Biological Psychiatry: Celebrating 50 Years

Archival Report

# Adolescent Alcohol Exposure Epigenetically Suppresses Amygdala *Arc* Enhancer RNA Expression to Confer Adult Anxiety Susceptibility

Evan J. Kyzar, Huaibo Zhang, and Subhash C. Pandey

## ABSTRACT

**BACKGROUND:** Adolescent intermittent ethanol (AIE) exposure is an emerging risk factor for adult psychopathology, such as anxiety disorders. Enhancer RNAs (eRNAs) are short noncoding RNAs transcribed from enhancer regions that regulate synaptic plasticity–associated gene expression, including *Arc*, but their role in AIE-induced susceptibility to anxiety in adulthood is unknown.

**METHODS:** Rats were exposed to AIE (ethanol exposure 2 days on/off) or intermittent normal saline during postnatal days 28 to 41 and allowed to grow to adulthood for analysis of behavior and biochemical measures. Some AIE rats and rats with intermittent normal saline exposure were exposed to an acute challenge with ethanol in adulthood. Cohorts of alcohol-naïve adult rats were cannulated in the central nucleus of amygdala and infused with either *Kdm6b* small interfering RNA or an antisense locked nucleic acid oligonucleotide specific to *Arc* eRNA before behavioral and biochemical analysis.

**RESULTS:** AIE adult rats displayed heightened anxiety and decreased *Arc* eRNA expression, which is regulated epigenetically through decreased *Kdm6b* expression. This triggered condensed chromatin at the synaptic activity response element site and promoter of the *Arc* gene, facilitating increased negative elongation factor binding to the *Arc* promoter and decreasing *Arc* expression in the amygdala. Knockdown of *Kdm6b* or *Arc* eRNA expression in the central nucleus of amygdala provoked anxiety in alcohol-naïve adult rats and recapitulated the molecular and epigenetic phenotypes of AIE.

**CONCLUSIONS:** These data suggest that eRNA regulation via epigenetic reprogramming in the amygdala, particularly at the *Arc* synaptic activity response element site, contributes to adult anxiety after adolescent alcohol exposure.

***Keywords:*** Adolescent alcohol, Amygdala, Anxiety, *Arc*, Enhancer RNA, epigenetic

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The adolescent brain undergoes structural and molecular changes during development (1–3). Both clinical and preclinical findings indicate that binge drinking during adolescence increases risk for alcohol use disorder (AUD) and affective disorders in adulthood (4–8). Patients with a diagnosis of AUD are more than twice as likely to have an anxiety disorder (7,9), and preclinical models reveal that exposure to ethanol during adolescence increases anxiety-like behaviors and alcohol preference in adulthood (10–12). These behavioral changes are associated with neurochemical alterations, including increased neuroimmune activation, decreased neurotrophin signaling, and altered epigenetic mechanisms (1,13,14).

Epigenetics refers to modifications to histone proteins and DNA itself that alter gene expression without altering the underlying DNA sequence (15). Additionally, noncoding RNAs can directly and indirectly regulate gene expression and serve as epigenetic modifiers (16). Adolescent intermittent ethanol (AIE) exposure causes long-lasting epigenetic alterations in the amygdala into adulthood specifically at the promoter regions of the synaptic plasticity–associated genes *Bdnf* and *Arc*, possibly explaining the decreased dendritic spine density and increased anxiety and alcohol intake seen in these animals, as this brain region is crucial for affective regulation (10,11). Interestingly, *Arc* transcription is tightly regulated by the synaptic activity response element (SARE) site located approximately 7-kb upstream of the transcription start site (17); this site also regulates an RNA product known as *Arc* enhancer RNA (eRNA) (18,19). eRNAs are a recently discovered class of noncoding RNAs bidirectionally transcribed from active enhancer regions, and some of these RNAs have functional roles in the regulation of target gene expression (19,20). *Arc* eRNA functions to decoy the negative elongation factor (NELF) protein away from the promoter of *Arc*, allowing for poised RNA polymerase II to begin elongating and transcribing *Arc* messenger RNA (mRNA) (18,21). The epigenetic regulation of *Arc* eRNA in the amygdala and its role in AIE-induced anxiety-like behavior are currently unknown.

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Several epigenetic marks at histone 3 lysine 27 (H3K27) and H3K4 are critical for regulating enhancer sequences and eRNA expression (19,22,23). Acetylated H3K27 (H3K27ac) is rapidly recruited to enhancers of immediate-early genes, including *Arc* in response to neuronal activity, leading to eRNA synthesis that precedes mRNA transcription (18,24). Additionally, H3K27 trimethylation (H3K27me3) is a repressive mark present at bivalent promoters (25) and poised enhancers (26). Polycomb repressive complex 2 adds methyl groups to H3K27, whereas lysine demethylase 6A (KDM6A) and KDM6B are responsible for H3K27me2/3 demethylation (27,28). KDM6B complexes with members of the cyclic adenosine monophosphate response element binding protein (CREB) pathway such as CREB binding protein (CBP) to activate expression of activity-regulated genes in response to neuronal depolarization (29,30). We recently observed that AIE exposure decreases CBP via deficits in H3K9/14 acetylation in the amygdala (31). We hypothesized that KDM6B and CBP may epigenetically regulate amygdala eRNA expression to drive changes in gene expression and anxiety susceptibility after AIE in adulthood. We therefore explored in an animal model whether AIE produces an enduring impact on amygdaloid histone modifications at the *Arc* SARE enhancer site and promoter region, thus altering higher-order chromatin interactions leading to aberrant *Arc* expression, synaptic remodeling, and anxiety susceptibility.

## METHODS AND MATERIALS

Male Sprague-Dawley adolescent rats (postnatal days 28–41) were exposed to ethanol (2 g/kg intraperitoneal, 2 days on/off; AIE rats) or intermittent normal saline (adolescent intermittent saline [AIS] rats) and allowed to mature into adulthood (10,11). A cohort of AIE and AIS adult rats was also challenged with an acute dose of ethanol (2 g/kg) or normal saline. All rats were used for anxiety measurement and biochemical analysis in the amygdala. In mechanistic experiments, alcohol-naïve adult rats were cannulated targeting the central nucleus of amygdala (CeA) and infused with either *Kdm6b* small interfering RNA (siRNA) or an antisense locked nucleic acid (LNA) oligonucleotide specific to *Arc* eRNA and subjected to anxiety measures followed by molecular analysis in the amygdala. Detailed methods, materials, and statistical analyses are provided in the Supplement.

## RESULTS

### AIE Exposure Decreases Synapse Number and Expression of *Arc* mRNA and eRNA in the Adult Amygdala

To measure changes in synapse number after AIE exposure (Figure 1A) in the adult amygdala, we quantified synaptophysin immunostaining, which labels presynaptic terminals (32), in the CeA, medial nucleus of amygdala (MeA), and basolateral amygdala (BLA) in adulthood after AIE exposure. AIE adult rats showed decreased synaptophysin immunoreactivity in the CeA ($p < .05$) and MeA ($p < .05$), but not the BLA, compared with AIS adult rats (Figure 1B, C; Supplemental Figure S1). To explore the transcriptional mechanisms underlying the synaptic alterations, we measured the expression of several genes involved in synaptic plasticity and maintenance (32), finding that AIE significantly ($p < .05$ to $p < .001$) decreased *Arc*, *Bdnf* exon IV, *Homer1*, *NeuroD1*, *NeuroD2*, *Nrgn*, *Syp*, and *Syt1* mRNA levels in the adult amygdala (Figure 1D). We generated a gene network with GeneMANIA (33) to prioritize highly interactive genes (Supplemental Figure S2A) for detailed epigenetic studies. *Arc* was the hub gene in our network of synaptic plasticity–associated genes downregulated in adulthood after AIE exposure (Supplemental Figure S2B, C). Studies have indicated that the *Arc* SARE site (Figure 1E) is highly conserved and involved in the fine-tuning of *Arc* mRNA transcription in response to environmental stimuli (17,24,34). We found that levels of *Arc* plus (+) eRNA and *Arc* minus (−) eRNA strands were significantly ($p < .05$ to $p < .01$) decreased in the adult amygdala after AIE exposure compared with AIS adult rats (Figure 1F).

We next investigated the epigenetic regulation of *Arc* transcription in the adult amygdala after AIE exposure. We found increased H3K27me3 occupancy at the *Arc* SARE site and five other sites across the *Arc* promoter and gene body in the amygdala of AIE adult rats compared with AIS rats (Supplemental Figure S3A, B). H3K27me3 occupancy was not altered between the groups at the most distal site in intron 2 of the *Arc* gene body (Supplemental Figure S3B). We found that mRNA levels of *Kdm6b*, but not *Kdm6a* or other members of polycomb repressive complex 2, were decreased in the adult amygdala after AIE exposure compared with AIS rats (Supplemental Figure S3C).

### Acute Alcohol in Adulthood Reverses AIE-Induced Anxiety-like Behavior and *Arc* eRNA and mRNA Transcriptional Changes in the Amygdala

Previous work indicates that acute ethanol exposure (2 g/kg) in adulthood in AIE rats decreases anxiety-like behavior in the elevated plus maze (10). We extended this finding using the same ethanol exposure paradigm (Figure 2A) but a different anxiety measurement, the light/dark box (LDB). We found that AIE rats spent more time than AIS rats in the dark compartment and less time in the light compartment ($p < .001$), indicative of an anxiety-like phenotype, which was attenuated by acute ethanol challenge in adulthood (Figure 2B). Acute ethanol challenge (2 g/kg) also led to anxiolytic-like effects in AIS rats. We found a main effect of acute alcohol exposure on total ambulation in the LDB as a measure of general activity and saw a significant ($p < .05$) increase in AIE animals exposed to acute alcohol compared with AIE animals exposed to saline (Supplemental Figure S4A).

We then determined whether the transcriptional deficits of *Arc* eRNA and mRNA in the AIE adult amygdala returned to baseline after an acute ethanol challenge in adulthood. Post hoc analysis after two-way analysis of variance (ANOVA) (Figure 2C) revealed that both (−) and (+) strand transcripts of *Arc* eRNA ($p < .05$) and *Arc* mRNA ($p < .01$) levels were decreased in the adult amygdala of AIE rats and were increased by acute ethanol in both AIS rats ($p < .05$ to $p < .001$) and AIE rats ($p < .01$ to $p < .001$). Post hoc comparison after two-way ANOVA (Figure 2C) showed that *Kdm6b* mRNA was decreased ($p < .05$) in the amygdala of AIE rats compared

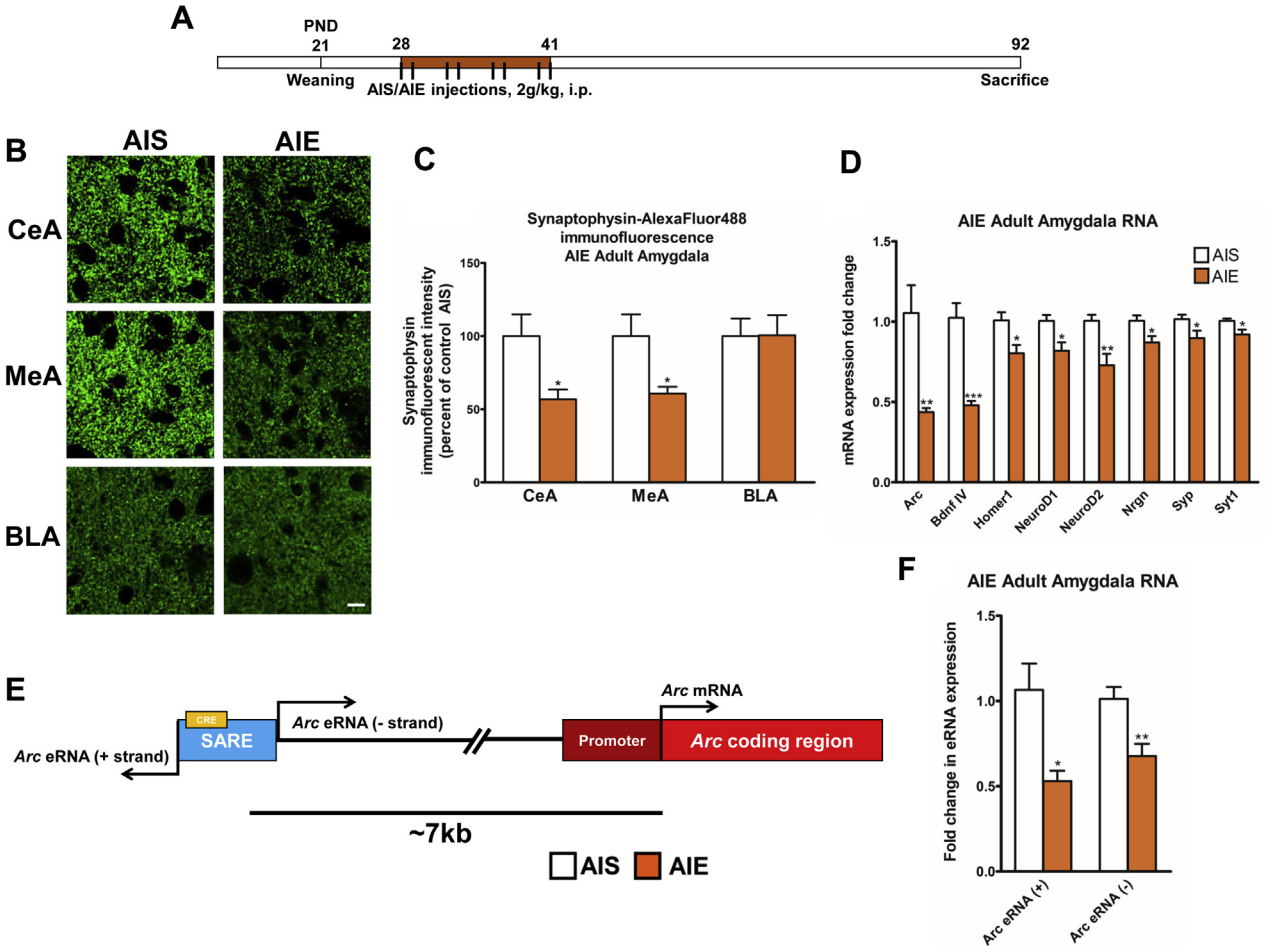


**Figure 1.** Adolescent intermittent ethanol (AIE) exposure leads to synaptic deficits and decreased *Arc* enhancer RNA (eRNA) and messenger RNA (mRNA) expression in the amygdala in adulthood. **(A)** Schematic of AIE and adolescent intermittent saline (AIS) treatment. **(B)** Representative photographs showing synaptophysin immunofluorescent staining visualized with confocal microscopy in the central nucleus of the amygdala (CeA), medial nucleus of the amygdala (MeA), and basolateral amygdala (BLA) of AIS and AIE animals (scale bar = 10 μm). **(C)** Bar diagram showing quantification of synaptophysin immunofluorescent staining. Data represent mean ± SEM derived from $n = 5$ rats in each group (CeA: $t_8 = 2.65$, $^*p < .05$, MeA: $t_8 = 2.51$, $^*p < .05$, by Student *t* test). **(D)** mRNA analysis of synaptic plasticity–associated genes in amygdala tissue obtained from AIS and AIE adult rats. Each gene represents an independent but not repeated measure. Data represent mean ± SEM derived from $n = 5$–8 rats in each group, (*Arc*: $t_9 = 3.85$, $^{**}p < .01$, *Bdnf* exon IV: $t_9 = 6.21$, $^{***}p < .001$, *Homer1*: $t_{11} = 2.79$, $^*p < .05$, *NeuroD1*: $t_{11} = 2.81$, $^*p < .05$, *NeuroD2*: $t_{11} = 3.27$, $^{**}p < .01$, *Nrgn*: $t_{11} = 2.58$, $^*p < .05$, *Syp*: $t_{14} = 2.18$, $^*p < .05$, *Syt1*: $t_{14} = 2.46$, $^*p < .05$, by Student *t* test). **(E)** Schematic representation of the *Arc* gene, including the synaptic activity response element (SARE) site, which contains a cyclic adenosine monophosphate-response element (CRE) for cyclic adenosine monophosphate response element binding protein binding and transcribes eRNA transcripts. **(F)** RNA analysis of *Arc* eRNA expression in the amygdala of AIS and AIE adult rats at baseline. Data represent mean ± SEM derived from $n = 5$–6 rats in each group (*Arc* eRNA plus (+) strand: $t_9 = 2.98$, $p < .05$), *Arc* eRNA minus (−) strand: $t_9 = 5.35$, $p < .01$, by Student *t* test). i.p., intraperitoneal; PND, postnatal day.

with AIS rats but returned to levels similar to AIS control rats after adult ethanol challenge in AIE rats (Figure 2C).

We next analyzed Arc protein and mRNA levels using gold immunolabeling and in situ polymerase chain reaction (PCR) histochemical procedures, respectively. Post hoc analysis after two-way ANOVA showed that in both the CeA and the MeA, AIE + saline rats showed decreased Arc mRNA and protein levels in the adult amygdala compared with AIS + saline rats ($p < .001$) (Figure 2D; Supplemental Figure S4B). Additionally, acute ethanol challenge in adulthood led to increased Arc mRNA and protein expression in both AIS + ethanol rats and AIE + ethanol rats compared with saline-exposed rats from both AIS and AIE groups ($p < .001$), respectively (Figure 2D; Supplemental Figure S4B). Our in situ PCR data confirmed the mRNA findings of decreased *Arc* mRNA in the AIE adult amygdala as measured by quantitative PCR (Figure 2C). We also performed gold immunolabeling to measure KDM6B protein levels in AIS and AIE animals exposed to adult acute ethanol challenge (Figure 2E). Post hoc analysis after two-way ANOVA indicated that KDM6B was decreased in AIE + saline rats in the CeA ($p < .001$) and MeA ($p < .01$) compared with AIS + saline rats and returned to levels similar to AIS control animals after acute ethanol exposure. KDM6B protein levels in the BLA did not significantly change between the groups (Figure 2E).

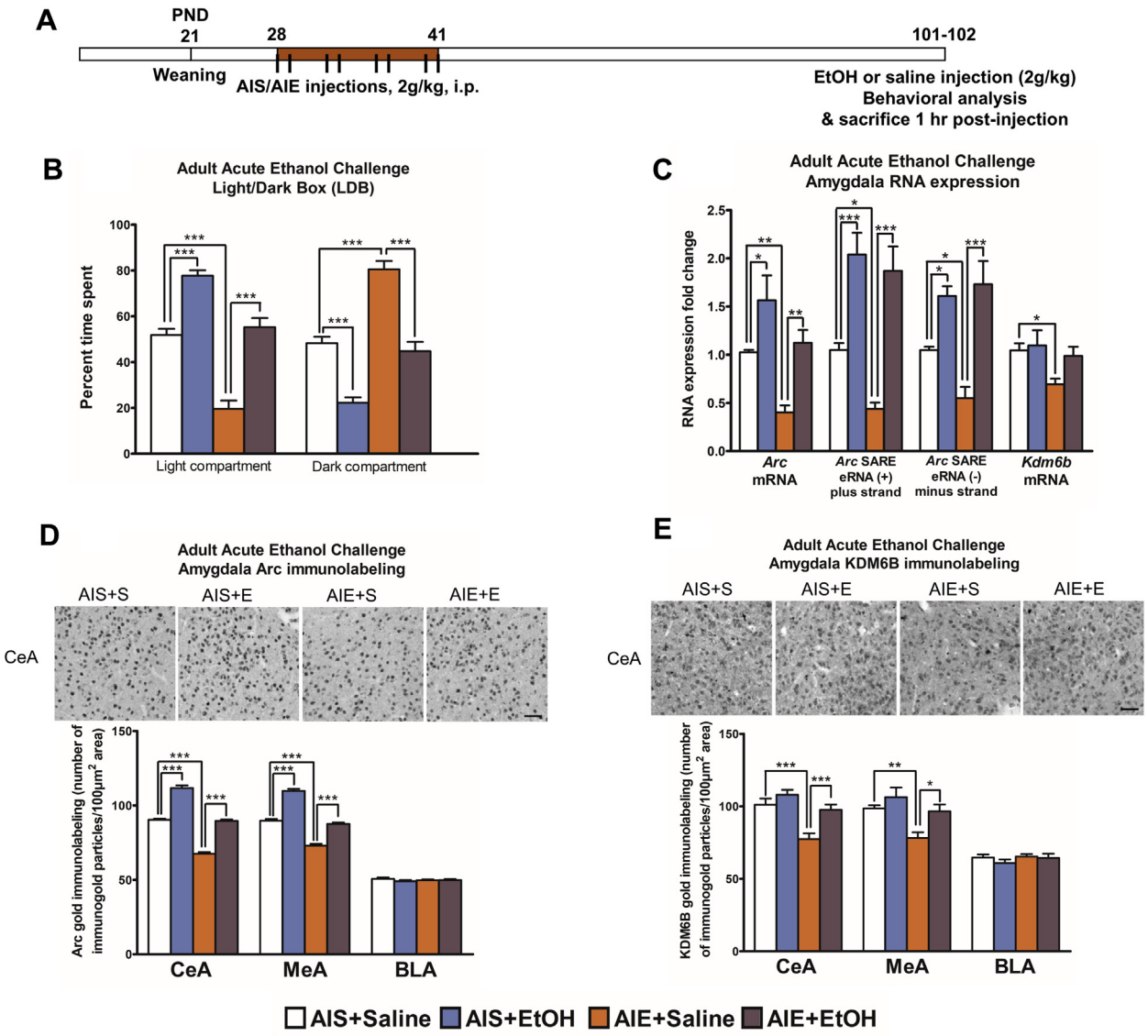


**Figure 2.** Adult ethanol (EtOH) challenge reverses adolescent intermittent ethanol (AIE)–induced anxiety-like behaviors and lysine demethylase 6B (KDM6B) and *Arc* expression deficits in the adult amygdala. **(A)** Schematic of AIE or adolescent intermittent saline (AIS) treatment followed by adult exposure to acute EtOH challenge (2 g/kg, intraperitoneal [i.p.] injection). **(B)** Light/dark box exploration test for anxiety measures in AIS and AIE adult rats exposed to an acute challenge of either ethanol or saline. Data represent mean ± SEM derived from $n$ = 9–10 rats in each group. ***$p < .001$ by two-way analysis of variance (ANOVA) (main effect of AIE treatment: $F_{1,33} = 67.3$, $p < .001$, main effect of acute ethanol exposure: $F_{1,33} = 85.4$, $p < .001$) followed by post hoc Tukey test. **(C)** RNA analysis of *Arc* enhancer RNA (eRNA), *Arc* messenger RNA (mRNA), and *Kdm6b* mRNA transcripts in the amygdala of rats exposed to either AIS or AIE followed by exposure to acute ethanol or saline in adulthood. Data represent mean ± SEM derived from $n$ = 5–6 rats in each group. *$p < .05$, **$p < .01$, ***$p < .001$ by two-way ANOVA (*Arc* mRNA levels, AIE treatment: $F_{1,20} = 12.6$, $p < .01$, acute alcohol exposure: $F_{1,20} = 17.8$, $p < .001$; *Arc* eRNA [+], AIE exposure: $F_{1,20} = 4.93$, $p < .05$, acute alcohol exposure: $F_{1,20} = 47.3$, $p < .001$; *Arc* eRNA [−], acute alcohol exposure: $F_{1,19} = 35.0$, $p < .001$, interaction between AIE treatment and acute alcohol exposure: $F_{1,19} = 4.44$, $p < .05$; *Kdm6b* mRNA levels, AIE treatment: $F_{1,20} = 4.90$, $p < .05$) followed by post hoc Tukey test. **(D)** Representative photomicrographs of gold immunolabeling showing activity-regulated cytoskeleton associated protein (ARC)-positive cells in the central nucleus of amygdala (CeA) and quantification of gold immunolabeling for ARC in the CeA, medial nucleus of amygdala (MeA), and basolateral amygdala (BLA) of AIE and AIS adult rats exposed to acute ethanol (E) or saline (S) challenge in adulthood (scale bar = 50 μm). Data represent mean ± SEM derived from $n$ = 5–6 rats in each group. ***$p < .001$ by two-way ANOVA (CeA, AIE exposure: $F_{1,19} = 349.1$, $p < .001$, acute ethanol challenge: $F_{1,19} = 327.1$, $p < .001$; MeA, AIE exposure: $F_{1,19} = 272.0$, $p < .001$, acute ethanol challenge: $F_{1,19} = 217.3$, $p < .001$) followed by post hoc Tukey test. **(E)** Representative photomicrographs of gold immunolabeling showing KDM6B-positive cells in the CeA and quantification of gold immunolabeling for KDM6B protein in the CeA, MeA, and BLA of AIE and AIS rats exposed to acute ethanol (E) or saline (S) in adulthood (scale bar = 50 μm). Data represent mean ± SEM derived from $n$ = 6 rats in each group. *$p < .05$, **$p < .01$, ***$p < .001$ by two-way ANOVA (CeA, AIE treatment: $F_{1,20} = 19.9$, $p < .001$, acute ethanol exposure: $F_{1,20} = 12.4$, $p < .01$; MeA, AIE exposure: $F_{1,20} = 10.5$, $p < .01$, acute ethanol exposure: $F_{1,20} = 8.00$, $p < .05$) followed by post hoc Tukey test. PND, postnatal day.

## Epigenetic Changes at the *Arc* SARE Site and Promoter in the Amygdala Correspond With *Arc* eRNA Transcript Levels

We investigated the occupancy of several epigenetic marks at the *Arc* SARE site by chromatin immunoprecipitation (ChIP) assay (Figure 3A). Post hoc testing after two-way ANOVA revealed that repressive H3K27me3 was decreased ($p < .05$) and the activating marks KDM6B ($p < .001$), CBP ($p < .001$), and H3K27ac ($p < .01$) were increased at the *Arc* SARE site in the amygdala of AIS + ethanol rats compared with AIS + saline rats. Interestingly, H3K27me3 ($p < .001$) was increased and KDM6B ($p < .05$), CBP ($p < .01$), and H3K27ac ($p < .05$) were decreased at the *Arc* SARE site in the amygdala of AIE + saline rats, and these four epigenetic marks returned to control-like levels after adult ethanol challenge (Figure 3A). We investigated chromatin occupancy at the *Arc* promoter (Figure 3B) and found increased occupancy of H3K27me3 along with decreased occupancy of KDM6B, CBP, and H3K27ac at the *Arc* promoter site in the amygdala of AIE + saline rats compared with AIS + saline rats. Changes in occupancy of these epigenetic marks returned to control-like levels in the amygdala of AIE adult rats after acute ethanol challenge (Figure 3B).

*Arc* eRNA (−) binds to the RNA recognition domain of the E subunit of NELF (NELF-E) to decoy the NELF complex away from the *Arc* promoter and allow polymerase II to transcribe *Arc* mRNA (18). Therefore, we measured the occupancy of NELF-E at the *Arc* promoter in AIS and AIE adult rats exposed to an acute ethanol challenge (Figure 3C). NELF-E occupancy at the *Arc* promoter site (−441 bp) was increased in the amygdala of AIE adult rats ($p < .001$) and normalized after acute ethanol exposure (Figure 2C).

## *Kdm6b* siRNA Infusion in the CeA Provokes Anxiety-like Behavior

To determine the direct contribution of *Kdm6b*-mediated chromatin remodeling and regulation of *Arc* eRNA and mRNA expression in the amygdala to anxiety-like behaviors, we cannulated alcohol-naïve AIS control rats in the CeA and infused either control or *Kdm6b* siRNA. Anxiety-like behaviors were measured using both the elevated plus maze and the LDB (Figure 4A) in these rats 24 hours after a single siRNA infusion in separate batches of rats. *Kdm6b* siRNA infusion into the CeA provoked anxiety-like behavior in the elevated plus maze as demonstrated by significant ($p < .001$) decrease in percentage of open arm entries and percent of time spent in the open arm compared with rats infused with vehicle (iFect solution; Neuromics, Edina, MN) or control siRNA (Figure 4B). There were no differences in closed arm entries as a measure of general activity of rats. Owing to the lack of effect of the control siRNA, we did not test this group in subsequent experiments. *Kdm6b* siRNA infusion additionally led to increased time spent in the dark compartment ($p < .001$) and decreased time spent in the light compartment ($p < .001$) in the LDB compared with vehicle-infused AIS rats without altering general activity (total ambulation) (Figure 4C). These data suggest that *Kdm6b* siRNA infusion

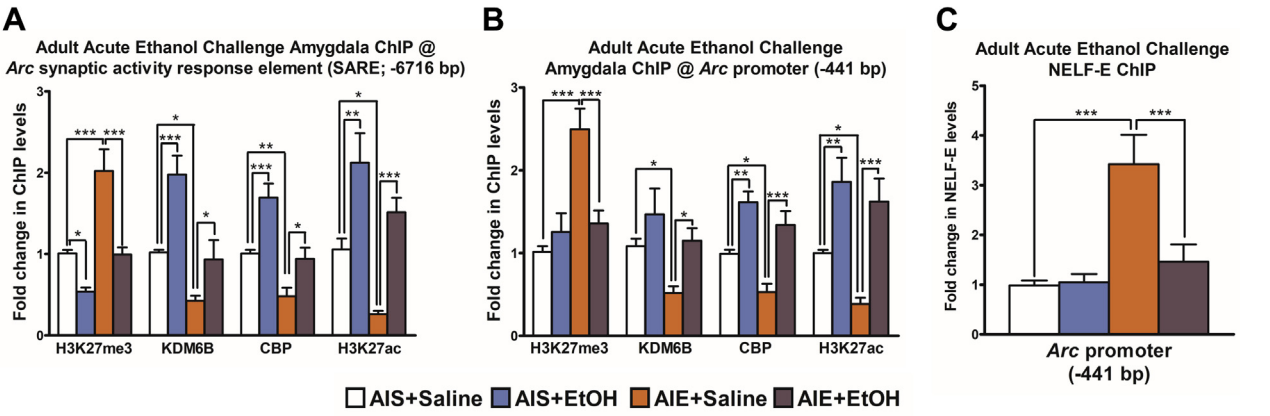


**Figure 3.** Adult ethanol (EtOH) challenge reverses adolescent intermittent ethanol (AIE)–induced chromatin remodeling at the *Arc* synaptic activity response element (SARE) site and promoter. **(A)** Chromatin immunoprecipitation (ChIP) analysis of H3K27 trimethylation (H3K27me3), lysine demethylase 6B (KDM6B), CREB binding protein (CBP), and acetylated H3K27 (H3K27ac) at the SARE site of the *Arc* gene in the amygdala of AIE or adolescent intermittent saline (AIS) adult rats exposed to acute ethanol or saline. Data represent mean ± SEM derived from $n = 5$–6 rats in each group.*$p < .05$, **$p < .01$, ***$p < .001$ by two-way analysis of variance (H3K27me3 occupancy, AIE treatment: $F_{1,20} = 25.5$, $p < .001$, acute ethanol exposure: $F_{1,20} = 26.6$, $p < .001$; KDM6B occupancy, AIE treatment: $F_{1,18} = 27.8$, $p < .001$, acute ethanol exposure: $F_{1,18} = 22.3$, $p < .001$; CBP occupancy, AIE treatment: $F_{1,19} = 25.3$, $p < .001$, acute ethanol exposure: $F_{1,19} = 20.5$, $p < .001$; H3K27ac occupancy, AIE treatment: $F_{1,19} = 10.2$, $p < .01$, acute ethanol exposure: $F_{1,19} = 27.8$, $p < .001$ at the *Arc* SARE site) followed by post hoc Tukey test. **(B)** ChIP analysis of H3K27me3, KDM6B, CBP, and H3K27ac at the *Arc* promoter (−441 bp) in the amygdala of AIS or AIE adult rats exposed to acute ethanol or saline. Data represent mean ± SEM derived from $n = 5$–8 rats in each group. *$p < .05$, **$p < .01$, ***$p < .001$ by two-way analysis of variance (H3K27me3, AIE treatment: $F_{1,24} = 17.7$, $p < .001$, acute ethanol exposure: $F_{1,24} = 5.7$, $p < .05$, AIE × acute ethanol interaction: $F_{1,24} = 13.5$, $p < .01$; KDM6B, AIE treatment: $F_{1,26} = 5.3$, $p < .05$, acute ethanol exposure: $F_{1,26} = 7.0$, $p < .05$; CBP, AIE treatment: $F_{1,19} = 8.9$, $p < .01$, acute ethanol exposure: $F_{1,19} = 33.6$, $p < .001$; H3K27ac, AIE treatment: $F_{1,19} = 4.7$, $p < .05$, acute ethanol exposure: $F_{1,19} = 28.3$, $p < .001$) followed by post hoc Tukey test. **(C)** ChIP analysis of the E subunit of negative elongation factor (NELF-E) at the *Arc* promoter region (−441 bp) in the amygdala of AIS or AIE adult rats exposed to acute ethanol or saline in adulthood. Data represent mean ± SEM derived from $n = 6$ rats in each group. ***$p < .001$ by two-way analysis of variance (AIE treatment: $F_{1,20} = 16.2$, $p < .001$, acute ethanol exposure: $F_{1,20} = 7.2$, $p < .05$, AIE × acute ethanol interaction: $F_{1,20} = 8.2$, $p < .05$) followed by post hoc Tukey test. CREB, cyclic adenosine monophosphate response element binding protein.

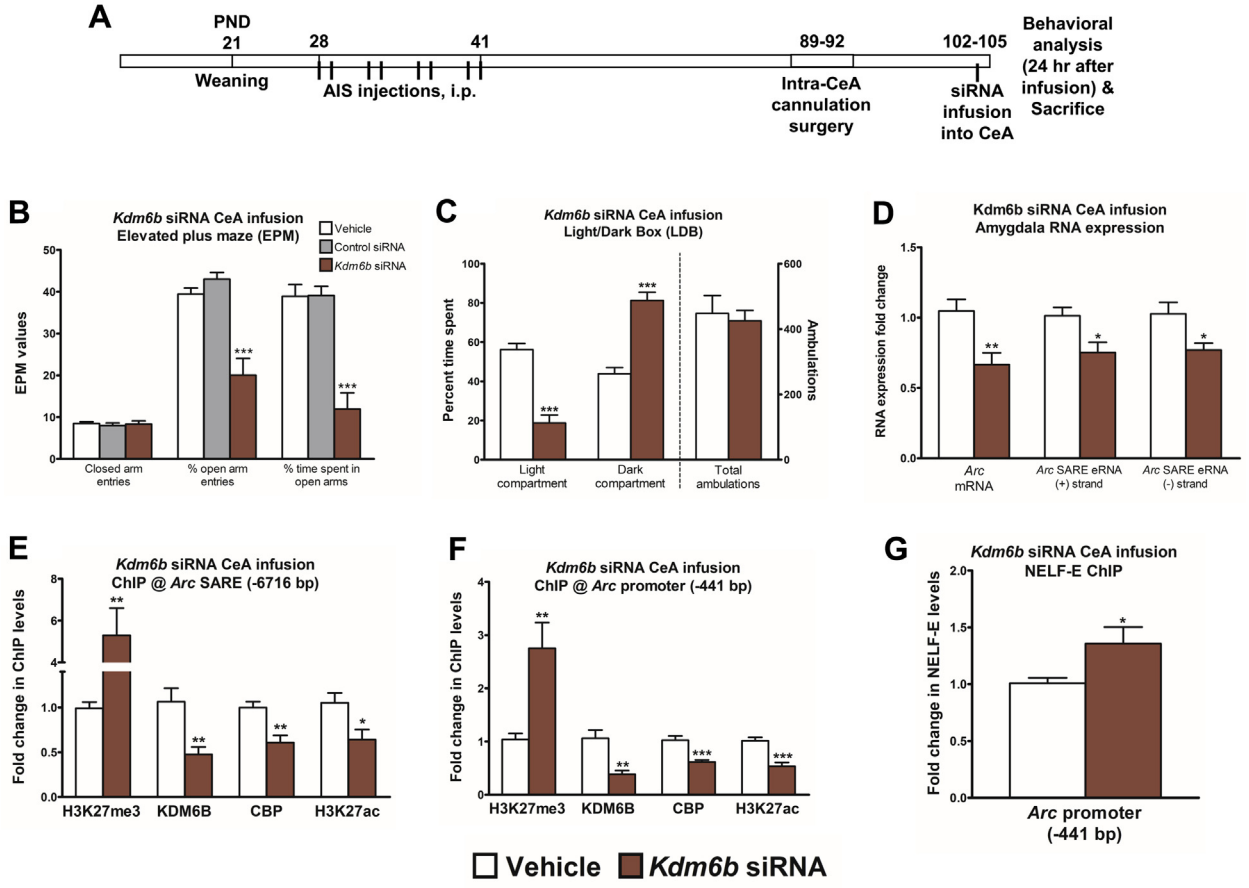


**Figure 4.** *Kdm6b* small interfering RNA (siRNA) infusion into the central nucleus of amygdala (CeA) provokes anxiety-like behavior and decreases expression of *Arc* enhancer (eRNA) and messenger RNA (mRNA). **(A)** Schematic representation of *Kdm6b* siRNA infusion directly into the CeA of adolescent intermittent saline (AIS)–exposed control adult rats. **(B)** Elevated plus maze (EPM) exploration test for anxiety-like behaviors in adult rats infused with *Kdm6b* siRNA, control siRNA, or vehicle (iFect solution) in the CeA. Data represent mean ± SEM derived from $n = 6$ rats in each group. $^{***}p < .001$ by one-way analysis of variance (percentage of open arm entries: $F_{2,15} = 21.8$, $p < .001$), percent of time spent in the open arm: $F_{2,15} = 26.3$, $p < .001$) followed by post hoc Tukey test. **(C)** Light/dark box exploration test for anxiety-like behaviors in adult control rats infused with *Kdm6b* siRNA or vehicle (iFect solution) in the CeA. Data represent mean ± SEM derived from $n = 8$ rats in each group. $^{***}p < .001$ by Student *t* test (time spent in the dark compartment: $t_{14} = -7.25$, $p < .001$, time spent in the light compartment: $t_{14} = 7.25$, $p < .001$). **(D)** RNA analysis of *Arc* eRNA (eRNA plus and minus strand) and *Arc* mRNA transcripts in the amygdala of AIS adult rats infused with *Kdm6b* siRNA or vehicle (iFect solution) in the CeA. Data represent mean ± SEM derived from $n = 7$–8 rats in each group. $^{*}p < .05$, $^{**}p < .01$ by Student *t* test (transcripts of *Arc* eRNA, eRNA plus strand: $t_{14} = 2.82$, $p < .05$, eRNA minus strand: $t_{14} = 2.69$, $p < .05$; *Arc* mRNA: $t_{12} = 3.25$, $p < .01$). **(E)** Chromatin immunoprecipitation (ChIP) analysis of H3K27 trimethylation (H3K27me3), lysine demethylase 6B (KDM6B), CREB binding protein (CBP), and acetylated H3K27 (H3K27ac) at the synaptic activity response element (SARE) site of the *Arc* promoter in the amygdala of AIS adult rats infused with *Kdm6b* siRNA or vehicle (iFect solution) in the CeA. Data represent mean ± SEM derived from $n = 7$–8 rats in each group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ by Student *t* test (H3K27me3 occupancy: $t_{13} = -3.08$, $p < .01$, KDM6B occupancy: $t_{13} = 3.54$, $p < .01$, CBP occupancy: $t_{14} = 3.77$, $p < .01$, H3K27ac occupancy: $t_{14} = 2.59$, $p < .05$). **(F)** ChIP analysis of H3K27me3, KDM6B, CBP, and H3K27ac at the *Arc* promoter (−441 bp) site in the amygdala of adult AIS rats infused with *Kdm6b* siRNA or vehicle (iFect solution) in the CeA. Data represent mean ± SEM derived from $n = 7$–8. $^{**}p < .01$, $^{***}p < .001$ by Student *t* test (H3K27me3 occupancy: $t_{13} = -3.26$, $p < .01$, KDM6B occupancy: $t_{13} = 4.21$, $p < .01$, CBP occupancy: $t_{14} = 4.56$, $p < .001$, H3K27ac occupancy: $t_{14} = 5.03$, $p < .001$). **(G)** ChIP analysis for the E subunit of negative elongation factor (NELF-E) at the *Arc* promoter (−441 bp) in the amygdala of rats infused with *Kdm6b* siRNA or vehicle (iFect solution) in the CeA. Data represent mean ± SEM derived from $n = 7$–8 rats in each group. $^{*}p < .05$ by Student *t* test ($t_{13} = -2.39$, $p < .05$). CREB, cyclic adenosine monophosphate response element binding protein; i.p., intraperitoneal; PND, postnatal day.

into the CeA in AIS adult rats leads to anxiety-like behavior in multiple behavioral tests, thus mimicking AIE-induced anxiety-like behaviors in adulthood.

*Kdm6b* siRNA infusion produced significantly ($p < .01$) decreased *Kdm6b* mRNA levels in the amygdala but no change in *Cbp* and *Kdm6a* mRNA levels compared with vehicle-infused control animals (Supplemental Figure S5A). We additionally performed confocal microscopy in control rats to colocalize KDM6B protein within the CeA, MeA, and BLA with NeuN and glial fibrillary acidic protein and found that KDM6B is predominately colocalized with NeuN, but not glial fibrillary acidic protein, in the CeA, MeA, and BLA (Supplemental Figure S5B), indicating that this protein is expressed at relatively high levels in neuronal populations. We have previously shown that siRNA infusion with iFect solution penetrates neurons (35), and we therefore suggest the possibility that *Kdm6b* siRNA exerts its main effects in neuronal cell populations in the CeA.

### *Kdm6b* siRNA Infusion Decreases *Arc* eRNA and mRNA Transcription via Epigenetic Changes at the SARE Site

We examined whether *Arc* eRNA transcripts were altered by *Kdm6b* siRNA infusion. Both (+) and (−) strand transcripts of *Arc* eRNA were significantly ($p < .05$) decreased in the amygdala of *Kdm6b* siRNA–infused rats (Figure 4D). Additionally, *Arc* mRNA, but not *Bdnf* exon IV and *Syp* mRNA (Supplemental Figure S5A), was decreased ($p < .01$) in the amygdala of *Kdm6b* siRNA–infused rats (Figure 4D). We next performed ChIP followed by quantitative PCR for H3K27me3, KDM6B, CBP, and H3K27ac occupancy at the *Arc* SARE site and promoter in rats infused with *Kdm6b* directly into the CeA. *Kdm6b* siRNA infusion into the CeA led to significantly increased ($p < .01$) H3K27me3 occupancy and decreased ($p < .05$ to $p < .01$) KDM6B, CBP, and H3K27ac occupancy at the *Arc* SARE site in the amygdala compared with vehicle-exposed rats (Figure 4E). A similar pattern of epigenetic modifications was observed at the *Arc* promoter site (Figure 4F).

Owing to the epigenetically encoded decrease in *Arc* eRNA in these rats, we tested whether NELF-E binding was altered in *Kdm6b* siRNA–infused rats in the amygdala. We observed increased ($p < .05$) NELF-E occupancy at the *Arc* promoter region, corresponding with decreased *Arc* eRNA and mRNA expression, in *Kdm6b* siRNA–infused rats compared with vehicle-infused rats (Figure 4G). We used the same primers (Supplemental Figure S6A) used to amplify ChIP pulldown from KDM6B and CBP that we used to amplify the *Arc* eRNA (−) strand transcript RNA product to determine whether the epigenetic changes seen at the SARE site may be solely responsible for regulating the *Arc* eRNA (−) strand transcript in our experiments (Supplemental Figure S6B). We did not find any alterations in KDM6B and CBP occupancy at the *Arc* eRNA (−) transcribed region (Supplemental Figure S6A, B), suggesting that epigenetic changes at the SARE site are likely responsible for the regulation of *Arc* eRNA expression.

### Inhibition of *Arc* eRNA (−) in the CeA Mimics AIE-Induced Anxiety-like Behaviors and Decreases *Arc* mRNA Expression

We infused a custom-designed *Arc* eRNA (−) antisense LNA or a control LNA oligonucleotide into the CeA of alcohol-naïve control adult rats twice per day for 2 days. On the third day (approximately 16 hours after the last LNA infusion), we measured anxiety-like behaviors in the LDB. Interestingly, we observed anxiety-like behaviors in rats infused with *Arc* eRNA (−) LNA in the CeA (Figure 5A), as represented by decreased percentage of time spent in the light compartment ($p < .001$) and increased percentage of time spent in the dark compartment ($p < .001$) compared with control LNA-infused rats. There were no alterations in total ambulation between the groups, indicating that general activity levels were unaltered (Figure 5A).

We measured the status of *Arc* eRNA transcripts (to confirm LNA knockdown) and *Arc* mRNA in the amygdala of rats infused with *Arc* eRNA (−) LNA in the CeA (Figure 5B). *Arc* eRNA (−) LNA infusion significantly decreased *Arc* eRNA (−) in the amygdala compared with control LNA-infused rats ($p < .05$) (Figure 5B), indicating that the *Arc* eRNA (−) LNA knockdown was effective. There was no effect on *Arc* eRNA (+) expression between the two groups. Interestingly, *Arc* mRNA (Figure 5B), but not *Bdnf* exon IV and *Syp* mRNA (Supplemental Figure S7A), transcription was significantly decreased ($p < .001$) in the amygdala of rats infused with *Arc* eRNA (−) LNA compared with control LNA-infused rats (Figure 5B).

Finally, we investigated whether NELF-E occupancy at the *Arc* promoter was altered following infusion of the *Arc* eRNA (−) LNA into the CeA using the ChIP assay. *Arc* eRNA (−) LNA infusion directly into the CeA led to a significant ($p < .01$) increase in NELF-E occupancy at the *Arc* promoter region in the amygdala compared with control LNA-infused rats (Figure 5C). Previous in vitro studies of eRNA function have shown that knockdown of eRNA transcripts can cause locus-specific chromatin remodeling (36), potentially representing a genomic effect of the probe rather than antisense inhibition of the eRNA transcript. To investigate this, we measured KDM6B and CBP occupancy at the *Arc* SARE site and promoter and H3K27ac levels at the *Arc* SARE site, *Arc* eRNA (−) transcribed region, and *Arc* promoter in the amygdala of rats infused with *Arc* eRNA (−) LNA or control LNA. We found no differences in the occupancy of these epigenetic marks between groups (Supplemental Figure S7B–D), suggesting that the inhibition of *Arc* eRNA (−) by the LNA oligonucleotide occurs posttranscriptionally.

## DISCUSSION

In this study, we show for the first time that deficits in KDM6B-mediated epigenetic reprogramming lead to long-lasting reductions in *Arc* eRNA and mRNA expression after AIE exposure in the adult amygdala, corresponding to decreased synapse number in the CeA and MeA and heightened anxiety-like behaviors. We show that H3K27me3 occupancy is increased and KDM6B, CBP, and H3K27ac occupancy is decreased at the SARE site of the *Arc* gene in AIE adult rats, which is the likely site of bidirectional transcriptional suppression of *Arc* eRNA expression. *Kdm6b* siRNA infusion into the CeA is sufficient to provoke anxiety-like behaviors in control rats and decreases *Arc* eRNA and mRNA expression via decreased KDM6B and CBP occupancy, the associated increased H3K27me3 and decreased H3K27ac at the SARE site, and a subsequent increase in NELF-E binding to the *Arc* promoter. Lastly, direct knockdown of *Arc* eRNA (−) in the CeA of control rats leads to anxiety-like behaviors, increased NELF-E occupancy at the *Arc* promoter, and decreased *Arc* mRNA expression in the amygdala. Our data suggest that epigenetic reprogramming due to deficits in KDM6B suppresses eRNA transcription and possibly is involved in the increased risk for anxiety after adolescent alcohol exposure. The data also provide evidence that KDM6B and CBP may form a complex in vivo in the amygdala to regulate neuronal gene transcription and synaptic function via epigenetic modifications. Furthermore, *Arc* eRNA expression in the CeA directly regulates anxiety-like behaviors (Figure 6).

AIE exposure leads to increased anxiety-like behavior in adulthood, confirming previous studies (11,37), and this anxiety is reversed by acute alcohol challenge similar to our previous findings (10). Human epidemiological studies show a

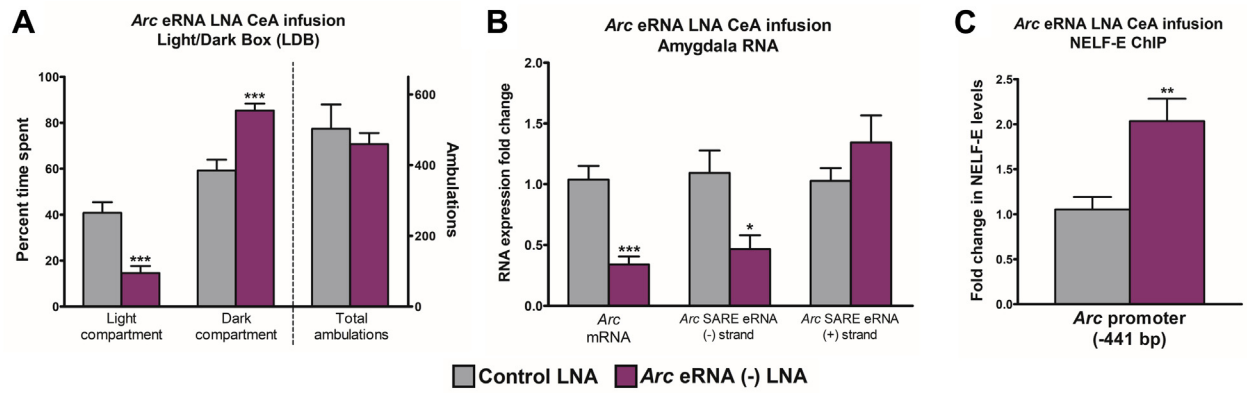


**Figure 5.** Minus (−) strand of the *Arc* enhancer RNA (eRNA), locked nucleic acid (LNA) oligonucleotide infusion into the central nucleus of amygdala (CeA) provokes anxiety-like behaviors and decreases *Arc* messenger RNA (mRNA) in alcohol-naïve rats. **(A)** Light/dark box exploration test for anxiety-like behaviors in adult alcohol-naïve control rats infused with LNA antisense oligonucleotide directed toward *Arc* eRNA (−) or control LNA in the CeA. Data represent mean ± SEM derived from $n = 7$ rats in each group. ***$p < .001$ by Student *t* test (percentage of time spent in the light compartment: $t_{12} = 4.69$, $p < .001$, percentage of time spent in the dark compartment: $t_{12} = -4.69$, $p < .001$). **(B)** RNA analysis in the amygdala of rats infused with *Arc* eRNA (−) LNA in the CeA. Data represent mean ± SEM derived from $n = 6–7$ rats in each group. *$p < .05$, ***$p < .001$ by Student *t* test (*Arc* eRNA (−) strand mRNA: $t_{12} = 2.88$, $p < .05$, *Arc* mRNA: $t_{12} = 5.31$, $p < .001$). **(C)** Chromatin immunoprecipitation (ChIP) analysis for the E subunit of negative elongation factor (NELF-E) at the *Arc* promoter (−441 bp) in the amygdala of rats infused with *Arc* eRNA (−) LNA in the CeA. Data represent mean ± SEM derived from $n = 7$ rats in each group. **$p < .01$ by Student *t* test ($t_{12} = -3.45$, $p < .01$).

relationship between increased anxiety risk and early-life alcohol consumption (6,38,39). Patients with a diagnosis of alcohol dependence are more likely to have an additional anxiety disorder diagnosis, and early onset of anxiety disorders reliably predicts the age of first alcohol use (7,9). Additionally, the presence of anxiety and/or depressive symptoms increases the likelihood of early alcohol dependence (6). Other groups have observed behavioral disinhibition in adult Wistar rats exposed to AIE, indicating that the lasting effects of adolescent alcohol exposure are dependent on genetic background (40,41). Notably, exposure to the same dosing regimen of alcohol in adulthood does not have lasting behavioral and physiological effects (42,43), highlighting adolescence as a critical period sensitive to ethanol exposure (1,13,14).

The anxiety-like behavior induced by *Kdm6b* siRNA infusion into the CeA indicates that this transcript is crucial for the expression of affective states via chromatin remodeling, mimicking the effects of AIE on KDM6B and related epigenetic processes at the *Arc* gene (Figure 6). *Kdm6b* siRNA infusion in the CeA did not alter the expression of *Bdnf* exon IV or *Syp* in control rats. These results suggest the possibility that *Arc* eRNA expression may be sensitive to *Kdm6b* reduction, whereas reductions in other synaptic genes lacking activity-dependent enhancers may require changes in additional epigenetic regulators. KDM6B may lie downstream of brain-derived neurotrophic factor (BDNF) action, as loss of KDM6B prevents BDNF-induced gene induction in cerebellar granule neurons (44). Importantly, several other genes involved in synaptic plasticity are also decreased, possibly owing to globally condensed chromatin architecture in the amygdala after AIE. Previously, we have shown that AIE treatment causes increased histone deacetylase 2 levels and decreased levels of a neuron-specific lysine-specific demethylase 1 isoform known as Lsd1+8a in the amygdala in adulthood, leading to altered histone modifications (H3K9ac and H3K9me2) both globally and at the promoter regions of *Bdnf* exon IV and *Arc* (10,11). Taken together, our work indicates that alterations in KDM6B, histone deacetylase 2, CBP, and lysine-specific demethylase 1 create an imbalance between active and inactive chromatin states, particularly on H3K9 and H3K27 residues, in the adult amygdala after AIE. Other brain circuits, such as dopaminergic transmission in the prefrontal cortex (45), are likely involved in the lasting effects of AIE, but it should be noted that the negative affective states associated with alcohol addiction, such as anxiety, have been identified as promising phenotypes to use for AUD drug development (46,47).

Enhancer RNAs were first discovered in large sequencing datasets and originally characterized as transcriptional noise or simply by-products of active enhancer regions (19,48). Comprehensive sequencing-based analyses indicate that eRNAs are relatively tissue specific and brain region specific, suggesting possible roles in cell fate determination and developmental processes (49,50). However, some eRNAs do serve biological functions, such as the ability of *Arc* eRNA to bind to the RNA recognition site of NELF-E and cause the NELF complex to leave the chromatin so poised polymerase II can begin actively transcribing *Arc* mRNA in vitro in neuronal culture cells (18). Our in vivo data in the CeA support this mechanism of *Arc* expression. The chromatin modifications on both the SARE and promoter sites seen here may act in tandem to tightly regulate *Arc* mRNA transcription, although our data suggest the possibility that chromatin alterations at the SARE site are primarily responsible for the changes in *Arc* eRNA transcription seen in this study (Figure 6). The associations between *Arc* eRNA levels, NELF-E binding at the *Arc* promoter, and *Arc* mRNA levels across multiple models and perturbations would seem to suggest that, at least in the case of the *Arc* eRNA transcribed from the SARE site, this eRNA has a transcription-independent function (18). Therefore, some eRNAs may be functional entities, whereas others simply have

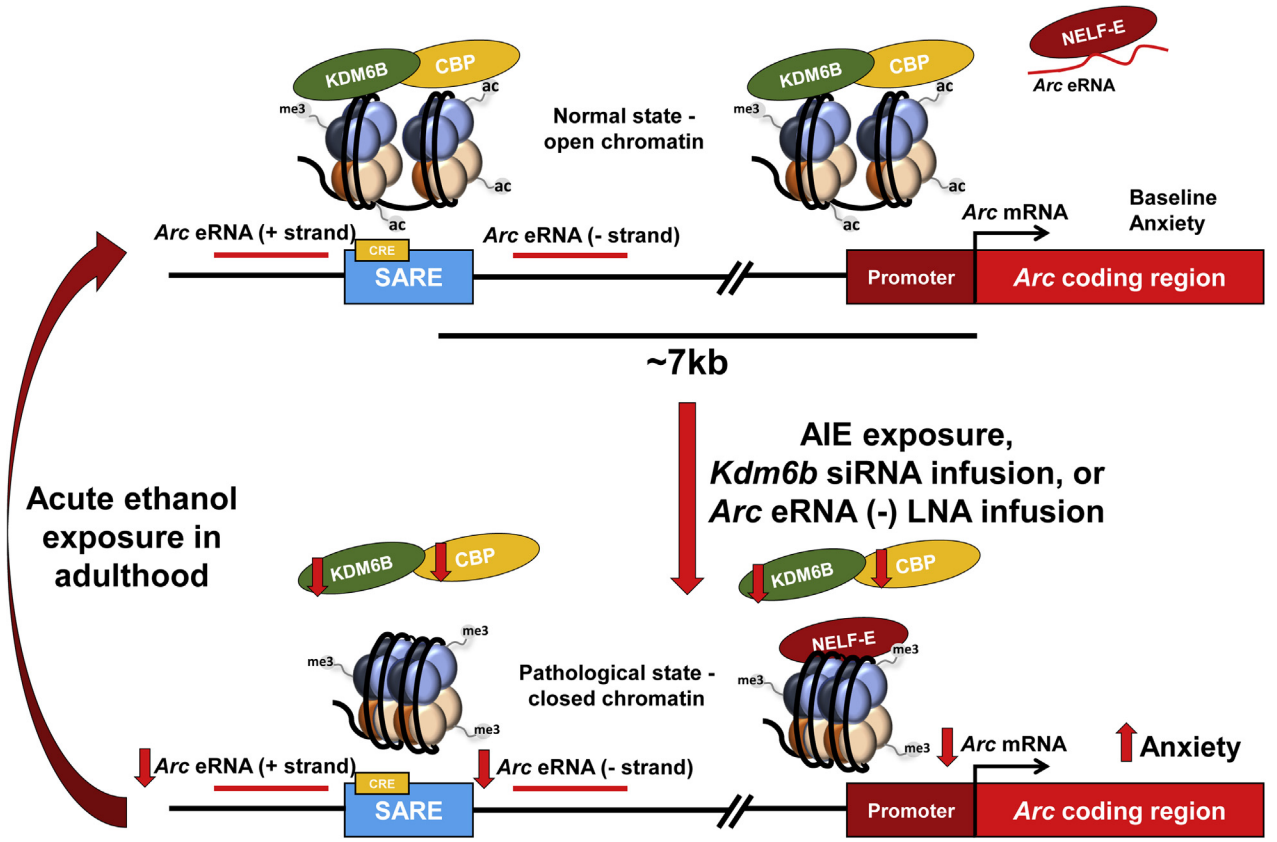


**Figure 6.** Adolescent intermittent ethanol (AIE) leads to adult anxiety susceptibility via epigenetic reprogramming at the *Arc* synaptic activity response element (SARE) site and decreased *Arc* enhancer RNA (eRNA) in the amygdala. AIE produces long-lasting impacts on epigenetic reprogramming and decreases synapse-related transcriptional events in the amygdala leading to anxiety-like behaviors in adulthood. AIE increases the repressive epigenetic mark H3K27 trimethylation (H3K27me3) and decreases lysine demethylase 6B (KDM6B), CREB binding protein (CBP), and H3K27 acetylation (H3K27ac) at the *Arc* promoter and SARE site. The closed chromatin architecture at the SARE site is associated with decreased *Arc* eRNA expression, increased E subunit of negative elongation factor (NELF-E) binding at the *Arc* promoter, and decreased *Arc* messenger RNA (mRNA) expression. These behavioral and epigenetic changes return to control-like levels after acute ethanol challenge in AIE adult rats. Notably, both *Kdm6b* small interfering RNA (siRNA) infusion or locked nucleic acid (LNA) knockdown of *Arc* eRNA minus (−) strand directly in the central nucleus of amygdala leads to marked anxiety-like behavior, reduced *Arc* eRNA and mRNA expression, and increased NELF-E binding to the *Arc* promoter. Taken together, these results indicate that epigenetic alterations resulting in decreased *Arc* eRNA are critical for the increased risk of anxiety in adulthood after adolescent alcohol exposure. CREB, cyclic adenosine monophosphate response element binding protein.

transcription-dependent functions, meaning that the act of transcribing the eRNA itself may be important for subsequent downstream activities, such as transcription factor binding (20,51). The act of eRNA transcription may also be important for maintaining enhancer-promoter looping, thus existing in a positive feedback loop to further promote target mRNA transcription. Our data involving *Kdm6b* siRNA infusion in the CeA suggest that *Arc* eRNA interacts with epigenetic machinery at the *Arc* promoter to decoy NELF and regulate *Arc* expression and anxiety-related phenotypes. *Arc* eRNA (−) inhibition by LNA infusion into the CeA increased NELF binding at the *Arc* promoter, decreased *Arc* mRNA expression, and provoked anxiety-like behaviors without altering the expression of *Bdnf* exon IV and *Syp* in the amygdala. Similarly, previous studies found that *Arc* eRNA inhibition decreased *Arc* mRNA expression without altering the expression of other immediate early genes (18). We also observed that *Arc* eRNA (−) inhibition by LNA did not alter the epigenetic dynamics at *Arc* SARE or promoter sites, suggesting that *Arc* expression may be regulated principally by eRNA-mediated decoying of NELF in this experimental condition (52). Mechanistic studies were performed in the CeA owing to its critical involvement in AUD (10,11,35,53), but this does not rule out the possibility that AIE-induced chromatin remodeling in the MeA may be involved in adult psychopathology. Therefore, future studies will increase the expression of *Arc* in both the CeA and the MeA to investigate the similarities and differences in the function of specific amygdaloid nuclei in AIE-induced anxiety-like behaviors.

The present study provides novel evidence that epigenetically regulated eRNA expression interacts with transcriptional machinery at the *Arc* gene to alter *Arc* expression in the CeA and orchestrate AIE-induced anxiety-like behaviors in adulthood (Figure 6). Future studies should continue to identify individual functional eRNAs as well as novel functions of eRNAs as a group, as these molecules represent a new frontier of epigenetics and may play important roles in neuronal function and psychiatric disorders.

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## ARTICLE INFORMATION

From the Center for Alcohol Research in Epigenetics (EJK, HZ, SCP), Department of Psychiatry, and Department of Anatomy and Cell Biology (SCP), University of Illinois at Chicago; and Jesse Brown Veterans Affairs Medical Center (EJK, HZ, SCP), Chicago, Illinois.

Address correspondence to Subhash C. Pandey, Ph.D., Center for Alcohol Research in Epigenetics, Department of Psychiatry, University of Illinois at Chicago, 1601 West Taylor Street (m/c 912), Chicago, IL 60612; E-mail: scpandey@uic.edu.

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## REFERENCES

1. Spear LP (2018): Effects of adolescent alcohol consumption on the brain and behaviour. Nat Rev Neurosci 19:197–214.
2. 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.
3. Lister R, Mukamel EA, Nery JR, Urich M, Puddifoot CA, Johnson ND, *et al.* (2013): Global epigenomic reconfiguration during mammalian brain development. Science 341:1237905.
4. DeWit DJ, Adlaf EM, Offord DR, Ogborne AC (2000): Age at first alcohol use: A risk factor for the development of alcohol disorders. Am J Psychiatry 157:745–750.
5. Brown SA, McGue M, Maggs J, Schulenberg J, Hingson R, Swartzwelder S, *et al.* (2009): Underage alcohol use: Summary of developmental processes and mechanisms: ages 16-20. Alcohol Res Health 32:41–52.
6. Boschloo L, Vogelzangs N, van den Brink W, Smit JH, Veltman DJ, Beekman AT, *et al.* (2013): Depressive and anxiety disorders predicting first incidence of alcohol use disorders: Results of the Netherlands Study of Depression and Anxiety (NESDA). J Clin Psychiatry 74:1233–1240.
7. Lai HM, Cleary M, Sitharthan T, Hunt GE (2015): Prevalence of comorbid substance use, anxiety and mood disorders in epidemiological surveys, 1990-2014: A systematic review and meta-analysis. Drug Alcohol Depend 154:1–13.
8. Grant BF, Goldstein RB, Saha TD, Chou SP, Jung J, Zhang H, *et al.* (2015): Epidemiology of DSM-5 Alcohol Use Disorder: Results from the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry 72:757–766.
9. 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.
10. 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.
11. 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.
12. Alaux-Cantin S, Warnault V, Legastelois R, Botia B, Pierrefiche O, Vilpoux C, *et al.* (2013): Alcohol intoxications during adolescence increase motivation for alcohol in adult rats and induce neuroadaptations in the nucleus accumbens. Neuropharmacology 67:521–531.
13. Crews FT, Vetreno RP, Broadwater MA, Robinson DL (2016): Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. Pharmacol Rev 68:1074–1109.
14. Kyzar EJ, Floreani C, Teppen TL, Pandey SC (2016): Adolescent alcohol exposure: Burden of epigenetic reprogramming, synaptic remodeling, and adult psychopathology. Front Neurosci 10:222.
15. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A (2009): An operational definition of epigenetics. Genes Dev 23:781–783.
16. Qureshi IA, Mehler MF (2011): Non-coding RNA networks underlying cognitive disorders across the lifespan. Trends Mol Med 17:337–346.
17. Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M, *et al.* (2009): Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. Proc Natl Acad Sci U S A 106:316–321.
18. Schaukowitch K, Joo JY, Liu X, Watts JK, Martinez C, Kim TK (2014): Enhancer RNA facilitates NELF release from immediate early genes. Mol Cell 56:29–42.
19. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, *et al.* (2010): Widespread transcription at neuronal activity-regulated enhancers. Nature 465:182–187.
20. Li W, Notani D, Rosenfeld MG (2016): Enhancers as non-coding RNA transcription units: Recent insights and future perspectives. Nat Rev Genet 17:207–223.
21. Rajarajan P, Gil SE, Brennand KJ, Akbarian S (2016): Spatial genome organization and cognition. Nat Rev Neurosci 17:681–691.
22. Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, *et al.* (2010): Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A 107:21931–21936.
23. Kouzarides T (2007): Chromatin modifications and their function. Cell 128:693–705.
24. Tyssowski KM, DeStefino NR, Cho JH, Dunn CJ, Poston RG, Carty CE, *et al.* (2018): Different neuronal activity patterns induce different gene expression programs. Neuron 98:530–546.
25. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, *et al.* (2007): Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448:553–560.
26. Zentner GE, Tesar PJ, Scacheri PC (2011): Epigenetic signatures distinguish multiple classes of enhancers with distinct cellular functions. Genome Res 21:1273–1283.
27. Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, *et al.* (2007): UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734.
28. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, *et al.* (2006): Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441:349–353.
29. Palomer E, Carretero J, Benvegnu S, Dotti CG, Martin MG (2016): Neuronal activity controls Bdnf expression via Polycomb de-repression and CREB/CBP/JMJD3 activation in mature neurons. Nat Commun 7:11081.
30. Bannister AJ, Kouzarides T (1996): The CBP co-activator is a histone acetyltransferase. Nature 384:641–643.
31. Zhang H, Kyzar EJ, Bohnsack JP, Kokare DM, Teppen T, Pandey SC (2018): Adolescent alcohol exposure epigenetically regulates CREB signaling in the adult amygdala. Sci Rep 8:10376.

32. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, *et al.* (2009): HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459:55–60.
33. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, *et al.* (2010): The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. Nucleic Acids Res 38:W214–W220.
34. Fukuchi M, Nakashima F, Tabuchi A, Shimotori M, Tatsumi S, Okuno H, *et al.* (2015): Class I histone deacetylase-mediated repression of the proximal promoter of the activity-regulated cytoskeleton-associated protein gene regulates its response to brain-derived neurotrophic factor. J Biol Chem 290:6825–6836.
35. Moonat S, Sakharkar AJ, Zhang H, Tang L, Pandey SC (2013): Aberrant histone deacetylase2-mediated histone modifications and synaptic plasticity in the amygdala predisposes to anxiety and alcoholism. Biol Psychiatry 73:763–773.
36. Pnueli L, Rudnizky S, Yosefzon Y, Melamed P (2015): RNA transcribed from a distal enhancer is required for activating the chromatin at the promoter of the gonadotropin alpha-subunit gene. Proc Natl Acad Sci U S A 112:4369–4374.
37. Sakharkar AJ, Vetreno RP, Zhang H, Kokare DM, Crews FT, Pandey SC (2016): A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood. Brain Struct Funct 221:4691–4703.
38. Birrell L, Newton NC, Teesson M, Tonks Z, Slade T (2015): Anxiety disorders and first alcohol use in the general population. Findings from a nationally representative sample. J Anxiety Disord 31:108–113.
39. Grant BF, Stinson FS, Harford TC (2001): Age at onset of alcohol use and DSM-IV alcohol abuse and dependence: A 12-year follow-up. J Subst Abuse 13:493–504.
40. Gilpin 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.
41. 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.
42. Vetreno RP, Broadwater M, Liu W, Spear LP, Crews FT (2014): Adolescent, but not adult, binge ethanol exposure leads to persistent global reductions of choline acetyltransferase expressing neurons in brain. PLoS One 9:e113421.
43. 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.
44. Wijayatunge R, Liu F, Shpargel KB, Wayne NJ, Chan U, Boua JV, *et al.* (2018): The histone demethylase Kdm6b regulates a mature gene expression program in differentiating cerebellar granule neurons. Mol Cell Neurosci 87:4–17.
45. Trantham-Davidson H, Centanni SW, Garr SC, New NN, Mulholland PJ, Gass JT, *et al.* (2017): Binge-like alcohol exposure during adolescence disrupts dopaminergic neurotransmission in the adult prelimbic cortex. Neuropsychopharmacology 42:1024–1036.
46. Koob GF, Mason BJ (2016): Existing and future drugs for the treatment of the dark side of addiction. Annu Rev Pharmacol Toxicol 56:299–322.
47. Pandey SC, Kyzar EJ, Zhang H (2017): Epigenetic basis of the dark side of alcohol addiction. Neuropharmacology 122:74–84.
48. Jensen TH, Jacquier A, Libri D (2013): Dealing with pervasive transcription. Mol Cell 52:473–484.
49. Andersson R, Gebhard C, Miguel-Escalada I, Hoof I, Bornholdt J, Boyd M, *et al.* (2014): An atlas of active enhancers across human cell types and tissues. Nature 507:455–461.
50. Yao P, Lin P, Gokoolparsadh A, Assareh A, Thang MW, Voineagu I (2015): Coexpression networks identify brain region-specific enhancer RNAs in the human brain. Nat Neurosci 18:1168–1174.
51. Sigova AA, Abraham BJ, Ji X, Molinie B, Hannett NM, Guo YE, *et al.* (2015): Transcription factor trapping by RNA in gene regulatory elements. Science 350:978–981.
52. Madabhushi R, Kim TK (2018): Emerging themes in neuronal activity-dependent gene expression. Mol Cell Neurosci 87:27–34.
53. Gilpin NW, Herman MA, Roberto M (2015): The central amygdala as an integrative hub for anxiety and alcohol use disorders. Biol Psychiatry 77:859–869.