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Original Research

miRNA and mRNA expression profiling in rat brain following alcohol dependence and withdrawal

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Abstract: Long- lasting alterations in brain gene expression in alcohol addiction have been determined although no clear mechanism has yet been elucidated. There exist many factors regulating the mechanism of gene expression. We aimed in this study to detect miRNA (microRNA) and mRNA expression profile at the specific brain regions regarding ethanol exposure and withdrawal. Rats were exposed to liquid alcohol consumption for 21 days. Oligonucleotides microarrays and bioinformatics analyses were used to identify gene expression, miRNA expression and their functions in the Prefrontal cortex, Hippocampus and Corpus striatum of wistar rats. A bioinformatics strategy with microarray analysis, quantitative real time PCR, bioinformatics and mRNA (messenger RNA) miRNA- miRNA integrative analyses revealed that expression models interact with neuroplasticity and synaptic processes. Those significantly changed after ethanol exposure and withdrawal processes included 160 mRNAs and 29 rat-miRNAs at prefrontal cortex, 142 mRNAs and 26 rat-miRNAs at hippocampus, and 143 mRNAs and 30 rat-miRNAs at corpus striatum. Gene ontology and ingenuity pathway analyses revealed that most of the altered genes were responsible for synaptic plasticity, neuron differentiation, chromatin organization and some certain important signaling pathways. In conclusion, consistent and integrated variations in miRNA expression and in their focus mRNAs in rat brain were noted after alcohol exposure and withdrawal. Besides, understanding the molecular mechanisms of alcohol abuse will no doubt guide to development of significant cure methods for addiction. We are of the opinion that our findings may shed light on classification of novel biomarkers.

Key words: Alcohol addiction; Withdrawal; Gene expression profiling; miRNA; Microarray.

Introduction

Alcoholism is a chronic and complicated disease effected by genetic, epigenetic and environmental factors (1). Alcohol addiction has now been known to cause aberrances in synaptic plasticity and neuronal functions. The neuroadaptation of brain is associated with long-lasting deregulation of gene transcription, protein expression and signaling systems in the central nervous system. miRNAs mediate the formation of various gene variation in brain if stimulated by alcohol. miRNAs are little, definitely preserved, noncoding RNA molecules that regulate gene expression at the post-transcriptional level. Also they are overexpressed in the brain and have significant roles in terms of biological functions, including neuronal distinction, synapse arrangement, homeostatic synaptic plasticity, neuronal degeneration, behavior and addiction. Evidence for miRNA-mediated regulation of gene expression by ethanol was first provided by ex-vivo studies. In neurosphere cultures derived from fetal mouse cortex, ethanol was found to cause up-regulation of *JAG1*, a notch receptor ligand along with suppression of miR-335, miR-21 and miR-153. Each miRNA can control hundreds of mRNAs and each mRNA can be targeted by multiple miRNAs. Moreover, multiple miRNAs can either collaborate or compete for the regulation of common mRNA targets (2-4). Systematic investigation of genome-wide effects of ethanol on miRNA expression in adult brain has recently provided novel insights to the regulation of protein-coding gene expression in the context of chronic ethanol exposure in the frontal cortex of alcoholics (2, 5). A total of 35 miRNAs were up-regulated in alcoholics and used for target prediction. Interestingly, predicted miRNA targets were considerably over-represented in the set of 217 genes which were previously recognized as down-regulated in the same postmortem samples, and most putative targets were adjusted by multiple miRNAs (2, 6). Conversely, there were 27 miRNAs whose predicted targets were over-represented among down-regulated transcripts. Many of the down-regulated transcripts were akin to lipid biosynthesis and metabolism along with cytoskeleton and cell cycle control. Interestingly, the down-regulated transcript targeted by the highest number of up-regulated miRNAs encodes Dicer, a key enzyme in the generation of mature miRNAs. In this study, our aim was to detect miRNA and mRNA expression profile in the setting of ethanol addiction and withdrawal. Addicted rats were exposed to ethanol in order to understand effects of alcohol addiction on brain in molecular basis. A total of 18 rats were used in three groups. After 21 days rats became addicted, and hippocampus, cortex and striatum were extracted and used for gene expression analysis. After isolation of RNA's from

brain tissues, samples were used for miRNA and mRNA microarray profile analyses. A bioinformatics strategy with microarray analysis together with quantitative real time PCR, bioinformatics analysis and messenger RNA miRNA- miRNA combinative analyses revealed disruption in neuroplasticity and synaptic processes. The results elucidated differentiated mRNA and miRNA expression profile in this alcohol dependence and withdrawal rat model. Some of the mRNAs and miRNAs were affirmed by qPCR. The study is the latest and the most comprehensive work in recent times. Gene ontology classes of differential expression indicated useful steps generally corresponding to neuroadaptation synaptic plasticity and neurotransmission. Further data as to molecular mechanism of alcohol addiction will guide improvement of possible treatment methods for this syndrome.

Materials and Methods

Animal subjects

All procedures in this work were in conformity with the Guide for the Care and Use of Laboratory Animals as followed by the National Institutes of Health (USA) and were accepted by the Local Ethics Committee for Animal Experimentation of Istanbul University (2013/104). 12 mature masculine Wistar rats (365-500g) were the subjects in this study. They each were encased in different heat & humidity monitored rooms (22+-3°C and 60+-5%, respectively) where a 12 h light/dark cycle was provided (7:00-19:00 h light). The subjects were divided into three groups (n=6 each): Alcohol addicted, withdrawal and control groups.

Chronic ethanol administration

The rats were given ethyl alcohol through a liquid diet (composition : cow milk 925 ml, 25–75 ml ethanol (96.5% ethyl alcohol), vitamin A 5000 IU, and sucrose 17g) for 21 days with respect to the protocol (7). At the beginning of the study, all rats (n=18) were fed with liquid diet without ethanol for a week. Then, they were divided in three groups (n=6 each). For alcoholic groups (n=6), a liquid diet of 2.4% (v/v) ethanol was applied for 3 days. The ethanol concentration was increased to 4.8% (v/v) for the next 4 days and eventually to 7.2% (v/v) for the next 14 days. For withdrawal group (n=6), everthing was same with alcoholic group except last day fed with normal diet without alcohol. Control group was fed with ethanol free liquid diet during whole procedure. At 22nd day, all rats were sacrificed by deprivation of O_2 followed by cervical dislocation and decapitation, and brain parts were removed. Each brain tissue sample was put into DNase–RNase free tubes placed into liquid nitrogen. Finally, all sample tubes were collected from liquid nitrogen and they were stored in −80°C until next experiment.

Determination of blood ethanol levels

Blood alcohol levels were determined in three different groups of the ethanol exposed rats (n = 6 for each group) at 7., 14. and 21. days of ethanol intake and at 12th and 24th hour of ethanol withdrawal. Blood samples were taken below the ether anesthesia. Serum alcohol levels were determined spectrophotometrically at 340 nm by use of an enzymatic kit (Sigma Chemical, USA).

RNA-miRNA isolation and preparation for microarray analysis

Total RNA was extracted from brain sample with Trizol reagent (Invitrogen Life Technologies, USA) and was purified by RNAeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. miRNAs were isolated using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Total RNA concentration was measured on the NanoDrop (ThermoScientific, USA).

mRNA and miRNA expression profiling

In this study, Affymetrix microarray system was used. Rat Genome 230 2.0 Array was used for mRNA expression profiling and GeneChip® miRNA Affymetrix 902018 was used for miRNA expression profiling. Samples were hybridized to an individual microarray chip for each brain regions. Posthybridization washing, staining and scanning of chips were performed with manufacturer's technical manual (Affymetrix, USA). After scanning, the data were log2 transformed and normalized. Statistical differences between groups were calculated using Partek Genomic Suite (Partek Inc, USA). RNA expression standards were provided and applied for different gene expressions utilizing two-sample *t*-test presuming irregular variations, with the $p < 0.05$ being the cut-off.

Validation of mRNA microarray expression data with qRT- PCR

From the each brain samples, a total of 100 ng isolated RNA was reverse transcribed to use "RevertAid™ First Strand cDNA Synthesis" kit (Thermo scientific, USA) according to manufacturer's instructions. Primers for every target were laid out according to National Center for Biotechnology Information (NCBI) reference sequence database and primer design program. Primer sequence in this study is shown in **Table 1.** Samples were analyzed in triplicate in a total volume of 15 ml utilizing SYBR Green based All-in-One™ qPCR Mix (Genecopoeia, USA) on Bio-Rad CFX 96 RT-PCR system (40 cycle of 95 C for 10 minutes for initial denaturation, 95 C for 10 seconds for denaturation, 58 C for 20 seconds for annealing and 72 C for 20 sec for extension steps). Gene expression level determination was done according to $2^{-\Delta\Delta CT}$ method (8). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was chosen as inner control. Each sample was normalized according to *GAPDH* expression. For visual explanation, the ΔΔCt values were converted (-x), therefore down regulated genes demonstrated $\Delta\Delta Ct < 0$ and up regulated genes $\Delta\Delta Ct > 0$ values. The ΔΔCt values were contrasted by student t-test for each gene ($P<0.05$ significance; $P<0.07$ trend).

Validation of miRNA microarray expression data with qRT-PCR

QuantaMir ™ miRNome miRNA profiler's kit (System bioscience, USA) was used for miRNA expression and qPCR according to their protocols. A total of 160 ng isolated RNA from each brain sample was used for miRNA qPCR. Experiments were stabilized to the *U6*

Table 1. Primers.

Gene Name	RefSeq ID	Forward Primer	Rewers Primer
Satb2	NM_001109306	CTTCCTCAACCTGCCTGAAG	GTTGTCGGTGTCGAGGTTTT
Nr4a2	NM_019328	CCAATCCGGCAATGACCAG	GATGATCTCCATAGAGCCAGTCAG
Rpl32	NM_013226	TGTCCTCTAAGAACCGAAAAGCC	CGTTGGGATTGGTGACTCTGA
Galr1	NM_012958	TCAGAGGCATCCAAGAAAAAG	AACTCAGCCCAGAGGTGGA
Ndufa2	NM_001106153	ACTGAGGACTGAACAAGCCCACCA	GCGACATCCCAGCGGGTAGC
Nr4a3	NM_031628	TACGGAGTCCGCACCTGCGA	CGACGTCTCTTGTCTACCGGGC
Nptxr	NM_030841	ATATCTGCATCGCCTGGACT	AGGATGCCATGTGGTTTGAT
Hmga1	NM_139327	CAACTCCGGGGAGGAAACCA	AGGACTCCTGGGAGATGC
Hmgb1	NM_012963	TGATTAATGAATGAGTTCGGGC	TGCTCAGGAAACTTGACTGTTT
Egr2	NM_053633	TCCGAGTTCTGAACCTTTGG	GGACACTTGCAACACCACTG
Slc17a7	NM_053859	TGCTGCTGGTGGTCGGATAC	AGGGGCGATGTCCAAGTGGT
Slc17a8	NM_153725	AAAACAGGACTGGGCTGATCC	GAGACCAAGATCCATACGCCC
Bdnf	NM_012513	CCATAAGGACGCGGACTTGTAC	GAGGAGGCTCCAAAGGCACTT
Arc	NM_019361	CCGTCCCCTCCTCTCTTGA	AAGGCACCTCCTCTTTGTAATCCTAT
H2AFV	NM_001106019.1	CTGATCGGAAAGAAGGGGCAGCAGA	CACACACAGTGAGGACAGCAGGTCA
GAPDH	NM_017008.4	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

endogenous control and comparisons among the groups were performed using the $2^{-\Delta\Delta CT}$ method. This kit included nearly 800 individual miRNA primers.

Determination of canonical pathways, gene networks and miRNA–mRNA interaction analyses

Applicant mRNAs and miRNAs were investigated using bioinformatics analysis. Ingenuity pathway analysis, IPA (Qiagen, USA) is an online bioinformatics tool which locates applicant mRNAs and miRNAs into canonical pathways, gene networks, and searches gene-gene and miRNA-gene relationships with statistical standards. Each obtained important biological data regarding gene and miRNA networks, canonical pathways and biological features were consequently validated using Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) (9), GO Consortium (www.geneontology.org) (10) and Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) (11). MiRNA.org was applied as handle database (12). Each mRNA-miRNA forecast analyses were carried out by miRwalk database, an combinative mRNA-miRNA interaction forecast database (http:// www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) (13,14), IPA software (15) and miRNA.org website.

Results

Blood Alcohol Concentration

Post-dependent animals took alcohol-including liquid diets for 21 days maintaining a BAC levels of between nearly 150-400 mg/dl (**Table.2**). At the end of these 21 days BAC levels were stable in the range of 150-300 mg/ dl. In the withdrawal period, both 12th and 24th hours levels decreased significantly as compared to the levels that measured before withdrawal. Also post- dependent animals showed increase in BAC levels compared to 7 days animals significantly.

mRNA and miRNA expression profiling

Affymetrix microarray system was used in this study. Rat Genome 230 2.0 ST Array was used for mRNA expression profiling and Gene Chip miRNA 4.0 Array for miRNA expression. The data were log2 stabilized. RNA and miRNA expression standards were provided and tried for different gene expression using two-sample t-test presuming irregular variations, at a $P< 0.05$ cut-off level. In this study, only three parts of brain were used: prefrontal cortex, corpus striatum and hippocampus, all of which host signal transduction resulting in change in the expression of genes in the process of alcohol dependence and withdrawal. 160 mRNAs and 29 rat-miRNAs in the prefrontal cortex, 142 mRNAs and 26 rat-miRNAs in the hippocampus, 143 mRNAs and 30 rat-miRNAs in the corpus striatum were significantly changed after ethanol exposure and withdrawal condition.(cut off = 4, $p<0,05$, fold change +/- 1,2) (**Figure 1**). In addition, more genes and miRNAs were up regulated than those down regulated by alcohol addiction and withdrawal (**Figure 2**).

Integrative analysis of miRNA- mRNA interaction

In our study we have tried to examine relationship of miRNA- mRNA interaction and expression changes induced by alcohol dependency and withdrawal. IPA (ingenuity pathway analysis) was used to determine the miRNA- mRNA interaction and find canonical path-

Table 2. BAC table.

Exposure Day	BAC (mg/dL)	SEM (±)	Range
Control	0	0	0
7 days	147,5	± 51,5	80-220
14 days	262,9	± 31,4	210-320
21 days	275,4	± 42,9	220-350
22 days 12th h	170,8	± 26,5	150-220
22 days 24th h	96,6	± 19,1	75-130

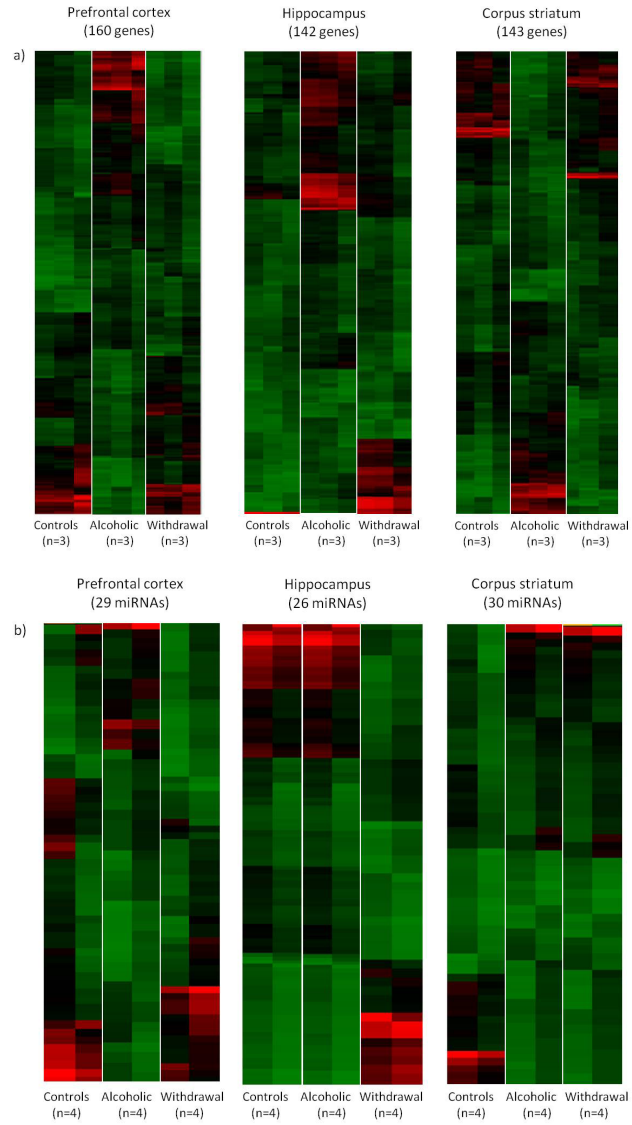

Figure 1. Hierarchical clustering of differential expressed miRNA and mRNA profiling from alcoholics, withdrawals and controls. Rows: individual miRNAs or mRNAs; columns: individual samples. Red within the heat map represents miRNA or mRNA upregulation, green within the heatmap represents miRNA or mRNA downregulation. a) Expression profiling of the total number of mRNAs linked with alcohol, withdrawal and control in preforontal cortex (160 genes), hippocampus (142 genes) and corpus striatum (143 genes) of wistar rats with significant different expression ($p < 0.05$). b) Expression profiling of the total number of miRNAs linked with alcohol, withdrawal and control in prefrontal cortex (29 miRNAs), hippocampus (26 miRNAs) and corpus striatum (30 miRNAs) of wistar rats with significant different expression ($p < 0.05$).

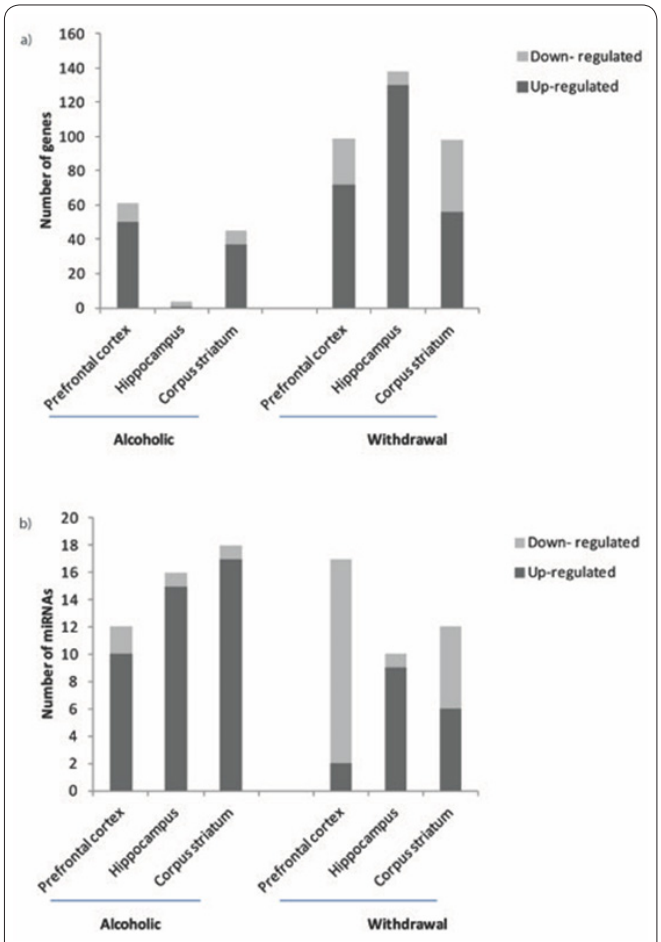

Figure 2. Number of mRNAs and miRNAs were significantly altered by alcohol and withdrawal conditions according to controls. a) Number of mRNAs were changed under alcoholic and withdrawal conditions in prefrontal cortex, hippocampus and corpus striatum. b) Number of miRNAs were changed under alcoholic and withdrawal conditions in prefrontal cortex, hippocampus and corpus striatum.

ways. GO Consortium (geneontoloy.org) and DAVID (database for annotation, visualization and integrative discovery; david.abcc.ncifcrf.gov) were used to identify the gene ontology. In addition, mirWalk and miRNA.org were used for additional analyses. miRNA-mRNA interaction was prepared with respect to all software results.

According to results in prefrontal cortex, 10 miRNA were found associated with 12 mRNA in alcohol addiction while 20 miRNA were with 39 mRNA in alcohol withdrawal. In hippocampus, 5 miRNA were associated with 5 mRNA in alcohol addiction while 44 miRNAs were with 299 mRNAs in alcohol withdrawal. In corpus striatum, 16 miRNAs were associated with 23 mRNAs in alcohol addiction while 23 miRNAs were with 37 mRNAs in alcohol withdrawal.

Validation of mRNA and miRNA microarray expression data with qRT- PCR

In order to confirm the expressed mRNAs, qRT- PCR were applied. The gene that was chosen to show opposite direction expression. That is mean gene expression was decrease and miRNA expression was increase. The same primer was used for both three regions and three situations. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was chosen as inner control for mRNA. *U6* primers were the inner control for miRNA. The fold change differences were calculated according to $2^{-\Delta\Delta CT}$ method. Figure 3 shows altered mRNA and miRNA validation. According to our results in prefrontal cortex, *GALR1* and *SLC17A8* decreased in alcoholic condition but increased in withdrawal conditions BDNF, ARC and *NR4A2* were found to increase significantly, whereas *C-FOS* decreased in both alcoholic and withdrawal conditions. In hippocampus; *GALR1, SLC17A8, BDNF, CREB, C-FOS* and *ARC* increased significantly in both alcoholic and withdrawal condition. In corpus striatum; *SATB2, NR4A2 and C-FOS* decreased in alcoholic condition while *GALR1, EGR2, SLC17A7* and *ARC* increased significantly in both alcoholic and with-

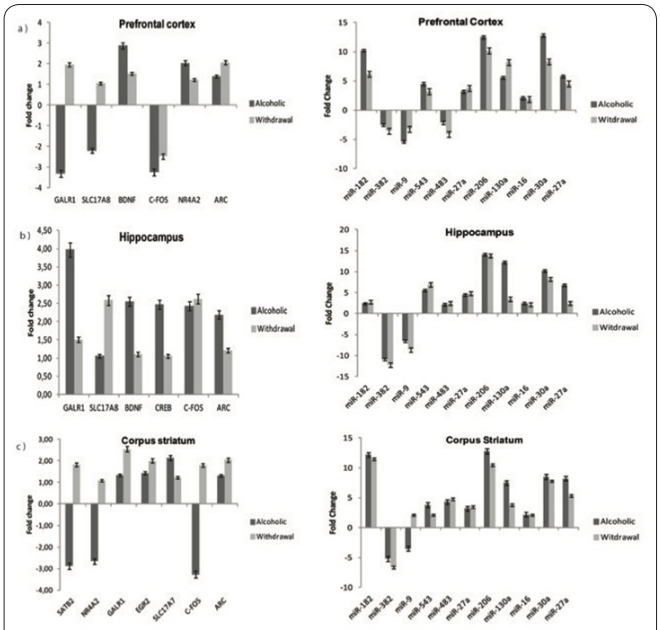

Figure 3. q-RT-PCR results for miRNA and mRNA confirmation. Confirmation of selected miRNA and mRNA with RT-PCR. a) Confirmation of some mRNA and miRNA in prefrontal cortex. b) Confirmation of some mRNA and miRNA in hippocampus. c) Confirmation of some mRNA and miRNA in corpus striatum. Downregulated genes show value < 0, upregulated genes are > 0. Significance was determined by $P < 0.05$.

drawal conditions (**Figure 3**).

According to results in prefrontal cortex; mir182, miR-130a, miR-16 and miR-27a significantly decreased but miR-9 increased in withdrawal condition. miR-206 and miR-30a were significantly higher in both conditions. In hippocampus, miR-382 decreased in all conditions significantly. However, miR-206 and miR-30a increased in both conditions. miR-130a and miR-27a decreased in withdrawal condition. In corpus striatum; while miR-182, miR-206 and miR-30a increased in all conditions, miR-130a and miR-27a decreased in withdrawal condition only (**Figure 3**).

Those genes seemed responsible for the plastic synapsisty along with behaviour as to psychological and neurological disease, cell cycle and neuron development (**Figure 4**).

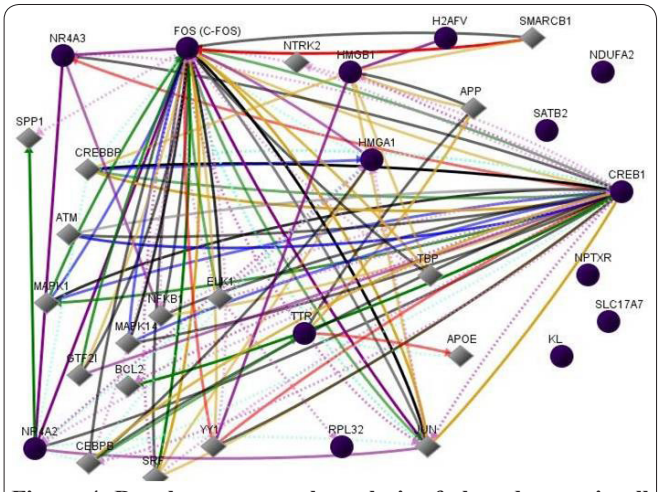

Figure 4. Regulatory network analysis of altered genes in all samples. This figure shows gene regulatory network built from alcoholic mRNA data in all samples represent regulatory interaction like protein- protein and protein – genes.

Integrative analysis of miRNA and mRNA

Integrative analysis and higher bioinformatics analysis were performed between alcohol related and other possible genes. IPA software was used for integrative analysis. As in other analysis alcoholic, withdrawal and alcoholic/ withdrawal groups were studied.

In prefrontal cortex - in the case of alcohol addiction - changing genes related not only to addiction-associated genes but also to some diseases and were responsible for some cell signaling pathways. However, in alcohol addiction, changing genes was noted to relate to neurological and cardiovascular disease genes along with those included in lipid metabolism, cancer and cell signaling. In prefrontal cortex, in case of withdrawal, some gene linkage was found to change. These genes related to DNA metabolism genes, hereditary disease genes and cell death pathway genes. In hippocampus in the case of alcohol addiction, changing genes was seen to relate to hematological disease genes, cell cycle genes and vitamin biosynthesis. In hippocampus in case of withdrawal, genes related to neurological disease, psychological disease, cell cycle and cell death genes. In corpus striatum in the case of alcohol addiction, changing genes were seen to relate to DNA replication, neurological disease, psychological disease, lipid metabolism, nerves system development, embryonic development genes. In corpus striatum in case of withdrawal, genes related to neurological disease, molecular transport and lipid metabolism.

In addition, according to GO analysis, changing genes related to different processes in our body. These processes includes behavior, synaptic plasticity, membrane potential, neuron differentiation, myelinizasion, cell cycle, chromatin organization and transcription. (**Table 3**).

Discussion

Some studies have discovered the effect of EtOH on the expression of miRNAs. Recent works reveal that the brain not only expresses varied miRNAs eseential for all cell varieties, but also a collection of neuronal-specific miRNAs in mice, rats and human beings throughout vegetative cell diversification (16-18). Thus, it is advocated that miRNA presents a mighty regulating mechanism in central nervous system. Moreover, this study suggests that mRNA- miRNA networks can be concerned in neuroadaptations linked to post-dependent condition and finally affect behavior (19). All results provide that goal of upcoming in vitro and in vivo research should be directed at identifying the practical duty of mRNA-miRNA networks in neuronal function and addiction associated behaviors. miRNA deregulation have been associated with cocaine consumption (20-22), nicotine excitement (23) and alcohol endurance (24). In our research, we found 11 miRNAs whose expression was changed after alcohol dependency in rats: miR-182, miR-382, miR-9, miR-543, miR-483, miR-206, miR-130a, miR-16, miR-30a and miR27a.

miR-9 was found down regulated in hippocampus, corpus striatum and cortex in addicted rats and up regulated in all brain regions in withdrawal condition in our study. In contrary, intense EtOH process was discovered

Table 3. Gene onthology analysis.

Gene Onthology Analysıs		
Term	**Term Descrıptıon**	**Genes**
GO:0048168	regulation of neuronal synaptic plasticity	ARC, EGR2
GO:0060079	regulation of excitatory postsynaptic membrane potential	BDNF,SLC17A7
GO:0043524	negative regulation of neuron apoptotic process	BDNF,NR4A3, NR4A2
GO:0030182	neuron differentiation	BDNF,NR4A2
GO:0007611	learning or memory	BDNF,EGR2
GO:0042493	response to drug	BDNF,HMGB1
GO:0006836	neurotransmitter transport	SLC17A8,SLC17A7
GO:0006814	sodium ion transport	SLC17A8,SLC17A7
GO:0045944	positive regulation of transcription from rna polymerase ii promoter	EGR2,HMGB1,NR4A3,SA TB2, NR4A2, FOS
GO:0045893	positive regulation of transcription dnadependent	EGR2,HMGA1,NR4A2,FO S
GO:0006355	regulation of transcription dna dependent	EGR2, NR4A2
GO:0007010	cytoskeleton organization	ARC
GO:0016477	cell migration	ARC
GO:0007612	Learning	ARC
GO:0007610	behavior	BDNF,NR4A3
GO:0021675	nerve development	BDNF
GO:0048167	regulation of synaptic plasticity	BDNF,EGR2
GO:0060079	regulation of excitatory postsynaptic membrane potential	SLC17A7
GO:0035249	synaptic transmission glutamatergic	SLC17A7
GO:0014037	schwann cell differentiation	EGR2
GO:0035284	brain segmentation	EGR2
GO:0042552	myelination	EGR2
GO:0001934	positive regulation of protein phosphorylation	HMGB1
GO:0002437	inflammatory response to antigenic stimulus	HMGB1
GO:0051384	response to glucocorticoid stimulus	HMGB1
GO:0031532	actin cytoskeleton reorganization	HMGB1
GO:0031175	neuron projection development	HMGB1
GO:0007399	nervous system development	HMGB1,FOS,NR4A2
GO:0045787	positive regulation of cell cycle	NR4A3
GO:0021766	hippocampus development	NR4A3
GO:0022900	electron transport chain	NDUFA2
GO:0008150	biological_process	NDUFA2,RLP32
GO:0007189	adenylate cyclase activating g protein coupled receptor signaling pathway	GALR1
GO:0007186	g protein coupled receptor signaling pathway	GALR1
GO:0021952	central nervous system projection neuron axonogenesis	NR4A2
GO:0021953	central nervous system neuron differentiation	NR4A2
GO:0042053	regulation of dopamine metabolic process	NR4A2
GO:0071542	dopaminergic neuron differentiation	NR4A2
GO:0006338	chromatin remodeling	SATB2
GO:0001764	neuron migration	SATB2

to trigger a quick boost in miR-9 expression in neuron culture from the striatum and allocated other neurons (24), however, we showed in addiction condition that miR-9 expression was down regulated but up regulated in withdrawal condition. This means miR-9 is potential miRNA for alcohol history and it is suggested that miR-9 may have the capability to entirely change the expression of a whole gene community included in the improvement of alcohol dependency. miR-206 expression assists to management of alcohol self-administration by the mPFC and classifies BDNF as an applicant arbiter of this process (22,25). miR-206 is well known as a skeletal muscle-specific miRNA that is required for renewal following intense nerve damage (22,25,26). It can be conluded that miR-206 is up regulated while BDNF is down regulated in ethanol addiction.

miR-182 was found up regulated in hippocampus, corpus striatum and prefrontal cortex in alcoholic condition but down regulated in prefrontal cortex in withdrawal condition. miR-182 was associated with depression-like behavior. Some researchers found increased serum levels of miR-182 in patients with depression (6, 27). miR-382 was down regulated in all brain parts and all conditions. miR-382 was discovered to be down regulated following use of alcohol. In order to identify the possible duty of miR-382 in alcohol consumption, the impact of miR-382 on the expression of DeltaFosB was identified (6, 28). miR-16 was up regulated in alcoholic condition but down regulated in withdrawal condition in prefrontal cortex. miR-16 was associated with depression (29). miR-27a was up regulated in alcoholic condition but down regulated in withdrawal condition in all brain regions. This miRNA is related to apoptosis and its target gene is *BCL-2*. The study showed that miR-27a focus on apoptosis response protein like *BCL-2* family and down regulation of miR-27a-3p following neuronal apoptosis in vitro increases *p53*- dependent apoptotic pathways and attributes to neuronal cell death in vivo and in vitro (30). We identified a lot of mRNAs with altered expression in alcoholic and withdrawal conditions. This mRNAs are *BDNF, SATB2, NRD4A2, GALR1, CREB, ARC*, and *SLC17A7-8*. Many mRNAs have been displayed to have a duty in drug abuse, addiction and neuropsychiatric disorders. These genes

are also involved in dopamine, serotonin, glutamate signaling pathway, G- protein coupled signaling pathway, growth hormone signaling pathway, *p53* signaling pathway, chromatin reorganization pathway, neurological disease, psychological disease and cardiovascular disease pathway. *SATB2* expression was up regulated in alcoholic condition. This chromatin-remodeling protein has significant function in neocortex development and neuron differentiation and, in alcoholic condition, some brain parts do not develop according to *SATB2* expression (2,31-33). *NR4A2* was down regulated in alcoholic conditions. This protein play a significant role in depression and drug abuse (34). *GALR1* was down regulated in alcoholism. *GALR1* plays a critical role in dopamine system by suppressing dopaminergic transmission (35, 36). Impact essential for the rewarding qualities of drugs of misuse (37). *CREB*- stimulated transcription is a significant element of a change from brief-phase to longer-phase plasticity and right *CREB* performance is required for longer-phase memory development (38-40).

In conclusion, we have determined consistent, integrated variations in miRNA expressions and in their focus mRNAs in rat brain after alcohol dependence. We found 11 miRNAs whose expression was altered after alcohol dependency and withdrawal in rats: miR-182, miR-382, miR-9, miR-543, miR-483, miR-206, miR-130a, miR-16, miR-30a and miR27a and 9 mRNA whose expression was altered after alcohol dependency and withdrawal in rats: *BDNF, SATB2*, *NRD4A2, GALR1, CREB, ARC, SLC17A7-8*. The miRNAs target many mRNAs that are linked to mechanisms included in reward pathways, synaptic plasticity and neurotransmission. The capability of miRNAs to manage behaviour of mRNAs managing complicated cellular functions in the brain in an integrated way recommends a potential duty for the pathophysiology of miRNAs in alcohol dependence. The integrated and extended persistent move in miRNA and mRNA expression might reconstitute the neural circuit function required in the intellectual managing brain consuming alcohol addiction. In addition, our results yield direct proof for miRNA roles and mRNA in alcoholic people. Understanding the molecular mechanisms of alcohol abuse will no doubt guide to development of significant cure methods for addiction.

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References

1. Crabbe JC, Phillips TJ, Harris RA, Arends MA, Koob GF. Alcohol-related genes: contributions from studies with genetically engineered mice. Addict Biol. 2006;11(3-4):195-269.

2. Alcamo EA, Chirivella L, Dautzenberg M, Dobreva G, Fariñas I, Grosschedl R, et al. Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. Neuron. 2008 7;57(3):364-77.

3. Sathyan P, Golden HB, Miranda RC. Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. J Neurosci. 2007 8;27(32):8546-57.

4. Smalheiser NR, Lugli G, Rizavi HS, Torvik VI, Turecki G, Dwivedi Y. MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects. PLoS One. 2012;7(3):e33201.

5. Lewohl JM, Nunez YO, Dodd PR, Tiwari GR, Harris RA, Mayfield RD. Up-regulation of microRNAs in brain of human alcoholics. Alcohol Clin Exp Res. 2011;35(11):1928-37.

6. Li J, Li J, Liu X, Qin S, Guan Y, Liu Y et al. MicroRNA expression profile and functional analysis reveal that miR-382 is a critical novel gene of alcohol addiction. EMBO Mol Med. 2013 Sep;5(9):1402-14. Uzbay IT, Kayaalp SO. A modified liquid diet of chronic ethanol administration: validation by ethanol withdrawal syndrome in rats. Pharmacol Res. 1995;31(1):37-42.

7. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.

8. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 2016 4;44(D1):D457-62.

9. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25-9.

10. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003;4(5):P3.

11. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res. 2008 Jan;36 (Database issue):D149-53.

12. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform. 2011;44(5):839-47.

13. Honardoost MA, Naghavian R, Ahmadinejad F, Hosseini A, Ghaedi K. Integrative computational mRNA-miRNA interaction analyses of the autoimmune-deregulated miRNAs and well-known Th17 differentiation regulators: An attempt to discover new potential miRNAs involved in Th17 differentiation. Gene. 2015 10;572(2):153-62.

14. Krämer A, Green J, Pollard J Jr, Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics. 2014 15;30(4):523-30.

15. Fiore M, Jaén CR, Baker TB, Bailey WC, Bennett G, Benowitz NL et al. Clinical Practice Guideline Treating Tobacco Use and Dependence 2008 Update Panel, Liaisons, and Staff. A clinical practice guideline for treating tobacco use and dependence: 2008 update. A U.S. Public Health Service report. Am J Prev Med. 2008;35(2):158-76.

16. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E et al. A MicroRNA feedback circuit in midbrain dopamine neurons. Science. 2007 31;317(5842):1220-4.

17. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 2004;5(3):R13.

18. Tapocik JD, Luu TV, Mayo CL, Wang BD, Doyle E, Lee AD et al.Neuroplasticity, axonal guidance and micro-RNA genes are associated with morphine self-administration behavior. Addict Biol. 2013;18(3):480-95.

19. Eipper-Mains JE, Kiraly DD, Palakodeti D, Mains RE, Eipper BA, Graveley BR. microRNA-Seq reveals cocaine-regulated expression of striatal microRNAs. RNA. 2011 Aug;17(8):1529-43.

20. Im HI, Hollander JA, Bali P, Kenny PJ. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. Nat Neurosci. 2010;13(9):1120-7.

21. Tapocik JD, Solomon M, Flanigan M, Meinhardt M, Barbier E, Schank JR et al. Coordinated dysregulation of mRNAs and microRNAs in the rat medial prefrontal cortex following a history of alcohol dependence. Pharmacogenomics J. 2013;13(3):286-96.
22. Huang W, Li MD. Nicotine modulates expression of miR-140*, which targets the 3'-untranslated region of dynamin 1 gene (Dnm1). Int J Neuropsychopharmacol. 2009;12(4):537-46.
23. Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM et al. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. Neuron. 2008 31;59(2):274-87.
24. Tapocik JD, Barbier E, Flanigan M, Solomon M, Pincus A, Pilling A et al. microRNA-206 in rat medial prefrontal cortex regulates BDNF expression and alcohol drinking. J Neurosci. 2014 26;34(13):4581-8.
25. Williams AH, Valdez G, Moresi V, Qi X, McAnally J, Elliott JL et al. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science. 2009 11;326(5959):1549-54.
26. Li Y, Li S, Yan J, Wang D, Yin R, Zhao L et al. miR-182 (microRNA-182) suppression in the hippocampus evokes antidepressant-like effects in rats. Prog Neuropsychopharmacol Biol Psychiatry. 2016 4;65:96-103.
27. Podda MV, Riccardi E, D'Ascenzo M, Azzena GB, Grassi C. Dopamine D1-like receptor activation depolarizes medium spiny neurons of the mouse nucleus accumbens by inhibiting inwardly rectifying K+ currents through a cAMP-dependent protein kinase A-independent mechanism. Neuroscience. 2010 19;167(3):678-90.
28. Baudry A, Mouillet-Richard S, Schneider B, Launay JM, Kellermann O. miR-16 targets the serotonin transporter: a new facet for adaptive responses to antidepressants. Science. 2010 17;329(5998):1537-41.
29. Sabirzhanov B, Zhao Z, Stoica BA, Loane DJ, Wu J, Borroto C et al. Downregulation of miR-23a and miR-27a following experimental traumatic brain injury induces neuronal cell death through activation of proapoptotic Bcl-2 proteins. J Neurosci. 2014 23;34(30):10055-71.
30. Britanova O, de Juan Romero C, Cheung A, Kwan KY, Schwark M, Gyorgy A et al. Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. Neuron. 2008 7;57(3):378-92.
31. Leone DP, Heavner WE, Ferenczi EA, Dobreva G, Huguenard JR, Grosschedl R et al. Satb2 Regulates the Differentiation of Both Callosal and Subcerebral Projection Neurons in the Developing Cerebral Cortex. Cereb Cortex. 2015;25(10):3406-19.
32. Srinivasan K, Leone DP, Bateson RK, Dobreva G, Kohwi Y, Kohwi-Shigematsu T et al. A network of genetic repression and derepression specifies projection fates in the developing neocortex. Proc Natl Acad Sci U S A. 2012 20;109(47):19071-8.
33. McCoy MT, Jayanthi S, Wulu JA, Beauvais G, Ladenheim B, Martin TA et al. Chronic methamphetamine exposure suppresses the striatal expression of members of multiple families of immediate early genes (IEGs) in the rat: normalization by an acute methamphetamine injection. Psychopharmacology (Berl). 2011;215(2):353-65.
34. Ericson E, Ahlenius S. Suggestive evidence for inhibitory effects of galanin on mesolimbic dopaminergic neurotransmission. Brain Res. 1999 20;822(1-2):200-9.
35. Tsuda K, Tsuda S, Nishio I, Masuyama Y, Goldstein M. Effects of galanin on dopamine release in the central nervous system of normotensive and spontaneously hypertensive rats. Am J Hypertens. 1998;11(12):1475-9.
36. Koob GF. Addiction is a Reward Deficit and Stress Surfeit Disorder. Front Psychiatry. 2013 1;4:72.
37. Barco A, Alarcon JM, Kandel ER. Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell. 2002 8;108(5):689-703.
38. Benito E, Barco A. CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. Trends Neurosci. 2010;33(5):230-40.
39. Li MD, van der Vaart AD. MicroRNAs in addiction: adaptation's middlemen? Mol Psychiatry. 2011;16(12):1159-68.