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Disconnect between alcohol-induced alterations in chromatin structure and gene transcription in a mouse embryonic stem cell model of exposure

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ABSTRACT

Alterations to chromatin structure induced by environmental insults have become an attractive explanation for the persistence of exposure effects into subsequent life stages. However, a growing body of work examining the epigenetic impact that alcohol and other drugs of abuse exert consistently notes a disconnection between induced changes in chromatin structure and patterns of gene transcription. Thus, an important question is whether perturbations in the 'histone code' induced by prenatal exposures to alcohol implicitly subvert gene expression, or whether the hierarchy of cellular signaling networks driving development is such that they retain control over the transcriptional program. To address this question, we examined the impact of ethanol exposure in mouse embryonic stem cells cultured under 2i conditions, where the transcriptional program is rigidly enforced through the use of small molecule inhibitors. We find that ethanol-induced changes in post-translational histone modifications are dose-dependent, unique to the chromatin modification under investigation, and that the extent and direction of the change differ between the period of exposure and the recovery phase. Similar to *in vivo* models, we find post-translational modifications affecting histone 3 lysine 9 are the most profoundly impacted, with the signature of exposure persisting long after alcohol has been removed. These changes in chromatin structure associate with dose-dependent alterations in the levels of transcripts encoding *Dnmt1*, *Uhrf1*, *Tet1*, *Tet2*, *Tet3*, and *Polycomb* complex members *Eed* and *Ezh2*. However, in this model, ethanol-induced changes to the chromatin template do not consistently associate with changes in gene transcription, impede the process of differentiation, or affect the acquisition of monoallelic patterns of expression for the imprinted gene *Igf2R*. These findings question the inferred universal relevance of epigenetic changes induced by drugs of abuse and suggest that changes in chromatin structure cannot unequivocally explain dysgenesis in isolation.

1. Introduction

Despite numerous preventative and educational strategies, gestational exposure to alcohol remains the most common cause of environmentally induced birth defects affecting humans. The worldwide prevalence of Fetal Alcohol Spectrum Disorders (FASDs) ranges from 6 to 55 children per 1000 live births, depending on the region examined (Roozen et al., 2016). While progress toward understanding the toxicological and neurological actions of ethanol have been made, the teratogenic actions of this agent remain very poorly understood (Memo, Gnoato, Caminiti, Pichini, & Tarani, 2013). In particular, it has been incredibly challenging to identify relevant molecular alterations arising between the period of initial exposure and the acquisition of developmental defects.

The separation between the period of exposure and the development of structural defects suggests that alcohol imparts a memory of exposure that influences the developmental trajectory of select cellular populations. In recent years, alterations to chromatin structure induced by environmental and/or nutritional insults have become an attractive explanation for the persistence of

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exposure effects into subsequent life stages (Feil & Fraga, 2011). Indeed, numerous studies have demonstrated that ethanol has the capacity to alter chromatin structure, suggesting that epigenetic mechanisms are relevant to the dysgenesis associated with FASDs (Basavarajappa & Subbanna, 2016; Mead & Sarkar, 2014; Resendiz, Mason, Lo, & Zhou, 2014; Ungerer, Knezovich, & Ramsay, 2013). For example, studies from a number of laboratories have demonstrated both acute and long-term ethanol exposure influences patterns of DNA methylation (Ungerer et al., 2013). Further, our group and others have reported persistent alterations in post-translational histone modifications both *in vitro* and *in vivo* (Bekdash, Zhang, & Sarkar, 2013; Finegersh et al., 2015; Govorko, Bekdash, Zhang, & Sarkar, 2012; Moonat, Sakharkar, Zhang, Tang, & Pandey, 2013; Pal-Bhadra et al., 2007; Pan et al., 2014; Subbanna & Basavarajappa, 2014; Veazey, Carnahan, Muller, Miranda, & Golding, 2013; Veazey, Parnell, Miranda, & Golding, 2015; Zhang, Ho, Vega, Burne, & Chong, 2015). Collectively, these published studies suggest alcohol has the capacity to induce alterations in chromatin structure and that in select instances, these changes are heritable through cell division, persist well beyond the initial encounter, and have the potential to contribute to the genesis of FASD birth defects.

Work by our group using an *in vitro* neurosphere model has previously identified significant ethanol-induced changes in chromatin structure within the regulatory regions of multiple genes controlling both neural precursor cell identity and the processes of cellular differentiation (Veazey et al., 2013). However, despite significant changes in chromatin structure, only a small subset of genes displayed altered patterns of transcription. Further, when ethanol was withdrawn and exposed neurospheres were allowed to recover, a distinct profile of chromatin alterations emerged, but yet no correlative alterations in transcription were observed (Veazey et al., 2015). These observations are significant as they suggest that the induced changes in chromatin structure do not affect the transcriptional programs governing cellular identity and would therefore have a limited capacity to impact subsequent developmental stages. Similar to our work, exposures to cocaine and other drugs of abuse are associated with widespread alterations in chromatin structure, yet in these studies, a significant number of genes show changes in expression opposite to those that would be predicted based upon the chromatin profiles, while most genes show no change at all (Nestler, 2014).

Recent studies in chromatin biology suggest that rather than being causal in controlling gene transcription, alterations to the chromatin template serve as a mechanism for the integration of cell signaling events with the biochemical machinery controlling transcription (Badeaux & Shi, 2013). In this view, multiple signaling pathways converge on the chromatin fiber, which serves as a regulatable platform to facilitate the integration of transcription factor regulatory networks with cell-specific cohorts of genes. The key distinction here is that while chromatin structure facilitates gene expression, protein transcription factors drive it. Thus, an important question that remains to be resolved is whether perturbations in the 'histone code' induced by prenatal exposure to drugs of abuse actually have the potential to alter gene transcription, or whether the extracellular and intracellular signaling programs regulating cellular identity maintain rigid control over cellular patterns of gene expression. As neurospheres are complex populations of cells, we elected to examine this question using mouse embryonic stem cells, which can be cultured with high uniformity in terms of cellular phenotype, chromatin profiles and transcriptional output (Galonska, Ziller, Karnik, & Meissner, 2015; Marks et al., 2012).

Embryonic Stem Cells (ESCs) have an innate cell signaling program maintaining pluripotency, which is balanced by differentiation-promoting signals from the *mitogen-activated protein kinase (MAPK)* and *glycogen synthase kinase 3* (*GSK3*) signaling pathways (Hirai, Karian, & Kikyo, 2011). When input from these differentiation inducing pathways is blocked, the networks governing cellular identity become locked into a transcriptional program promoting perpetual self-renewal and a ground state of pluripotency (Ying et al., 2008). Given the static state in which the transcriptional program of these cells is maintained, we sought to employ this model to determine the extent to which ethanol-induced modifications to chromatin structure could heritably alter transcription. To this end, we employed the *2i* embryonic stem cell culture system, which strictly enforces the undifferentiated state through the use of small molecule inhibitors 6-bromoindirubin-3′-oxime, a potent and reversible *GSK-3α/β* inhibitor, and PD0325901, which inhibits *MAPK*. Although not strictly necessary, Leukemia Inhibitory Factor (LIF) was also used for maximum pluripotency (Miyanari & Torres-Padilla, 2012). Cultures were monitored for changes in the enrichment of key post-translational histone modifications within the regulatory regions of genes controlling pluripotency and core aspects of neural differentiation, during both the period of exposure as well as during the recovery period.

Similar to our previous studies in cultured neural stem cells, we find that ethanol-induced changes in post-translational histone modifications are dose-dependent, unique to the chromatin modification under investigation, and finally that both the extent and direction of the change differ between the period of exposure and the recovery phase. However, these ethanol-induced changes to the chromatin template do not directly associate with changes in gene transcription. To assess the effect of ethanol induced changes on cellular differentiation and a measure of developmental programming, we examined the transcription of *Insulin Growth Factor 2 Receptor* (*Igf2R*), which is an imprinted gene that acquires monoallelic patterns of gene expression during embryonic stem cell differentiation (Nagano et al., 2008; Santoro et al., 2013). Genomic imprinting is an epigenetic mechanism of transcriptional regulation that restricts expression to either the maternally- or paternally-inherited copy of the gene; the opposite parental copy is silent (Bartolomei & Tilghman, 1997). During the production of eggs and sperm, a subset of genes is marked or "imprinted" with differential patterns of DNA methylation to mark whether they are transmitted from the mother or father, respectively. In developing embryos, expression of imprinted genes becomes restricted, based on these parental epigenetic marks (Mann et al., 2004). Disruptions in imprinting can have severe consequences for growth and development of the mammalian embryo, as proper acquisition and maintenance of genomic imprints are crucial for both fetal and placental development (McGrath & Solter, 1984). Importantly, alterations in *Igf2R* were recently identified in studies examining the impact of prenatal ethanol exposures on the developing brain using an *ex vivo* model of early neurulation (Liu, Balaraman, Wang, Nephew, & Zhou, 2009). In our model, ethanol exposure did not appreciably impede the process of differentiation nor affect the acquisition of *Igf2R* imprinted gene expression. Analysis of fully differentiated cells identifies similar trends, suggesting that while ethanol can alter chromatin structure, the induced changes do not necessarily affect the transcriptional program.

2. Materials and methods

2.1. Cell culture

Primary embryonic stem cells (ESCs) derived from B6XCAST F1 embryos were employed to examine allele specific patterns of gene expression in a parent of origin specific fashion (Golding et al., 2011). ESCs were maintained in ESC medium under *2i* conditions as previously described (Miyanari & Torres-Padilla, 2012). Cells were grown in DMEM culture medium (Catalog # D5671; Sigma)