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Persistence of cerebellar ataxia during chronic ethanol exposure is associated with epigenetic upregulation of *Fmr1* gene expression in rat cerebellum

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Abstract

Background: Alcohol intoxication produces ataxia by affecting the cerebellum which coordinates movements. Fragile X Mental Retardation (FMR) Protein is a complex regulator of RNA and synaptic plasticity implicated in Fragile X-associated Tremor /Ataxia Syndrome which features ataxia and increased *Fmr1* mRNA expression resulting from epigenetic dysregulation of FMRP. We recently demonstrated that acute ethanol-induced ataxia is associated with increased cerebellar *Fmr1* gene expression via histone modifications in rats, but similar behavioral and molecular changes following chronic ethanol exposure are unknown. Here, we investigated the effects of chronic ethanol exposure on ataxia and epigenetically regulated changes in *Fmr1* expression in the cerebellum.

Methods: Male adult Sprague-Dawley rats were trained on the accelerating rotarod and then fed with chronic ethanol or control Lieber-DeCarli diet while undergoing periodic ataxia behavior testing during ethanol exposure and withdrawal. Cerebellar tissues were analyzed for gene expression of the *Fmr1* gene and its targets using real-time quantitative polymerase chain reaction. The epigenetic regulation of Fmr1 gene was also investigated using chromatin immunoprecipitation assay.

Results: Ataxia behavior as measured by accelerating rotarod behavioral test developed during chronic ethanol treatment and persisted at both 8h and 24h withdrawal time-points when compared with control diet-fed rats. In addition, chronic ethanol treatment resulted in upregulated expression of *Fmr1* mRNA as well as increased activating epigenetic marks H3K27 acetylation and H3K4 trimethylation at two sites within the *Fmr1* promoter. Finally, we measured expression of relevant FMRP mRNA targets in the cerebellum and found that chronic ethanol upregulated cAMP response element binding (CREB) *Creb1*, *Psd95, Grm5,* and *Grin2b* mRNA expression without altering *Grin2a, Eaa1* or histone acetyltransferases CREB binding protein (*Cbp*) or *p300* mRNA transcripts.

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Conclusions: These results suggest that epigenetic regulation of *Fmr1* and subsequent FMRP regulation of target mRNA transcripts constitute neuroadaptations in the cerebellum that may underlie persistence of ataxic behavior during chronic ethanol exposure and withdrawal.

Keywords

Epigenetics; Ethanol; Ataxia; Cerebellum; FMR1

INTRODUCTION

Alcohol use disorder (AUD) is a chronic medical condition that can lead to persistent and debilitating motor deficits including ataxia (Scholz et al., 1986; Sullivan and Pfefferbaum, 2005). The cerebellum is crucial in mediating ethanol-induced motor disturbances, and ataxia is a consistent physical manifestation found in both ethanol intoxication as well as in AUD patients abstinent from ethanol (Dar, 2015; Scholz et al., 1986; Sullivan and Pfefferbaum, 2005). Despite the significance of the cerebellum in mediating ethanol-induced ataxia, the cellular and molecular mechanisms underlying ataxia remain poorly understood and most preclinical studies focus on acute ethanol-induced ataxia (Dar, 2015; Dulman et al., 2019; Auta et al., 2017). Further mechanistic comprehension of ethanol-induced ataxia will allow for better screening for AUD development susceptibility due to innately low sensitivity to ethanol-induced ataxia (Kaplan et al., 2013) and better treatment plans for AUD patients struggling with motor deficits as a consequence of excessive long-term alcohol drinking (Baker et al., 1999).

The chronic ethanol drinking behavior of AUD patients frequently leads to the development of common clinical symptoms of cerebellar dysfunction such as ataxia and gait disturbance (Jaatinen and Rintala, 2008). Structurally, magnetic resonance imaging studies have revealed that AUD patients with ataxia have gray matter cerebellar hemisphere volume deficits, while those with Korsakoff's syndrome have both gray and white matter cerebellar volume deficits and worse ataxia (Sullivan et al., 2000). Korsakoff's syndrome is a progression of untreated Wernicke's encephalopathy common in AUD patients wherein deficiency of thiamine produces neurological symptoms including confusion, amnesia, nystagmus, and ataxia (Baker et al., 1999; Zahr et al., 2011). Additional factors besides thiamine deficiency leading to ethanol-induced cerebellar degeneration include alterations in gene expression of neurotrophin and neurotransmitter systems, excitotoxicity, oxidative stress, mitochondrial damage and apoptosis (Jaatinen and Rintala, 2008).

Recently, epigenetics has become increasingly relevant to our understanding of the mechanisms underlying changes in gene expression during exposure to drugs of abuse including alcohol (Pandey et al., 2017; Renthal and Nestler, 2008). Epigenetic mechanisms provide a biological basis for the environmental influence on the development of psychiatric disorders since gene expression can be altered by the environment and thereby induce functional consequences (Kouzarides, 2007; Bohnsack and Pandey, 2021). Our lab has consistently shown that exposure to ethanol can alter chromatin architecture via histone acetylation changes with subsequent effects on both gene expression and behavior (Pandey et al., 2017; Bohnsack and Pandey, 2021). Specifically, we have shown that withdrawal from

chronic ethanol exposure consistently results in marked anxiety-like behavior concomitant with decreased histone acetylation and CREB (cAMP-response element binding) binding protein (CBP) expression in the amygdala (Pandey et al., 2008). Such chromatin remodeling via CREB pathways provides a biological basis for the negative affective states seen in AUD (Asher et al., 2002; Pandey et al., 2017). Furthermore, CREB is known to regulate ethanol sensitivity in the mouse cerebellum (Acquaah-Mensah et al., 2006) and phosphorylated CREB is decreased in the rat cerebellum following chronic ethanol exposure (Yang et al., 1998), yet comprehensive behavioral effects of the cerebellar changes to chronic ethanol exposure are poorly characterized.

We focus on the FMR1 gene, since it is a well-known epigenetic target which, when altered, yields functional consequences in humans and because of its roles in synaptic regulation and an ataxic clinical syndrome (Hagerman, 2013). Fragile-X Mental Retardation Protein (FMRP) is a complex regulator of RNA that shapes synaptic protein expression and dendritic spine density by binding important glutamatergic transcripts including a subtype of metabotropic glutamate receptor, *Grm5*, ionotropic glutamate N-Methyl D-Aspartate (NMDA) receptor subunits *Grin2a* and *Grin2b,* and post-synaptic density scaffolding protein, *Psd95* (Ascano et al., 2012; Darnell et al., 2011; Zalfa et al., 2007). FMRP can affect translation by either stalling or stabilizing bound mRNA transcripts to prevent or initiate synthesis of new proteins vital for synaptic plasticity (Todd et al., 2003; Zalfa et al., 2007). FMRP also can bind many epigenetic targets including histone acetyltransferases *Cbp* and *P300* that were identified in the two most comprehensive characterizations of FMRP's mRNA targets (Ascano et al., 2012; Darnell et al., 2011).

Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) results from a pre-mutation condition with 55–200 CGG trinucleotide repeats at the FMR1 promoter, and is characterized by both increased *FMR1* mRNA expression and cerebellar ataxia and tremor (Hagerman and Hagerman, 2013; Hagerman, 2013; Jacquemont et al., 2003). FXTAS patients have altered cortical and cerebellar expression of the FMRP target *Grm5* (Lohith et al., 2013; Pretto et al., 2014). Another glutamatergic FMRP target, *Eaa1* is the main cerebellar excitatory amino acid transporter and is implicated in hereditary ataxia (Choi et al., 2017). Moreover, alcohol misuse is known to underlie earlier onset and greater severity of FXTAS, possibly due to cerebellum-specific effects (Dorn et al., 1994; Kogan et al., 2008). Given this information regarding FXTAS, we characterized the role of cerebellar FMRP in the context of acute ethanol-induced ataxia, where we showed that ethanol-induced ataxic behavior may be partially mediated by epigenetic regulation of *Fmr1* expression and subsequent stabilization of target glutamatergic transcripts (Dulman et al., 2019). Here, we extended these studies by investigating the effects of chronic ethanol exposure on ataxic behavior and epigenetic regulation of *Fmr1* expression in the cerebellum.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN). Rats were maintained on a 12:12 hour light/dark cycle and were given *ad libitum* access to food and water until liquid diet procedures were initiated. Rats arrived at Post-natal Day (PD) 80

and were allowed 10 days to acclimate to the facility. Animals were group-housed during acclimation and then single-housed for chronic ethanol diet feeding (Pandey et al., 2008). Rats underwent comprehensive motor behavior testing throughout the chronic diet procedure and they were habituated to and trained on the rotarod before liquid diet and single housing began. A separate cohort of rats underwent only chronic ethanol diet treatment for biochemistry experiments without behavioral measurements featuring a control diet-fed group and two ethanol diet-fed groups: one with 0h withdrawal (ethanol on board) and the other with 24h withdrawal from ethanol. All animal procedures followed Institutional Animal Care and Use Committee Guidelines and NIH directives for the Care and Use of Laboratory Animals.

Measurement of ataxia using rotarod test

Rats were trained on the accelerating rotarod (IITC Life Science ,Woodland Hills, CA) for 3 days starting at PD90 with 3 trials of 180 seconds each day, as previously described (Dulman et al., 2019). Briefly, the rotarod cylinder apparatus is 9.5 cm in diameter and 15 cm wide; the rotarod starts spinning at 5 rotations per minute and accelerates to 20 rotations per minute over the course of the full 180-second trial. Latency to fall is recorded for every trial and is the primary outcome measure. Time spent on the rod is calculated as an average latency to fall across the three trials measured within each rotarod test session. Rats were tested at the same time every day at 10AM, with the exception of the acute withdrawal 8-hour timepoint when rats were tested at 6PM. Schematic diagram of periodic rotarod testing and chronic diet procedure is shown in Figure 1. During training, rats were placed back on the rotarod apparatus if they fell off before the full trial was completed. By the end of training, all rats were able to remain on the rod for the entire 180 second rotarod session in nearly every trial. Once the rats were trained, we controlled for motor behavior “overtraining” (Luong et al., 2011; Scholz et al., 2015; Tung et al., 2014) by only conducting rotarod testing every three days throughout the chronic diet procedure.

Chronic ethanol exposure procedure

Rats were offered 80 mL of nutritionally complete Lieber-DeCarli diet 82 (Bio-Serv, Frenchtown, NJ) as their only source of food and fluid while being single-housed, as previously described (Pandey et al., 2008; You et al. 2014). Rats were subdivided into control and ethanol groups. The control group received control liquid diet throughout the entire study. After 3 days of access to this control diet, rats from the ethanol group were gradually habituated to a liquid diet containing ethanol that increased in concentration from 1.8% to 9% over the course of 7 days and then maintained on 9% ethanol diet for 15 days. On the final day, ethanol-fed rats were given control liquid diet to induce a state of acute withdrawal. For the ataxic behavior measurements, only two groups (ethanol and control) were used since the repeated measures behavioral testing paradigm allows for assessment at multiple withdrawal time-points such that multiple ethanol groups are not needed. For the biochemistry experiments, a three-group design was employed as previously reported by us (Pandey et al. 2008).

Cerebellum brain tissue collection

At the end of the chronic ethanol diet procedure, animals were decapitated under isoflurane anesthesia. Brain tissue, specifically the whole cerebellum, was quickly dissected, frozen and kept at −80°C until used for biochemical studies. The whole cerebellum was crushed on dry ice with a mortar and pestle; aliquots of pulverized cerebellar tissue were used for parallel mRNA quantification and chromatin immunoprecipitation analyses.

Blood collection

Blood samples were collected from the lateral tail vein following completion of the rotarod testing session 11 and session 14. Following rotarod session 16 at 24-hour withdrawal, rats were sacrificed, and trunk blood was collected. Blood samples were kept on ice and spun down to collect plasma for measurement of blood ethanol concentrations (BECs) using the AM1 Alcohol Analyzer (Analox, Lunenberg, MA).

mRNA quantification by real-time PCR

Total RNA was isolated from rat cerebellum with Trizol using the miRNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from RNA using random primers and reverse transcriptase from a Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). Quantitative real-time PCR (qPCR) was performed using a CFX Connect qPCR machine, SYBR Green master mix, and CFX Manager software for data analysis (BioRad, Hercules, CA). Primers corresponding to mRNAs for the genes of interest (Table 1) were added to the master mix, and Hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*) was utilized as a reference gene. qPCR thermal conditions were a 2-minute hold at 95 °C, followed by 40 cycles of 95°C for 5 seconds and 60 °C for 30 seconds, and finally a melt step with 5 second plate-reads for every 0.5°C increase from 65°C to 95°C. Target cDNA (*Fmr1, Creb1, Cbp, P300, Psd95, Grm5, Eaa1, Grin2a, Grin2b*) was measured alongside *Hprt1*, with every sample run in triplicate. Relative mRNA levels normalized to *Hprt1* using the ΔΔCt method (Livak and Schmittgen 2001) and results are expressed as fold change in mRNA levels compared with control diet-treated animals.

Measurement of histone acetylation or methylation occupancy at Fmr1 promoter

To examine levels of histone acetylation and methylation at specific genomic locations in the cerebellum of rats exposed to chronic ethanol, we performed chromatin immunoprecipitation (ChIP) assays using antibodies against specific histone residues as previously described (Kyzar et al., 2017). Briefly, homogenized cerebellar tissue was fixed in methanol-free formaldehyde and then DNA was sonicated. The resulting sheared DNA-chromatin complexes were immunoprecipitated with an antibody directed to acetylated H3K27 (Cell Signaling, #4353) or to trimethylated H3K4 (Cell Signaling, #9727). The precipitated DNA was then quantified using qPCR with Biorad SYBR Green master mix and primers targeting two locations within the *Fmr1* promoter (Forward: 5'-CAC GAC CTG TTA CTA CCC TTT G-3', Reverse: 5'-GGT AAC TGG GCA GAT ACT TTG-3' [site 1] and Forward: 5'- CCT CGC TTC CTC CTG TAC AA-3', Reverse: 5'-GCG CAA AGG GTA GTA ACA GG -3' [site 2]). Refer to Figure 2A for *Fmr1* gene map featuring these ChIP primer locations. Immunoprecipitated DNA levels were normalized to input DNA

levels using the ΔΔCt method to calculate fold change in the levels of H3K27 acetylation (H3K27Ac) or H3K4 trimethylation (H3K4Me3) at the *Fmr1* gene (Livak and Schmittgen, 2001).

Statistical analysis

All statistical analyses were conducted in Prism 9 (GraphPad, San Diego, CA) or SigmaStat. To assess the effects of chronic ethanol treatment on rotarod behavior, repeated measures two-way ANOVA were used with diet group as the between subjects factor and time on the rotarod as the within subjects factor. Bonferroni tests were employed for post-hoc pairwise comparisons. For correlations between blood ethanol data and rotarod behavioral performance, simple linear regressions were performed to calculate Pearson's correlation coefficient and the general linear F-statistic was calculated to assess if the slope of the linear regressions were significantly non-zero. For biochemical data, 1-way ANOVAs were conducted to assess the effect of the diet group (Control, Ethanol, and Withdrawal) when data passed tests of normality and equal variance. In a few cases, nonparametric Kruskal Wallis tests were employed due to non-normal data or unequal variances. Tukey post-hoc pairwise tests were used for parametric data whereas Dunn's tests were used for nonparametric data. A $p<0.05$ for all experiments was considered to be statistically significant.

RESULTS

Motor behavior during chronic ethanol exposure

Rotarod performance was compared at various points during the liquid diet procedure to compare motor behavior between rats fed the control diet and rats fed the ethanol diet (Figure 1A). Two-way repeated measures ANOVA analyzing rotarod behavioral performance was significant for the effect of chronic ethanol diet treatment ($F(1,10) = 19.57$; $p<0.01$), time ($F(15, 150) = 5.096$; $p<0.001$), and the interaction of treatment and time ($F(15, 150) = 9.942$; $p<0.001$). Bonferroni multiple comparisons tests for post-hoc significance between treatment groups shows that ethanol diet-fed rats did not differ from control-diet fed rats throughout rotarod training or early in the chronic diet procedure but that throughout the latter half of the chronic diet procedure, the ethanol diet-fed group consistently shows significantly impaired rotarod performance compared to the control diet-fed group including at both withdrawal time-points (Figure 1B). Blood was collected immediately following rotarod testing sessions 11, 14, and 16, and as expected, ethanol-diet fed rats have considerable blood ethanol levels after sessions 11 and 14 (Figure 1C), and negligible BECs at the 24-hour withdrawal time-point. Although the rats reached considerable blood ethanol levels during the chronic ethanol exposure, there was no correlation between blood ethanol concentration and rotarod behavioral performance for either session 11 ($r^2<0.0001$; $F(1,4) = 0.0003$; $p>0.05$) or session 14 ($r^2=0.229$; $F(1,4) = 1.19$; $p>0.05$).

Effects of chronic ethanol exposure and withdrawal on Fmr1 expression and histone modifications

The impact of the chronic ethanol exposure was evaluated for effects on *Fmr1* expression and histone modifications within the *Fmr1* promoter in the cerebellum (Figure 2A). It was found that chronic ethanol treatment significantly alters cerebellar expression of *Fmr1* mRNA ($F(2,24) = 21.58$; $p<0.001$) with both the ethanol and withdrawal groups showing significant *Fmr1* upregulation compared to the control diet-fed group (Figure 2B). We also measured occupancy of H3K27Ac and H3K4Me3 at two sites of the *Fmr1* promoter. It was found that H3K27Ac occupancy at *Fmr1* promoter site 1 is also significantly affected by chronic ethanol diet treatment ($H(2) = 13.89$, $p<0.001$) with both the ethanol and withdrawal groups showing significantly greater acetylation compared to the control diet group (Figure 2C). The occupancy of H3K27Ac at *Fmr1* promoter site 2 is significantly affected by chronic ethanol diet treatment ($H(2) = 8.074$, $p<0.05$) with only the ethanol group but not the withdrawal group showing significantly greater acetylation compared to the control diet group (Figure 2D). The occupancy of H3K4Me3 at *Fmr1* promoter site 1 is significantly affected by chronic ethanol diet treatment ($F(2,15) = 5.530$, $p<0.05$) with both the ethanol and withdrawal groups showing significantly greater H3K4Me3 levels compared to the control diet group (Figure 2E). At *Fmr1* promoter site 2, occupancy of H3K4Me3 is significantly affected by chronic ethanol diet treatment ($H(2) = 9.556$, $p<0.01$) where both ethanol and withdrawal groups show significantly greater H3K4Me3 levels compared to the control diet group (Figure 2F).

Correlation between FMR1 mRNA and histone modifications

Since mRNA expression and chromatin occupancy dynamics of *Fmr1* gene derive from the same set of animals, we performed correlation analyses of mRNA and epigenetic measures for each rat. These data show that for all four ChIP assays, the fold changes in acetylated or methylated histone protein occupancy significantly correlated with the fold changes in *Fmr1* mRNA expression [Figure 3A H3K27Ac at site 1: $r^2 = 0.3603$; $F(1,25) = 14.08$; $p<0.001$; Figure 3B H3K27Ac at site 2: $r^2 = 0.2346$; $F(1,25) = 7.661$; $p<0.05$; Figure 3C H3K4Me3 at site 1: $r^2 = 0.2331$; $F(1,16) = 4.864$; $p<0.05$; Figure 3D H3K4Me3 at site 2: $r^2 = 0.2888$; $F(1,16) = 6.496$; $p<0.05$].

Expression of FMRP mRNA targets in the cerebellum

We also profiled cerebellar mRNA expression of CREB-related histone acetyltransferase genes and select glutamatergic FMRP targets following chronic ethanol exposure that we previously examined in the context of acute ethanol exposure (Dulman et al., 2019). We found that chronic ethanol exposure alters cerebellar *Creb1* expression ($F(2,24) = 3.543$; $p<0.05$) with significant upregulation in the withdrawal group but not the ethanol group compared to the control group. There were no significant differences in *Cbp* or *P300* expression in the cerebellum following chronic ethanol exposure (Figure 4A). Furthermore, chronic ethanol treatment altered metabotropic glutamate receptor *Grm5* mRNA expression ($H(2) = 6.804$; $p<0.05$) in the cerebellum with a significant increase in *Grm5* mRNA levels in the ethanol group compared to the withdrawal group but not with the control group (Figure 4B). The expression of the major glutamate transporter in the cerebellum

Eaa1 did not change after chronic ethanol exposure and withdrawal. Next, *Psd95* expression was altered by chronic ethanol exposure ($F(2,24) = 9.503$; $p<0.001$) with the ethanol diet group having significantly greater *Psd95* expression than both control and withdrawal groups (Figure 4B). Finally, *Grin2a* expression was not affected by chronic ethanol exposure but Grin2b expression was altered by chronic ethanol exposure ($H(2) = 6.804$; $p<0.05$) with the ethanol group showing significantly upregulated *Grin2b* expression compared to the withdrawal group but not with the control group (Figure 4B).

DISCUSSION

The present study shows that chronic ethanol exposure causes persistent ataxia on the accelerated rotarod in rats during intoxication and withdrawal that is associated with epigenetic activation of *Fmr1* and alterations in glutamatergic FMRP targets and *Creb1* expression. These results suggest that epigenetic regulation of *Fmr1* expression via H3K27Ac and H3K4Me3 at the *Fmr1* promoter and subsequent FMRP regulation of target mRNA transcripts constitute key gene expression changes in the cerebellum that may underlie chronic ethanol-induced ataxic behavior (Figure 5). The upregulation of *Fmr1* mRNA expression in the cerebellum following chronic ethanol extends our previous findings following acute ethanol exposure and ataxia (Dulman et al., 2019) and agrees with several hippocampal findings that also show *Fmr1* induction by acute ethanol exposure (Wolfe et al., 2016), chronic ethanol exposure (Spencer et al., 2016), and adolescent intermittent ethanol exposure (Mulholland et al., 2018). The increased occupancy of H3K27Ac and H3K4Me3 at the *Fmr1* promoter following chronic ethanol is consistent with the increased mRNA expression as both epigenetic marks are activating and associated with increased transcription (Kouzarides, 2007). Accordingly, correlations for these epigenetic measures and the *Fmr1* mRNA expression for each animal are strong, suggesting that the greater the occupancy of active epigenetic marks of transcription, the greater transcription of the target mRNA. Our epigenetic findings also agree with the earlier studies which found that adolescent intermittent ethanol or adult acute ethanol treatment could epigenetically regulate *Fmr1* expression in the hippocampus (Mulholland et al., 2018) and the cerebellum (Dulman et al., 2019). The increased H3K4Me3 in the cerebellum following chronic ethanol exposure agrees with findings of decreased S-adenosyl-homocysteine levels and increased S-adenosyl-methionine/S-adenosyl-homocysteine ratio in the cerebellum following chronic ethanol exposure, a “methylation index” suggestive of increased methylation (Auta et al., 2017).

The behavioral data shown here represent, to our knowledge, the first preclinical findings of longitudinally diminished motor behavior representing ongoing ataxia throughout both the course of chronic ethanol treatment and during acute withdrawal after chronic ethanol exposure. Many previous studies have examined acute ethanol-induced ataxia (See Dar 2015 for review), and a few have examined ataxia in the context of chronic ethanol (da Silva et al., 2018; Jung et al., 2002; Mitra and Nagaraja, 2008; Pascual et al., 2007; Servais et al., 2005; Teixeira et al., 2014; Zimitat et al., 1990), however these studies focused on motor behavior or motor learning at the end of chronic ethanol exposure. In contrast, by using the repeated measure rotarod assessment and by training the rats in the task before any ethanol administration, we are able to track the development of motor dysfunction by examining

the ataxia throughout the course of chronic ethanol exposure. We observed that rats start to have motor behavior impairment on the rotarod by the time they start consuming 9% ethanol. The reduced rotarod performance in the ethanol group compared to the control diet group was maintained throughout the last four time-points of the exposure period and notably persisted through acute withdrawal at both 8-hour and 24-hour withdrawal time-points. This finding contradicts an earlier study that only saw motor-impairment in mice following chronic ethanol exposure in a naïve motor task, but not in a prior learned one (Servais et al., 2005). However, our behavioral results agree with another study using Wistar rats in a different motor behavioral assessment that found development of ataxia after a few weeks of chronic ethanol exposure (Zimitat et al., 1990). Since motor behavior on the rotarod is a learned task, and most of these other studies with chronic ethanol and rotarod did not pretrain animals before ethanol exposure or during ethanol exposure, it is difficult to compare behavioral results as these other experiments were designed to assess if motor learning is impaired by chronic ethanol exposure while we investigated if a learned motor behavior skill deteriorates throughout chronic ethanol exposure or during acute withdrawal. Nonetheless, the finding of ataxia during withdrawal from chronic ethanol is consistent in both preclinical and clinical literature (Dar, 2015; Servais et al., 2005; Sullivan and Pfefferbaum, 2005). Cerebellar deficits that are observed in AUD subjects even persist during abstinence (Sullivan et al, 2002; Sullivan and Pfefferbaum, 2005). Ethanol-induced cerebellar ataxia in rodents represents a relevant clinical correlate of human AUD (Hammoud and Jimenez-Shahed, 2019; Dar, 2015; Fitzpatrick et al., 2012). Furthermore, FXTAS is associated with increased *FMR1* mRNA expression and cerebellar ataxia and tremor (Hagerman and Hagerman, 2013; Jacquemont et al., 2003). These clinical findings are well supported by data presented here showing the association between ataxia during chronic ethanol exposure and increased *Fmr1* mRNA expression in the cerebellum.

Interestingly, ataxia at 24 hours into acute withdrawal is perhaps the most notable data point because there is no ethanol on board at 24-hour withdrawal and yet the rats still have significant motor impairment and increased Fmr1 mRNA expression in the cerebellum. Ataxia during withdrawal in rats greatly resembles the ataxia associated with withdrawal seen in AUD patients (Deshmukh et al., 2002; Scholz et al., 1986). At the two time-points during chronic ethanol exposure where blood was also collected, we find considerable levels of blood ethanol intoxication in the ethanol diet-fed rats. We find that the BECs do not correlate with the rotarod performance. This is perhaps an indication that neuroadaptations to the constant presence of chronic ethanol have already begun. The negligible BECs at 24-hour withdrawal concur with the results of many studies we and others have done using this alcohol treatment paradigm, but the BECs during the diet are typically higher than those we report here (Auta et al., 2017; Baldwin et al., 1991; Hunter et al., 1975; Pandey et al., 2008; You et al., 2014). These differences may be related to differences in the timing of the diet provision, the behavioral testing and blood collection. Fresh diet was provided daily at 6PM when the animals began their dark cycle, rotarod testing was conducted at 10AM, and blood was collected after rotarod testing so as not to stress the animals prior to their behavioral test; furthermore, animals did not have access to diet during rotarod testing, thereby preventing any ethanol drinking immediately before blood collection.

Ethanol produces distinct brain region-dependent transcriptional changes associated with intoxication, tolerance, and withdrawal (Contet, 2012), and findings of increased cerebellar *Fmr1* expression during acute and chronic ethanol exposure and acute withdrawal strongly suggests a role in cerebellar ataxia. Since *Fmr1* is abundantly expressed in neurons (Devys et al., 1993), the protein features many RNA-binding motifs (Anderson et al., 2016), and epigenetic modifications at the *Fmr1* promoter produces functional consequences (Hagerman and Hagerman, 2013; Jacquemont et al., 2003), induction of increased gene expression by ethanol exposure is likely to significantly alter synaptic neurotransmission. We therefore hypothesized that chronic ethanol exposure would produce similar upregulations of glutamatergic targets as we saw after acute ethanol exposure (Dulman et al., 2019), and we found similar changes suggestive of a hyperglutamatergic state in the cerebellum most likely due to increases in *Grm5, Grin2b,* and *Psd95* expression. It is interesting to point out that mRNA levels of these genes are increased during chronic ethanol exposure but normalized during withdrawal. However, ataxic behaviors were observed during ethanol exposure that persisted into withdrawal. It is possible that NMDA receptor binding sites or protein levels may be increased during ethanol exposure and remain upregulated at early phases of withdrawal as a result of neuroadaptations. This notion is supported by the findings of increased NMDA receptor binding by MK-801 but no change in NMDA receptor subunit mRNA levels in several brain regions after chronic ethanol exposure and early withdrawal in animals (Gulya et al.,1991; Grant et al.,1990; Morrow et al., 1994). Others have reported that NR2A and NR2B protein expression was increased in the hippocampus after chronic ethanol exposure but normalized during withdrawal in adult rats (Pian et al., 2010). It is also possible that cytoarchitechtonic changes in dendritic spine density that FMRP is known to dynamically regulate (Grossman et al., 2006; Mulholland et al., 2018) may lead to changes in synaptic connectivity and strength that persist longer into withdrawal than transcriptional changes (Sidorov et al., 2013; Irwin et al., 2000). The association of hyperglutamatergic neurotransmission and symptoms associated with chronic ethanol exposure is further supported by a study of human cerebrospinal fluid which found that AUD patients had a higher ratio of excitatory to inhibitory neurotransmitters compared to controls (Tsai et al., 1998). Future experiments will examine changes in mRNA and protein levels of other FMRP targets as well as dendritic spine density both within the cerebellum and across other brain regions following chronic ethanol exposure to assess if the neuronal hyperexcitability associated with withdrawal may be mediated by epigenetic induction of *Fmr1* and subsequent synaptic dysregulation of FMRP targets.

Fmr1 is a dynamic epigenetic target that can alter expression of hundreds of mRNA transcripts, especially those trafficked to the synapse and crucial for synaptic plasticity (Todd et al., 2003; Zalfa et al., 2007). In the case of the cerebellum, a recent review suggests the motoric deficit found in ethanol withdrawal results from the interplay of aberrant gene modifications via epigenetic changes with excessive glutamate release and resulting high intracellular calcium levels that overload mitochondria promoting release of cytochrome c and ultimately apoptosis (Jung, 2015). This latter consequence of cerebellar degeneration was found in preclinical chronic ethanol studies which found both ataxia and decreased numbers of cerebellar Purkinje cells, although months of forced ethanol diet was needed for such degeneration (Jung et al., 2002; Mitra and Nagaraja, 2008). Interestingly,

FMRP may indeed have a role in cell death as the protein notably regulates cell viability following rat neuron apoptosis induced by excess glutamate (Jeon et al., 2012), is involved in sensitivity to DNA damage underlying cell cycle progression (Liu et al., 2012), and even plays a part in melanoma progression (Zalfa et al., 2017). While the regulon of FMRP remains both poorly understood and astonishingly vast, the emerging impact of ethanol on *Fmr1* gene expression is enticing as it provides a substrate for incorporating the behavioral phenotypes of ethanol exposure, particularly its ataxic effects but also its anti-depressant effects (Wolfe et al., 2016), with the molecular and epigenetic changes in the brain that lead to dysregulated neural circuits characteristic of AUD. In our studies in the cerebellum, increased *Fmr1* expression is associated with acute ethanol-induced ataxia (Dulman et al., 2019) and ataxic behavior that persists during chronic ethanol exposure and acute withdrawal. Ethanol exposure and ataxia are associated with increased active transcriptional histone modifications at the *Fmr1* promoter and increased *Fmr1* expression in the cerebellum (Figure 5). These results suggest that epigenetic regulation of *Fmr1* expression and changes in FMRP target mRNA transcripts may constitute important molecular mechanisms in the cerebellum that may underlie ethanol-induced ataxic behavior. Overall, these studies further the understanding of potential epigenetic mechanisms in the cerebellum underlying ethanol-induced ataxia, illuminating gene candidate FMR1 as a potential epigenetic and neuroregulatory target for treating alcohol use disorder-associated ataxia.

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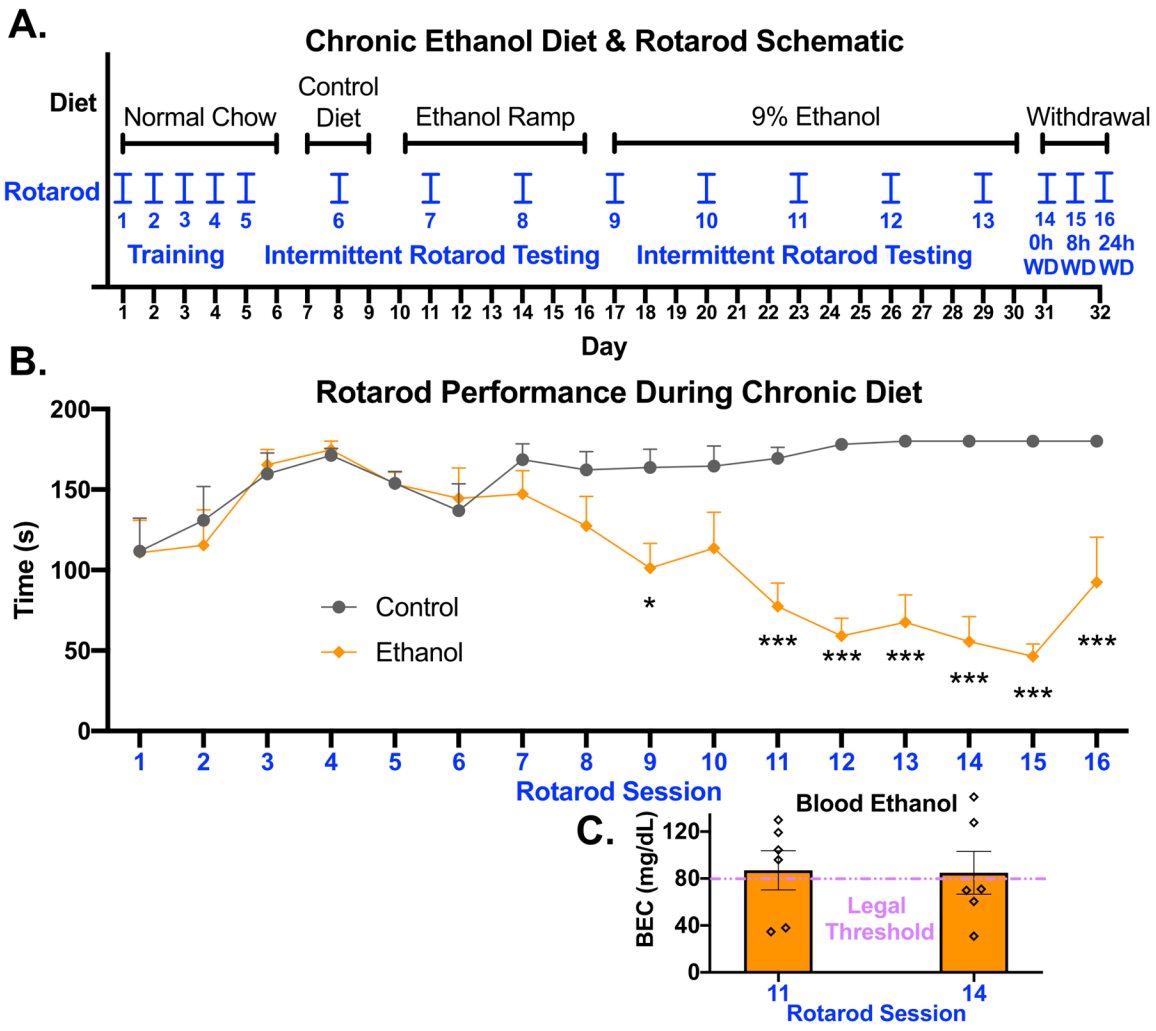

Figure 1:

This schematic for the main behavioral experiment shows the progression of both the chronic ethanol diet as well as the rotarod training and testing (A). Following rotarod training, rats initiated the chronic ethanol or control diet feeding and were subsequently tested on the rotarod every three days as well as multiple times throughout the day when ethanol was withdrawn (0hr, 8hr, and 24hr) to induce acute withdrawal (WD). Effects of chronic ethanol diet feeding on ataxia behavior measured by accelerating rotarod behavior test (B). Effects of chronic ethanol diet on blood ethanol concentrations (C). Blood was collected following rotarod test sessions 11 and 14 and used for BEC measurement. Data are represented as mean ± SEM (n = 6 per group) and individual values are shown with open circles. *p<0.05, ***p<0.001 significantly different from control diet-fed group.

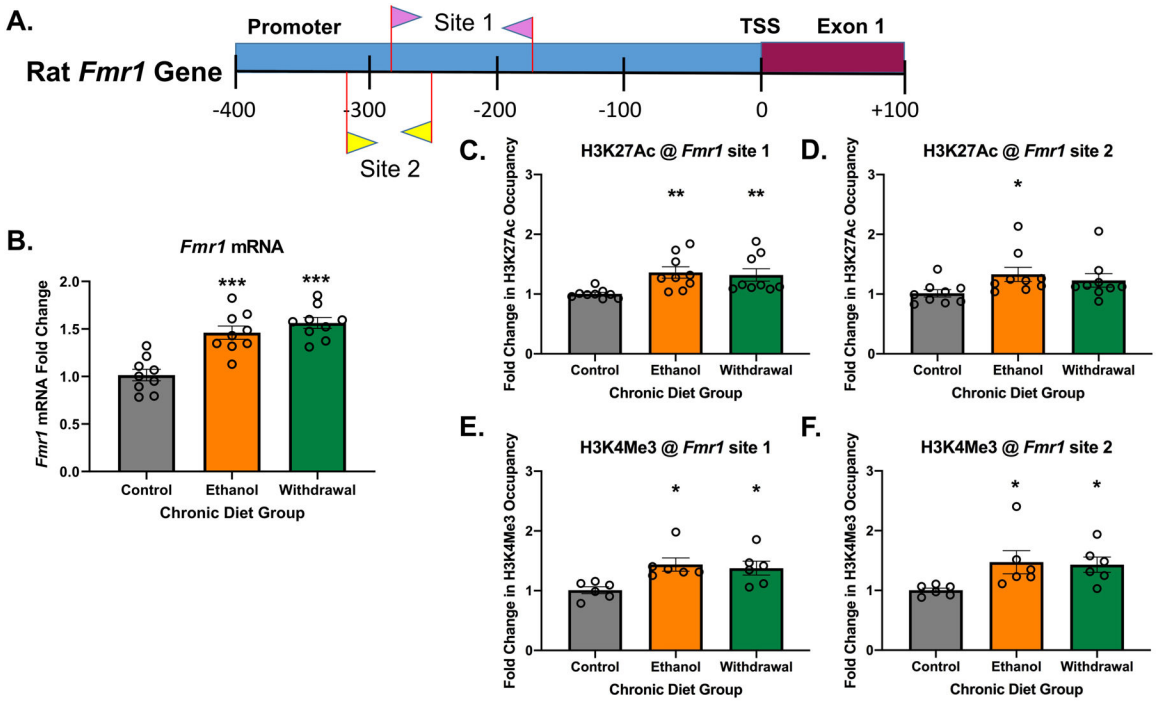

Figure 2:

Gene map of *Fmr1* ChIP primer site locations (A) and effects of chronic ethanol diet on the *Fmr1* gene mRNA expression in the cerebellum of rats (B). Chromatin immunoprecipitation experiments assess *Fmr1* epigenetic regulation at two sites within the promoter for activating histone marks H3K27 acetylation (H3K27Ac) (C and D) and for H3K4 trimethylation (H3K4Me3) (E and F). Data are represented as mean ± SEM (n = 6–9 per group) and individual values are shown with open circles. *p<0.05, **p<0.01, ***p<0.001 significantly different from control diet-fed group.

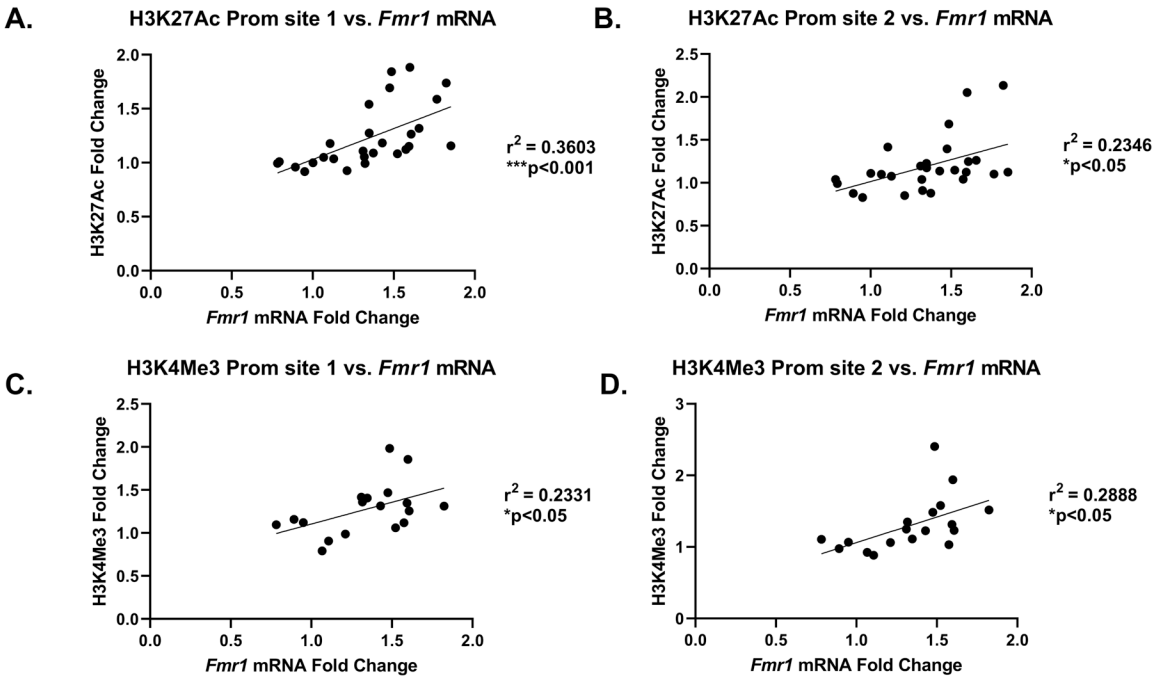

Figure 3:

Correlations between *Fmr1* mRNA fold changes and H3K27 acetylation (H3K27ac) ChIP fold changes for *Fmr1* promoter site 1 (A) and site 2 (B) and correlations between *Fmr1* mRNA fold changes and H3K4 trimethylation (H3K4me3) ChIP fold changes for *Fmr1* promoter site 1 (C) and site 2 (D). r^2 values and p values are derived from simple linear regressions.

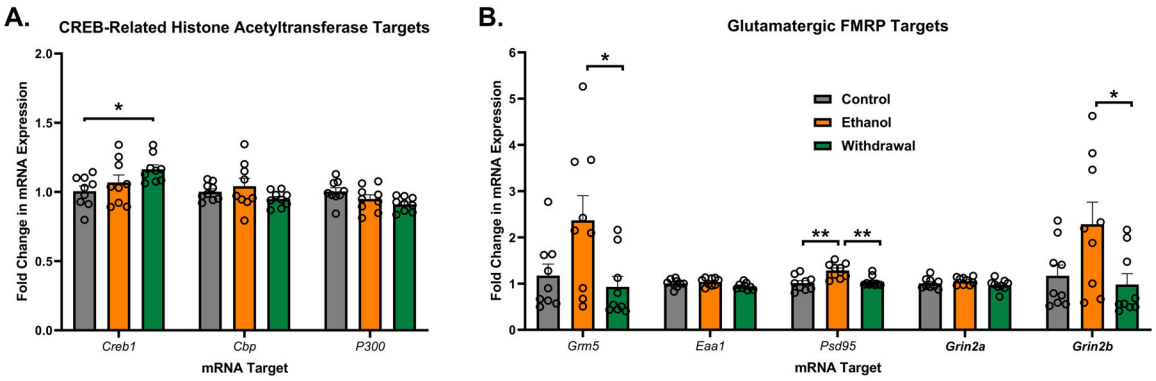

Figure 4:

Effects of chronic ethanol exposure on cerebellar mRNA expression of histone acetyltransferase complex *Creb1*, *Cbp*, and *P300* (A) and of selected glutamatergic targets of FMRP *Grm5, Eaa1, Psd95, Grin2a,* and *Grin2b* (B). Data are represented as mean ± SEM (n = 9 per group) and individual values are shown with open circles. *p<0.05; **p<0.01 significantly different post-hoc comparison as indicated.

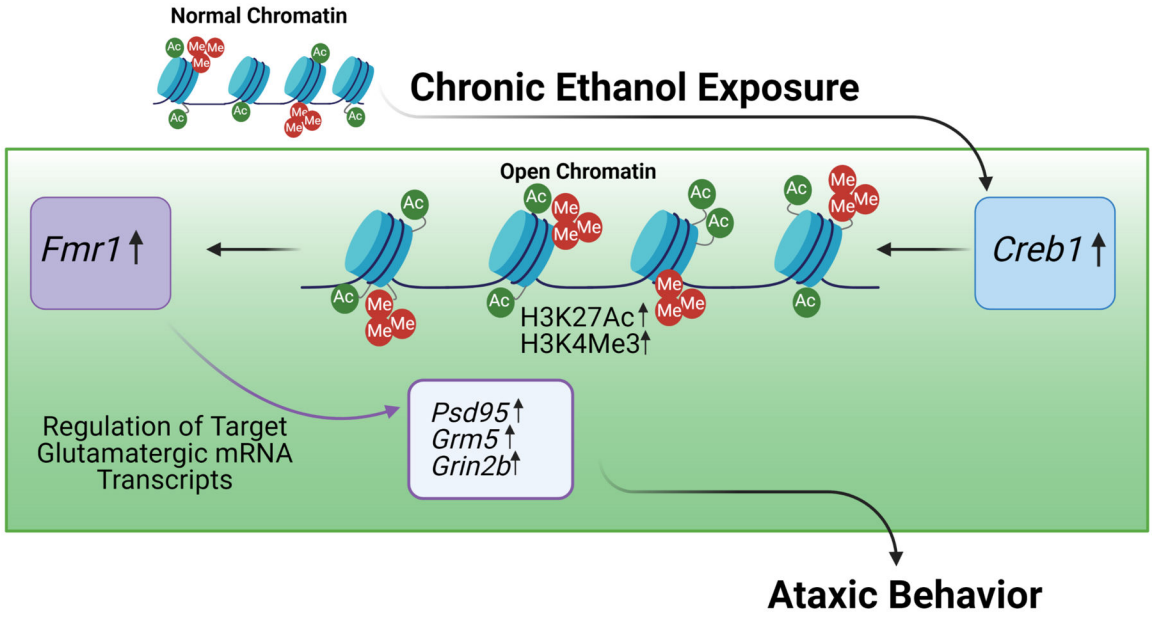

Figure 5:

Schematic presentation showing effects of chronic ethanol exposure on epigenetic regulation of *Fmr1* and its expression in the cerebellum and possible involvement in ataxic behavior. This model also depicts FMRP targets including glutamatergic transcripts *Grm5, Grin2b,* and *Psd95* have increased expression, representing neuroadaptive changes leading to hyperglutamatergic function in the cerebellum following chronic ethanol exposure.

Table 1:

The forward and reverse primer sequences utilized in qPCR reactions for amplification of cDNA corresponding with target mRNAs.

Primer Sequence	
Fmr1 Forward	5'-AAA GTC CAG AGG GGG ATG GT-3'
Fmr1 Reverse	5'-TCT CTC CAA ACG CAA CTG GT-3'
Creb1 Forward	5'-AGA AGC AGC ACG AAA GAG AG-3'
Creb1 Reverse	5'-CAC TGC CAC TCT GTT CTC TAA A-3'
Cbp Forward	5'-TAA TGG AGG CTG CCC AGT GTG TAA-3'
Cbp Reverse	5'-CTG GCG GAG CTT GTG TTT GAT GTT-3'
P300 Forward	5'-AAA CAC CAG CAA CGA GAG TAC CGA-3'
P300 Reverse	5'-TCC ATG GTG GCG TAC AGT TTC TGA-3'
Psd95 Forward	5'-TCA TAA CTC CCC ATG CCA TT-3'
Psd95 Reverse	5'-CTC ATG CAA ACC AGC AAA GA-3'
Grm5 Forward	5'-ATG CAT GTA GGA GAC GGC AA-3'
Grm5 Reverse	5'-TTT CCG TTG GAG CTT AGG GTT T-3'
Eaa1 Forward	5'-AGT AAG TGG CGG TTT CGG AG-3'
Eaa1 Reverse	5'-TGG TGT TAG AGA GGA CAA CTT TTC T-3'
Grin2a Forward	5'-CTG TCC GGA GTG GAA CAG AAA-3'
Grin2a Reverse	5'-CCG CGA CTC TCA GAC CTC AT-3'
Grin2b Forward	5'-GGG CTT GGC TCA ATG GAG AA-3'
Grin2b Reverse	5'-TCA GTC TCT CGG GTT GGG AA-3'