Neuropsychopharmacology (2016) 41, 538–548

www.neuropsychopharmacology.org

Synaptic microRNAs Coordinately Regulate Synaptic mRNAs: Perturbation by Chronic Alcohol Consumption

Dana Most[1,2], Courtney Leiter[1], Yuri A Blednov[1], R Adron Harris[1] and R Dayne Mayfield[1]

[1]Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, Austin, TX, USA; [2]Institute for Neuroscience (INS), University of Texas at Austin, Austin, TX, USA

Local translation of mRNAs in the synapse has a major role in synaptic structure and function. Chronic alcohol use causes persistent changes in synaptic mRNA expression, possibly mediated by microRNAs localized in the synapse. We profiled the transcriptome of synaptoneurosomes (SN) obtained from the amygdala of mice that consumed 20% ethanol (alcohol) in a 30-day continuous two-bottle choice test to identify the microRNAs that target alcohol-induced mRNAs. SN are membrane vesicles containing pre- and post-synaptic compartments of neurons and astroglia and are a unique model for studying the synaptic transcriptome. We previously showed that chronic alcohol regulates mRNA expression in a coordinated manner. Here, we examine microRNAs and mRNAs from the same samples to define alcohol-responsive synaptic microRNAs and their predicted interactions with targeted mRNAs. The aim of the study was to identify the microRNA–mRNA synaptic interactions that are altered by alcohol. This was accomplished by comparing the effect of alcohol in SN and total homogenate preparations from the same samples. We used a combination of unbiased bioinformatic methods (differential expression, correlation, co-expression, microRNA-mRNA target prediction, co-targeting, and cell type-specific analyses) to identify key alcohol-sensitive microRNAs. Prediction analysis showed that a subset of alcohol-responsive microRNAs was predicted to target many alcohol-responsive mRNAs, providing a bidirectional analysis for identifying microRNA–mRNA interactions. We found microRNAs and mRNAs with overlapping patterns of expression that correlated with alcohol consumption. Cell type-specific analysis revealed that a significant number of alcohol-responsive mRNAs and microRNAs were unique to glutamate neurons and were predicted to target each other. Chronic alcohol consumption appears to perturb the coordinated microRNA regulation of mRNAs in SN, a mechanism that may explain the aberrations in synaptic plasticity affecting the alcoholic brain.
Neuropsychopharmacology (2016) **41,** 538–548; doi:10.1038/npp.2015.179; published online 5 August 2015

INTRODUCTION

Local translation of synaptic mRNAs is essential for the functional properties of brain cells (Raab-Graham *et al,* 2006; Wang *et al,* 2009). The extensive neuroadaptations associated with alcohol dependence are likely caused by persistent changes in the expression of hundreds of mRNAs (Mayfield *et al,* 2002; Ponomarev *et al,* 2012; Nunez *et al,* 2013). Many of the alcohol-responsive adaptations are related to synaptic structure and function and may be caused by coordinated changes in local mRNA translation (Wang *et al,* 2010; Mayfield and Nunez, 2012). MicroRNAs are short, non-coding RNAs that can regulate the translation of many target mRNAs, and this process is known to occur in the synaptic compartments of the cell (Lugli *et al,* 2005,2008; Smalheiser and Lugli, 2009; Sosanya *et al,* 2013). The ability of microRNAs to regulate mRNAs provides a localized regulatory system that may be important in the treatment of alcoholism.

*Correspondence: D Most, Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, 2500 Speedway, Austin, TX 78712, USA, Tel: +512 232 2520, Fax: +512 232 2525, E-mail: danamost@gmail.com
Received 24 June 2014; revised 27 May 2015; accepted 6 June 2015; accepted article preview online 24 June 2015

Exposure to alcohol and other drugs of abuse modulates microRNA expression in the brain (Pietrzykowski *et al,* 2008; He *et al,* 2010; Eipper-Mains *et al,* 2011; Lewohl *et al,* 2011; Tapocik *et al,* 2013). MicroRNAs have important roles in learning and memory (Konopka *et al,* 2010), and are also altered in addiction-related behaviors, such as cocaine-conditioned place preference (Chandrasekar and Dreyer, 2011), cocaine-seeking behavior (Novak *et al,* 2010), and self-administration of alcohol (Tapocik *et al,* 2014). Little is known about the microRNAs involved in the regulation of synaptic mRNA translation during alcohol dependence. Studies investigating the effects of chronic alcohol consumption in standard tissue (total homogenate, TH) preparations found a persistent change in the expression of microRNAs and their target mRNAs in humans, mice, and rats (Lewohl *et al,* 2011; Gorini *et al,* 2013; Nunez *et al,* 2013; Tapocik *et al,* 2013). However, the standard TH preparation likely underestimates the number and magnitude of alcohol-responsive transcripts localized in the synapse (Lewohl *et al,* 2011; Most *et al,* 2014).

Synaptoneurosomes (SN) (Hollingsworth *et al,* 1985; Quinlan *et al,* 1999; Raab-Graham *et al,* 2006; Sosanya *et al,* 2013) contain membrane vesicles of pre- and post-synaptic compartments of neurons as well as peri-synaptic

compartments of astrocytes and microglia and offer an improved model for studying the synaptic transcriptome. We recently showed that alcohol-induced mRNA changes are greater in SN compared with TH (Most *et al,* 2014). Here, we profiled the microRNA transcriptomes from paired SN and TH samples of mouse amygdala after a voluntary alcohol consumption paradigm. We identified alcohol-induced microRNAs that were correlated with alcohol consumption and also identified their predicted mRNA synaptic targets.

MATERIALS AND METHODS

Animal Housing and Alcohol Consumption

Adult (two-month-old) C57BL/6J female mice were maintained at the University of Texas at Austin Animal Resources Center. Mice were group-housed and given a 1-week acclimation period in combined housing and another week to acclimate to the bottle positions in individual housing. Food and water were provided *ad libitum* and monitored daily, as were the temperature and reverse light/dark cycle. Mice underwent a 30-day two-bottle choice paradigm with continuous (24-h) access to one bottle of 20% ethanol (referred to as alcohol) and one bottle of water (Blednov *et al,* 2012). A control group of mice received two bottles of water. Bottle weights were recorded daily, mice were weighed every four days, and the amount of alcohol consumed was calculated as g/kg/24 h (see Supplementary Figure 1 for amounts of alcohol consumed). Alcohol bottle positions were alternated daily to control for position preferences. The stage of the menstrual cycle was not determined. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and adhere to NIH Guidelines for the ethical care and use of animals in research.

RNA Extraction

As described previously (Most *et al*, 2014), after 30 days of the two-bottle choice drinking, 8 alcohol-consuming and 12 control mice were killed by cervical dislocation and decapitated at the beginning of the light phase of the light/dark cycle. Brains were removed and washed for 1 min with 1 ml of ice-cold homogenizing buffer (HB) containing 20 mM HEPES, 1 mM EDTA (pH 7.4), 40 U/ml RNAseOut (Invitrogen, CA), phosphatase inhibitor cocktail 3 (Sigma, MO) and protease inhibitors 'Complete' (Roche, IN). Brains were then placed in a coronal Zivic mouse brain slicer with a 0.5 mm resolution (Zivic Instruments, PA) and sliced in the following coordinates in order to isolate extended amygdala: coronal level 56-66 (Bregma (−0.18)-(−1.155)) and 66–80 (Bregma (−1.155)-(−2.55)). The extended amygdala was dissected, placed in ice-cold HB (250 µl), and homogenized for 1 min using a VWR homogenizer and pestle (VWR, PA). To minimize homogenate loss, pestles were washed with 50 µl HB after use and the wash was collected and added to the sample. Ten percent of the homogenate (30 µl) were snap frozen in liquid nitrogen and stored at − 80 °C for subsequent RNA TH analysis. Paired SN samples were isolated from the remaining 270 µl of the homogenate. Homogenates were filtered through a 100-µm-pore filter (Millipore, MA) and subsequently through a 5-µm-pore filter (Millipore); filters were washed with HB before use for protection from RNAse. To maximize yield, the filters were washed with 50 µl HB after use and the wash was collected and added to the homogenate. The homogenate was then centrifuged at 14 000 *g* for 20 min at 4 °C in order to pellet the cell fraction containing SN. The supernatant was removed and the pellet was snap frozen and stored at − 80 °C for SN RNA analysis. Microscopy was used to further characterize the SN preparation (Most *et al,* 2014).

RNA was extracted from 20 paired SN and TH samples using the Direct-Zol RNA extraction kit (Zymo, Japan) with small IC extraction columns according to the manufacturer's instructions. The RNA was quantified using a NanoDrop1000 (Thermo Fisher Scientific Inc., IL) and assayed for quality using an Agilent 2100 Tape Station (Agilent Technologies, CA; Supplementary Figure 2A). The criteria for RNA quantity and quality were as follows: total amount of RNA >500 ng, 280/260 >1.7, and RIN >6.5. We measured RIN of five of the control samples using the Bioanalyzer Nano kit (Agilent). To ensure that samples with high RINs also included high quality and quantity of microRNAs, the same five control samples were subjected to a small RNA analysis using the Bioanalyzer Small kit (Agilent). MicroRNAs comprised $>10\%$ of the small RNA population in the samples (Supplementary Figure 2B).

Microarray Hybridization, Data Quality Assessment, and Analysis

Total RNA was extracted from the TH and SN samples. The homogenates were divided into two parts (90% was used for SN and 10% for TH). The 20 samples from the SN and 20 from the TH (40 total) were hybridized to 40 microRNA microarrays. Previously, we used 40 mRNA microarrays from these same mice (Most *et al*, 2014). Four samples per mouse were hybridized (SN microRNA, SN mRNA, TH microRNA, and TH mRNA). The RNA samples were divided for mRNA (Most *et al*, 2014) and microRNA (this study) analyses. The RNA targeted for microRNA analysis was labeled with flash-tag biotin HSR (Affymetrix, CA) and hybridized to GeneChip microRNA 3.0 Arrays (Affymetrix) at the University of Texas Southwestern Medical Center microarray facility in Dallas. Affymetrix microarrays show a high correlation with results obtained from other platforms such as qPCR (Kolbert *et al*, 2013; Mestdagh *et al,* 2014). This microarray platform uses annotations from miRBase version 17 and contains 19 724 probes for mature microRNAs from 153 organisms. For this study, we focused on the 1111 mature mouse microRNAs detected on the array. The mRNA and microRNA data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al,* 2002) and are accessible through GEO Series accession number GSE51730.

We analyzed the array data using R programming language and Bioconductor packages (http://www.bioconductor.org). Preprocessing (RMA, background correction, and quantile normalization) of the microRNA data was performed with the Bioconductor Oligo package. Quality measures were taken before and after preprocessing using the Array Quality Metrics package (Kauffmann *et al,* 2009; Kauffmann and Huber, 2010) to generate the principal

component analysis (PCA). For all further analyses, only the mouse mature microRNA data were used. The microarray expression data were then analyzed using the Bioconductor Limma package according to the author instructions (Smyth, 2004). This analysis is the main approach currently used for studying thousands of genes from microarrays. We used differential expression analysis between paired SN and TH samples (paired/dependent *t*-test; $n=20$ per group). For comparison of SN and TH expression levels, fold changes were calculated as the ratio of SN to TH. Fold changes greater than 1 are referred to as 'SN-enriched' and fold changes less than 1 are referred to as 'SN-depleted'.

Independent *t*-tests were used to compare the alcohol ($n=8$) and control ($n=12$) groups to determine the effect of alcohol within SN and TH groups. These tests were performed in two separate analyses (one for each preparation). Equal sample sizes are not required for this analysis. For comparison of alcohol and control expression levels, fold changes were calculated as the ratio of alcohol to control expression levels. Fold changes greater than 1 are referred to as 'upregulated' and fold changes less than 1 are referred to as 'downregulated'. *P*-values <0.05 were considered significant for all analyses in the study.

For microRNA co-expression analyses, we used weighted gene correlation (co-expression) network analysis (WGCNA; Langfelder and Horvath, 2008). Individual mice can consume slightly different amounts of ethanol, and we were interested in the time period that represented a consistent amount of drinking and chose the average consumption per mouse from day 10 to day 30. The alcohol consumption was correlated with the expression data using Pearson correlation ($n=8$). However, a WGCNA network was calculated using the 20 SN samples, including both alcohol and control samples. We then determined which of the microRNA modules correlated with alcohol consumption. The WGCNA parameters were as follows for the SN: power 12, signed network, cutHeight 0.995, minModsize 60. For cell type enrichment analysis, we used lists from CamkIIa+ glutamate neurons and Gad+, somatostatin+ and parvalbumin+ GABA neurons (He *et al,* 2012).

We combined the microRNA data (see Table 1 for experimental design) with our previously published mRNA results (Most *et al,* 2014), which were obtained from the same samples. To determine alcohol-sensitive SN and TH modules, we used the 'alcohol-responsive mRNA' lists from Most *et al,* 2014. All *P*-values from the bioinformatic analyses were adjusted using the Benjamini–Hochberg method. For cell types and immune response enrichment, we used the following lists of genes: neurons, astrocytes and oligodendrocytes (Cahoy *et al,* 2008), microglia (cured from Oldham *et al,* 2008), and glutamate/GABA (Sugino *et al,* 2006). For cross species comparison, we used lists from alcohol vapor-treated rats (Tapocik *et al,* 2013) and human alcoholics (Lewohl *et al,* 2011) to identify conserved alcohol-responsive microRNAs. Drugs that potentially target the alcohol-responsive mRNAs and the 'mRNA targets of the alcohol-responsive microRNAs' were found using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Qiagen, CA). See Supplementary Table 1 for a detailed flowchart and the extensive battery of bioinformatic analyses used in the study.

Table 1 Experimental Design of the Study

A	B	C
Treatment	**Preparation**	**40 microRNA microarray samples**
Alcohol-consuming mice, $N=8$	Synaptoneurosome	$N=8\times2=16$
	Total homogenate	
Control mice, $N=12$	Synaptoneurosome	$N=12\times2=24$
	Total homogenate	

Each mouse was allocated to either the alcohol or control group (A) and each contributed an amygdala sample that was split into two parts for SN and TH (B). Total RNA was extracted from each of these preparations and was hybridized to microRNA microarrays (C). The total number of samples was 40 (8 SN and 8 TH for a total of 16 alcohol samples) and 24 control samples (12 SN and 12 TH), and these were individually hybridized to 40 microarrays.

Classification of microRNA–mRNA Interactions

Mouse target predictions for microRNAs and mRNAs were extracted from microRNA.org (version 8-2010). The microRNA.org resource comprises predictions computed by the miRanda algorithm (John *et al,* 2004; Betel *et al,* 2010). The algorithm predicts microRNAs according to the number of putative target sites and the sum of alignment scores determined by both seed match type and seed match context. The predictions that were considered were those annotated as 'conserved microRNA' and 'good mirSVR score'. The individual binding locations can be found in http://www.microRNA.org. We then overlapped the list of mRNAs that were predicted to be targeted by alcohol-responsive microRNAs with the list of microRNAs that were predicted to target the alcohol-responsive mRNAs. Prediction analysis showed that a subset of alcohol-responsive microRNAs were predicted to target certain alcohol-responsive mRNAs and vice versa, providing a bidirectional analysis for identifying microRNA–mRNA interactions using a novel approach.

Quantitative Real-Time PCR (qPCR)

qPCR was used to validate the array data of microRNA expression levels in the same SN samples. First, RNA was DNase treated using the DNA-free Kit (Ambion, TX) and reverse transcribed into cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, CA). Samples were then evaluated for the presence of genomic DNA by comparing GAPDH Cq values from RT+ and RT− reactions, using single threshold Cq determination. Then, RNA (4.5 ng) from four of the SN alcohol samples and five of the SN control samples was used to synthesize cDNA, using the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan PreAmp Master Mix, according to the manufacturer's protocol for creating custom RT and preamplification pools (Applied Biosystems, NY). qPCR was performed in triplicates using TaqMan Universal PCR Master Mix (no AmpErase UNG). FAM-labeled TaqMan MicroRNA Assays (Applied Biosystems) were used to amplify mmu-miR-137 (Assay ID 001129) and hsa-miR-9* (Assay ID 002231). Normalized relative expression was determined with respect to the most stably expressed small nucleolar RNAs, 234 (Assay ID 001234) and 202 (Assay ID 001232), as determined by geNorm analysis (Vandesompele *et al*, 2002).

qPCR results were imported into qBase+ software, version 2.5 (Biogazelle, BE), where the ΔΔCt method was used (Hellemans *et al,* 2007). Statistical analysis was completed in GraphPad Prism software, version 6.

RESULTS

SN and TH microRNA Transcriptomes are Different in Control and Alcohol Samples

We studied the microRNA transcriptomes in paired SN and TH preparations to compare synaptic vs total cell microRNA expression. PCA revealed a distinct clustering of microRNA expression in the two preparations, while showing a homogenous sample population (no outliers detected) within each preparation (Figure 1a). The clustering was evident along the first principal component, indicating the largest variation stems from the distinct expression profiles of the preparations. A comparison of the individual microRNA expression levels on the arrays showed similar values in the SN and TH preparations as well as microRNAs enriched in SN ('SN-enriched') and microRNAs depleted in SN ('SN-depleted'; Figure 1b). We compared the mature mouse microRNA expression levels in SN and TH (non-treated control) and found 180 differentially expressed microRNAs. Eighty-one microRNAs were 'SN-enriched' showing up to an eightfold change in expression, and ninety-nine were 'SN-depleted' (Supplementary Table 2, columns I and J).

We then compared SN and TH samples (referred to as SN-alcohol and TH-alcohol) from mice that chronically consumed 20% ethanol in a two-bottle choice paradigm and found 153 differentially expressed microRNAs, 96 of which were enriched in SN (Supplementary Table 2, columns K and L). It is possible that alcohol exposure changes the expression or trafficking of microRNAs to the synapse, resulting in different SN-enriched microRNAs in alcohol samples compared with control. We assessed the overlap between the differentially expressed microRNAs in SN and TH under alcohol and control conditions and found 63 overlapping microRNAs present in both conditions (Figure 1c).

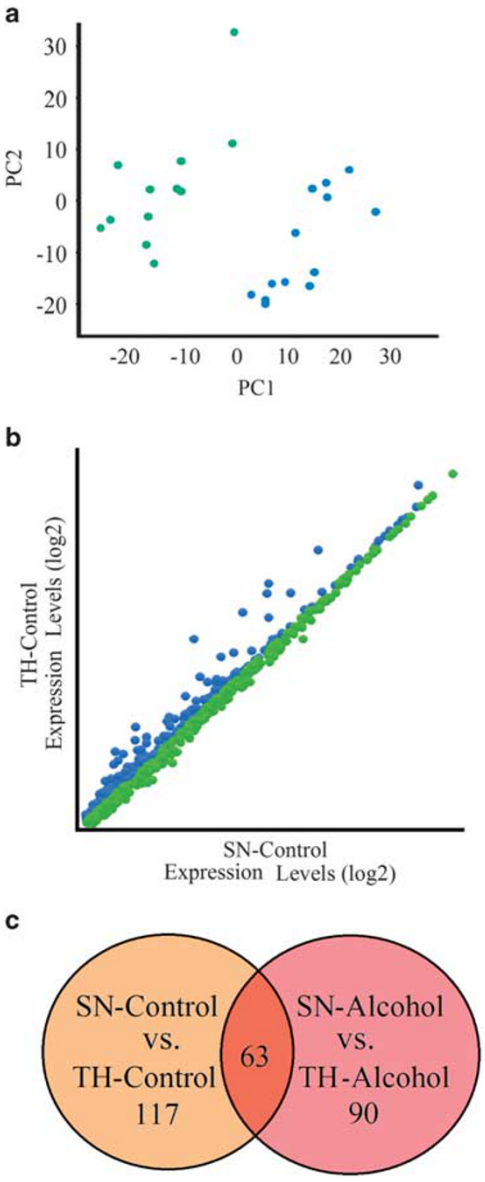

Figure 1 Difference in expression profiles in SN and TH preparations. (a) Principal component analysis of paired SN (green) and TH samples (blue). All microRNAs on the array (including those from different species) were used for this analysis (25 119 probes). The primary purpose for this analysis is quality control to show the overall detection of transcripts on the array and to facilitate comparison of the preparations. Only the mouse mature microRNAs will be discussed and presented in subsequent tables and figures. (b) Expression levels of microRNAs in SN and TH preparations (control group only). For comparison of SN and TH expression levels, fold changes were calculated as the ratio of SN to TH. MicroRNAs below the diagonal are enriched in SN relative to TH and have a fold change greater than 1 (referred to as 'SN-enriched'; shown in green). MicroRNAs above the diagonal are depleted in the SN relative to the TH and have a fold-change less than 1 (referred to as 'SN-depleted'; shown in blue). (c) Venn diagram showing the number of differentially expressed microRNAs from the SN-control/TH-control analysis and the SN-alcohol/TH-alcohol analysis, and the overlap between them. *P*-values <0.05 were considered significant.

Alcohol Consumption Alters microRNA Expression in SN and TH

We investigated alcohol's effects on SN microRNA expression and identified 65 mature mouse microRNAs that were differentially expressed between the alcohol and control samples (Table 2; Supplementary Table 2, columns E and F). Seventy-seven alcohol-sensitive microRNAs were differentially expressed in TH samples (Supplementary Table 2, columns G and H). Twenty microRNAs with the greatest fold changes are shown for SN and TH in Table 3.

There were 23 and 39 upregulated microRNAs with average fold-change magnitudes of 33% and 26% in SN and TH, respectively (Figure 2a). There were 42 and 38 downregulated microRNAS with average fold-change magnitudes of 25% and 27% in SN and TH, respectively. The average fold changes between the preparations were not statistically significant (Supplementary Table 3). The fold changes in SN and TH were not influenced by inherent bias associated with either preparation, as shown in a volcano plot of the fold changes and *P*-values for SN and TH (Figure 2b).

Two microRNAs were chosen for qPCR validation: miR-137, which was found to be alcohol-responsive in SN, and miR-9*, which was not alcohol responsive in SN. Pearson correlation for expression levels between the qPCR

Table 2 Alcohol-Sensitive microRNAs in SN and TH

A	B	C	D	E
MicroRNA type	**Number of microRNAs investigated**	**Number of alcohol-responsive microRNAs in SN**	**Number of alcohol-responsive microRNAs in TH**	**Overlap between SN and TH**
All species	25 119	1377	1623	77
Mouse mature	1111	65	77	1

The number of microRNAs from all species (mature and premature/precursor mRNA) and the mouse (mature) microRNAs (A), the number of microRNAs in each group (B), the number of significant (*P*-values < 0.05) alcohol-responsive microRNAs in SN (C) and TH (D), and the total number of microRNAs that were alcohol-sensitive in both preparations (E) are shown.

Table 3 Alcohol-sensitive microRNAs with the Largest Fold Change in SN (left) and TH (right)

A	B	C	D	E	F	G	H	I	J
Alcohol-responsive microRNAs in SN	**SN fold change**	**SN *P*-value**	**TH fold change**	**TH *P*-value**	**Alcohol-responsive microRNAs in TH**	**SN fold change**	**SN *P*-value**	**TH fold change**	**TH *P*-value**
mmu-miR-1893	1.62	**2.71E-02**	0.91	6.36E-01	mmu-miR-1965	1.06	7.66E-01	1.63	**1.44E-02**
mmu-miR-875-3p	1.61	**4.45E-03**	0.78	9.24E-02	mmu-miR-207	0.99	9.48E-01	1.61	**1.98E-04**
mmu-miR-187*	1.56	**2.87E-02**	1.44	1.67E-01	mmu-miR-467d*	0.68	1.04E-01	1.57	**1.38E-02**
mmu-miR-187	1.51	**1.68E-02**	1.33	1.76E-01	mmu-miR-193*	1.27	2.42E-01	1.53	**1.44E-02**
mmu-miR-92a-2*	1.47	**4.53E-02**	1.18	4.59E-01	mmu-miR-3113*	0.84	2.07E-01	1.45	**1.98E-04**
mmu-miR-466n-3p	1.43	**3.83E-04**	0.87	2.84E-01	mmu-miR-574-5p	0.63	8.91E-02	1.39	**1.61E-02**
mmu-miR-669d-2*	1.38	**1.67E-02**	1.06	4.32E-01	mmu-miR-200c	1.28	8.78E-02	1.35	**2.95E-02**
mmu-miR-216b	1.38	**8.53E-03**	0.98	8.67E-01	mmu-let-7b*	0.78	9.23E-02	1.32	**2.11E-02**
mmu-miR-501-5p	1.37	**4.41E-02**	0.80	1.09E-01	mmu-miR-322	0.99	9.32E-01	1.30	**3.78E-02**
mmu-miR-5115	1.34	**3.51E-02**	0.79	2.15E-01	mmu-miR-30c-2*	1.03	8.43E-01	1.28	**2.79E-02**
mmu-miR-18a	0.65	**1.41E-02**	1.00	9.98E-01	mmu-miR-9	1.18	3.64E-01	0.63	**1.93E-02**
mmu-miR-377*	0.65	**1.73E-02**	1.13	4.23E-01	mmu-miR-539-5p	0.90	5.57E-01	0.62	**1.40E-03**
mmu-miR-466g	0.65	**4.11E-02**	1.33	1.97E-01	mmu-miR-411*	0.83	1.73E-01	0.62	**7.47E-03**
mmu-miR-135a-2*	0.64	**4.82E-03**	0.74	2.09E-01	mmu-miR-3068	0.95	8.25E-01	0.61	**2.32E-02**
mmu-miR-466f	0.64	**3.33E-02**	0.85	3.12E-01	mmu-miR-3473	1.03	8.39E-01	0.60	**1.67E-02**
mmu-miR-5099	0.63	**4.18E-02**	0.90	3.74E-01	mmu-miR-5100	1.28	9.73E-02	0.60	**5.64E-04**
mmu-miR-344c	0.59	**8.78E-04**	0.83	2.63E-01	mmu-miR-5097	1.23	2.25E-01	0.58	**1.07E-02**
mmu-miR-34c*	0.58	**3.78E-02**	0.77	2.98E-01	mmu-miR-132*	0.96	6.99E-01	0.54	**9.32E-03**
mmu-miR-1187	0.58	**2.09E-03**	1.48	1.75E-01	mmu-miR-720	1.20	3.46E-01	0.52	**3.52E-03**
mmu-miR-466f-3p	0.43	**3.83E-04**	1.59	1.37E-01	mmu-miR-1298	0.56	8.19E-02	0.37	**1.78E-02**

The top 10 most upregulated microRNAs (top rows) and the top 10 most downregulated microRNAs (bottom rows) in SN (columns A–E) and TH (columns F–J) are shown. Columns B and G show the fold changes and columns C and H show the *P*-values in SN. Columns D and I show the fold changes and columns E and J show the *P*-values in TH. For comparison of alcohol and control expression levels, fold changes were calculated as the ratio of alcohol to control expression levels. Fold changes greater than 1 are referred to as 'upregulated' and fold changes less than 1 are referred to as 'downregulated'. *P*-values < 0.05 were considered significant and are in bold. The microRNAs are organized from top to bottom by the top 10 up- and downregulated microRNAs. For example, miR-1893 is upregulated in SN, but is downregulated in TH following alcohol treatment.

and arrays for miR-137 showed $r = 0.69$. A one-tailed Students' *t*-test was used to test the significance of the correlation between the array and qPCR data. The correlation for miR-137 was significant ($p = 0.021$). Pearson correlation for expression levels between qPCR and microarrays for miR-9* showed $r = 0.70$ and this was also significant ($p = 0.018$).

Synaptic microRNAs Coordinately Regulate Synaptic mRNAs Following Alcohol Consumption

Co-expressed microRNAs may regulate their mRNA targets in response to alcohol treatment. We used WGCNA to create a co-expression network to group microRNAs with similar patterns of expression into modules. We then identified the modules that were significantly correlated with alcohol consumption and found 610 microRNAs that were co-expressed within six different modules (Supplementary Table 2, column B). Sixty-five microRNAs were differentially expressed in SN (Table 2, columns E and F), and 35 were co-expressed within these modules (Figure 3a). We identified microRNAs that correlated with alcohol consumption and found 74 in SN (Supplementary Table 2, columns C and D; average correlation: $r > 0.78$), 48 of which were co-expressed within the six microRNA modules.

We next utilized mRNA expression data (alcohol-responsive modules of co-expressed mRNAs) from our previous study (Most *et al,* 2014) to identify the alcohol-responsive microRNAs co-expressed with the mRNA modules. The modules were defined as astrocytic, microglial, or neuronal if

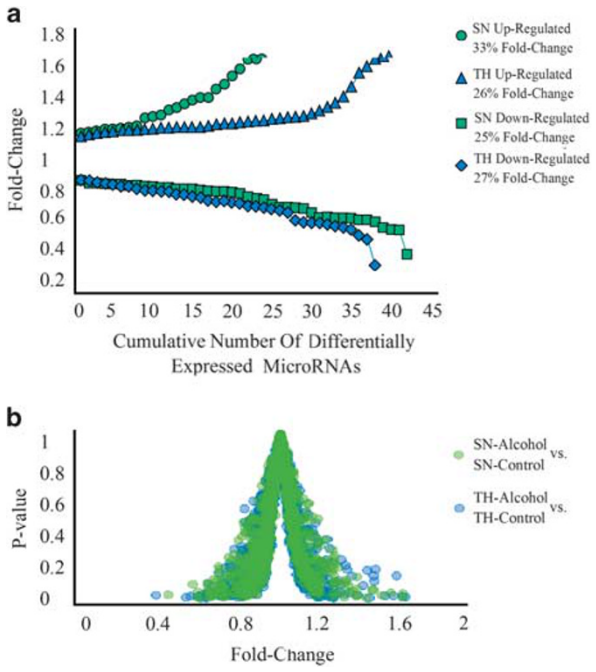

Figure 2 Alcohol-induced microRNAs are different in SN and TH. (a) The number of alcohol-responsive microRNAs in SN and TH. Alcohol induced fold changes are shown on the *y*-axis for microRNAs. For comparison of alcohol and control expression levels in each of the preparations, fold changes were calculated as the ratio of alcohol to control expression levels (SN-alcohol/SN-control and TH-alcohol/TH-control). Fold changes greater than 1 are referred to as 'upregulated' and fold-changes less than 1 are referred to as 'downregulated'. (b) Volcano plot (scatter plot) of fold changes and *P*-values of the effects of alcohol on microRNAs in SN and TH.

they contained a significant number of the cell type-associated mRNAs based on a hypergeometric distribution. The astrocyte modules overlapped with the microglia ones and were therefore combined. Cell type-specific analysis revealed 16 microRNAs that were significantly correlated with the alcohol-responsive mRNA modules (Figure 4).

Synaptic microRNA–mRNA Interactions are Regulated by Alcohol

MicroRNA–mRNA interactions were constructed using target predictions from the 2010 miRanda database for 'good mirSVR scores' and 'conserved microRNAs' (http://www.microrna.org). From these predicted interactions, the alcohol-responsive microRNAs were predicted to target 1039 target mRNAs of the 1531 that were identified as alcohol-sensitive in Most *et al*, 2014. The alcohol-responsive mRNAs were predicted to be targeted by 15 of the 65 alcohol-responsive microRNAs (Table 4). The 15 microRNAs showed 15–51% change. We found that 250 of the mRNAs were predicted to be targeted by more than five of the alcohol-responsive microRNAs (Supplementary Table 4).

We identified mRNAs cooperatively targeted by co-expressed microRNAs by combining the mRNA predicted target analysis with the microRNA co-expression data (Table 5). *MiR-106b, miR-203,* and *miR-374* are examples of co-expressed microRNAs that were predicted to target 61 overlapping targets (Figure 3b). MicroRNAs can be negatively or positively correlated with their mRNA targets; eg, *miR-106b, miR-203,* and *miR-374* were all downregulated and were predicted to target both up- and downregulated mRNAs (Figure 3c).

The relationship among the alcohol-responsive microRNAs and the cell type specific glutamate and GABA microRNAs was examined using microRNA records (He *et al,* 2012). We identified 10 differentially expressed microRNAs that were unique to CamkIIa+ glutamate neurons, five unique to Gad+ GABA neurons, 9 unique to somatostatin+ GABA neurons but none unique to parvalbumin+ GABA neurons. In addition, 9 of the 15 alcohol-sensitive microRNAs were previously shown to be specific to glutamate neurons, and 8 of the 9 were highly predicted to target the alcohol-responsive glutamate mRNAs (Gria2, Grina, Grm7, and Grip1; Table 6).

DISCUSSION

The compartmentalization of RNA in cells allows for rapid responses to stimuli and may be important for the neuroadaptations in response to chronic alcohol consumption. The aim of this study was to identify the synaptic microRNAs that are altered by alcohol consumption and to propose microRNA–mRNA synaptic interactions that may be changed by chronic alcohol. We compared the effect of alcohol in paired SN vs TH samples and identified those changes that were specific to the SN. Our data indicate that the microRNAs in the SN and TH respond differently to alcohol exposure: there was only one common alcohol-responsive microRNA, *miR-411*, between the preparations (Supplementary Table 3). Such a small overlap was unexpected and underscores the advantage of the SN in examining discrete, localized responses to alcohol. We identified microRNAs that were SN enriched in alcohol but not control samples and vice versa. If only the magnitude of treatment fold changes for the same microRNA was larger in SN compared with TH, this might suggest that synaptic enrichment was responsible for the differences. However, changes in magnitude do not explain all of the differences that we observed which appear to be both qualitative (different microRNAs) and quantitative (different amounts of the same microRNA). The differences between SN and TH are likely due to localized effects of alcohol and it is possible that alcohol changes the trafficking of microRNAs to the synapse, resulting in unique SN-enriched microRNAs.

This regimen of alcohol consumption causes extensive and coordinated changes in gene expression in the brain, suggesting a network regulator such as a microRNA may be involved (Lewohl *et al,* 2011). The question remains regarding how, or if, alcohol affects synaptic pathways through synaptic microRNA regulation.

This is the first study to use SN profiling of microRNA and mRNA obtained from the same samples, enabling detection of alcohol-responsive synaptic microRNAs and mRNAs and the predicted interactions between them. We used a combination of unbiased methods to reveal key microRNAs and their targets. Chronic alcohol consumption caused robust changes in synaptic microRNA expression levels consistent with those seen in human alcoholics (Lewohl *et al,* 2011) and in other animal models of dependence

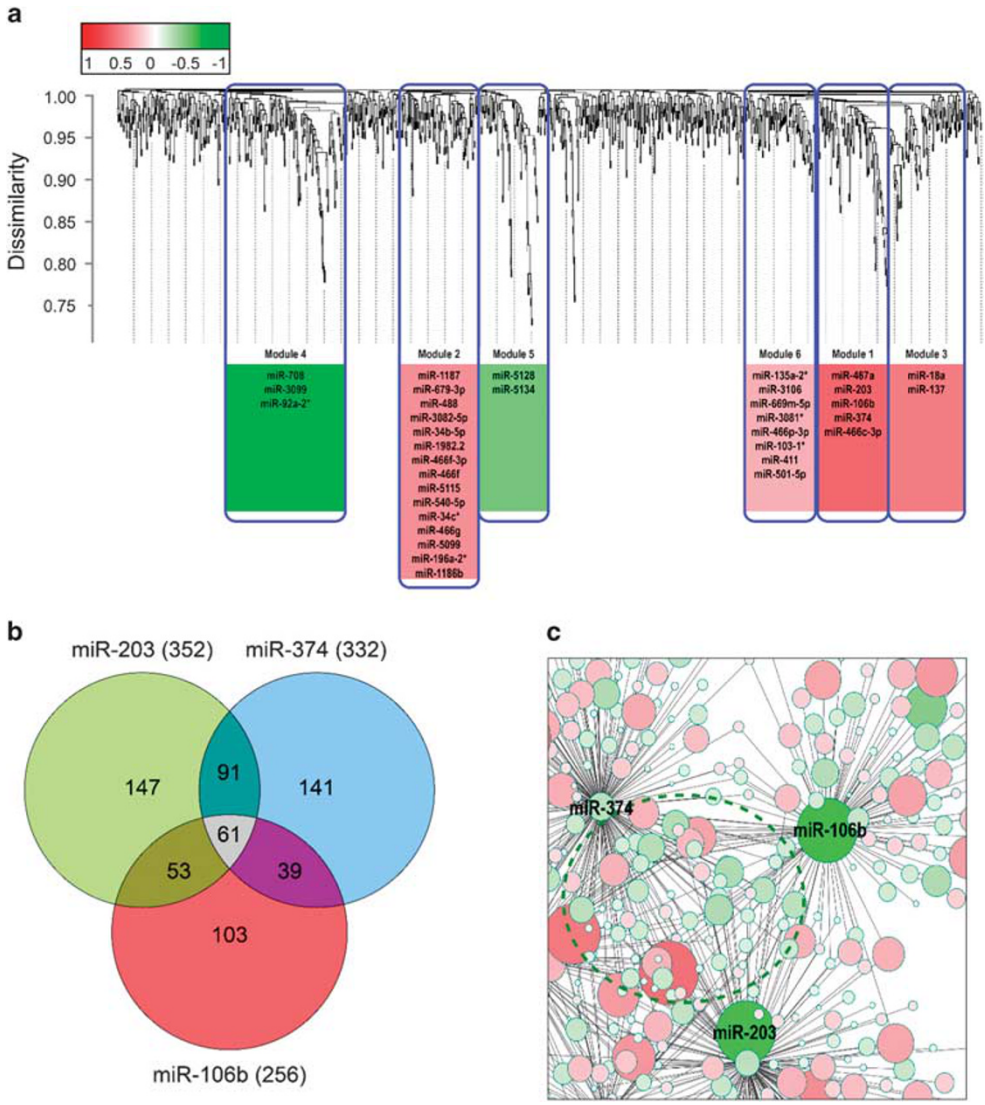

Figure 3 Alcohol induces coordinated expression of microRNAs that are correlated with alcohol consumption. (a) Hierarchical clustering of microRNAs from SN, including both alcohol and control data. The microRNAs are arranged by covariance similarity; thus, microRNAs under the same branch have greater expression similarity than those outside the branch. The dissimilarity among microRNAs is represented in the *y*-axis. The six different modules are shown in boxes. The microRNAs represent the co-expressed microRNAs overlapping with alcohol-responsive microRNAs. The width of the box represents the number of microRNAs co-expressed in that module. The correlation of each module with alcohol consumption is shown as a heat map (red represents positive correlation with consumption and green represents negative). The microRNAs in the gap between the modules are ones that did not pass the co-expression threshold as defined by the WGCNA and were not included in any module. (b) Examples of three co-expressed microRNAs and the number of overlapping predicted alcohol-responsive mRNAs. (c) Examples of microRNA predicted interactions. The greater the color intensity, the greater the fold-change magnitude (red is upregulated and green is downregulated). The unmarked circles represent mRNAs. The dotted circle emphasizes mRNAs that are co-targeted by the illustrated microRNAs.

(Gorini *et al*, 2013; Nunez *et al*, 2013; Tapocik *et al*, 2013). We further identified microRNAs with overlapping patterns of expression that correlated with alcohol consumption.

Previous studies used different experimental conditions, such as alcohol paradigms, species, gender, and brain regions, and we were interested in identifying potential conserved microRNAs that extend across all these different conditions. We suggest that a conserved microRNA could potentially be important in human disease. We employed many different bioinformatic approaches, such as co-expression and co-targeting, to identify the overlapping microRNAs and found some evidence that these are conserved among different species and genders. Further studies will be needed to validate the individual interactions. Nevertheless, the combined approaches provide a list of potential alcohol-sensitive interactions in the synapse that are candidates for further study.

We found the following conserved microRNAs in our study and that of an alcohol vapor exposure study in rats: *miR-137, miR-187, miR-18a, miR-34c*, miR-369*, miR-374, miR-382*, miR-423, miR-488,* and *miR-92b* (Tapocik *et al*, 2013). Differentially expressed SN microRNAs in the current study also overlapped with differentially expressed

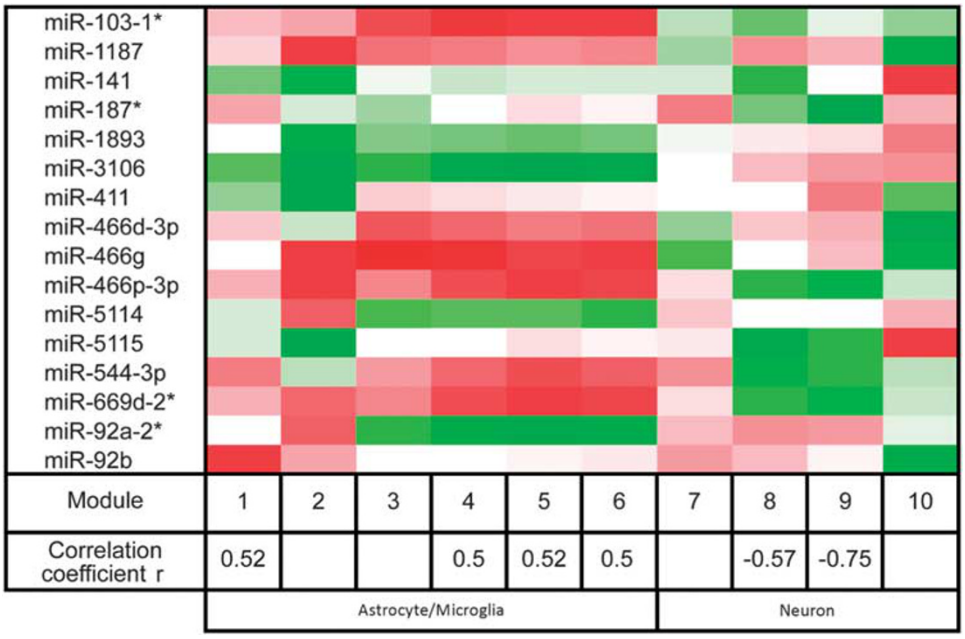

Figure 4 MicroRNA–mRNA interactions are coordinately expressed in response to alcohol and are associated with specific cell types. Shown are the alcohol-responsive mRNA modules and their correlation to individual microRNAs found in SN. The 10 alcohol-responsive mRNA modules are shown. The modules' correlation with consumption for the six alcohol-responsive modules is shown as *r*-values (Pearson's correlation coefficient). Alcohol-responsive mRNA module correlation to individual microRNAs is represented as a heatmap, with red representing a positive correlation and green representing a negative. Cell type mRNA enrichment is also shown. Modules 1–6 were enriched with astrocytic/microglial mRNAs, whereas modules 7–10 were enriched with neuronal mRNAs. The 16 microRNAs that were significantly correlated with at least one mRNA module are shown (mRNA data are from Most *et al*, 2014).

Table 4 Number of Alcohol-Sensitive mRNAs, microRNAs and their Predicted Interactions in SN

A	B	C
Number of alcohol-responsive RNAs	**Number of predicted targets**	**Overlap between alcohol-responsive and predicted tragets**
1531 mRNA	13 857 mRNAs	1039 mRNAs
65 microRNAs	238 microRNAs	15 microRNAs

(A) The number of alcohol-responsive RNAs. (B) The number of predicted participants as defined by the miRanda mouse database for interactions. (C) The number of predicted participants that overlap with the alcohol-responsive mRNAs/microRNAs. The overlapping participants between the alcohol-responsive list and the predicted interaction list are termed 'bidirectional'. The data for the alcohol-responsive mRNAs are from Most *et al,* 2014. *P*-values < 0.05 were considered significant.

microRNAs from human alcoholics (Lewohl *et al,* 2011). These were *miR-18a, miR-203, miR-369, miR-374, miR-92a,* and *miR-423*.

We analyzed the differentially expressed mRNAs as potential targets for known drugs using IPA software and found three of the few drugs currently used to treat alcohol dependence (baclofen, disulfiram, and acamprosate) in the list. Using the differentially expressed microRNAs for the IPA analysis, we identified 26 drugs, 10 of which were also in the list of drugs identified using differentially expressed mRNAs. Seven of the ten drugs are FDA approved (Table 7), with several of these having links with alcohol actions. For example, aminophylline blocks the behavioral effects of alcohol in mice (Soares *et al*, 2009) and has antidepressant effects after alcohol exposure (Escudeiro *et al,* 2013). Theophylline blocks alcohol withdrawal-induced hyperalgesia (Gatch and Selvig, 2002). Other drugs discovered from IPA were rasagiline (treatment for Parkinson's disease) and vorinostat, regorafenib, gemcitabine (paclitaxel), and romidepsin (treatments for cancer). The mechanisms of these drugs include histone deacetylase inhibition, adenosine receptor antagonism, phosphodiesterase inhibition, anti-inflammatory actions, and inhibition of monoamine oxidase and tyrosine kinase.

We used a bidirectional approach to predict synaptic microRNA–mRNA interactions that were sensitive to alcohol (1039 mRNAs and 15 microRNAs). Of these RNAs, 99 mRNAs and 9 microRNAs were unique to glutamate neurons. Notably, eight of these nine microRNAs were predicted to target alcohol-responsive mRNAs, such as CamkII, Gria2, Grina, Grm7, and Grip 1. Moreover, *miR-203, miR-18a*, and *miR-374* were among the glutamate microRNAs that overlapped with the human data set (Lewohl *et al,* 2011). These results suggest that alcohol regulates synaptic microRNAs, which in turn affect the expression of mRNAs in glutamate synapses and may partially explain the glutamate system dysregulation seen in alcoholics (Tsai and Coyle, 1998). We used the miRanda database to examine the most probable interactions that are regulated by alcohol consumption. This provides further support for the predicted interactions, but it is important to note that these require direct validation to define their role in alcohol consumption.

Table 5 Co-expressed Alcohol-Responsive microRNAs and their Overlapping mRNA Targets

A	B	C	D	E	F	G
MicroRNA	**MicroRNA fold change**	**MicroRNA *P*-value**	**Number of mRNA targets**	**Sum of scores**	**Co-expression**	**Overlapping targets for the co-expressed microRNAs**
miR-203	0.66	1.63E-02	393	−157.76	Module 1	6330408A02Rik, Aebp2, Ank2, Ankfy1, Apc Asph, Camta1, Cdh8, Cobll1, Crbn, Dclk2, Dph3, Dpp3, Eif4a2, Etl4, Fam135a, Foxj3, Gria2, Hace1, Hnrnpa2b1, Hsd17b11, Huwe1, Ivns1abp, Maf, Mbd3l2, Mbd5, Mef2c, Mll3, Mtdh, Myt1l, Napepld, Narg2, Ndrg3, Necap1, Neto2, Oat, Ocrl, Osbp2, Pbrm1, Pbx1, Pja2, R3hdm1, Rgs7bp, Runx1t1, Scn1a, Sgk3 Ski, Slc30a1, Slc39a10, Srpk2, Syap1, Syncrip, Tmem209, Tmem87b, Trim37, Tshz3, Ube3a, Unc80, Zc3h6, Zfp644, Zxda
miR-374	0.85	2.90E-02	366	−181.00		
miR-106b	0.83	3.43E-02	282	−129.25		
miR-488	0.83	1.20E-02	290	−123.16	Module 2	1600014C10Rik, 4833424O15Rik, AI314180, Acot11, Ahcyl1, Ankfy1, Ap2b1, Atl2, Camta1, Capn6, Car10, Cdc37, Cdc37l1, Cinp, Crbn, Cyld, Dgcr6, Etl4, Fam126b, Fbxo3, Gabrb2, Glce, Grm7, Gtf3c2, Hps5, Kif1b, Med18, Mllt3, Msi2, Mtdh, Myt1l, Ndst1, Pcdh10, Pja2, Pkp4, Prkd1, R3hdm1, Rab21, Rapgef4, Rarb, Rbbp9, Rftn2, Rnf4, Sec16a, Sesn1, Slc22a17, Slc35e3, Smc6, Snx12, Spnb2, Stx8, Tbxas1, Tcf25, Tle1, Tmem209, Tprkb, Tshz1, Tshz3, Tspan7, Unc80, Usp13, Utp6, Vps41, Vwa5b2, Zc3h14, Zfp644
miR-34b-5p	0.66	1.48E-02	246	−84.36		
miR-137	0.65	2.03E-02	243	−133.62	Module 3	2700060E02Rik, 4732418C07Rik, Aebp2, Ahcyl1, Ank2, Ank3, Asph, Atl2, Cdk13, Crtc3, Ctdspl, Dgcr6, Dut, E2f6, Fry, Gatad1, Gigyf1, Hnrpdl, Kdm5b, Khdrbs3, Lingo2, Lrrc16a, Maf, Mbnl2, Mfsd6, Mll1, Msi2, Nme7, Nrg1, Osbp2, Ppp2r5c, Rab8a, Rin2, Sae1, Scamp2, Scn1a, Seh1l, Slc13a3, Smc6, Sntg2, Snurf, Spag9, Stx8, Syncrip, Tmem87b, Ybx1, Zfp804a
miR-18a	0.65	1.35E-02	198	−75.92		
miR-708	0.84	1.51E-02	192	−70.24	Module 4	Asph, Azi2, E2f6, Foxj3, Gpt2, Gucy1a3, Hspa9, Lipa, Mia3, Mtpap, Ocrl, Palld, Phf17, Pja2, Prkar1a, Pros1, Rapgef6, Rfx1, Robo2, Seh1l, Sgms1, Socs5, Txnip, Unc5c, Ybx1, Zfp804a
miR-92b	1.19	4.40E-02	190	−90.99		
miR-365	1.18	4.45E-02	156	−58.25	Module 5	
miR-411	0.84	3.89E-02	153	−66.06	Module 6	
miR-141	0.87	4.10E-02	338	−144.24	NA	
miR-216b	1.35	1.49E-02	230	−101.41		
miR-92a	1.19	3.62E-02	201	−94.12		
miR-187	1.50	1.85E-02	64	−22.28		

Fifteen microRNAs that are regulated by alcohol (A), their fold change (B) and *P*-values (C) are shown. For comparison of alcohol and control expression levels, fold changes were calculated as the ratio of alcohol to control expression levels. Fold changes greater than 1 are referred to as 'upregulated' and fold changes less than 1 are referred to as 'downregulated'. *P*-values < 0.05 were considered significant. The number of predicted target mRNAs (alcohol-responsive mRNAs identified in Most *et al*, 2014) is shown for mRNAs that were also regulated by alcohol (column D) and was calculated using the miRanda database. The sum of each of the mirSVR scores per microRNA is shown for all predicted interactions per microRNA (column E), with the scores representing the relative probability of occurrence of the microRNA–mRNA interactions. The WGCNA co-expression module for each of the microRNAs (column F) and the number of overlapping predicted mRNA targets for the microRNAs in the same module (column G) are shown.

Table 6 Alcohol-Sensitive microRNAs and their mRNA Targets that are Specific to Glutamate Neurons

MicroRNA	Grm7	Gria2	Grina	Grip1
miR-106b	−0.58	−1.03	NA	NA
miR-137	−0.69	NA	NA	NA
miR-18a	NA	−0.23	−0.34	−0.25
miR-203	NA	−0.52	NA	−0.39
miR-374	−0.77	−0.27	NA	NA
miR-411	NA	−0.68	NA	NA
miR-708	NA	NA	NA	NA
miR-92a	−0.70	NA	NA	NA
miR-92b	−0.70	NA	NA	NA

Nine glutamate-specific microRNAs of the 15 predicted microRNAs that are alcohol-responsive are shown. The numbers in the table represent the mirSVR scores for the predicted interactions between a microRNA and its target mRNA (as defined by the miRanda mouse database for interactions). NA means no interactions were predicted. mRNA gene symbols are shown. The data for the alcohol-responsive mRNAs are from Most *et al*, 2014. Grm7 (glutamate receptor, metabotropic 7), Gria2 (glutamate receptor, ionotropic, AMPA 2), Grina (glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1, glutamate binding), and Grip1 (glutamate receptor-interacting protein 1).

Table 7 Drugs Identified by IPA that Potentially Target Alcohol-Responsive mRNAs

A	B	C
Drugs for alcohol targets	**Molecular mechanisms**	**Potential uses**
Belinostat	Histone deacetylase inhibitor	Cancer
Pyroxamide		
Romidepsin		
Vorinostat		
Gemcitabine/ paclitaxel	DNA polymerase inhibitor	
Tributyrin	Adenosine receptor antagonist	
Regorafenib	Tyrosine kinase receptor inhibitor	
Rasagiline	Monoamine oxidase inhibitor	Parkinson's disease
Aminophylline	Phosphodiesterase inhibitor and adenosine receptor antagonist	Respiratory diseases
Theophylline		

The alcohol-responsive mRNAs and the 'mRNA targets of the alcohol-responsive microRNAs' were overlapped to identify drugs known to affect these targets. Drug names (A), molecular mechanisms (B), and their current potential uses (C) are shown. The data for the alcohol-responsive mRNAs are from Most *et al*, 2014. The seven drugs in bold are FDA approved.

A single microRNA has the potential to target many alcohol-responsive mRNAs (Mayfield and Nunez, 2012; Tapocik *et al*, 2013). This mechanism may be of particular importance in the synaptic proteome where slight adaptions can greatly impact synaptic plasticity. Alcohol-responsive microRNAs were significantly correlated with astrocytic, microglial, and neuronal modules. The co-expression of a microRNA with a network of alcohol-responsive mRNAs supports the role of microRNAs as master regulators in the synapse. The biological pathways associated with the mRNA modules include long-term potentiation and depression, glutamate and neuroimmune signaling, RNA processing, etc., suggesting the regulation of microRNAs in multiple processes. As for many other diseases (Maciotta *et al*, 2013), the neuroadaptations associated with alcohol dependence likely rely on many mRNAs. A subset of the mRNA changes may be driven by only a small number of microRNAs, each with the ability to target multiple mRNAs, thereby impacting alcohol-mediated responses and therapeutic strategies.

FUNDING AND DISCLOSURE

The work published in this paper represents original research, not previously submitted or published elsewhere. This work was supported by the National Institutes of Health grants AA022557, AA013520, AA012404, RC2AA019382, and AA020683. The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Jody Mayfield for many valuable comments and scientific editing.

AUTHOR CONTRIBUTIONS

Dana Most designed, performed experiments, and wrote the paper; Courtney Leiter performed the PCR experiments; Yuri A. Blednov designed the behavioral experiments; R. Adron Harris designed the experiments and edited the paper; R Dayne Mayfield designed experiments and edited the paper.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)