

Taper off treatment can improve executive functions and regulate expression of *BDNF* and *5HTTPLR* genes in opium abusers after six months period

Ani Dezhakam

Congress 60 Non-governmental organization

Hossein Dezhakam

2) Congress 60 Non-governmental organization

Arvin Haghighatfard (✉ arvinland@yahoo.com)

Khwarizmi Institute of science and technology <https://orcid.org/0000-0002-2179-9822>

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Abstract

Opium is the dried latex obtained from the opium poppy. Opium addiction is the most prevalent addiction in Iranian society. During the last two decades "congress 60" a nongovernmental organization, has been performed a taper off treatment of opium associated with a package of psychological treatment group classes. While the effectiveness of taper off method in opium addiction has been confirmed, molecular mechanisms of this treatment in addicts are not clarified. *BDNF* gene is a brain-derived neurotrophic factor which is related to several molecular mechanisms of the brain including memory. 5-*HTTPLR* is a transporter of serotonin that is related to the decision-making process.

In present study mRNA level of *BDNF* and 5-*HTTPLR* genes in peripheral blood of 20 non-psychiatric persons and 79 opium addicted individuals before and after six months period of taper off treatment was examined by using Real-Time PCR and confirmed by protein level analysis using Western blotting. In addition, executive functions including memory and decision making were analyzed in all participants.

Results showed significant down expression of *BDNF* and 5-*HTTPLR* in addict persons vs. non psychiatric persons. Also significantly increase detected in *BDNF* ($p < 0.01$) and 5-*HTTPLR* ($p < 0.01$) expression level in addicts after six months of therapy period. Similar results with gene expression results revealed in protein level analysis. Also, significant improvements in memory and decision making were revealed in addicts after therapy and these improvements were correlated with the expression level of *BDNF* and 5-*HTTPLR*.

Findings revealed the effect of opium abuse and taper off treatment on the expression of *BDNF* and 5-*HTTPLR*. In addition, the association of *BDNF* mRNA level with psychological states of addict's individuals detected. Results of the present study may help to a better understanding of molecular and neuropsychological mechanisms of opium addiction and taper off treatment. Also, present *BDNF* and 5-*HTTPLR* as potential markers for screening the effectiveness of different kinds of addiction treatment.

Introduction

Addiction is a chronic and severe psychiatric disease that may cause major health, social and economic problems and causing 12.4% of deaths worldwide. Opioids are a class of addictive drugs that affect the nervous system and use to produce euphoric feelings as well as pain relief .(1)

Opioid addiction is characterized by a powerful, compulsive urge to use opioid drugs. Individuals who become addicted may prioritize drug abuse over all other activities in their lives that in turn may impact badly to their professional and personal life (2). Methadone maintenance treatment (MMT) is the most widely used treatment for opiate addiction that is designed to normalization of many physiological abnormalities caused by chronic use of short-acting opiates (3). MMT have potential side effects such as methadone dependence, low bone density, and even hepatotoxicity(4).

Addictions heritability in different drugs and behaviors are different but ranged from 39 to 72 percent; which means that addiction could consider as a multifactorial genetic disease (5). Animal studies helped better understanding addiction neurobiology and the identification of several genes that mediate variation in drug preference and response. Several physiological and psychological pathways were found to euphoric and addictiveness of opioids. Most of these pathways are related to dopamine signaling. By the way, it is not completely clear that why the tendency to a drug, severity of addictive behavior, and abstaining traits are different between people (6). The neurobiological pathways that modulate reward, stress resiliency and behavior inhibition are among those that also underlie general addiction liability .(7)

Studies of polymorphisms in the endogenous genes of opioid and monoaminergic systems, which encode the receptor target of some endogenous opioids, heroin, or morphine such as μ opioid receptor, have contributed substantially to knowledge of genetic influences on opiate addiction (8). Variants of dopamine β -hydroxylase and dopamine, serotonin, and

norepinephrine transporters have also been reported related to the neurobiology of addiction as well as anxiety and stress (9, 10).

Brain-derived neurotrophic factor (*BDNF*) regulates the survival and growth of neurons and influences synaptic efficiency and plasticity. The human *BDNF* genes located in 11p14.1 consists of 11 exons, and alternative promoters and splicing produces distinct *BDNF* transcripts (11). Most of the *BDNF* transcripts can be detected not only in the brain but also in the blood cells, and make *BDNF* a potential good peripheral biomarker for several psychiatric diseases, behavioral abnormalities, and neuronal activities (12, 13).

5-*HTTLPR* (serotonin transporter linked polymorphic region) is a degenerate repeat polymorphic region in the *SLC6A4* gene located in the 17q11.1-q12 human chromosome and encodes the serotonin transporter (14). 5-*HTT* variants are associated with depressive responses to life stress and several other psychological situations and traits (15). Independent lines of evidence were showed *SLC6A4* is significantly involved with decision-making processes the function that is strongly affected in addiction (16, 17).

Several treatment methods for opium addictions were not completely successful because these methods just focusing on the acute phase of illness and are not personalized. The personalized and targeted treatments based on genetic and neurobiological predictor biomarkers are highly needed to follow up the treatment methods during weeks and months (18). Molecular biology may help to reduce the side effects of treatments as well as choosing the personalized treatment methods for each subject by analysis of gene expression and protein level changes (19, 20).

During the last two decades "congress 60" a nongovernmental organization in the Islamic Republic of Iran, has been performed a taper off treatment of opium associated with a package of comprehensive psychological treatments, group classes, social caring, and harm reduction methods for patients and their family members. Opium addicts who became members of "congress 60" called passengers do not take any medication besides opium tincture (OT). During the 12 months, passengers will reduce the dosage of OT use while they are participating in the classes and group training of congress 60 which is including sports training, passing the courses about the reason of human tendency to addiction, and some cognitive-behavioral therapies. It is supposed that passengers finish the process and leave opium abuse completely after twelve months. More than 5000 Iranians were successfully finished the 12 months of treatments of congress 60 (called DST method) and cut the opium abuse until now. Five years follow-up in the DST method was shown one of the lowest rates of relapse in this method compared with other opioid addiction treatment methods. The present study aimed to examine the effectiveness of the DST method by using molecular biology and neuropsychological testing.

Material And Methods

Subjects recruitments and Samplings periods:

The present study is six months follow-up of addicts who registered for taper treatments in congress 60. In the beginning, 200 male subjects with opium addiction that were registered in congress 60, have been recruited. At the end of the study, 79 opium-addicted individuals who completed all the examinations before and after six months period of taper off-treatment were included in the data analysis. Twenty non-psychiatric male subjects from family members of addicts were recruited for the control group. The Control group completed only the first time examinations. Subjects with IQ total score (Wechsler Abbreviated Intelligence Scale, 1999) lower than < 80 were excluded from the study. The control group selected with age, race, somatic properties, socioeconomic situation, familial situation, and education matched to patients group and with no history of any psychological or somatic problem in their family. None of the subjects had a current or history of any severe medical condition, neurological disorder, history of head trauma with loss of consciousness, and any psycho-stimulant drug abuse and alcohol dependence. For better presentation of data test groups shoed by this order: N (normal group), C (addicts before admission in congress 60), and PC (addicts after six months of treatment in congress 60). All subjects have explained the purpose of the study and next, written informed consent has been provided.

Neuropsychological tests:

N-Back and spatial N-Back:

The n-back and spatial n-Back tasks are a continuous performance task that is commonly used to the examination of [working memory](#) and spatial working memory respectively. The n-back was introduced in 1958 and meta-analysis have shown that it could be used to analysis of brain region (dorsal [cingulate](#) and medial, premotor cortex, lateral [posterior parietal, etc.](#)) activities (21). In recent decades spatial n-Back a novel and simple task designed to measure spatial WM in adults and children (22). Participants were presented with a sequence of stimuli, and the task consisted of indicating when the current stimulus was matched the one from n steps earlier in the sequence.

Digit span:

The digit-span task is one of the subsets of [the Wechsler Adult Intelligence Scale](#) (WAIS) and was commonly used to measure [working memory](#)'s number storage capacity in psychiatric disorders and addiction (23, 24). All Subjects were sitting in an isolated room and hear a sequence of numerical digits and asked to recall the sequence correctly, with increasingly longer sequences being tested in each trial. Digit-span tasks were performed forwards and backward, to evaluate the verbal and spatial working memory respectively.

GO no GO:

Go/no Go testing refers to a pass/fail test to evaluate sustained [attention](#) and decision-making abilities (25). A computational form of the test had performed and subjects were using [binary classification](#). The test is passed only when the Go condition is met and also the No go condition fails.

Gene expression analysis by quantitative PCR:

Blood (5 ml) was collected from the cubital vein without a tourniquet between 8.00 and 10.00 AM. Total RNA was extracted from peripheral blood samples according to standard protocols using by RNA Purification kit (GeneJET™ RNA Purification Kit#K0732, Thermo scientific, Latvia). The cDNA was synthesized using a Transcription First Strand cDNA Synthesis Kit (RevertAid Premium First Strand cDNA Synthesis Kit #K1652, Thermo scientific, Latvia) according to the manufacturer's protocol. We performed quantitative RT-PCR by using CFX96 Touch Real-Time PCR Detection System (BIO-RAD, [California, United States](#)) and SYBR green kit (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) #K0221, Thermo scientific, Latvia) according to a previous study (26). Specific primers for target and reference genes were designed by "oligo7" software and blasted on the NCBI website. The GAPDH gene was used for normalization as an internal control gene.

Western blotting:

Lymphocyte pellets were thawed on ice and immediately lyzed in VRL buffer: 50 mM HEPES (pH 7.5), 250 mM sucrose, 5 mM MgCl₂, 100 mM KAc, 2 mM PMSF (all Sigma–Aldrich, Germany), 2x Pro-tease Inhibitor (Roche, Germany) supplemented with 1% TritonX-100, 1 mM PMSF (Sigma–Aldrich, Germany) and 40 U/mL DNaseI (Roche, Germany). After 40 min on ice, for DNA digestion the lysate was incubated for 40 min at 37°C. Then, the protein content of the lysate was detected by using the DC Protein Assay Kit (Bio-Rad, Germany) and for each sample, 30 micrograms of total protein were loaded onto 10% SDS-PAGE gels. After blotting, the membranes were directly treated with 100 mM KOH for 5 min at room temperature and subsequently blocked with 5% milk in PBS with 0.05% Tween-20 (PBST). Immunoreactivity was tested with the human anti-BDNF Antibody (#ANT-010) and human [anti-Serotonin Transporter \(SERT\)](#) (#AMT-004) that are highly specific antibodies. Analysis was done blind with regard to diagnosis. Protein level analysis was conducted based on previous studies (27).

Statistical analysis:

Descriptive data are expressed as mean \pm SD (range) and the level of statistical significance was set at $P < 0.05$. Compliance with normal distribution for continuous variables was assessed via the Kolmogorov-Smirnov test by using SPSS version 23. One-way ANOVA and Pearson correlation analysis was conducted to determine the relationship between the 2 independent variables.

Results

Neuropsychological examinations:

Significant memory deficiencies and abnormalities in sustained attention and decision-making were detected in the addict group compared with a non-psychiatric control group. In addition, significant improvement in verbal and spatial memory and decision making were detected in addict subjects after the six months of treatment periods. Neuropsychological test results were presented in Table 2.

Table 1
sequences of primers used to analysis of gene expression by
Real time PCR

Forward Primer <i>BDNF</i>	5'CTGTAGTCGCCAAGGTGGTT3'
Reverse Primer <i>BDNF</i>	5'AAGTGCTAGGAAGAGCCGTG3'
Forward primer <i>GAPDH</i>	5'AAATCCGTTGACTCCGACCT3'
Reverse primer <i>GAPDH</i>	5'CACTAGGCGCTCACTGTTCTC3'
Forward primer <i>5-HTT</i>	5'CCTGGCGAGCGCAACC3'
Reverse primer <i>5-HTT</i>	5'GCGCGGCCGTATTTGTACC3'

Table 2
Demographic data and neuropsychological examinations results

GROUPs	Age	spatial N back result	Spatial N back time	N back result	N back time	Go/no	Go/no	Go/no	Go/no	Digit span forward	Digit span backward
						Go FA	Go	Go	Go		
						M	I		time		
N	42.1 \pm 3.5	22.14 \pm 1.2	721.1 \pm 36.6	100.4 \pm 11.1	620.3 \pm 22.5	0.8 \pm 0.26	3.14 \pm 2.5	36.04 \pm 2.1	415.9 \pm 14.4	8.19 \pm 1.4	4.57 \pm 2.2
C	38.2 \pm 5.6	25.8 \pm 3.2	782.5 \pm 33.7	87.4 \pm 13.1	689.3 \pm 33.7	0.96 \pm 0.23	4.95 \pm 1.2	34.07 \pm 3.4	430.4 \pm 23.1	6.27 \pm 2.3	3.73 \pm 1.4
PC	38.2 \pm 5.6	23.9 \pm 1.2	734.1 \pm 35.2	84.2 \pm 10.7	631.6 \pm 20.4	0.88 \pm 0.31	3.8 \pm 1.6	35.74 \pm 1.7	422.3 \pm 26.2	7.83 \pm 3.8	4.12 \pm 2.2

Gene expression findings:

Expression analyses were showed significant down regulation of *BDNF* and *5-HTT* genes in the addict group compared with the normal group. In addition, mRNA levels of *BDNF* and *5-HTT* were significantly increased in addicts after the six months of treatments with the DST method. Gene expression results including statistical data and ratio ($2^{\Delta\Delta Ct}$) were presented in Table 3 and Fig. 1. Also, several significant correlations were found between the expression level of target genes and neuropsychological data. Statistical results of correlations for *BDNF* within each group were presented in Table 4 and Figs. 2 and *5-HTT* within each group Table 5 and Fig. 3 respectively.

Table 3
Gene expression results, statistical and ratio ($2^{\Delta\Delta Ct}$) data

GROUP COMPARISONS	BDNF gene ratio	BDNF gene p value	5-HTT gene ratio	5-HTT gene p value
C vs. N	0.24	0.0001	0.2	0.0001
PC vs. C	8.94	0.0001	12.44	0.0001
PC vs. N	0.87	0.16	0.8	0.12

Table 4
Correlation analysis of BDNF gene expression (\log_2 fold) results with neuropsychological results

GROUPs	spatial N back result	Spatial N back time	N back result	N back time	Go/no Go time	Digit span forward	Digit span backward
N	P value = 0.15 r=-0.26	P value = 0.41 r= -0.18	P value = 0.55 r = 0.18	P value = 0.04 r=- 0.36	P value = 0.09 r= -0.23	P value = 0.02 r = 0.55	P value = 0.07 r = 0.49
C	P value = 0.05 r=-0.47	P value = 0.07 r=-0.42	P value = 0.03 r = 0.65	P value = 0.002 r=- 0.78	P value = 0.07 r= -0.31	P value = 0.003 r = 0.73	P value = 0.001 r = 0.76
PC	P value = 0.02 r=-0.54	P value = 0.08 r=-0.46	P value = 0.06 r = 0.42	P value = 0.003 r= -0.63	P value = 0.14 r= -0.2	P value = 0.005 r = 0.62	P value = 0.004 r = 0.68

Table 5
Correlation analysis of 5-HTTPLR gene expression (\log_2 fold) results with neuropsychological results

GROUPs	spatial N back result	Spatial N back time	N back result	N back time	Go/no Go time	Digit span forward	Digit span backward
N	P value = 0.23 r=- 0.12	P value = 0.18 r = 0.16	P value = 0.09 r = 0.33	P value = 0.12 r=- 0.28	P value = 0.01 r= -0.62	P value = 0.04 r = 0.61	P value = 0.003 r = 0.56
C	P value = 0.1 r=- 0.34	P value = 0.003 r = 0.72	P value = 0.08 r = 0.46	P value = 0.01 r=- 0.67	P value = 0.003 r= -0.64	P value = 0.001 r = 0.73	P value = 0.006 r = 0.6
PC	P value = 0.09 r= -0.36	P value = 0.004 r = 0.68	P value = 0.05 r = 0.52	P value = 0.02 r= -0.59	P value = 0.002 r= -0.7	P value = 0.008 r = 0.66	P value = 0.02 r = 0.61

Western blotting results:

In order to confirmation of gene expression results; protein levels of both *BDNF* and *5-HTT* have been examined in three addict subjects and three non-psychiatric control groups. Findings were confirmed the gene expression data. *BDNF* and *5-HTT* protein levels were lower in addicts compared with normal control. The protein level of both *BDNF* and *5-HTT* were significantly increased in addicts after the six months of treatment. Results of protein level assessment were presented in Table 6.

Table 6
Data of protein level assessment by western blotting

SAMPLES	BDNF percentage	5HTT percentage	GAPDH percentage
N	27.91 ± 2.31	22.48 ± 4.22	11.61 ± 2.1
C	3.90 ± 0.37	8.12 ± 1.2	9.66 ± 1.13
PC	23.04 ± 3.42	21.33 ± 2.8	12.05 ± 1.52

Discussion

Cross-inheritance twin studies showed that substance-specific and substance-nonspecific genetic risk factors are both associated with addictions. Also, linkage studies have determined genes that are shared biomarkers in addiction and psychiatric disorders (5). *BDNF* and *5-HTT* are both candidate genes for several psychiatric disorders and several kinds of addictions (28–31).

Results in the first period of sampling were showed a dramatic down-regulation of mRNA level in *BDNF* and *5-HTT* in opioid addicts. Reduction in expression of these genes may cause a lack of cognitive and executive functions and lack of decision-making functions respectively. On the other hand, neuropsychological examinations have been detected memory and decision-making disabilities in addicts before starting the DST treatment method. After the six months of DST treatment which is half of all treatment periods, mRNA levels of *BDNF* and *5-HTT* were significantly increased in addicts along with improvement of memory and decision-making abilities. Several correlations were found between *BDNF* and *5-HTT* expression levels and memory testing results (digit span, spatial n-back, and n-back tests) and sustained attention and decision-making test (GO/no GO) respectively.

BDNF Recent evidence implicates epigenetic mechanisms in drug-associated memory processes. *BDNF/TrkB* activation could lead to overcoming memory and neuroplasticity reduction (32). Previous studies were revealed that *BDNF* plays an essential role in learning and memory including aversive memories associated with conditioned drug withdrawal. *BDNF* downregulation was found associated with memory deficiency and relapse of morphine (33). *BDNF* is also involved in neural circuits for visual long-term memory formation that may explain the correlations were found between mRNA level of *BDNF* and memory tests(34). Altogether it has been found that learning and memory and drug addiction are modulated by share neurotrophic factors, signaling pathways, and transcription factors. Addiction and memory are strongly related together with the same neuronal morphology adaptations and synaptic plasticity situations such as long-term potentiation (LTP) and long-term depression (LTD) in glutamatergic synapses (35). It may suggest that improvement of memory deficiencies which is common in addicts is a key to treatment of addiction and prevention of relapse. The study showed that both verbal and spatial memory of addicts were improved after six months and approached normal subjects. These improvements are highly correlated with the increase of *BDNF* expression.

Previous reports indicated that serotonin transporters are altered by chronic cocaine use, while also being involved in WM processing. A cohort study was showed that the variable number of tandem repeats and single-nucleotide polymorphisms in and high mRNA expression of *5-HTTLPR* are associated with the worse executive working memory performance in cocaine users but with increased performance in controls (36). Other lines of evidence indicated that genetic variants and down expression of *5-HTT* are detected in addiction as well as attention deficits and hyperactivity disorders (37, 38). The present study has reported a significant down regulation of *5-HTT* in addicts that was associated with lack of functions in memory, sustain attention, and decision making. While increased *5-HTT* activity improves cognitive functioning, gene × environment interaction may cause complexity of the *5-HTT* systems in different psychological situations and addictions (39). By the way, the DST method has reregulated the *5-HTT* expression level as a biomarker for the number of psychiatric disorders compared with normal subjects.

It seems that DST as a package of psychological caring, group educations similar to cognitive behavioral therapy along with the taper method instead of using synthetic materials such as methadone could be a reliable alternative treatment for opioid addiction. Also, DST is not just a treatment method to reduce or cut the dependency of addicts on opioids but it can improve the cognitive problems, made by opioid abuse, as well. Improvement of cognitive abilities in addiction may consider as a harm reduction tool that also helps the addict to back to normal life (40).

Conclusion

The present study was a follow-up of a new alternative method for the treatment of opioid addiction. We aimed to assess the rate of the change of neuropsychological and molecular situation of same subjects during the treatment to a better understanding of the nature of DST effectiveness. It seems that the DST method has successfully helped addicts to taper off and leave the opioid abuse without any second dependence on other substances or somatic or psychological side effects of a sudden leave of opioid abuse. In addition, psychological caring and educational programs for under treatment addicts or "passengers" have helped them to improve their cognitive abilities that are confirmed at both molecular and clinical levels. The study also confirmed the role of *BDNF* and serotonin transporters in memory functions and decision-making processes respectively.

Declarations

Statement of ethics approval: All participants filled out a written consent form after explaining the aim of the study for each subject in private. Informed consent was obtained for all participants after at least two meetings, along with 40 minutes for personal question and answer time at the end of the session. Study levels and the consent forms were approved by the central ethical committee of Islamic Azad University based on Helsinki declaration obligations under reference number: 49920E3.

Competing interests The authors declare that there are no competing interests.

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Figures

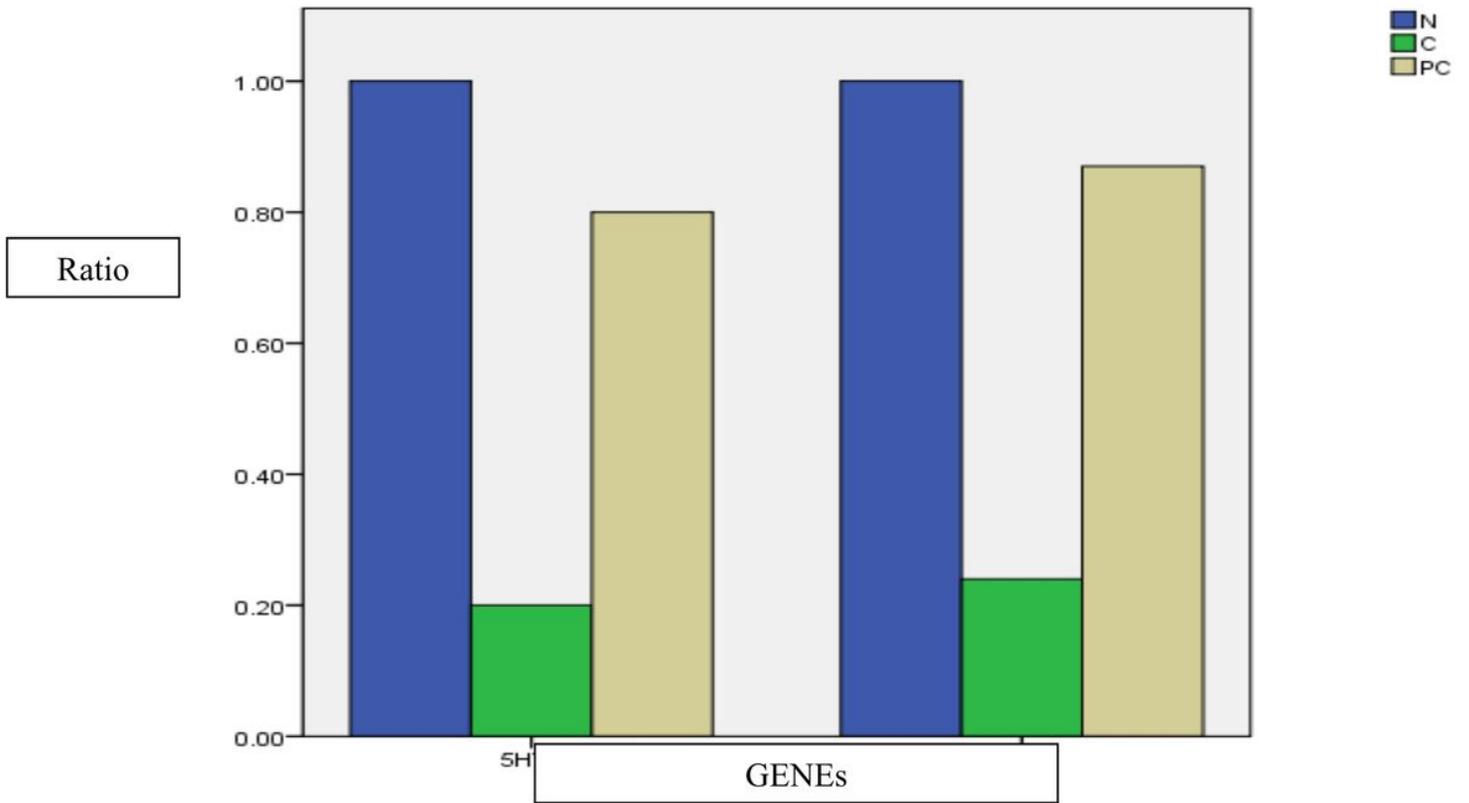


Figure 1

Ratio ($2^{\Delta\Delta Ct}$) of target genes expression level in three groups: N (normal group) was showed by blue color, C (addicts before admission in congress 60) was showed by green color and PC (addicts after six months of treatment in congress 60) was showed by beige color.

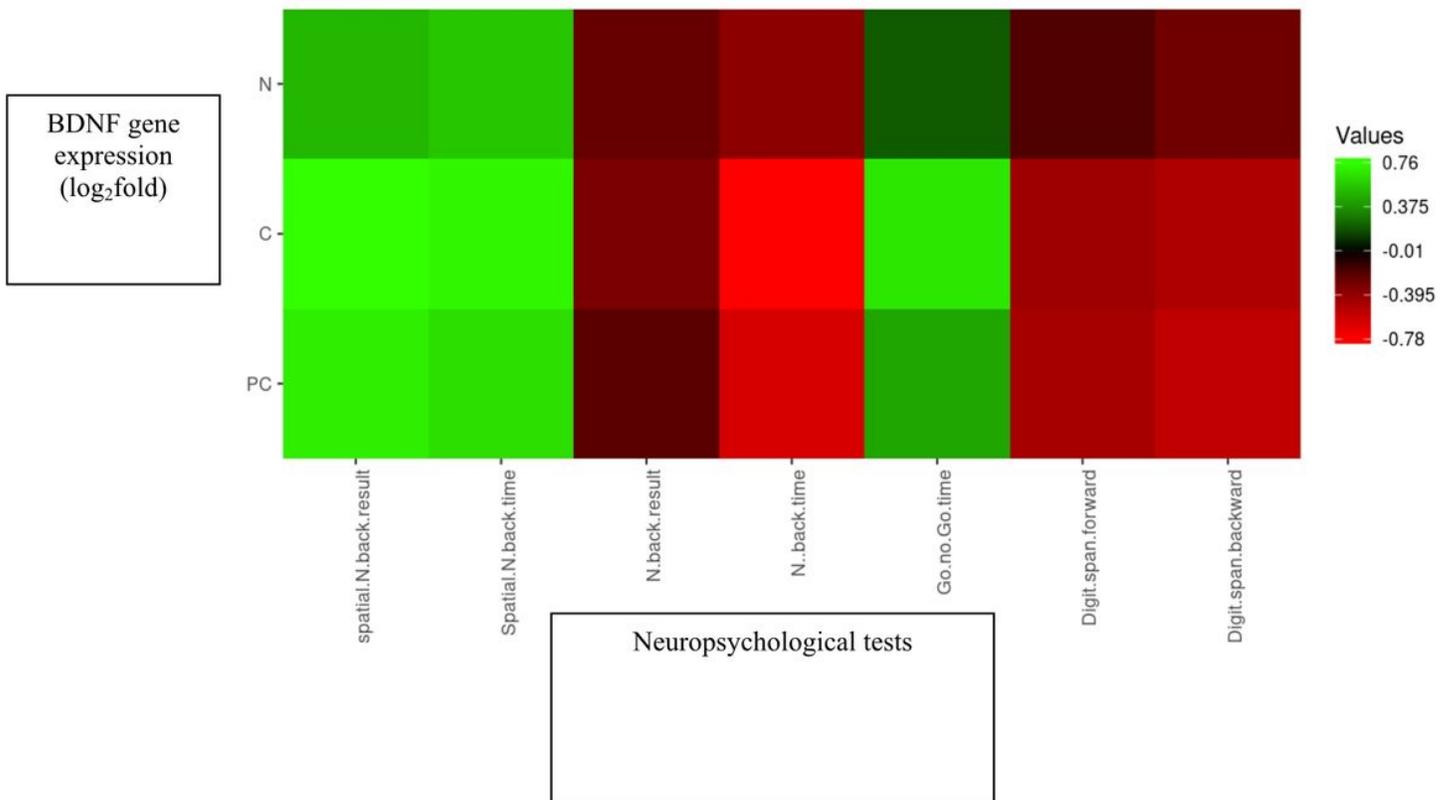


Figure 2

Heat map of r (-1 to +1) data of Pearson correlations between BDNF gene expression level and results of neuropsychological tests in N (normal group), C (addicts before admission in congress 60) and PC (addicts after six months of treatment in congress 60). Red color represents negative correlation and green color represents positive correlation.

Figure 3

Heat map of r (-1 to +1) data of Pearson correlations between 5HTT gene expression level and results of neuropsychological tests in N (normal group), C (addicts before admission in congress 60) and PC (addicts after six months of treatment in congress 60). Red color represents negative correlation and green color represents positive correlation.