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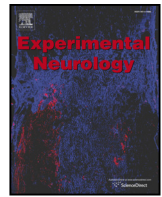

Regular Article

# 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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## 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, 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., 2013a, 2013b, 2014; 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, 2010b; Perkins et al., 2013; Subbanna et al., 2013b, 2014), which may be responsible for the development of ethanol associated brain disorders (Mattson et al., 2010, 2011). Recent studies focus on the importance of

*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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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 regulates 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 h light/12 h 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, 2013b). For blood ethanol levels (BEL), pups were euthanized by decapitation; truncal blood was collected at 3 and 9 h 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 10 μl 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 to saline-treated mice and did not cause any inflammation or bleeding in any of the organs (Subbanna et al., 2013b, 2014). 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., 2013a, 2013b, 2014). 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 2.5×, or 40× 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, 2013b). Cytosolic and nuclear fractions from tissue homogenates were prepared as described in our recent publications (Basavarajappa and Subbanna, 2014; Basavarajappa et al., 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, 1:1000), 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% $O_2$ and 5% $CO_2$. 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

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