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Pre-administration of G9a/GLP Inhibitor during Synaptogenesis Prevents Postnatal Ethanol-induced LTP Deficits and Neurobehavioral Abnormalities in Adult Mice

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Abstract

It has been widely accepted that deficits in neuronal plasticity underlie the cognitive abnormalities observed in fetal alcohol spectrum disorder (FASD). Exposure of rodents to acute ethanol on postnatal day 7 (P7), which is equivalent to the third trimester of fetal development in human, induces long-term potentiation (LTP) and memory deficits in adult animals. However, the molecular mechanisms underlying these deficits are not well understood. Recently, we found that histone H3 dimethylation (H3K9me2), which is mediated by G9a (lysine dimethyltransferase), is responsible for the neurodegeneration caused by ethanol exposure in P7 mice. In addition, pharmacological inhibition of G9a prior to ethanol treatment at P7 normalized H3K9me2 proteins to basal levels and prevented neurodegeneration in neonatal mice. Here, we tested the hypothesis that pre-administration of G9a/GLP inhibitor (Bix-01294, Bix) in conditions in which ethanol induces neurodegeneration would be neuroprotective against P7 ethanol-induced deficits in LTP, memory and social recognition behavior in adult mice. Ethanol treatment at P7 induces deficits in LTP, memory and social recognition in adult mice and these deficits were prevented by Bix pretreatment at P7. Together, these findings provide physiological and behavioral evidence that the long-term harmful consequences on brain function after ethanol exposure with a third trimester equivalent have an epigenetic origin.

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Disclosure
The authors declare no conflict of interest.

Keywords

Synaptic plasticity; FASD; methyltransferase; memory loss; epigenetics; histones; Bix; postnatal development; ethanol; H3K9

Introduction

Ethanol exposure during pregnancy causes birth defects (Jones and Smith, 1973) and can lead to fetal alcohol spectrum disorders (FASDs) (Streissguth, et al., 1990). FASD symptoms generally include growth deficiency and brain damage. FASD is one of the major contributors to intellectual disability in the Western world (Mattson, et al., 2011). Some of the most persistent deficits are neurobehavioral hallmarks, such as learning and memory deficits (Goodman, et al., 1999, Mattson, et al., 1999). As many as 1 in 100 children born in the United States and Canada (Chudley, et al., 2005, May and Gossage, 2001) are estimated to be diagnosed with FASD, whereas heavily afflicted areas of South Africa exhibit the most pervasive diagnoses of FASD in around 10.9 per 100 children (May, et al., 2000, May, et al., 2007, Urban, et al., 2008). The developing brain is so sensitive to ethanol exposure that even a single exposure can produce massive losses of neurons in several brain regions (Ikonomidou, et al., 2000) during the first few postnatal days in neonatal mice (postnatal days 4–10 [P4–10]), a developmental period which corresponds with the third trimester pregnancy in humans (Bayer, et al., 1993). Excessive acute ethanol intoxication in P7 mice prompts neurodegeneration in vital brain regions including the hippocampus and cortex (Ikonomidou, et al., 2000, Sadrian, et al., 2012, Subbanna, et al., 2014, Subbanna, et al., 2013a, Subbanna, et al., 2013b, Wilson, et al., 2011), as well as impairments in LTP (Izumi, et al., 2005, Sadrian, et al., 2012, Subbanna, et al., 2013a, Wilson, et al., 2011) and spatial memory task performance in adult mice (Subbanna, et al., 2013a). Similarly, the local and interregional brain circuitry of the olfacto-hippocampal pathway in adult mice is compromised when P7 mice are exposed to acute ethanol (Sadrian, et al., 2012, Wilson, et al., 2011).

Increasing evidence suggests that ethanol exposure during brain development induces chromatin dysregulation in numerous brain regions (Bekdash, et al., 2013, Kaminen-Ahola, et al., 2010a, Kaminen-Ahola, et al., 2010b, Perkins, et al., 2013, Subbanna, et al., 2014, Subbanna, et al., 2013b), which may be responsible for the development of ethanol associated brain disorders (Mattson, et al., 2011, Mattson, et al., 2010). Recent studies focus on the importance of post-translational modification of histone proteins on the regulation of normal brain function and the development of several human developmental disorders (Campuzano, et al., 1996, Gavin and Sharma, 2010, Makedonski, et al., 2005, Petronis, 2003, Ryu, et al., 2006, Warren, 2007). In addition to acetylation and phosphorylation, histone methylation is one of the most extensively investigated histone modification mechanism in the central nervous system (CNS) (Tsankova, et al., 2006). Histone H3K9 dimethylation is correlated with transcriptional inhibition, whereas histone H3 trimethylation at lysine 4 (H3K4me3) is linked to active transcription (Schneider, et al., 2004). The dimethylation of histone H3K9 is catalyzed by the euchromatic histone methyltransferases (EHMTases), including G9a (Tachibana, et al., 2002) and the G9a-

related protein (GLP) (Ogawa, et al., 2002); these can repress gene expression by inducing local dimethylation of H3K9 at target promoters. Consequentially, G9a/GLP regulate neuronal function during brain development (Schaefer, et al., 2009). Recently, we reported that histone H3K9 dimethylation by G9a was responsible for postnatal ethanol-induced neurodegeneration (Subbanna, et al., 2013b). In addition, in the presence of ethanol, the G9a exon itself is regulated by epigenetic modification of histone proteins during early brain development (Subbanna, et al., 2014). The present study evaluated the neuroprotective role of G9a inhibition on postnatal ethanol-induced long-lasting neurobehavioral deficits in adult mice.

Materials and methods

Animals and treatment

Animal care and handling procedures followed Institutional (NKI IACUC) and National Institutes of Health guidelines. C57BL/6J mice were housed in groups under standard laboratory conditions (12 hr light / 12 hr dark cycle) with food and water available ad libitum. An ethanol treatment paradigm, which has been previously shown to induce robust apoptotic neurodegeneration in P7 mice (Olney, et al., 2002) and causes no lethality, was used in the current study. Litters of mice were culled to four to six pups per litter, and on the day of treatment, half of the pups (male) in each litter were treated subcutaneously (s. c.) with saline and the other half with ethanol at P7 (based on the day of birth) (2.5 g/kg s. c. at 0 h and again at 2 h) in their home cage with the dam as described previously by our laboratory (Subbanna, et al., 2013a, Subbanna, et al., 2013b). For blood ethanol levels (BEL), pups were euthanized by decapitation ; truncal blood was collected at 3 and 9 hr following the first ethanol injection. The concentrations of ethanol in pup serum were then determined using a standard alcohol dehydrogenase-based method (Lundquist, 1959). For the Bix experiments, Bix-01294 (2-(Hexahydro-4-methyl-1 *H*-1,4-diazepin-1-yl)-6,7-dimethoxy-*N*-[1-(phenylmethyl)-4-piperidinyl]-4-quinazolinamine trihydrochloride) (Cayman, Michigan, USA) was dissolved in 10μl of ethanol followed by 2–3 drops of Tween 80 (10 μl) and then volume was made up with sterile saline solution. The Bix solution was administered by s. c. injection at a volume of 5 μl/g body weight 30 min before ethanol treatment. The Bix vehicle solution was injected as a control. Bix treatment did not alter P7 ethanol induced intoxication (sleeping time) and Bix alone treated P7 mice were similar like saline treated mice and did not cause any inflammation or bleeding in any of the organs (Subbanna, et al., 2014, Subbanna, et al., 2013b). Mice were kept with the dams until they were weaned. Three months old mice derived from different litters after P7 treatment [saline + vehicle (S + V), ethanol + vehicle (E + V), saline + Bix (S + Bix) and ethanol + Bix (E + Bix)] were used for several analyses, as described below. Five to 8 animals were used for each data point. In the current study, male mice were used for behavioral analysis to avoid the hormonal fluctuation that occurs during the estrous cycle; this could potentially affect animal behavior, thus complicating the data interpretation . Separate sets of animals were subjected to each behavioral study (n=8/group).

Immunohistochemistry

Mice were anesthetized and perfused with a solution containing 4% paraformaldehyde and 4% sucrose in 0.05 M cacodylate buffer (pH 7.2), 8 h after the first ethanol dose treatment . It has been shown that this time point is optimal to induce maximum caspase-3 activation in one or more brain regions (Ikonomidou, et al., 2000, Subbanna, et al., 2013b, Wilson, et al., 2011). The brains were further processed according to our previously described protocols (Subbanna, et al., 2014, Subbanna, et al., 2013a, Subbanna, et al., 2013b). Free-floating sections were obtained from ethanol- and saline-exposed brains (8 h of exposure) and immunostained using an antibody against anti-rabbit cleaved caspase-3 (Asp175) (CC3) (# 9661 , Cell Signaling Technology, Danvers, MA, USA) with ABC reagents (Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA) and a peroxidase substrate (DAB) kit (Vector Labs) to label neurodegenerating neurons. The primary antibodies were omitted from the reactions as a control for secondary antibody specificity. In addition, pre-incubation with blocking peptides for the anti-CC3 (GenScript, Piscataway, NJ, USA) completely blocked the immunostaining of CC3 antibody. All photomicrographs were taken through a 2.5X, or 40X objective with a Nikon Eclipse TE2000 inverted microscope attached to a digital camera (DXM1200F, Morrell Instrument Company, Melville, NY, USA).

Electrophoresis and immunoblot

For Western blot analysis, homogenates from the hippocampus and cortex of the pups were processed 4–24 h after saline or ethanol (first ethanol dose) injection as described previously (Lubin and Sweatt, 2007, Subbanna, et al., 2013a, Subbanna, et al., 2013b). Cytosolic and nuclear fractions from tissue homogenates were prepared as described in our recent publications (Basavarajappa, et al., 2014, Basavarajappa and Subbanna, 2014). The samples were prepared in a sample buffer as previously described by our laboratory (Basavarajappa, et al., 2008). The blots were incubated with the following primary antibodies: anti-rabbit-CC3 (Asp175) (polyclonal, #9661, 1:1000), anti-mouse-β-actin (monoclonal, #3700, 1:1,000), anti-rabbit-H3K9me2 (monoclonal, # 4658, 1: 1000) and anti-rabbit-H3 (polyclonal, # 9715, 1:1000) (Cell Signaling Technology, Danvers, MA, USA). The blots were incubated with the primary antibodies for 3 h at room temperature or overnight at 4 °C and processed as previously described by our laboratory (Basavarajappa, et al., 2008). Incubation with a secondary antibody alone did not produce any bands (data not shown).

LTP

Three-month-old male mice, treated at P7 with S + V, E + V, S + Bix, or E + Bix (n=5/group), were sacrificed by cervical dislocation followed by decapitation . Hippocampi were quickly removed. Transverse hippocampal slices (400 μm) were cut and recorded according to standard procedures (Basavarajappa and Subbanna, 2014, Sadrian, et al., 2012, Subbanna, et al., 2013a, Vitolo, et al., 2002). Following cutting, hippocampal slices were transferred to a recording chamber where they were maintained at 29° C and perfused with artificial cerebrospinal fluid (ACSF) continuously bubbled with 95% O2 and 5% CO2. The ACSF composition in mM was: 124.0 NaCl, 4.4 KCl, 1.0 Na2HPO4, 25.0 NaHCO3, 2.0 CaCl2,

2.0 MgSO4, 10.0 glucose, osmolarity 290–300. CA1 fEPSPs were recorded by placing both the stimulating and the recording electrodes in CA1 *stratum radiatum* . Basal synaptic transmission (BST) was determined by plotting the fiber volley amplitude against slopes of field-excitatory-post-synaptic potential (fEPSP). For LTP experiments, a 10 min baseline was recorded every min at an intensity that evokes a response ~35% of the maximum evoked response. LTP was induced using theta-burst stimulation (4 pulses at 100 Hz, with the bursts repeated at 5 Hz, and each tetanus including 3× 10-burst trains separated by 15 seconds). Responses were recorded for 2 hrs after and measured as fEPSP slope expressed as percentage of baseline.

Object recognition memory

Novel object recognition memory was evaluated as described before (Basavarajappa and Subbanna, 2014, Ennaceur and Delacour, 1988, Subbanna, et al., 2013a), which is based on the natural tendency of rodents to explore a novel object more than a familiar one. In brief, three month-old male mice, treated at P7 with S + V, E + V, S + Bix and E + Bix (n=8/group) were submitted individually to a habituation session where they were allowed to freely explore the open field for 3 min twice a day for two days . No objects were placed in the box during the habituation trial. Twenty-four hours after habituation, training (T1) was conducted by placing individual mice for 3 min in the open field, in which two identical objects (objects a1 and a2) were positioned in two adjacent corners at 10 cm distance from the walls. In a short-term recognition memory test given at 1 or 4 h (retention) after the training (T2), the mice explored the open field for 3 min in the presence of one familiar (a1) and one novel (b1, 1h; b2, 4h) object. In a long-term recognition memory test given at 24h (retention) after training (T2), the mice explored the open field for 5min in the presence of one familiar (a1) and one novel object (b3; different from b1 and b2). All objects had similar textures and sizes but had distinctive shapes and colors. Between trials, the objects were washed with 10% ethanol solution. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. e1 and e2 are measures of the total exploration time of both objects during T1 and T2, respectively. d2 was considered as index measures of discrimination between the new and the familiar objects. d2 is a relative measure of discrimination which corrects the difference between exploring the old and the novel object for exploration activity (e2) and appears to be independent of the total exploration times (Sik, et al., 2003).

Spontaneous alternation Y maze task

Spontaneous alternation was tested as described previously (Basavarajappa and Subbanna, 2014, Holcomb, et al., 1998) to assess spatial working memory that is dependent upon the hippocampus. In brief, three month old male mice, treated at P7 with S + V, E + V, S + Bix and E + Bix (n=8/group) were used in this test. Each mouse was placed in the center of the Y maze and was allowed to explore freely through the maze during an 8 min session. The sequence and total number of arms entered was recorded. Arm entry was considered to be completed when the hind paws of the mouse had been completely placed in the arm. Percentage alternation is the number of triads containing entries into all three arms divided by the maximum possible alternations (the total number of arms entered minus 2) × 100.

Spatial recognition memory using Y maze

Spatial recognition memory using Y maze was tested as described previously (Basavarajappa and Subbanna, 2014, Sarnyai, et al., 2000). Briefly, three-month-old male mice, treated at P7 with S + V, E + V, S + Bix and E + Bix (n=8/group) were used in this test. Briefly, each mouse was placed into one of the arms of the Y maze (start arm) and allowed to explore the maze with one of the arms closed for 10 min (training trial). After a 1h intertrial interval, mice were returned to the Y maze by placing them in the start arm. Then, the mice were allowed to explore freely all three arms of the maze for 3 min (test trial). The number of entries into and the time spent in each arm, and the first choice of entry were registered manually and from video recordings by an observer blind to the treatment or genotype of the mice.

Social recognition memory

The social recognition test was performed as previously described (Kogan, et al., 2000, Thor, et al., 1982). Three-month-old male mice were treated on P7 with S + V, E + V, S + Bix and E + Bix (n=8/group). Each mouse was placed in an individual cage in an observation room with dim light immediately prior to the experimental sessions. The mice were allowed to habituate to the new environment for 15 min. The experimental cages were identical to those in which the animals were normally housed (plastic, 27 cm long × 16 cm wide × 12 cm high). A male, juvenile mouse (3–4 weeks old) was placed into a cage with an adult for an initial interaction trial (2 min duration). Following the intertrial delay, the same juvenile mouse was placed back into the adult's cage for a 2-min test trial. The animals were returned to their home cages during the interim between the initial and the test trials. A trained observer continuously timed the duration of the social investigation (with a hand-held stopwatch). The scored social investigative behaviors have been previously described and include the following: direct contact with the juvenile while inspecting any part of the body surface (including grooming, licking, and pawing); sniffing of the mouth, ears, tail, ano-genital area; and close following (within 1 cm) of the juvenile (Kogan, et al., 2000, Thor, et al., 1982). If the adult mouse did not investigate the juvenile mouse for a minimum of 24 s during the initial trial (i.e., 20% of trial time), they were retested once with another juvenile. The trials with initial investigation times less than 24 s were excluded from the analysis. Any aggressive encounter between animals was an immediate cause for termination of the experiment, and these data were excluded from the analysis. The percentage of social investigation was calculated by dividing the investigation time during the second exposure by the initial investigation time × 100.

Statistical analysis

All of the data are presented as the mean ± SEM. A statistical comparison of the data was performed by either a student's *t* test or one-way analysis of variance (ANOVA) or a two-way ANOVA with Bonferroni's *post hoc* test. In all of the comparisons, $p < 0.05$ was considered to indicate statistical significance. The statistical analyses were performed using the Prism software (GraphPad, San Diego, CA).

Results

Pre-administration of G9a/GLP inhibitor before ethanol treatment in P7 mice prevents neurodegeneration in neonatal mice and LTP deficits in adult mice

Ethanol administration (2.5 g/kg s. c. at 0 h and again at 2 h) to mouse pups at P7 resulted in a blood ethanol level (BEL) of ~0.46 ± 0.22 g/dL at 3 h (first ethanol dose) that gradually decreased to 0.28 ± 0.05 g/dL at 9 h (first ethanol dose) . This ethanol paradigm produced a widespread pattern of neurodegeneration throughout the forebrain [hippocampus ($F_{1, 11}$ = 60, p<0.001) and cortex ($F_{1, 11}$ = 100, p<0.001) regions] (one-way ANOVA). The neurodegeneration was measured by caspase-3 activation [formation of cleaved caspase-3 (CC3)] in the ethanol-exposed brains (Fig. 1A). We also evaluated neurodegeneration in the hippocampal and neocortical protein extracts by Western blot analysis. Comparisons with a one-way ANOVA and Bonferroni's *post hoc* tests indicated that the 4 h (first ethanol dose) ethanol-treated group was not significantly different compared to saline (0 h) group. The 8 and 24 h ethanol-treated (first ethanol dose) groups exhibited significantly greater proportions of neuronal death in both the hippocampus ($F_{3, 28}$ = 55, p < 0.001) and neocortex ($F_{3, 28}$ = 60, p < 0.001) (Fig. 1B) compared to saline (0 h) group . Taken together, our experimental conditions demonstrated the apoptotic patterns and severity that has been previously described for this ethanol treatment paradigm in the developing brain (Ikonomidou, et al., 2000, Olney, et al., 2002, Subbanna, et al., 2013a, Subbanna, et al., 2013b).

In our previous studies, we found that Bix pretreatment inhibited ethanol-induced caspase 3 activation in the neocortex in a dose-dependent manner (0.25, 0.5, and 1 mg/kg) (Subbanna, et al., 2013b). The administration of Bix at the maximum dose (1 mg/kg) 30 min before ethanol treatment (first ethanol dose) was more effective in inhibiting G9a-mediated ethanol-induced caspase-3 activation (Fig. 1C) than co-treatment (ethanol and Bix together) or post-treatment [administration of Bix 1 h after ethanol treatment (first ethanol dose)] (Subbanna, et al., 2013b). Pre-administration of Bix also rescued ethanol-induced loss of H3K9me2 and H3 as observed in the previous study (p < 0.001) (Fig. 1D) (Subbanna, et al., 2013b). Furthermore, the administration of Bix (1 mg/kg) before ethanol treatment does not alter the BELs (Subbanna, et al., 2013b) [BEL peaked at 3 h (first ethanol dose) at 0.43 ± 0.20 g/dl and was gradually reduced to 0.23 ± 0.09 g/dl at 9 h (first ethanol dose)], which indicates that Bix pretreatment inhibited neurodegeneration without modulating the ethanol metabolism.

We first determined the input/output (I/O) responses and the LTP in the Schaffer collateral pathway of hippocampal slices (Fig. 2A) prepared from the adult animals treated with S + V, E + V, S + Bix and E + Bix at P7. The robust I/O responses were evoked by increasing the stimulus intensity in all groups. The I/O curve was not altered by S + V, E + V, S + Bix and E + Bix treatment (p>0.05) (Fig. 2B). Prior to tetanic stimulations, the baseline fEPSP was recorded at 60 s intervals with a stimulation intensity equivalent to ~35% of the maximum evoked response. The tetanic stimulation evoked typical LTP (Fig. 2C) in slices from adult mice treated on P7 with S + V, E + V, S + Bix and E + Bix . These responses were stable over 120 min. However, the tetanic stimulation evoked a significantly reduced LTP in slices

(n = 10 slices/5 mice/group) prepared from P7 E + V animals compared to S + V animals ($p < 0.001$) with a significant group interaction (two-way ANOVA) ($F_{1, 36} = 26$, $p< 0.001$; *post-hoc* test): S + V vs. E + V significantly different at all post-tetanic stimulation time intervals) ($p < 0.001$). The LTP in slices prepared from S + Bix-treated animals did not differ significantly from those in S + V-treated mice ($p > 0.05$). Bix pre-treatment completely rescued the P7 ethanol-induced LTP defects ($p < 0.001$) (Fig. 2D).

Pre-administration of G9a/GLP inhibitor at P7 rescues P7 ethanol-induced memory loss in adult mice

First, we investigated object recognition memory (ORM) to examine whether G9a/GLP inhibitor , which protects neurons from P7 ethanol-induced neurodegeneration (Subbanna, et al., 2013b) and LTP deficits, also rescues ethanol-induced memory impairments. The results indicate that P7 S + V, E + V or S + Bix, E + Bix treatment has no significant effect on exploration times (e1 or e2) in the ORM task (e1; $F_{3, 28} = 1.2$, $p > 0.05$; e2; $F_{3, 28} = 1.2$, $p > 0.05$; one-way ANOVA) (Fig. 3A). The ethanol (E + V) treatment at P7 impaired both short- (1+ 4 h retention, combined) (Fig. 3B) ($F_{3, 45} = 43$, $p < 0.001$) and long- (24 h retention) (Fig. 3C) ($F_{3, 21} = 21$, $p < 0.001$) (Two-way ANOVA) term ORM performance. Bix treatment before P7 ethanol administration (E + Bix) rescued ORM performance as observed with LTP. The ORM performance by S + Bix-treated animals was not significantly different from saline-treated mice ($p > 0.05$). Therefore, blocking G9a function before P7 ethanol exposure prevents ORM deficits in the adult mice.

In our second behavioral test, the adult mice treated with P7 S + V, E + V, S + Bix and E + Bix were tested with a spontaneous alternation in the Y maze (Lalonde, 2002). P7 S + V, E + V, S + Bix and E + Bix treatment had no significant effect on the exploratory activities as assessed by the number of arm entries (Fig. 4A) and time spent (Fig. 4B) in each arm during the Y-maze test. Consistent with the ORM performance, two-way ANOVA revealed that the ethanol-treated mice (E + V) exhibited significantly reduced spontaneous alternation performance compared to saline-treated mice (S + V), and Bix treatment (E + Bix) rescued these deficits [$F_{3,21} = 10$, $p < 0.001$] (Fig. 4C). P7 mice treated with Bix alone (S + Bix) showed no significant difference in their spontaneous alternation performance ($p > 0.05$). Taken together, these findings suggest that Bix pretreatment rescues P7 ethanol-induced (E + Bix) deficits in spontaneous alteration performance in adult mice.

We tested spatial recognition memory using a Y-maze in our third behavioral test. A two-way ANOVA revealed that S + V and S + Bix-treated mice entered more frequently into (Arm Entry: 1 h [$F_{3,21} = 18$, $p < 0.01$; 24 h [$F_{3,21} = 23$, $p < 0.01$]) (Fig. 5 A and B) and spent more time in (Dwell Time: 1 h [$F_{3,21} = 51$, $p < 0.01$; 24 h [$F_{3,21} = 25$, $p < 0.01$]) (Fig. 5 C and D) the novel, previously unvisited arm of the maze. In contrast, P7 E + V-treated mice showed a reduced preference toward the novel arm ($p<0.01$) and spent less time (Dwell time) ($p<0.01$) in the novel arm compared to the P7 S + V mice in both 1 h (Fig. 5 A and B) and 24 h (Fig. 5 C and D) retention. Bix pretreatment rescued ethanol-induced impairments (E + Bix) with a preference toward exploration of the novel arm ($p<0.01$) and time spent ($p<0.01$) in the novel arm in both 1 h and 24 h retention. Although all S + V, S + Bix and E + Bix-treated mice (combined 1 and 24 h) selected the novel arm as the first choice, E + V-

treated animals showed a reduced preference for the novel arm (Fig. 5E), and this was prevented by Bix pretreatment (E + Bix) [$F_{3,45} = 50$, $p < 0.01$]). These findings suggest that G9a inhibition before P7 ethanol treatment rescues the spatial recognition memory loss in adult mice.

The initial experiments evaluated the social investigation of the adult mouse with 1, 4 and 24 h intervals between the initial and test trials. The social investigation was higher with a 1 h intertrial delay compared to a 4 and 24 h intertrial delay (data not shown). The social investigation results revealed that E + V-treated mice exhibited significantly reduced short (Fig. 6 A) and long-term (Fig. 6 B) social recognition memory performance compared to the S + V-treated mice. A two-way ANOVA revealed that Bix pretreatment rescued the ethanol-induced (E + Bix) short- [$F_{3,21} = 18$, $p < 0.01$] and long-term [$F_{3,21} = 14$, $p < 0.01$] social recognition memory deficits compared to the ethanol (E + Bix) treated mice. In addition, S + Bix alone had no significant effect ($p > 0.05$) on the social recognition memory and these mice exhibited normal social recognition memory. These findings suggest that G9a inhibition by Bix before P7 ethanol treatment rescues social recognition memory loss in adult mice.

Discussion

Our current research suggests that blocking the loss of H3K9me2 by pre-administration of G9a/GLP inhibitor before P7 ethanol treatment rescues long-lasting synaptic dysfunction in adult mice . Most importantly, we have shown that the pre-administration of G9a/GLP inhibitor before ethanol exposure in P7 mice, which rescues H3K9me2 degradation, was adequate to prevent LTP deficits in adult mice. Although the molecular mechanism is not clear, there is growing evidence that neuronal plasticity is persistently impaired in animal models of FASD [for references see (Subbanna, et al., 2013a)]. It is possible that ethanol-induced activation of G9a and histone H3 modification during development disrupts the specific process involved in refinement of neuronal circuits, which leads to persistent synaptic dysfunction in adulthood. This could explain why some cortical maps (Margret, et al., 2006, Medina, et al., 2005, Powrozek and Zhou, 2005, Zhou, et al., 2005) and olfacto-hippocampal networks (Sadrian, et al., 2012, Wilson, et al., 2011) are altered in FASD models. Moreover, G9a-deficient mice display signs of severe developmental growth retardation and generally die between the embryonic days 9.5 and 12.5 (Tachibana, et al., 2002, Tachibana, et al., 2005). In a recent study, conditional inactivation of G9a/GLP in large numbers of functionally diverse neurons in the postnatal forebrain significantly erased euchromatic H3K9 dimethylation (Schaefer, et al., 2009). This lack of euchromatic H3K9me2 led to an intellectual-disability-like phenotype in adult mice (Schaefer, et al., 2009). In humans, genetic alterations of the GLP gene are associated with a severe intellectual disability syndrome that is further characterized by craniofacial abnormalities and a gradual decline in goal-directed cognition and behavior (Kleefstra, et al., 2006, Kleefstra, et al., 2009, Kramer and van Bokhoven, 2009). Intellectual disability in humans is distinguished by decreased cognitive function, as well as impaired adaptive behaviors in response to environmental triggers including perinatal trauma or intra-uterine infections, maternal and early childhood nutritional deficits, and maternal alcohol abuse (APA., 2000). Consistent with this notion, the deficiency of G9a/GLP leads to loss of H3K9me2 in

postnatal neurons causing severe defects in learning and memory (Schaefer, et al., 2009). Furthermore, our study is also consistent with other study which suggest that loss of H3K9me2 due to genetic ablation of G9a leads to reduced higher order dendrite branching and impaired learning and memory in Drosophila (Kramer, et al., 2011). In another study, loss of H3K9me2 due to partial ablation of G9a leads to the developmental delay, hypotonia, and cranial abnormalities, which are three of the core features of specific intellectual disability syndrome called Kleefstra syndrome (Balemans, et al., 2014) . Thus, it seems possible that genetically predetermined or environmentally (postnatal ethanol) induced epigenetic changes of the enzymes controlling H3K9 dimethylation (Subbanna, et al., 2014, Subbanna, et al., 2013b) may induce long-lasting learning and memory deficits as found in many postnatal ethanol studies (Izumi, et al., 2005, Sadrian, et al., 2012, Subbanna, et al., 2013a, Wilson, et al., 2011). Although more studies are warranted, in P7 ethanol model, the loss of H3K9me2 was initiated with enhanced G9a expression and dimethylation sensitive degradation of H3K9me2 protein (Subbanna, et al., 2013b) and suggest the G9a function is dependent on the level of expression, context and its H3K9 dimethylation activity. The observed loss of dimethylated H3K9 due to proteolytic degradation (Subbanna, et al., 2013b) during the synaptogenic period may partially explain the observed neuronal dysfunction in the present study as well as in several animal models of postnatal ethanol (Izumi, et al., 2005, Sadrian, et al., 2012, Subbanna, et al., 2013a, Wilson, et al., 2011) .

The current findings also reveal significant learning and memory impairments in the adult mice exposed to ethanol at P7 compared to controls. These findings go in line with previous literature showing that mice exposed to an acute dose of ethanol at P7 show impaired synaptic plasticity (Izumi, et al., 2005, Sadrian, et al., 2012, Subbanna, et al., 2013a) and olfacto-hippocampal (Sadrian, et al., 2012, Wilson, et al., 2011) and hippocampal memory (Subbanna, et al., 2013a) in adult mice. Most importantly, we have shown that pharmacologically inhibiting G9a via Bix pretreatment before ethanol exposure can rescue ethanol-induced neuronal deficiencies ranging anywhere from neuronal survival (Subbanna, et al., 2013b) to LTP to learning and memory behavior. Several rodent models also show impaired learning and memory in the adult rodents exposed to acute or chronic ethanol at pre- or postnatal stages of development (Christie, et al., 2005, Girard, et al., 2000, Iqbal, et al., 2006, Pick, et al., 1993, Ryan, et al., 2008, Savage, et al., 2002, Thomas, et al., 2008, Thomas, et al., 1997, Zimmerberg, et al., 1991). There is also growing evidence that heavy prenatal alcohol exposure leads to widespread cognitive deficits in children across several domains, including memory, social and adaptive functioning (Mattson, et al., 2011, Mattson, et al., 1998, Norman, et al., 2013). The children exposed to prenatal alcohol showed impaired verbal and nonverbal learning and memory (Mattson, et al., 1996, Mattson and Roebuck, 2002, Roebuck-Spencer and Mattson, 2004), reduced academic accomplishment, and higher rates of learning disabilities than non-exposed children (Howell, et al., 2006).

The findings that ethanol exposure at P7 also caused deficits in social recognition memory in adult mice generally agree with a previous study in which exposure to acute ethanol during neurogenesis at gestation day 12 (G12) caused pronounced and permanent deficits in social behavior throughout ontogeny (Mooney and Varlinskaya, 2011). Similar social recognition deficits were also established in another animal model of FASD (Shirasaka, et al., 2012) as well as a CD38 knockout autism model (Jin, et al., 2007). Using the optimum

dose of Bix treatment (recognized for preventing neurodegeneration in neonatal mice) to pharmacologically inhibit G9a prevented the development of ethanol-induced deficits in social recognition memory in adult mice. It is widely accepted that retaining normal social memories throughout ontogeny is crucial for establishing relationships within a group or between partners, along with developing the ability to recognize families [For references see (Cushing and Kramer, 2005)]. Stable social recognition memories in rodents function to facilitate several complex social and reproductive processes, not limited to pair relationship formation in monogamous species (Demas, et al., 1997), as well as selective pregnancy termination in mice (Kaba, et al., 1989, Keverne, 1998). It is well known that two brain regions, the olfactory system (Sanchez-Andrade and Kendrick, 2009) and the limbic system (Baron-Cohen, et al., 1994, Brothers, et al., 1990) underlie social behavior. It possible that improper processing of socially relevant olfactory stimuli might produce the observed deficit in social recognition memory in P7 ethanol treated adult mice. Early ethanol exposure damages olfactory neuroanatomy and physiology in both humans and rodents [For references see (Wilson, et al., 2011)]. Because the olfactory system provides a major input to the hippocampal formation (Wilson and Sullivan, 2011), and this structure is involved in integrating the complex stimuli necessary for the recognition process (Alvarez, et al., 2002, Ross and Eichenbaum, 2006), the hippocampus might be required for social memory (Kogan, et al., 2000). Consistent with this notion, our previous findings suggest that P7 ethanol treatment significantly modifies olfacto-hippocampal system function in adult mice (Sadrian, et al., 2012, Wilson, et al., 2011). Currently, there are no studies that investigate the epigenetic mechanisms leading to deficits in social recognition memory. However, one study has correlated the early social experience with a change in histone acetylation and DNA methylation status at the promoter region of the glucocorticoid receptor gene (Weaver, et al., 2004). While more research must be conducted, our data emphasizes the importance of specific epigenetic changes mediated by G9a due to ethanol exposure during the postnatal period, which is key for establishing adult social behavior expression (Gordon, 1998, Nelson and Luciana, 2001, Stiles, 2000).

The molecular mechanism(s) by which developmental ethanol exposure induces long-lasting behavioral and cognitive deficits are not fully understood. However, evidence suggests that the observed learning and memory deficits may be a result of the well-known toxic effects of ethanol on CNS development through several pathways, which leads to increased apoptotic neurodegeneration (Ikonomidou, et al., 2000, Sadrian, et al., 2012, Subbanna, et al., 2013a, Subbanna, et al., 2013b, West, et al., 1986), decreased cell motility (Carter, et al., 2008), decreased neurogenesis (Nixon and Crews, 2002), decreased pro-survival signaling (Eade, et al., 2010, Young, et al., 2008), and changes in dendritic tree complexity and the spine shape of hippocampal neurons (Abdollah, et al., 1993, Bonthius, et al., 2001, Butters, et al., 2000, Hayward, et al., 2004, Tarelo-Acuna, et al., 2000). Our previous and current observation directly pinpoints the participation of the epigenetically regulated G9a-induced loss of H3K9me2. A recent study suggests that the inhibition of G9a/GLP in adult rats with the infusion of Bix inhibit the formation H3K9me2 in area CA1 of the hippocampus and long-term memory (Gupta-Agarwal, et al., 2012). Although the mechanisms need to be further established, this evidence suggests that H3K9me2 levels regulate the memory process. Collectively, our studies emphasize the importance of epigenetic mechanisms in the

regulation of normal brain developmental processes and how changes in these pathways caused by environmental exposures, such as postnatal ethanol, can possibly trigger a cascade of neurological damage and dysfunction that may have immediate effects (Subbanna, et al., 2014, Subbanna, et al., 2013b) or manifest later in life. Furthermore, it remains unclear whether the observed epigenetic modifications of the G9a gene and the upregulation of G9a expression during postnatal development lasts until adulthood, or if it is even inheritable. Such epigenetic studies will help develop G9a based intervention to treat cognitive deficits in ethanol exposed offspring.

In conclusion, our study suggests an expanded epigenetic regulatory model for the neurodegenerative process in postnatal ethanol teratogenesis. Postnatal ethanol exposure leads to the abnormal alteration of the G9a-dependent H3K9me2 epigenetic system. We propose that the global loss of G9a-mediated H3K9me2 affecting neuronal survival in the postnatal ethanol-treated brain. The identification of a role for G9a in the neurodegenerative process, as well as the long-lasting consequence of postnatal ethanol exposure during synaptogenesis, expand our understanding of the dynamic changes underlying histone modifications and suggests new therapeutic targets for treatment. Currently, no pharmacotherapy aimed at counteracting either the neurodevelopmental or the neurodegenerative component of early-life ethanol exposure has been approved. Understanding the complex epigenetics of early-life ethanol exposure will undoubtedly shed new light on the mechanisms of developmental ethanol neurotoxicity. Most importantly, it will help us identify epigenetic mechanisms for early-life ethanol exposure, which will be essential for designing novel therapeutic strategies to improve specific aspects of the symptomatology of ethanol-induced neurobehavioral teratogenicity.

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Abbreviations

H3K4me3	histone H3 trimethylation at lysine 4
H3K9me2	histone H3 dimethylation at lysine 9
FASDs	fetal alcohol spectrum disorders
LTP	long-term potentiation
EHMTases	euchromatic histone methyltransferases
GLP	G9a-related protein
BEL	blood ethanol levels
CC3	cleaved caspase-3
fEPSP	field-excitatory-post-synaptic potential
ORM	object recognition memory
G12	gestation day 12

P7 postnatal day 7

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HIGHLIGHTS

- Ethanol treatment at P7 induces apoptosis, loss of H3K9me2 and LTP deficits as observed in FASD.
- Bix 01294 administration before P7 ethanol treatment rescues LTP and memory deficits in adult mice.
- P7 ethanol induces while Bix pretreatment rescues social recognition memory deficit in adult mice.
- G9a regulated H3K9me2 may serve as an important and potential therapeutic target against FASD.

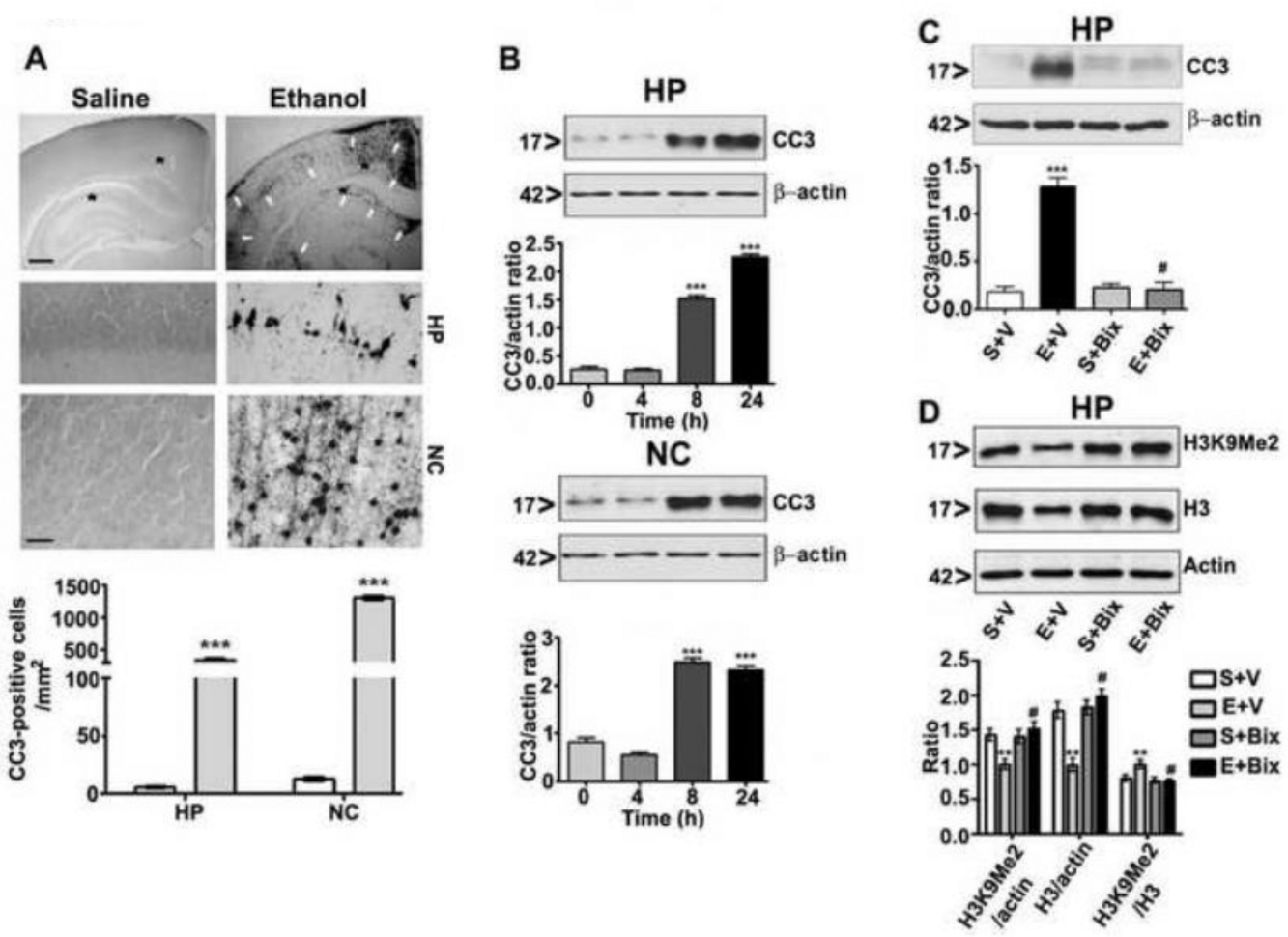

Fig. 1.

Ethanol induces apoptotic neurodegeneration in the P7 mouse brain and pharmacological inhibition of G9a rescues P7 ethanol-induced neurodegeneration and H3K9me2 in the neonatal mouse brain. (A) Coronal brain sections (hippocampus and retrosplenial cortex) from saline- and ethanol-treated animals were immunostained with an anti-rabbit CC3 antibody. The white arrows indicate the CC3-positive neurons in the hippocampus and retrosplenial cortex. Scale bars = 200 μm. The respective images were enlarged to show the CC3-positive cells (*). The scale bars represent 50 μm. CC3-positive cells were quantified in the hippocampus and retrosplenial cortex (n = 10 pups/group). Student's *t* test: ***p < 0.001 vs. respective saline group . Each point is presented as the mean ± SEM. (B) Western blot analysis of CC3 using cytosolic extracts (20 μg) of hippocampal and cortical samples from the saline- and ethanol-treated groups (n = 8 pups/group). The graphs represent the ratio of the proteins normalized to the expression of β-actin. ***p < 0.001 vs. 0 h (respective saline control) . Each point is presented as the mean ± SEM. (C and D) Mice pre-treated (30 min) with Bix (1 mg/kg) or vehicle were exposed to ethanol, and hippocampal extracts from S + V, E + V, S + Bix and E + Bix (n = 6 pups/group) were collected 8 h after first dose of ethanol treatment and processed for Western blotting to analyze CC3, H3K9me2 and H3 levels. β-actin was used as a loading control. Representative blots are shown for the

hippocampal cytosolic (CC3) and nuclear (H3K9 and H3) extracts. HP, hippocampus; NC, neocortex. Each point is presented as the mean ± SEM. One-way ANOVA with Bonferroni's *post hoc* tests; ***p < 0.001, **p < 0.01 vs. S + V; #p < 0.001 vs. E + V .

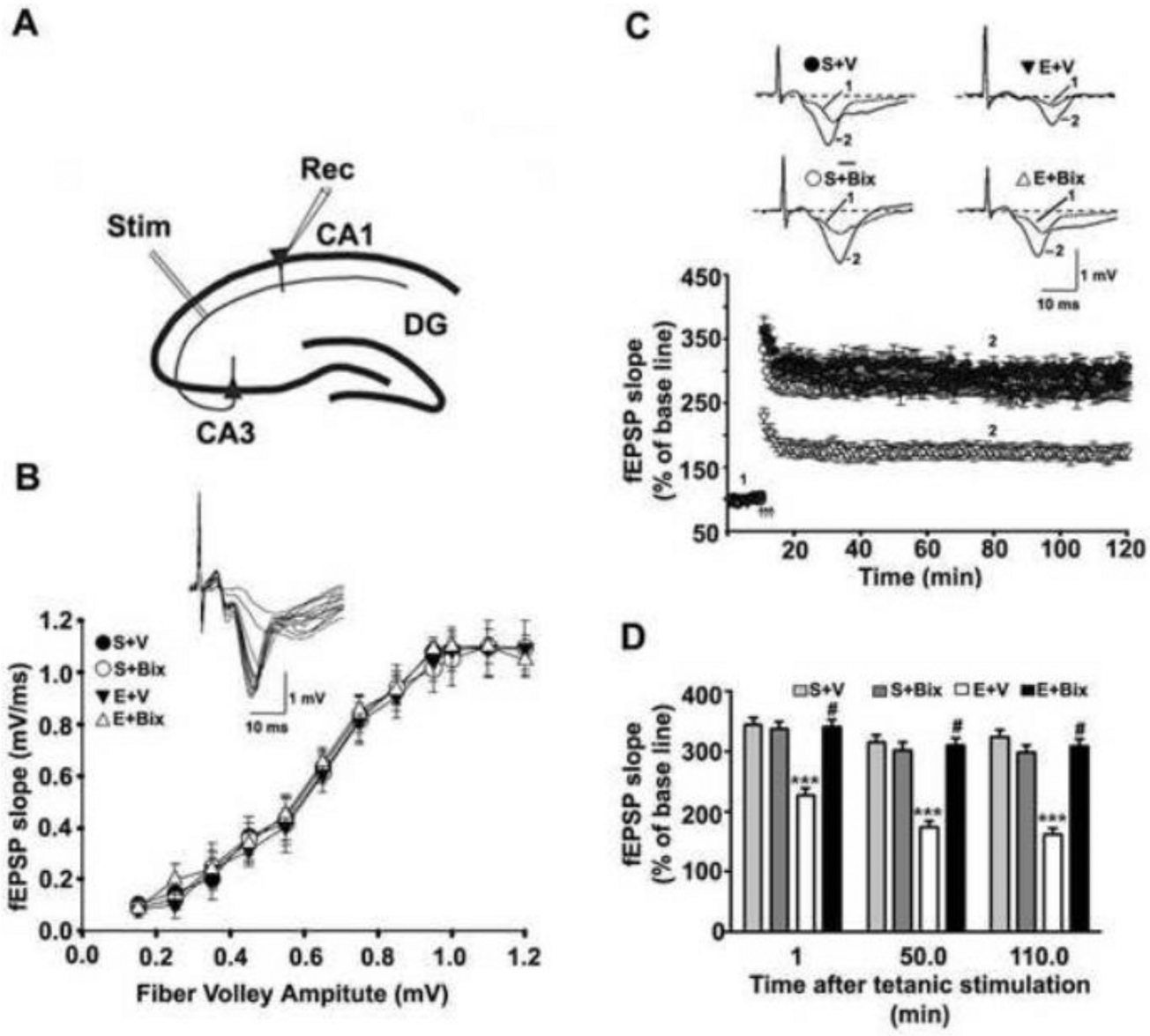

Fig. 2.

Inhibition of G9a before ethanol treatment in P7 pups prevents long-lasting synaptic deficits in adult mice. (A) A schematic diagram showing the stimulating and recording electrode positions in the CA1 region of the hippocampus. (B) A **s**ummary graph showing the field input/output relationships for P7 treated S + V, E + V, S + Bix and E + Bix adult mice. *Insert:* An example of traces taken from representative experiments from input/output relationships for S + V. Although not shown other groups also exhibited similar pattern . (C) Time course of the averages of the fEPSP slopes in slices obtained from S+V, E+V, S + Bix- and E + Bix-treated mice. The fEPSP slopes were normalized to the average value 10 min before stimulation in each experiment. Arrows denote the time of tetanic stimulation (4 pulses at 100 Hz, with bursts repeated at 5 Hz, and each tetanus including three 10-burst trains separated by 15 s). Representative traces of fEPSPs before (trace 1) and after (trace 2)

induction of LTP in hippocampal slices from S +V, E + V, S + Bix and E + Bix mice . (D) A combined plot of the averages of the fEPSP slopes at several time points. Each point is presented as the mean ± SEM (n= 5 mice/group; 10 slices/group). Two-way ANOVA with Bonferroni's *post hoc* tests; ***p < 0.001 vs. S + V; #p < 0.001 vs. E + V .

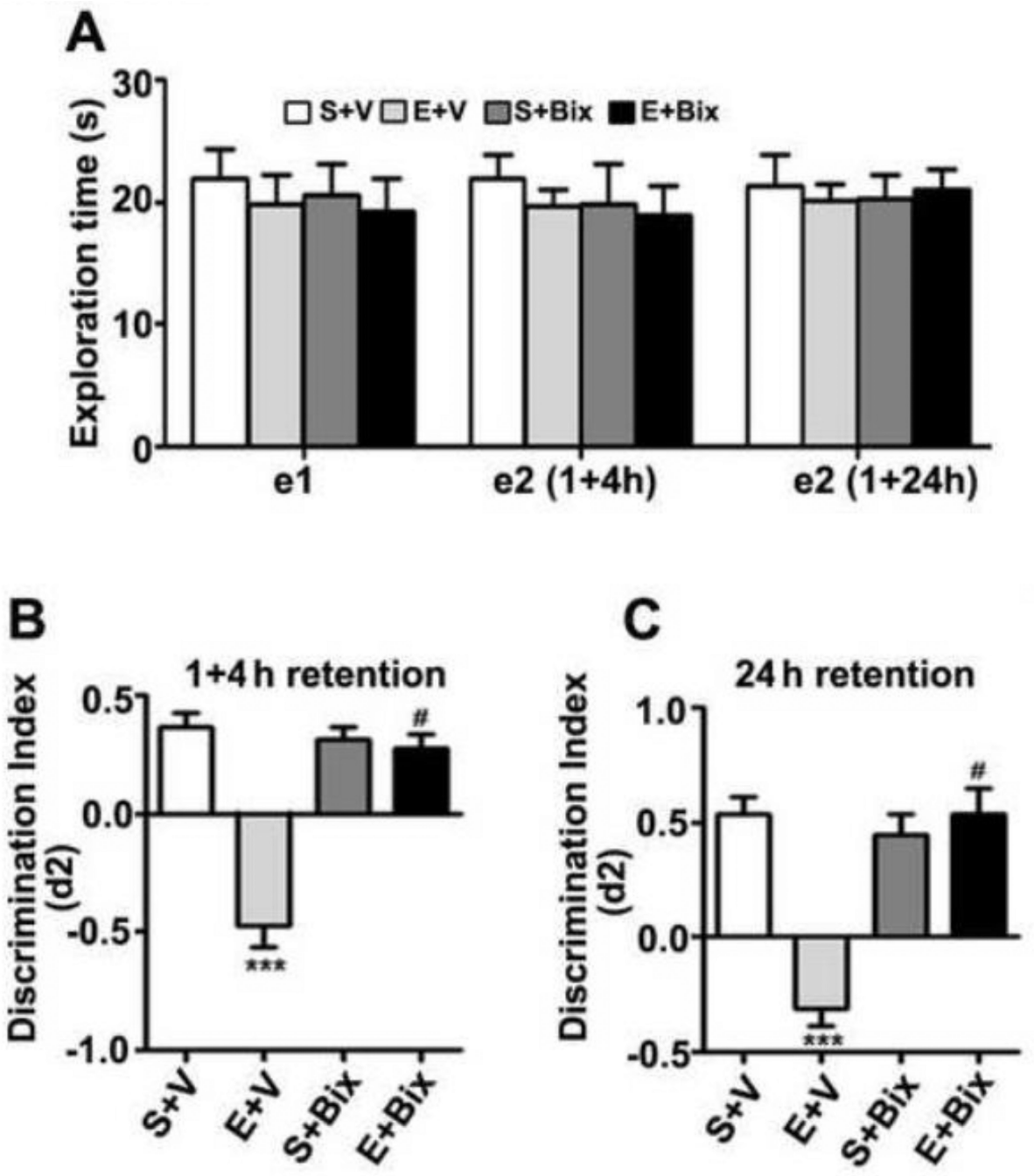

Fig. 3.

Inactivation of G9a activity before ethanol treatment at P7 prevents object recognition memory loss in adult mice. (A) Level of exploration was measured at el and e2, respectively: the time spent exploring the two objects in T1 and T2 (at 1+4 and 24 h) by S +V, E+V, S+ Bix, and E+ Bix-treated mice. (B) Discrimination indices (d2) obtained from the S+V, E+V, S+ Bix, and E+ Bix-treated mice after 1and 4 h retention intervals. (C) Discrimination indices (d2) obtained from the S+V, E+V, S+ Bix, and E+ Bix-treated mice

after 24 h retention intervals. Each point is the mean + SEM (n= 8 mice/group). One-way ANOVA with Bonferroni's *post hoc* test; ***p < 0.001 vs. S + V; #p < 0.001 vs. E + V .

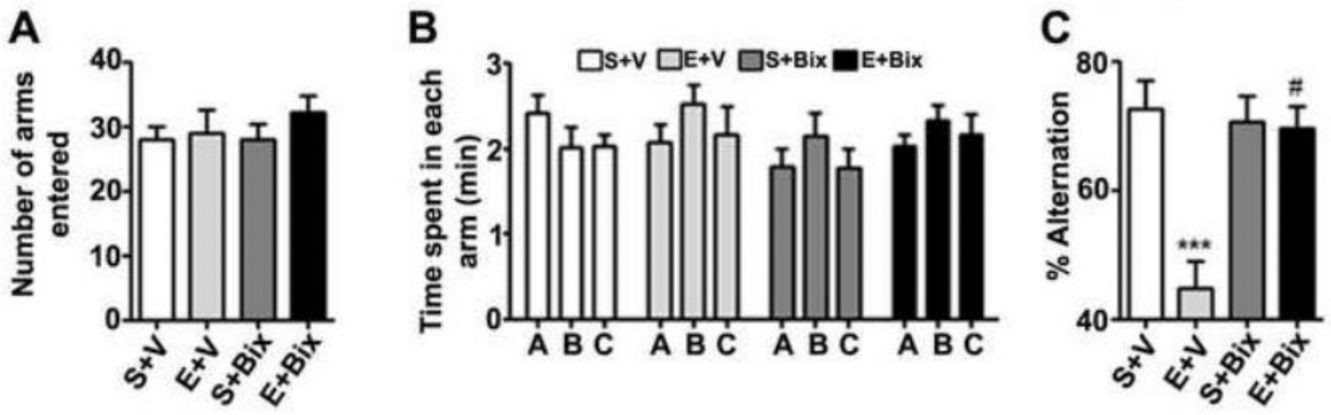

Fig. 4.

P7 ethanol treatment impairs and prior administration of G9a/GLP inhibitor prevents the spontaneous alternation performance deficit in adult mice. (A) Total number of arm entries reflecting exploratory activities of mice in the Y-maze does not differ between the four groups ($p > 0.05$). (B) The time spent in each arm was not different between four groups ($p > 0.05$). (C) The spontaneous alternation performance was reduced in by ethanol (E+V) and was rescued by Bix treatment (E+ Bix). Alternation performance was not affected by saline (S+V) and Bix (S+Bix) treatment. Each point is the mean ± SEM (n= 8 mice/group). (***$p < 0.001$ vs. S+V; #$p < 0.001$ vs. E+V) . One-way ANOVA with Bonferroni's *post hoc* test.

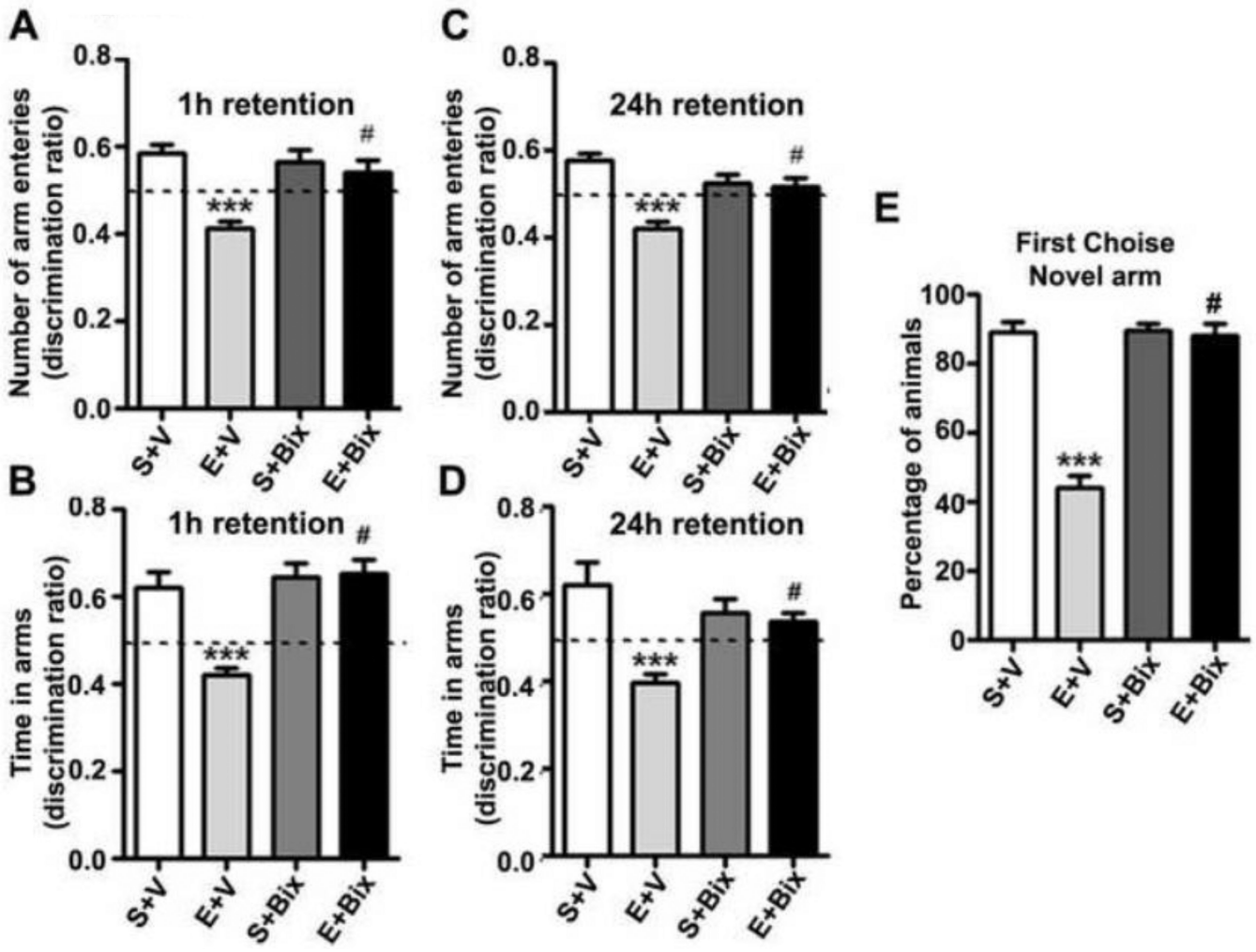

Fig. 5.

P7 ethanol treatment impairs and Bix pretreatment rescues impaired spatial memory performance as measured by Y maze. (A–D) Discrimination ratio [preference for the Novel arm over the familiar Other arm (Novel/Novel + Other)] for arm entries (A and C, 1 h and 24 h) and dwell time (B and D, 1 h and 24 h) of S+V and E+V mice treated with or without Bix (S+Bix and E+Bix), 1 or 24 h after the first encounter with the partially opened maze. The dashed line indicates chance performance (0.5). (E) The percentage of animals selecting the novel arm as the first choice is shown for S+V and E+V mice treated with or without Bix (S+Bix and E+Bix), 1 and 24 h after the first encounter with the partially opened maze. Each point is the mean + SEM (n= 8 mice/group). Two-way ANOVA with Bonferroni's *post hoc* test; ***$p < 0.001$ vs. S + V; # $p < 0.01$ vs. E + V .

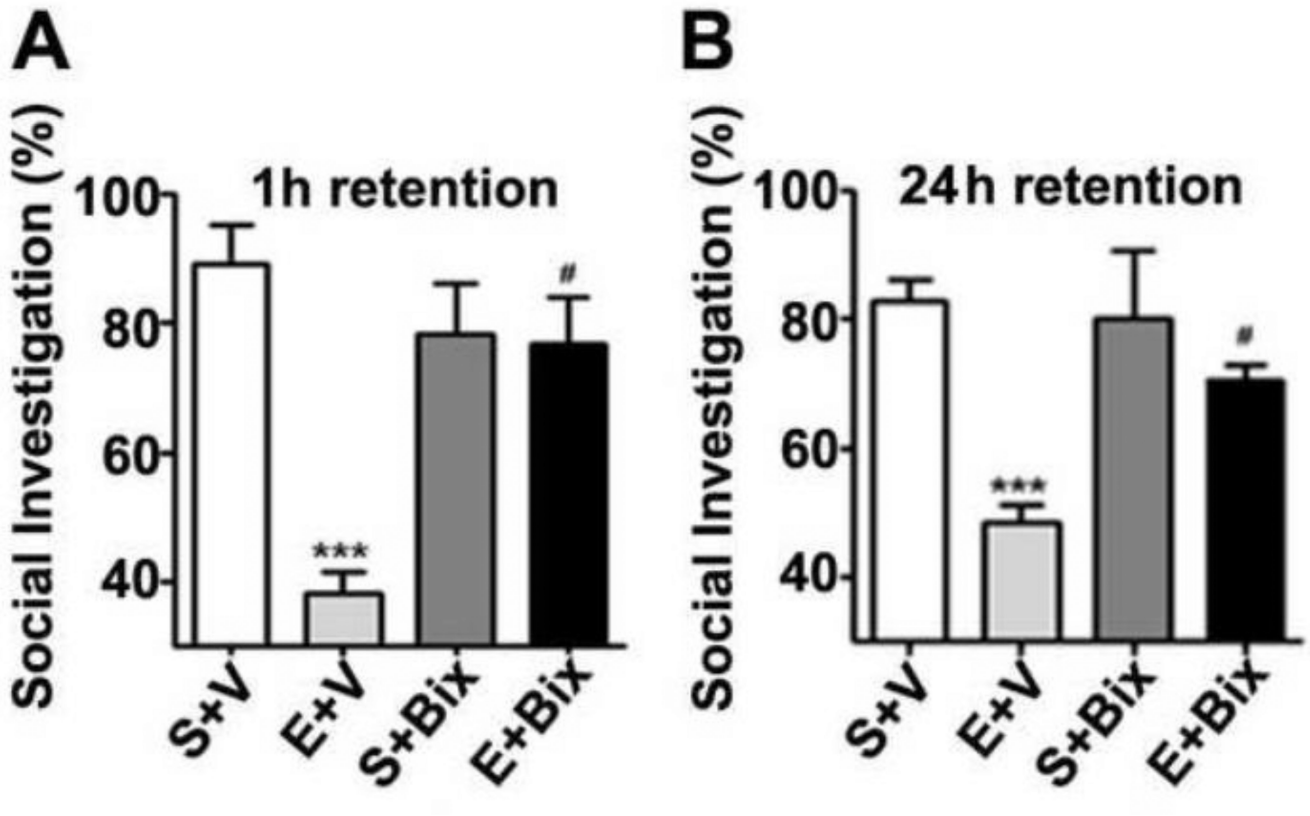

Fig. 6.

Bix pretreatment rescues P7 ethanol-induced social recognition memory loss in the adult mice. (A and B) Percent of social investigation is shown for S+V, E+V, S+ Bix, and E+ Bix-treated mice, 1 (A) and 24 h (B) after the first encounter with same juvenile mice. Each point is the mean ± SEM (n= 8 mice/group). Two-way ANOVA with Bonferroni's *post hoc* test. ***$p < 0.001$ vs. S + V; # $p < 0.01$ vs. E + V .