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Up-regulation of MicroRNAs in Brain of Human Alcoholics

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Abstract

Background—MicroRNAs (miRNAs) are small, non-coding oligonucleotides with an important role in post transcriptional regulation of gene expression at the level of translation and mRNA degradation. Recent studies have revealed that miRNAs play important roles in a variety of biological processes, such as cell proliferation, neuronal differentiation, developmental timing, synapse function and neurogenesis. A single miRNA can target hundreds of mRNA transcripts for either translation repression or degradation, but the function of many human miRNAs is not known.

Methods—MiRNA array analysis was performed on the prefrontal cortex of 27 individual human cases (14 alcoholics and 13 matched controls). Target genes for differentially expressed miRNAs were predicted using multiple target prediction algorithms and a consensus approach, and predicted targets were matched against differentially expressed mRNAs from the same samples. Over- and under-representation analysis was performed using hypergeometric probability and z-score tests.

Results—Approximately 35 miRNAs were significantly up-regulated in the alcoholic group compared with controls. Target prediction showed a large degree of overlap with our published cDNA microarray data. Functional classification of the predicted target genes of the regulated miRNAs includes apoptosis, cell cycle, cell adhesion, nervous system development and cell-cell signaling.

Conclusions—This data suggests that the reduced expression of genes in human alcoholic cases may be due to the up-regulated miRNAs. Cellular processes fundamental to neuronal plasticity appear to represent major targets of the suggested miRNA regulation.

Keywords

Alcohol; Brain; Gene Expression; miRNA; Microarray

Introduction

In the brain, the acute effects of alcohol are mediated by direct interaction with neurotransmitter and signaling systems resulting in changes in the number and type of

receptors, and in the amount of neurotransmitter released into the synapse. In contrast, chronic alcohol abuse, results in persistent changes in brain function which are mediated, at least in part, by changes in gene expression (Nestler and Aghajanian, 1997).

Gene and protein expression profiling techniques have been used to identify alcohol-induced changes in gene expression in both cultured cells and animal models of alcoholism (reviewed in (Mayfield et al., 2008)). Alcohol alters the expression of genes involved in a wide variety of cellular functions including catecholamine metabolism, signal transduction cascades, protein trafficking, and oxidative stress responses. These studies suggest that specific patterns of gene expression may underlie alcohol-related phenotypes. In previous studies, we and others have used cDNA and oligonucleotide microarrays to identify genes with altered expression following long-term alcohol consumption (Lewohl et al., 2000; Liu et al., 2004; Liu et al., 2006; Mayfield et al., 2002) as well as the effects of concomitant diseases such as liver cirrhosis (Liu et al., 2007). These studies identified genes that likely underlie the adaptive response of neurons in the prefrontal cortex, a region of the brain that is particularly susceptible to the effects of long-term alcohol abuse and include genes involved in myelination, ubiquitination, apoptosis, cell adhesion, neurogenesis, and neural disease. Genes involved in neurodegenerative diseases such as Alzheimer disease were also significantly altered, suggesting a link between alcoholism and other neurodegenerative conditions. The mechanism by which chronic alcohol abuse results in these changes in gene expression remains unknown.

MicroRNAs (miRNAs) are small, non-coding RNAs that are thought to act as post-transcriptional modulators of gene expression, binding to miRNA Recognition Elements (MREs) in the 3'UTR of their target genes resulting in either the suppression of translation or degradation of mRNA transcripts or both (Filipowicz et al., 2008). However, recent reports have shown that MREs may also be found in the upstream elements (5'UTR) of a target mRNA (Lee et al., 2008; Lytle et al., 2007; Orom and Lund, 2010). Generally, miRNAs function as endogenous repressors of target mRNA expression and/or translation although there are also instances where miRNAs have been shown to enhance mRNA translation (Manakov et al., 2009; Nielsen et al., 2009; Orom and Lund, 2010). The compounding effect of the multiple regulatory programs ultimately determines the protein profile of the cell.

Recent studies have revealed that miRNAs are highly abundant in brain and play important roles in a variety of biological processes such as neuronal differentiation (Cheng et al., 2009), brain development (Siegel et al., 2009), synapse formation and plasticity (Schratt et al., 2006), neuronal survival (Schaefer et al., 2007), and neurodegenerative diseases (Bushati and Cohen, 2008). MiRNAs may also mediate the cellular adaptations that occur in response to drug abuse and addiction. Specifically, changes in miRNA expression occur as a result of exposure to a number of drugs of abuse, including nicotine (Huang and Li, 2009; Shan et al., 2009), cocaine (Chandrasekar and Dreyer, 2009), morphine (Wu et al., 2008; Wu et al., 2009), and alcohol (Pietrzykowski et al., 2008; Sathyan et al., 2007; Tang et al., 2008).

Pietrzykowski et al (Pietrzykowski et al., 2008) showed that alcohol, acting via a specific miRNA, miR-9, regulates the expression of alternatively spliced mRNAs encoding the large-conductance calcium- and voltage-activated potassium channel (BK), which is a known target of alcohol's actions in mediating molecular alcohol tolerance. Alcohol caused a rapid up-regulation in miR-9 expression, resulting in selective degradation of BK mRNAs containing a miR-9 target site in their 3'UTRs. The selective degradation of some splice variants but not others altered the profile of BK channels, consistent with the development of tolerance to alcohol (Pietrzykowski et al., 2008). This represents a new mechanism of

gene regulation of splice variants that may underlie the neuroadaptive changes that occur at a cellular level to the long-term alcohol exposure.

In the present study, we used miRNA arrays to identify miRNAs with altered expression in the frontal cortex of 14 alcoholics and 13 age- and sex-matched controls that were used in our microarray studies (Liu et al., 2006). Since miRNAs are known to act in part as modulators of gene expression, we correlated mRNA and miRNA profiles across the entire genome to determine whether differentially expressed miRNAs could be influencing the mRNA expression changes observed in the frontal cortex of alcoholics. The predicted mRNA targets for each differentially expressed miRNA were determined and compared with the list of differentially expressed mRNAs reported in Liu et al (Liu et al., 2006) from the same samples.

Materials and Methods

Case selection

Tissues were received from the Queensland Brain Bank, School of Molecular & Microbial Sciences, The University of Queensland in collaboration with the Australian Brain Bank Network which is supported by the National Health and Medical Research Council (NHMRC) as well as the Australian Brain Donor Programs NSW Tissue Resource Centre which is supported by The University of Sydney, NHMRC, Schizophrenia Research Institute, National Institute of Alcoholism and Alcohol Abuse and New South Wales Department of Health. The brain samples were collected by qualified pathologists under full ethical clearance and informed written consent from the next of kin. This study was approved by the Institutional Review Board (IRB) of the University of Texas at Austin.

A total of 27 cases including 14 alcoholic and 13 control cases were selected for analysis. The cases were identical to those used in previous microarray studies (Liu et al., 2006). Alcoholics and controls were selected on the basis of alcohol intake. Controls were defined as individuals who consumed less than 20 g of ethanol per day on average. Alcoholics were defined by NHMRC/WHO criteria as individuals who consumed more than 80 g of ethanol per day for most of their adult lives. Many of the alcoholics used in this study had consumed over 200 g of ethanol per day and had been drinking for over 20 years. Alcoholics with a history of poly-drug abuse or other complicating conditions such as cirrhosis of the liver, Wernicke Korsakoff syndrome or Hepatic Encephalopathy were excluded.

MiRNA extraction and hybridization

MiRNAs were extracted using the *mirVana*[™] miRNA Isolation Kit (Ambion, Austin TX, USA) according to the manufacturer's instructions. Yield and quality of the miRNA-enriched fraction was determined using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

Microarray printing and hybridization were performed at the NIH-Duke University microarray facility. miRNA samples from 13 controls and 14 alcoholics were labeled with the *mirVana* miRNA Labeling Kit (Ambion) according to the manufacturer's protocol. Expression profiling was performed using the miRNA-AI (Ambion *mirVana* set2 + Invitrogen NCode multispecies miRNA microarray V2), which contains probes targeting all of the known mature miRNAs in mirBase 9.0 for human, mouse, rat, *Drosophila*, *C.elegans*, and zebrafish. Images were analyzed using Axon GenePix 6.0.

MiRNA microarray data analysis

MiRNA array data analysis. Data preprocessing included normexp background correction (Ritchie et al., 2007) and print-tip loess normalization (Smyth and Speed, 2003). Statistical differences between groups was performed using the Bioconductor package limma (Smyth et al., 2005) using an empirical Bayes approach. Since each probe was spotted in duplicate on the arrays, within-array replication was assessed using the limmaduplicate Correlation function (Smyth et al., 2005). False discovery rate was assessed using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). The raw data has been deposited in MIAME compliant Longhorn Array Database (LAD) (Killion et al., 2003) with the access number in progress.

Validation by real time RT-PCR analysis

Single-stranded cDNA was synthesized from total RNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Following reverse transcription, quantitative RT-PCR (qRT-PCR) was performed in duplicate, using TaqMan® MicroRNA Assays (Applied Biosystems) following manufacturer's instructions. Assays used were: hsa-miR-7 (P/N: 4427975, ID: 000268), hsa-let-7g (P/N: 4427975, ID: 002282), hsa-miR-152 (P/N: 4427975, ID: 000475), hsa-miR-15b (P/N: 4427975, ID: 000390), hsa-miR-301a (P/N: 4427975, ID: 000528), hsa-miR-369-3p (P/N: 4427975, ID: 000557), RNU44 (P/N: 4427975, ID: 001094), and RNU6B (P/N: 4427975, ID: 001093). qRT-PCR reactions were carried out in a 7900HT Fast Real-Time PCR System and data collected using SDS software (Applied Biosystems). qRT-PCR results (absolute Ct data) were imported into GenEx software (MultiD Analyses AB, Gothenburg, Sweden). Data assembled from multiple experimental plates was subjected to normalization to common reference samples, autoscaling, and normalization to the average of two endogenous control genes (SNORD44 and U6BsnRNA). Kolomogorov-Smirnov test was performed to assess normality of the data and t-test to assess statistical significance.

MiRNA target prediction

Gene targets of the differentially expressed miRNAs were predicted using the Predicted Targets component of miRecords ((Xiao et al., 2009), <http://miRecords.biolead.org>), which integrates the predicted targets produced by 11 established miRNA target prediction programs (DIANA-microT, Micro Inspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargetScanS). Predictions were filtered to only consider those targets predicted by at least 4 out of the 11 prediction algorithms.

Integrative analysis of miRNA and mRNA microarray data

Hypergeometric tests for multiple experimental proportions extracted from the integrated dataset containing information about differentially expressed miRNAs, differentially expressed mRNAs, and putative miRNA targets were implemented in the R environment (R Development Core Team (2010); R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria. Available from: <http://www.R-project.org>). Hypergeometric tests for each differentially expressed miRNA were conducted to determine whether corresponding putative targets were over- or under-represented among inversely correlated differentially expressed mRNAs from the same samples. Hypergeometric tests for each differentially expressed mRNA that is predicted to be targeted by more than one miRNA were conducted to determine whether any of such mRNAs could be subjected to "over-targeting" by miRNAs.

To verify the results from the hypergeometric tests on the experimental data, we generated 1000 randomized samples of non-modulated miRNAs and 1000 randomized samples of non-modulated mRNAs. The size of the samples were fixed and determined by the number of corresponding modulated miRNAs and mRNAs in the experimental dataset. These randomized samples were paired into a 1000 sets of randomized miRNAs and mRNAs and iteratively subjected to the same statistical analysis workflow as the experimental dataset. These represent a set of 1000 randomized trials, which represent our randomized control group. Proportions of interest (proportion of putative targets among randomly selected mRNAs; proportion of randomly selected mRNAs being targeted by more than 1 randomly selected miRNAs; proportion of randomly selected miRNAs that have an over-represented number of randomly selected mRNA targets; and proportion of randomly selected mRNAs that have an over-represented numbers of randomly selected miRNAs targeting them) were recorded for each trial, summarized after completion of 1000 trials, and statistically compared to corresponding experimental proportions using the z-score test (z-test), also implemented in the R environment. The download of miRecords' predicted target information for every human miRNA accessible through the website was done using the RCurl package implemented in R.

Gene list functional enrichment analysis

Lists of statistically significant miRNAs and mRNAs were subjected to functional enrichment analysis using GO categories and KEGG pathways in DAVID (Dennis et al., 2003) and ToppFun(Chen et al., 2009).

Results

MiRNA up-regulation in the frontal cortex of alcoholics

MiRNA microarrays were used to measure miRNA expression profiles in the frontal cortex of control and well-characterized alcoholic cases. The cases used in the present study are the same as those used in previous cDNA microarray studies (Liu et al., 2006) allowing direct comparison between mRNA expression profiles and those of the predicted target mRNAs.

Approximately 48 miRNAs were up-regulated in the frontal cortex of alcoholics ($p < 0.005$, $FDR < 15\%$) with a fold change of between 16% and 72% (mean = $27\% \pm 11\%$). Interestingly, miRNA down-regulation was not observed at this level of significance. The miRNA microarray contains probes targeting miRNAs for human, mouse, rat, *Drosophila*, *C.elegans*, and zebrafish; and, several miRNAs that met our significance cutoff for differential expression were from species other than human. For each of these miRNAs, the sequence of the probe representing the miRNA on the array was compared with all known miRNA sequences in miRBase using BLAST to identify the human equivalent. Where there was a greater than 90% homology in miRNA sequence across species, the human homologs for the miRNAs were used for target prediction. Six of the differentially expressed miRNAs had no homology to any known human miRNA and were not included in subsequent analyses.

The miRNA microarray also included a set of probes corresponding to unpublished miRNA sequences from Ambion. Seven of these miRNAs were differentially expressed in the frontal cortex of alcoholics (ambi_miR_12897, ambi_miR_9451, ambi_miR_8488, ambi_miR_9125, ambi_miR_7058, ambi_miR_8518, and ambi_miR_13196). The sequences corresponding to the probes on the microarray for each of these miRNAs were compared with the sequences of the known miRNAs registered in miRBase using the SSEARCH algorithm (<http://www.ebi.ac.uk>). Only one, ambi_miR_7058, showed homology to a known miRNA. This miRNA sequence exhibited a

100% match to the hsa-miR-423-5p sequence. The predicted targets for this miRNA were included in subsequent analyses. Thus, a total of 35 differentially expressed miRNAs were used for target predictions (Table 1).

Predicted miRNA targets are over-represented among down-regulated mRNAs

Genome-wide prediction algorithms can provide a variable picture of miRNA behavior, although it is difficult to assess which method is best for identifying miRNA targets. We relied on a consensus-based approach to increase our prediction confidence. For the 35 up-regulated miRNAs, a total of 8,200 targets were predicted by a consensus of any 4 of the 11 prediction tools provided by miRecords (see Materials and Methods).

Global expression profiling identified 531 differentially expressed mRNAs in the frontal cortex of alcoholics. Annotations are currently available for 342; 217 were down-regulated and 125 were up-regulated (Liu et al., 2006). In general, the expression levels of miRNAs and their mRNA targets are inversely correlated (Guo et al., 2010), with up-regulation of miRNAs correlated with down-regulation of their respective targets. Because all of the differentially expressed miRNAs in our dataset were up-regulated, we first considered only those mRNAs which were down-regulated in our microarray study and conducted over-representation analysis of predicted miRNA target sites in our set of down-regulated mRNAs. Predicted miRNA targets were significantly over-represented in our set of down-regulated mRNAs (experimental proportion 131/217 vs. mean randomized trial proportion 95/217, z-test $P = 0.00059$). There was no such over-representation amongst the set of up-regulated mRNAs. This finding supports a role for miRNA-regulation of these genes that is consistent with the current understanding that miRNA expression levels are generally inversely correlated with the expression levels of their respective mRNA targets (Guo et al., 2010).

Down-regulated mRNAs may be combinatorially regulated by multiple miRNAs

We used our list of consensus predictions to determine the proportion of down-regulated mRNAs potentially targeted by more than one of the up-regulated miRNAs. Of the 131 down-regulated mRNAs, 102 (78%) were predicted to have miRNA recognition sequences for more than one miRNA. This proportion is significantly higher than expected by chance (hypergeometric $P = 2.7 \times 10^{-5}$). Analysis of the corresponding proportion on the randomized control group (54/95, 57%) additionally evidenced the significant over-representation of putative targets regulated by multiple miRNAs (z-test $P = 0.00068$). There was no such over-representation amongst the set of up-regulated mRNAs. This suggests that post-transcriptional mRNA processing in the frontal cortex of alcoholics may be the result of the combined effects of multiple miRNAs acting on the same mRNA.

Further analysis showed that the predicted targets of 27 up-regulated miRNAs were over-represented in the group of down-regulated mRNAs (hypergeometric $p < 0.05$, FDR $< 5\%$, Table 2). This was also corroborated by the analysis on the randomized control group (z-test $P = 0.00099$), where only 14 out of 35 randomly selected miRNAs display the equivalent behaviour. Remarkably, this core group of 27 miRNAs is predicted to regulate 128 out of the 131 (98%) down-regulated mRNAs that are putative miRNA-targets. This underscores the central role played by these 27 miRNAs in regulating gene expression in the frontal cortex in response to chronic alcohol consumption.

There is increasing evidence to suggest that multiple miRNAs can work together either cooperatively or competitively to alter the expression of their mRNA targets (Grimson et al., 2007; Hua et al., 2006; Nachmani et al., 2010; Sathyan et al., 2007). Supporting this idea, we have shown that combinatorial miRNA targeting events are over-represented among

down-regulated mRNAs. To identify the down-regulated mRNAs that may be targeted by multiple miRNAs in the frontal cortex of alcoholics we performed an over-representation analysis of miRNA targeting events for each individual down-regulated mRNA. Out of the 102 downregulated mRNAs that are putative targets of multiple miRNAs, 17 were predicted to be targeted by a significantly higher number of up-regulated miRNAs than expected by chance (hypergeometric $p < 0.05$ and FDR $< 25\%$, Table 3). This proportion is also significantly different (z-test $P = 0.00086$) from the equivalent one obtained in the random control group, where only 1 out of 54 relevant mRNAs displayed such behaviour. These results suggest that, similarly to the upregulated miRNA core group, this downregulated “over-targeted” mRNA group may be important in mediating the effects of miRNA regulation due to chronic alcohol consumption.

Functional Enrichment Analysis

To investigate the functional significance of the group of 131 downregulated mRNA that are putative targets of the 35 upregulated miRNAs, and the functional significance of the core of 17 downregulated mRNAs that appear combinatorially over-targeted by the same miRNAs, we performed enrichment analysis using ToppFun (Table 4) and DAVID (Table 5).

Among the relevant categories highlighted by the analysis of the 131 downregulated target list (Table 4.A) is the process of cellular component morphogenesis, particularly in apical parts of the cells, which is intrinsically related to neuronal development and structural plasticity (Russo et al., 2010). Interestingly, Hamilton and collaborators have recently reported that alcohol exposure decreases dendritic complexity and density while consolidating pre-existing synapses in prefrontal cortex of postnatal rats (Hamilton et al., 2010). Our data shows additional significant enrichment in the growth cone cellular component category for the core of 17 over-targeted mRNAs (Table 4.B), which underscore the impact that miRNAs may play in this process. Vesicle-related and the endomembrane system categories, accounting for about 20% of the downregulated targets in the group of 131 downregulated mRNAs, also showed highly significant enrichments. This could represent additional evidence for the role that neuronal remodelling and plasticity might play during neuroadaptation to chronic alcohol consumption. Furthermore, ubiquitination-related activities were highlighted as significantly enriched molecular functions in the core of 17 over-targeted mRNAs, which underscores the importance of the proteasome system. An unexpected result from the functional enrichment analysis was the inverse relationship detected between a subset of 27 downregulated mRNA targets and upregulated Alzheimer’s disease genes (Blalock et al., 2004) (Table 4, Coexpression Gene Sets). Interestingly, there is growing body of evidence describing a neuroprotective role for moderate alcohol drinking that significantly reduces the risk of either cognitive decline or dementia, including Alzheimer’s disease (Collins et al., 2009). Table 5 shows the lists of downregulated genes that scored significantly enriched in DAVID GO categories and KEGG pathways.

Validation by real time RT-PCR analysis

To validate results from our integrated miRNA-mRNA microarray analysis we performed qRT-PCR. Due to limited sample availability, a representative subset (6 out of 27) of up-regulated miRNAs was assessed. Four of the six miRNAs (67%) were also found significantly upregulated by qRT-PCR: hsa-let-7g ($P = 0.00196$), hsa-miR-15b ($P = 0.02394$), hsa-miR-152 ($P = 0.02605$), and hsa-miR-7 ($P = 0.04647$). Two miRNAs, hsa-miR-301a and hsa-miR-369-3p, did not produce statistically significant differences. Although hsa-miR-301a did not achieve the level of significance desired, we were able to detect an expected 1.57 fold increase in expression in the alcoholic samples as compared to the control group. This particular miRNA assay was negatively affected by the distribution of the control group results, which failed the Kolmogorov-Smirnov test for normality.

Additional validation of these results was recently obtained by our group while conducting miRNA microarray studies in a mouse model of high ethanol consumption (unpublished data). Mouse homologs of 5 out of the 6 human miRNAs assessed by qRT-PCR (hsa-let-7g, hsa-miR-15b, hsa-miR-152, hsa-miR-7, and hsa-miR-301a) were also found significantly upregulated in the mouse microarray study. This conservation across species represents further invaluable validation of the role played by these miRNAs in alcohol-related cellular mechanisms.

Discussion

Global microarray and proteomics studies have identified a number of gene groups and gene families that are differentially expressed in the prefrontal cortex of alcoholics. These gene groups include those involved in myelination, ubiquitination, apoptosis, cell adhesion, neurogenesis, and neural disease (Liu et al., 2006). The mechanism by which chronic alcohol abuse results in selective changes in gene expression in the prefrontal cortex is not known. Here we present the results of miRNA microarray analysis of the same samples used in our previous microarray studies. The aim of the study was to identify miRNAs which are differentially expressed in the prefrontal cortex. By profiling global miRNA expression changes and mRNA profiles in the same samples, we were able to determine the extent to which down-regulated genes may be targeted by miRNAs. In striking correspondence with the relatively small but abundant changes detected in mRNA expression, we now find that many miRNAs (approximately 35) change expression levels in the frontal cortex of the human alcoholics and that these changes are consistently small across the whole set of differentially expressed miRNAs. In addition, 27 of the differentially expressed miRNAs had an over-represented number of predicted targets correspondingly changing expression in the alcoholic group.

Using miRNA target prediction programs in combination with previous mRNA profiling, more than half of the annotated differentially expressed mRNAs (55%) were identified as potential targets. Putative targets of miRNAs were significantly over-represented among downregulated genes in alcoholics. This may indicate an inverse relationship among the over-expressed miRNAs and respective down-regulated target genes. Several studies have recently reported similar negatively correlated expression patterns relationships (Farh et al., 2005; Manakov et al., 2009; Nielsen et al., 2009; Sood et al., 2006), which may indicate a characteristic behavior of miRNA-mRNA regulatory networks. However, miRNAs may additionally function indirectly over specific targets, e.g. through complex feed-forward and feedback loops (Hobert, 2008; Sales et al., 2010), or different combinations of miRNAs may coordinately regulate specific target genes by unknown mechanism to promote positively correlated mRNA expression patterns.

Among major findings reported by our group in Liu et al. (Liu et al., 2006) was the consistent down-regulation of particular biological processes such as myelination and cell adhesion, which play critical roles in the development of the CNS and synapse formation. These observations were supported by previous morphological and expression profiling studies (Charness et al., 1994; Lewohl et al., 2000; Liu et al., 2004; Mayfield et al., 2002). We have now found that up-regulated miRNAs appear to impact the down-regulation of genes relevant to the same processes, for example miR-203/miR-586/miR-146a targeting proteolipid protein 1 (PLP1), a major component of the myelin sheath; miR-194 targeting myelin gene regulatory factor C11ORF9 (a putative transcription factor); and miR-519b-3p/miR665 targeting minor myelin sheath component 2',3'-cyclic nucleotide 3'phosphodiesterase (CNP). Important cell adhesion genes such as activated leukocyte cell adhesion molecule (ALCAM) and scavenger receptor class B, member 2 (SCARB2) are apparently targeted by a myriad of up-regulated miRNAs including miR-586/miR-144/

miR-34c-5p/miR-18a and miR-203/miR-586/miR-580/miR-144/miR-380/miR-802/miR-7/miR-339-5p respectively. Genes involved in cytoskeleton (CDC42, FERMT2, LMO7, ABI2, TNKS, CNP, CAPN3, DST, SPAST, SGBC) and cell projection (ALCAM, CDC42, CCDC88A, ULK2, ABI2, RDX, CNP, DST) organization were also among the functional groups enriched among the differentially expressed miRNA targets. Other neurogenesis-related genes such as TIMP metalloproteinase inhibitor 2 (TIMP2) and TGFB2 appear to be regulated by multiple miRNAs as well. These results support the consensus idea that remodelling of synaptic connections, dependent upon changes in gene expression in response to alcohol, is responsible for the structural and functional alterations seen in alcohol dependence (Pignataro et al., 2009; Wilke et al., 1994). It has also been suggested that the adaptations to alcohol resemble to some extent the brain plasticity taking place during long-term exposure to other drug of abuse such as cocaine and heroin (Pignataro et al., 2009).

In addition, genes involved in lipid metabolism (PLP1, SPTLC2, SCD, PIK3C2B, SGMS1, FAR1, PLCL1, TEX2, ELOVL5, ELOVL4, LA SS2, ELOVL7, OSBPL11), ubiquitination (UFBP2, BIRC6, FBXL3, USP31), and cell death (SGK1, PLP1, CDKN1B, SPG20, RYBP, BIRC6, RIPK2, SGMS1, DDO1, SPAST, TP53, NP1, TGFB2) (see Table 5) are differentially expressed and over-represented in our genome-wide mRNA profiling study as well as over-represented as predicted targets of miRNA changing expression in the same samples. Furthermore, out of a list of 27 genes previously identified in other alcohol studies using autopsy human brain (Flatscher-Bader et al., 2005; Lavoie and Butterworth, 1995; Lewohl et al., 2000; Liu et al., 2006; Mayfield et al., 2002; Sokolov et al., 2003), about half of them (13/27) were also identified as potential targets of differentially expressed miRNAs. These included: Calpain 3 (p94) (CAPN3); Lysosomal-associated membrane protein 2 (LAMP2); EGF-like repeats and discoidin I-like domains 3 (EDIL3); sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1 (SPOCK1); TIMP metalloproteinase inhibitor 2 (TIMP2), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 (SMARCA5); Calnexin (CANX); Unc-51-like kinase 2 (ULK2); proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated) (PLP1); 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP); transketolase (Wernicke-Korsakoff syndrome) (TKT); stearyl-CoA desaturase (delta-9-desaturase) (SCD); and acidic (leucine-rich) nuclear phosphoprotein 32 family, member E (AMP32E).

A recurring theme evidenced in our analysis is the extensive combinatorial targeting by which miRNA apparently exert an “over-zealous” control to fine tune the expression of specific targets. A combinatorial paradigm in miRNA function could help explain how miRNA could be achieving process-related specificity despite the fact that they are known to target a wide variety and number of distinct targets. Support for this idea has started to accumulate and its impact only recently highlighted. For example, while studying the global and local architecture of the mammalian miRNA-transcription factor (TF) regulatory network, Shalgi and collaborators found extensive interactions between miRNAs and between miRNAs and TFs, and suggested that “combinatorics may also have the advantage of allowing multiple sources of information, each represented by a single miR, to be integrated into the regulation of individual transcripts” (Shalgi et al., 2007). The potential combinatorial regulation of DICER1, the down-regulated alcohol-responsive gene with the greatest over-representation of miRNA targeting events in our dataset, could have important implications for understanding the regulatory events taking place under chronic alcohol abuse. Dicer is a key ribonuclease that processes precursor miRNAs and siRNA into their short mature forms and its knockdown has resulted in reduced expression of mature miRNA (Kumar et al., 2007). Targeting and down-regulation of Dicer by miRNAs could represent an important negative feedback mechanism by which miRNAs control their own availability

and avoid potentially detrimental effects of excess deregulation. Supporting this line of reasoning is the recent report by Tokumaru et al. (Tokumaru et al., 2008) describing the possible existence of a novel negative feedback loop between let-7 and Dicer that may play a role in the tuning of mature miRNA expression and carcinogenesis. In addition, it has been reported that the magnitude of change in levels of endogenous miRNAs that mediate regulation of gene expression often occur over a narrow range of only 20-30% (Hobert, 2007; Peter, 2010). In our study we also found that the alcohol-induced up-regulation of miRNAs was constrained in a similar range, with only a few miRNAs varying levels in more than 30%. We speculate that negative feedback loops between Dicer and specific miRNAs could serve as a general mechanism for tight, global regulation of miRNA levels. The importance of feedback loops in homeostasis and cellular differentiation programs has been highlighted (Alon, 2007; Martinez et al., 2008) and the role of Dicer as a stress response component suggested (Wiesen and Tomasi, 2009). We reason that remodeling of cellular homeostasis could be taking place in the brain of chronic alcohol abusers as an adaptive mechanism, particularly in this specific cohort of alcoholic individuals, who had spent most of their adult lives consuming high quantities of ethanol without developing complicated alcohol-related disorders an indication of benign adaptation to the increased concentrations of alcohol in brain and other tissues.

This study highlights the relevant impact that miRNAs could be having in the development of alcohol-related changes in human brain. In general, our results suggest that up-regulation of miRNAs in the frontal cortex of human alcoholics may contribute to the deterioration and concomitant adaptation of neuronal functioning observed in cases of alcohol abuse. The overlap between putative targets of miRNAs and the differentially expressed genes from our previous study provides information on the biological functions and networks of genes regulated by specific miRNAs in human alcoholism. In the future, biochemical validation should be used to determine the extent to which gene regulation by miRNAs could be impacting specific protein levels.

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Table 1

Differentially expressed miRNAs used for target prediction.

miRNA	% Change	P.Value	Adj. P.Value
miR-553	25	0.00009	0.0425
miR-369-3p	29	0.00014	0.0425
miR-18a	24	0.00014	0.0425
miR-339-5p	29	0.00015	0.0425
miR-1	45	0.00018	0.0440
miR-7	24	0.00023	0.0440
miR-196a	28	0.00026	0.0440
miR-301a	25	0.00031	0.0453
miR-144	24	0.00036	0.0453
let-7g	23	0.00043	0.0453
miR-153	26	0.00044	0.0453
let-7f	27	0.00050	0.0490
miR-203	21	0.00054	0.0500
miR-34c-5p	23	0.00068	0.0554
miR-101	25	0.00080	0.0554
miR-376c	27	0.00080	0.0554
miR-665	26	0.00084	0.0554
miR-152	33	0.00085	0.0554
miR-194	21	0.00098	0.0597
miR-423-5p	27	0.00122	0.0715
miR-515-3p	31	0.00135	0.0744
miR-374b	26	0.00149	0.0794
miR-140	72	0.00223	0.1065
miR-519b-3p	17	0.00256	0.1152
miR-586	20	0.00282	0.1162
miR_135b	25	0.00284	0.1162
miR-92a	51	0.00333	0.1283
miR-15b	18	0.00338	0.1283
miR-580	23	0.00350	0.1283
miR-146a	19	0.00391	0.1283
miR-454-3p	16	0.00398	0.1283
miR-380	26	0.00402	0.1283
miR-652	16	0.00404	0.1283
miR-802	18	0.00409	0.1283
miR-196b	20	0.00481	0.1432

Table 2

miRNAs whose targets are over-represented among down-regulated mRNAs.

Up-regulated miRNAs	Down-regulated Targets	%	All Predicted Targets	%	Hyper-geometric P. Value	Adjusted P. Value
hsa-miR-203	49	37.4	1759	15.1	8.3E-11	2.9E-09
hsa-miR-454	22	16.8	428	3.7	3.7E-10	6.4E-09
hsa-miR-586	37	28.2	1185	10.2	1.4E-09	1.6E-08
hsa-miR-301a	23	17.6	542	4.7	7.0E-09	6.1E-08
hsa-miR-144	22	16.8	547	4.7	3.9E-08	2.8E-07
hsa-miR-152	17	13.0	455	3.9	3.3E-06	1.9E-05
hsa-miR-380	31	23.7	1248	10.7	5.9E-06	2.9E-05
hsa-miR-374b	10	7.6	204	1.8	1.9E-05	8.2E-05
hsa-miR-101	16	12.2	513	4.4	6.2E-05	0.00024
hsa-miR-519b-3p	24	18.3	1003	8.6	0.00012	0.00042
hsa-miR-18a	8	6.1	193	1.7	0.00033	0.00100
hsa-miR-196a	8	6.1	194	1.7	0.00034	0.00100
hsa-miR-802	22	16.8	962	8.3	0.00043	0.00116
hsa-miR-1	13	9.9	453	3.9	0.00058	0.00145
hsa-miR-7	11	8.4	355	3.1	0.00066	0.00155
hsa-miR-196b	8	6.1	222	1.9	0.00091	0.00198
hsa-miR-15b	18	13.7	779	6.7	0.00114	0.00234
hsa-miR-153	10	7.6	375	3.2	0.00340	0.00661
hsa-miR-580	18	13.7	869	7.5	0.00393	0.00723
hsa-miR-665	7	5.3	228	2.0	0.00425	0.00744
hsa-miR-376c	12	9.2	544	4.7	0.00833	0.01388
hsa-let-7g	12	9.2	551	4.7	0.00922	0.01467
hsa-miR-194	8	6.1	352	3.0	0.01805	0.02746
hsa-let-7f	11	8.4	547	4.7	0.02052	0.02984
hsa-miR-135b	11	8.4	550	4.7	0.02132	0.02984
hsa-miR-92a	10	7.6	491	4.2	0.02279	0.03069
hsa-miR-369-3p	7	5.3	329	2.8	0.03259	0.04225

Table 3

mRNA targets with an over-represented number of miRNA targeting events.

Down-regulated Target	Up-regulated miRs	%	All Predicted miRs	%	Hyper-geometric P. Value	Adjusted P. Value
CXCR4	3	8.6	5	1.4	0.00036	0.01227
DICER1	13	37.1	59	16.5	0.00037	0.01227
BIRC6	8	22.9	29	8.1	0.00072	0.01875
SCARB2	8	22.9	36	10.1	0.00405	0.07250
EDIL3	3	8.6	8	2.2	0.00407	0.07250
SLAIN1	5	14.3	18	5.0	0.00445	0.07250
SSR1	8	22.9	37	10.3	0.00498	0.07250
GART	2	5.7	5	1.4	0.00750	0.09831
FAM108B1	5	14.3	21	5.9	0.01041	0.11368
RNF103	2	5.7	6	1.7	0.01400	0.13897
FBXL3	6	17.1	29	8.1	0.01485	0.13897
TEX2	5	14.3	23	6.4	0.01671	0.14592
FRYL	6	17.1	30	8.4	0.01799	0.14728
PAPD4	5	14.3	24	6.7	0.02069	0.15943
SESTD1	5	14.3	25	7.0	0.02528	0.18069
RIPK2	2	5.7	8	2.2	0.03413	0.21897
CDKN1B	4	11.4	20	5.6	0.03510	0.21897

Table 4

ToppFun functional enrichment analysis (Bonferroni FDR < 0.2). Categories with 2 or more terms in query.

A. List of 131 downregulated genes**GO Biological Process**

ID	Name	P-value	Term in Query	Term in Genome
GO:0032989	cellular component morphogenesis	0.0219	20	987
GO:0048468	cell development	0.0416	21	1120
GO:0002064	epithelial cell development	0.1847	4	37

GO Cellular Component

ID	Name	P-value	Term in Query	Term in Genome
GO:0012505	endomembrane system	0.0004	27	1481
GO:0016023	cytoplasmic membrane-bounded vesicle	0.0017	17	719
GO:0045177	apical part of cell	0.0796	8	253
GO:0012506	vesicle membrane	0.1167	8	268
GO:0005794	Golgi apparatus	0.1802	17	1056

Coexpression Gene Sets

Source	Name	P-value	Term in Query	Term in Genome
mSigDB	POD1_KO_DN	0.0002	22	696
mSigDB	ASTON_DEPRESSION_DN	0.0004	10	141
mSigDB	ET743_SARCOMA_DN	0.0595	10	248
mSigDB	ALZHEIMERS_DISEASE_UP	0.1318	27	1424

B. List of 17 over-targeted downregulated mRNAs**GO Molecular Function**

ID	Name	P-value	Term in Query	Term in Genome
GO:0016879	ligase activity, forming carbon-nitrogen bonds	0.0063	4	290
GO:0004842	ubiquitin-protein ligase activity	0.0063	3	204
GO:0030145	manganese ion binding	0.0092	2	162

GO Cellular Component

ID	Name	P-value	Term in Query	Term in Genome
GO:0030426	growth cone	0.1135	2	89

Table 5

DAVID functional enrichment analysis on list of 131 downregulated mRNA

Term	Count	Fold	PValue	Genes
fatty acid biosynthetic process	6	9.8	0.0003	FAR1, PLP1, ELOVL5, ELOVL4, SCD, ELOVL7
lipid biosynthetic process	9	3.6	0.0034	FAR1, PLP1, ELOVL5, SPTLC2, ELOVL4, LASS2, SCD, ELOVL7, SGMS1
cytoskeleton organization	10	3.0	0.0064	CDC42, FERMT2, LMO7, ABI2, TNKS, CNP, CAPN3, DST, SPAST, SGCB
cell cycle arrest	5	6.3	0.0081	KHDRBS1, CDKN1B, DST, TP53INP1, TGFB2
fatty acid metabolic process	6	3.9	0.0183	FAR1, PLP1, ELOVL5, ELOVL4, SCD, ELOVL7
sphingolipid metabolic process	4	6.9	0.0200	TEX2, SPTLC2, LASS2, SGMS1
cell death	12	2.2	0.0221	SGK1, PLP1, CDKN1B, SPG20, RYBP, BIRC6, RIPK2, SGMS1, DIDO1, SPAST, TP53INP1, TGFB2
ensheathment of axons in the central nervous system	2	85.9	0.0229	PLP1, C11ORF9
myelination in the central nervous system	2	85.9	0.0229	PLP1, C11ORF9
cell projection organization	8	2.8	0.0233	ALCAM, CDC42, CCDC88A, ULK2, ABI2, RDX, CNP, DST
sphingolipid biosynthetic process	3	12.5	0.0235	SPTLC2, LASS2, SGMS1
membrane lipid metabolic process	4	6.4	0.0244	TEX2, SPTLC2, LASS2, SGMS1
regulation of organelle organization	6	3.6	0.0260	CDC42, CDKN1B, CCDC88A, RDX, TNKS, DST
actin cytoskeleton organization	6	3.4	0.0302	CDC42, FERMT2, LMO7, ABI2, CAPN3, DST
Sphingolipid metabolism	3	8.9	0.0425	SPTLC2, PPAP2C, SGMS1
phosphorylation	12	1.9	0.0432	SRPK2, SGK1, ULK2, PIK3C2B, AAK1, GAB1, ABI2, RIPK2, TNKS, CDK7, MON2, TGFB2