Disorders of the Nervous System

MicroRNA-137 Drives Epigenetic Reprogramming in the Adult Amygdala and Behavioral Changes after Adolescent Alcohol Exposure

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Abstract

Adolescent binge drinking is a serious public health concern and a risk factor for alcohol use disorder (AUD) and comorbid anxiety in adulthood. Chromatin remodeling mediated by epigenetic enzymes including lysine-specific demethylase 1 (LSD1) due to adolescent alcohol exposure may play a role in adult psychopathology. The mechanism by which adolescent alcohol exposure mechanistically regulates epigenetic reprogramming and behavioral changes in adulthood is unknown. We investigated the role of microRNA-137 (miR-137), which is crucial for normal neurodevelopment and targets LSD1, in adolescent intermittent ethanol (AIE) exposure-induced anxiety-like and alcohol-drinking behaviors and related epigenetic reprogramming in the amygdala in adulthood. Adolescent rats were exposed to 2 g/kg ethanol (2 d on/off; AIE) or adolescent intermittent saline (AIS) during postnatal days (PND)28–PND41 and allowed to grow to adulthood for analysis of behavior, miRNA expression, and epigenetic measures in the amygdala. Interestingly, miR-137 was increased and its target genes Lsd1 and Lsd1 + 8a were decreased in the AIE adult amygdala. Infusion of miR-137 antagonim directly into the central nucleus of the amygdala (CeA) rescues AIE-induced alcohol-drinking and anxiety-like behaviors via normalization of decreased Lsd1 expression, decreased LSD1 occupancy, and decreased Bdnf IV expression due to increased H3K9 dimethylation in AIE adult rats. Further, concomitant Lsd1 small interfering RNA (siRNA) infusion into the CeA prevents the miR-137-mediated reversal of AIE-induced adult anxiety and chromatin remodeling at the Bdnf IV promoter. These novel results highlight miR-137 as a potential therapeutic target for anxiety and AUD susceptibility after adolescent alcohol exposure in adulthood.

Key words: adolescent alcohol; amygdala; anxiety; BDNF; epigenetics; microRNA-137

Significance Statement

Adolescent alcohol exposure is a serious public health problem and contributes to alcohol use and anxiety disorders later in life. In this study, we identify microRNA-137 (miR-137), a small non-coding RNA, in the central nucleus of the amygdala (CeA) as a crucial regulator of increased alcohol consumption and anxiety-like behavior in adult rats after adolescent intermittent ethanol (AIE) exposure. Inhibition of miR-137 in the CeA reverses increased alcohol intake and anxiety-like behavior, and this effect is mediated by lysine-specific demethylase 1 (LSD1), a miR-137 target gene that regulates epigenetic programming. Thus, we have identified miR-137 and its target LSD1 in the CeA that play a mechanistic role in the pathogenesis of increased adult anxiety and alcohol consumption after adolescent alcohol exposure.
Introduction
Adolescence is an important developmental period for neuromaturation, as numerous biological changes including gene expression and synaptic remodeling in discrete brain regions inform typical adolescent behaviors like impulsivity and novelty-seeking (Keshavan et al., 2014; Spear, 2018). The long-term consequences of adolescent binge drinking also include increased risk of alcohol use disorder (AUD) and other psychiatric disorders including anxiety and depression (Swendsen et al., 1998; Chassin et al., 2002; Jennison, 2004). Physiologic changes in the developing brain are at least partly mediated by epigenetic mechanisms (Lister et al., 2013), which constitute changes in genomic architecture that alter gene expression without altering the underlying DNA sequence (Berger et al., 2009). Rodent models reveal that exposure to alcohol during the crucial developmental period of adolescence leads to increased anxiety-like behaviors, alcohol preference, and epigenetic changes in brain regions including the amygdala (Pascual et al., 2009; Pandey et al., 2015; Kyzar et al., 2016, 2017; Montesinos et al., 2016; Trantham-Davidson et al., 2017). The amygdala is a hub regulator of anxiety and negative affective states that plays a crucial role in maintaining and escalating addictive behaviors (Koob and Volkow, 2010; Pandey et al., 2017).

MicroRNAs (miRNAs) are small, non-coding RNAs that are ~22–23 nucleotides long and function to negatively regulate mRNA stability and translation (Bartel, 2009). miRNAs play critical roles in neurodevelopment and dendritic spine outgrowth (Miska et al., 2004; Schratt et al., 2006; Im and Kenny, 2012), and contribute to the pathogenesis of psychiatric disorders (Lewohl et al., 2011; Muiños-Gimeno et al., 2011; Issler and Chen, 2015; Warника et al., 2015). miRNAs interact with other epigenetic factors, such as histone modifications and DNA methylation, to tightly regulate gene expression in the brain in response to environmental stimuli (Kenny, 2014). Altered miRNA expression in specific brain circuits regulates alcohol-related behaviors (Tapocik et al., 2014; Darcq et al., 2015; Teppen et al., 2016), and miRNAs are potential biomarkers for the effects of developmental alcohol exposure (Balaraman et al., 2014).

Materials and Methods
AIE exposure and behavioral studies
All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago and complied with National Institutes of Health guidelines for animal use. Timed-pregnant Sprague Dawley (SD) rats or dams with pups were ordered from a vendor (Harlan Laboratories). Rats were kept on a 12/12 h light/dark cycle and provided chow and water ad libitum. Rats were weaned at postnatal day (PND)21 and were exposed to either AIE or adolescent intermittent saline (AIS; 0.9% NaCl) beginning at PND28. As described previously (Pandey et al., 2015; Kokare et al., 2017; Kyzar et al., 2017, 2019), male rats were randomly assigned to groups and exposed to either ethanol (2 g/kg, i.p.; AIE) or volume-matched saline (AIS) on a 2-d on/off schedule from PND28 to PND41, for a total of five injections. Rats were allowed to grow to adulthood (PND92–PND98) and amygdaloid tissues (predominantly the lateral, medial, and basolateral amygdala) were used for biochemical measures (RNA expression, and chromatin occupancy). A subset of both AIS and AIE male rats was exposed to an acute challenge of 2 g/kg ethanol (intraperitoneal) or volume-matched saline in adulthood (PND101–PND102) prior to sacriﬁce 1 h after injection and extraction of the amygdala for the measurement of miR-137 levels as described below.

While aspects of epigenetic regulation such as histone modifications and DNA methylation are altered in the adult amygdala following adolescent alcohol exposure (Pandey et al., 2015; Kyzar et al., 2017, 2019; Sakhrakar et al., 2019), the contribution of amygdala miRNAs to chromatin remodeling and the observed phenotype of increased anxiety and alcohol consumption in adulthood after adolescent alcohol exposure remains unknown. We recently reported that lysine-specific demethylase 1 (LSD1; also known as KDM1A) is decreased in the adult amygdala, but not in the bed nucleus of the stria terminalis (BNST), after adolescent alcohol exposure in male rats, and this is associated with increased repressive H3K9 dimethylation (H3K9me2) levels particularly at crucial synaptic plasticity related genes such as brain-derived neurotrophic factor (Bdnf) and unchanged levels of H3K4me2 (Kyzar et al., 2017). Interestingly, microRNA-137 (miR-137) is the only miRNA empirically shown to target LSD1 and is implicated in psychiatric illness including schizophrenia (Sun et al., 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Mahmoudi and Cairns, 2017). However, miR-137 expression and its effect on epigenetic and behavioral outcomes in adulthood after adolescent alcohol exposure is not currently known. Therefore, we examined the effects of adolescent intermittent ethanol (AIE) on miR-137 and target gene expression. The mechanistic role of miR-137 in the regulation of LSD1-mediated epigenetic effects in the amygdala and anxiety-like and alcohol-drinking behaviors in adulthood after AIE was also investigated.
Central nucleus of the amygdala (CeA) cannulation and infusion of miR-137 antagonist

Separate cohorts of AIS and AIE male rats were bilaterally cannulated directly targeting the CeA under anesthesia with inhaled isoflurane during adulthood (PND > 80). Rats were allowed at least one week to fully recover from surgery. Animals were placed in a stereotaxic apparatus and bilaterally implanted with CMA/11 guide cannulae (CMA Microdialysis) as previously published (Moonat et al., 2013). The coordinates for the CeA were 2.5 mm posterior to bregma, 4.2 mm lateral to the bregma, and 5.1 mm ventral from the point of cannula entry at the skull surface. Rats were singly-housed after surgery and monitored daily for recovery. Following recovery, rats were bilaterally infused in the CeA using a Hamilton syringe (Hamilton) to inject 400 pmol (in 0.5 µl) of an antisense locked nucleic acid (LNA) antagonist construct specific to miR-137 (Exiqon), scrambled LNA (Negative Control B; Exiqon), or vehicle (iFect solution; Neuromics). The sequence of the miR-137 antagonist construct is G+T+A* T*T+C+T+A*A+G+C+G+T*A*T, with asterisks representing phosphorothioated nucleotides. Rats were infused twice per day (9 A.M. and 5 P.M.) for 2 d, similar to our previous studies using antagonist technology (Teppen et al., 2016). Another batch of AIS- and AIE-exposed animals were exposed to both miR-137 antagonist and Lsd1 small interfering RNA (siRNA); or control siRNA, miR-137 antagonist was administered similarly with 400 pmol of antagonist given bilaterally twice per day for 2 d (9 A.M. and 5 P.M.), and Lsd1 siRNA (0.5 µg/0.5 µl per side) was concomitantly given once along with the 9 A.M. infusion of miR-137 antagonist on the second day of infusions. The sequence of Lsd1 siRNA is sense; 5’-CAACGCUCCUCAA UAAUAATT-3’, antisense; 5’-UUUUAUUAUGAGGACGUU GAA-3’ (QIAGEN), and the negative control siRNA used was obtained from QIAGEN (AllStars Negative Control siRNA). On the third day, animals were tested for behavior in the elevated plus maze (EPM) and their amygdala tissue was immediately collected for downstream biochemical processing. The timing of antagonist and siRNA infusion was chosen based on the effects of similar constructs on the behavior of adult rats in prior studies (Moonat et al., 2013; Teppen et al., 2016; Kyzar et al., 2019). A separate batch of rats was generated for infusion of miR-137 antagonist into the CeA to examine the effect on alcohol consumption measured using a two-bottle free-choice voluntary ethanol drinking paradigm, similar to our previous studies (Pandey et al., 2015). Following cannulation surgery on PND95–PND96, AIS and AIE adult rats were single-housed and after recovery received water in two bottles until no significant bottle preference was detected, which took two weeks. Subsequently, rats received water in one bottle and increasing concentrations (w/v) of ethanol (3% of ethanol for 3 d, 7% of ethanol for 3 d and 9% of ethanol for 9 d) in the other bottle. AIS and AIE rats were then bilaterally infused with miR-137 antagonist or vehicle directly into the CeA twice per day (9 A.M. and 5 P.M.) on the fourth and fifth day of 9% alcohol intake, and we continued to monitor their 9% ethanol and water intake. After miR-137 antagonist or vehicle treatment was terminated, rats continued to receive 9% ethanol in one bottle and water in the other bottle to determine whether the alcohol preference would return to pre-treatment levels. All rats received fresh bottles each day in the evening between 5 and 6 P.M., and ethanol and water intake was measured (ml/d) at this time. The position of the bottles was exchanged every day to avoid preference formation for bottle position.

Behavioral studies

For measurement of anxiety-like behavior, animals were tested in the EPM as described previously (Pandey et al., 2015; Kyzar et al., 2017, 2019). The batch of adult rats exposed only to miR-137 antagonist were tested 16 h after last miR-137 antagonist (or scrambled/vehicle) infusion at PND110–PND111. The batch of adult rats exposed to both miR-137 antagonist and Lsd1 siRNA were tested 16 h after last miR-137 antagonist (or scrambled) infusion and 24 h after last Lsd1 siRNA (or scrambled) infusion at PND118–PND119. The percentage of open arm entries and the percentage of time spent in the open arm represent anxiety-related endpoints, while the number of closed arm entries is a measure of general activity (File, 1993).

A cohort of AIS and AIE male adult rats were exposed to miR-137 antagonist or vehicle during a two-bottle free-choice voluntary ethanol drinking paradigm, similar to our previous studies (Pandey et al., 2015). Following cannulation surgery on PND95–PND96, AIS and AIE adult rats were single-housed and after recovery received water in two bottles until no significant bottle preference was detected, which took two weeks. Subsequently, rats received water in one bottle and increasing concentrations (w/v) of ethanol (3% of ethanol for 3 d, 7% of ethanol for 3 d and 9% of ethanol for 9 d) in the other bottle. AIS and AIE rats were then bilaterally infused with miR-137 antagonist or vehicle directly into the CeA twice per day (9 A.M. and 5 P.M.) on the fourth and fifth day of 9% alcohol intake, and we continued to monitor their 9% ethanol and water intake. After miR-137 antagonist or vehicle treatment was terminated, rats continued to receive 9% ethanol in one bottle and water in the other bottle to determine whether the alcohol preference would return to pre-treatment levels. All rats received fresh bottles each day in the evening between 5 and 6 P.M., and ethanol and water intake was measured (ml/d) at this time. The position of the bottles was exchanged every day to avoid preference formation for bottle position.

miRNA measurement by qPCR

TaqMan mature microRNA qPCR assays (Thermo Fisher Scientific) were purchased for miR-137 (for primer sequences, see Table 1), and qPCR was performed according to the assay protocol using TaqMan Universal PCR Master Mix (no UNG) as described previously (Teppen et al., 2016) using the Mx3000P qPCR system (Agilent Technologies) and MxPro software. U6 was used as a

| Primer name | Sequence |
|-------------|----------|
| TaqMan miRNA primers miR-137-3p | UUAUUGCUUAGAUAAGCGGUAG AGAAGATTCGATGGCCCTT |
| U6 miRNA primers | CGCCACGCGTCTAATCACTT GCCAAGAACCTGTGGCTTA |
| Lsd1 forward | GAGTAACCTGCGTCTGCAGT |
| Lsd1 reverse | GAGGAATCCCATGGCTGT |
| Lsd1 - 8a forward | GAACTTCAGACGTGAGCTT |
| Bdnf IV mRNA forward | TTCGCTAGGACTTGGAGATT |
| Bdnf IV mRNA reverse | TCCTGAGACCTTTTCGC |
| Hprt1 forward | TCATCACTAACTACAGGACGTTG |
| Hprt1 reverse | ChIP qPCR primers |
| Bdnf IV promoter forward | CCTGTCCTGAAATAAGAC |
| Bdnf IV promoter reverse | CGCCACGCGTCTAATCACTT |

Table 1. Primers sets used for miRNA analysis (using Taq-Man probe assays), qPCR analysis of mRNA transcripts, and ChIP assay using qPCR
reference gene for miRNA qPCR analysis. The Ct value of U6 was subtracted from the Ct value of target miRNA, and fold changes were calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001; Kyzar et al., 2017). Data are expressed as fold change of AIS control rats.

mRNA quantification by qPCR

Quantification of mRNA was conducted using reverse transcription PCR and primers specifically designed for rat mRNAs Lsd1, Lsd1 + 8a, and Bdnf exon IV (Table 1). RNA was isolated from amygdala tissue as described previously (Kyzar et al., 2017, 2019) and then reverse transcribed in duplicate using mixed random primers and MuLV reverse transcriptase (Life Technologies). Quantitative real-time PCR was performed using either the Mx3000P qPCR system (Agilent Technologies) and SYBR Green master mix (Fermentas) or a CFX Connect qPCR system with iQ SYBR SuperMix (Bio-Rad). Data were analyzed using the 2−ΔΔCT method (Livak and Schmittgen, 2001). Hprt1 was used as a reference gene (Kyzar et al., 2017, 2019). Data are represented as fold change compared to control AIS groups.

Chromatin immunoprecipitation (ChiP) assay

The ChIP assay was used to measure occupancy of H3K4me2, H3K9me2, and LSD1 proteins (Kyzar et al., 2017, 2019; Bohnsack et al., 2019). Amygdala tissue was fixed in methanol-free formaldehyde, and chromatin was then sheared to 200- to 500-bp fragments by sonication with the Covaris ME220 (Covaris). The chromatin complex was immunoprecipitated with specific antibodies to H3K9me2 (Abcam ab1220, RRID: AB_449854, 3 μg/sample), LSD1 (Abcam ab17721, RRID: AB_443964, 3 μg/sample), and H3K4me2 (Abcam ab32356, RRID: AB_732924, 3 μg/sample). The precipitated DNA was quantified by qPCR using a CFX Connect qPCR system with iQ SYBR SuperMix (Bio-Rad) and primers designed to specific genomic regions (Table 1). Rabbit IgG (Millipore; catalog #NI01; 3 μg/sample) was used as a negative control antibody but did not amplify any genomic region tested in qPCR. Input DNA Ct value was subtracted from the Ct value of each respective sample. The 2−ΔΔCT method (Livak and Schmittgen, 2001) was used to determine the fold change compared to control AIS groups.

Experimental design and statistical analysis

Two group experiments involving AIS- and AIE-exposed rats were analyzed using an independent sample two-tailed (Student’s ) t test. Experiments involving animals exposed to AIS or AIE in adolescence followed by an acute ethanol or saline challenge in adulthood were analyzed with two-way ANOVA. This was followed by post hoc comparison using Tukey’s test. For miR-137 antagonist experiments involving five groups, one-way ANOVA was employed to test for differences between groups followed by a post hoc Tukey’s test. For miR-137 antagonist experiments in the two-bottle free choice alcohol-drinking paradigm, two-way repeated measures ANOVA was performed. All experiments were performed in biological replicates indicated by the “n” value indicated in each figure legend. At least two technical replicates were averaged for each biochemical measure. We did not perform power analysis to predetermine group size and instead based sample sizes on previous experiments performed using similar protocols and effect sizes in the lab. Data are presented as mean ± SEM. Significance for all experiments was set at p < 0.05. Exact p values are reported up to p < 0.001.

Results

AIE exposure leads to increased miR-137 and decreased Lsd1 expression in the amygdala

We examined effects of AIE exposure (Fig. 1A) on RNA levels of miR-137 in the adult amygdala. It was found that AIE exposure increased miR-137 expression (t(10) = -3.07, p = 0.013 by unpaired t test) in the amygdala of AIE adult rats (PND94; Fig. 1B). miR-137 is a brain-enriched miRNA (Landgraf et al., 2007) that is crucial for neuronal development. Gene ontology (GO) analysis of predicted miR-137 target genes, performed using the Enrichr software (Kuleshov et al., 2016), reveals five of the top ten over-represented pathways involved in transcriptional regulation and contain the epigenetic enzyme Lsd1 (Fig. 1C, D). Extended Data Fig. 1-1), and bioinformatics analysis reveals a well-conserved miR-137 binding site in the 3′UTR of Lsd1 (Fig. 1C). We observed decreased expression of Lsd1 (t(9g) = 4.91, p < 0.001 by unpaired t test) and Lsd1 + 8a (t(9) = 2.49, p = 0.034 by unpaired t test) mRNA in the amygdala of AIE adult rats compared to AIS adult rats (Fig. 1E). We then investigated the binding of Lsd1 to the promoter of Bdnf exon IV and found decreased Lsd1 binding to the Bdnf IV promoter (t(9g) = 7.31, p < 0.001 unpaired t test) in the AIE adult amygdala compared to AIS rats (Fig. 1F). Notably, we have previously shown that BDNF protein and Bdnf IV mRNA levels are decreased in the amygdala of adult AIE rats compared to adult AIS rats (Pandey et al., 2015), suggesting that decreased Lsd1 may be involved in the regulation of BDNF expression after AIE in adulthood.

miR-137 inhibition in the CeA rescues AIE-induced anxiety-like behavior

We next explored if increased expression of miR-137 in the CeA is responsible for AIE-induced anxiety-like behaviors by examining the effects of direct inhibition of miR-137 in the CeA on anxiety-like behaviors in AIE adult rats (Fig. 2A). Interestingly, infusion of miR-137-specific antagonist, but not scrambled antagonist, directly in the CeA significantly attenuated AIE-induced anxiety-like behaviors (Fig. 2B), as one-way ANOVA revealed a group level effect for percentage of open arm entries (F(4,28) = 25.8, p < 0.001) and time spent in the open arms (F(4,28) = 27.7, p < 0.001) but no change in the number of closed arm entries. Post hoc analysis showed that AIE animals exposed to either vehicle (AIE+ vehicle) or control antagonist (AIE+ scrambled) showed significantly decreased percentage of open arm entries and time spent in the open arms compared to AIE adult rats infused with vehicle (p < 0.001 by Tukey’s post hoc test). AIE rats infused with miR-137 antagonist (AIE+ miR-137 antagonist) show
significantly increased percentage in entries and time spent in the open arms compared to AIE vehicle and AIE scrambled groups (p < 0.001 by Tukey’s post hoc test) but not compared to AIS vehicle groups. AIS rats infused with the miR-137 antagonim (AIS+ miR-137 antagonist) do not show any significant differences from AIS vehicle rats in anxiety measures. These results suggest that infusion of miR-137 antagonim into the
Figure 2. miR-137 antagomir infusion directly into the CeA reverses AIE-induced anxiety-like behaviors and target mRNA expression. 

A, Schematic of AIE or adolescent intermittent saline (AIS) exposure followed by cannulation surgery and infusion of an antagomir specific to miR-137, scrambled antagomir, or vehicle directly into the CeA of adult male rats. 

B, Bar diagram showing anxiety-like behaviors (measured in the EPM test) in AIS and AIE adult rats infused with miR-137 antagomir, scrambled antagomir, or vehicle (iFect solution) directly into the CeA (n = 6–7, ***p < 0.001 vs AIS+ vehicle, ###p < 0.001 vs AIE+ vehicle, $$$p < 0.001 vs AIE+ scrambled by one-way ANOVA followed by Tukey’s post hoc test).

C, qPCR analysis of the miR-137 target genes Lsd1 and Lsd1 + 8a mRNA in the amygdala of AIS and AIE adult rats infused with miR-137 antagomir, scrambled antagomir, or vehicle directly into the CeA (n = 6–7, p < 0.05 vs AIS+ vehicle, p < 0.01 vs AIS+ vehicle, p < 0.01 vs AIE+ vehicle, $p < 0.05 vs AIE+ scrambled by one-way ANOVA followed by Tukey’s post hoc test).

D, ChIP analysis of LSD1 occupancy of the Bdnf exon IV promoter in the amygdala of AIS and AIE adult rats infused with miR-137 antagomir, scrambled antagomir, or vehicle directly into the CeA (n = 6–7, p < 0.05 vs AIS+ vehicle, p < 0.01 vs AIS+ vehicle, #p < 0.01 vs AIE+ vehicle, $p < 0.05 vs AIE+ scrambled by one-way ANOVA followed by Tukey’s post hoc test).

E, ChIP analysis of H3K9me2 and H3K4me2 occupancy of the Bdnf exon IV promoter region in the amygdala of AIS and AIE adult rats infused with miR-137 antagomir, scrambled antagomir, or vehicle directly into the CeA (n = 6–7, p < 0.05 vs AIS+ vehicle by one-way ANOVA followed by Tukey’s post hoc test).
continued

H3K4me2 occupancy of the Bdnf exon IV promoter region in the amygdala of AIS and AIE adult rats infused with miR-137 antagonist, scrambled antagonist, or vehicle into the CeA (n = 6–7, *p < 0.05 vs AIS+ vehicle, **p < 0.01 vs AIE+ vehicle, $p < 0.05$ vs AIE+ scrambled by one-way ANOVA followed by Tukey’s post hoc test). F, qPCR analysis of Bdnf exon IV (Bdnf IV) mRNA in the amygdala of AIS- and AIE-exposed adult rats infused with miR-137 antagonist, scrambled antagonist, or vehicle (iFect solution) into the CeA (n = 6–7, *p < 0.05 vs AIS+ vehicle by one-way ANOVA followed by Tukey’s post hoc test). Data expressed as mean ± SEM.

CeA was able to attenuate anxiety-like behaviors in AIE adult rats.

miR-137 inhibition in the CeA rescues AIE-induced epigenetic changes

We next measured mRNA expression of miR-137 target genes in the amygdala of adult rats after AIS or AIE exposure and infusion of miR-137 antagonist directly in the CeA. One-way ANOVA revealed a group level effect on Lsd1 mRNA ($F_{(4,27)} = 6.16$, $p = 0.001$) and Lsd1 + 8a mRNA ($F_{(4,27)} = 7.00$, $p < 0.001$). Lsd1 mRNA is decreased in the amygdala in AIE+ vehicle ($p = 0.021$ by Tukey’s post hoc test) and AIE+ scrambled ($p = 0.040$ by Tukey’s post hoc test) adult rats compared to AIS+ vehicle control rats (Fig. 2C). After miR-137 antagonist infusion in AIE rats, Lsd1 mRNA expression is increased compared to AIE+ vehicle ($p = 0.005$ by Tukey’s post hoc test) and AIE+ scrambled ($p = 0.011$ by Tukey’s post hoc test) rats but is not different from AIS+ vehicle control rats. Similarly, Lsd1 + 8a mRNA is decreased in the amygdala in AIE+ vehicle ($p = 0.001$ by Tukey’s post hoc test) and AIE+ scrambled ($p = 0.002$ by Tukey’s post hoc test) adult rats compared to AIS+ vehicle control rats (Fig. 2C). Lsd1 + 8a mRNA levels are not significantly different from AIS+ vehicle levels in the amygdala of AIE rats infused with miR-137 antagonist.

LSD1 is an important epigenetic regulator that can remove methyl groups from both H3K4me1/2 and H3K9me1/2, depending on the genomic context and the binding of various nuclear cofactors (Shi et al., 2004; Metzger et al., 2005). Additionally, the neuron-specific splice variant LSD1 + 8a preferentially acts to demethyl- ate H3K9me1/2 (Laurent et al., 2015). We determined whether the transcriptional downregulation of Lsd1/ Lsd1 + 8a and subsequent rescue by inhibition of miR-137 (Fig. 2C) was accompanied by alterations in chromatin dynamics at the Bdnf exon IV (Bdnf IV) promoter, mRNA levels of which are decreased in the AIE adult amygdala (Pandey et al., 2015; Kyzar et al., 2017, 2019). Occupancy of Lsd1 ($F_{(4,27)} = 5.30$, $p = 0.003$ by one-way ANOVA) and H3K9me2 ($F_{(4,27)} = 6.17$, $p = 0.001$ by one-way ANOVA), but not H3K4me2, were altered in AIE adult rats infused with miR-137 antagonist in the CeA in adulthood (Fig. 2D,E). Post hoc analysis revealed a significant deficit in Lsd1 binding to the Bdnf IV promoter region in adult AIE rats infused with vehicle ($p = 0.022$ by Tukey’s post hoc test) or scrambled antagonist ($p = 0.007$ by Tukey’s post hoc test) compared to AIE+ vehicle control rats, but no significant difference between the AIE+ vehicle and AIE+ miR-137 antagonist adult rats (Fig. 2D). H3K9me2 occupancy was increased in the amygdala of both the AIE+ vehicle ($p = 0.023$ by Tukey’s post hoc test) and AIE+ scrambled ($p = 0.046$ by Tukey’s post hoc test) adult rats compared to AIE+ vehicle control rats (Fig. 2E).

AIE+ miR-137 antagonist rats showed levels of H3K9me2 occupancy at Bdnf IV that were not different from AIS+ vehicle control rats and were significantly lower compared to both AIE+ vehicle ($p = 0.007$ by Tukey’s post hoc test) and AIE+ scrambled ($p = 0.015$ by Tukey’s post hoc test) adult rats.

We next determined whether the effect of altered LSD1 and H3K9me2 occupancy on Bdnf IV mRNA expression. Bdnf IV mRNA levels were significantly altered between groups ($F_{(4,27)} = 7.47$, $p < 0.001$ by one-way ANOVA) in AIS or AIE adult rats infused with either vehicle, scrambled antagonist, or miR-137 antagonist in the CeA. Bdnf IV mRNA is decreased in the amygdala in AIE+ vehicle ($p = 0.016$ by Tukey’s post hoc test) and AIE+ scrambled ($p = 0.026$ by Tukey’s post hoc test) adult rats compared to AIS+ vehicle control rats (Fig. 2F), but Bdnf IV mRNA levels are not significantly different between AIS+ vehicle and AIE+ miR-137 antagonist rats. These results suggest that infusion of miR-137 antagonist into the CeA was able to correct the deficits in Bdnf IV mRNA expression due to normalization of decreased LSD1 expression and the associated increase in H3K9me2 occupancy at the Bdnf IV promoter in AIE adult rats.

Increased miR-137 expression in the CeA regulates anxiety-like behaviors and epigenetic changes after AIE in adulthood via LSD1

miR-137 has numerous verified target genes other than LSD1 (Mahmoudi and Cairns, 2017), and we therefore investigated the direct role of LSD1 in the miR-137 antagonist-mediated reversal of anxiety phenotype and epigenetic changes induced by AIE in adulthood. For this purpose, we cannulated AIS and AIE adult rats targeting the CeA and allowed them to recover. Subsequently, miR-137 antagonist infusion in the CeA was performed as shown in Figure 3A except Lsd1 siRNA was co-infused once with the miR-137 antagonist on the third infusion (of four total antagonist infusions), 24 h before behavioral testing in the EPM and subsequent amygdala dissection for biochemical measures (Fig. 3A). This experiment allowed us to determine whether Lsd1 mRNA rescue was crucial to the reversal of AIE-induced behavioral and epigenetic changes.

Concurrent inhibition of Lsd1 by siRNA in the CeA was sufficient to prevent miR-137 antagonist-mediated rescue of the anxiety-like behaviors seen in the EPM in AIE adult rats (Fig. 3B), as one-way ANOVA revealed a group level effect for percentage of open arm entries ($F_{(4,27)} = 32.8$, $p < 0.001$), time spent in the open arms ($F_{(4,27)} = 15.7$, $p < 0.001$), and number of closed arm entries ($F_{(4,27)} = 3.77$, $p = 0.015$). Post hoc analysis showed that AIE rats infused with control siRNA and scrambled antagonist controls (AIE+ scrambled antagonist/control siRNA) show de-
Figure 3. Lsd1 siRNA infusion into the CeA prevents reversal of AIE-induced behavioral and epigenetic changes by miR-137 antagonim. A, Schematic of AIE or adolescent intermittent saline (AIS) exposure followed by cannulation surgery and infusion of miR-137 antagonim or scrambled antagonim along with Lsd1 siRNA or control siRNA into the CeA during adulthood. B, Anxiety-like behavioral analysis in the EPM test in AIS and AIE adult rats infused with miR-137 antagonim or scrambled antagonim along with Lsd1 siRNA or control siRNA directly into the CeA (n = 6–7, *p < 0.05, ***p < 0.001 vs AIS+ scrambled antagonim/control siRNA, #p < 0.01, ###p < 0.001 vs AIS+ scrambled antagonim/control siRNA, $p < 0.05, $$$p < 0.001 vs AIS+ miR-137 antagonim/control siRNA by one-way ANOVA followed by Tukey’s post hoc test). Data expressed as mean ± SEM. C, qPCR analysis of Lsd1 and Lsd1 + 8a mRNA in the amygdala of AIS and AIE rats infused with miR-137 antagonim or scrambled antagonim along with Lsd1 siRNA or scrambled siRNA directly into the CeA (n = 6–7, ***p < 0.001 vs AIS+ scrambled antagonim/control siRNA, ###p < 0.001 vs AIE+ scrambled antagonim/control siRNA, $$$p < 0.001 vs AIE+ miR-137 antagonim/control siRNA by one-way ANOVA followed by Tukey’s post hoc test).
increased time spent in the open arms and decreased percentage of entries into the open arms compared to AIS infused with scrambled antagonir and control siRNA rats ($p < 0.001$ by Tukey’s post hoc test). Decreased time spent in the open arms and decreased percentage of entries into the open arms, indicative of anxiety-like behavior, was also observed in AIE rats but was not modulated by Lsd1 siRNA and scrambled antagonir infusion into CeA (AIE+ Lsd1 siRNA/scrambled; $p = 0.005$ by Tukey’s post hoc test) and percentage of entries into the open arms ($p < 0.001$ by Tukey’s post hoc test) compared to AIE+ scrambled antagonir/control siRNA rats, indicating a rescue of anxiety-like behavior as shown previously in Figure 2B.

Interestingly, infusion with Lsd1 siRNA together with miR-137 antagonir in AIE rats (AIE+ miR-137 antagonir/Lsd1 siRNA) was able to block the miR-137 antagonir-mediated rescue of anxiety-like behaviors as indicated by decreased time spent in the open arms ($p = 0.011$ by Tukey’s post hoc test) and decreased percentage of entries into the open arms ($p < 0.001$ by Tukey’s post hoc test) compared to AIE+ miR-137 antagonir/control siRNA rats. AIE+ miR-137 antagonir/Lsd1 siRNA rats also displayed increased closed arm entries compared to AIS control rats ($p = 0.047$ by Tukey’s post hoc test).

We found that co-infusion of Lsd1 siRNA prevented the miR-137 antagonir-related normalization of epigenetic alterations in the AIE adult amygdala. One-way ANOVA revealed a group level effect on miR-137 target genes Lsd1 mRNA ($F_{(4,27)} = 20.1$, $p < 0.001$) and Lsd1 + 8a mRNA ($F_{(4,27)} = 17.2$, $p < 0.001$). Lsd1 and Lsd1 + 8a show decreased mRNA expression in AIE+ scrambled antagonir/control siRNA and AIE+ Lsd1 siRNA/scrambled antagonir rats compared to AIS controls, and this decrease returns to baseline-like levels in AIE+ miR-137 antagonir/control siRNA rats ($p < 0.001$ by Tukey’s post hoc test). However, AIE+ miR-137 antagonir/Lsd1 siRNA rats continue to display significantly decreased Lsd1 and Lsd1 + 8a mRNA levels compared to both AIS+ antagonir scrambled/control siRNA and AIE+ miR-137 antagonir/control siRNA rats ($p < 0.001$ by Tukey’s post hoc test). We observed a similar pattern for Lsd1 occupancy by ChIP at the Bdnf IV promoter ($F_{(4,27)} = 20.8$, $p < 0.001$ by one-way ANOVA; Fig. 3D), where AIE+ scrambled antagonir/control siRNA and AIE+ scrambled antagonir/Lsd1 siRNA show decreased Lsd1 occupancy that is rescued in the AIE+ miR-137 antagonir/control siRNA rats which is prevented in AIE+ miR-137 antagonir/Lsd1 siRNA-infused rats ($p < 0.001$ by Tukey’s post hoc test).

We additionally observed altered H3K9me2 occupancy at the Bdnf IV promoter ($F_{(4,27)} = 22.1$, $p < 0.001$ by one-way ANOVA), with increased H3K9me2 occupancy in the amygdala of AIE+ scrambled antagonir/control siRNA and AIE+ scrambled antagonir/Lsd1 siRNA rats compared to AIS adult rats infused with scrambled antagonir/control siRNA ($p < 0.001$ by Tukey’s post hoc test; Fig. 3D). H3K9me2 occupancy at Bdnf IV in the amygdala returns to control-like levels after miR-137 antagonir infusion into the CeA, but the normalization is prevented by co-infusion of Lsd1 siRNA ($p < 0.001$ by Tukey’s post hoc test). Lastly, one-way ANOVA revealed a group level effect on Bdnf IV mRNA ($F_{(4,27)} = 19.4$, $p < 0.001$; Fig. 3E).

We observed that Bdnf IV mRNA expression is decreased in the AIE+ scrambled antagonir/control siRNA and AIE+ scrambled antagonir/Lsd1 siRNA groups, returns to control-like levels in the AIE+ miR-137 antagonir/control siRNA, and decreased compared to both the AIE+ miR-137 antagonir/control siRNA and AIS control rats in animals co-infused with Lsd1 siRNA and miR-137 antagonir ($p < 0.001$ by Tukey’s post hoc test). These results suggest that targeting of Lsd1 mRNA by miR-137 and subsequent chromatin remodeling in the CeA is crucial in AIE-induced anxiety-like behaviors in adulthood.

miR-137 inhibition in the CeA rescues AIE-induced increases in voluntary ethanol consumption

We next determined whether inhibition of miR-137 could also rescue increased alcohol consumption seen in AIE adult rats (Pandey et al., 2015). AIS and AIE adult rats were cannulated targeting the CeA and, after one week of recovery, habituated to drinking 9% ethanol (w/v). AIS and AIE rats were infused with either vehicle or miR-137 antagonir twice per day for 2 d (days 10–11) while their ethanol consumption was monitored (Fig. 4A). We observed significant effects of group ($F_{(3,495)} = 52.1$, $p < 0.001$ by two-way repeated measures ANOVA), day ($F_{(15,495)} = 20.4$, $p < 0.001$), and the interaction between group and day ($F_{(45,495)} = 5.06$, $p < 0.001$) on ethanol consumption (g/kg/d; Fig. 4B). We also found significant effects of group ($F_{(3,495)} = 26.1$, $p < 0.001$ by two-way repeated measures ANOVA), day ($F_{(15,495)} = 13.4$, $p < 0.001$), and the interaction between group and day ($F_{(45,495)} = 4.31$, $p < 0.001$) on ethanol preference index (Fig. 4C), and of group ($F_{(3,495)} = 3.83$, $p = 0.019$ by two-way repeated measures ANOVA), day ($F_{(15,495)} = 14.6$, $p < 0.001$), and the interaction between group and day ($F_{(45,495)} = 2.17$, $p < 0.001$) for total volume consumed (Fig. 4D). Post hoc analysis shows that AIE+ vehicle infused rats consume significantly more ethanol than AIS+ vehicle controls (g/kg/d) on days 1 ($p = 0.040$).
by Tukey’s post hoc test and 3–16 (p = 0.004-p < 0.001, Tukey’s post hoc test; Fig. 3B). AIE rats infused with miR-137 antagonist (AIE+ miR-137 antagonist) consume significantly more ethanol than AIE+ vehicle control rats on days 3–9 before miR-137 antagonist infusion, as well as on day 10 (p = 0.032- p < 0.001, Tukey’s post hoc test). However, AIE+ miR-137 antagonist infusion significantly decreased ethanol intake on the second day of miR-137 antagonist infusion (day 11; p < 0.001, Tukey’s post hoc test) and on days 12–14 after cessation of the infusions (p = 0.001- p < 0.001, Tukey’s post hoc test) as compared to AIE+ vehicle infused rats. AIE+ miR-137 antagonist infused rats resume drinking significantly more ethanol than AIE+ vehicle control rats 4 d following the cessation of last miR-137 antagonist infusion (p < 0.001 by Tukey’s post hoc test).
When evaluated for ethanol preference, the results are similar to total ethanol intake (Fig. 4C), with AIE+ vehicle rats showing increased ethanol preference compared to AIS+ vehicle controls (p < 0.001 by Tukey’s post hoc test) for the duration of the study. AIE+ miR-137 antagonir rats show increased ethanol preference compared to AIS+ vehicle rats on days 1–10 (p < 0.001 by Tukey’s post hoc test) before the miR-137 antagonist infusions are complete, and these rats then show decreased ethanol preference compared to AIS+ vehicle rats on days 11–13 (p < 0.001 by Tukey’s post hoc test). AIE+ miR-137 antagonist rats regain increased ethanol preference compared to AIS+ vehicle rats on days 15–16 (p < 0.001 by Tukey’s post hoc test), 4 d following the cessation of last miR-137 antagonist infusion. Post hoc differences in total volume consumed (Fig. 4D) during the 9% ethanol period of the study showed only decreased consumption in the AIS+ miR-137 antagonist group compared to the AIS+ vehicle rats on days 11 (p = 0.027 by Tukey’s post hoc test) and 13 (p = 0.040 by Tukey’s post hoc test). Importantly, the AIS+ miR-137 antagonist group showed no alterations in ethanol preference or ethanol consumption. These results suggest that AIE increases alcohol intake and is attenuated by the inhibition of miR-137 in the CeA of rats, suggesting that AIE-induced increases in miR-137 expression in the amygdala may drive increases in ethanol consumption in adulthood.

Effects of acute ethanol challenge on the AIE-induced increase in RNA levels of miR-137 in the adult amygdala

A previous study showed that the increased anxiety-like behavior seen in AIE adult rats, the decrease in Lsd1 + 8a mRNA in the AIE adult amygdala, and the increased H3K9me2 at the Bdnf exon IV promoter regions all return to control-like levels following an acute challenge with ethanol (2 g/kg) in adulthood (Kyzar et al., 2017; Fig. 5A). We extended these studies and examined if AIE rats may consume higher amounts of ethanol to inhibit miR-137 levels in the amygdala. We found that miR-137 expression in the amygdala (F(1,20) = 5.82, p = 0.026 by two-way ANOVA: AIE treatment x adult acute ethanol interaction) is increased in AIE+ saline rats (p = 0.026 by Tukey’s post hoc test) and returns to control-like levels in the amygdala of AIE+ EtOH rats (p = 0.033 by Tukey’s post hoc test; Fig. 5B). These results suggest that ethanol exposure in AIE rats, but not in AIS rats, was able to inhibit the increased RNA levels of miR-137 in the amygdala and further supports the hypothesis that an increase in miR-137 levels in the amygdala is involved in AIE-induced alcohol-drinking behaviors in adulthood.

Discussion

Our study shows that miR-137 is increased and its target Lsd1 is decreased in the amygdala leading to epigenetic alterations contributing to the risk of anxiety-like and alcohol-drinking behaviors after AIE in adulthood. Inhibition of miR-137 in the CeA reversed AIE-associated anxiety and alcohol consumption phenotypes, as well as the altered LSD1-mediated chromatin remodeling at the Bdnf IV promoter. Concurrent inhibition of Lsd1 mRNA in the CeA prevents the miR-137 antagonist-mediated rescue of AIE-associated behavioral and epigenetic changes. To our knowledge, this is the first investigation of miR-137 in the pathophysiology of alcohol-related behaviors, as well as the first demonstration of reversal of adolescent alcohol-related phenotypes in adulthood by targeting miR-137 in the CeA and mechanistically linking this to LSD1-mediated epigenetic reprogramming. Together, these data suggest that increased miR-137-mediated epigenetic reprogramming via decreased expression of LSD1 in the amygdala is involved in anxiety-like and alcohol-drinking behaviors after AIE in adulthood (Fig. 6).
The increased miR-137 seen in the AIE adult amygdala alters chromatin dynamics at the Bdnf IV promoter via downregulation of LSD1. We have previously shown that LSD1 protein levels are decreased in the CeA of AIE-exposed adult rats, and this decrease is associated with increased global levels of H3K9me2 protein but unchanged H3K4me2 protein levels (Kyzar et al., 2017). The current study shows that LSD1 protein occupancy is decreased and H3K9me2 is increased at the Bdnf IV promoter region, which is associated with decreased Bdnf IV mRNA expression in the amygdala of AIE adult rats. Interestingly, BDNF expression is decreased specifically in human postmortem amygdala of patients with AUD who began drinking before the age of 21, and levels of an antisense BDNF transcript (BDNF-as) are inversely correlated with BDNF expression (Bohsnack et al., 2019). Additionally, adult AIE rats show decreased lysine demethylase 6B (KDM6B) expression and altered KDM6B-mediated histone modifications at the activity-regulated cytoskeleton-associated (Arc) enhancer region in the amygdala, leading to decreased Arc enhancer RNA and mRNA expression (Kyzar et al., 2019). AIE appears to alter specific histone-modifying enzymes, such as LSD1 and KDM6B, that act on critical regulators of dendritic outgrowth and synaptic plasticity including BDNF and Arc. These important epigenetic regulators alter gene expression via multiple modalities including non-coding RNAs such as miR-137 and by the alteration of histone methylation and acetylation in the amygdala after AIE in adulthood.

We have reported decreased dendritic spine density and synaptophysin immunolabeling in the CeA and MeA following AIE exposure (Pandey et al., 2015; Kyzar et al., 2019), and miR-137 is a negative regulator of dendritic outgrowth and spine development by targeting the E3 ubiquitin-protein ligase Mib1 (Smrt et al., 2010). miR-137 overexpression leads to deficits in synaptic transmission including decreased spontaneous neurotransmitter release accompanied by decreased synaptogenesis in mouse hippocampal neurons (He et al., 2018). Germline deletion of miR-137 results in embryonic lethality, but conditional haploinsufficiency of miR-137 in the nervous system leads to altered synaptic plasticity and behavior (Cheng et al., 2018). miR-137 is known to gradually increase across development (Hollins et al., 2014), and our results here suggest that adolescent alcohol exposure leads to an aberrant upregulation of miR-137 in adulthood that regulates epigenetic processes and adult psychopathology.

Previous studies have shown that the lasting behavioral effects seen after AIE are both strain-dependent and dependent on the method of alcohol exposure (Pascual et al., 2009; Gilpin et al., 2012; Alaux-Cantin et al., 2013; Ehlers et al., 2013; Varlinskaya et al., 2014; Pandey et al., 2015; Kyzar et al., 2017). Future studies should also probe

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**Figure 6.** Conceptual model of the molecular and behavioral effects of AIE on miR-137 and LSD1-mediated chromatin remodeling in the adult amygdala. Adolescent intermittent exposure (AIE) leads to increased expression of miR-137, thereby decreasing expression of LSD1 and its neuron specific variant (LSD1 + 8a) in the adult rat amygdala. Increased expression of miR-137 is associated with decreased levels of LSD1 and increased levels of H3K9me2 at the Bdnf exon IV promoter, leading to decreased Bdnf IV mRNA expression and BDNF protein levels (Pandey et al., 2015). Blockade of the upregulation of miR-137 via antagomir in the CeA rescues these epigenetic modifications and deficits in Bdnf expression. miR-137 antagonist infusion in the CeA also attenuate the phenotypes of heightened anxiety and higher alcohol intake induced by AIE in adult rats. Concomitant inhibition of Lsd1 by siRNA in the CeA blocks the miR-137 antagonist-mediated rescue of AIE-induced behavioral phenotypes and altered chromatin at the Bdnf exon IV promoter. These results identify miR-137 as an important epigenetic regulator in the CeA that mechanistically regulates AIE-induced anxiety-like and alcohol-drinking behaviors.
different rat strains and exposure paradigms to determine the risk of specific genetic variants of miR-137 and its target genes. Interestingly, the deficits in BDNF and Arc in the adult amygdala in the AIE model (Pandey et al., 2015; Kyzar et al., 2019) used here are similar to molecular findings in human postmortem amygdala of AUD patients who started drinking before the age of 21 when compared with control subjects (Bohsnack et al., 2019). Downstream biochemical measures focused on the target gene LSD1 due to its known downregulation in the AIE adult amygdala (Kyzar et al., 2017) and also its enrichment in critical biological processes as determined by GO. It is important to point out that we cannot rule out the possible contributions of other verified miR-137 targets (Mahmoudi and Cairns, 2017) in the pathogenesis of AIE-induced anxiety and alcohol consumption, and future studies should investigate the role of these genes and validate additional targets. However, epigenetic effectors such as LSD1, as well as other proteins identified in our previous studies including KDM6B and HDAC2 (Pandey et al., 2015; Kyzar et al., 2019), are candidates for therapeutic intervention following early-life alcohol exposure due to the existence of pharmacologic agents targeting these proteins. Epigenetic proteins additionally bind to the genome and lead to ubiquitous downstream effects on transcription at many different loci, and as such they are likely involved in the altered expression of genes seen after adolescent alcohol exposure (Pandey et al., 2015, 2017; Kyzar et al., 2016, 2019). Finally, other miRNAs are likely involved in AIE-induced adult pathology, and these should be investigated in detail in future studies.

Another interesting finding of present study is that inhibition of miR-137 in the CeA of AIE adult rats was able to attenuate alcohol-drinking behaviors in a two-bottle choice drinking experiment. One hypothesis as to why miR-137 inhibition may be effective in decreasing drinking behaviors is that AIE rats drink higher amounts of ethanol to suppress miR-137 levels in the amygdala, thereby normalizing the AIE-induced deficits in LSD1 and subsequent epigenetic changes. We tested this possibility and found that acute ethanol challenge suppressed the AIE-induced increase in miR-137 levels in the amygdala, while the same dose of ethanol did not alter miR-137 levels in AIS rats. Earlier, we reported that acute ethanol challenge was also able to normalize Lsd1 +/−a mRNA levels and decrease repressive H3K9me2 occupancy of Bdnf exon IV in the amygdala of AIE, but not AIS, adult rats (Kyzar et al., 2017). Furthermore, acute ethanol challenge in AIE adult rats also attenuates anxiety-like behaviors (Kyzar et al., 2017, 2019). These data suggest that increased consumption of alcohol by AIE adult rats may act to normalize miR-137 levels, Lsd1 +/−a mRNA levels, H3K9me2 levels at Bdnf exon IV, and anxiety-like behaviors.

In summary, these results suggest that adolescent alcohol exposure causes enduring effects on miR-137 leading to downstream effects on chromatin remodeling through LSD1, heightened anxiety-like behavior, and higher alcohol consumption (Fig. 6). miR-137 antagonom infused directly into the CeA normalizes the aberrant AIE-induced behavioral and epigenetic effects, and this normalization is prevented by co-infusion of Lsd1 siRNA. Our results highlight miR-137 and its target gene LSD1 in the amygdala as a potential therapeutic target for susceptibility to anxiety and AUDs in adulthood after adolescent ethanol exposure.

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References

Alaux-Cantin S, Warnault V, Legasteois R, Rotia B, Pierreiche O, Vilpoux C, Naassilla M (2013) Alcohol intoxications during adolescence increase motivation for alcohol in adult rats and induce neuroadaptations in the nucleus accumbens. Neuropsychopharmacology 67:521–531.
Balararaman S, Lunde ER, Sawant O, Cudd TA, Washburn SE, Miranda RC (2014) Maternal and neonatal plasma microRNA biomarkers for fetal alcohol exposure in an ovine model. Alcohol Clin Exp Res 38:1390–1400.
Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233.
Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A (2009) An operational definition of epigenetics. Genes Dev 23:781–783.
Bohsnack JP, Teppen T, Kyzar EJ, Dzitoyeva S, Pandey SC (2019) The IncRNA BDNF-AS is an epigenetic regulator in the human amygdala in early onset alcohol use disorders. Transl Psychiatry 9:34.
Chassin L, Pitts SC, Prost J (2002) Binge drinking trajectories from adolescence to emerging adulthood in a high-risk sample: predictors and substance abuse outcomes. J Consult Clin Psychol 70:67–78.
Cheng Y, Wang ZM, Tan W, Wang X, Li Y, Bai B, Li Y, Zhang SF, Yan HL, Chen ZL, Liu CM, Mi TW, Xia S, Zhou Z, Liu A, Tang GB, Liu C, Dai ZJ, Wang YY, Wang H, et al. (2018) Partial loss of psychiatric risk gene Mir137 in mice causes repetitive behavior and impairs sociability and learning via increased Pde10a. Nat Neuroscience 21:1689–1703.
Darceq E, Warnault V, Phamluong K, Besserer GM, Liu F, Ron D (2015) MicroRNA-30a-5p in the prefrontal cortex controls the transition from moderate to excessive alcohol consumption. Mol Psychiatry 20:1261.
Ehlers CL, Liu W, Wills DN, Crews FT (2013) Periadolescent ethanol vapor exposure persistently reduces measures of hippocampal neurogenesis that are associated with behavioral outcomes in adulthood. Neuroscience 244:1–15.
File SE (1993) The interplay of learning and anxiety in the elevated plus-maze. Behav Brain Res 58:199–202.
Glipin NW, Karanikas CA, Richardson HN (2012) Adolescent binge drinking leads to changes in alcohol drinking, anxiety, and amygdalar corticotropin releasing factor cells in adulthood in male rats. PLoS One 7:e31466.
He E, Lozano MAG, Stringer S, Watanabe K, Sakamoto K, den Oudsten F, Koopmans F, Giambardino SN, Hammenschlag A, Comelisse LN, Li KW, van Weering J, Posthuma D, Smit AB, Sullivan PF, Verhage M (2018) MIR137 schizophrenia-associated locus controls synaptic function by regulating synaptogenesis, synapse maturation and synaptic transmission. Hum Mol Genet 27:1879–1891.
Hollins SL, Goldie BJ, Carroll AP, Mason EA, Walker FR, Eyles DW, Cairns MJ (2014) Ontogeny of small RNA in the regulation of mammalian brain development. BMC Genomics 15:777.
Im HI, Kenny PJ (2012) MicroRNAs in neuronal function and dysfunction. Trends Neurosci 35:325–334.
Lasleser Q, Chen A (2015) Determining the role of microRNAs in psychiatric disorders. Nat Rev Neurosci 16:201–212.
Jennison KM (2004) The short-term effects and unintended long-term consequences of binge drinking in college: a 10-year follow-up study. Am J Drug Alcohol Abuse 30:659–684.
Kenny PJ (2014) Epigenetics, microRNA, and addiction. Dialogues Clin Neurosci 16:335–344.
Keshavan MS, Giedd J, Lau JY, Lewis DA, Paus T (2014) Changes in the adolescent brain and the pathophysiology of psychotic disorders. Lancet Psychiatry 1:549–558.

Kokare DM, Kyzar EJ, Zhang H, Sakharkar AJ, Pandey SC (2017) Adolescent alcohol exposure-induced changes in alpha-melanocyte stimulating hormone and neuropeptide Y pathways via histone acetylation in the brain during adulthood. Int J Neuropsychopharmacol 20:758–768.

Koob GF, Volkow ND (2010) Neurocircuits of addiction. Neuropsychopharmacology 35:217–238.

Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, Koplev S, Jenkins SL, Jagodnik KM, Lachmann A, McDermott MG, Monteiro CD, Gundersen GW, Ma’ayan A (2016) Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res 44:W90–W97.

Kyzar EJ, Zhang H, Pandey SC (2019) Adolescent alcohol exposure epigenetically suppresses amygdala arc enhancer RNA expression to confer adult anxiety susceptibility. Biol Psychiatry 85:904–914.

Kyzar EJ, Florenai C, Teppen TL, Pandey SC (2016) Adolescent alcohol exposure: burden of epigenetic reprogramming, synaptic remodeling, and adult psychopathology. Front Neurosci 10:222.

Kyzar EJ, Zhang H, Sakharkar AJ, Pandey SC (2017) Adolescent alcohol exposure alters lysine demethylase 1 (LSD1) expression and histone methylation in the amygdala during adulthood. Addict Biol 22:1191–1204.

Landgraf P, Rusu M, Sheridan R, Sewer A, Aravin A, Pfeffer R, Gratacòs M, Metspalu A, Hovatta I, Estivill X (2011) Human alcoholism. Biol Psychiatry 73:763–773.

Kokare DM, Kyzar EJ, Zhang H, Sakharkar AJ, Pandey SC (2017) Involvement of TLR4 in the long-term epigenetic changes, and synaptic plasticity in the amygdala predisposes to anxiety and alcoholism. Biol Psychiatry 73:763–773.

Laurent B, Ruitu L, Murn J, Hempel K, Ferrao R, Xiang Y, Liu S, Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res 44:W90–W97.

Lister R, Mukamel EA, Nery JR, Urich M, Puddifoot CA, Johnson ND, Lewohl JM, Nunez YO, Dodd PR, Tiwari GR, Harris RA, Mayfield RD (2014) Global epigenomic reconfiguration during mammalian brain development. Science 341:1237905.

Lucero J, Huang Y, Dwork AJ, Schultz MD, Yu M, Tonti-Filippini J, Alarcón MTX and LSD1 in neural stem cells. NatCommun 2:529.

Mahmoudi E, Cairns MJ (2014) MiR-137: an important player in the adolescent brain and the pathophysiology of psychotic disorders. Biol Psychiatry 69:526–533.

Pandey SC, Zhang H, Roy A, Misra K (2006) Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. J Neurochem 96:8320–8331.

Pandey SC, Sakharkar AJ, Tang L, Zhang H (2015) Potential role of adolescent alcohol exposure-induced amygdaloid histone modifications in anxiety and alcohol intake during adulthood. Neurobiol Dis 82:607–619.

Pandey SC, Kyzar EJ, Zhang H (2017) Epigenetic basis of the dark side of alcohol addiction. Neuropharmacology 122:74–84.

Pascual M, Boix J, Felipo V, Guerri C (2009) Repeated alcohol administration during adolescence causes changes in the melanocortinergic and glutamatergic systems and promotes alcohol intake in the adult rat. J Neurochem 108:920–931.

Sakharkar AJ, Kyzar EJ, Gavin DP, Zhang H, Chen Y, Krishnan HR, Grayson DR, Pandey SC (2019) Altered amygdala DNA methylation mechanisms after adolescent alcohol exposure contribute to adult anxiety and alcohol drinking. Neuropharmacology 157:107679.

Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014) Biological insights from 108 schizophrenia-associated genetic loci. Nature 511:421–427.

Schatt RM, Teding F, Nigh EA, Kane GC, Sabatini ME, Kiebler M, Greenberg ME (2006) A brain-specific microRNA regulates dendritic spine development. Nature 439:283–289.

Shi Y, Lan F, Matson C, Mulligan P, Whetsell JW, Cole PA, Casero RA, Shi Y (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953.

Smrt RD, Szulwach KE, Pfeiffer RL, Li X, Guo W, Pathania M, Teng ZQ, Luo Y, Peng J, Bordey A, Jin P, Zhao X (2010) MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. Stem Cells 28:1060–1070.

Spear LP (2018) Effects of adolescent alcohol consumption on the brain and behaviour. Nat Rev Neurosci 19:197–214.

Sun G, Ye P, Murali K, Lang MF, Li S, Zhang H, Li W, Fu C, Yin J, Wang A, Ma X, Shi Y (2011) miR-137 forms a regulatory loop with nuclear receptor TLX and LSD1 in neural stem cells. Nat Commun 2:529.

Swendsen JD, Merikangas KR, Canino GJ, Kessler RC, Rubio-Stipec M, Angst J (1998) The comorbidity of alcoholism with anxiety and depressive disorders in four geographic communities. Compr Psychiatry 39:176–184.

Tапопик Дж, Барбери Е, Фланиган М, Соломон М, Пинкус А, Пиллинг А, Сон Х, Шаэнк ЖР, Кинг С, Хейлг М (2014) microRNA-206 в рад медиальных префронтальных корковых регуляторов BDNF экспрессии и алкогольной зависимости. J Neurosci 34:4581–4588.

Teppen TL, Krishnan HR, Zhang H, Sakharkar AJ, Pandey SC (2016) The potential role of amygdaloid microRNA-494 in alcohol-induced anxiolysis. Biol Psychiatry 80:711–719.

Trantham-Davidson H, Centanni SW, Garr SC, New NN, Mulolland PJ, Gass JT, Glover EJ, Floresco SB, Crews FT, Krishnan HR, Pandey SC, Chandler LJ (2015) Binge-like alcohol exposure during adolescence disrupts dopaminergic neurotransmission in the adult prelimbic cortex. Neuropsychopharmacology 42:1024–1036.

Varlinskaya EI, Truxell E, Spear LP (2014) Chronic intermittent ethanol exposure during adolescence: effects on social behavior and ethanol sensitivity in adulthood. Alcohol 48:433–444.

Wannica W, Merico D, Costain G, Alfred SE, Wei J, Marshall CR, Scherer SW, Bassett AS (2015) Copy number variable microRNAs in schizophrenia and their neurodevelopmental gene targets. Biol Psychiatry 77:158–166.

Zhang H, Sakharkar AJ, Shi G, Ugale R, Prakash A, Pandey SC (2010) Neuropeptide Y signaling in the central nucleus of the amygdala regulates alcohol-drinking and anxiety-like behaviors of alcohol-prefering rats. Alcohol Clin Exp Res 34:451–461.