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Alcohol induces parallel changes in hippocampal histone H3 phosphorylation and c-fos protein expression in male rats

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Abstract

Background—Changes in gene expression associated with alcohol-induced neuroadaptations are controlled in part by post-translational histone modifications. Serine 10 phosphorylation of histone H3 (H3S10ph) has been implicated in drug-induced changes in gene expression; however, ethanol's effects on H3S10ph have yet to be examined in brain. Therefore, hippocampal H3S10ph was examined after acute alcohol exposure and alcohol dependence.

Methods—Adult male Sprague-Dawley rats received an acute exposure of ethanol (0–5 g/kg) via gavage. Or, rats were made alcohol dependent by administering 25% w/v ethanol every 8 hours for 4 days following a modified Majchrowicz protocol. In both cases, rats were perfused transcardially and paraformaldehyde-fixed brains were collected and processed for immunohistochemistry for H3S10ph or the immediate early gene, c-fos.

Results—Acute ethanol exposure dose-dependently altered the number of H3S10ph-positive (+) cells in the hippocampus. Specifically, 1 g/kg ethanol increased the number of H3S10ph+ cells in all neuronal layers, while 2.5 and 5 g/kg ethanol reduced the number of H3S10ph+ cells, an effect that was confined to the granule cell layer (GCL). In ethanol dependent rats, the number of H3S10ph+ cells in the GCL was reduced by 66% during intoxication; however, H3S10ph+ cells were increased in all neuronal layers during peak withdrawal. Subsequent examination of c-fos, a gene known to be regulated by H3S10ph, revealed that ethanol and withdrawal-associated changes in c-fos closely paralleled changes in H3S10ph.

Conclusions—These results suggest that H3S10ph regulates ethanol-mediated changes in c-fos expression, effects that likely have important implications for ethanol-induced changes in hippocampal neuronal plasticity.

Keywords

Alcohol use disorder; c-Fos; Epigenetics; Hippocampus; Histone

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Introduction

Excessive alcohol intake, the hallmark of alcohol use disorders (AUDs), results in widespread neuroadaptations that involve significant region and cell-type specific changes in gene expression (Ponomarev et al., 2012). The mechanisms by which alcohol alters gene expression are poorly understood; however, accumulating evidence indicates that epigenetic regulation of chromatin structure plays a central role (Krishnan et al., 2014; Ponomarev, 2013). Chromatin is organized into fundamental structural units called nucleosomes, which consist of an octamer of histone proteins (2 each of histone H2A, H2B, H3, and H4) around which DNA is wrapped. The globular head domains of histone proteins interact with each other and DNA to form the nucleosome core, while the highly conserved histone tail domains protrude from the nucleosome where they are subjected to enzyme-mediated covalent post-translational modifications, such as acetylation, methylation, and phosphorylation. Together, these modifications strongly influence chromatin structure throughout the cell cycle and form a code read by transcriptional regulators (Strahl and Allis, 2000).

In the central nervous system, ethanol-induced changes in histone acetylation have received the most attention. In particular, the acetylation state of histone H3 appears key to ethanol's effects on anxiety. Pandey and colleagues have demonstrated in rodent models that acute ethanol intoxication inhibits histone deacetylase activity leading to enhanced acetylation of histone H3 at lysine 9 (H3K9ac), which was correlated with ethanol's anxiolytic effects (Krishnan et al., 2014). Changes in H3K9ac are neuronal-specific and lead to upregulation of brain-derived neurotrophic factor (BDNF), Arc, prodynorphin, and pronociceptin expression, all of which likely contribute to ethanol's effects on anxiety (D'Addario et al., 2013; Krishnan et al., 2014). Most importantly, treatment with histone deacetylase inhibitors prevented the development of rapid tolerance to alcohol's anxiolytic effects and blocked withdrawal-induced anxiety, showing the potential utility of pharmacological manipulation of the histone code in blocking alcohol-induced neuroadaptations (Krishnan et al., 2014). Ethanol's effects on H3K9ac extend beyond the amygdala. In cultured cortical neurons, withdrawal from chronic intermittent ethanol exposure increases H3K9ac in the promoter of the plasticity-associated NMDA receptor 2B gene resulting in up-regulated expression (Qiang et al., 2011). Moreover, ethanol's effects on H3K9ac in the prefrontal cortex and nucleus accumbens are age-dependent, adding another layer of complexity to understanding how ethanol alters the histone code (Pascual et al., 2009; Pascual et al., 2012).

A primary site of ethanol action is the hippocampus, where it produces significant structural and functional changes. Structurally, cell death and other neuroplastic changes in neurons and glia vary across the stages of the addiction cycle (e.g. Figure 8; Kelso et al., 2011). For example, ethanol intoxication-induced reductions in neurogenesis impacts the dentate gyrus, while glial responses and reactive (increased) neurogenesis occur during protracted withdrawal (Geil et al., 2014; Kelso et al., 2011; Marshall et al., 2013). A relationship between aberrant histone acetylation and impairments in mitochondrial respiration also supports a link between histone modifications and neurodegenerative processes (Jung and Metzger, 2015). Functionally, induction of long-term potentiation in hippocampal cornu ammonis (CA) 1 neurons is potently inhibited by ethanol (Durand and Carlen, 1984).

Alcohol is well known to impair hippocampus-dependent learning and memory (reviewed in Geil et al., 2014). Involvement of chromatin remodeling in mediating ethanol-induced hippocampal dysfunction seems likely. Chronic alcoholism is associated with altered H3K4me3 in hundreds of expressed genes in the hippocampus (Zhou et al., 2011). However, H3K4me3 was not correlated with changes in gene expression, suggesting involvement of other histone modifications (Ponomarev, 2013; Zhou et al., 2011). In support of this view, ethanol increases global hippocampal histone H3 acetylation (Finegersh and Homanics, 2014), and ethanol-induced changes in hippocampal BDNF expression require both altered histone H3 acetylation and methylation in the gene's promoter region (Stragier et al., 2015).

Potential contributions of other histone modifications in ethanol-induced changes in hippocampal gene expression have yet to be examined. The effects of ethanol on the phosphorylation of histone H3 at serine 10 (H3S10ph) is of particular interest as it has been implicated in hippocampal-dependent memory function (Reul, 2014). H3S10ph plays two seemingly contradictory functions in chromatin remodeling. On one hand, global H3S10ph occurring towards the end of the G2 phase of the cell cycle may initiate chromosome condensation (Healy et al., 2012). Global H3S10ph lasts through metaphase, which explains its use as a marker for dividing cells during hippocampal neurogenesis (e.g. McClain et al., 2011). On the other hand, discrete H3S10ph is associated with chromatin unwinding and, in tandem with H3K14 acetylation, is recognized by transcriptional regulators (Healy et al., 2012). This later function of H3S10ph is critical for induction of immediate early genes (IEGs) such as c-fos in response to a variety of stimuli (Healy et al., 2012). In fact, H3S10ph is required for the induction of c-fos expression in granule cell neurons (Crosio et al., 2003), an effect shown to mediate the adaptive response of rodents in a forced-swim test (Chandramohan et al., 2008). In addition, H3S10ph has been linked to c-fos induction in models of drug abuse, indicating that this histone modification may be important for drug-induced neuroadaptations (Brami-Cherrier et al., 2005; Ciccarelli et al., 2013). Evidence linking H3S10ph to control of gene expression involved in hippocampal-dependent memory formation and drug-induced neuroadaptations, coupled with our unexpected observation of H3S10ph expression in non neurogenic regions, led us to hypothesize that ethanol would disrupt neuronal H3S10ph in the hippocampus.

Materials and Methods

Animals

Adult, male Sprague-Dawley rats (275–300g; Charles River Laboratories, Raleigh, NC) were singly housed and maintained on a 12 h light/dark cycle. Only males were used in this study, as the original observation of H3S10ph expression in non neurogenic regions occurred in a separate study that was also conducted in males. Food and water were available *ad libitum* during a 5-day acclimatization period then food was removed during alcohol administration. Experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee and adhered to the Guide for the Care and Use of Laboratory Animals (NRC 1996).

Acute Ethanol Exposure

Ethanol (25% w/v in 0.9% saline; Aaper, Shelbyville, KY) was administered via intragastric gavage in a single 1, 2.5, or 5 g/kg dose at the start of the light cycle. Controls received saline (0.9%) in an equivalent volume to the 5 g/kg ethanol dose. Rats (n = 8–10/group) were overdosed 2 h post-gavage with sodium pentobarbital (Nembutal®, MWI Veterinary Supply, Boise, ID or Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI) and transcardially perfused with phosphate buffered saline then 4% paraformaldehyde. Blood ethanol concentrations (BECs) were measured from trunk blood collected at perfusion and assayed on an AM1 Alcohol Analyzer (Analox, Lunenburg, MA).

4-Day Binge Model of Ethanol Dependence

Rats were made ethanol dependent via a model modified from Majchrowicz (1975), as described previously (Morris et al., 2010b). Briefly, rats were administered 25% (w/v) ethanol in Vanilla Ensure Plus® (Abbott Labs, Chicago, IL) every 8 h for 4 days via gavage. After a priming dose of 5 g/kg, subsequent doses were determined based on a 6-point intoxication scale [0 = normal animal (5g/kg), 1 = hypoactive, mild ataxia (4g/kg), 2 = ataxia (3g/kg), 3 = delayed righting reflex, ataxia with no abdominal elevation (2g/kg), 4 = loss of righting reflex (1g/kg), 5 = loss of eye blink reflex (0g/kg)]. Controls were gavaged with isocaloric diet containing dextrose. Tail blood was collected 90 min after the 7th ethanol dose and BECs determined using an AM1 Alcohol Analyzer. Rats (n = 8–10/group) were overdosed with sodium pentobarbital and transcardially perfused as above at 2 or 16 h after the last ethanol dose. As the 16 h time point corresponds to the time of peak withdrawal in this model, rats in this group were observed and scored for withdrawal behavior 10–16 hours following the last ethanol dose (Morris et al., 2010b; Penland et al., 2001; see Supplemental Table 1).

Immunohistochemistry

Following perfusion, brains were harvested, postfixed 24h in 4% paraformaldehyde, then sectioned (1:12, 40µm thick series), as described previously (McClain et al., 2011). Standard free-floating immunohistochemistry procedures were used to detect H3S10ph and c-fos protein expression (see Table 1 for primary antibody details; McClain et al., 2011). Briefly, endogenous peroxidase activity was quenched with 0.6% H₂O₂. For H3S10ph detection, antigen unmasking was accomplished with 1 h incubation in 1x Citra Solution (Biogenex; Fremont, CA) at 65°C. Tissue sections were blocked with 3% normal goat serum (Vector Laboratories; Burlingame, CA) in 0.1% Triton X-100/TBS (Bio-Rad; Hercules, CA) and incubated 16–18 h (H3S10ph) or 46 h (c-fos) in primary antibody solution. After incubation in biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories), tissue sections were incubated in avidin-biotin-peroxidase complex (ABC Elite kit, Vector Laboratories). A nickel-enhanced diaminobenzidine (PolySciencences, Warrington, PA) reaction was used to develop and visualize immunoreactivity. Sections were mounted to glass slides and coverslipped in Cytoseal® (Richard Allen Scientific; Kalamazoo, MI).

Immunostained tissue sections were analyzed on a BX-51 microscope (Olympus, Center Valley, PA) equipped with a ProScan II motorized stage (Prior Scientific, Rockland, MA), DP-70 digital camera (Olympus), and 60x oil immersion lens (PlanApo N, 1.42 numerical

aperture, Olympus). H3S10ph and c-fos immunoreactive cells detected in the dentate gyrus granule cell layer and CA1-3 pyramidal neuron layers were counted across the dorsal hippocampus at 600× magnification. Specifically, immunoreactive granule cells were quantified in all sections (n= 7–9 sections per brain) between Bregma –1.92 and –5.52 (Paxinos and Watson, 2009), which spans from the anterior appearance of the inferior blade caudally to the point just before the dorsal inferior and superior blades meet their ventral counterparts. Immunoreactive CA pyramidal neurons were quantified in sections located between Bregma –1.92 and –4.56, which spans from the anterior appearance of CA3, caudally to the point just prior to where the dorsal and ventral hippocampal CA3 pyramidal layer merge. Due to their heterogeneous distribution and relatively low number of immunopositive cells, all cells were counted and reported as mean cells/section (Crews et al., 2004; Noori and Fornal, 2011).

Double-label Immunofluorescence

Dual fluorescent immunohistochemistry was conducted similar to previous reports (Morris et al., 2010a). Following, TBS rinses and incubation in primary antibody, sections were washed 3 times with blocking buffer (3% normal goat serum in 0.1% Triton X-100) and then incubated in AlexaFluor 488 goat anti-mouse IgG and AlexaFluor 546 goat anti-rabbit IgG secondary antibodies (Life Technologies, Grand Island, NY). A sequential primary incubation protocol was used to double-label tissue for H3S10ph and c-fos, as both primary antibodies were raised in rabbit. On day 1, antigen retrieval was performed with 1x Citra Solution at 65°C for 1h. Following blocking, tissue was incubated in rabbit anti-c-fos (1:2500) for 46 h at 4°C. On day 3, tissue was washed 3 times in blocking buffer and incubated in AlexaFluor 546 conjugated goat anti-rabbit IgG. Following 3 TBS washes, tissue was incubated in 5% normal rabbit serum for 2 h at room temperature in order to block any open binding sites on the secondary antibody with unlabeled IgG. After 3 additional TBS washes, tissue was incubated overnight at 4°C in AffiniPure goat anti-rabbit IgG F_{ab} fragments (Jackson Laboratories, West Grove, PA). This step blocks open binding sites on the rabbit IgG to prevent unwanted binding of the second secondary antibody. On day 4, tissue was washed in TBS, blocked, and incubated for 46 h at 4°C in rabbit anti-H3S10ph (1:400). On day 6, tissue was washed in blocking buffer, incubated in AlexaFluor 488 conjugated goat anti-rabbit IgG for 1 h, washed in TBS, and mounted with ProLong Gold anti-fade media (Life Technologies).

Antibody co-localization in the dentate gyrus granule cell layer was examined using a TCS SP5 inverted laser scanning confocal microscope (Leica, Wetzlar, Germany). Z-plane stacks were collected in 0.8 µm planes throughout the thickness of the section and cells were examined in multiple sections. Z-stacks were reconstructed and rotated in 3-D for the final determination of co-localization, similar to previous reports (Morris et al., 2010a).

Statistical Analysis

GraphPad Prism (version 5.04, GraphPad Software, La Jolla, CA) was used to run all statistical tests. For the acute study, BEC measurements and cell count data were analyzed with one-way ANOVA, followed by the post-hoc Tukey's test. For the ethanol dependence study, mean intoxication scores were compared by the non-parametric Mann-Whitney test,

while BECs and ethanol doses were compared by two-tailed, Student's t-tests. Cell count data was analyzed using two-way ANOVA followed by planned post-hoc t-tests to compare ethanol and controls within each time point. Pearson (parametric) or Spearman (nonparametric) correlations were conducted for subject data and cells counts as appropriate. Data are presented as mean $\pm$ SEM.

Results

Intoxication Parameters

Rats treated with a single 1, 2.5, or 5 g/kg dose displayed the expected dose-dependent effect on BECs measured from trunk blood samples collected at the time of perfusion ($F_{2,23} = 68391, p < 0.001$). Mean BECs for each group were as follows: 68 ± 5.9 , 191 ± 10.5 mg/dL and 267 ± 16.0 mg/dL for the 1, 2.5, and 5 g/kg groups, respectively. For rats subjected to the 4-day binge model of alcohol dependence, no difference was seen in mean intoxication or daily ethanol dose between rats euthanized 2 and 16 h following the last ethanol dose (Table 2). Although BEC differed between the 2 and 16 h groups ($p < 0.001$), ethanol dependent rats were equally intoxicated throughout the 4-day ethanol exposure period and received the same daily ethanol dose. Mean and peak withdrawal scores were consistent with previous experiments using this model (Morris et al., 2010b).

Effect of acute ethanol intoxication on hippocampal H3S10ph

In vehicle-treated control rats, H3S10ph immunoreactivity was observed in two distinct patterns. Densely stained irregular-shaped cell clusters were detected along the subgranular zone as expected, consistent with other cell cycle markers that label the dividing neural progenitor cells located in this region (McClain et al., 2011). However, immunoreactivity was also found scattered throughout the granule cell layer and CA pyramidal neuron layer in cells with large, oval-shaped nuclei, resembling those of neurons. Confocal microscopy performed on tissue double-labeled with NeuN and H3S10ph confirmed that these cells were mature neurons and not a mitotically active cell type (Fig. S1). Only immunoreactive cells with this neuronal nuclear morphology were included in cell counts. Acute ethanol exposure dose-dependently altered the number of H3S10ph+ in the dentate gyrus granule cell layer ($F_{3,30} = 28.0, p < 0.001$). Specifically, 1 g/kg ethanol increased the number of H3S10ph+ granule cells by 30% compared to vehicle-treated controls, while 2.5 and 5 g/kg ethanol reduced the number of H3S10ph+ cells by 40% and 57%, respectively (Fig. 1a–d, i). Acute ethanol intoxication also dose-dependently affected the number of H3S10ph+ cells in the CA pyramidal neuron layer ($F_{3,30} = 20.4, p < 0.001$). Similar to the granule cell layer, 1 g/kg ethanol significantly increased the number of H3S10ph+ pyramidal neurons by 224% compared to vehicle-treated controls; however, 2.5 and 5 g/kg ethanol had no effect on the number of H3S10ph+ cells (Fig. 1e–h, j).

Dose-dependent changes in hippocampal H3S10ph are paralleled by changes in c-fos protein expression

Targeted S10 histone H3 phosphorylation helps direct the rapid transactivation of IEGs in response to mitogenic stimulation, stress, and activity (Clayton and Mahadevan, 2003). Therefore, we measured c-fos protein expression following acute ethanol intoxication. The

staining pattern for c-fos in vehicle-treated control rats closely matched the pattern seen for H3S10ph, except that c-fos was confined to the granule cell layer and CA pyramidal layer and was not seen in clusters of dividing neural progenitor cells in the subgranular zone. Acute ethanol intoxication dose-dependently altered granule cell layer c-fos expression ($F_{3,30} = 29.3, p < 0.001$). Compared to vehicle-treated controls, the number of c-fos+ granule cells increased by 71% in response to 1 g/kg ethanol, but decreased by 42% after exposure to 5 g/kg ethanol (Fig. 2a–d,i). Ethanol also affected CA pyramidal neuron c-fos expression in a dose-dependent manner ($F_{3,30} = 7.6, p < 0.001$). Specifically, 1g/kg ethanol exposure increased the number of c-fos+ pyramidal neurons by 512% compared to vehicle-treated controls, while higher doses had no effect (Fig. 2e–h,j). Parallel changes in H3S10ph and c-fos protein expression were further supported by the strong positive correlation between these two markers in both the granule cell layer ($r^2 = 0.75, p < 0.001$; Fig. 2k) and CA pyramidal neuron layer ($r^2 = 0.38, p < 0.001$; Fig. 2l).

Alcohol dependence similarly alters hippocampal H3S10ph and c-fos

The effects of alcohol dependence on hippocampal H3S10ph and c-fos protein expression were examined using the 4-day binge model of alcohol dependence. Two-way ANOVA conducted on granule cell H3S10ph data revealed a significant main effect of diet ($F_{1,33} = 5.5, p = 0.026$) and time ($F_{1,33} = 35.5, p < 0.001$) as well as a significant diet by time interaction ($F_{1,33} = 24.0, p < 0.001$). Specifically, the number of H3S10ph+ granule cells decreased by 66% at the 2 h time point ($p < 0.001$), but increased 146% at the peak withdrawal time point ($p = 0.002$) measured 16 h after the final ethanol dose (Fig. 3a–e). Interestingly, the robust increase in H3S10ph was confined entirely to the suprapyramidal blade of the granule cell layer (Fig. 3f). Two-way ANOVA performed on pyramidal neuron H3S10ph cell counts revealed a significant main effect of diet ($F_{1,33} = 24.6, p < 0.001$) and time ($F_{1,33} = 25.6, p < 0.001$) as well as a significant diet by time interaction ($F_{1,33} = 25.7, p < 0.001$; Fig. 3g–k). Interestingly, no difference was detected at the 2 h time point; however, a robust, 16-fold increase in CA pyramidal neuron H3S10ph ($p < 0.001$) accompanied peak ethanol withdrawal (Fig. 3i, j). In contrast to the regional specificity of the H3S10ph effect in the granule cell layer, the increase in H3S10ph was distributed across both the CA1/2 ($p < 0.001$) and CA3 ($p = 0.001$; Fig. 3l).

Correlations between subject data collected as part of the 4-day binge and the number of H3S10ph+ cells in the granule cell layer or CA fields were conducted in order to offer insight into the aspects of alcohol dependence that may contribute to these changes in pH3H3 expression. Mean intoxication score (0 – 5), last intoxication score (12th feeding), mean withdrawal score and peak withdrawal score (if applicable) were correlated with the number of H3S10ph+ cells by Spearman's r (r_s) whereas Pearson's r was used for BEC and H3S10ph+ cell data. Peak withdrawal correlated negatively with the number of granule cell layer H3S10ph+ cells ($r_s = -0.58, p = 0.0357$), though no other correlations were significant.

Changes in hippocampal c-fos expression closely paralleled changes described for H3S10ph in alcohol dependent rats. Two-way ANOVA carried out on granule cell c-fos+ cell count data revealed a significant main effect of diet ($F_{1,33} = 5.6, p = 0.025$) and time ($F_{1,33} = 13.3, p < 0.001$) and a significant diet by time interaction ($F_{1,33} = 26.0, p < 0.001$). The number of

c-fos+ granule cells decreased by 62% ($p = 0.008$) 2 h after the last ethanol dose, but increased by 170% ($p = 0.009$) at peak withdrawal (Fig. 4a–e). Similar to H3S10ph, the effect of peak withdrawal on granule cell c-fos expression was confined to the suprapyramidal blade (Fig. 4f). Two-way ANOVA conducted on pyramidal neuron c-fos cell count data revealed a significant main effect of diet ($F_{1,33} = 33.2, p < 0.001$) and time ($F_{1,33} = 32.4, p < 0.001$) and a significant diet by time interaction ($F_{1,33} = 31.7, p < 0.001$). Compared to control rats, pyramidal neuron c-fos was unaltered at the 2 h time point, but was clearly induced in numerous neurons during peak ethanol withdrawal (Fig. 4g–k). In addition, the increase in c-fos at peak withdrawal occurred in both the CA1/2 and CA3 pyramidal neuron layer (Fig. 4l). Correlations between subject data and the number of c-fos+ cells in the granule cell layer or CA fields revealed that peak withdrawal correlated negatively with the number of granule cell layer c-fos+ cells ($r_s = -0.58, p = 0.0357$; statistics were identical to H3S10ph+). No other correlations were significant.

The close relationship between changes in H3S10ph and c-fos expression in ethanol dependent rats is supported by the strong positive correlation between these two markers in both the granule cell ($r^2 = 0.95, p < 0.001$; Fig. 5a) and pyramidal neuron layer ($r^2 = 0.63, p < 0.001$; Fig. 5b). Furthermore, c-fos co-localized extensively with hippocampal neuronal H3S10ph (Fig. 5c). Control experiments verified that there was no unwanted capture of the rabbit anti-H3S10ph/goat anti-rabbit IgG-AF488 antibody combination by the rabbit anti-c-fos/goat anti-rabbit IgG-AF546 antibody combination (Fig. S2).

Discussion

Our work demonstrates a clear, robust correlation between H3S10ph and c-fos expression in hippocampal neurons during ethanol intoxication and withdrawal. Although ethanol has been shown to directly impact H3S10ph in hepatocytes (James et al., 2012), to our knowledge, this is the first report to suggest that ethanol alters histone phosphorylation in the brain. Interestingly, H3S10ph increases in hepatocytes following acute exposure to 1.75–3.5 g/kg ethanol (James et al., 2012). With the exception of low, acute doses, ethanol intoxication inhibits granule cell neuron H3S10ph while only at the peak of withdrawal was a striking increase in H3S10ph observed. These results support the emerging idea that ethanol exerts tissue-specific effects on post-translational histone modifications, which likely strongly influence ethanol-induced changes in gene expression and neuroadaptations (Ponomarev, 2013).

As an IEG, c-fos transcription is regulated by signal transduction pathways activated in response to many stimuli (O'Donnell et al., 2012). The c-fos gene encodes an important AP-1 family transcription factor capable of regulating genes responsible for phenotypic responses to stimuli (Perez-Cadahia et al., 2011). In the brain, c-fos expression marks active neurons, making it useful for identifying neurons that participate in learning or respond to drugs of abuse. In particular, ethanol intoxication and withdrawal affect c-fos expression in a number of neuronal populations; consistent with the idea that ethanol has wide-reaching impacts on brain physiology (Knapp et al., 2001; Vilpoux et al., 2009). These data concur with previous reports that intoxicating doses of ethanol disrupt c-fos expression in the hippocampus, while robust increases in hippocampal c-fos occur during ethanol withdrawal

(Ryabinin et al., 1995). Curiously, the greater the severity of withdrawal symptoms (i.e. peak withdrawal score achieved), the lower the number of c-fos+ or H3S10ph+ cells. These data suggest that c-fos and other IEGs mediate withdrawal symptoms arising from neuronal hyperexcitability (Dave et al., 1990; Matsumoto et al., 1993; Morgan et al., 1992). Indeed, c-fos and other IEGs are thought to participate in signaling pathways that lead to long-term drug-induced neuroadaptations (Vilpoux et al., 2009).

Induction of c-fos transcription is accompanied by H3S10ph in nucleosomes associated with the c-fos gene sequence and its promoter (Healy et al., 2012). Stimulus-mediated activation of MAPK signal transduction pathways controls this process. Specifically, activation and nuclear translocation of extracellular-signal regulated kinase 1 and 2 (ERK1/2) activates mitogen- and stress-activated protein kinase 1 and 2 (MSK1/2), the kinases directly responsible for interphase H3S10ph (Healy et al., 2012; Soloaga et al., 2003). In the hippocampus, activation of ERK1/2 with subsequent MSK1/2 activation coordinates increased granule cell H3S10ph and c-fos expression in response to psychological stressors, such as forced swimming and predator presence (Reul, 2014). Furthermore, alcohol also modifies hippocampal ERK signaling: 3.5 g/kg ethanol strongly inhibits ERK phosphorylation (Chandler and Sutton, 2005), while withdrawal following chronic ethanol administration increases ERK activity (Sanna et al., 2002). Consistent with alcohol's effects on hippocampal ERK signaling, we found that acute ethanol inhibited H3S10ph in granule cells, while ethanol withdrawal had the opposite effect. Thus, signaling through the ERK pathway likely influences these ethanol-mediated changes in hippocampal H3S10ph, though experiments are needed to confirm this connection. The parallel changes in c-fos and H3S10ph expression support the concept that ethanol-induced changes in c-fos expression are controlled by signaling pathways leading to alterations in chromatin structure.

The striking increase in hippocampal c-fos expression during withdrawal is well-established in models of alcohol dependence (Dave et al., 1990; Morgan et al., 1992). The dependence of this effect on NMDA receptor activation has led some to suggest that upregulated c-fos may be involved in withdrawal-induced excitotoxicity (Bouchenafa and Littleton, 1998; Matsumoto et al., 1993). The 4-day binge model used in these studies induces neurodegeneration across corticolimbic regions; however, hippocampal cell death is restricted mostly to the ventral dentate gyrus (Collins et al., 1996; Crews et al., 2000; Kelso et al., 2011). The dorsal hippocampus and dentate gyrus, the region of interest in the current study, are mostly spared. Therefore, while increased NMDA receptor activity mediates withdrawal-induced c-fos expression in hippocampal granule cell and pyramidal neurons, the increased excitation is not sufficient to induce widespread neurotoxicity in these two populations in the 4-day model. Taken together, the evidence hints that c-fos-expressing neurons survive ethanol withdrawal, indicating that c-fos and the pathways that regulate its expression likely serve other functions.

IEGs regulate neuroplasticity and mediate the establishment of long-term neuroadaptations induced by drug exposure (Perez-Cadahia et al., 2011). Adult granule cell neurogenesis represents an important form of hippocampal neuroplasticity. Ethanol intoxication potently inhibits adult neurogenesis; however, a large, transient burst of neural progenitor cell proliferation begins as early as 5 days following the last ethanol exposure (Hayes et al., in

revision). Interestingly, the activity of mature granule cells appears to have regulatory control over neural progenitor cell proliferation. For instance, deep brain stimulation targeting the entorhinal cortex activates the perforant pathway and increases the activity of granule cell neurons, an effect that is followed by enhanced neural progenitor cell proliferation 3–5 days later (Stone et al., 2011). Similarly, reactive neurogenesis in our model of ethanol dependence occurs 5–7 days following peak ethanol withdrawal. Although the timing of neural progenitor cell proliferation differs slightly, qualitatively, this proliferative effect is identical. This raises the possibility that withdrawal-induced activity of granule cell neurons underlies the transient increase in neurogenesis observed in protracted withdrawal. As H3S10ph regulates c-fos expression, modulation of this histone modification may provide an opportunity for uncovering the mechanism and functional significance of alcohol-induced reactive neurogenesis.

In the dentate gyrus, increased phosphorylated H3S10 and c-fos expression associated with peak ethanol withdrawal was restricted to the suprapyramidal blade of the granule cell layer. Similar blade-specific expression patterns for H3S10ph and c-fos expression occur in rodents subjected to restraint stress or hippocampal-dependent learning tasks (Chandramohan et al., 2008; Fevurly and Spencer, 2004). Few studies have examined the mechanisms responsible for these observations; however, evidence suggests that the suprapyramidal blade may be more sensitive to glucocorticoid and NMDA receptor-dependent signaling (Chandramohan et al., 2008; Fevurly and Spencer, 2004). Significant elevations in corticosterone and enhanced NMDA receptor activity are both experienced during ethanol withdrawal (Matsumoto et al., 1993; Sharrett-Field et al., 2013). Thus, blade-specific differences in H3S10ph and c-fos expression observed during peak ethanol withdrawal may be related to this differential sensitivity to glucocorticoid and NMDA receptor signaling.

It must be noted that this work was conducted in male rats only. Although, striking sex differences have not been observed for the various neurodegeneration and adult neurogenesis endpoints measured in this model (e.g. Leasure & Nixon), because the exact study has not been performed in female rats, it is possible that these findings will not generalize to females.

It is increasingly clear that ethanol-induced changes in CNS gene expression are controlled by altering the histone code (Ponomarev, 2013). Ethanol targets histone acetylation in the amygdala, an effect with strong ties to the anxiolytic effects of ethanol (D'Addario et al., 2013; Krishnan et al., 2014). Altered gene expression in the cerebral cortex and hippocampus has been linked to ethanol-induced changes in histone methylation and acetylation (Finegersh and Homanics, 2014; Ponomarev et al., 2012; Stragier et al., 2015; Zhou et al., 2011). In addition, histone phosphorylation correlates with ethanol-mediated changes in hippocampal c-fos expression. The mechanisms by which ethanol leads to altered post-translational histone modifications represents an exciting area of research that promises to yield critical information regarding how ethanol promotes neuroadaptations that lead to the development of dependence. Targeting mechanisms controlling the histone code holds promise for the development of novel therapeutic strategies aimed at preventing or reversing ethanol-induced neuroadaptations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Acute ethanol intoxication dose-dependently alters the number of phosphorylated histone H3+ cells in the hippocampus. Representative images show H3S10ph+ cells in the dentate gyrus granule cell layer (a–d) and hippocampal CA pyramidal neuron layer (e–h) after an acute dose of saline (n = 8), 1 (n = 8), 2.5 (n = 9), or 5 g/kg (n = 9) ethanol. Arrows show regions in insets. In the granule cell layer (i), H3S10ph+ cells increased 2 h after 1 g/kg ethanol, but decreased 2 h following 2.5 and 5 g/kg ethanol. In the pyramidal neuron layer (j), 1g/kg ethanol increased H3S10ph+ cells compared to controls, while 2.5 and 5 g/kg ethanol had no effect. Values are mean $\pm$ SEM. Scale bars = 100 μ m (10 μ m, insets). * $p < 0.05$ versus control (Tukey's post-hoc test). CA = cornus ammonis, GCL = granule cell layer, PyL = pyramidal cell layer.

Fig. 2.

Acute ethanol intoxication dose-dependently alters hippocampal c-fos protein expression. Representative images show c-fos+ cells in the dentate gyrus granule cell layer (ad) and hippocampal CA pyramidal neuron layer (e–h) after an acute dose of saline (n = 8), 1 (n = 8), 2.5 (n = 9), or 5 g/kg (n = 9) ethanol. Arrows show regions in insets. In the granule cell layer, the number of c-fos+ cells increased 2 h after 1 g/kg ethanol, but decreased 2 h following 5 g/kg ethanol (i). In the pyramidal neuron layer, 1 g/kg ethanol increased c-fos compared to controls, while 2.5 and 5 g/kg ethanol had no effect. * $p < 0.05$ versus control (Tukey's post-hoc test). H3S10ph and c-fos protein expression correlated in both the granule cell layer (k) and pyramidal neuron layer (l). Closed circles = saline, squares = 1 g/kg, triangles = 2.5 g/kg, open circles = 5 g/kg. Values in i, j are mean $\pm$ SEM. Scale bars = 100 μ m (10 μ m for insets). CA = cornus ammonis, GCL = granule cell layer, PyL = pyramidal cell layer.

Fig. 3.

Ethanol dependence induces temporally-specific changes the number of phosphorylated histone H3+ cells in the hippocampus. Representative images show H3S10ph+ cells in the dentate gyrus granule cell layer (a–d) and hippocampal CA pyramidal neuron layer (g–j) 2 h (n = 10 control, 8 ethanol) and 16 h (n = 10 control, 8 ethanol) following the last control or ethanol diet dose, quantified in (e, f, k, l). The increase in H3S10ph+ cells at peak withdrawal was confined to the suprapyramidal layer of the granule cell layer (f). In the pyramidal neuron layer, H3S10ph+ cells were unaltered at 2 h, but increased at 16 h in ethanol dependent rats (k). The increase in H3S10ph+ cells at peak withdrawal was detected in both the CA1/2 and CA3 subfields (l). Values are mean ± SEM. Scale bar = 100 μm. CA = cornus ammonis, Con = control, DG = dentate gyrus, EtOH = ethanol, GCL = granule cell layer, IB, Infra = infrapyramidal, PyL = pyramidal cell layer, SB, supra = suprapyramidal.

Fig. 4.

Ethanol dependence induces temporally-specific changes in c-fos protein expression. Representative images show c-fos+ cells in the dentate gyrus granule cell layer (a–d) and hippocampal CA pyramidal neuron layer (g–j) 2 h (n = 10 control, 8 ethanol) and 16 h (n = 10 control, 8 ethanol) following the last control or ethanol diet dose, quantified in (e, f, k, l). The effect at peak withdrawal was restricted to the suprapyramidal blade (f). In the pyramidal neuron layer, ethanol dependent rats had significantly more c-fos+ cells at peak withdrawal compared to the control group (k). The effect at peak withdrawal occurred along the entire pyramidal neuron layer across all 3 CA subfields (l). Values are mean $\pm$ SEM. Scale bar = 100 μ m. CA = cornus ammonis, Con = control, DG = dentate gyrus, EtOH = ethanol, GCL = granule cell layer, IB, Infra = infrapyramidal, PyL = pyramidal layer, SB, supra = suprapyramidal.

Fig. 5.

Evidence that c-fos expression overlaps with H3S10ph in hippocampal neurons. The number of c-fos+ cells correlated strongly with the number of H3S10ph+ cells in both the granule cell layer (a) and pyramidal cell layer (b). Squares = 2 h control, triangles = 2 h ethanol, + = 16 h control, circles = 16 h ethanol. Double-label immunofluorescence shows a high degree of overlap between H3S10ph+ cells (c) and c-fos+ cells (d) in the granule cell layer (merged in e). A representative single, 0.8 μm optical slice of H3S10ph+ cells (f) and c-fos+ cells (g) are merged in (h) to show a representative orthogonal view of a H3S10ph+ and c-fos+ co-positive cell reconstructed from Z-plane stacks (slices). GCL = granule cell layer.

Table 1

Primary Antibody Information

Antibody	Species	Dilution	Antigen	Source, Catalog no.
H3S10ph	Rabbit	1:1,000-IHC 1:400-IF	Peptide corresponding to amino acids 7-20 of human S10 phosphorylated histone H3	Millipore, 06-570
c-fos	Rabbit	1:10,000-IHC 1:2,500-IF	Peptide corresponding to amino acids 4-17 of human c-fos	Calbiochem, PC38
NeuN	Mouse	1:500	Purified mouse brain nuclei	Millipore, MAB377
GFAP	Rabbit	1:2000	Bovine spinal cord GFAP	Dako, Z 0334

NeuN = neuronal nuclei, GFAP = glial fibrillary acidic protein

Table 2

4-Day Binge Subject Data

Time Point (h post-EtOH)	n	Intoxication Scores	Daily EtOH Dose (g/kg/d)	BEC (mg/dL)	Peak Withdrawal	Mean Withdrawal
2	9	2.3 ± 0.1	8.6 ± 0.3	370 ± 18.4	N/A	N/A
16	8	2.5 ± 0.1	8.0 ± 0.3	473 ± 6.1 *	3.1 ± 0.2	2.1 ± 0.2

BEC = Blood ethanol concentration, EtOH = ethanol, Values are mean ± SEM.

* $p<0.05$