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Adolescent alcohol exposure epigenetically suppresses amygdala *Arc* enhancer RNA expression to confer adult anxiety susceptibility

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Abstract

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

METHODS: Rats were exposed to ethanol (2 days on/off; AIE) or intermittent n-saline (AIS) during postnatal days (PND) 28–41 and allowed to grow to adulthood for analysis of behavior and biochemical measures. Some AIE and AIS rats were exposed to an acute challenge with ethanol in adulthood. Cohorts of alcohol-naïve adult rats were cannulated in the central nucleus of amygdala (CeA) and infused with either lysine demethylase 6B (*Kdm6b*) small interfering RNA (siRNA) or an antisense locked nucleic acid (LNA) oligonucleotide specific to *Arc* eRNA prior to behavioral and biochemical analysis.

RESULTS: AIE adult rats display heightened anxiety and decreased *Arc* eRNA expression, which is regulated epigenetically through decreased KDM6B expression. This triggers condensed chromatin at the synaptic activity response element (SARE) site and promoter of the *Arc* gene, facilitating increased negative elongation factor (NELF) binding to the *Arc* promoter and decreasing *Arc* expression in the amygdala. Knockdown of *Kdm6b* or *Arc* eRNA expression in the CeA provokes anxiety in naïve adult rats and recapitulates the molecular and epigenetic phenotypes of AIE.

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

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Keywords

Adolescent alcohol; Amygdala; Anxiety; *Arc*; Enhancer RNA; epigenetic

INTRODUCTION

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 diagnosed with 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, non-coding 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 brain-derived neurotrophic factor (*Bdnf*) and activity-regulated cytoskeleton-associated protein (*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 ~7kb upstream of the transcription start site (TSS) (17), and this site also regulates an RNA product known as the *Arc* enhancer RNA (eRNA) (18, 19). eRNAs are a recently discovered class of non-coding 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 (Pol II) to begin elongating and transcribing *Arc* mRNA (18, 21). The epigenetic regulation of *Arc* eRNA in the amygdala and its role in AIE-induced anxiety-like behavior is currently unknown.

Several epigenetic marks at histone 3 lysine 27 (H3K27) and H3K4 are critical for regulating enhancer sequences and eRNA expression (19, 22, 23). H3K27ac is rapidly recruited to enhancers of immediate-early genes (IEGs) 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). The Polycomb repressive complex 2 (PRC2) adds methyl groups to H3K27, while lysine demethylase 6A (KDM6A) and KDM6B are responsible for H3K27me2/3 demethylation (27, 28). KDM6B complexes with members of the cyclic AMP-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 hypothesize 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 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 using an animal model.

METHODS AND MATERIALS

Male Sprague-Dawley adolescent rats [postnatal days (PND) 28–41] were exposed to ethanol (2g/kg intraperitoneal, 2 days on/off; AIE) or intermittent n-saline (AIS) 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 (2g/kg) or n-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 Supplemental Materials.

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 (Figure 1A) in the adult amygdala, we quantified synaptophysin (Syp)-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 show decreased synaptophysin immunoreactivity in the CeA ($p < 0.05$) and MeA ($p < 0.05$), but not the BLA, compared to adolescent intermittent saline (AIS) exposed adult rats (Figure 1B, C, S1). In order 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 ($p < 0.05$ – 0.001) decreased *Arc*, *Bdnf* exon IV, *Homer1*, *NeuroD1*, *NeuroD2*, neurogranin (*Nrgn*), *Syp*, and synaptotagmin 1 (*Syt1*) mRNA levels in the adult amygdala (Figure 1D). We generated a gene network with GeneMANIA (33) to prioritize highly interactive genes (Figure S2A) for detailed epigenetic studies. *Arc* is the ‘hub gene’ in our network of synaptic plasticity-associated genes downregulated after AIE in adulthood (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* eRNA (+) and *Arc* eRNA (–) are significantly ($p < 0.05$ – 0.01) decreased in the AIE adult amygdala compared to AIS adult rats (Figure 1F).

We next investigated the epigenetic regulation of *Arc* transcription in the adult amygdala after AIE. 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 to AIS rats (Figure S3A, B). H3K27me3 occupancy was not altered between the groups at the

most distal site in intron 2 of the *Arc* gene body (Figure S3B). We found that mRNA levels of *Kdm6b*, but not *Kdm6a* or other members of the PRC2, were decreased in the AIE adult amygdala compared to AIS rats (Figure S3C).

Acute alcohol in adulthood reverses AIE-induced anxiety-like behavior and *Arc* eRNA & mRNA transcriptional changes in the amygdala

Previous work indicates that acute ethanol exposure (2g/kg) in adulthood in AIE rats decreases anxiety-like behavior in the elevated plus maze (EPM) (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 spend more time than AIS rats in the dark compartment and less time in the light compartment ($p < 0.001$), indicative of an anxiety-like phenotype, which was attenuated by acute ethanol challenge in adulthood (Figure 2B). Acute ethanol challenge (2g/kg) also leads 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 ($p < 0.05$) increase in AIE animals exposed to acute alcohol compared to AIE animals exposed to saline (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 ANOVA (Figure 2C) revealed that both (–) and (+) strand transcripts of *Arc* eRNA ($p < 0.05$) and *Arc* mRNA ($p < 0.01$) levels are decreased in the adult amygdala of AIE rats and are increased by acute ethanol in both AIS ($p < 0.05$ – 0.001) and AIE rats ($p < 0.01$ – 0.001). Post-hoc comparison after two-way ANOVA (Figure 2C) shows that *Kdm6b* mRNA is decreased ($p < 0.05$) in the amygdala of AIE rats compared to AIS rats but returns 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* PCR histochemical procedures, respectively. Post-hoc analysis after two-way ANOVA shows that, in both the CeA and the MeA, AIE+ Saline rats show decreased *Arc* mRNA and protein levels in the adult amygdala compared to AIS+ Saline rats ($p < 0.001$; Figure 2D, S4B). Additionally, acute ethanol challenge in adulthood leads to increased *Arc* mRNA and protein expression in both AIS+ EtOH and AIE+ EtOH rats compared to saline-exposed rats from AIS and AIE groups ($p < 0.001$), respectively (Figure 2D, S4B). Our *in-situ* PCR data confirms the mRNA findings of decreased *Arc* mRNA in the AIE adult amygdala as measured by qPCR (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 indicates that KDM6B is decreased in AIE+ Saline rats in the CeA ($p < 0.001$) and MeA ($p < 0.01$) compared to AIS+ Saline rats, which returns to levels similar to AIS controls after acute ethanol exposure. KDM6B protein levels in the BLA did not significantly change between the groups (Figure 2E).

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 is decreased ($p<0.05$), and the activating marks KDM6B ($p<0.001$), CBP ($p<0.001$), and H3K27 acetylation (H3K27ac) ($p<0.01$) are increased, at the *Arc* SARE in the amygdala of AIS+ EtOH rats compared to AIS+ Saline rats. Interestingly, H3K27me3 ($p<0.001$) is increased and KDM6B ($p<0.05$), CBP ($p<0.01$), and H3K27ac ($p<0.05$) are decreased at the *Arc* SARE site in the amygdala of AIE+ Saline rats, and these four epigenetic marks return 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 to AIS+ Saline rats. Changes in occupancy of these epigenetic marks return 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 NELF-E subunit to decoy the NELF complex away from the *Arc* promoter and allow Pol 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) is increased in the amygdala of AIE adult rats ($p<0.001$) 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 EPM and LBD (Figure 4A) in these rats 24 hrs after a single siRNA infusion in separate batches of rats. *Kdm6b* siRNA infusion into the CeA provoked anxiety-like behavior in the EPM as demonstrated by significant ($p<0.001$) decrease in percentage of open arm entries and percent of time spent in the open arm compared to rats infused with vehicle (iFect solution) or control siRNA (Figure 4B). There were no differences in closed arm entries as a measure of general activity of rats. Due to the lack of effect of the control siRNA, we did not test this group in subsequent experiments. *Kdm6b* siRNA infusion additionally leads to increased time spent in the dark compartment ($p<0.001$) and decreased time spent in the light compartment ($p<0.001$) in the LBD compared to vehicle-infused AIS rats without altering general activity (total ambulation) (Figure 4C). These data suggest that *Kdm6b* siRNA infusion 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 produces significantly ($p<0.01$) decreased *Kdm6b* mRNA levels in the amygdala, but no change in *Cbp* and *Kdm6a* mRNA levels, compared to vehicle-infused controls (Figure S5A). We additionally performed confocal microscopy in control rats to

colocalize KDM6B protein within the CeA, MeA, and BLA with NeuN and GFAP and found that KDM6B is predominately colocalized with NeuN, but not GFAP, in the CeA, MeA, and BLA (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 & mRNA transcription via epigenetic changes at the SARE site

We examined whether *Arc* eRNA transcripts were altered by *Kdm6b* siRNA infusion. Both the plus and minus strand transcripts of *Arc* eRNA were significantly ($p<0.05$) decreased in the amygdala of *Kdm6b* siRNA-infused rats (Figure 4D). Additionally, *Arc* mRNA, but not *Bdnf* exon IV and *Syp* mRNA (Figure S5A), was decreased ($p<0.01$) in the amygdala of *Kdm6b* siRNA-infused rats (Figure 4D). We next performed ChIP followed by qPCR for H3K27me3, KDM6B, CBP, and H3K27ac occupancy at the *Arc* SARE site and promoter in rats infused with *Kdm6b* siRNA directly into the CeA. *Kdm6b* siRNA infusion into the CeA leads to significantly increased ($p<0.01$) H3K27me3 occupancy and decreased ($p<0.05$ – 0.01) KDM6B, CBP, and H3K27ac occupancy at the *Arc* SARE site in the amygdala compared to vehicle-exposed rats (Figure 4E). A similar pattern of epigenetic modifications was observed at the *Arc* promoter site (Figure 4F).

Due 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 ($p<0.05$) NELF-E occupancy at the *Arc* promoter region, corresponding with decreased *Arc* eRNA and mRNA expression, in *Kdm6b* siRNA-infused rats as compared with vehicle-infused rats (Figure 4G). We used the same primers (Figure S6A) used to amplify ChIP pulldown from KDM6B and CBP that we used to amplify the *Arc* eRNA (–) RNA product to determine whether the epigenetic changes seen at the SARE site might be solely responsible for regulating the *Arc* eRNA (–) transcript in our experiments (Figure S6B). We did not find any alterations in KDM6B and CBP occupancy at the *Arc* eRNA (–) transcribed region (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 two days. On the third day (approximately 16 hrs 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 ($p<0.001$) and increased percentage of time spent in the dark compartment ($p<0.001$) compared to 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 when compared to control LNA-infused rats ($p < 0.05$; 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 (Figure S7A), transcription was significantly decreased ($p < 0.001$) in the amygdala of rats infused with *Arc* eRNA (-) LNA compared to 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 ($p < 0.01$) increase in NELF-E occupancy at the *Arc* promoter region in the amygdala compared to 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 as well as 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 (Figure S7B–D), suggesting that the inhibition of *Arc* eRNA (-) by the LNA oligonucleotide occurs post-transcriptionally.

DISCUSSION

Here, 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 suggests that epigenetic reprogramming due to deficits in KDM6B suppresses eRNA transcription and possibly involved in the risk for anxiety due to adolescent alcohol exposure. It also provides 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 relationship between increased anxiety risk and early-life alcohol consumption (6, 38, 39). Patients diagnosed with 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 upon 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 CeA indicates that this transcript is crucial for the expression of affective states via chromatin remodeling, thus 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 BDNF action, as loss of KDM6B prevents BDNF-induced gene induction in cerebellar granule neurons (CGNs) (44). Importantly, several other genes involved in synaptic plasticity are also decreased, possibly due to globally condensed chromatin architecture in the amygdala after AIE. Previously, we have shown that AIE treatment causes increased histone deacetylase 2 (HDAC2) levels and decreased levels of a neuron-specific lysine-specific demethylase 1 (LSD1) 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, HDAC2, CBP, and LSD1 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 utilize for AUD drug development (46, 47).

Enhancer RNAs were first discovered in large sequencing datasets and originally characterized as transcriptional noise or simply byproducts 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 Pol II can begin actively transcribing *Arc* mRNA *in vitro* in neuronal culture cells (18). Our *in vivo* data in the CeA supports 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

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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 while others simply have transcription-dependent functions, meaning that the act of transcribing the eRNA itself may be important for subsequent downstream activities such as transcription factor (TF) 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 due 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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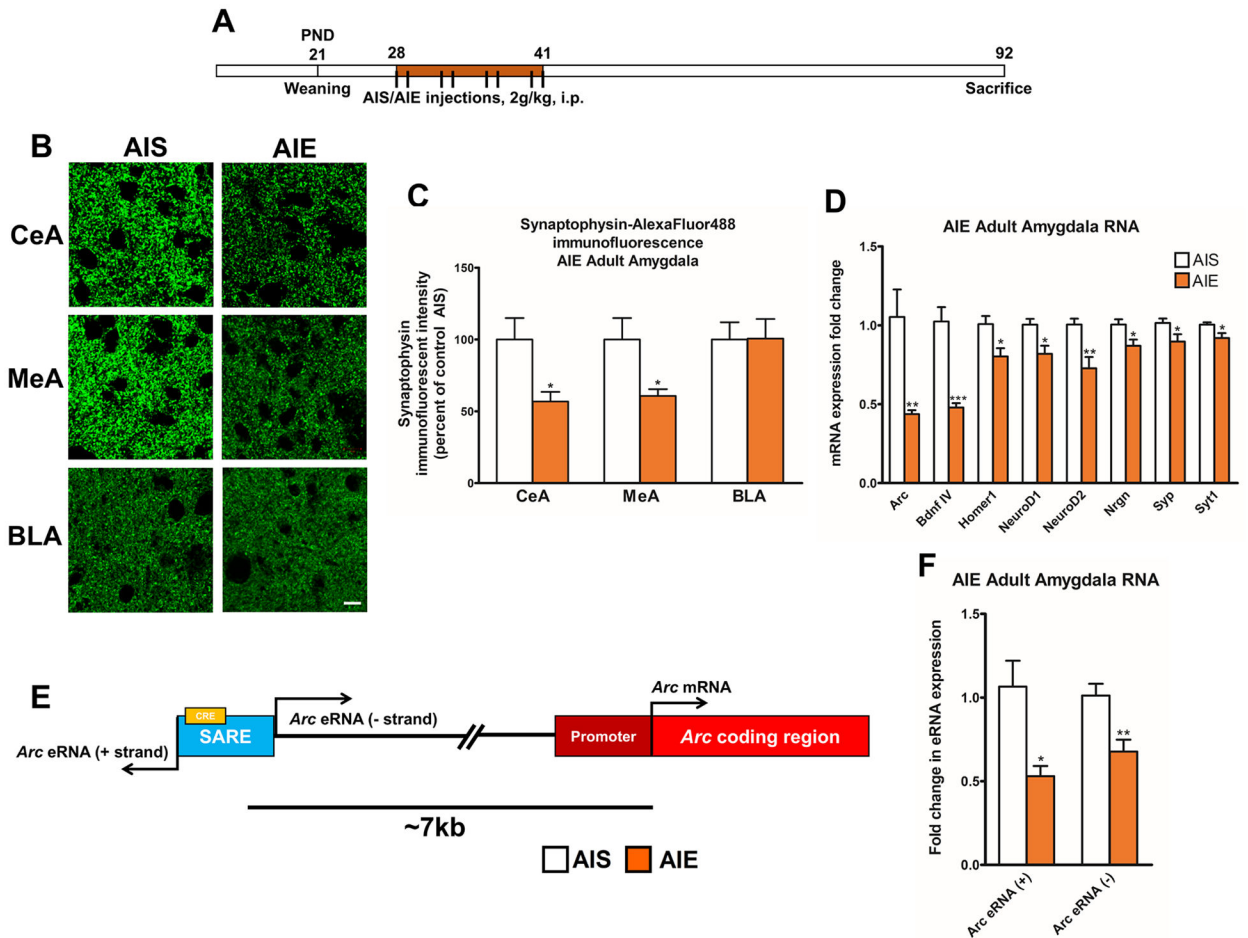


Figure 1. Adolescent intermittent ethanol (AIE) exposure leads to synaptic deficits and decreased *Arc* eRNA and mRNA expression in the amygdala at adulthood.

A) Schematic of AIE (or adolescent intermittent saline [AIS]) treatment.

B) Representative photographs showing synaptophysin (Syp)-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 μ m).

C) Bar diagram showing quantification of Syp-immunofluorescent staining. Data represent mean \pm SEM [n = 5 rats in each group, CeA ($t(8) = 2.65$, $*p < 0.05$) and MeA ($t(8) = 2.51$, $*p < 0.05$) by Student's 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 \pm SEM derived from n = 5–8 rats in each group [*Arc*, $t(9) = 3.85$, $**p < 0.01$; *Bdnf* exon IV, $t(9) = 6.21$, $***p < 0.001$; *Homer1*, $t(11) = 2.79$, $*p < 0.05$; *NeuroD1*, $t(11) = 2.81$, $*p < 0.05$; *NeuroD2*, $t(11) = 3.27$, $**p < 0.01$; *Nrgn* (neurogranin), $t(11) = 2.58$, $*p < 0.05$; *Syp*, $t(14) = 2.18$, $*p < 0.05$; *Syt1* (synaptotagmin 1), $t(14) = 2.46$, $*p < 0.05$ by Student's t -test].

E) Schematic representation of the *Arc* gene, including the synaptic activity response element (SARE) site, which contains a cAMP response element (CRE) for CREB binding and transcribes enhancer RNA (eRNA) transcripts.

F) RNA analysis of *Arc* eRNA expression in the amygdala of AIS and AIE adult rats at baseline. Data represent mean \pm SEM derived from n=5–6 rats in each group [*Arc* eRNA (+) strand, $t(9) = 2.98$, $p < 0.05$]; *Arc* eRNA (-) strand, $t(9) = 5.35$, $p < 0.01$) by Student's *t*-test].

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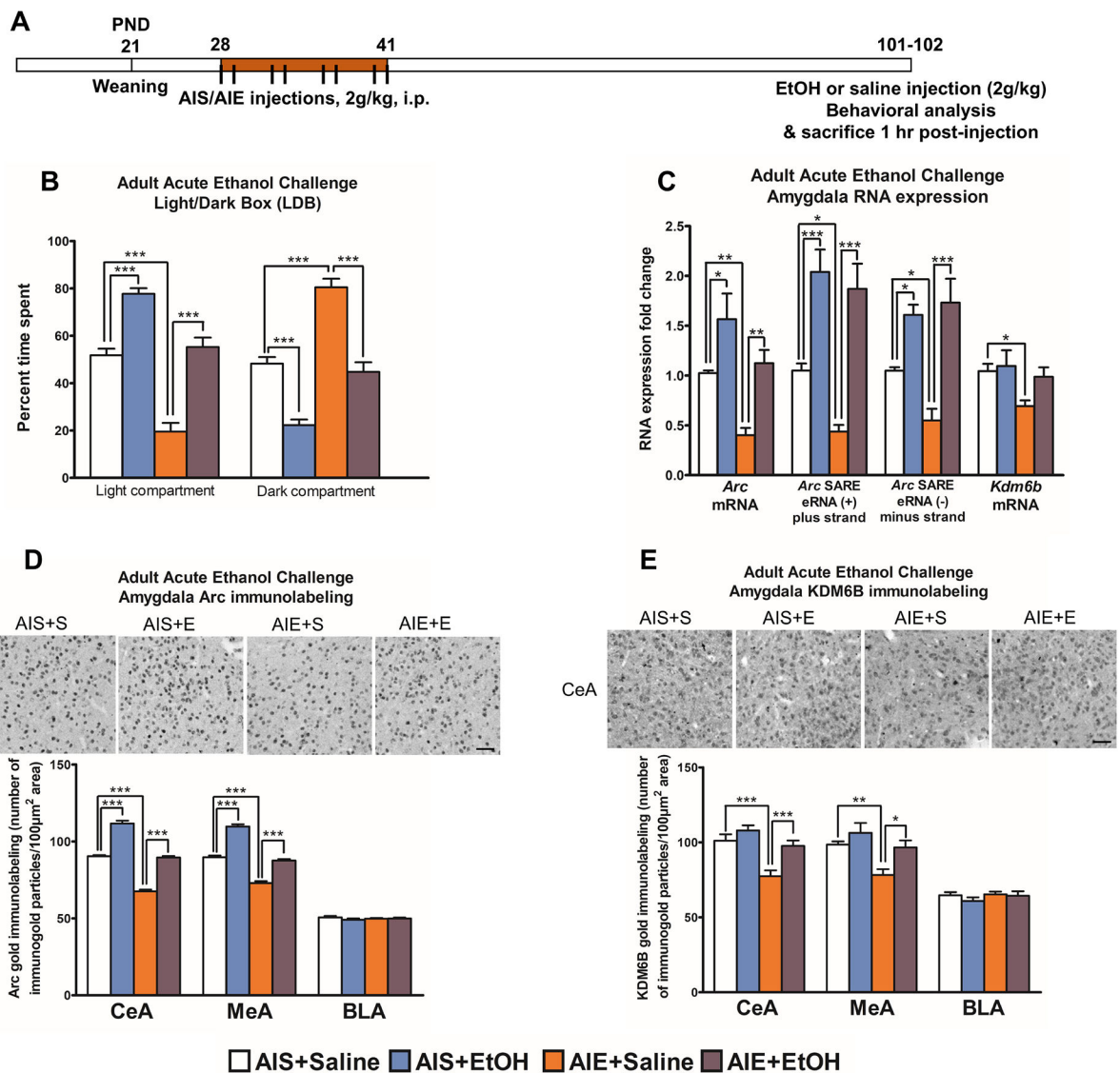


Figure 2. Adult ethanol challenge reverses AIE-induced anxiety-like behaviors and KDM6B and Arc expression deficits in the adult amygdala.

A) Schematic of adolescent intermittent ethanol (AIE) or saline (AIS) treatment followed by adult exposure to acute ethanol challenge (2 g/kg, intraperitoneal injection).

B) Light/dark box (LDB) exploration test for anxiety measures in AIS and AIE adult rats exposed to an acute challenge of either ethanol or saline. Data represent mean \pm SEM derived from $n=9-10$ rats in each group. *** $p<0.001$ by two-way ANOVA [main effect of both AIE treatment ($F_{1,33}=67.3$, $p<0.001$) and acute ethanol exposure ($F_{1,33}=85.4$, $p<0.001$)] followed by post-hoc Tukey test.

C) RNA analysis of Arc enhancer RNA (eRNA), Arc mRNA, and Kdm6b mRNA transcripts in the amygdala of rats exposed to either AIS or AIE exposed to acute ethanol or saline in adulthood. Data represent mean \pm SEM derived from $n=5-6$ rats in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by two-way ANOVA [Arc mRNA levels, AIE treatment ($F_{1,20}=12.6$, $p<0.01$), acute alcohol exposure ($F_{1,20}=17.8$, $p<0.001$); Arc eRNA (+), AIE exposure

($F_{1,20}=4.93$, $p<0.05$), acute alcohol exposure ($F_{1,20}=47.3$, $p<0.001$); *Arc* eRNA (-), acute alcohol exposure ($F_{1,19}=35.0$, $p<0.001$), interaction between AIE treatment and acute alcohol exposure ($F_{1,19}=4.44$, $p<0.05$), *Kdm6b* mRNA levels, AIE treatment ($F_{1,20}=4.90$, $p<0.05$) followed by post-hoc Tukey test.

D) Representative photomicrographs of gold immunolabeling showing Arc-positive cells in the central nucleus of amygdala (CeA) and quantification of gold-immunolabeling for Arc protein 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 \pm SEM (n=5–6 rats in each group). *** $p<0.001$ by two-way ANOVA [CeA, AIE exposure ($F_{1,19}=349.1$, $p<0.001$), acute ethanol challenge ($F_{1,19}=327.1$, $p<0.001$); MeA, AIE exposure ($F_{1,19}=272.0$, $p<0.001$), acute ethanol challenge ($F_{1,19}=217.3$, $p<0.001$)] 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 \pm SEM derived from n=6 rats in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by two-way ANOVA [CeA, AIE treatment ($F_{1,20}=19.9$, $p<0.001$), acute ethanol exposure ($F_{1,20}=12.4$, $p<0.01$), MeA, AIE exposure ($F_{1,20}=10.5$, $p<0.01$), acute ethanol exposure ($F_{1,20}=8.00$, $p<0.05$)] followed by post-hoc Tukey test.

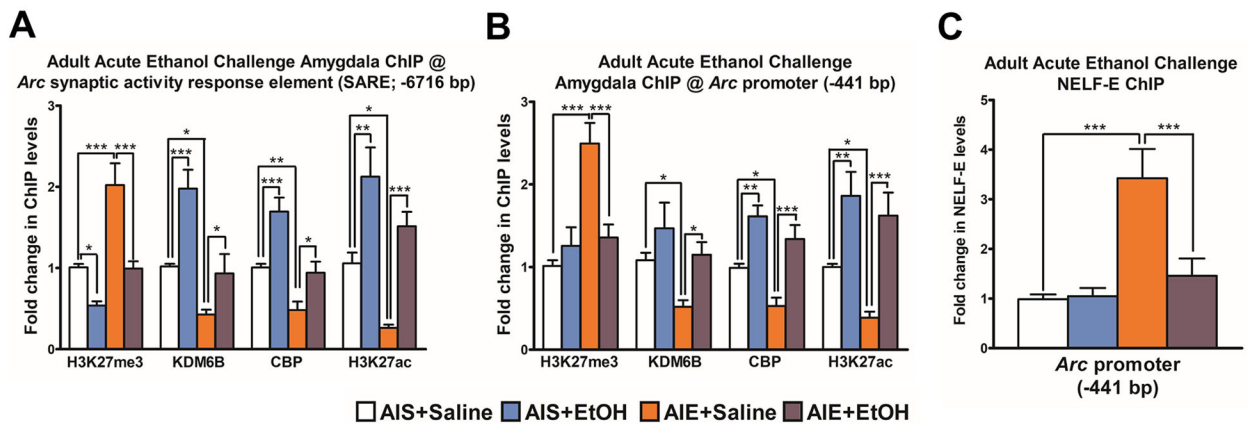


Figure 3. Adult ethanol challenge reverses AIE-induced chromatin remodeling at the *Arc* SARE site and promoter.

A) Chromatin immunoprecipitation (ChIP) analysis of H3K27me3, KDM6B, CREB binding protein (CBP) and acetylated H3K27 (H3K27ac) at the synaptic activity response element (SARE) site of the *Arc* gene in the amygdala of adolescent intermittent ethanol (AIE) or saline (AIS) adult rats exposed to acute ethanol or saline. Data represent mean \pm SEM derived from $n=5-6$ rats in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by two-way ANOVA [H3K27me3 occupancy, AIE treatment ($F_{1,20}=25.5$, $p<0.001$), acute ethanol exposure ($F_{1,20}=26.6$, $p<0.001$); KDM6B occupancy, AIE treatment ($F_{1,18}=27.8$, $p<0.001$), acute ethanol exposure $F_{1,18}=22.3$, $p<0.001$); CBP occupancy, AIE treatment ($F_{1,19}=25.3$, $p<0.001$), acute ethanol exposure ($F_{1,19}=20.5$, $p<0.001$); H3K27ac occupancy, $F_{1,19}=10.2$, $p<0.01$), acute ethanol exposure ($F_{1,19}=27.8$, $p<0.001$) 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 of AIE adult rats exposed to acute ethanol or saline. Data represent mean \pm SEM derived from $n=5-8$ rats in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by two-way ANOVA (H3K27me3, AIE treatment ($F_{1,24}=17.7$, $p<0.001$), acute ethanol exposure ($F_{1,24}=5.7$, $p<0.05$), AIE x acute ethanol interaction ($F_{1,24}=13.5$, $p<0.01$); KDM6B, AIE treatment ($F_{1,26}=5.3$, $p<0.05$), acute ethanol exposure ($F_{1,26}=7.0$, $p<0.05$); CBP, AIE treatment ($F_{1,19}=8.9$, $p<0.01$), acute ethanol exposure ($F_{1,19}=33.6$, $p<0.001$); H3K27ac, AIE treatment ($F_{1,19}=4.7$, $p<0.05$), acute ethanol exposure ($F_{1,19}=28.3$, $p<0.001$)] 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 of AIE adult rats exposed to acute ethanol or saline in adulthood. Data represent mean \pm SEM ($n=6$ rats in each group). *** $p<0.001$ by two-way ANOVA [AIE treatment ($F_{1,20}=16.2$, $p<0.001$), acute ethanol exposure ($F_{1,20}=7.2$, $p<0.05$), and the interaction between the two factors ($F_{1,20}=8.2$, $p<0.05$)] followed by post-hoc Tukey test.

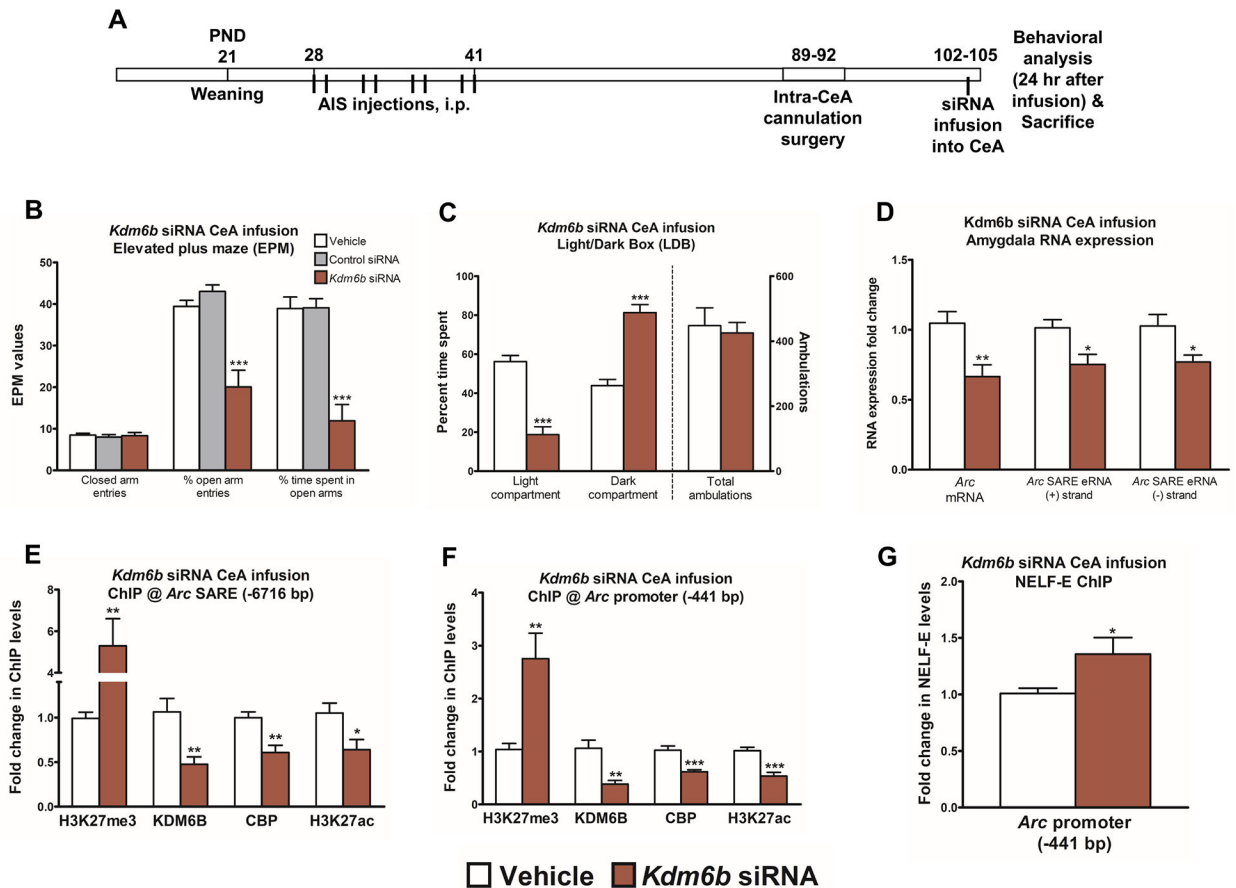


Figure 4. *Kdm6b* siRNA infusion into the CeA provokes anxiety-like behavior and decreases expression of *Arc* eRNA and mRNA.

A) Schematic representation of *Kdm6b* siRNA infusion directly into the central nucleus of amygdala (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 \pm SEM (n=6 rats in each group). *** p <0.001 by one-way ANOVA [percentage of open arm entries ($F_{2,15}=21.8$, p <0.001) and percent of time spent in the open arm ($F_{2,15}=26.3$, p <0.001)] followed by post-hoc Tukey test.

C) Light/dark box (LDB) exploration test for anxiety-like behaviors in adult control rats infused with *Kdm6b* siRNA or vehicle (iFect solution) in the CeA. Data represent mean \pm SEM (n=8 rats in each group). *** p <0.001 by Student's t -test [time spent in the dark compartment ($t(14)=-7.25$, p <0.001), time spent in the light compartment ($t(14)=7.25$, p <0.001)].

D) RNA analysis of *Arc* enhancer RNA [eRNA (+) and (-) 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 \pm SEM (n=7–8 rats in each group). * p <0.05, ** p <0.01 by Student's t -test [eRNA plus ($t(14)=2.82$, p <0.05) and minus strand ($t(14)=2.69$, p <0.05) transcripts of *Arc* eRNA; *Arc* mRNA ($t(12)=3.25$, p <0.01)].

E) Chromatin immunoprecipitation (ChIP) analysis of H3K27me3, 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 \pm SEM (n=7–8 rats in each group). * p <0.05, ** p <0.01, *** p <0.001 by Student's *t*-test [H3K27me3 occupancy (t (13) = -3.08, p <0.01), KDM6B (t (13) = 3.54, p <0.01), CBP (t (14) = 3.77, p <0.01), and H3K27ac (t (14) = 2.59, p <0.05) occupancy].

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 \pm SEM (n=7–8). ** p <0.01, *** p <0.001 by Student's *t*-test [H3K27me3 occupancy (t (13) = -3.26, p <0.01), KDM6B (t (13) = 4.21, p <0.01), CBP (t (14) = 4.56, p <0.001), and H3K27ac (t (14) = 5.03, p <0.001) occupancy].

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 \pm SEM (n=7–8 rats in each group). * p <0.05 by Student's *t*-test [t (13) = -2.39, p <0.05].

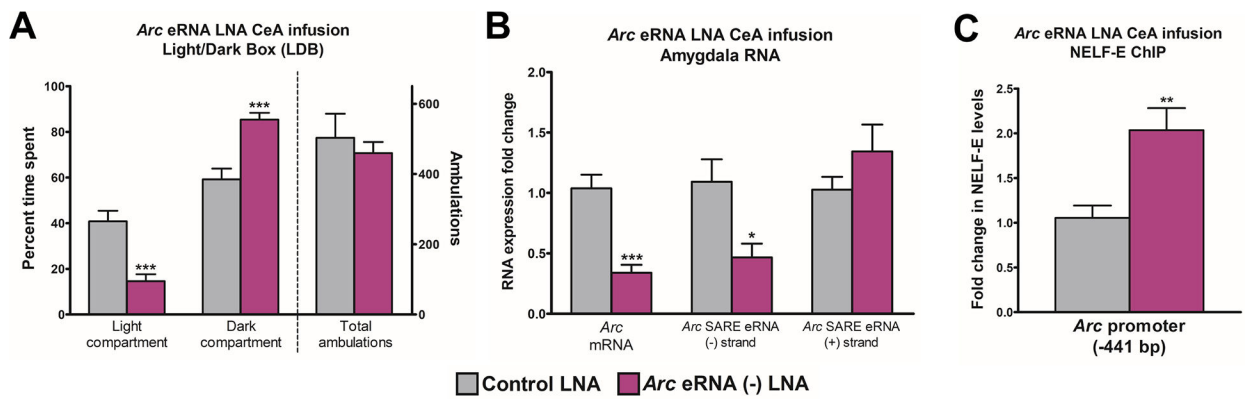


Figure 5. *Arc* eRNA (-) LNA oligonucleotide infusion into the CeA provokes anxiety-like behaviors and decreases *Arc* mRNA in alcohol-naïve rats.

A) Light/dark box (LDB) exploration test for anxiety-like behaviors in adult alcohol-naïve control rats infused with a locked nucleic acid (LNA) anti-sense oligonucleotide directed toward the minus strand of the *Arc* enhancer RNA [*Arc* eRNA (-)] or control LNA in the central nucleus of amygdala (CeA). Data represent mean \pm SEM (n=7 rats in each group). *** p <0.001 by Student's *t*-test [percentage of time spent in the light compartment ($t(12) = 4.69$, p <0.001), percentage of time spent in the dark compartment ($t(12) = -4.69$, p <0.001)].

B) RNA analysis in the amygdala of rats infused with *Arc* eRNA (-) LNA in the CeA. Data represent mean \pm SEM (n=6–7 rats in each group), * p <0.05, *** p <0.001 by Student's *t*-test [*Arc* eRNA (-) strand mRNA ($t(12) = 2.88$, p <0.05), *Arc* mRNA ($t(12) = 5.31$, p <0.001)].

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 \pm SEM (n=7 rats in each group). ** p <0.01 by Student's *t*-test [($t(12) = -3.45$, p <0.01)].

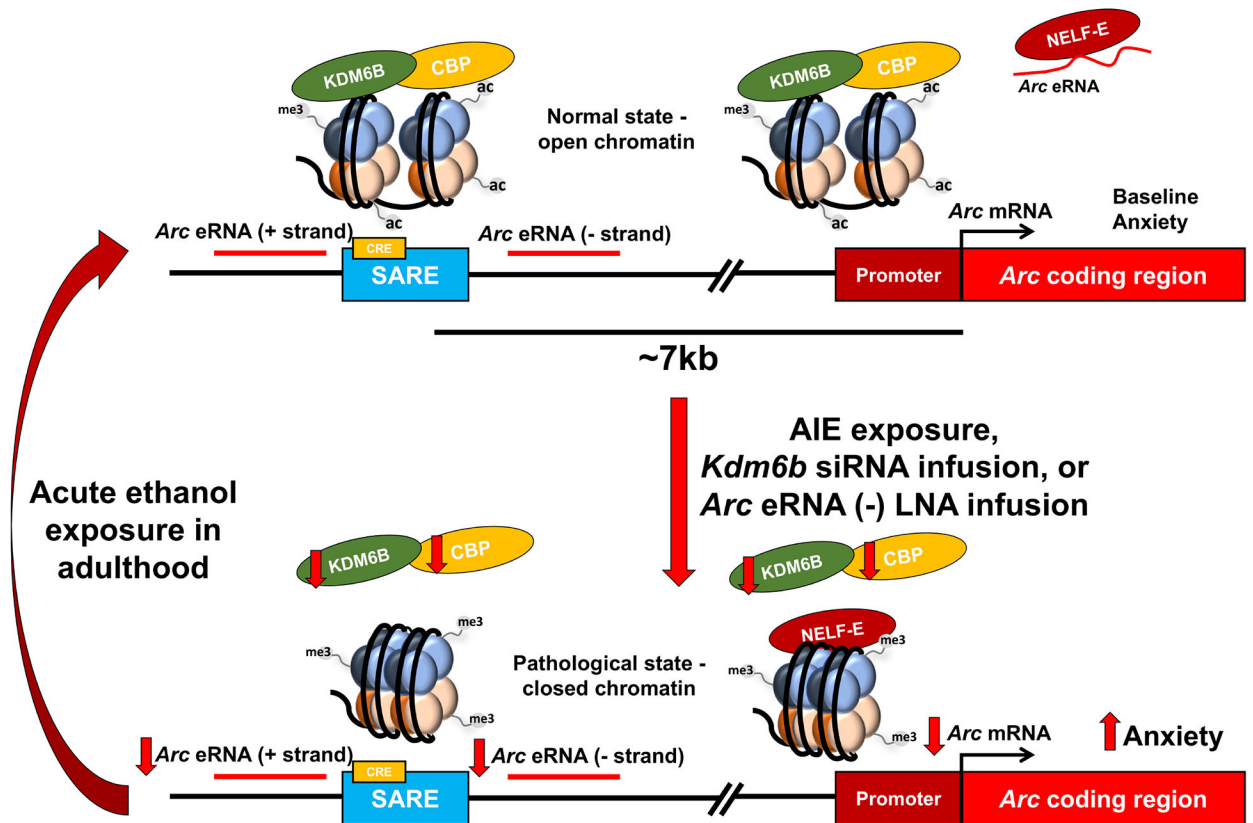


Figure 6. Adolescent intermittent ethanol (AIE) leads to adult anxiety susceptibility via epigenetic reprogramming at the *Arc* synaptic activity response element (SARE) site and decreased *Arc* enhancer RNA (eRNA) in the amygdala.

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 negative elongation factor (NELF) binding at the *Arc* promoter, and decreased *Arc* mRNA expression. These behavioral and epigenetic changes return to control-like levels after acute ethanol challenge in AIE adult rats. Notably, both *Kdm6b* siRNA infusion or locked nucleic acid (LNA) knockdown of *Arc* eRNA (-) strand directly in the central nucleus of amygdala leads to marked anxiety-like behavior, reduced *Arc* eRNA and mRNA expression, and increased NELF 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.