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Immediate-early alcohol-responsive miRNA expression in *Drosophila*

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Abstract

At the core of the changes characteristic of alcoholism are alterations in gene expression in the brain of the addicted individual. These changes are believed to underlie some of the neuroadaptations that promote compulsive drinking. Unfortunately, the mechanisms by which alcohol consumption produces changes in gene expression remain poorly understood. MicroRNAs (miRNAs) have emerged as important regulators of gene expression because they can coordinately modulate the translation efficiency of large sets of specific mRNAs. Here we investigate the early miRNA responses elicited by an acute sedating dose of alcohol in the *Drosophila* model organism. In our analysis, we combine the power of next generation sequencing with *Drosophila* genetics to identify alcohol-sensitive miRNAs and to functionally test them for a role in modulating alcohol sensitivity. We identified 14 known *Drosophila* miRNAs, and 13 putative novel miRNAs that respond to an acute sedative exposure to alcohol. Using the GeneSwitch Gal4/UAS system, a subset of these ethanol-responsive miRNAs was functionally tested to determine their individual contribution in modulating ethanol sensitivity. We identified two microRNAs that when overexpressed significantly increased ethanol sensitivity: miR-6 and miR-310. MicroRNA target prediction analysis revealed that the different alcohol-responsive miRNAs target overlapping sets of mRNAs. Alcoholism is the product of accumulated cellular changes produced by chronic ethanol consumption. Although, all of the changes described herein are extremely rapid responses evoked by a single ethanol exposure, understanding the gene expression changes that occur in the first few minutes after ethanol exposure will help us to categorize ethanol responses into those that are near instantaneous and those that are emergent responses produced only by repeated ethanol exposure.

Keywords

Alcoholism; Neuroadaptation; miRNA-seq; Genomics; miR-6; miR-310

Introduction

MicroRNAs (miRNAs) are endogenous RNAs of approximately 22 nucleotides that have been implicated in the post-transcriptional regulation of complex biological networks. These small noncoding RNAs are thought to play a crucial role in shaping the function of tissues and organs in both health and disease. In general, miRNAs suppress gene expression by repressing the translation of specific mRNAs and/or by message destabilization (Bethune *et al.*, 2012; Gurtan & Sharp, 2013).

Canonical miRNAs are produced from long primary RNA transcripts (pri-miRNA) that by cleavage in the nucleus by the enzyme Drosha to form ~60-70 nt precursor miRNA hairpin loops (pre-miRNA). Individual pri-miRNAs have been observed to encode as many as 6 different pre-miRNAs. Each pre-miRNA hairpin loop is then transported to the cytoplasm where they are processed into small ~22 nt double stranded RNA duplexes by the enzyme Dicer (Bartel, 2004). Although, both strands of the duplex are necessarily produced in equal amounts by transcription, their accumulation is known to be asymmetric. The most abundant strand from each duplex will become the biologically active strand that is incorporated into the RNA-Induced Silencing Complex (RISC) and serves as the guide strand during miRNA-directed silencing. The other strand, which is considered the inactive strand and is called the miRNA* (miRNA star) or passenger strand, is typically degraded (Meijer *et al.*, 2014). The miRNA/RISC complex then binds to specific sequences predominantly found in the 3' UTR of protein-coding mRNAs triggering inhibition of protein synthesis and mRNA degradation, depending on the degree of complementarity between the miRNA and the target mRNA (Fang & Rajewsky, 2011). Nonetheless, a strong complementarity within positions 2 to 8 of the 5' end of the miRNA (known as the "seed") is considered critical for recognition of target mRNA and its regulation (Bartel, 2009; Doench & Sharp, 2004; Lai, 2004). Both the mechanism of generating miRNAs and the miRNAs themselves are highly conserved from flies to mammals, therefore, analysis of miRNA function in the *Drosophila* model system should translate well to mammals (Ibanez-Ventoso *et al.* 2008).

Recent evidence suggests that some miRNAs are alcohol-responsive and play important regulatory roles in neuroadaptations that mediate the development of alcohol addiction. The best example so far of miRNA regulation that alters an alcohol response is the miR-9 regulation of *slo* gene expression. The *slo* gene encodes BK-type Ca²⁺-activated K⁺ channels whose activity is sensitive to ethanol. In mammalian hypothalamo-neurohypophysial system neurons, acute alcohol exposure up-regulates miR-9 to mediate selective destabilization of mRNA splice variants encoding ethanol-sensitive BK channels. This favors the production of more ethanol-resistant channels, thereby contributing to the development of functional alcohol tolerance (Pietrzykowski *et al.*, 2008). Importantly, the *slo* gene is also involved in the development of functional ethanol tolerance in *Drosophila*. In this model system, ethanol affects *slo* transcription in a way that contributes to behavioral functional tolerance (Cowmeadow *et al.*, 2005; Cowmeadow *et al.*, 2006). However, miR-9 is not the only alcohol-responsive miRNA nor is *slo* the only protein-coding gene involved in alcohol responses. There is a growing body of evidence indicating that alcohol regulates the expression of other miRNAs and other protein-coding genes (Bala & Szabo, 2012; Li *et al.*, 2013; Nunez *et al.*, 2013; Tapocik *et al.*, 2013).

Here, we use the genetically malleable *Drosophila* model system and next generation sequencing to identify miRNAs whose abundance changes within the first 30 minutes after a sedative alcohol exposure. Our central hypothesis is that changes in the expression of specific miRNAs induced by alcohol exposure contribute to the neuroadaptations that lead to alcohol dependence. Changes in miRNA expression that contribute to alcoholism-related phenotypes in flies will be a useful guide for identifying the relevant miRNA mechanisms that contribute to alcoholism-related phenotypes in mammals. The advantage of doing this work in *Drosophila* is that large numbers of candidate miRNAs can be rapidly and functionally tested for their role in producing ethanol tolerance and dependence by controlled miRNA overexpression or inhibition.

Methods

Stocks and Fly Husbandry

All flies used in this study were maintained on a standard cornmeal/molasses/agar medium under 12:12 light:dark conditions. Experiments were conducted using 3-5 day-old female flies. Only females were used because all previous directly comparable studies used only females. Canton S was the wild-type stock used for the RNA-seq analysis. All transgenic stocks were obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537). These are (stock numbers in parenthesis):

W[1118]; P{UAS-LUC-mir-1003.T}attP2 (41220)

w[1118]; P{UAS-LUC-mir-124.T}attP2 (41126)

w*; P{UAS-mir-308.D}10 (41809)

w[1118]; P{UAS-LUC-mir-6-1.mir-6-2.mir-6-3.T}attP2 (41136)

w[1118]; P{UAS-LUC-mir-304.T}attP2 (41170)

w[1118]; P{UAS-LUC-mir-310.T}attP2/TM3, Sb1 (41155)

w[1118]; P{UAS-LUC-mir-310.mir-311.mir-312.mir-313.T}attP2/TM3, Sb1 (41135)

y[1] w[*]; P{w[+mC]=elav-witch.O}GSG301 (43642)

The GeneSwitch system was used to specifically overexpress each UAS-miRNA in the nervous system (Osterwalder *et al.*, 2001; Roman *et al.*, 2001). Inducible transheterozygous animals were produced by crossing each of the UAS-miRNA stocks to the neuronal specific “elav-Switch” driver stock. To activate transgene expression RU486 (Cayman Chemical, Ann Arbor, MI, USA) was supplied to the transgenic flies in their food as described in Troutwine *et al.* (2016). RU486-laced food was generated by adding 25 mM RU486 in 80% ethanol to molten fly food to a final concentration of 200 μ M RU486 (after dilution in food, final ethanol concentration is below 0.64%). Control food was also melted and mixed with an equivalent amount of the carrier solution (80% ethanol). The RU486 food was distributed to fly vials and allowed to cool and dry for at least one hour before flies were allowed to

feed. Flies were kept on RU486 or carrier-containing food for three consecutive days before being subjected to our ethanol resistance assay.

Ethanol treatment, RNA Isolation and Next-Generation Sequencing

Prior to Next-Generation Sequencing miRNA analysis, wild-type Canton S flies were treated with alcohol vapor using the inebriator as described previously (Cowmeadow *et al.*, 2005; Krishnan *et al.*, 2012). Briefly, approximately 180 flies were divided in 6 vials of ~30 flies each and exposed to an air stream saturated with ethanol vapor until all flies were sedated (typically 12-15 minutes). After sedation, the ethanol-saturated air stream was replaced with a stream of humidified air for 30 minutes and then the animals were sacrificed for RNA extraction. An untreated group, which was exposed to an ethanol-free stream of humidified air, was prepared in parallel. This group served as control in the miRNA-seq analysis. Preparation of RNA and quality control was performed as described in Troutwine *et al.* (2016). Small RNA libraries were generated from each RNA sample using Illumina Small RNA Sample Preparation kit according to the manufacturer's instructions and sequenced with the Illumina HiSeq 2000 at the Beijing Genomics Institute (Shenzhen, China). Sequence depth was set to 20M reads using single-end chemistry and 50-bp cycles. Raw sequences have been deposited in the NCBI: Gene Expression Omnibus (GEO) public functional genomics data repository. Data can be found on the GEO website (<http://www.ncbi.nlm.nih.gov/geo/>) using accession number GSE89137. All essential sample annotation and experimental design information including sample data relationships have been included in the repository according to the Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma *et al.*, 2001).

miRNA Sequence Analysis and Differential Expression

rRNAs, tRNAs, snRNAs and low-quality reads (containing adapters or high content of unknown bases) were filtered out by aligning to Rfam and Genbank databases (Griffiths-Jones *et al.*, 2003). miRNA-seq reads were aligned and mapped to the *Drosophila* reference genome (BDGP Release 5) using SOAPaligner/SOAP2 (Li *et al.*, 2009), allowing no more than 5 bp mismatches. Known miRNAs were identified by alignment to miRBase21.0 (Kozomara & Griffiths-Jones, 2014). Expression levels for miRNAs were calculated using the CLC Genomics Workbench (CLC bio, Boston, MA). Differential expression analysis was conducted using Cluster 3.0, and Java TreeView software (Eisen *et al.*, 1998; Saldanha, 2004) and expressed as log₂ Ratios (EtOH/Ctrl). We used FDR = 0.0001 and an absolute value of log₂ Ratio ≥ 1 as the threshold to judge the significance of expression difference.

The identification of putative novel miRNA were based on the analysis recommendations of the Beijing Genomics Institute (Shenzhen, China). This institute sequenced the miRNAs and provided an initial analysis. In brief the following criteria were used to identify novel miRNAs. After removing the known miRNAs, RNA sequences with a length of 19 to 26 nucleotides were examined further. Those that matched the sense strand of a mRNA, rRNA, tRNA, snoRNA, or a snRNA gene were filtered out. The remaining RNAs were mapped to the genome and the mirReap algorithm was used to show that a stem-loop structure of the appropriate loop size, without any internal bulges, could be formed by a transcript produced by the genomic locus. The putative hairpin structure of a possible precursor is relatively

stable with an energy of hybridization less than or equal to -18 kcal/mol. Each predicted precursor has a 3 n depth for the Drosha/Dicer cutting site. All putative miRNAs also must have an arbitrary cutoff count of no fewer than 5 reads per million. None of the putative novel miRNAs have been functionally tested for miRNA activity. The structure of the putative precursor miRNA is shown in Supplemental Figure 1.

miRNA Target Analysis

Prediction of miRNA targets was performed using TargetScanFly (Ruby *et al.*, 2007b). Gene ontology analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) web-accessible tool, version 6.7 (Huang da *et al.*, 2009). For gene ontology annotation search and clustering, significant gene categories in “Biological Process” for each cluster were identified using default High Classification Stringency parameters (Kappa Similarity Term Overlap: 3; Similarity Threshold: 0.85; Initial Group Membership: 3; Final Group Membership: 3; Multiple Linkage Threshold: 0.5) and official gene symbols as input.

Ethanol Resistance Assay

To test for ethanol resistance, all transgenic stocks tested were subjected to the inebriator as described above (*Ethanol treatment, RNA Isolation and Next-Generation Sequencing*). This time however, six sets of 10 flies from the RU486-treated group and six sets of 10 flies from the control group were placed in plastic vials (one set per vial) and exposed to an ethanol-saturated air stream in parallel, until all flies were sedated (Sedation was determined visually as when flies lose positional control and remain on their backs with only occasional twitching. This typically occurs within 12-15 minutes). After sedation, the ethanol-saturated air stream was replaced with a stream of humidified air and recovery from sedation was monitored over a 90-minute period in 3-minute intervals. Flies that regain postural control were considered recovered. The number of recovered flies was plotted against time to generate a recovery curve. For each vial, the 50% recovery time (RT50) was calculated using Prism 6 (GraphPad Software, Inc.) by nonlinear regression curve fit of the recovery curves to the Richard's five-parameter dose response equation. Statistically significant differences in RT50 between induced and uninduced lines were determined using Student t-tests. Error bars represent standard error of the mean. The magnitude of the difference in ethanol sensitivity between induced and uninduced transgenic lines was calculated using the following formula:

$$\frac{(\text{Average RT50})_{\text{induced}} - (\text{Average RT50})_{\text{uninduced}}}{(\text{Average RT50})_{\text{uninduced}}}$$

All experiments were performed between 11:00 and 16:00 (zeitgeber time 3-8) using age-matched, ethanol-naïve female flies. We did not observe death of flies after a single sedation (examples – Krishnan *et al.* 2012, 2016) nor did we observe death in the 14 behavioral resistance assays in Figure 2 (7 with and 7 without RU486).

Results

Identification of Ethanol Responsive miRNAs

To examine early ethanol-induced changes in miRNA abundance in the nervous system of *Drosophila*, we conducted a genome-wide survey of microRNAs from ethanol-treated flies. For this, we extracted total RNA from the heads of two groups of age-matched flies. One group was exposed to a single sedating dose of ethanol vapor, while the second group was left untreated (the untreated group was used for comparison purposes). RNA was extracted 30 minutes after onset of sedation (~45 minutes from the initiation of the ethanol vapor exposure). At this point, all flies in the treated group were still sedated. miRNAs were isolated from both samples by size selection and sequenced using whole genome RNA sequencing. Differential expression between the two treatment groups was determined to identify ethanol-responsive miRNAs. Analysis of protein coding mRNAs was also performed in parallel, and has been previously reported in Troutwine et al. (2016).

We observed significant changes ($P < 0.05$, FDR < 0.005 , log₂ ratio > 1) in multiple miRNAs and mRNAs. Out of 466 miRNAs annotated in the *Drosophila* genome (miRBase21.0, BDGP5.0), acute alcohol exposure induced abundance changes in only fourteen miRNAs. Of these, 7 miRNAs were upregulated, and 7 were downregulated by ethanol (Figure 1). In addition, we identified 13 putative novel miRNA species that were also alcohol responsive (5 upregulated, 8 downregulated). All previously annotated miRNAs with significant ethanol-induced changes in expression are listed in Table 1. In Troutwine et al. (2016), we also identified 137 protein-coding transcripts that respond to the ethanol treatment. Of these, 89 increased whereas 48 decreased in abundance after the ethanol treatment. Between miRNAs and mRNAs, a total of 166 transcripts were affected.

Of the fourteen annotated ethanol-responsive miRNAs, four belong to a family that share the same seed and therefore recognize the same mRNA targets. These are miR-6-3p, miR-11-5p, miR13b-1-5p, and miR-308-3p, which belong to the miR-2a-1/6/11/13/308 family. Members of this family share a common seven nucleotide seed sequence: 5'-AUCACAG-3' (Aravin et al., 2003). However, the mature miRNAs produced from the 5' arm of the miR-11 and miR-13b-1 identified in our study are very likely the passenger strand and are destined for degradation as they do not carry the seed sequence (Aravin et al., 2003; Ruby et al., 2007b; Stark et al., 2007). We observed that forty-five minutes after the onset of a sedating dose of alcohol vapor that miR-6-3p was increased in abundance but that the abundance of miR-308-3p was reduced. While this data may seem contradictory at first, it is possible that these two miRNAs reflect an ethanol response in different parts of the brain, and thus do not necessarily negate each other's abundance change. The miR-6-3p miRNA is a *Drosophila*-specific miRNA and is transcribed as a precursor containing the miRNAs mir-6-1, mir-6-2, and mir-6-3. The mature products generated from the 3' arm of each mir-6 precursor hairpin have identical sequences, and have been experimentally validated to act as guide miRNAs. The mature miR-6 miRNAs have been shown to target apoptotic genes such as *hid*, *grim* and *rpr* (Lagos-Quintana et al., 2001; Ruby et al., 2007b; Sempere et al., 2003; Stark et al., 2007). Similarly, the 3' mature product of mir-308 is a validated guide miRNA with the same targets.

A second well-conserved miRNA family represented in our ethanol-responsive data set is the miR-92/310/311/312/313 family. We observed that both miR-310-3p and miR-313-5p are affected by acute alcohol exposure. miR-310-3p is upregulated by ethanol whereas miR-313-5p is downregulated. In this case, only miR-310-3p has been validated as a guide miRNA, while miR-313-5p according to miRBase analysis is most likely to serve as a passenger strand with an unknown role (Kozomara & Griffiths-Jones, 2014).

With the exception of miR-277-5p, all the other ethanol-responsive miRNAs have been validated as guide miRNAs (Aravin *et al.*, 2003; Berezikov *et al.*, 2011; Lai *et al.*, 2003; Ruby *et al.*, 2007b; Stark *et al.*, 2003; Stark *et al.*, 2007). The miR-124 miRNA is well conserved and has been identified in flies, worms, mice and humans. The mature miR-124-3 originates from the 3' arm of the precursor hairpin and is perhaps the most abundant miRNA expressed in neuronal cells. It is believed to participate in neuronal differentiation (Sun *et al.*, 2012; Wang *et al.*, 2014; Weng & Cohen, 2012). miR-1003-3p, miR-1009-3p and miR-1008-3p miRNAs, which are generated from intronic microRNA precursors that bypass Drosha processing, are not conserved in mammals (Ruby *et al.*, 2007a).

In our genome-wide survey for alcohol-responsive miRNAs we also detected new microRNAs that were not reported before in the fruit flies. A temporary name was assigned to each of the putative novel miRNAs. The sequence, fold change, and chromosomal locations of these miRNAs are reported in Table 2.

Functional Testing of miRNAs on alcohol sensitivity

To test the effects of ethanol-responsive miRNAs on behavioral ethanol sensitivity, we overexpressed six miRNAs for which overexpression lines were available (mir-6, mir-124, mir-304, mir-308, mir-310 and mir-1003). These overexpression lines carry UAS-controlled transgenes encoding the specific precursor miRNAs (Bejarano *et al.*, 2012). To specifically drive expression of alcohol-responsive miRNAs in the *Drosophila* nervous system, we used the conditional tissue-specific transgene expression system called GeneSwitch (Osterwalder *et al.*, 2001; Roman *et al.*, 2001). This system makes use of a fusion of Gal4 and progesterone receptor domains to generate a transcription factor that activates the miRNA-expressing UAS transgene only in the presence of RU486 (a.k.a. mifepristone). Additionally, because we use the neuronal-specific *elav-Switch* driver of the Gal4-fusion transgene, expression of miRNAs is specific to the nervous system. Of the six tested miRNAs, miR-6 was available as a transgenic cluster of mir-6-1, mir-6-2 and mir-6-3. Thus, when transgene expression was activated, all 3 mir-6 paralogs were expressed together. These three paralogs contain identical mature miRNA sequences. On the other hand, miR-1003, miR-310, miR-304, miR-308, and miR-124, were all tested independently using transgenic lines individually expressing each precursor sequence. Additionally, miR-310 was also tested a second time as a cluster of four different miRNA precursor sequences (mir-310, mir-311, mir-312 and mir-313), all of which share the same seed sequence. While miR-313 is also represented in this cluster, only the passenger strand from this pre-miRNA was found to be alcohol-responsive.

All overexpression lines were induced by feeding RU486 to the transgenic flies for 3 consecutive days, before testing for their effect on alcohol sensitivity using a behavioral

assay. In this assay, flies were exposed to a sedating dose of alcohol vapor until knockdown, after which the ethanol was switched to fresh air. At this point, recovery from ethanol was monitored to calculate the 50% recovery time (RT50). The RT50 of miRNA-induced flies was compared to that of their uninduced counterparts. The uninduced flies were fed food without RU486 but were otherwise treated identically. Also, because these flies share the same genetic background, a direct comparison was possible. We observed that the overexpression of the miR-6 cluster as well as the mir-310 precursor — either individually or as a cluster — significantly increased ethanol sensitivity. These two miRNAs were both induced by ethanol. However, miR-1003 — also induced by ethanol — or the ethanol-down regulated miR-304, miR-308, and miR-124, had no effect on ethanol sensitivity (Figure 2).

Gene Ontology Analysis of Predicted Targets

Predicted targets for miR-6 and miR-310 were obtained using the TargetScanFly (release 6.2) web-based algorithm (Ruby *et al.*, 2007b). To categorize the predicted targets of each of the identified miRNA groups according to their Gene Ontology annotations, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) web-accessible tool, version 6.7 (Huang da *et al.*, 2009). This model categorizes a set of genes based on their functional annotation and assigns each category a score that corresponds to the significance of enrichment. For miR-6, TargetScanFly predicts a total of 377 targets, and DAVID functional annotation results in a total of 34 gene ontology clusters. The first term of the top ten clusters of miR-6 targets is displayed in Table 3. A significant enrichment in genes involved in organ morphogenesis, including neuronal morphogenesis was found. This is consistent with previous reports for this miRNA. For miR-310, TargetScanFly predicts a total of 349 targets and DAVID functional annotation resulted in a total of 35 gene ontology clusters. The first term of the top ten clusters of miR-310 targets is displayed in Table 4. Again, a significant enrichment in genes involved in organ morphogenesis, including neuronal morphogenesis was found. A list of all target genes for miR-6 and miR-310, and a list of the complete GO terms clusters for miR-6 and miR-310, can be found in the supplementary information (Supplementary Data 2 and Supplementary Data 3, respectively).

It is important to note that the sets of putative targets for miR-6 and miR-310 show a significant overlap. We found a group of 30 genes in common between the two datasets, 26 of which are neurally-expressed genes. A list of these genes can be found in Supplementary Data 2. When subjected to Gene Ontology Clustering analysis, this group of genes showed significant enrichment in transcription regulator activity, with 8 genes showing a direct association with the GO term (Enrichment Score: 2.63, 30%, P-Value 3.69E-05). These genes are: *salm*, *caup*, *en*, *nub*, *Eip93F*, *aop*, *nab*, *kay*. From the overlapping set of putative miRNA targets, none of them overlap with changes in mRNA abundance noted in Troutwine *et al.* (2016). However, this does not mean very much because 1) miRNAs can alter translatability of a mRNA before mRNA destabilization occurs, and 2) any destabilization wrought by a miRNA will take time to occur and both of these samples (Troutwine's mRNA and Ghezzi's miRNA) were taken at the same time point.

Discussion

It is generally held that changes in gene expression in the brain contribute to alcoholism. Alcohol-induced changes in miRNA expression are believed to be responsible for many changes in gene expression that underlie alcohol-response adaptations. While some of these changes have been documented in mammals, it is currently expensive and time consuming to determine the functional consequence of each change in miRNA expression in a mammalian model system. Because all mammalian miRNA families are represented in *Drosophila*, experiments in flies can be highly informative and applicable to the biology of higher organisms, including humans (Ibanez-Ventoso *et al.*, 2008). Here we investigate the consequences of alcohol-induced changes in miRNA expression in the genetically tractable *Drosophila melanogaster* model system. We have shown that 30 minutes after ethanol sedation there are significant changes in miRNA abundance in the fly head. The sedation protocol we use has been shown to also produce significant long-term changes in alcohol related behaviors and physiological alterations. This single ethanol exposure results in functional behavioral tolerance that is detectable 4 hrs after sedation and persists for 7–10 days (Cowmeadow *et al.*, 2005; Krishnan *et al.*, 2016). Similarly, symptoms of ethanol withdrawal to the same dose of ethanol are detectable 24 hours after exposure and also persist for 7–10 days (Ghezzi *et al.*, 2014). At this early 30-minute post-sedation time point we have observed changes in 14 previously identified miRNAs as well as changes in 13 novel miRNAs (27 total). While this is perhaps only a snapshot of all changes induced by ethanol sedation, we hypothesize that the immediate-early changes in miRNA abundance detected here are an important part of gene expression changes that modulate future alcohol responses. At this time, we cannot formally distinguish which of these changes are the product of ethanol exposure, ethanol sedation, or just sedation.

Among the ethanol responsive miRNAs, we identified four mature miRNA*s that have not been previously considered to serve as guide miRNAs. These are presumed to be inactive complementary passenger strands. This is intriguing because miRNA* strands are usually degraded. The fact that we observed significant accumulation changes in these miRNA*s suggest that they have regulatory potential. Evidence for functional miRNA* strands exists. In *Drosophila* for instance, certain miRNA* strands have been found to associate with Argonaute2 (Ago2) but not with Argonaute1 (Ago1) (Ghildiyal *et al.*, 2010). Ago1 is the central protein component of the microRNA (miRNA) pathway, while Ago2 is central to the RNA interference pathway (siRNAs). The evidence suggests that in some instances that miRNA* strands can be recycled into the RNA interference pathway thereby increasing the complexity of regulatory networks (Okamura *et al.*, 2008).

A key aspect of this work is that in *Drosophila*, alcohol-responsive miRNAs can be functionally tested to evaluate their contribution to ethanol sensitivity. Furthermore, because in this study each miRNA was expressed using a conditional tissue-specific transgene expression system, we limited the analysis to the adult nervous system. This is important because by activating the transgenes at a specific developmental stage, we could avoid occluding developmental effects. Using this system, we identified two conserved *Drosophila* miRNAs with a significant role in modulating ethanol sensitivity: miR-6 and miR-310. Both miRNAs were upregulated by ethanol. None of the ethanol-downregulated miRNAs had a

significant effect on alcohol sensitivity. Our evidence, so far, indicates that the downregulated miRNAs do not play a role in modulating alcohol sensitivity, however, it is possible that overexpression is not effective for functionally validating the role of downregulated miRNAs. Future studies should explore this issue further by transgenically inhibiting microRNA activity. Efforts in creating collections of transgenic miRNA sponge lines should soon provide the tools needed for this purpose (Kluiver *et al.*, 2012; Loya *et al.*, 2009). miR-6 and miR-310 are well-studied miRNAs in *Drosophila*. Both have been associated with neural morphogenesis during development. Mutations in miR-6 have been shown to cause defects in the central nervous system and embryonic lethality (Ge *et al.*, 2012). These effects have been directly linked to the regulation of the proapoptotic genes, *reaper (rpr)*, *head involution defective (hid)*, *grim* and *sickle (skl)*. Upregulation of these genes results in elevated apoptosis within the central nervous system in the mutants. On the other hand, miR-310 has been associated with the proliferation and differentiation of early germ and somatic progenitor cells through the regulation of the Wntless pathway members *arm* and *pangolin* (Pancratov *et al.*, 2013). miR-310 was also shown to participate in the regulation of synaptic transmission at the *Drosophila* larval neuromuscular junction (Tsurudome *et al.*, 2010). Perhaps most significant with regards to the effects of alcohol, loss of the miR-310-313 cluster has been shown to lead to a significant enhancement of neurotransmitter release, as well as a significant increase in the active zone protein Bruchpilot (encoded by *brp*). Interestingly, *brp* was shown to be upregulated by alcohol and to participate in alcohol tolerance in flies (Ghezzi *et al.*, 2013). More recently, miR-310 was also shown to orchestrate Hedgehog signaling strength in response to diet, implicating miRNA-directed silencing as an important regulator of *Drosophila* metabolic state (Çiçek *et al.*, 2016). These connections between miR-310 and synaptic activity and its response to changes in diet supports our interpretation that miR-310 plays an important role in the response to alcohol.

Among the gene targets of miR-6 and miR-310, we have identified a series of interesting candidates. These include genes with known links to alcohol related behaviors. Potential miR-310 targets include: *Arfip*, a dynactin binding protein associated with positive regulation of synaptic growth at neuromuscular junction recently shown to increase ethanol sensitivity when mutated (Chang *et al.*, 2013; Peru *et al.*, 2012); *CASK*, a calcium/calmodulin-dependent protein kinase associated with the regulation of neurotransmitter secretion and reduced ethanol tolerance when mutated (Lloyd *et al.*, 2000; Maiya *et al.*, 2012); *Toll*, a transmembrane receptor associated with activation of the innate immune response —activation of *Toll* has been recently linked to increased ethanol resistance (Tauszig *et al.*, 2000; Troutwine *et al.*, 2016); *kn*, a transcription factor also associated with the innate immune response and the development of ethanol tolerance (Ghezzi *et al.*, 2013); and *tao*, a protein serine/threonine kinase involved in promoting axon guidance and the ethanol-induced hyperactivity response (King *et al.*, 2011; King & Heberlein, 2011). Meanwhile, miR-6 can potentially regulate: *AcCoAS*, an acetyl-coenzyme A synthetase associated with many biochemical pathways, including those for ethanol metabolism, and is thought to contribute to ethanol-induced hyperactivity (Kong *et al.*, 2010); *rho*, a serine-type endopeptidase associated with axogenesis and also linked to increased ethanol sensitivity when mutant (Berger *et al.*, 2008; Grueber *et al.*, 2007); and *Teh2*, putative sodium channel

and BK channel auxiliary subunit associated with the development of ethanol tolerance (Derst *et al.*, 2006; Ghezzi *et al.*, 2013).

Additionally, we also detected a series of immune response genes (*imd*, *PGRP-LC*, *Tollo*, *upd3*, *crq*), ion channels alpha and beta subunit genes (*Ca-alpha1T*, *SK*, *Slob*, *Teh3*, *Ih*, *SKIP*, *Best2*), synaptic genes (*Syt12*, *Prosap*, *Dscam3*), as well as transcription factor and co-factor genes (*CrebA*, *kay*, *Sirt2*, *Sin3A*). While these genes have not been directly linked to the alcohol responses, they fall within important gene categories associated with the behavioral responses to alcohol. Among the immune genes, *PGRP-LC* and *imd* are particularly interesting candidates, as both belong to the innate immune IMD pathway that results in activation of the *Drosophila* NFkB homolog Rel. In a recent study we showed that overexpression of the active form of Rel increases resistance to ethanol (Troutwine *et al.*, 2016). In the ion channel category, the gene *Slob* is another salient example. *Slob* encodes a well-known modulatory subunit of the BK channel gene *slo* (Shahidullah *et al.*, 2009). In both flies and mammals the BK channel has been strongly linked to alcohol tolerance (for review, see: Ghezzi & Atkinson, 2011), and modulation of its activity by *Slob* can have a strong impact on alcohol sensitivity. Finally the transcriptional activator encoded by *kay* (*kayak*) is the *Drosophila* homolog of AP1/c-FOS in mammals (Perkins *et al.*, 1990). In mammals, alcohol has been shown to selectively attenuate stress-induced c-fos expression (Ryabinin *et al.*, 1995), and has also been implicated in the persistent changes underlying cocaine addiction (Larson *et al.*, 2010; Xu, 2008). It is thus not surprising that collectively, modulation of these genes by miRNAs can severely affect the neural responses to alcohol.

A complicating factor for work of this kind is that the behaviors of interest might be the product of more than one miRNA. Furthermore, multiple miRNAs can regulate partially overlapping sets of mRNA and might all be necessary to see an effect. Furthermore, because miRNA functionality does not require strict sequence complementarity to the mRNA target for its regulation, our prediction of targets may have misidentified or not identified some targets. Although, an individualistic approach to miRNA analysis may not completely recapitulate the natural response, nonetheless this approach has already identified alcohol-responsive miRNAs that share a common biological output — the regulation of neural morphogenesis. Furthermore, we have connected these miRNAs to new interesting gene candidates with potential roles in ethanol neuroadaptation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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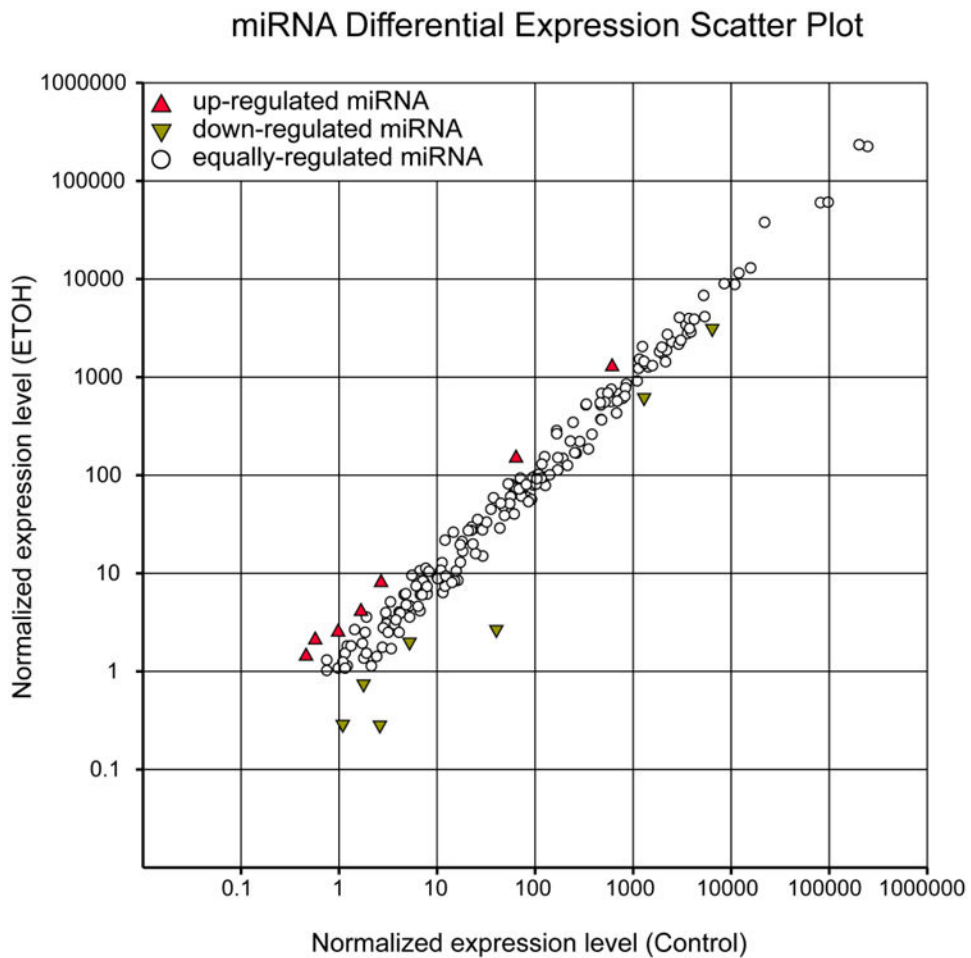


Figure 1. Scatter plot showing a comparison of the relative abundance of the 426 known *Drosophila* miRNAs. The comparison is between miRNAs isolated from heads 30 minutes post ethanol sedation and miRNAs isolated from non-ethanol treated matched controls. The normalized count is reads per million [(actual count/total reads)* 10^6]. Open circles represent miRNAs whose abundance did not differ between the two treatments. Upward-pointing triangles represent miRNAs whose abundance increases while downward-pointing triangles represent miRNAs whose abundance decreases at least two fold. Values for the relative abundances of these miRNAs are found in Supplementary Data 1.

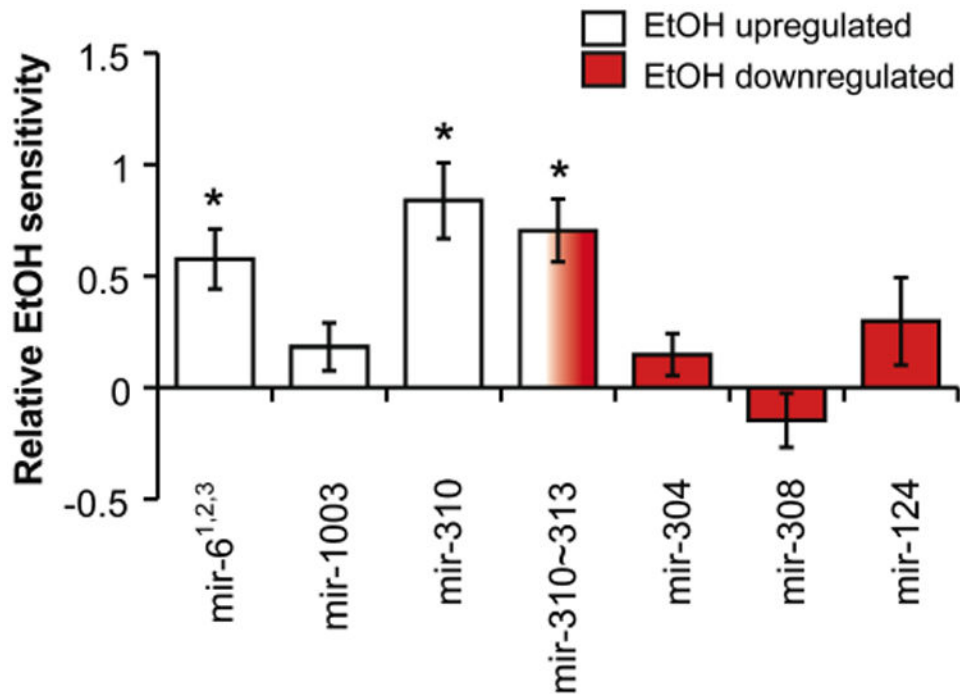


Figure 2.

Feeding of RU486 to GeneSwitch-elav/UAS-miRNA transgenic stocks alters the ethanol sensitivity of three out of seven tested miRNA overexpression lines (relative to stock not fed RU486). Shown is the fold difference in ethanol sensitivity between the RU486-fed and not fed stocks calculated using the formula in the Methods section (error bars are SEM, * indicates a P value of < 0.05). miRNAs found to be induced by ethanol are shown by open bars while miRNAs normally suppressed by ethanol are by closed bars. The open/closed bar is a transgene that encodes four miRNAs: miR-310 was induced by ethanol and miR-313 was suppressed by ethanol. mir-311 and mir-312 were not found to be ethanol-responsive. The mir-310~313 transgene induces all four of these miRNAs. The mir-6^{1,2,3} transgene induces all 3 mir-6 paralogs.

Table 1

Known miRNAs with significant changes after ethanol exposure

miRNA	EtOH	Fold-change	P-value	Sequence	Guide miRNA	Behavioral EtOH test
miR-6-3p	Up	1.87	7.65E-05	<u>U</u> AUCACAGUGGCUGUUCUUUUU	Yes [‡]	Sensitive
miR-310-3p	Up	1.62	3.38E-03	UAUUGCACACUCCCGCCUUU	Yes	Sensitive
miR-304-5p	Up	1.56	5.66E-12	<u>U</u> AAUCUCAAUUUGUAAAUGUGAG	Yes	No change
miR-1009-3p	Up	1.35	6.18E-04	UCUCAAAAAUUGUUACAUUCAG	Yes	
miR-1008-3p	Up	1.27	3.12E-05	<u>U</u> CACAGCUUUUGUGUUUACA	Yes	
miR-1003-3p	Up	1.24	3.11E-139	<u>U</u> CACACAUUACAUAUUCACAG	Yes	No change
miR-193-3p	Up	1.08	0.00E+00	<u>U</u> ACUGGCCUACUAAAGUCCCAAC	Yes	
miR-124-3p	Down	-1.03	0.00E+00	<u>U</u> AAGGCACGCGGUGAAUGCCAAG	Yes	No change
miR-277-5p	Down	-1.07	0.00E+00	CGUGUCAGGAGUGCAUUUGCA	No	
miR-313-5p	Down	-1.28	5.67E-03	UGCUGCGGAUGGGGGCAGUACU	No	No change
miR-11-5p	Down	-1.40	2.80E-07	CAAGAACUUUCUCUGUGACCCG	No	
miR-13b-1-5p	Down	-1.95	3.62E-03	UCGUUAAAUGUUUGUGAACUU AUG	No	
miR-4976-5p	Down	-3.19	1.70E-09	<u>U</u> CCAGGCUGUGUCCCAUGGAGU	Yes	
miR-308-3p	Down	-3.94	8.93E-153	<u>AA</u> UCACAGGAUUAUACUGUGAG	Yes [‡]	No change

Ethanol-induced changes are shown relative to control. Fold change is expressed as log₂ values (EtOH/Ctrl). The underlined region in each sequence designates the seed region.

[‡] denotes miRNAs that share the same seed sequence. Information of whether a miRNA is known to serve as a guide miRNA is included. The effects of miRNA overexpression on alcohol sensitivity are also reported for all miRNAs tested in the present study. The relative abundance of each miRNA, measured as normalized reads per million before and after ethanol treatment, can be found in the Supplementary Data 1.

Table 2
Putative novel miRNAs with significant changes after ethanol exposure

miRNA	EtOH	Fold-change	P-value	Sequence	Chromosomal location
novel_miR_1	Up	10.92	2.93E-102	GCGUCCGGUGGUAUUGGUAG	3R 29555866:29555607
novel_miR_2	Up	8.09	5.14E-15	GUGGGCGGUGGAGAGGUGGAG	3L 935306:935326
novel_miR_3	Up	8.09	5.14E-15	GUGGGUGGGGUGGACGGGCCA	X 9176644:9176663
novel_miR_4	Up	7.51	2.98E-10	UCGGUCCACCCGGGGCGGCCA	2L 1856619:1856599 / 2R 9632238:9632218
novel_miR_5	Up	7.09	7.19E-08	UUGGGGGCGGGGUGGUGG	X 9057913:9057893
novel_miR_6	Down	-1.02	6.81E-03	AGAGCGACGAAGACUGAGCUG	3L 14420650:14420630
novel_miR_7	Down	-6.78	1.64E-06	CCGGUGUGUGGGAUUGGGUA	3R 21410603:21410623
novel_miR_8	Down	-7.11	4.93E-08	UUGGGGGCGGGGUGGUGGC	X 9057913:9057893
novel_miR_9	Down	-7.17	2.45E-08	UGUGGCAGGCGAAUUGAUGUGG	2L 4458246:4458225
novel_miR_10	Down	-7.34	2.99E-09	GUUGAACGGUCGUUGAACAAU	X 13635866:13635886
novel_miR_11	Down	-7.43	7.36E-10	GCGGUCGGAGUGGGUGGUGG	2L 16283135:16283115
novel_miR_12	Down	-9.05	9.98E-29	UUGGGCGGGACGGGCGGCCG	3R 3300451:3300431
novel_miR_13	Down	-10.82	5.54E-96	GUGGAGCGGUGGGCGGAGCG	3L 4576278:4576298

Ethanol-induced changes are shown relative to control. Fold change is expressed as log₂ values (EtOH/Ctrl). The relative abundance of each miRNA, measured as normalized reads per million before and after ethanol treatment, can be found in the Supplementary Data 1. All of these match criteria for a miRNA (see Methods) but none have been functionally tested to confirm that functionally act as a miRNA.

Table 3

Gene ontology annotations for miR-6 targets

Annotation cluster	Enrichment score	GO Term: Biological Function	Gene Count	% of total	P-Value	FE	BS	FDR
1	3.84	instar larval or pupal morphogenesis	23	6.42	0.00	2.62	0.00	0.10
2	3.63	neuron projection morphogenesis	20	5.59	0.00	2.76	0.00	0.17
3	3.46	post-embryonic organ morphogenesis	19	5.31	0.00	2.64	0.01	0.47
4	2.93	imaginal disc-derived appendage morphogenesis	16	4.47	0.00	2.64	0.02	1.66
5	2.39	imaginal disc-derived wing morphogenesis	14	3.91	0.00	2.54	0.05	5.43
6	1.66	negative regulation of transcription	12	3.35	0.02	2.29	0.16	22.50
7	1.54	negative regulation of macromolecule biosynthetic process	13	3.63	0.02	2.09	0.21	30.00
8	1.50	leg segmentation	4	1.12	0.01	9.29	0.10	12.63
9	1.37	response to other organism	9	2.51	0.02	2.73	0.17	24.13
10	1.35	cell migration	10	2.79	0.03	2.26	0.26	41.86

Significant biological function categories were clustered based on enrichment scores using DAVID. The top term in each of the top-10 cluster is listed. The 'Gene Count' column displays number of genes in each cluster associated with a particular GO term, as well as the % of the total number of genes in the cluster is shown. P-value, Fold Enrichment (FE), Benjamini Score (BS) and False Discovery Rate (FDR) are also shown.

Gene ontology annotations for miR-310 targets

Table 4

Annotation cluster	Enrichment score	GO Term: Biological Function	Gene Count	% of total	P-Value	FE	BS	FDR
1	6.06	morphogenesis of an epithelium	21	6.33	0.00	3.86	0.00	0.00
2	4.69	maginal disc-derived appendage morphogenesis	19	5.72	0.00	3.29	0.00	0.03
3	3.43	branching involved in open tracheal system development	7	2.11	0.00	7.43	0.01	0.50
4	2.90	imaginal disc-derived wing hair organization	6	1.81	0.00	7.52	0.02	1.72
5	2.41	neuron projection morphogenesis	16	4.82	0.00	2.32	0.05	5.99
6	2.32	positive regulation of cellular biosynthetic process	11	3.31	0.00	2.98	0.04	6.02
7	2.15	muscle cell development	6	1.81	0.00	5.52	0.05	6.82
8	2.11	positive regulation of transcription	9	2.71	0.01	3.21	0.07	10.54
9	1.73	establishment of planar polarity	6	1.81	0.02	4.07	0.13	22.45
10	1.46	epithelial cell migration	4	1.20	0.03	5.71	0.22	41.09

Significant biological function categories were clustered based on enrichment scores using DAVID. The top term in each of the top-10 cluster is listed. The 'Gene Count' column displays number of genes in each cluster associated with a particular GO term. Percentage of the total number of genes in the cluster, P-value, Fold Enrichment (FE), Benjamini Score (BS) and False Discovery Rate (FDR) are also shown.