IDENTIFYING THE PUTATIVE G PROTEIN COUPLED RECEPTOR/S CANDIDATES OF COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT (CART) PEPTIDE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MERVE KASAP

IN PARTIAL FULLFILMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

FEBRUARY 2014

Approval of the thesis:

IDENTIFYING THE PUTATIVE G PROTEIN COUPLED RECEPTOR/S CANDIDATES OF COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT (CART) PEPTIDE

submitted by **MERVE KASAP** in partial fulfillment of the requirements for the degree of **Masters 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. Gülay Özcengiz
Head of Department, **Biological Sciences** _____

Assoc. Prof. Dr. Çağdaş D. Son
Supervisor, **Biological Sciences Department**, **METU** _____

Examining Committee Members:

Prof. Dr. Ufuk Gündüz
Biological Sciences Department, METU _____

Assoc. Prof. Dr. Çağdaş D. Son
Biological Sciences Department, METU _____

Assoc. Prof. Dr. Mesut Muyan
Biological Sciences Department, METU _____

Assoc. Prof. Dr. Tülin Yanık
Biological Sciences Department, METU _____

Assist. Prof. Dr. Bala Gür Dedeoğlu
Biotechnology Institute, Ankara University _____

Date:

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: Merve KASAP

Signature:

ABSTRACT

IDENTIFYING THE PUTATIVE G PROTEIN COUPLED RECEPTOR/S CANDIDATES OF COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT (CART) PEPTIDE

Kasap, Merve

M. Sc. Department of Biology
Supervisor, Assoc. Prof. Çağdaş D. SON

February, 2014. 80 pages

Cocaine and amphetamine regulated transcript (CART) peptides are neurotransmitters and endocrine factors in the nervous system and periphery. *CARTPT* codes for a neuropeptide hormone with a number of biological roles which are important for the mammalian physiology such as controlling feeding behavior, drug reward, bone remodeling, sensory processing, neuroendocrine function, stress anxiety, cardiovascular function, gastrointestinal motility and development. In hypothalamus, behavioral and drug studies showed that CART can suppress the feeding behavior. Also, due to having neuroprotective and survival promoting properties, and ability to initiate differentiation of neurons *in vitro,* CART peptides appear early in the development of the CNS and other tissues. It is suggested that they have a role in the development of specific regions of the brain, GI tract, pancreas and ovary. Although CART peptide plays very important roles and has important physiological functions, CART receptor/s has not been identified yet. There are two forms of the CART peptide: CART-I (55–102) and CART-II (61–102). These active forms cause an increase in c-Fos levels in a variety of neurons.

Because of the observed effects of CART peptide on differentiation and development, CART 55–102 effects are examined to alter the MAPK cascade and the phosphorylation state of ERK1 and 2. ERKs are known to be activated by a variety of growth factors, hormones and neurotransmitters. It is found that CART peptide stimulation strongly regulates ERK activity in pituitary-derived cell lines. The CART effect is blocked by inhibitors such as U0126, genistein and pertussis toxin, indicating the involvement of the upstream kinases, MEK1 and 2. Therefore, the involvement of a G_i/G_0 coupled GPCR in CART signaling is considered as possibility. G_i/G_0 is a member of α subunit of heterotrimeric G-protein that inhibits the production of cAMP from ATP. Also, previous electrophysiological studies suggested that the effects of CART may involve G-proteins, but concrete biochemical evidence for a G-protein-mediated pathway activated by CART has been lacking. The CART receptor is suspected to be a GPCR, possibly one that coupled to G_i/G_0.

Keywords:

CART peptide, receptor, ERK phosphorylation, AtT-20 cell line, N2a cell line

ÖZ

"KOKAİN VE AMFETAMİN İLE REGÜLE EDİLEN TRANSKRİPT" (CART) PEPTİDİNİ TANIDIĞI VARSAYILAN G-PROTEİNE KENETLİ RESEPTÖR ADAYLARININ TAYİNİ

Kasap, Merve.

Yüksek Lisans, Biyoloji Bölümü
Tez Yürütücüsü: Doç. Dr. Çağdaş D. SON

Subat 2014, 80 Sayfa

CART (**K**okain ve **A**mfetamin ile **R**egüle Edilen **T**ranskript) peptidi; yeme ve ödül mekanizmalarında, ilaç bağımlılığı, stres, kardiovasküler aktiviteler ve kemik yenilenmesinde görev alan önemli nörohormon/modülatörlerden biridir. Hipotalamustaki davranış ve ilaç çalışmaları CART peptidinin yeme alışkanlığını baskıladığını göstermiştir. Nöronlarda koruyucu etki göstermesi ve nöron başkalaşmasını sağlama özelliklerinden dolayı gelişme evresinin erken süreçlerinde merkezi sinir sisteminde önemli rol oynamaktadır. Ayrıca beyinin belli bazı bölgelerinde, hipofizde, adrenal bezlerde, pankreasta, yumurtalıklarda ve mide-bağırsak yolunda yüksek miktarlarda sentezlenmektedir. Bu önemli fizyolojik fonksiyonlara sahip olmasına rağmen CART reseptör ya da reseptörleri henüz belirlenememiştir. CART peptidinin; CART-I (55–102) ve CART-II (62–102) olmak üzere iki aktif formu bulunmaktadır. Bu aktif peptidler pek çok nöronda c-Fos seviyesini arttırarak sinyal yolaklarını indüklemektedir. Gelişme ve başkalaşma evrelerinde önemli rol oynamasından dolayı, MAPK yolağındaki yer alan ERK 1-2'nin fosforilasyonuna etkileri incelenmiştir. Hipofizden alınmış hücre

hatlarında CART; büyüme faktörlerini, hormonları ve nörotransmiterleri aktive etmekten sorumlu ERK (Hücre dışı sinyalle regüle edilen kinaz) aktivitesini uyarmaktadır. ERK1 ve 2'nin aktifleşmesini indükleyen CART'ın etkisi; boğmaca toksini, genistein ve U0126 gibi MEK (MAPK/ERK kinaz) inhibitörleri tarafından bloke edilmekte, ayrıca MEK1 ve 2 kinazları da bu oluşuma katılmaktadır. Böylece, fosfo-ERK (PERK) oluşumunun engellenmesiyle, CART sinyalinin G_i/G_o, G proteine kenetli reseptör alt biriminin, katılımıyla gerçekleştiği düşünülmektedir. G_i/G_o alt birimi ATP'den cAMP oluşumunu engellemektedir. Elektrokimyasal çalışmalar sonucunda CART etkisinin G proteine bağlanan reseptörler (GPCR) üzerinde gerçekleştiği düşünülse de, deneysel analizlerin yetersizliğinden reseptörler tam olarak tanımlanamamıştır. Bugüne kadar yapılan araştırmalarda CART reseptörünün özellikle G_i/G_o kenetli bir reseptör GPCR olduğu düşünülmektedir.

Anahtar Kelimeler: CART peptidi, reseptör, ERK fosforilasyonu, AtT-20 hücre hattı, N2a hücre hattı

To my dearest beautiful blue eyed friend-my beloved mother…

ACKNOWLEDGEMENTS

In a long night of this August, I try to write all over again once more. Unbelievably, I am writing for the end of a chapter of my life this time. There were a lot of people to thank… First and above all, I pay my sincere respect to my cheerful and patient advisor Assoc. Prof. *Çağdaş Devrim SON*. Whenever I knock his already welcoming door, I received infinite support. Little or huge from every kind, problems have just flown away with his guidance and supervision. I am honored to be his student.

I want to thank to Assoc. Prof. *Tülin YANIK* for her warm welcome. I had the chance to meet this project thanks to her.

When I found myself in the analysis part, actually the rough part of this study, I thought that I was in a nightmare because I had no idea what to do. Then, I saw two shining stars; Assist. Prof. *Bala Gür DEDEOĞLU,* and Assist. Prof. *Yeşim Aydın SON*. For more than three months, I was welcomed with my all kinds of questions. My greatest appreciation is obviously for them. Their contributions are irreplaceable for this study.

Then, I'd like to inform all the readers of this thesis that I was nothing without the help of my two teachers, my dear faithful friends *Gizem KURT* and *Aktan ALPSOY*. They were always there for me, when I got stuck with my q-results, western blot gels and whatsoever. I owe these two beautiful friends more than a simple "thank you". Wherever I go it doesn't matter, I will have two more cups of coffee for them in case they come.

Moreover, there is someone who deserves the gold medal for his endless company, my dear friend *Hikmet Taner TEKER*. He is the origin of philosophy of my thesis, translation of the naivety, and reminder of that there is still good in people. He makes me see "the

good” in every living on earth. I always appreciate what he’s done for me. He is the invisible hero of this thesis.

For the passing seven years, from waking me up in the mornings to dragging me to library when I feel that I cannot move anymore, I had her: *Seren ATILGAN*. I will always be the lucky one for having her by my side. She’s the only friend. She’s that *song* playing in the depths of my mind. I want to thank her for saving my soul and of course in a mediate manner for saving this thesis.

There are two guys also; I cannot forget to mention them here: my little brother *Şerif Mert KASAP* and my dear father *Abdullah KASAP*. I’d like to thank them for their fabulous support; especially, for the online lecture on Microsoft Office.

For now and forever, I’d like to thank to my dear friend: my beautiful mother. She is the creator of this thesis from many aspects. She always knew when my primers were coming, or where I was wrong. I hope I can make her proud by dedicating this study to her.

To conclude, as in the song: “The longer I run, the lesser I find”. This is the storyline of this study. How happy I would be, if I could contribute to unknowns’ world of science by adding it one more.

Merve Kasap

February, 2014.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Aa	Amino Acid
AgRP	Aguti-Related Peptide
ATP	Adenosine Tri Phosphate
AtT-20	Mouse pituitary epithelial-like tumor cell line
BLAST	Basic Local Alignment Search Tool
BRB ArrayTools	Biometric Research Branch Array Tools
Bp	Base pairs
cAMP	Cyclic adenosine mono phosphate
CART	Cocaine and Amphetamine Regulated Transcript
CATH	*M. musculus* pituitary derived cell line
cDNA	Complementary deoxyribonucleic acid
CEL	File that stores the results of the intensity calculations on the pixel values of the DAT file
CELSR1	Cadherin EGF LAG seven-pass G-type receptor 1
cGMP	Cyclic guanosine mono phosphate
CG	Chorionic gonadotrophin
CH3	Lymphatic endothelial cell line 3
CIRL1	Ca^{2+} -Independent Receptor of α-Latrotoxin 1
CNS	Central Nervous System
CRD	Cysteine rich domain
CRF	Corticotropin-releasing factor
CRF_1R	Corticotropin-releasing factor 1 receptor
CRLR	Calcitonin-receptor-like receptor
Cy3	Cyanine dye 3

Cy5	Cyanine dye 5
C5a	Complement factor 5a
DKK1	Dickkopf 1
DNA	Deoxyribonucleic acid
DMD	Digital micromirror device
DMEM	Dulbecco's Modified Eagle Medium
ECL	Extracellular loop
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-7TM	Epidermal growth factor 7-trans membrane
ERK	Extracellular-signal Regulated Kinase
FBS	Fetal Bovine Serum
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FSH	Follicle Stimulating Hormone
FR7	Antagonized protein of Dipkoff 1
F-12K	Kaighn's Modification of Ham's Medium
GABA	γ-amino-butyric acid
GAPs	GTPase-activating-proteins
GDP	Guanosine di-phosphate
GDS	Graphic Data System File
GEO	Gene Expression Omnibus
GEO2R	GEO dataset analysis tool
GnRH	Gonadotropin-releasing hormone
Ghsr	Growth hormone secretagogue receptor
GI	Gastrointestinal
G_i/G_0	G_i alpha subunit
GLP1	Glucagon-like peptide-1

GPL	GEO Platform of microarray
GPR	G protein receptor
GPR file	GenePix Results File
GPCR	G protein coupled receptor
GSE	GEO Series Number
GSE file	GeneSys Export File
HBD	Hormone binding domain
HEK293	Human Embryonic Kidney 293 cells
ICL	Intracellular loop
JNK	c-Jun N-terminal kinase
LH	Luteinizing hormone
LRR	Leucine-rich repeat
LGR	leucine-rich repeat containing receptors
LN-7TM	Long N-termini seven transmembrane
LRP5	Dickkopf 1's accessory protein
MAPK	Mitogen-activated protein kinase
Mc3r	Melanocortin 3 receptor
Mc4r	Melanocortin 4 receptor
MEK	A dual-specificity protein kinase of the STE7 kinase family
MIAME	Minimum Information About a Microarray Experiment
MAS	Maskless Array Synthesizer
Nac	Nucleic Accumbens
NCBI	National Center for Biotechnology Information
NPY	Neuropeptide Y
N2a	Neuro 2a
NTC	No Template Control

NTD	N Terminal Domain
oGPCR	Orphan G protein coupled receptor
PAC1	Pituitary adenylate cyclase-activating peptide ligand/type 1 receptor
PACAP	Pituitary adenylate cyclase-activating peptide
PC12	Pheochromocytoma 12
PERK	Phosphorilated Extracellular Regulated Kinase
Pen/Strep	Penicillin/Streptomycin
PI3Ks	Phosphoinositide 3-kinase
POMC	Pro-opiomelanocortin
PTH	Parathyroid hormone
PTX	Pertussis Toxin
q-RT-PCR	Quantitative Real Time Polymerase Chain Reaction
RANKL	Receptor Activator of Nuclear factor Kappa-B Ligand
rlCART	Rat-long-CART
TMH	Transmembrane Helices
T1R	Taste 1 Receptor
T2R	Taste 2 Receptor
TRF	Thyrotropin-Releasing Factor
TSH	Thyroid Stimulating Hormone
U0126	A selective inhibitor of both MEK1 and MEK2
WEBGESTALT	WEB-based GEne SeT AnaLysis Toolkit
VTA	Ventral Tegmental Area
V1R	Thyrotropin-releasing factor
Y_1r	Neuropeptide 1 receptor
7TM	Seven transmembranes

CHAPTER 1

INTRODUCTION

1.1.Cocaine and Amphetamine Regulated Transcript

During the addiction studies, a group in Oregon found out that cocaine administration caused an increase in an mRNA level in animals (Douglass *et al*, 1995). Product of this mRNA is called “Cocaine and Amphetamine Regulated Transcript (CART)”. When injected to animals, it produced similar effects like cocaine and amphetamine. However, when they co-administered together, CART blocked the effect of cocaine and amphetamine.

Research showed that CART is abundantly found in throughout the body, not just in brain. Therefore, it is thought to be involved in drug addiction, endocrine control, feeding, stress, and many other functions. When the findings of that hypothalamic CART causes anorexigenic effect revealed in 1998, CART peptide became a hot topic in research.

Further studies showed that CART is an important neuropeptide playing key roles in a variety of biological mechanisms such as the control of feeding behavior, drug reward, bone remodeling, sensory processing, and modulation of stress, anxiety, cardiovascular function, gastrointestinal motility and development.

Although it is an important neuropeptide, its receptor has not been identified yet.

1.1.1. *CARTPT* Gene

In human, CART is encoded by *CARTPT*. CARTPT is localized on a segment of genomic DNA which is approximately two kb on

chromosome 5. Transcript is 900-nucleotide-long and has 3 distinctive exons (Kuhar, *et al*., 2002).

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
	5' upstream sequence						.....cctggaacccggcgggcattgacgtcaagcgccggcggagcgctgcctac
1	ENSE00001306358	71,014,990	71,015,279	-	0	290	AGACGGTTGACCCGGGCCCTCCTCCACACCCCCTTCCTTCTTCGCCTCCTCCCTCTTTCC TGCACGGGGGCTCGGGCTCACTATAAAAGGTGGGAGCGCGTGGTGCCCCAGCAACGACGA GTTTCAGAACGATGGAGAGCTCCCGCGTGAGGCTGCTGCCCCTCCTGGGCGCCGCCCTGC TGCTGATGCTACCTCTGTTGGGTACCCGTGCCCAGGAGGACGCCGAGCTCCAGCCCCGAG CCCTGGACATCTACTCTGCCGTGGATGATGCCTCCCACGAGAAGGAGCTG
	Intron 1-2	71,015,280	71,015,706			427	gtcggtattcccctcgctctcgacc.....caggctccgaagcggtgtgttgcag
2	ENSE00001083530	71,015,707	71,015,790	0	0	84	ATCGAAGCGCTGCAAGAAGTCTTGAAGAAGCTCAAGAGTAAACGTGTTCCCATCTATGAG AAGAAGTATGGCCAAGTCCCCATG
	Intron 2-3	71,015,791	71,016,334			544	gtaaggtttgtggtcactcccttcc.....atgaccacacattttgttgtttcag
3	ENSE00003474230	71,016,335	71,016,875	0	-	541	TGTGACGCCGGTGAGCAGTGTGCAGTGAGGAAAGGGGCAAGGATCGGGAAGCTGTGTGAC TGTCCCCGAGGAACCTCCTGCAATTCCTTCCTCCTGAAGTGCTTATGAAGGGGCGTCCAT TCTCCTCCATACATCCCCATCCCTCTACTTTCCCCAGAGGACCACACCTTCCTCCCTGGA GTTTGGCTTAAGCAACAGATAAAGTTTTTATTTTCCTCTGAAGGGAAAGGGCTCTTTTCC TGCTGTTTCAAAAATAAAAGAACACATTAGATGTTACTGTGTGAAGAATAATGCCTTGTA TGGTGTTGATACGTGTGTGAAGTATTCTTATTTTATTTGTCTGACAAACTCTTGTGTACC TTTGTGTAAAGAAGGGAAGCTTTGTTTGAAAATTGTATTTTTGTATGTGGCATGGCAGAA TGAAAATTAGATCTAGCTAATCTCGGTAGATGTCATTACAACCTGGAAAATAAATCACCC TAAGTGACACAAATTGAAGCATGTACAAATTATACATAATAAAGTGTTTTTAATAATTGC C
	3' downstream sequence						catagtgcactgctgttttcatataagtaatttaagtggaaatggtgaga.....

Figure 1 Ensembl exon-intron view of human *CARTPT*

Similar to mouse (m) and rat (r), in human there are two alternatively spliced variants of *CARTPT*: 129 aa long (l) peptide, and 116 aa short (s) form. On exon 2, rlCART is spliced by missing 39 nucleotides resulting to 116 aa peptide. This is found in human mouse and rat species. However, 129 aa peptide found in just mouse and rat (Kuhar, *et al*., 2002).

1.1.2. CART Peptide Structure

Post-translational process gives rise to two biologically active peptides: CART 55-102, CART 61-102, **CART I** and **CART II** respectively.

```
         10         20         30         40         50         60
MESSRVRLLP LLGAALLLML PLLGTRAQED AELQPRALDI YSAVDDASHE KELIEALQEV

         70         80         90        100        110
LKKLKSKRVP IYEKKYGQVP MCDAGEQCAV RKGARIGKLC DCPRGTSCNS FLLKCL
```

Figure 2 Amino acid sequence of CART from UniProtKB, ID number: Q16568 (Last modified on November 1, 1996. Version 1)

CART is conserved within species. Coding region shows 98% identity among rat and mouse. This conservation suggests that tertiary structure of the peptide may be conserved and stabilized by disulfide bonds (Thim *et al.*, 2001).

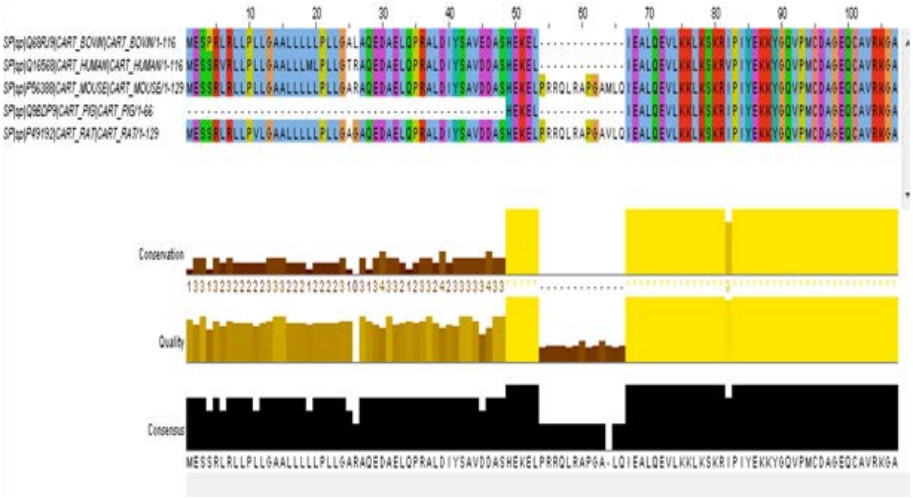


Figure 3 Human CART shows a 95% homology with rat and mouse

Crystallization studies show that CART has two antiparallel β sheets and three disulfide bonds in **Figure 4**.

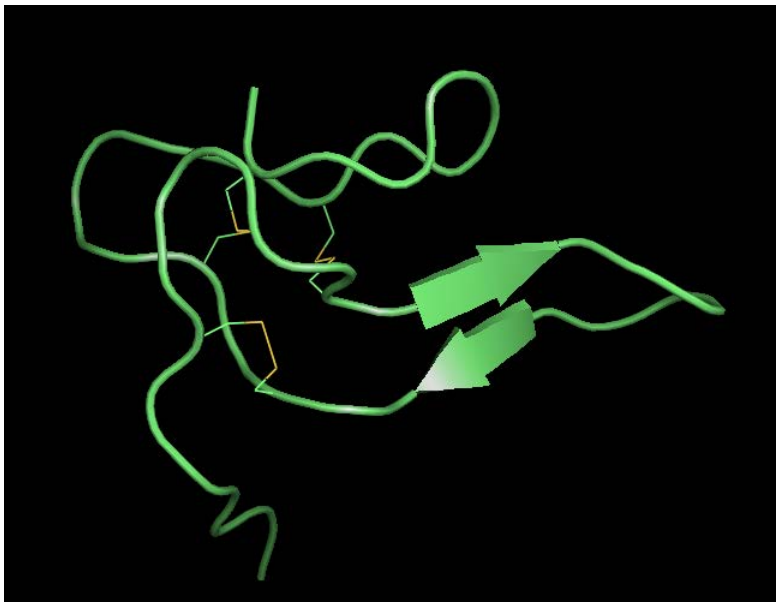


Figure 4 3D structure of CART peptide from PyMOL v1.6. Arrows show antiparallel β sheets, and yellow lines represent disulfide bonds.

1.1.3. Tissue Distribution of the Peptide

CART is synthesized in brain, mesolimbic dopamine system (VTA and Nac), several nuclei of hypothalamus, pituitary, hindbrain, several islet types, sympathetic preganglionic neurons and adrenal glands and myentric neurons in gastrointestinal tract.

1.1.4. Biogenic Actions of the CART Peptide

1.1.4.1. In Energy Homeostasis

In arcuate nucleus there are two neuronal cells, AgRP/NPY and POMC/CART neurons. AgRP is agouti relating protein, and NPY is neuropeptide Y. These are neuropeptides stimulating the food intake while decreasing the energy expenditure. On the other hand, proopiomelanocortin, POMC, and CART are neuropeptides inhibiting food intake and increasing the energy consumption. Insulin and leptin inhibit AgRP/NPY neurons while stimulating the POMC/CART neurons. Likewise, lower insulin and leptin cause to activation of AgRP/NPY. There is ghrelin, a circulating peptide secreted from the

stomach, also activates Agrp/Npy neurons. Therefore, ghrelin plays a role as stimulant of food intake (**Figure 5**). It is seen that plasma ghrelin levels are increased before a meal and decreased after a meal (Cummings *et al.*, 2001).

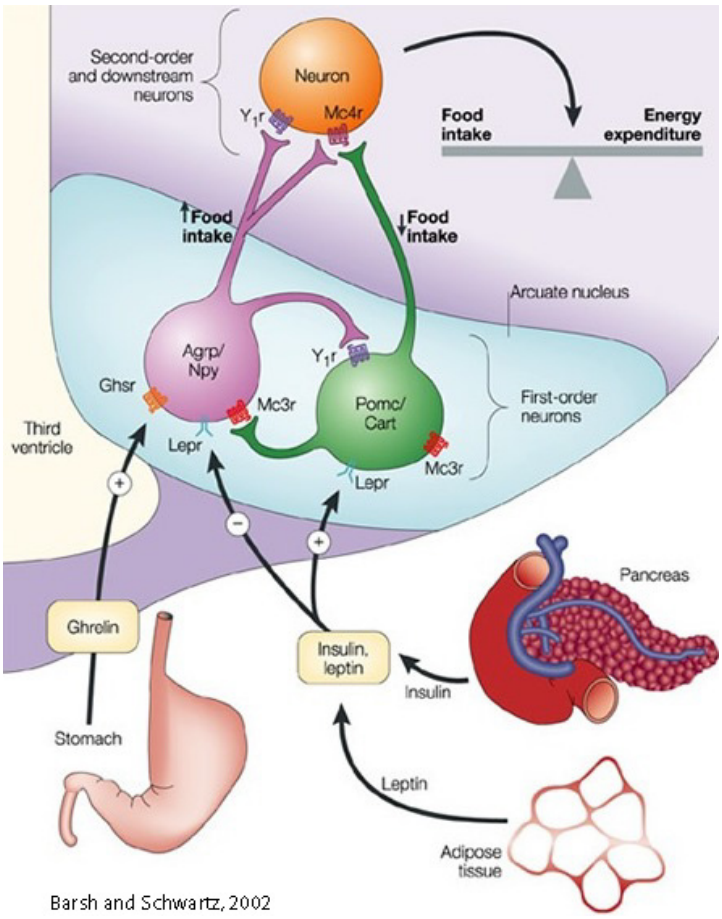


Figure 5 Energy Homeostasis (Here, Ghsr is growth hormone secretagogue receptor; Lepr is leptin receptor; Mc3r/Mc4r is melanocortin 3/4 receptor; and Y_1r is neuropeptide Y_1 receptor.) (Cummings *et al*, 2001).

1.1.4.2. In Bone Remodeling

Bone remodeling is the process that the skeleton adaptation happens through time. Within the adulthood, bone forming osteoblasts and bone-resorbing osteoclasts interact with each other to keep bone mass stable. Regulations of these cells are quite complex. Current studies

showed that it is regulated via neuronal pathways. Leptin, for example, signals to osteoblast receptors in sympathetic pathway directly driven by hypothalamus. Since leptin is related with energy homeostasis pathways, it is found out that CART and serotonin interact with the brainstem and hypothalamus to regulate bone formation resorption in cancellous bone (Driessler & Baldock, 2010). Likewise, NPY acts via hypothalamic Y_2r to modulate the skeleton-wide osteoblast activity by coordinating body weight and bone mass.

Since CART is broadly expressed in the hypothalamus and the peripheral organs such as the pancreas and adrenal glands, and CART knockout mice shows decreased levels of CART while shows increased resorption, it is implicated that, CART may be a potential regulator of bone resorption. Consistently, these animals are also osteoporotic due to an increase in bone resorption (Elefteriou, et al., 2005). Moreover, CART deficient mice express higher levels of RANKL (Receptor activator of nuclear factor kappa-B ligand, a member of tumor necrosis factor) in bone than wild-type mice. The effect of CART on bone is not cell autonomous, suggesting that there is a local mechanism for the central CART changes (Driessler & Baldock, 2010).

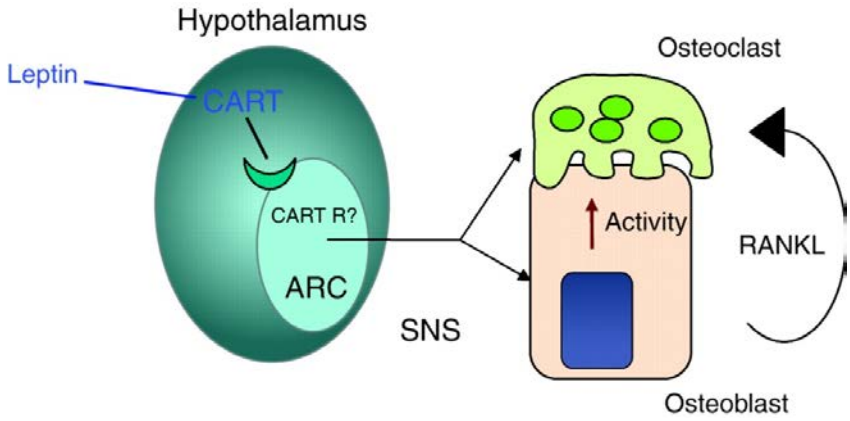


Figure 6 Bone remodeling via CART signaling regulation. CART expression can change according to serum leptin. When expression is

low, an increase in bone resorption is induced via higher levels of RANKL, whereas increased expression of CART creates a higher bone-mass (Driessler & Baldock, 2010).

1.1.4.3. In Stress Response

Any factor causing to a threat to organism disturbing the homeostasis is called stressor, and the resulting responses counteract this threat to regain the homeostasis is called stress response. Stress response composes neuronal and neuroendocrinal routes. Hypothalamo-pituitary pathway has an important role leading these routes (Pacak & Palkovits, 2001) . HPA axis has the major role in stress response; however, hypothalamo-pituitary control contributes it significantly. In this extent, CART's remarkable expression in HPA axis suggests a role for CART peptide in stress response mediation (Pogun *et al*, 2006).

There is a strong relation between stress response, eating disorders and the psycostimulant actions. Stress is one of the major factors in drug abuse also. According to a study, food shock stress redeemed cocaine seeking behavior in male rats; however, this effect was not observed in adrenalectomized animals (Piazza & Le Moal, 1998). Stress-drug abuse relation is due to increased sensitivity of glucocorticoid-exposed dopaminergic neurons (Piazza & Le Moal, 1998).

In drug addicts, HPA axis more active than in normal subjects, while there is a directly proportional relation between HAP axis activation and CRF levels in addiction (Goeders, 2003).

CART gene activation, thus CART peptide synthesis and secretion in hypothalamus and pituitary are regulated by glucocorticoids. Blood CART levels may be affected by the interaction between the HPA axis activity and glucocorticoids (Pogun *et al*, 2006).

Due to unknown molecular pathways of CART mechanism, these cannot be understood clearly although evidences show the relations.

1.1.5. CART Induced Signaling

In fact, little is known about the pathway of CART interacting with its receptor(s), or how it initiates a downstream cascade (Zhang, Han & Xu, 2012).

It is known that CART I and II cause an increase in c-Fos levels in many neurons. Due to the observations of CART peptide effects on differentiation and development, CART 55–102 is tested whether it causes any changes in the MAPK cascade and the phosphorylation of ERK 1 and 2 proteins (Extracellular signal Regulated Kinases 1 and 2). ERKs are known to be activated by a variety of growth factors, hormones and neurotransmitters. Then, it is found out that CART peptide stimulation strongly regulates ERK activity in pituitary-derived *in vitro* cell lines. The CART effect can be blocked by inhibitors such as U0126 (artificial, highly selective MAPK/ERK inhibitor), genistein and pertussis toxin (PTX), indicating the involvement of the upstream kinases, MEK1 and 2. Therefore, the involvement of a G_i/G_o coupling via GPCR in CART signaling is considered as possibility by Kuhar team (2005).

Previous electrophysiological studies suggested that CART effect may involve G-proteins. However, solid evidences are still missing for a G-protein-mediated pathway. It is strongly suggested that CART receptor or receptors should be a GPCR specifically the one coupled to G_i/G_o. As a GPCR using G_i/G_o works, **CART I** also inhibits voltage-dependent intracellular calcium signaling (Kuhar *et al*, 2005).

In order to find ERK related pathway relation, many cell types examined whether **CART I** application cause a response as giving increased levels of phosphorylated ERKs (P-ERKs). HEK293, PC12, N2a and CATH.a cell lines were used for this purpose. Only pituitary-derived ones AtT20 and GH3 gave significant responses (Dominguez,

Lakatos, & Kuhar, 2002), (Kuhar *et al.*, 2005). PERK levels are tested by western blot over time and results showed that PERK levels changed significantly over time (Kuhar *et al.*, 2005).

1.1.6. CART Receptor(s)

Both by inhibiting the voltage-dependent intracellular Ca^{++} signaling and activating ERK1 and 2 specifically in AtT-20 cell line, CART I shows evidences of having a GPCR. CART II also has the same specific binding in AtT-20 cell line. Studies also showed that CART I and II binding are saturable and have a high affinity for a single site in the same cell line (Jones, Lakatos, & Vicentic, 2006). Binding of the CART peptides also depends on temperature, pH, protein concentration, and time. These data shows that CART has a specific receptor binding in AtT-20 cell line.

With different relative potencies for CART I and II are seen in food consumption (Thim *et al*, 1998; Douglas *et al*, 2001) increased plus maze activity (Okuyama *et al*, 2003), hot plate antinociception, acoustic startle response, and prepulse inhibition (Douglas et al, 2001). Since potencies for CART peptides differ in magnitude and direction, it is possible that CART peptides induce multiple signal transduction mechanisms. Moreover, this may be due to multiple receptor subtypes (Kuhar *et al*, 2005).

Since evidences indicate the involvement of a G_i/G_o coupling, CART signaling might be induced via a G protein coupled receptor. Receptor or receptors of the CART peptides might be one of the orphan GPCRs.

Orphan GPCRs (*o*GPCRs) are the GPCR class that is revealed after genome project is completed. Which endogenous ligand belongs to these orphan GPCRs is not known. However, apart from their molecular structure; there is no information related with their physiological roles and what roles they might have in disease state

(Tang *et al.*, 2012). Most of these *o*GPCRs are seen to be expressed in nervous tissues.

*o*GPCRs can be from one of seven GPCR classes: A, B, large N-terminal family B-7 TMH, C, Frizzled/Smoothened, taste 2, and vomeronasal 1 receptors (Kristiansen, 2004). Distribution of these *o*GPCR classes are shown in figure.

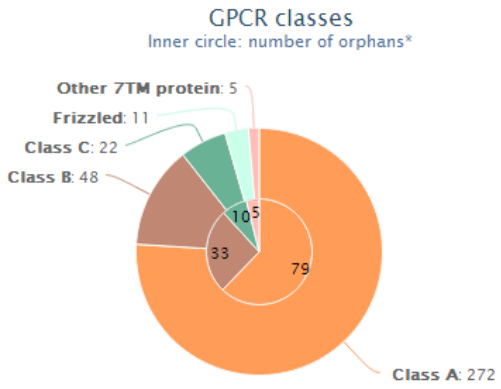


Figure 7 Pie chart of gene-product representation for oGPCRs. Outer Circle constitutes genes of related GPCRs, and the inner circle represents receptor numbers of *o*GPCRs.

It is possible that receptor(s) of CART peptides is one these orphan G protein coupled receptors based on the assumption that CART peptide induces a GPCR signaling pathway. When examined in detail by means of different subclass types:

- A Class GPCRs comprise the largest group of GPCRS. Ligands of A Class GPCRs differ in structure and character; however, amino acid sequences of receptors are very similar and share a common structure constituting 7TM helices. Peptide receptors of class A GPCRs compose many important physiological functions that are performed by acting as neurotransmitters, hormones, and paracrines. Ligands of peptide receptors of A Class GPCRs are angiotensin II, arginine vasopressin/oxytocin, bradykinin, cholecystokinin/ gastrin,

chemokines, complement factor 5a (C5a), fMLP, gonadotropin releasing hormone (GnRH), neurotensin, NPY, opioids, somatostatin, and TSH (Gether, 2000). When compared to these ligands of A Class GPCRs, the possibility of CART's receptor belonging to A Class GPCRs is relatively weak due to being a smaller peptide.

- B Class GPCRs, also known as secretin-like family receptors, activate adenylyl cyclase and IP3-Ca^{++} signaling pathway. During the signal transduction, ERK1&2 might be stimulated via induction of a B Class GPCR. It may point out that CART might have receptor from B Class GPCRs. Also, B class GPCRs may act by splitting the corticotropin-releasing factor (CRF) (Sakmar, 2011). CRF should include both a C-terminal region binding the N terminal domain (NTD) of receptor with high affinity, and N-terminal region activating the receptor by interacting helical bundles with high potency (Sakmar, 2011). For example, adenylyl cyclase activating poly peptide (PACAP) receptor (PAC1), glucagon, and GLP1 receptors cannot account alone for the binding affinity of their ligands. According to a recent study, PACAP 6-38 is defined as an antagonist of CART I (Kuhar *et al*, 2011). This finding increases the probability of CART's receptor belonging to B Class receptors.

- Having similarities to B class GPCRs, Large N-terminal Family B-7 Transmembrane Helix (LNB-7TM) Class GPCRs have large N-termini playing roles in cell-to-cell adhesion and cell migration via attachment to the extracellular matrix components or interaction of other cells' membrane proteins (Stacey *et. al*, 2000). It seems that interactions among these domains from different receptors can be resulted in homomultimerization or heteromultimerization of same cells' such other receptors. These receptors are related with the biological roles such as the controlling of brain angiogenesis, synaptic exocytosis, immune

system regulations, cell polarity and synaptogenesis (Foord *et al.*, 2002). Plenty of orphan GPCRs belong to this family such as BAI1 – 3 (Brain-specific Angiogenesis Inhibitor), CIRL1 – 3 (Ca^{2+} -Independent Receptor of α-Latrotoxin), GPR113, GPR124, and GPR125. Only one GPCR is demonstrated to have an intracellular ligand (Hamman *et al*, 1996), others remain to be undefined. Therefore, only link of this subclass with CART peptide is the physiological relations. That is, CART is known to be inducing neuronal differentiation in early development (Sundler *et al*, 2004). It is a probability that CART can induce this effect via one of this LNB-7TM Class GPCRs.

- C Class GPCRs have large N termini by having 11 subunits. Their well-known endogenous ligand-bound receptors are metabotropic glutamate (mGlu1–8) receptors, receptors of major excitatory neurotransmitter in CNS, and the γ -aminobutyric acid (GABA) and the Ca^{2+} sensing receptor (Kristiansen, 2004). Ca^{++} sensing receptor is highly related with neuroendocrine pathways. They may work by cooperation with other GPCRs. For example, the mGluR2 came into prominence as a potential drug target and it cooperates with 5-HT2A serotonin receptors, which is an A Class GPCR (Bruno *et. al*, 2012). Since CART is also active in both brain and CNS, and blocks Ca^{++} signaling by activating ERK1 and 2, it is probable that CART have a receptor from C Class GPCRs.

- F/S Class GPCRs and Vomeronasal -Taste Receptors are slightly small when compared to A Class GPCRs. Their ligands are called “Wnts”. Wnts are the products of more than 16 vertebrate genes, nearly 350-amino-acid long proteins (Kristiansen, 2004). Frizzled receptors do not often signal via G-proteins and only some of the members show G-protein signaling. On the contrary, Smoothened receptors may activate G-proteins. However, unlike other GPCRs this activation is independent of ligand binding. Although it is suggested that different Wnts can

activate the same Frizzled receptor, it is seen that they activate different pathways: ‘agonist trafficking’ (Holbrook *et. al*, 2002). Frizzled receptor responses may depend on accessory proteins. Their smaller sizes make them probable targets for CART peptide. Also, like the other examples of this class CART peptides may induce a pathway via an accessory protein.

In the light of this information, orphan GPCRs can be used to search for the correct match of CART peptide receptor.

By reaching a comprehensive study of orphan GPCRs and their related products published in 2011, it became possible to find full repertoire orphan GPCRs - nearly 360 - expressed among the most commonly used cell lines: HEK293, AtT-20, BV2 and N18 (Atwood *et al*, 2011).

BV2 cells have 108, N18 cells 105, AtT-20 cells 79 and HEK293 cells 73 *o*GPCRs expressed. From the previous studies it’s known that CART peptide has a specific binding in the cell lines of AtT-20, GH3 and differentiated PC12.

In this study of microarrays carried out by Atwood *et al*, A Class *o*GPCRs are found in the greatest abundance among four cell lines. As most widely cited cell line, HEK293 has numerous identified *o*GPCRs. AtT-20 cell line is known having the least expression of GPCRs and GPCR-related gene product among these four cell lines. Significant expression of nearly 45 GPCR related gene products are found in AtT-20 cell line, whereas other cell lines have 72 or more related gene products. N18 cells have numerous GPCRs, GPCR-related proteins and having the highest GPCR expression among these four cell lines. On the other hand, BV2 cells which are commonly used in microglial studies have predominantly A Class GPCRs and related proteins. AtT20 cells have wide range of A Class *o*GPCRs, large number of BAI (Brain Angiogenesis Inhibitor) as B Class *o*GPCRs, six frizzled receptors but no smoothened receptors for

Frizzled/Smoothened Class GPCRs. Therefore, revelation of this study makes enable to study CART's receptor by using these CART responsive and non-responsive cell lines.

1.2.G Protein Coupled Receptors

G protein coupled receptors, GPCRs, also known as seven transmembrane domain receptors, serpentine-like receptors, or heptahelical receptors, are the largest family of membrane receptors. Although these other names are used, GPCR term is preferred today because of the presence of other heptahelical proteins such as bacteriorhodopsin that is unrelated with GPCRs in function and also evolution (Pierce *et al*., 2002). GPCRs are found in eukaryotic cells like cyanoflagellates, yeast and higher eukaryote species. GPCRs are found in the interface of a cell's external and internal environments. A specific ligand, that is binding and activating the receptors, may range from an ion to nucleoside, amine, peptide, protein, and lipid or, for optical receptors to light. These ligands cause a conformational change in receptor, leading to an active state. This triggers G-proteins inside the cell to release the components that activate cellular mechanisms (Filmore, 2004). There is an alternative model suggested called "three-state model" (Bouvier, 2013). The receptor is found in intermediate state at resting while showing some activity. When the ligand binds to receptor, it became more stable and the active form showing higher activity. In **Figure 8**, conformation ensembles are shown for this model.

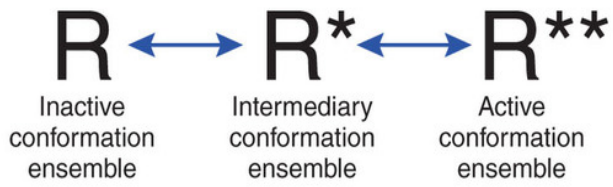


Figure 8 Three-state model (Bouvier, 2013)

The intracellular signaling molecules triggered by GPCRs are arachidonic acid, cytosolic ions, cAMP, cGMP and inositol phosphates. Sometimes, receptors other than GPCRs can activate G-proteins. It is implicated that signal transduction on tyrosine kinase receptors such as epidermal growth factor, insulin, insulin-like growth factor I and II and colony-stimulating factor-1 can also activate G-proteins not related with GPCRs (Kuemmerle & Murthy, 2001).

The superfamily of GPCRs is the receptor of many neurotransmitters, neuromodulators, hormones, and paracrines (local hormones) with important physiological functions. They are active in just about every organ and system. Their dysfunction cause many diseases including central nervous system disorders, cancer, cardiac dysfunction, diabetes, inflammation, obesity, and pain. Therefore, approximately 40% of the drugs and pharmaceuticals of modern medicine target GPCRs (Filmore, 2004).

1.2.1. Classification

Among mammalian species, which is the focus of this thesis, there are seven sub-classes of GPCRs: A, B, large N-terminal family B-7 TMH, C, Frizzled/Smoothened, taste 2, and vomeronasal 1 receptors (Kristiansen, 2004). Members of this superfamily classified according to their native ligands, phylogenetic analysis of their amino acid sequences, analysis of clustering of genes in the human genome, and analysis of globular domains and motifs in the N terminus of large N-terminal family B-7TM receptors. Previously, there were 6 different families of GPCRs (Kolakowski, 1994). Each member of this superfamily has more than 20% amino acid sequence identity by transmembrane helices. Only A, B and C class of GPCRs are represented among multicellular animals (Kolakowski, 1994). Since then, other mammalian GPCR families are identified such as Frizzled/Smoothened (F/S), vomeronasal 1 receptor (V1R), and taste 2 receptor T2R) (**Figure 9**). In addition, with the human genome project

it is found out that there are still many *orphan GPCRs (oGPCRs).* These are the GPCRs having no defined ligands yet.

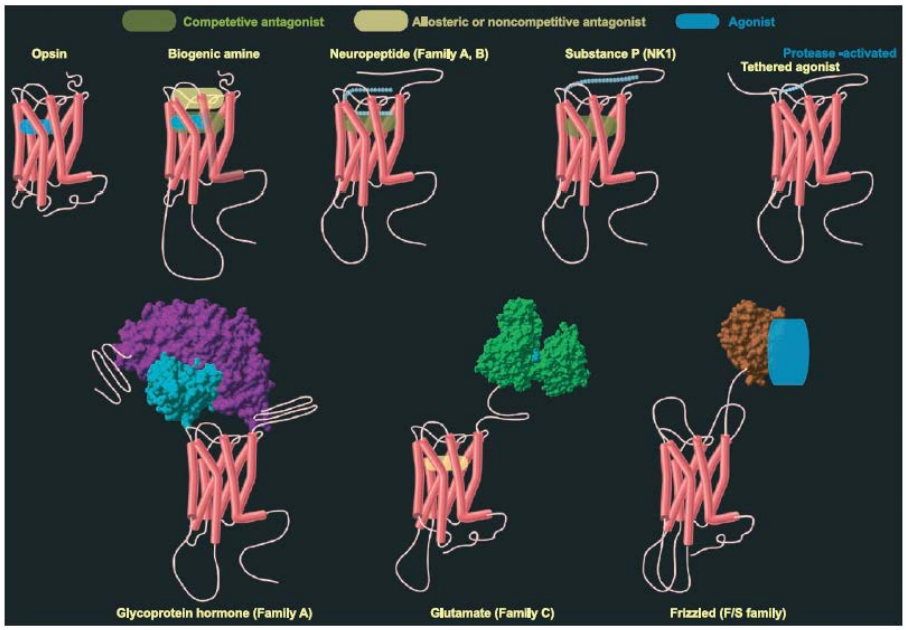


Figure 9 Examples to different classes of GPCRs and their ligands (Kristiansen, 2004)

1.2.1.1. Class A GPCRs

Among others, class A GPCRs are the largest and most studied GPCRs. Family A receptors include opsins, some odorant receptors, small endogenous antagonists, peptide receptors, leucine-rich motif including receptors, and protease activating receptors (Kristiansen, 2004).

In order to study Class A receptors, “position identifiers” are used. Position identifiers are representing the positions of residues to a reference amino acid appearing in the TMH. The most conserved amino acid residue in the THM is taken as a reference residue.

When compared with other Class A receptors, it will be seen that opsins are activated by photons. Therefore, they have a chromophore attachment so that they can response to absorption of light (Filipek *et. al*, 2003).

Peptide receptors of class A GPCRs compose the largest sub-group and many important physiological functions are performed by acting as neurotransmitters, hormones, and paracrines. With the peptide receptors of A Class like angiotensin II, arginine vasopressin/oxytocin, bradykinin, cholecystokinin/ gastrin, chemokines, complement factor 5a (C5a), fMLP, gonadotropin releasing hormone (GnRH), neurotensin, NPY, opioids, somatostatin, and TSH it is shown that they interact directly with the residues of N terminus and ECLs of receptors (Gether, 2000).

Protease-activated receptors (PARs), another Class A receptor, are activated by proteolytic cleavage. PAR_1 and PAR_2 are thrombin receptors, PAR_2 is serine protease receptor, and PAR_4 is both serine and protease receptors working by cleavage of the receptor while creating a new N-terminus (Macfarlane *et al*, 2001).

1.2.1.2. Class B GPCRs

It is considered that there are 15 receptors in B class GPCRs in humans, also known as secretin-like family receptors. Among them, glucagon and glucagon-like peptide 1 (GLP1) receptors play roles in glucose metabolism; calcitonin and PTH receptors regulate Ca^{++} homeostasis; and the corticotropin-releasing factor receptor (CRF_1R) regulates the hypothalamic-pituitary-adrenal axis. All B class GPCRs bind to endogenous large peptide hormones ranging between 30–40 residues and had long extracellular N-terminal tail domains (NTDs) (Alexander-Thomas & Manfred, 2011). When compared to A Class GPCRs, TMHs of the B class show very little primary structural conservation.

Characterized by nearly 120-amino-acids-long extracellular N-terminal hormone binding domain (HBD), B class GPCRs act by splitting the corticotropin-releasing factor (CRF) (Sakmar, 2011). HBD varies in length from 35 residues to 350 residues (Kristiansen, 2004). CRF, on the other hand, is 41-amino-acids-long peptide which

binds and activates its receptor, CRF_1R. CRF should include both a C-terminal region binding the NTD of receptor with high affinity, and N-terminal region activating the receptor by interacting helical bundles with high potency (Sakmar, 2011). HBD of PTH and adenylyl cyclase activating poly peptide (PACAP) receptor (PAC1), glucagon, and GLP1 receptors cannot account alone for the binding affinity of their ligands.

1.2.1.3. Large N-terminal Family B-7 Transmembrane Helix

It is reported that at least 30 receptors of six novel members of GPCRs have long N-termini, which are previously termed EGF-7TM, LNB-7TM, B2 or LN-7TM (Foord *et. al.*, 2002). These receptors have similarities to B class in their THMs. Their N-termini, however, play roles in cell-to-cell adhesion and cell migration via attachment to the extracellular matrix components or interaction of other cells' membrane proteins (Stacey *et. al*, 2000).

These receptors are related with the biological roles such as the controlling of brain angiogenesis, synaptic exocytosis, immune system regulations, cell polarity and synaptogenesis (Foord *et al.*, 2002).

Plenty of orphan GPCRs belong to this family such as BAI1 – 3 (Brain-specific Angiogenesis Inhibitor), CIRL1 – 3 (Ca^{2+} - Independent Receptor of α-Latrotoxin), CELSR1 – 3 (Cadherin EGF Laminin a G-type repeat 7-pass Receptor), GPR113, GPR124, and GPR125.

1.2.1.4. Frizzled/Smoothened receptors

10 members of Frizzled and only one member of Smoothened receptors have many similarities with B class GPCRs. Their ligands are called "Wnts". Wnts are the products of more than 16 vertebrate genes, 350-amino-acid long proteins (Kristiansen, 2004).

Frizzled receptors do not often signal via G-proteins and only some of the members show G-protein signaling. On the contrary, Smoothened receptors may activate G-proteins. However, unlike other GPCRs this activation is independent of ligand binding. Although it is suggested that different Wnts can activate the same Frizzled receptor, it is seen that they activate different pathways: 'agonist trafficking' (Holbrook *et. al*, 2002). Frizzled receptor responses may depend on accessory proteins. These accessory proteins are the keys of another control level.

Known Wnt inhibitors are CRBs, Cerberus, Dickkopf 1 (DKK1) and Wif. These inhibitors may be specific for each Wnts. For example, Dickkopf 1 acts to antagonize the association between FR7 and its accessory protein LRP5, therefore prevents the activation of Wnt.

1.2.1.5. Class C GPCRs

Receptors belong to C class GPCRs form constitutive dimers. Constitutive dimerization of C Class occurs in both forms of homo- and heteromers (Bruno *et. al*, 2012). Nevertheless, antagonist activation association of the receptor dimerization remains to be unclear (Lane & Canals, 2012). Similar to class A, C class GPCRs also have TMDs composed of seven hydrophobic helices.

By having 11 subunits, endogenous ligand-bound receptors of C class are metabotropic glutamate (mGlu1–8) receptors, receptors of major excitatory neurotransmitter in CNS, and the γ -aminobutyric acid (GABA) and the Ca^{2+} sensing receptor (Kristiansen, 2004). For example, the mGluR2 came into prominence as a potential drug target. It cooperates with 5-HT2A serotonin receptors, which is an A Class GPCR (Bruno *et. al*, 2012).Umami taste receptors also belong to C Class GPCRs (Nelson *et al.*, 2001).

1.2.1.6. Taste 2 and Vomeronasal 1 receptors

Chemical signals, like pheromones, are thought to be detected by vomeronasal organ located on the base of nasal septum. There are three receptors known to be related with vomeronasal organ: V1Rs, V2Rs and V3Rs, all GPCRs. It is thought that human V1R genes mostly are pseudogenes (Pantages & Dulac, 2000). Expression of these V1Rs in human has not been clarified yet. On the other hand, taste 2 receptors (T2R) are encoded for bitter taste stimulating molecules.

This receptor family is characterized by a very large NTD that likely acts an ECL. However, all members of V1R and T2R families have short N-terminal segments. Therefore, it is suggested that the ligand binding pocket is buried in between 7TMHs (Kristiansen, 2004).

1.2.2. GPCR Signaling Mechanisms

There are two types of G-proteins: heterotrimeric G-proteins interacting with GPCRs by a signal transduction pathway, and small cytoplasmic G-proteins. Heterotrimeric G-proteins have α (39-45 kDa), β (35-39 kDa), and γ (6-8 kDa) subunits. β and γ subunits often regarded as one functional unit due to their tight association. Not including the splicing variants, there are 5 different β and 12 different γ subunits described.

All these heterotrimeric G-proteins follow up same cycle of activation-deactivation signal transmission in a specific and reversible fashion. GDP binds to α subunit, and then βγ complex joins to form an inactive heterotrimeric G-protein.

With binding of an agonist receptor is activated and undergone a conformational change. This change initiates an increase affinity for G-protein by allowing the release of GDP from α subunit. Physiologically, GDP is replenished by GTP immediately after the concentration of GDP is exceeded by several folds. The active state

continues till the GTP is hydrolyzed by Gα subunits' GTPase activity. Moreover, GTPase-activating-proteins (GAPs) can inactivate the G-proteins. Some proteins can enhance the activation of G-proteins, called as effectors. To turn off the signal transduction some processes like desensitization, neurotransmitter-reuptake, and extracellular degradation of neuropeptides can take place (Kristiansen, 2004).

Majority of GPCR signaling is rather linear and sequential. Also, one G-protein can interact directly with various effectors; therefore, cause a change in second messenger concentration. Second messengers may alter the activity of effectors by integration of highly complex signaling networks.

GPCRs can activate mitogen-activated protein kinases (MAPKs) via Ca^{++}, Ras (small GTPase family), phospholipase C, cAMP, phosphatidylinositide 3 kinases (PI3Ks), and tyrosine kinases.

Many GPCRs are known for making complexes with PAR_2 (protease activated receptor), ERK1/2, and JNK (c-Jun N-terminal kinase), which are the components of MAPKs.

1.3. Aim of the Study

In this study, from the microarray data supplied in the research of Atwood *et al*, mRNA levels of a comprehensive profile of non-chemosensory orphan GPCRs and over a hundred GPCR signaling related gene products are analyzed to choose 10 candidate genes that can be CART peptide receptor. Microarray data were derived from four cell lines: HEK293, AtT-20, BV2, and N18. As mentioned previously, CART peptide causes an increase in PERK levels in AtT-20, GH3 and differentiated PC12 cell lines, but not induces such an increase in N2a, HEK293, and CATH cell lines.

The hypothesis based in this study is that between the CART responsive cell lines and CART nonresponsive cell lines, one or more of differentially expressed GPCR genes induce ERK1 and 2 signaling pathways by interaction, therefore cause an increase in PERK levels. This gene or genes might be CART receptor(s) depending on the findings that CART receptor can be a GPCR. In order to test this hypothesis, candidate genes are determined by analyzing microarray data supplied in the study of Atwood *et al.* in 2011. Most differentially expressed genes in CART responsive cell lines when compared to nonresponsive cell lines are selected from the microarray data. After mining out candidate genes, expressions of these candidate genes are validated by q-RT-PCR. As a starting point, differentiated PC12 and AtT-20 cell lines are tested for increasing the PERK levels as found in the study carried out by Kuhar *et al*, in 2005. Then, as CART responsive cell line AtT-20 cell line and as CART nonresponsive cell line N2a are used. The results obtained from this presented study might narrow down the candidate genes for CART peptide receptor. As further studies, by transfecting these candidate genes to N2a cell line, candidate gene products can be investigated if they are the receptors of CART peptide. Here, aim is to identify CART receptor candidates which in turn helps to identify unknown CART functions, CART related pathways and related drug design investigations.

CHAPTER 2

MATERIALS AND METHODS

2.1. Microarray Data Analysis

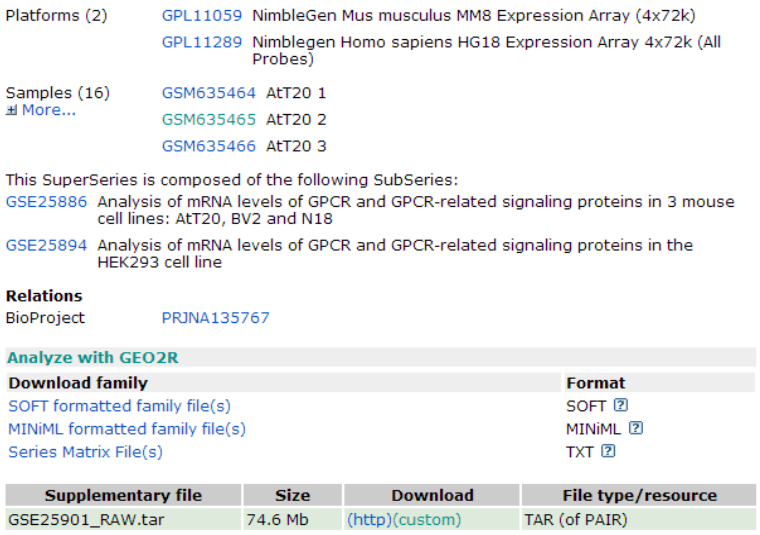
There are limited numbers of microarray analyzing software. The numbers of free-web based ones are even less. Also, most of them are designed to analyze the most commonly used microarrays like Affymetrix. In this thesis study, the microarray data that is going to be used for analysis belong to Nimblegen. Since there is no analyzing tool developed for this data, following steps are used for the analysis.

Data set is obtained from GEO (gene expression omnibus database), NCBI. GEO accession number of the data set is GSE25901 showed as in **Figure 10**.

Series GSE25901	Query DataSets for GSE25901
Status	Public on Feb 01, 2011
Title	Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis
Organisms	Homo sapiens; Mus musculus
Experiment type	Expression profiling by array
Summary	This SuperSeries is composed of the SubSeries listed below.
Overall design	Refer to individual Series
Citation(s)	Atwood BK, Lopez J, Wager-Miller J, Mackie K et al. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 2011 Jan 7;12:14. PMID: 21214938
Submission date	Dec 07, 2010
Last update date	Dec 27, 2012
Contact name	Jacqueline Lopez
E-mail	jacqueline.ann.lopez@gmail.com
Phone	574-631-7875
Organization name	University of Notre Dame
Department	Biology
Lab	Pfrender Laboratory
Street address	112 Galvin Life Science Center
City	Notre Dame
State/province	Indiana
ZIP/Postal code	46556
Country	USA

Figure 10 View of data set GSE25901 in GEO

It is performed on NimbleGen 4x72k platforms GPL11059 designed for *Mus musculus*, and GPL11289 designed for human. As shown in the bottom part of **Figure 11**, supplementary files are downloaded accordingly.



Platforms (2) GPL11059 NimbleGen Mus musculus MM8 Expression Array (4x72k)
GPL11289 Nimblegen Homo sapiens HG18 Expression Array 4x72k (All Probes)

Samples (16) GSM635464 AtT20 1
More...
GSM635465 AtT20 2
GSM635466 AtT20 3

This SuperSeries is composed of the following SubSeries:
GSE25886 Analysis of mRNA levels of GPCR and GPCR-related signaling proteins in 3 mouse cell lines: AtT20, BV2 and N18
GSE25894 Analysis of mRNA levels of GPCR and GPCR-related signaling proteins in the HEK293 cell line

Relations
BioProject PRJNA135767

Analyze with GEO2R

Download family	Format
SOFT formatted family file(s)	SOFT
MINiML formatted family file(s)	MINiML
Series Matrix File(s)	TXT

Supplementary file	Size	Download	File type/resource
GSE25901_RAW.tar	74.6 Mb	(http)(custom)	TAR (of PAIR)

Figure 11 View of data set GSE25901 in GEO continues

Since the dataset has .gz extensions, in order to display the files GEO2R (under NCBI server) is used (**Figure 12**). There are 12 sample groups. Since interested ones are AtT-20 and N2a, blue and pink labeled data sets are used for further studies in this thesis.

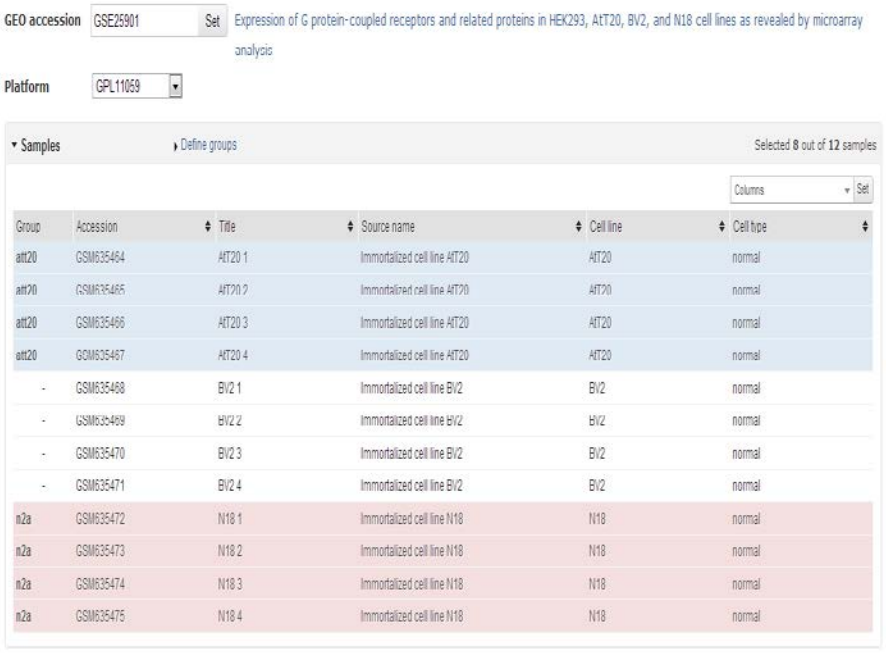


Figure 12 GEO2R Server Data Representation of the platform GPL11059

In order to analyze microarray data “BRB ArrayTools Version 4.3.0”, an analysis program developed by Biometric Research Branch of National Cancer Institute is used.

Data is imported via general importer of ArrayTools add-in as in following Figures.

BRB ArrayTools appears as an add-in within Excel after setup. This program perfectly analyses GDS files. Since GDS are curated datasets, the data within the project has all required information. However, GSE data sets are user-submitted data sets. Therefore, “general format importer” is used as in **Figure 13**.

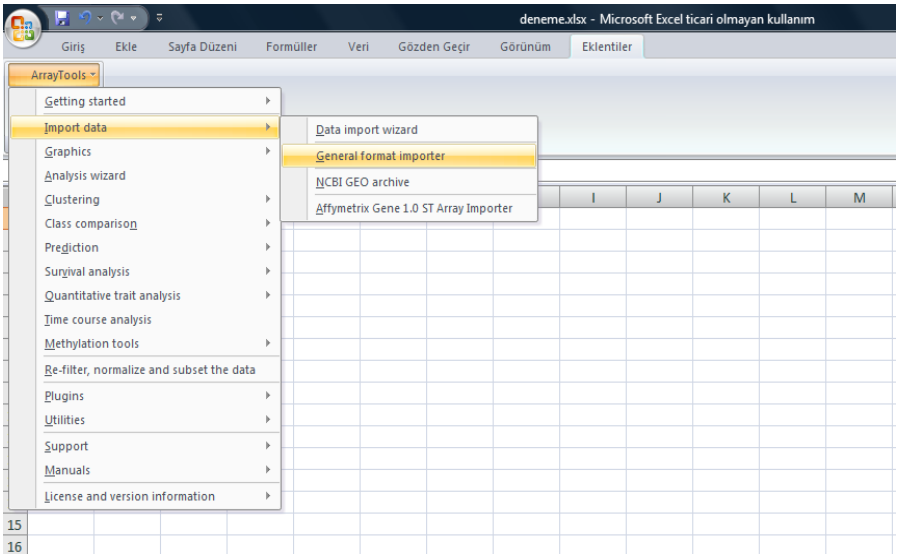


Figure 13 Screenshot of BRB ArrayTools Add-in Excel

Downloaded separate files from NCBI GEO are imported via “Arrays in Separate Files” tab, as can be seen in **Figure 14**.

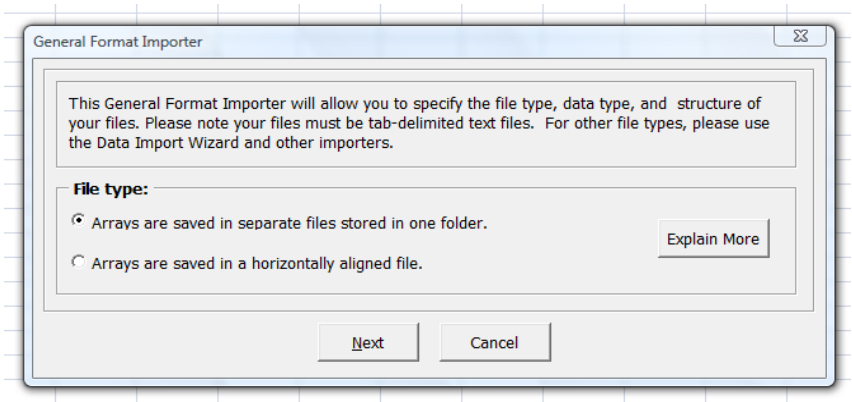


Figure 14 A screenshot in BRB ArrayTools Array Importer Wizard.

There were 8 sub-groups of data set four coming from AtT-20 cell line, and other four coming from the N2a. Log files of these data sets are imported accordingly, as it is shown in **Figure 15**.

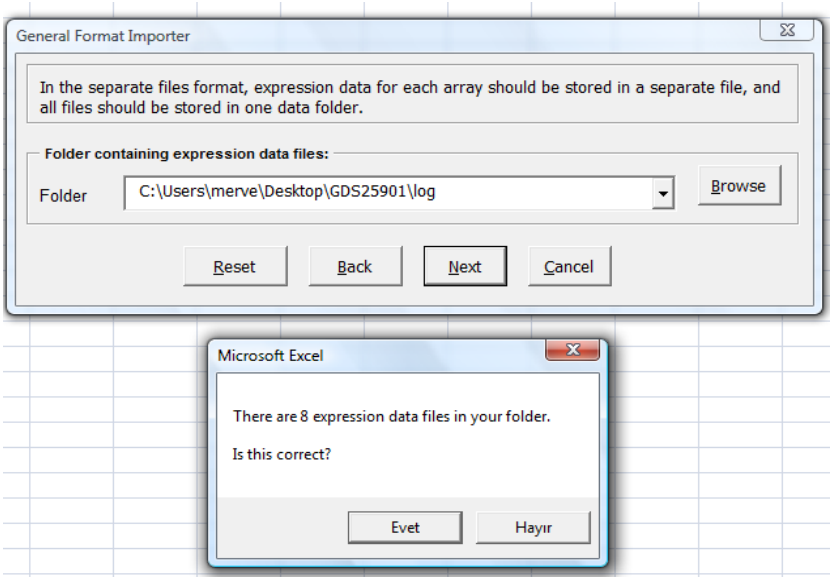


Figure 15 Data Folder Tab of BRB ArrayTools.

Since NimbleGen array platform information says that it is a dual channel data, in order to choose correct platform feature “Dual-channels data” is clicked on as it appears on **Figure 16**.

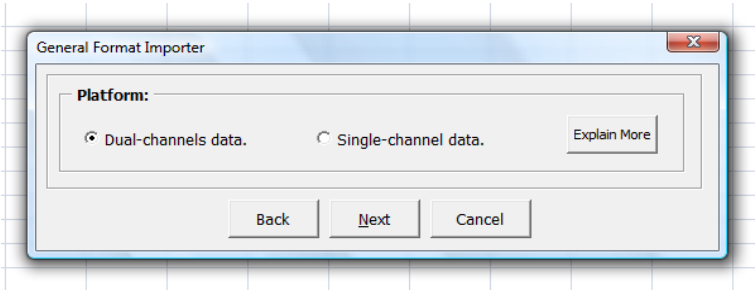


Figure 16 Platform Preferences of BRB ArrayTools

Due to using log files of data sets, dual channel data information is set as “log transformation is already transformed” as in **Figure 17**.

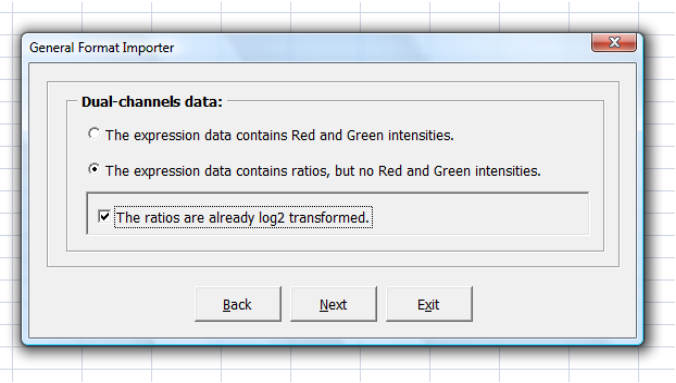


Figure 17 Dual-channels Preference of BRB ArrayTools.

Header line is choosen as the tab showing column ID. First, data line is set as the second line just appears after the header line. Then, log ratios is set as Log2 values (**Figure 18**).

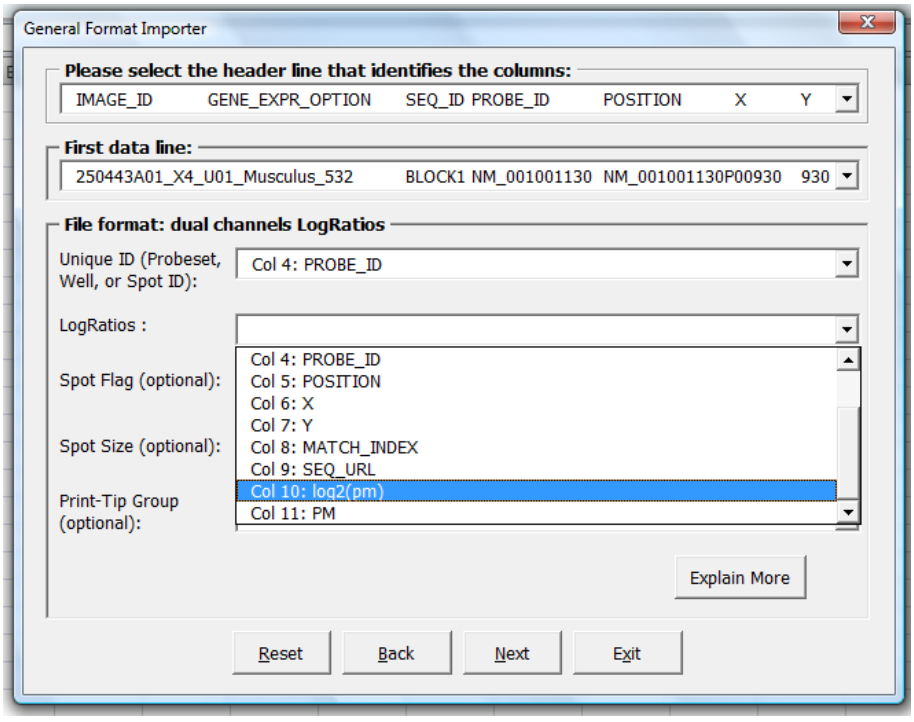


Figure 18 Column Selection for the data import in BRB ArrayTools.

Next, gene identifiers are set. In the provided data, probe IDs designed using Refseq gene IDs. Columns of Probe IDs are set to “EnterezID”,

since there is not any other option (**Figure 19a**). Annotation of the data will be provided after importation. Since annotation could not be handled, program creates a template with the array IDs (**Figure 19b**).

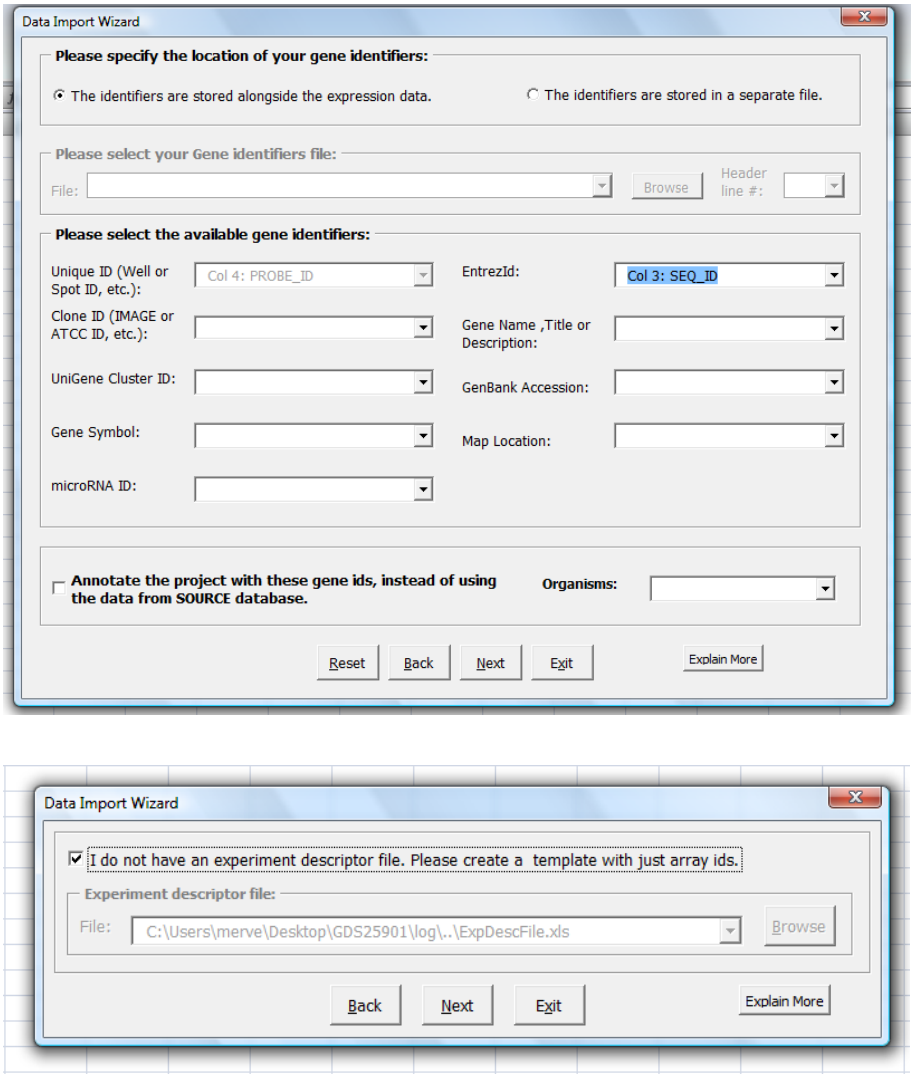


Figure 19a, b Annotation tab of the BRB ArrayTools.

Importer progresses the array by colltaing them in few minutes, then asks the spotting of the filters, how to normalize the submitted data and entering desired gene filters as in **Figure 20 a, b** and **c**.

Since it is a done-experiment, its intensity optimizaiton is already set. Therefore, it is enough to click “Average the Replicate Spots” tab (**Figure 20a**). Secondly, in order to normalize the data “Normalize each array” with “Using median over entire array” for log 2 transformed data as in **Figure 20b**. When come to “Gene Filter” option, in order to increase significance, log-ratio variation is pulled to 90 percentile in Figure **20c**.

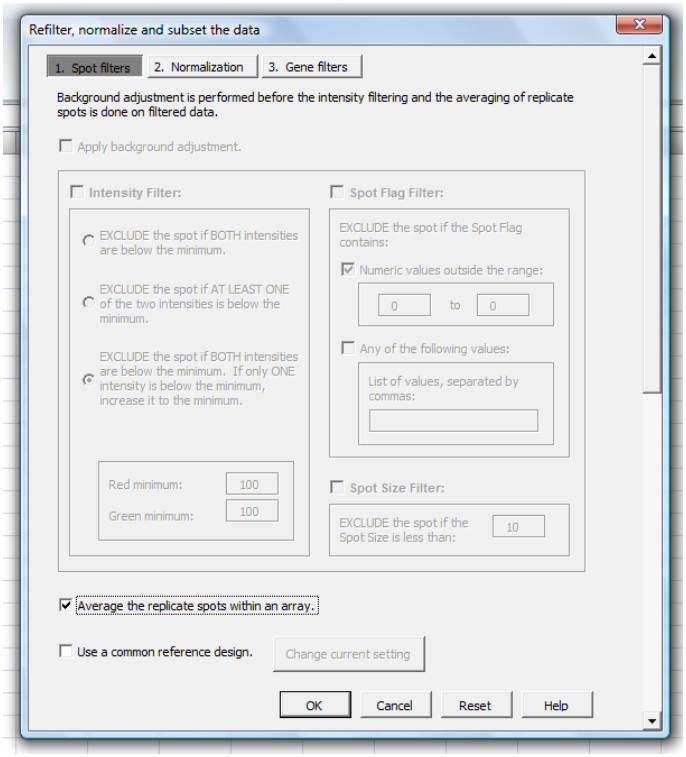


Figure 20 a Spot filters Option of BRB ArrayTools.

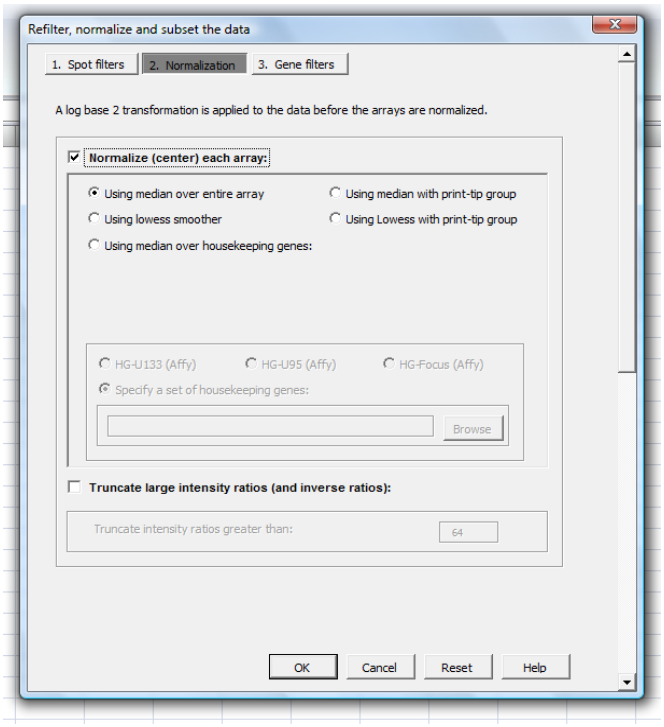


Figure 20 b Normalization tab of the BRB Arraytools

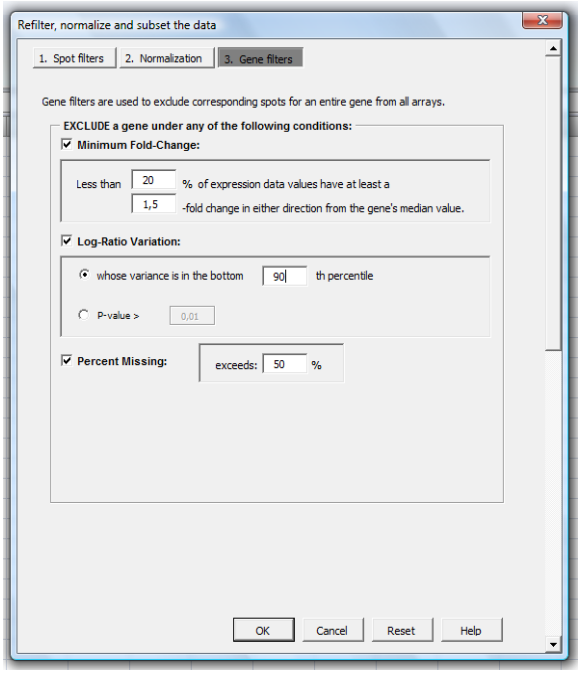


Figure 20 c Gene Filters Option of BRB ArrayTools

Coming data appears on Excel as four data sheets: “Experiment descriptors”, “Gene annotations”, “Filtered log ratio”, and “Gene identifiers” as indicated with red rectangular in **Figure 21**. On the first data sheet, there are descriptors of the experiment. There were approximately 72000 genes in data set first. After the filtering data according to significance, 1512 genes are appeared as current data set. Significance filtration is made due to pick the genes having most significant changes. In order to annotate this data as a part of it appears on **Figure 21**; WEBGESTALT (WEB-based GEne SeT AnaLysis Toolkit) web page is set.

Figure 21 After successful import of the data, excel sheet view

WebGestalt is one of the popular software tools since 2005. For the gene lists derived from large-scale genetic, transcriptomic and proteomic studies, latest version of WebGestalt enables to interpret 8 organisms and 201 gene identifiers from various databases & different technology platforms (Zhan *et al*, 2013). Since the annotation of the data couldn’t be made via BRB Array Tools, WebGestalt is used for this purpose. The *.ndf* file was old version of annotation file seen in **Figure 22**. From here, a *.txt* file is created and uploaded to WebGestalt in order to annotate the data (**Figure 23**).

```
#ID = PROBE_ID from .ndf file
#DESIGN_ID =
#PROBE_DESIGN_ID =
#GB_ACC = GenBank Accession number; NimbleGen SEQ_ID
#SEQUENCE = probe sequence
ID      DESIGN_ID       PROBE_DESIGN_ID GB_ACC  SEQUENCE
XM_892799P00106 5045    5045_0135_0003  XM_892799       TCTCTGGGAGACAGAATAACCATCACTTGCCAGGCAACTCAAGACATTGTTAAGAATTTA
NM_146837P00579 5045    5045_0137_0003  NM_146837       GGCCTGTGGAGATACTTCAATACACGAGTTGTCTGTCTACTTAGTAGCTATGCTGTTTGT
NM_028835P02890 5045    5045_0139_0003  NM_028835       AACACAGATGCTGCAACAAGCGGCCCTACCATCTGTGCAAGGCTCCCCACAAGTAGCCAG
NM_008323P00170 5045    5045_0141_0003  NM_008323       AAACAATTCCTCCATCTGCTAAGTATGGTGGGCGGCATACAGTGACTATGATCCCAGGGG
NM_016799P02836 5045    5045_0143_0003  NM_016799       AAGGGACAGCACAGGGCCTTTTGCTGTATGTAGATTGGGCAATGTATGCTACCACTGAGC
NM_177799P00877 5045    5045_0145_0003  NM_177799       CTTTCCCGGTGCCTGCTGATAATCCCTGACACTGTTTGGAGGAATGCGTCACTTTCTCAA
NM_026685P01286 5045    5045_0147_0003  NM_026685       TAGAAATTTCCCTGCACGGCGCTGAACCTTCCGCAGCAGAGTGACTTATCTTAGACAACT
NM_001033527P00303      5045    5045_0149_0003  NM_001033527    GGAACCGGTGCGGCACTCAAAGTTAACTCGAACACAGCCTGCAAACTTCTGGGCTGCAGC
NM_001033769P00120      5045    5045_0151_0003  NM_001033769    TGAGAATTTCTGGATTCTTGACTCTGCTTCCTATCCAGCTATTGCGGAATAAGTTCAACT
NM_019951P01025 5045    5045_0153_0003  NM_019951       TCGTTCCTCCTTACAGTAGGAATGGCACATATTGAGGCGATCATAAGTCTTAACTTTTCA
XM_975703P00001 5045    5045_0155_0003  XM_975703       ATGGAGTCAGGAATCCAGTTCGTGGATGAGTCGGAAATGGCAGTGTTTCTGCAGAACTCC
NM_177236P03755 5045    5045_0157_0003  NM_177236       TGGAGAAACCAGAATCCAAGAGTTGCATCCATAACTTCATGGCAACACCCGAGTTTTTGA
NM_145580P01149 5045    5045_0159_0003  NM_145580       AGGTGCTATCTAAACTTGGCTGAGTCAGAGTTTGCTGGGGCTTACTACTCCATCAATAAA
NM_025605P00449 5045    5045_0161_0003  NM_025605       TCTCTGACTTGCAGCAGGTCTCCAGGATAGCTGCGTATGCCTATAGTGCACTTTCTCAGA
XM_894787P00291 5045    5045_0163_0003  XM_894787       AAGCATTTGGAATAATGATAACTACTACAACCCATCTTTGAAGAGCCGGCTCACAATCTC
NM_007760P04123 5045    5045_0165_0003  NM_007760       GTTTTTGACAGTAGCACAGACCAGACATAGCGGCATAGCTATGTACCAGAGTGTACCAGA
XM_001003382P00151      5045    5045_0167_0003  XM_001003382    ATTCCCTTAATATCAGATCCCAAGCGCACCATTGCTCAGGATTATGGAGTCTTAAAGGCT
NM_146891P00001 5045    5045_0169_0003  NM_146891       ATGAACAACCAGAGCTGTGTCACTGAGTTCATATTCCTGGGACTTTCACAGAACTCAAAA
NM_011070P00090 5045    5045_0171_0003  NM_011070       TAATTGCTGGCTTTAATCGCCTTCGGCAGGAACAGAGGGGCTTGGCTTCCAAAGCAGCTG
XM_135479P04329 5045    5045_0173_0003  XM_135479       GAGACTGCATATCTAGGCTCTTAGGCAGATGGAAAATTTACGTAGTACAGATATTGTAAG
NM_183149P01867 5045    5045_0175_0003  NM_183149       AGCAAAGCTGAAGTGCCAGTAACCATCGTTGTCAATGGACATTCCGAAGGGTCAGCCCTG
XM_985551P00495 5045    5045_0177_0003  XM_985551       TGTGAAAGTGGCTTGCAAAATTACGAGCAATGTTGCCCTGCTCGGGTATCAGTAGTTTTG
NM_023119P01058 5045    5045_0179_0003  NM_023119       AGTGACCAACCCTAAGCGGATTGCCAAGGCTGCGAGCGAGAAGTCCTGCAACTGCCTCTT
NM_199422P00655 5045    5045_0181_0003  NM_199422       TGGGCAGAAGACATGGCTCCTGCCTTCAGTGATACCTTGCTTGAATACCGGGGGGAATAC
NM_011480P04062 5045    5045_0183_0003  NM_011480       TGTGCACCAGCATAGGCGAAGGATCAGGGCCCATTAGTGAGTAGACTGGGAACCCTGTTT
NM_028357P00134 5045    5045_0185_0003  NM_028357       AGGCAGACTGGGATGCCACATTTAGCTCATCCACAAAGCTTTGGATACTATGAGAAGTTG
NM_133355P02559 5045    5045_0187_0003  NM_133355       TGTGTTGGCCATGGGTAACTATCTCAACGATGGACAGCCCAAGACCAACAAGACCACTGG
NM_023440P00066 5045    5045_0189_0003  NM_023440       TTATCAGCAAGTGCCAGACAAGAGCAGGCCTTGCGTGAGCCCTGTTATCAGCCCCAGAGT
```

Figure 22 Annotation File of Platform opened in GEO2R.

User ID information table

Mapped User IDs back				
NM_001001881	72190	ENSMUSG00000043391	2510009E07Rik	RIKEN cDNA 2510009E07 gene
NM_001001179	232400	ENSMUSG00000047228	BC048546	cDNA sequence BC048546
NM_001001882	269400	ENSMUSG00000038685	Rtel1	regulator of telomere elongation helicase 1
NM_001001714	227631	ENSMUSG00000059625	Sohlh1	spermatogenesis and oogenesis specific basic helix-loop-helix 1
NM_001001490	239283	ENSMUSG00000044819	Oxgr1	oxoglutarate (alpha-ketoglutarate) receptor 1
NM_001001446	226143	ENSMUSG00000025197	Cyp2c44	cytochrome P450, family 2, subfamily c, polypeptide 44
NM_001001804	384214	ENSMUSG00000033805	Ephx4	epoxide hydrolase 4
NM_001001737	414758	NA	Zfp826	zinc finger protein 826
NM_001001152	238690	ENSMUSG00000055480	Zfp458	zinc finger protein 458
NM_001001187	408068	ENSMUSG00000048280	Zfp738	zinc finger protein 738
NM_001001452	387514	ENSMUSG00000046652	Tas2r143	taste receptor, type 2, member 143
NM_001001318	209380	NA	Gm4759	GTPase, very large interferon inducible 1 pseudogene
NM_001001320	109575	ENSMUSG00000037477	Tbx10	T-box 10
NM_001001803	408198	ENSMUSG00000060201	Spink7	serine peptidase inhibitor, Kazal type 7 (putative)
NM_001001489	215928	ENSMUSG00000038528	BC021785	cDNA sequence BC021785
NM_001001444	75400	ENSMUSG00000044249	Defb29	defensin beta 29
NM_001001805	258248	NA	Olfr576	olfactory receptor 576
NM_001001332	100038854	ENSMUSG00000079594	BC117090	cDNA sequence BC1179090
NM_001001321	70484	ENSMUSG00000033114	Slc35d2	solute carrier family 35, member D2
NM_001001496	414089	ENSMUSG00000055691	Gja6	gap junction protein, alpha 6
NM_001001183	407831	ENSMUSG00000024168	Tmem204	transmembrane protein 204
NM_001001883	329152	ENSMUSG00000042807	Hecw2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2
NM_001001326	76954	ENSMUSG00000031024	St5	suppression of tumorigenicity 5
NM_001001451	387513	ENSMUSG00000058250	Tas2r138	taste receptor, type 2, member 138
NM_001001334	381350	ENSMUSG00000037708	BC061194	cDNA sequence BC061194
NM_001001322	279028	ENSMUSG00000014852	Adamts13	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 13
NM_001001453	387515	ENSMUSG00000051917	Tas2r144	taste receptor, type 2, member 144
NM_001001559	384701	ENSMUSG00000057321	Dub2a	deubiquitinating enzyme 2a
NM_001001807	258502	ENSMUSG00000054036	Olfr279	olfactory receptor 279
NM_001001499	414105	NA	4732465J04Rik	RIKEN cDNA 4732465J04 gene
NM_001001495	414084	ENSMUSG00000044162	Tnip3	TNFAIP3 interacting protein 3
NM_001001449	380878	NA	AF067063	cDNA sequence AF067063

Figure 23 Webgestalt Annotation of the data set

2.2. Cell Culture

In this thesis study, two cell lines are used: Neuro 2a (N2a), and AtT-20. N2a cell line is derived from *Mus musculus* brain. These cells are adherent neuroblasts. In order to culture this cell line, Dulbeco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin are used according to **Table 1**.

Table 1 Growth Media of N2a cells

Components of Culture Media	**Final Concentration**
FBS	10 %
Pen/Strep	1 %
DMEM	q.s.p.

Cells are cultured in an atmospheric environment composed of 5 % CO_2 and 95% air in 37 °C.

In sub-culturing, 0.25% (w/v) Trypsin - 0.53 mM EDTA solution is used to remove cells. Sub-culturing is carried in a ratio of 1:6.

AtT-20 cell line is derived from pituitary of *Mus musculus*. They are small rounded cells, but loosely adherent. For the growth of AtT-20 cell line, F-12K selective medium, horse serum, FBS and penicillin/streptomycin are combined as in **Table 2**.

Table 2 Growth Media Components of AtT-20 cell line

Components of Culture Media	Final Concentration
Horse Serum	15 %
FBS	2.5 %
Pen/Strep	1 %
F-12K Selective Media	q.s.p.

In sub-culturing AtT-20 clumps are transferred to another flask filled with growth media. Therefore, there is no cultivation ratio. Cells live sustainably in clusters.

Atmospheric environment composed of 5 % CO_2 and 95 % air is required for AtT-20 cell growth in 37 °C.

2.3. RNA Extraction

In order to obtain RNAs from cell lines, iNtRON Biotechnology RNA-spin™ Total RNA Extraction Kit [for Cell/Tissue] is used (Cat. No: 17211). For the N2a cell line, from over-cultured T-75 flask 4 tubes of 0.5 mL cells are obtained. On the other hand, for the AtT-20 cell line RNA extraction, cells are collected to one tube only. Then, the protocol within the kit is strictly followed.

2.4. DNase treatment

In order to remove genomic DNA from RNAs, DNAse treatment is applied to total RNAs obtained previously by Invitrogen's Ambion DNA-free™ Kit (Cat. No: AM1906). The protocol within the kit is strictly followed.

2.5. Agarose Gel Electrophoresis for total RNA

After DNase treatment of total RNAs, products are run in 1% agarose gel.

2.6. cDNA Synthesis

As the crucial step of q-RT-PCR, cDNA synthesis is carried out by Thermo Scientifics' RevertAid First Strand cDNA Synthesis Kit (Cat. No: K1622). Since random hexamer multiplies every RNA molecules found in the sample, random hexamer primed reverse transcription is preferred. Therefore, by obtaining a random hexamer primed cDNA sample can be used as a source for every desired gene. The protocol within the kit of "RevertAid First Strand cDNA Synthesis" is strictly followed.

2.7. Primer Design

Because both cell lines are derived from mouse species, primers are designed in reference to *Mus musculus* genome. Genes selected using the microarray data are: *Crhr1, Glp1r, Glp2r, Sctr, Bai1, Bai3, Gpr113, Gpr116, Gpr133, Casr, Grm1, and Fzd10.*

Here as an example primer design for *Crhr1* gene will be examined. First, gene sequence is obtained from NCBI.

"Mus musculus corticotropin releasing hormone receptor 1 (Crhr1), mRNA

NCBI Reference Sequence: NM_007762.4

>gi|145966876|ref|NM_007762.4| Mus musculus corticotropin releasing hormone receptor 1 (Crhr1), mRNA

AGACTTGCTCGCGCAGGGCGAGCCAGAGCCTGCCGGTGGGGC
GGGGCGGGAGGGTGCGAAGCCGGGCGCCTGGGAGCAGCTCAG
CGACCCGGGAGACTGGCCGCCCAACGGAGACCGCAGCCGCCC
GCTCTTCGCTCTGGGATGTCGGAGCGATCCTGGCATCCAGGAC
GCTGACAGAGCGAGCCCGAGGATGGGACAGCGCCCGCAGCTC

CGGCTCGTGAAGGCCCTTCTCCTTCTGGGGCTGAACCCCGTCTC
CACCTCCCTCCAGGATCAGCAGTGTGAGAGCCTGTCCCTGGCC
AGCAATGTCTCTGGCCTGCAGTGCAATGCCTCCGTGGACCTCA
TTGGCACCTGCTGGCCCAGGAGCCCTGCAGGGCAGTTGGTGGT
TCGGCCCTGCCCTGCCTTTTTCTACGGTGTCCGCTACAACACCA
CAAACAATGGCTACCGGGAATGCCTGGCCAACGGCAGCTGGG
CAGCCCGTGTGAATTATTCTGAGTGCCAGGAGATTCTCAACGA
AGAGAAGAAGAGCAAAGTGCACTACCACATTGCCGTCATCATC
AACTACCTGGGCCACTGCATCTCCCTGGTGGCCCTCCTGGTGGC
CTTTGTCCTCTTCCTGCGGCTCAGGAGCATCCGGTGCCTGAGGA
ACATCATCCACTGGAACCTCATCTCGGCTTTCATCCTGCGCAAC
GCCACGTGGTTTGTGGTCCAGCTCACCGTGAGCCCCGAGGTCC
ACCAGAGCAACGTGGCCTGGTGCAGGCTGGTGACAGCCGCCTA
CAACTACTTCCACGTAACCAACTTCTTCTGGATGTTCGGTGAGG
GCTGCTACCTGCACACAGCCATCGTACTCACGTACTCCACCGA
CCGTCTGCGCAAGTGGATGTTCGTCTGCATCGGCTGGGGTGTG
CCTTTCCCCATCATTGTGGCTTGGGCCATTGGGAAACTTTACTA
CGACAATGAAAAGTGCTGGTTTGGCAAACGTCCTGGAGTATAT
ACTGACTACATCTACCAGGGCCCCATGATCCTGGTCCTGCTGAT
CAACTTTATCTTTCTCTTCAACATTGTCCGCATCCTCATGACCA
AACTCCGAGCATCCACCACATCTGAGACTATTCAGTACAGGAA
GGCTGTGAAGGCCACTCTGGTGCTCTTGCCCCTCCTGGGCATCA
CCTACATGTTGTTCTTCGTCAACCCTGGGGAGGACGAGGTCTCC
AGGGTTGTCTTCATCTACTTCAACTCTTTCCTGGAGTCCTTTCA
GGGCTTCTTCGTGTCTGTGTTCTATTGTTTTCTGAACAGTGAGG
TCCGCTCTGCCATCCGGAAGAGGTGGCGGCGATGGCAGGACAA
GCACTCCATCAGAGCCCGAGTGGCCCGCGCCATGTCCATCCCC
ACCTCCCCCACCAGAGTCAGCTTCCACAGCATCAAGCAGTCCA
CAGCAGTGTGAACCTCAGGCCACAGAGCAGCCCCCAAGACCC
GAGGCTGGGGAAATGATGCAAGCTCACTAGCGAGCCTGTCTGC

AGAGGCAGGCAGCCTTCCCATCCCTGCCCCTGGGATGCAGACC
TGTAAGCCTGCCCAGCCGTGGACAAAGCCCATAGCACTGGGGT
GGGCCCTTGGCATCTAGCTCCCTGCTGCCCATTCTCCCTGGGAA
GTTGAAATGGGCATTGGGGGCTGGAAACCCTGCAGCAGTTTGA
TGGGCCTGTGAGCGCTGTCTTCTCCCAGAGCAGCTTACTGAAG
ATCTGTCGTCTTCAGGAGCTGTTGGGGAGGCCAACTGTTACCCT
GGGGCATCATGGAAAACTCCCTTCTGAGACTGTAAAGTCTCTG
AGTGTTAGCGATGCCTTGGGATGCTACCGAGGACCAACATGGT
CCAGTCAGGAGACCGGGAGATAGCGGTAGAAATCTGGGAACG
TCATCAGATGGCACTCCACCTCCCTACAAGTCACTCCTGAGCC
ACCAGGATTTCATCAGCACTGTGGCACTGCCACTGGAAAGCCC
TGCCTTGCTGCTTTGCTGCCCTGCACCTTTAGACATTTACTATTC
TGCAGGCCAAGCCAGCTTTCTGTCACTTATCCACTGACAGCAA
CGGTCCCCTCGCCCCCAAATCCTCCCACCTCTGGGTATCTTCTA
ACCTGTGAGAAGATGGGGGTCGGGAAGGGGACTTGAGTTGCC
AGGAACCAGAGTGGGCCCAGTCTATGAGGAAGGAGTGGCCCC
TGGGTACCCAGGCCACTGGCTTCAGTGGCTGGCCTCTTGAACA
CAGTCACAAGCTGGGGGAAGGATCTATTCAAGTGCCCTGACCA
GCGACAGGTGGCTCCTGGGACAACTAACTAACTAAGCCCTTGC
TCCCAGGCTTGGAATGGCCCAGTCCTCAGTGGGTAGGAGAGCT
GAGGAGCCGCAGCAGGACTGAGGTGGGGGTGATATAAATAAT
ATTTATCTTTTC”

Then, “NCBI Primer-BLAST” under the website of “http://www.ncbi.nlm.nih.gov/tools/primer-blast/” and “Primer3” under the website of “http://bioinfo.ut.ee/primer3-0.4.0/primer3/” are used to pick primers.

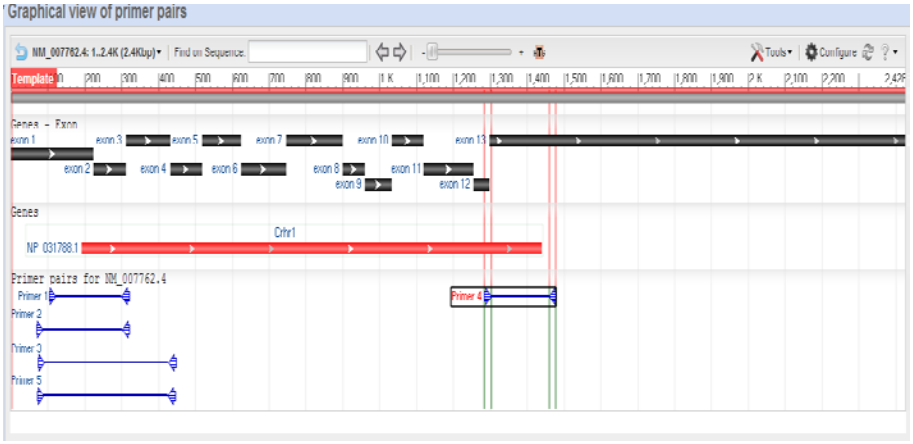


Figure 24 NCBI Primer-BLAST results for Crhr1 gene

Since there is not any other variant of Crhr1 gene for *Mus musculus*, 4th primer may be appropriate due to its short product size which is 193 bp, and exon-exon junction fit. When examined by clicking the tab on fourth primer as in **Figure 24**, details are as shown below:

Primer pair 4

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTCTGAACAGTGAGGTCCGC	Plus	20	1284	1303	59.97	55.00	5.00	3.00
Reverse primer	CAGCCTCGGGTCTTGGG	Minus	17	1476	1460	59.34	70.59	4.00	0.00
Product length	193								
Exon junction	1297/1298 (forward primer) on template NM_007762.4								

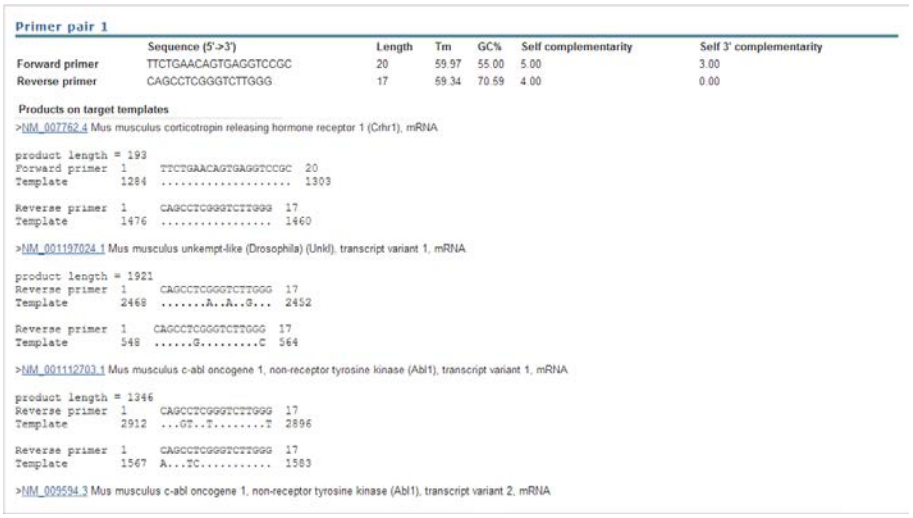
Products on intended target

>NM_007762.4 Mus musculus corticotropin releasing hormone receptor 1 (Crhr1), mRNA

```
product length = 193
Forward primer  1     TTCTGAACAGTGAGGTCCGC  20
Template        1284  ....................  1303

Reverse primer  1     CAGCCTCGGGTCTTGGG  17
Template        1476  .................  1460
```

Figure 25 Primer details of Crhr1 gene

In order to see whether it hits another sequences in the genome, BLAST search is made as in **Figure 25**.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTCTGAACAGTGAGGTCCGC	20	59.97	55.00	5.00	3.00
Reverse primer	CAGCCTCGGGTCTTGGG	17	59.34	70.59	4.00	0.00

Products on target templates

>NM_007762.4 Mus musculus corticotropin releasing hormone receptor 1 (Crhr1), mRNA

```
product length = 193
Forward primer  1     TTCTGAACAGTGAGGTCCGC  20
Template        1284  ....................  1303

Reverse primer  1     CAGCCTCGGGTCTTGGG  17
Template        1476  .................  1460
```

>NM_001197024.1 Mus musculus unkempt-like (Drosophila) (Unkl), transcript variant 1, mRNA

```
product length = 1921
Reverse primer  1     CAGCCTCGGGTCTTGGG  17
Template        2468  .......A..A..G...  2452

Reverse primer  1    CAGCCTCGGGTCTTGGG  17
Template        548  ......G.........C  564
```

>NM_001112703.1 Mus musculus c-abl oncogene 1, non-receptor tyrosine kinase (Abl1), transcript variant 1, mRNA

```
product length = 1346
Reverse primer  1     CAGCCTCGGGTCTTGGG  17
Template        2912  ...GT..T........T  2896

Reverse primer  1     CAGCCTCGGGTCTTGGG  17
Template        1567  A...TC...........  1583
```

>NM_009594.3 Mus musculus c-abl oncogene 1, non-receptor tyrosine kinase (Abl1), transcript variant 2, mRNA

Figure 26 BLAST results of the picked primer

There are 10 hits elsewhere in the genome although not perfectly match with sequences. Therefore, “Primer3” search is made in order to make a comparison. Sequence is pasted to “Primer3” this time, and the results were:

Primer3 Output

```
PRIMER PICKING RESULTS FOR gi|145966876|ref|NM_007762.4| Mus musculus corticotropin
releasing hormone receptor 1 (Crhr1), mRNA

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%  any th  3' th hairpin seq
LEFT PRIMER       1666   20   58.97   50.00   27.77   0.00    0.00 GAAACCCTGCAGCAGTTTGA
RIGHT PRIMER      1834   20   58.99   55.00    0.00   0.00    0.00 CCAAGGCATCGCTAACACTC
SEQUENCE SIZE: 2428
INCLUDED REGION SIZE: 2428

PRODUCT SIZE: 169, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

    1 AGACTTGCTCGCGCAGGGCGAGCCAGAGCCTGCCGGTGGGGCGGGGCGGGAGGGTGCGAA

   61 GCCGGGCGCCTGGGAGCAGCTCAGCGACCCGGGAGACTGGCCGCCCAACGGAGACCGCAG

  121 CCGCCCGCTCTTCGCTCTGGGATGTCGGAGCGATCCTGGCATCCAGGACGCTGACAGAGC

  181 GAGCCCGAGGATGGGACAGCGCCCGCAGCTCCGGCTCGTGAAGGCCCTTCTCCTTCTGGG

  241 GCTGAACCCCGTCTCCACCTCCCTCCAGGATCAGCAGTGTGAGAGCCTGTCCCTGGCCAG

  301 CAATGTCTCTGGCCTGCAGTGCAATGCCTCCGTGGACCTCATTGGCACCTGCTGGCCCAG
```

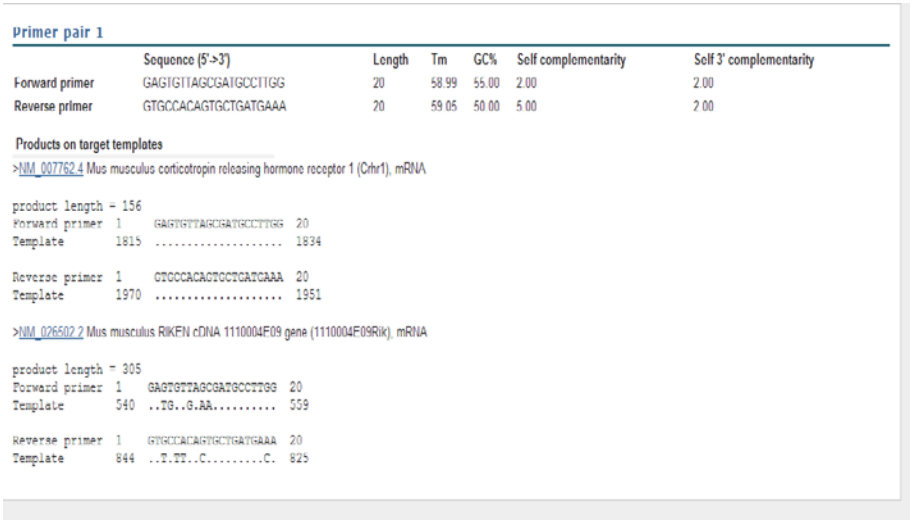
.

```
    1441 CTCAGGCCACAGAGCAGCCCCCAAGACCCGAGGCTGGGGAAATGATGCAAGCTCACTAGC

    1501 GAGCCTGTCTGCAGAGGCAGGCAGCCTTCCCATCCCTGCCCCTGGGATGCAGACCTGTAA

    1561 GCCTGCCCAGCCGTGGACAAAGCCCATAGCACTGGGGTGGGCCCTTGGCATCTAGCTCCC

    1621 TGCTGCCCATTCTCCCTGGGAAGTTGAAATGGGCATTGGGGGCTGGAAACCCTGCAGCAG
                                                    >>>>>>>>>>>>>>>

    1681 TTTGATGGGCCTGTGAGCGCTGTCTTCTCCCAGAGCAGCTTACTGAAGATCTGTCGTCTT
         >>>>>
    1741 CAGGAGCTGTTGGGGAGGCCAACTGTTACCCTGGGGCATCATGGAAAACTCCCTTCTGAG

    1801 ACTGTAAAGTCTCTGAGTGTTAGCGATGCCTTGGGATGCTACCGAGGACCAACATGGTCC
                      <<<<<<<<<<<<<<<<<<<

    1861 AGTCAGGAGACCGGGAGATAGCGGTAGAAATCTGGGAACGTCATCAGATGGCACTCCACC

    1921 TCCCTACAAGTCACTCCTGAGCCACCAGGATTTCATCAGCACTGTGGCACTGCCACTGGA

    1981 AAGCCCTGCCTTGCTGCTTTGCTGCCCTGCACCTTTAGACATTTACTATTCTGCAGGCCA


KEYS (in order of precedence):
>>>>>> Left primer
<<<<<< Right primer

ADDITIONAL OLIGOS
                    start  len      tm      gc%   any th  3' th  hairpin  seq

 1 LEFT PRIMER        764   20    59.03    50.00    0.00   0.00     0.00
ACAGCCGCCTACAACTACTT
   RIGHT PRIMER       989   20    59.02    50.00    0.00   0.00     0.00
ATACTCCAGGACGTTTGCCA
   PRODUCT SIZE: 226, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

 2 LEFT PRIMER       1815   20    58.99    55.00    0.00   0.00     0.00
GAGTGTTAGCGATGCCTTGG
   RIGHT PRIMER      1970   20    59.05    50.00    0.00   0.00     0.00
GTGCCACAGTGCTGATGAAA
   PRODUCT SIZE: 156, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

 3 LEFT PRIMER       2059   20    59.03    50.00    0.00   0.00     0.00
TATCCACTGACAGCAACGGT
   RIGHT PRIMER      2260   20    59.05    55.00    0.00   0.00     0.00
CCCAGCTTGTGACTGTGTTC
   PRODUCT SIZE: 202, PAIR ANY_TH COMPL: 6.31, PAIR 3'_TH COMPL: 1.38

 4 LEFT PRIMER        995   20    58.95    60.00   16.46  16.46     0.00
GACTACATCTACCAGGGCCC
   RIGHT PRIMER      1223   20    58.94    55.00    0.00   0.00     0.00
TGAAGACAACCCTGGAGACC
   PRODUCT SIZE: 229, PAIR ANY_TH COMPL: 0.08, PAIR 3'_TH COMPL: 0.00
```

Figure 27 Primer 3 Output of crhr1 gene

Primer3 output results give more stable primers due to GC content and secondary structure that can occur. In order to get a successful q-RT-PCR result, short products should be targeted. Smaller than 300bp products would be synthesized ideally. Since 2nd pair is the shortest one here, it is checked in BLAST once more to see where it hits in the genome. As

shown in **Figure 27**, there are two hits in the mouse genome, first is the targeted gene, and second one is a bad match with a cDNA clone.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAGTGTTAGCGATGCCTTGG	20	58.99	55.00	2.00	2.00
Reverse primer	GTGCCACAGTGCTGATGAAA	20	59.05	50.00	5.00	2.00

Products on target templates

>NM_007762.4 Mus musculus corticotropin releasing hormone receptor 1 (Crhr1), mRNA

```
product length = 156
Forward primer  1     GAGTGTTAGCGATGCCTTGG  20
Template        1815  ....................  1834

Reverse primer  1     GTGCCACAGTGCTGATGAAA  20
Template        1970  ....................  1951
```

>NM_026502.2 Mus musculus RIKEN cDNA 1110004F09 gene (1110004F09Rik), mRNA

```
product length = 305
Forward primer  1    GAGTGTTAGCGATGCCTTGG  20
Template        540  ..TG..G.AA.........  559

Reverse primer  1    GTGCCACAGTGCTGATGAAA  20
Template        844  ..T.TT..C.........C.  825
```

Figure 28 BLAST result of the 2nd primer from Primer3 output

In order to check secondary structures, oligo analyzer under the website of IDT, Integrated DNA Technologies, is used to see a detailed probable hairpin structures, internal and hetero dimers as shown in the **Figure 29**.

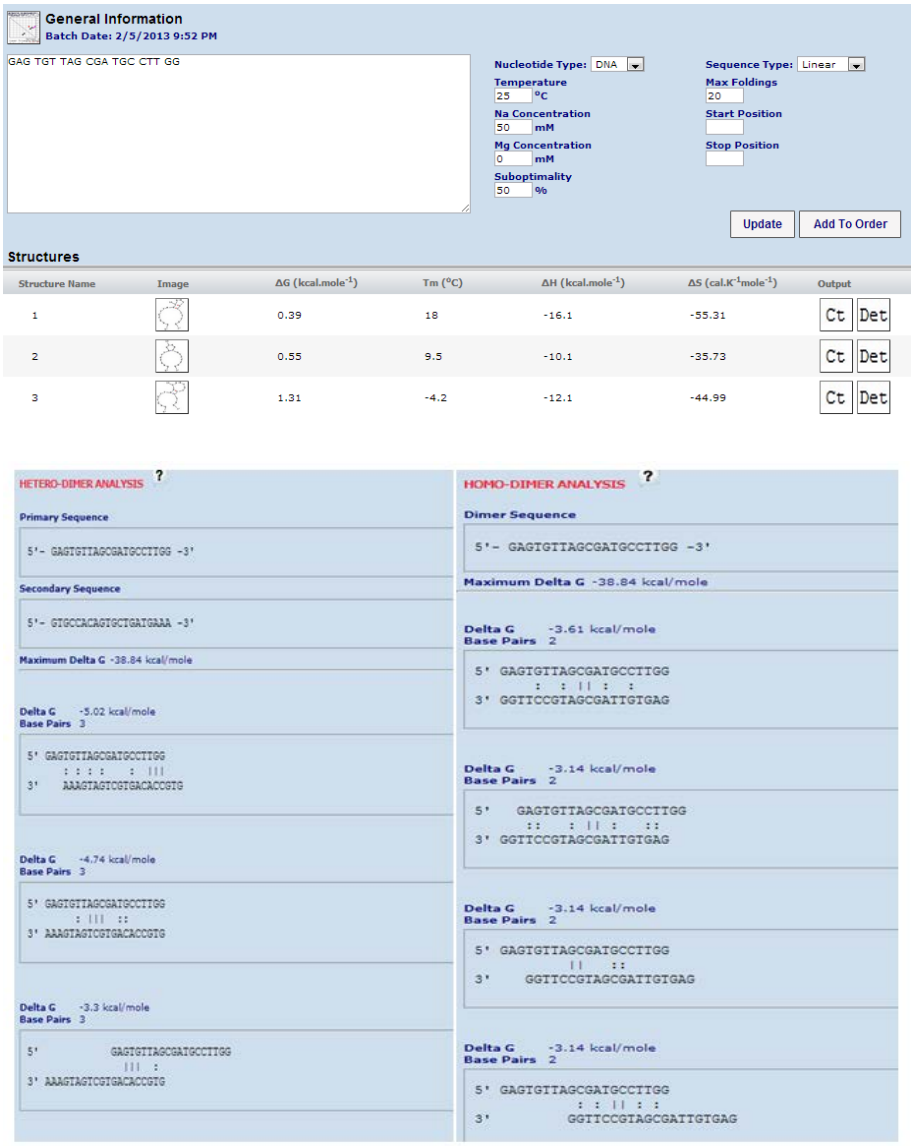


Figure 29 Detailed report of the desired primer pair in "OligoAnalyzer 3.1"

Since showed hairpins are insignificant in terms of structures and Tm's, and also heterodimer and selfdimer check results seem insignificant, as a conclusion this primer pair is chosen for RT-PCR studies. Whole table of targeted gene primers are shown in the **Table 3**.

Table 3 Primer Sequences designed for the targeted genes and the product size

Gene Name	Direction	Primer Sequence	P. size
Bai1	F 5'→ 3'	CCCAGCTCATGACCGACTTT	192
	R 5'→ 3'	TGGACACATTAGCTGGCAGG	
Bai3	F 5'→ 3'	CTGCCTGGCCTTGATAACTC	164
	R 5'→ 3'	TCGTGCAGATGCTCTTATTGTG	
Casr	F 5'→ 3'	TTGCCTTGTGATCCTCTTTCCA	199
	R 5'→ 3'	TCATGTAGAGACTGCCCGAGA	
Crhr1	F 5'→ 3'	GAGTGTTAGCGATGCCTTGG	156
	R 5'→ 3'	GTGCCACAGTGCTGATGAAA	
Fzd10	F 5'→ 3'	AAGAGAGTCACTTCCCCAGC	159
	R 5'→ 3'	TGTTTTGCTAGGGAGAGGGG	
Glp1r	F 5'→ 3'	ATGTCAGCTGCCCCTGGTA	329
	R 5'→ 3'	AGGTGGATGTAGTTCCTGGT	
Glp2r	F 5'→ 3'	GCTGGTTTCCATCAAGCAAG	268
	R 5'→ 3'	GTGTCTGTAGGCCCTTCCTG	
Gpr113	F 5'→ 3'	ATTTGTCCCCACAGCCCAAT	97
	R 5'→ 3'	GACCAGCACTGATTCCCCTT	
Gpr116	F 5'→ 3'	CTCCCGATTGTCTGTGAGGT	262
	R 5'→ 3'	TGGAGAATTGAAGGTTGAACAATGG	
Gpr133	F 5'→ 3'	ACCTACTGCTTTCAGGCACC	232
	R 5'→ 3'	TGAGTGCTACACACCTGGAC	
Grm1	F 5'→ 3'	TCACTACCTGCTTCGCAGTG	233
	R 5'→ 3'	GCCGTTAGAATTGGCGTTCC	
Sctr	F 5'→ 3'	CTTCGGATGGGGTTCTCCAG	106
	R 5'→ 3'	CCAGATGGAAGCGTTGGAGT	
GAPDH	F 5'→ 3'	CAAGGTCATCCATGACAACTTTG	496
	R 5'→ 3'	GTCCACCACCCTGTTGCTGTAG	

2.8. q-RT-PCR

From obtained cDNAs 1:10, 1:100, and 1:1000 dilutions are prepared for both AtT-20 and N2a derived ones in order to calculate the concentration accurately as cDNA concentration cannot be measured due to RNA presence in the samples. It is known that nearly 20% of RNA is converted into cDNA during a reverse transcriptase reaction. Three replicates of samples are used with a no template control (NTC) as shown in **Figure 30**.

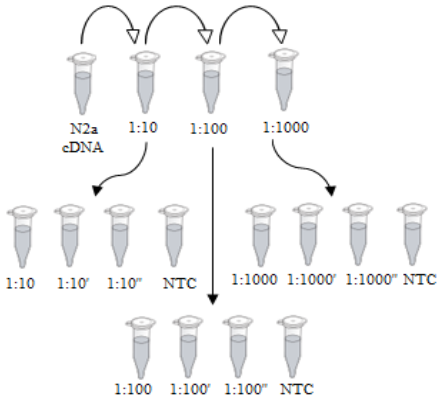


Figure 30 Experimental Design of cDNA dilutions for N2a samples

In order to prepare a master mix, Roche's FastStart Universal SYBR Green Master (Rox) (Cat. No: 04 913 850 001) is used. Components of the master mix are SYBR green mix, nuclease free water, forward primer and reverse primer according to **Table 4**.

Table 4 SYBR Green Mixture Components for q-RT-PCR

COMPONENTS	**Volume for One Tube**
SYBR green mix	9.7 µL
Nuclease free water	11.3 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Total	**22 µL**

When 3 µL cDNA is added to mix finally, 25 µL reaction tubes are placed onto QIAGEN.

Two-step reaction is designed accordingly:

Table 5 2-step Reaction Conditions for q-RT-PCR experiment

95 °C	10 min		**Hold**
95 °C	15 sec	40 cycles	**Annealing**
60 °C	45 sec		
50 °C – 99 °C			**Melting**

CHAPTER 3

RESULTS & DISCUSSION

3.1. Microarray Data Analysis Results

After first analysis made by GEO2R, N18 and At-T20 cell line samples are obtained as in **Figure 32**. High red bars show four samples genes that are expressed in At-T20 cell line. However, there are no bars on N18 representing these could be the candidate genes for our hypothesis. The detailed analysis revealed after BRB Array Tools analysis (**Figure 33**).

To scatter the plot of differentially expressed genes, the excel sheet obtained after the microarray analysis result (**Figure 31**) is used. Then by using the V-look-up function of Microsoft Excel, the genes are determined as in **Table 7** in the order of significance.

	Display the data	GSM635464	GSM6354	GSM6354	GSM6354	GSM6354	Rank	Mean	T-test	Fold
1	UniqueID	_250443A01	65_25044	66_25044	67_25044	72_25044				Change
2	NM_175642.4	515733888	5.21E+08	-1.1E+09	4.96E+08	3.81E+08	1	4.55125	8.50250E-19	4.5513
3	XM_134865P01843	515643136	4.95E+08	4.45E+08	5.1E+08	2.49E+08	2	4.15125	8.50250E-19	4.1513
4	XM_138109P00228	515633536	5.06E+08	4.22E+08	4.92E+08	1.95E+08	3	3.234854	8.50250E-19	3.2349
5	NM_177218P01390	515515392	5.17E+08	-9.9E+08	4.91E+08	-1.2E+09	4	2.334854	8.12325E-19	2.3349
6	NM_030024P00173	515461248	4.87E+08	-9.9E+08	4.68E+08	2.66E+08	5	2.231258	4.40700E-19	2.2313
7	NM_001029837P03556	515438848	-9.2E+08	4.54E+08	5.13E+08	3.65E+08	6	2.134112	4.11820E-19	2.1341
8	NM_011475P00397	515366656	5.13E+08	-9.9E+08	5.16E+08	-1E+09	7	2.030975	6.41347E-19	2.0310
9	XM_901559P01106	515143808	4.91E+08	-9.9E+08	5.09E+08	-1.1E+09	8	1.994854	6.34789E-19	1.9949
10	NM_009026P00742	515123456	5.04E+08	4.25E+08	-1.1E+09	1.8E+08	9	1.994854	4.50250E-20	1.9949
11	NM_013654P00285	515075968	5.21E+08	4.49E+08	-9.2E+08	3.56E+08	10	1.936755	4.50250E-19	1.9368
12	NM_007812P01723	515038464	5.21E+08	4.54E+08	-9.2E+08	-1.1E+09	11	1.934854	7.50250E-19	1.9349
13	XM_129479P04733	514510336	4.99E+08	4.54E+08	5.02E+08	2.65E+08	12	1.932485	6.50250E-19	1.9325
14	NM_029849P02447	514276352	5.15E+08	4.54E+08	5.13E+08	-1.6E+08	13	1.916792	5.04570E-19	1.9168
15	XM_899399P00424	514115072	5.19E+08	4.39E+08	4.01E+08	-1.2E+08	14	1.911585	2.50250E-19	1.9116
16	XM_205276P04133	514074624	-9.2E+08	4.53E+08	-9.3E+08	2.71E+08	15	1.903877	2.50250E-19	1.9039
17	NM_053217P02356	514068224	5.21E+08	4.54E+08	5.19E+08	3.87E+08	16	1.900877	2.50250E-19	1.9009
18	NM_199422P00470	513745408	5.21E+08	4.54E+08	5.14E+08	3.89E+08	17	1.894854	2.50250E-19	1.8949
19	XM_488314P00296	513670784	5.17E+08	4.54E+08	5.19E+08	3.89E+08	18	1.890695	2.50250E-19	1.8907
20	NM_175485P06491	513590016	5.1E+08	4.23E+08	5.16E+08	3.26E+08	19	1.890129	2.50250E-19	1.8901
21	XM_486268P05198	513438976	5.03E+08	4.19E+08	4.61E+08	2.9E+08	20	1.884854	2.50250E-19	1.8849
22	NM_023814P01904	513396736	5.21E+08	4.54E+08	5.12E+08	-1.1E+09	21	1.881345	2.50250E-19	1.8813
23	NM_007988P09016	513310720	5.21E+08	4.54E+08	5.19E+08	3.54E+08	22	1.880347	2.50250E-19	1.8803
24	XM_148862P00054	513168384	5.02E+08	4.13E+08	5.07E+08	3.42E+08	23	1.880046	2.50250E-19	1.8800
25	NM_172454P01022	512394112	5.21E+08	4.51E+08	5.15E+08	-1.1E+09	24	1.879835	2.50250E-19	1.8798

Figure 31 Raw Data Result after BRB ArrayTool analysis

ID	adj.P.Val	P.Value	t	B	logFC	GB_ACC	SEQUENCE
NM_009022P01390	1.28e-184	1.78e-189	29.4	422	7.01	NM_009022	TGCAAGCTGGGACAG...
XM_973193P00843	7.74e-164	2.15e-168	27.7	374	6.61	XM_973193	TGATCATAAGAGCAC...
NM_010910P01279	9.86e-161	4.11e-165	27.4	366	6.54	NM_010910	GCTCAGTTTCACCAG...
NM_145629P01944	1.16e-152	6.46e-157	26.7	348	6.37	NM_145629	GATGACAAGCACAATA...
NM_133729P01279	2.78e-141	1.93e-145	25.7	321	6.13	NM_133729	CTGTTTGTCTATGATA...
NM_026286P00304	1.12e-131	9.30e-136	24.8	299	5.92	NM_026286	TATCAACCGCCAAAT...
NM_010910P00926	3.39e-123	3.30e-127	24	279	5.73	NM_010910	AGCCGCTTCACCGT...

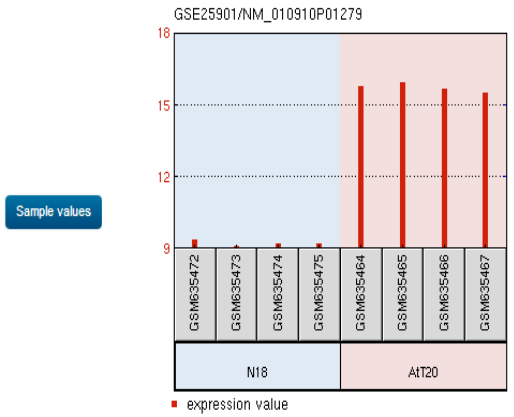


Figure 32 A sample from GEO2R Top 250 genes selection

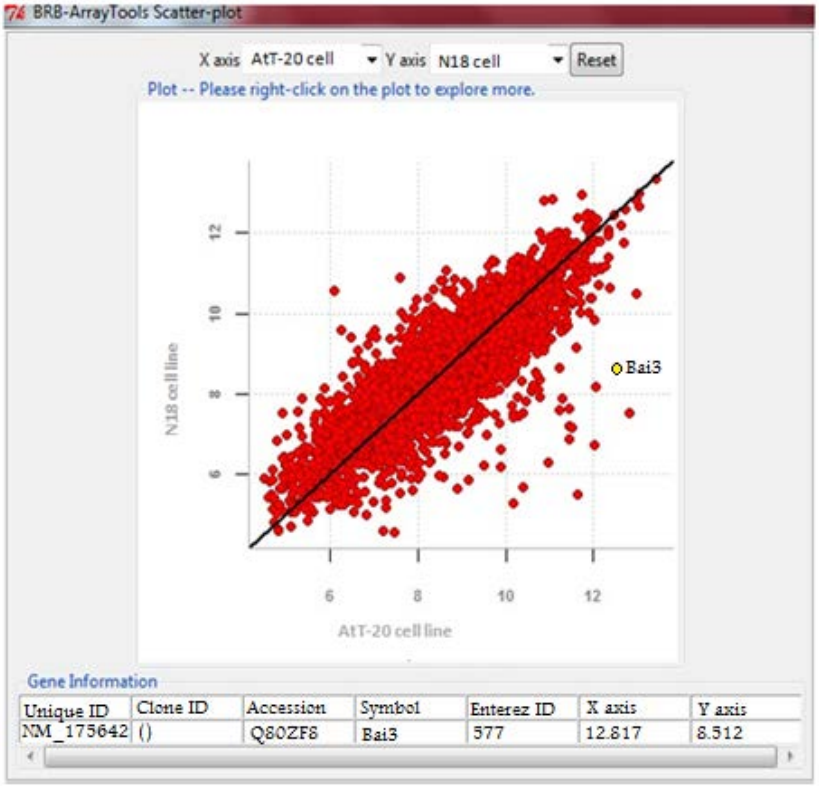


Figure 33 Scatter Plot of Data Analysis by BRB ArrayTools

It is seen that there are differentially expressed genes by AtT-20 cell line when compared to N18 cell line. In order to choose top genes whose expressions are nearly zero in N18, but having highest expression in AtT-20 cell line – also shown in **Figure 33** the analysis revealed by GEO2R - BRB ArrayTools scatter plot gave the detailed result.

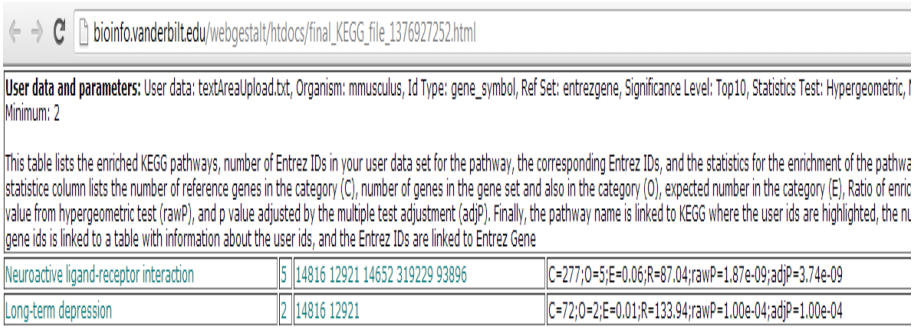
Final list of the selected genes are listed in **Table 6**.

Table 6 Genes Selected By Microarray Data Analysis

Bai3	ENSMUSG00000033569	Brain-specific angiogenesis inhibitor 3
Glp2r	ENSMUSG00000049928	Glucagon-like peptide 2 receptor
Fzd10	ENSMUSG00000081683	Frizzled homolog 10 (Drosophila)
Gpr116	ENSMUSG00000056492	G protein-coupled receptor 116
Bai1	ENSMUSG00000034730	Brain-specific angiogenesis inhibitor 1
Gpr133	ENSMUSG00000044017	G protein-coupled receptor 133
Gpr113	ENSMUSG00000067642	G protein-coupled receptor 113
Glp1r	ENSMUSG00000024027	Glucagon-like peptide 1 receptor
Sctr	ENSMUSG00000026387	Secretin receptor
Casr	ENSMUSG00000051980	Calcium-sensing receptor
Crhr1	ENSMUSG00000018634	Corticotropin releasing hormone receptor 1
Grm1	ENSMUSG00000019828	Glutamate receptor, metabotropic 1

As the final step of microarray data analysis, KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Analysis results revealed that genes of interest are related with two pathways (**Figure 34**):

- Neuroactive ligand-receptor interaction (**Figure 35**)
- Long-term depression (**Figure 36**)



bioinfo.vanderbilt.edu/webgestalt/htdocs/final_KEGG_file_1376927252.html

User data and parameters: User data: textAreaUpload.txt, Organism: mmusculus, Id Type: gene_symbol, Ref Set: entrezgene, Significance Level: Top10, Statistics Test: Hypergeometric, Minimum: 2

This table lists the enriched KEGG pathways, number of Entrez IDs in your user data set for the pathway, the corresponding Entrez IDs, and the statistics for the enrichment of the pathwa statistice column lists the number of reference genes in the category (C), number of genes in the gene set and also in the category (O), expected number in the category (E), Ratio of enric value from hypergeometric test (rawP), and p value adjusted by the multiple test adjustment (adjP). Finally, the pathway name is linked to KEGG where the user ids are highlighted, the nu gene ids is linked to a table with information about the user ids, and the Entrez IDs are linked to Entrez Gene

Neuroactive ligand-receptor interaction	5	14816 12921 14652 319229 93896	C=277;O=5;E=0.06;R=87.04;rawP=1.87e-09;adjP=3.74e-09
Long-term depression	2	14816 12921	C=72;O=2;E=0.01;R=133.94;rawP=1.00e-04;adjP=1.00e-04

Figure 34 KEGG Pathway Analysis Results

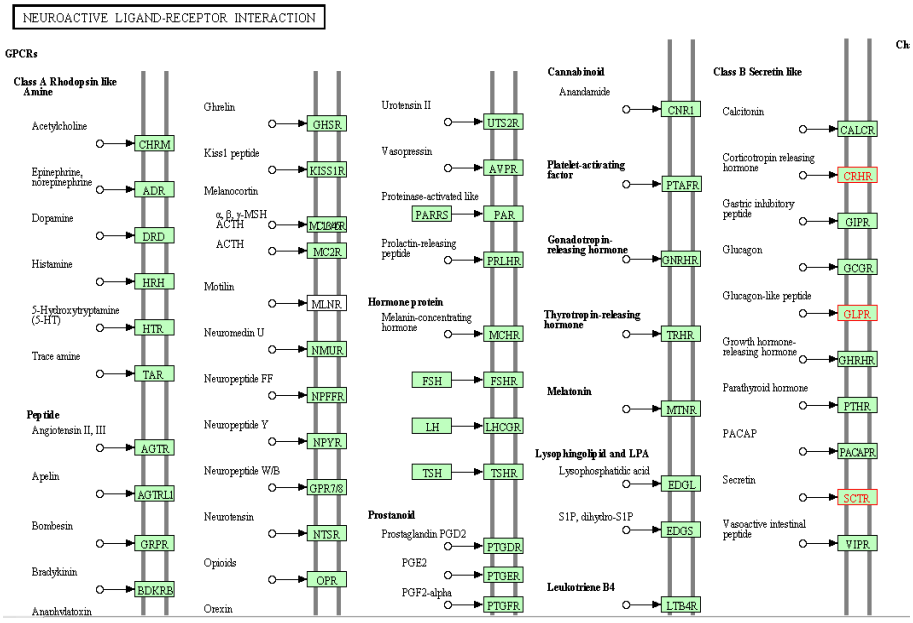


Figure 35 Neuroactive ligand-receptor interactions

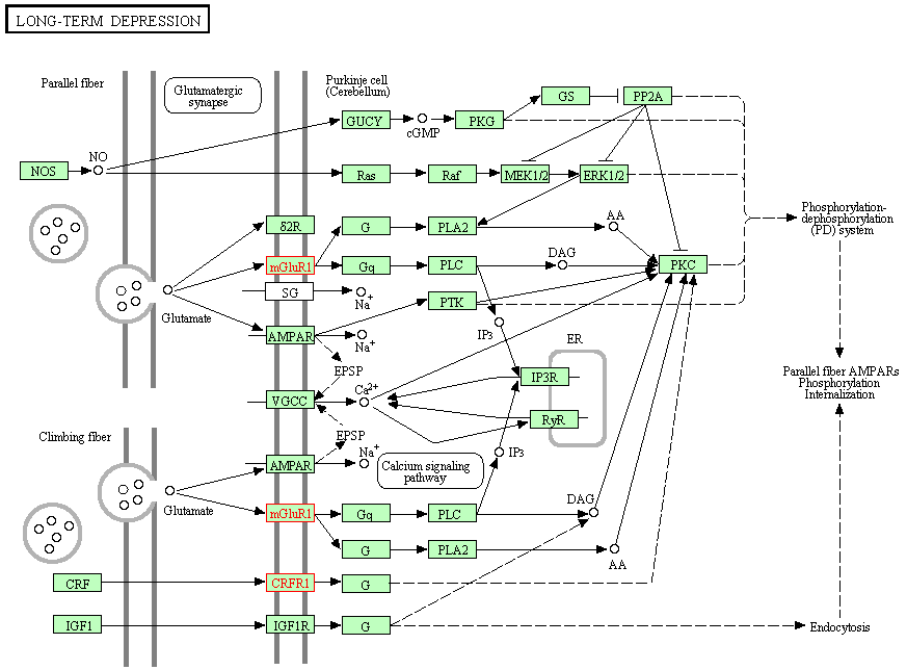


Figure 36 Long-Term Depression

3.2. q-RT-PCR Results

For determination of the efficiency of q-RT-PCR reactions, calculated efficiencies by Rotor-Gene Q Series Software on slope of standard curves are used.

R^2 represented on the standard curves describes the proportion of the variability in a q-RT-PCR reaction. When R^2 value reaches to 1, variance of the reaction decreases means a better reaction.

The Ct value is the threshold cycle, the increase that can be detected in the amount of fluorescence. Starting template amount for reaching the expression can be calculated by Ct value.

In the melt curves, T_m values depend on the length of DNA sample that is used. Different peaks in different temperatures show different DNA samples.

In order to calculate relative changes, $2^{-\Delta\Delta Ct}$ formula is used as described in the study of Livak & Schmittgen, in 2001.

3.2.1. Results for *Bai 3* gene

Samples (**Figure 37**) are represented in colors in the quantitation graph and melting curve for *Bai3* gene.

C	ID	Name	Type
	1	n2a-bai3	Standard
	2	n2a-bai3	Standard
	3	n2a-bai3	Standard
	4	n2a-bai3	Standard
	5	n2a-bai3	Standard
	6	n2a-bai3	Standard
	7	ntc	NTC
	8	n2a-gapdh	Standard
	9	n2a-gapdh	Standard
	10	n2a-gapdh	Standard
	11	n2a-gapdh	Standard
	12	n2a-gapdh	Standard
	13	n2a-gapdh	Standard
	14	ntc	NTC
	15	att20-bai3	Standard
	16	att20-bai3	Standard
	17	att20-bai3	Standard
	18	att20-bai3	Standard
	19	att20-bai3	Standard
	20	att20-bai3	Standard
	21	ntc	NTC
	22	att20-gapdh	Standard
	23	att20-gapdh	Standard
	24	att20-gapdh	Standard
	25	att20-gapdh	Standard
	26	att20-gapdh	Standard
	27	att20-gapdh	Standard
	28	ntc	NTC

Figure 37 Samples with color interpretations

Fluorescence levels of all N2a derived *Bai3* samples and no template controls (NTC) are depleted in 10 cycles due to lack of products as expected (**Figure 38**).

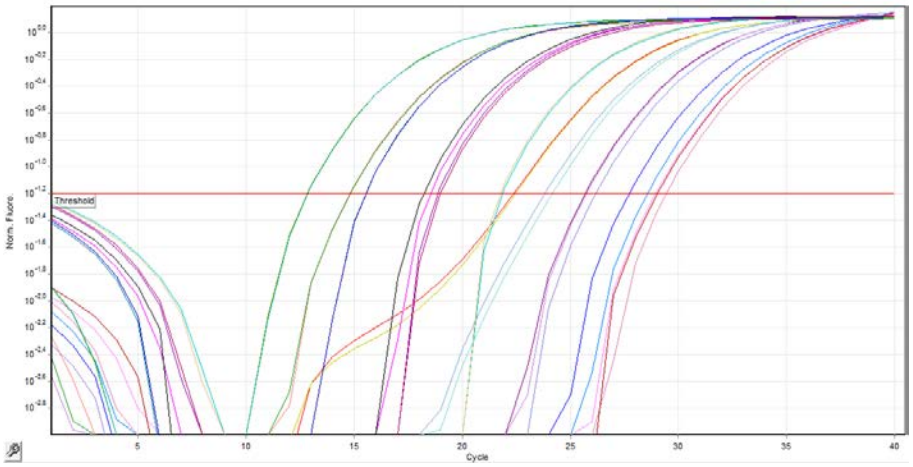


Figure 38 Quantitation of *Bai3* concentrations

In the melt curve of *Bai3* gene, two peaks are observed. The first peak is around 78° which belongs to AtT-20 *Bai3* samples. This means all AtT-20 derived Bai3 samples have a single product because there is a single unique peak. All GAPDH samples obtained from both N2a and AtT-20 cell lines gave a second single peak around 87°. That means they have a single unique product for GAPDH gene.

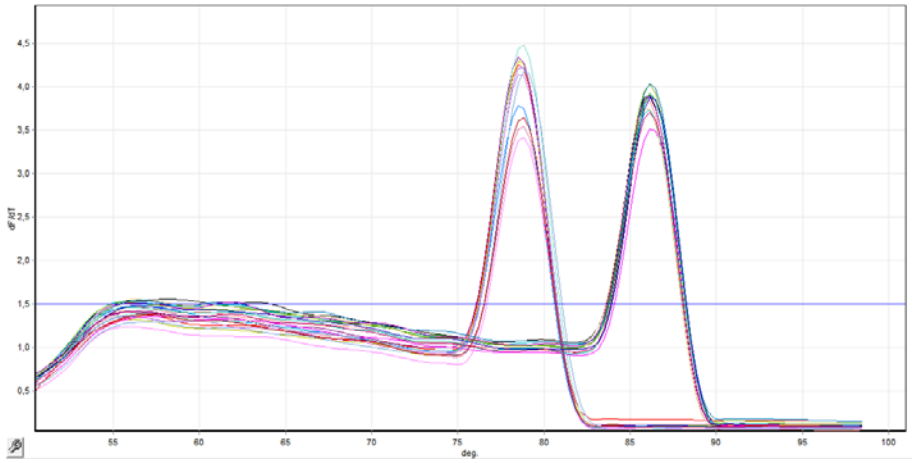


Figure 39 Melt Curve of *Bai3*

Standard curve for Bai3 gene shows that sample duplicates are close each other by means of concentration. Moreover, concentration dilutions are

successfully made which can be seen from R value (**Figure 40**). It is, the Pearson Coefficient of Determination, the value indicating how well the data fits a line or curve. The closer the value is to 1, the better the data fits the curve. Here, R value is 0.9778290. That indicates dilutions were successfully made. In order to say effective to this q-RT-PCR run, the efficiency value should be between 90–100%, which means slope should be within the range of (−3.6, −3.3). If the efficiency is 100% in a perfect run, the CT values of the 10 fold dilution will be 3.3 cycles apart due to a 2-fold change for each change in CT. Therefore, if the slope is below –3.6, then the PCR has poor efficiency. Here, slope of standard curve is -6.35, which means that the efficiency is low for this q-RT-PCR run. It is also indicated as E value, which is 0.43. That means the efficiency of the run is 43%, which is low. This could be due to the replicates quality, which is pretty high in this case, indicating a good precision. There is one outlier in the bottom probably causing the deviation. If it is omitted, more efficient standard curve can be obtained.

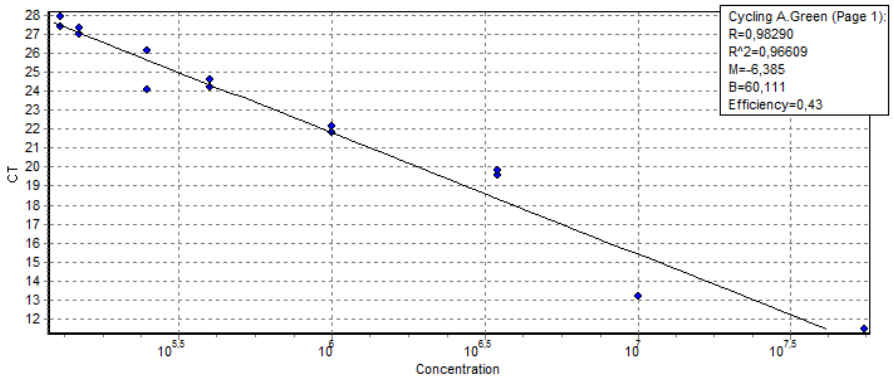


Figure 40 Standard Curve of *Bai3*.

In **Figure 41**, agarose gel electrophoresis result is seen for Bai3 gene products. Loading information of the samples is found in **Table 8**. According to agarose gel electrophoresis result, we see two distinct bands. Lower product is around on the line of 150, and bigger product is

around 500bp. Bai3 gives 159bp-product and GAPDH should give 496bp-product based on primer design, which is held previously. They perfectly match with what is expected theoretically.

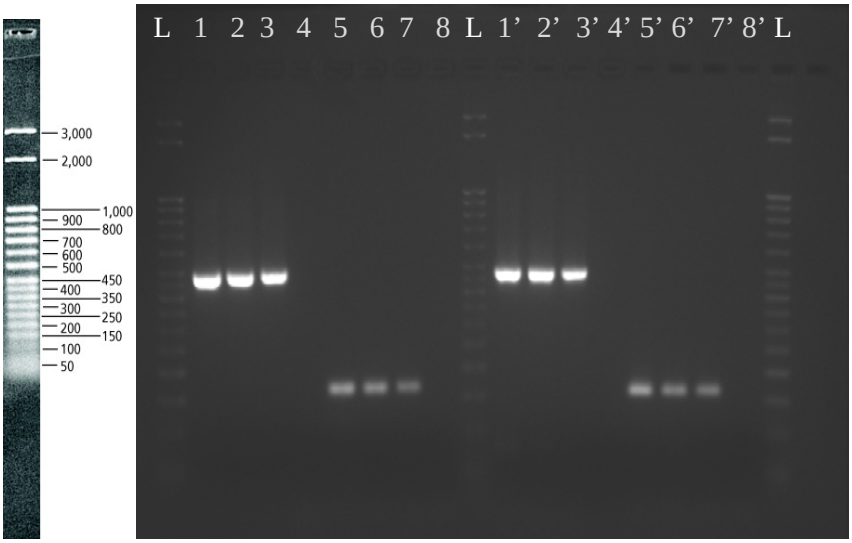


Figure 41 Gel view of *Bai3* and GAPDH q-RT-PCR products. **Table 7** shows content of each lane.

Table 7 Bai3 and GAPDH products loaded in agarose gel

Sample #	Sample Name	Product Length (bp)
1	1:10 dilution AtT-20 GAPDH product	~ 500
2	1:100 dilution AtT-20 GAPDH product	~ 500
3	1:1000 dilution AtT-20 GAPDH product	~ 500
4	NTC of AtT-20 GAPDH product	-
5	1:10 dilution AtT-20 Bai3 product	~150
6	1:100 dilution AtT-20 Bai3 product	~150
7	1:1000 dilution AtT-20 Bai3 product	~150
8	NTC of AtT-20 Bai3 product	-
1’	1:10 dilution N2a GAPDH product	~ 500
2’	1:100 dilution N2a GAPDH product	~ 500
3’	1:1000 dilution N2a GAPDH product	~ 500
4’	NTC of N2a GAPDH product	-
5’	1:10 dilution N2a Bai3 product	~150
6’	1:100 dilution N2a Bai3 product	~150
7’	1:1000 dilution N2a Bai3 product	~150
8’	NTC of N2a Bai3 product	-

Here, the standard curve and gel view of *Bai3* gene are examined in detail as an example. Rest of the genes that are examined for this thesis study can be found in **Appendix A**.

3.2.2. Fold Changes of Selected Gene Expressions

Fold change graph is structured via “GraphPad Prism Version 6.02”. GAPDH gene is used for relative gene expression calculations. When the fold changes of 12 genes are compared, it is seen that *Bai1, Casr, Fzd10, Glp1r, Glp2r, Gpr116, Gpr133, Grm1* and *Sctr* are significantly expressed in AtT-20 cell line as conforming microarray data analysis (Data are mean ± SEM from 2 cell lines per gene group, $p < 0.001$).

However, *Bai3*, *Crhr1* and *Gpr113* are not as it is expected as highlighted with red asterisks (**Figure 42**). *Bai3* and *Crhr1* genes are expressed in both N2a and AtT-20 cell lines. On the other hand, Gpr113, interestingly, is expressed in N2a but not in AtT-20 cell line.

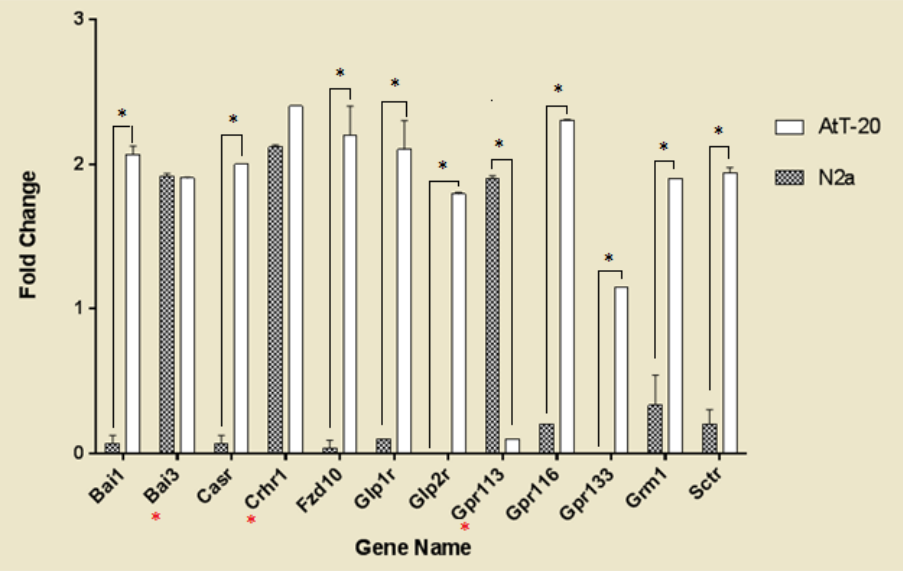


Figure 42 Fold Changes of Selected Genes (mean ± SEM from N2a cell line and AtT-20 cell line per gene group, $p < 0.001$)

In microarray data there were nearly 72000 GPCR and GPCR-related genes. Since the microarray data solely cannot be counted as solid evidence, these q-RT-PCRs are carried out.

In microarray study, the cell line that was used is N18. N18 is a cell line of mouse neuroblastoma and rat glioma hybrid. However, the study revealed by Kuhar *et al.* claimed that the cell line not responding to CART are N2a, HEK293 and not differentiated PC12 cell lines. In order to perform this study and make a comparison between cell lines, obtained mRNAs should be from the same organism, to make a conclusion. Therefore, due to derivation from *Mus musculus* N2a and AtT-20 cell

lines are used. Moreover, although HEK293 cell line both takes part in western blot and microarray studies as CART nonresponsive cell line, it is derived from human. Therefore, a proper comparison was not possible in AtT-20 cell line. As a result, these three genes *Bai3, Crhr1* and *Gpr113* might have given such different expressions from the microarray data.

Nevertheless, in order to be a CART receptor candidate, selected genes should be expressed in AtT-20 cell line while not being expressed in N2a cell line. Thus, *Bai3*, *Crhr1* and *Gpr113* are eliminated for being the candidate of CART's receptor at the end of this analysis.

Also, it should not be forgotten to mention that lack of mRNAs doesn't indicate the lack of translated protein. mRNAs might have been degraded shortly after their synthesis. However, it is possible to say that present mRNAs indicate that a specified protein is translated from its mRNA. Therefore, presence of mRNAs that are showed in this study indicates the protein presence.

CHAPTER 4

CONCLUSION

In this study, for the determination of CART receptor candidates; 12 genes are selected via microarray data analysis. These genes are: *Bai1, Bai3, Casr, Crhr1, Fzd10, Glp1r, Glp2r, Gpr113, Gpr116, Gpr133, Grm1* and *Sctr. Bai1, Bai3, Crhr1, Glp1r, Glp2r, Gpr113, Gpr116, Gpr133* and *Sctr* are from B Class *o*GPCRs, *Casr* and *Grm1* from C Class *o*GPCRs, Fzd10 is from F/S Class *o*GPCRs.

Due to being a small peptide, CART's receptor might be one of *o*GPCRs except the A Class. A Class GPCRs are huge receptors in size. Nevertheless, this does not entirely eliminate the probability that CART peptide has an A Class GPCR.

In microarray study, the cell lines that were used are HEK293, N18, AtT-20, and BV2. As mentioned previously, CART responsive cell lines were AtT-20 and differentiated PC-12 cells. Non responsive cell lines for CART are N2a, undifferentiated PC12, GH3, CATII and HEK293 cells. As CART responsive cell line AtT-20, as nonresponsive cell line N2a are used.

After q-RT-PCR studies carried out, it is found out that *Bai1, Casr, Fzd10, Glp1r, Glp2r, Gpr116, Gpr133, Grm1* and *Sctr* genes are expressed in high amount in AtT-20 cell line, while *Bai3* and *Crhr1* show low expression in N2a cell line. Interestingly, *Gpr113* is expressed in N2a, but not expressed in AtT-20 cells.

To conclude, *Bai1, Casr, Fzd10, Glp1r, Glp2r, Gpr116, Gpr133, Grm1* and *Sctr* genes can be candidates for CART's receptor(s). It is also possible that there can be more than one receptor for the CART peptide.

For further studies, these genes that are determined can be tested. After cloning the candidates to N2a cell line, it is possible to investigate CART's response via western blotting. Likewise, if a modified version of CART peptide can be synthesized, it is possible to pull down the receptor-peptide complex and analyze it for further.

REFERENCES

Alexander-Thomas, H. &. (2011, July). Chemical probes: Sharpen your epigenetic tools. *Nat Chem Biol 7* , pp. 499-500.

Atwood, B., Lopez, J., Wager-Miller, J., Mackie, K., & Straiker, A. (2011). Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* , pp. 1-14.

Bouvier, M. (2013, May 20). Unraveling the structural basis of GPCR activation and inactivation. *Nature Structure Moleculer Biology* , pp. 539-41.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., et al. (2001). Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. *Nature Genetics 29* , pp. 365 - 371 .

Bruno, A., Costantino, G., Fabritiis, G., Pastor, M., & Selent, J. (2012, August). Membrane-Sensitive Conformational States of Helix 8 in the Metabotropic Glu2 Receptor, a Class C GPCR. *PloS One, Volume 7, Issue 8* , pp. 1-9.

Chang, G. W. (2003). Proteolytic cleavage of the EMR2 receptor requires both the extracellular stalk and the GPS motif. *FEBS Lett 547* , pp. 145–150.

Dominguez, G., Lakatos, A., & Kuhar, M. (2002). Characterization of the cocaine- and amphetamine-regulated transcript (CART) peptide gene promoter and its activation by a cyclic AMP-dependent signaling pathway in GH3 cells. *Journal of Neurochemistry (80)* , pp. 885-893.

Driessler, F., & Baldock, P. A. (2010, October 1). Hypothalamic Regulation of Bone. *Journal of Molecular Endocrinology 45* , pp. 175-181.

Elefteriou, F., Ahn JD, J., Takeda, S., Starbuck, M., Yang, X., Liu, X., et al. (2005). Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature 434* , pp. 514–520.

Filipek, S. S. (2003). G protein-coupled receptor rhodopsin: a prospectus. *Annual Reviews Physiology, 65* , pp. 851– 879.

Filmore, D. (2004). It's a GPCR world. *American chemical society modern drug discovery* , pp. 24-28.

Foord, S. M., Jupe, S., & Holbrook, J. (2002). Bioinformatics and type II G-protein-coupled receptors. *Biochemical Society Transactions Volume 30, part 4* , pp. 473-479.

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrinology Revision 2* , pp. 90– 113.

Goeders, N. E. (2003). The impact of stress on addiction. *Neuropsychopharmacol* , pp. 13:435–41.

International HapMap Project. (n.d.). *Bead Arrays*. Retrieved August 16, 2013, from NCBI: http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechBeadArray.shtml

Jones, D., Lakatos, A., & Vicentic, A. (2006). The CART receptors: Background and recent advances. *P e p t i d e s 2 7* , pp. 1 9 34 – 1 93 7.

Kim, J. H. (1996). Glutamate residues in the second extracellular loop of the human A2a adenosine receptor are required for ligand recognition. *Molecular Pharmacology 49* , pp. 683– 691.

Kobe, B. &. (2001, December). The leucine-rich repeat as a protein recognition motif. *Current Opinion in Structural Biology* , pp. 725–732.

Kolakowski, J. L. (1994). GCRDb: a G protein-coupled receptor database. *Recept Channel 2* , pp. 1–7.

Koylu, E. O., Balkan, B., Kuhar, M. J., & Pogun, S. (2006, July 5). Cocaine and amphetamine regulated transcript (CART) and the stress response. *p e p t i d e s 2 7* , pp. 1 9 56 – 1 96 9.

Koylu, E., Balkan, B., Kuhar, M., & Pogun, S. (2006). Cocaine and amphetamine regulated transcript (CART) and the stress response. *Peptides (27)* , pp. 1956-1969.

Kristiansen, K. (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Science Direct - Pharmacology & Therapeutics,103* , pp. 21– 80.

Kroeze, W. K. (2002). Molecular biology of serotonin receptors structure and function at the molecular level. *Top Med Chem 2* , pp. 507– 528.

Kuemmerle, J. F. (2001). Coupling of the insulin-like growth factor-I receptor tyrosine kinase to Gi2 in human intestinal smooth muscle: Gbetagamma-dependent mitogen-activated protein kinase activation and growth. *J Biol Chem* , pp. 276, 7187–7194.

Kuhar, M., Adams, G., Dominguez, J., Jaworski, J., & Balkan, B. (2002). CART peptides. *NEuropeptides 36* , pp. 1-8.

Kuhar, M., Lakatos, A., Prinster, S., Vicentic, A., & Hall, R. (2005). Cocaine- and amphetamine-regulated transcript (CART) peptide activates the extracellular signal-regulated kinase (ERK) pathway in AtT20 cells via putative G-protein coupled receptors. *Neuroscience Letters (384)* , pp. 198–202.

Kuhar, M., Lin, Y., & Hall, R. (2011, August 19). CART peptide stimulation of G protein-mediated signaling in differentiated PC12 cells: identification of PACAP 6-38 as a CART receptor antagonist. *Neuropeptides* , pp. 351-8.

Lane, J., & Canals, M. (2012, November). Sequential conformational rearrangements dictate the dynamics of class C GPCR activation. *Science Signalling* .

Langer, I. V. (2003). Lysine 195 and aspartate 196 in the first extracellular loop of the PAC1 receptor are essential for high affinity binding of agonists but not of antagonists. *Neuropharmacology 44* , pp. 125–131.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 22DDCT Method. *METHODS 25* , pp. 402–408.

Macfarlane, S. R. (2001). Proteinase-activated receptors. *Pharmacology Review 53,* , pp. 245–282.

Oikawa, M. S. (2013, March). Effects of protease-activated receptors (PARs) on intracellular calcium dynamics of acinar cells in rat lacrimal glands. *Histochemistry and Cell Biology* , pp. DOI 10.1007/s00418-013-1082-0.

Okada, T. F. (2002). Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proc Natl Acad Sci USA 99* , pp. 5982– 5987.

Pacak, K., & Palkovits, M. (2001). Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocrinology Reviews* , pp. 22:502–48.

Pantages, E., & Dulac, C. (2000). A novel family of candidate pheromone receptors in mammals. *Neuron, Vol 28, Issue 3* , pp. 835–845.

Pfaffl, M. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Oxford Journals Nucleic Acids Research* , pp. Volume 29, Issue 9 45.

Piazza, P., & Le Moal, M. (1998). The role of stress in drug selfadministration. *Trends Pharmacological Sciences* , pp. 19:67–74.

Pierce, K. L. (2002). Seven-transmembrane Receptors. *Nat Rev Mol Cell Biol 3* , pp. 639–650.

Roth, C., & Yarmush, M. (1999). Nucleic acid biotechnology. *Annual Review of Biomedical Engineering 1* , pp. 265-297.

Sakmar, P. T. (2011, August). Clicking class B GPCR ligands. *Nature Chemical Biology Vol 7* , pp. 500-502.

Tang, X. W. (2012, Feb 27). Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. *Acta Pharmacologica Sinica 33* , pp. 363–371.

Thim, L., Kristiensen, P., Wulff, B., & Clausen, J. (1999). Tissue Specific Processing of Cocaine and Amphetamine Regulated Transcript Peptides in Rat. *Proc Natl Acad Sci USA* , s. 2722-2727.

Vischer, H. F. (2003). Ligand selectivity of gonadotropin receptors. Role. *Biol Chem 278* , s. 15505– 15513.

Wang, J., Duncan, D., Shi, Z., & Zhan, B. (2013). WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Research 41(Web Server issue):W77* .

Wierup N, K. M. (2004, Feb). Cocaine- and amphetamine-regulated transcript (CART) is expressed in several islet cell types during rat development. *J Histochem Cytochem.* , pp. 52(2):169-77.

Zhang, M., Han, L., & Xu, Y. (2012, Jun). Roles of cocaine- and amphetamine-regulated transcript in the central nervous system. *Clinical and Experimental Pharmacology Physiology 39(6)* , pp. 586-592.

APPENDIX

A

A1. Casr

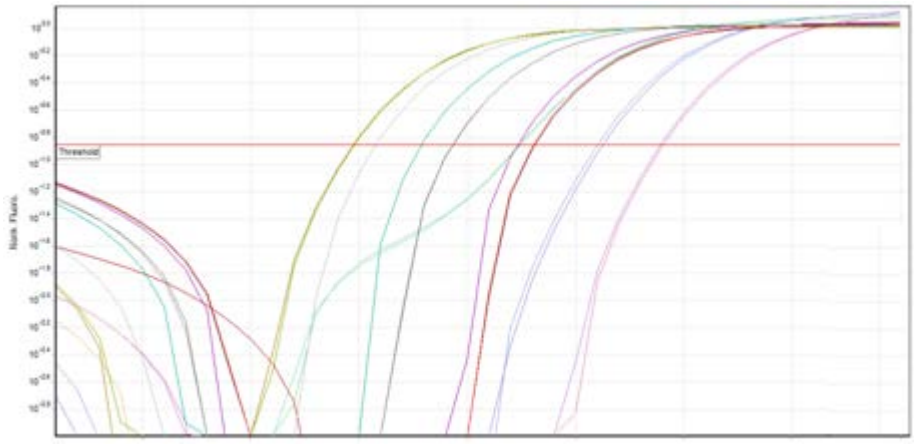


Figure 43 Quantitation of *Casr* concentration

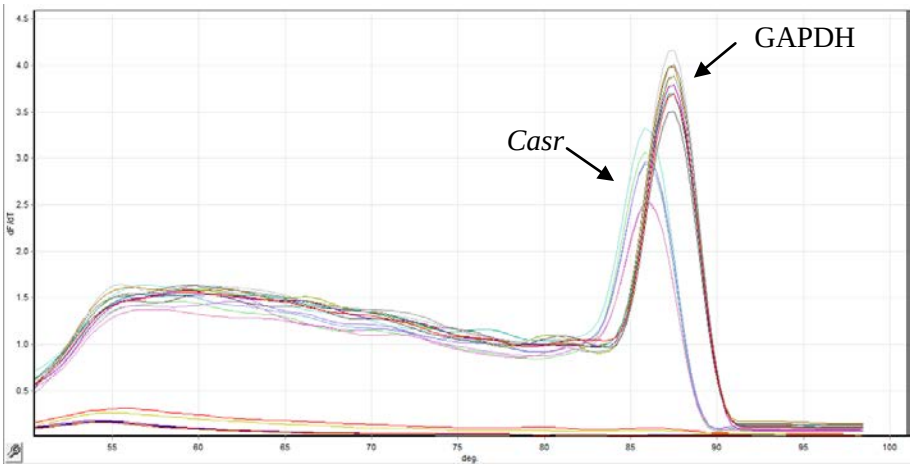


Figure 44 Melt Curve of *Casr*

A2. Crhr1

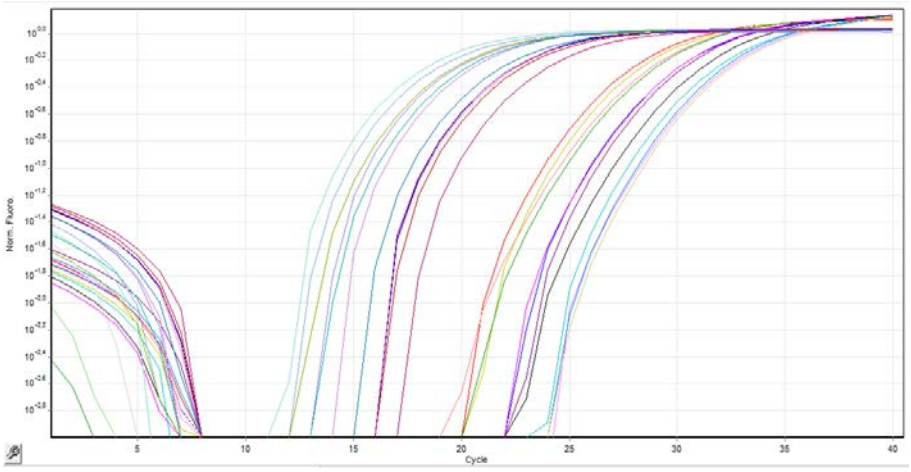


Figure 45 Quantitation of *Crhr1* concentration

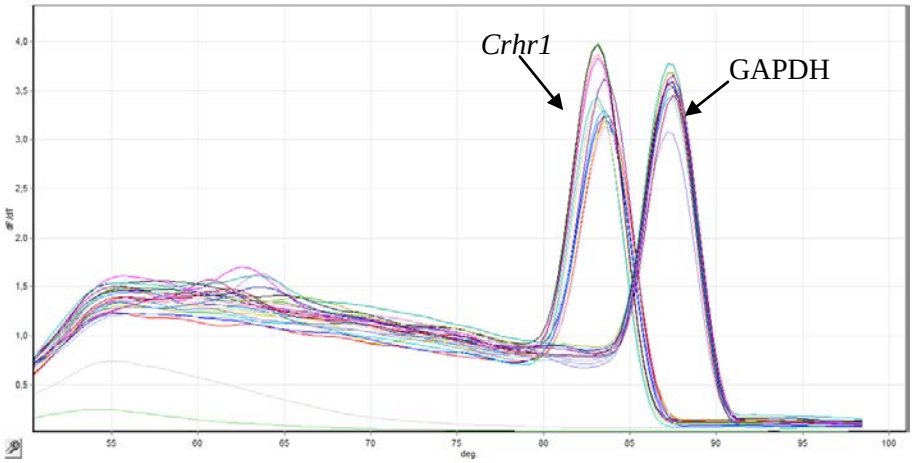


Figure 46 Melt Curve of *Crhr1*

A3. Fzd10

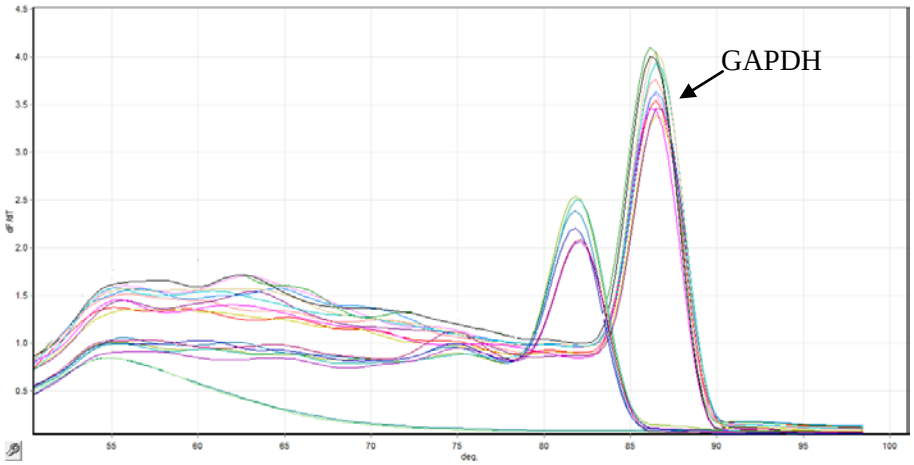


Figure 47 Quantitation of *Fzd10* Concentration

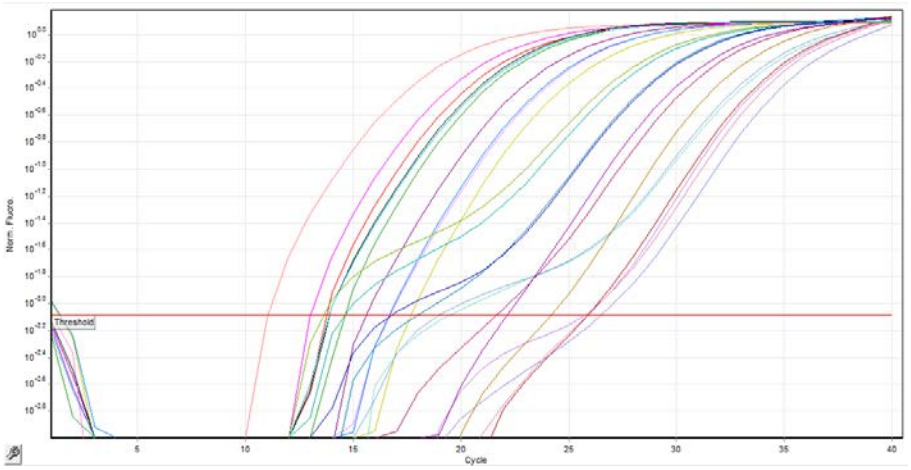


Figure 48 Melt Curve of *Fzd10*

A4. Glp1r

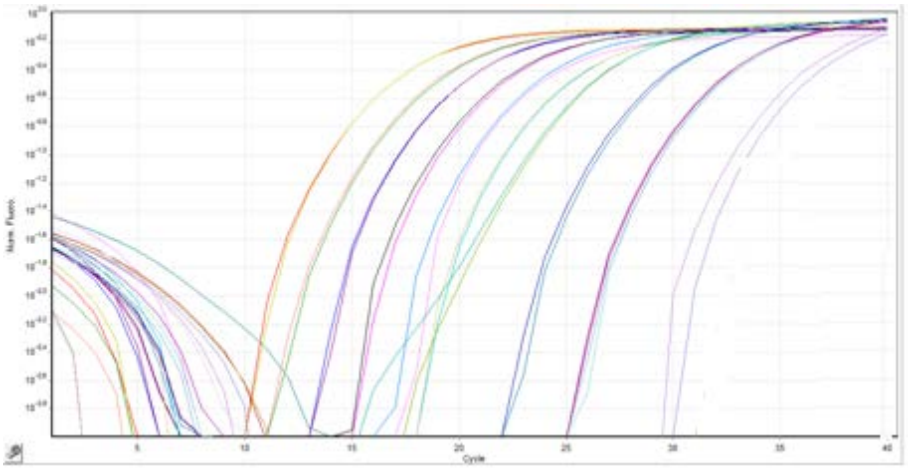


Figure 49 Quantitation of *Glp1r* Concentration

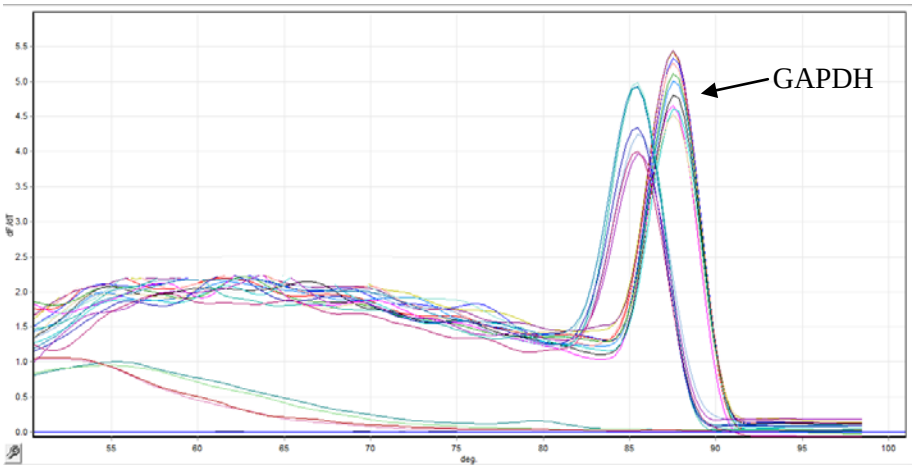


Figure 50 Melt Curve of *Glp1r*

A5. Glp2r

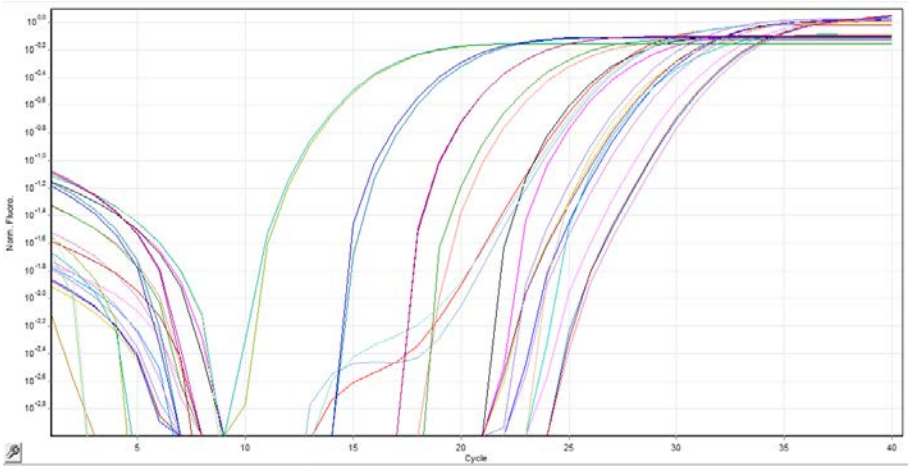


Figure 51 Quantitation of *Glp2r* Concentration

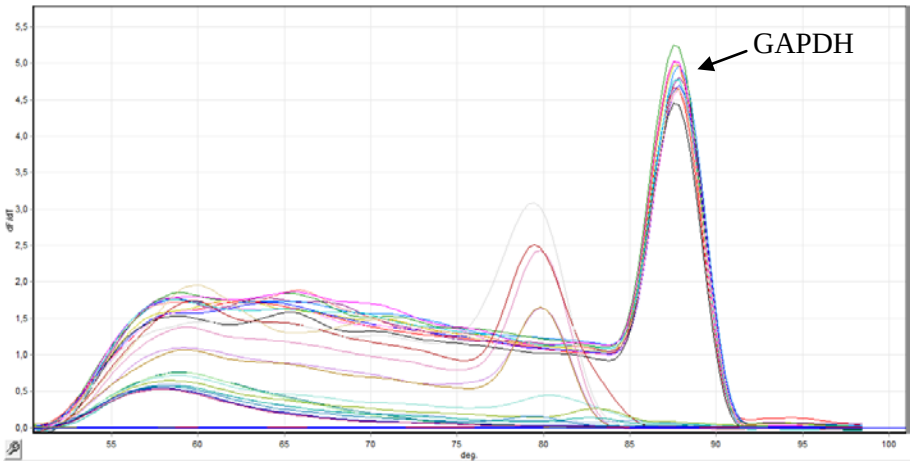


Figure 52 Melt Curve of *Glp2r*

A6. Gpr113

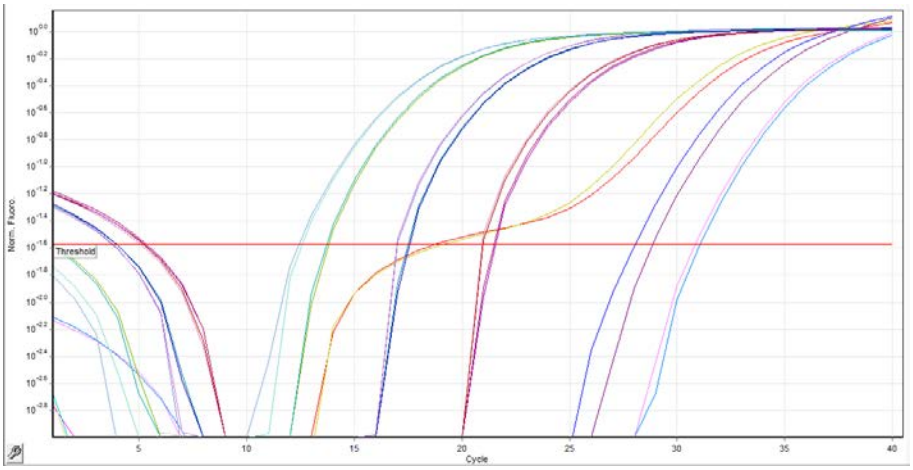


Figure 53 Quantitation of *Gpr113* Concentration

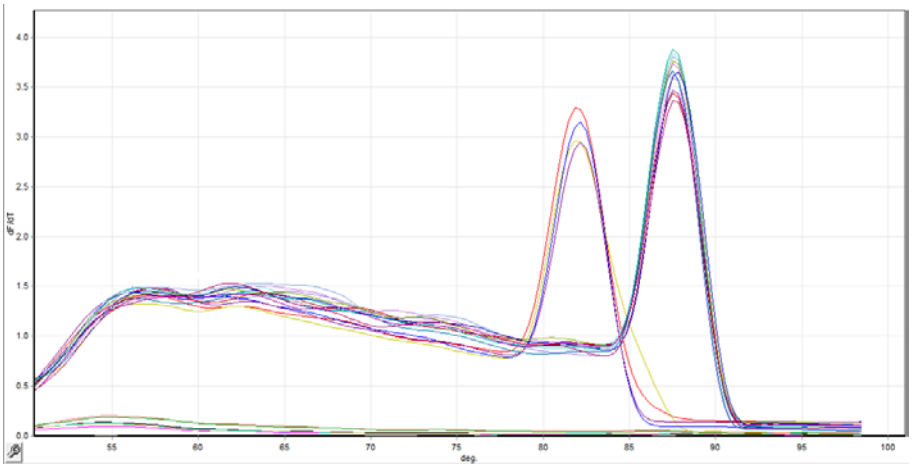


Figure 54 Melt Curve of *Gpr113*

A7. Gpr116

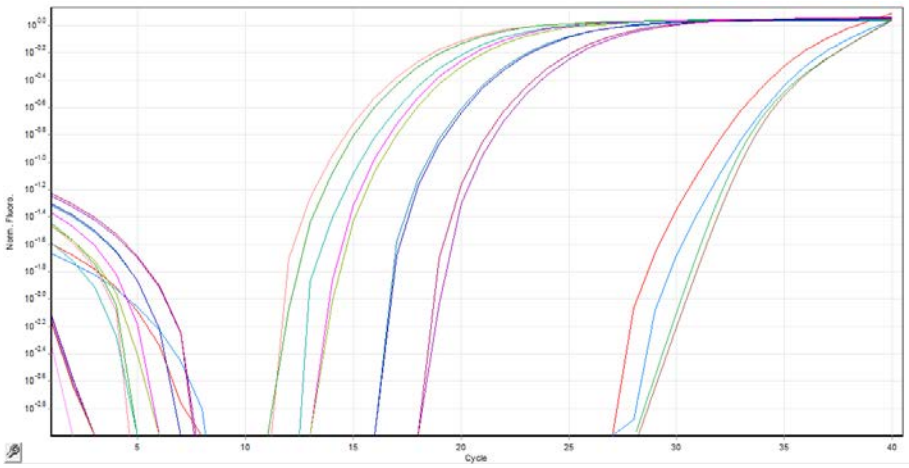


Figure 55 Quantitation of *Gpr116* Concentration

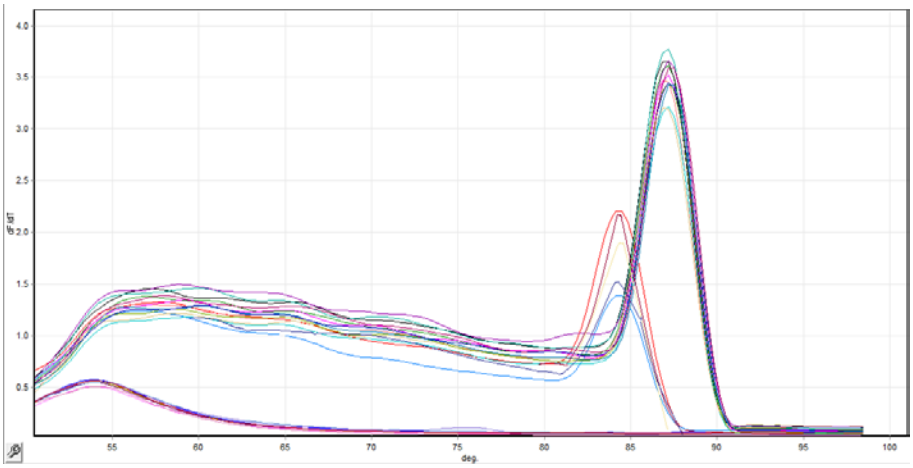


Figure 56 Melt Curve of *Gpr116*

A8. Gpr133

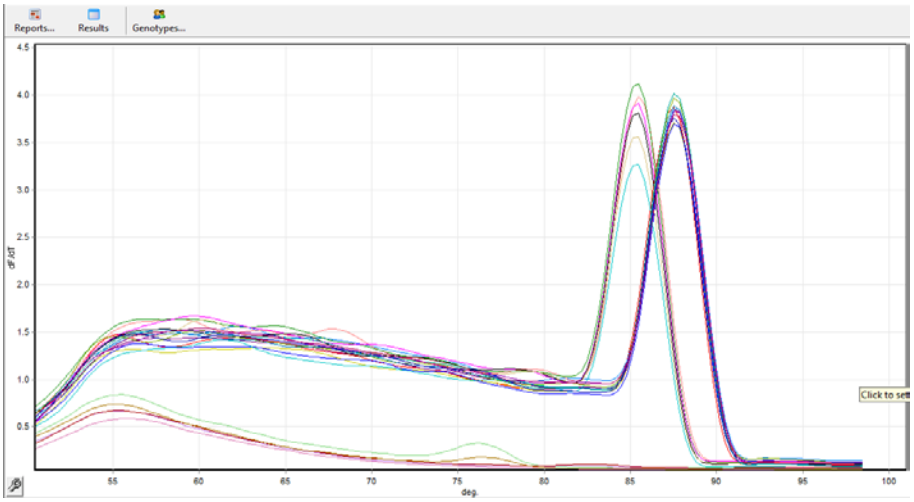


Figure 57 Quantitation of *Gpr133* Concentration

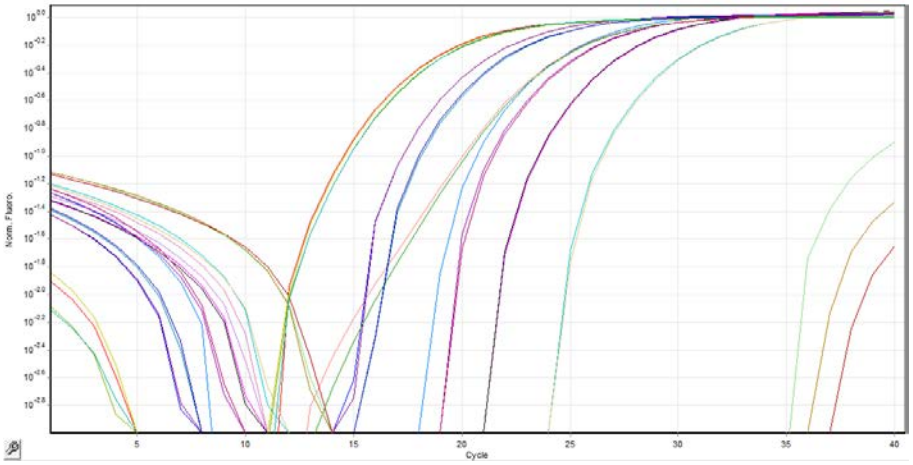


Figure 58 Melt Curve of *Gpr133*

A9. Grm1

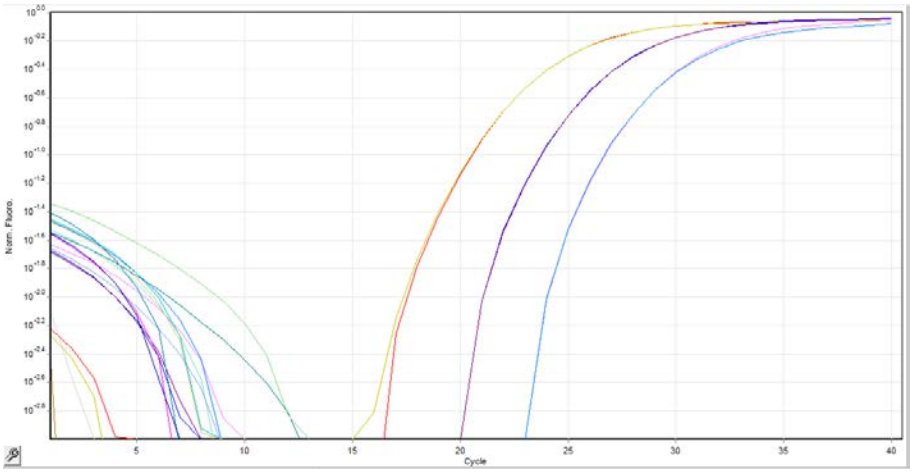


Figure 59 Quantitation of *Grm1* Concentration

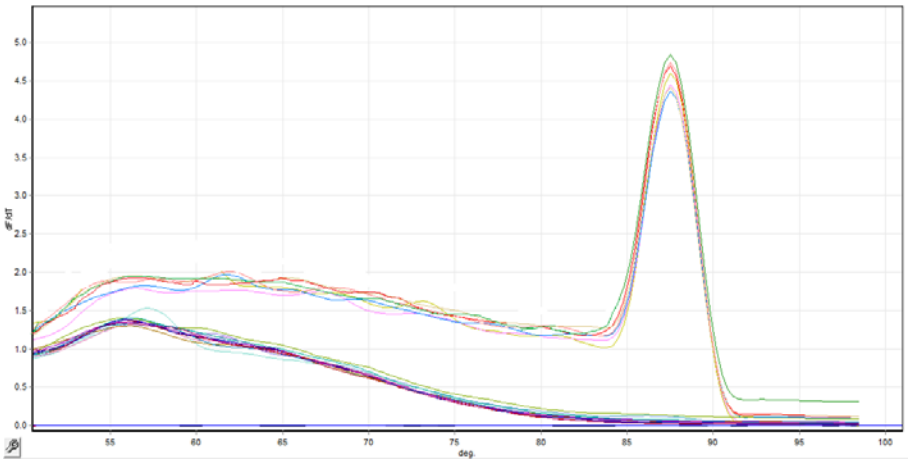


Figure 60 Melt Curve of *Grm1*

A10. Sctr

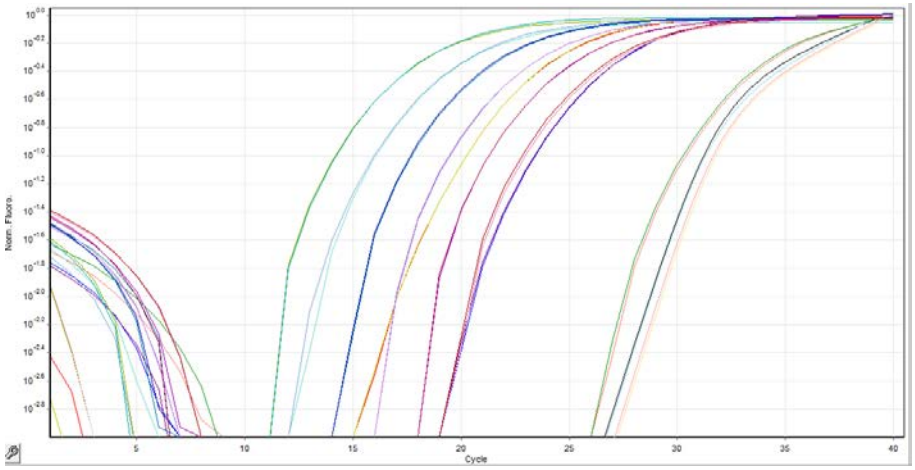


Figure 61 Quantitation of *Sctr* Concentration

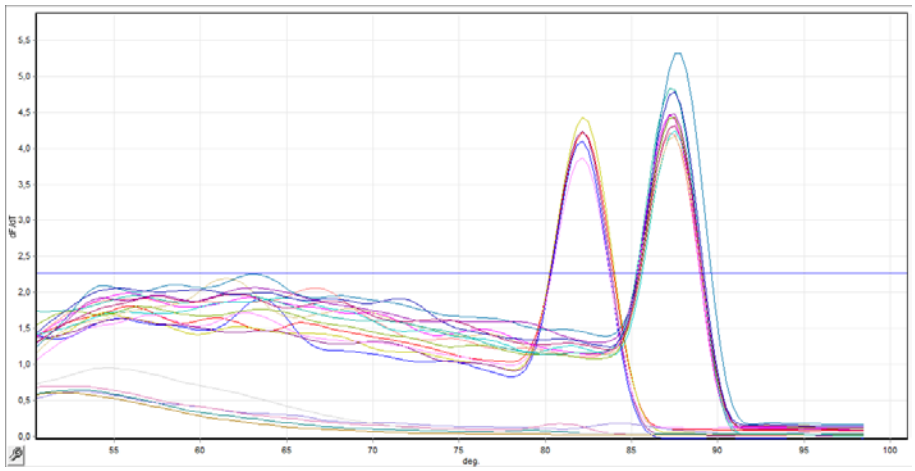


Figure 62 Melt Curve of *Sctr*