, which is 3µl, of 10x DNase I buffer and 1µl of rDNase I (recombinant deoxyribonuclease I enzyme) was added to each sample and gently mixed. The samples were incubated at 37^{0}C for 30 minutes. Then, 3µl of the DNase inactivation reagent was added and the samples were incubated at room temperature by inversions. The samples were centrifuged at 10.000 x g for 1.5 minutes to remove the inactivation reagent. After the centrifugation, the supernatant containing the RNA was transferred to a new tube and then again the concentrations of the RNA samples were measured by using the NanoDrop 2000 (Thermo Scientific, US). Those measurement results are shown in Table 3.1. The concentrations were measured and, A_{260}/A_{280} and A_{260}/A_{230} ratios were determined to check the purity of the RNA samples. Moreover, the samples were run on 1% agarose gel to qualify the RNA samples purity and integrity (Fig.3.8 and 3.9). The values of A_{260}/A_{280} between 1.80 - 2.00 indicate the purity of RNA and the absence of DNA contamination. If this value is less than 1.80 it shows protein contamination. All RNA samples' A_{260}/A_{280} ratio is greater than 1.8, which indicates that there is no protein contamination in the samples. Furthermore, A_{260}/A_{230} ratio is also used to check the presence of contaminants, such as EDTA, TRI reagent, that give peak at 230 nm wavelength and the purity is determined with a A_{260}/A_{230} ratio of approximately 2.0.

2.3.7 Agarose Gel Electrophoresis of DNase treated RNA samples

Firstly, for the preparation of agarose gels, 50x stock Tris-Acetate-EDTA (TAE) buffer was prepared. 121 g of Tris and 37.2 g of EDTA were put into a beaker. By adding a little amount of distilled water, Tris and EDTA was dissolved on the magnetic stirrer by heating. Then, 28.55 ml of glacial acetic acid was added (pH 8.4), then pH was adjusted to pH 8.0 with HCl. The volume was completed to 500ml by adding distilled water. 1X TAE was prepared by adding 490ml of distilled water to 10ml of 50x stock TAE. 1X TAE was used to prepare 1% agarose (Sigma Aldrich, Germany) gels.

For the final preparation of the gels, firstly 0.5g of agarose (Sigma Aldrich, Germany) was dissolved in 50ml of 1X TAE buffer by mixing in an erlenmayer flask. Then, the flask was put into microwave oven and heated to dissolve the agarose completely for two minutes. Then, the flask was removed from the oven and it was allowed to cool down by continuous shaking. 1μl of ethidium bromide solution (AppliChem, Germany) was added to the gel and mixed for the homogenous distribution of ethidium bromide. The gel solution was poured onto the tank and the comb was placed on it. After the gel was solidified, the comb was removed and 1x TAE buffer was added to the electrophoresis tank in the way that the gel was completely in the buffer. Then the samples were loaded into the wells by mixing the samples with 6x gel loading solution (Fermentas, Germany). 2.5μl of the sample was mixed with 0.5μl of the loading solution on the parafilm and loaded into the wells. Moreover, 100bp DNA ladder (Sigma Aldrich, Germany) was also added to the first well to control the run of the gel. The gel was run with 100V current for 45 minutes.

2.3.8 cDNA preparation

After the RNA isolation procedure, in order to check the expression level, first step is the synthesis of the complementary DNA (cDNA) samples, since the polymerase chain reaction (PCR) is the amplification reaction of DNA template.

2.3.8.1 Primer Synthesis

The primers were synthesized according to their NCBI accession numbers and purchased from Sigma Aldrich, Germany. The primer synthesis was done as the primers bypasses the introns, and they bind to the end of the exon and the beginning of the next exon. The primers were too specific not to bind any possible contaminating DNA sample since the contaminating DNA samples would have the intronic sequences, and the designed primer bypasses the introns and only binds to the exons. The illustiration of the *GAPDH* primer design is shown in Appendix part A.10-A.12. All other genes' primers were designed as described for *GAPDH* and selected primers were listed in Table 2.1 representing the NCBI accession numbers, the oligonucleotide sequences, the T_m values, and the distilled water volumes for preparation of 100µM stock primers. Those primers were both used for cDNA synthesis and the quantitative real time polymerase chain reaction (qRT-PCR) experiments. Moreover, from the 100µM stock, firstly a 25µM stock was prepared by adding 75µl of dH_2O to 25µl of stock primer. Then, a 5µM working solution was prepared by adding 80µl of distilled water to 20µl of 25µM stock. All the experiments were performed by using 5µM working primer solution.

Table 2. 1 Primers used in cDNA Synthesis and qPCR experiments (RP: reverse primer, FP: forward primer)

Gene name and NCBI accession number	Sequence	T_m values	Distilled water added for 100µM stock	Product Length
GAPDH (NM_017008)	**FP**: 5'TCCCATTCTTCCACCTTT3'	59.5 ^{0}C	504 µl	92 bp
	RP: 5'TAGCCATATTCATTGTCATACC3'	58 ^{0}C	438 µl	
POMC (NM_139326)	**FP**: 5'AACATCTTCGTCCTCAGA3'	56.6 ^{0}C	413 µl	80 bp
	RP: 5'CGACTGTAGCAGAATCTC3'	53.7 ^{0}C	420 µl	
NPY (NM_012614)	**FP**: 5'AATGAGAGAAAGCACAGAAA3'	55.5 ^{0}C	411 µl	89 bp
	RP: 5' AAGTCAGGAGAGCAAGTT3'	52.9 ^{0}C	512 µl	
AgRP (NM_033650)	**FP**: 5'GAGTTCTCAGGTCTAAGTCT3'	51.4 ^{0}C	396 µl	97 bp
	RP: 5' GTGGATCTAGCACCTCTG3'	55.5 ^{0}C	537 µl	
CART (NM_017110)	**FP**: 5'TGAAGATTAGACCTAGTTAA3'	48.1 ^{0}C	500 µl	108 bp
	RP: 5' ATGCTTTATTGGGTGTAA3'	52.8 ^{0}C	460 µl	

2.3.8.2 cDNA Synthesis

cDNA preparation was done by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Germany). After the determination of the RNA sample concentrations, the concentration of the RNA to be used in cDNA preparation was adjusted as indicated in Table 2.2. Because of the lower concentrations of some RNA samples, the final concentrations varied during cDNA preparation, but they were adjusted as equal in qRT-PCR studies.

Table 2. 2 RNA concentrations and volumes used in cDNA Synthesis

Sample	Concentration (ng/µl)	Used volume for cDNA synthesis	Final concentration of RNA
V1	74.70	2.68 µl	200 ng/µl
V2	68.40	2.92 µl	200 ng/µl
V3	159.30	1.26 µl	200 ng/µl
V4	141.30	1.42 µl	200 ng/µl
V5	784.30	1.28 µl	1000 ng/µl
V6	439.00	2.28 µl	1000 ng/µl
V7	835.50	1.20 µl	1000 ng/µl
V8	764.50	1.31 µl	1000 ng/µl
V9	541.00	1.85 µl	1000 ng/µl
R1	836.00	1.20 µl	1000 ng/µl
R2	872.40	1.15 µl	1000 ng/µl
R3	640.50	1.56 µl	1000 ng/µl
R4	385.20	2.60 µl	1000 ng/µl
R5	174.00	2.87 µl	500 ng/µl
R6	743.30	1.35 µl	1000 ng/µl
R7	194.20	2.57 µl	500 ng/µl
R8	366.00	2.73 µl	1000 ng/µl
R9	1045.70	0.96 µl	1000 ng/µl
R10	1156.60	0.86 µl	1000 ng/µl

The cDNA synthesis was done according to the manufacturer's instructions. The volumes and the reagents that were used in cDNA synthesis reaction are depicted in Table 2.3. When all mixtures were prepared, the samples were incubated at 42^0C for 60 minutes and the reaction was terminated by incubating them at 70^0C for 5 minutes. All the primers used in this reaction were gene-specific, so for all samples, cDNA samples were prepared for each 5 gene, to increase the specificity.

Table 2. 3 cDNA synthesis reagents

The reagent	Volume
Template RNA	As in Table 2.2
Gene-specific primer	4µl (1µM)
5x Reaction buffer	4µl
RNase Inhibitor	1µl
10 mM dNTP solution	2µl
Reverse Transcriptase Enzyme (M-Mul-V)	1µl
Nuclease-free water	Complete to 20µl
Total volume	**20µl**

2.3.9 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR reactions were performed by using the SybrGreen Quantitative RT-PCR kit (Sigma Aldrich, Germany) according to the manufacturer's instructions. The reactions were run by the Corbett Rotor-Gene 6000 (Qiagen, Germany) and the instruments software system was used to report the results. Melting curves for each gene were analyzed to ensure specific amplicon replication, and all reactions performed in triplicates. The each reaction included no template control. Moreover, all the cDNA samples were diluted to get 100ng/µl final concentration, and these diluted samples were used in all qRT-PCR reactions. The reactions were optimized for each primer and gene for the efficient qRT-PCR reactions and the optimized reactions are shown at the following (Table 2.4 – 2.8).

Table 2. 4 The optimization of the GAPDH gene

Reaction component	Volume	Reaction conditions		
SybrGreen Mix	10 µl	94^0C	15 minutes	
Reverse primer (0.4 µM)	1.6 µl	94^0C	30 seconds	
Forward primer (0.4 µM)	1.6 µl	57^0C	30 seconds	35 cycle
cDNA	2 µl	72^0C	30 seconds	
Nuclease free water	4.8 µl	Melt (50^0C-99^0C)		
Final volume	**20 µl**			

Table 2. 5 The optimization of the POMC gene

Reaction component	Volume	Reaction conditions		
SybrGreen Mix	10 µl	94^0C	15 minutes	
Reverse primer (0.2 µM)	0.8 µl	94^0C	30 seconds	
Forward primer (0.2 µM)	0.8 µl	55^0C	30 seconds	35 cycle
cDNA	2 µl	72^0C	30 seconds	
Nuclease free water	6.4 µl	Melt (50^0C-99^0C)		
Final volume	**20 µl**			

Table 2. 6 The optimization of the NPY gene

Reaction component	Volume	Reaction conditions		
SybrGreen Mix	10 µl	94^0C	15 minutes	
Reverse primer (0.2 µM)	0.8 µl	94^0C	30 seconds	
Forward primer (0.2 µM)	0.8 µl	56^0C	30 seconds	40 cycle
$MgCl_2$ (5mM)	1.6 µl	72^0C	30 seconds	
cDNA	2 µl	Melt (50^0C-99^0C)		
Nuclease free water	4.8 µl			
Final volume	**20 µl**			

Table 2. 7 The optimization of the AgRP gene

Reaction component	Volume	Reaction conditions		
SybrGreen Mix	10 µl	94^0C	15 minutes	
Reverse primer (0.2 µM)	0.8 µl	94^0C	30 seconds	40 cycle
Forward primer (0.2 µM)	0.8 µl	55^0C	30 seconds	
cDNA	2 µl	72^0C	30 seconds	
Nuclease free water	6.4 µl	Melt (50^0C-99^0C)		
Final volume	**20 µl**			

Table 2. 8 The optimization of the CART gene

Reaction component	Volume	Reaction conditions		
SybrGreen Mix	10 µl	94^0C	15 minutes	
Reverse primer (0.4 µM)	1.6 µl	94^0C	30 seconds	35 cycle
Forward primer (0.4 µM)	1.6 µl	51^0C	30 seconds	
cDNA	2 µl	72^0C	30 seconds	
Nuclease free water	4.8 µl	Melt (50^0C-99^0C)		
Final volume	**20 µl**			

After all the qRT-PCR reactions were optimized and performed for each sample, the threshold cycles (Ct's) were determined for each sample. In order to determine the relative expression levels of the genes of interest relative to housekeeping gene, *GAPDH*, the calculation was done according to the described equation for the relative quantification described in Pfaffl, 2001. This equation allows doing the calculation even if the efficiencies are not equal for the housekeeping gene and the gene of interest. The equation is as following:

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct \text{ Gene of interest (control – sample)}}}{(E_{housekeeping})^{\Delta Ct \text{ housekeeping (control – sample)}}} \quad (2.1)$$

E_{target} = the efficiency of the reaction of gene of interest

$E_{housekeeping}$ = the efficiency of the reaction of housekeeping gene (*GAPDH*)

ΔCt = the difference between the Ct values of the control and samples. All the expression changes were calculated according to above efficiency corrected equation.

2.4 Enzyme Linked Immunosorbent Assay (ELISA) of all plasma samples

To determine the peripheral levels of the hypothalamic neuropeptides and leptin, Enzyme Linked Immunosorbent Assays (ELISA) was performed. All the ELISA reactions were done by using commercial kits (Phoenix Pharmaceuticals Inc., Germany) and according to the manufacturers' procedures. The human and the rats' plasma samples were analyzed using human and rat kits for the candidate genes.

The kit for the determination of the plasma leptin concentration was a sandwich enzyme immunoassay type. Before the kit procedures, all solutions were prepared according to the protocol. Biotin-labeled antibody was prepared by the addition of 100µl of biotin-labeled antibody to 900µl of biotin-antibody diluents. The wash buffer was prepared by mixing 100ml of the wash buffer with 900ml of the distilled water. All the other solutions were ready-to-use in the kit. First, the calibrators were prepared as shown in the Table 2.9. 100µl of the samples, calibrators, and the positive control were added to each well as duplicates of the 96-well plate, by leaving the first two well as blank. The plates were incubated at room temperature for one hour by shaking at 300 rpm on the orbital microplate shaker. After the incubation period, the wells were washed with the 200µl wash solution per well for three times. After the washing step, 100µl of the diluted biotin-labeled anti-mouse leptin antibody solution was added to the each well. The plates were again incubated at room temperature for one hour by shaking at 300 rpm on the orbital microplate

shaker. The washing step was repeated for three times. Then, 100µl of the Streptavidin-HRP (horse-radish peroxidase) conjugate solution was added to each well. The incubation was for 30 minutes on the orbital microplate shaker at 300 rpm at room temperature. The washing step was again repeated for three times. 100µl of the substrate solution was added to each well and the plated was sealed by aluminum foil to keep the wells away from the light. The plate was incubated for 10 minutes at room temperature. Finally, the 100µl of the stop solution was added to each well to stop the reaction and the absorbance was immediately measured by using the ELISA plate reader (SPECTRAmax 340PC, USA) at 450 nm. The standard curve was constructed by using the concentrations and the absorbances of the calibrators. The standard curve was constructed as absorbance at 450 nm of the calibrators (Y axis) vs. concentrations of the calibrators (X axis) in the logarithmic scale. The standard curve of the leptin ELISA is shown in Appendix A.

Table 2. 9 The preparation of the calibrators for leptin ELISA kit.

#	Volume of the calibrator	Dilution buffer (µl)	Concentration (pg/ml)
1	4000pg/ml (ready)	-	4000
2	250µl from calibrator #1	250	2000
3	250µl from calibrator #2	250	1000
4	250µl from calibrator #3	250	500
5	250µl from calibrator #4	250	250
6	250µl from calibrator #5	250	125
7	250µl from calibrator #6	250	62.5

The ELISA kits for the α-MSH, NPY, CART and AgRP were in same principle and same protocol, which were the competitive enzyme immunoassay. First, the 20X assay buffer concentrate was diluted to 1X by the addition of 950ml of distilled water. The peptide standards were diluted as shown in the Table 2.10. The primary antibody was rehydrated by the addition of 5ml of 1X assay buffer as well as biotinylated peptide. The positive controls were rehydrated by the addition of 200µl of 1X assay buffer. By leaving the first two wells as blank, 50µl of the samples, standards and positive controls were added to each well in duplicates. Then, 25µl of the primary antibody and after that 25µl of biotinylated peptide were added to each well in order, except for the blank wells. The plate was incubated at room temperature for 2 hours on the orbital microplate shaker at 300 rpm. After the incubation, the wells were washed with 200µl of 1X assay buffer per well, for four times. Then, the Streptavidin-HRP (SA-HRP) conjugate was diluted as adding 12µl of SA-HRP into 12ml of 1x assay buffer. This diluted SA-HRP was added to each well at a volume of 100µl after the washing step and the plate was incubated for 1 hour at room temperature on the orbital microplate shaker at 300 rpm. After the incubation period, the washing step was repeated for four times. Then, 100µl of the TMB substrate solution was added to each well and the plates were sealed by aluminum foil to keep the wells away from the light. The plates were incubated for 1 hour at room temperature and at the end of the incubation; 100µl of 2N HCl solution was added to each well to stop the reaction. The absorbance was immediately measured by using the ELISA plate reader at 450 nm. The standard curve was constructed by using the concentrations and the corresponding absorbances of the calibrators. The standard curve was constructed as absorbance at 450 nm of the calibrators (Y axis) vs. concentrations of the calibrators (X axis). The standard curve is shown in Appendix A.

Table 2. 10 The preparation of the standards for α-MSH, NPY, CART and AgRP ELISA kits.

#	Volume of the standard	1x Assay Buffer (µl)	Concentration (ng/ml)
Stock	1000µl stock	-	1000
1	100µl from the stock	900	100
2	100µl from standard #1	900	10
3	100µl from standard #2	900	1
4	100µl from standard #3	900	0.1
5	100µl from standard #4	900	0.01

2.5 Statistical Analysis

Frequency distribution for descriptive statistics, arithmetic mean, and standard deviation for continuous variables were calculated. For all studies, significance value (*p*), which was *$p < 0.05$*, obtained using a paired two-tailed Student´s t-test. Data are expressed as mean ± standard error.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 The Body Mass Index (BMI) of patients after the risperidone treatment

The 4-weeks risperidone treatment of schizophrenic patients resulted in a statistically significant increase (***$p<0.001$) in their BMI values (Fig.3.1). The control group's BMI was 23.30±0.92, while patients' BMI was 22.25±0.61 before the treatment and it increased to 25.08±0.71 with the treatment. Patients' average weight gain was 4.55 kg within the treatment.
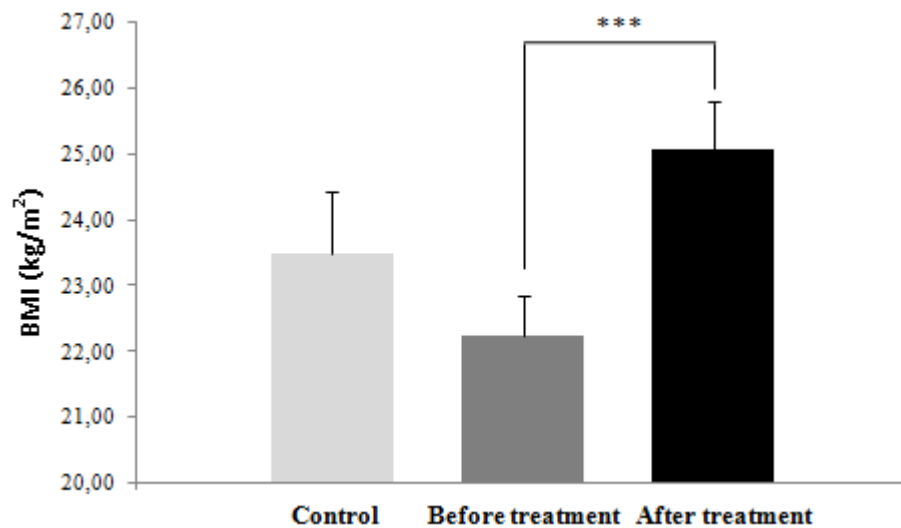

Figure 3. 1 BMI levels of control group and patients before and after the risperidone treatment. BMI was increased significantly in the patients after the treatment (***$p<0.001$) (all groups, n=17).

3.2 The plasma levels of peripheral and CNS neurohormones that affect food intake and appetite

3.2.1 Plasma leptin levels

Plasma leptin levels in schizophrenic patients increased significantly after the treatment with risperidone (*$p<0.05$). Control group's plasma leptin level was 1.53 ± 0.29 ng/ml. Patients plasma leptin level was 0.99 ± 0.17 ng/ml before the treatment and it increased to 1.68 ± 0.35 ng/ml after 4-weeks risperidone treatment. This indicates approximately a 60% increase in the plasma leptin levels of patients (Fig.3.2).
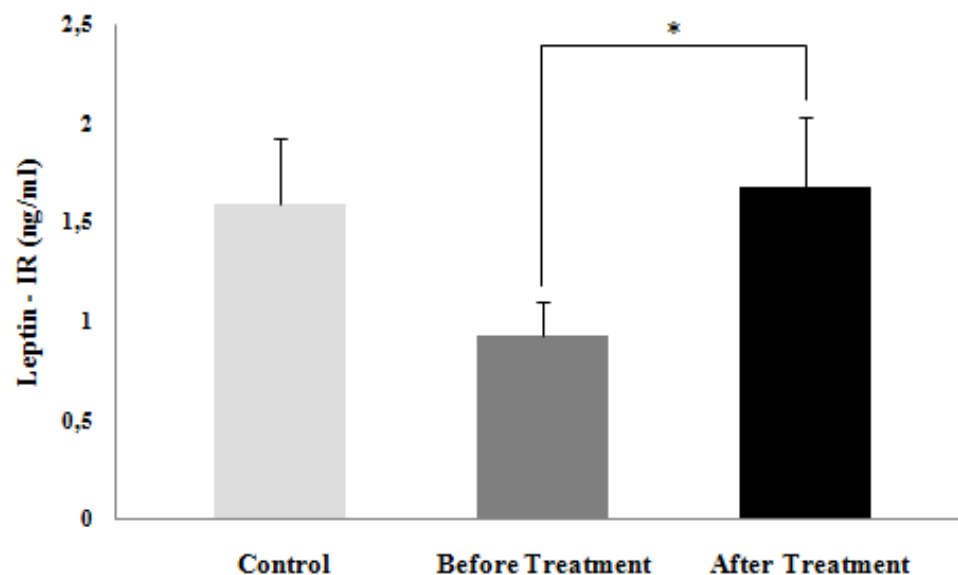

Figure 3. 2 ELISA analyses of plasma leptin concentrations in schizophrenic patients before and after the treatment. A significant increase was shown in patients after the treatment (1.68 ± 0.35 ng/ml) when compared with before treatment values (0.99 ± 0.17 ng/ml), *$p<0.05$. The control group value was 1.53 ± 0.29 ng/ml (n=17).

3.2.2 Plasma NPY levels

There is no statistically significant change in the plasma NPY levels of patients between before and after the treatment. Nevertheless, schizophrenic patients before treatment had significantly less NPY levels in the plasma when compared to control group (*$p<0.05$). The plasma NPY levels of patients were 0.73±0.13 ng/ml before the treatment and 0.66±0.14 ng/ml after the treatment while control group had 1.31±0.23 ng/ml (Fig.3.3).
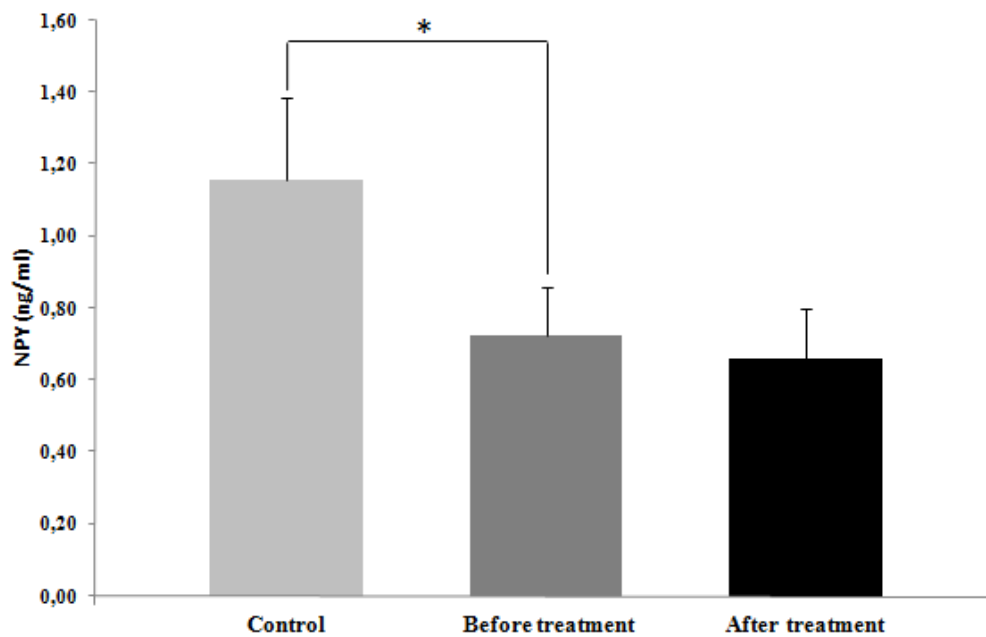

Figure 3. 3 The plasma NPY levels determined by ELISA in controls vs patients. The NPY levels were reduced in patients before treatment when compared with controls (*$p<0.05$). The NPY levels were 1.31±0.23 ng/ml in controls, 0.73±0.13 and 0.66±0.14 ng/ml in patients before and after treatment, respectively.

3.2.3 Plasma α-MSH levels

Schizophrenic patients before treatment had statistically significant less α-MSH levels in the plasma as compared to control group (*$p<0.05$). Although the plasma levels of α-MSH did not reach a significant value in the patients after the treatment, risperidone treatment kept less α-MSH plasma levels in patients. The plasma α-MSH levels of control group was 1.47±0.27 ng/ml, and the patients' plasma α-MSH levels was 0.58±0.17 ng/ml before treatment and it was 0.41±0.12 ng/ml after the treatment (Fig.3.4).
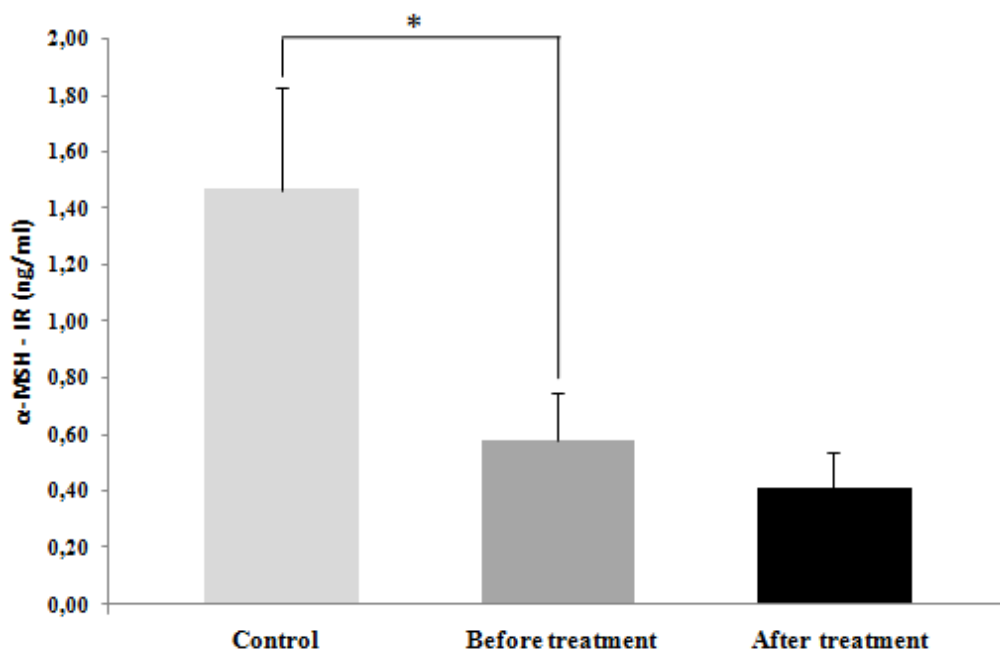

Figure 3. 4 The α-MSH plasma concentrations determined by ELISA in controls and patients. The α-MSH plasma levels of patients before treatment (0.58 ± 0.17 ng/ml) were 2.5 times lower than the controls (1.47 ± 0.27 ng/ml), *$p<0.05$. After the treatment, it was 0.41± 0.12 ng/ml.

3.2.4 Plasma CART levels

The plasma CART levels of schizophrenic patients were significantly higher than the levels of control group (*$p<0.05$). Control group's plasma CART levels were 0.90±0.16 ng/ml while the schizophrenic patients had 1.36±0.18 ng/ml plasma CART levels. However, after four-week risperidone treatment the plasma CART levels of patients decreased to 1.22±0.14 ng/ml, which did not indicate a statistical significance (Fig.3.5).
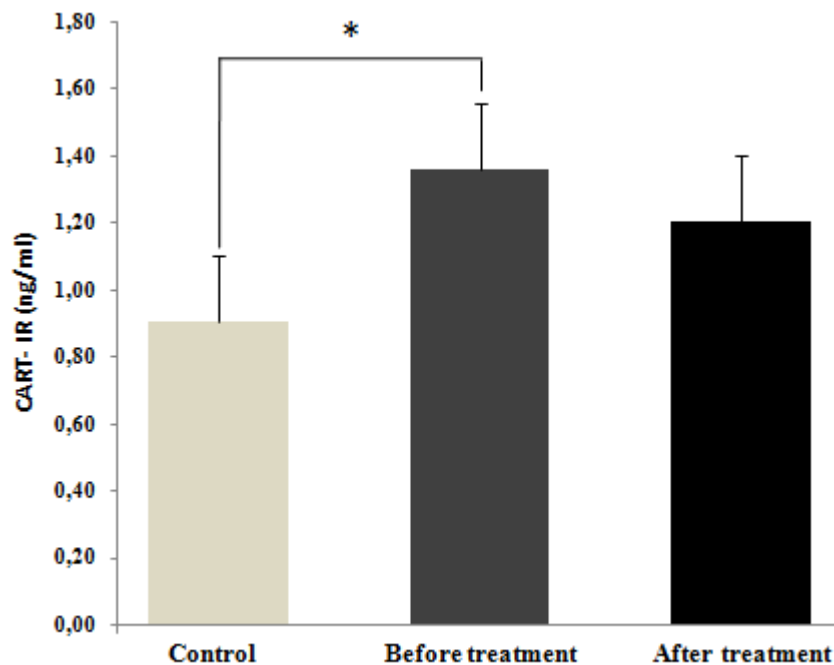

Figure 3. 5 The concentrations of plasma CART in controls (n=15) and patients (n=15). The CART levels were significantly changed between the control group (0.90±0.16 ng/ml) and before treatment (1.36±0.18 ng/ml) (*$p<0.05$). The levels of CART in after treatment were 1.22±0.14 ng/ml.

3.3 Rat Studies

After the analysis of the human plasma samples, to determine both the hypothalamic tissue specific expression levels, and the alterations in peripheral levels of the neurohomones affecting the appetite oral risperidone administered to the Wistar rats.

3.3.1 Change of weight and food consumption of rats

Rats were weighed once a week and the results indicated that the risperidone administered group gained more weight than the sucrose administered vehicle group. The vehicle group gained 72.30±10.59 g at the end of the 4-weeks of whereas the risperidone administered rats gained 91.58±5.84 g (Fig.3.6).
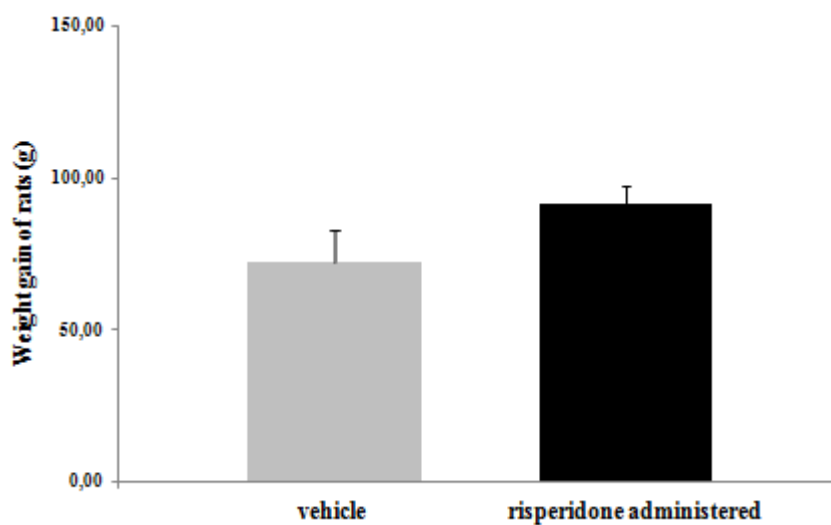

Figure 3. 6 Weight gain of the vehicle vs. risperidone administered rats. Vehicle group's mean weight gain was 72.30±10.59 g in the 4-weeks period, while risperidone administered rats gained 91.58±5.84 g at the end of the study.

The consumed food per cage was also measured every day. The risperidone administered rats consumed more food during 4-weeks. The food consumption per cage was calculated comparing the trend line of the consumed food (g) vs days of experiment graph. Risperidone administered rats trended to have more of food consumption that indicates increase in their appetite (Fig.3.7).
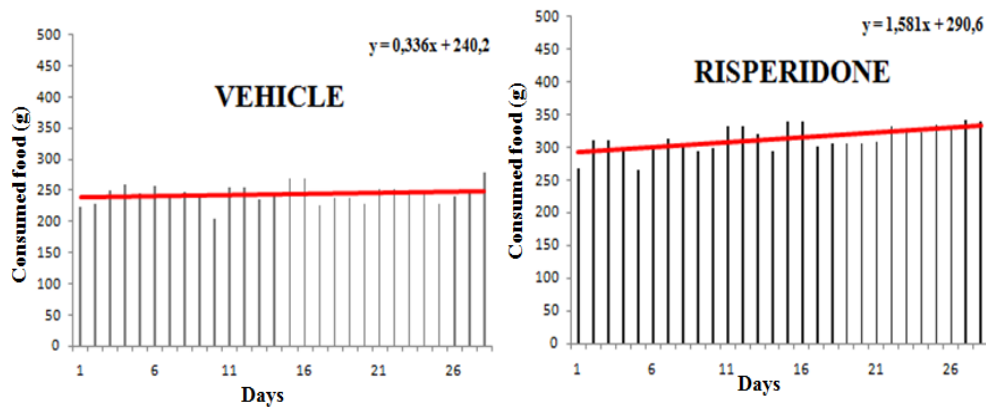

Figure 3. 7 The consumed food by the vehicle and risperidone administered group in the 4-weeks period per cage. The slope of the trend line is 0.336 for the vehicle group while the slope of the trend line for the risperidone treated group is 1.581. These slope values indicate that the risperidone administered group consumed more food in the 4-weeks period.

3.3.2 RNA concentrations and agarose gel electrophoresis results

Concentrations of isolated RNA from the rats' hypothalamic tissues were measured and ratios of A_{260}/A_{280} and A_{260}/A_{230} were detected. The results are shown in the Table 3.1. RNA concentrations were expressed in ng/µl and the concentrations were high enough to use in cDNA preparation and qRT-PCR experiments. Moreover, the values of A_{260}/A_{280} between 1.80 - 2.00 indicate the purity of RNA and the absence of DNA contamination.

Table 3. 1 RNA concentrations and A_{260}/A_{280} and A_{260}/A_{230} ratios of vehicle group (V) and risperidone adminestered group (R).

Sample	Concentration (ng/µl)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio
V1	74.70	1.93	1.33
V2	68.40	1.88	0.89
V3	159.30	1.98	1.35
V4	141.30	1.96	1.01
V5	784.30	1.91	1.20
V6	439.00	2.00	1.89
V7	835.50	2.04	1.95
V8	764.50	2.02	1.95
V9	541.00	1.99	1.93
R1	836.00	2.05	1.81
R2	872.40	2.07	1.92
R3	640.50	2.05	1.30
R4	385.20	2.01	0.98
R5	174.00	1.95	1.19
R6	743.30	2.04	1.51
R7	194.20	1.97	1.62
R8	366.00	2.00	1.11
R9	1045.70	2.03	1.89
R10	1156.60	2.02	2.01

The RNA concentrations and their A_{260}/A_{280} and A_{260}/A_{230} ratios were measured to check the quality of the RNA samples after the DNase treatment. The data show that the RNA samples are not containing contaminants and they are pure.

The hypothalamic RNA samples of all animals were run on the 1% agarose gel after the DNase treatment and the three bands of RNA (28S, 18S, 5S) were detected (Fig.3.8 is for vehicle and Fig.3.9 is for risperidone). The gel images show that expected RNA was intact with three bands of RNA (28S, 18S, and 5S) and free of DNA contamination that might interfere with the qRT-PCR studies otherwise.
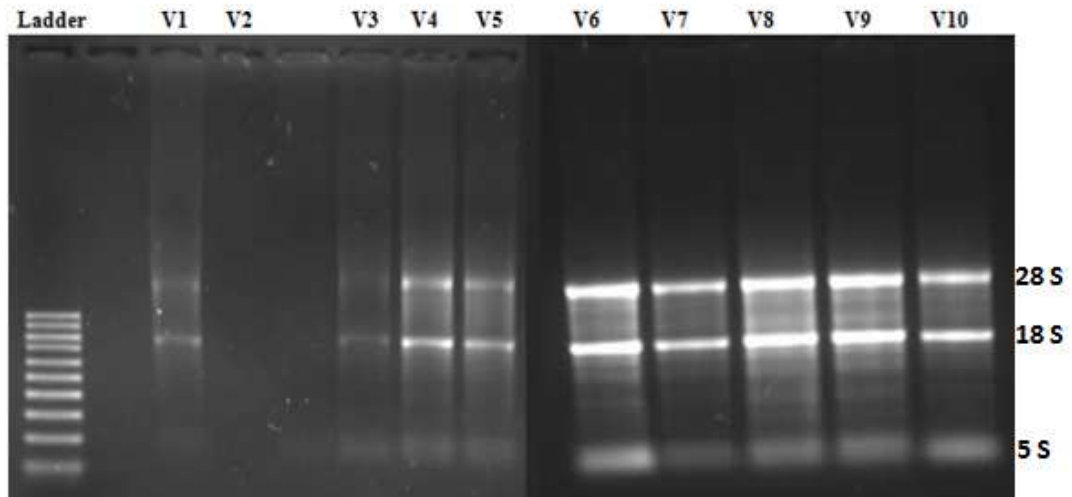

Figure 3. 8 Total RNA gel electrophoresis of the vehicle group. The RNA bands are 28S, 18S, and 5S, which shows quality of the samples.
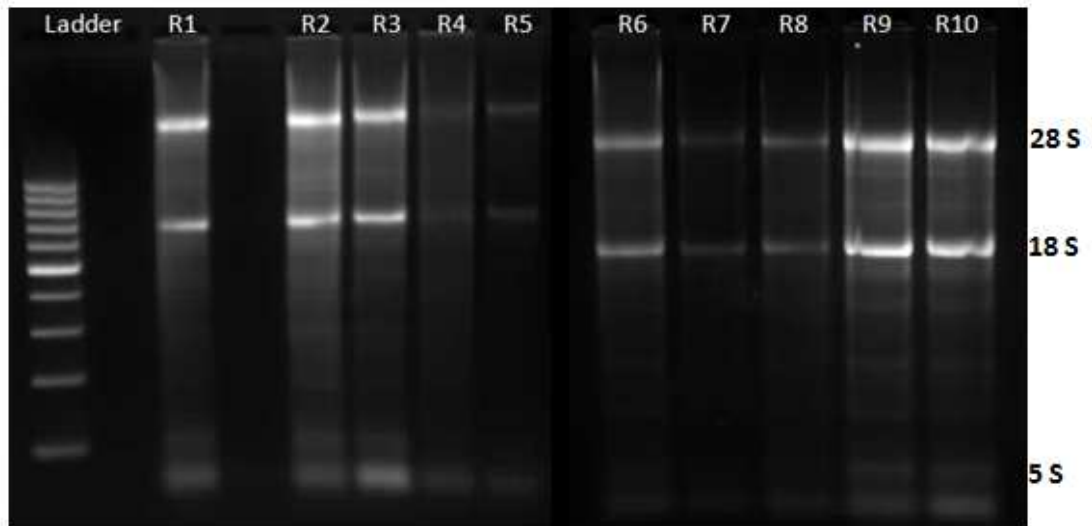

Figure 3. 9 Total RNA gel electrophoresis of the risperidone administered rats. The RNA bands are 28S, 18S, and 5S bands.

3.3.3 Quantitation results of the rats' hypothalamic samples

3.3.3.1 Analysis of qRT-PCR data

There are a number of criteria in the determination of the quality of qPCR. The most critical factor is the efficiency (E). When the amount of PCR product doubles in each cycle, this indicates a 100% efficiency of the reaction. The efficiency is determined by the slope of the standard curve. Standard curves with the slope of -3.322, gives the 100% efficiency, which is equal to 1. The efficiency of the reaction can be calculated by the following equation:

$$\text{qPCR efficiency (\%)} = 10^{(-1/\text{Slope})}-1) \times 100 \qquad (3.1)$$

The slopes of the standard curves between –3.3 and –3.8 are acceptable as efficient reactions (Pfaffl, 2001).

Another important determination factor for the qRT-PCR is the R^2 value which can be described as the proportion of the variability in the experiment. It might be explained as the variability of the samples' which are run in triplicates. The minimum variability gives the optimum predictable results from that reaction. R^2 value should be 1 for no variability, which is the case for only two samples. When the sample size increases, the variability also increases and the R^2 value deviates from 1. The acceptable R^2 value should be approximately 0.999. The closer the value to 1, the less the variance and the better the reaction.

In addition, the threshold cycle (Ct) which might be explained as the cycle when the detectable increase in the amount of the fluorescent is obtained, need to be determined. Ct is used for the calculation of the starting template amount to reach the expression.

Finally, the melt curve analysis is required to determine the specificity of the reaction. Each double stranded DNA, formed in the qPCR reaction has a melting temperature (T_m), at which the 50% of the double stranded DNA molecules become single stranded. The T_m value depends on the GC content of the DNA sample; hence, the source of the DNA. In the melt curve analysis, the SybrGreen dye relieves from the DNA when the 50% of the DNA dissociates to single strand. Thus, when the fluorescence decreases, and the middle point of the decrease line gives a peak which is specific for each length of the DNA produced. When there are different peaks in different temperatures, it means there are other contaminating DNA samples or primer dimers in the reaction. It is expected that there sould be only one peak at the same temperature for the all samples to indicate the specific expected product.

3.3.3.2 Standard curves for the quantitation of the candidate genes

3.3.3.2.1 The housekeeping gene, *GAPDH* standard curve

The standard concentrations of *GAPDH* were used at 400ng/rxn, 200ng/rxn, 20ng/rxn and 2 ng/rxn, whose Ct values are indicated on the quantitation curve with the arrows (Fig.3.10).
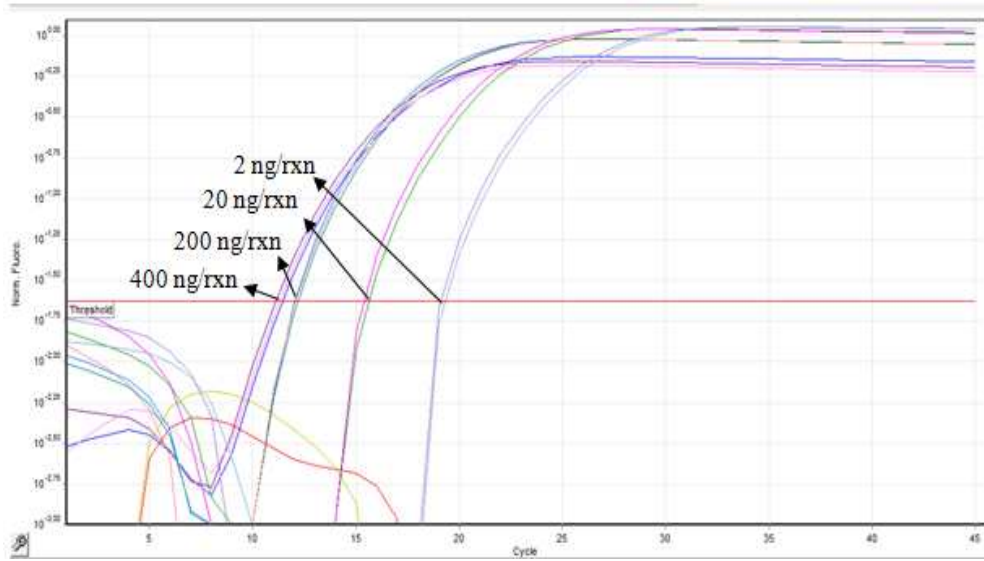
Figure 3. 10 The quantitation data of the GAPDH for the standard curve concentrations.
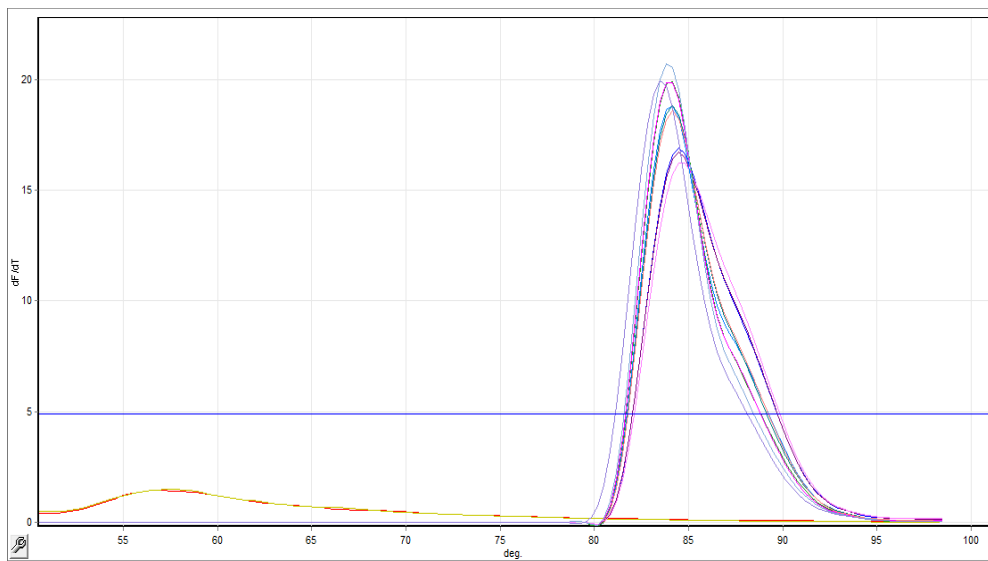

Figure 3. 11 The melt curve analysis of the GAPDH standards for the standard curve construction. All the standards give the peak at approximately at 84^0C.
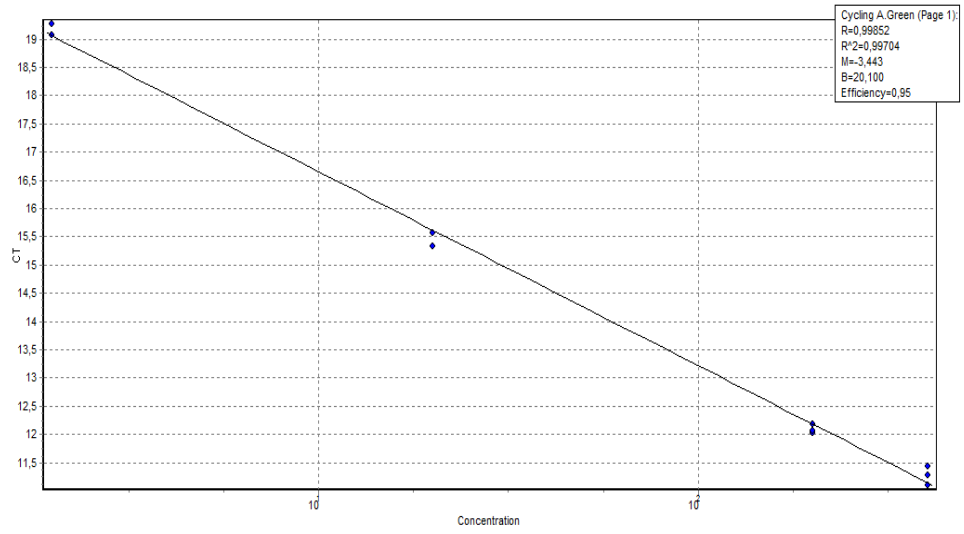
Figure 3. 12 The standard curve of the GAPDH. The efficiency of the reaction was 0.95 and the R^2 value was 0.99704.

3.3.3.2.2 The *NPY* standard curve

The standard curve for the *NPY* was obtained by using the standards 1000
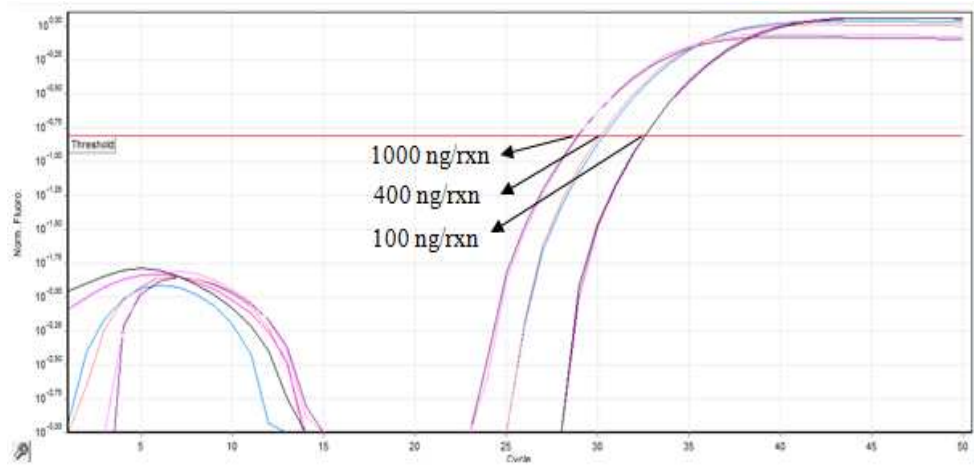ng/rxn, 400 ng/rxn and 100 ng/rxn (Fig.3.15).

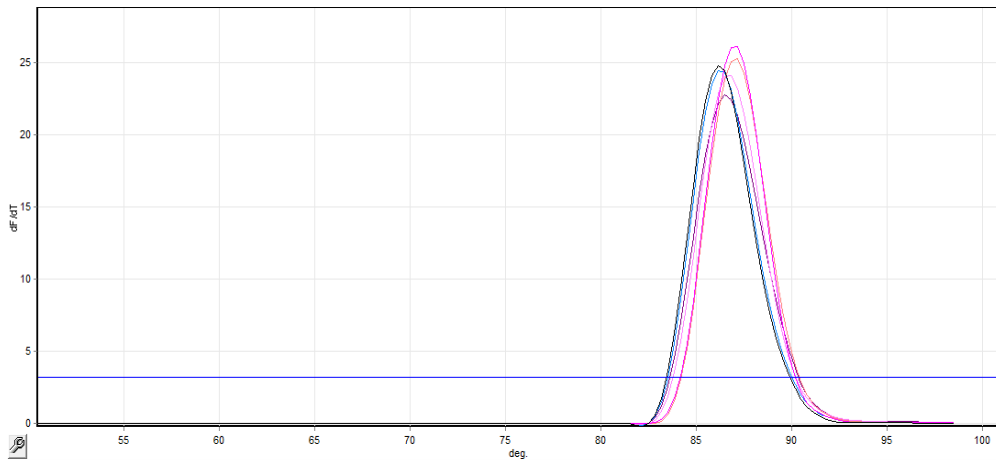**Figure 3. 13** The quantitation of NPY for the standard curve

Figure 3. 14 The melt curve analysis of NPY standards for the standard curve
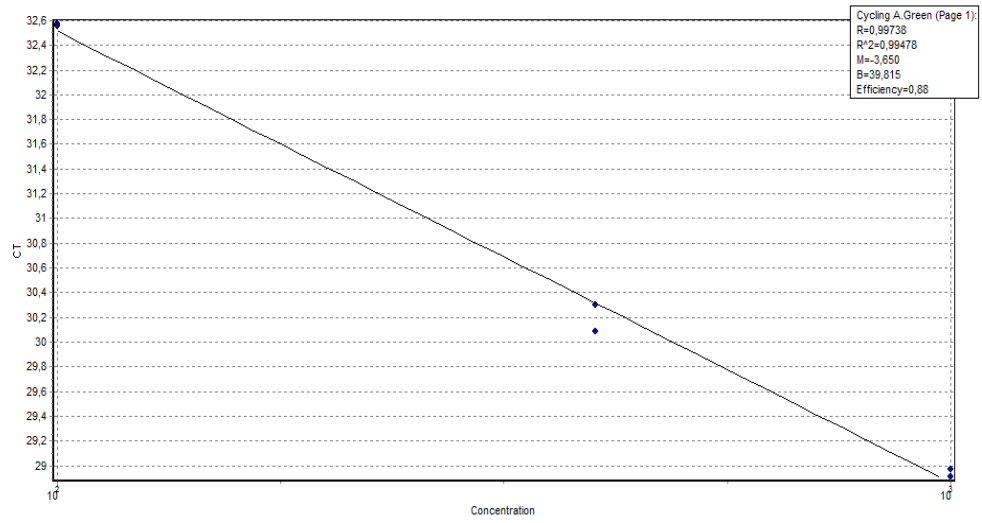construction. All the standards give the peak approximately at 86.5^0C.

Figure 3. 15 The standard curve of NPY. The efficiency of the reaction was 0.88 and the R^2 value was 0.99478.

3.3.3.2.3 The *AgRP* standard curve

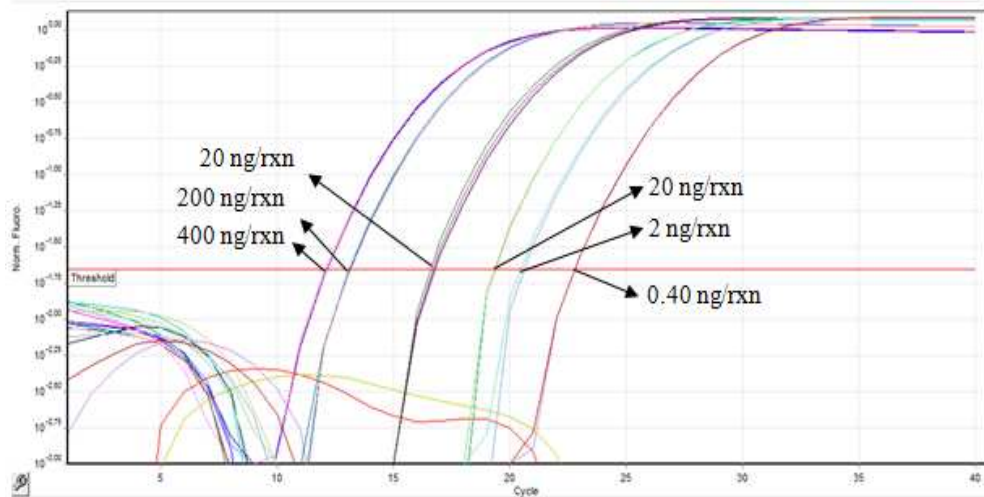The *AgRP* standard curve was resulted in using the standards 400, 200, 20, 4, 2, and 0.40 ng/rxn (Fig.3.18).

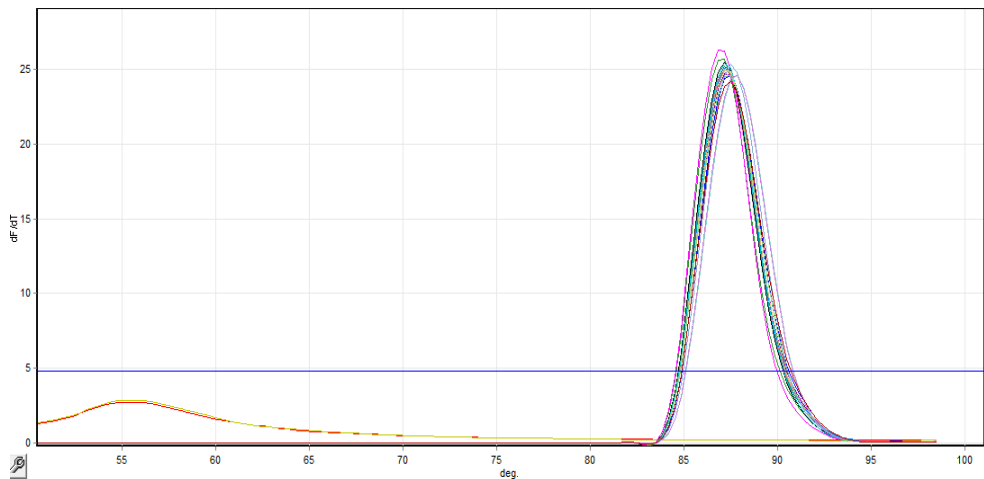**Figure 3. 16** The quantitation data of the AgRP for the standard curve construction.

Figure 3. 17 The melt curve analysis of the AgRP standards for the standard curve construction. All the standards give the peak approximately at 87^{0}C.
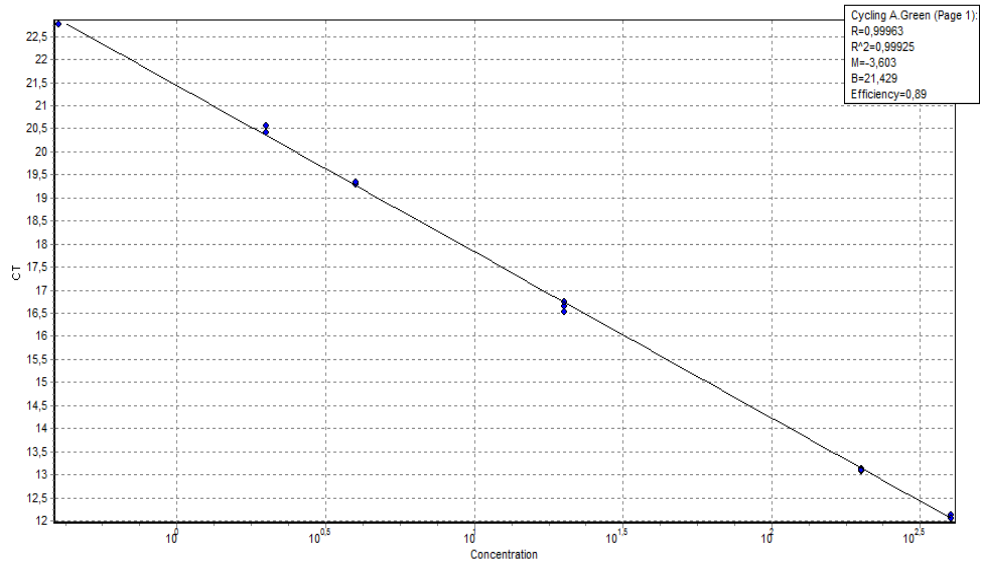
Figure 3. 18 The standard curve of the AgRP. The efficiency of the reaction was 0.89 and the R^2 value was 0.99925.

3.3.3.2.4 The *POMC* standard curve

The standard curve of *POMC* was constructed by using the standards 400, 200, 20, 4, 2, 0.40 ng/rxn (Fig.3.21).
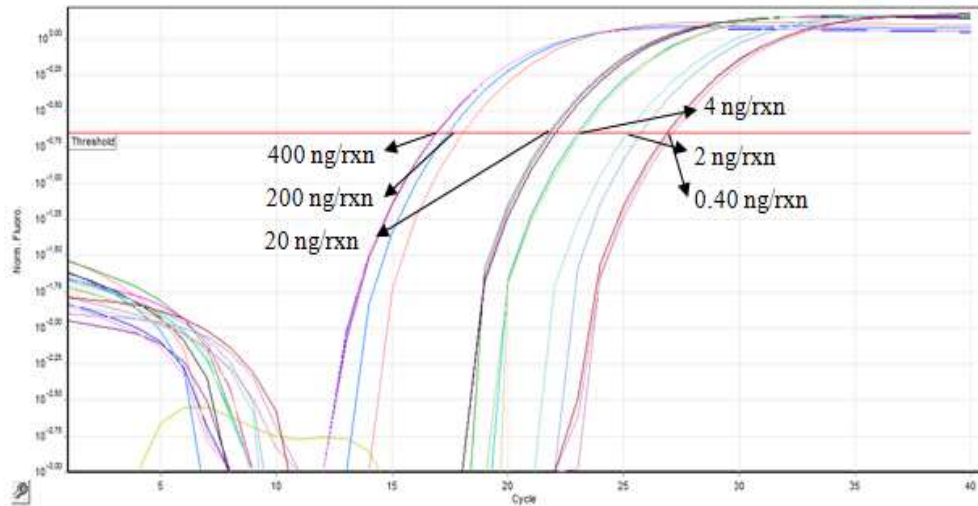

Figure 3. 19 The quantitation data of the POMC for the standard curve construction.

Figure 3. 20 The melt curve analysis of the POMC standards for the standard curve construction. All the standards give the peak approximately at 84^0C..

Figure 3. 21 The standard curve of POMC. The efficiency of the reaction was 0.97 and the R^2 value was 0.98239.

3.3.3.2.5 The *CART* standard curve

The standard curve for *CART* was attained by using the standards 400, 200, 20, 4, and 2 ng/rxn (Fig.3.24).

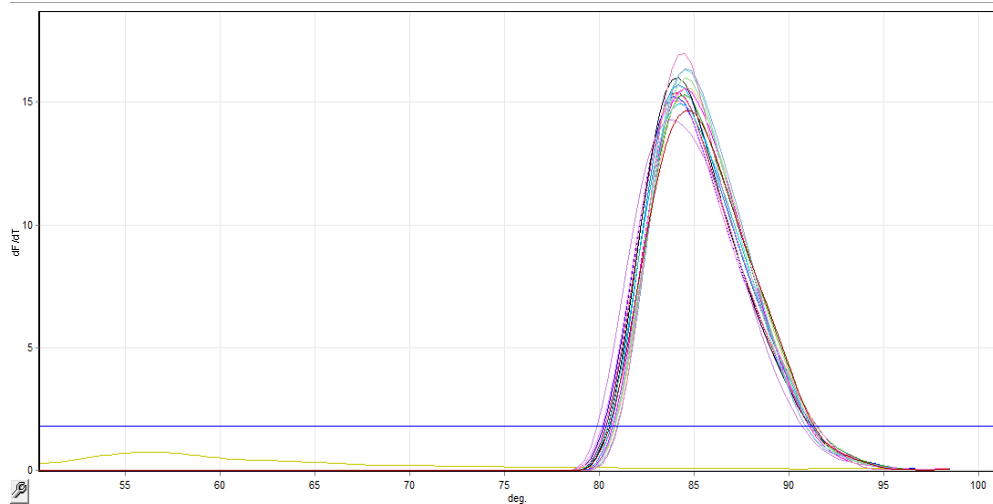**Figure 3. 22** The quantitation data of the CART for the standard curve construction.
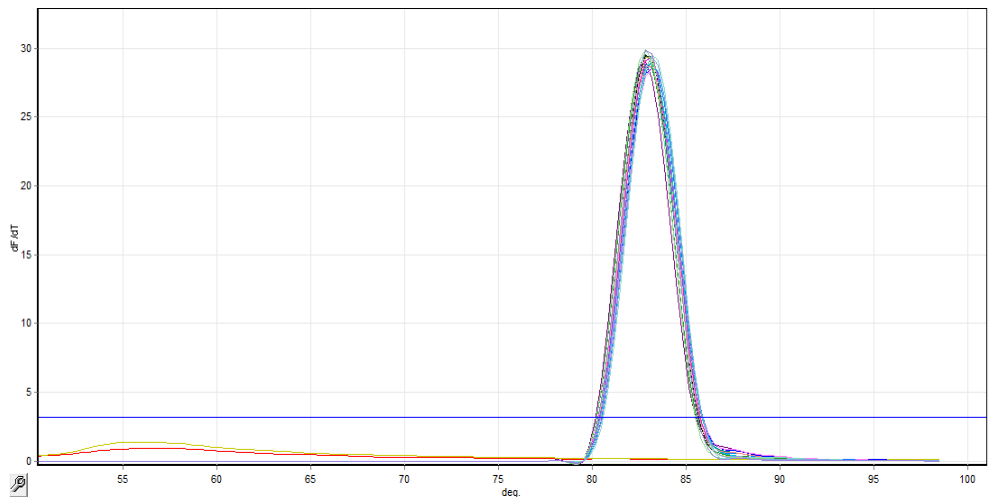
Figure 3. 23 The melt curve analysis of the CART standards for the standard curve construction. All the standards give the peak approximately at 83^0C.
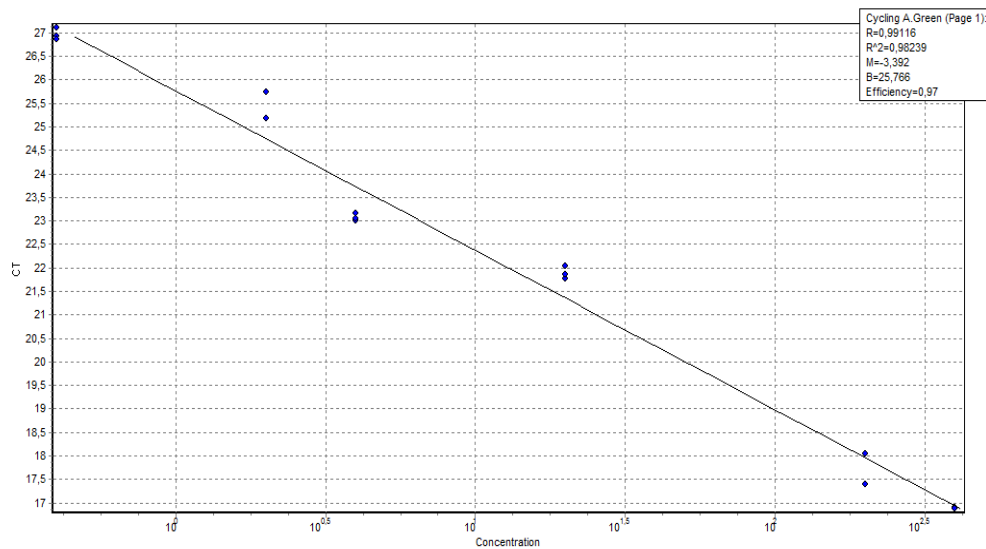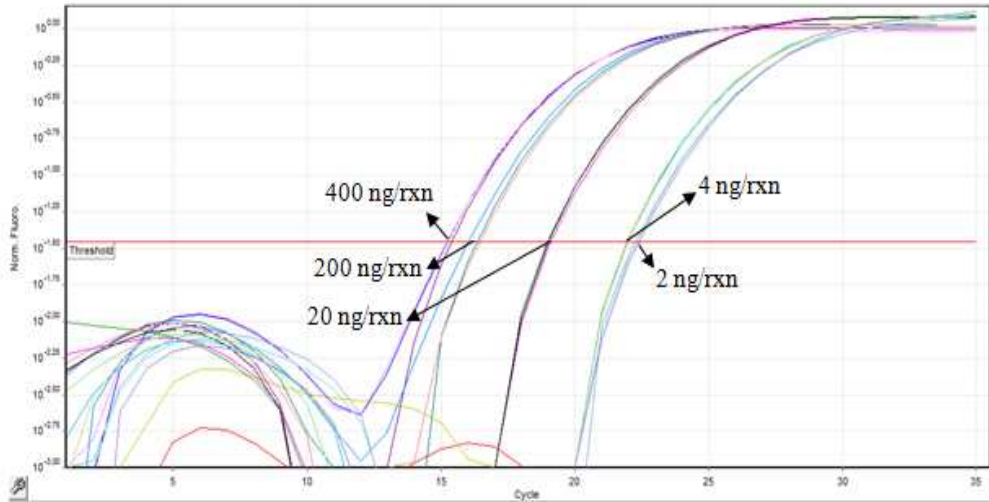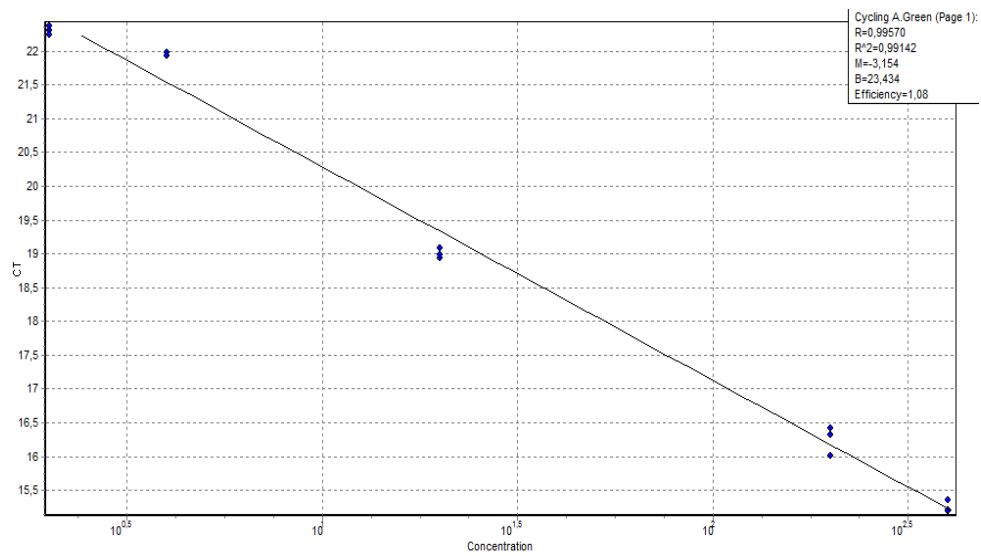
Figure 3. 24 The standard curve of POMC. The efficiency of the reaction was 1.08 and the R^2 value was 0.99142.

3.3.3.3 Quantitation results of vehicle group's genes

3.3.3.3.1 *GAPDH*

The quantitation of expression (Fig.3.25) and the melt curve analysis (Fig.3.26) of *GAPDH* for the vehicle group. The standard curve of *GAPDH* is imported for the calculation of the C_T values of the samples.
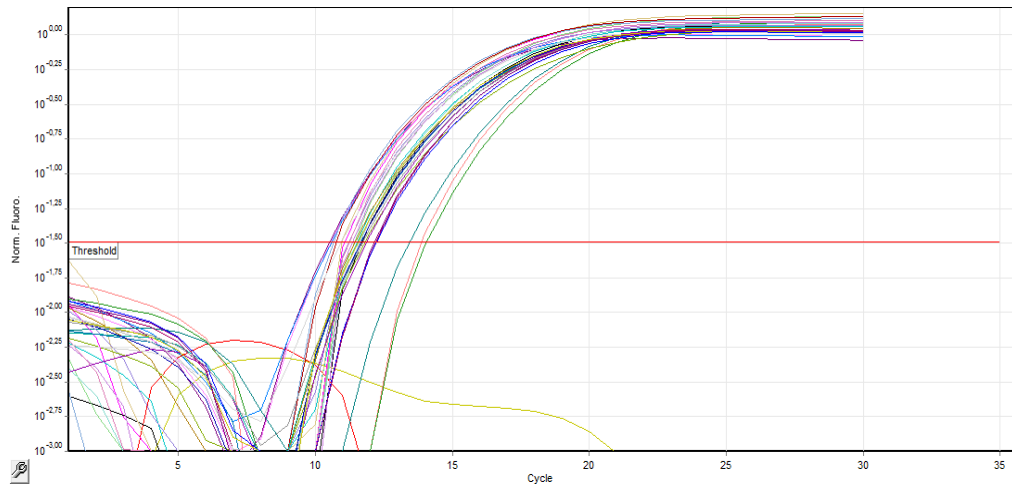
Figure 3. 25 The quantitation of GAPDH for the vehicle group.

Figure 3. 26 The melt curve analysis of *GAPDH* for the vehicle group. All the samples give the peak approximately at 84.5^0C.

3.3.3.3.2 *NPY*

Fig.3.27 shows the quantificaton of *NPY* expression and the melt curve (Fig.3.28) for the vehicle group. The standard curve of *NPY* is imported for the calculation of the C_T values of samples.

Figure 3. 27 The quantitation data of the NPY for the vehicle group.

Figure 3. 28 The melt curve analysis of the *NPY* for the vehicle group. All the samples give the peak approximately at 86.5^0C.

3.3.3.3.3 *AgRP*

AgRP qPCR results are shown in Fig.3.29 and 3.30, where the standard curve of *AgRP* is imported for the calculation of the C_T values of samples.
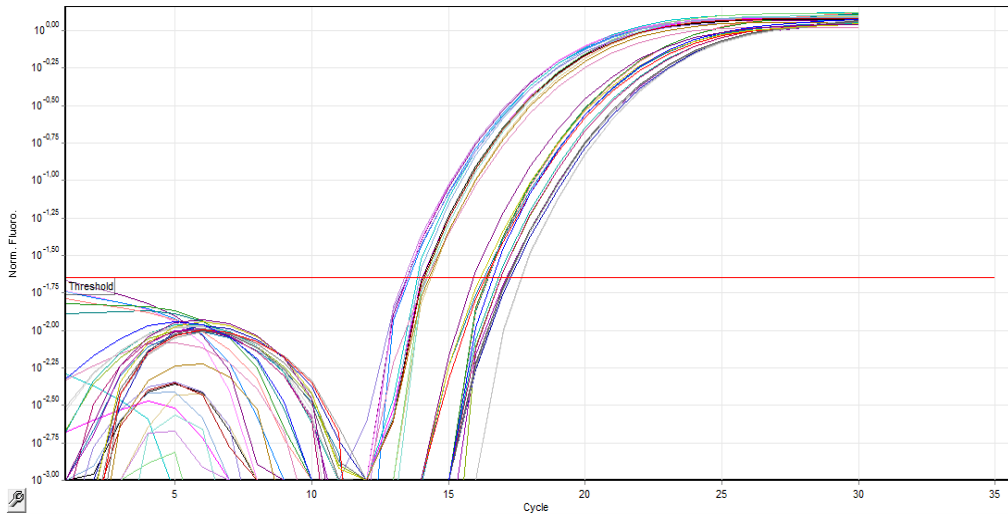
Figure 3. 29 The quantitation data of the AgRP for the vehicle group.
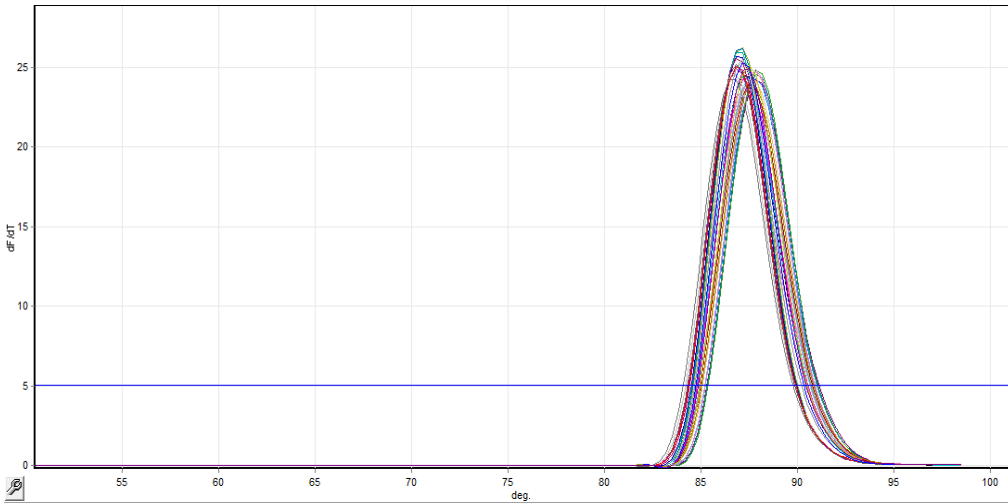
Figure 3. 30 The melt curve analysis of the *AgRP* for the vehicle group. All the samples give the peak approximately at 87.5^0C.

3.3.3.3.4 *POMC*

POMC qPCR results for the vehicle group are shown in the Figure 3.31 and 3.32. The data was obtained by importing the standard curve of the *POMC*.
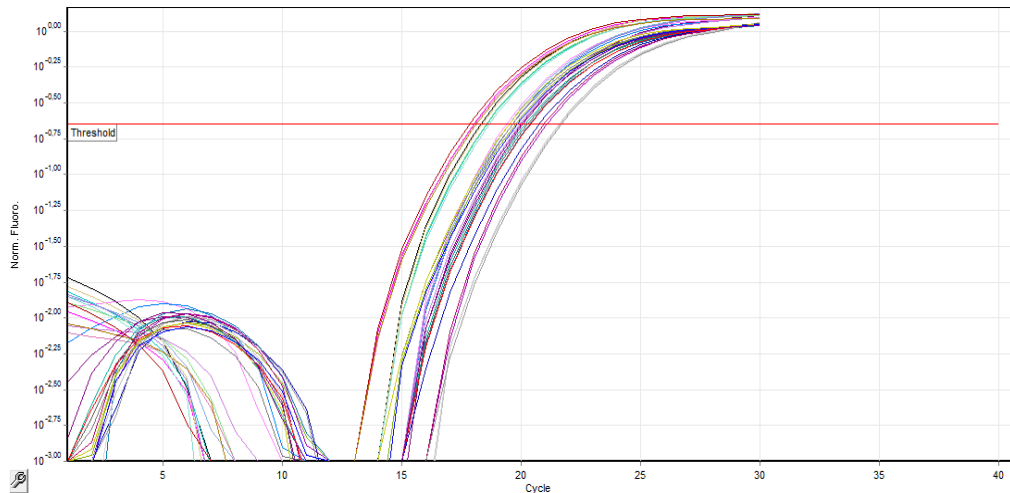
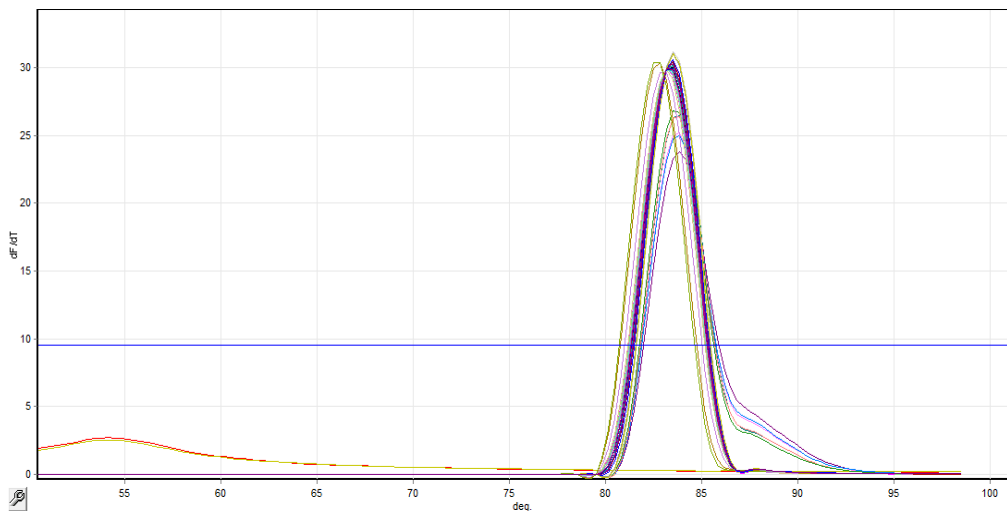**Figure 3. 31** The quantitation data of the POMC for the vehicle group.
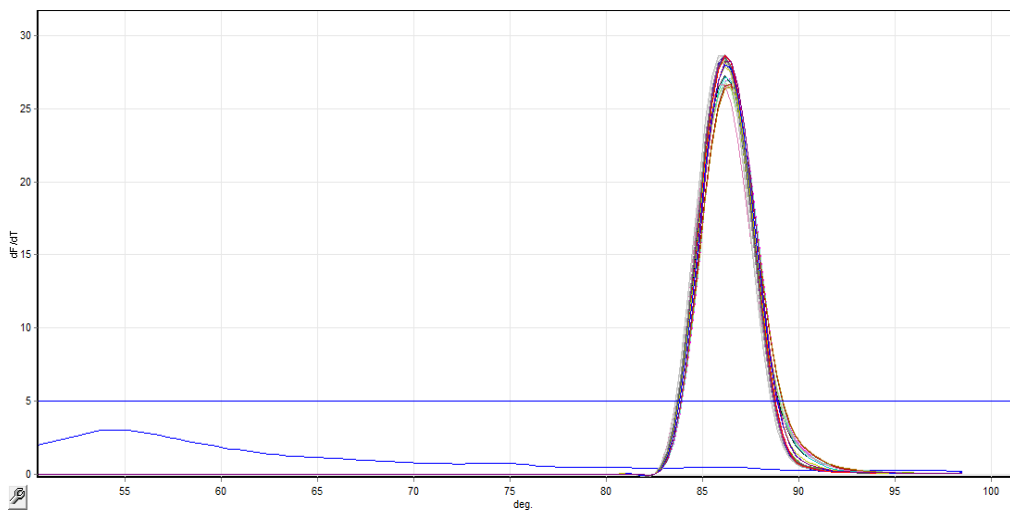
Figure 3. 32 The melt curve analysis of the *POMC* for the vehicle group. All the samples give the peak approximately at 84^{0}C.

3.3.3.3.5 *CART*

The vehicle group's *CART* qPCR results are shown in the Fig.3.33 and 3.34. The data were calculated by importing the standard curve generated for *CART*.
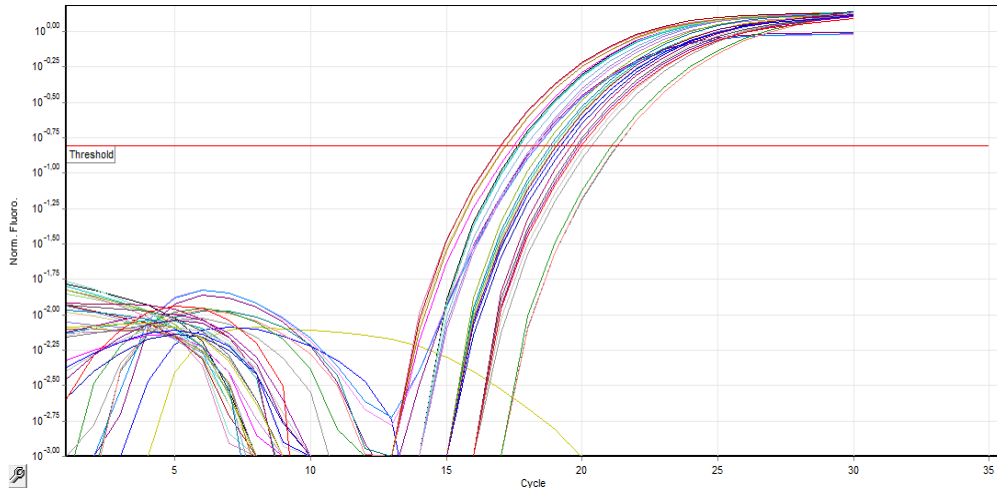

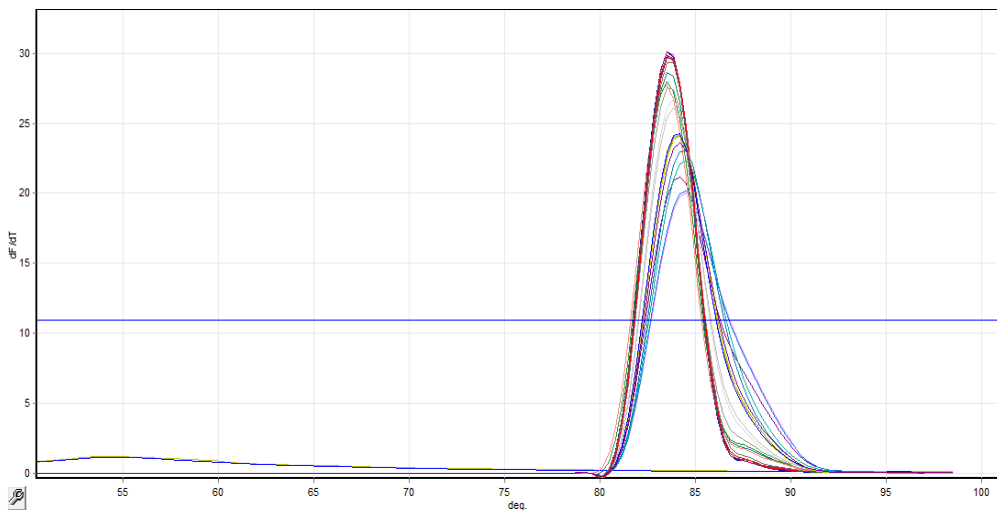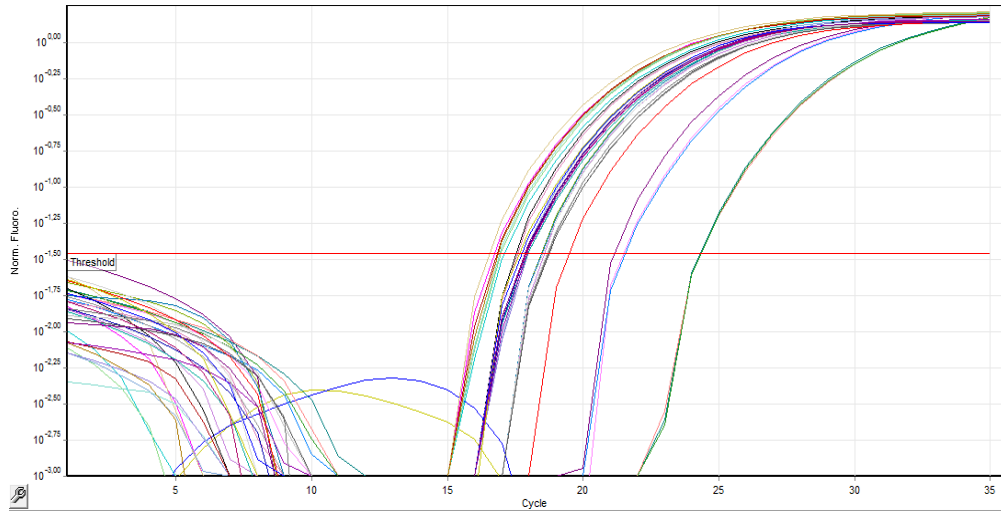**Figure 3. 33** The quantitation data of the CART for the vehicle group.
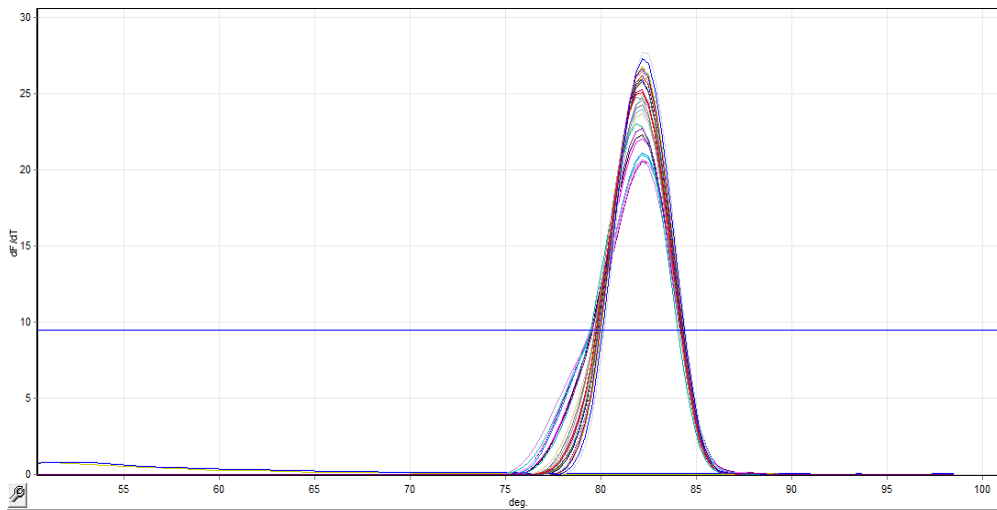
Figure 3. 34 The melt curve analysis of the *CART* for the vehicle group. All the samples give the peak approximately at 83^{0}C.

3.3.3.4 Quantitation results of risperidone administered rats' candidate genes

3.3.3.4.1 *GAPDH*

The risperidone administered rats' *GAPDH* qPCR results are shown in Fig.3.35 and 3.36. The resulting data are calculated by importing the standard curve.

Figure 3. 35 The quantitation data of the *GAPDH* for the risperidone administered group.

Figure 3. 36 The melt curve analysis of the *GAPDH* for the risperidone administered group. All the samples give the peak approximately at $84.5\,^{0}$C.

3.3.3.4.2 *NPY*

NPY qPCR result for the risperidone administered group is shown in the Fig.3.37 and 3.38. The *NPY* standard curve is imported for the determination of the results.
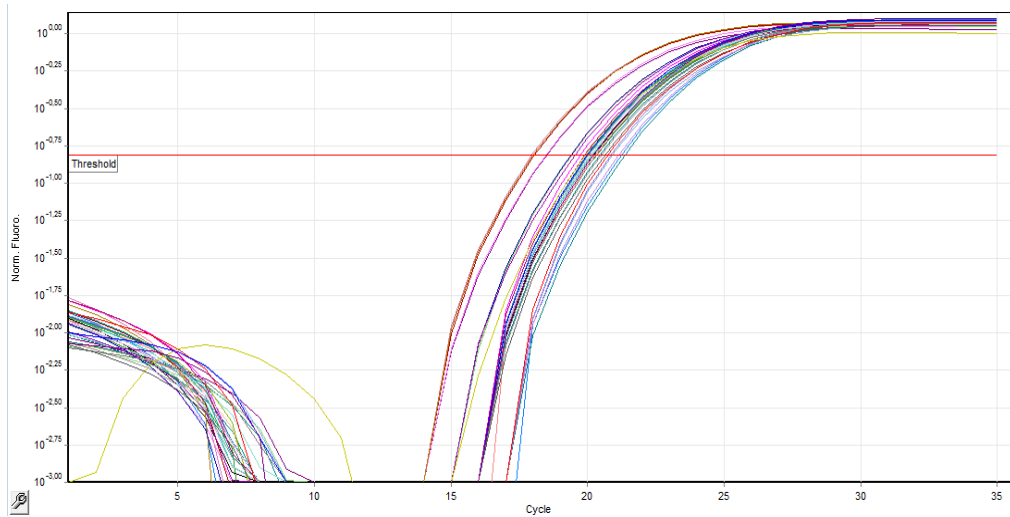
Figure 3. 37 The quantitation data of the *NPY* for the risperidone administered group.
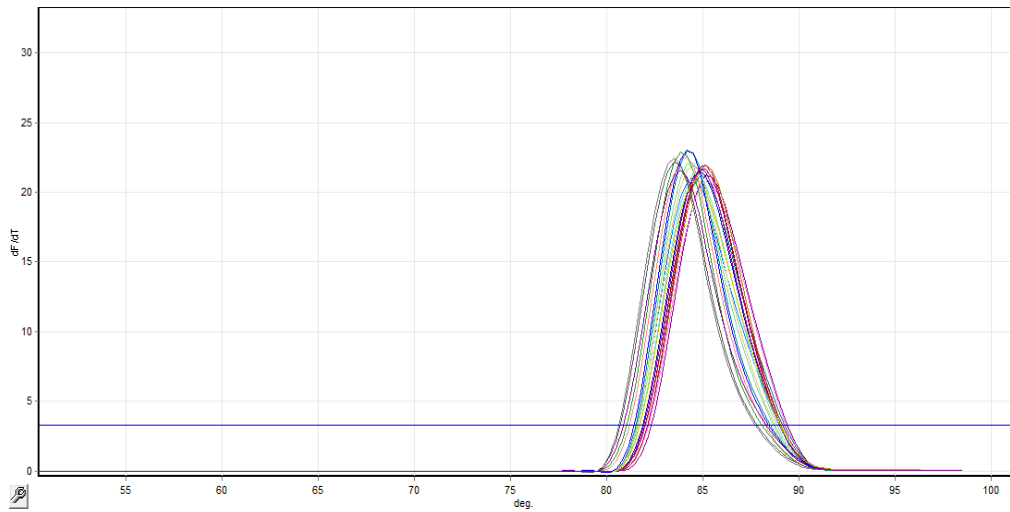
Figure 3. 38 The melt curve analysis of the NPY for the risperidone administered group. All the samples give the peak approximately at 86.5^0C.

3.3.3.4.3 *AgRP*

The qPCR results of *AgRP* of the risperidone administered rats are shown in the Fig.3.39 and 3.40. The standard curve of *AgRP* is imported for the data calculation.
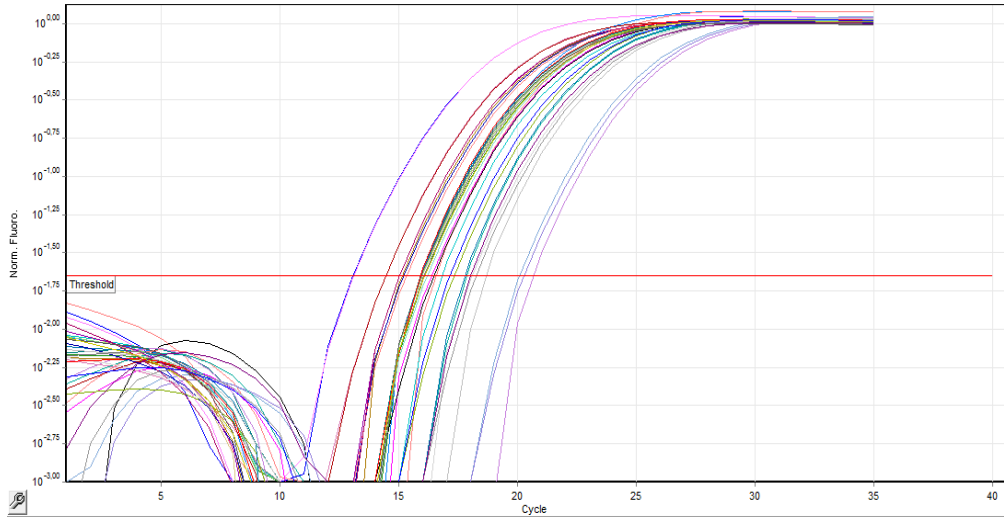
Figure 3. 39 The quantitation data of the *AgRP* for the risperidone administered group.
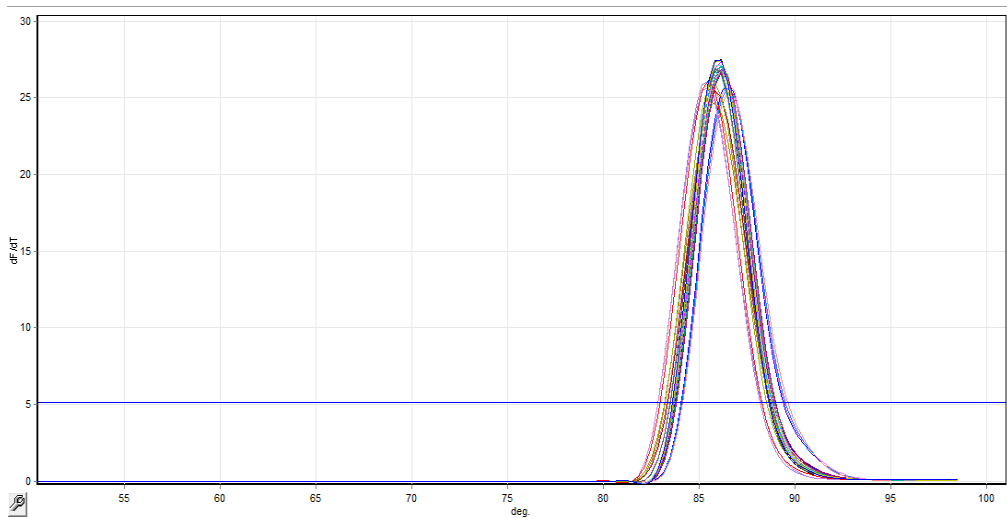
Figure 3. 40 The melt curve analysis of the *AgRP* for the risperidone administered group. All the samples give the peak approximately at 87.5^{0}C.

3.3.3.4.4 *POMC*

POMC qPCR result is shown in the Fig.3.41 and 3.42, where the standard curve of the *POMC* is imported.
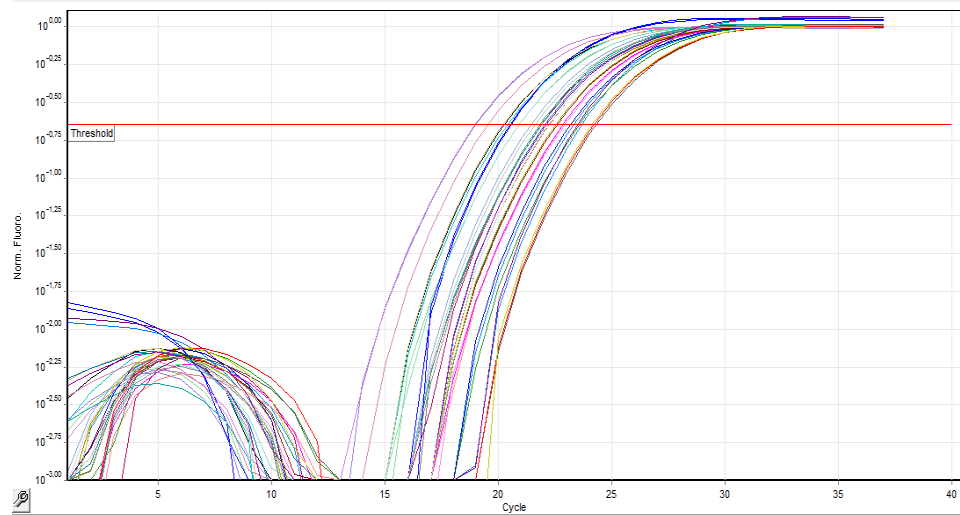
Figure 3. 41 The quantitation data of the *POMC* for the risperidone administered group.
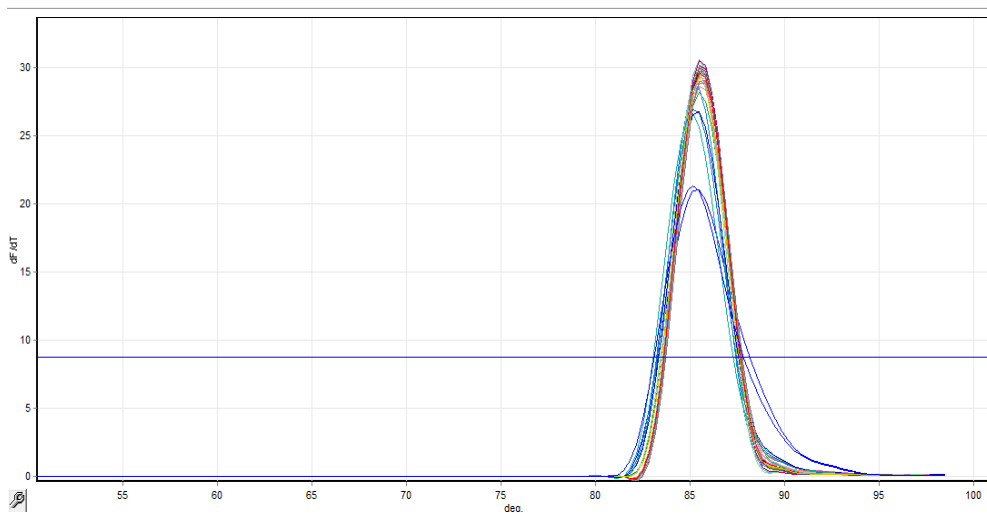
Figure 3. 42 The melt curve analysis of the *POMC* for the risperidone administered group. All the samples give the peak approximately at $84^{0}C$.

3.3.3.4.5 *CART*

The qPCR results of *CART* of the risperidone administered rats are shown in the Fig.3.43 and 3.44. The standard curve of *CART* is imported for the data calculation.
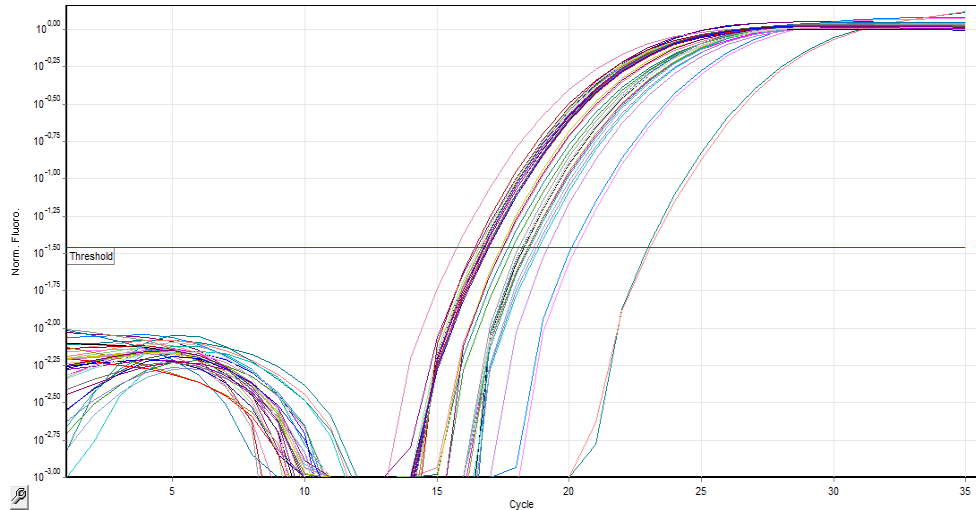
Figure 3. 43 The quantitation data of the *CART* for the risperidone administered group.

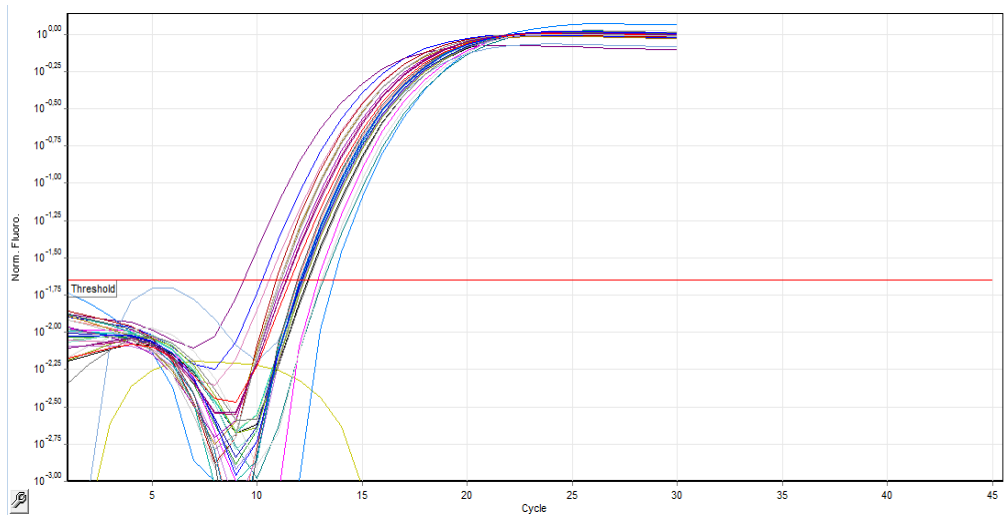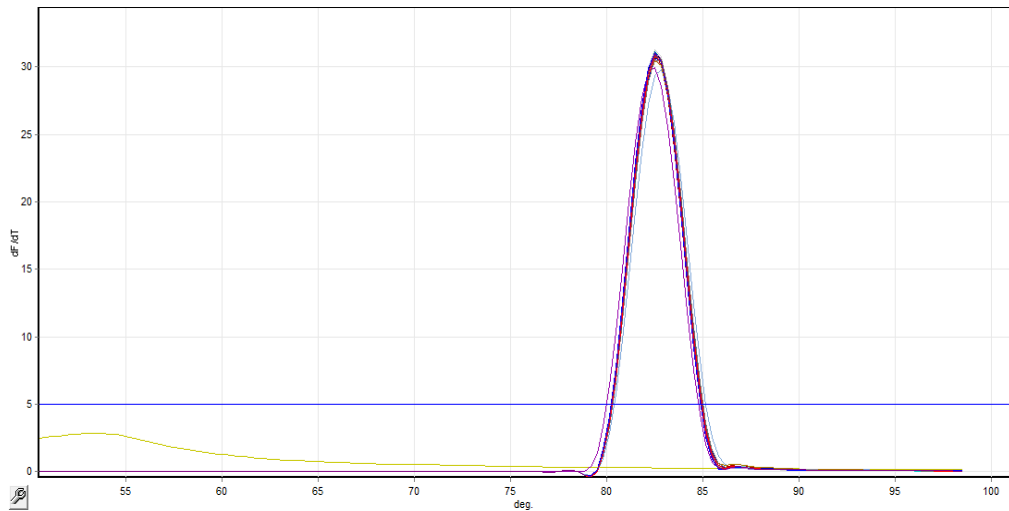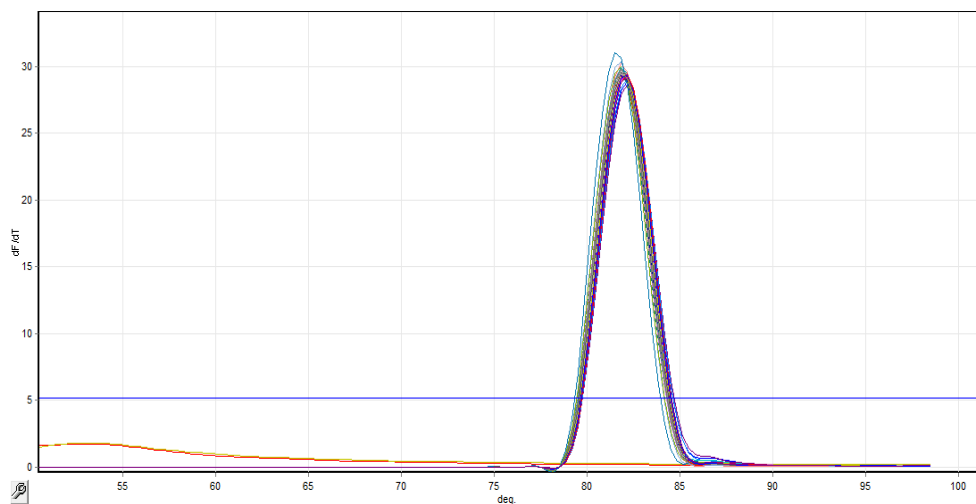**Figure 3. 44** The melt curve analysis of the *CART* for the risperidone administered group. All the samples give the peak approximately at 83^0C.

3.3.3.5 Fold changes in the expression levels of the candidate genes

The calculation of the fold change was done as relative to the housekeeping gene, *GAPDH*, for both vehicle and risperidone administered group according to the efficicency corrected equation described in Pfaffl, 2001. The differences in the gene expressions are depicted in Fig.3.45 as bar graphs. There is a significant decrease in the expression levels of *POMC* and *NPY* in the risperidone administered groups when compared to the vehicle group, while the expression of *CART* increased significantly. Although there is no statictical significance, there is a 38% decrease in the *AgRP* gene expression of the risperidone administered rats vs. vehicle group.
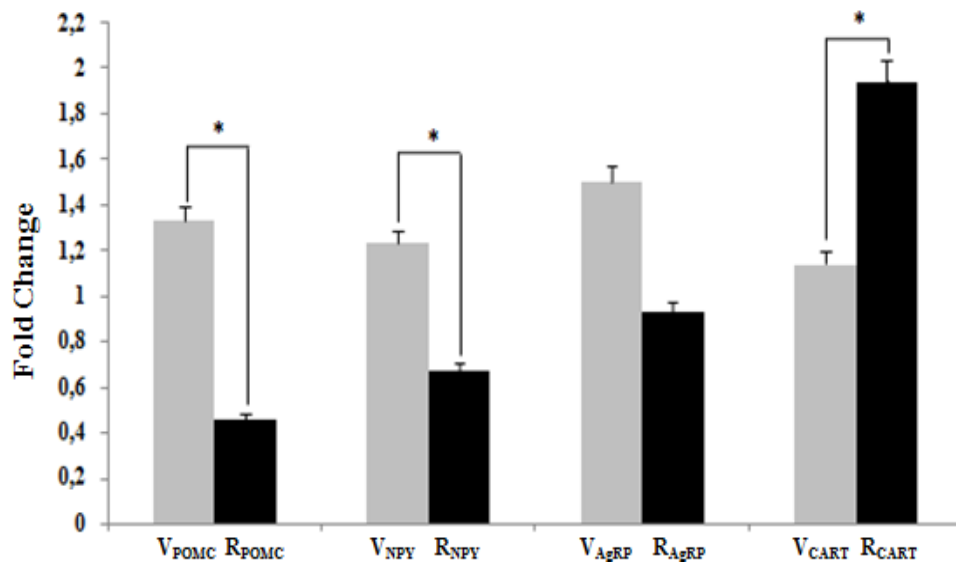
Figure 3. 45 The fold changes of the candidate genes. V is for the vehicle groups and R is for the risperidone administered groups of the genes.

3.3.4 The detection of plasma levels of peripheral and CNS neurohormones that affect food intake in rats by using ELISA

3.3.4.1 Leptin plasma levels

The plasma leptin levels of vehicle group and risperidone administered group are shown in Fig.3.46. Vehicle group had 103.59 ± 5.35 pg/ml leptin in their plasma while risperidone administered rats had 122.34 ± 6.81 pg/ml leptin, which indicated a significant increase in the plasma leptin levels due to risperidone administration (*$p<0.05$).
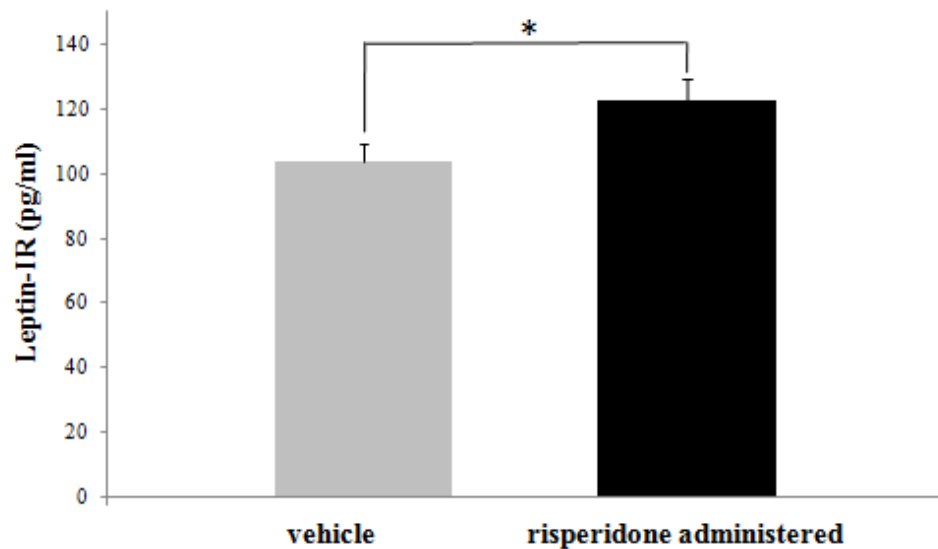
Figure 3. 46 The plasma leptin levels of the vehicle group vs. risperidone administered group. The vehicle group had 103.59 ± 5.35 pg/ml leptin level whereas the risperidone administered group showed a statistically significant higher leptin levels in the plasma (122.34 ± 6.81 pg/ml) (*$p<0.05$).

3.3.4.2 NPY plasma levels

The plasma NPY level of the vehicle group was 99.36±2.28 ng/ml and the risperidone administered group had 91.53±2.57 ng/ml NPY levels in their plasma. Although NPY levels are shown to be decreasing slightly by the risperidone administration, there is no significant difference between the vehicle group and the risperidone administered group NPY levels (Fig.3.47).
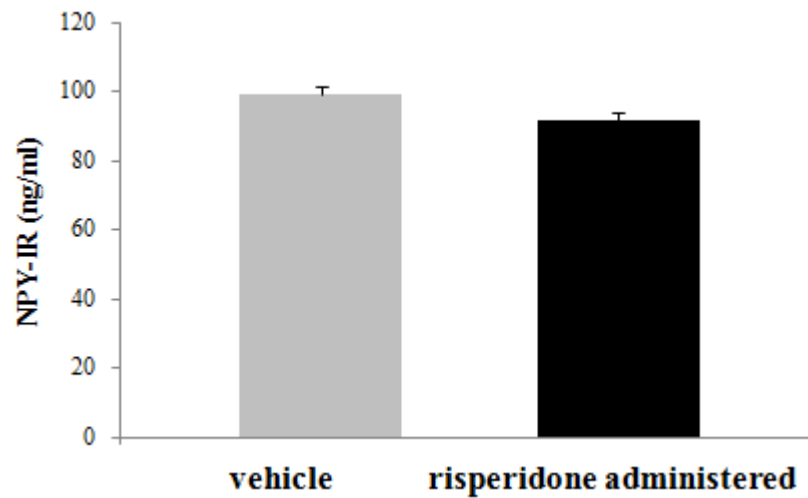
Figure 3. 47 The plasma NPY levels of vehicle group vs. risperidone administered group. The vehicle group had 99.36±2.28 ng/ml NPY level and the risperidone administered group had 91.53±2.57 ng/ml plasma NPY level.

3.3.4.3 AgRP plasma levels

Vehicle group's plasma AgRP concentration was 21.82±0.67 ng/ml while the risperidone group had 18.53±0.90 ng/ml AgRP in their plasma. The risperidone administration led to a statistically significant decrease in the AgRP plasma levels of risperidone administered rats when compared to the vehicle group (*$p<0.05$) (Fig.3.48).
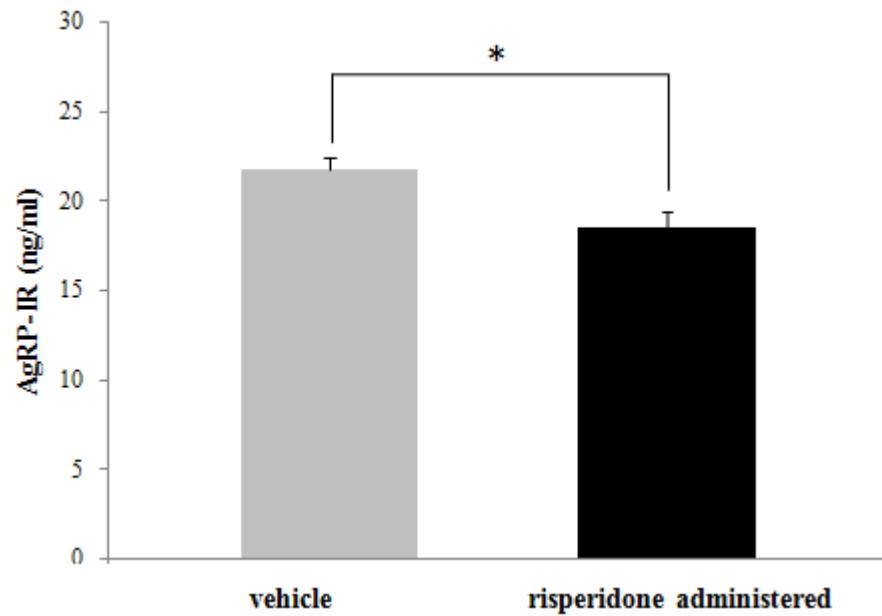
Figure 3. 48 The plasma AgRP levels of vehicle group vs. risperidone administered group. The concentration of AgRP was 21.82±0.67 ng/ml in the vehicle group and the risperidone administered group had 18.53±0.90 ng/ml plasma AgRP level, which indicates a statistically significant decrease in plasma AgRP level due to the risperidone administration (*$p<0.05$).

3.3.4.4 α-MSH plasma levels

The vehicle group's plasma α-MSH concentration was 22.95±3.11 ng/ml while the risperidone administered group had 14.26±2.45 ng/ml plasma α-MSH concentration. The risperidone administration caused a statistically significant decrease in the plasma α-MSH concentration of the treated rats when compared to vehicle group (*$p<0.05$) (Fig.3.49).
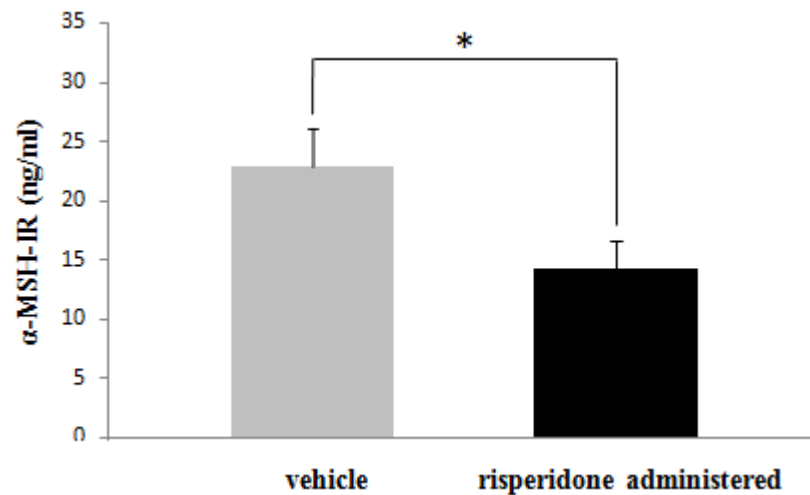
Figure 3. 49 The plasma α-MSH levels of vehicle group vs. risperidone administered group. The vehicle group's was 22.95±3.11 ng/ml the risperidone administered group's plasma concentration was 14.26±2.45 ng/ml. The difference between the vehicle and risperidone administered group is indicating a statistically significant decrease in plasma α-MSH concentration in the risperidone administered group (*$p<0.05$).

3.3.4.5 CART plasma levels

The plasma CART levels of the vehicle group was 12.97±3.57 ng/ml while the risperidone administered group showed a significantly reduced plasma CART levels, 5.54±0.45 ng/ml (*$p<0.05$) (Fig.3.50).
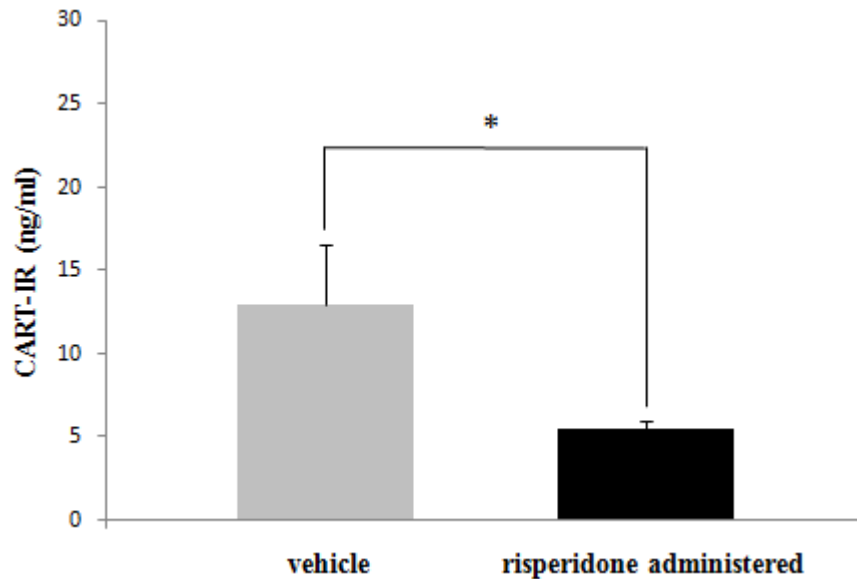
Figure 3. 50 The plasma CART levels of vehicle group vs. risperidone administered group. The vehicle group's plasma CART concentration was 12.97±3.57 ng/ml while the risperidone administered group's plasma CART concentration was 5.54±0.45 ng/ml. There is a statistically significant decrease in the plasma CART concentration in the risperidone administered group when compared to vehicle group (*$p<0.05$).

Risperidone is known to have a moderate weight gain effect in the long-term use (Russell and Mackell, 2001). In this study, the schizophrenic patients used risperidone for 4-weeks and consequently, they gained weight and showed a significant increase in their BMI. Although patients received routine hospital food with 2500 kcal/day, but they reported that their appetite had increased; therefore used the hospital cafeteria for extra food. Although it was impossible to detect the extra food intake, the patients indicated that they preferred eating junk food during their daily examinations. This might be the reason for their weight when compared to control group.

To explore the tissue specific alterations in the candidate genes' expression levels, risperidone was administered to rats and their hypothalamic tissue was removed. Together with the expression study, the peripheral levels of those neuropeptides were also detected. Wistar-rats received risperidone for 4-weeks period showed an increase in their appetite, hence a slightly more increase in their weights compared to the vehicle group. Number of studies indicate that the risperidone administration to the rats with different kinds route of administration increases the food intake and body weight gain in 4-weeks periods (Baptista *et al.*, 2002; Ota *et al.*, 2002).

Leptin is known to increase the weight gain and cause obesity due to an increase in the white adipose tissue mass (Considine and Caro, 1999). Interestingly, the schizophrenic patients have lower leptin levels in the plasma when compared to healthy individuals (Kraus *et al.*, 2001; Atmaca *et al.*, 2003). In this study, the results indicate that the patients before treatment had lower plasma leptin levels when compared to controls, but the 4-weeks risperidone treatment increased the leptin levels significantly. Schizophrenic patients using risperidone in the long-terms, had higher plasma leptin levels when compared to the normal individuals (Cummings and Schwartz, 2003). Despite the fact that this study is a short term trial only, higher plasma leptin levels of the patients had been observed. It might be inferred from the data that if those patients continue using risperidone longer time, their plasma leptin might stay increased or increase more.

The plasma leptin levels of the risperidone administred rats were also increased significantly compared with the vehicle group. Increased appetite and body weight of the risperidone administered rats confirms the increased leptin plasma levels in rats.

In this study, the plasma NPY levels of the schizophrenic patients were also significantly lower than the control group. Data are consistent with the previous studies reporting that schizophrenic patients have lower NPY levels in their different brain regions (Kuromitsu *et al.*, 2001; Frederiksen *et al.*, 1991; Gabriel *et al.*, 1996). NPY is known to have a signficant role in the stress response in the body (Redrobe *et al.*, 2003) and Morgan *et al.* (2000), showed that the humans during their military service had significantly higher NPY levels together with increased cortisol levels. On the contrary, a study exposing the rats to different stress conditions for 8-weeks resulted in reduction of NPY levels in different brain regions (Hyunyoung *et al.*, 2003). Moreover, NPY gene expression was shown to increase in the ARC of the rat brain hypothalamus but it was shown to reduce in the locus coeruleus region under the stress conditions (Makino *et al.*, 2000). Our data showed that there is a 53% decrease in their NPY levels of the schizophrenic patients. On the other hand, there was no change in the plasma NPY levels of the patients after the four-week risperidone treatment. It is a known fact that leptin has an inhibitory effect on the hypothalamic *NPY* expression (Weigle, *et al.*, 1995). Our data indicating the increase in the plasma leptin levels due to risperidone treatment. The increased leptin might be the cause of the low plasma NPY levels of the patients even after the treatment and despite the increase in the appetite. It might be due to the fact that leptin might be a more potent regulator on the *NPY* expression than the antagonism of the risperidone through the serotonergic receptors on NPY neuron group.

The plasma NPY levels of the risperidone administered rats showed a decreasing trend compared to the vehicle group. The decrease in the plasma NPY levels was parallel to the decrease in the *NPY* expression in the hypothalamus of the risperidone administered rats. The drug administration of rats lasted for only four weeks and caused a significant decrease in the *NPY* expression. Therefore, we might speculate more of decreased levels of *NPY*

expression in longer drug administration; in addition increased plasma leptin levels might be affecting the NPY neurons to suppress its expression, consequently its plasma levels. The expression level of *NPY* was found to be decreasing 41% in the risperidone administered group when compared to the vehicle group. As discussed in the previous parts, human plasma NPY levels were also slightly decreasing after the risperidone treatment. The *NPY* expression might be under control of the increased plasma leptin concentrations of the risperidone administered rats, instead of the serotonergic antagonism of the risperidone. Obuchowicz and Turchan (1999) demonstrated that treatment with an atypical antipsychotic, clozapine, results with decrease in th NPY expression in the nucleus accumbens of the wistar rats. Moreover, microdylasates from the brain tissues of the wistar rats showed a significant decrease in the NPY levels after thirty-day risperidone administration (Gruber and Mathe, 2000).

Also, α-MSH, an anorexigenic product of POMC, plasma levels reflect the brain expression levels. POMC has a variety of roles besides food intake in the body, including the stress response (Baubet *et al.*, 1994). The HPA axis, in which α-MSH exerts its functions, was shown to have affected from the pathophysiology of the depression (Van Praag, 2004). In this study, the schizophrenic patients have a 2.5 fold decreased plasma α-MSH levels compared with the control group which might suggest the disrupted HPA axis in these individuals. Moreover, there is a slight decrease (30%) in the plasma α-MSH levels of the patients after the risperidone treatment. Since risperidone is known as the antagonist of 5-HT_{2C} receptor (Miyamoto *et al.*, 2005) and POMC neurons have that receptor on their cell membrane, the slight decrease in the plasma α-MSH levels of the patients after the treatment period might be due to the antagonism effect of risperidone on the POMC neurons, leading to less α-MSH synthesis.

The α-MSH levels of the risperidone administered rats were shown to decrease significantly after 4-weeks risperidone administration. Due to the decreased *POMC* expression in the hypothalamic tissue of the risperidone administered rats, lowered α-MSH levels confirms the increased appetite in these animals. The 5-HT$_{2C}$ receptor on the POMC neurons, owing to the antagonistic effect of risperidone, might be the cause of the decreased α-MSH levels, which is the result of the decreased *POMC* expression. *POMC* expression in the risperidone administered rats were decreased 71% when compared to the vehicle group. This significant decrease might be the result of the antagonism of risperidone on the 5-HT$_{2C}$ receptor, so that the downstream pathways of the serotonergic receptor are blocked and this caused a reduction in the *POMC* expression. This data is, also, consistent with the increase in the appetite and the body weight of the risperidone administered rats, since POMC is an anorexigenic peptide, and its decrease causes increase in the appetite. Moreover, the human patients' plasma levels of α-MSH were found to be decreased after the risperidone administration. It was shown that 5-days treatment of the rats with one of the atypical antipsychotics, olanzapine, caused a significant decrease in the *POMC* expression in the ARC of the hypothalamus (Fernø *et al.*, 2011).

Circulating CART levels were higher in the schizophrenic patients than healthy individuals. The patients reported that they had decreased appetite during their diagnosis period. This might be the result of the increased plasma CART levels, since CART is an anorexigenic peptide (Harrold and Halford, 2006). Moreover, Rosse *et al.* (2005), showed that cocaine-induced psychotic patients had low BMI but high plasma CART levels, which is consistent with the data of this study. On the other hand, four-week risperidone treatment caused a slight decrease in the plasma CART levels of the patients, which might be due to the antagonistic effect of risperidone on the 5-HT$_{2C}$ receptor CART neurons (Miyamoto *et al.*, 2005).

Plasma levels of CART neuropeptide of the risperidone administered rats decreased significantly when compared to the vehicle group. The significant increase of *CART* expression in the hypothalamus of risperidone administered rats is not reflecting the plasma CART levels, which might be due to the different forms of CART circulating in the bloodstream. The risperidone administration caused a significant increase in the risperidone administered rats' *CART* expression (1.70 fold increase) when compared to the vehicle group.

The significant decrease of the AgRP in the plasma of the risperidone administered rats compared to the vehicle group might be the result of the decrease in the *AgRP* expression in their hypothalamus. Furthermore, this study showed a significant increase in the plasma leptin levels, which might affect the AgRP neurons negatively, to reduce the *AgRP* expression and its plasma levels.

AgRP expression in the risperidone administered rats decreased 10%, which was not a significant decrease, when compared to the vehicle group. The AgRP gene is expressed in the same neuron group with *NPY* in the ARC, therefore, change of the *AgRP* expression might be correlated with the *NPY* expression. It might be suggested that the weight gain effect of the risperidone is not through the NPY/AgRP neuron group, due to the fact that those neuropeptides are orexigenic, and their decrease results with a decrease in the appetite. However, both the risperidone treated patients' and rats' appetite and the body weight increased significantly. The decrease in the *NPY* and *AgRP* expressions might be due to the increased leptin, and it also might be speculated that, after a longer risperidone administration, the weight gain becomes more severe, leptin resistance occurs, so that leptin cannot inhibit the expression of NPY and AgRP further. Thus, the leptin control's lost on orexigenic peptide synthesizing neurons lead to increased appetite and obesity.

CHAPTER 4

CONCLUSION

- This study showed the effect of atypical antipsychotic, risperidone on the food intake and appetite mechanism. Both the patients and the orally risperidone administered rats showed hyperphagic behavior which caused a significant increase in their body weight. The significant increase in the body weight of both patients and risperidone administered rats resulted in higher plasma leptin levels, peripheral signal for food intake.

- The schizophrenic patients had significantly lower NPY levels before the risperidone treatment, which may also reflect their psychotic episodes. After 4-weeks risperidone treatment, patients and rats demonstrated reduced NPY levels, both in their plasma, and the hypothalamic NPY expression in the rats. This decrease might be the result of the increased leptin concentration, which might have lowered the *NPY* expression. Therefore, this might suggest that risperidone is not directly acting on the NPY neurons to alter its levels, but the NPY decrease is the result of the leptin increase due to risperidone administration. The reduced α-MSH levels in both patients and the risperidone administered rats may signify that risperidone acts through the 5-HT$_{2C}$ receptor antagonism, decreasing the POMC expression in the ARC of the hypothalamus. AgRP was found to be decreasing in rats after risperidone administration both in the expression level and its

plasma levels. This might, again, indicate that the increased leptin levels might be the reason of the decreased AgRP expression and levels in the plasma. The CART levels of both patients and risperidone administered rats showed decreased CART levels in their plasma, but a significant increase was found in the expression levels of CART in the hypothalamus of the rats. The increase in the expression might be the result of the increased leptin. Further elevation of plasma leptin levels might result with the leptin resistance, leading to obesity.

- In conclusion, this study proved that one of the weight gain mechanism of risperidone administration, which cause a significant weight gain and increased BMI both in schizophrenic patients and Wistar-rats, is because of increased food intake and appetite. The increased levels of body weight mechanisms could be alter the levels of both the expression and plasma levels of the neuropeptides; POMC, NPY, CART, and AgRP responsible for the appetite regulation in the ARC of the hypothalamus as summarized in Fig.4.1.

- Future studies are further warranted to clarify the cellular mechanisms of the alterations of the NPY, AgRP, POMC and CART expression levels. The treatment does not rescue these hormones levels in the plasma which may contribute to their increased appetite; hence, increased body weight.
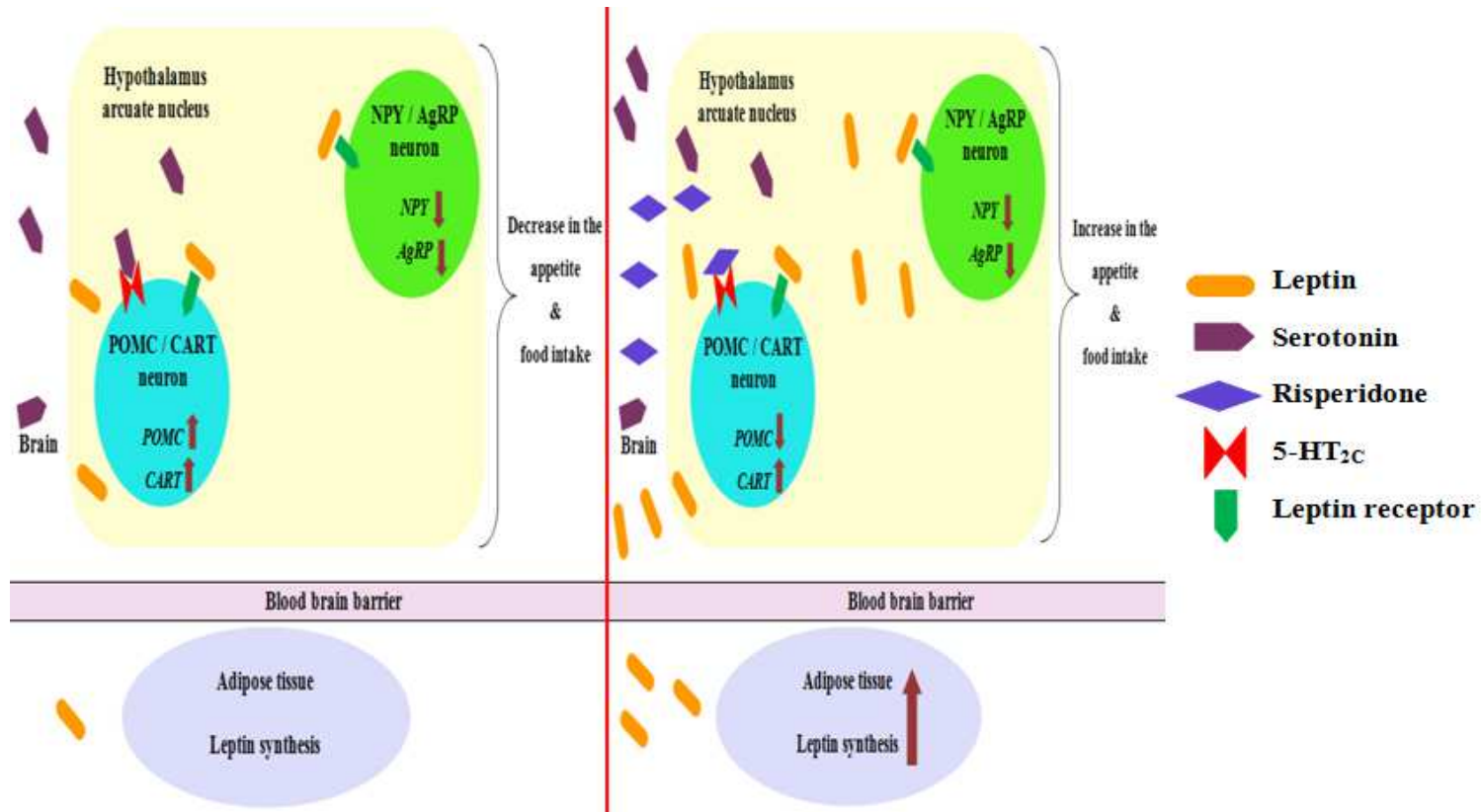
Figure 4. 1 Appetite regulating pathways in the ARC of hypothalamus. The left is the normal mechanism and the right is proposed changed pathways in risperidone treatment.

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Standard curves of ELISA

$$y = -0,003x^2 + 0,255x + 0,145$$
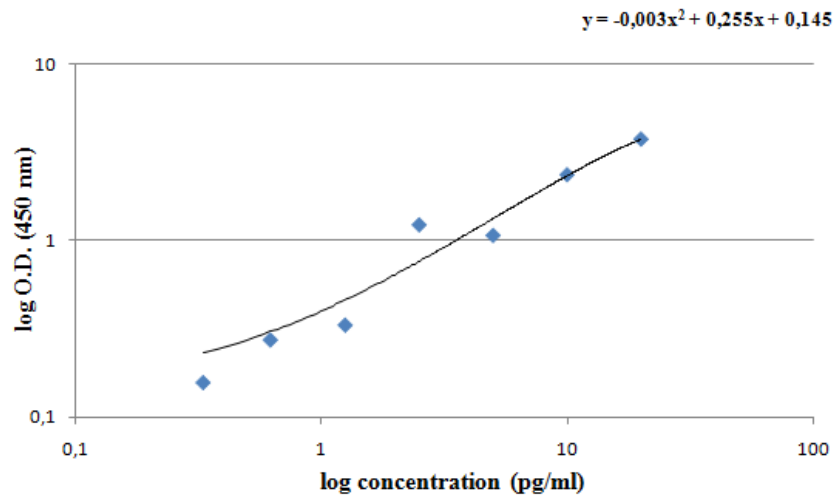
Figure A. 1 Leptin standard curve for human samples

$$y = 0,00052x^{0,99702}$$
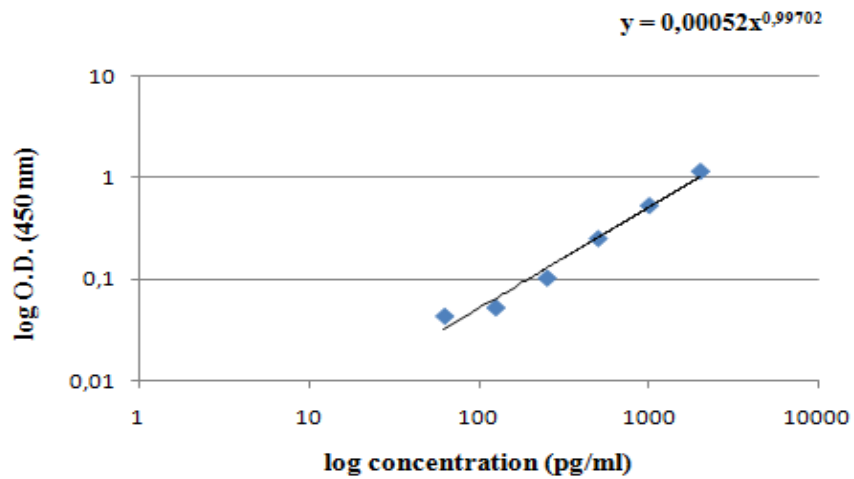
Figure A. 2 Leptin standard curve for rat samples
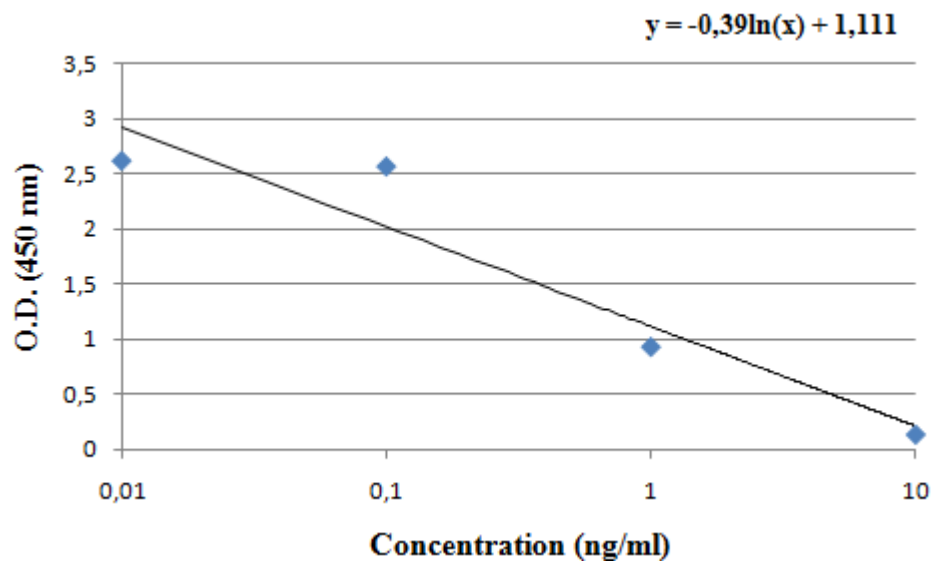
Figure A. 3 NPY standard curve for human samples
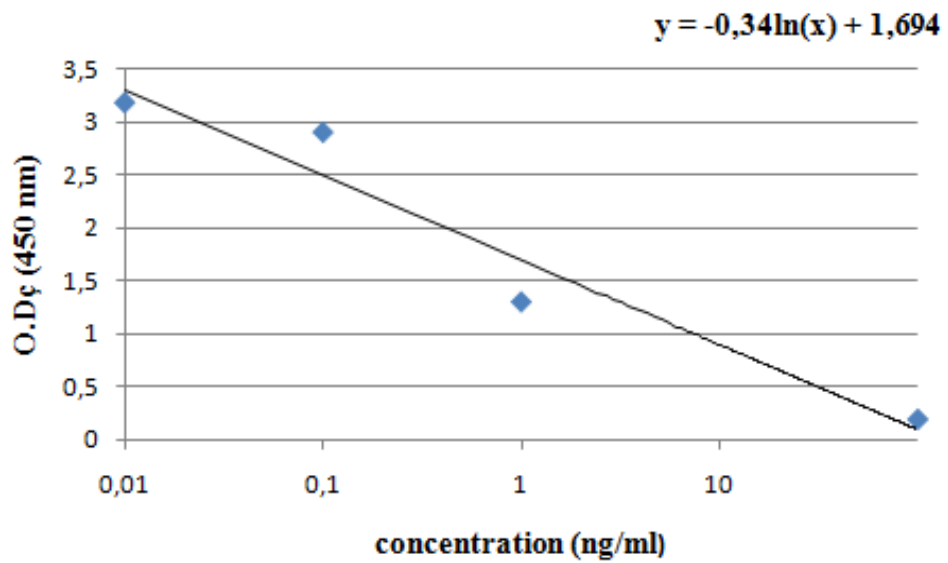
Figure A. 4 NPY standard curve for rat samples
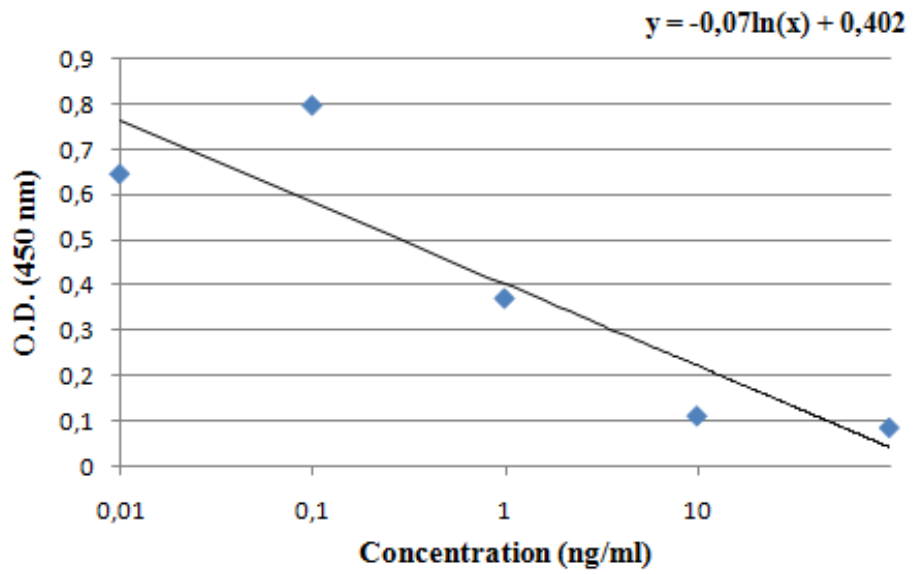
$$y = -0,07\ln(x) + 0,402$$

Figure A. 5 α-MSH standard curve for human samples

$$y = -0,38\ln(x) + 1,492$$
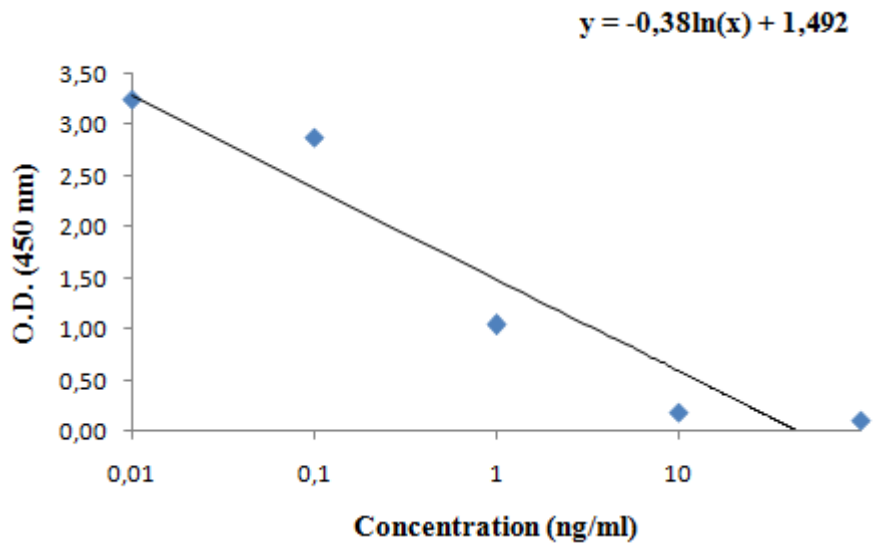
Figure A. 6 α-MSH standard curve for rat samples
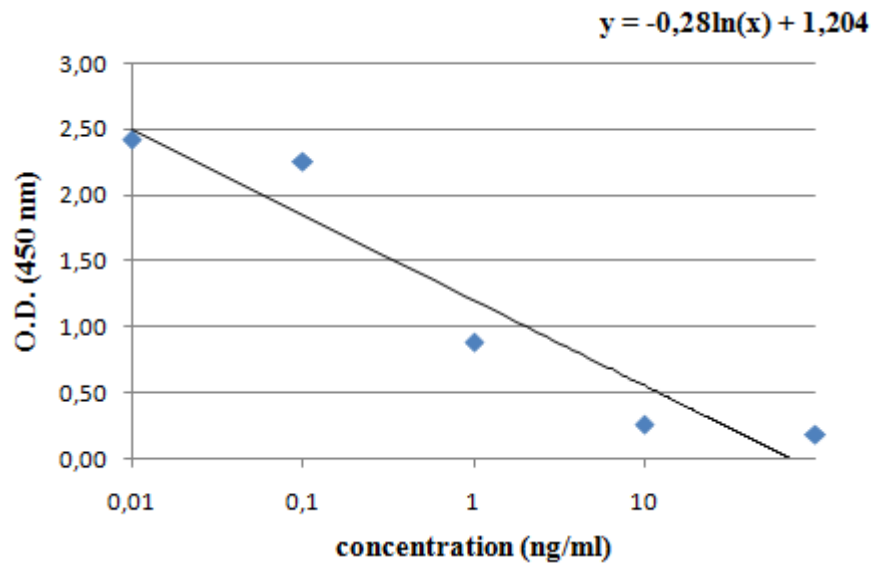
Figure A. 7 CART standard curve for human samples
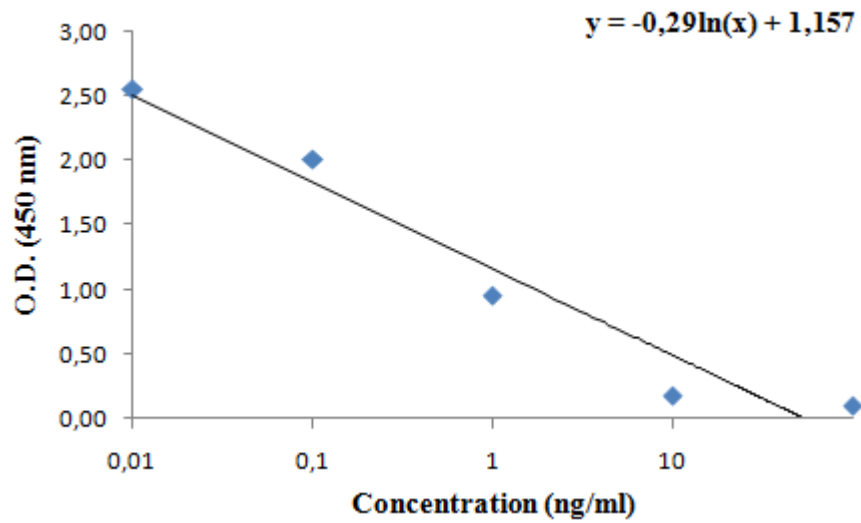
Figure A. 8 CART standard curve for rat samples
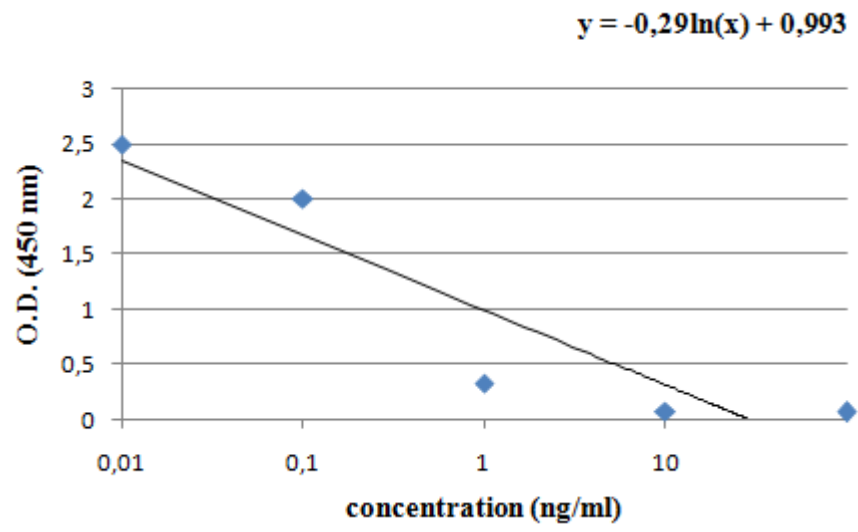
Figure A. 9 AgRP standard curve for rat samples

PRIMER DESIGN

GGTGCTCTCTGCTCCTCCCTGTTCTAGAGACAGCCGCATCTTCTTGTGCAGTGCCAGGTGAAAAAGAAAAGAAAAAAAAGGACTGGGCCGCAGGAGGCCG
GAGACGAATGGAAATTAGGAATGGGGGGAAGGACGCTGTACGGGTTTAGGGGCGCTGGTGCGAGGTCCGGAAGCCGAGCCCAGGCTCCGCATTGCAGAG
GATGGTAGAGGACGTGATGGGGCATGCGGCGGGAATGGAGGCGGGTGGGGGGAGGGGACTGGCCACGCTAATCTGACTTTCTTCTCCCGCAGCCTCGTCT
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CGTAATCCCCAGGCTGGGGCTTCTTTCTTTACTTTCGCGCCCAGAGGAGTCACGTGCCAGAAGGGGAGCCCCTCCCCCATCGTCCCCTTCCTCAGGGTCGG
TGGCACGGGGGTGTGAGGTGCATACCTTTGCGCATCTTCTAAGGGCTTCACTTATGGTAACTGGCCGCCGCCCATGTTGCAAACGGGAAGGAAATGAATGA
ACCACCGTTAAGAAATCTTCCTTCGGCCTTCCTCATTCTTAGCTTGTGACTAACTTTCTCATTCCTCTCAGCTGGGTGGAGTGTCCTTTATTCTGTAGGCCAG
GTGATGGATGGCAGGGTTTCCGTGCTCTCATTAGAGCTCTACTATACAATCCGTCTCTCGTCAGCCTTTATAGGCCTCCAGCCTTATTTCCCTCGGGCATAA
CTTATATTCTAGATTGTTCTCTGGAAGTCGAAGTGCATTTACAGAGGTCTACTTGGCCTCCCAGCCCCGGAGGTGCGGTAGCAATGGCGTAGTGCCGAGCC
CGGGGGTGGGGTGGGGAGCTGAGTCATGATGGTCCTGAAAAGAAATTTTCTGCCACAAAATGGCTCCGGTGGTAGCAGCCCCCTCCCTCCAGGGCCTCCA
CTTCCATCCCAGCTCAGCACTGACCCAAACCCTATAGGCCAGGATGTAAAGGTCACTAAGAGGATTGGGTGTCTCTGGAAGTTCACTAAGAGGATTGGGTGTCTCTGGAAGTGCATTTAC... (sequence continues)

Figure B.1 DNA sequence of *GAPDH*. The exons are highlighted and the unmarked regions represent the introns.

GGTGCTCTCTGCTCCTCCCTGTTCTAGAGACAGCCGCATCTTCTTGTGCAGTGCCAGCCTCGTCTCATAGACAAGATGGTGAAGGTC
GGTGTGAACGGATTTGGCCGTATCGGACGCCTGGTTACCAGGGCTGCCTTCTCTTGTGACAAAGTGGACATTGTTGCCATCAACGAC
CCCTTCATTGACCTCAACTACATGGTCTACATGTTCCAGTATGACTCTACCCACGGCAAGTTCAACGGCACAGTCAAGGCTGAGAAT
GGGAAGCTGGTCATCAACGGGAAACCCATCACCATCTTCCAGGAGCGAGATCCCGCTAACATCAAATGGGGTGATGCTGGTGCTGA
GTATGTCGTGGAGTCTACTGGCGTCTTCACCACCATGGAGAAGGCTGGGGCTCACCTGAAGGGTGGGGCCAAAAGGGTCATCA
CCGCCCCTTCCGCTGATGCCCCCATGTTTGTGATGGGTGTGAACCACGAGAAATATGACAACTCCCTCAAGATTGTCAGCAATGCAT
CCTGCACCACCAACTGCTTAGCCCCCCTGGCCAAGGTCATCCATGACAACTTTGGCATCGTGGAAGGGCTCATGGTATGTAGGCAATGGGAGACAGCTCATGCATGAGTGGA
CCATCACTGCCACTCAGAAGACTGTGGATGGCCCCTCTGGGAAAGCTGTGGCGTGATGGCCGTGGGGCAGCCCAGAACATCATCCCT
GCATCCACTGGTGCTGCCAAGGCTGTGGGCAAGGTCATCCATGACAACTTTGGCATCGTGGAAGGGCTCATGGTATGTAGGCAATGGGAGACAGCTCATGCATGAGTGGA
CCGAGGGCCCACTAAAGGGCATCCTGGGCTACACTGAGGCACCAGGTTGTCTCCTGGTGACTTCAACAGCAAC*TCCCATTCTTC
CACCTTT*GATGCTGGGGCTGGCATTGCTCTCAATGACAACTTTGTGAAGCTCATTTCCT*GGTATGACAATGAATATG
GCTA*CAGCAACAGGGTGGTGGACCTCATGGCCTACATGGCCTCCAAGGAGTAAGAAACCCTGGACCACCCAGCCCAGCAAGGAT
ACTGAGAGCAAGAGAGAGGCCCTCAGTTGCTGAGGAGTCCCCATCCCAACTCAGCCCCCAACACTGAGCATCTCCCTCACAATTCC
ATCCCAGACCCCATAACAACAGGAGGGGCCTGGGGAGCCCTCCCTTCTCTCGAATACCATCAATAAAGTTCGCTGCACCCTC

Figure B.2 *GAPDH* has 8 exons. Pink and gren boxes were shown only the sequential exons. The primer sequences were represented as bold and with bigger fonts.

*TCCCATTCTTCCACCTTT*GATGCTGGGGCTGGCATTGCTCTCAATGACAACTTTGTGAAGCTCATTTCCT*GGTATGACAATGAATATGGCTA*

Figure B.3 The amplified region by the synthesized primer in GAPDH gene.