Published in final edited form as:

Neuroscience. 2018 November 10; 392: 230–240. doi:10.1016/j.neuroscience.2018.07.011.

Activity-Dependent Signaling and Epigenetic Abnormalities in Mice Exposed to Postnatal Ethanol

Shivakumar Subbanna¹, Vikram Joshi¹, and Balapal S. Basavarajappa^{1,2,3,*}

¹Division of Analytical Psychopharmacology, Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA

²Department of Psychiatry, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA

³Department of Psychiatry, New York University Langone Medical Center, New York, NY, USA

Abstract

Postnatal ethanol exposure has been shown to cause persistent defects in hippocampal synaptic plasticity and disrupt learning and memory processes. However, the mechanisms responsible for these abnormalities are less well studied. We evaluated the influence of postnatal ethanol exposure on several signaling and epigenetic changes and on expression of the activity-regulated cytoskeletal (Arc) protein in the hippocampus of adult offspring under baseline conditions and after a Y-maze spatial memory (SP) behavior (activity). Postnatal ethanol treatment impaired pCaMKIV and pCREB in naïve mice without affecting H4K8ac, H3K14ac and H3K9me2 levels. The Y-maze increased pCaMKIV, pCREB, H4K8ac and H3K14ac levels in saline-treated mice but not in ethanol-treated mice; while H3K9me2 levels were enhanced in ethanol-exposed animals compared to saline groups. Like previous observations, ethanol not only reduced Arc expression in naïve mice but also behaviorally induced Arc expression. ChIP results suggested that reduced H3K14ac and H4K8ac in the Arc gene promoter is because of impaired CBP, and increased H3K9me2 is due to the enhanced recruitment of G9a. The CB1R antagonist and a G9a/GLP inhibitor, which were shown to rescue postnatal ethanol-triggered synaptic plasticity and learning and memory deficits, were able to prevent the negative effects of ethanol on activity-dependent signaling, epigenetics and Arc expression. Together, these findings provide a molecular mechanism involving signaling and epigenetic cascades that collectively are responsible for the neurobehavioral deficits associated with an animal model of fetal alcohol spectrum disorders (FASD).

*To whom correspondence should be addressed: Balapal S. Basavarajappa, Division of Analytical Psychopharmacology, Nathan Kline Institute for Psychiatric Research, 140 Old Orangeburg Rd, Orangeburg, NY 10962, USA. Tel: 845-398-3234; Fax: 845-398-5451; Basavaraj.Balapal@nki.rfmh.org.

AUTHOR CONTRIBUTIONS

BSB conceived and designed the experiments. SS and VJ performed the experiments and contributed to data analysis. BSB and SS wrote the manuscript. BSB contributed to study supervision.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest: The authors declare no competing financial interests.

Graphical abstract:

Postnatal ethanol exposure causes activity dependent signaling defects and epigenetic abnormalities in adult mice and CB1R antagonist or G9a/GLP inhibitor rescues these defects. Y-maze spatial memory behavior was used as activity-dependent event and current findings suggest that P7 ethanol treatment impair activity-dependent recruitment of CBP and G9a on Arc gene promoter region, inhibit Arc expression and CB1R antagonist (SR) and/or G9a/GLP inhibitor (Bix) rescue these signaling and epigenetic remodeling and Arc expression.

Keywords

CB1R; G9a; Synaptic Plasticity; FASD; Behavior; Histone

Introduction

Exposure to ethanol during development causes long-lasting neurobehavioral defects including reduced intelligence quotient (IQ), and synaptic, learning and memory impairments. The extent of ethanol's negative impact on the developing fetus is associated with the amount, pattern (Bailey et al., 2004) and developmental period (Kelly et al., 2000, Sood et al., 2001) of ethanol exposure. The various adverse neurobehavioral outcomes due to ethanol have been called fetal alcohol spectrum disorder (FASD). At least 10% of women in the overall global population, and up to 25% in Europe, continue to expose their unborn fetuses to alcohol (Popova et al., 2017a). In the United States, the pooled prevalence of

FASD was estimated to be approximately 15 per 1,000 among the general population (Popova et al., 2017b) and the majority have no physical features but exhibit significant persistent cognitive deficits through adulthood (Kodituwakku et al., 2006, Green et al., 2009, Panczakiewicz et al., 2016, Gross et al., 2017, Wozniak et al., 2017). These long-lasting cognitive functional deficits such as learning and memory behaviors have been demonstrated by numerous independent investigators using different animal models of FASD (Sutherland et al., 1997, Savage et al., 2002, Vaglenova et al., 2008, Noel et al., 2011, Wilson et al., 2011, Sanchez Vega et al., 2013, Subbanna et al., 2013a, Sadrian et al., 2014, Subbanna et al., 2015, Schambra et al., 2017, Subbanna et al., 2018) as well as hippocampal synaptic plasticity (Sutherland et al., 1997, Savage et al., 2002, Wilson et al., 2011, Brady et al., 2013, Subbanna et al., 2013a, Sadrian et al., 2014, Subbanna et al., 2015, Subbanna et al., 2018), a significant process associated with learning. These studies demonstrate a persistent effects of ethanol exposure during different stages of development on synaptic, learning and memory deficits in adulthood.

Recent studies have established that binge-like ethanol exposure during postnatal development contributes to long-lasting synaptic dysfunction leading to behavioral abnormalities (Noel et al., 2011, Wilson et al., 2011, Subbanna et al., 2013a, Subbanna et al., 2015, Subbanna et al., 2018) in adulthood through cannabinoid receptor type-1 (CB1R)-mediated (Subbanna et al., 2013a) deficits in the phosphorylation of cAMP response element binding protein (pCREB) (Subbanna et al., 2015, Subbanna et al., 2018). Postnatal ethanol-induced pCREB deficits were long-lasting into adulthood and inhibited *Arc* expression (Subbanna et al., 2015, Subbanna et al., 2018) through a reduction in histone acetylation at the *Arc* gene promoter region (Subbanna et al., 2015, Subbanna et al., 2018). It is well known that *Arc* expression is activity-dependent and is involved in activity-dependent synaptic plasticity (Lyford et al., 1995, Guzowski et al., 2000, Waltereit et al., 2001, Hong et al., 2005, Tzingounis and Nicoll, 2006, Cohen and Greenberg, 2008, Kawashima et al., 2009, Ivanova et al., 2011). Further, activity-dependent signaling changes and gene activation are prerequisites in the persistent modification of neural circuits (Arancio et al., 1995, Taha and Stryker, 2002, Lu, 2003, Tabuchi, 2008, Kawashima et al., 2009). Interestingly, synaptic activity during brain development has been shown to control the expression of many genes (West and Greenberg, 2011), including *Arc*, through specific signaling events (Plath et al., 2006, Rial Verde et al., 2006, Chotiner et al., 2010). However, the signaling (Subbanna et al., 2018) and epigenetic mechanisms fundamental for the deficits in activity-dependent plasticity changes as a result of postnatal ethanol exposure are not well understood. In the present study, employing spatial memory with a Y-maze as a behavioral activation paradigm, we evaluated the hypothesis that postnatal ethanol exposure would impair spatial memory-induced elevations of several signaling events, global and *Arc* gene promoter-specific epigenetic changes, and *Arc* expression and the CB1R antagonist or G9a/GLP inhibitor preadministration rescue aforementioned defects.

MATERIALS AND METHODS

Experimental animals

C57BL/6J mice were generated from our breeding colony at the Nathan Kline Institute for Psychiatric Research (NKI). C57BL/6J mice were kept in groups in standard cages at 21-24°C with 40-60% humidity, a 12-h light/12-h dark cycle. Mouse food and tap water were provided *ad libitum*. Animal handling and care protocols were approved by the NKI IACUC and followed the National Institutes of Health guidelines.

Experimental ethanol exposure model

We used a postnatal day seven animal model that mimics third-trimester alcohol exposure in humans. P7 (day of birth as labeled as P0) mouse pups were equally divided into male and female groups (each litter) and subcutaneous (s. c.) injection of saline and ethanol (2.5 g/kg s. c. at 0 h and again at 2 h) was performed as described previously (Subbanna et al., 2013a, Subbanna et al., 2018). This ethanol administration paradigm does not cause lethality and was demonstrated to induce significant apoptotic neuronal death in P7 mice (Olney et al., 2002, Subbanna et al., 2013b) and persistent neurobehavioral abnormalities in adulthood (Noel et al., 2011, Wilson et al., 2011, Sadrian et al., 2012, Subbanna et al., 2013a, Sadrian et al., 2014, Subbanna and Basavarajappa, 2014, Subbanna et al., 2015, Subbanna et al., 2018). The pups and dams were kept together until weaning. In some experiments, the blood ethanol levels (BELs) were determined as described previously (Subbanna et al., 2013a) and BELs were found consistent with our previous findings (Subbanna et al., 2013a).

Drug treatment

SR141716A (SR) or Bix-01294 (Bix) was dissolved in ethanol (10 µl) and Tween 80 (10 µl) solution, and the volume was made with sterilized saline. The optimum dose of SR or Bix (1 mg/kg), based on our previous dose-response studies (Subbanna et al., 2013a, Subbanna et al., 2013b), was subcutaneously administered 30 min before ethanol exposure at a volume of 5 µl/g body weight. The solution containing mixture of ethanol (10 µl), Tween 80 (10 µl) and saline was injected as a vehicle. Until weaning, the pups remained with the dams. As found in our previous studies (Subbanna et al., 2013a, Subbanna et al., 2013b), SR or Bix preadministration failed to affect ethanol-induced intoxication (sleeping time) and produced no visible damage to any organs. Three-month-old male and female mice were subjected to all analyses.

Spatial memory (SM) using the Y-maze

P7 mice exposed to saline, ethanol, saline + SR/Bix or ethanol + SR/Bix at P7 were subjected to an SM task (Dellu et al., 1992), which was performed (Sarnyai et al., 2000) using a symmetrical Y-maze precisely as described previously (Subbanna and Basavarajappa, 2014, Subbanna et al., 2015, Subbanna et al., 2018). Using a sheet of opaque paper, entry to one arm (the novel arm) of the Y-maze was blocked throughout the training duration (10 min). The mice were permitted to explore all three arms (3 min, preference trial) after a 24 h intertrial interval. The number of arm entries and the total time spent in each arm were noted manually using video recordings by an experimenter blind to the

treatment conditions of the mice. The discrimination ratio for arm entries and the dwell time were evaluated by the following formula: [preference for the novel arm over the familiar other arm (Novel/Novel + Other)].

Protein extraction, electrophoresis, immunoblotting, activity-dependent regulation of signaling and epigenetic events

The mice were sacrificed instantly after performing the Y-maze behavior. Hippocampi were dissected and homogenized. Phosphatase and protease inhibitor mixtures (Roche, Indianapolis, IN, USA) were added to the tissue homogenates and were subjected for cytosolic and nuclear fractionation. The nuclear fraction was further extracted using nuclear extraction buffer (Grabowski, 2005) in agreement to the manufacturer's manual (Thermo Fisher Scientific, Suwanee, GA, USA) and processed for analysis of several signaling and epigenetic histone marks levels using western blotting, as described previously (Subbanna and Basavarajappa, 2014, Subbanna et al., 2015, Subbanna et al., 2018). The western blots were incubated with anti-mouse CaMKIV (monoclonal, # 610275, 1:1000, BD Biosciences, San Jose CA, USA), anti-rabbit phospho-CaMKIV (Polyclonal # sc28443, 1:1000), anti-mouse Arc (monoclonal, # sc17838, 1:1000), anti-rabbit p-CaMKII (polyclonal, # Sc-12886-R, 1:1000), anti-mouse CaMKII (monoclonal, # Sc-5306-R, 1:1000) (Santa Cruz Biotechnology, CA, USA), anti-rabbit CREB (monoclonal, # 9197, 1:1000), anti-rabbit phospho-CREB (monoclonal, # 9198, 1:1000), anti-rabbit p44/42 MAPK (ERK1/2) (polyclonal, # 9102, 1:2000), anti-rabbit-phospho-p44/42 MAPK (polyclonal, # 9101, 1:1000), anti-rabbit-H3K9me2 (monoclonal, # 4658, 1:1000), anti-rabbit-Histone H3 (polyclonal # 9715, 1:1000), anti-rabbit-H3K14ac (monoclonal, # 7627, 1:1000), anti-mouse β -actin (monoclonal, # 3700, 1:1000), anti-rabbit-H4K8ac (polyclonal, # 2594, 1:1000) (Cell Signaling Technology, Denver, MA USA) and anti-rabbit-Histone H4 (polyclonal # 07-108, 1:1000), (Millipore, Billerica, MA, USA) antibodies at room temperature for 3 h or overnight at 4°C, and further processed as previously described by our laboratory (Basavarajappa et al., 2008, Basavarajappa et al., 2014). Western blots incubated with a secondary antibody (goat anti-mouse peroxidase conjugate, # AP 124P, 1:5000, Millipore; goat anti-rabbit, # AP132P, 1:5000, Millipore) alone produced no bands.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was evaluated as described previously (Subbanna et al., 2014, Subbanna et al., 2015, Subbanna et al., 2018). Briefly, adult mouse hippocampus (HP) tissue (25 mg) was fixed with formaldehyde (1%), homogenized, and subjected to DNA shearing. The sample amount was normalized to contain equivalent protein amounts. A small aliquot of precleaned chromatin was used as the input. Chromatin immunoprecipitation was performed with an antibody recognizing normal rabbit IgG as a negative control and with anti-rabbit-H3K14ac (monoclonal, # 7627), anti-rabbit-H4K8ac (polyclonal, # 2594), anti-rabbit-G9a (monoclonal, # 3306), anti-rabbit-H3K9me2 (monoclonal, # 4658) (Cell Signaling Technology, Denver, MA USA), and anti-rabbit-CBP (polyclonal, # ab2832, Abcam, Cambridge, MA, USA) antibodies. The real-time qPCR with mouse primers (forward, 5'-AGTGCTCTGGCGAGTAGTCC-3' and reverse, 5'-TCGGGACAGGCTAAGAACTC-3') designed to amplify *Arc* gene promoter regions was followed to quantify the immunoprecipitated DNA and data were normalized to the total DNA (input). Relative

enrichment of H3K14ac, H4K8ac, CBP, H3K9me2 and G9a on the *Arc* gene promoter in the saline and treatment groups was determined by taking the ratio between immunoprecipitated chromatin and input chromatin (Hendrickx et al., 2014). Data presented as % input.

Statistical analysis

The experiments were conducted with an equal number of mice/treatment. All the data are presented as the mean $\pm$ SEM. The data were statistically analyzed by one-way or two-way analysis of variance (ANOVA) with Bonferroni's post hoc test. Statistical significance was set at $P < 0.05$. The statistical analyses were performed using Prism software (GraphPad, San Diego, CA).

Results

Treatment of P7 mice with the CB1R antagonist, SR141716A, before ethanol treatment rescues the activity-dependent signaling abnormalities in mice exposed to postnatal ethanol.

The schematic diagram in Fig. 1A shows the experimental strategy for this study. SR preadministration rescued P7 ethanol-induced spatial memory deficits in adult mice. Results revealed that postnatal saline-treated (arm entry: $F_{3,33} = 28$, $p < 0.01$) mice with or without SR entered (Fig. 1Bi) more frequently and spent more time (dwell time: $F_{3,33} = 27$, $p < 0.01$) (Fig. 1Bii) in the novel, previously unvisited arm of the maze (Two-way ANOVA with Bonferroni's *post hoc* test). Although P7 ethanol-exposed mice displayed an impaired preference for the novel arm ($p < 0.01$), they spent less time (dwell time) ($p < 0.01$) in the novel arm compared with the saline-exposed mice after 24 h of retention. Postnatal ethanol and SR pre-administered (interaction: $F_{3,33} = 12$, $p < 0.01$) mice exhibited a greater preference toward exploration of the novel arm ($p < 0.01$) and an increased time spent ($p < 0.01$) in the novel arm compared to the other arms. To further evaluate the function of intracellular signaling in the persistent effects of ethanol, we assessed the levels of many signaling molecules (pERK1/2, pCaMKII, pCaMKIV, pCREB) in the HP tissue extracts. One-way ANOVA with Bonferroni's *post hoc* analysis is used in these studies. In naïve animals, the results suggested that ethanol (vs. saline) significantly impaired pCaMKIV ($F_{3,33} = 32$, $p < 0.01$) and pCREB ($F_{3,33} = 20$, $p < 0.01$) levels (Fig. 1C). However, ethanol failed to affect pERK and pCaMKII levels ($p > 0.05$) in naïve animals. In saline-treated animals, the Y-maze behavior (activity) significantly enhanced pCaMKIV ($F_{3,33} = 30$, $p < 0.01$), pCREB ($F_{3,33} = 28$, $p < 0.01$) and pCaMKII ($F_{3,33} = 20$, $p < 0.01$) levels whereas in ethanol-treated animals, the Y-maze behavior failed to enhance pCaMKIV, pCREB and pCaMKII ($p > 0.05$) levels. SR pre-administration before ethanol exposure prevented the loss of pCREB in naïve animals (without Y-maze behavior) in our previous studies (Subbanna et al., 2015). Consistent with these previous findings, preadministration of SR before ethanol treatment ($F_{3,33} = 18$, $p < 0.01$) significantly (pCaMKIV: $F_{3,33} = 13$, $p < 0.01$; pCREB: $F_{3,33} = 17$, $p < 0.01$; pCaMKII: $F_{3,33} = 22$, $p < 0.01$) rescued ethanol-induced, activity-dependent (Y-maze behavior) signaling defects in P90 mice. Saline or ethanol-treated adult animals with or without the Y-maze behavior failed to affect pERK1/2 levels ($p > 0.05$) (Fig. 1C).

Administration of SR141716A before ethanol exposure prevents the impaired activity-dependent epigenetic marks in adult mice exposed to postnatal ethanol.

To examine the role of global histone marks on ethanol-induced persistent synaptic and behavioral deficits, we determined the levels of many histone marks in the HP tissue extracts. One-way ANOVA with Bonferroni's *post hoc* analysis was used in these studies. Findings suggested that P7 ethanol treatment failed to affect H4K8ac, H3K14ac and H3K9me2 ($p > 0.05$) levels in adult naïve animals (Fig. 2). In P7 saline-treated adult animals, the Y-maze behavior significantly enhanced H4K8ac ($F_{3,33} = 22$, $p < 0.01$) and H3K14ac ($F_{3,33} = 19$, $p < 0.01$) levels. However, in P7 saline-treated adult mice, the Y-maze behavior failed to affect H3K9me2 ($p > 0.05$) levels. The Y-maze behavior in P7 ethanol-treated adult mice significantly impaired H4K8ac ($F_{3,33} = 15$, $p < 0.01$) and H3K14ac ($F_{3,33} = 25$, $p < 0.01$) levels and enhanced H3K9me2 ($F_{3,33} = 51$, $p < 0.01$) levels. Administration of SR before ethanol treatment ($F_{3,33} = 13$, $p < 0.01$) significantly prevented the loss of H4K8ac ($F_{3,33} = 19$, $p < 0.01$) and H3K14ac ($F_{3,33} = 18$, $p < 0.01$) levels and normalized H3K9me2 ($F_{3,33} = 22$, $p < 0.01$) levels (Fig. 2).

Administration of P7 mice with SR141716A before ethanol treatment rescues the activity-dependent Arc expression defects in adult mice.

In naïve animals, ethanol (vs. saline) significantly impaired Arc ($F_{3,33} = 41$, $p < 0.01$) expression (Fig. 3A). In saline-treated animals, the Y-maze behavior significantly enhanced Arc ($F_{3,33} = 47$, $p < 0.01$) expression, whereas in ethanol-treated animals, the Y-maze behavior failed to enhance Arc ($p > 0.05$) expression. In our previous studies (Subbanna et al., 2013a, Subbanna et al., 2015, Subbanna et al., 2018), SR preadministration prior to ethanol exposure prevented the loss of Arc expression in naïve animals (without the Y-maze behavior). In the present study, SR preadministration significantly ($p < 0.01$) rescued the ethanol-induced activity-dependent (Y-maze behavior) ($F_{3,33} = 23$, $p < 0.01$) Arc expression defects in P90 mice (One-way ANOVA with Bonferroni's *post hoc* analysis).

Preadministration of SR141716A rescues the postnatal ethanol-induced chromatin remodeling in the Arc gene promoter region in adult mice.

To further examine the mechanism through which ethanol caused the differential regulation of H3K14ac and H3K9me2 on the Arc promoter region, we determined the histone marks and the enrichment of their enzymes at the Arc gene promoter region using a ChIP assay. Two-way ANOVA with Bonferroni's *post hoc* analysis indicated that ethanol in naïve mice (vs. saline in naïve mice) significantly reduced H3K14ac ($F_{3,33} = 15$, $p < 0.01$) and CBP ($F_{3,33} = 20$, $p < 0.01$). However, P7 ethanol treatment failed to affect H4K8ac ($p > 0.05$) levels in adult naïve mice (Fig. 3Bi). Further, ethanol in naïve mice (vs. saline in naïve mice) significantly increased H3K9me2 ($F_{3,33} = 25$, $p < 0.01$) and G9a ($F_{3,33} = 30$, $p < 0.01$) levels (Fig. 3Bii). The Y-maze behavior in P7 saline-treated adult mice significantly enhanced H3K14ac ($F_{3,33} = 31$, $p < 0.01$), H4K8ac ($F_{3,33} = 20$, $p < 0.01$) and CBP ($F_{3,33} = 21$, $p < 0.01$) levels. The Y-maze behavior in P7 ethanol-treated adult mice significantly impaired H3K14ac ($F_{3,33} = 14$, $p < 0.01$), H4K8ac ($F_{3,33} = 11$, $p < 0.01$) and CBP ($F_{3,33} = 16$, $p < 0.01$). No significant change in H3K9me2 and G9a levels were found in saline (Y-maze behavior) groups. However, the Y-maze behavior in P7 ethanol-treated adult mice

significantly enhanced H3K9me2 ($F_{3,33} = 34$, $p < 0.01$) and G9a ($F_{3,33} = 22$, $p < 0.01$) compared to the saline (activity) groups. Administration of SR prior to P7 ethanol exposure significantly (interaction: $F_{3,33} = 16$, $p < 0.01$) rescued the loss of H3K14ac ($F_{3,33} = 29$, $p < 0.01$), H4K8ac ($F_{3,33} = 19$, $p < 0.01$), CBP ($F_{3,33} = 23$, $p < 0.01$) and increased H3K9me2 ($F_{3,33} = 33$, $p < 0.01$) and G9a ($F_{3,33} = 28$, $p < 0.01$) levels (Fig. 3B).

Preadministration of a G9a/GLP inhibitor prior to ethanol treatment rescues the impaired activity-dependent epigenetic marks and Arc expression in adult mice exposed to postnatal ethanol.

Preadministration of Bix (G9a/GLP inhibitor) prevented postnatal ethanol-induced spatial memory deficits in adult mice. The results suggested that postnatal saline-exposed mice with or without Bix entered more frequently (arm entry: $F_{3,33} = 31$, $p < 0.01$) (Fig. 4Ai) and spent more time (dwell time: $F_{3,33} = 32$, $p < 0.01$) (Fig. 4Aii) in the novel, previously unvisited arm of the maze (Two-way ANOVA with Bonferroni's *post hoc* test) compared to the other arms. Nonetheless, P7 ethanol-exposed mice displayed an impaired preference to the novel arm ($p < 0.01$) and spent less time (dwell time) ($p < 0.01$) in the novel arm compared with the saline-exposed mice after 24 h of retention. Postnatal ethanol injected mice pre-administered with Bix showed (interaction: $F_{3,33} = 18$, $p < 0.01$) a heightened preference for the novel arm ($p < 0.01$) and increased time spent ($p < 0.01$) in the novel arm. One-way ANOVA with Bonferroni's *post hoc* analysis suggested that P7 ethanol treatment failed to affect H3K9me2 ($p > 0.05$) levels in adult naïve animals (Fig. 4B). The Y-maze behavior in P7 saline-treated adult mice failed to affect H3K9me2 ($p > 0.05$) levels. The Y-maze behavior in P7 ethanol-treated adult mice significantly enhanced H3K9me2 ($F_{3,33} = 31$, $p < 0.01$) levels. Administration of Bix prior to P7 ethanol exposure significantly ($F_{3,33} = 12$, $p < 0.01$) normalized H3K9me2 ($F_{3,33} = 34$, $p < 0.01$) levels.

In naïve mice, ethanol (vs. saline) significantly reduced Arc ($F_{3,33} = 47$, $p < 0.01$) expression (Fig. 5). In saline-treated mice, exposure to the Y-maze behavior significantly increased Arc ($F_{3,33} = 43$, $p < 0.01$) expression (vs. saline naïve), whereas in ethanol-treated mice, the Y-maze behavior failed to increase Arc ($p > 0.05$) expression. Bix preadministration before ethanol exposure significantly ($F_{3,33} = 27$, $p < 0.01$) prevented ethanol-induced activity-dependent (Y-maze behavior) Arc expression deficits in P90 mice (One-way ANOVA with Bonferroni's *post hoc* analysis).

Discussion

An understanding of how neuronal activity modifies signaling, epigenetic events and gene expression may help to elucidate the mechanisms of long-term neuronal plasticity in the brain. The mechanisms by which developmental ethanol impairs the maturation of the central nervous system (CNS) remain unclear. Using a Y-maze spatial memory task as a model for neuronal activity (Subbanna et al., 2018), we have identified some of the early signaling and epigenetic changes that may be responsible for gene expression in an animal model of FASD. The evidence presented here supports three critical observations. Postnatal ethanol treatment impairs activity-dependent increases in pCaMKIV, pCaMKII, pCREB levels and Arc expression in the HP. However, spatial memory, as evaluated by the Y-maze,

failed to increase pERK1/2 levels. Although the impact of developmental ethanol on activity-dependent signaling changes is studied less, our findings from naïve saline-treated mice are consistent with many previous activity-dependent studies. It has been shown that the activity-dependent signaling through pCaMKII (Vaillant et al., 2002, Gaudilliere et al., 2004), pCaMKIV and pCREB is required to promote activity-dependent increases in dendritic length (Redmond et al., 2002) and is critical for long-term neuronal plasticity (Ma et al., 2014). A learning and memory behavioral paradigm has been shown to enhance CREB phosphorylation in the HP (Porte et al., 2008). Further, consistent with CREB activation, expression of the immediate-early gene *Arc* has been shown to increase immediately after spatial memory usage in a water maze task (Ramirez-Amaya et al., 2005, Ramirez-Amaya et al., 2013). *Arc* protein plays a vital role in synaptic plasticity through the regulation of AMPA-type glutamate receptor trafficking via recruiting endosomal pathways (Chowdhury et al., 2006, Shepherd et al., 2006) and is required for learning and memory (Zhang et al., 2015). Additionally, *Arc* binds to CaMKII in the kinase-inactive state, and this interaction targets *Arc* to synapses and helps in the homeostatic maintenance of synaptic strength (Okuno et al., 2012). Expression of CaMKII along with *Arc* was enhanced in the HP immediately after an object recognition test (Pollak et al., 2005, Soule et al., 2008). A spatial memory test in the Morris water maze also enhanced pCaMKII in the HP (Soule et al., 2008). Several studies demonstrate that the reduction in ERK activity disrupts associative fear-conditioned memory formation (Blum et al., 1999, Selcher et al., 2003). The ERK1/2 signaling is essential for the activation of transcription during long-term memory formation (Chwang et al., 2006, Chotiner et al., 2010). Additionally, contextual fear conditioning induces ERK2 activation in the HP (Atkins et al., 1998). Although inhibition of ERK1/2 activation by postnatal ethanol was found to be critical for causing neurodegeneration in neonatal mice (Subbanna et al., 2013a), ERK1/2 activation was not affected in adult mice and was not enhanced by the Y-maze behavior either in adult mice exposed to postnatal saline or ethanol. Together, these observations suggest that the activity-dependent fine-tuning of neural circuits during development is compromised due to impaired signaling-mediated gene expression in postnatal ethanol-exposed adult mice, leading to synaptic and behavioral abnormalities as found in animal models of FASD (Abel et al., 1983, Bonthius and West, 1991, Bellinger et al., 1999, Berman and Hannigan, 2000, Bellinger et al., 2002, Alati et al., 2006, Brown et al., 2007, Brady et al., 2013, Subbanna et al., 2013a, Subbanna and Basavarajappa, 2014, Basavarajappa, 2015, Subbanna et al., 2015).

In our second observation, we found that spatial memory in postnatal saline-treated adult naïve mice enhanced several histone marks such as H4K8ac and H3K14ac that promote relaxed chromatin and gene expression, which are essential for learning and memory (Bousiges et al., 2010, Zovkic et al., 2013). However, in postnatal ethanol-treated adult mice, spatial memory failed to enhance H4K8ac, and H3K14ac levels. Additionally, in postnatal ethanol-treated adult mice, spatial memory enhanced H3K9me2 levels, which induces compact chromatin and inhibits gene expression. Consistent with these observations, we found that postnatal ethanol treatment persistently impaired *Arc* expression through the enhanced occupation of H3K9me2 in the *Arc* gene promoter (Subbanna et al., 2018) and caused defective synaptic and learning and memory behavior (Subbanna et al., 2013a, Subbanna and Basavarajappa, 2014, Subbanna et al., 2015, Subbanna et al., 2018) in adult

mice. While the influence of developmental ethanol on activity-dependent epigenetic changes is not known, in general, our saline-treated naïve mice data are consistent with many studies involving HP-dependent tasks that have been linked with global increases of relaxed chromatin-related modifications of histones that promote gene expression. For example, contextual fear conditioning paradigm enhanced H3K14ac levels in the HP (Levenson et al., 2004, Chwang et al., 2006). In contrast to our Y-maze spatial memory data with H3K9me2, contextual fear conditioning was shown to increase H3K9me2 levels (Gupta et al., 2010, Gupta-Agarwal et al., 2012). Further, spatial memory in the Morris water maze also enhanced H4K12ac and pan-acetylation of H2B (tetra-acetylated-H2BK5K12K15K20) (Bousiges et al., 2010). Furthermore, studies with genetically modified HAT and HDAC animal models have consistently reported impaired object recognition memory (Barrett and Wood, 2008), indicating the relative significance of HAT catalytical activity and histone acetylation process for an object recognition memory task. Together, these observations suggest that activity-dependent histone modifications are vital for the expression of genes that are central for maturation of synaptic circuits (Katz and Shatz, 1996, Khazipov and Luhmann, 2006) during development and inhibition of these epigenetic machineries in postnatal ethanol-exposed adult mice may cause neurobehavioral abnormalities (Abel et al., 1983, Bonthius and West, 1991, Bellinger et al., 1999, Berman and Hannigan, 2000, Bellinger et al., 2002, Alati et al., 2006, Brown et al., 2007, Brady et al., 2013, Subbanna et al., 2013a, Subbanna and Basavarajappa, 2014, Basavarajappa, 2015, Nagre et al., 2015) similar to those in FASD.

In our third observation, we found that preadministration of SR or Bix rescued ethanol-induced activity-dependent signaling events, epigenetic and *Arc* expression deficits in adult mice. These findings provided additional strengths to our previous discoveries. In our earlier studies, it was shown that both CB1R and G9a/GLP play an essential role in postnatal ethanol-induced neurodegeneration in neonatal mice in addition to synaptic plasticity and learning and memory defects in adult mice. Blockade of either CB1R with SR (Subbanna et al., 2013a, Subbanna et al., 2015) or G9a with Bix (Subbanna et al., 2013b, Subbanna and Basavarajappa, 2014, Subbanna et al., 2014) prevented neurodegeneration and neurobehavioral abnormalities in adult mice. Furthermore, in our earlier study, postnatal ethanol exposure impaired the expression of *Arc* via the reduced activation of pCREB followed by reduced H3K14ac or increased activation of G9a followed by increased H3K9me2 in the *Arc* gene promoter (Subbanna et al., 2018). Our current ChIP studies suggest that reduced pCREB may be responsible for reduced recruitment of CBP in the *Arc* gene promoter region that leads to reduced H3K14ac and H4K8ac in the *Arc* gene promoter region. Additionally, enhanced H3K9me2, in fact, is mediated by the increased recruitment of G9a in the *Arc* gene promoter region. This postnatal ethanol-induced condensed chromatin in the *Arc* promoter region inhibited the expression of *Arc* and blocking CB1R-mediated signaling or G9a could rescue these defects. Here, we show that the ethanol-induced defects in the activity-dependent induction of the *Arc* gene are regulated by reduced CBP and increased G9a, two chromatin-modifying enzymes. However, less is known about the mechanism by which enzymes related to acetylation and methylation cooperate during neuronal activity-dependent gene transcription. These findings together support the suggestion that the activity-dependent regulation of signaling followed by dynamic

modifications in chromatin structure, as shown by the dysregulation of the factors/enzymes (CBP and G9a) responsible for chromatin remodeling, may be a vital, distinctive feature of mental disorders (Ma et al., 2014, Oey et al., 2015). These changes may also be responsible for some of the intellectual disabilities found in FASD. In addition, together, the previous (Subbanna et al., 2013a, Subbanna et al., 2015) and current data strongly suggest that characterizing the CB1R downstream signaling events that regulate pCREB-mediated epigenetic remodeling and gene expression and understanding the influence of developmental ethanol exposure on these CB1R-mediated actions may eventually help advance therapeutic drugs to treat FASD.

In summary, the current study supports the idea that postnatal ethanol activates two chromatin-modifying enzymes CBP and G9a and may aid as epigenetic modulators to memory formation processes by controlling histone acetylation and methylation, a learning-induced, activity-dependent chromatin remodeling that allows activity-dependent Arc expression. Future research into the modulation of CB1R/G9a-mediated epigenetic changes may have potential applications in the development of novel therapeutics to treat neurobehavioral defects found in FASD.

ACKNOWLEDGMENTS

The current study was supported by the NIH/NIAAA grant AA019443 (BSB). We would like to thank Neha Balapal for editing the final version of the manuscript. The authors also declare no competing financial interests.

References

- Abel EL, Jacobson S, Sherwin BT (1983) In utero alcohol exposure: functional and structural brain damage. *Neurobehav Toxicol Teratol* 5:363–366. [PubMed: 6877477]
- Alati R, Al Mamun A, Williams GM, O'Callaghan M, Najman JM, Bor W (2006) In utero alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. *Arch Gen Psychiatry* 63:1009–1016. [PubMed: 16953003]
- Arancio O, Kandel ER, Hawkins RD (1995) Activity-dependent long-term enhancement of transmitter release by presynaptic 3',5'-cyclic GMP in cultured hippocampal neurons. *Nature* 376:74–80. [PubMed: 7596438]
- Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. *Nat Neurosci* 1:602–609. [PubMed: 10196568]
- Bailey BN, Delaney-Black V, Covington CY, Ager J, Janisse J, Hannigan JH, Sokol RJ (2004) Prenatal exposure to binge drinking and cognitive and behavioral outcomes at age 7 years. *Am J Obstet Gynecol* 191:1037–1043. [PubMed: 15467586]
- Barrett RM, Wood MA (2008) Beyond transcription factors: the role of chromatin modifying enzymes in regulating transcription required for memory. *Learn Mem* 15:460–467. [PubMed: 18583646]
- Basavarajappa BS (2015) Fetal Alcohol Spectrum Disorder: Potential Role of Endocannabinoids Signaling. *Brain sciences* 5:456–493. [PubMed: 26529026]
- Basavarajappa BS, Nagre NN, Xie S, Subbanna S (2014) Elevation of endogenous anandamide impairs LTP, learning, and memory through CB1 receptor signaling in mice. *Hippocampus* 24:808–818 [PubMed: 24648181]
- Basavarajappa BS, Ninan I, Arancio O (2008) Acute Ethanol Suppresses Glutamatergic Neurotransmission through Endocannabinoids in Hippocampal Neurons. *J Neurochem* 107:1001–1013. [PubMed: 18796007]
- Bellinger FP, Bedi KS, Wilson P, Wilce PA (1999) Ethanol exposure during the third trimester equivalent results in long-lasting decreased synaptic efficacy but not plasticity in the CA1 region of the rat hippocampus. *Synapse* 31:51–58. [PubMed: 10025683]

- Bellinger FP, Davidson MS, Bedi KS, Wilce PA (2002) Neonatal ethanol exposure reduces AMPA but not NMDA receptor levels in the rat neocortex. *Brain Res Dev Brain Res* 136:77–84. [PubMed: 12036520]
- Berman RF, Hannigan JH (2000) Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. *Hippocampus* 10:94–110. [PubMed: 10706221]
- Blum S, Moore AN, Adams F, Dash PK (1999) A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J Neurosci* 19:3535–3544. [PubMed: 10212313]
- Bonthius DJ, West JR (1991) Permanent neuronal deficits in rats exposed to alcohol during the brain growth spurt. *Teratology* 44:147–163. [PubMed: 1925974]
- Bousiges O, Vasconcelos AP, Neidl R, Cosquer B, Herbeaux K, Panteleeva I, Loeffler JP, Cassel JC, Boutillier AL (2010) Spatial memory consolidation is associated with induction of several lysine-acetyltransferase (histone acetyltransferase) expression levels and H2B/H4 acetylation-dependent transcriptional events in the rat hippocampus. *Neuropsychopharmacology* 35:2521–2537. [PubMed: 20811339]
- Brady ML, Diaz MR, Iuso A, Everett JC, Valenzuela CF, Caldwell KK (2013) Moderate prenatal alcohol exposure reduces plasticity and alters NMDA receptor subunit composition in the dentate gyrus. *J Neurosci* 33:1062–1067. [PubMed: 23325244]
- Brown KL, Calizo LH, Goodlett CR, Stanton ME (2007) Neonatal alcohol exposure impairs acquisition of eyeblink conditioned responses during discrimination learning and reversal in weanling rats. *Dev Psychobiol* 49:243–257. [PubMed: 17380527]
- Chotiner JK, Nielson J, Farris S, Lewandowski G, Huang F, Banos K, de Leon R, Steward O (2010) Assessment of the role of MAP kinase in mediating activity-dependent transcriptional activation of the immediate early gene *Arc/Arg3.1* in the dentate gyrus in vivo. *Learn Mem* 17:117–129. [PubMed: 20154358]
- Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, Kuhl D, Huganir RL, Worley PF (2006) *Arc/Arg3.1* interacts with the endocytic machinery to regulate AMPA receptor trafficking. *Neuron* 52:445–459. [PubMed: 17088211]
- Chwang WB, O’Riordan KJ, Levenson JM, Sweatt JD (2006) ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. *Learn Mem* 13:322–328. [PubMed: 16741283]
- Cohen S, Greenberg ME (2008) Communication between the synapse and the nucleus in neuronal development, plasticity, and disease. *Annu Rev Cell Dev Biol* 24:183–209. [PubMed: 18616423]
- Dellu F, Mayo W, Cherkaoui J, Le Moal M, Simon H (1992) A two-trial memory task with automated recording: study in young and aged rats. *Brain Res* 588:132–139. [PubMed: 1393562]
- Gaudilliere B, Konishi Y, de la Iglesia N, Yao G, Bonni A (2004) A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. *Neuron* 41:229–241. [PubMed: 14741104]
- Grabowski PJ (2005) Splicing-active nuclear extracts from rat brain. *Methods* 37:323–330. [PubMed: 16314261]
- Green CR, Mihic AM, Nikkel SM, Stade BC, Rasmussen C, Munoz DP, Reynolds JN (2009) Executive function deficits in children with fetal alcohol spectrum disorders (FASD) measured using the Cambridge Neuropsychological Tests Automated Battery (CANTAB). *J Child Psychol Psychiatry* 50:688–697. [PubMed: 19175817]
- Gross LA, Moore EM, Wozniak JR, Coles CD, Kable JA, Sowell ER, Jones KL, Riley EP, Mattson SN (2017) Neural correlates of verbal memory in youth with heavy prenatal alcohol exposure. *Brain imaging and behavior*.
- Gupta-Agarwal S, Franklin AV, Deramus T, Wheelock M, Davis RL, McMahon LL, Lubin FD (2012) G9a/GLP Histone Lysine Dimethyltransferase Complex Activity in the Hippocampus and the Entorhinal Cortex Is Required for Gene Activation and Silencing during Memory Consolidation. *J Neurosci* 32:5440–5453. [PubMed: 22514307]
- Gupta S, Kim SY, Artis S, Molfese DL, Schumacher A, Sweatt JD, Paylor RE, Lubin FD (2010) Histone methylation regulates memory formation. *J Neurosci* 30:3589–3599. [PubMed: 20219993]
- Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA (2000) Inhibition of activity-dependent *arc* protein expression in the rat hippocampus impairs the

maintenance of long-term potentiation and the consolidation of long-term memory. *J Neurosci* 20:3993–4001. [PubMed: 10818134]

- Hendrickx A, Pierrot N, Tasiaux B, Schakman O, Kienlen-Campard P, De Smet C, Octave JN (2014) Epigenetic regulations of immediate early genes expression involved in memory formation by the amyloid precursor protein of Alzheimer disease. *Plos One* 9 e99467. [PubMed: 24919190]
- Hong EJ, West AE, Greenberg ME (2005) Transcriptional control of cognitive development. *Curr Opin Neurobiol* 15:21–28. [PubMed: 15721740]
- Ivanova TN, Matthews A, Gross C, Mappus RC, Gollnick C, Swanson A, Bassell GJ, Liu RC (2011) Arc/Arg3.1 mRNA expression reveals a subcellular trace of prior sound exposure in adult primary auditory cortex. *Neuroscience* 181:117–126. [PubMed: 21334422]
- Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. *Science* 274:1133–1138. [PubMed: 8895456]
- Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M, Takemoto-Kimura S, Worley PF, Bito H (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. [PubMed: 19116276]
- Kelly SJ, Day N, Streissguth AP (2000) Effects of prenatal alcohol exposure on social behavior in humans and other species. *Neurotoxicol Teratol* 22:143–149. [PubMed: 10758343]
- Khazipov R, Luhmann HJ (2006) Early patterns of electrical activity in the developing cerebral cortex of humans and rodents. *Trends Neurosci* 29:414–418. [PubMed: 16713634]
- Koditwaku PW, Adnams CM, Hay A, Kitching AE, Burger E, Kalberg WO, Viljoen DL, May PA (2006) Letter and category fluency in children with fetal alcohol syndrome from a community in South Africa. *J Stud Alcohol* 67:502–509. [PubMed: 16736069]
- Levenson JM, O’Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD (2004) Regulation of histone acetylation during memory formation in the hippocampus. *J Biol Chem* 279:40545–40559. [PubMed: 15273246]
- Lu B (2003) BDNF and activity-dependent synaptic modulation. *Learn Mem* 10:86–98. [PubMed: 12663747]
- Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG, Gilbert DJ, Jenkins NA, Lanahan AA, Worley PF (1995) Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 14:433–445. [PubMed: 7857651]
- Ma H, Groth RD, Cohen SM, Emery JF, Li B, Hoedt E, Zhang G, Neubert TA, Tsien RW (2014) gammaCaMKII shuttles Ca(2+)(+)/CaM to the nucleus to trigger CREB phosphorylation and gene expression. *Cell* 159:281–294. [PubMed: 25303525]
- Nagre NN, Subbanna S, Shivakumar M, Psychoyos D, Basavarajappa BS (2015) CB1-receptor knockout neonatal mice are protected against ethanol-induced impairments of DNMT1, DNMT3A, and DNA methylation. *J Neurochem* 132:429–442. [PubMed: 25487288]
- Noel M, Norris EH, Strickland S (2011) Tissue plasminogen activator is required for the development of fetal alcohol syndrome in mice. *Proc Natl Acad Sci U S A* 108:5069–5074. [PubMed: 21383198]
- Oey NE, Leung HW, Ezhilarasan R, Zhou L, Beuerman RW, VanDongen HM, VanDongen AM (2015) A Neuronal Activity-Dependent Dual Function Chromatin-Modifying Complex Regulates Arc Expression. *eNeuro* 2.
- Okuno H, Akashi K, Ishii Y, Yagishita-Kyo N, Suzuki K, Nonaka M, Kawashima T, Fujii H, Takemoto-Kimura S, Abe M, Natsume R, Chowdhury S, Sakimura K, Worley PF, Bito H (2012) Inverse synaptic tagging of inactive synapses via dynamic interaction of Arc/Arg3.1 with CaMKIIbeta. *Cell* 149:886–898. [PubMed: 22579289]
- Olney JW, Tenkova T, Dikranian K, Qin YQ, Labruyere J, Ikonomidou C (2002) Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Brain Res Dev Brain Res* 133:115–126. [PubMed: 11882342]
- Panczakiewicz AL, Glass L, Coles CD, Kable JA, Sowell ER, Wozniak JR, Jones KL, Riley EP, Mattson SN (2016) Neurobehavioral Deficits Consistent Across Age and Sex in Youth with Prenatal Alcohol Exposure. *Alcohol Clin Exp Res* 40:1971–1981. [PubMed: 27430360]

- Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Welzl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A, Therstappen E, Cooke SF, Blanquet V, Wurst W, Salmen B, Bosl MR, Lipp HP, Grant SG, Bliss TV, Wolfer DP, Kuhl D (2006) Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron* 52:437–444. [PubMed: 17088210]
- Pollak DD, Herkner K, Hoeger H, Lubec G (2005) Behavioral testing upregulates pCaMKII, BDNF, PSD-95 and egr-1 in hippocampus of FVB/N mice. *Behav Brain Res* 163:128–135. [PubMed: 15927279]
- Popova S, Lange S, Probst C, Gmel G, Rehm J (2017a) Global Prevalence of Alcohol Use and Binge Drinking During Pregnancy and Fetal Alcohol Spectrum Disorder. *Biochem Cell Biol*.
- Popova S, Lange S, Probst C, Parunashvili N, Rehm J (2017b) Prevalence of alcohol consumption during pregnancy and Fetal Alcohol Spectrum Disorders among the general and Aboriginal populations in Canada and the United States. *European journal of medical genetics* 60:32–48. [PubMed: 27638329]
- Porte Y, Buhot MC, Mons N (2008) Alteration of CREB phosphorylation and spatial memory deficits in aged 129T2/Sv mice. *Neurobiol Aging* 29:1533–1546. [PubMed: 17478013]
- Ramirez-Amaya V, Angulo-Perkins A, Chawla MK, Barnes CA, Rosi S (2013) Sustained transcription of the immediate early gene Arc in the dentate gyrus after spatial exploration. *J Neurosci* 33:1631–1639. [PubMed: 23345235]
- Ramirez-Amaya V, Vazdarjanova A, Mikhael D, Rosi S, Worley PF, Barnes CA (2005) Spatial exploration-induced Arc mRNA and protein expression: evidence for selective, network-specific reactivation. *J Neurosci* 25:1761–1768. [PubMed: 15716412]
- Redmond L, Kashani AH, Ghosh A (2002) Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron* 34:999–1010. [PubMed: 12086646]
- Rial Verde EM, Lee-Osbourne J, Worley PF, Malinow R, Cline HT (2006) Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. *Neuron* 52:461–474. [PubMed: 17088212]
- Sadrian B, Lopez-Guzman M, Wilson DA, Saito M (2014) Distinct neurobehavioral dysfunction based on the timing of developmental binge-like alcohol exposure. *Neuroscience* 280:204–219. [PubMed: 25241068]
- Sadrian B, Subbanna S, Wilson DA, Basavarajappa BS, Saito M (2012) Lithium prevents long-term neural and behavioral pathology induced by early alcohol exposure. *Neuroscience* 206:122–135. [PubMed: 22266347]
- Sanchez Vega MC, Chong S, Burne TH (2013) Early gestational exposure to moderate concentrations of ethanol alters adult behaviour in C57BL/6J mice. *Behav Brain Res* 252:326–333. [PubMed: 23756143]
- Sarnyai Z, Sibille EL, Pavlides C, Fenster RJ, McEwen BS, Toth M (2000) Impaired hippocampal-dependent learning and functional abnormalities in the hippocampus in mice lacking serotonin(1A) receptors. *Proc Natl Acad Sci U S A* 97:14731–14736. [PubMed: 11121072]
- Savage DD, Becher M, de la Torre AJ, Sutherland RJ (2002) Dose-dependent effects of prenatal ethanol exposure on synaptic plasticity and learning in mature offspring. *Alcohol Clin Exp Res* 26:1752–1758. [PubMed: 12436066]
- Schambra UB, Lewis CN, Harrison TA (2017) Deficits in spatial learning and memory in adult mice following acute, low or moderate levels of prenatal ethanol exposure during gastrulation or neurulation. *Neurotoxicol Teratol* 62:42–54. [PubMed: 28476615]
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* 3:1101–1108. [PubMed: 18546601]
- Selcher JC, Weeber EJ, Christian J, Nekrasova T, Landreth GE, Sweatt JD (2003) A role for ERK MAP kinase in physiologic temporal integration in hippocampal area CA1. *Learn Mem* 10:26–39. [PubMed: 12551961]
- Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Huganir RL, Worley PF (2006) Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron* 52:475–484. [PubMed: 17088213]

- Sood B, Delaney-Black V, Covington C, Nordstrom-Klee B, Ager J, Templin T, Janisse J, Martier S, Sokol RJ (2001) Prenatal alcohol exposure and childhood behavior at age 6 to 7 years: I. dose-response effect. *Pediatrics* 108:E34. [PubMed: 11483844]
- Soule J, Penke Z, Kanhema T, Alme MN, Laroche S, Bramham CR (2008) Object-place recognition learning triggers rapid induction of plasticity-related immediate early genes and synaptic proteins in the rat dentate gyrus. *Neural Plast* 2008:269097. [PubMed: 19190776]
- Subbanna S, Basavarajappa BS (2014) Pre-administration of G9a/GLP inhibitor during Synaptogenesis Prevents Postnatal Ethanol-induced LTP Deficits and Neurobehavioral Abnormalities in Adult Mice. *Exp Neurol* 261 34–43. [PubMed: 25017367]
- Subbanna S, Nagre NN, Shivakumar M, Joshi V, Psychoyos D, Kutlar A, Umapathy NS, Basavarajappa BS (2018) CB1R-Mediated Activation of Caspase-3 Causes Epigenetic and Neurobehavioral Abnormalities in Postnatal Ethanol-Exposed Mice. *Frontiers in molecular neuroscience* 11:45. [PubMed: 29515368]
- Subbanna S, Nagre NN, Shivakumar M, Umapathy NS, Psychoyos D, Basavarajappa BS (2014) Ethanol induced acetylation of histone at G9a exon1 and G9a-mediated histone H3 dimethylation leads to neurodegeneration in neonatal mice. *Neuroscience* 258:422–432. [PubMed: 24300108]
- Subbanna S, Nagre NN, Umapathy NS, Pace BS, Basavarajappa BS (2015) Ethanol exposure induces neonatal neurodegeneration by enhancing CB1R Exon1 histone H4K8 acetylation and up-regulating CB1R function causing neurobehavioral abnormalities in adult mice. *Int J Neuropsychopharmacol* 18: 1–15
- Subbanna S, Shivakumar M, Psychoyos D, Xie S, Basavarajappa BS (2013a) Anandamide-CB1 Receptor Signaling Contributes to Postnatal Ethanol-Induced Neonatal Neurodegeneration, Adult Synaptic and Memory Deficits. *Journal of neuroscience* 33:6350–6366.
- Subbanna S, Shivakumar M, Umapathy NS, Saito M, Mohan PS, Kumar A, Nixon D, RA, Verin AD, Psychoyos Basavarajappa BS (2013b) G9a-Mediated Histone Methylation Regulates Ethanol-Induced Neurodegeneration in the Neonatal Mouse Brain. *Neurobiol Dis* 54 475–485. [PubMed: 23396011]
- Sutherland RJ, McDonald RJ, Savage DD (1997) Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. *Hippocampus* 7:232–238. [PubMed: 9136052]
- Tabuchi A (2008) Synaptic plasticity-regulated gene expression: a key event in the long-lasting changes of neuronal function. *Biol Pharm Bull* 31:327–335. [PubMed: 18310887]
- Taha S, Stryker MP (2002) Rapid ocular dominance plasticity requires cortical but not geniculate protein synthesis. *Neuron* 34:425–436. [PubMed: 11988173]
- Tzingounis AV, Nicoll RA (2006) Arc/Arg3.1: linking gene expression to synaptic plasticity and memory. *Neuron* 52:403–407. [PubMed: 17088207]
- Vaglenova J, Pandiella N, Wijayawardhane N, Vaithianathan T, Birru S, Breese C, Suppiramaniam V, Randal C (2008) Aniracetam reversed learning and memory deficits following prenatal ethanol exposure by modulating functions of synaptic AMPA receptors. *Neuropsychopharmacology* 33:1071–1083. [PubMed: 17609677]
- Vaillant AR, Zanassi P, Walsh GS, Aumont A, Alonso A, Miller FD (2002) Signaling mechanisms underlying reversible, activity-dependent dendrite formation. *Neuron* 34:985–998. [PubMed: 12086645]
- Waltereit R, Dammermann B, Wulff P, Scafidi J, Staubli U, Kauselmann G, Bundman M, Kuhl D (2001) Arg3.1/Arc mRNA induction by Ca²⁺ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. *J Neurosci* 21:5484–5493. [PubMed: 11466419]
- West AE, Greenberg ME (2011) Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harbor perspectives in biology* 3:1–21.
- Wilson DA, Peterson J, Basavaraj BS, Saito M (2011) Local and regional network function in behaviorally relevant cortical circuits of adult mice following postnatal alcohol exposure. *Alcoholism Clin and Exp Res* 35:1974–1984.
- Wozniak JR, Mueller BA, Mattson SN, Coles CD, Kable JA, Jones KL, Boys CJ, Lim KO, Riley EP, Sowell ER (2017) Functional connectivity abnormalities and associated cognitive deficits in fetal

alcohol Spectrum disorders (FASD). *Brain imaging and behavior* 11:1432–1445. [PubMed: 27734306]

Zhang W, Wu J, Ward MD, Yang S, Chuang YA, Xiao M, Li R, Leahy DJ, Worley PF (2015) Structural basis of arc binding to synaptic proteins: implications for cognitive disease. *Neuron* 86:490–500. [PubMed: 25864631]

Zovkic IB, Guzman-Karlsson MC, Sweatt JD (2013) Epigenetic regulation of memory formation and maintenance. *Learn Mem* 20:61–74. [PubMed: 23322554]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HIGHLIGHTS:

- Postnatal ethanol treatment impairs spatial memory behavior on Y-maze in adult mice.
- Ethanol impairs, and SR rescues Y-maze enhanced pCaMKIV, pCaMKII, pCREB.
- Ethanol impairs, and SR prevents Y-maze enhanced H3K14/H4K8ac and Arc expression.
- Y-maze enhances H3K9me2 in postnatal ethanol exposed adult mice and was rescued by SR.
- Arc expression is regulated by epigenetic modification on Arc gene promoter and rescued by Bix.

Fig. 1.

SR preadministration rescues postnatal ethanol-inhibited activation of activity-dependent signaling events in adult mice. (A) Schematic diagram representing the experimental strategy to determine the activity-dependent events in adulthood. (B) The spatial memory of adult mice treated with saline + vehicle (S + V), ethanol + vehicle (E + V), S + SR, or E + SR at P7 was determined using a Y-maze (* $p < 0.01$ vs. S + V; @ $p < 0.01$ vs. E + V). (C) HP tissues were processed immediately after intertrial interval (24 h) when the mice completed exploring all three arms (3 min, preference trial, test trial). The signaling proteins

(i) pCaMKIV, (ii) pCREB, (iii) pERK1/2 and (iv) pCaMKII were evaluated in nuclear or cytosolic fractions from naïve saline (S + V), ethanol (E + V) and Y-maze-subjected S + V, E + V, S + SR, or E + SR groups through western blot analysis. Ponceau S staining was done to establish identical protein loading. As a loading control β -actin was used. [$*p < 0.01$ vs. S + V (Naïve); $\#p < 0.01$ vs. S + V (Naïve); $\$p < 0.01$ vs S + V (Y-maze); $@p < 0.01$ vs. E + V (Y-maze)]. Error bars, SEM (n = 8 mice/group).

Fig. 2.

Postnatal ethanol impairs activity-dependent modulation of global epigenetic changes and SR preadministration rescues these defects in ethanol-exposed adult mice. HP tissues were processed immediately after intertrial interval (24 h) as described in Fig. 1A. The histone proteins (i) H4K8ac, (ii) H3K14ac, (iii) H3K9me2 were evaluated in nuclear extracts from naive saline + vehicle (S + V), ethanol + vehicle (E + V) and Y-maze-subjected S + V, E + V, S + SR, or E + SR groups through western blot analysis. Ponceau S staining was done to establish identical protein loading. As a loading control β -actin was used. [$\#p < 0.01$ vs. S +

V (Naïve); \$p < 0.01 vs. S + V (Y-maze); @p < 0.01 vs E + V (Y-maze)]. Error bars, SEM (n = 8 mice/group).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Postnatal ethanol impairs activity-dependent Arc expression, *Arc* gene promoter epigenetic changes and SR preadministration rescues these defects in ethanol-exposed adult mice. (A) HP tissues were processed immediately after intertrial interval (24 h) as described in Fig. 1A. The Arc protein was determined via western blot analysis from naïve saline + vehicle (S + V), ethanol + vehicle (E + V) and Y-maze-subjected S + V, E + V, S + SR, or E + SR groups. Ponceau S staining was done to establish identical protein loading. As a loading control β -actin was used. [*p < 0.01 vs. S + V (naïve); #p < 0.01 vs. S + V (naïve); \$p < 0.01

vs S + V (Y-maze); @p < 0.01 vs E + V (Y-maze)] (n = 16 mice/group). **(B)** ChIP analysis of the *Arc* gene promoter in HP tissues from the six treatment groups (naïve: S + V, E + V; Y-maze: S + V, E + V, S + SR, or E + SR) with anti-H3K14ac, anti-H4K8ac, anti-CBP, anti-H3K9me2 or anti-G9a antibodies. Enrichment of *Arc* gene promoter chromatin in the IPs were measured by RT-qPCR [*p < 0.01 vs. S + V (naïve); #p < 0.01 vs. S + V (naïve); \$p < 0.01 vs S + V (Y-maze); @p < 0.01 vs E + V (Y-maze)]. Error bars, SEM (n = 16 mice/group).

Fig. 4.

Bix preadministration prevents ethanol-induced spatial memory defects and the Y-maze behavior enhanced H3K9me2 in P7 ethanol treated adult mice. **(A)** The spatial memory of adult mice treated with saline + vehicle (S + V), ethanol + vehicle (E + V), S + Bix, or E + Bix at P7 was evaluated using a Y-maze (* $p < 0.01$ vs. S + V; @ $p < 0.01$ vs. E + V). **(B)** HP tissues were processed immediately after a 24 h intertrial interval as described in Fig 1A. The H3K9me2 protein was determined from naïve saline (S), ethanol (E) and Y-maze-subjected S + V, E + V, S + Bix, or E + Bix groups via western blot analysis. Ponceau S

staining was done to establish identical protein loading. As a loading control β -actin was used. [$p < 0.01$ vs. S + V (Y-maze); @ $p < 0.01$ vs E + V (Y-maze)]. Error bars, SEM (n = 8 mice/group).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. G9a/GLP inhibitor administration before postnatal ethanol exposure prevents ethanol-induced defects in activity-dependent Arc expression in adult mice. HP tissues were processed immediately after a 24 h intertrial interval as described in Fig. 1A. The Arc protein was determined from naïve saline + vehicle (S + V), ethanol + vehicle (E + V) and Y-maze-subjected S + V, E + V, S + Bix, or E + Bix groups via western blot analysis. Ponceau S staining was done to establish identical protein loading. As a loading control β-actin was used. [*p < 0.01 vs. S + V (naïve); #p < 0.01 vs. S + V (naïve); \$p < 0.01 vs S + V (Y-maze); @p < 0.01 vs E + V (Y-maze)]. Error bars, SEM (n = 8 mice/group).