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## The potential role of amygdaloid microRNA-494 in alcohol-induced anxiolysis

Tara L. Teppen<sup>#1,3</sup>, Harish R. Krishnan<sup>#1,3</sup>, Huaibo Zhang<sup>1,3</sup>, Amul J. Sakharkar<sup>1,3</sup>, and Subhash C. Pandey<sup>1,2,3</sup>

<sup>1</sup>Center for Alcohol Research in Epigenetics, Department of Psychiatry, University of Illinois at Chicago, Chicago IL 60612

<sup>2</sup>Anatomy and Cell Biology, University of Illinois at Chicago, Chicago IL 60612

<sup>3</sup>Jesse Brown Veterans Affairs Medical Center Chicago, IL 60612

<sup>#</sup> These authors contributed equally to this work.

### Abstract

**Background**—The anti-anxiety effects of ethanol appear to be a crucial factor in promoting alcohol intake. Regulation of gene expression by microRNA (miRNA) is an important epigenetic mechanism that affects neuronal pathways and behaviors. Here, we investigated the role of miRNAs underlying the mechanisms of ethanol-induced anxiolysis.

**Methods**—Acute ethanol-induced anxiolysis was measured in adult rats and amygdaloid tissues were used for miRNA profiling by microarray analysis. The expression of miR-494 and its target genes in the amygdala was measured using real-time quantitative PCR. The direct role of miR-494 in the anxiety phenotype was also investigated via infusion of a miR-494 antagomir into the central nucleus of amygdala (CeA).

**Results**—Microarray profiling of miRNAs in the amygdala showed significant alteration of several miRNA expression levels by acute ethanol exposure. Expression of miR-494 was significantly decreased whereas expression of CREB binding protein (CBP), p300 and CBP/p300-interacting transactivator 2 (*Cited2*) were increased in the amygdala during ethanol-induced anxiolysis. Interestingly, inhibition of miR-494 in the CeA, through infusion of a specific antagomir, provoked anxiolysis thus mimicking the action of ethanol. Also expression of *Cited2*, CBP and p300 as well as histone H3-K9 acetylation were significantly increased by miR-494 antagomir infusion, indicating their regulation by miR-494 in the amygdala.

**Conclusions**—These novel results suggest that acute ethanol-induced reduction in miR-494 expression in the amygdala can serve as a key regulatory mechanism for chromatin remodeling possibly leading to anxiolysis.

Address for Correspondence: Dr. Subhash C. Pandey, Department of Psychiatry, University of Illinois at Chicago and Jesse Brown VA Medical Center, 1601 West Taylor Street, Chicago, Illinois 60612. Fax: 312-996-7658 Phone: 312-413-1310 scpandey@uic.edu.

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## Keywords

Amygdala; Alcohol; Anxiety; Anxiolysis; CBP; Cited2; microRNAs; microRNA-494; p300

## Introduction

The anxiolytic-like effects of alcohol may play an important role in the initiation and maintenance of alcohol drinking behaviors (1-4). Both clinical and pre-clinical studies have shown that activation of brain stress circuitry, in concordance with the negative emotional state produced by alcohol dependence, produces anxiety and drives higher alcohol intake to self-medicate, thereby maintaining the status of addiction (4-10). Alcoholism is a complex disease that results from the long-term plasticity-dependent dysregulation of key neuroanatomical circuits (2,8-10). As the critical brain structure involved in fear and anxiety, the amygdala, specifically the central (CeA) and medial nucleus of the amygdala (MeA), has been implicated in the anxiolytic effects of acute ethanol exposure (2,4,5,10-13). Recently, it was shown that acute ethanol regulates anxiolytic-like effects through epigenetic modifications such as histone H3-K9 acetylation mechanisms via histone deacetylase (HDAC)-2 regulation in the CeA (4,8,9,14). Acute ethanol was shown to inhibit HDAC activity in the amygdala and increase cAMP responsive-element binding (CREB) phosphorylation and CREB binding protein (CBP) protein levels as well as histone H3-K9 acetylation in the CeA and MeA, producing anxiolytic-like effects in rats (8,14). However, the mechanism by which acute ethanol rapidly modifies CBP and histone acetylation in the amygdala and produces the anxiolytic response is not well explored.

Another epigenetic factor, which modulates gene expression, is microRNA (miRNA) mediated post-transcriptional regulation (15-17). These small (~21-23 nt) non-coding RNA molecules regulate genes at the post-transcriptional level via recruitment of protein complexes to target mRNAs, resulting in either degradation and/or translational inhibition and thus lowering mRNA and protein levels of target genes (15-19). Highlighting their crucial role in gene transcription, more than half of all protein-coding genes in mammals may be targets of miRNAs, indicating that the majority of the protein-coding transcriptome is regulated by this mechanism (18). In the brain, miRNAs have been shown to regulate synaptic plasticity and dendritic spine density (19-24). Moreover, complex phenotypes produced by drugs of abuse including cocaine and alcohol have been shown to be due to altered miRNAs and related biological pathways (25-30). Thus, regulation by microRNAs represent a newly emerging yet vital epigenetic mechanism capable of shaping the output of the neuronal transcriptome and plasticity (31,32). Despite the arising importance of brain miRNA pathways, the potential involvement of amygdaloid specific miRNAs in acute ethanol-induced behavioral effects such as anxiolysis remains unknown. We therefore investigated miRNA pathways and subsequent downstream targets that are operative in the amygdala to regulate the acute effects of ethanol using an animal model. We performed a complete miRNA profiling via microarray and identified a novel miRNA, miR-494, that is down regulated by acute ethanol and regulates CREB signaling pathways and histone acetylation leading to anti-anxiety effects of ethanol.

## Materials and Methods

### Acute ethanol exposure paradigm

Adult male Sprague-Dawley rats used in this study were purchased from Harlan (Indianapolis, IN), and group housed in a temperature-controlled room with a 12/12-hr light/dark cycle, with food and water provided ad libitum. All rat procedures were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. As reported earlier (8,9,14), rats were injected with either normal saline (5µl/g body weight) or ethanol (1g/kg; 20% w/v). One-hour post-injection anxiety indices were measured using the elevated plus maze (EPM) or light/dark box (LDB) exploration tests as described below. Immediately following behavioral paradigms, animals were anesthetized with sodium pentobarbital (50 mg/kg) and amygdaloid tissue was dissected out and quickly stored at -80°C until further processed for microRNAs and gene expression studies. Blood alcohol levels were measured using an Analox Alcohol Analyzer (Lunenburg, MA).

### Cannula implantation and miR-494 antagomir infusion

In regards to the microRNA antagomir infusion experiment, *in vivo* delivery in the brain has been accomplished via several different dosing regimens ranging from continuous infusions (33,34) to acute single or multiple injections (25,35). Our approach is based on these publications. Rats were anesthetized with sodium pentobarbital (50mg/kg, i.p.) and implanted bilaterally with CMA/11 guide cannulae targeted 3mm above the central nucleus of amygdala (2.5 mm posterior; ± 4.2 mm lateral; 5.1 mm ventral) as described by us previously (4,12). One week after recovery, rats were infused bilaterally twice per day (9:00 am and 5:00 pm) with 0.5µl of aCSF, 500pmol/0.5µl of miR-494 antagomir (5'-TCCCGTGTATGTTTC-3'; Exiqon Woburn, MA) in aCSF, or 500pmol/0.5µl of scrambled antagomir (5'-ACGTCTATACGCCCA-3'; Exiqon) in aCSF for two consecutive days. On the morning of the third day, 16 to 18 hours after the last infusion, anxiety indices were measured, rats were anesthetized and either perfused (4,8,12) to collect brains for histochemistry or decapitated to dissect out amygdaloid tissue and were quickly frozen for miRNA and related gene expression studies.

### Behavioral measurements

To measure anxiety-like behaviors, the elevated plus-maze exploration test was employed, as previously described by our laboratory (8,9,12,36). The results are expressed as the mean ± SEM of the percent of open-arm entries and the mean percent of time spent on the open arms (open-arm activity). The total number of arm entries was used to represent the general activity level of each rat. Anxiety-like behaviors were also measured using the light/dark box exploration test, which has been described by us previously (4,8,14). Animal activity was computer monitored and time spent in light and dark compartments was recorded via an infrared beam. Data is presented as percentage of time spent in either the dark or light compartment. Total ambulation was used to measure general activity of rats.

### miRNA profiling in the amygdala using microarray

RNA was extracted from amygdala tissue from six control and six acute ethanol treated rats using the QIAgen RNeasy Mini Kit. RNA was then pooled so that each sample sent for analysis was the combination of two animals from the same treatment group. Results are therefore presented as n of 3 per group. Microarrays and subsequent analysis was performed by LC Sciences (Houston, TX) using a  $\mu$ Paraflo<sup>®</sup> Microfluidic biochip using hybridization RNA probes, that utilized version 18 of the rat miRBase database. The detailed procedure has been described in supplemental methods. The results are presented as a heat map, organized with individual RNA samples on the horizontal axis and miRNAs listed according to magnitude of significance on the vertical axis. The mean data for each miRNA for control and ethanol group has been provided in Supplemental Table 2. Microarray data for miRNA profiling have been deposited at NCBI gene expression omnibus (GEO; accession number GSE68278).

### Validation of miRNA profiling data

miRNA profiling results were reviewed and the miRNAs with the highest statistical significance (Figure 1) were chosen for further validation: *miR-494*, *miR-130a*, and *miR-191*. TaqMan primers were purchased (see Supplemental Table 1 for primer sequences) and real-time PCR was performed in accordance with the TaqMan assay protocol (Life Technologies).

### RNA isolation and Real-Time quantitative Polymerase Chain Reaction

Total RNA was extracted from amygdaloid tissues as described above. Fifteen nanograms of total RNA was reverse transcribed using the GeneAmp RNA PCR Core Kit (Life Technologies) and the reaction was stopped by 5-minute incubation at 85°C. Aliquots from each cDNA preparation were amplified by Real-Time PCR using RT2 SYBR Green (Qiagen) performed with the Mx3000P qPCR system (Agilent Technologies) and analyzed with MxPro software. Expression of *CBP*, *p300* and *Cited2* mRNA was examined, and either *GAPDH* or *Hprt1* were used as internal controls. PCR conditions for all primers were 30s at 95°C, 30s at 58°C and 30s at 72°C for 40 cycles (see Supplemental Table 1 for primer sequences). The Ct values from the genes of interest were normalized to internal control genes. Relative expression was then determined using the  $2^{-Ct}$  method (37) and presented as fold change per experimental condition.

### Gold immunolabeling of CBP, p300 and acetylated histone H3 proteins

The protein levels of CBP and p300 were examined using anti-CBP (1:200 dilution), p300 (1:500 dilution) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and histone H3-K9 acetylated (1:500) antibodies (EMD Millipore, Billerica, MA) by gold immunolabeling histochemistry as reported previously by our laboratory (4, 8,14). Gold-immunolabeled proteins were quantified using Image Analysis System connected to a light microscope and we calculated the number of gold particles/ 100  $\mu\text{m}^2$  at high magnification (100 $\times$ ) within the amygdaloid brain regions. The gold particles in the defined amygdaloid nuclei (3 object fields within each region of 3 adjacent brain sections from bregma levels 2.3-2.8 mm, totaling 9 object fields per region per rat) were calculated and values were

averaged for each rat. Results are represented as the number of immunogold particles/100 $\mu\text{m}^2$  area.

### Statistical analysis

Statistical differences between the data of two groups including miRNA profiling data were evaluated by student's t test. The false discovery rate was not used in microarray data analysis. Data derived from more than three groups were analyzed using one-way ANOVA followed by Tukey's *post hoc* multiple comparisons between groups. All differences were considered significant when *p* values were <0.05.

## Results

### Acute ethanol elicits an anxiolytic-like response and alters miRNA expression in the amygdala

We first examined the effects of acute ethanol on anxiety-like behaviors by employing two separate behavioral paradigms, the light-dark box and elevated plus maze exploration tests. Previously reported in several studies (8,9,14) by our lab and here again, we confirmed that acute ethanol exposure produced anxiolytic-like effects in rats (Data not shown). The average blood ethanol level (mg%) of ethanol treated rats (at the time of brain collections) was  $94 \pm 1.4$  (n=12; mean $\pm$  SEM), which is consistent with the range of our previous studies (8,9,14). There were no significant differences in the mean body weights (gm) among the groups (Control=  $318 \pm 2$ ; Ethanol=  $320 \pm 2$ ; n=12; mean $\pm$  SEM)

A miRNA profiling microarray revealed altered miRNA expression patterns in the amygdala of acute ethanol treated rats as compared with controls (Figure 1). Expression of seven miRNAs was found to be significantly ( $p < 0.05$ ) changed in the amygdala following acute ethanol exposure; two miRs were decreased (miR-494 and miR-130a) and five were increased (miR-191, miR-204, miR-361, miR-674-5p and miR-337\*). Additionally, twenty miRs were found to be differentially ( $p < 0.1$ ) expressed following acute ethanol exposure (Figure 1). For a complete list of miRNA profiling data see Supplemental Table 2.

### Effects of acute ethanol exposure on miRNA expression in the amygdala

We validated the miRNA microarray profiling data of the top miRNAs altered by acute alcohol exposure in the amygdala (Figure 2). Using a TaqMan qRT-PCR miRNA expression assay, we measured the transcript abundance of *miR-494*, *miR-191*, and *miR-130a* in the amygdala of control and acute ethanol exposed rats. Expression of *miR-494* ( $p < 0.001$ ) and *miR-130a* ( $p < 0.001$ ) were significantly reduced and expression of *miR-191* ( $p < 0.05$ ) was significantly increased in the amygdala following acute alcohol exposure, thereby validating the findings obtained by the miRNA profiling microarray method.

### Downstream target genes of miR-494 regulate the CREB pathway

We next investigated the downstream gene targets of miR-494 whose expression was significantly decreased in the amygdala by acute ethanol exposure. The predicted gene targets of miR-494 were obtained from the well-reviewed miRNA target prediction program at microRNA.org (38,39). Gene ontology analysis revealed functional classification of novel

miR-494 targets associated with chromatin remodeling and synaptic plasticity (40,41), several of which are presented in Supplemental Table 3. Some of the predicted *in silico* miR-494 targets (*Cited2* and *CBP*) appear to be related to the CREB pathway (Figure 3A), that has been highly implicated in anxiety and alcoholism (8,36, 42). We therefore examined for changes in the expression of *Cited2* and *CBP*, and the CBP associated gene *p300* in the amygdala after acute ethanol exposure. It was found that mRNA levels of *Cited2*, *CBP* and *p300* were significantly ( $p<0.05$ ) higher in the amygdala by ethanol (Figure 3B) implicating involvement of the CREB pathway in the actions of alcohol via targeting of miR-494. These results indicate that ethanol-induced reductions in the expression of miR-494 may increase the mRNA levels of *Cited2*, *CBP* and *p300* in the amygdala and regulate anxiolytic-like effects.

### **Infusion of a miR-494 antagomir mimics the ethanol-induced anxiolysis**

We next evaluated the behavioral effects of *in vivo* infusion of a locked nucleic acid (LNA<sup>TM</sup>) antisense oligonucleotide directed at miR-494 into the CeA. Inhibition of CeA miR-494 function was able to mimic the anxiolytic-like effects produced by acute ethanol in both tests of anxiety i.e. light/dark box and elevated plus-maze exploration tests (Figure 4A&B). Rats infused with miR-494 antagomir into the CeA spent significantly ( $p<0.01$ ) more time in the light compartment and less time in the dark compartment of the light/dark box, indicating that inhibition of miR-494 in the CeA mimics the alcohol-induced anxiolysis. Data from the elevated plus-maze test reiterated these findings and revealed that infusion of a miR-494 antagomir mimics the behavioral effects of acute ethanol by producing significant anxiolytic-like effects ( $p<0.001$ ) as evidenced by the increased percentage of open arm entries and time spent in the open arms as compared to rats infused with aCSF (control group). Infusion with a scrambled antagomir did not elicit anxiolytic-like effects, indicating the observed behavioral effect is specific to miR-494 infusion. No difference was observed in the number of total arm entries in elevated plus-maze or total ambulation in light/dark box exploration test between treatment groups (Figure 4A&B), indicating that miR-494 antagomir infusion did not have an effect on the general activity of rats.

### **Inhibition of miR-494 up-regulates the expression of CREB signaling related genes**

Similar to ethanol, inhibition of miR-494 by antagomir infusion into CeA also significantly ( $p<0.05$ - $0.001$ ) increased the expression of *Cited2*, *CBP* and *p300* in the amygdala (Figure 5A). Infusion of a scrambled antagomir did not alter mRNA levels of the aforementioned genes as compared to control aCSF infused rats. These results mimic those observed following acute alcohol exposure, suggesting that ethanol possibly acts via miR-494 to regulate the expression of genes related to CREB pathway and synaptic plasticity.

### **Inhibition of miR-494 up-regulates protein levels of CBP, p300 and increased histone H3-K9 acetylation**

We have previously shown that protein levels of CBP and acetylated histone H3-K9 are up-regulated in the CeA and MeA of rats following acute ethanol exposure (8,14). Here we measured CBP, p300 and acetylated histone H3-K9 protein levels using gold-immunolabeling in the amygdaloid brain structures of miR-494 antagomir or aCSF infused rats. We observed that they were significantly ( $p<0.001$ ) increased in the CeA, but not in the



surrounding MeA or BLA following antagomir infusion (Figure 5B&C). These results suggest that increased levels of CBP and p300 due to inhibition of miR-494 may be responsible for the increased histone H3-K9 acetylation leading to chromatin remodeling.

## Discussion

The major finding of the present study is that amygdaloid miR-494 plays an essential role in the anxiolytic-like effects of ethanol. Microarray profiling of miRNAs within the amygdala revealed differential expression of numerous miRNAs in response to acute ethanol exposure. We validated the changes in the expression of several miRNAs including miR-494, which is significantly decreased in the amygdala by acute ethanol exposure. Based on network analysis we chose miR-494 and its putative targets, *Cited2* and CBP, which could possibly regulate the anxiolytic-like effects of ethanol. An *in vivo* infusion of an antagomir against miR-494 into the CeA was able to mimic ethanol's anti-anxiety behavioral effects indicating that miR-494 may be involved in ethanol-induced anxiolysis. Inhibition of miR-494 via antagomir infusion was able to increase the mRNA expression of *Cited2*, mRNA and protein levels of CBP and p300 and protein levels of acetylated histone H3-K9 (Figure 5A,B &C), similar to what we observed after an acute ethanol exposure (Figure 3; 8,14). Together, these results suggest that miR-494 acting via downstream regulation of mRNA levels of *Cited2*, CBP and p300 in the CeA may regulate histone acetylation thereby resulting in the anxiolytic-like effects observed after acute ethanol (Figure 6). These data also indicate that miR-494 has the ability to regulate chromatin structure via modulating histone acetylation in the central nucleus of amygdala, thus identifying novel mechanisms in the rapid action of acute ethanol and its behavioral effects.

Chromatin remodeling mechanisms in the amygdala have been implicated in modulating the expression of genes that are involved in regulating anxiolytic and anxiety-like behaviors during ethanol treatment and its withdrawal (4,8,14). It is well known that phosphorylated CREB (pCREB) recruits CBP and modifies the expression of downstream cAMP-inducible genes (43-45). CBP and its paralog p300, via their intrinsic histone acetyltransferase (HAT) activity, regulate histone acetylation and thereby gene transcription (46,47). Acute ethanol increases CREB phosphorylation and CBP levels as well as histone H3-K9 and H4-K8 acetylation in the CeA and MeA of rats (8,9). These changes are associated with increased expression of CREB target genes, such as neuropeptide Y, brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc) in the CeA and MeA (8,9,48,49). Studies from our lab have shown that acute ethanol increases CREB phosphorylation through activation of extracellular signal-regulated kinases (Erk1/2), leading to increased expression of BDNF and Arc as well as increased dendritic spines in the CeA and MeA, but not in the BLA of rats. The alterations in these molecular and synaptic remodeling mechanisms were additionally shown to be associated with anxiolytic-like effects observed after acute ethanol exposure (9). Studies from other laboratories have revealed that acute ethanol significantly increased phosphorylated Erk1/2 levels in the CeA, but not BLA of mice (50). In addition to the aforementioned modulation of miR-494 by acute ethanol, other mechanisms such as increased phosphorylation of Erk1/2 may also regulate CREB pathway and alcohol-induced anxiolysis. Furthermore, deficits in CREB levels in the amygdala have been associated with heightened anxiety and excessive alcohol

intake (36,42). The data collected in the current study extend previous findings by identifying miR-494 in the CeA as an additional molecular regulator of the CREB signaling pathway and anxiolytic-like effects of ethanol.

Using an unbiased approach, microarray data generated in the current study identified many interesting miRNA candidates significantly altered by acute ethanol treatment, some of which were validated using qRT-PCR. Based on *in silico* target gene predictions of miR-494 (38, 39) using the microRNA.org database we identified several gene targets (Supplemental Table 3) that may be involved in alcohol-induced anxiolysis. However, we chose to perform detailed studies on the identified target genes *CBP* and *Cited2*, as CBP, in association with p300, exhibits HAT activity and regulates chromatin remodeling subsequently affecting brain function (46, 51). CBP has also been implicated in ethanol related phenotypes (8). *Cited2* is emerging as an important regulator of neuronal function and *Cited2* deficits have been associated with neural crest defects and neurulation during development (52). Moreover, *Cited2* also has been shown to regulate NF-kappa B, an important transcription factor (53) that has been implicated in the neuroinflammatory mechanisms of alcohol exposure (54,55). Here we show for the first time a possible direct role of miRNA mediated CBP and *Cited2* regulation in the amygdala that regulates anxiolytic-like effects following acute ethanol exposure. Also we demonstrated that miR-494 can regulate histone H3-K9 acetylation in the amygdala opening the possibility that acute ethanol-induced increases in H3-K9 acetylation in the CeA could be partly related to decreased expression of miR-494. Numerous studies have investigated the role of miRNAs in synaptic plasticity and related phenotypes of addictive behavior. For example, cocaine-induced changes in plasticity related gene expression and behavioral effects have been shown to be regulated by miRNAs (25, 26, 56, 57). Several other miRNAs that are altered by ethanol exposure play critical roles in tolerance and the pathophysiology of fetal alcohol spectrum disorders (27, 29, 58-60). The miRNA, miR-9, has been shown to affect ethanol tolerance via regulation of large conductance calcium- and voltage-gated potassium channel (BK) function in mammalian brain (29). In the medial pre-frontal cortex of rats, miR-206 and miR-30a-5p have been shown to promote alcohol drinking and dependence, acting via BDNF signaling pathways (30, 61). Our data suggest a fascinating link between miR-494 and downstream CREB pathway genes in the amygdala and suggest that an acute ethanol-induced reduction in miR-494 levels in the CeA may regulate this pathway and histone acetylation, ensuing in the anxiolytic-like effects of ethanol (Figure 6). Furthermore, miR-191 and miR-130a were also significantly altered in the amygdala following acute ethanol exposure and appear to be implicated in the synaptic plasticity of brain disorders (62,63). Future studies will investigate the role of these miRNAs in the anxiolytic effects of acute ethanol exposure.

In summary, we demonstrate that amygdaloid miR-494 regulates the components of the CREB signaling pathway in the amygdala and is mechanistically involved in the regulation of alcohol-induced anxiolysis. Importantly, this work provides insights into epigenetic pathways that are regulated by miR-494, which play a role in the anxiolytic-like effects of acute ethanol. This rapid action of a non-sedative dose of acute alcohol has clinical implications in social alcohol drinking populations via modulation of alcohol's negative reinforcing properties (1,5,6).



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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S.C.P. reports that a US patent application entitled “Histone acetyltransferase activators and histone deacetylase inhibitors in the treatment of alcoholism” (serial number 60/848237 filed on September 29<sup>th</sup>, 2006) is currently pending.

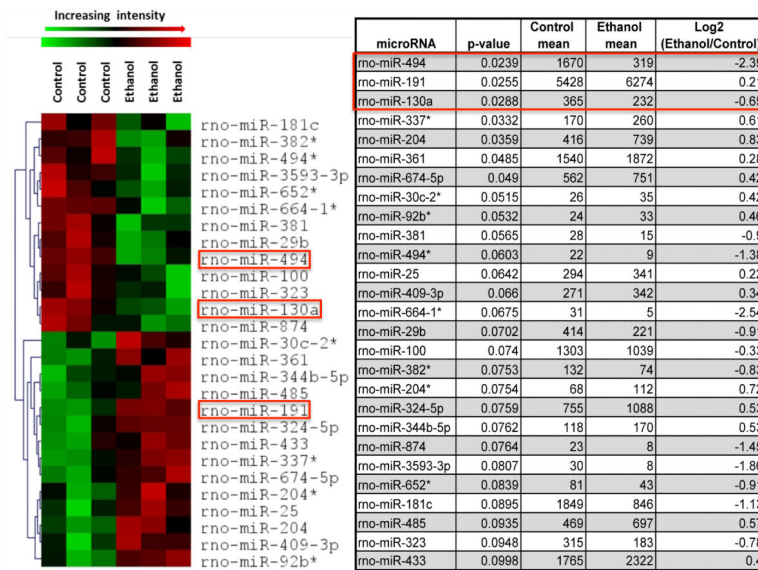
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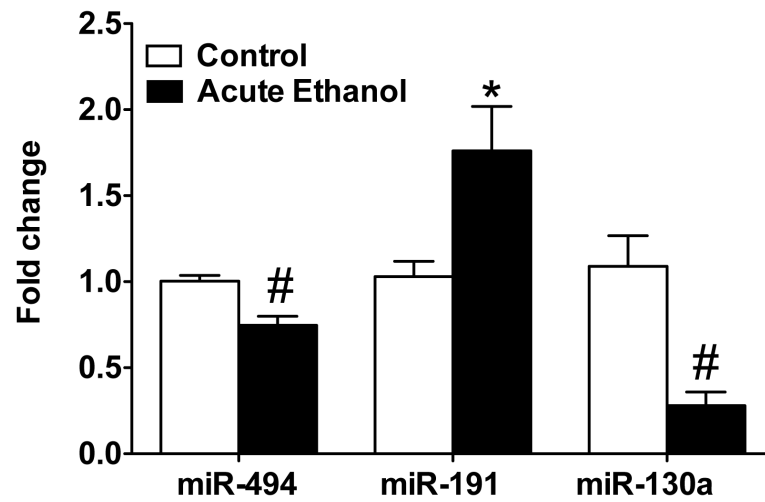
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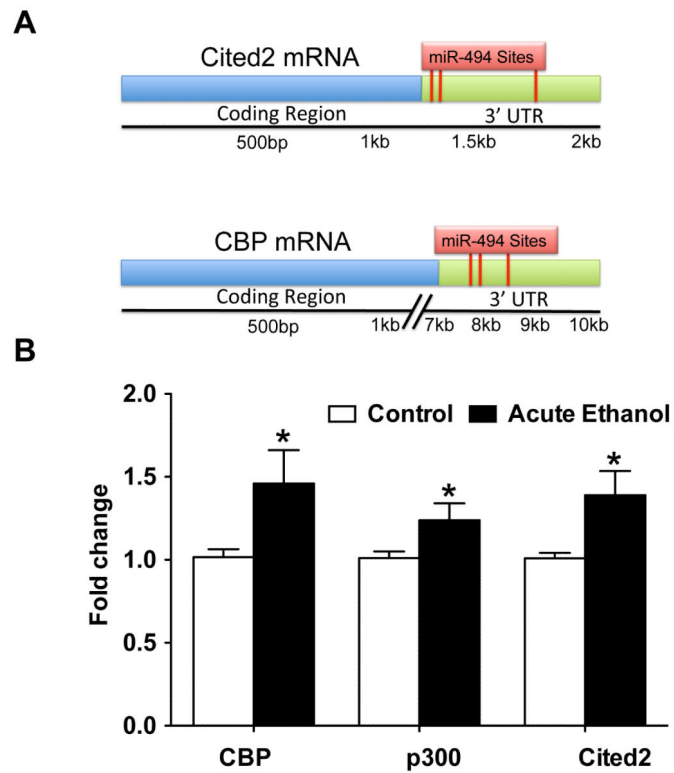
**Figure 1. Hierarchical clustering of differentially expressed miRNAs in the amygdala obtained from normal saline (control) and acute ethanol treated rats**  
Individual miRNAs from miRNA profiling data are represented in rows while individual rats are represented in columns (n=3). The heat map contains seven miRNAs with significantly altered expression ( $p < 0.05$ ; raw p values) and twenty additional miRNAs differentially expressed with a significance of  $p < 0.1$ . The color red represents increase and color green represents decrease in miRNA expression (rno= Rattus Norvegicus; miRNA with asterisk derived from opposite arm of the precursor). The mean miRNA value in control and ethanol groups and associated raw p values derived from t test analysis has been shown in the adjoining table.



**Figure 2. Validation of expression of selected miRNAs (miR-494, miR-191 and miR-130a) as identified via miRNA microarray**

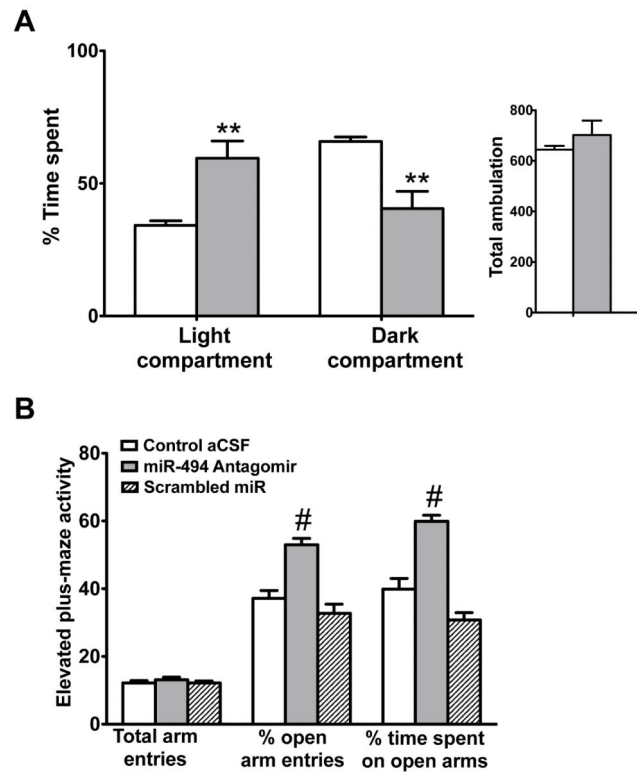
Expression of miRNAs significantly affected by acute ethanol exposure as measured by qPCR. Data represents the mean  $\pm$  SEM of seven to eleven rats per group. Significantly different than saline treated control rats (\* $p < 0.05$ ; # $p < 0.001$ ; Student's t test).





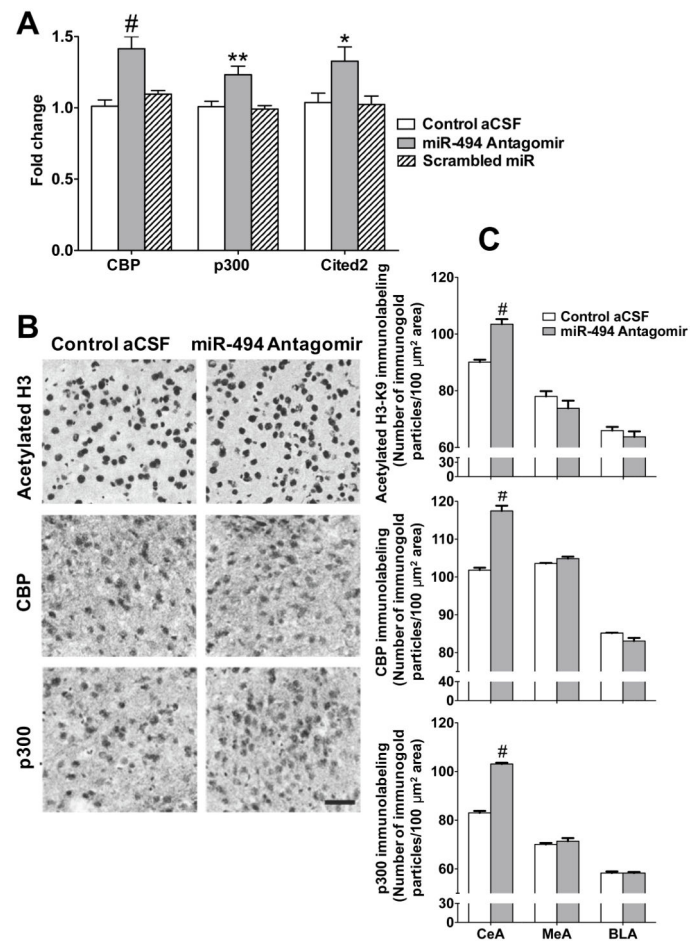
**Figure 3. Chromatin remodeling genes are up-regulated by acute ethanol exposure**

Map of CBP and Cited2 mRNA showing the coding region and the 3' UTR (untranslated region). The predicted binding site of miR-494 is in the 3' UTR for both mRNAs (A). Expression of *CBP*, *p300* and *Cited2* were significantly increased in the amygdala following acute ethanol exposure as measured by qPCR (B). Data represents the mean  $\pm$  SEM of eight to ten rats per group. Significantly different than normal saline treated control rats (\* $p < 0.05$ ; Student's t test).



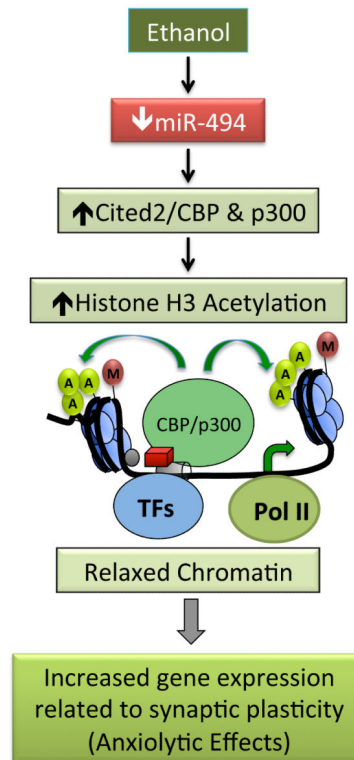
**Figure 4. Inhibition of miR-494 in the central nucleus of amygdala (CeA) elicits an anxiolytic response**

CeA infusion of miR-494 antagomir produces anxiolytic-like effects as demonstrated in the light/dark box exploration test (A) and on open arm activity in the elevated plus maze test (B). Data represents the mean  $\pm$  SEM from six to nine rats per group. Significantly different than control rats infused with aCSF [\*\*\* $p < 0.01$ ; Student's  $t$  test; # $p < 0.001$ , one-way ANOVA (% time spent on open arms,  $F_{2, 19} = 40.6$ ,  $p < 0.001$ ; % open arm entries  $F_{2, 19} = 23.8$ ,  $p < 0.001$ ) followed by Tukey's test].



**Figure 5A.**

Expression of *CBP*, *p300* and *Cited2* were significantly upregulated in the amygdala following miR-494 antagomir CeA infusion. Data represents the mean  $\pm$  SEM of six to nine rats per group. Significantly different than control rats infused with aCSF [ $*p < 0.05$ ,  $**p < 0.01$ ,  $\#p < 0.001$ , one-way ANOVA (CBP,  $F_{2, 20} = 12.9$ ,  $p < 0.001$ ; p300,  $F_{2, 21} = 8.3$ ,  $p < 0.01$ ; Cited2,  $F_{2, 21} = 4.5$ ,  $p < 0.05$  followed by Tukey's test]. **B.** Representative low magnification (scale bar, 40  $\mu$ m) photomicrographs of CBP, p300 and acetylated histone H3-K9 gold immunolabeling protein in the central nucleus of amygdala in aCSF and miR-494 antagomir infused rats. **C.** Effect of CeA miR-494 antagomir infusion on protein levels of CBP, p300 and acetylated histone H3-K9 in the CeA, MeA and BLA. Data represents the mean  $\pm$  SEM from four to six rats per group. Significantly different than control rats infused with aCSF ( $\#p < 0.001$ ; Student's *t* test).



**Figure 6. The potential molecular pathways by which ethanol exposure may regulate downstream epigenetic modifier proteins and chromatin remodeling via inhibition of miR-494 thereby inducing anxiolysis**

Acute ethanol administration down-regulates miR-494 while concurrently up-regulating expression of *Cited2*, CBP and p300 and also as published earlier increased histone H3-K9 acetylation (8,14). Interestingly, inhibition of miR-494 via infusion of an antagomir into the central nucleus of amygdala was able to mimic these effects of acute ethanol. Furthermore, these data suggest the possibility that miR-494 signaling may be involved in the regulation of synaptic plasticity associated genes (8,9) via chromatin remodeling leading to anxiolytic-like effects following ethanol exposure (TFs: transcription factors; Pol II: RNA polymerase II; CBP: CREB binding protein; Cited2: CBP/p300-interacting transactivator 2).