

ALTERATIONS OF HYPOTHALAMIC NEUROPEPTIDES INVOLVED IN  
FOOD INTAKE AND APPETITE IN OLANZAPINE MONOTHERAPY

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DENİZ SEZLEV

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IN FOOD INTAKE AND APPETITE IN OLANZAPINE MONOTHERAPY**

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

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

Prof. Dr. Musa Doğan \_\_\_\_\_  
Head of Department; **Biological Sciences**

Assist. Prof. Dr. Tülin Yanık \_\_\_\_\_  
Supervisor, **Biological Sciences Dept., METU**

**Examining Committee Members:**

Prof. Dr. Emel Arınç \_\_\_\_\_  
Biological Sciences Dept., METU

Assist. Prof. Dr. Tülin Yanık \_\_\_\_\_  
Biological Sciences Dept., METU

Assoc. Prof. Dr. Ewa Dođru \_\_\_\_\_  
Biological Sciences Dept., METU

Assist. Prof. Dr. Çađdaş Son \_\_\_\_\_  
Biological Sciences Dept, METU

Assist. Prof. Dr. Mehmet Ak \_\_\_\_\_  
Psychiatry Dept., GATA

**Date: 03.09.2012**

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

Name, Last name : Deniz Sezlev

Signature :

## **ABSTRACT**

### **ALTERATIONS OF HYPOTHALAMIC NEUROPEPTIDES INVOLVED IN FOOD INTAKE AND APPETITE IN OLANZAPINE MONOTHERAPY**

Sezlev, Deniz

M. Sc., Department of Biology

Supervisor: Assist. Prof. Dr. Tülin Yanık

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The mechanism of weight gain due to treatment with olanzapine, a serotonin receptor antagonist, has not been fully understood. Weight gain and food intake are under the control of neuropeptides/hormones, POMC (proopiomelanocortin), CART (cocaine and amphetamine regulated transcript), AgRP (Agouti-related peptide) and NPY (neuropeptide Y) that are synthesized and secreted from the arcuate nucleus (ARC) of hypothalamus. In this study, the alteration of the ARC neuropeptide/hormone levels both in humans and rats were determined as one of the weight gain mechanism. To examine olanzapine's weight gain effects, male first attack psychotic patients (pre-treatment), were hospitalized and treated for 4 - weeks (post-treatment), (n = 22), and healthy control group (n = 26) were included to the study. Case-control association design was used to analyze the changes in body mass index (BMI), peripheral leptin and the ARC neuropeptides levels. In patients, after 4-weeks of the olanzapine treatment; BMI and the waist circumference were significantly increased with average weight gain of 4.33 kg.

In pre-treatment group, NPY levels were significantly lower while  $\alpha$ -MSH, the anorexigenic product of POMC levels were significantly higher vs. control. At post-treatment, both leptin and NPY levels were significantly increased but the CART levels did not change. To further understand the underlying mechanism of olanzapine induced weight gain, the drug was orally administered to 10 healthy male Wistar rats to analyze both the hypothalamic gene expression and peripheral levels of those candidate neuropeptides. In rats food consumption was increased and hypothalamic mRNA levels of *NPY*, *AgRP* and *POMC* were decreased while *CART* levels did not show any alteration. Consistent with the expression data, circulating levels of NPY, AgRP and  $\alpha$ -MSH decreased significantly but *CART* levels were also reduced unexpectedly. In conclusion, it may be presumed that the antagonistic effect of olanzapine on the ARC neurons might be the basis for a dysregulation of the neurohormones secretion which may cause weight gain in the treated psychotic patients.

**Keywords:** olanzapine, weight gain, leptin, neuropeptide Y, Pro-opiomelanocortin

## ÖZ

# OLANZAPİN MONOTERAPİSİNDE BESİN ALIMI VE İŞTAH DUZENLENMESİNDE GÖREV ALAN HİPOTALAMİK NÖROPEPTİTLERİN DEĞİŞİMİ

Sezlev, Deniz

Yüksek Lisans, Biyoloji Bölümü

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Serotonin reseptör antagonisti olan olanzapinin neden olduğu kilo alımının mekanizması tam olarak anlaşılmamıştır. Besin ve kilo alımı, hipotalamusun arkuat çekirdeğinde (ARC) sentezlenen ve buradan salgılanan nöropeptitler/hormonların, POMC (proopiomelanokortin), CART (kokaine ve amfetamin le regüle edilen transkript), AgRP (agouti peptit) ve NPY (nöropeptit Y), kontrolünde gerçekleşmektedir. Bu çalışmada, kilo alım mekanizması olarak, ARC nöropeptit/hormon seviyelerindeki değişim, hem insanlarda hem de sıçanlarda belirlenmiştir. Olanzapin'in kilo aldırma nedenlerini incelemek için, hastaneye yatırılan erkek ilk atak psikotik hastalar (tedavi-öncesi) 4 hafta boyunca olanzapin ile tedavi edilmiştir (tedavi-sonrası) (n=22). Ayrıca kontrol grubu (n=26) da çalışmaya dahil edilmiştir. Hasta-kontrol düzenlemesi yapılarak vücut kitle indexi (BMI), periferik leptin ve ARC nöropeptit seviyeleri incelenmiştir. Dört haftalık olanzapin tedavisi sonucunda, BMI ve bel çevresi değerleri anlamlı

olarak artmış ve hastalar ortalama 4.33 kg almıştır. Tedavi öncesi değerleri kontrol grubu değerleri ile karşılaştırıldığında, NPY seviyeleri istatistiksel olarak anlamlı bir şekilde az iken, POMC nöropeptinin anoreksijenik ürünü olan  $\alpha$ -MSH ( $\alpha$ -melanosit stimüle eden hormon) seviyeleri anlamlı şekilde yüksek olduğu bulunmuştur. Tedavi sonrasında, leptin ve NPY seviyeleri anlamlı bir şekilde artarken, CART seviyelerinde bir değişiklik bulunmamıştır. Olanzapin'in neden olduğu kilo alımının mekanizmasının daha ayrıntılı araştırılması için, ilaç oral yol ile sağlıklı 10 Wistar sıçana uygulanarak, aday genlerin hipotalamik gen ifadeleri ve periferdeki seviyeleri belirlenmiştir. Sıçanlarda besin tüketimi artmış, NPY, AgRP ve POMC hipotalamik mRNA seviyeleri azalmış, ancak CART seviyelerinde herhangi bir değişiklik görülmemiştir. Gen ifadesi verileri ile uyumlu olarak, plazma NPY, AgRP ve  $\alpha$ -MSH seviyeleri istatistiksel olarak anlamlı bir şekilde azalmıştır. CART seviyelerinin de beklenmedik bir şekilde azaldığı belirlenmiştir. Sonuç olarak, olanzapin'in ARC nöronlarındaki antagonistik etkisi, nörohormon salgılanmasının etkilenmesinin temelini oluşturarak, tedavi edilen hastaların kilo alımına sebep olduğu düşünülebilir.

**Anahtar Kelimeler:** olanzapin, kilo alımı, leptin, nöropeptid-y, pro-opiomelanocortin

*To my beloved family*

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## TABLE OF CONTENTS

ABSTRACT .....	IV
ÖZ .....	VI
ACKNOWLEDGEMENTS .....	IX
TABLE OF CONTENTS .....	XI
LIST OF TABLES .....	XV
LIST OF FIGURES .....	XVI
LIST OF ABBREVIATIONS .....	XX
CHAPTERS.....	1
1.INTRODUCTION .....	1
1.1. SCHIZOPHRENIA .....	1
1.1.1. ETIOLOGY OF SCHIZOPHRENIA.....	2
1.1.2. PREVALENCE.....	3
1.2. CHANGES IN BRAIN ANATOMY IN SCHIZOPHRENIA.....	4
1.3. CHANGES IN BRAIN BIOCHEMISTRY IN SCHIZOPHRENIA .....	5
1.3.1. SEROTONINERGIC PATHWAY .....	6
1.3.1.1. SEROTONIN AND SCHIZOPHRENIA.....	9
1.4. TREATMENT OF SCHIZOPHRENIA.....	9
1.4.1. ATYPICAL ANTIPSYCHOTIC DRUG: OLANZAPINE .....	10
1.4.1.1. METABOLISM AND DISPOSITION OF OLANZAPINE.....	11
1.5. OBESITY .....	14
1.5.1. DIAGNOSTICS AND CLASSIFICATION .....	15
1.5.2. ETIOLOGY OF OBESITY .....	16
1.5.3. PREVALENCE OF OBESITY .....	17
1.6. REGULATION OF EATING MECHANISM AND NEUROPEPTIDES INVOLVED .....	18

1.6.1. REGULATION OF FOOD INTAKE .....	19
1.6.2. HYPOTHALAMIC NEUROPEPTIDES INVOLVED IN FOOD INTAKE ..	22
1.6.2.1. PROOPIOMELANOCORTIN (POMC).....	22
1.6.2.2. AGOUTI-RELATED PEPTIDE (AGRP) .....	27
1.6.2.3. COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT (CART) .....	28
1.6.2.4. NEUROPEPTIDE Y (NPY) .....	31
1.6.2.5. INTERACTION OF NEUROHORMONES INVOLVED IN THE REGULATION OF FOOD INTAKE .....	34
1.7. <i>IN VIVO</i> STUDIES OF OLANZAPINE.....	35
1.8. THE AIM OF THE STUDY .....	37
2. MATERIALS & METHODS .....	39
2.1. DIAGNOSIS AND TREATMENT OF PATIENTS .....	39
2.1.1. PLASMA PREPARATION FROM PATIENTS.....	40
2.2. RAT STUDIES .....	40
2.2.1. PREPARATION AND ADMINISTRATION OF DRUG SOLUTION .....	41
2.2.2. ANALYZING WEIGHT GAIN AND FOOD CONSUMPTION.....	42
2.2.3. ANIMAL SACRIFISE AND HYPOTHALAMUS EXTRACTION .....	42
2.2.3.1. HYPOTHALAMUS EXTRACTION .....	43
2.2.4. PLASMA PREPARATION FROM RATS .....	44
2.2.5. TOTAL RNA ISOLATION FROM RAT HYPOTHALAMUS .....	44
2.2.6. DNASE-I TREATMENT TO ISOLATED RNA SAMPLES .....	45
2.2.7. AGAROSE GEL ELECTROPHORESIS .....	45
2.2.8. COMPLEMENTARY DNA (CDNA) PREPARATION FROM ISOLATED RNA SAMPLES .....	46
2.2.8.1. PRIMER DESIGN .....	46
2.2.8.2. CDNA SYNTHESIS .....	47
2.2.9. QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (QRT- PCR).....	48
2.2.9.1. THE REACTION CONDITIONS OF THE CANDIDATE GENES .....	49

2.2.9.2. QUANTIFICATION OF QRT-PCR.....	50
2.3. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) .....	51
2.3.1. QUANTIFICATION OF PEPTIDES LEVELS FROM BOTH HUMAN AND RATS .....	51
2.4. STATISTICAL ANALYSIS.....	53
3. RESULTS AND DISCUSSION .....	54
3.1. HUMAN EXPERIMENTS .....	54
3.1.1. BODY MEASUREMENTS.....	54
3.1.2. PLASMA LEVELS OF NEUROHORMONES IN HUMAN SAMPLES .....	55
3.1.2.1. PLASMA LEPTIN LEVELS.....	55
3.1.2.2. PLASMA NPY LEVELS.....	56
3.1.2.3. PLASMA A-MSH LEVELS.....	57
3.1.2.4. PLASMA CART LEVELS .....	58
3.2. RAT STUDIES .....	59
3.2.1. WEIGHT GAIN AND FOOD CONSUMPTION .....	59
3.2.2. RNA CONCENTRATION AND AGAROSE GEL ELECTROPHORESIS ..	60
3.2.3. QRT-PCR OF CANDIDATE GENES IN RAT HYPOTHALAMIC SAMPLES.....	63
3.2.3.1. STANDARD CURVES OF GENES .....	65
3.2.3.1.1. <i>GAPDH</i> STANDARD CURVE.....	65
3.2.3.1.2. <i>NPY</i> STANDARD CURVE .....	67
3.2.3.1.3. <i>AGRP</i> STANDARD CURVE .....	68
3.2.3.1.4. <i>POMC</i> STANDARD CURVE.....	70
3.2.3.1.5. <i>CART</i> STANDARD CURVE.....	72
3.2.3.2. QUANTITATION OF THE CANDIDATE GENES EXPRESSION FOR THE VEHICLE GROUP .....	73
3.2.3.2.1 <i>GAPDH</i> EXPRESSION .....	74
3.2.3.2.2 <i>NPY</i> EXPRESSION .....	75
3.2.3.2.3 <i>AGRP</i> EXPRESSION .....	76
3.2.3.2.4 <i>POMC</i> EXPRESSION .....	77

3.2.3.2.5 <i>CART</i> EXPRESSION.....	78
3.2.3.3. QUANTITATION OF THE CANDIDATE GENES EXPRESSION FOR OLANZAPINE-TREATED GROUP .....	79
3.2.3.3.1 <i>GAPDH</i> .....	79
3.2.3.3.2 <i>NPY</i> .....	80
3.2.3.3.3 <i>AGRP</i> .....	81
3.2.3.3.4 <i>POMC</i> .....	82
3.2.3.3.5 <i>CART</i> .....	83
3.2.3.4. FOLD CHANGES IN EXPRESSION .....	84
3.2.4. RAT PERIPHERAL CONCENTRAIONS OF HYPOTHALAMIC PEPTIDES THAT REGULATE FOOD INTAKE .....	85
3.2.4.1. PLASMA LEPTIN LEVELS .....	85
3.2.4.2. PLASMA <i>NPY</i> LEVELS.....	86
3.2.4.3. PLASMA <i>AGRP</i> RESULTS.....	87
3.2.4.4. PLASMA <i>A-MSH</i> RESULTS .....	87
3.2.4.5. PLASMA <i>CART</i> RESULTS.....	88
4. CONCLUSION.....	96
REFERENCES.....	100
APPENDICIES .....	128
A. STANDARD CURVES OF ELISA.....	128
B. VOLUME OF RNA FOR CDNA SYNTHESIS.....	133

## LIST OF TABLES

### TABLES

<b>Table 2. 1</b> Administered olanzapine amounts based on the body weight of rats. ....	<b>41</b>
<b>Table 2. 2</b> Primer pairs used for the cDNA synthesis and the qRT-PCR amplification. (FP: forward primer; RP: reverse primer). ....	<b>47</b>
<b>Table 2. 3</b> Reaction mixtures of the candidate genes and the internal control.....	<b>48</b>
<b>Table 2. 4</b> The reaction conditions of the GAPDH gene.....	<b>49</b>
<b>Table 2. 5</b> The reaction conditions of the NPY gene. ....	<b>49</b>
<b>Table 2. 6</b> The reaction conditions of the POMC gene. ....	<b>49</b>
<b>Table 2. 7</b> The reaction conditions of the AgRP gene.....	<b>50</b>
<b>Table 2. 8</b> The reaction conditions of the CART gene.....	<b>50</b>
<b>Table 3. 1</b> Body Measurements of the Controls and Patients .....	<b>55</b>
<b>Table 3. 2</b> The concentration, 260/280 and 260/230 ratios of the vehicle RNA. O: olanzapine-treated rats; V: vehicle-treated rats .....	<b>61</b>
<b>Table B. 1</b> Volume of RNA used for cDNA synthesis. O: olanzapine rats; V: vehicle rats .....	<b>133</b>

## LIST OF FIGURES

### FIGURES

<b>Figure 1. 1</b> Distribution of schizophrenia worldwide .....	<b>4</b>
<b>Figure 1. 2</b> Brain areas containing serotonergic neurons. ....	<b>6</b>
<b>Figure 1. 3</b> Synthesis of serotonin.....	<b>8</b>
<b>Figure 1. 4</b> Chemical structure of the drug olanzapine .....	<b>11</b>
<b>Figure 1. 5</b> The metabolic pathway of olanzapine .....	<b>13</b>
<b>Figure 1. 6</b> The mean change of BMI worldwide since 1980-2008.....	<b>18</b>
<b>Figure 1.7</b> Leptin effect on hypothalamic appetite regulation.....	<b>19</b>
<b>Figure 1. 8</b> Leptin signaling pathway (Heshka and Jones, 2001). ....	<b>20</b>
<b>Figure 1. 9</b> Regulation of neuropeptides through leptin signaling.....	<b>22</b>
<b>Figure 1. 10</b> Processing of POMC into its products and enzymes involved.....	<b>23</b>
<b>Figure 1. 11</b> PI3K pathway induced by leptin on POMC neurons.....	<b>24</b>
<b>Figure 1.12</b> Activation of POMC neurons by 5HT-2C receptor.....	<b>25</b>
<b>Figure 1. 13</b> Cleavage of pro-AgRP to active form AgRP83-132. ....	<b>27</b>
<b>Figure 1. 14</b> Interactions of NPY/AgRP and POMC/CART neurons in the arcuate nucleus of hypothalamus.....	<b>34</b>
<b>Figure 2. 1</b> The administration of drug. ....	<b>42</b>
<b>Figure 2. 2</b> The dissected rat brain. ....	<b>43</b>
<b>Figure 3. 1</b> Human plasma leptin levels in controls and patients.....	<b>55</b>
<b>Figure 3. 2</b> Human plasma NPY levels in controls and patients.....	<b>56</b>
<b>Figure 3. 3</b> Human plasma $\alpha$ -MSH concentrations in controls and patients.....	<b>57</b>
<b>Figure 3. 4</b> Human plasma CART levels. ....	<b>58</b>
<b>Figure 3. 5</b> Weight gain of rats.....	<b>59</b>
<b>Figure 3. 6</b> The food consumption of the vehicle group. ....	<b>60</b>
<b>Figure 3. 7</b> The food consumption of the olanzapine treated group. ....	<b>60</b>

<b>Figure 3. 8</b>	Total RNA gel electrophoresis of the vehicle group (V).....	<b>62</b>
<b>Figure 3. 9</b>	Total RNA gel electrophoresis of olanzapine group (O).....	<b>63</b>
<b>Figure 3. 10</b>	The amplification data of GAPDH standard curve.....	<b>65</b>
<b>Figure 3. 11</b>	Melting curve analysis of GAPDH standard curve. ....	<b>66</b>
<b>Figure 3. 12</b>	The standard curve of GAPDH .....	<b>66</b>
<b>Figure 3. 13</b>	The amplification data of NPY standard curve .....	<b>67</b>
<b>Figure 3. 14</b>	Melting curve analysis of NPY.....	<b>67</b>
<b>Figure 3. 15</b>	The standard curve of NPY. ....	<b>68</b>
<b>Figure 3. 16</b>	Amplification of AgRP standard curve .....	<b>68</b>
<b>Figure 3. 17</b>	Melting curve analysis of AgRP standard curve. ....	<b>69</b>
<b>Figure 3. 18</b>	The standard curve of AgRP.....	<b>69</b>
<b>Figure 3. 19</b>	The quantification data of POMC standards. ....	<b>70</b>
<b>Figure 3. 20</b>	Melting curve analysis of POMC standard curve. ....	<b>71</b>
<b>Figure 3. 21</b>	The standard curve of AgRP.....	<b>71</b>
<b>Figure 3. 22</b>	The amplification data of CART. ....	<b>72</b>
<b>Figure 3. 23</b>	Melting curve analysis of CART standard curve. ....	<b>72</b>
<b>Figure 3. 24</b>	The standard curve of CART.....	<b>73</b>
<b>Figure 3. 25</b>	The amplification data of GAPDH from vehicle samples.....	<b>74</b>
<b>Figure 3. 26</b>	Melting curve analysis of GAPDH of vehicle samples. ....	<b>74</b>
<b>Figure 3. 27</b>	The amplification data of NPY from vehicle samples.....	<b>75</b>
<b>Figure 3. 28</b>	Melting curve of NPY in vehicle hypothalamic samples. ....	<b>75</b>
<b>Figure 3. 29</b>	The quantification data of AgRP from vehicle samples. ....	<b>76</b>
<b>Figure 3. 30</b>	Melting curve analysis of AgRP of vehicle samples. ....	<b>76</b>
<b>Figure 3. 31</b>	The quantification data of POMC from vehicle samples. ....	<b>77</b>
<b>Figure 3. 32</b>	Melting curve analysis of POMC of vehicle samples. ....	<b>77</b>
<b>Figure 3. 33</b>	The quantification data of CART from vehicle samples. ....	<b>78</b>
<b>Figure 3. 34</b>	Melting curve analysis of CART of the vehicle group.....	<b>78</b>
<b>Figure 3. 35</b>	The amplification of GAPDH from olanzapine-treated group samples.....	<b>79</b>
<b>Figure 3. 36</b>	Melting curve analysis of GAPDH of the olanzapine treated group ....	<b>79</b>

<b>Figure 3. 37</b> nThe quantification data of NPY from olanzapine-treated group samples.....	<b>80</b>
<b>Figure 3. 38</b> Melting curve analysis of NPY of the olanzapine treated group. The melt curve analysis showed the same peak at approximately at 86.5 <sup>0</sup> C. ....	<b>80</b>
<b>Figure 3. 39</b> The amplification of AgRP from olanzapine-treated group samples. ..	<b>81</b>
<b>Figure 3. 40</b> Melting curve analysis of AgRP of the olanzapine treated group .....	<b>81</b>
<b>Figure 3. 41</b> The quantification data of POMC from olanzapine-treated group samples.....	<b>82</b>
<b>Figure 3. 42</b> Melting curve analysis of POMC of the olanzapine treated.....	<b>82</b>
<b>Figure 3. 43</b> The amplification data of CART from olanzapine-treated group samples.....	<b>83</b>
<b>Figure 3. 44</b> Melting curve analysis of POMC of the olanzapine treated.samples ...	<b>83</b>
<b>Figure 3. 45</b> Fold changes in the expressions of the candidate genes compared with the vehicle group. ....	<b>84</b>
<b>Figure 3. 46</b> Plasma leptin levels of rats. ....	<b>85</b>
<b>Figure 3. 47</b> Circulating NPY levels of rats. ....	<b>86</b>
<b>Figure 3. 48</b> Circulating AgRP levels of rats. ....	<b>87</b>
<b>Figure 3. 49</b> Plasma $\alpha$ -MSH levels of rats. ....	<b>88</b>
<b>Figure 3. 50</b> Plasma CART levels of rats.....	<b>89</b>
<b>Figure 3.51</b> The overall alterations in the neuropeptide levels in schizophrenic patients and rats.....	<b>90</b>
<b>Figure 4. 1</b> The proposed interactions of leptin and serotonin (5-HT) on the neurons of candidate genes involved in the appetite regulation. ....	<b>99</b>
<b>Figure A. 1</b> Human Leptin Standard Curve .....	<b>128</b>
<b>Figure A. 2</b> Rat Leptin Standard Curve.....	<b>129</b>
<b>Figure A. 3</b> Human NPY Standard Curve.....	<b>129</b>
<b>Figure A. 4</b> Rat NPY Standard Curve .....	<b>130</b>
<b>Figure A. 5</b> Human $\alpha$ -MSH Standard Curve.....	<b>130</b>

<b>Figure A. 6</b> Rat $\alpha$ -MSH Standard Curve.....	<b>131</b>
<b>Figure A. 7</b> Human CART Standard Curve .....	<b>131</b>
<b>Figure A. 8</b> Rat CART Standard Curve .....	<b>132</b>

## LIST OF ABBREVIATIONS

<b>5-HT</b>	Serotonin, 5-hydroxytryptamine
<b>5-HT<sub>1</sub> - 5-HT<sub>7</sub></b>	Serotonin receptors
<b>AgRP</b>	Agouti-related peptide
<b>BBB</b>	Blood Brain Barrier
<b>BMI</b>	Body Mass Index
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CART</b>	Cocaine and amphetamine regulated transcript
<b>cDNA</b>	complementary deoxyribonucleic acid
<b>Ct</b>	Threshold cycle
<b>DEPC</b>	Diethylpyrocarbonate
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>GAPDH</b>	Glyceraldehyhde-3-phosphate dehydogenase
<b>GPCR</b>	G-protein coupled receptor
<b>JAK</b>	Janus kinase
<b>MAO</b>	Monoamine oxidase
<b>MC-R</b>	melanocortin receptor
<b>M-Mul-V</b>	Moloney Murine Leukemia Virus
<b>NPY</b>	Neuropeptide Y
<b>Ob-R</b>	Leptin receptor
<b>PC</b>	prohormone convertase
<b>PI3-K</b>	phosphatidylinositol-3-kinase
<b>PIP2</b>	phosphatidylinositol-4,5-bisphosphate
<b>PIP3</b>	phosphatidylinositol-3,4,5-trisphosphate
<b>POMC</b>	Pro-opiomelanocortin
<b>qPCR</b>	quantitative real time polymerase chain reaction
<b>RNA</b>	ribonucleic acid
<b>RNase</b>	ribonuclease
<b>RT-PCR</b>	reverse transcriptase polymerase chain reaction
<b>SERT</b>	Serotonin reuptake protein
<b>SOCS</b>	suppressor of cytokine signaling 3
<b>STAT</b>	Signal transducer and activator of transcription
<b>Y.R</b>	NPY receptors
<b><math>\alpha</math>-MSH</b>	alpha melanocyte stimulating hormone

## CHAPTER 1

### INTRODUCTION

#### 1.1. Schizophrenia

Syndromes recognized as types of psychosis appeared firstly in the writings of Hippocrates (Andreasen, 1995). The disease that is now referred to as “schizophrenia” first received its definition in the late nineteenth century under the name of “dementia praecox” (early dementia) in the writings of Emil Kraepelin in 1887 (Kraepelin *et al*, 1919). His definition separated “dementia praecox” from manic depressive illness and from the other forms of dementia occurring elderly, which was later on renamed as Alzheimer’s disease (Andreasen, 1995). In Krapelin’s definition, “dementia praecox” could begin early in life but had a remitting course; which was disproved in the early twentieth century by Bleuler, as the illness was not a dementia, it did not always lead to mental deteriorantation, could either occur late or early in life and the illness causes a severe fragmentation of thinking and personality (Schizo = split, phrenia = mind) (Bleuler, 1950). Bleuler also introduced the concept of positive and negative symptoms to the definition of schizophrenia (Kaplan & Sadock’s, 2000). Today, according to World Health Organization (WHO), schizophrenia is defined as a severe mental disorder which is characterized by disruptions in thinking, affecting sense of self, language and perception and also associated with some psychotic experiences such as delusions or visual hallucinations. In other words, it is a clinical syndrome with variable but detractive psychopathology

involving thought, emotion, movement, perception and behavior (Kaplan & Sadock's, 2000).

Being a clinically confusing illness, the diagnostic criteria of schizophrenia was not cleared out (Andreasen, 1995) until the WHO and the American Psychiatric Association suggested criterion-based systems for diagnosing schizophrenia, the fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual which was incorporated in the recent versions of their diagnostic systems; the tenth International Classification of Disease (Kaplan & Sadock's, 2000).

According to International Classification of Disease and American Psychiatric Association's Diagnostic and Statistical Manual, the core properties of schizophrenia are (1) showing at least one or two of a list of psychotic, which are also called positive symptoms such as tactile, auditory or visual hallucinations, delusions, disorganized thought and/or speech (Kneisl and Trigoboff, 2004) and negative symptoms such as alogia, affective blunting, anhedonia, asociality and avolition (Velligon and Alphs, 2008), (2) decline in social functioning and self-care, (3) "exclusion criteria" where other disorders such as mood disorder, must be ruled out before assigning a diagnosis of schizophrenia (4) an adequate duration time of six months for American Psychiatric Association's Diagnostic and Statistical Manual or one month for International Classification of Disease (Andreasen, 1995 and Barbato, 1998).

### **1.1.1. Etiology of schizophrenia**

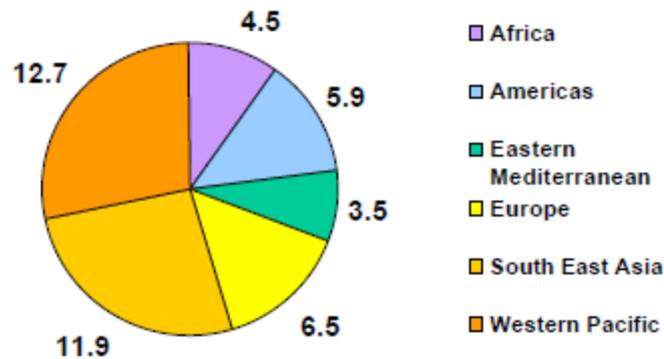
Whether the reason of developing schizophrenia is the failure of the normal development of the brain or it is the disease itself that in time, changes the normally developed brain is still not known. Even though the etiological process causing the pathophysiology of schizophrenia is still not known, the risk factors associated with the development of schizophrenia have been identified (Kaplan &

Sadock's,2000). Family and twin studies suggest strong evidence that genetic factors contributes to schizophrenia (Gottesmen, 1991). Linkage analysis for instance, reveals a potential location on chromosome 6 (Kenneth *et al*, 1996) or an association with chromosomes 4, 13, 22 (Shaw *et al*, 1998), 10 (Schwab *et al*, 2000) and 15 (Freedman *et al*, 2001). Being vulnerable to schizophrenia may also be due to environmental factors (Tsuang *et al*, 2001). Some environmental factors such as place and season of birth may play role in the development of schizophrenia (Mortensen *et al*, 1999). A study performed by Brown et al, 2004 clearly suggested that, prenatal exposure to viruses such as influenza, especially in the first trimester of pregnancy, is associated with schizophrenia, because, the fever during infection affects the normal brain development, particularly at the time when active neuronal cell migration takes place.

### **1.1.2. Prevalence**

According to WHO (World Health Organization), in the age group of 15-35 years-old adult population, about 7 per thousand are suffer from schizophrenia which is approximately 24 million people worldwide (WHO-Global burden of disease, 2004) (Fig. 1.1).

**Number of people with schizophrenia world-wide  
(in millions)**



**Figure 1. 1** Distribution of schizophrenia worldwide (WHO-Global burden of disease 2004).

As shown in Fig. 1.1., the distribution of the illness throughout the world, prevalence of schizophrenia is higher in the Western Pacific region of WHO, followed by South East Asia, Europe, Eastern Mediterranean, America and with the lowest rate in Africa region.

## **1.2. Changes in brain anatomy in schizophrenia**

Schizophrenia is the illness that disrupts some functions of the brain. Therefore it is reasonable to presume that alterations in volumes of specific areas or neuronal circuits of the brain for instance, are associated with the pathophysiology of schizophrenia. Those alterations would change the processing of the physiological information and thus emerging of the symptoms of schizophrenia (Kaplan & Sadock's, 2000).

The changes in brain anatomy could be analyzed by postmortem tissue examinations (Laplan & Sadok's 2000, and Brown *et al*, 1986). With the development of structural imaging systems, such as computerized-axial tomography (CT) and magnetic resonance imaging (MRI), and functional *in-vivo*

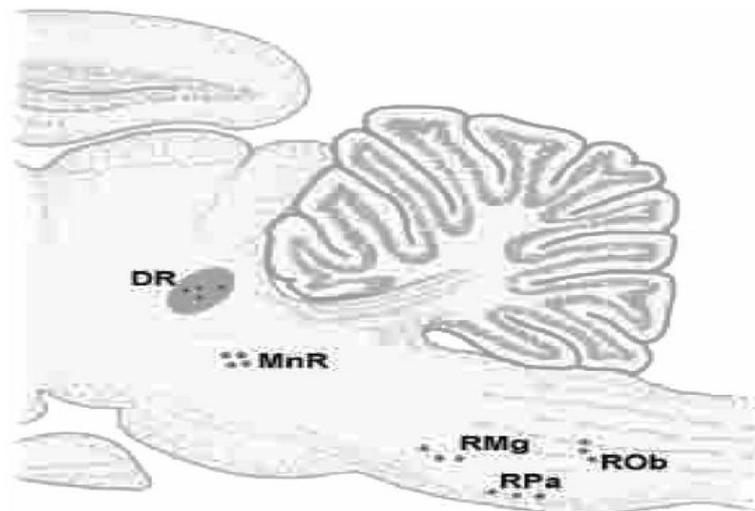
imaging systems such as, positron emission tomography (PET), single photon emission computerized tomography (SPECT) and functional MRI, made it possible to visualize the structure and physiology of the brain in living patients (Kaplan & Sadock's, 2000). Results of some imaging studies indicate that anatomical brain abnormalities play an important role in the pathology of schizophrenia. The most consisting morphological abnormalities found in schizophrenic patients are lateral ventricle enlargement which is an indicator of reduced brain volume (Suddaht *et al*, 1989), third ventricle enlargement (Bornstein *et al*, 1991), decreased volume of cortical gray and white matter (Suddaht *et al*, 1989 and Canon *et al*, 1998) and volume reductions in specific brain regions such as frontal lobes, thalamus and some limbic structures such as amygdala and hippocampus (Kaplan & Sadock's, 2000).

### **1.3. Changes in brain biochemistry in schizophrenia**

Other than having changes in brain anatomy, schizophrenic patients have abnormalities in their several pathways; such as, dopaminergic pathways, GABAergic pathways and serotonergic pathways. Patients with schizophrenia exert increased dopamine receptor (D2) transmission (Davis *et al*, 1991) and hypersensitivity to dopamine (DA) which results in the positive symptoms of schizophrenia (Seeman *et al*, 2005). The other pathway that exerts abnormalities is the GABAergic pathway.  $\gamma$ -Aminobutyrate (GABA), on the other hand, is one of the important inhibitory neurotransmitter in mammalian central nervous system (CNS) and almost all neurons are inhibited by GABA (Watanabe *et al*, 2002) and also is associated with schizophrenia. For instance, increased GABA<sub>A</sub> receptor density and binding in the prefrontal cortex (Benes, 1996), and reduced GABA uptake sites in hippocampus (Reynolds, 1990) have been suggested. There are also studies showing decreased GABA cell number or GABA transmission (Kaplan and Sadock's, 2000).

### 1.3.1. Serotonergic pathway

When first discovered in 1947, serotonin (5-hydroxytryptamine, 5-HT) was considered to be a substance which modulates vasoconstriction, but later on it is defined to be a neurotransmitter. Serotonin is in the class of monoamine transmitters and its biosynthesis resembles that of catecholamines'. The localization of serotonergic neurons is spread throughout central nervous system (CNS). Being predominantly found in raphe nuclei, serotonergic receptors are localized relatively in higher densities in areas such as cerebral cortex, hippocampus and amygdala.



**Figure 1. 2** Brain areas containing serotonergic neurons. The serotonergic innervation of the brain consists mainly of projections originating in the nuclei of the dorsal raphe and the medial raphe. DR=dorsal raphe; MnR=medial raphe; RMg=nucleus raphe magnus; ROb=nucleus raphe obscurus; RPa=nucleus raphe pallidus (Dermietzel& Halbach, 2006).

To date, seven subtypes of 5-HT receptors (5-HT<sub>1</sub> to 5-HT<sub>7</sub>), and three different molecular structures of these receptors have been identified. The largest group of serotonergic system receptors is G-coupled receptors; however, there are subtypes of 5-HT receptors which are either transporter or ligand-gated ion channel. 5-HT

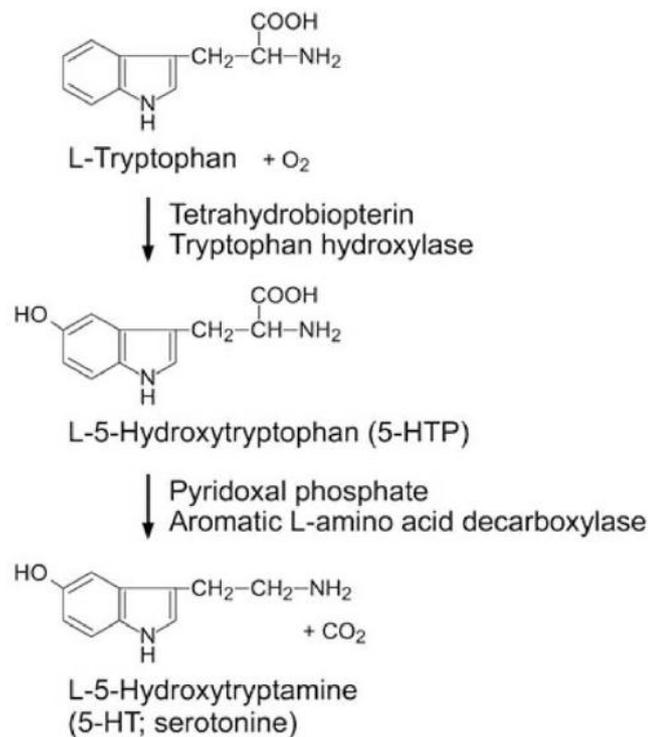
receptors are further categorized due to their pharmacological and structural properties (Table 1.1).

**Table 1. 1.** Subtypes of serotonin (5-HT) receptors

G-coupled receptors	5-HT <sub>1</sub> receptor family	5-HT <sub>1A</sub> , 5-HT <sub>1B</sub> , 5-HT <sub>1D</sub> , 5-HT <sub>1E</sub> , 5-HT <sub>1F</sub>
	5-HT <sub>2</sub> receptor family	5-HT <sub>2A</sub> , 5-HT <sub>2B</sub> , 5-HT <sub>2C</sub>
	Other receptors	5-HT <sub>4S</sub> , 5-HT <sub>4L</sub> , 5-HT <sub>5A</sub> , 5-HT <sub>5B</sub> , 5-HT <sub>6</sub> , 5-HT <sub>7</sub>
Transporters		5-HT “uptake site”
Ligand-gated ion channels		5-HT <sub>3</sub>

5-HT<sub>1</sub> receptors have seven different members (1A, 1B, 1D, 1E, 1F, 1P and 1S), show high affinity for serotonin, unlike 5-HT<sub>2</sub> family or 5HT<sub>4</sub> receptors, they generate inhibitory effects on signal transduction. 5-HT<sub>2</sub> receptors, on the other hand, display excitatory effects and are coupled to phospholipase C resulting in the activation of cAMP-independent protein kinase C. 5-HT<sub>2</sub> receptor family has three members; 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub>. 5-HT<sub>3</sub> receptors are not coupled to G proteins. They belong to ligand-gated ion channels and permeable to sodium (Na) and potassium (K) ions. The activation of the receptor leads to an increase in Na<sup>+</sup> and K<sup>+</sup> permeability and makes the membrane impermeable to divalent cations, thus, depolarization of the neuron. 5-HT<sub>4</sub> receptors differ from 5-HT<sub>1</sub> receptors by activating cAMP. 5-HT<sub>5</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors are positively coupled to adenylatecyclase.

Serotonin is synthesized from tryptophan; which is the rate limiting step. The synthesis pathway of serotonin is shown in Fig. 1.3.



**Figure 1. 3** Synthesis of serotonin (Dermietzel& Halbach, 2006). In the existence of the cofactor (tetrahydropterine) of the enzyme and oxygen molecules tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. 5-HTP is then decarboxylized to 5-HT by an aminoacid decarboxylase.

The degradation of 5-HT is performed by the serotonin transporter (SERT). SERT, causes the re-uptake of extracellular serotonin into cytosol to be repackaged or MAO enzymatically inactivates serotonin. 5-hydroxyindolic acid (5-HIAA), the resulting metabolite is then eliminated via urinary tract.

Serotonergic system has influences on dopaminergic system. For instance, somatodendritic 5-HT<sub>2</sub> receptors regulate dopaminergic firing and striatal nerve terminal of serotonin receptors inhibit dopamine release. Also, together with dopamine, frontal serotonin plays an important role in the modulation of arousal and attention.

### **1.3.1.1. Serotonin and schizophrenia**

Serotonin's possible role in schizophrenia was first recognized in 1950, when researchers discovered that serotonin and lysergic acid diethylamide (LSD), a potent hallucinogenic drug that competes for and occupies serotonin receptor sites with high potency, possess common functional features (Dermitzel&Halbach, 2006). Studies performed with schizophrenic patients revealed a reduction in the density of 5-HT<sub>2</sub> receptor in prefrontal cortex. Moreover, in frontal cortex, the density of reuptake sites is also found to be decreased, but the levels of serotonin were found to be increased in some brain areas such as prefrontal cortex (Kapur& Remington, 1996). Also, receptor sensitization occurs in schizophrenia. Studies indicate that, serotonergic pathway arising from dorsal raphe and projecting to substantianigra, inhibit dopamine neurons and it is hypothesized that elevated levels of serotonin in the prefrontal cortex leads to lower levels of dopamine in that area and that the reduced levels of dopamine in the area causes the negative symptoms of schizophrenia and leads to increased levels of dopamine in other dopaminergic pathways, resulting in the positive symptoms of schizophrenia.

### **1.4. Treatment of schizophrenia**

For the treatment of psychosis, particularly schizophrenia is accomplished by the use of antipsychotics. Antipsychotics are divided into two groups as typical and atypical antipsychotics. Until 1952, all the drugs were used for the treatment is thought to have same effect on the disease but then many clinicians believed that different subtypes of schizophrenia respond to the treatment differently. However, the invention of the first typical drug "chlorpromazine" was a breakthrough in the treatment of schizophrenia. Typical antipsychotics are also called "dopamine receptor antagonist" or "neuroleptics". They have very high occupancy to dopamine receptors (Geddes *et al*, 2000) with a little regional specificity which was a problem because the selectivity of the antagonism to dopaminergic system was poor resulting in communication and activity impairment (Ereshefsky and

Lacombe, 1993). They are effective in controlling the positive symptoms of schizophrenia but create extrapyramidal side effects (EPS). More importantly, they caused lethal complications such as neuroleptic malignant syndrome and with the long term usage, tardive dyskinesia (Kaplan and Sadock, 1995).

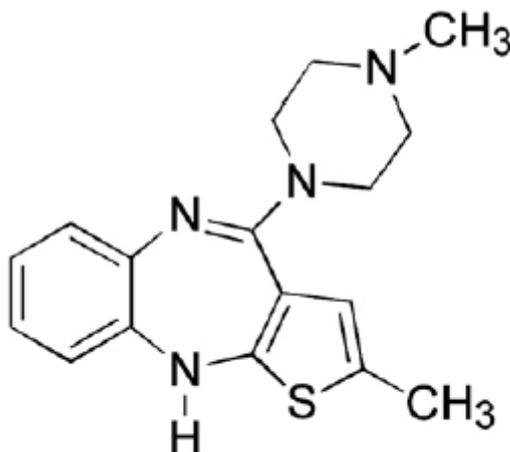
Because of these negative advantages of traditional antipsychotic, atypical antipsychotics were generated. Atypical antipsychotics are also called “serotonin-dopamine antagonists” (Kaplan and Sadock, 1995). They differ in their actions on serotonergic and dopaminergic receptors resulting in fewer side effects. They have less occupancy for D2 receptors. They bind to D2 receptor more loosely and they are more quickly released (Keefe *et al*, 2006). Moreover, since typical antipsychotics remain attached to D2 receptors, they accumulate in the brain tissue, which eventually leads to tardive dyskinesia. However, second generation antipsychotics, atypical ones, are released from the receptor very rapidly, which decreases the risk of causing tardive dyskinesia (Seeman, 2004). Even though, preventing these kinds of side effects of typical antipsychotic, atypical antipsychotics create some other adverse effects like weight gain and metabolic abnormalities including insulin resistance, type II diabetes, hypoglycemia, hyperlipidemia and with long term usage cardiovascular diseases (Sertie *et al*, 2011).

#### **1.4.1. Atypical antipsychotic drug: Olanzapine**

One of the atypical antipsychotic drug olanzapine (Zyprexa®) which belongs to thienobenzodiazepine class is known to have a potent effect on weight gain, indeed has the highest ability to induce weight gain after clozapine which was the main reason why its mechanism causing weight gain was investigated in this study.

The systematic name of olanzapine is 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b] [1,5]benzodiazepine and its molecular formula is C<sub>17</sub>H<sub>20</sub>N<sub>4</sub>S. Its

molecular weight is 312.44 g (Wawrzycka-Gorcyca et al, 2003). The chemical structure of olanzapine is shown in Fig. 1.4.



**Figure 1. 4** Chemical structure of the drug olanzapine (Olanzapine - prescribing information, 2010)

Just like other atypical antipsychotics, olanzapine binds to D<sub>2</sub> and 5-HT<sub>2C</sub> receptors. It has also affinities for 5-HT<sub>2A</sub>, 5-HT<sub>6</sub>, D<sub>1-4</sub>, histaminergic H<sub>1</sub>, adrenergic  $\alpha$ -1 receptors. Its moderate antagonistic effect is on 5-HT<sub>3</sub> and muscarinergic receptors M<sub>1-5</sub>. Moreover it binds weakly to GABA<sub>A</sub> and 5-HT<sub>1B</sub> receptors (Olanzapine- prescribing information, 2010 and Bymaster *et al*, 1996).

With clinical studies performed by people suffering from schizophrenia, olanzapine was found to be very effective in the treatment of schizophrenia with fewer extra pyramidal side effects (EPS) and the antipsychotic efficacy of olanzapine is found to be 5-20 mg/day (Bymaster *et al*, 1996).

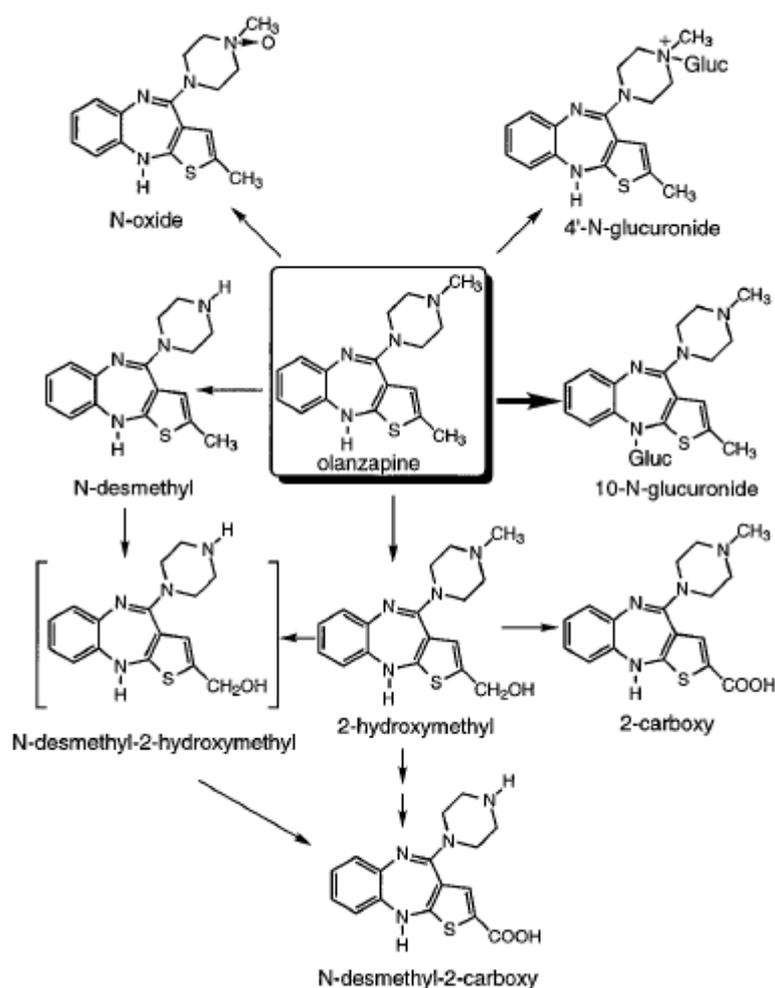
#### **1.4.1.1. Metabolism and disposition of olanzapine**

The disposition of olanzapine in human was evaluated with a study performed by six healthy volunteers (Kassahun *et al*, 1997). By using radioactive <sup>14</sup>C, they determined that, around 50% of the drug is excreted from urine and approximately 30% is from feces within 15 to 21 days right after administration of a single dose

of 12.5 mg of olanzapine. Moreover, the plasma  $^{14}\text{C}$  made a peak at 4 to 6 hours after the administration. *In-vitro* studies in the same study revealed that, olanzapine was highly bound to albumin, moderately to  $\alpha$ -1 glycoprotein and minimally to  $\gamma$ -globulins and that the half-life of olanzapine was 30 hrs in human (Kassahun *et al*, 1997)

One study concerning the disposition of olanzapine in rats, the half-life of olanzapine and tissue distribution after receiving a single oral dose of 6 mg/kg were analyzed. It was shown that olanzapine was present in the rat brain even after 48 hrs of the dose applied. Also, the concentrations of olanzapine after the 6 mg/kg/day of oral dosing and intraperitoneal injection (i.p.) for consecutive 15 days were evaluated. They found out that with oral dosing, the concentration of olanzapine in the brain was  $151 \pm 87.0$  vs. i.p. injection which was  $63.1 \pm 14.7$  (Aravagiri *et al*, 1999).

The metabolism of olanzapine is performed by the many enzymes including the cytochrome P450 enzymes; CYP1A2 and CYP2D6, flavin mono-oxygenase (FMO) and uridinedisphosphateglucuronyltransferase (UDPGT) (Callaghan *et al*, 1999) which is illustrated in Fig. 1.5.



**Figure 1. 5** The metabolic pathway of olanzapine (Kassahun *et al*, 1997).

In humans, olanzapine is mostly metabolized to 10-N-glucuronide, N-desmethyl olanzapine, olanzapine N-oxide and 2-hydroxymethyl olanzapine (Kassahun *et al*, 1997) and. Olanzapine reacts with UDPGT to form 10-N-glucuronide and 4-N-glucuronide which is the prominent pathway and 10-N-glucuronide is the major circulating metabolite. CYP1A2 and FMO reactions result with the formation of N-desmethyl olanzapine and olanzapine-N-oxide; respectively. The formation of 2-hydroxymethyl via CYP2D6 is the minor pathway and it is the minor metabolite of olanzapine. In rats, olanzapine accounted for 38 %, 2-hydroxymethyl

olanzapine for 8% and N-desmethyl olanzapine for 5% of the plasma (Chiu and Franklin, 1996).

### **1.5. Obesity**

Even though having completely different definitions being “overweight” or “obese” are two terms that could be confused easily. Being overweight is the condition in which a person has a body weight that is beyond the standard weight of a particular person, based on his or her height and frame size. It is possible to be overweight according to those standard values but have a total body fat which is average or below average. It is also possible to be within the normal range of those standards and yet has excessive amount of body fat (Wilmore J, 1994). Obesity, on the other hand, is the excess amount of body fat accumulation in an individual’s body (Haslam *et al*, 2006), which means that to diagnose obesity, the amount of body fat in an individual’s body, or its percentage of a person’s weight must be determined (Wilmore J, 1994).

Many diseases results from being overweight or obese for a long time leads to weakness, decrease in quality of life (Manson *et al*, 1995), health problems such as diabetes mellitus, insulin resistance, dyslipidemia, hypercholesterolemia and even diseases results in death such as prostate and kidney cancer and coronary heart diseases (Kopelman P, 2000, Munsch *et al*, 2005). The distribution of body fat is critical for developing risk of health problems associated with obesity. Having android distribution of body fat, the fat that has accumulated mainly in the upper part of the body such as abdomen, chest or neck, leads an increase in the risk of developing diabetes and heart diseases, while gynoid type of fat distribution, the fat which is accumulated around the hips and bottom, has a lower risk for mentioned diseases (Bray G, 2004).

### 1.5.1. Diagnostics and classification

For both diagnostics and classification the degrees of overweight, the most widely used parameter is Body Mass Index (BMI) (Wabitsch M, 2000) which is calculated by dividing the weight (kg) by the height square of the adult person (m<sup>2</sup>) (Munsch and Beglinger, 2005). According to WHO, the National Institute of Health (NIH) and the German Obesity Society (Deutsche Adipositas-Gesellschaft – DAG), to specify the degree of overweight, age and gender-independent BMI should be used. The classification of obesity on based on BMI is shown in Table 1.2.

**Table 1. 2.** The BMI values used to classify the obesity

	BMI (kg/m <sup>2</sup> )
Normal Weight	20-25
Overweight	25-30
Obesity I	30-35
Obesity II	35-40
Obesity III (Morbid obesity)	>40

Another important parameter that is taken into account when measuring the obesity risk is waist-to-hip ratio (WHR) which is calculated by dividing the circumference of waist by the circumference of hip (Aronne N, 2002). WHR is an indicator of fat distribution of an individual's body, android or gynoid type (Bray G, 2004). For women and men, having a waist circumference higher than 88 cm and 102 cm, respectively, shows the presence of abdominal obesity (Munsch and Beglinger, 2005) and indicates the risk of developing health problems (Kopelman P, 2000).

### 1.5.2. Etiology of obesity

The etiology of obesity is multifactorial. In other words, it can be caused by one or a combination of many factors (Wilmore J, 1994) such as genetic factors, (Cummings and Schwartz, 2003, Barsh *et al*, 2000) prenatal factors (Gorski *et al*, 2006), dietary habits, physical inactivity (Munsch *et al*, 2005) and eating behavior (Wabitsch M, 2000). It could be the consequence of some diseases like diabetes (Kahn and Flier, 2000) and polycystic ovary syndrome (Barber *et al*, 2006) or drug usage for treatment of diseases such as schizophrenia (Ratzoni *et al*, 2002).

Not only life-style but also some genetic or prenatal factors may lead to obesity. According to a study performed by Yeo *et al.*, 1998, a frameshift mutation in MC4 (melanocortin 4) receptor is associated with morbid obesity (BMI >40) (Vaisse *et al*, 1998). Also, Clement *et al.*, 1998, showed that a mutation in leptin receptor gene, results with early onset obesity. It was also shown that mutations in leptin gene (Montague *et al*, 1997) and prohormoneconvertase 1 gene (Jackson *et al*, 1997) were associated with obesity in humans; however, not all obese individuals have these mutations (Munsh and Beglinger, 2005), suggesting that obesity is dependent on many genetic variations of an individual.

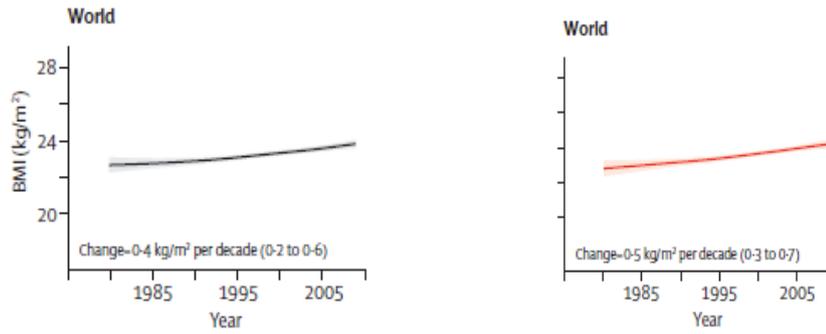
Prenatal factors, on the other hand, causes offspring to be prone to obesity. For instance a rat study performed by Levin and Govek, 1998, showed that, the high energy diet during gestation causes an increase in the food intake of offspring. Horton *et al*, 1995, showed that the type of diet has influences on developing obesity, indeed, not high carbohydrate but high fat diet leads to obesity. Although carbohydrates are also converted to fats in the body by lipogenesis, fat leads more fat accumulation in body than does the carbohydrate. It is known that obesity results in low responsiveness to internal satiety signals but high responsiveness external appetite signals. Also it is also suggested that normal-weight individuals

have a set level of fat stored in their body (Munsh and Beglinger, 2005). This level is regulated by increasing the appetite when there is a decrease in the fat percentage and by suppressing it if the fat percentage is elevated. The set point of obese people; however, is higher than that of normal weight, leading to abnormal appetite when they are below their own set point (Munsh and Beglinger, 2005). For etiology of obesity the interaction between genetics and environment is also important. It is claimed that being genetically predisposed to develop obesity, does not necessarily means that individuals will be obese later in life; because, that genotype may be only be expressed under certain environmental conditions, such as high fat diet or physically inactive lifestyles (Stunkard A, 1998).

### **1.5.3. Prevalence of obesity**

The prevalence of being overweight or obese is increasing year by year, worldwide. According to WHO, nearly 1 billion people were overweight and approximately 300 million were obese in 2005 (WHO, 2009). It is estimated that, obesity rates will increase in almost all countries, to 1.5 billion people (Tunstall-Pedoe H, 2006) in 2015.

The prevalence of obesity is associated with the income level of the country that the rate of obesity seen in countries with high income is twice that of seen in countries with low income (WHO bulletin, 2011). According to the WHO 2009 bulletin, the mean increase in the change of BMI per decade in women is more than men, between 1980 and 2008, worldwide (Fig 1.6).



**Figure 1. 6** The mean change of BMI worldwide since 1980-2008 (WHO bulletin, 2009).

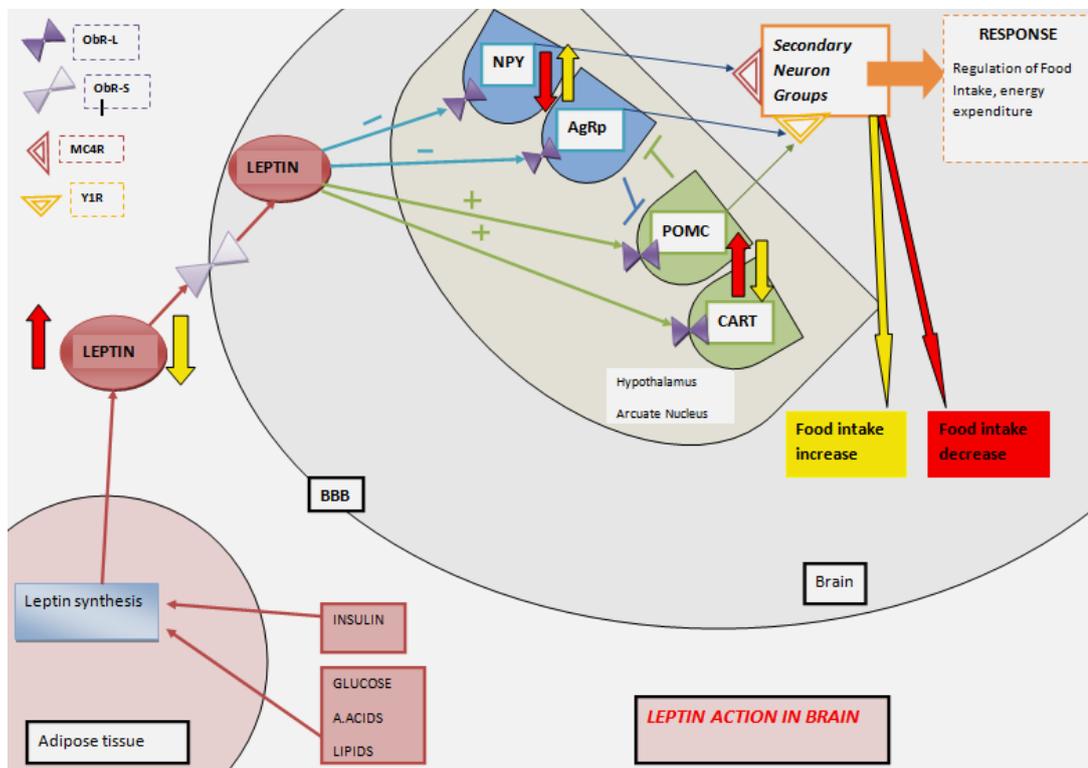
In Turkish population, the prevalence of obesity seen in women increased 5.1% from 1998 to 2008 (Turkey Demographic and Health Survey, 2008). Moreover, 18.8% of women were obese in 1998 and the number increased to %23.9 in 2008 (The Health Ministry of Turkish Republic, The General Management of Basic Health Service, 2010). In 2000-2005, nearly 14.000 individuals from 6 cities, İstanbul, Konya, Denizli, Gaziantep, Kastamonu and Kırklareli were evaluated for their body weight that 39.6 % of them overweight and 29.5 % of them were obese (Bagriacik *et al*, 2009).

### **1.6. Regulation of eating mechanism and neuropeptides involved**

Eating mechanism and energy homeostasis are controlled with the involvement of many factors which are mainly leptin, an adipose tissue derived hormone which can pass blood-brain-barrier (BBB), and neuropeptides that are synthesized and secreted from the arcuate nucleus of hypothalamus, proopiomelanocortin (POMC), agouti-related peptide (AgRP), neuropeptide Y (NPY) and lastly, cocaine and amphetamine regulated transcript (CART). The interactions between those peripheral and central nervous system (CNS) signals will be discussed in the next sections.

### 1.6.1. Regulation of food intake

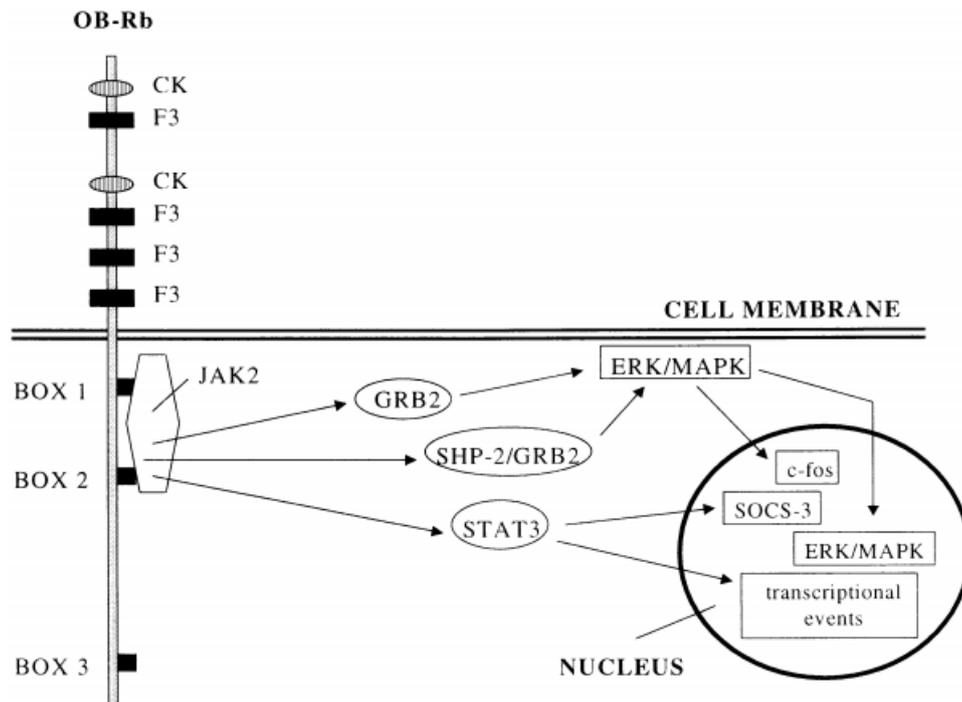
Appetite control is under control of many peripheral and CNS factors such as leptin, insulin, ghrelin and peptide YY (PYY). However, the most important regulator of food intake is leptin (Schwartz *et al*, 2000). Leptin is a product of *ob* gene which is located on chromosome 7 in humans and chromosome 6 in rats (Zhang *et al*, 1994). It is a 16 kDa protein that and mainly synthesized and secreted from white adipose tissue which however is not the only source of leptin. Gastric mucosa, skeletal muscle, placenta, bone marrow and pituitary also synthesize and secrete leptin (Ahima and Flier, 2000 and Wauters *et al*, 2000).



**Figure 1.7** Leptin effect on hypothalamic appetite regulation

Leptin acts through its receptor encoded by *ob* gene. There are six forms of leptin identified but only the long form (Ob-Rb) and short form (Ob-a) from which the former form is known to be involved in the regulation of the biological effects of

leptin which is mainly localized in the arcuate nucleus (ARC) of hypothalamus (Heshka and Jones, 2001). Ob-Rb is expressed from the neurons that are involved in the control of food intake and energy expenditure (Schwartz *et al*, 2000) and the downstream signaling pathway of leptin plays a crucial role in the regulation in the secretion of those neuropeptides. Leptin signaling is shown in Fig. 1.7.



**Figure 1. 8** Leptin signaling pathway (Heshka and Jones, 2001).

Binding of leptin to its receptor (Ob-Rb) causes a conformational change of the receptor and triggers two mechanisms. To start with, Janus kinase 2 (JAK2) family of tyrosine kinases are transphosphorylated and therefore activated. The activation of JAK2 is followed by the phosphorylation of the tyrosine residues that are found on the intracellular domain of Ob-Rb. Phosphorylation of tyrosine residues on the tail of the Ob-Rb and JAK2 leads to the phosphorylation of an adaptor protein that is involved in the signal transduction, growth factor receptor binding protein (GRB 2). Moreover, the JAK2 phosphorylation also results in

tyrosine phosphatase, SHP2 phosphorylation resulting in the phosphorylation of GRB2 (Heshka and Jones, 2001).

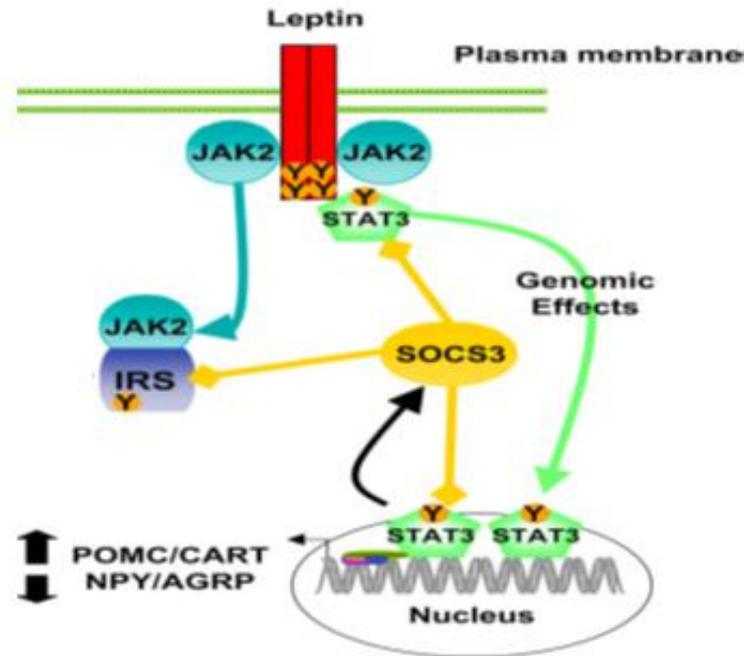
The second leptin signaling mechanism is STAT3 (Signal Transducers and activators of transcription) activation via JAK2. The STAT is a group of protein which contains serine rich homology sequences that make them possible to interact with both receptor molecules through their phosphorylated tyrosine domains and act as a DNA binding transcription factor. With the activation of STAT3, translocation to the nucleus and modulation of target genes are started (Heshka and Jones, 2001). It is even suggested that STAT3 pathway is the dominant pathway of leptin action in hypothalamus, since STAT3 is found to be present in high levels in the hypothalamus (Stromberg *et al*, 2000 and Hakansson and Meister 1998).

Binding of leptin to OB-Rb receptor also activates a tyrosine kinase-based signaling system, namely, extracellular factor-regulated kinases (ERKs). ERK pathway consists of serine-threonine kinases which include mitogen-activated protein kinases (MAPKs) (Takahashi *et al*, 1997). After the JAK2 or SHP2 induced GRB 2 tyrosine phosphorylation MAPKs translocate into the nucleus to mediate gene expression of the target gene (Banks *et al*, 2000).

Moreover, it is identified that suppressor of cytokine signaling (SOCS) is also involved in the signaling of leptin. Leptin induces SOCS-3 expression and SOCS-3 negatively regulates leptin signaling by binding to JAK2 and inhibiting tyrosine phosphorylation of OB-Rb (Bjorbaek *et al*, 1999).

It is a known fact that leptin has some neuronal groups in the arcuate nucleus of hypothalamus that have OB-Rb receptor on them. These neuronal groups are anorexigenic peptide group, POMC/CART and orexigenic peptide group

NPY/AgRP (Schwartz *et al*, 1996), which will be discussed in detail in the next sections.



**Figure 1. 9** Regulation of neuropeptides through leptin signaling (Ahima and Antwi *et al*, 2008).

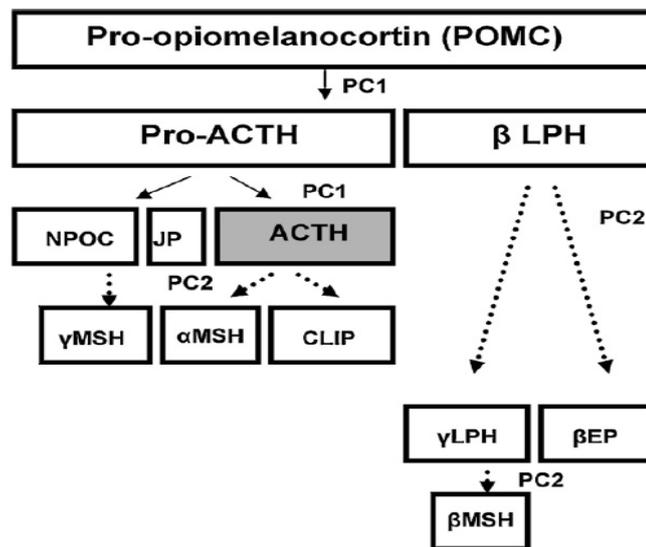
## 1.6.2. Hypothalamic neuropeptides involved in food intake

### 1.6.2.1. Proopiomelanocortin (POMC)

POMC is a satiety neurohormone and a precursor peptide for many peptides such as N-terminal peptide of proopiomelanocortin (NPP or NPOC), adrenocorticotrophic hormone (ACTH), alpha, beta and gamma-melanocyte stimulating hormone ( $\alpha$ -MSH,  $\beta$ -MSH and  $\gamma$ -MSH),  $\beta$ -endorphin, lipotropin gamma ( $\gamma$ -LPH), beta lipotropin ( $\beta$ -LPH) and corticotropin-like intermediate peptide (CLIP). It is located in the chromosome 2p23 and synthesized as pre-POMC with 285 amino-acid; however 44-amino-acid signal peptide is cleaved

during translation remaining with a 241 amino acid peptide (Dermietzel and Halbach, 2006). It consists of 7665 bp with two introns and three exons. The first exon (87bp) is the untranslated region; the second exon (152bp) encodes for the signal peptide and the first amino acids for the N-terminal and the third exon (833bp) codes for the translated region (Raffin-Sanson *et al*, 2003).

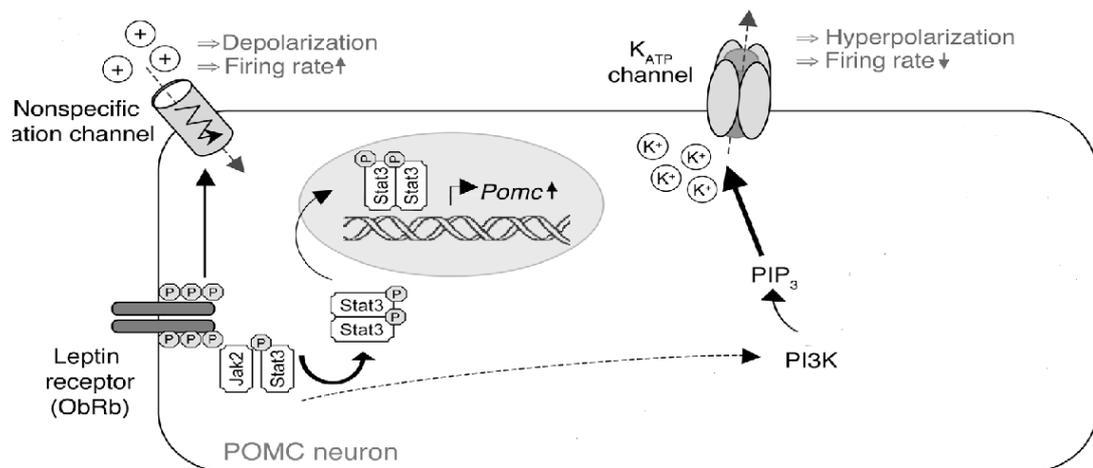
After POMC is synthesized as precursor protein, it is been processed to create different products, such as ACTH,  $\alpha$ -MSH,  $\gamma$ -MSH and  $\beta$ -lipotropin ( $\beta$ -LPH) by prohormoneconvertase 1 and 2 (PC1 and PC2) (Stovold *et al*, 2012). PC1 cleaves POMC to pro-ACTH and  $\beta$  LPH. Then pro-ACTH is converted to active ACTH, a jointing peptide (JP) and N-proopiomelanocortin (NPOC) by PC1. However, PC2 cleaves ACTH to  $\alpha$ -MSH and  $\beta$  LPH to  $\gamma$  LPH and  $\beta$ -MSH (Fig. 1.9).



**Figure 1. 10** Processing of POMC into its products and enzymes involved (Stovold *et al*, 2012).

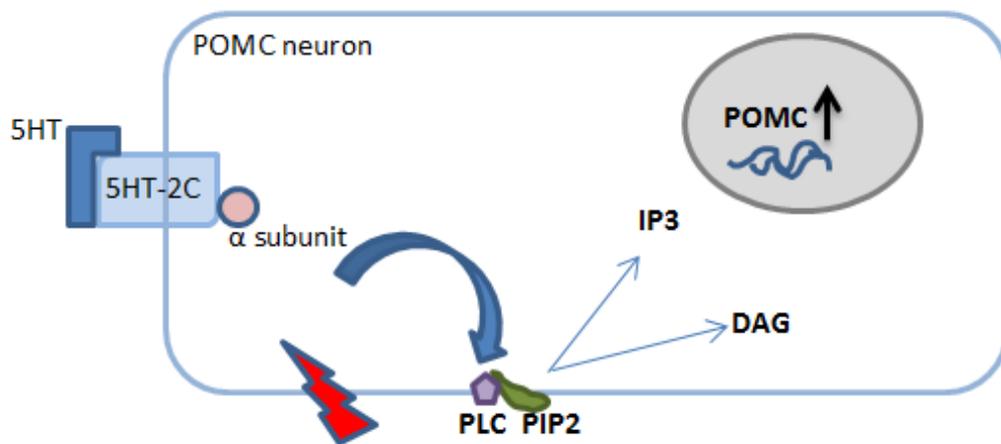
POMC is synthesized in many parts of the brain and also throughout the body (Raffin-Sanson *et al*, 2003) such as placenta, melanocytes, both in the form of POMC and  $\alpha$ -MSH, endothelial cells and keratinocytes (Raffin-Sanson *et al*, 2003). In the brain, on the other hand, it is synthesized mainly in the pituitary gland, and the ARC of hypothalamus (Dermietzel and Halbach, 2006).

POMC neuronal groups have both leptin receptor (Cheung *et al*, 1997) and 5HT-2C (Heisler *et al*, 2002) on their cell body. Therefore, POMC neuron activation is both through leptin (JAK/STAT pathway) and 5HT-2C (cAMP pathway) receptor signaling. By leptin receptor signaling POMC is upregulated and so are the products that are the cleavage products of POMC (Schindler and Darnell, 1995). Another mechanism is the phosphatidylinositol 3-kinase (PI3K) pathway, which is shown in Fig. 1.10. Binding of leptin to its receptor results in the activation of PI3K pathway. Stimulation of PI3K results in the phosphorylation the lipid phosphatidylinositol-4-5-bisphosphate (PIP<sub>2</sub>) that is found on membrane lipids to PIP<sub>3</sub>. Increased PIP<sub>3</sub> leads to the exclusion of FOXO1 from the nucleus, which has a stimulatory effect on POMC expression. Thereby, the POMC expression begins.



**Figure 1. 71** PI3K pathway induced by leptin on POMC neurons (Modified from Plum *et al*, 2006).

Another mechanism of POMC expression increase from POMC neurons in the ARC is through the 5HT-2C receptor which is found on the cell bodies of the POMC neurons (Fig. 1.12). Binding of 5HT to the 5HT-2C results in the activation of the  $\alpha$ -subunit of the GPCR. The activated  $\alpha$ -subunit stimulates phospholipase C which results in the formation of IP3 and DAG from the membrane bound PIP2.  $Ca^{+2}$  influx occurs and the neuron is activated.



**Figure 1. 12** Activation of POMC neurons by 5HT<sub>2C</sub> receptor (Modified from Plum et al, 2006).

Effects of POMC and its products on food intake are studied in many studies. For instance, intracerebroventricular (i.c.v.) injections of  $\alpha$ -MSH, the main satiety product of POMC, results in decreased food intake (Fan *et al*, 1997). It has also been shown that after 8 weeks of high-fat dieting, there was no change in POMC mRNA levels between high and low fed groups, after 19 weeks of high-fat dieting POMC mRNA levels were reduced approximately 55 % (Lin *et al*, 2000). They concluded their study by speculating a possible leptin resistance; which might be due to decreased leptin transport through BBB (El-Haschimi *et al*, 2000) or via the activation of inhibitory pathways in leptin signaling such as SOCS-3 (Ernst *et al* 2009), occurred in the brain, therefore leptin failed to increase POMC neurons and thus, POMC mRNA levels were decreased. POMC neuron activation

via 5HT-2C receptor, on the other hand, is through the increase in cAMP via G-coupled protein receptor. Serotonin, when binds to 5HT-2C receptor on POMC neurons are activated resulting in the increased POMC mRNA expression, and by extrapolation,  $\alpha$ -MSH synthesis and release (Lam *et al*, 2008 and Nanogaki *et al*, 2006). While leptin signaling and 5HT<sub>2CR</sub> function activates POMC neurons, GABAergic pathway and 5HT<sub>1BR</sub> inhibits them (Foster-Schubert and Cummings, 2006).

$\alpha$ -MSH exerts its satiety effects via two receptors (MC3 and MC4) (Rah *et al*, 2006). MC4<sup>-/-</sup> mice, for instance, has hyperphagia, increased adiposity, hyperinsulinemia and hyperleptinemia. Moreover, leptin administration to MC4<sup>-/-</sup> mice did not lead to a reduction in food intake (Marsh *et al*, 1999) suggesting that MC4 receptor signaling is important for the control of appetite. Just like MC4 receptor deletion, MC3 receptor deletion also results in increased adiposity (Butler *et al*, 2000) but they did not show any hyperphagic behavior. This might be due the role of MC3 receptor on POMC neurons which is found as an autoreceptor. Since MC3 receptor limits POMC neuronal activity, then absence of MC3 receptor would result in the increased POMC expression, therefore the hyperphagic behavior would be disrupted (Rah *et al*, 2006).

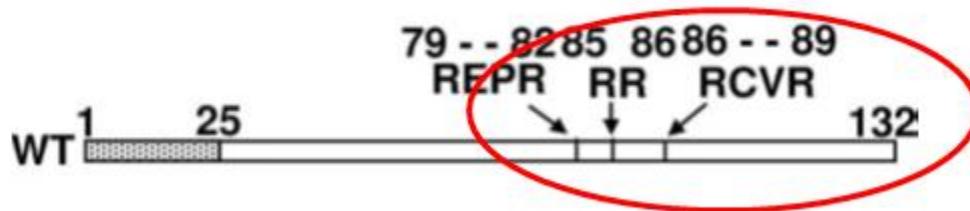
Increased POMC levels in plasma are also indicated as a response to stress. For instance, a study performed with soldiers who are taking several exams implicated that POMC and the products of POMC such as ACTH and  $\beta$ -LPH levels in blood samples of the soldiers were significantly higher than normal people who did not take any examination. Moreover, after the exam, soldiers' POMC levels were reduced to a normal value (Meyerhoff *et al*, 1988). It is stated that when rats who were exposed to electrical food shock stress, hypothalamic expression of *POMC* and *MC4 receptor* genes were increased significantly, indicating that melanocortin system, particularly *POMC* and *MC4 receptor* gene expression may be inferred as the response to stress conditions (Yamano *et al*, 2004). On the

contrary, the chronic immobilization stress led to a decrease in *POMC* gene expression in the prefrontal cortex, pituitary and hippocampus (Chen *et al*, 2008).

### 1.6.2.2. Agouti-related Peptide (AgRP)

AgRP is an orexigenic peptide with 132 aminoacid sequence and has four exons and four introns that is found on humans' 16q22 chromosome. Rat AgRP, shares 81% aminoacid identity to human AgRP with 131 aminoacid long and found on the 8D1-D2 chromosome (Shatter *et al*, 1997).

After pro-AgRP is synthesized it is predominantly cleaved by PC1/3 to create the active form of AgRP, AgRP<sub>83-132</sub>, that is involved in feeding mechanism regulation in the hypothalamus. However, in vitro studies indicate that in the absence of PC1/3, PC2 and PC5/6A can cleave pro-AgRP to active form of AgRP (Creemers *et al*, 2006). Processing sites of AgRP is shown in Fig. 1.11.



**Figure 1. 13** Cleavage of pro-AgRP to active form AgRP<sub>83-132</sub>. Cleavage sites are indicated as Arg79-X-X-Arg82 (REPR), Arg85-Arg86 (RR) and Arg86-X-X-Arg89 (RCVR). PC1/3 enzymes recognizes REPR site (Creemers *et al*, 2006).

In humans, AgRP is synthesized from other tissues than brain. AgRP mRNA was found at higher levels in adrenal cortex, followed by adrenal medulla, hypothalamus subthalamic nucleus, testis, lung and kidney, which the similar pattern for rats (Shatter *et al*, 1997).

It is known that, leptin inhibits orexigenic peptides signaling in the arcuate nucleus of hypothalamus. The expression of pattern of AgRP was examined in

study carried out by Morrison *et al*, 2005, suggesting that leptin administration to rats resulted in the increased SOCS-3 mRNA levels in the hypothalamus. As discussed earlier, SOCS-3 negatively affects leptin signaling by binding to JAK2 and inhibiting the phosphorylation of OB-Rb receptor (Morrison *et al*, 2005). In other words, increased expression of SOCS-3, results in the inhibition of phosphorylation of OB-Rb which in turn leads to reduced levels of AgRP expression. Another mechanism for the inhibition of AgRP neurons is through the inhibitory effect of 5HT-1B, which was explained earlier, in detail, on those neuron groups.

AgRP exerts its orexigenic effects via the antagonism of MC3 and MC4 receptors of POMC (Lu *et al*, 1994). However, AgRP<sub>83-132</sub> antagonism on MC4 receptor is suggested to be more potent than that of MC3 receptor (Harrold *et al*, 1999).

When it AgRP is synthesized in the arcuate nucleus of hypothalamus it increases food intake. For instance, a study performed by Tang-Christensen *et al*, 2004, revealed that central administration of AgRP<sub>83-132</sub> led to an increase in food intake and also with fasting, AgRP levels are shown to be increased. In addition to these, it is stated that the increase in AgRP levels are associated with decreased leptin levels and that the increased levels of AgRP is under the control of other factors independent from low leptin such as blood glucose levels (Mizuno *et al*, 1999).

### **1.6.2.3. Cocaine and Amphetamine Regulated Transcript (CART)**

CART is an anorexigenic peptide which is abundantly expressed in the hypothalamic tissue, arcuate nucleus. In both rats and human, CART gene has 3 exons and in rats it is expressed in two forms, long form; consisting of 129 aminoacid, and the short form; 116 aminoacid. The 27 aminoacid sequence is considered to be the leader sequence which results in 102 aminoacid and 89 aminoacid sequence forms, long form and short form, respectively. In humans, on

the other hand, only the short form is shown to be expressed (Thim *et al*, 1999). CART gene is found to be located on chromosome 13 (Adams *et al*, 1999).

Like all other peptides, CART is also synthesized as pro-CART and cleaved by prohormoneconvertases to its active form. It is evaluated that PC2 and PC1/3 are involved in the processing mechanism, as PC2 is known to be more potent (Dey *et al*, 2003).

CART expression is not exclusively present in the arcuate nucleus. It is also found in ganglion cells of retina, spinal cord, olfactory bulb, anterior pituitary, adrenal medulla (Couceyro *et al*, 1997) and in brain regions such as nucleus accumbens and amygdala (Koylu *et al*, 1998). Moreover, CART immunoreactivity is found to be positive in the PVN, LHA, posterior hypothalamus and arcuate nucleus, in humans (Elias *et al*, 2001).

In rats' the arcuate nucleus of hypothalamus, POMC and CART neurons are colocalized and just like POMC neurons CART neurons also have OB-Rb on their cell body (Elias *et al*, 1998). As discussed earlier, STAT3 activation is involved as an excitatory pathway in leptin signaling. Since these two neuropeptides are coexpressed in the arcuate nucleus, it is found that the action of leptin on CART neurons through OB-Rb includes STAT3 pathway (Dominguez *et al*, 2001). It was also found that intravenous injection of leptin results in the increased c-fos activation indicating in the activation of the neuron itself (Elias *et al*, 2001).

Very little is known about the cellular mechanisms of CART. No receptor has been identified for the pathway of CART to exert its effects (Lakatos *et al*, 2005). However, receptor studies performed indicate that, voltage gated calcium signaling might be involved in the modulation (Yermolaieva *et al*, 2001) and recently it is suggested that the receptor of CART might be a G-coupled protein receptor modulating the Gi/o mechanism that involved MAPK (Lakatos *et al*, 2005).

When CART is synthesized in the arcuate nucleus of hypothalamus, it exerts a satiety signal. For instance, icv injection of CART peptide fragments results in the inhibition of feeding and that CART antiserum leads to an increase in food intake (Kristensen *et al*, 1998). Vrang *et al*, 1999 identified that CART inhibits the expression of NPY in rats and that CART induces c-fosimmunoreactivity in the brain areas that are involved in the regulation of food intake (Vrang *et al*, 1999). Moreover, it was found that CART expression was reduced in leptin deficient mice (ob/ob) and was normalized with leptin repletion (Kristensen *et al*, 1998). Lastly, it is known that CART expression increases extracellular 5-HT in rats (Ma *et al*, 2007). This might be the result or the cause of the decreased levels of NPY/AgRP peptide levels with increased CART expression, because of the involvement of 5HT-1B receptor on those neurons.

Expression of CART is localized in many parts of brain that are involved in stress response, such as hippocampus, raphe nucleus, PVN, locus coeruleus (LC) arcuate nucleus and pituitary (Koylu *et al*, 2006). Therefore, the relationship of CART to stress has been evaluated by many studies. Kask *et al*, 2000, performed icv injection of CART<sub>83-102</sub> increased anxiety responses determined by plus-maze test, a test to measure anxiety related responses, in rats suggesting that CART is involved in stress response. In the same study it is suggested that CART peptide exert its anorexic effect due to its actions on stress response mechanism (Kask *et al*, 2000). In another study, it was suggested that icv injection of a fragment of CART peptide resulted in decreased social interaction in mice and that the icv injection lead to an increase in the firing rate of the neurons that are found in LC which indicate the involvement of CART on stress response (Chaki *et al*, 2003).

#### 1.6.2.4. Neuropeptide Y (NPY)

NPY is the most potent peptide for feeding mechanism and stress response (Mintz *et al*, 1986). NPY gene is located on chromosome 7 and has four exons. The first and fourth exon are the nontranslated regions, the second and a part of third exon are the translated exons that create pro-NPY. NPY is derived from 69 amino acid length peptide, pro-NPY. Tissue specific cleavage of NPY with PC1/3 and PC2, leads to the conversion of pro-NPY to the active form, NPY-36 (Paquet *et al*, 1996 and Brackh *et al*, 1997). Approximately 40 % of NPY is synthesized and secreted from the ARC (Ramos *et al*, 2005) followed by medulla which is responsible for receiving afferent nerves from gastrointestinal tract and projecting them to PVN and dorsomedial hypothalamus (Bai *et al*, 1985). NPY and GABA are shown to be colocalized in the hypothalamus (Dermietzel and Halbach, 2006).

The inhibition of NPY neurons is same as that of AgRP. SOCS-3 mRNA expression is increased with the binding of leptin to its OB-Rb receptor on NPY/AgRP neurons. SOCS-3 is phosphorylated and it inhibits leptin receptor phosphorylation and STAT3 activated expression of the target gene (Morrison *et al*, 2005).

NPY exerts its effects via its five receptor subtypes; Y<sub>1</sub>, Y<sub>2</sub>, Y<sub>4</sub>, Y<sub>5</sub>, and Y<sub>6</sub>, however, Y<sub>6</sub> receptors is absent in humans and rats (Malenka R.C., 2009). All of the NPY receptors are G-coupled protein receptors that show their responses via the inhibition of the accumulation of cAMP (Blomqvist *et al*, 1997). Among these receptor subtypes, Y<sub>1</sub>, Y<sub>2</sub> and Y<sub>5</sub> receptors are shown to be associated with appetite regulation mechanisms.

Y<sub>1</sub> receptor (Y<sub>1</sub>R) is mainly found in the arcuate nucleus of hypothalamus. Moreover, in rat brain Y<sub>1</sub>R is shown to be located on anterior thalamus, cerebral

cortex and amygdala. The receptor is also been shown on blood vessels and in colon, kidney, heart, placenta and adrenal glands (Malenka R.C., 2009). Y<sub>1</sub>R shows its effects by the stimulation of MAPK. The distribution of the receptor on neurons might be both pre- or post-synaptically and is thought to be associated with showing anxiety like behavior (Carola *et al*, 2006). For instance, Karl *et al*, 2006 showed that central administration of Y<sub>1</sub>R agonists revealed a significant anxiolytic effect; however, Y<sub>1</sub>R gene lacking mice showed anxiety-like behavior. Moreover, in a study, it was shown that many POMC neurons and also all of the NPY neurons' cell membrane in the arcuate nucleus have Y<sub>1</sub>R mRNA and protein which indicates the involvement of the receptor on food intake (Broberger *et al*, 1997). Kanatani *et al*, 2000, created a mice lacking Y<sub>1</sub>R and revealed that NPY-induced appetite was reduced in this mice, indicating that Y<sub>1</sub>R activation results in increased food intake.

Y<sub>2</sub> receptor (Y<sub>2</sub>R) expression in brain is pronounced in the hypothalamus, hippocampus and amygdala and also in peripheral system such as intestine and on blood vessels (Malenka R.C., 2009). In a study performed by Broberger *et al*, 1997 showed that all NPY neurons in the arcuate nucleus of hypothalamus have Y<sub>2</sub>R. The presynaptically located Y<sub>2</sub>R receptor acts as an autoreceptor in order to inhibit the further release of NPY and other neurotransmitters such as GABA (Walther *et al*, 2010). For instance deletion in Y<sub>2</sub>R receptor caused increased NPY expression in the arcuate nucleus (Sainsbury *et al*, 2002) which is probably due to the lack of inhibitory action of Y<sub>2</sub>R on NPY neurons. Interestingly, in the same paper it was suggested that Y<sub>2</sub>R gene deletion, in leptin receptor deficient mice leads to an increase in the POMC expression in the arcuate nucleus. In another study, knocking-out the Y<sub>2</sub>R in mice resulted in elevated body weight, increased appetite and fat deposition. Also, this mice type showed decreased response to leptin administration and the lack of Y<sub>2</sub>R also altered heart rate (Naveilhan *et al*, 1999). Also the effect of diet type on Y<sub>2</sub>R binding was studied. For instance a study performed by Xu-Feng *et al* 2008, revealed that NPY binding to Y<sub>2</sub>R was

significantly reduced with changing from high-fat diet to low-fat diet. In addition to its role on food intake the relation of Y<sub>2</sub>R with the stress response was analyzed in many studies. For instance, in a study performed by Nakashima *et al*, 1997, it was suggested that the anxiolytic effect of NPY is not only produced via Y<sub>1</sub>R receptor but also via Y<sub>2</sub>R and that the effects of antidepressant drugs leads to a reduction in the Y<sub>2</sub>R in many brain regions (Widdowson and Halaris, 1991).

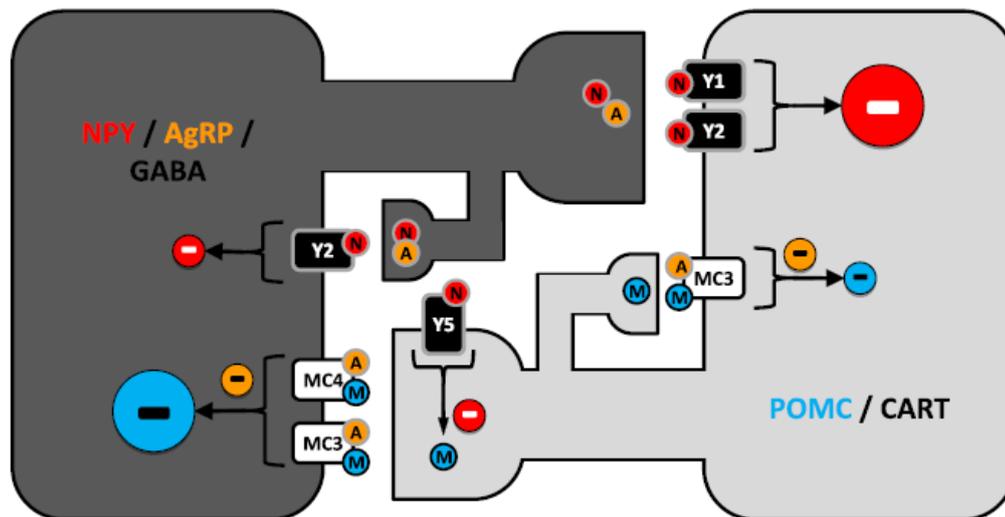
Y<sub>5</sub> receptor (Y<sub>5</sub>R) is mainly localized to olfactory bulb, anteroventral thalamic nucleus, ventral hippocampus and with very low densities in hypothalamus in rat brain (Dumont *et al*, 1998). In addition to these, in the body, Y<sub>5</sub>R is also found intestine, pancreas, kidney, liver and heart (Malenka R.C., 2009). The effect of Y<sub>5</sub>R on food intake and its interactions with Y<sub>1</sub>R receptor is contradictory. For instance, knockout mice for either Y<sub>1</sub>R or Y<sub>5</sub>R did not show any alterations in food intake. Indeed the NPY administration to wild type (WT) mice showed obesity symptoms in terms of increased fat pad and leptin concentration. Moreover a reduction in POMC expression was also detected; however, knockout mice with either Y<sub>1</sub>R or Y<sub>5</sub>R did not show such response with NPY administration (Raposo *et al*, 2004). In another study, on the other hand, it was shown that Y<sub>5</sub>R knockouts display an elevation in the expression of orexigenic peptides NPY and AgRP and reduction in the anorexigenic peptides POMC and CART (Hiroshi *et al*, 2008). Nguyen *et al*, 2012 concluded that both Y<sub>1</sub>R and Y<sub>5</sub>R are both required for the food intake regulation, by using both Y<sub>1</sub>R and Y<sub>5</sub>R knockouts. These rats showed reduced food intake due to the effect of both receptors on appetite increase. No association of Y<sub>5</sub>R was found with anxiety or designation with anxiety-like behavior (Kask *et al*, 2001)

In addition to the association of NPY receptors on stress response, the relation of NPY peptide in stress response has been studied. In a study performed by Hashimoto *et al*, 1996 found that NPY levels were reduced in the patients with major depressive disorder. Another study performed with flinder sensitive line

rats, which is a genetic model of depression, revealed that NPY mRNA levels were significantly reduced in some parts of brain, such as, hippocampus, meso and neocortex and nucleus accumbens (Caberlotto *et al*, 1998). Not only depression, but also in schizophrenia an alteration of the NPY gene expression is shown. A study performed with 15 schizophrenic patients revealed the significantly decreased NPY mRNA levels in the frontal cortex. The same reduction was shown in the bipolar depression patients too (Kuromitsu *et al*, 2001). The genetic reason that may be involved in the susceptibility to schizophrenia has also been implicated. For instance, in 2003, a mutation at the promoter region of NPY gene (C to T change) resulted in the significantly reduced transcriptional activity which was suggested to be the decreased NPY mRNA expression in schizophrenic patients (Itokawa *et al*, 2003).

### 1.6.2.5. Interaction of neurohormones involved in the regulation of food intake

Figure 1.14 indicates the interactions of neurohormones that are involved in the appetite regulation with each other through their receptors.



**Figure 1. 14** Interactions of NPY/AgRP and POMC/CART neurons in the arcuate nucleus of hypothalamus. NPY release: in red; melanocortin release: in blue; release of AgRP: orange. (Mercer *et al*, 2011).

Synthesis and secretion of NPY from the NPY/AgRP neuron group directly inhibits POMC/CART neurons through Y<sub>1</sub>R and Y<sub>2</sub>R. Also the secreted NPY finds its Y<sub>5</sub> receptor on the POMC/CART presynaptic neuron site and causes the inhibition of melanocortin release ( $\alpha$ -MSH) from POMC/CART neuron group. With the inhibition on the release of POMC, AgRP secreted together with NPY binds to the melanocortin receptors on the NPY/AgRP neurons. Binding of AgRP on MC3 and MC4 receptors on NPY neurons creates negative feedback on NPY neurons leading to reduced NPY/AgRP neurohormone secretion. Binding of either  $\alpha$ -MSH or AgRP to the MC3 receptor also results in the inhibition of POMC neurons. (Mercer *et al*, 2011).

### **1.7. *In vivo* studies of olanzapine**

Many studies have been performed in attempt to understand both the causes and the results of weight gaining side effect of the atypical antipsychotic drug olanzapine. For instance, a study to analyze the alterations on the feeding behavior of rats due to chronic olanzapine treatment revealed that 26 days of 2mg/kg/day treatment resulted in increased the number of meals but decreased time interval between meals, ingestion rate and the meal size (Victoriano *et al*, 2009), indicating that olanzapine treatment causes less but frequent eating. In another study, it was found that, 19 days of chronic olanzapine treatment led to an elevation in body weight and that the weight gain pattern reached to a plauto after 10 days of the treatment. However, they suggested that the weight gain was irreversible. A single day of withdrawal resulted in a significant weight loss (Goudie *et al*, 2002). Kirk *et al*, 2009 proved that the weight gaining effect of olanzapine was trough 5-HT<sub>2C</sub> antagonism, in the presence of D2 receptor antagonism by administering just olanzapine and co-administration of olanzapine with the inverse agonism of the receptors.

Albaugh *et al*, 2011, revealed that with three days of olanzapine treatment (10mg/day) to 8 male and 7 female healthy volunteers who had an average BMI value of 18.5-25.0 and at the age of 18-30 years old, leptin levels were increased by 24 %. However, in another study designed, two groups of 60 days-old male rats received either olanzapine with a normal diet or olanzapine with hypercaloric diet. Interestingly, leptin levels of both olanzapine and normal diet and hypercaloric diet groups were reduced compared with the control group; however, they had significantly increased body weight. (Zugno *et al*, 2012). A study concerning the effect of olanzapine on leptin levels in both female and male rats revealed a gender difference response to olanzapine in terms of weight gain. They could not find any significant increase in leptin levels neither in female nor in male rat, on the other hand, only female rats showed an olanzapine induced weight gain (Davey *et al*, 2012).

In order to understand the weight gaining mechanism of olanzapine, many studies were evaluated for both anorexigenic and orexigenic peptide expressions. For instance, female rats that were exposed to 6 mg/kg/day olanzapine for 6 days showed significant decrease in the POMC expression which was detected by using in-situ hybridization technique (Ferno *et al*, 2011). However, acute oral olanzapine administration (1mg/kg) to eight female Sprague-Dawley rats showed no alteration in their POMC gene expression when compared with the control group (Davoodi *et al*, 2009). Martins *et al*, 2010 also revealed that administration of the drug olanzapine to eight male Sprague-Dawley rats by *icv* injection resulted in the elevation of POMC mRNA levels (Martins *et al*, 2010). Also, CART mRNA expressions were studied and no statistically significant change was observed with the olanzapine treatment (Ferno *et al*, 2011).

Guesdon *et al*, 2010 studied the effects of olanzapine on the orexigenic peptides NPY and AgRP in male rats (n=6). The rats were exposed to 1mg/kg olanzapine via the medium fat diet. They analyzed NPY and AgRP expression levels by in-

situ hybridization technique and found that expressions of those orexigenic peptides were not affected with the treatment of olanzapine. On the contrary, Martins *et al*, 2010 revealed the increased levels of rat brain NPY and AgRP mRNA expressions when olanzapine was injected (*icv.*). Inconsistency of the results would be due the route of the drug administration. Consistent with Martins *et al* 2010; it was suggested that 6mg/kg/day olanzapine administration resulted in the increased mRNA levels of NPY and AgRP (Ferno *et al*, 2011).

### **1.8. The Aim of the Study**

The aim of this study was to examine atypical antipsychotic, olanzapine effects on the body weight changes in humans and rats. It was hypothesized that the hypothalamic neurohormones' mRNA and protein levels that induce food intake and appetite during the drug administration may be affected by the antagonistic effect of olanzapine on serotonergic receptors on those neuron bodies; thus change the body weight. The weight gain is controlled by the interactions of the hypothalamic orexigenic neuropeptides/hormones; NPY and AgRP and the anorexigenic neuropeptides/hormones; POMC and CART. They are synthesized and secreted from the ARC of hypothalamus and modulated by leptin, adipose tissue hormone (Schwartz *et al*, 2000). Olanzapine has high binding affinities for many receptors, particularly 5-HT<sub>2C</sub> and weak binding affinity for 5-HT<sub>1B</sub> which are found on those hypothalamic neurons that are responsible for the modulation of appetite control. Binding of olanzapine to its receptors on the ARC neurons creates antagonistic effects for the downstream pathways and changes the expression levels of the candidate genes that are regulated by leptin for the food intake regulation.

In this study, we aimed to determine the possible alterations of the orexigenic (NPY/AgRP) and anorexigenic peptides (POMC/CART) due to the antagonistic effect of olanzapine on 5-HT<sub>2C</sub> receptors which are found on POMC neurons. It was hypothesized that the antagonistic effect of olanzapine on 5-HT<sub>2C</sub> receptors

might change the  $\alpha$ -MSH secretion from POMC neurons; thus may affect the balance among the neuropeptides which may change the food intake behavior. When body fat increase, the leptin levels increase which ultimately change the other ARC neuropeptide mRNA expressions. To test this hypothesis, the plasma concentrations of the neuropeptides in the first attack male psychotic disorder patients who were treated with olanzapine for 4 weeks were determined. Based on the peripheral data; we tested these neurohormone circulating levels and mRNA hypothalamic expressions in male wistar-rats. Therefore, the drug was administered to rats for 28 days; then the expression profile of the candidate genes by qRT-PCR and their peripheral hormone levels by ELISA were analyzed. The results showed us that the olanzapine induced weight gain is through serotonergic receptor signaling that are particularly found on the candidate hypothalamic neurohormones involved in the appetite regulation which might elucidate one of the weight gain molecular mechanisms.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Diagnosis and treatment of patients

The study consists of two different groups; control and patient groups. The patient group's results were taken before and after the treatment, which were referred as "pre-treatment" and "post-treatment" groups. Patients (n=22) with a mean age of  $21.46 \pm 1.1$  who had applied to Gulhane School of Medicine (GATA), Ankara, Turkey and diagnosed with first attack disorder were included in the study. The patients did not have an antipsychotic treatment history which may lead to metabolic side effects that might interfere with the results of the study. They were hospitalized for four weeks. The control group, were consisted of 26 healthy individuals with no psychological or endocrinological health problems. All individuals have similar age interval and sociodemographical features.

The diagnosis, severity and improvements of psychotic symptoms were analyzed by the Scale for Assessment of Negative Symptoms (SANS), Scale for Assessment of Positive Symptoms (SAPS) and Brief Psychiatric Rating Scale (BPRS). After the diagnosis of the patients, their weight and height were documented and blood samples were taken to EDTA-preserved tube for hormone tests; which created the pre-treatment values. After 4 weeks of treatment, their weight and height were again measured and blood samples were collected, which created the post-

treatment values. A dose of 5-10 mg/day of olanzapine was administered at the beginning of the study; however according to the response of the patient to the treatment, dose change was applied. The final dose was 10-20mg/day, with a mean value of  $12.08 \pm 3.8$  mg/day.

During hospitalization, the patients received but not restricted to approximately 2500 kcal/day of hospital food. They had free access to obtain extra food from hospital facilities such as cafeteria.

### **2.1.1. Plasma preparation from patients**

Fresh blood samples were taken from GATA (GülhaneAskeri Tıp Akademisi), Psychiatry Department. Blood samples were taken into EDTA-preserved tubes and were centrifuged at 14000 rpm for 10 minutes to obtain plasma and stored at  $-80^{\circ}\text{C}$  until used.

### **2.2. Rat studies**

In order to further understand the weight gaining mechanism of olanzapine, the drug was administered to male Wistar-rats for 28 days. Four-weeks-old 25 male Wistar rats (100-130g), were kindly obtained from the Department of Laboratory Animals, GATA, Ankara and were randomly divided into two groups as the vehicle group (n=10) which was used as the control and the treatment group (n=15). Animals were group-housed three per cage, under a 12-hour light/dark cycle (lights on at 7 a.m.), in a room maintained at a temperature of  $25 \pm 2^{\circ}\text{C}$  and a humidity of 60%, with free access to water and 200 g/day normal rat chow.

At the beginning of the study there were 15 rats in the olanzapine-treatment group. However, one of them died during drug administration period and two of them were used for the optimization studies for qRT-PCR experiments. Two of the animals' RNA samples were excluded from the study because of the low integrity and lower concentrations compared to other RNA samples isolated.

### 2.2.1. Preparation and administration of drug solution

Olanzapine was a kind gift from Eli Lilly. Each rat received olanzapine at a dose of 4mg/kg/day for 28 days. Since the half-life of olanzapine in rats is 4-6 hours (Olanzapine- prescribing information, 2010), the drug administration was carried out twice a day at 10 a.m. and at 6 p.m. at half doses, 2mg/kg/dose.

At the beginning of the study the mean weight of the olanzapine group was 132.67 g. Therefore, on 0-14 days of the study, for one dose, 6 mg of olanzapine was dissolved in 1 mL of 0.1 M acetic acid solution and was added to 19 mL 10% sucrose solution. The pH was adjusted to 7.5-8.00 with 1M NaOH solution. 1 ml from a total volume of 20 ml drug solution was administered to each rat in the olanzapine group. As the animals gained weight, recalculation of the mg drug to be used in the administration was done (Table 2.1).

**Table 2. 1** Administered olanzapine amounts based on the body weight of rats.

Days of study	Mean weight of animals (g)	Drug (mg)	ml of acetic acid solution	ml of sucrose solution	Total volume (ml)
0-14	132,67	6	1	19	20
15-28	191,86	8	1	19	20
28-33	229	9	1	19	20

Drug administration was performed orally by the help of a syringe in 1 ml of 10% sucrose solution (figure 2.1). The vehicle group received only 1 ml 10% sucrose solution in order to eliminate the interference of sucrose on weight gain and to eliminate the interference of stress to the results of the study (Schleimer *et al*, 2005).



**Figure 2. 1** The administration of drug.

### **2.2.2. Analyzing weight gain and food consumption**

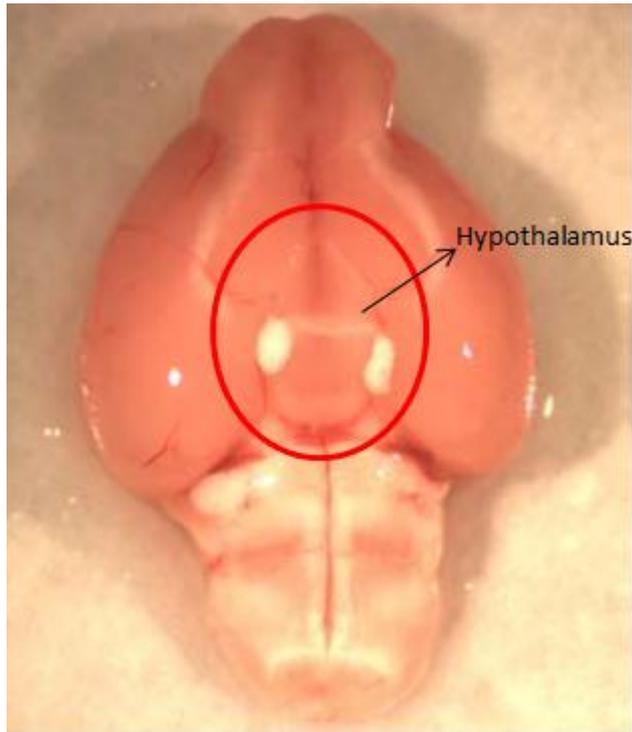
For weight gain determination, all animals were individually weighed every week and groups' mean weight was documented. For food consumption analysis, every day, 200 g of food was given to animals and the remaining food was weighed the next day and the difference between two days was recorded as the amount of food consumed. This way, the food consumption per cage was evaluated. The food consumption differences between vehicle and olanzapine treated group was determined via the slope of the trend line plotted to the graph showing the food consumption (g) per day.

### **2.2.3. Animal sacrifice and hypothalamus extraction**

All equipments were treated with diethylpyrocarbonate (DEPC) in order to avoid the RNase contamination. 1 ml of DEPC (Sigma Aldrich, Germany) was added to 1 L of dH<sub>2</sub>O water and was shaken thoroughly. The forceps, scissors, eppendorf tubes, pipette tips that will be used in animal sacrifice were incubated in DEPC-treated water for 16 h. under the hood and were then autoclaved.

### 2.2.3.1. Hypothalamus extraction

The animals were sacrificed by decapitation using the guillotine. In order to minimize the stress during decapitation and the possible changes in the expression levels of stress-related peptides such as NPY, the animals were exposed to CO<sub>2</sub> in a desiccator for 5-10 sec.



**Figure 2. 2** The dissected rat brain. The region in red circle represents the hypothalamus.

For the hypothalamus extraction, after the removal of whole brain from the skull, the brain was washed with 1X phosphate buffered saline (PBS) (Sigma Aldrich, Germany) which was prepared by dissolving one tablet containing 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, with a pH of 7.4, in 200 ml of distilled water, in order not to let the tissue dry while taking out the hypothalamus. Extracted hypothalamus were incubated at +4°C in

RNAlater™, RNA stabilization reagent (Qiagen, GmbH., Germany) overnight and then were stored at -80°C until used.

#### **2.2.4. Plasma preparation from rats**

The trunk blood was collected into blood collection tubes and immediately centrifuged at 14000 rpm for 10 minutes to obtain plasma. The plasma samples were stored at -80°C until used.

#### **2.2.5. Total RNA isolation from rat hypothalamus**

For RNA isolation TRIreagent (Sigma Aldrich, Germany) was used. All centrifuge steps were performed at 4°C unless indicated. The protocol of Chomczynski, 1993 was used with some modifications in order to clean up the RNAlater™ RNA stabilization reagent. The hypothalamus was centrifuged in a banchtop microcentrifuge at 11000 rpm for 30 sec in 1 ml 1 X PBS before homogenizing the tissue. The tissue was taken into a new microcentrifuge tube having 500 µl of TRIreagent and waited for 1-2 minutes on ice. Then, it was homogenized in the glass-teflon homogenization system in 1 ml TRIreagent, incubated for 10 minutes at room temperature (RT) and centrifuged at 12.000 x g for 10 minutes. After centrifugation, the supernatant was taken into a new microcentrifuge tube and 200 µl ice-cold chloroform (Sigma Aldrich, Germany) was added. After 30 sec of vortexing and incubation 3 minutes at RT, the mixture was centrifuged at 12.000 x g for 15 minutes. The aqueous phase was mixed with 200 µl ice-cold chloroform and then incubated at RT for 7 min. After centrifugation at 12.000 x g for 15 minutes the aqueous phase was taken to a new microcentrifuge tube and 500 µl ice-cold isopropanol (Sigma Aldrich, Germany) was added and inverted gently until the cloudy appearance disappear. Then 2 µl of glycogen was added to the mixture and incubated at -20°C for 40 minutes. After incubation, the mixture was centrifuged at 12.000 x g for 25 minutes and the supernatant was discarded. The pellet was washed with 1 ml of 75% ice-cold ethanol (Sigma Aldrich, Germany) and inverted gently until the pellet floats. After

the centrifugation at 7.500 x g for 5 minutes, the pellet was dissolved in 30  $\mu$ l nuclease free water. Finally, the RNA sample was incubated at 65<sup>0</sup>C for 10 minutes; RNA was treated with DnaseI and stored at -80<sup>0</sup>C until used.

After total RNA isolation, RNA sample concentrations were determined by loading 2  $\mu$ l RNA sample to NanoDrop2000 (Thermo Scientific, US). The concentration, 260/280 and 260/230 ratios of RNA samples were documented to check the purity. The RNA samples having lower 260/280 ratio and 260/230 ratio than 2; which indicates the DNA contamination and any possible remaining contaminants were excluded from the study.

#### **2.2.6. DNase-I treatment to isolated RNA samples**

To clean up the contaminating DNA in RNA samples, DNase-I treatment was performed using DNA-free<sup>TM</sup> Kit (Ambion, Invitrogen, Germany). Briefly, 30 $\mu$ l of RNA sample contaminated with DNA was incubated with 3  $\mu$ l of DNase I buffer and 1 $\mu$ l of rDNase I for 30 minutes at 37<sup>0</sup>C. Then, 3 $\mu$ l of DNase inactivation reagent was added to the solution and incubated for 2 minutes at RT, mixed occasionally during the incubation. Lastly, the solution was centrifuged at 10,000 x g for 1.5 min., the supernatant was taken into a DEPC treated tube and stored -80<sup>0</sup>C until used.

#### **2.2.7. Agarose gel electrophoresis**

DNase-I treated RNA samples were run on 1% Agarose gel in order to check both the RNA integrity and possible remaining DNA contamination. Briefly, 0.5 g of agarose (Sigma Aldrich, Germany) was dissolved in 1X Tris Acetate EDTA (TAE) that contains 121 g Tris, 37.2 g EDTA (50 X TAE). The agarose mixture was heated in the microwave by shaking in time intervals for 2 min. When agarose was dissolved completely in the TAE solution, 1  $\mu$ l of Etidium Bromide (EtBr) (Applicam, Germany) was added to see the bands while UV exposure.

Finally, the solution was poured into the electrophoresis tank and the comb was placed into it. For electrophoresis, 0.5 µl of the samples were mixed with 2.5 µl 6 X loading dye (Fermentas, Germany) for a final volume of 3 µl and 100bp DNA ladder (Sigma Aldrich, Germany) were loaded onto the gel and run for 45 minutes at 100V.

### **2.2.8. Complementary DNA (cDNA) preparation from isolated RNA samples**

For relative quantification of the candidate genes by quantitative real time polymerase chain reaction (qRT-PCR), total RNA samples were converted to cDNA by using the gene specific primers.

#### **2.2.8.1. Primer design**

Primers, used to analyze the expression levels of NPY, AgRP, POMC and CART genes from hypothalamus of rats, were designed according to mRNA sequences of *Rattus norvegicus*. (NCBI accession numbers: NM\_012614, NM\_033650, NM\_139326 and NM\_017110, respectively). As the housekeeping gene GAPDH mRNA sequence of *Rattus norvegicus* was used. (NCBI accession number: NM\_017008). The primers were purchased from Sigma Aldrich, Germany. The primers sequences used for both cDNA synthesis and qRT-PCR analysis are shown in table 2.2.

**Table 2. 2** Primer pairs used for the cDNA synthesis and the qRT-PCR amplification. (FP: forward primer; RP: reverse primer).

Gene	Primer	Oligonucleotide sequence	Size of PCR amplicon
NPY	FP	5' - AATGAGAGAAAGCACAGAAA - 3'	89 bp
	RP	5' - AAGTCAGGAGAGCAAGTT - 3'	
AgRP	FP	5' - GAGTTCTCAGGTCTAAGTCT - 3'	97 bp
	RP	5' - GTGGATCTAGCACCTCTG - 3'	
POMC	FP	5' - AACATCTTCGTCCTCAGA - 3'	80 bp
	RP	5' - CGACTGTAGCAGAATCTC - 3'	
CART	FP	5' - TGAAGATTAGACCTAGTTAA - 3'	108 bp
	RP	5' - ATGCTTTATTGGGTGTAA - 3'	
GAPDH	FP	5' - TCCATTCTTCCACCTTT - 3'	92 bp
	RP	5' - TAGCCATATTCATTGTCATACC - 3'	

#### 2.2.8.2. cDNA synthesis

The cDNA synthesis from DNase-I treated RNA samples were performed by using Thermo Scientific RevertAid first strand cDNA synthesis kit (Thermo Scientific, US). The synthesis was carried out by using the manufacturer's protocol. Briefly, 1  $\mu$ M of primers were added to 1  $\mu$ g of total RNA and the solution was completed to 12  $\mu$ l with nuclease free water. However, some samples from vehicle group have low concentrations, which was not possible to adjust to 1  $\mu$ g RNA concentration. Therefore, the concentration of those samples was adjusted to 200 ng/ $\mu$ l which is shown in detail in appendix B. After adjusting the total volume of samples to 12  $\mu$ l, 4  $\mu$ l of 5 X reaction buffer, 1  $\mu$ l of RiboLockRNase inhibitor, 2  $\mu$ l of 10 mM dNTP mix and 1  $\mu$ l M-MuLV reverse transcriptase (200 u/ $\mu$ l) were added and 20  $\mu$ l final volume was achieved. The samples were then incubated at 42°C for 60 min. and the reaction was terminated by heating at 70°C for 5 min.

### 2.2.9. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

For the quantification of the mRNA levels of the candidate genes, qRT-PCR experiments were performed with the oligonucleotide primers (Table 2.2) purchased from Sigma Aldrich, Germany. The reaction mixture of the candidate genes and the housekeeping gene were shown in table 2.3.

**Table 2. 3** Reaction mixtures of the candidate genes and the internal control

Genes Ingredients	POMC	AgRP	CART	NPY	GAPDH
SybrGreen Mix	10 µl	10 µl	10 µl	10 µl	10 µl
Sense Primer (5 µM)	0,8 µl	0,8 µl	1,6 µl	0,8 µl	1.6 µl
Anti-sense Primer (5 µM)	0,8 µl	0,8 µl	1,6 µl	0,8 µl	1.6 µl
MgCl <sub>2</sub> (25mM)	-	-	-	1,6 µl	-
cDNA (100ng/µl)	2 µl	2 µl	2 µl	2 µl	2 µl
Water	6,4 µl	6,4 µl	4,8 µl	4,8 µl	6,4 µl
Final Volume	20 µl	20 µl	20 µl	20 µl	20 µl

### 2.2.9.1. The Reaction Conditions of the Candidate Genes

The reaction conditions of the housekeeping gene GAPDH and the candidate genes, NPY, POMC, AgRP and CART were shown in table 2.4, table 2.5, table 2.6, table 2.7 and table 2.8; respectively.

**Table 2. 4** The reaction conditions of the GAPDH gene.

	Temperature	Time	Repeated Cycle
Initial denaturation	94 <sup>0</sup> C	15 min	
Denaturation	94 <sup>0</sup> C	30 sec	} 45 cycle
Annealing	57 <sup>0</sup> C	30 sec	
Extension	72 <sup>0</sup> C	30 sec	
Melt	50 <sup>0</sup> C - 99 <sup>0</sup> C	-	

**Table 2. 5** The reaction conditions of the NPY gene.

	Temperature	Time	Repeated Cycle
Initial denaturation	94 <sup>0</sup> C	15 min	
Denaturation	94 <sup>0</sup> C	30 sec	} 50 cycle
Annealing	56 <sup>0</sup> C	30 sec	
Extension	72 <sup>0</sup> C	30 sec	
Melt	50 <sup>0</sup> C - 99 <sup>0</sup> C	-	

**Table 2. 6** The reaction conditions of the POMC gene.

	Temperature	Time	Repeated Cycle
Initial denaturation	94 <sup>0</sup> C	15 min	
Denaturation	94 <sup>0</sup> C	30 sec	} 40 cycle
Annealing	55 <sup>0</sup> C	30 sec	
Extension	72 <sup>0</sup> C	30 sec	
Melt	50 <sup>0</sup> C - 99 <sup>0</sup> C	-	

**Table 2. 7** The reaction conditions of the AgRP gene.

	Temperature	Time	Repeated Cycle
Initial denaturation	94 <sup>0</sup> C	15 min	} 40 cycle
Denaturation	94 <sup>0</sup> C	30 sec	
Annealing	55 <sup>0</sup> C	30 sec	
Extension	72 <sup>0</sup> C	30 sec	
Melt	50 <sup>0</sup> C - 99 <sup>0</sup> C	-	

**Table 2. 8** The reaction conditions of the CART gene.

	Temperature	Time	Repeated Cycle
Initial denaturation	94 <sup>0</sup> C	15 min	} 35 cycle
Denaturation	94 <sup>0</sup> C	30 sec	
Annealing	51 <sup>0</sup> C	30 sec	
Extension	72 <sup>0</sup> C	30 sec	
Melt	50 <sup>0</sup> C - 99 <sup>0</sup> C	-	

### 2.2.9.2. Quantification of qRT-PCR

The calculation of the fold changes of the candidate genes compared with the housekeeping gene, GAPDH, was performed according to the formulation of Pfaffl, 2001, which made it possible to calculate the fold changes of the target genes even if the reaction efficiencies are not the same as the GAPDH reaction efficiency. For the calculation of the gene expression changes, equation indicated below was used in which E is the reaction efficiency and  $\Delta C_t$  is the difference of the  $C_t$ 's between the control and the sample.

$$\text{Ratio} = \frac{(E_{\text{Target}})^{\Delta\text{Ct Gene of Interest (Control-Sample)}}}{(E_{\text{Housekeeping}})^{\Delta\text{Ct Housekeeping (Control-Sample)}}} \quad (1)$$

### 2.3. Enzyme-linked Immunosorbent Assay (ELISA)

Plasma collected from both humans and rats were used to determine NPY, AgRP,  $\alpha$ -MSH, CART and leptin levels in peripherally. For human ELISA experiments, all the kits were purchased from Phoenix Pharmaceutical, Germany, except for leptin which was purchased from DRG Instruments GmbH, Germany. Rat NPY, AgRP,  $\alpha$ -MSH, CART and leptin ELISA kits were purchased from Phoenix Pharmaceutical, Germany and experiments were performed according to manufactures protocols. The samples were run in duplicate.

#### 2.3.1. Quantification of peptides levels from both human and rats

For determination of peptide levels in plasma samples, sandwich model ELISA kits (Phoenix Pharmaceuticals, INC, Germany) were used. The assay procedures were carried out at RT unless indicated. For NPY levels, 50  $\mu$ l plasma samples, positive control and 5 standards were added to secondary antibody coated wells followed by the addition of 25  $\mu$ l primary antibody and biotinylated peptide. After incubation of covered plates for 2 hours on an orbital shaker, 300-400 rpm, the plates were washed with 350  $\mu$ l of 1X assay buffer (provided by the kit) by inverting and blot drying the plate for 4 times. After the washing step 100  $\mu$ l of SA-HRP solution was added to each well and incubated for 1 hour on the shaker. After the incubation step, 100  $\mu$ l of TMB substrate solution provided by the kit was added and the plate was covered with aluminium foil to protect the plate from light interference followed by another incubation step for one hour. The reaction was stopped with the addition of 100  $\mu$ l 2N HCl into each well and the absorbance O.D was read at 450 nm by ELISA plate reader (SPECTRAMax 340PC, USA).

For CART and  $\alpha$ -MSH determination, the same procedure of NPY kit was used.

For human leptin detection, 15  $\mu$ l of each standard, controls and samples were loaded into secondary antibody coated wells which was followed by the addition of 100  $\mu$ l assay buffer, mixed for 10-15 sec and incubated for 2 hours on bench top. After the incubation, the wells were washed 3 times with 300  $\mu$ l wash buffer, and 100  $\mu$ l of antiserum to each well were added. The plates were incubated for 30 min on bench top washed 3 times with wash buffer. After, 100  $\mu$ l of enzyme complex was added into each well. After incubation step for 30 min the wells were washed with wash buffer. Finally, 100  $\mu$ l of substrate solution was added into each well, the plate was covered with aluminum foil and incubated for 15 min. The reaction was stopped by adding 50  $\mu$ l of 0.5M H<sub>2</sub>SO<sub>4</sub> to each well and absorbance was read at 450nm by ELISA plate reader (SPECTRAMax 340PC, USA).

To determine the leptin levels of rats, ELISA kit from Phoenix Pharmaceutical, Germany was used. Briefly, 100  $\mu$ l of standards, positive control and samples were added to secondary antibody coated wells and incubated on an orbital shaker for 1 hour at 300 rpm. The wells were washed with 200  $\mu$ l of washing solution (provided with the kit) for 3 times. After the addition of 100  $\mu$ l of the biotin-labelled anti-mouse leptin antibody solution, the plates were incubated on an orbital shaker at 300 rpm for 1 hour. The plates were again washed and 100  $\mu$ l streptavidin-HRP conjugate was added to the wells, followed by incubation on an orbital shaker for 30 min at 300 rpm. The plates were washed last time with 200  $\mu$ l of washing solution and 100  $\mu$ l of substrate solution was added to the wells. The plates were covered with aluminum foil to protect from light and incubated 10 min at RT. The reaction was stopped with the addition of 100  $\mu$ l stop solution to the wells. The absorbance (OD) of the wells was read on ELISA reader. The standard curves of all ELISA plates are shown in Appendix A.

## **2.4. Statistical Analysis**

Statistical significance in qRT-PCR and ELISA was determined by the two-tailed Student's t-test. The significance value (p) was  $*p < 0.05$ . Data are expressed as mean  $\pm$  standard error.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1. Human Experiments

The analysis of olanzapine effects on body measurements and hypothalamic neuropeptides were examined in male psychotic patients. The body measurements were documented in pre- and post-treatment periods.

##### 3.1.1. Body Measurements

The control group had a mean body weight of  $71.52 \pm 12.23$  g and the pre-treatment group had  $66.38 \pm 8.9$  g; however after 4 weeks of olanzapine treatment, body weight of the patient group increased to  $70,71 \pm 9.0$  g which was statistically significant compared with pre-treatment values ( $***p < 0.001$ ) (table 3.1). The BMI of the control group was  $23.93 \pm 3.92$ , and the pre-treatment and post-treatment groups were  $21.74 \pm 2.2$  and  $23.18 \pm 2.3$ ; respectively. The elevation of BMI in post-treatment group was statistically significant compared to pre-treatment group ( $***p < 0.001$ ). Another indicator of weight gain, the waist circumference was increased to  $86.00 \pm 7.2$  in post-treatment group compared to  $81.25 \pm 7.3$  in pre-treatment group ( $***p < 0.001$ ). The mean waist circumference of the control group was  $81.72 \pm 7.70$ .

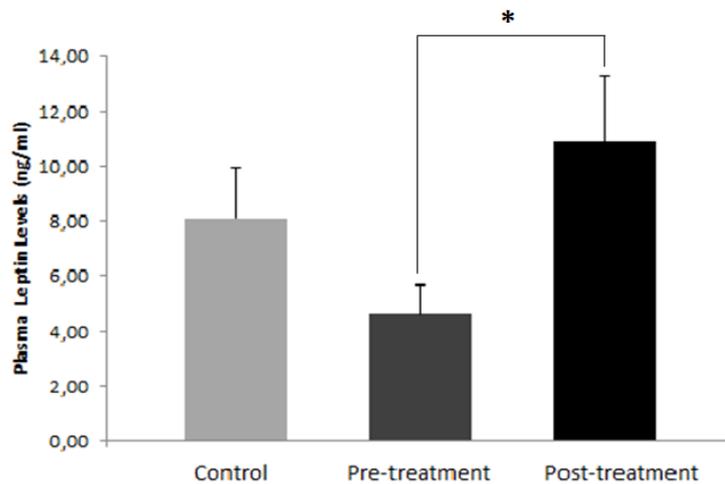
**Table 3. 1** Body Measurements of the Controls and Patients

	Control (n=26)	Pre-treatment (n=22)	Post-treatment (n=22)	p value
Weight (kg)	71.52±12.23	66.38±8.9	70,71±9.0	<0.001***
BMI (kg/m <sup>2</sup> )	23.93±3.92	21.74± 2.2	23.18±2.3	<0.001***
Waist circumference	81.72±7.70	81.25±7.3	86.00±7.2	<0.001***

### 3.1.2. Plasma levels of neurohormones in human samples

#### 3.1.2.1. Plasma Leptin Levels

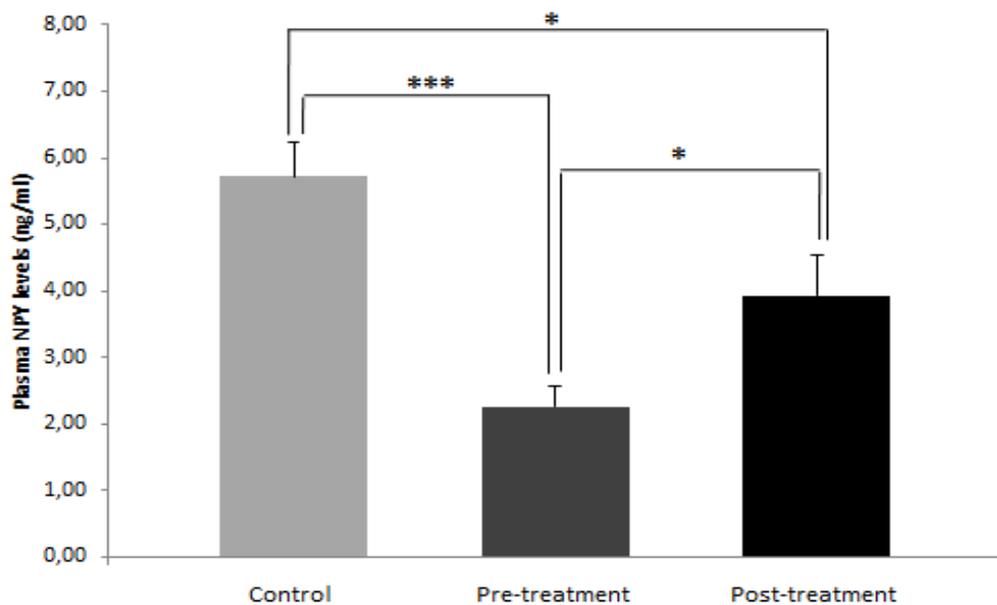
Plasma leptin levels of pre-treated patients increased significantly from  $4.64 \pm 1.83$  ng/ml to  $10.89 \pm 2.35$  ng/ml after the treatment ( $*p < 0.05$ ). The control group had a mean value of  $8.10 \pm 1.83$  ng/ml.



**Figure 3. 1** Human plasma leptin levels in controls and patients. Plasma leptin levels were significantly elevated in post-treatment values ( $10.89 \pm 2.35$ ng/ml) compared to pre-treatment values ( $4.64 \pm 1.83$ ng/ml), ( $*p < 0.05$ ). No significant change was detected in the leptin levels between the pre-treatment groups vs. the control group ( $8.10 \pm 1.83$ ng/ml).

### 3.1.2.2. Plasma NPY Levels

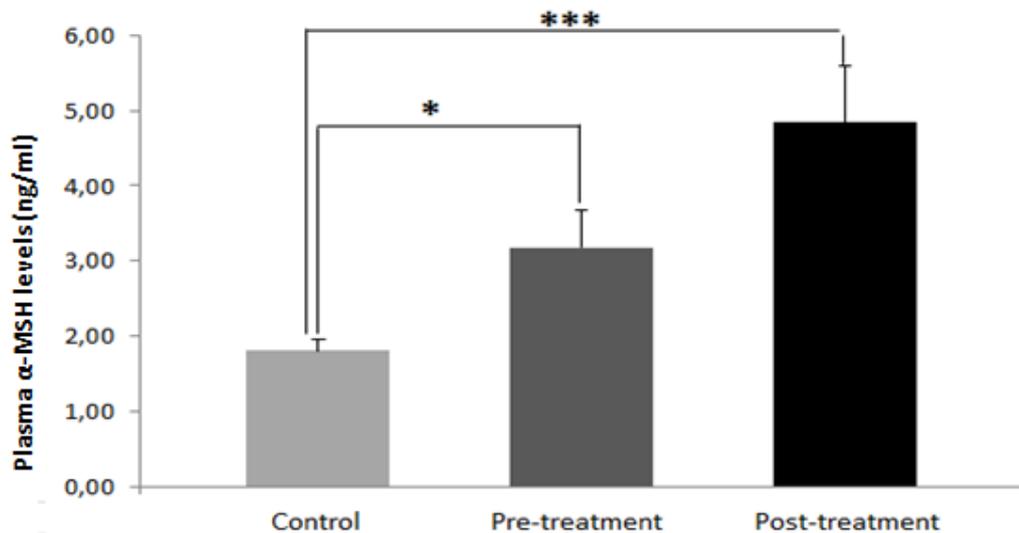
Plasma NPY levels in the psychotic patients ( $2.27 \pm 0.29$  ng/ml) were statistically significant ( $***p < 0.001$ ) when compared to the control group ( $5.72 \pm 0.5$  ng/ml). After 4-weeks of olanzapine treatment the NPY levels increased significantly to  $3.94 \pm 0.60$  ( $*p < 0.05$ ) compared with pre-treatment group. The NPY levels after the treatment also indicated a statistically significant increase ( $*p < 0.05$ ) when compared with control group.



**Figure 3. 2** Human plasma NPY levels in controls and patients. Plasma NPY levels were significantly reduced both in pre-treatment ( $2.27 \pm 0.29$ ng/ml) ( $***p < 0.001$ ) and post-treatment ( $3.94 \pm 0.60$  ng/ml) patients ( $*p < 0.05$ ) vscontrol ( $5.72 \pm 0.5$ ng/ml). After the treatment NPY levels were significantly low when compared to control group ( $*p < 0.05$ ).

### 3.1.2.3. Plasma $\alpha$ -MSH Levels

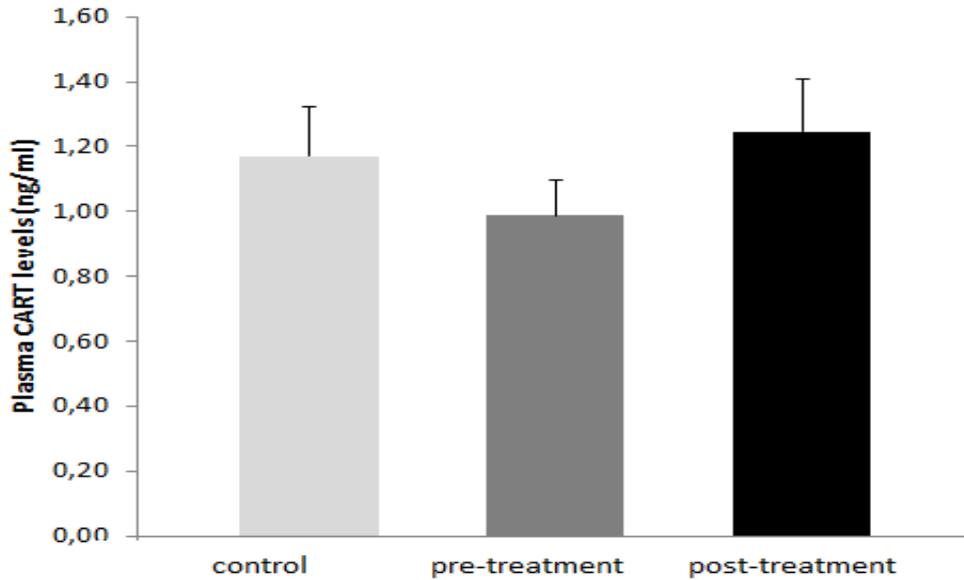
Plasma  $\alpha$ -MSH levels in the pre-treatment period ( $3.18 \pm 0.49$  ng/ml) were significantly higher than the control values ( $1.80 \pm 0.16$  ng/ml) ( $*p < 0.05$ ). Four weeks of olanzapine treatment did not alter  $\alpha$ -MSH levels significantly compared with pre-treatment group values ( $4.84 \pm 0.74$  ng/ml); but it led to a significant increase in  $\alpha$ -MSH levels when compared with the control group ( $***p < 0.001$ ).



**Figure 3. 3** Human plasma  $\alpha$ -MSH concentrations in controls and patients.  $\alpha$ -MSH levels were significantly elevated in pre-treatment group ( $3.18 \pm 0.49$  ng/ml) vs. control ( $1.80 \pm 0.16$  ng/ml) ( $*p < 0.05$ ).  $\alpha$ -MSH concentration was also significantly higher in the post-treatment group ( $4.84 \pm 0.74$  ng/ml) vs control group ( $***p < 0.001$ ).

### 3.1.2.4. Plasma CART Levels

Plasma CART levels did not show any statistically significant value between the control ( $1.17 \pm 0.15$  ng/ml) and patient group; pre-treatment  $0.99 \pm 0.11$  ng/ml and post-treatment  $1.25 \pm 0.16$  ng/ml.

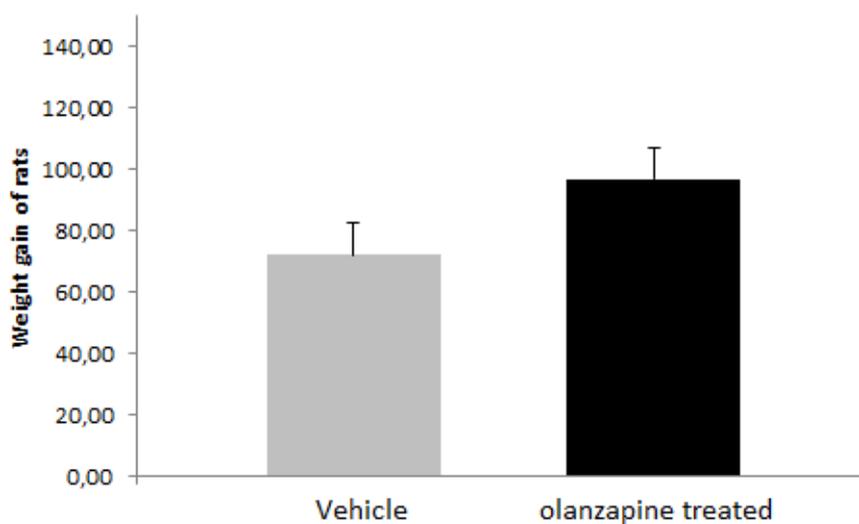


**Figure 3. 4** Human plasma CART levels. No significant alteration was observed between the plasma CART levels before ( $0.99 \pm 0.11$  ng/ml) and after the treatment ( $1.25 \pm 0.16$  ng/ml) in patients vs controls ( $1.17 \pm 0.15$  ng/ml).

## 3.2. Rat Studies

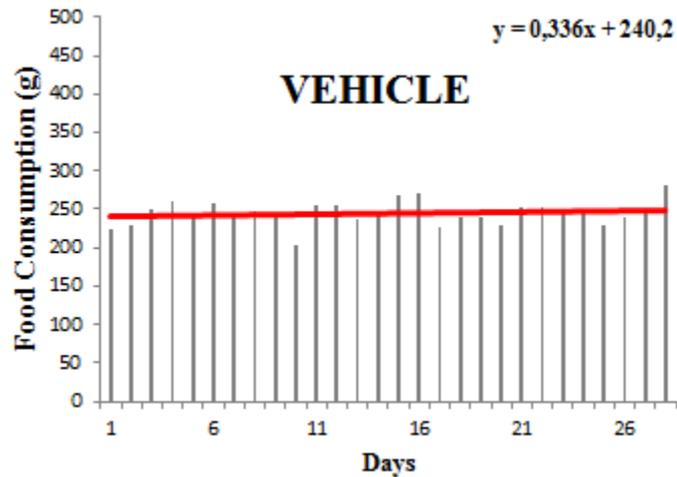
### 3.2.1. Weight Gain and Food Consumption

The difference in the mean weight gain of the vehicle group during sucrose administration ( $72.30 \pm 10.59$ ) and the olanzapine treatment ( $96.85 \pm 10.21$ ) was not statistically significant. (Fig. 3.5).

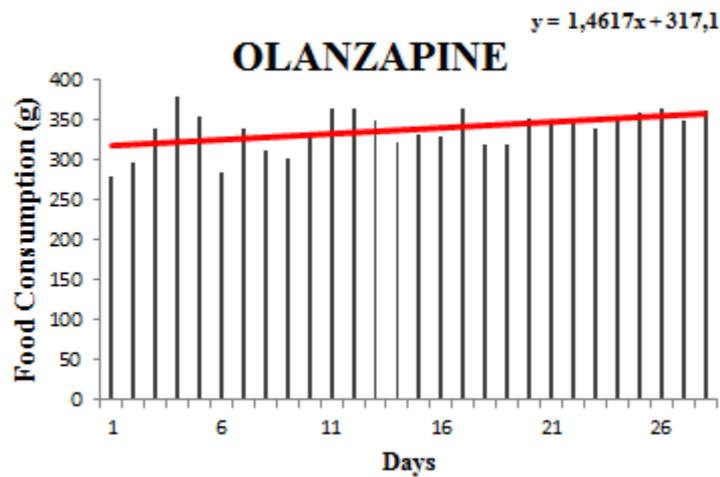


**Figure 3. 5** Weight gain of rats. The weight gain of olanzapine treated rats was  $96.85 \pm 10.21$  and  $72.30 \pm 10.59$  for vehicle group. No statistically significant alteration was observed between the groups.

As indicated earlier, the food consumption values of vehicle and olanzapine treated group was analyzed via the slope of the trend line plotted to the graph showing the food consumption (g) per day. Figure 3.6 shows the food consumption of vehicle group and the olanzapine treated group was shown in figure 3.7.



**Figure 3. 6** The food consumption of the vehicle group.



**Figure 3. 7** The food consumption of the olanzapine treated group.

### 3.2.2. RNA Concentration and Agarose Gel Electrophoresis

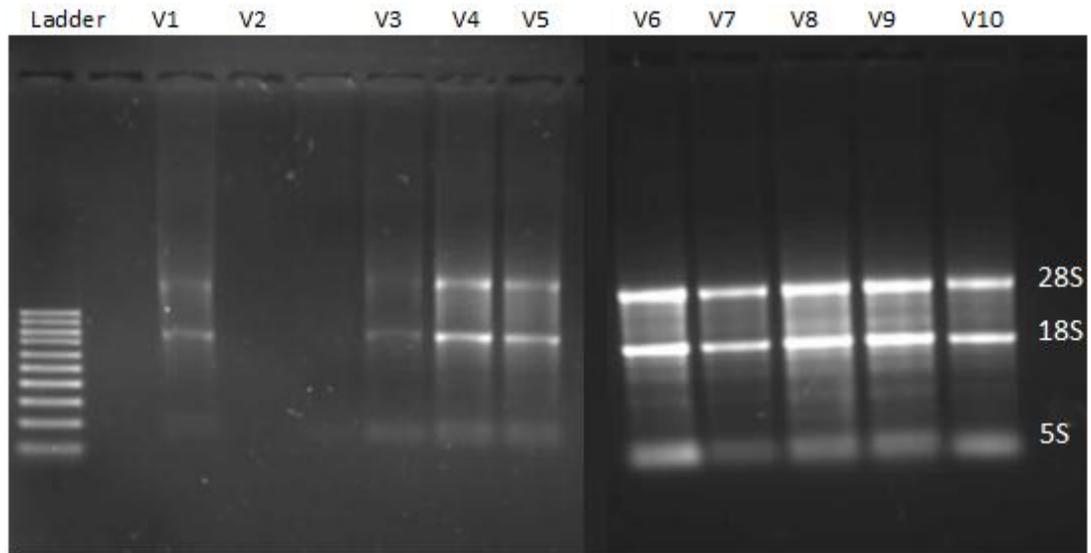
For the determination of mRNA levels of the candidate genes, the concentrations and the integrity of the isolated RNA sample were documented. The concentration and the 260/280 and 260/230 ratios of the RNA isolated from the hypothalamus of vehicle group and olanzapine treated group is shown in table 3.2.

The quality of RNA is determined by the ratios of A260/A280 and A260/A230 ratios. The RNA having the highest quality has a ratio of A260/A280 should be between 1.8 - 2.0 and the A260/A230 ratio should be 2.0. Those ratios indicate the purity of the RNA sample. If the A260/A280 ratio is lower than 1.8 it indicates protein contamination. Having a lower ratio for A260/A230, indicates possible contaminants that is being been absorbed at A230 such as EDTA or Trizol. As shown in table 3.2, the ratios of the RNA isolated from brain tissues have ratios close to those values.

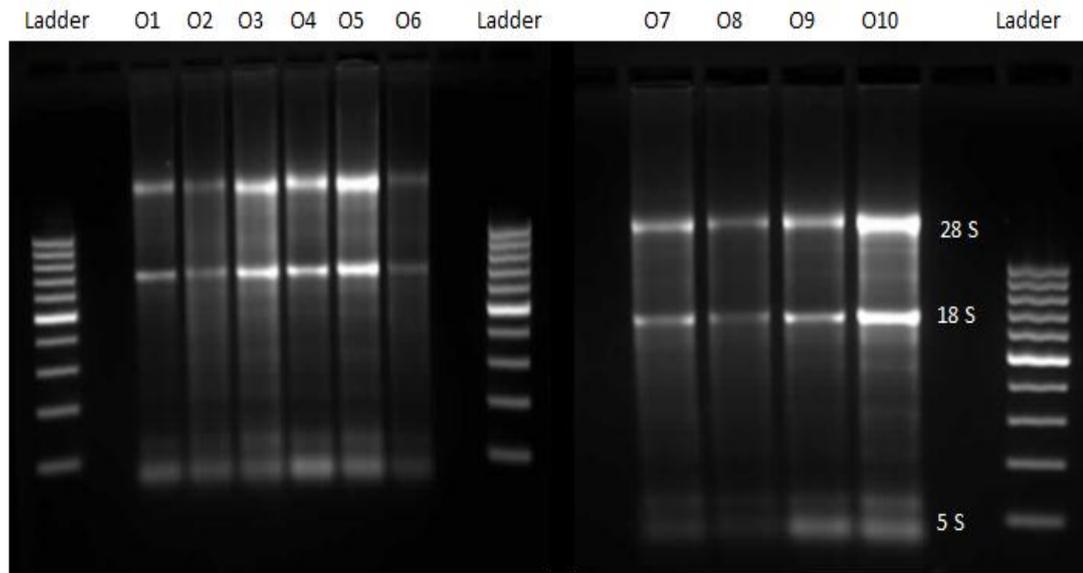
**Table 3. 2** The concentration, 260/280 and 260/230 ratios of the vehicle RNA. O: olanzapine-treated rats; V: vehicle-treated rats

<b>Samples</b>	<b>Concentration (ng/ul)</b>	<b>A260/A280</b>	<b>A260/A230</b>
<b>V1</b>	74,70	1,93	1,33
<b>V2</b>	68,40	1,88	0,89
<b>V3</b>	159,30	1,98	1,35
<b>V4</b>	141,30	1,96	1,01
<b>V5</b>	784,30	1,91	1,20
<b>V6</b>	439,00	2,00	1,89
<b>V7</b>	835,50	2,04	1,95
<b>V8</b>	764,50	2,02	1,95
<b>V9</b>	541,00	1,99	1,93
<b>O1</b>	430,60	1,98	1,48
<b>O2</b>	476,50	1,98	1,42
<b>O3</b>	782,90	2,05	1,89
<b>O4</b>	654,60	2,04	1,73
<b>O5</b>	889,40	2,05	1,63
<b>O6</b>	480,90	1,82	0,86
<b>O7</b>	624,30	2,03	1,68
<b>O8</b>	428,20	1,99	1,19
<b>O9</b>	646,80	2,02	1,42
<b>O10</b>	1.109,00	2,02	2,05

After DNase I treatment, the isolated RNA samples were also run on 1% agarose gel to check the integrity and any possibility of remaining contaminated DNA that might affect the qRT-PCR results. As shown in figure 3.8 and figure 3.9, the total RNA was intact; having the three rRNA bands (28S, 18S and 5S) with no DNA contamination remained.



**Figure 3. 8** Total RNA gel electrophoresis of the vehicle group (V). The bands show 28S, 18S and 5S of RNA. Any contamination of DNA was not observed. V2 was omitted from the study. The samples were run on different electrophoresis tanks and were merged to indicate them in one figure.



**Figure 3. 9** Total RNA gel electrophoresis of olanzapine group (O). The bands (28S, 18S and 5S) indicates the integrity of total RNA. No DNA contamination was observed. The figure was constructed by merging the gel photos of different electrophoresis tanks.

### 3.2.3. qRT-PCR of candidate genes in Rat Hypothalamic Samples

To calculate accurate results for the expression analysis, there are important points to consider while analyzing the quality of the qRT-PCR. One of them, the efficiency (E) indicates the duplication of a product in each cycle of the reaction. In this case, when the reaction efficiency is 100 %, which is equals to 1, the slope of the curve (S) gives you a value of -3.322. The efficiency of the qRT-PCR reaction is calculated by the following formula;

Calculation of PCR efficiency:

$$(\%) = 10^{(-1/S) - 1} \times 100 \quad (2)$$

In this study the efficiencies calculated from slope values between -3.3 to -3.8 are accepted as the efficient reaction to be used in the quantification.

Another important point is the “coefficient of determination” ( $R^2$ ). This value is described as the variability in a data set.  $R^2$  value from a qRT-PCR reaction should be 1; which can only be obtained for two samples. Having  $R^2$  value of approximately 0.99 indicates that the amount of the product in each sample replicates of each individual tube in the reaction set is be more or less equal to each other, resulting in the  $R^2$  value of 0.99.

The other critical factor of the qRT-PCR reaction is the “threshold cycle”,  $C_t$ .  $C_t$  is defined as the cycle number in which a significant detectable elevation in the fluorescence is observed.

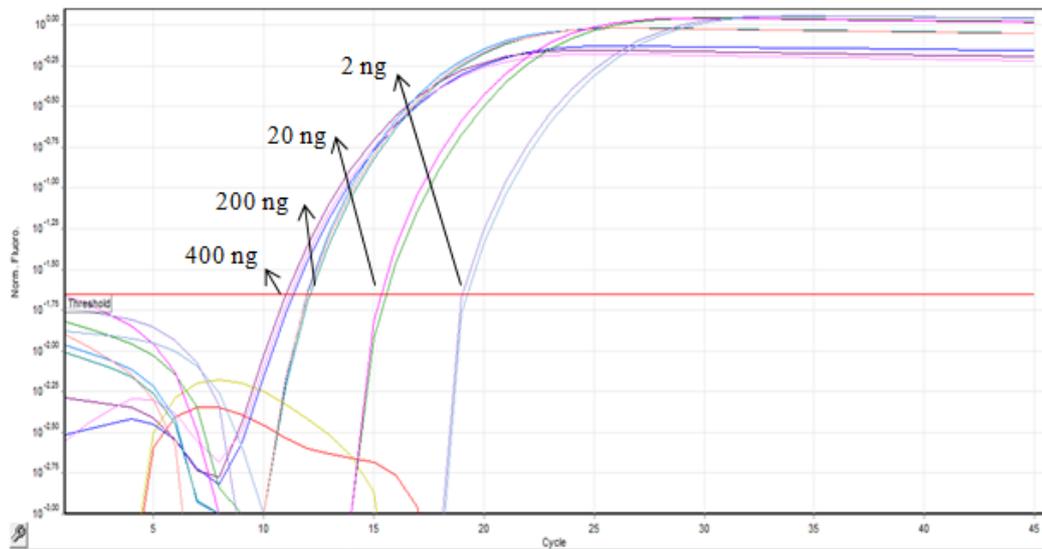
In addition, the melt curve analysis is performed in order to understand the specificity of the reaction. Melting temperature can be described as the temperature at which the 50% of the double stranded DNA become single stranded. The products that are formed during amplification should be at the same size if the primers are specific to the product of interest. However, if some nonspecific products are formed in the reaction, all the products formed during the reaction will have different melting temperatures ( $T_m$ ). In the qPCR reactions, when the double stranded DNA molecules become single stranded, the fluorescence decreases since the SybrGreen dye dissociates from the DNA sample. The middle point of this decrease curve gives a peak at a temperature, which indicates the specific product. If there is any contamination of genomic DNA, or formation of primer dimers or nonspecific products in the reaction, there would be more than one peak, which is not desired. On the other hand, the existence of only one peak demonstrates the specific product and confirms the specificity of the reaction.

### 3.2.3.1. Standard Curves of Genes

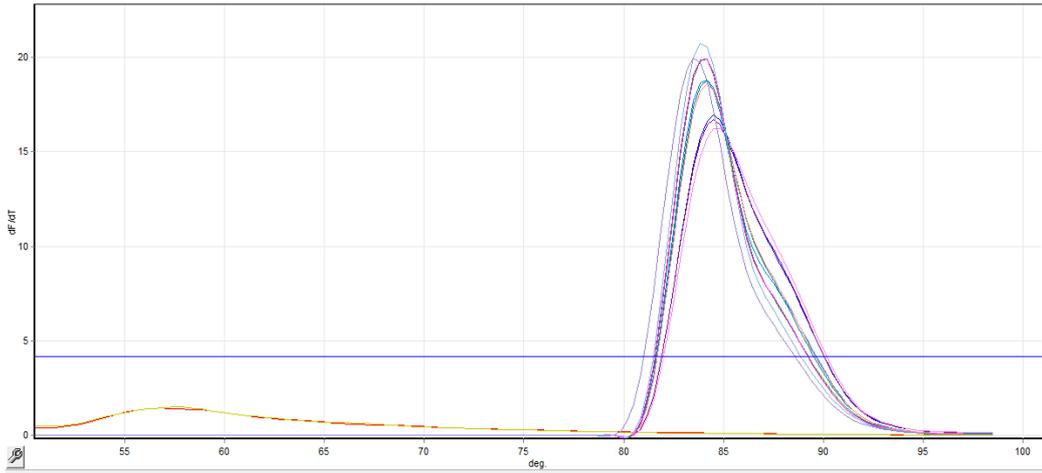
Standard curves were created for all the genes of interest and for the housekeeping gene; *GAPDH*, to calculate the concentrations of the expressed mRNA in the rat hypothalamic tissue.

#### 3.2.3.1.1. *GAPDH* Standard Curve

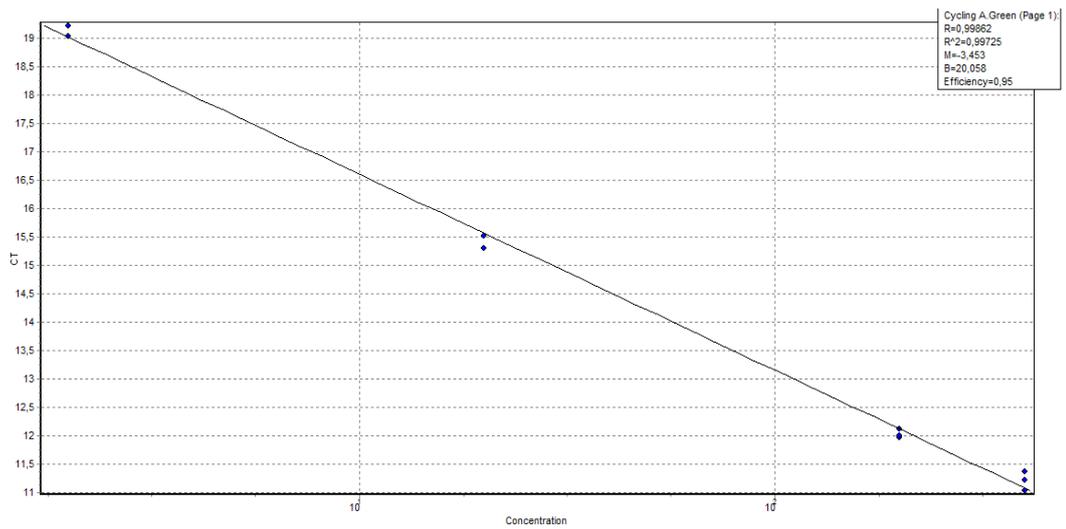
The standard curve of *GAPDH* is shown in figure 3.12. The concentrations used to construct the standard curve were samples having 400 ng/rxn, 200 ng/rxn, 20 ng/rxn and 2 ng/rxn as the final concentration. Fig. 3.10 indicates the amplification of *GAPDH*. Fig. 3.11 illustrates the melt curve of the reaction showing one specific product peak approximately at 84.5°C.



**Figure 3. 10** The amplification data of *GAPDH* standard curve



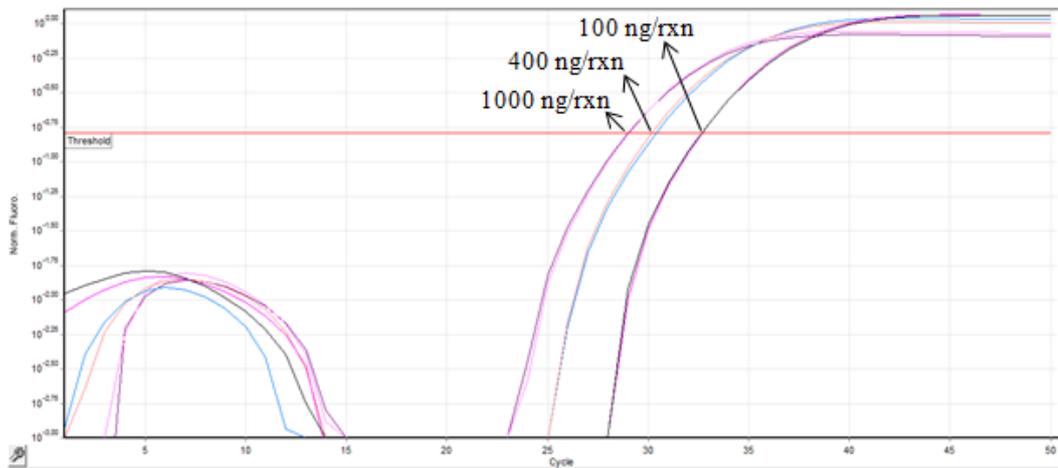
**Figure 3. 11** Melting curve analysis of GAPDH standard curve. The melt curve analysis showed the similar peak at approximately 84.5<sup>0</sup>C.



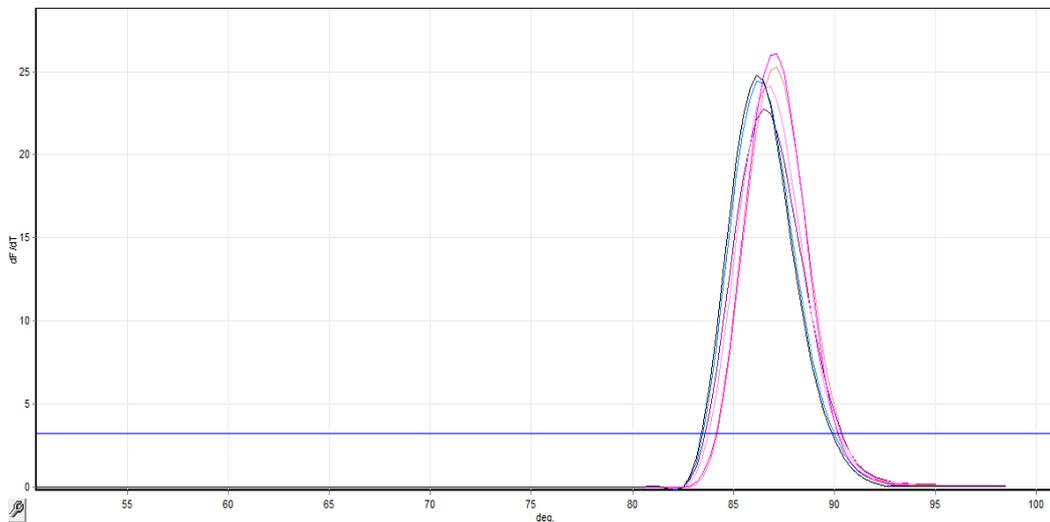
**Figure 3. 12** The standard curve of GAPDH . The reaction efficiency was 0.95 and the R<sup>2</sup> was 0.99.

### 3.2.3.1.2. *NPY* Standard Curve

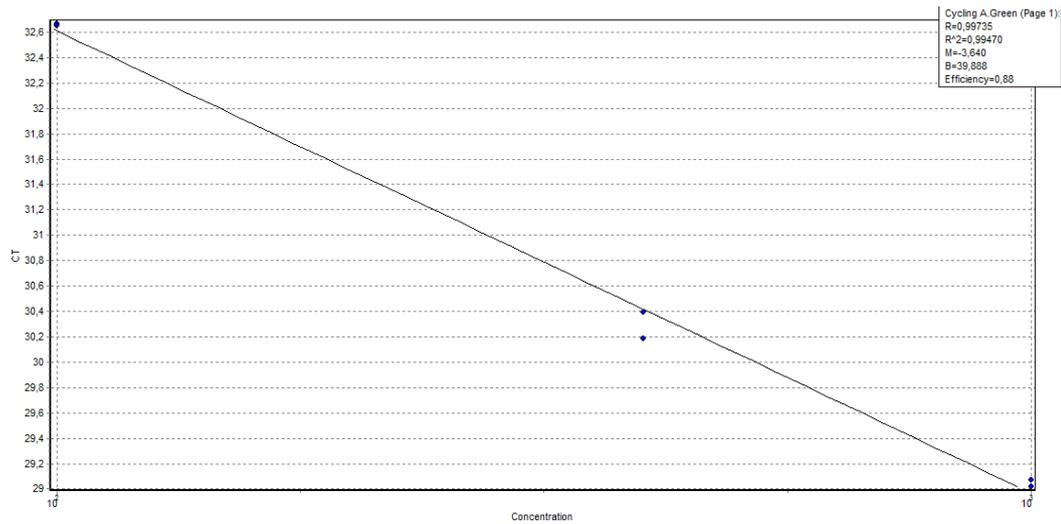
The standard curve of *NPY* is shown in Fig. 3.15. The concentration of the samples used to construct the standard curve of *NPY* was 1000 ng/rxn, 400 ng/rxn and 100 ng/rxn. Fig. 3.13 shows the amplification of *NPY* and Fig. 3.14 indicates the melt curve of samples for *NPY* quantification.



**Figure 3. 13** The amplification data of *NPY* standard curve



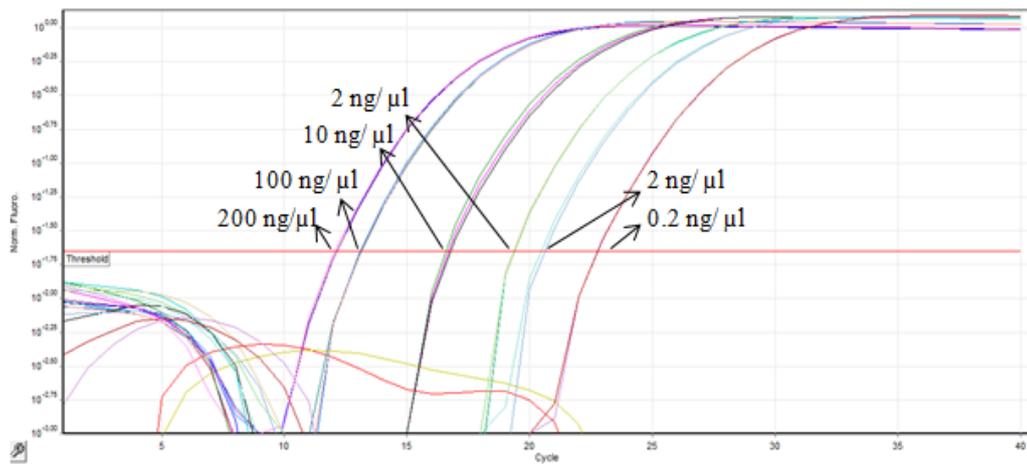
**Figure 3. 14** Melting curve analysis of *NPY*. The melt curve analysis showed the same peak at approximately 86.5°C which shows the reaction specificity.



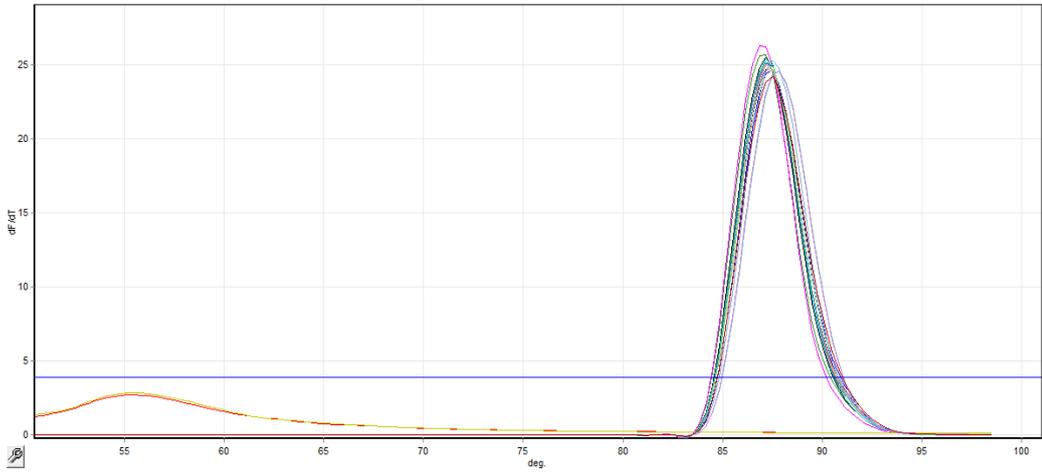
**Figure 3. 15** The standard curve of NPY. The reaction efficiency was 0.88 and the  $R^2$  was 0.99.

### 3.2.3.1.3. AgRP Standard Curve

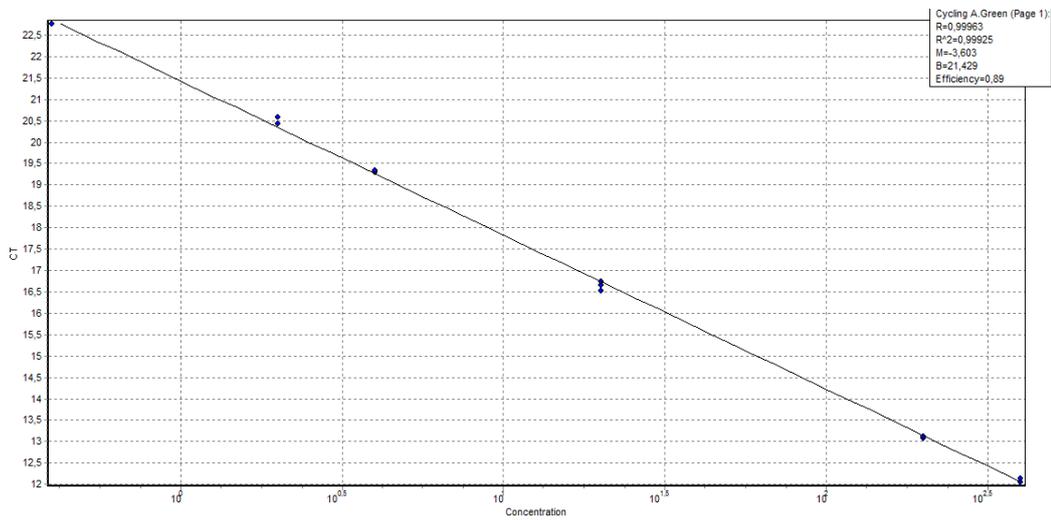
AgRP standard curve is shown in figure 3.18. The samples' concentration used to construct the curve was 200 ng/ $\mu$ l, 100 ng/ $\mu$ l, 10 ng/ $\mu$ l, 2 ng/ $\mu$ l, 1 ng/ $\mu$ l and 0,2ng/ $\mu$ l. The amplification and melt curve data are shown in Fig. 3.16 and Fig 3.17. Melt curve analysis shows the specific product approximately at 87.5<sup>0</sup>C.



**Figure 3. 16** Amplification of AgRP standard curve



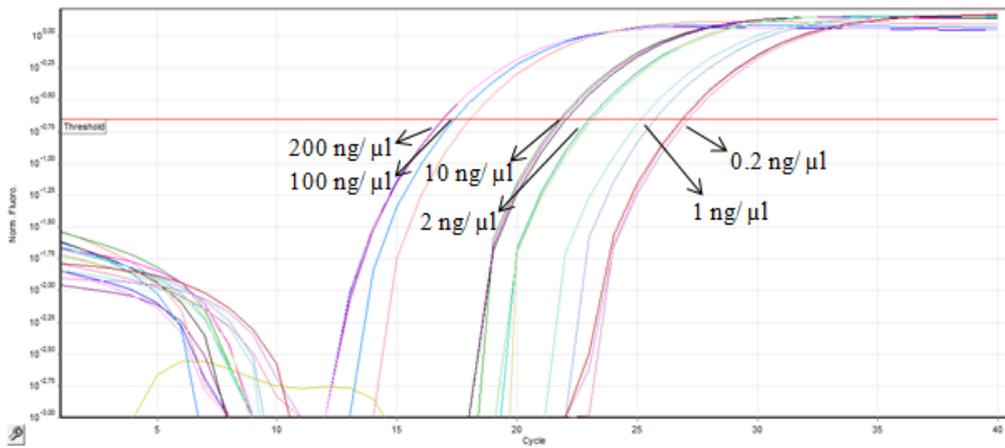
**Figure 3. 17** Melting curve analysis of AgRP standard curve. The melt curve analysis showed the similar peak at approximately 87.5<sup>o</sup>C.



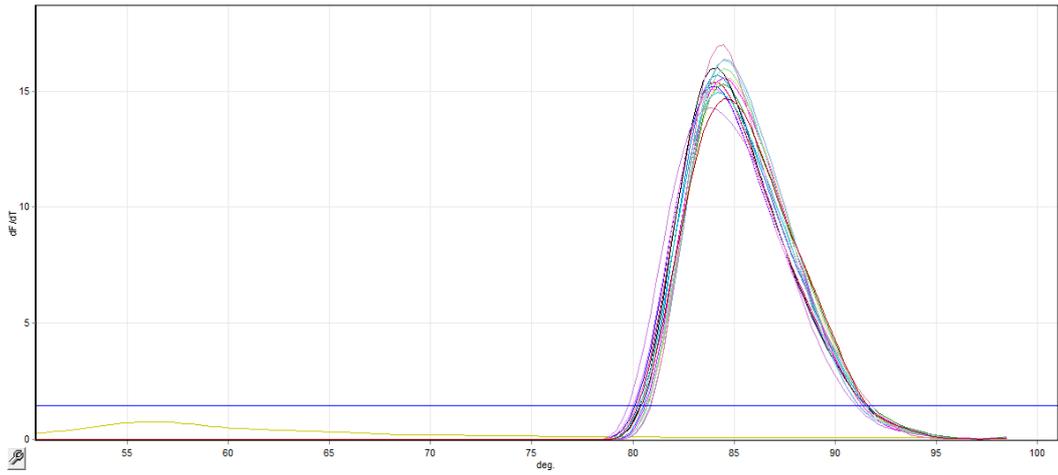
**Figure 3. 18** The standard curve of AgRP. The efficiency of the reaction was 0.89 and the  $R^2$  was 0.99.

### 3.2.3.1.4. *POMC* Standard Curve

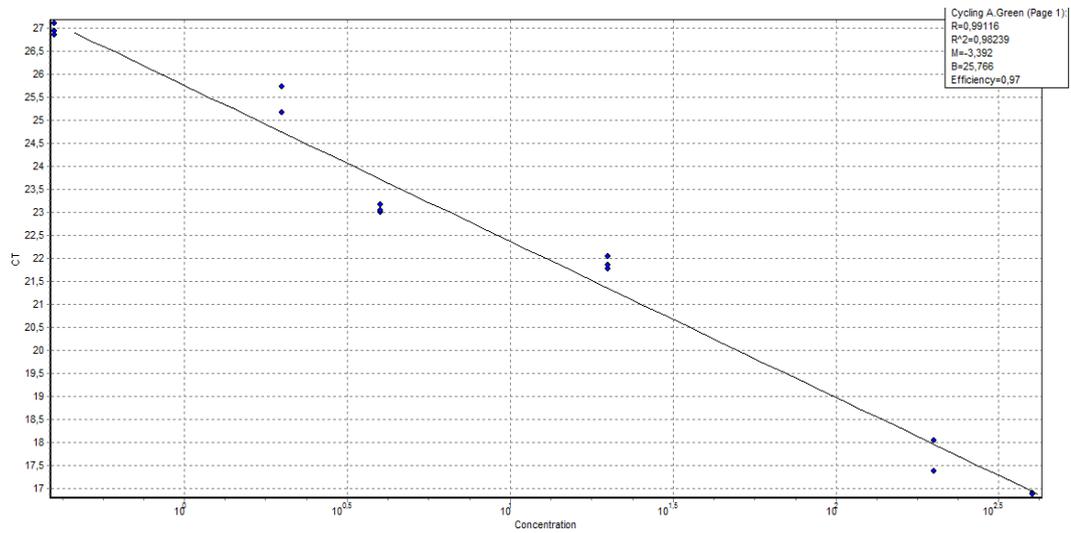
The standard curve for *POMC* is shown in Fig 3.21. The concentration of the cDNA used to construct the standard curve was 200 ng/ul, 100 ng/ul, 10 ng/ul, 2 ng/ul, 1 ng/ul and 0.2 ng/ul. Fig. 3.19 indicates the amplification data of *POMC*. The standards used in the construction of the curve are illustrated in the Fig. 3.19. The less the concentration of the cDNA used for the reaction, the late the  $C_t$  value is obtained. The melt curve analysis is shown in Fig. 3.20.



**Figure 3. 19** The quantification data of *POMC* standards.



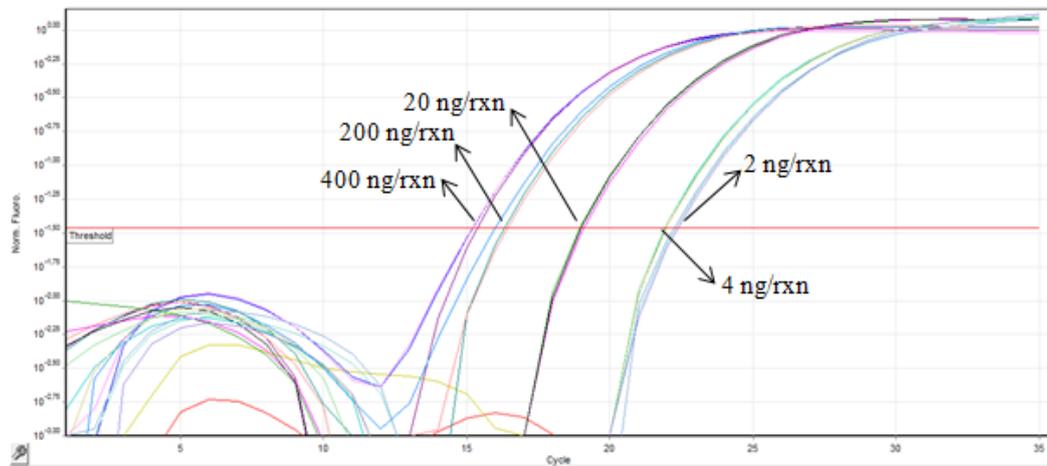
**Figure 3. 20** Melting curve analysis of POMC standard curve. The melt curve analysis showed the same peak at approximately 84<sup>0</sup>C.



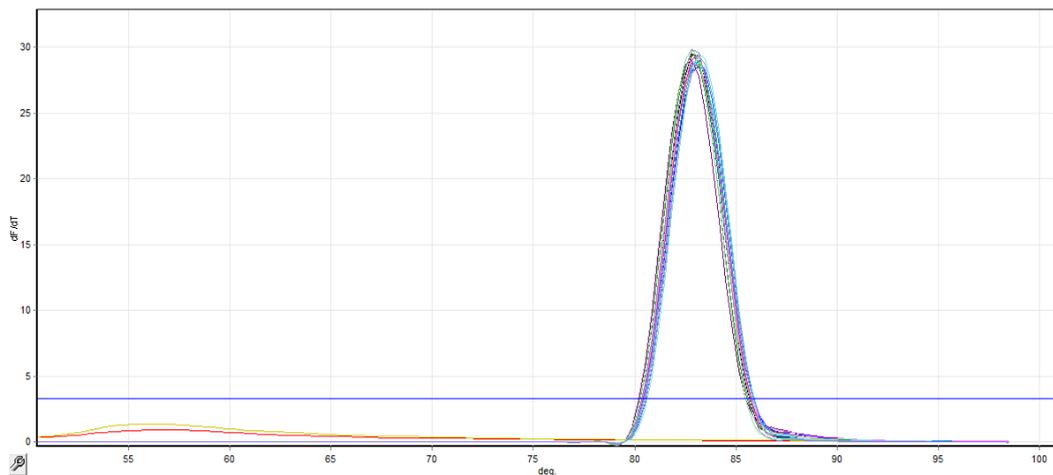
**Figure 3. 21** The standard curve of AgRP. The efficiency of the reaction was 0.97 and the R<sup>2</sup> value was 0.98.

### 3.2.3.1.5. *CART* Standard Curve

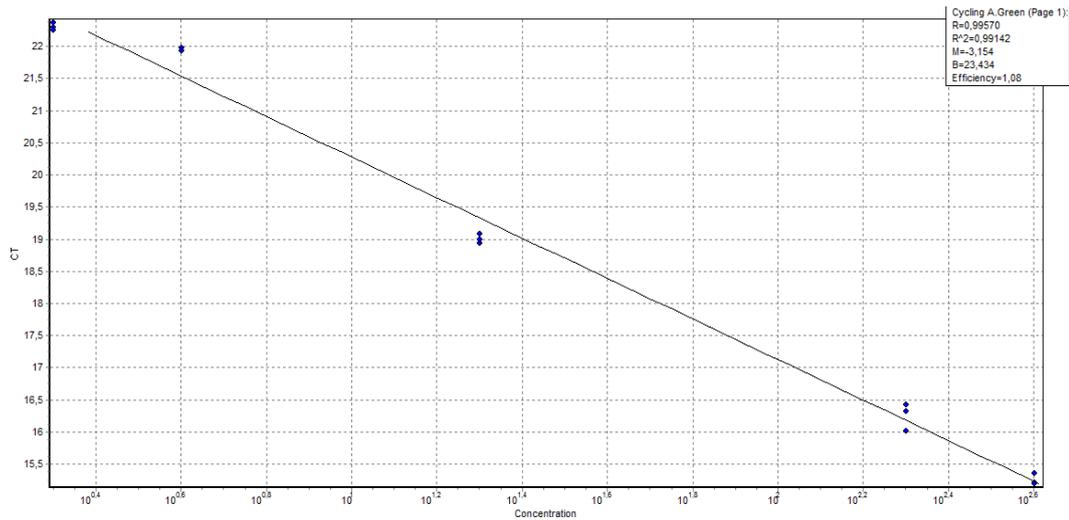
*CART* standard curve is shown in figure 3.24. The concentration of samples used in the construction of the curve was 400 ng/rxn, 200 ng/rxn, 20 ng/rxn, 4 ng/rxn, 2 ng/rxn. Amplification data of the standards is illustrated in Fig. 3.22. Melt curve analysis of *CART* standards gave the similar peak approximately at 83°C indicating the specificity of the reaction (Fig. 3.23).



**Figure 3. 22** The amplification data of *CART*.



**Figure 3. 23** Melting curve analysis of *CART* standard curve. The melt curve analysis showed the specific product peak at approximately at 83°C.



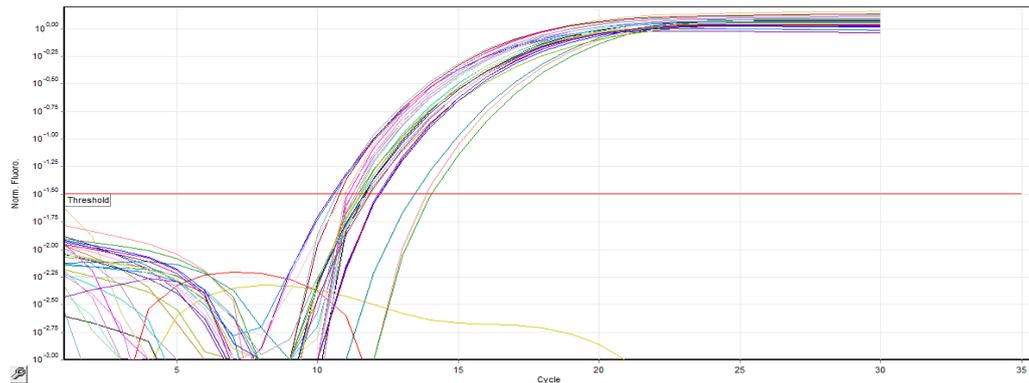
**Figure 3. 24** The standard curve of CART. The reaction efficiency was 1.08 and the R<sup>2</sup> was 0.99.

### 3.2.3.2. Quantitation of the candidate genes expression for the Vehicle Group

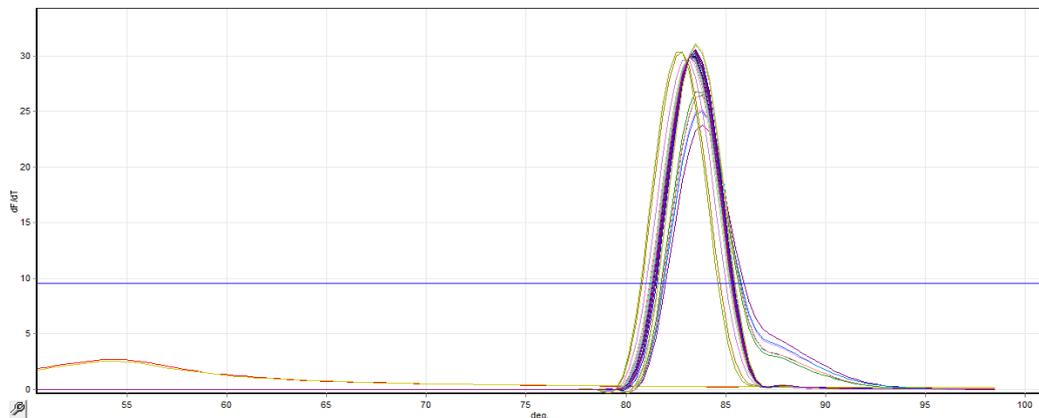
The runs of the vehicle group’s candidate genes and *GAPDH* were imported to the related gene’s standard curves to calculate the Ct values. For all the reactions of vehicle group that will be shown below, the reaction was accepted to be used in the calculation of the expressed mRNA in the conditions where the efficiency and R<sup>2</sup> values of the run were in between 80% - 115% range of the constructed standard curve.

### 3.2.3.2.1 *GAPDH* expression

The housekeeping gene *GAPDH* was amplified in each vehicle rat according to optimized conditions above. The amplification of the vehicle group's *GAPDH* mRNA is shown in Fig. 3.25. Melt curve analysis of *GAPDH* indicated the specific product peak approximately at 84.5<sup>0</sup>C (Fig. 3.26).



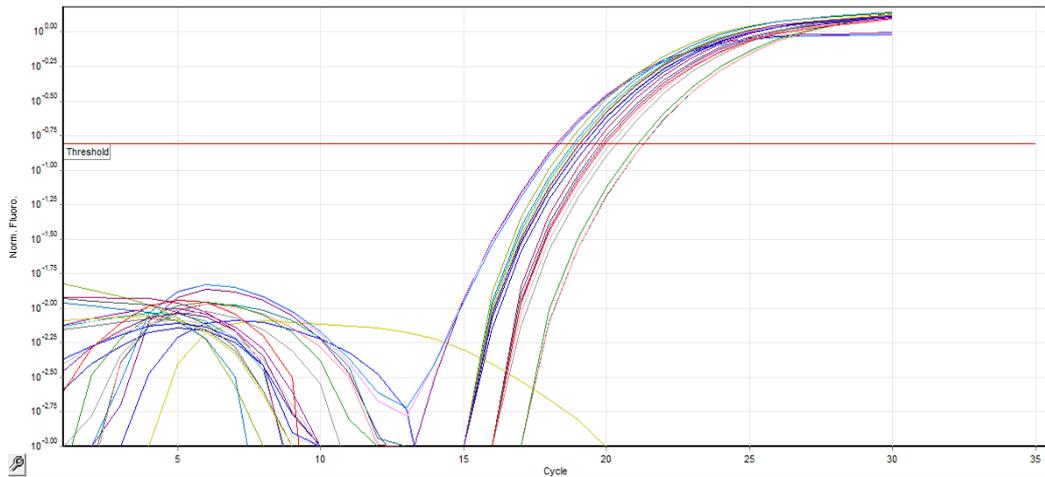
**Figure 3. 25** The amplification data of *GAPDH* from vehicle samples



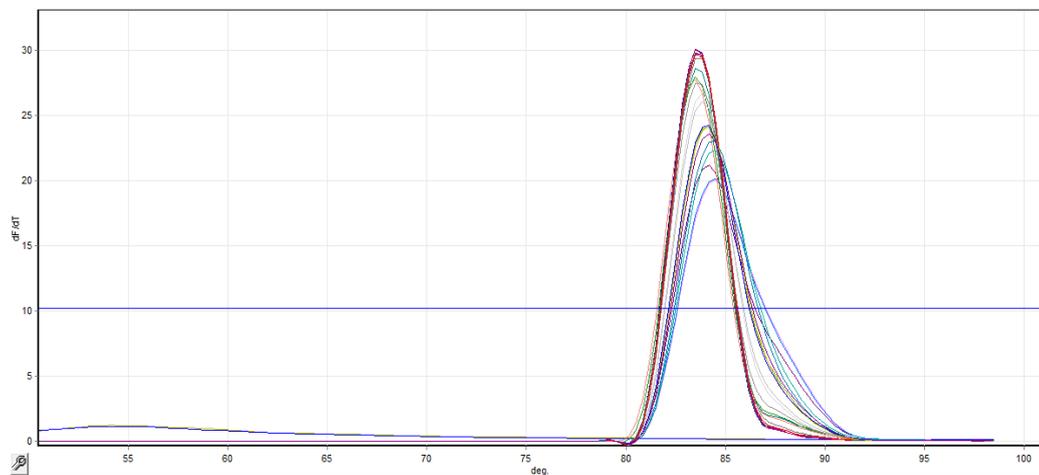
**Figure 3. 26** Melting curve analysis of *GAPDH* of vehicle samples. The melt curve analysis showed the same peak at approximately at 84.5<sup>0</sup>C.

### 3.2.3.2.2 *NPY* expression

*NPY* amplification and melt curve analysis of the vehicle group is shown in Fig. 3.27 and Fig. 3.28. The single peak in melt curve analysis of the *NPY* reaction indicates the specificity.



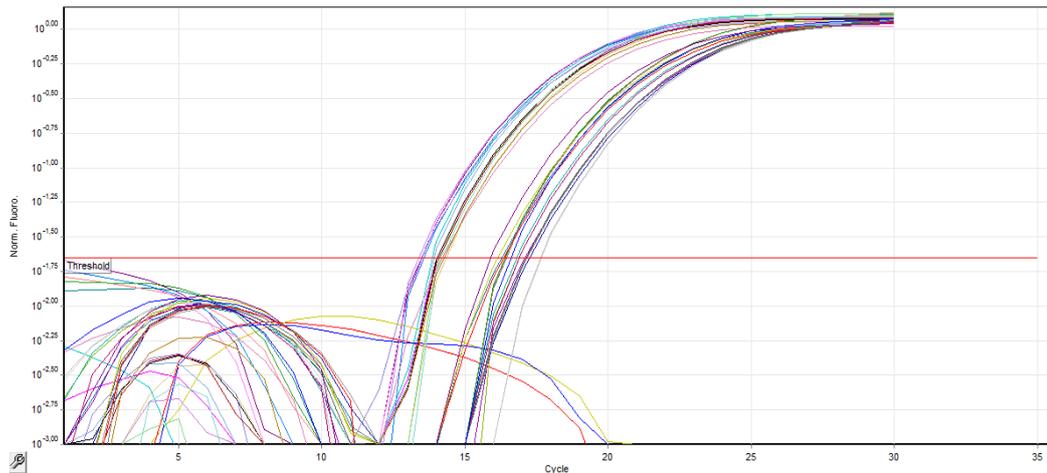
**Figure 3. 27** The amplification data of *NPY* from vehicle samples



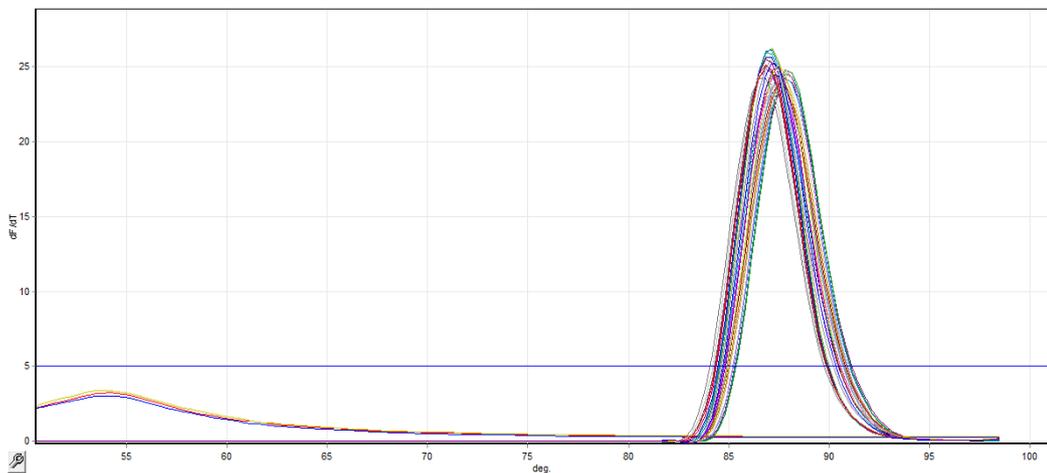
**Figure 3. 28** Melting curve of *NPY* in vehicle hypothalamic samples. The melt curve analysis showed the same peak at approximately at 86.5<sup>0</sup>C.

### 3.2.3.2.3 *AgRP* expression

The amplification and melt curve analysis of *AgRP* gene of vehicle group is indicated in Fig. 3.29. and Fig. 3.30. The amplification data shows the duplication of *AgRP* in every cycle. Melt curve of the *AgRP* indicated the specific amplicon had been amplified during the reaction.



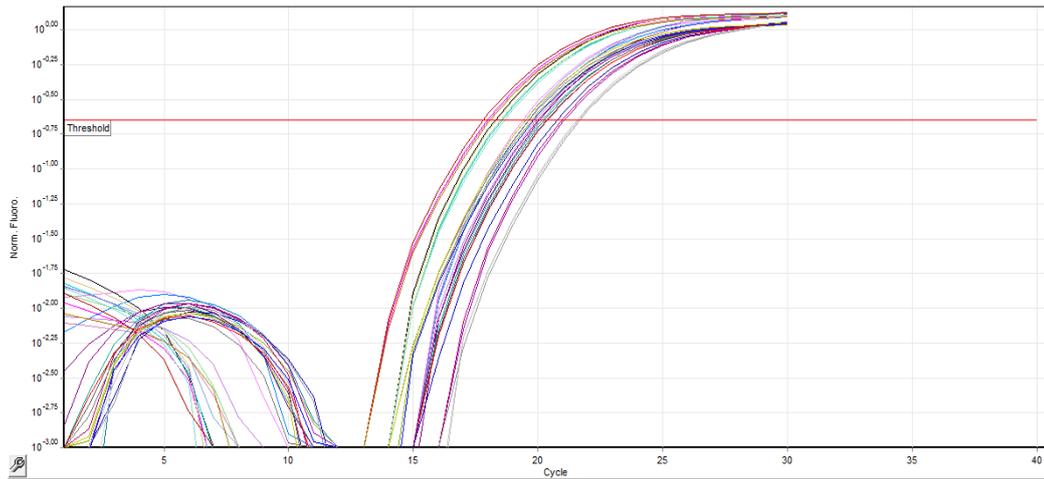
**Figure 3. 29** The quantification data of *AgRP* from vehicle samples.



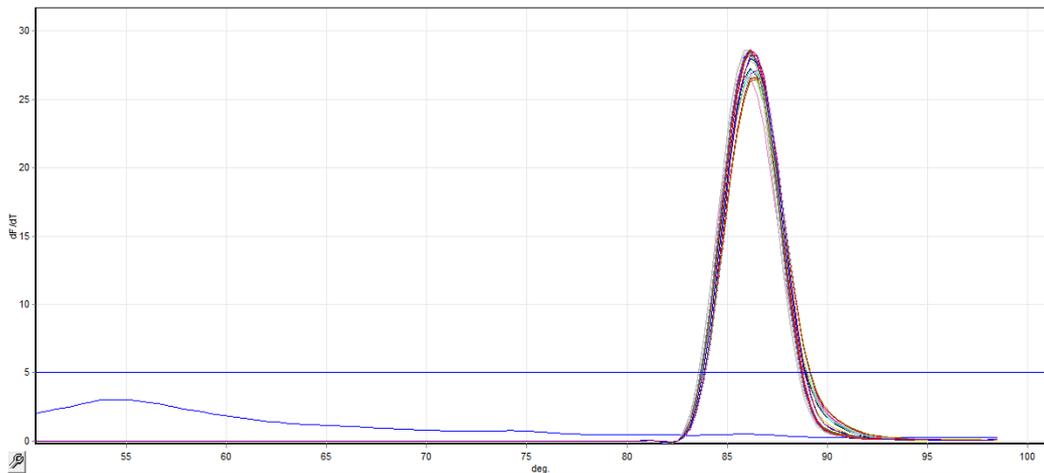
**Figure 3. 30** Melting curve analysis of *AgRP* of vehicle samples. The melt curve analysis showed the same peak at approximately at 87.5°C.

### 3.2.3.2.4 POMC expression

POMC gene amplification data and melt curve analysis are shown in Fig. 3.31 and Fig. 3.32; respectively. Melt peaks of all the vehicle hypothalamic samples at 84°C indicated that the reaction was specific for *POMC*.



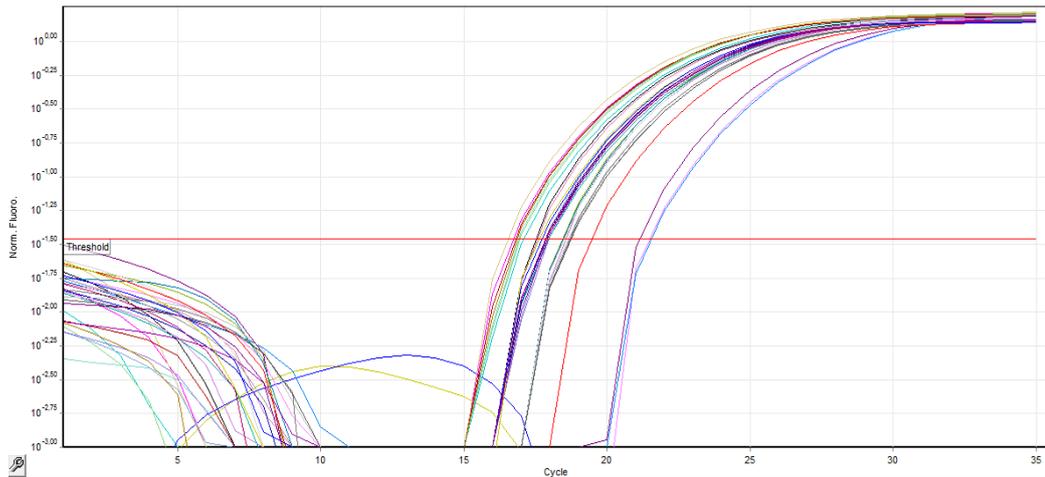
**Figure 3. 31** The quantification data of POMC from vehicle samples.



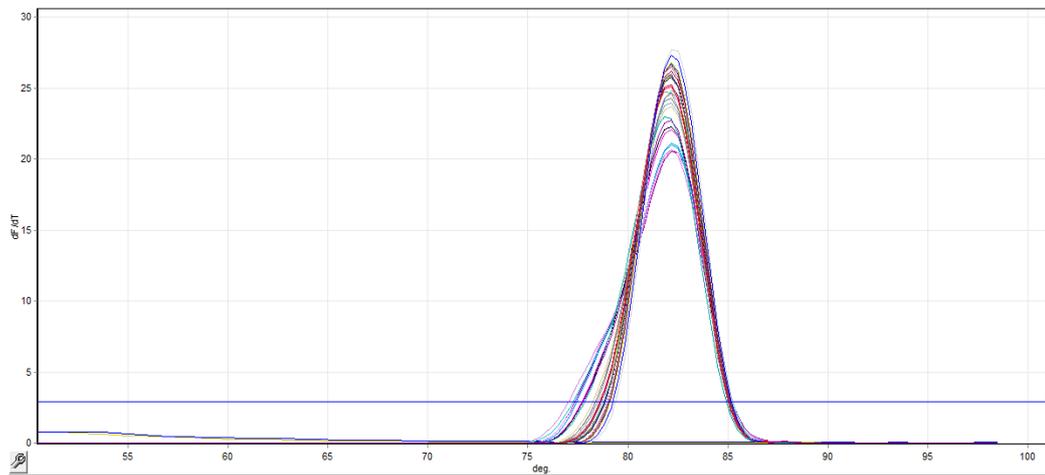
**Figure 3. 32** Melting curve analysis of POMC of vehicle samples. The melt curve analysis showed the same peak at approximately at 84°C.

### 3.2.3.2.5 *CART* expression

Amplification data and melt curve analysis of *CART* gene of the vehicle group are indicated in Fig. 3.33. and Fig. 3.34. *CART* expression amplification was specific as it was understood from the melt curve analysis indicating only the single peak at 83<sup>0</sup>C.



**Figure 3. 33** The quantification data of *CART* from vehicle samples.

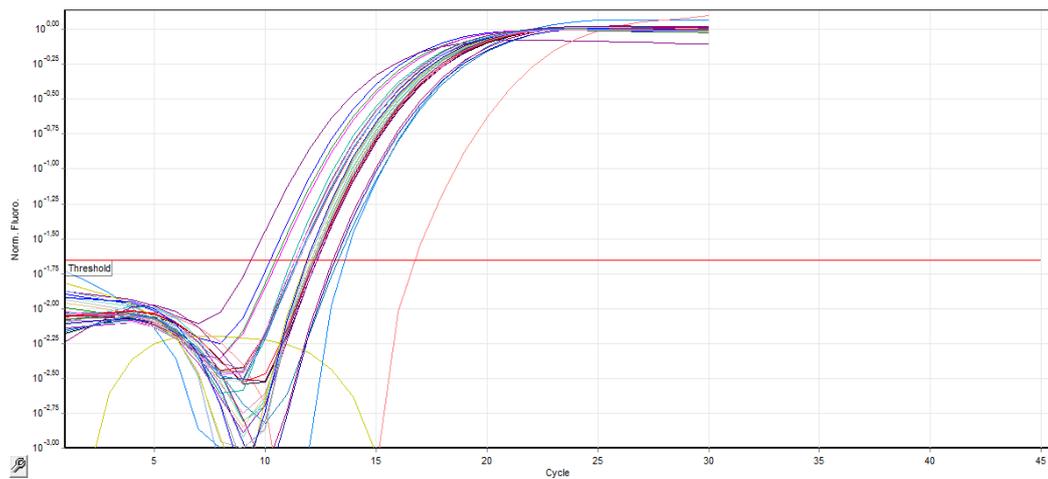


**Figure 3. 34** Melting curve analysis of *CART* of the vehicle group. The melt curve analysis showed the same peak at approximately at 83<sup>0</sup>C.

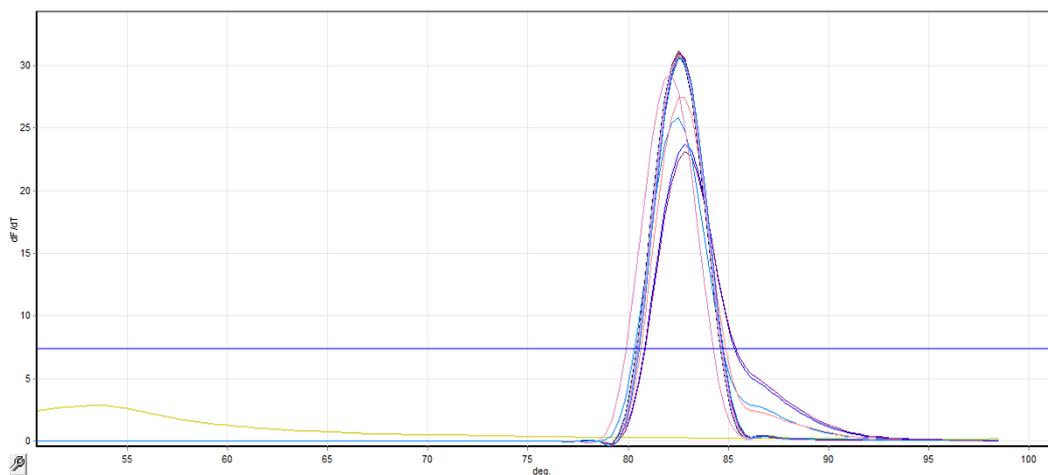
### 3.2.3.3. Quantitation of the candidate genes expression for Olanzapine-treated Group

#### 3.2.3.3.1 GAPDH

The housekeeping gene *GAPDH* amplification of the olanzapine-treated group is indicated in Fig. 3.35. The melt curve analysis indicated that the reaction for *GAPDH* gene was specific (Fig. 3.36).



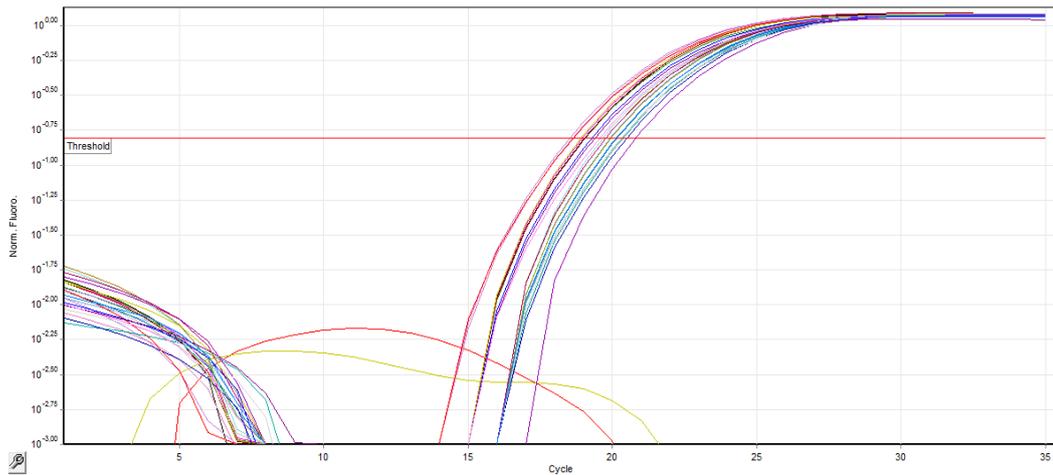
**Figure 3. 35** The amplification of GAPDH from olanzapine-treated group samples.



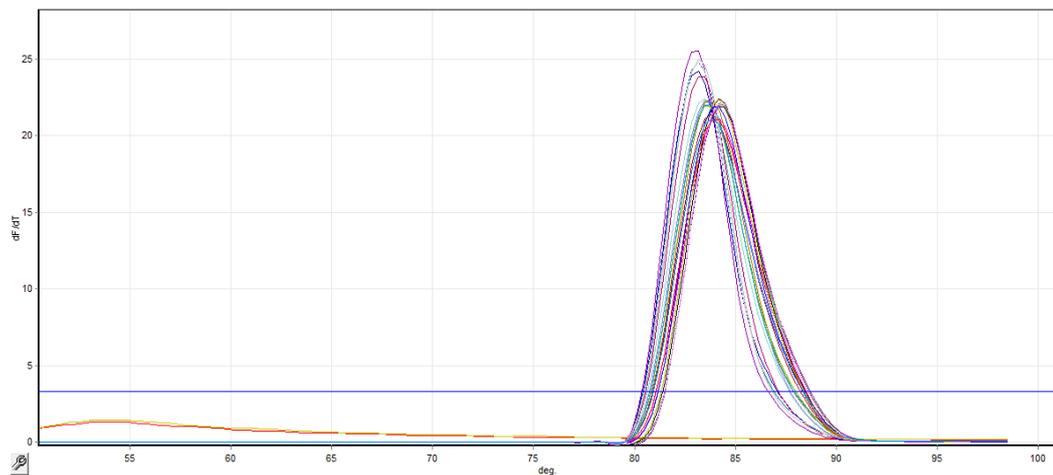
**Figure 3. 36** Melting curve analysis of GAPDH of the olanzapine treated group. The melt curve analysis showed the same peak at approximately at 84.5<sup>0</sup>C.

### 3.2.3.3.2 *NPY*

The amplification and melt curve analysis of the *NPY* gene of olanzapine-treated group is shown in Fig. 3.37 and Fig. 3.38. The amplification of the gene was duplicated in each cycle as shown in Fig. 3.37. The melt curve indicated single peak approximately at 86.5<sup>0</sup>C.



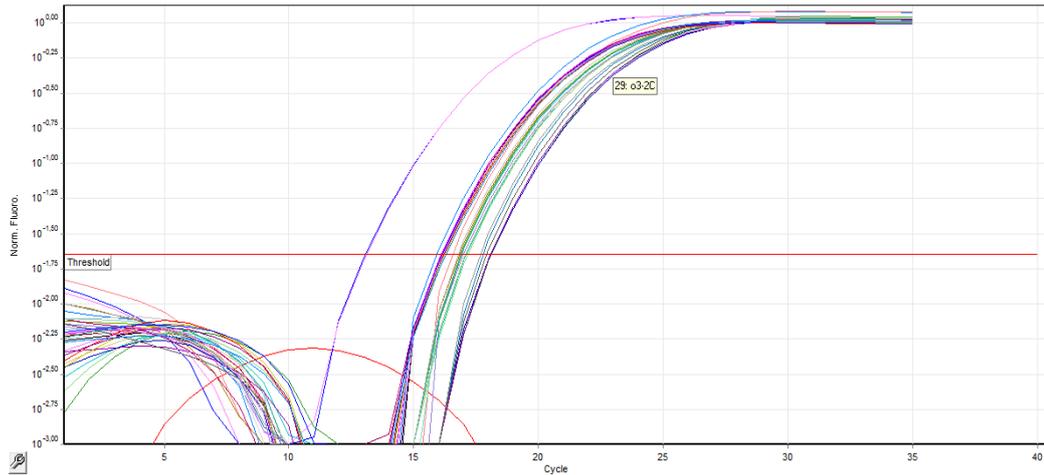
**Figure 3. 37** The quantification data of *NPY* from olanzapine-treated group samples.



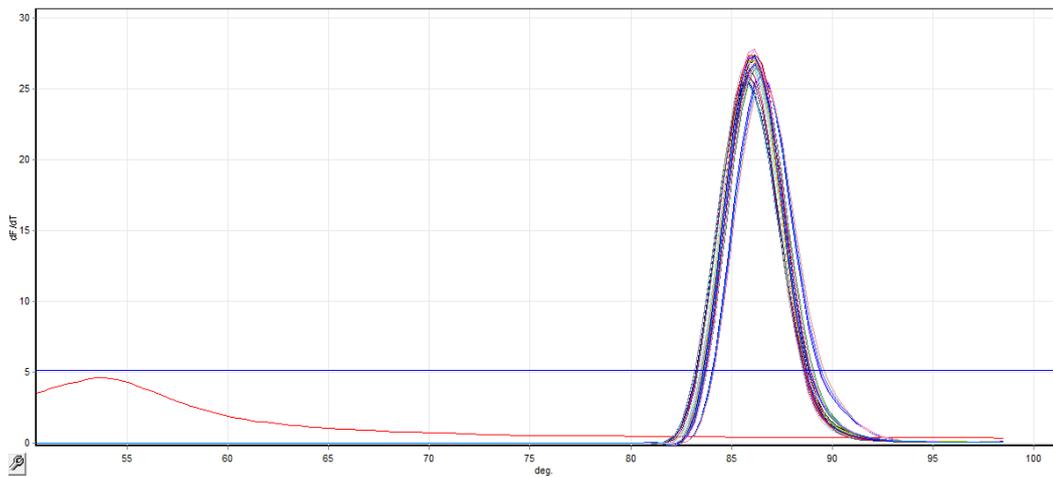
**Figure 3. 38** Melting curve analysis of *NPY* of the olanzapine treated group. The melt curve analysis showed the same peak at approximately at 86.5<sup>0</sup>C.

### 3.2.3.3.3 *AgRP*

*AgRP* amplification is illustrated in Fig. 3.38. which indicates the curve of duplication in each cycle. The melt curve showed similar peak for the *AgRP* reaction (Fig. 3.39).



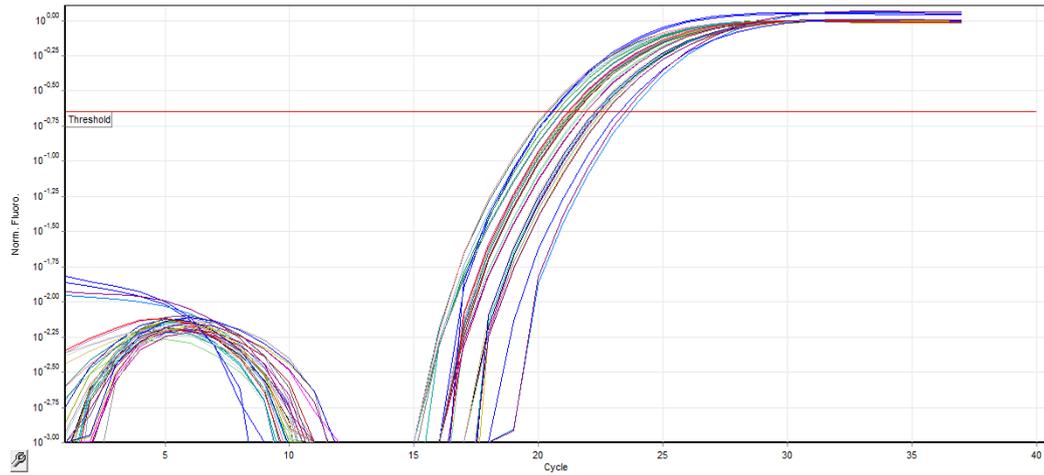
**Figure 3. 39** The amplification of *AgRP* from olanzapine-treated group samples.



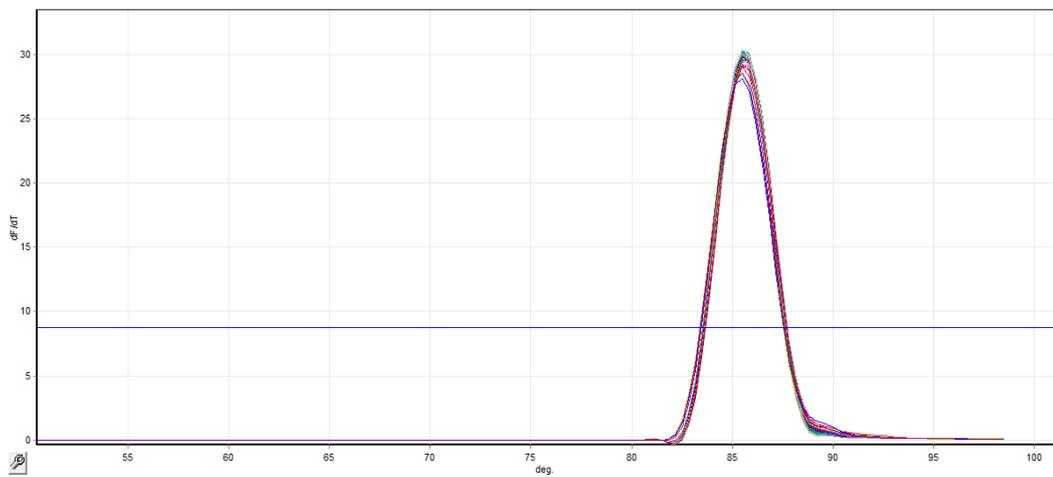
**Figure 3. 40** Melting curve analysis of *AgRP* of the olanzapine treated group. The melt curve analysis showed the same peak at approximately at 87.5<sup>0</sup>C indicating the specific amplification of the gene.

### 3.2.3.3.4 POMC

The amplification and melt curve analysis of *POMC* gene of olanzapine treated group is indicated in Fig. 3.41. and Fig. 3.42. Melt curve indicates the specific reaction conditions for POMC gene.



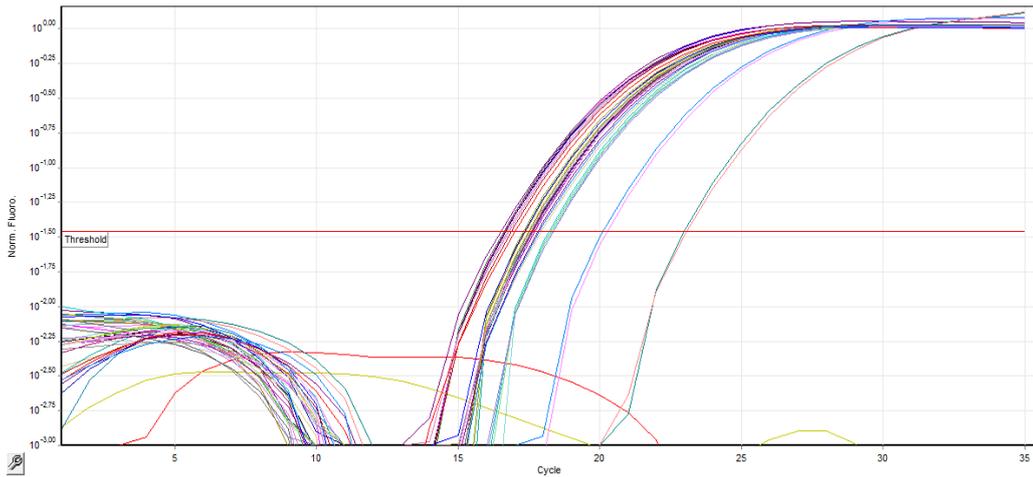
**Figure 3. 41** The quantification data of POMC from olanzapine-treated group samples.



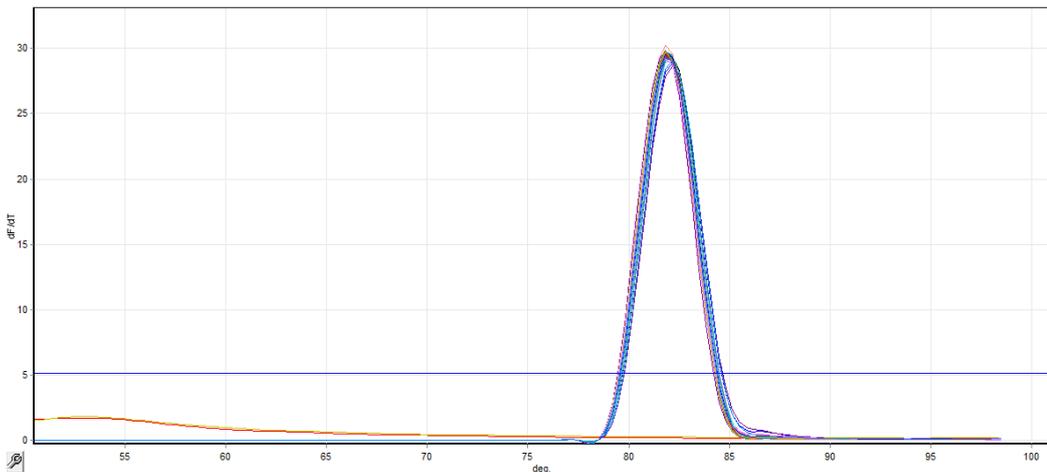
**Figure 3. 42** Melting curve analysis of POMC of the olanzapine treated. The melt curve analysis showed the same peak at approximately at 84<sup>0</sup>C.

### 3.2.3.3.5 CART

CART amplification analysis indicates the mRNA was duplicated in each cycle (Fig. 3.43) and this amplification was specific to CART mRNA sequence that could be proved with the melt curve of the reaction (Fig. 3.44).



**Figure 3. 43** The amplification data of CART from olanzapine-treated group samples.

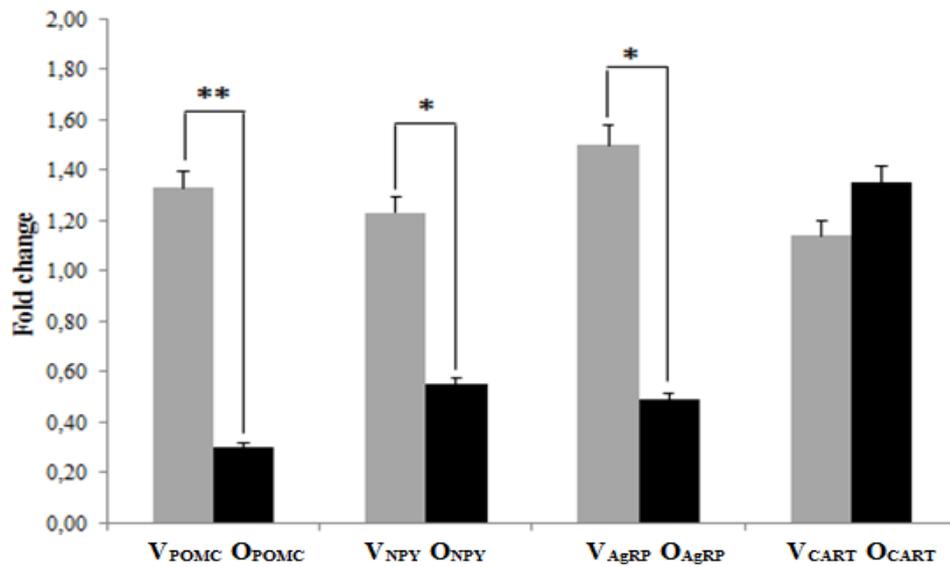


**Figure 3. 44** Melting curve analysis of POMC of the olanzapine treated. The melt curve analysis showed the same peak at approximately at 83<sup>0</sup>C.

To calculate the fold changes of the candidate genes,  $\Delta C_t$  values of every gene of interest was divided by the  $\Delta C_t$  values of the housekeeping gene, GAPDH.  $\Delta C_t$  values were obtained by subtracting the  $C_t$  values of olanzapine treated group from the  $C_t$  values of the vehicle group.

### 3.2.3.4. Fold Changes in Expression

The fold change of the candidate genes, POMC, NPY, AgRP and CART was calculated and is shown in Fig. 3.45.



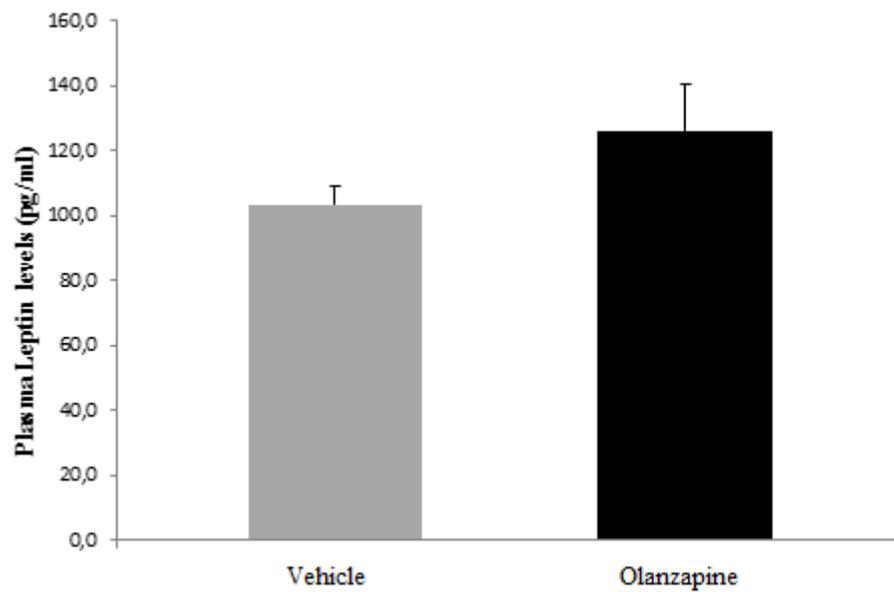
**Figure 3. 45** Fold changes in the expressions of the candidate genes compared with the vehicle group. V: vehicle; O: olanzapine-treated group; the genes of interests are indicated as subscription.

### 3.2.4. Rat peripheral concentrations of hypothalamic peptides that regulate food intake

The circulating levels of hypothalamic peptides that are involved in the appetite regulation were analyzed to compare with the hypothalamic expression data.

#### 3.2.4.1. Plasma leptin levels

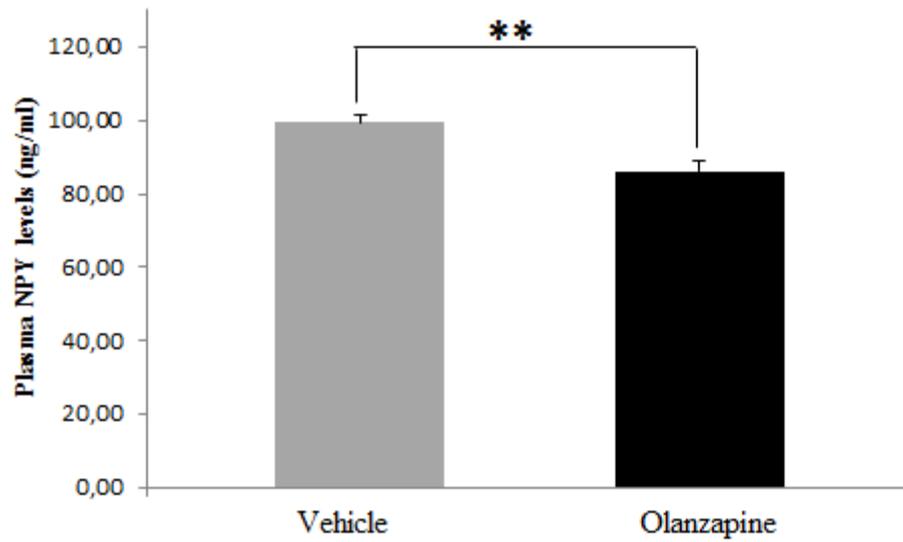
Since the peptides that regulate the food intake are under the control of peripheral leptin, the circulating leptin levels were determined. The plasma leptin concentration in olanzapine group ( $123.15 \pm 14.33$  pg/ml) was higher than vehicle group ( $103.59 \pm 5.35$  pg/ml) (Fig. 3.46). Although the leptin levels were trended higher in the drug treated group, it did not reached statistical significance.



**Figure 3. 46** Plasma leptin levels of rats. The vehicle group and olanzapine group had a mean value of  $103.59 \pm 5.35$  pg/ml and  $123.15 \pm 14.33$  pg/ml; respectively.

### 3.2.4.2. Plasma NPY levels

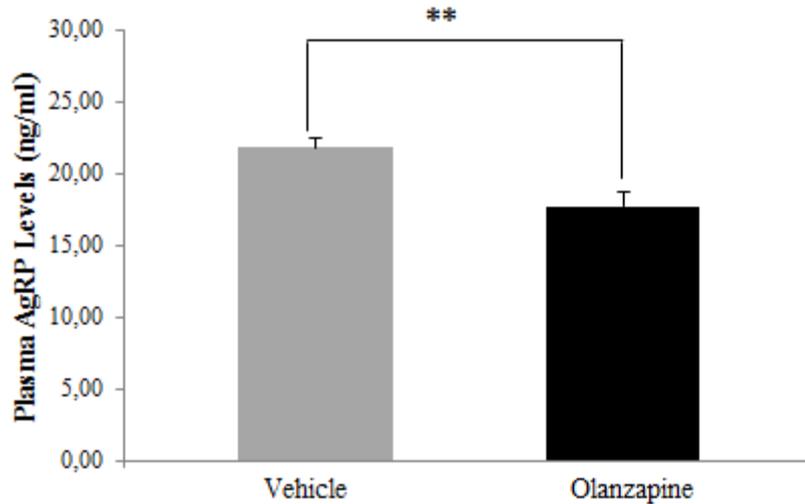
The circulating levels of the orexigenic peptide NPY was determined. Plasma NPY levels of the vehicle and control groups are shown in Fig. 3.47. Olanzapine group had statistically significant (\*\* $p < 0.005$ ) less NPY levels in their plasma ( $86.61 \pm 2.94$  ng/ml) than vehicle group ( $99.36 \pm 2.28$  ng/ml).



**Figure 3. 47** Circulating NPY levels of rats. Peripheral NPY levels in olanzapine group were  $86.61 \pm 2.94$  ng/ml and the vehicle group NPY levels were  $99.36 \pm 2.28$  ng/ml. The difference between two group was statistically significant (\*\* $p < 0.005$ ).

### 3.2.4.3. Plasma AgRP Results

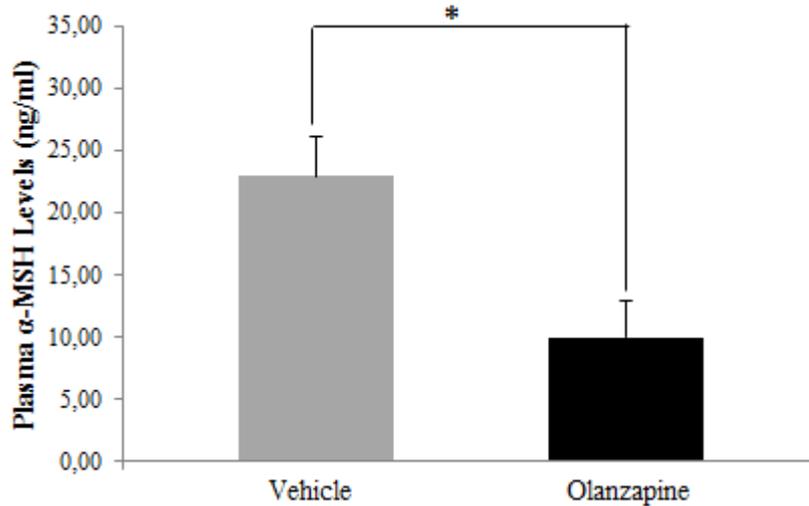
Peripheral AgRP levels of both vehicle and olanzapine groups are shown in Fig. 3.48. Plasma AgRP levels of olanzapine group ( $17.72 \pm 0.98$  ng/ml) were significantly (\*\* $p < 0.005$ ) lower than that of vehicle group ( $21.82 \pm 0.62$  ng/ml).



**Figure 3. 48** Circulating AgRP levels of rats. AgRP concentration of olanzapine group was  $17.72 \pm 0.98$  ng/ml. The vehicle group AgRP value was  $21.82 \pm 0.62$  ng/ml. The reduction in the AgRP levels of the olanzapine group was significantly lower than the vehicle group (\*\* $p < 0.005$ ).

### 3.2.4.4. Plasma $\alpha$ -MSH Results

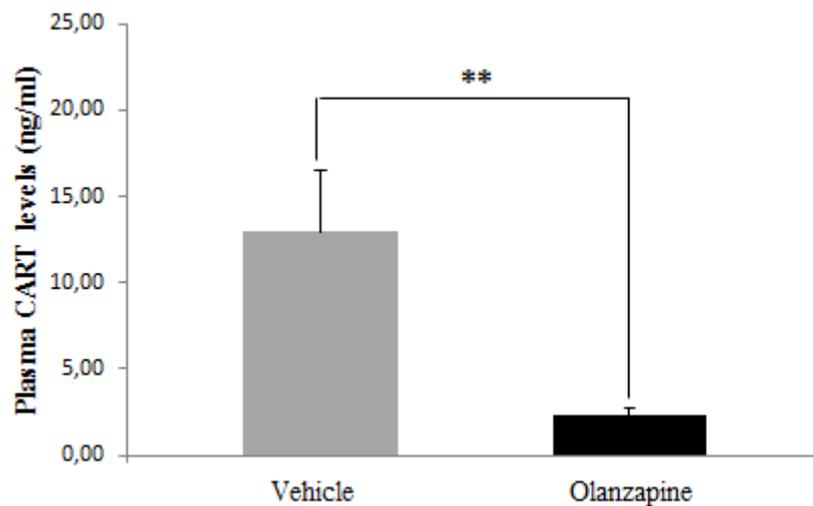
Vehicle group had  $22.95 \pm 3.11$  ng/ml  $\alpha$ -MSH concentration in their plasma; however, the olanzapine treated group value was  $10.03 \pm 2.91$  ng/ml. The decrease in the peripheral  $\alpha$ -MSH levels of olanzapine group was statistically significant compared with the vehicle group ( $*p < 0.05$ ). Plasma  $\alpha$ -MSH levels of the groups are shown in Fig. 3.49.



**Figure 3. 49** Plasma  $\alpha$ -MSH levels of rats. Peripheral  $\alpha$ -MSH value in olanzapine group was  $10.03 \pm 2.91$  ng/ml. The vehicle group had  $22.95 \pm 3.11$  ng/ml. The difference between two group was statistically significant ( $*p < 0.05$ ).

#### 3.2.4.5. Plasma CART Results

The anorexigenic peptide CART levels in the circulatory system were determined. Plasma CART levels in olanzapine group were reduced significantly compared with the vehicle group ( $**p < 0.005$ ). Vehicle group had a mean value of  $12.97 \pm 3.57$  ng/ml concentration in their plasma samples. The olanzapine-treated group, on the other hand, had a mean value of  $2.32 \pm 0.40$  ng/ml. The bar-graph of the plasma CART levels of the groups are indicated in figure 3.50.



**Figure 3. 50** Plasma CART levels of rats. Peripheral CART value in olanzapine treated group was  $12.97 \pm 3.57$  ng/ml. The vehicle group had  $2.32 \pm 0.40$  ng/ml. CART levels were reduced significantly (\*\* $p < 0.005$ ) in olanzapine group compared with the vehicle group.

The overall changes in the expressions and plasma levels of neuropeptides are shown in Fig. 3.51.

	Schizophrenic Patients			Rats	
	Pre-treatment vs. control	Post-treatment vs. control	Pre-treatment vs. post-treatment	Olanzapine administered group vs. vehicle group	
	Plasma levels	Plasma levels	Plasma levels	Plasma levels	mRNA levels
Leptin	↓	↑ <sup>a</sup>	↑	↑ <sup>a</sup>	X
NPY	↓	↓	↑	↓	↓
AgRP	X	X	X	↓	↓
POMC/α-MSH	↑	↑	↑ <sup>a</sup>	↓	↓
CART	↓ <sup>a</sup>	↑ <sup>a</sup>	↑ <sup>a</sup>	↓	↑ <sup>a</sup>

**Figure 3. 51** The overall alterations in the neuropeptide levels in schizophrenic patients and rats. Superscription of a: values detected not statistically significant; nevertheless, there was a trend of change.

Based on the results of the study, four weeks of olanzapine treatment to first attack psychotic patients lead to a significant increase in their body weight, waist circumference and BMI values. These findings are correlated with previous studies (Raposo *et al*, 2011; Chen *et al*, 2011). The patients in the study group gained 4.33 kg during the treatment and their BMI values increased 1.44 kg/m<sup>2</sup> which are the important signs of weight gain. The increase in waist circumference is also accepted to be an indicator of sign of the weight gain and obesity. The statistically significant increase in the waist circumference (4.75 cm, \*\*\* $p < 0.001$ ) may accepted to be as a symptom of the increase in the abdominal fat of these patients. Even though it was impossible to detect the extra food consumption of the patients, the patients indicated to the clinicians in their daily visits that their appetite was increased. Therefore, we might state that the treatment of olanzapine to first attack psychotic disorder patients led to an increased appetite and resulted in weight gain. Besides the data obtained from human plasma samples, even though it was not statistically significant ( $p = 0.09$ ), 33% difference in weight gain in the olanzapine administered group was detected when compared with the vehicle group. By increasing the number of rats used for the study, the statistical significance may be seen. The data suggests that the rats gained  $96.85 \pm 10.21$  gr in this period which is consistent with previous studies (Pouzet *et al*, 2003). The differences in the slopes of the trendlines indicate that the drug administered group's weight gain was more than that of the vehicle group. The slope of olanzapine treated group (1.46) was higher than that of vehicle group (0.34), which indicates that the olanzapine treatment resulted in increased appetite in rats leading to the elevation in the food consumption compared with the vehicle group which showed a more stable food consumption which was also shown in other studies concerning the olanzapine induced weight gain (Goudie *et al*, 2002).

The significant increase in leptin levels in the plasma is also an indicator of the weight gain (Ahima and Flier, 2000a). Since the adipose tissue increase with the increased body weight and waist circumference, the hormone synthesized from

adipose tissue also increase (Ahima and Flier, 2000b). In this study, we determined significantly higher leptin concentrations in the plasma after the treatment compared to the pre-treatment values are correlated with previous studies (Jin *et al*, 2008; Eder *et al*, 2001). Moreover, the leptin levels in pre-treatment period were less than the control group, which was also correlated with the increased body weight. The reduced leptin levels in the plasma of the psychotic patients were correlated with previous studies (Atmaca *et al*, 2003; Krous *et al*, 2001). Even though the olanzapine treatment to schizophrenic patients was for only four weeks, it led to a significant increase in the leptin levels. Even though it did not reach to a significant value ( $p=0.08$ ) in rat studies, plasma leptin levels were treated to increase after four weeks of olanzapine treatment when compared to the vehicle group. The reason why leptin levels did not reach to a significant value might be due the treatment period which was only 4-weeks. With longer periods of olanzapine administration, the leptin levels would found to be increased significantly. The elevation in the plasma leptin levels of rats is correlated with the increase in appetite and also with human ELISA results of leptin.

In the pre-treatment group, there was statistically significant less amount of plasma NPY concentration compared to the controls. This reduced NPY levels in psychotic patients are thought to be a sign of the pathophysiology of psychotic disorder. In 2001, Kuromitsu *et al*, revealed that there is a decreased NPY mRNA expression in the schizophrenic patients. Also Itokowa *et al*, 2003 indicated that there is hypoactivity of NPY neurons in the brain of psychotic patients. Therefore, the reduced NPY levels in psychotic pre-treatment group might be due to the psychotic pattern. In our study, four weeks of olanzapine treatment resulted in significant increase in NPY levels. The significant increase in plasma levels of NPY in post-treatment group compared with the pre-treatment group might cause elevation in the appetite. It was indicated that the antipsychotic treatment leads to increased NPY levels (Itokowa *et al*, 2003 and Kraus *et al*, 2001). The increase in

NPY levels was also shown in a study that olanzapine treatment (Raposo *et al*, 2011). Even though the effect of olanzapine treatment on NPY levels has led to a rise, it was still significantly less than the control values, which also implies the reduced NPY levels in psychotic patients as indicated in Kuromitsu *et al*, 2001 that reduction in NPY levels are considered to be a symptom of psychotic disorder.

In addition, there was a significant decrease in the plasma NPY levels compared with vehicle group in rats. The reduction of the plasma NPY levels in the olanzapine treated group of rats was consistent with the decreased hypothalamic NPY expression. The decrease of both plasma and hypothalamic NPY levels might be due to the leptin's negative effect on the NPY neurons in the ARC. The trended increase in leptin levels in rats' plasma might cause the inhibition of NPY expression by binding to Ob-Rb receptor on NPY neurons (Morrison *et al*, 2005). In rats, the statistically significant decrease in the plasma AgRP levels compared with the vehicle group was also consistent with decreased hypothalamic AgRP expression in rats. Reduced AgRP levels could be also explained with the trended increase in leptin levels. Since in the ARC NPY/AgRP neurons are colocalized (Menyhe'rt *et al*, 2007), the reductions of both NPY and AgRP gene expression and plasma protein levels were observed as expected. Both NPY and AgRP gene expressions revealed 55% and 77% reductions; respectively, which was statistically significant. This reduction in the NPY mRNA levels is consistent Davoodi *et al*, 2009. They showed decreased mRNA levels of both POMC and NPY. It is important to emphasize that NPY and AgRP neurons in the ARC are colocalized and coexpressed from the same neuron group (Chen *et al*, 1998). In this study, the decrease in both NPY and AgRP mRNA expression was consistent with each other and can be explained by the effects of leptin on the NPY/AgRP levels in the ARC of hypothalamus. Since leptin inhibits NPY and AgRP expression (Morrison *et al*, 2005), it could be suggested that the reduction in the mRNA levels of those neuropeptides might be due to the significantly increased

leptin levels in humans and trended increase in rats which were treated with olanzapine. The decrease in rat *NPY* expression and circulating *NPY* levels are found to be different from the plasma *NPY* levels in human.

$\alpha$ -MSH, the anorexigenic product of POMC which have a role on food intake and appetite. Psychotic patients' serotonin (5HT) levels are increased in the extra hypothalamic brain areas (Kapur & Remington, 1996); in the view of the fact that, POMC neurons have 5HT-<sub>2C</sub> receptors on the neuron body (Heisler *et al.*, 2002; Heisler *et al.*, 2006), the increased levels of 5HT in psychotic patients might result in the increased POMC levels; hence the increased levels of  $\alpha$ -MSH. The study group patients indicated that they had less appetite prior to the treatment; therefore, it might be deduced that increased  $\alpha$ -MSH levels could be due to binding of 5HT to 5HT-<sub>2C</sub> receptors on the POMC neurons, eventually might cause decreased appetite. In addition, the significant increase in  $\alpha$ -MSH levels in the post-treatment group compared with controls might be explained by the positive effects of leptin on the POMC neurons of the ARC. The  $\alpha$ -MSH levels in the post-treatment group showed a trended increase ( $p = .006$ ) when compared with the pre-treatment group. Since human leptin levels were found to be significantly increased with the four weeks of olanzapine treatment, POMC neuron activation due to leptin may results in increased levels of  $\alpha$ -MSH levels. Therefore, it may be suggested that leptin has a more potent effect on the POMC neurons than olanzapine's antagonistic effect, which generates elevated levels of plasma  $\alpha$ -MSH in post-treatment group. However, plasma  $\alpha$ -MSH levels of the olanzapine group of rats were found to be significantly lower than the the vehicle group which might be due to binding of olanzapine to the 5HT<sub>2C</sub> receptor and creating an antagonistic effect on the POMC neurons which in turn lead to diminished  $\alpha$ -MSH levels. Since the plasma levels of  $\alpha$ -MSH reflect the hypothalamic sources (Thornton *et al.*, 1997), it can be stated that POMC neuron activation due to leptin and 5HT results in the increased levels of  $\alpha$ -MSH. In addition, *POMC* expression levels in olanzapine group were decreased 93%

which was statistically significant when compared with the vehicle group. The decrease of *POMC* expression in rats was consistent with a study performed by Davoodi *et al*, 2009. They showed a decrease in the *POMC* expression; however they failed to find any significant result of this reduction. The reason might be due to the short period of drug administration which was 7 days for that study and 28 days for our study. The reduction in *POMC* mRNA levels might be explained via the 5-HT<sub>2C</sub> receptor antagonism of olanzapine. Since POMC neurons have 5-HT<sub>2C</sub> receptor on their cell bodies (Heisler *et al*, 2002), the binding of 5-HT to its receptor on those neurons would increase the expression level of *POMC* from the neurons; however, olanzapine, as an antagonist, exerts its effects on the receptor negatively, thus the expression of *POMC* might be abolished. The reduction of *POMC* expression may also explain the olanzapine induced appetite (Kluge *et al*, 2007). The reduced rat *POMC* mRNA levels were correlated with rat  $\alpha$ -MSH plasma levels.

The peripheral concentration of CART in humans did not show any significant result between groups. However, Fatemi *et al*, 2006 showed up regulation of CART at least two fold change in the olanzapine treated Sprague-Dawley rats' frontal cortex. Even though it was not statistically significant, the result of Fatemi *et al*, 2006 is consistent with the hypothalamic expression data in our study. Statistically significant reduction in the plasma concentrations of CART were detected in rats treated with olanzapine when compared with the vehicle rats which may not seem to be consistent with the increased CART expression in the hypothalamic tissue. Nevertheless, different forms of CART (CARTI, CARTII) may have been detected in periphery (Thim *et al*, 1999) Also, we believe that circulating forms of CART might be majorly from the endocrine glands; such as pituitary and may be adrenal glands and hypothalamic CART expression may not be reflecting the circulating CART. Hypothalamic CART may not be reaching to the periphery. Although rat CART mRNA expression was not statistically significant in olanzapine administered group compared with vehicle, a trended

increase of 1.2 fold was detected after four weeks of olanzapine administration. This elevation of CART mRNA levels might be due to the leptin's stimulatory effect on CART neurons in the ARC. The leptin receptors on CART neurons (Elias et al, 1998) might have more effect on CART neurons than that of 5HT<sub>2C</sub>. As a result of this the leptin action of leptin on CART neurons, the CART gene expression might increase and may reach to statistical significance with longer terms of olanzapine administration.

## CHAPTER 4

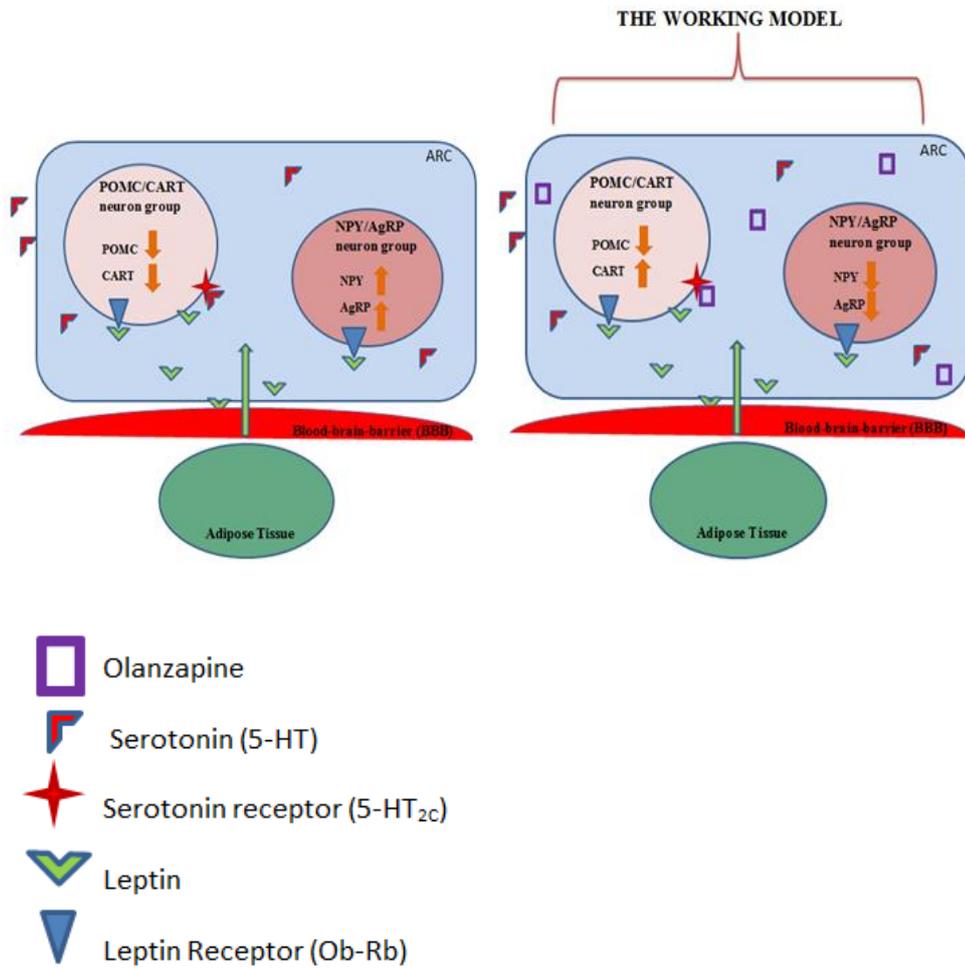
### CONCLUSION

- Even though it was impossible to detect the hyperphagic behavior of psychotic patients, they had implied increased appetite during the 4 weeks of hospitalization on olanzapine treatment. Additionally, 4 weeks of olanzapine treatment to healthy male rats resulted in increased food intake. Therefore, it can be suggested that, due to the increase in food intake, humans' body weight were significantly increased and rats exerted 33% increase in their body weight when compared with the vehicle. The increased leptin levels of humans and trended leptin levels in rats confirms the body weight gain and may indicate increase food intake and appetite.
- The reduced NPY levels in psychotic patients are considered to be the symptom of schizophrenia (Kuromitsu *et al*, 2001) and may lead to reduced appetite. However, the elevated NPY levels after the treatment may be the reason of increased appetite during treatment period which is consistent with increased body weight and increased leptin levels. The rat data, on the other hand, showed significant reduction in both mRNA levels and plasma concentrations. This might be due to either leptin's inhibitory effect on the NPY neurons or the increased levels of extracellular 5HT resulting in binding of serotonin to 5HT-1B receptors on the NPY/AgRP

neurons. However, further studies are needed to clarify the difference of NPY levels in humans and in rats.

- The decreased expression of AgRP mRNA in rats' hypothalamus was correlated with reduced AgRP plasma levels. Like NPY, the decreased concentration of AgRP might be related to the negative effect of leptin on the NPY/AgRP neurons. Also, it might be related to binding of serotonin on 5HT-1B receptors on the NPY/AgRP neurons.
- The reduction of *POMC* in rats with four weeks of olanzapine treatment might be associated with the antagonistic effect of olanzapine on 5HT<sub>2C</sub> receptors on the POMC neurons. Decreased plasma  $\alpha$ -MSH levels support the mRNA expressions since peripheral  $\alpha$ -MSH reflects hypothalamic  $\alpha$ -MSH levels (Thornton *et al.*, 1997). Human plasma levels of POMC were not similar with rat POMC plasma levels.
- Although it was not statistically significant the rat mRNA expression levels of *CART* there was an increasing trend which would be consequence of positive modulation of leptin on the POMC/CART neurons. Nevertheless, this phenomenon was not observed in the plasma since the hypothalamic CART may not be reflected on the plasma levels.
- In summary, this study revealed the weight gaining mechanism of olanzapine in humans and rats. In humans, increased weight gain, BMI and waist circumference were detected. Together with these results, circulating levels of the appetite regulatory neurohormones were analyzed. The orexigenic peptide NPY levels were increased and even though they were not statistically significant a trend in the elevation of CART and  $\alpha$ -MSH, the anorexigenic product of POMC was detected after the treatment of olanzapine for 4 weeks. In rats, increased food consumption and 33% weight gain difference in olanzapine administered group compared with

the vehicle were observed. Expression studies indicated that olanzapine exerts its effects on the hypothalamic neurons through serotonergic system on POMC neurons but not on NPY/AgRP neurons and leptin has more potent effect on NPY/AgRP neurons than that of olanzapine as summarized in Fig. 4.1.



**Figure 4. 1** The proposed interactions of leptin serotonin (5-HT) and olanzapine on the expressions of candidate genes involved in the appetite regulation.

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## APPENDIX A

### STANDARD CURVES OF ELISA

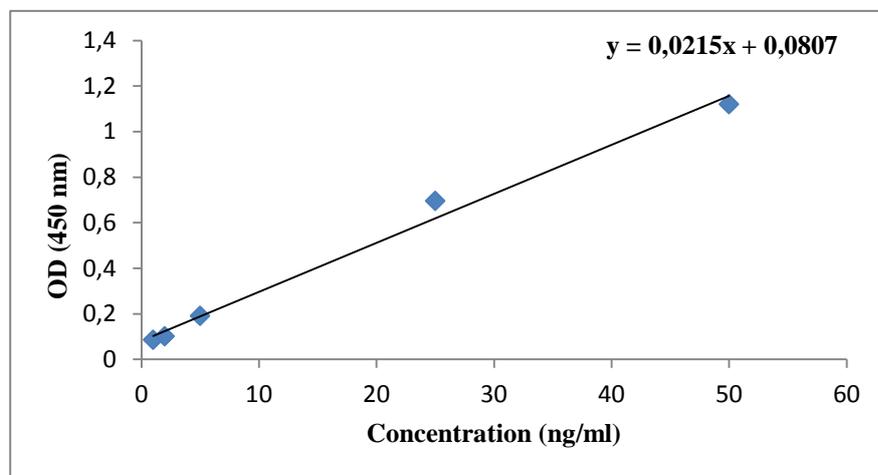
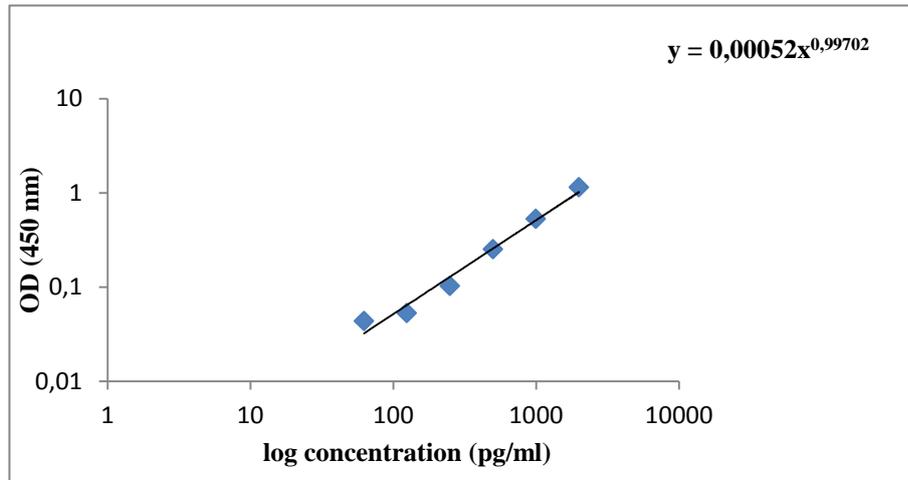
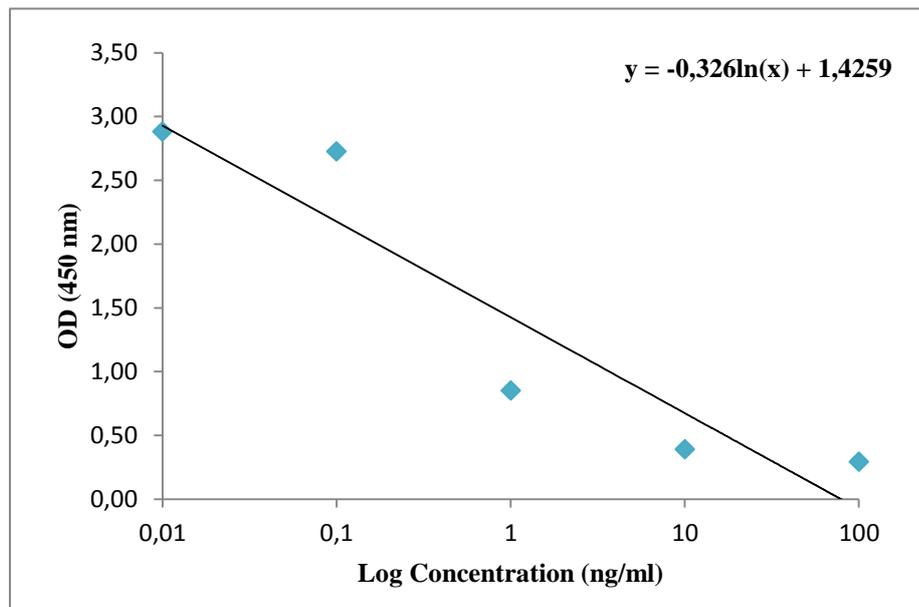


Figure A. 1 Human Leptin Standard Curve



**Figure A. 2** Rat Leptin Standard Curve



**Figure A. 3** Human NPY Standard Curve

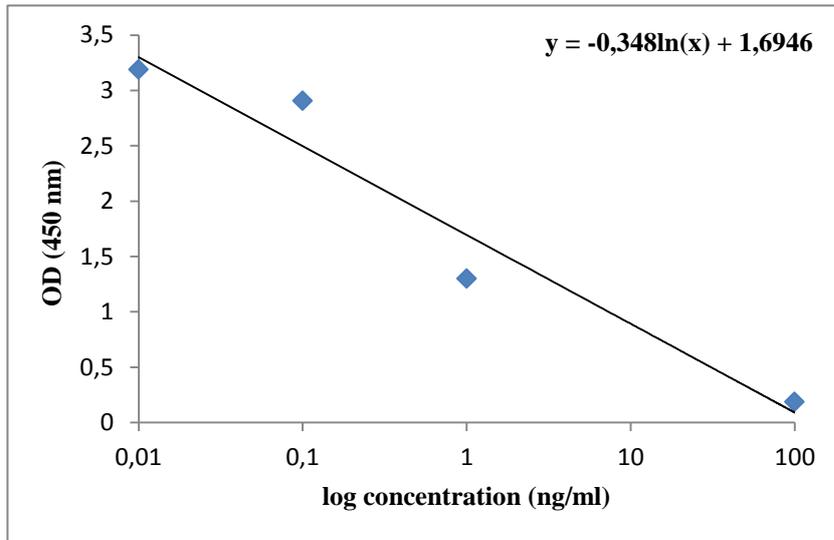


Figure A. 4 Rat NPY Standard Curve

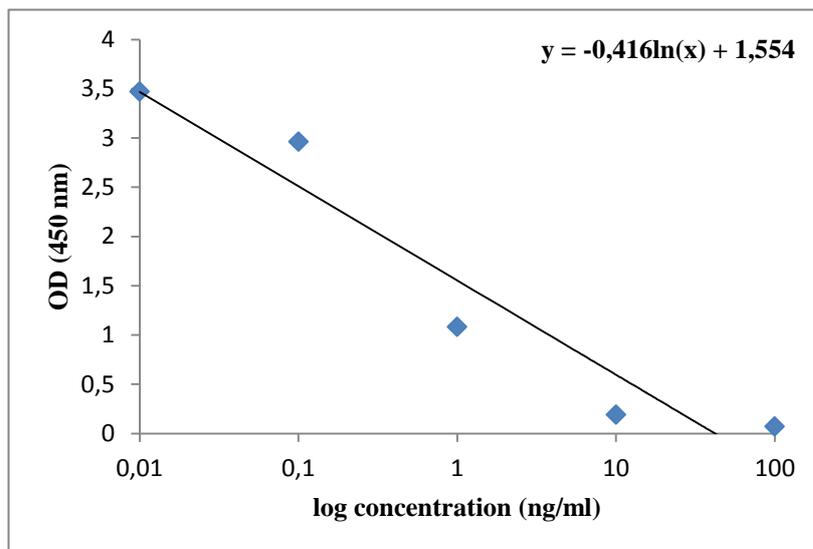
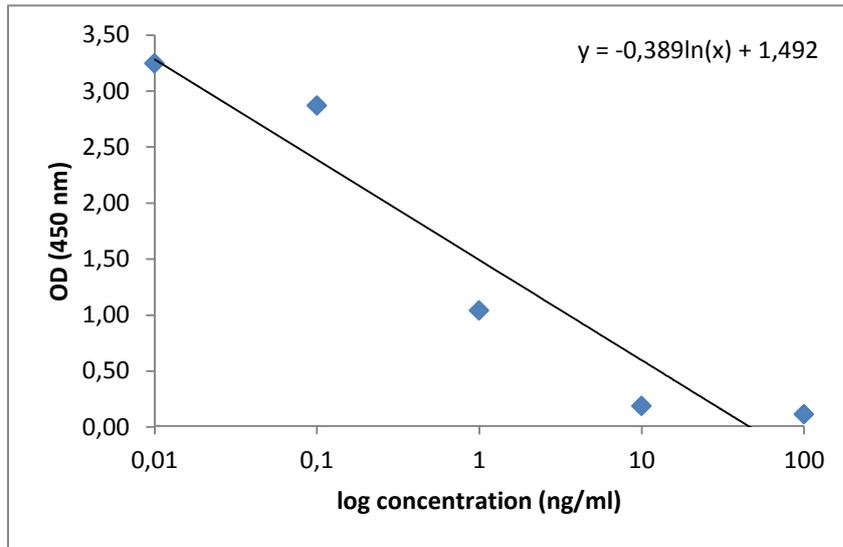
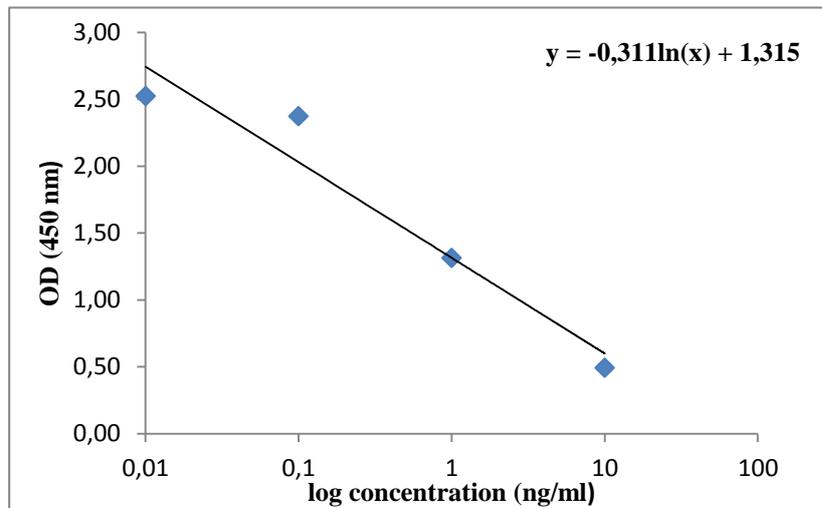


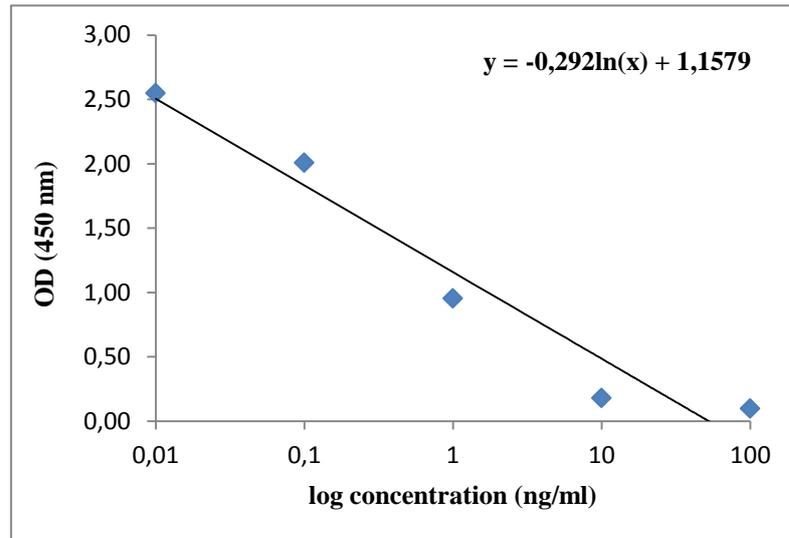
Figure A. 5 Human  $\alpha$ -MSH Standard Curve



**Figure A. 6** Rat  $\alpha$ -MSH Standard Curve



**Figure A. 7** Human CART Standard Curve



**Figure A. 8** Rat CART Standard Curve

## APPENDIX B

### VOLUME of RNA for cDNA SYNTHESIS

**Table B. 1** Volume of RNA used for cDNA synthesis. O: olanzapine rats; V: vehicle rats

Samples	Concentration of total RNA (ng/ $\mu$ l)	Volume of RNA used in cDNA synthesis ( $\mu$ l)
O-1	430,60	2.32
O-2	476,50	2.10
O-3	782,90	1.15
O-4	654,60	1.53
O-5	889,40	1.12
O-6	480,90	2.08
O-7	624,30	1.60
O-8	428,20	2.34
O-9	646,80	1.55
O-10	1.109,00	0.90
V-1	74,70	2.68
V-3	68,40	2.92
V-4	159,30	1.26
V-5	141,30	1.42
V-6	784,30	1.28
V-7	439,00	2.28
V-8	835,50	1.20
V-9	764,50	1.31
V-10	541,00	1.85