GENETICS AND EPIGENETICS OF FETAL ALCOHOL SPECTRUM DISORDERS

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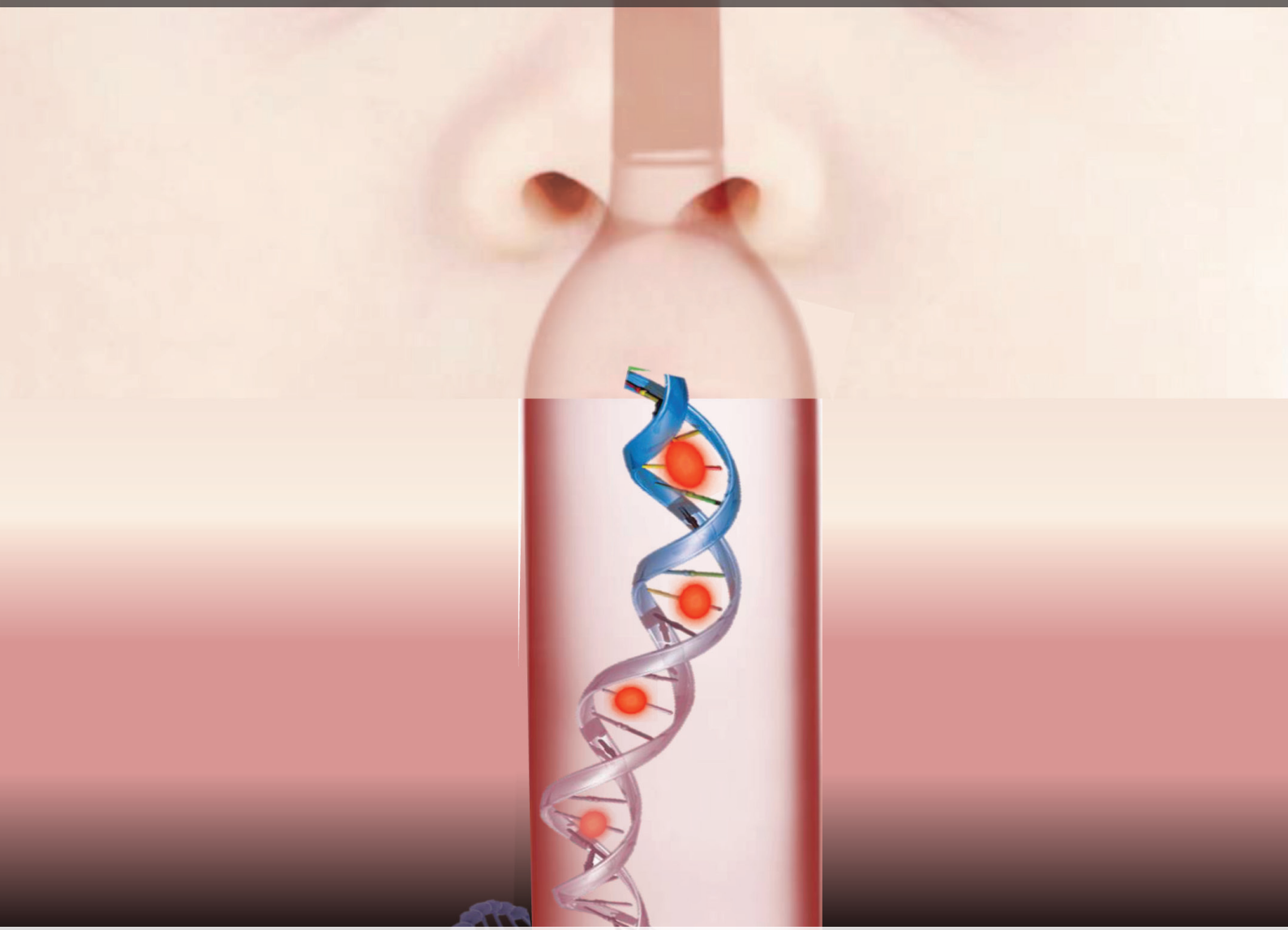

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GENETICS AND EPIGENETICS OF FETAL ALCOHOL SPECTRUM DISORDERS

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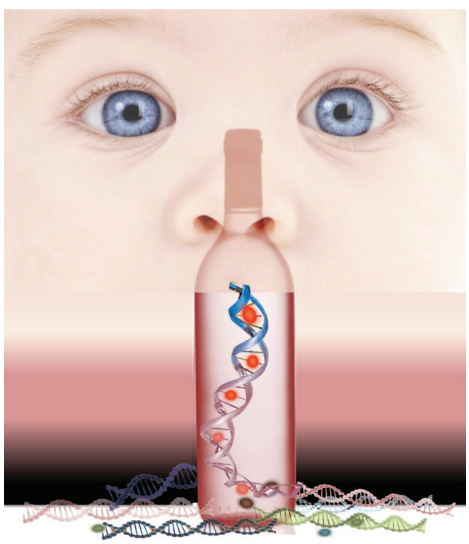

Pregnancy drinking unknowingly subjects her child to second-hand drinking. Prenatal alcohol leads to wide spectrum of growth retardation, brain dysfunction, and learning and mental disability. This diverse vulnerability is in part contributed by the differential genetic background. It is now understood that besides affecting through the genetic make-up and disturbing cell physiology, alcohol can directly access DNA and chromatin throughout the genome and alter epigenetics for DNA interpretation and subsequently gene transcription. This eBook address the triangle of alcohol, gene, and epigenetic interactions affecting the brain development of the once innocent baby.
The conceptual composite was designed and created by Feng C. Zhou, Ph.D. Part of the imagery has been adopted from HD Wallpapers and Darryl Leja, NHGRI over genome.gov.

Women drinking during pregnancy can result in Fetal Alcohol Spectrum Disorder (FASD), which may feature variable neurodevelopmental deficits, facial dysmorphology, growth retardation, and learning disabilities. Research suggests the human brain is precisely formed through an intrinsic, genetic-cellular expression that is carefully orchestrated by an epigenetic program. This program can be influenced by environmental inputs such as alcohol. Current research suggests the genetic and epigenetic elements of FASD are heavily intertwined and

highly dependent on one another. As such, now is the time for investigators to combine genetic, genomic and epigenetic components of alcohol research into a centralized, accessible platform for discussion.

Genetic analyses inform gene sets which may be vulnerable to alcohol exposure during early neurulation. Prenatal alcohol exposure indeed alters expression of gene subsets, including genes involved in neural specification, hematopoiesis, methylation, chromatin remodeling, histone variants, eye and heart development. Recently, quantitative genomic mapping has revealed loci (QTLs) that mediate alcohol-induced phenotypes identified between two alcohol-drinking mouse strains. One question to consider is (besides the role of dose and stage of alcohol exposure) why only 5% of drinking women deliver newborns diagnosed with FAS (Fetal Alcohol Syndrome)? Studies are ongoing to answer this question by characterizing genome-wide expression, allele-specific expression (ASE), gene polymorphisms (SNPs) and maternal genetic factors that influence alcohol vulnerability.

Alcohol exposure during pregnancy, which can lead to FASD, has been used as a model to resolve the epigenetic pathway between environment and phenotype. Epigenetic mechanisms modify genetic outputs through alteration of 3D chromatin structure and accessibility of transcriptional machinery. Several laboratories have reported altered epigenetics, including DNA methylation and histone modification, in multiple models of FASD. During development DNA methylation is dynamic yet orchestrated in a precise spatiotemporal manner during neurulation and coincidental with neural differentiation. Alcohol can directly influence epigenetics through alterations of the methionine pathway and subsequent DNA or histone methylation/acetylation. Alcohol also alters noncoding RNA including miRNA and transposable elements (TEs). Evidence suggests that miRNA expression may mediate ethanol teratology, and TEs may be affected by alcohol through the alteration of DNA methylation at its regulatory region. In this manner, the epigenetic and genetic components of FASD are revealing themselves to be mechanistically intertwined.

Can alcohol-induced epigenomic alterations be passed across generations? Early epidemiological studies have revealed infants with FASD-like features in the absence of maternal alcohol, where the fathers were alcoholics. Novel mechanisms for alcohol-induced phenotypes include altered sperm DNA methylation, hypomethylated paternal allele and heritable epimutations. These studies predict the heritability of alcohol-induced epigenetic abnormalities and gene functionality across generations.

We opened a forum to researchers and investigators the field of FASD to discuss their insights, hypotheses, fresh data, past research, and future research themes embedded in this rising field of the genetics and epigenetics of FASD. This eBook is a product of the collective sharing and debate among researchers who have contributed or reviewed each subject. We organized them here into four parts: Editorial and Opinion, Genetics, Epigenetics, and the Interaction of Genetics and Epigenetics. We would like to thank all the contributors, the avid and never-compromising reviewers, and Frontiers of Genetics, who provided the platform and maintained a high standard for the discussion forum. Our special thanks is additionally extended to Drs. Tobias Preuten and Victoria Newman for their tireless assistance in the production of this eBook.

Feng C. Zhou, Ph.D. at Indiana University School of Medicine.

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EDITORIAL
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Editorial: Genetics and epigenetics of fetal alcohol spectrum disorders

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Children born to mothers who drink during pregnancy are at risk for growth retardation, memory, learning, and cognitive deficits under a lifelong disability known as Fetal Alcohol Spectrum Disorders (FASD), which occurs at a high rate in the US (∼1/100 live births) and worldwide. There are three outstanding features of FASD. (a) Humans have been associated with alcohol consumption dated as far back as 7000 BC (McGovern et al., 2004) and there is no sign of waning. (b) FASD can range across a large spectrum of severity, from the more severe Fetal Alcohol Syndrome (FAS) (encompassing facial, brain, and gross deformity) to the hard to detect, subtle mental dysfunctions. The Centers for Disease Control and Prevention (CDC) indicates that approximately 7–8% of pregnant women consume alcohol in US, but diagnosed FASD occurs in a much smaller percentage (CDC, 2012). There is strong evidence to indicate that genetic makeup is a major contributing factor to the differential vulnerability to FASD. (c) Alcohol's deleterious effect has recently been found to go beyond cellular toxicity, to affect epigenetics. The epigenetic chemical code, methylation and acetylation written on top of genomic base elements (e.g., DNA cytosine and histone tails), can confer 3D DNA packaging and fundamentally alter gene transcription. Alcohol has recently been recognized to have strong influences on methylation and acetylation (see Resendiz et al., 2014a) via alcohol metabolism. Furthermore, current evidence points to alcohol's influence on the interaction of genetic and epigenetic factors. These fascinating new views are the center of this eBook, which includes new data and an in-depth discussion of the recent findings and expert opinions. It is hoped that by elucidating the genetic x epigenetic (GxE) interaction at the center of fetal alcohol exposure, new insight will lead the community of scientists toward a greater understanding of this disease, and lay a foundation for prospective new treatments and interventions.

FASD is, theoretically, an avoidable disease. Unfortunately, there are many misconceptions and a lack of general public awareness of the profound causality of alcohol on developmental dysfunction. This eBook includes a public awareness effort with an *Opinion* from Singh et al. (2014), challenging the research community to convey the message that "at no time during pregnancy is alcohol 100% safe to drink."

Is the differential vulnerability of mother and offspring to alcohol derived from the genetic difference rendered by the physiological environment (e.g., differential maternal placenta) or the fetal genotypes themselves? The forum begins with a study to tease out this entanglement. Gilliam (2014) analyzes this question by experimentally transferring the fertilized blastocysts of the alcohol sensitive C57BL/6N (B6) mice to that of alcohol resistant DBA/2 (D2) dams, and vice versa. The finding is intriguing, and the result is supported by another design directly exposing the embryos of B6 and D2 (with identical alcohol conditions) in an incubator thereby bypassing maternal influence (e.g., Ogawa et al., 2005; Chen et al., 2011) and further by *in vivo* study between lines of genetically diverse mice (see Loucks and Carvan, 2004; Anthony et al., 2010; Downing et al., 2012). These studies indicated that abnormal neurodevelopment depends not

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only on the dose and pattern of alcohol exposure but also on interactions among environmental, genetic, and maternal factors. Which genes underlie the abnormal development featured in FASD? The Smith et al. (2014) chapter has a succinct discussion of the multiple genomic factors that can increase vulnerability to facial deficits through pathways such as calcium-mediated neural crest apoptosis. Additionally, multiple genomic factors acting on different genetic backgrounds (e.g., in W98S vs. W98D chicks) may contribute to the differential sensitivity of the ethanol-imposed apoptosis. What differences lie within the genome that really impact the genetic contribution to alcohol vulnerability and resistance? To ultimately answer this question requires an interrogation of the entire genome at single nucleotide resolution. The paper "Genomic signatures in the differential vulnerability to fetal alcohol in B6 and D2 mice" did just that (Lossie et al., 2014). The single nucleotide variant (SNV) analysis captured ∼900 genes on promoter regions where transcription factors bind (e.g., *Eya2, Csmd3*) that may affect transcription and on non-coding regions that may result in missense mutations leading to abnormal protein formation.

How does alcohol utilize GxE to turn neuroprogenitor cells away from their normal course? Goldowitz et al. (2014) used a B6XD2 mice crossing to demonstrate that alcohol exposure alters the γH2AX histone to mediate differential cerebral cortical apoptosis between genetic lines. It appears the neuroprogenitor cells used an intricate epigenetic program to guide neuronal differentiation and maturation. Resendiz et al. (2014b) present a timely and in-depth discussion of the normal neurodevelopmental epigenetic program and how alcohol deregulates this program. Alcohol exposure alters gene promoter methylation, histone modification, and deregulates non-coding RNA that challenges canonical gene expression and can result in an observable phenotype. Imprinted genes are known to play a particularly important role in human growth and development. Masemola et al. (2015) examined human blood cells to show that an FAS cohort in South Africa undergo CpG methylation changes in imprinting control regions (ICRs) (e.g., lower KvDMR1 and PEG3 DMR) that control allele specific gene expression. If such changes are also carried in the brain, they may contribute to neurodevelopmental abnormalities seen in FASD.

As discussed in Kleiber et al. (2014), genetics and epigenetics are intertwined and changes to this dynamic may last into adulthood, contributing to FASD (Lo and Zhou, 2014). These GxE interactions are also elucidated through examples presented in the previously introduced papers (Lossie et al., 2014; Smith et al., 2014). The complex interplay of genetic and epigenetic factors are also showcased in the altered hypothalamic-pituitary-axis axis, which may account for causality in mental impairment and cancer (Mead and Sarkar, 2014). Additionally, DNA methylation changes, if carried through the germ line, may affect multiple generations subsequent to alcohol exposure (Mead and Sarkar, 2014). Such complex transmission may be established through imprinting of parental genes, which defies Mendelian inheritance (Tunc-Ozcan et al., 2014). Further, validations of transgenerational effects of alcohol exposure are needed, and are in progress.

In summary, this eBook reveals that FASD has a genetic propensity perturbed by an environmental input which may in part be registered through epigenetics. Abnormal epigenetic marks may be accumulated over time or even generations. This emphasizes our original message that no specific level of drinking is ever safe during pregnancy. Finally, treatment and prevention of FASD would be best addressed by taking both genetic and epigenetic factors into consideration.

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References

Anthony, B., Vinci-Booher, S., Wetherill, L., Ward, R., Goodlett, C., and Zhou, F. C. (2010). Alcohol-induced facial dysmorphology in C57BL/6 mouse models of fetal alcohol spectrum disorder. *Alcohol* 44, 659–671. doi: 10.1016/j.alcohol.2010.04.002

CDC. (2012). *Alcohol Use and Binge Drinking Among Women of Childbearing Age–United States, 2006–2010*. MMWR Morbidity and Mortality Weekly Report. Available online at: http://www.cdc.gov/mmwr

Chen, Y., Ozturk, N. C., Ni, L., Goodlett, C., and Zhou, F. C. (2011). Strain differences in developmental vulnerability to alcohol exposure via embryo culture in mice. *Alcohol. Clin. Exp. Res.* 35, 1293–1304. doi: 10.1111/j.1530-0277.2011.01465

Downing, C., Flink, S., Florez-McClure, M. L., Johnson, T. E., Tabakoff, B., and Kechris, K. J. (2012). Gene expression changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 36, 1519–1529. doi: 10.1111/j.1530-0277.2012.01757

Gilliam, D. (2014). Embryo transfers between C57BL/6J and DBA/2J mice: examination of a maternal effect on ethanol teratogenesis. *Front. Genet.* 5:436. doi: 10.3389/fgene.2014.00436

Goldowitz, D., Lussier, A. A., Boyle, J. K., Wong, K., Lattimer, S. L., Dubose, C., et al. (2014). Molecular pathways underpinning ethanol-induced neurodegeneration. *Front. Genet.* 5:203. doi: 10.3389/fgene.2014.00203

Kleiber, M. L., Diehl, E. J., Laufer, B. I., Mantha, K., Chokroborty-Hoque, A., Alberry, B., et al. (2014). Long-term genomic and epigenomic dysregulation as a consequence of prenatal alcohol exposure: a model for fetal alcohol spectrum disorders. *Front. Genet.* 5:161. doi: 10.3389/fgene.2014.00161

Lo, C. L., and Zhou, F. C. (2014). Environmental alterations of epigenetics prior to the birth. *Int. Rev. Neurobiol.* 115, 1–49. doi: 10.1016/B978-0-12-801311-3.00001-9

Lossie, A. C., Muir, W. M., Lo, C.-L., Timm, F., Liu, Y., Gray, W., et al. (2014). Implications of genomic signatures in the differential vulnerability to fetal alcohol exposure in C57BL/6 and DBA/2 mice. *Front. Genet.* 5:173. doi: 10.3389/fgene.2014.00173

Loucks, E., and Carvan, M. J. III. (2004). Strain-dependent effects of developmental ethanol exposure in zebrafish. *Neurotoxicol. Teratol.* 26, 745–755. doi: 10.1016/j.ntt.2004.06.017

Masemola, M. L., van der Merwe, L., Lombard, Z., Viljoen, D., and Ramsay, M. (2015). Reduced DNA methylation at the PEG3 DMR and KvDMR1 loci in children exposed to alcohol in utero: a South African Fetal Alcohol Syndrome cohort study. *Front. Genet.* 6:85. doi: 10.3389/fgene.2015.00085

McGovern, P. E., Zhang, J., Tang, J., Zhang, Z., Hall, G. R., Moreau, R. A., et al. (2004). Fermented beverages of pre- and proto-historic China. *Proc. Natl. Acad. Sci. U.S.A.* 101, 17593–17598. doi: 10.1073/pnas.0407921102

Mead, E. A., and Sarkar, D. K. (2014). Fetal alcohol spectrum disorders and their transmission through genetic and epigenetic mechanisms. *Front. Genet*. 5:154. doi: 10.3389/fgene.2014.00154

Ogawa, T., Kuwagata, M., Ruiz, J., and Zhou, F. C. (2005). Differential teratogenic effect of alcohol on embryonic development between C57BL/6 and DBA/2 mice: a new view. *Alcohol. Clin. Exp. Res*. 29, 855–863. doi: 10.1097/01.ALC.0000163495.71181.10

Resendiz, M., Lo, C.-L. S., Badin, J. K., and Zhou, F. C. (2014a). "Alcohol metabolism and epigenetic methylation and acetylation," in *Molecular Aspects of Alcohol and Nutrition, 1st Edn*, ed V. Patel (Elsevier).

Resendiz, M., Mason, S., Lo, C.-L., and Zhou, F. C. (2014b). Epigenetic regulation of the neural transcriptome and alcohol interference during development. *Front. Genet*. 5:285. doi: 10.3389/fgene.2014.00285

Singh, S. M., Laufer, B. I., and Kapalanga, J. (2014). Fetal alcohol and the right to be born healthy. *Front. Genet*. 5:356. doi: 10.3389/fgene.2014.00356

Smith, S. M., Garic, A., Berres, M. E., and Flentke, G. R. (2014). Genomic factors that shape craniofacial outcome and neural crest vulnerability in FASD. *Front. Genet.* 5:224. doi: 10.3389/fgene.2014.00224

Tunc-Ozcan, E., Sittig, L. J., Harper, K. M., Graf, E. N., and Redei, E. E. (2014). Hypothesis: genetic and epigenetic risk factors interact to modulate vulnerability and resilience to FASD. *Front. Genet*. 5:261. doi: 10.3389/fgene.2014.00261

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OPINION ARTICLE
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Fetal alcohol and the right to be born healthy...

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Keywords: teratogen, spectrum disorders, neurodevelopment, neuroepigenomics, pregnancy, exposure, environment, fetal alcohol syndrome

Finding the cause and applying the insights toward prevention and treatment forms the ultimate goal of most disease research. This strategy has been successfully used to make diseases like Scurvy and Smallpox, a history. The impact of this research during the last few years has been nothing less than miraculous. More and more people are living longer with healthy and productive lives, well into the 80's and 90's. Discovery of the cause(s) of disease(s) however is a demanding, time consuming, and expensive exercise. Also, there is no guarantee for success. Yet, even the modest success in search for causes have the potential to change the outcome and perception. Early diagnosis of a number of cancers for example is now viewed as treatable with reasonable chance of recovery. Also, some heart diseases are being managed and treated with high rate of success. Given this record of success, the research on disease causations continues to increase and the results have begun to pay increasing dividend. Unfortunately, there are cases of diseases where even full understanding of the cause has not resulted in the prevention or treatment of some common and devastating diseases. One such disease is the fetal alcohol spectrum disorder (FASD).

FASD is caused by the exposure of developing fetus to alcohol via maternal drinking during pregnancy (Jones and Smith, 1973). It represents the biggest single cause of mental retardation and developmental disabilities among babies born in the Western World (Barry et al., 2009). In the U.S. more than 50,000 babies are born with FASD every year (May and Gossage, 2001) and the annual cost of treating FASD in Canada and U.S. exceeds $6 and $8 billions, respectively (Lupton et al., 2004; Popova et al., 2013). Although, the prevention of FASD is a high priority, the failure to prevent it is attributed to our alcohol culture. Most people drink for social and recreational purposes. Others are addicted to alcohol.

As it stands, there is no consensus on whether there is a "safe" limit for alcohol consumptions during pregnancy. Recent research involving animal (mice) models has shown that continuous exposure of low-to-moderate dose of alcohol during pregnancy impacts behavioral and cognitive outcomes of resulting pups (Kleiber et al., 2011) and even a single binge dose of alcohol at any time during pregnancy results in alterations in gene expression (Kleiber et al., 2012, 2013) and associated FASD related phenotypes. Furthermore, the molecular alterations may be initiated and maintained for life by alcohol's effect on epigenetic features that includes DNA methylation (Laufer et al., 2013). The results on animal models argue that clinical features of FASD represent "tip of the iceberg." They are also backed by results on humans. For example, exposure of human embryonic stem cells to low alcohol can alter gene expression leading to the abnormal development of prefrontal cortex (Krishnamoorthy et al., 2010). Also, fetal alcohol exposed school children show "a small but potentially important detrimental effect" on educational outcomes (Zuccolo et al., 2013) as well as generalized deficit of conceptualization (Quattlebaum and O'Connor, 2013).

We feel that such results deserve due consideration given that Royal College of Obstetrics and Gynecologists (Royal College of Obstetrician and Gynaecology, 2006) states that, "there is no evidence of harm from low levels of alcohol consumption, defined as no more than one or two units of alcohol once or twice a week." Also, "there is considerable doubt as to whether infrequent and low level of alcohol consumption during pregnancy convey any long-term harm"—in other words they suggest a safe amount of alcohol consumption in pregnancy. Unfortunately, this limit has not been defined and may vary from individual to individual. Individual women process alcohol differently. Also, the age of the mother, the timing and regularity of the alcohol ingestion, and whether the mother has eaten any food while drinking may be important. We argue that there is no logistic evidence to define this limit. What is needed is to undertake thorough studies on neurodevelopment and assess the significance of such factors as maternal and fetal genotype, stress during pregnancy and childbirth, prenatal drinking patterns (mild, medium, heavy), post-natal environment, and socioeconomic status, as most of these may contribute to the manifestation of the effect of prenatal alcohol on the newborn. We note that some of these studies will be problematic if not impossible on humans. The rational question is "does no evidence of harm from low levels of alcohol consumption means 100% exclusion of the possibility of any harm to the fetus?" To the best of our understanding the answer is "no."

The issue is particularly problematic as there is a rise in heavy drinking by young people, particularly women. Often, it is

framed, as freedom of choice or "a single drink will not harm." Not surprisingly, 1 in 8 adult women and 1 in 5 high school girls binge drink (CDC, 2013) and there is ample evidence from animal experiments, which argue for a life-long effect of even a single exposure of alcohol during pregnancy. The developing brain is a sequential, multistage, closely orchestrated, and highly sensitive to stresses. Also, any aberration could lead to life-long abnormality. For now, it is prudent to prevent a brain disorder than to attempt to ameliorate or cure it. Preventing a single case of FASD will save the society $1 million. More importantly it will save a productive life. The business as usual model is not helpful. It continues to result in births with alcohol effects. Any harm caused by prenatal alcohol is currently not reversible. It will affect the child for life.

With the current knowledge of what causes FASD it is prudent to stay on the safe side and avoid any drinking during and around the pregnancy. FASD is an alcohol problem. It is possible to prevent this calamity by avoiding alcohol during pregnancy and the time is now! FASD is a preventable disease—by not drinking during pregnancy. On the other hand, finding "cure" will be much more challenging, costly and time taking. It is critical to undertake active measures to reduce the occurrence of this disorder by a message of "no alcohol dose is guaranteed to be 100% safe for the embryo/fetus." Also, "no time during pregnancy is 100% safe to drink." Any adult has the right to drink if they so wish. Also, every child has the right to be born healthy!

REFERENCES

Barry, K. L., Caetano, R., Chang, G., DeJoseph, M. C., Miller, L. A., O'Connor, M. J., et al. (2009). *Reducing Alcohol- Exposed Pregnancies: A Report of the National Task Force on Fetal Alcohol Syndrome and Fetal Alcohol Effect*. Atlanta, GA: Centers for Disease Control and Prevention.

CDC. (2013). Vital signs: binge drinking among women and high school girls—United States, 2011. *MMWR Morb. Mortal. Wkly. Rep.* 62, 9–13. Available online at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6201a3.htm

Jones, K. L., and Smith, D. W. (1973). Recognition of the fetal alcohol syndrome in early infancy. *Lancet* 302, 999–1001. doi: 10.1016/S0140-6736(73)91092-1

Kleiber, M. L., Laufer, B. I., Wright, E., Diehl, E. J., and Singh, S. M. (2012). Long-term alterations to the brain transcriptome in a maternal voluntary consumption model of fetal alcohol spectrum disorders. *Brain Res.* 1458, 18–33. doi: 10.1016/j.brainres.2012.04.016

Kleiber, M. L., Mantha, K., Stringer, R. L., and Singh, S. M. (2013). Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure. *J. Neurodev. Disord.* 5:6. doi: 10.1186/1866-1955-5-6

Kleiber, M. L., Wright, E., and Singh, S. M. (2011). Maternal alcohol drinking in C57BL/6J mice: advancing a model for fetal alcohol spectrum disorder. *Behav. Brain Res.* 223, 376–387. doi: 10.1016/j.bbr.2011.05.005

Krishnamoorthy, M., Gerwe, B. A., Scharer, C. D., Heimburg-Molinaro, J., Gregory, F., Nash, R. J., et al. (2010). Low ethanol concentration alters CHRNA5 RNA levels during early human development. *Reprod. Toxicol.* 30, 489–492. doi: 10.1016/j.reprotox.2010.04.011

Laufer, B. I., Mantha, K., Kleiber, M. L., Diehl, E. J., Addison, S. M., and Singh, S. M. (2013). Long lasting alterations to DNA methylation and ncRNAs could underlie the effect of fetal alcohol exposure in mice. *Dis. Model. Mech.* 6, 977–992. doi: 10.1242/dmm.010975

Lupton, C., Burd, L., and Harwood, R. (2004). Cost of fetal alcohol spectrum disorders. *Am. J. Med. Genet. C Semin. Med. Genet.* 127C, 42–50. doi: 10.1002/ajmg.c.30015

May, P. A., and Gossage, J. P. (2001). Estimating the prevalence of fetal alcohol syndrome - a summary. *Alcohol Res. Health* 25, 159–167. Available online at: http://pubs.niaaa.nih.gov/publications/arh25-3/159-167.htm

Popova, S., Lange, S., Burd, L., Chudley, A. E., Clarren, S. K., and Rehm, J. (2013). Cost of fetal alcohol spectrum disorder diagnosis in Canada. *PLoS ONE* 8:e60434. doi: 10.1371/journal.pone.0060434

Quattlebaum, J. L., and O'Connor, M. J. (2013). Higher functioning children with prenatal alcohol exposure: is there a specific neurocognitive profile? *Child Neuropsychol.* 19, 561–578. doi: 10.1080/09297049.2012.713466

Royal College of Obstetrician and Gynaecology. (2006). *Alcohol Consumption and the Outcome of Pregnancy.* London, UK: ROGC Statement No 5.

Zuccolo, L., Lewis, S. J., Smith, G. D., Sayal, K., Draper, E. S., Fraser, R., et al. (2013). Prenatal alcohol exposure and offspring cognition and school performance. A mendelian randomization natural experiment. *Int. J. Epidemiol.* 42, 1358–1370. doi: 10.1093/ije/dyt172

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Embryo transfers between C57BL/6J and DBA/2J mice: Examination of a maternal effect on ethanol teratogenesis

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Genetic factors influence fetal alcohol spectrum disorders (FASDs) in both humans and animals. Experiments using inbred and selectively bred mouse stocks that controlled for (1) ethanol dose, (2) maternal and fetal blood ethanol levels, and (3) fetal developmental exposure stage, show genotype can affect teratogenic outcome. Other experiments distinguish the teratogenic effects mediated by maternal genotype from those mediated by fetal genotype. One technique to distinguish maternal versus fetal genotype effect is to utilize embryo transfers. This study is the first to examine ethanol teratogenesis – fetal weight deficits and mortality, and digit, kidney, and vertebral malformations – in C57BL/6J (B6) and DBA/2J (D2) fetuses that were transferred as blastocysts into B6 and D2 dams. We hypothesized that, following maternal alcohol exposure, B6 and D2 fetuses gestating within B6 mothers, as compared to D2 mothers, will exhibit a higher frequency of malformations. On day 9 of pregnancy, females were intubated (IG) with either 5.8 g/kg ethanol (E) or maltose-dextrin (MD). Other females were mated within strain and treated with either ethanol or maltose, or were not exposed to either treatment. Implantation rates were affected by genotype. Results show more B6 embryos implanted into D2 females than B6 females ($p < 0.05$; 47% vs. 23%, respectively). There was no difference in the percentage of D2 embryos implanting into B6 and D2 females (14 and 16%, respectfully). Litter mortality averaged 24% across all experimental groups. Overall, *in utero* ethanol exposure reduced mean litter weight compared to maltose treatment ($E = 1.01$ g; MD = 1.19 g; $p < 0.05$); but maltose exposed litters with transferred embryos weighed more than similarly treated natural litters (1.30 g vs. 1.11 g; $p < 0.05$). Approximately 50% of all ethanol exposed B6 fetuses exhibited some malformation (digit, vertebral, and/or kidney) regardless of whether they were transferred into a B6 or D2 female, or were naturally conceived. This suggests the D2 maternal uterine environment did not offer any protection against ethanol teratogenesis for B6 fetuses. One of the questions remaining is the how the B6 uterine environment affects D2 teratogenesis. No definitive conclusions can be drawn because too few viable D2 litters were produced.

Keywords: fetal alcohol spectrum disorders/genetics, mice, inbred strains, teratogenesis, embryo transfer

INTRODUCTION

Women who drink alcohol while pregnant risk having children with congenital malformations (Jones and Smith, 1973, 1975). In the most extreme cases Fetal Alcohol Syndrome (FAS) may be diagnosed. The syndrome is hallmarked by pre- and postnatal growth retardation, craniofacial abnormalities, and central nervous system dysfunction including behavioral abnormalities. The designation fetal alcohol spectrum disorder (FASD) is now used as an umbrella term covering all outcomes associated with prenatal alcohol exposure. Not all children exposed to alcohol *in utero* display FASD characteristics. This indicates individual differences (both maternal and fetal) in ethanol teratogenesis susceptibility. Many risk factors play a role in FASD development and several studies point to genetic differences in susceptibility.

Mice are useful in studying FASD. All hallmark features of FASD can be replicated in mice. Experiments using inbred and selectively bred mouse stocks show genotype can affect teratogenic outcome (Warren and Li, 2005). The deleterious outcomes under genetic control include differing levels of embryo lethality; brain morphology; fetal weight gain; and digit, skeletal, ocular, renal, and heart anomalies; and behavioral anomalies (Gilliam et al., 1987, 1989; Goodlett et al., 1989; Gilliam and Kotch, 1990, 1992, 1996; Boehm et al., 1997).

Some teratogenic effects are mediated by maternal genotype and may be distinguished from those mediated by fetal genotype (Gilliam and Irtenkauf, 1990; Gilliam et al., 1997, 2011; Downing and Gilliam, 1999). Reciprocal breeding between distinct mouse stocks identify these distinctions. A maternal effect is indicated when genetically identical heterozygous offspring differ in responses based on which homozygous mouse stock is used as the mother in the reciprocal cross (Biddle and Fraser, 1977). If the difference is limited to male offspring or observed at a higher rate in males, the maternal effect may be attributed to X-linked genes. When the difference is not male-specific, maternal

effects are presumably due to cytoplasmic inheritance, maternal physiology, or epigenetic phenomena.

When C57BL/6J (B6) mice are crossed with any other mouse stock, having a B6 mother significantly increases malformation frequency compared to not having a B6 mother (Gilliam and Irtenkauf, 1990; Gilliam et al., 1997, 2011; Downing and Gilliam, 1999). In one study (Downing and Gilliam, 1999) we found a maternal genetic effect on vertebral malformations in reciprocal crosses of B6 and DBA/2J (D2) mice following alcohol exposure. Furthermore, the source of the maternal effect could not be ascribed to sex-linked genes or factors transmitted through the egg cytoplasm. Two other factors may account for a maternal effect. They are maternal uterine environment or epigenetic phenomena. In the present study we used embryo transfers to examine the independent effect of maternal uterine environment on ethanol teratogenesis. Genetically identical embryos can be implanted into pseudo-pregnant recipient females with differing genotypes. If treatment effects on these offspring differ, the difference can be attributed to maternal genotype. Conversely, embryos of differing genotype can be implanted into genetically identical recipient females. If treatment effects on these offspring differ, it can be attributed to fetal genotype. Because we previously ruled out sex-linked genes and cytoplasmic factors accounting for the maternal effect in crosses of B6 and D2 mice, we hypothesized that the B6 uterine environment is responsible for increased vertebral malformation in D2 fetuses. This is the first study to examine how maternal uterine environment affects ethanol teratogenesis using embryo transfers.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND EMBRYO TRANSFER PROCEDURES

Experimental animals were B6 and D2 mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a normal light/dark cycle (lights on at 0700 h). All embryo transfers were completed by James Gross, Mouse Genetics Core Facility, National Jewish Health (NJH), 1400 Jackson St., Denver, CO 80206, USA. Seven or eight 3.5-day blastocysts of the same genotype were obtained from approximately 10-week old donor females. These were implanted into each oviduct of pseudo-pregnant recipient 10-week old recipient females (2.5 days *post coitus*). By design, donor and recipient female ages were consistent with those used in our previous studies (Boehm et al., 1997; Downing and Gilliam, 1999). Subjects recovered from surgery at NJH and were transported to the Animal Facility at the University of Northern Colorado (UNCO) on day 7 of pregnancy.

EXPERIMENTAL TREATMENTS

Between 1230 and 1330 h on day 9 of pregnancy, females were intragastrically intubated with either 5.8 g/kg ethanol (20% w/v) or an isocaloric amount of a maltose-dextrin solution (35% w/v). Naturally conceived (no embryo transplant) fetal groups were established by mating females with same-strain males. These females were treated with either ethanol or maltose-dextrin as described above. Three B6 females mated to a B6 male were inadvertently not intubated. They remained in the experiment as an unexposed control group.

TERATOLOGICAL ASSESSMENT

On day 18 of pregnancy females were killed by CO_2 inhalation and necropsied between 1400 and 1700 h. Uterine horns were exposed and a count made of live and resorbed fetuses. Live fetuses were sexed, weighed, and prepared for either skeletal or soft tissue examination, as previously described Gilliam et al. (2011). All live fetuses were examined for digit malformations (ectrodactyly, syndactyly). Approximately half of each litter was examined for vertebral malformations (missing or fused vertebral arches and/or centra), while the remaining half was examined for kidney malformations (hydronephrosis, missing kidney). Examination of uterine horn contents and fetal malformations was conducted by the same person (DG), who was blind to prenatal treatment and transferred embryo genotype.

PROTOCOL APPROVAL

Experimental procedures were approved by the Institutional Animal Care and Use Committees of the University of Northern Colorado (Protocol 239-01) and National Jewish Health (Protocol AS2788-05-11). All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al., 2011].

STATISTICAL ANALYSES

Mean litter weight and percent litter mortality were examined by ANOVA with implant status (naturally conceived and transferred), maternal genotype (B6 and D2), fetal genotype (B6 and D2), and prenatal treatment (maltose, ethanol, and non-intubated) as grouping factors. The proportion of successful implantations was calculated. The total number of embryos successfully implanted was divided by the total number transferred. Successful-implantation proportions for the same embryo genotype (either B6 or D2) were compared between maternal genotypes (B6 vs. D2) using the z-test for comparing two proportions, as described by Bluman (2012). Since ethanol or maltose-dextrin treatments were given 2–3 days after embryos normally achieve uterine implantation, treatment effects on implantation success were not examined. Comparisons of malformations were conducted using the Fisher's Exact Test Calculator for 2 × 2 Contingency Tables at: http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/fisherexacttest/.

RESULTS

TRANSFER SUCCESS RATE

A total of 294 B6 blastocysts and 196 D2 blastocysts were transferred to B6 or D2 oviducts (**Table 1**). More B6 embryos successfully implanted into D2 dams than into B6 dams (z-test for comparing two proportions $= -4.29$, $p < 0.05$). In contrast, there were no differences in the proportion of D2 embryos that successfully implanted into D2 or B6 dams ($z = 0.27$; $p > 0.05$). Teratogenic effects are often calculated on a per litter basis, therefore only litters with more than one implantation site are typically included in tallying fetal mortality and/or malformations. **Table 1** includes both the number of successful implants and proportions of litters with more than one successful implant. Verification

Table 1 | Embryo transfer and implantation success rates.

	B6 Dams[a]				D2 Dams[b]			
	Total blastocysts transferred	**Successful implants**	**Total litters**	**Total litters with > 1 implant**	**Total blastocysts transferred**	**Successful implants**	**Total litters**	**Total litters with > 1 implant**
B6 Embryos	150	35 (23.3%)	10*	6	144	68 (47.2%)	9	7
D2 Embryos	126	18 (14.3%)	9	2	70	11 (15.7%)	5*	3

[a]*Three B6 dams had zero B6 implants and seven had zero D2 implants.*
[b]*Two D2 dams had zero B6 implants and one had zero D2 implants.*
**One B6 litter had only one successful B6 embryo implantation and one D2 litter had only one successful D2 embryo implantation.*

Table 2 | Mean percent litter mortality (±SEM).

	B6 Transferred		D2 Transferred		B6 Natural			D2 Natural	
	Maltose ($n = 3$)	**Ethanol ($n = 4$)**	**Maltose**	**Ethanol ($n = 2$)**	**Unexposed ($n = 3$)**	**Maltose ($n = 7$)**	**Ethanol ($n = 8$)**	**Maltose ($n = 2$)**	**Ethanol ($n = 3$)**
B6 Dam	33.3 (16.7)	39.4 (16.3)	NA	44.4 (0)	11.1 (11.1)	20.2 (8.2)	10.1 (3.9)	22.7 (22.7)	14.3 (14.3)
	Maltose ($n = 2$)	**Ethanol ($n = 5$)**	**Maltose ($n = 2$)**	**Ethanol ($n = 2$)**					
D2 Dam	43.7 (6.2)	44.9 (14.5)	0	12.5 (12.5)					

NA – data not available. Of the 9 B6 dams implanted with D2 embryos, three were treated with maltose, but none had any implants or live pups.

of zero implantations was made by compressing uterine horns between two microscope slides.

LITTER MORTALITY

Litter mortality data were calculated by tabulating early and late resorptions, as differentiated by Gleich and Frohberg (1977), and dividing by total implantations. Average percent litter mortality for each treatment and genotype combination is shown in **Table 2**. Mortality tended to be higher for litters with transferred embryos (34%) than for naturally conceived litters (15%). However, analysis (ANOVA) of litter mortality indicated no effects of pregnancy type (transferred vs. natural), maternal genotype, fetal genotype, or treatment, nor were there any interactions.

LITTER WEIGHT

A total of 43 litters had one or more live pups (see **Table 3** for n's). Each pup was weighed on an electronic balance to 0.001 g. Treatment effects on average litter weight for transferred embryos and naturally conceived litters (within strain mating) for each genotype combination are shown in **Table 3**.

Analysis of mean litter weight showed a main effect of treatment [ethanol < maltose; $F(2,42) = 4.58$, $p < 0.05$] and an interaction between pregnancy type (transferred vs. natural) and treatment [ethanol vs. maltose; $F(1,42) = 4.48$, $p < 0.05$]. Litter weight for both transferred and natural litters was similarly decreased by ethanol exposure. In contrast, maltose exposed litters with transferred embryos weighed more than similarly treated natural litters ($p < 0.05$; **Figure 1**).

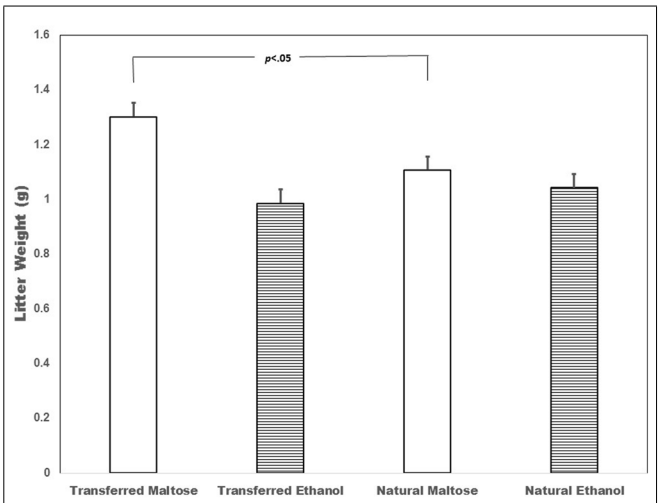

FIGURE 1 | Fetal weight (grams) was similarly affected by ethanol treatment in both transferred and naturally conceived fetuses ($p > 0.05$), while maltose-exposed fetuses resulting from embryo transfers weighed more than similarly treated naturally conceived fetuses ($p < 0.05$).

Table 3 | Mean litter weight in grams (±SEM).

	B6 Transferred		D2 Transferred		B6 Natural			D2 Natural	
	Maltose ($n = 3$)	**Ethanol ($n = 4$*)**	**Maltose**	**Ethanol ($n = 2$)**	**Unexposed ($n = 3$)**	**Maltose ($n = 7$)**	**Ethanol ($n = 8$)**	**Maltose ($n = 2$)**	**Ethanol ($n = 3$)**
B6 Dam	1.333 (0.098)	1.064 (0.085)	NA	0.830 (0.078)	1.205 (0.027)	1.171 (0.039)	1.064 (0.037)	0.884 (0.021)	0.984 (0.090)
	Maltose ($n = 2$)	**Ethanol ($n = 5$*)**	**Maltose ($n = 2$)**	**Ethanol ($n = 2$)**					
D2 Dam	1.292 (0.001)	0.954 (0.052)	1.267 (0.060)	1.061 (0.270)					

NA – data not available. Of the 9 B6 dams implanted with D2 embryos, three were treated with maltose, but none had any implants or live pups.
**Includes one litter with more than one implant but only one live pup.*

Table 4 | Digit, kidney, and vertebral malformations.

		B6 Transferred		D2 Transferred		B6 Natural			D2 Natural	
		Maltose	**Ethanol**	**Maltose**	**Ethanol**	**Unexposed**	**Maltose**	**Ethanol**	**Maltose**	**Ethanol**
B6 Dam	Digit	0/6	9/18	NA	0/10	0/25	0/42	20/69	0/11	0/16
	Kidney	0/1	1/5	NA	1/4	0/13	2/24	9/38	1/5	2/9
	Vertebral	0/5	2/13	NA	4/6	0/12	0/28	7/31	1/6	0/7
	ANY	0/6	9/18		5/10	0/25	2/42	30/69	2/11	2/16
D2 Dam	Digit	0/7	10/33	0/6	0/4					
	Kidney	0/3	5/15	0/3	1/1					
	Vertebral	0/4	5/18	0/3	0/3					
	ANY	0/7	16/33	0/6	1/4					

Table numbers represent number of fetuses with a malformation (numerator) divided by total number of fetuses examined (denominator).
NA – data not available. Of the 9 B6 dams implanted with D2 embryos, three were treated with maltose, but none had any implants or live pups.

FETAL MALFORMATIONS

Malformations were tabulated for digits, vertebra, and kidneys and are shown in **Table 4**. As expected, ethanol exposed B6 fetuses exhibited more malformations than ethanol exposed D2 fetuses (Fisher's exact test, $p = 0.06$) regardless of implantation status (naturally conceived or transferred). In fact, approximately 50% of all ethanol exposed B6 fetuses had some malformation. Importantly, malformation frequency for B6 fetuses did not depend on whether they were naturally conceived or transferred into D2 or B6 dams. This suggests the D2 uterine environment does not provide B6 fetuses protection from *in utero* alcohol exposure, meaning no maternal genotype effect. In contrast to B6 fetuses, ethanol exposed D2 fetuses showed very few malformations. The one exception was ethanol exposed D2 fetuses transferred to B6 dams; 50% had some malformation. In particular, *in utero* ethanol increased vertebral malformations from 0% in D2 fetuses transferred to D2 dams to 67% in D2 fetuses transferred to B6 dams. This suggests the B6 uterine environment may increase malformation frequency in D2 fetuses, a maternal genotype effect. However, given the low numbers of ethanol exposed D2 embryos, malformation frequency was not significantly different from other ethanol exposed D2 groups.

DISCUSSION

These results suggest no protective effect of the D2 uterine environment on ethanol teratogenesis. However there is some evidence to suggest the B6 uterine environment can increase susceptibility. But the lack of power due to genotype-dependent implantation failure limits the reliability of this finding. Vertebral malformations were increased in D2 fetuses transferred to B6 dams, but this result was not statistically significant because of too few viable D2 litters. *In utero* alcohol exposure produced similar malformation frequencies among B6 fetuses regardless of whether embryos were transferred into a B6 or D2 dam or they resulted from natural mating. This suggests ethanol exposure occurred during the same critical period of development among all B6 fetal groups. That is, the transfer procedure did not change a key developmental stage sensitive to digit, vertebral, and kidney malformations. Only fifteen percent of D2 blastocysts successfully implanted and only 35% of B6 blastocysts successfully implanted. A similar difference was observed in the percentage of viable pups produced

when D2 or B6 blastocysts were implanted into pseudo-pregnant CByB6F1/J mice (Byers et al., 2006). Viable pup percentages were 25% for D2 implants but 53% for B6 implants. To assure meaningful comparisons on sample sizes, future studies should at least quadruple the number of litters with transferred D2 blastocysts and double the number of litters with transferred B6 blastocysts. Also, a focus on only skeletal (vertebral) malformations and not soft-tissue (kidney) malformations would increase sample size. A maternal effect for vertebral malformations and not kidney malformations was previously observed (Downing and Gilliam, 1999).

Susceptibility to specific ethanol teratogenic effects in mice appear to be due to both fetal and maternal genetic influences. By making assumptions about additive and dominance genetic effects, variation in total malformation frequency can be partitioned into what is due to embryonic genotype and what is due to maternal genotype. Interestingly, when maternal genetic influences are observed they account for more than half of the total malformation frequency (Gilliam and Irtenkauf, 1990; Gilliam et al., 1997, 2011; Downing and Gilliam, 1999). Recently, using stocks related to B6 and D2 mice, Lossie et al. (2014) identified specific genes showing up-regulation in B6 embryos but down-regulation in D2 embryos after ethanol exposure. These findings are consistent with others when using embryo cultures (Ogawa et al., 2005; Chen et al., 2011). Determining how these genes are differentially regulated by maternal factors would shed light on the maternal contribution to ethanol teratogenesis. Uncovering the reasons for a maternal effect in mice may lead to a better understanding of why only certain babies have FAS or FASD.

Because of the increased vertebral malformations observed in ethanol exposed D2 embryos transferred to B6 dams, it is tempting to speculate that the B6 uterine environment somehow changed fetal D2 gene expression. In support of this hypothesis, Downing et al. (2012) measured gene expression changes following *in utero* alcohol exposure in four embryonic genotypes: true-bred B6 and D2, and reciprocally bred B6D2 and D2B6. The mating period, time of intubation, and ethanol and maltose dosages were all nearly identical to those used in the present study. Of specific interest was the finding that 22 genes were differentially expressed in B6D2 embryos gestating within B6 dams but not in genetically identical D2B6 embryos gestating within D2 dams following *in utero* alcohol exposure. B6D2 fetuses also show significantly higher rates of ethanol teratogenesis than do D2B6 fetuses (Downing and Gilliam, 1999). These results implicate the B6 uterine environment as increasing FASD risk. These effects could also be due to changes in fetal and/or placental genomic imprinting status (Nelissen et al., 2011). The interplay between maternal genetic factors and fetal genetic factors that increase FASD susceptibility need further study to unravel the underlying mechanisms responsible.

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REFERENCES

Biddle, F. G., and Fraser, F. C. (1977). "Maternal and cytoplasmic effects in experimental teratology," in *Handbook of Teratology*, Vol. 3, eds J. G. Wilson and F. C. Fraser (New York, NY: Plenum Press), 3–33.

Bluman, A. G. (2012). *Elementary Statistics: A Step by Step Approach.* New York: McGraw-Hill.

Boehm, S. L. II, Lundahl, K. R., Caldwell, J., and Gilliam, D. M. (1997). Ethanol teratogenesis in the C57BL/6J, DBA/2J, and A/J inbred mouse strains. *Alcohol* 14, 389–395. doi: 10.1016/S0741-8329(97)87950-5

Byers, S. L., Payson, S. J., and Taft, R. A. (2006). Performance of ten inbred mouse strains following assisted reproductive technologies (ARTs). *Theriogenology* 65, 1716–1726. doi: 10.1016/j.theriogenology.2005.09.016

Chen, Y., Ozturk, N. C., Ni, L., Goodlett, C., and Zhou, F. C. (2011). Strain differences in developmental vulnerability to alcohol exposure via embryo culture in mice. *Alcohol. Clin. Exp. Res.* 35, 1293–1304. doi: 10.1111/j.1530-0277.2011.01465.x

Downing, C., Flink, S., Florez-Mcclure, M. L., Johnson, T. E., Tabakoff, B., and Kechris, K. J. (2012). Gene expression changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 36, 1519–1529. doi: 10.1111/j.1530-0277.2012.01757.x

Downing, C., and Gilliam, D. (1999). Cytoplasmic factors do not contribute to a maternal effect on ethanol teratogenesis. *Behav. Genet.* 29, 31–39. doi: 10.1023/A:1021485821842

Gilliam, D. M., and Irtenkauf, K. T. (1990). Maternal genetic effects on ethanol teratogenesis and dominance of relative embryonic resistance to malformations. *Alcohol. Clin. Exp. Res.* 14, 539–545. doi: 10.1111/j.1530-0277.1990.tb01196.x

Gilliam, D. M., and Kotch, L. E. (1990). Alcohol-related birth defects in long- and short-sleep mice: postnatal litter mortality. *Alcohol* 7, 483–487. doi: 10.1016/0741-8329(90)90036-C

Gilliam, D. M., and Kotch, L. E. (1992). Developmental thermoregulatory deficits in prenatal ethanol exposed long- and short-sleep mice. *Dev. Psychobiol.* 25, 365–373. doi: 10.1002/dev.420250507

Gilliam, D. M., and Kotch, L. E. (1996). Dose-related growth deficits in LS but not SS mice prenatally exposed to alcohol. *Alcohol* 13, 47–51. doi: 10.1016/0741-8329(95)02010-1

Gilliam, D. M., Kotch, L. E., Dudek, B. C., and Riley, E. P. (1989). Ethanol teratogenesis in selectivity bred long-sleep and short-sleep mice: a comparison to inbred C57BL/6J mice. *Alcohol. Clin. Exp. Res.* 13, 667–672. doi: 10.1111/j.1530-0277.1989.tb00402.x

Gilliam, D. M., Mantle, M. A., Barkhausen, D. A., and Tweden, D. R. (1997). Effects of acute prenatal ethanol administration in a reciprocal cross of C57BL/6J and short-sleep mice: maternal effects and nonmaternal factors. *Alcohol. Clin. Exp. Res.* 21, 28–34. doi: 10.1097/00000374-199702000-00004

Gilliam, D. M., Stilman, A., Dudek, B. C., and Riley, E. P. (1987). Fetal alcohol effects in long- and short-sleep mice: activity, passive avoidance, and in utero ethanol levels. *Neurotoxicol. Teratol.* 9, 349–357. doi: 10.1016/0892-0362(87)90030-4

Gilliam, D., Valdez, N., Branson, S., Dixon, A., and Downing, C. (2011). Maternal effects on ethanol teratogenesis in a cross between A/J and C57BL/6J mice. *Alcohol* 45, 441–449. doi: 10.1016/j.alcohol.2011.02.308

Gleich, J., and Frohberg, H. (1977). "General teratological techniques," in *Methods in Prenatal Toxicology*, eds D. Neubert, H. J. Merker, and T. E. Kwasigroch (Stuttgart: Georg Thieme).

Goodlett, C. R., Gilliam, D. M., Nichols, J. M., and West, J. R. (1989). Genetic influences on brain growth restriction induced by development exposure to alcohol. *Neurotoxicology* 10, 321–334.

Jones, K. L., and Smith, D. W. (1973). Recognition of the fetal alcohol syndrome in early infancy. *Lancet* 2, 999–1001. doi: 10.1016/S0140-6736(73)91092-1

Jones, K. L., and Smith, D. W. (1975). The fetal alcohol syndrome. *Teratology* 12, 1–10. doi: 10.1002/tera.1420120102

Lossie, A. C., Muir, W., Lo, C.-L., Timm, F., Liu, Y., Gray, W., et al. (2014). Implications of genomic signatures in the differential vulnerability to fetal alcohol exposure in C57BL/6 and DBA/2 mice. *Front. Gen.* 5:173 doi: 10.3389/fgene.2014.00173

National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al. (2011). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academies Press.

Nelissen, E. C. M., Van Montfoort, A. P. A., Dumoulin, J. C. M., and Evers, J. L. H. (2011). Epigenetics and the placenta. *Hum. Reprod. Update* 17, 397–417. doi: 10.1093/humupd/dmq052

Ogawa, T., Kuwagata, M., Ruiz, J., and Zhou, F. C. (2005). Differential teratogenic effect of alcohol on embryonic development between C57BL/6 and DBA/2 Mice: a new view. *Alcohol. Clin. Exp. Res.* 29, 855–863. doi: 10.1097/01.ALC.0000163495.71181.10

Warren, K. R., and Li, T.-K. (2005). Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Res. A Clin. Mol. Teratol.* 73, 195–203. doi: 10.1002/bdra.20125

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Implications of genomic signatures in the differential vulnerability to fetal alcohol exposure in C57BL/6 and DBA/2 mice

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Maternal alcohol consumption inflicts a multitude of phenotypic consequences that range from undetectable changes to severe dysmorphology. Using tightly controlled murine studies that deliver precise amounts of alcohol at discrete developmental stages, our group and other labs demonstrated in prior studies that the C57BL/6 and DBA/2 inbred mouse strains display differential susceptibility to the teratogenic effects of alcohol. Since the phenotypic diversity extends beyond the amount, dosage and timing of alcohol exposure, it is likely that an individual's genetic background contributes to the phenotypic spectrum. To identify the genomic signatures associated with these observed differences in alcohol-induced dysmorphology, we conducted a microarray-based transcriptome study that also interrogated the genomic signatures between these two lines based on genetic background and alcohol exposure. This approach is called a gene x environment (GxE) analysis; one example of a GxE interaction would be a gene whose expression level increases in C57BL/6, but decreases in DBA/2 embryos, following alcohol exposure. We identified 35 candidate genes exhibiting GxE interactions. To identify *cis*-acting factors that mediated these interactions, we interrogated the proximal promoters of these 35 candidates and found 241 single nucleotide variants (SNVs) in 16 promoters. Further investigation indicated that 186 SNVs (15 promoters) are predicted to alter transcription factor binding. In addition, 62 SNVs created, removed or altered the placement of a CpG dinucleotide in 13 of the proximal promoters, 53 of which overlapped putative transcription factor binding sites. These 53 SNVs are also our top candidates for future studies aimed at examining the effects of alcohol on epigenetic gene regulation.

Keywords: fetal alcohol syndrome, gene x environment interactions, genomics, gene expression, next generation sequencing, genetic association, epigenetics

INTRODUCTION

Women who drink during pregnancy place their unborn children at risk of acquiring clinical features of fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorder (FASD). FASD encompasses all patients displaying some of the clinical features of fetal alcohol exposure and is much more prevalent than FAS. These syndromes comprise an array of phenotypes that include: cognitive deficits, intrauterine and post-natal growth retardation, memory deficits, poor motor skills, facial dysmorphology and social/behavioral problems. On one end of the spectrum (mild FASD; ∼1 in 100 live births), children present with mild to moderate mental deficits that are difficult to diagnose by appearance, while those on the other end (FAS; ∼1 in 1000 live births) exhibit a readily identifiable facial dysmorphology coupled with a complement of severe neuropsychological sequelae and neurobehavioral deficits (Abel, 1995; Stratton et al., 1996; Sampson et al., 1997; Jacobson, 1998; Astley et al., 2002; Niwa et al., 2002; Hoyme et al., 2005; Moore et al., 2007).

Several factors contribute to the phenotypic variability observed within FAS/FASD, including quantity, frequency and duration of alcohol exposure (Coles, 1993; Abel, 1995; Maier and West, 2001; May et al., 2013), developmental stage at the time of consumption and underlying maternal factors and/or genetic background influences, as only 5–10% of women with a positive drinking history give birth to children displaying features of FASD (Abel, 1995; Stratton et al., 1996). Furthermore, monozygotic twins display phenotypes that are more similar than dizygotic twins, suggesting that genetic background affects the incidence of FAS/FASD (Christoffel and Salafsky, 1975; Chasnoff, 1985; Streissguth and Dehaene, 1993; Riikonen, 1994). In addition, allelic variation within the alcohol dehydrogenase gene, *ADH1B*, can lead to varying teratogenic outcomes in different

ethnic groups (Warren and Li, 2005). These reports support the hypothesis that environmental elements, plus an individual's genetic background, contribute significantly to his or her susceptibility to the teratogenic effects of alcohol.

There are challenges to studying the pathogenesis of FAS/FASD in the human population; the clinical drinking history is not always complete or reliable, and it is unethical to conduct fetal alcohol studies in humans. To address these challenges, several groups performed studies describing the morphological changes that occur following alcohol exposure in various strains of mice. Two inbred mouse strains, C57BL/6 and DBA/2, differ widely in their response to maternal consumption of alcohol (Gilliam et al., 1988; Goodlett et al., 1989; Ogawa et al., 2005; Downing et al., 2009, 2012; Chen et al., 2011; Zhou et al., 2011). Previous studies by our group demonstrated that while DBA/2 animals are resistant to the teratogenic effects of fetal alcohol exposure in embryonic cultures, C57BL/6 mice are quite susceptible, exhibiting phenotypic abnormalities that affect embryonic lethality, brain morphology, fetal weight gain, behavior, as well as formation of the digits, skeleton, eyes, kidneys and heart (Ogawa et al., 2005; Chen et al., 2011; Zhou et al., 2011). Using a whole embryo culture approach that strictly controls the timing and dose of alcohol exposure, without interference from the maternal environment, we demonstrated that genetic background was a driving factor in the teratogenicity of alcohol in C57BL/6 embryos (Ogawa et al., 2005; Chen et al., 2011).

In this report, we sought to better understand the underlying genomic vulnerabilities to the teratogenic effects of alcohol observed between C57BL/6 and DBA/2 cultured embryos. We hypothesized that by strictly controlling confounding physiological factors (i.e., the maternal environment, intrauterine position effects, etc.), these studies would identify underlying genetic differences between C57BL/6 and DBA/2 that contribute to the observed differential vulnerability to the teratogenic effects of alcohol. Furthermore, we proposed that polymorphisms between C57BL/6 and DBA/2 in regulatory elements and other factors that influence RNA expression are responsible for much of these morphological differences. We used a two-pronged approach that takes advantage of the strain-specific genomic signatures and gene expression profiles to identify candidate *cis*-regulatory elements with the potential to drive differential expression between C57BL/6 and DBA/2 embryonic cultures. First, we used a gene x environment (GxE) approach to identify transcripts that were affected by both genetic background (i.e., C57BL/6 vs. DBA/2) and alcohol exposure (presence vs. absence of alcohol) to identify genes that are candidates for the morphological diversity between the these two inbred strains following exposure of equimolar ratios of alcohol in cultured embryos. Since the GxE approach identifies differentially expressed genes that are candidates for mediating the interaction, we then sought to uncover underlying single nucleotide variants (SNVs) in *cis*-regulatory elements within the proximal promoters of these candidate genes. SNVs within the promoters are excellent candidates for regulating expression of the nearby altered genes, as regulatory SNVs can alter the location and function of enhancers and promoters (De Gobbi et al., 2006) that change transcriptional levels (Munkhtulga et al., 2010) in specific cell types (Poitras et al., 2010) and lead to allele-specific changes in expression (Azad et al., 2013). SNV studies narrowed the list to 16 genes that showed both a GxE interaction based on gene expression studies and an SNV driven to fixation in opposite directions in the proximal promoter. Pathway analysis of this set of genes enhanced the understanding of the pathophysiology of alcohol exposure. Lastly, we investigated how these SNVs may lead to altered DNA methylation and transcription factor binding, with 53 SNVs potentially affecting both DNA methylation and transcription factor binding. This study provides a framework for future experiments aimed at understanding the combinatorial effects of these SNVs on susceptibility to FAS/FASD, and these two strains provide a tractable model system for identifying the complement of genetic loci and epigenetic events that contribute to the teratogenesis that occur along the spectrum of FAS/FASD.

MATERIALS AND METHODS

MOUSE STRAINS USED IN THIS STUDY

The Harlan strains used in the microarray study (C57BL/6NHsd and DBA/2NHsd), as well as the lines used to identify the potential regulatory SNVs (C57BL/6NJ and DBA/2J), descended from the original C57BL/6J (http://jaxmice.jax.org/strain/000664.html) and DBA/2J (http://jaxmice.jax.org/strain/000671.html) lines created at The Jackson Laboratories (Zurita et al., 2011). The C57BL/6 founder line originated from one of Abbie Lathram's fancy mouse stocks in 1921 by CC Little, and was maintained by brother-sister mating at the Jackson Laboratories until 1951 (at generation F32), when a sub-line, designated C57BL/6N, was sent to the NIH (Zurita et al., 2011). This sub-line is a founder line for C57BL/6NHsd and C57BL/6NJ. Harlan acquired these animals from the NIH in 1983 and has maintained them continuously in their colony; this sub-line is designated C57BL/6NHsd (order code 044; http://www.harlan.com). The C57BL/6NJ (http://jaxmice.jax.org/strain/005304.html) sub-line consists of embryos that were frozen at the NIH in 1997 and sent back to the Jackson Laboratory in 2005. The Jackson Laboratory thawed this line, expanded it and froze it down immediately. New stock animals are continually thawed to maintain the genetic integrity of the C57BL/6NJ sub-line. CC Little started inbreeding DBA/2 mice in 1909, and DBA/2J animals have been maintained continuously at The Jackson Laboratories. A sub-line (DBA/2N) was sent to the NIH in 1951 (at generation F34). Harlan later acquired this line, designated DBA/2NHsd (order code 042; http://www.harlan.com).

ALCOHOL EXPOSURE IN EMBRYONIC CULTURES

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine (Indianapolis, IN) and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (National-Academy-of-Sciences, 2010).

Previous studies from our lab measured the phenotypic variations between the C57BL/6 and DBA/2 inbred strains resulting from alcohol exposure in embryonic cultures (Ogawa et al., 2005; Chen et al., 2011). The technique for whole embryo culture was

described previously (Ogawa et al., 2005), based on methods by New (New, 1978). Briefly, 2-month-old C57BL/6NHsd and DBA/2NHsd mice from Harlan, Inc. (Indianapolis, IN) were individually housed and acclimated for at least 1 week prior to mating. Two females were placed with one male of the same strain for 2 h. When a vaginal plug was detected after the mating period, it was designated as embryonic day 0 (E0). On E8.25, females were sacrificed by CO_2 asphyxiation for morphological and transcriptome studies.

The alcohol exposure paradigm that yielded the highest degree of differential vulnerability was used for gene expression analysis (Chen et al., 2011). The gravid uterus was removed at 37°C; each embryo, plus the visceral yolk sac and a small piece of the ectoplacental cone, was carefully removed. Three embryos from each C57BL/6NHsd or DBA/2NHsd dam, bearing 3–5 somites (∼E8.25, age of the embryos is determined by the somites), were collected. Four groups of embryos, C57BL/6NHsd with or without alcohol and DBA/2NHsd with or without alcohol ($n = 6$ each) were incubated in 20 mL of medium, consisting of 70% immediately centrifuged heat-inactivated rat serum and 30% phosphate buffered saline supplemented with 20 units/ml penicillin and 20 units/ml streptomycin (Sigma, St. Louis, MO). Culture bottles were gassed for 2 h with 5% O_2, 5% CO_2 and 90% N_2 in a rotating culture system (B.T.C. Precision Incubator Unit, B.T.C. engineering, Cambridge, England, 36 rpm). After a 1–2 h acclimation period, a 6 h treatment was initiated by incubating embryos in fresh medium with or without 88 mM ethanol in isotonic buffer. After the 6-h alcohol treatment, all embryos were transferred to alcohol-free culture media for 15–16 h, for a total of 24 h in culture. The 6 h exposure allows a precise delineation of the developmental time period in which various teratogenic effects can be induced, which facilitates a rigorous examination of the temporal windows of vulnerability (Chen et al., 2011).

In previous studies, we demonstrated distinct morphological differences between these two lines following 44 h of continuous ethanol treatment (Ogawa et al., 2005) and 42 h after a 6 h ethanol treatment (Chen et al., 2011). In both studies, the C57BL/6 embryonic cultures were more susceptible to the teratogenic effects of alcohol than the DBA/2 animals. However, the two lines showed some differential dysmorphology after alcohol exposure. For example, C57BL/6 animals showed increased developmental delay compared to DBA/2 animals, although both are delayed compared to controls (Chen et al., 2011). In the same study, we cataloged the embryonic structures that showed morphological evidence of developmental delay in C57BL/6 and DBA/2 cultures. For example, dysmorphology is prominent in the heart, forebrain, midbrain, hindbrain, caudal neural tube, optic vesicle and hindlimb in C57BL/6 cultures, but restricted to the forebrain and optic vesicle in DBA/2 cultures. Furthermore, C57BL/6 embyros contained a large number of cleaved(c)-caspase 3 positive (+) cells (i.e., apoptotic cells) in the optic vesicles, several brain regions, the craniofacial primordial, as well as cranial nerve nuclei V, VII, VIII, and IX following alcohol exposure. In contrast, alcohol-exposed DBA/2 embryos only contained a small number of c-caspase 3^+ cells (Chen et al., 2011).

In the present study, our goal was to capture the transcriptome changes that occur prior to development of the morphological changes in order to gain an understanding of the genetic pathways that are targeted by alcohol exposure, not necessarily the alterations in expression that occur after manifestation of the dysmorphology. We hypothesized that collecting embryos at 15–16 h post-alcohol treatment would give the culture system time to eliminate the alcohol and reduce potential alcohol-induced artifacts. Therefore, all cultures were terminated 24 h after the start of alcohol treatment to reveal the upstream genetic pathways. Embryos were alive at termination, which was confirmed by observing a beating heart and circulating blood in the yolk sac. We conducted morphological examinations of the embryos at embryonic day (E) 8.25 (when culture initiated), +1 day in culture (i.e., E8.25 + 1); E8.25 + 1 is analogous to an embryo examined at E9.0. We observed several morphological changes in the embryos, which are analogous to those observed at the later time points (Ogawa et al., 2005; Chen et al., 2011). In all cases, the C57BL/6NHsd embryos demonstrated more morphological changes and developmental delay compared to the DBA/2NHsd strain. In addition, we noticed two distinct subtypes of embryos in C57BL/6NHsd cultures. Approximately 50% of the ethanol-treated C57BL/6NHsd cultures still had an open neural tube. This could be due to delayed embryonic development or to the teratological effects of alcohol. Therefore, we stratified the embryonic cultures into two subtypes, neural tube closed (NTC) and neural tube open (NTO) in an attempt to capture distinct genetic differences potentially caused by alcohol. All control embryos from both genotypes (C57BL/6NHsd and DBA/2NHsd) had a closed neural tube. Following alcohol treatment, we observed that the majority of the DBA/2NHsd embryos also had a closed neural tube. However, the neural tube failed to close in a small number of DBA/2NHsd and ∼50% of the C57BL/6NHsd embryos, resulting in an NTO morphology. Therefore, we subclassified each embryo into NTO and NTC bins. Embryos were segregated into six subtypes based on neural tube dysmorphology (NTO vs. NTC), inbred strain (C57BL/6NHsd or DBA/2NHsd) and alcohol exposure [with EtOH vs. without (Ctrl)]: NTO-B6-EtOH, NTC- B6-EtOH, NTC-B6-Ctrl, NTO-D2-EtOH, NTC-D2-EtOH and NTC-D2-Ctrl, where B6 indicates C57BL/6NHsd and D2 designates DBA/2NHsd. For more strict and detailed comparison, we analyzed the NTC and NTO embryos separately compared to their respective NTC control embryos. Due to the small numbers of NTO embryos in the DBA/2NHsd background, a large number of DBA/2NHsd animals were used in this study.

AFFYMETRIX GENE EXPRESSION

The comparative expression studies presented here were conducted on heads instead of whole embryos to focus on the cranial neural crest and brain. At this stage (∼E9.0), further dissection of parts of the head or brain would not yield enough tissue for microarray studies. The head of each embryo was dissected above the otic vesicle, immediately immersed in 0.7 ml TRIzol (Invitrogen, Carlsbad, CA) and homogenized to extract total RNA for RT-PCR and microarray studies. Total RNA was isolated from 36 individual embryos (6 vehicle control, 6 NTC alcohol treated and 6 NTO alcohol treated per strain) as previously described (Zhou et al., 2011). The quality of RNA was assessed by the Agilent Bioanalyzer (Agilent Technologies, Waldbronn,

Germany) and by spectrophotometry (220 nm to 350 nm); concentration was determined from the values at A260. Microarray hybridizations and bioinformatics analyses were performed at the Center for Medical Genomics at the Indiana University School of Medicine. Labeling and hybridization to Affymetrix Mouse Genome 430A GeneChips® (Affymetrix, Santa Clara, CA) were carried out following the manufacturer's suggested procedure. The Mouse Genome 430A chip contains over 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes. Fragmented biotinylated RNA from each embryo was hybridized to its own GeneChip for 17 h at 42°C. We previously demonstrated that the microarray analysis revealed striking differences between the embryos with open neural tubes (EtOH-NTO) and those with closed neural tubes (EtOH-NTC) (Zhou et al., 2011). Control embryos had closed neural tubes. Therefore, the transcriptome analysis was conducted based on the embryonic morphological phenotype.

The data from independent arrays (each with RNA from a single embryo) for each treatment were extracted using the Affymetrix Microarray Suite 5.0 (MAS5) algorithm. Data for both experiments have been deposited in GEO/NCBI and have been assigned series accession number GSE9545 and sample numbers GSM241642 through GSM241660. To minimize false positive results, only genes detected ("present" by the MAS5 algorithm) on at least half of all individual arrays in at least one experimental condition were retained for further analysis. This avoids data that primarily represent noise (McClintick and Edenberg, 2006).

B6 AND D2 GENOMIC SEQUENCE

We remapped the recently established genomic sequence available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/) for both C57BL/6NJ (http://www.ebi.ac.uk/ena/data/view/ERS076384&display=html) and DBA/2J (http://www.ebi.ac.uk/ena/data/view/ERS075663&display=html) onto release 66 of the *M. musculus* genome from Ensemble (http://www.ensembl.org) using CLC Genomics Workbench V4.7 (http://www.clcbio.com). We then called SNVs using the quality based variant detection tool with the neighborhood radius set to 5, maximum gap and mismatch count set to 2, minimum neighborhood quality of PHRED 15, and minimum central quality of PHRED 20. Variants in non-specific regions were ignored. Across all reads, a minimum variant frequency greater than 10% was required.

GENE X ENVIRONMENT (GxE) INTERACTIONS

The transcriptome of these lines, as impacted by alcohol exposure, was quantified by microarray analysis. We designed this experiment to analyze expression based on embryo neural tube dysmorphology (NTO and NTC), genetic background (C57BL/6NHsd or DBA/2NHsd) and alcohol exposure (88 mm EtOH or vehicle) in six groups as indicated in the experimental design above. Five embryos from each category were used for microarray studies. Since there were no control animals with the neural tube open morphology, all NTO studies used the control-NTC animals for comparison.

We analyzed the data using the Affymetrix Expression Console 1.1.2800.19935. Intensities were normalized using quantile normalization and the adjusted data were analyzed using the probe logarithmic intensity error (PLIER) method. According to the Affymetrix manual (http://media.affymetrix.com/support/technical/technotes/plier_technote.pdf). *The PLIER method produces a more accurate probe set signal by employing feature responses to interpret intensity data, dynamic weighting by empirical feature performance, and handling error appropriately across low and high target abundance.* The results from the PLIER analysis were variance stabilized using $\log_2$ transformation and analyzed by gene for significant differences using a 2 factor analysis of variance adjusted for multiple testing with a false discover rate of 5% (Benjamini and Hochberg, 1995). The main effects were line (DBA/2J vs. C57BL/6NHsd) and exposure to alcohol. Of primary interest were those genes where a differential response to alcohol was dependent on the line, i.e., differential expression in response to alcohol is dependent on the genetic background.

IDENTIFYING CANDIDATE SNVs

Since the C57BL/6NHsd and DBA/2NHsd genomes are not available, we chose closely related sub-strains (C57BL/6NJ and DBA/2J) for the genomic analyses. To define potential SNVs driving the differential phenotype following alcohol exposure, we cataloged all SNVs between C57BL/6NJ and DBA/2J located within 5 kb upstream of the transcriptional start site(s) of each candidate gene. To define a SNV, we calculated the difference in allele frequency for the same allele between C57BL/6NJ and DBA/2J, and concentrated on alleles driven to opposite fixation. SNVs were defined as alleles that were fixed at $\geq$0.90 (i.e., the frequency was $\geq$90% in one strain and $\leq$10% in the other). To identify putative transcription factor binding sites, we retrieved 20 bp of flanking sequence (10 bp on each side of the SNV; 21 bp total) from the NCBI37/mm9 mouse genome assembly, and put the sequence into PROMO (http://alggen.lsi.upc.es/) (Messeguer et al., 2002; Farre et al., 2003). PROMO uses version 8.3 of Transfac (Knuppel et al., 1994; Wingender, 2008) to identify putative transcription factor binding sites. For genes expressed from the Watson strand, we used the published sequence; for genes expressed on the Crick strand, we used the reverse complement. This ensured that we analyzed the same strand that the transcriptional machinery uses to transcribe the gene. The C57BL/6NJ and DBA/2J sequences were analyzed separately for putative transcription factor binding sites and additions or loss of CpG dinucleotides.

RESULTS

LINE-SPECIFIC DIFFERENCES IN GENE EXPRESSION

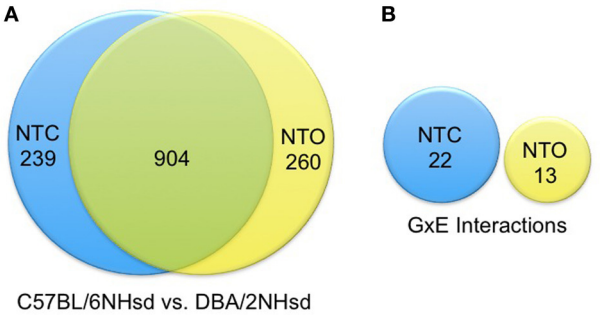
We first compared gene expression profiles between all C57BL/6NHsd and all DBA/2NHsd cultured embryos without taking alcohol exposure into consideration. We combined all C57BL/6NHsd embryos with an NTC morphology, which included those exposed and not exposed to alcohol, and compared them to the DBA/2NHsd embryos with a NTC phenotype, including those exposed and not exposed to alcohol. We repeated this study with embryos displaying NTO. However, in this analysis, the controls had a NTC phenotype, as none of the control animals displayed an NTO morphology. This analysis was used

to determine the subset of genes that are differentially expressed between the two lines. Using a false discovery rate (FDR) of 5% (Benjamini and Hochberg, 1995), we identified 1143 genes that were differentially expressed in NTC embryos and 1164 genes that were differentially expressed in NTO embryos, for a total of 1403 differentially expressed genes between these two inbred strains (**Figure 1A**). In these two independent experiments, 904 differentially expressed transcripts were detected in both the NTC and NTO embryos; ∼718 are significant at an FDR of $\sim 10^{-5}$. To confirm that these demonstrate statistical significance, we performed a hypergeometric distribution and a binomial distribution. Using a hypergeometic distribution, we found that the probability of drawing 904 genes that would be in the union by chance with independent draws is less than 0.015. A more informative metric might be the number one would expect by chance. This can be approximated with the binomial distribution because the numbers are large, i.e., sampling with replacement gives probabilities similar to sampling without replacement. The proportion of genes found significant in each experiment was 0.0327 and 0.0324 respectively. With independence, the proportion expected in the union is 2(0.0327) (0.0324) × 35,561 = 75. We observed about 12 times that number.

Many genes in this cohort are key to the development of the nervous system including: channel proteins and receptors, e.g., *Kcnmb1* (potassium large conductance calcium-activated channel), *Chrnb1* [cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)]; neural transcription factors and homeobox genes, e.g., *Nkx1-2* [NK1 transcription factor related, locus 2 (Drosophila)], *Igf1* (insulin-like growth factor 1), *Gbx2* (gastrulation brain homeobox 2), *Sox21* (SRY (sex determining region Y)-box 21); epigenetic components, e.g., *Hist1h2ab* (histone cluster 1, H2ab), *Setd7* (SET domain containing lysine methyltransferase 7); heat shock proteins, e.g., *Dnajc13*, *Dnajc21* DnaJ (Hsp40) homolog, subfamily C, member 13 and 21; genes involved in apoptosis, e.g., *Bnip3l* (BCL2/adenovirus E1B interacting protein 3-like), *Gadd45gip1* (growth arrest and DNA-damage-inducible, gamma interacting protein 1), *E2f5* (E2F transcription factor 5); and structural proteins, *Bean1* (brain expressed, associated with Nedd4, 1), *Tuba3a* (tubulin, alpha 3A), *Optc* (opticin).

Furthermore, Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) of this set of 904 genes found in both the NTC and NTO experiments indicates that the most significant pathways are: inflammatory response; cell death and survival; energy production; lipid metabolism; embryonic development; and cancer, hematological disease. We picked three networks as examples. The genes delineating these networks are listed in **Table 1**.

Notably among the 1403 differentially expressed genes, a number of microRNAs were identified: *miR206, miR31, miR463, miR551b, miR669a-2, miR142, miR211, miR22, miR27b, miR326, miR429, miR488, miR672, miRlet7a-2, miRlet7d, miRlet7e, miRlet7i, miR330, miR683-1*. Many of these miRNAs display functions relevant to development. For example *miR-142* regulates the formation and differentiation of hematopoietic stem cells in vertebrates (Lu et al., 2013). *miR-31*, which plays multiple roles during development and in cancer progression, is over expressed in myoblasts from patients with Duchene Muscular Dystrophy,



C

NTC Genes		NTO Genes
Csmd3	*Rhoc*	*Apold1*
Cxadr	*Rpl36a*	*Eya4*
D5Ertd577e	*Tac2*	*Kera*
Eno2	*Tspan2*	*Leo1*
Eya2	*Vmn1r37*	*Olfr312*
Fam174b	*Zfp157*	*Olfr975*
Mageb18	*4930579K19 Rik*	*Pkm2*
Marveld2		*Psme4*
Mcat	*7420416P09 Rik*	*Qars*
Nudt8		*Snord38a*
Olfr1248	*B230319C09 Rik*	*Tnfrsf22*
Olfr148		*Vipr1*
Pdxp		*1110014N23Rik (Vps51)*

FIGURE 1 | Summary of the differentially expressed genes. **(A)** Line-specific differences in gene expression. We conducted this analysis in two separate experiments that captured two embryonic phenotypes: neural tube closed (NTC) and neural tube open (NTO). This analysis was conducted by examining the gene expression changes solely between C57BL/6NHsd and DBA/2NHsd; alcohol exposure is not taken into consideration. Therefore, in the NTC group, all C57BL/6NHsd embryos (with and without alcohol) were measured as one cohort, while the DBA/2NHsd embryos (with and without alcohol) comprised the second group. The NTO cohort was analyzed in the same manner. This depicts the innate changes in gene expression between the two genetic backgrounds, plus the changes that are due to alcohol exposure, but cannot assess if there are interactions between the genetic background and alcohol exposure. There are 1403 genes that are differentially expressed between the C57BL/6NHsd and DBA/2NHsd embryos; 904 of these are differentially expressed in both the NTO and NTC studies. **(B)** Differential gene expression as result of genetic and alcohol interactions (GxE). As above, we analyzed gene expression in the NTC and NTO cohorts separately. In this study, we measured the potential interactions between alcohol exposure and genetic background. We found 22 genes that demonstrated GxE interactions in the NTC group and 13 genes showing GxE interactions in the NTO group. **(C)** Genes subject to GxE Interactions. The genes subject to GxE interactions are delineated.

compared to control individuals (Cacchiarelli et al., 2011) and thought to act as an inhibitor of metastasis in breast cancer cells (Valastyan et al., 2011). While *miR-206* regulates angiogenesis in zebrafish (Stahlhut et al., 2012) the miRlet7 family is essential for cell fate determination in C. elegans (Reinhart et al., 2000) and has been implicated in impairment of tumorigenesis (Johnson et al., 2005; Kumar et al., 2008). Finally, *miR-27b* regulates CYP1B1 expression post-transcriptionally in cancer tissues (Tsuchiya et al., 2006; Chuturgoon et al., 2014).

DIFFERENTIAL GENE EXPRESSION AS RESULT OF GENETIC AND ALCOHOL INTERACTIONS (GxE)

To further understand the genes that are responsive to alcohol and display a genetic basis, we determined which transcripts showed an interaction between these two conditions (i.e., a gene

Table 1 | Networks of NTO/NTC overlapping genes between C57BL/6NHsd and DBA/2NHsd.

Molecules in network	Score	# Target genes	Top diseases and functions
Ager, Atp5f1, Atp5g2, B3gnt2, Bcl2l14, Ccl19, Ccl21, Ccl27, Clcn7, Cyba, Epx, Fgf18, Gfi1, Gipc1, Gp49a/Lilrb4, Gzmk, Hmgb2, Itgb1bp1, Metap2, Mgat3, Mkl2, Nod1, Ntsr2, Oscp1, Ppp2r2d, Prg3, S100a1, S100a9, Smg1, Sytl1, Tff2, Tpm4	50	32	Inflammatory Response, Cellular Function and Maintenance, Cellular Movement
Akt2, Arhgap23, Atf6, Birc6, Camkk1, Chkb, Cox7a2l, Dcps, Ddx24, Dennd4a, Gapdh, Hmgb1, Hpcal1, Kdelr1, Map3k5, Myef2, Pank2, Prex1, Smek2, Smndc1, Stxbp4, Syncrip, Tinf2, Tomm22, Vdac3, Vps28, Ywhaq, Znrf2	40	28	Cellular Compromise, Cellular Function and Maintenance, Cell Death and Survival
Actg2, Anapc1, Blvra, Cdc23, Cml3, Cpd, Cxxc4, Daz2, Dlx5, Dvl1, Ercc4, Gde1, Hbg2, Hmbs, Hsd3b4, Kirrel, Let-7, Mir-204, Runx2, Sbf1, Sfrp4, Smad4, Sprr1a, Tob2, Trim32	34	25	Embryonic Development, Organ Development, Organismal Development

x environment (GxE) interaction). We compared the ethanol-treated animals with a NTC subtype from each genotype to their respective NTC controls. However, since none of the control animals had an NTO phenotype, NTC embryos from the same strain were used as a control, as follows: the Control-NTC vs. Alcohol-NTC group and the Control-NTO vs. Alcohol-NTO group. Accepting an FDR of 5%, we identified 35 candidates that demonstrate a GxE interaction; 22 were in the NTC subtype and 13 were in the NTO subtype (**Figures 1B,C**). IPA studies indicate that the top networks are cancer; respiratory disease; cell-to-cell signaling; gene expression; cell death and survival; and cellular compromise (**Table 2**). These 35 genes are top candidates underlying the divergent morphological changes observed between C57BL/6Hsd and DBA/2Hsd embryonic cultures that were exposed to alcohol.

Genes exhibiting a GxE interaction show not only differential expression, but also opposite patterns of expression that are dependent upon treatment. For example, in NTC embryos, *Eya2* [eyes absent 2 homolog (drosophila)] expression changes from an average of 7.26 $\pm$ 0.045 in control C57BL/6NHsd embryos to 7.52 $\pm$ 0.004 in ethanol-treated embryos. Conversely, control DBA/2NHsd embryos show an expression value of 7.63 $\pm$ 0.007, which drops to 7.40 $\pm$ 0.04 following incubation in alcohol (**Figure 2**). The Affymetrix PLIER expression levels are shown on the Y-axis. The black circles represent gene expression levels in control embryos, while the triangles represent gene expression levels in the alcohol-treated animals. There is a clear inverse correlation between the two genotypes following exposure to alcohol, as expression of *Eya2* increases following treatment in C57BL6NHsd embryos, while it decreases in DBA/2Hsd embryos.

We plotted the GxE interactions for the remaining genes. Twelve of the NTC genes (*Csmd3, Cxadr, D5ert577e, Eno2, Mageb18, Olfr1248, Olfr148, Rhoc, Rpl36a, Tspan2, Vmn1r37, and 7402416P09Rik*) showed strong GxE interactions (Supplemental Data, **Figure S1**), as measured by an intersecting line demonstrating inverse correlation with gene expression; in a weak interaction, the lines do not intersect, while nine NTC genes (*Fam174b, Marveld2, Mcat, Nudt8, Pdxp, Tac2, Zfp157, 4930579K19Rik, and B230319C09Rik*) showed weak GxE interactions (Supplemental Data, **Figure S1**). Eleven of the NTO genes (*Apold1, Eya4, Kera, Leo1, Olfr312, Olfr975, Psme4, Qars, Snord38a, Tnfrsf22, and Vps51*) showed strong GxE interactions and two (*Pkm2* and *Vipr1*) showed weak GxE interactions (Supplemental Data, **Figure S2**).

One concern for gene expression studies using microarrays is whether or not there is inherent expression bias due to SNVs within the probe-sets that hamper binding. This is potentially exacerbated when comparing changes in expression from a strain that is closely related to the reference sequence (i.e., C57BL/6NHsd) to a strain that is more distantly related (i.e., DBA/2NHsd). A hallmark of biased expression would be a consistent observation of decreased signal from the DBA/2NHsd animals in transcripts harboring SNVs in the probe-sets. In **Figure 2**, the DBA/2NHsd animals show higher levels of *Eya2* expression under control conditions. Therefore, it is highly unlikely that SNVs within the *Eya2* probe-sets are causing biased detection. Examination of the remaining GxE genes shows that 3/35 genes demonstrating GxE interactions potentially exhibit biased expression; *Mcat* and *Zfp157*, which were detected in NTC animals (**Figure S1**), and *Pkm2*, which was detected in NTO studies (**Figure S2**), are expressed at lower levels in DBA/2NHsd embryos under both conditions. Examination of the probe-sets for these three genes indicates that there is low likelihood that SNVs within the probe-sets account for the decreased expression in DBA/2NHsd cultures. There are 25 probe-sets that span *Mcat*; 2 of these harbor SNVs. Similarly, 23 probe-sets span *Zfp157*, 2 of which have SNVs. *Pkm2* has 15 probesets, but non-contain any informative SNVs.

GENOMIC SIGNATURES OF THE DIFFERENTIALLY EXPRESSED GxE GENES

To better understand the genetic basis driving these GxE interactions, we systematically identified the single nucleotide variants (SNVs) between C57BL/6 and DBA/2 using a genomics approach. However, the strains used for the microarray

Table 2 | Genes Demonstrating a GxE Interaction with Associated SNVs in the Promoter.

Gene	Name	Function	Expression during mouse development	Relevance to neural development	References
Eya2	*Eyes Absent Homolog2 (Drosophila)*	Tyrosine phosphatase, transcription activator	Cranial placodes, branchial arches and CNS during organogenesis	Regulate preplacedal ectoderm (PPE) differentiation	Xu et al., 1997; Grifone et al., 2007; Sato et al., 2010
Csmd3	*CUB and Sushi multiple domains 3*	Hydrolyzing O-glycosyl compounds	E14.5 to adult—Cardiovascular and nervous system (mainly in human adult and fetal brains)	Candidate gene for Autism Spectrum Disorders (ASDs)	Shimizu et al., 2003; Floris et al., 2008
Mcat	*Malonyl CoA:ACP Acyltransferase (Mitochondrial)*	Transfer a malonyl group from malonyl-CoA to the mitochondrial acyl carrier protein	E14.5 by RT-PCR	N/A	Smith et al., 2012
Tac2	*Tachykinin-2*	Neural peptide signaling	E11.5 to adult—CNS	May involve pain modulation	Mar et al., 2012
Vps51	*Vacuolar protein sorting 51*	Transport of endosomes to the trans-Golgi network (TGN)	N/A	N/A	Skarnes et al., 2011
Apold1	*Apolipoprotein L Domain Containing 1*	Angiogenesis and activity-dependent changes of brain vasculature	E14.5—Heart ventricle	May affect blood-brain permeability	Diez-Roux et al., 2011; Zhou et al., 2013
Leo1	*Paf1/RNA Polymerase II Complex Component*	Gene transcription regulation and chromatin remodeling	E9.5, E11.5, and E14.5—urogenital system	Cardiac and neural crest development in zebrafish	Wertz and Herrmann, 2000
Psme4	*Proteasome (Prosome, Macropain) Activator Subunit 4*	Proteasome activator, involves in histone degradation and DNA damage response	E14.5—Nervous system, sensory organs, alimentary system, and skeletal system	N/A	Ustrell et al., 2002; Qian et al., 2013
Vipr1	*Vasoactive Intestinal Peptide Receptor 1*	VIP receptor, modulated by G-proteins	E12-E18.5 by RT-PCR; detected at E13.5—cerebral cortex; Adult rat: cerebral cortex and hippocampus	Promote neuronal survival	Delgado et al., 1996; Fabricius et al., 2011
Fam174b	*Family with Sequence Similarity 174, Member B*	Membrane protein of unknown function	E14.5—alimentary system, limbs, head, skeleton system, sensory organs	Deleted in a patient with behavioral disturbance, autism, and epilepsy.	Diez-Roux et al., 2011; Sarahan et al., 2011; Kamien et al., 2014
Mageb18	*Melanoma-associated antigen B18*	Infection, inflammation, regulation of cell proliferation and apoptosis	Post-natal brain, testis and adipose tissue	N/A	Diez-Roux et al., 2011; Lin et al., 2012
Pdxp	*Pyridoxal (pyridoxine, vitamin B6) phosphatase*	Vitamin B6 Metabolism Required for normal progress through mitosis and normal cytokinesis.	E14.5—Nervous system, sensory organs, cardiovascular system, alimentary system	Activates cofilin through dephosphorylation	Niwa et al., 2002; Jang et al., 2003

(Continued)

Table 2 | Continued

Gene	Name	Function	Expression during mouse development	Relevance to neural development	References
Rpl36a	*60S ribosomal protein L36a*	Structural constituent of ribosome	E2 to E4.5—blastocyst E12.5 to E18.5—brain	Down-regulated in spinal cord of Amyotrophic Lateral Sclerosis (ALS) model	de Oliveira et al., 2013; MGI database
Tspan2	*Tetraspanin-2*	Regulation of cell development, activation, growth and motility	E14.5—pituitary Post-natal—CNS	Involved in early differentiation of oligodendrocytes	Birling et al., 1999; Diez-Roux et al., 2011
Zfp157	*Zinc finger protein 157*	Stat6-regulated KRAB domain zinc finger protein, binds DNA and regulate transcription	E8.5—head fold, neural tube at mid-gestation—brain, neural tube, mammary placodes, limbs		Oliver et al., 2013
Snord38a	*Small nucleolar RNA, C/D Box 38A*	2′O-ribose methylation of RNA	N/A	N/A	Nicoloso et al., 1996

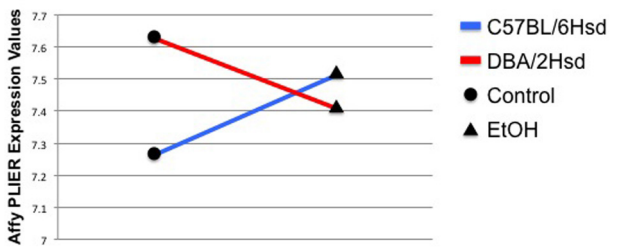


FIGURE 2 | *Eya2* demonstrates a strong GxE interaction. The Affymetrix PLIER expression levels are shown on the Y-axis. The black circles represent gene expression levels in control embryos, while the triangles represent gene expression levels in the alcohol-treated animals. Two conditions are measured in this experiment, gene expression levels in controls and gene expression levels in EtOH-treated animals. The blue line depicts the changes in gene expression found between the C57BL/6NHsd control embryos (black circle) and their EtOH-treated test embryos (black triangle). The red line depicts the changes in in gene expression found between the DBA/2NHsd control embryos (black circle) and their EtOH-treated test embryos (black triangle). The interaction is depicted by showing an inverse correlation between the gene expression profiles of the two inbred strains.

study (C57BL/6NHsd and DBA/2NHsd) were raised at Harlan and have not been added to the mouse sequence database. The closest matches in the mouse database are C57BL/6NJ (Yalcin et al., 2012) and DBA/2J. Therefore, we delineated the SNVs between C57BL/6NJ and DBA/2J in the promoters (5 kb upstream of each transcriptional start site) of all 35 genes showing GxE interactions. We found that 16 of these genes contain SNVs between C57BL/6NJ and DBA/2J: *Eya2, Csmd3, Mcat, Tac2, Vps51, Apold1, Leo1, Psme4, Vipr1, Fam174b, Megab18, Pdxp, Rpl36a, Tspan2, Zfp157, and Snord38a* (**Table 2**). A number of olfactory receptor genes were also identified, but were placed on the backburner since they display high sequence identity among orthologs, confounding SNV interpretation.

One gene identified in the NTC subtype, *Eya2*, stood out for further examination. *Eya2* exhibits strong GxE interactions and is expressed in the cranial placodes, branchial arches and the CNS during organogenesis (Xu et al., 1997), areas known to be affected clinically in FAS/FASD. There are three alternative transcriptional start sites associated with *Eya2*. To ensure complete analysis, we examined the most proximal 5 kb from all three promoters. The genomic signature profile demonstrates that there are 21 SNVs (seven are in dbSNP Build 138) between C57BL/6NJ and DBA/2NJ within the three promoters (see **Table 3**). **Table 3** provides the genomic location of each SNV, its relative position within the locus and the allele frequency. In addition, **Table 3** shows the genomic sequence surrounding each SNV and illustrates changes in putative transcription factor (TF) binding sites and CpG dinucleotides.

Sixteen of the twenty-one SNVs identified in the three promoters of *Eya2* potentially lead to the loss or gain of putative transcription factor (TF) binding sites. For example, there are no predicted TF binding sites at SNVs 1, 4, 7, 10, 13, 14, and 16 in the C57BL/6NJ allele, while SNVs 4, 7, 10, 13, and 16 are predicted to contain one or more TF binding sites in the DBA/2J allele. Given the potential for these SNVs to function not only in a genetic manner, but also epigenetically through alterations in DNA methylation, we report that 5 of the 21 SNVs within the three promoters of *Eya2* change a CpG dinucleotide. Four of these (SNV 2, 4, 21, and 22) lead to the loss of a CpG dinucleotide in the DBA/2J background. Examination of putative TF binding sites in these four SNVs shows that there are fewer potential TF binding sites in the C57BL/6NJ allele compared to the DBA/2J allele. *Eya2* shows increased expression in C57BL/6NHsd embryos following alcohol treatment, while the DBA/2NHsd animals exhibit a decrease in expression following alcohol exposure. This would be consistent with increased TF binding in C57BL/6NHsd embryos compared to DBA/2NHsd cultures, and provides a putative mechanism for the GxE interactions.

Table 3 | SNVs near *Eya2*. *Eya2* is on MMU 2 and expressed from the Watson (+) strand.

	dbSNP	Genomic	Start site			CpG Site	Putative TF binding sites			C57BL/6NJ		DBA/2J		NCBI37/mm9
#	Build 138	Location	TSS1	TSS2	TSS3	B6/D2	B6	D2	Flanking sequence	NT	%	NT	%	NT
1		165417649	Promoter	Promoter	Promoter		N/A	N/A	GTGTACATGG[T/C]ATATGTATGG	T	100	C	100	T
2	rs33341731	165419967	Promoter	Promoter	Promoter	CG/CA	Hes1	Hoxa5/Hes1	GAAACCTTGC[G/A]TTGGGGGGTG	G	100	A	100	G
3		165429834	Intron 1	Promoter	Promoter		SRF	N/A	TATAACCCGA[G/A]CTTAAAGGCC	G	100	A	100	G
4	rs33448850	165431347	Intron 1	Promoter	Promoter	CG/TG	N/A	TFE3-S/c-Fos	TATACTCACA[C/T]GAAAAAAAAC	C	100	T	100	C
5		165465686	Intron 1	Promoter	Promoter		C/EBPbeta	c-Fos	ACACACACAC[A/T]CACACACACA	A	100	T	100	A
6		165466018	Intron 1	Promoter	Promoter		C/EBPbeta c-Fos	TCF-1(P) c-Fos	TCTCTCTCTC[A/T]CACACACACA	A	100	T	100	A
7		165475306	Intron 1	Promoter	Promoter		N/A	C/EBPbeta	GGGATGGAGG[G/A]CAGATAAGAA	G	100	A	100	G
8		165475312	Intron 1	Promoter	Promoter		GATA-1 GATA-2	Tal-1 RelA NF-kappaB COE1	GAGGGCAGAT[AA/GG]GAAGGGACA	A	100	G	100	A
9		165475313	Intron 1	Promoter	Promoter					A	100	T	100	A
10		165475318	Intron 1	Promoter	Promoter		N/A	TCF-1(P);	AGATAAGAAG[G/A]GACAGGGAGA	G	100	A	100	G
11		165475328	Intron 1	Promoter	Promoter		TCF-1(P)	c-Fos DEC2	GGACAGGGAG[A/G]TGAATGGGAT	A	100	G	100	A
12	rs27298255	165485303	Intron 1	Intron 1	Promoter		N/A	HES-1 myogenin MyoD	GTGTTAACAG[G/C]AAGTTGTGAA	G	100	C	100	G
13		165485789	Intron 1	Intron 1	Promoter		N/A	N/A	CCACCACCAC[T/C]ATACAAAGGA	T	100	C	100	T
14		165486306	Intron 1	Intron 1	Promoter		TCF-1(P)	C/EBPbeta TCF-1(P)	CTCTCTCTCT[C/G]TGTGTGTGTG	C	100	G	100	C
15		165488467	Intron 1	Intron 1	Promoter		N/A	c-Fos DEC2	AGATGGGTTG[G/A]AGCTAGCCTG	G	100	A	100	G
16		165488503	Intron 1	Intron 1	Promoter	GG/CG	LyF-1	LyF-1	GTCGTTGTTT[G/C]GGGAGAAATC	G	100	C	100	G
17	rs3718350	165488796	Intron 1	Intron 1	Promoter		GR C/EBPbeta	TCF-1(P)	CTTAACAAGA[A/G]CAGTATGCAC	A	100	G	100	A
18	rs27298227	165489658	Intron 1	Intron 1	Promoter		TFE3-S	TFE3-S	GTCTCACATG[GG/CA]TAAGGCAAGA	G	100	C	100	G
19		165489659	Intron 1	Intron 1	Promoter					G	100	A	100	G
20	rs27298226	165489698	Intron 1	Intron 1	Promoter	CG/CA	TCF-1(P)	c-Fos TCF-1(P)	TAGCCCTCTC[G/A]GTACTTAGCA	G	100	A	100	G
21	rs27298225	165489830	Intron 1	Intron 1	Promoter	CG/AG	HES-1 Nkx2-1	HES-1 Nkx2-1 c-Fos	AAGGCACTTG[C/A]GAAACAGTAT	C	100	A	100	C

Similar SNV analyses of the other 15 genes are documented in the Supplemental Files (Table S1). There are four genes that show strong GxE interactions that also have SNVs within the proximal promoter: *Apold1* (Apolipoprotein L domain containing 1), *Leo1* (Paf1/RNA Polymerase II Complex Component), *Psme4* (Proteasome Activator Subunit 4) and *Snord38a* (Small nucleolar RNA, C/D box 38A) (**Table 2**). *Apold1* is thought to be involved in angiogenesis in the brain vasculature (Diez-Roux et al., 2011; Zhou et al., 2013); *Leo1*, which important in chromatin remodeling, is involved in neural crest development (Wertz and Herrmann, 2000); *Psme4* is a component of the proteasome that specifically targets histones for degradation following DNA damage (Ustrell et al., 2002; Qian et al., 2013); and *Snord38a* is a C/D box small nucleolar RNA that is important for 2′O-ribose methylation of rRNAs (Nicoloso et al., 1996).

A summary of the putative regulatory implications of these 241 SNVs is found in **Table 4**. Ten genes were found in the NTC subtype, while six were detected in the NTO embryos. Out of the 241 SNVs identified between C57BL/6NJ and DBA/2J, 186 have the possibility to alter TF binding sites, while 62 cause the addition or removal of a CpG dinucleotide. Out of the 62 SNVs that lead to CpG changes, 34 (55%) led to the loss of a CpG dinucleotide, 26 (42%) led to the *de novo* creation of a CpG site and 2 (3%) changed the position of a CpG site (i.e., CCG to CGG). In addition, the vast majority of SNVs that alter a CpG site [53/62 (85%)] are associated with potential additions, losses and/or substitutions in TF binding sites, indicating that both genetic (SNVs) and epigenetic (CpG methylation) could be implicated in the GxE interactions.

To better understand the genetic variability between the C57BL/6 sub-strains used for gene expression studies (C57BL/6NHsd) and SNV analysis (C57BL/6NJ), we used the SNV Data from the Broad Institute (Broad2) to identify SNVs between C57BL/6NHsd and C57BL/6NJ in the "Compare Two or More Strains" option from the Mouse Phenome Database (http://phenome.jax.org/db/q?rtn=strains/search&compare2=1). We discovered that there are 119590 SNVs between C57BL/6NHsd and C57BL/6NJ, which given a genome of $\sim 2.7 \times 10^9$ bp, corresponds to a mutation frequency of 4.4×10^{-5} mutations/bp, or one SNV in every ∼22,500 bp. In this study, we analyzed 5000 bp from 18 promoters of 16 genes, including three promoters for *Eya2*. This totals 90,000 bp of sequence. Given a mutation frequency of 4.4×10^{-5}, we would expect to find four SNVs that could be attributed to background alterations between C57BL/6NHsd and C57BL/6NJ in our sample. Although we need to estimate the background mutation frequency between DBA/2NHsd (expression studies) and DBA/2NJ (SNV analysis), the DBA/2NHsd line has not been sequenced nor has it been included in any of the available SNP chip repositories. Therefore, we assumed a similar mutation frequency between the two DBA/2 lines (i.e., four mutations in 90,000 bp). Taking these numbers as an estimate, we would expect to find eight total SNVs (four for the C57BL/6 lines and four for the DBA/2 lines) that could be due within sub-line mutations. However, we found 241 SNVs between C57BL/6NJ and DBA/2J in this study, which corresponds to a mutation frequency of 2.7×10^{-3} mutations/bp, indicating that likelihood of seeing a GxE interaction due to false positives (∼0.033) falls within a 0.05 confidence interval.

DISCUSSION

This study was conducted to better understand the genetic underpinnings underlying the susceptibility to alcohol exposure during fetal development. We pursued this by identifying the intersected set of genes that: (1) Are differentially expressed in response to alcohol treatment during neurulation; and (2) Demonstrate a genetic interaction between two strains of mice (C57BL/6 and DBA/2) that respond very differently to prenatal alcohol exposure. While C57BL/6 animals are highly sensitive to the teratogenic effects of alcohol, DBA/2 animals are remarkably resistant, as shown by studies using gavage feeding (Gilliam et al., 1988; Downing et al., 2009, 2012) and our prior work in cultured embryos that found alcohol-vulnerability in C57BL/6 embryo cultures exposed to a short alcohol incubation period (Ogawa et al., 2005; Chen et al., 2011; Zhou et al., 2011).

These comparative expression studies reveal a cohort of ∼1400 genes that are preferentially expressed in either the C57BL/6NHsd or DBA/2NHsd strains during the beginning stages of neurulation. These genes may represent a first tier contribution to either protect (DBA/2NHsd) or increase (C57BL/6NHsd) the vulnerability of the embryo to the effects of alcohol exposure. Because this set of transcripts does not take alcohol exposure into consideration (e.g., C57BL/6NHsd ± EtOH vs. DBA/2NHsd ± EtOH), it represents the cohort of transcripts that differ between these two strains in two independent experiments. Genes of particular interest are the neural transcription factor and homeobox genes (*Nkx1-2, Sox21, Igf1*, and *Gbx2*), as they can mediate neural progenitor cell fate determination and neural tube patterning. The potassium channel (*Kcnmb1*) and cholinergic receptor (*Chnb1*) genes can mediate timely signal transduction during neural differentiation, while *Hist1h2ab* and *Setd7* have the potential to differentially affect the epigenetic response of the developing brain cells to environmental assaults, including alcohol. The apoptosis, cell cycle and heat shock proteins act to influence cell number, and could contribute to neural tube deficits between the two lines.

For stringent comparison, the closed neural tube (NTC) and open neural tube (NTO) embryonic subtypes were analyzed independently, with an FDR of 5% as the cut off. These differentially expressed genes potentially network to alter inflammatory response, cell death and survival, energy production, embryonic development and hematopoiesis between the two inbred lines. This cohort of differentially expressed transcripts likely results from combinations of factors including genetic background, innate programming, reactivation of transposable elements and epigenetic inheritance and responses. Since this study was conducted on embryonic cultures, it eliminates maternal elements (e.g., physiology, maternal care, circulation factors) that would have normally acted during this critical developmental window. However, since we analyzed whole brains, we were not able to assess tissue-specificity or cell-specific differences in gene expression, and it is plausible that we missed rare transcripts with large effects or primary cell-specific transcripts that are driving the genetic differences in the sensitivity of these two inbred strains to alcohol exposure. Therefore, it is likely that our results are

Table 4 | Summary of the potential regulatory effects of the SNVs between C57BL/6NJ and DBA/2J in genes demonstrating GxE interactions.

NTC/ NTO	Symbol	Gene	MMU	Coordinates	Strand	B6/D2 SNV	Altered TFB	Potential epigenetic gene regulation changes			
								CpG Change	CG to NN	NN to CG	Altered TFB
NTC	*Csmd3*	CUB and Sushi multiple domains 3	15	47412184-48623535	Reverse	11	8	1	0	1	1
NTC	*Eya2*	Eyes Absent Homolog2 (Drosophila)	2	165420528-165597131	Forward	21	16	5	4	1	4
NTC	*Fam174b*	Membrane protein FAM174B precursor	7	80885193-80921805	Forward	1	0	0	0	0	0
NTC	*Mageb18*	Melanoma-associated antigen B18	X	89364218-89844911	Forward	1	1	0	0	0	0
NTC	*Mcat*	Malonyl CoA:ACP Acyltransferase (Mitochondrial)	15	83377227-83386141	Reverse	54	42	12	6	5	9
NTC	*Pdxp*	Pyridoxal phosphate phosphatase	15	78744349-78749947	Forward	18	14	9	5	4	8
NTC	*Rpl36a*	60S ribosomal protein L36a	X	131120193-131122601	Forward	3	2	1	1	0	1
NTC	*Tac2*	Tachykinin-2	10	127162448-127168824	Forward	9	7	3	1	2	3
NTC	*Tspan2*	Tetraspanin-2	3	102538693-102576233	Forward	1	1	0	0	0	0
NTC	*Zfp157*	Zinc finger protein 157	5	138882704-138901922	Forward	5	3	2	2	0	1
NTO	*Apold1*	Apolipoprotein L Domain Containing 1	6	134932019-134936854	Forward	51	43	12	6	6	11
NTO	*Leo1*	Paf1/RNA Polymerase II Complex Component	9	75289331-75314239	Forward	6	6	3	1	2	3
NTO	*Psme4*	Proteasome (Prosome, Macropain) Activator Subunit 4	11	30671775-30780361	Forward	16	10	2	1	1	2
NTO	*Snord38a*	Small nucleolar RNA, C/D box 38A	4	116827121-116827179	Reverse	32	24	8	4	3	7
NTO	*Vipr1*	Vasoactive Intestinal Peptide Receptor 1	9	121551834-121582072	Forward	8	6	3	2	1	2
NTO	*Vps51*	Vacuolar protein sorting 51	19	6067842-6077187	Reverse	4	3	1	1	0	1
Total						241	186	62	34	26	53

an under-representation of the total number of genes that are differentially expressed between these two lines.

Genes exhibiting GxE interactions demonstrate differential expression that inversely correlates with genotype and exposure to the environmental factor (i.e., alcohol). With an FDR of 5%, we identified 35 genes that exhibited GxE interactions, as measured by an inverse correlation in gene expression (based on genotype) in response to alcohol. Examination of potential mechanisms for GxE interactions indicates that 16 genes have SNVs in their proximal promoters. Several of these 16 candidates are strongly associated with brain and cranial development, including preplacedal ectoderm differentiation (*Eya2*) (Xu et al., 1997; Grifone et al., 2007; Sato et al., 2010), cardiac and neural crest development (*Leo1*) (Wertz and Herrmann, 2000), neuronal survival (*Vipr1*) (Delgado et al., 1996; Fabricius et al., 2011) and oligodendryocyte differentiation (*Tspan2*) (Birling et al., 1999; Diez-Roux et al., 2011). Others are implicated in behavioral disorders including autism spectrum disorders (*Csmd3* and *Fam147b*) (Shimizu et al., 2003; Floris et al., 2008; Diez-Roux et al., 2011; Sarahan et al., 2011; Kamien et al., 2014), Amytrophic Lateral Sclerosis (*Rps36a*) (de Oliveira et al., 2013) and pain modulation (*Tac2*) (Mar et al., 2012). Three genes in the NTO subtype are involved in gene regulation, *Leo1* (chromatin structure and gene regulation; Wertz and Herrmann, 2000), *Psme4* (proteasome

degradation of histones; Ustrell et al., 2002; Qian et al., 2013) and *Snord38a* (modifications of ribosomal RNA; Nicoloso et al., 1996). Although it is not clear if there are any specific pathways that are affected by these four genes, several have the ability to disrupt expression and protein function on a global scale. Most of these are downregulated in C57BL/6NHsd animals following alcohol exposure, indicating that global disruption to the proteasome pathway, chromatin remodeling and rRNA methylation may be a factor in the increased developmental delay in the NTO embryonic cultures.

It is becoming increasingly clear that phenotypic variability and causative lesions are not restricted to the protein-coding regions of the genome. Several GWAS studies have demonstrated that SNVs in non-coding regions of the DNA are strongly associated with disease processes (De Gobbi et al., 2006; Enjuanes et al., 2006; Choi et al., 2009; Glinskii et al., 2009; Heckmann et al., 2010; Maceachern et al., 2011a,b; Brown et al., 2013; Fan et al., 2013; Perumbakkam et al., 2013; Renteria et al., 2013).

To understand the possible regulatory consequences associated with these gene expression changes, we took this analysis one step further by cataloging the SNVs between the parental strains in the promoter regions of the 35 genes and determining potential regulatory changes associated with the SNVs. Sixteen of these genes had a least one SNV in the proximal promoter (i.e., within 5 kb of the transcriptional start site), for a total of 241 SNVs within 90,000 bp of promoter sequence. The majority of SNVs (77%; 186/241) altered the genomic DNA in a way that led to the predicted addition or subtraction or a potential transcription factor binding site, indicating that these underlying genetic differences could play significant roles in the differential phenotypes we detected in this study. In addition, ∼25% (62/241) of the SNVs have the potential to affect DNA methylation by either creating or eliminating a CpG dinucleotide. Since DNA methylation is affected by alcohol exposure *in utero* (Ramsay, 2010; Laufer et al., 2013; Resendiz et al., 2013; Ungerer et al., 2013) and epigenetic gene regulation is critical for neuronal development (Chen et al., 2014), the underlying genetic differences between the C57BL/6 and DBA/2 inbred strains that impact CpG dinucleotides could create epigenetic consequences that predispose the C57BL/6 embryos to the teratogenic effects of alcohol, while protecting DBA/2 from this vulnerability.

CONCLUSIONS

By using a multi-pronged approach in two separate experiments (neural tube open and neural tube closed phenotypes), we captured over 900 genes that have the potential to contribute to the cascade that leads to differential dysmorphology in embyros from the two genetically contrasting lines. These first tier genes are good candidates for further understanding the phenotypic variability associated with *in utero* alcohol exposure. Exploration of the genetic and environmental factors led us to a cohort of 35 genes subject to GxE interactions; these genes represent the best candidates for driving the downstream morphological changes observed following alcohol exposure. Examination of potential regulatory SNVs associated with these 35 candidates indicates that the differences between the C57BL/6 and DBA/2 inbred strains could be due to a variety of factors, including binding of *cis*-acting elements (e.g., transcription factors), expression of *trans*-acting factors, epigenetic events, evolutionary consequences and combinations of these different factors.

These experiments lay the groundwork for future studies aimed at testing the causality of these different SNVs. Although a seemingly daunting task, with emerging genome modification tools, such as zinc finger, TALEN and CRISPR modification systems (Gaj et al., 2013) under development and refinement, it may be possible to start systematically disrupting multiple SNVs with exquisite sensitivity. Other possible approaches include using selection to screen for animals containing the most predictive SNVs and determining which cohorts of SNVs recapitulate the susceptible phenotype in the resistant DBA/2NHsd population. Studies in chicken demonstrate the feasibility and elegance of this approach (Maceachern et al., 2011a,b; Perumbakkam et al., 2013). Although we cannot rule out that the alterations in gene expression are not due to the SNVs identified here, this study provides the top 35 genes showing GxE interactions following alcohol exposure, providing a strong platform for future studies aimed at understanding the roles of these genetic and transcriptional changes associated with vulnerability to the teratogenic effects of fetal alcohol exposure.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00173/abstract

Figure S1 | Gene x Environment interactions of the genes identified in the embryos with closed neural tubes (NTC). The Affymetrix PLIER expression levels are shown on the Y-axis. The black circles represent gene expression levels in control embryos, while the triangles represent gene expression levels in the alcohol-treated animals. Two conditions are measured in this experiment, gene expression levels in controls and gene expression levels in EtOH-treated animals. The blue line depicts the changes in gene expression found between the C57BL/6NHsd control embryos (black circle) and their EtOH-treated test embryos (black triangle). The red line depicts the changes in in gene expression found between the DBA/2NHsd control embryos (black circle) and their EtOH-treated test embryos (black triangle). The interaction is depicted by showing an inverse correlation between the gene expression profiles of the two inbred strains.

Figure S2 | Gene x Environment interactions of the genes identified in the embryos with open neural tubes (NTO). The Affymetrix PLIER expression levels are shown on the Y-axis. The black circles represent gene expression levels in control embryos, while the triangles represent gene expression levels in the alcohol-treated animals. Two conditions are measured in this experiment, gene expression levels in controls and gene

expression levels in EtOH-treated animals. The blue line depicts the changes in gene expression found between the C57BL/6NHsd control embryos (black circle) and their EtOH-treated test embryos (black triangle). The red line depicts the changes in in gene expression found between the DBA/2NHsd control embryos (black circle) and their EtOH-treated test embryos (black triangle). The interaction is depicted by showing an inverse correlation between the gene expression profiles of the two inbred strains.

REFERENCES

Abel, E. L. (1995). An update on incidence of FAS: FAS is not an equal opportunity birth defect. *Neurotoxicol. Teratol.* 17, 437–443. doi: 10.1016/0892-0362(95)00005-C

Astley, S. J., Stachowiak, J., Clarren, S. K., and Clausen, C. (2002). Application of the fetal alcohol syndrome facial photographic screening tool in a foster care population. *J. Pediatr.* 141, 712–717. doi: 10.1067/mpd.2002.129030

Azad, A. K., Curtis, A., Papp, A., Webb, A., Knoell, D., Sadee, W., et al. (2013). Allelic mRNA expression imbalance in C-type lectins reveals a frequent regulatory SNP in the human surfactant protein A (SP-A) gene. *Genes Immun.* 14, 99–106. doi: 10.1038/gene.2012.61

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B* 57, 289–300. doi: 10.2307/2346101

Birling, M. C., Tait, S., Hardy, R. J., and Brophy, P. J. (1999). A novel rat tetraspan protein in cells of the oligodendrocyte lineage. *J. Neurochem.* 73, 2600–2608. doi: 10.1046/j.1471-4159.1999.0732600.x

Brown, C. D., Mangravite, L. M., and Engelhardt, B. E. (2013). Integrative modeling of eQTLs and cis-regulatory elements suggests mechanisms underlying cell type specificity of eQTLs. *PLoS Genet.* 9:e1003649. doi: 10.1371/journal.pgen.1003649

Cacchiarelli, D., Incitti, T., Martone, J., Cesana, M., Cazzella, V., Santini, T., et al. (2011). miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy. *EMBO Rep.* 12, 136–141. doi: 10.1038/embor.2010.208

Chasnoff, I. J. (1985). Fetal alcohol syndrome in twin pregnancy. *Acta Genet. Med. Gemellol. (Roma).* 34, 229–232.

Chen, Y., Damayanti, N. P., Irudayaraj, J., Dunn, K., and Zhou, F. C. (2014). Diversity of two forms of DNA methylation in the brain. *Front. Genet.* 5:46. doi: 10.3389/fgene.2014.00046

Chen, Y., Ozturk, N. C., Ni, L., Goodlett, C., and Zhou, F. C. (2011). Strain differences in developmental vulnerability to alcohol exposure via embryo culture in mice. *Alcohol. Clin. Exp. Res.* 35, 1293–1304. doi: 10.1111/j.1530-0277.2011.01465.x

Choi, S. J., Marazita, M. L., Hart, P. S., Sulima, P. P., Field, L. L., McHenry, T. G., et al. (2009). The PDGF-C regulatory region SNP rs28999109 decreases promoter transcriptional activity and is associated with CL/P. *Eur. J. Hum. Genet.* 17, 774–784. doi: 10.1038/ejhg.2008.245

Christoffel, K. K., and Salafsky, I. (1975). Fetal alcohol syndrome in dizygotic twins. *J. Pediatr.* 87(6 Pt 1), 963–967. doi: 10.1016/S0022-3476(75)80919-X

Chuturgoon, A. A., Phulukdaree, A., and Moodley, D. (2014). Fumonisin B1 modulates expression of human cytochrome P450 1b1 in human hepatoma (Hepg2) cells by repressing Mir-27b. *Toxicol. Lett.* 227, 50–55. doi: 10.1016/j.toxlet.2014.02.026

Coles, C. D. (1993). Impact of prenatal alcohol exposure on the newborn and the child. *Clin. Obstet. Gynecol.* 36, 255–266. doi: 10.1097/00003081-199306000-00007

De Gobbi, M., Viprakasit, V., Hughes, J. R., Fisher, C., Buckle, V. J., Ayyub, H., et al. (2006). A regulatory SNP causes a human genetic disease by creating a new transcriptional promoter. *Science* 312, 1215–1217. doi: 10.1126/science.1126431

Delgado, M., Martinez, C., Johnson, M. C., Gomariz, R. P., and Ganea, D. (1996). Differential expression of vasoactive intestinal peptide receptors 1 and 2 (VIP-R1 and VIP-R2) mRNA in murine lymphocytes. *J. Neuroimmunol.* 68, 27–38. doi: 10.1016/0165-5728(96)00063-X

de Oliveira, G. P., Alves, C. J., and Chadi, G. (2013). Early gene expression changes in spinal cord from SOD1(G93A) Amyotrophic Lateral Sclerosis animal model. *Front. Cell. Neurosci.* 7:216. doi: 10.3389/fncel.2013.00216

Diez-Roux, G., Banfi, S., Sultan, M., Geffers, L., Anand, S., Rozado, D., et al. (2011). A high-resolution anatomical atlas of the transcriptome in the mouse embryo. *PLoS Biol.* 9:e1000582. doi: 10.1371/journal.pbio.1000582

Downing, C., Balderrama-Durbin, C., Hayes, J., Johnson, T. E., and Gilliam, D. (2009). No effect of prenatal alcohol exposure on activity in three inbred strains of mice. *Alcohol. Alcohol.* 44, 25–33. doi: 10.1093/alcalc/agn082

Downing, C., Flink, S., Florez-McClure, M. L., Johnson, T. E., Tabakoff, B., and Kechris, K. J. (2012). Gene expression changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 36, 1519–1529. doi: 10.1111/j.1530-0277.2012.01757.x

Enjuanes, A., Garcia-Giralt, N., Supervia, A., Nogues, X., Ruiz-Gaspa, S., Bustamante, M., et al. (2006). A new SNP in a negative regulatory region of the CYP19A1 gene is associated with lumbar spine BMD in postmenopausal women. *Bone* 38, 738–743. doi: 10.1016/j.bone.2005.10.010

Fabricius, D., Karacay, B., Shutt, D., Leverich, W., Schafer, B., Takle, E., et al. (2011). Characterization of intestinal and pancreatic dysfunction in VPAC1-null mutant mouse. *Pancreas* 40, 861–871. doi: 10.1097/MPA.0b013e318214c783

Fan, J., Dy, J. G., Chang, C. C., and Zhou, X. (2013). Identification of SNP-containing regulatory motifs in the myelodysplastic syndromes model using SNP arrays and gene expression arrays. *Chin. J. Cancer* 32, 170–185. doi: 10.5732/cjc.012.10113

Farre, D., Roset, R., Huerta, M., Adsuara, J. E., Rosello, L., Alba, M. M., et al. (2003). Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Res.* 31, 3651–3653. doi: 10.1093/nar/gkg605

Floris, C., Rassu, S., Boccone, L., Gasperini, D., Cao, A., and Crisponi, L. (2008). Two patients with balanced translocations and autistic disorder: CSMD3 as a candidate gene for autism found in their common 8q23 breakpoint area. *Eur. J. Hum. Genet.* 16, 696–704. doi: 10.1038/ejhg.2008.7

Gaj, T., Gersbach, C. A., and Barbas, C. F. 3rd. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31, 397–405. doi: 10.1016/j.tibtech.2013.04.004

Gilliam, D. M., Kotch, L. E., Dudek, B. C., and Riley, E. P. (1988). Ethanol teratogenesis in mice selected for differences in alcohol sensitivity. *Alcohol* 5, 513–519. doi: 10.1016/0741-8329(88)90091-2

Glinskii, A. B., Ma, J., Ma, S., Grant, D., Lim, C. U., Sell, S. et al. (2009). Identification of intergenic trans-regulatory RNAs containing a disease-linked SNP sequence and targeting cell cycle progression/differentiation pathways in multiple common human disorders. *Cell Cycle* 8, 3925–3942. doi: 10.4161/cc.8.23.10113

Goodlett, C. R., Gilliam, D. M., Nichols, J. M., and West, J. R. (1989). Genetic influences on brain growth restriction induced by development exposure to alcohol. *Neurotoxicology* 10, 321–334.

Grifone, R., Demignon, J., Giordani, J., Niro, C., Souil, E., Bertin, F., et al. (2007). Eya1 and Eya2 proteins are required for hypaxial somitic myogenesis in the mouse embryo. *Dev. Biol.* 302, 602–616. doi: 10.1016/j.ydbio.2006.08.059

Heckmann, J. M., Uwimpuhwe, H., Ballo, R., Kaur, M., Bajic, V. B., and Prince, S. (2010). A functional SNP in the regulatory region of the decay-accelerating factor gene associates with extraocular muscle pareses in myasthenia gravis. *Genes Immun.* 11, 1–10. doi: 10.1038/gene.2009.61

Hoyme, H. E., May, P. A., Kalberg, W. O., Kodituwakku, P., Gossage, J. P., Trujillo, P. M., et al. (2005). A practical clinical approach to diagnosis of fetal alcohol spectrum disorders: clarification of the 1996 institute of medicine criteria. *Pediatrics* 115, 39–47. doi: 10.1542/peds.2005-0702

Jacobson, S. W. (1998). Specificity of neurobehavioral outcomes associated with prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 22, 313–320. doi: 10.1111/j.1530-0277.1998.tb03654.x

Jang, Y. M., Kim, D. W., Kang, T. C., Won, M. H., Baek, N. I., Moon, B. J., et al. (2003). Human pyridoxal phosphatase. Molecular cloning, functional expression, and tissue distribution. *J. Biol. Chem.* 278, 50040–50046. doi: 10.1074/jbc.M309619200

Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., et al. (2005). RAS is regulated by the let-7 microRNA family. *Cell* 120, 635–647. doi: 10.1016/j.cell.2005.01.014

Kamien, B., Harraway, J., Lundie, B., Smallhorne, L., Gibbs, V., Heath, A., et al. (2014). Characterization of a 520 kb deletion on chromosome 15q26.1 including ST8SIA2 in a patient with behavioral disturbance, autism spectrum disorder, and epilepsy. *Am. J. Med. Genet. A* 164, 782–788. doi: 10.1002/ajmg.a.36345

Knuppel, R., Dietze, P., Lehnberg, W., Frech, K., and Wingender, E. (1994). TRANSFAC retrieval program: a network model database of eukaryotic transcription regulating sequences and proteins. *J. Comput. Biol.* 1, 191–198. doi: 10.1089/cmb.1994.1.191

Kumar, M. S., Erkeland, S. J., Pester, R. E., Chen, C. Y., Ebert, M. S., Sharp, P. A. et al. (2008). Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3903–3908. doi: 10.1073/pnas.071232110

Laufer, B. I., Mantha, K., Kleiber, M. L., Diehl, E. J., Addison, S. M., and Singh, S. M. (2013). Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis. Model. Mech.* 6, 977–992. doi: 10.1242/dmm.010975

Lin, Y., Wen, T., Meng, X., Wu, Z., Zhao, L., Wang, P., et al. (2012). The mouse Mageb18 gene encodes a ubiquitously expressed type I MAGE protein and regulates cell proliferation and apoptosis in melanoma B16-F0 cells. *Biochem. J.* 443, 779–788. doi: 10.1042/BJ20112054

Lu, B., Nagappan, G., Guan, X., Nathan, P. J., and Wren, P. (2013). BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. *Nat. Rev. Neurosci.* 14, 401–416. doi: 10.1038/nrn3505

Maceachern, S., Muir, W. M., Crosby, S., and Cheng, H. H. (2011a). Genome-wide identification of allele-specific expression (ASE) in response to Marek's disease virus infection using next generation sequencing. *BMC Proc.* 5(Suppl. 4):S14. doi: 10.1186/1753-6561-5-S4-S14

Maceachern, S., Muir, W. M., Crosby, S. D., and Cheng, H. H. (2011b). Genome-wide identification and quantification of cis- and trans-regulated genes responding to marek's disease virus infection via analysis of allele-specific expression. *Front. Genet.* 2:113. doi: 10.3389/fgene.2011.00113

Maier, S. E., and West, J. R. (2001). Drinking patterns and alcohol-related birth defects. *Alcohol. Res. Health* 25, 168–174.

Mar, L., Yang, F. C., and Ma, Q. (2012). Genetic marking and characterization of Tac2-expressing neurons in the central and peripheral nervous system. *Mol. Brain* 5:3. doi: 10.1186/1756-6606-5-3

May, P. A., Blankenship, J., Marais, A. S., Gossage, J. P., Kalberg, W. O., Joubert, B., et al. (2013). Maternal alcohol consumption producing fetal alcohol spectrum disorders (FASD): quantity, frequency, and timing of drinking. *Drug Alcohol. Depend.* 133, 502–512. doi: 10.1016/j.drugalcdep.2013.07.013

McClintick, J. N., and Edenberg, H. J. (2006). Effects of filtering by Present call on analysis of microarray experiments. *BMC Bioinformatics* 7:49. doi: 10.1186/1471-2105-7-49

Messeguer, X., Escudero, R., Farre, D., Nunez, O., Martinez, J., and Alba, M. M. (2002). PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* 18, 333–334. doi: 10.1093/bioinformatics/18.2.333

Moore, E. S., Ward, R. E., Wetherill, L. F., Rogers, J. L., Autti-Ramo, I., Fagerlund, A., et al. (2007). Unique facial features distinguish fetal alcohol syndrome patients and controls in diverse ethnic populations. *Alcohol. Clin. Exp. Res.* 31, 1707–1713. doi: 10.1111/j.1530-0277.2007.00472.x

Munkhtulga, L., Nagashima, S., Nakayama, K., Utsumi, N., Yanagisawa, Y., Gotoh, T., et al. (2010). Regulatory SNP in the RBP4 gene modified the expression in adipocytes and associated with BMI. *Obesity (Silver Spring)* 18, 1006–1014. doi: 10.1038/oby.2009.358

National-Academy-of-Sciences. (2010). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

New, D. A. (1978). Whole-embryo culture and the study of mammalian embryos during organogenesis. *Biol. Rev. Camb. Philos. Soc.* 53, 81–122. doi: 10.1111/j.1469-185X.1978.tb00993.x

Nicoloso, M., Qu, L. H., Michot, B., and Bachellerie, J. P. (1996). Intron-encoded, antisense small nucleolar RNAs: the characterization of nine novel species points to their direct role as guides for the 2′-O-ribose methylation of rRNAs. *J. Mol. Biol.* 260, 178–195. doi: 10.1006/jmbi.1996.0391

Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K., and Uemura, T. (2002). Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* 108, 233–246. doi: 10.1016/S0092-8674(01)00638-9

Ogawa, T., Kuwagata, M., Ruiz, J., and Zhou, F. C. (2005). Differential teratogenic effect of alcohol on embryonic development between C57BL/6 and DBA/2 mice: a new view. *Alcohol. Clin. Exp. Res.* 29, 855–863. doi: 10.1097/01.ALC.0000163495.71181.10

Oliver, C. H., Nichols, J., and Watson, C. J. (2013). The KRAB domain zinc finger protein, Zfp157, is expressed in multiple tissues during mouse embryogenesis and in specific cells in adult mammary gland and skin. *Genesis* 51, 179–186. doi: 10.1002/dvg.22367

Perumbakkam, S., Muir, W. M., Black-Pyrkosz, A., Okimoto, R., and Cheng, H. H. (2013). Comparison and contrast of genes and biological pathways responding to Marek's disease virus infection using allele-specific expression and differential expression in broiler and layer chickens. *BMC Genomics* 14:64. doi: 10.1186/1471-2164-14-64

Poitras, L., Yu, M., Lesage-Pelletier, C., Macdonald, R. B., Gagne, J. P., Hatch, G., et al. (2010). An SNP in an ultraconserved regulatory element affects Dlx5/Dlx6 regulation in the forebrain. *Development* 137, 3089–3097. doi: 10.1242/dev.051052

Qian, M. X., Pang, Y., Liu, C. H., Haratake, K., Du, B. Y., Ji, D. Y., et al. (2013). Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. *Cell* 153, 1012–1024. doi: 10.1016/j.cell.2013.04.032

Ramsay, M. (2010). Genetic and epigenetic insights into fetal alcohol spectrum disorders. *Genome Med.* 2:27. doi: 10.1186/gm148

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 403, 901–906. doi: 10.1038/35002607

Renteria, M. E., Coolen, M. W., Statham, A. L., Choi, R. S., Qu, W., Campbell, M. J., et al. (2013). GWAS of DNA methylation variation within imprinting control regions suggests parent-of-origin association. *Twin. Res. Hum. Genet.* 16, 767–781. doi: 10.1017/thg.2013.30

Resendiz, M., Chen, Y., Ozturk, N. C., and Zhou, F. C. (2013). Epigenetic medicine and fetal alcohol spectrum disorders. *Epigenomics* 5, 73–86. doi: 10.2217/epi.12.80

Riikonen, R. S. (1994). Difference in susceptibility to teratogenic effects of alcohol in discordant twins exposed to alcohol during the second half of gestation. *Pediatr. Neurol.* 11, 332–336. doi: 10.1016/0887-8994(94)90012-4

Sampson, P. D., Streissguth, A. P., Bookstein, F. L., Little, R. E., Clarren, S. K., Dehaene, P., et al. (1997). Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology* 56, 317–326. doi: 10.1002/(SICI)1096-9926(199711)56:5%3C317::AID-TERA5%3E3.3.CO;2-T

Sarahan, K. A., Fisler, J. S., and Warden, C. H. (2011). Four out of eight genes in a mouse chromosome 7 congenic donor region are candidate obesity genes. *Physiol. Genomics* 43, 1049–1055. doi: 10.1152/physiolgenomics.00134.2010

Sato, S., Ikeda, K., Shioi, G., Ochi, H., Ogino, H., Yajima, H., et al. (2010). Conserved expression of mouse Six1 in the pre-placodal region (PPR) and identification of an enhancer for the rostral PPR. *Dev. Biol.* 344, 158–171. doi: 10.1016/j.ydbio.2010.04.029

Shimizu, A., Asakawa, S., Sasaki, T., Yamazaki, S., Yamagata, H., Kudoh, J., et al. (2003). A novel giant gene CSMD3 encoding a protein with CUB and sushi multiple domains: a candidate gene for benign adult familial myoclonic epilepsy on human chromosome 8q23.3-q24.1. *Biochem. Biophys. Res. Commun.* 309, 143–154. doi: 10.1016/S0006-291X(03)01555-9

Skarnes, W. C., Rosen, B., West, A. P., Koutsourakis, M., Bushell, W., Iyer, V., et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 474, 337–342. doi: 10.1038/nature10163

Smith, S., Witkowski, A., Moghul, A., Yoshinaga, Y., Nefedov, M., de Jong, P., et al. (2012). Compromised mitochondrial fatty acid synthesis in transgenic mice results in defective protein lipoylation and energy disequilibrium. *PLoS ONE* 7:e47196. doi: 10.1371/journal.pone.0047196

Stahlhut, C., Suarez, Y., Lu, J., Mishima, Y., and Giraldez, A. J. (2012). miR-1 and miR-206 regulate angiogenesis by modulating VegfA expression in zebrafish. *Development* 139, 4356–4364. doi: 10.1242/dev.083774

Stratton, K., Howe, C., and, Battaglia, F. (1996). *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment*. Washington, DC: National Academy Press.

Streissguth, A. P., and Dehaene, P. (1993). Fetal alcohol syndrome in twins of alcoholic mothers: concordance of diagnosis and IQ. *Am. J. Med. Genet.* 47, 857–861. doi: 10.1002/ajmg.1320470612

Tsuchiya, Y., Nakajima, M., Takagi, S., Taniya, T., and Yokoi, T. (2006). MicroRNA regulates the expression of human cytochrome P450 1B1. *Cancer Res.* 66, 9090–9098. doi: 10.1158/0008-5472.CAN-06-1403

Ungerer, M., Knezovich, J., and Ramsay, M. (2013). *In utero* alcohol exposure, epigenetic changes, and their consequences. *Alcohol. Res.* 35, 37–46.

Ustrell, V., Hoffman, L., Pratt, G., and Rechsteiner, M. (2002). PA200, a nuclear proteasome activator involved in DNA repair. *EMBO J.* 21, 3516–3525. doi: 10.1093/emboj/cdf333

Valastyan, S., Chang, A., Benaich, N., Reinhardt, F., and Weinberg, R. A. (2011). Activation of miR-31 function in already-established metastases elicits metastatic regression. *Genes Dev.* 25, 646–659. doi: 10.1101/gad.2004211

Warren, K. R., and Li, T. K. (2005). Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Res. A Clin. Mol. Teratol.* 73, 195–203. doi: 10.1002/bdra.20125

Wertz, K., and Herrmann, B. G. (2000). Large-scale screen for genes involved in gonad development. *Mech. Dev.* 98, 51–70. doi: 10.1016/S0925-4773(00)00452-4

Wingender, E. (2008). The TRANSFAC project as an example of framework technology that supports the analysis of genomic regulation. *Brief. Bioinformatics* 9, 326–332. doi: 10.1093/bib/bbn016

Xu, P. X., Woo, I., Her, H., Beier, D. R., and Maas, R. L. (1997). Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. *Development* 124, 219–231.

Yalcin, B., Adams, D. J., Flint, J., and Keane, T. M. (2012). Next-generation sequencing of experimental mouse strains. *Mamm. Genome* 23, 490–498. doi: 10.1007/s00335-012-9402-6

Zhou, F. C., Zhao, Q., Liu, Y., Goodlett, C. R., Liang, T., McClintick, J. N., et al. (2011). Alteration of gene expression by alcohol exposure at early neurulation. *BMC Genomics* 12:124. doi: 10.1186/1471-2164-12-124

Zhou, L., Wang, L. M., Song, H. M., Shen, Y. Q., Xu, W. J., Xu, J. H., et al. (2013). Expression profiling analysis of hypoxic pulmonary disease. *Genet. Mol. Res.* 12, 4162–4170. doi: 10.4238/2013.October.7.2

Zurita, E., Chagoyen, M., Cantero, M., Alonso, R., Gonzalez-Neira, A., Lopez-Jimenez, A., et al. (2011). Genetic polymorphisms among C57BL/6 mouse inbred strains. *Transgenic Res.* 20, 481–489. doi: 10.1007/s11248-010-9403-8

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Genomic factors that shape craniofacial outcome and neural crest vulnerability in FASD

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Prenatal alcohol exposure (PAE) causes distinctive facial characteristics in some pregnancies and not others; genetic factors may contribute to this differential vulnerability. Ethanol disrupts multiple events of neural crest development, including induction, survival, migration, and differentiation. Animal models and genomic approaches have substantially advanced our understanding of the mechanisms underlying these facial changes. PAE during gastrulation produces craniofacial changes corresponding with human fetal alcohol syndrome. These result because PAE reduces prechordal plate extension and suppresses sonic hedgehog, leading to holoprosencephaly and malpositioned facial primordia. Haploinsufficiency in sonic hedgehog signaling increases vulnerability to facial deficits and may influence some PAE pregnancies. In contrast, PAE during early neurogenesis produces facial hypoplasia, preceded by neural crest reductions due to significant apoptosis. Factors mediating this apoptosis include intracellular calcium mobilization, elevated reactive oxygen species, and loss of trophic support from β-catenin/calcium, sonic hedgehog, and mTOR signaling. Genome-wide SNP analysis links PDGFRA with facial outcomes in human PAE. Multiple genomic-level comparisons of ethanol-sensitive and – resistant early embryos, in both mouse and chick, independently identify common candidate genes that may potentially modify craniofacial vulnerability, including ribosomal proteins, proteosome, RNA splicing, and focal adhesion. In summary, research using animal models with genome-level differences in ethanol vulnerability, as well as targeted loss-and gain-of-function mutants, has clarified the mechanisms mediating craniofacial change in PAE. The findings additionally suggest that craniofacial deficits may represent a gene–ethanol interaction for some affected individuals. Genetic-level changes may prime individuals toward greater sensitivity or resistance to ethanol's neurotoxicity.

Keywords: fetal alcohol spectrum disorders, neural crest, sonic hedgehog, apoptosis, β-catenin, CaMKII, ribosome biogenesis

INTRODUCTION

It is indisputable that genetic factors modulate individual risk for alcoholism (see Edenberg and Foroud, 2013, for a recent review). For example, allelisms in alcohol and aldehyde dehydrogenases control blood alcohol levels and alcohol clearance rates. Allelisms in neurotransmitter receptors and their downstream effectors shape neuronal responses to alcohol reward, tolerance, and withdrawal. However, our understanding of how genetic factors might shape individual risk for fetal alcohol spectrum disorders (FASD) is less well defined. It is without question that prenatal alcohol exposure (PAE) is teratogenic; indeed, it is the most common teratogen exposure in western societies. However, there is appreciable variability in the severity of individual responses to PAE, even after controlling for the pattern, timing, and dose of alcohol intake. Some of this variance is due to environmental factors that include maternal nutrition and socioeconomic status. Evidence has been accumulating that genetic factors can also shape individual risk for FASD (Warren and Li, 2005). Concordance for FASD risk is greater in monozygotic than in dizygotic twins (Streissguth and Dehaene, 1993). The potential existence of genetic modifiers is strongly endorsed by animal models of FASD. Much of this animal work emphasizes morphological rather than behavioral outcomes. When ethanol exposure is held constant, genetic factors modulate the risk for cardiac, craniofacial, skeletal, and central nervous system defects in the developing offspring, and this is documented for both mammalian (primates, rats, mice) and non-mammalian (chick, zebrafish) developmental models (Goodlett et al., 1989; Gilliam and Kotch, 1996; Boehm et al., 1997; Chen et al., 2000; Debelak and Smith, 2000; Su et al., 2001; Loucks and Carvan, 2004; Downing et al., 2009, 2012a,b; Swartz et al., 2014). Because much of this work is summarized in other contributions to this volume, we will not further detail those findings here. Apart from allelisms that modulate alcohol clearance rates, the identities of genes that modify fetal vulnerability to FASD are not well characterized.

ETHANOL INFLUENCES CRANIOFACIAL OUTCOMES IN FASD

Insights into how genetic factors may modify vulnerability to PAE are informed by an understanding of ethanol's mechanisms of action. Unlike other teratogens, ethanol does not have a single receptor but instead affects cellular activity though structural

interactions with diverse proteins that include ligand-gated ion channels, G-protein-coupled receptors, and intracellular signaling proteins (Howard et al., 2011). This diversity of targets enables ethanol to alter multiple signaling pathways and processes that are essential for normal development. Ethanol's disruption of these pathways dysregulates cellular events that are central to morphogenesis, such as proliferative expansion, migration, differentiation, and survival. One well-characterized target of PAE is the developing face. PAE can cause facial characteristics that have diagnostic utility and these include a flattened midface, micrognathia, smooth philtrum, thin upper lip, and short palpebral fissures (Klingenberg et al., 2010). During development, the facial cartilage and bone are derived from the cranial neural crest, a stem cell population that originates at the neuroectoderm–ectoderm boundary during the process of neurulation (Sauka-Spengler and Bronner-Fraser, 2008). Shortly thereafter, cranial neural crest progenitors transform from an epithelial to mesenchymal phenotype, delaminate, and migrate laterally and ventrally to form the facial primordia (frontonasal, maxilla, and mandible), branchial arches (connective tissue of the thymus, thyroid, and cardiac outflow tract), cranial nerve elements, and melanocytes. At the same time, medial–lateral expansion of the underlying forebrain, or prosencephalon, further defines the size and relative positioning of the facial elements (frontonasal, maxilla, mandible, and hyoid). In humans, these events begin at day 17–18 post-fertilization, before the pregnancy is typically recognized. Animal models reveal that ethanol adversely affects many events of craniofacial development including neural crest induction, survival, migration, and expansion, as well as cranial midline development (Hassler and Moran, 1986; Cartwright et al., 1998; Dunty et al., 2001; Ahlgren et al., 2002; Rovasio and Battiato, 2002; Yan et al., 2010; Oyedele and Kramer, 2013). The mechanisms by which ethanol alters these events are the focus of a recent review (Smith et al., 2014), and the reader is referred to that article for a detailed discussion of those findings. This review instead emphasizes current knowledge of genetic influences upon craniofacial outcomes in PAE. It should be noted that women who abuse alcohol typically do so throughout pregnancy. Thus, it is likely that multiple mechanisms contribute to the craniofacial dysmorphology that partly typifies FASD.

GENETIC BACKGROUND INFLUENCES CRANIOFACIAL OUTCOME IN FASD

Evidence that genome-level differences can influence craniofacial outcomes in PAE emerges largely from animal models. Mouse strains are especially powerful tools for this work, and numerous ethanol-sensitive and relatively resistant strains have been characterized. With respect to cranial development, the inbred strain C57BL/6J is considered ethanol sensitive, whereas DBA/J is considered ethanol resistant. Quantitative trait locus (QTL) mapping of the BxD recombinant lines derived from these strains established that vulnerability to ethanol's teratogenicity has strong heritability and identified several large QTLs that may be contributory (Downing et al., 2012a). The related inbred strains C57BL/6J and C57BL/6N also generate different facial dysmorphologies in response to PAE, although some of this may be due to differences in alcohol consumption (Anthony et al., 2010), as also found for the "U" and "N" rat strains (Wentzel and Eriksson, 2008). Non-mammalian embryos complement this rodent work because maternal influences are removed and direct embryonic effects can be investigated. The zebrafish strains AB, Ekkwill, and Tuebingen differ in their levels of cranial cell death, craniofacial dysmorphology, and overall survival in response to equivalent ethanol challenge (Loucks and Carvan, 2004). Commercial chick strains show similar variability, wherein broiler strains had greater cranial reductions compared with layer strains (Bupp Becker and Shibley, 1998), and layer strains themselves have differing facial apoptosis patterns and dysmorphologies in response to equivalent ethanol exposure (Debelak and Smith, 2000; Su et al., 2001). As detailed below, these genetic models have informed the mechanisms by which PAE disrupts craniofacial development.

GENETIC INFLUENCES UPON HOLOPROSENCEPHALY IN FASD

The physical size and relationship of craniofacial structures are influenced by the underlying prosencephalon or forebrain. During gastrulation, anterior extension of the prechordal mesendoderm induces the overlying ectoderm to form neuroepithelium (Sauka-Spengler and Bronner-Fraser, 2008). Neural crest is specified at this ectoderm/neuroectoderm boundary. Simultaneous with this, the prechordal mesoderm induces sonic hedgehog (*shh*) within the neuroepithelial midline. Subsequently, *shh* activity at the prosencephalon midline drives expansion not only of the forebrain but also the overlying facial primordial. Thus, craniofacial development is intimately linked with brain induction and expansion.

Ethanol exposure at gastrulation disrupts midline formation and thereby craniofacial development. Ethanol exposure at gastrulation activates the *shh* suppressor protein kinase A and the increased protein kinase A activity downregulates *shh* at the embryo's midline (Aoto et al., 2008). Ethanol-induced apoptosis within the anterior prechordal plate, as well as its reduced expansion, further limits neuroepithelial size and the neural crest induction field (Blader and Strahle, 1998; Aoto et al., 2008). Consequently, the prosencephalon expansion is reduced and the overlying facial primordia are malpositioned. Additionally, as shown in zebrafish, PAE also reduces cholesterol ester pools and thereby limits substrate availability for the covalent modification of the nascent N-terminal shh protein, which is necessary for the protein's membrane association and *shh* signaling (Li et al., 2007). The reduced *shh* expression along the prosencephalon midline persists developmentally, as do reductions in additional inductive signals including *goosecoid*, *Foxa2*, and *Fgf8* (Li et al., 2007; Aoto et al., 2008; Hong and Krauss, 2012). Work in mice reveals that targeted ethanol exposure during these gastrulation-stage events generates the "classic" FAS face, including elongated upper lip, flattened philtrum, and reduced midface. These changes represent holoprosencephaly (Sulik, 1984; Lipinski et al., 2012) and are recreated in both mammalian and non-mammalian models of FASD (Sulik, 1984; Su et al., 2001; Carvan et al., 2004; Li et al., 2007; Aoto et al., 2008; Hong and Krauss, 2012; Lipinski et al., 2012). PAE at mouse e8.5 instead produces a distinct facial outcome that lacks these holoprosencephalic features, suggesting that

criteria for recognizing facial dysmorphology in FASD may need expansion.

Genetic-level alterations within the *shh* signaling pathway increase vulnerability to facial dysmorphology in PAE. Mice that are haploinsufficient in *Shh*, *Gli2*, or *Cdon* generally have normal crania due to compensation from the remaining allele. However, ethanol exposure of these same heterozygotes at gestational day 7.0 (e7) causes holoprosencephaly, demonstrating this pathway's mechanistic role in producing FASD facial changes (Hong and Krauss, 2012; Kietzman et al., 2014). It also suggests that haploinsufficiency in this pathway increases risk for ethanol-induced holoprosencephaly. In humans, holoprosencephaly is estimated to affect 1/16,000 live births and 1/250 conceptuses (Dubourg et al., 2007); thus, heterozygous carriers at risk for ethanol-induced damage may be more common in the general population than appreciated. It is possible that even mild ethanol exposure during this critical period increases the frequency and severity of human holoprosencephalic disorders.

GENETIC INFLUENCES UPON NEURAL CREST APOPTOSIS IN FASD

Ethanol also alters craniofacial development through its induction of significant cell death within neural crest populations. This occurs at clinically relevant ethanol exposures from 20 to 100 mM (∼0.1 to ∼0.4 mg%). It has been documented for mouse, chick, and zebrafish models of PAE (Sulik et al., 1981; Cartwright et al., 1998; Carvan et al., 2004), suggesting that neural crest sensitivity is conserved across vertebrates and most likely also occurs in human exposure. This cell death is apoptotic, as the cells are positive for extracellular Annexin-V or terminal deoxynucleotidyl transferase (TUNEL), and their death is prevented by pretreating the cells with caspase inhibitors (Cartwright et al., 1998; Dunty et al., 2001; Carvan et al., 2004; Reimers et al., 2006). The apoptosis significantly reduces cranial neural crest numbers and contributes to craniofacial deficits (Sulik et al., 1981; Cartwright and Smith, 1995; Carvan et al., 2004; Garic et al., 2011; Flentke et al., 2014b). Multiple mechanisms contribute to this apoptosis, including the production of reactive oxygen species (Chen et al., 2013), generation of intracellular calcium transients (Debelak-Kragtorp et al., 2003), and the loss of tropic support from β-catenin (Flentke et al., 2011) and/or from sonic hedgehog (Ahlgren et al., 2002). The details of these mechanisms have been recently reviewed (Smith et al., 2014).

Genetic models offer insights into the mechanisms mediating this apoptosis. For example, neural crest populations from the mouse strain C56BL/6J have much greater apoptosis than do ICR neural crest at equivalent ethanol exposures (50–200 mM; Chen et al., 2000). The membrane content of GM1 ganglioside is enriched in ICR cells compared with those from C57BL/6J, and addition of GM1 ganglioside attenuates ethanol's damage, suggesting that differences in GM1 content may affect ethanol vulnerability. Screens of zebrafish mutants have identified multiple genes that influence craniofacial outcomes in ethanol exposure including *pdgfra*, *plk1*, *hinfp*, *mars*, *vangl2*, and *foxi1* (McCarthy et al., 2013; Swartz et al., 2014). These are all thought to be loss-of-function mutations and all worsened the craniofacial dysmorphology in response to ethanol. At least two of these, *pdgfra* and *plk1* loss-of-function, also enhance apoptosis within ethanol-treated cranial regions. Importantly, PDGFRA also could be linked to craniofacial defects in individuals with FASD (McCarthy et al., 2013). It should be noted that loss-of-function of additional craniofacial genes did not modify the risk for ethanol-induced facial defects in this model, including *cyp26b1*, *gata3*, *smad5*, *smoothened*, *mitfa*, and *neurog1*, among others. This endorses that ethanol's mechanism is specific rather than generalized.

NEURAL CREST APOPTOSIS – INSIGHTS FROM THE CHICK MODEL

The developing chick embryo is advantageous for neural crest investigations due to its ready accessibility and ease of experimental manipulation. Much of the mechanism that governs neural crest apoptosis within this ethanol exposure model is now understood and this pathway is shown in **Figure 1**. In these cells, ethanol interacts with a G-protein-coupled receptor of yet-unknown identity and having a binding pocket that accommodates aliphatic alcohols from C1 through C5 (Garic-Stankovic et al., 2005, 2006). Alcohol binding stimulates G-protein signaling in which a pertussis toxin-sensitive Gβγ dimer activates phospholipase Cβ, likely the β4 isozyme that is expressed in neural crest (Garic-Stankovic et al., 2005). Within seconds of ethanol exposure, the resulting production of phosphoinositides initiates calcium mobilization from intracellular stores and the capacitative entry of extracellular calcium (**Figures 2A,B**; Debelak-Kragtorp

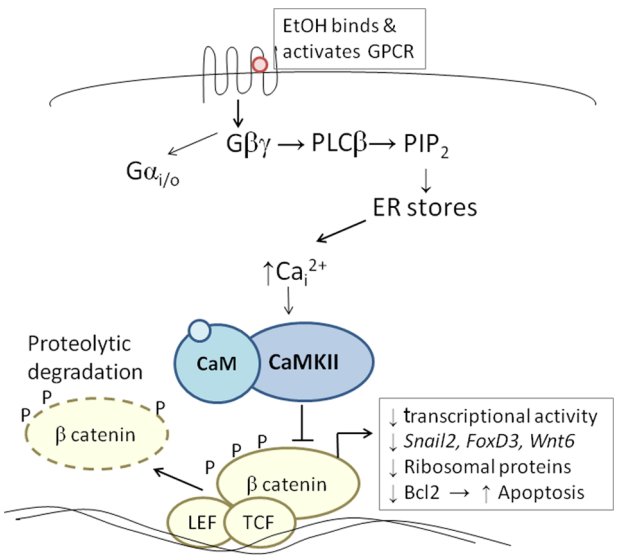

FIGURE 1 | Diagram of the calcium/β-catenin pathway by which ethanol causes the apoptosis of cranial neural crest populations, as deciphered for chick embryos having 1–10 somites; additional details in text. Ethanol's interaction with a G-protein-coupled receptor of unknown identity activates a calcium transient that originates from Gβγ, PLCβ, and phosphoinosityl phosphate release. The calcium transient stimulates calmodulin and CaMKII. CaMKII phosphorylates and thereby destabilizes β-catenin, abrogating the latter's transcriptional activity. This removes trophic support from neural crest including genes critical for neural crest development (*snai2*, *FoxD3*, *Wnt6*). Accompanying reductions in bcl2 and ribosomal proteins may also be contributory.

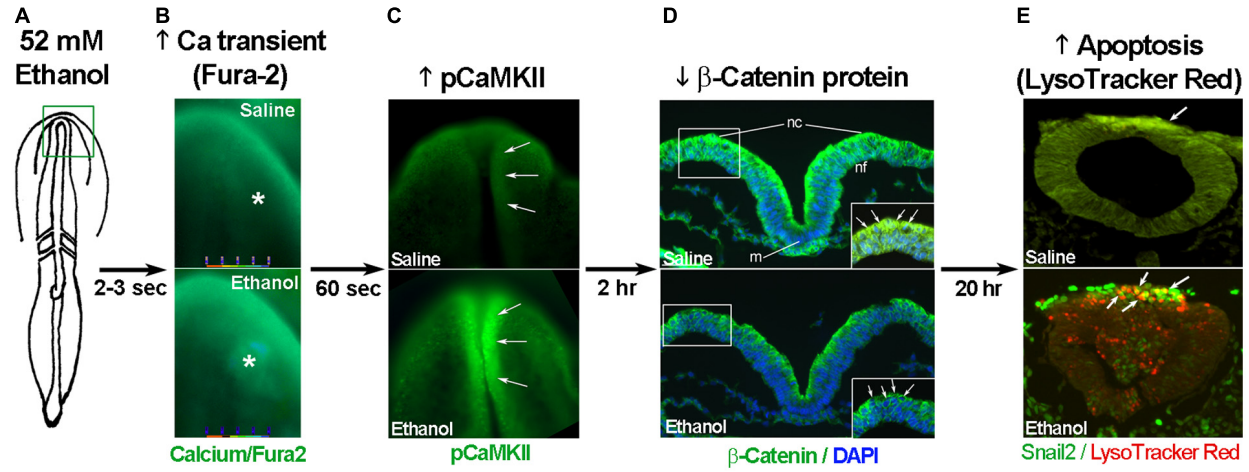

FIGURE 2 | Key events in ethanol-induced apoptosis of chick cranial neural crest. (A) Diagram of chick embryo having 3 somites (stage 8-). **(B)** Exposure to 52 mM ethanol instigates the mobilization of intracellular calcium stores (*) within the early headfold (boxed region) as quantified using Fura2. Shown is dorsal view with anterior to the top. **(C)** The calcium transient selectively activates CaMKII within the anterior neural folds including neural crest (arrows) as detected using antibody directed against phospho-CaMKII (green signal, arrows). A dorsal view of the headfolds is depicted, anterior to the top. **(D)** Among other targets, CaMKII phosphorylates and destabilizes β-catenin protein (green signal at green arrows in boxed region) within the dorsal neural folds enriched in neural crest. Shown is a transverse section, dorsal at top, through the headfold of embryos having three somites; blue indicates DAPI-stained nuclei. **(E)** Subsequently, there is significant apoptosis (red signal) within ethanol-exposed dorsal neuroprogenitors of the hindbrain including neural crest (strong green signal), detected using antibody against the neural crest marker *snail2*. The saline-treated control hindbrain displays little cell death. Shown is a transverse section through rhombomere 4, which normally lacks appreciable cell death, of embryos having 16–18 somites; dorsal roof is at the top. Chick embryos normally have a low-level green autofluorescent background.

et al., 2003; Garic-Stankovic et al., 2005). This calcium transient stimulates calmodulin and, within 1 min of ethanol exposure, the calmodulin-dependent kinase CaMKII is activated within neural crest and neuroprogenitors within the dorsal headfolds (**Figure 2C**, Garic et al., 2011). This action of ethanol is specific to these cells and CaMKII is not activated in ventral neural populations or in the presomitic mesoderm. CaMKII activation converts the short-lived calcium transient into a longer lived effector of neuroprogenitor fate, as CaMKII phosphorylates a number of downstream proteins within the cell. The induction of an intracellular calcium transient and CaMKII activity is essential and sufficient to produce neural crest apoptosis (**Figure 2E**).

Trophic support for early neural crest progenitors is provided by canonical Wnt signaling and its transcriptional effector β-catenin, which interacts with TCF/LEF proteins to induce gene expression (Kohn and Moon, 2005; MacDonald et al., 2009). Transcriptionally active β-catenin is indispensible for neural crest survival (Brault et al., 2001). Its transcriptional activity is negatively regulated through phosphorylation, which targets β-catenin for ubiquitination and proteolytic degradation. Ethanol's calcium transient destabilizes nuclear β-catenin within 2 h of ethanol addition (**Figure 2D**) and significantly reduces its transcriptional activity, as measured using TopFlash reporter constructs and quantitation of known Wnt target genes (Flentke et al., 2011). β-Catenin over-expression in ethanol-treated neural crest is sufficient to rescue their survival and prevent their apoptosis (Flentke et al., 2011), and agents that sequester calcium similarly stabilize β-catenin in ethanol's presence. Calcium is known to destabilize transcriptional β-catenin through multiple mechanisms including direct phosphorylation by protein kinase C, cleavage by calpain proteases, and indirectly through CaMKII phosphorylation of TCF/LEF. The GSK3β and JNK kinases can also directly phosphorylate and destabilize β-catenin (Kohn and Moon, 2005). Of those known effectors, only CaMKII inhibition stabilizes β-catenin and its transcriptional activity in ethanol-treated neural crest (Flentke et al., 2014a). Inhibiting other β-catenin effectors does not affect the protein's stability or cell survival in ethanol-treated cells. Moreover, CaMKII directly phosphorylates β-catenin at three evolutionarily conserved and previously uncharacterized sites at T332, T472, and S552. Thus, β-catenin is a novel target for CaMKII's kinase activity. Blocking any of the above steps within ethanol-exposed cells, using small molecules or targeted misexpression of loss/gain-of-function mutants within this pathway, fully prevents the apoptosis triggered by ethanol exposure (Debelak-Kragtorp et al., 2003; Garic-Stankovic et al., 2005; Flentke et al., 2011, 2014a; Garic et al., 2011).

Chick neural crest is not the only embryonic cell population in which an ethanol-induced calcium transient initiates apoptosis. Key elements of this pathway (calcium release, CaMKII activation **Figure 2E**) also mediate ethanol-induced apoptosis within zebrafish neural crest progenitors from equivalent developmental stages (Flentke et al., 2014b). Ethanol similarly invokes a pro-apoptotic, intracellular calcium transient within the gastrulating mouse, as well as in human cytotrophoblast cells and in mouse cerebellar neurons (Kilburn et al., 2006; Kouzoukas et al., 2013; Bolnick et al., 2014). Thus, this pro-apoptotic mechanism of ethanol's action appears to be evolutionarily conserved and occurs in diverse cell lineages, at least within the embryo and fetus. Ethanol also invokes this same phosphoinositide-stimulated calcium transient in the mouse morula/blastocyst; however, it does

not cause apoptosis and instead stimulates pathways that govern implantation and proliferative expansion (Winston and Maro, 1995). This suggests that how cells interpret ethanol's calcium transient is lineage dependent.

GENOMIC FACTORS MODIFY CALCIUM-MEDIATED NEURAL CREST APOPTOSIS

The serendipitous discovery of ethanol-sensitive and -resistant chicken strains provided novel insights into the mechanisms of this ethanol-mediated apoptosis. Layer flocks in the authors' poultry facility are replaced annually and one such exchange revealed that the birds' genetic background affects neural crest vulnerability to ethanol-induced apoptosis. As with inbred mice, commercial layer strains reproducibly fall along a continuum of ethanol responses. Some strains, such as Hy-Line W98 and W36, display high levels of neural crest apoptosis and a pronounced craniofacial dysmorphology, whereas other strains have little cell death and a relatively normal face (Debelak and Smith, 2000; Su et al., 2001). Vulnerability to ethanol-induced cardiac defects is similarly shaped by genetics (Cavieres and Smith, 2000). The ethanol content of eggs and embryos is equivalent and cannot account for this differential vulnerability. Because commercial chick strains are derived from hybrid crosses of sib-grandparent stocks, it is impractical to identify potential loci using traditional genetic breeding approaches. We therefore turned to deep DNA/RNA sequencing to characterize the transcriptomes of ethanol-sensitive and -resistant strains. The *Gallus gallus* genome was among the first to be sequenced and it is sufficiently annotated for detailed genetic analysis.

This comparison was accomplished using a unique genetic resource, two related chicken lines of the Hy-Line W98 strain that were selected for multiple traits affecting egg production. These lines were maintained as distinct closed flocks for perhaps as many as 40 generations. Line W98S (so designated because it originated from Hy-Line's Spencer, IA, facility) displays a robust calcium transient and high apoptosis in response to ethanol challenge (**Figure 3**; Garic et al., 2014), and it was utilized in many of the authors' ethanol publications in the decade following 2000. In contrast, the related W98 line W98D (originating from Hy-Line's Dallas Center, IA, facility) has ethanol-invoked calcium transients that are 30–40% lower than those of W98S at exposure

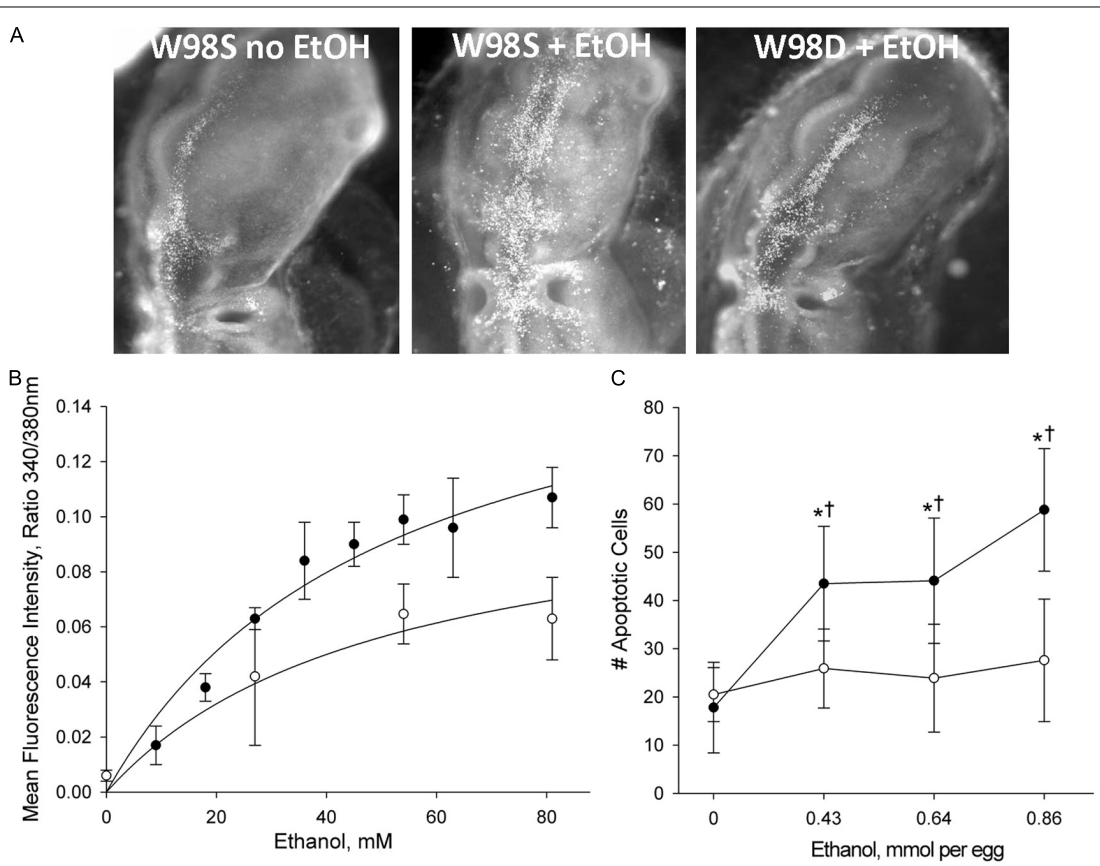

FIGURE 3 | Chick lines of the W98 background differ in their sensitivity to ethanol-induced apoptosis. (A) There is appreciably more ethanol-induced cell death (white dots) in neural crest and neuroprogenitors in line W98S as compared with ethanol-treated W98D. Cell death levels in the latter do not differ from that in saline-treated controls. Here, cell death is visualized using acridine orange, which detects apoptosis in this model; dorsal view is shown. **(B)** W98S embryos (•) mobilize greater intracellular calcium concentrations (quantified using Fura2) in response to ethanol challenge than do W98D embryos (○) at equivalent ethanol exposures. Their calcium release is dose-dependent. **(C)** W98S (•) has significantly more apoptotic cells than does W98D (○) in response to equivalent ethanol exposures. Values are mean ± SD for 3–8 embryos/treatment; $^{*}p < 0.001$ vs. 0 mM ethanol for within-strain comparison; $^{\dagger}p < 0.001$ between W98S/W98D at equivalent ethanol dose.

to equivalent ethanol concentrations. Both strains achieve different plateau values, indicating that their differential response is not due to a shifted dose–response curve. Non-linear regression of these dose-response curves finds that their ethanol-induced calcium responses share similar Kds (51 mM vs. 55 mM) and different maxima (**Figure 3B**; Garic et al., 2014). This suggests that their neural crest progenitors possess ethanol-binding sites with similar affinities and that they differ in transduction of the ethanol signal.

High-throughput transcriptome sequencing (RNA-Seq) of neural crest-enriched headfolds from W98S and W98D identified genomic difference that might influence ethanol sensitivity. Cells were not exposed to ethanol, so as to identify baseline differences that might potentially shape calcium and/or β-catenin signaling. The analysis identified 363 genes that are differentially expressed between W98S and W98D (Garic et al., 2014). Of these, 171 genes (47.1%) are increased in W98S and 192 genes (52.9%) are significantly decreased. Additionally, 18 genes within the Wnt/β-catenin signaling pathway have significantly differential expression. Importantly, the ethanol-sensitive W98S cells have significantly reduced expression of β-catenin itself (0.916-fold vs. W98D, $p = 0.00588$), as well as the two calmodulin isoforms that detect the calcium transient in these cells (CALM, 0.898-fold, $p = 0.0170$; CALM2, 0.836-fold, $p = 0.060$). W98S also has increased expression of two distinct Wnt/β-catenin antagonists, SHISA2 (1.106-fold, $p = 2.34 \times 10^{-7}$) and the secreted frizzled receptor protein SFRP2 (1.193-fold, $p = 7.08 \times 10^{-8}$), which is normally enriched in hindbrain neural crest progenitors fated for apoptosis (Ellies et al., 2002). Although these different expression levels represent transcripts and not protein, it suggests that cells derived from these two lines have foundational differences in how they perform Wnt/β-catenin-dependent signaling (**Figure 4**, left). In ethanol-sensitive W98S, the reduced β-catenin expression coupled with elevations in two canonical Wnt antagonists could dampen β-catenin's transcriptional activity within its neural crest as compared with W98D. Similarly, their lower content of both calmodulin isoforms could alter their respective calcium signaling dynamics. Taken together, these changes could shift the ethanol dose–response curve such that W98D cells are buffered against β-catenin transcriptional losses, whereas W98S is more vulnerable to those losses. One caveat to this analysis is that these headfolds are composed of neural crest and neuroprogenitors and thus not all these genes are present in neural crest. However, Wnt signaling genes such as β-catenin and SFRP2 are restricted to neural crest at these stages and their identification in this model likely informs their cellular responses.

Additional analysis of this gene set using the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed 10 biologically characterized pathways with significantly differential representation between the two lines (**Table 1**; Garic et al., 2014). These pathways, in turn, largely contribute to two major cellular networks. In the first, four of these pathways mediate the flow of cellular information from nucleus to cytosol and included the spliceosome ($p = 7.02 \times 10^{-8}$), RNA transport ($p = 0.00676$), ribosome ($p = 1.85 \times 10^{-47}$), and protein processing in the endoplasmic reticulum ($p = 0.00109$) gene clusters. The second differentially represented network includes two pathways that govern energy generation and include metabolism ($p = 0.00983$) and oxidative phosphorylation ($p = 1.10 \times 10^{-11}$), and near

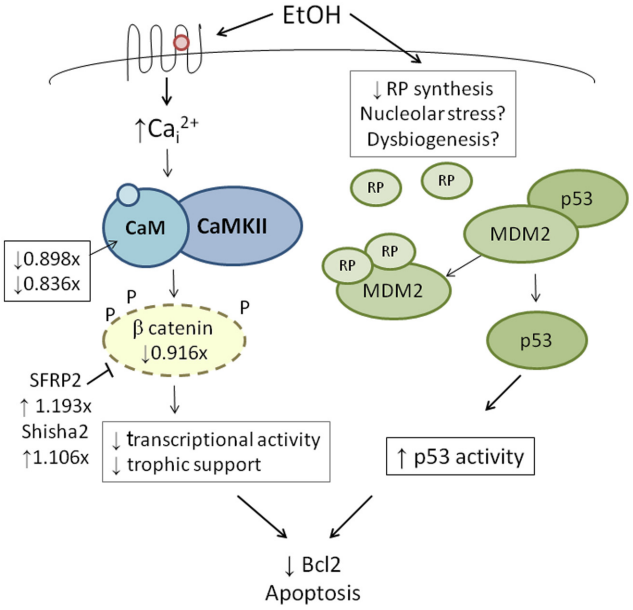

FIGURE 4 | Integration of genetic findings with known ethanol-induced signals in neural crest. The left panel shows how the expressions of genes that participate in ethanol's apoptosis are altered in within ethanol-sensitive W98S as compared against ethanol-resistant W98D. Fold-changes in expression are presented. The right panel proposes a mechanism by which ribosome dysgenesis could interact with MDM2 to stimulate p53-mediated apoptosis. In response to cellular stress such as ethanol, a decrease in ribosome synthesis leads to ribosome proteins interacting with MDM2. MDM2 no longer silences p53 and permits p53-mediated activities, including apoptosis, to increase within cells.

Table 1 | KEGG pathway enrichments in W98S vs. W98D.

KEGG	Identity	No. of genes	Significance
3010	Ribosomal	38	1.85×10^{-47}
190	Oxidative phosphorylation	18	1.10×10^{-11}
4260	Cardiac muscle	10	5.71×10^{-9}
3040	Spliceosome	13	7.02×10^{-8}
4141	Protein processing in ER	9	0.00109
4530	Epithelial tight junctions	6	0.00309
4114	Oocyte meiosis	6	0.00585
3013	RNA transport	7	0.00676
1100	Metabolism	25	0.00983
4110	Cell cycle	6	0.0140
10	Glycolysis/gluconeogenesis	18	0.0530
4145	Phagosome	5	0.0600

Data from Garic et al. (2014).

significance for glycolysis/gluconeogenesis ($p = 0.0530$). Also differentially represented are KEGG pathways involving the cell cycle ($p = 0.0140$) and epithelial tight junctions ($p = 0.00309$).

NEURAL CREST AND RIBOSOME BIOGENESIS

The most compelling finding from the RNA-Seq comparison of ethanol-vulnerable/resistant neuroprogenitors is the significant differential enrichment of 38 genes that encode ribosome proteins. This includes 27 large and 11 small ribosomal subunit proteins (**Figure 5**), as well as two additional genes that participate in ribosome biogenesis, pescadillo (PES-1, 1.198-fold, $p = 0.0145$) and NSA2 (1.340-fold, $p = 3.81 \times 10^{-6}$). Of these ribosomal proteins, 25 were decreased and 13 increased in ethanol-sensitive W98S. Remarkably, ribosomal gene clusters were also significantly altered in two independent studies of ethanol exposure to mouse neural folds of comparable developmental stages to our chick embryos (Green et al., 2007; Downing et al., 2012b; see this volume). Ribosomal gene clusters additionally emerged from independent studies of ethanol-treated neuronal cultures (Rahman and Miles, 2001; Gutala et al., 2004). Perhaps, further supporting its importance, haploinsufficiency in the methionyl tRNA synthetase (*mars*), which donates the first amino acid to initiate protein translation, also heightens embryo sensitivity to ethanol-induced craniofacial deficits (Swartz et al., 2014). The repeated emergence of ribosomal protein clusters from multiple comparisons of ethanol-treated neuroprogenitors suggests that ribosomal activity may be an integral component of cellular ethanol responses.

Why are ribosomal proteins potentially relevant for neural crest apoptosis? Ribosome synthesis is complex (Granneman and Baserga, 2004). Ribosomes convert messenger

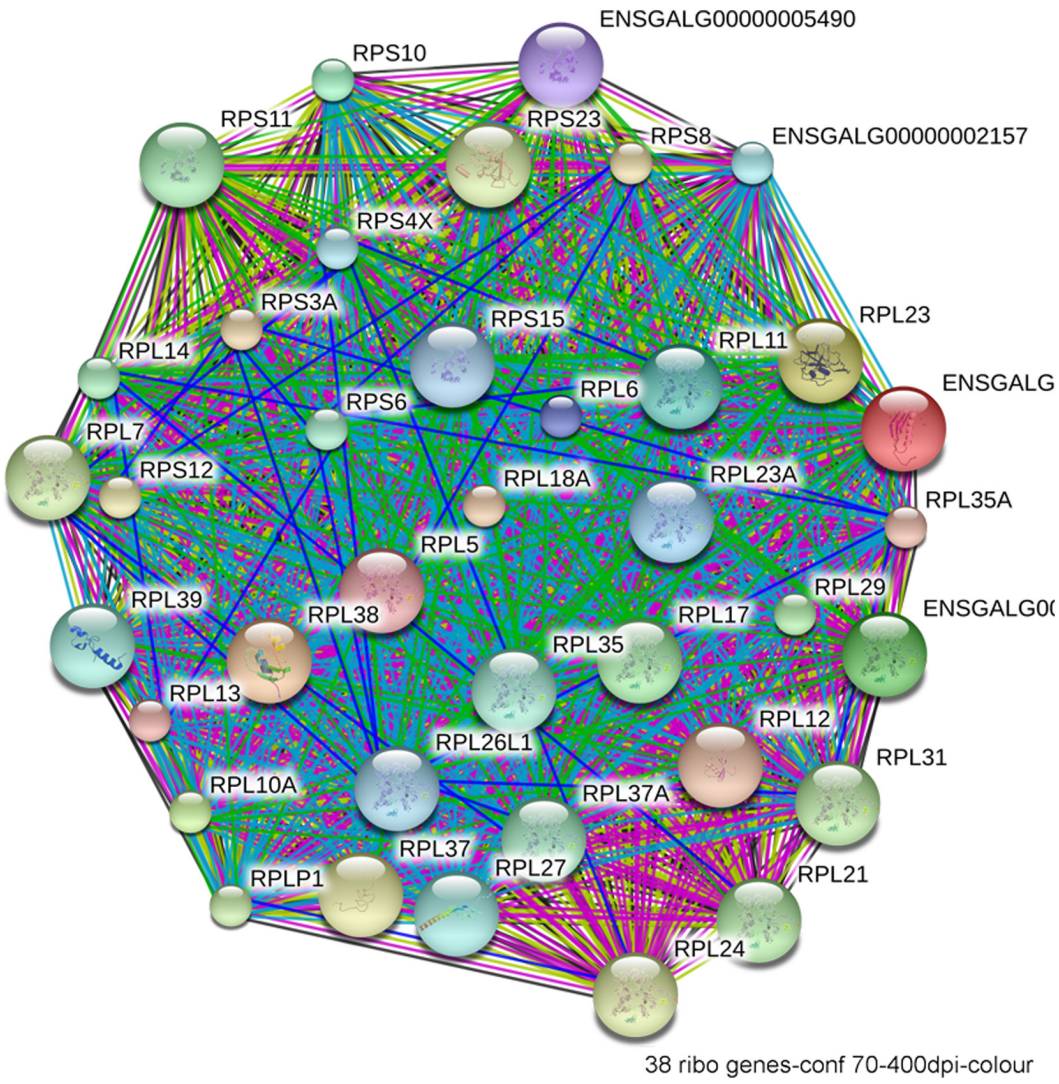

FIGURE 5 | Network interactions from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) depict the relationships between the 38 ribosomal proteins that have significantly different expression between ethanol-sensitive W98S and ethanol-resistant W98D neural folds.

RNA (mRNA) information into protein. Composed of four ribosomal RNAs (rRNA) and approximately 80 proteins, ribosomes bind mRNA, provide docking sites for codon recognition by amino acyl-transfer RNA, and catalyze the synthesis of nascent protein. The cell nucleolus denotes locations of polycistronic rRNA synthesis, processing, and ribosome assembly. The rRNA emerges as a single transcript that is then modified by small nucleolar RNAs (snoRNAs) and protein cofactors that mediate cleavage, methylation, and pseudouridylation of the rRNA. Ribosome biogenesis imposes a significant energy demand and, for rapidly proliferating cells, occupies as much as 80% of the energy budget because cells must disassemble the nucleolus and replenish their ribosomes with each mitotic division (Schmidt, 1999).

Given its high use of cellular resources, ribosome biogenesis has been recently recognized as an important sensor of cellular stress through its regulation of p53 activity (**Figure 4**, right; Kruse and Gu, 2009; Fumagalli and Thomas, 2011). The tumor suppressor protein p53 accumulates in response to stress to effect cell cycle arrest, apoptosis, DNA repair, and other processes. Interactions with the E3 ubiquitin ligase MDM2 silence p53 through MDM2-catalyzed ubiquitination and proteosomal destruction. When ribosome biogenesis is perturbed, as through reduced ATP availability or imbalanced ribosome protein content, ribosomal proteins such as RPL5, RPL11, RPL23, and RPS7 instead interact with MDM2 to suppress its ubiquitinase activity and thereby stabilize p53 and stimulate p53-mediated activities including cell cycle arrest and apoptosis.

Defects in ribosome biogenesis are clinically significant. Human ribosomopathies are not embryolethal and are associated with anemia, short stature, limb and heart defects, and, of relevance to this discussion, can feature significant craniofacial deficits including a flattened nasal bridge, micrognathia, epicanthal folds, cleft lip/palate, and altered palpebral fissues (Narla and Ebert, 2010; Fumagalli and Thomas, 2011). These facial changes echo those of FASD and suggest that cranial neural crest is especially sensitive to ribosome dysbiogenesis. Animal models confirm that loss-of-function mutations in ribosome proteins or effectors of ribosome biogenesis can cause cell cycle arrest and p53-mediated apoptosis within neural crest progenitors, the best described of these being Treacher-Collins syndrome (Trainor, 2010). Five of the differentially expressed ribosome proteins in W98S/D headfolds are known to regulate MDM2/p53 interactions (**Table 2**; RPL5, RPL11, RPL12, RPS15, RPL23). Additionally, nine of these ribosomal proteins are causative in the craniofacial ribosomopathy Diamond–Blackfan anemia (RPL5, RPL11, RPL26, RPL27, RPL35A, RPL36, RPS10, RPS15, RPS17). Additional attention to ribosome biogenesis arises from the analysis of these same cell populations 6 h following ethanol challenge, wherein the greatest gene cluster change again involves ribosome biogenesis ($p = 2.2 \times 10^{-21}$; Garic et al., 2014). Similar to the afore-mentioned reductions in *shh* signaling (Ahlgren et al., 2002) or in *Pdgfra* (McCarthy et al., 2013), genomic-level alterations in ribosomal proteins might disturb the balance of MDM2/p53 regulation that, in of itself, is insufficient to initiate apoptosis, but does so upon the additional stress of ethanol challenge (**Figure 4**). Indeed, interactions between mTOR and ribosome biogenesis control both cellular anabolism and decisions regarding p53 activity (reviewed in Shimobayashi and Hall, 2014), and it is tempting to speculate that these mTOR and ribosome/p53 findings have identified different aspects of the same mechanism. Given the established role for calcium/β-catenin signals in this model of ethanol-induced apoptosis, we additionally speculation that the combination of diminished β-catenin activity and ribosome dysbiogenesis may interact to stimulate these cells' pro-apoptotic fate. Studies are underway to evaluate this hypothesis in detail.

Table 2 | Differentially expressed RPs linked with human ribosomopathies.

Gene	Fold-change W98S/D	*p*-Value adjusted	MDM2/p53 effector	DBA-linked
RPL5	1.24	4.72×10^{-19}	X	X
RPL11	0.88	0.00555	X	X
RPL12	0.75	4.63×10^{-23}	X	–
RPL23	0.87	2.56×10^{-6}	X	–
RPL26	0.89	0.00182	–	X
RPL27	0.83	1.27×10^{-21}	–	X
RPL35A	0.85	5.01×10^{-8}	–	X
RPL36	0.49	3.69×10^{-34}	–	X
RPS10	0.93	0.0226	–	–
RPS15	1.17	1.67×10^{-5}	X	X
RPS17	0.765	6.43×10^{-13}	–	X

Adapted from Narla and Ebert (2010), Fumagalli and Thomas (2011), and Garic et al. (2014).

ENERGY METABOLISM AND APOPTOSIS

Excessive ethanol suppresses energy metabolism through the competition for cellular reducing equivalents, through inhibition of lipolysis, and through direct effects upon mitochondrial activity (Bunout, 1999). Embryos have a high energy demand due to their obvious anabolic and pro-proliferative state. The avian embryo's primary energy source is triglyceride β-oxidation and thus oxidative phosphorylation; unlike mammals, yolk-bearing embryos can convert acetyl-CoA subunits into glucose via gluconeogenesis. Ethanol-vulnerable cells from W98S had significant up-regulation of multiple components of oxidative phosphorylation including many proteins in complex I/NADH dehydrogenase, cytochrome c oxidase, and the ATP synthase (**Figure 6**). However, several components of the cytochrome c reductase and cytochrome c oxidase were significantly lower compared with W98D. It is possible that these changes altered the metabolic flux within W98S relative to W98D, but unfortunately those embryos are no longer available to test this hypothesis. These metabolic differences might also be linked to the ribosomal changes between these strains, given the high energy cost of ribosome biogenesis and its role as a cellular stress sensor. It is likely that these differences in energy metabolism resulted from commercial pressures during line selection, because efficiency in nutrient

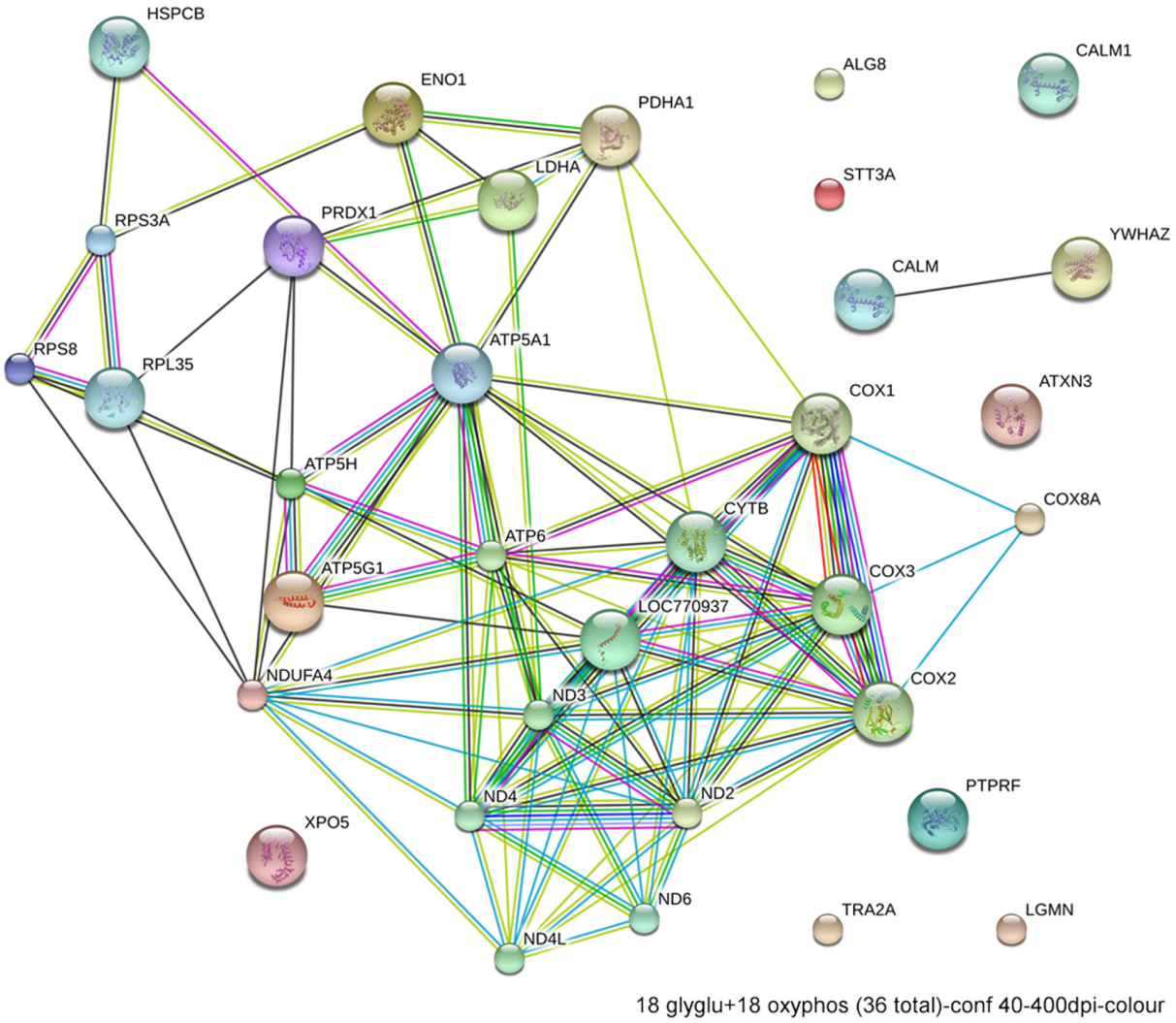

FIGURE 6 | Network interactions from DAVID depict the relationships between the 18 glycolytic and 18 oxidative phosphorylation genes that have significantly different expression between ethanol-sensitive W98S and ethanol-resistant W98D neural folds.

utilization and feed conversion are desirable agricultural traits to improve growth and production. Similarly, the emphasis on improved growth may have also created efficiencies in ribosome biogenesis. Given the importance of energy generation for the rapidly growing embryo, relative differences in energy metabolism may have influenced cellular vulnerability to ethanol-induced stress.

SUMMARY

In summary, genetic approaches have informed the multiple mechanisms by which ethanol disrupts craniofacial morphogenesis. The use of haploinsufficient mice has demonstrated how PAE suppresses shh signaling and generates holoprosencephalic features when exposure occurs during gastrulation. Zebrafish mutants implicate additional mechanisms through effects upon mTOR signaling and cell cycle regulation. Transcriptome sequencing of both mouse and avian embryos with differing ethanol sensitivities has further identified novel potential mechanisms including energy metabolism and/or ribosome biogenesis; in turn, these could be related to each other as well to contributions from mTOR. That ribosomal dysregulation also emerged from multiple, independent analyses of ethanol responsive genes in mouse neural folds make ribosome biogenesis a strong candidate to modify and contribute to ethanol's damage. Our findings suggest that genetic-level differences in neural crest vulnerability to PAE may partially explain why only a percentage of ethanol-exposed pregnancies exhibit a postnatal craniofacial dysmorphology. Next-generation sequencing offers a rapid and comparatively affordable approach to identify additional genomic candidates that modify craniofacial responses to ethanol. Together with advances in epigenetics, this research will greatly advance our understanding of how genes and environment interact to shape individual outcomes in FASD.

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REFERENCES

Ahlgren, S. C., Thakur, V., and Bronner-Fraser, M. (2002). Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10476–10481. doi: 10.1073/pnas.162356199

Anthony, B., Vinci-Booher, S., Wetherill, L., Ward, R., Goodlett, C., and Zhou, F. C. (2010). Alcohol-induced facial dysmorphology in C57BL/6 mouse models of fetal alcohol spectrum disorder. *Alcohol* 44, 659–671. doi: 10.1016/j.alcohol.2010.04.002

Aoto, K., Shikata, Y., Higashiyama, D., Shiota, K., and Motoyama, J. (2008). Fetal ethanol exposure activates protein kinase A and impairs shh expression in prechordal plate mesoderm cells in the pathogenesis of holoprosencephaly. *Birth Defects Res. A Clin. Mol. Teratol.* 82, 224–231. doi: 10.1002/bdra.20447

Blader, P., and Strahle, U. (1998). Ethanol impairs migration of the prechordal plate in the zebrafish embryo. *Dev. Biol.* 201, 185–201. doi: 10.1006/dbio.1998.8995

Boehm, S. L. 2nd, Lundahl, K. R., Caldwell, J., and Gilliam, D. M. (1997). Ethanol teratogenesis in the C57BL/6J, DBA/2J, and A/J inbred mouse strains. *Alcohol* 14, 389–395. doi: 10.1016/S0741-8329(97)87950-5

Bolnick, J. M., Karana, R., Chiang, P. J., Kilburn, B. A., Romero, R., Diamond, M. P., Smith, S. M., et al. (2014). Apoptosis of alcohol-exposed human placental cytotrophoblast cells is downstream of intracellular calcium signaling. *Alcohol. Clin. Exp. Res.* 38, 1646–1653. doi: 10.1111/acer.12417 [Epub ahead of print].

Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D. H., McMahon, A. P., et al. (2001). Inactivation of the β-catenin gene by Wnt-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* 128, 1253–1264.

Bunout, D. (1999). Nutritional and metabolic effects of alcoholism: their relationship with alcoholic liver disease. *Nutrition* 15, 583–589. doi: 10.1016/S0899-9007(99)00090-8

Bupp Becker, S. R., and Shibley, I. A. Jr. (1998). Teratogenicity of ethanol in different chicken strains. *Alcohol Alcohol.* 33, 457–464. doi: 10.1093/alcalc/33.5.457

Cartwright, M. M., and Smith, S. M. (1995). Stage dependent effects of ethanol on cranial neural crest cell development: partial basis for the phenotypic variations observed in fetal alcohol syndrome. *Alcohol. Clin. Exp. Res.* 19, 1454–1462. doi: 10.1111/j.1530-0277.1995.tb01007.x

Cartwright, M. M., Tessmer, L. A., and Smith, S. M. (1998). Ethanol-induced neural crest apoptosis is coincident with their endogenous death but is mechanistically distinct. *Alcohol. Clin. Exp. Res.* 22, 142–149. doi: 10.1111/j.1530-0277.1998.tb03630.x

Carvan, M. J. III, Loucks, E., Weber, D. N., and Williams, F. E. (2004). Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. *Neurotoxicol. Teratol.* 26, 757–768. doi: 10.1016/j.ntt.2004.06.016

Cavieres, M. F., and Smith, S. M. (2000). Genetic and developmental modulation of cardiac deficits in prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 24, 102–109. doi: 10.1111/j.1530-0277.2000.tb04559.x

Chen, S. Y., Periasamy, A., Yang, B., Herman, B., Jacobson, K., and Sulik, K. K. (2000). Differential sensitivity of mouse neural crest cells to ethanol-induced toxicity. *Alcohol* 20, 75–81. doi: 10.1016/S0741-8329(99)00058-0

Chen, X., Liu, J., and Chen, S. Y. (2013). Over-expression of Nrf2 diminishes ethanol-induced oxidative stress and apoptosis in neural crest cells by inducing an antioxidant response. *Reprod. Toxicol.* 42, 102–109. doi: 10.1016/j.reprotox.2013.08.003

Debelak, K. A., and Smith, S. M. (2000). Avian genetic background modulates the neural crest apoptosis induced by ethanol exposure. *Alcohol. Clin. Exp. Res.* 24, 307–314. doi: 10.1111/j.1530-0277.2000.tb04612.x

Debelak-Kragtorp, K. A., Armant, D. R., and Smith, S. M. (2003). Ethanol-induced cephalic apoptosis requires phospholipase C-dependent intracellular calcium signaling. *Alcohol. Clin. Exp. Res.* 27, 515–523. doi: 10.1097/01.ALC.0000056615.34253.A8

Downing, C., Balderrama-Durbin, C., Broncucia, H., Gilliam, D., and Johnson, T. E. (2009). Ethanol teratogenesis in five inbred strains of mice. *Alcohol. Clin. Exp. Res.* 33, 1238–1245. doi: 10.1111/j.1530-0277.2009.00949.x

Downing, C., Balderrama-Durbin, C., Kimball, A., Biers, J., Wright, H., Gilliam, D., et al. (2012a). Quantitative trait locus mapping for ethanol teratogenesis in BXD recombinant inbred mice. *Alcohol. Clin. Exp. Res.* 36, 1340–1354. doi: 10.1111/j.1530-0277.2012.01754.x

Downing, C., Flink, S., Florez-McClure, M. L., Johnson, T. E., Tabakoff, B., and Kechris, K. J. (2012b). Gene expression following changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 36, 1519–1529. doi: 10.1111/j.1530-0277.2012.01757.x

Dubourg, C., Bendavid, C., Pasquier, L., Henry, C., Odent, S., and David, V. (2007). Holoprosencephaly. *Orphanet. J. Rare Dis.* 2:8. doi: 10.1186/1750-1172-2-8

Dunty, W. C. Jr., Chen, S. Y., Zucker, R. M., Dehart, D. B., and Sulik, K. K. (2001). Selective vulnerability of embryonic cell populations to ethanol-induced apoptosis: implications for alcohol-related birth defects and neurodevelopmental disorder. *Alcohol. Clin. Exp. Res.* 36, 1340–1354.

Edenberg, H. J., and Foroud, T. (2013). Genetics and alcoholism. *Nat. Rev. Gastroenterol. Hepatol.* 10, 487–494. doi: 10.1038/nrgastro.2013.86

Ellies, D. L., Tucker, A. S., and Lumsden, A. (2002). Apoptosis of premigratory neural crest cells in rhombomeres 3 and 5: consequences for patterning of the branchial region. *Dev. Biol.* 251, 118–128. doi: 10.1006/dbio.2002.0815

Flentke, G. R., Garic, A., Amberger, E., Hernandez, M., and Smith, S. M. (2011). Calcium-mediated repression of β-catenin and its transcriptional signaling mediates neural crest cell death in an avian model of fetal alcohol syndrome. *Birth Defects Res. A Clin. Mol. Teratol.* 91, 591–602. doi: 10.1002/bdra.20833

Flentke, G. R., Garic, A., Hernandez, M., and Smith, S. M. (2014a). CaMKII represses transcriptionally-active β-catenin to mediate acute ethanol neurodegeneration and can phosphorylate β-catenin. *J. Neurochem.* 128, 523–535. doi: 10.1111/jnc.12464

Flentke, G. R., Klingler, R. H., Tanguay, R. L., Carvan, M. J. III, and Smith, S. M. (2014b). An evolutionarily-conserved mechanism of calcium-dependent neurotoxicity in a zebrafish model of FASD. *Alcohol. Clin. Exp. Res.* 38, 1255–1265. doi: 10.1111/acer.12360

Fumagalli, S., and Thomas, G. (2011). The role of p53 in ribosomopathies. *Sem. Hematol.* 48, 97–105. doi: 10.1053/j.seminhematol.2011.02.004

Garic, A., Berres, M. E., and Smith, S. M. (2014). High-throughput transcriptome sequencing identifies candidate genetic modifiers of vulnerability to fetal alcohol spectrum disorders. *Alcohol. Clin. Exp. Res.* 38, 1874–1882. doi: 10.1111/acer.12457

Garic, A., Flentke, G. R., Amberger, E., Hernandez, M., and Smith, S. M. (2011). CaMKII activation is a novel effector of alcohol's neurotoxicity in neural crest stem/progenitor cells. *J. Neurochem.* 118, 646–657. doi: 10.1111/j.1471-4159.2011.07273.x

Garic-Stankovic, A., Hernandez, M. R., Chiang, P. J., Debelak-Kragtorp, K. A., Flentke, G. R., Armant, D. R., et al. (2005). Ethanol triggers neural crest apoptosis through the selective activation of a pertussis toxin-sensitive G protein and a phospholipase Cβ-dependent Ca2+ transient. *Alcohol. Clin. Exp. Res.* 29, 1237–1246. doi: 10.1097/01.ALC.0000172460.05756.D9

Garic-Stankovic, A., Hernandez, M., Flentke, G. R., and Smith, S. M. (2006). Structural constraints for alcohol-stimulated Ca2+ release in neural crest, and dual agonist/antagonist properties of n-octanol. *Alcohol. Clin. Exp. Res.* 30, 552–559. doi: 10.1111/j.1530-0277.2005.00061.x

Gilliam, D. M., and Kotch, L. E. (1996). Dose-related growth deficits in LS but not SS mice prenatally exposed to alcohol. *Alcohol* 13, 47–51. doi: 10.1016/0741-8329(95)02010-1

Goodlett, C. R., Gillam, D. M., Nichols, J. M., and West, J. R. (1989). Genetic influences on brain growth restriction induced by developmental exposure to alcohol. *Neurotoxicity* 10, 321–334.

Granneman, S., and Baserga, S. J. (2004). Ribosome biogenesis: of knobs and RNA processing. *Exp. Cell Res.* 296, 43–50. doi: 10.1016/j.yexcr.2004.03.016

Green, M. L., Singh, A. V., Zhang, Y., Nemeth, K. A., Sulik, K. K., and Knudsen, T. B. (2007). Reprogramming of genetic networks during initiation of the fetal alcohol syndrome. *Dev. Dyn.* 236, 613–631. doi: 10.1002/dvdy.21048

Gutala, R., Wang, J., Kadapakkam, S., Hwang, Y., Ticku, M., and Li, M. D. (2004). Microarray analysis of ethanol-treated cortical neurons reveals disruption of

genes related to the ubiquitin-proteasome pathway and protein synthesis. *Alcohol. Clin. Exp. Res.* 28, 1779–1788. doi: 10.1097/01.ALC.0000148117.17707.B4

Hassler, J. A., and Moran, D. J. (1986). Effects of ethanol on the cytoskeleton of migrating and differentiating neural crest cells: possible role in teratogenesis. *J. Craniofac. Genet. Dev. Biol.* (Suppl. 2), 129–136.

Hong, M., and Krauss, R. S. (2012). Codon mutation and fetal ethanol exposure synergize to produce midline signaling defects and holoprosencephaly spectrum disorders in mice. *PLoS Genet.* 8:e1002999. doi: 10.1371/journal.pgen.1002999

Howard, R. J., Slesinger, P. A., Davies, D. L., Das, J., Trudell, J. R., and Harris, R. A. (2011). Alcohol-binding sites in distinct brain proteins: the quest for atomic level resolution. *Alcohol. Clin. Exp. Res.* 35, 561–1573. doi: 10.1111/j.1530-0277.2011.01502.x

Kietzman, H. W., Everson, J. L., Sulik, K. K, and Lipinski, R. J. (2014). The teratogenic effects of prenatal ethanol exposure are exacerbated by Sonic Hedgehog or GLI2 haploinsufficiency in the mouse. *PLoS ONE* 9:e89448. doi: 10.1371/journal.pone.0089448

Kilburn, B. A., Chiang, P. J., Wang, J., Flentke, G. R., Smith, S. M., and Armant, D. R. (2006). Rapid induction of apoptosis in gastrulating mouse embryos by ethanol and its prevention by HB-EGF. *Alcohol. Clin. Exp. Res.* 30, 127–134. doi: 10.1111/j.1530-0277.2006.00008.x

Klingenberg, C. P., Wetherill, L., Rogers, J., Moore, E., Ward, R., Autti-Rämö, I., et al. (2010). Prenatal alcohol exposure alters the patterns of facial asymmetry. *Alcohol* 44, 649–457. doi: 10.1016/j.alcohol.2009.10.016

Kohn, A. D., and Moon, R. T. (2005). Wnt and calcium signaling: β-catenin-independent pathways. *Cell Calcium* 38, 439–446. doi: 10.1016/j.ceca.2005.06.022

Kouzoukas, D. E., Li, G., Takapoo, M., Moninger, T., Bhalla, R. C., and Pantazis, N. J. (2013). Intracellular calcium plays a critical role in the alcohol-mediated death of cerebellar granule neurons. *J. Neurochem.* 124, 323–335. doi: 10.1111/jnc.12076

Kruse, J. P., and Gu, W. (2009). Modes of p53 regulation. *Cell* 137, 3907–3920. doi: 10.1016/j.cell.2009.04.050

Li, Y. X., Yang, H. T., Zdanowicz, M., Sicklick, J. K., Qi, Y., Camp, T. J., et al. (2007). Fetal alcohol exposure impairs Hedgehog cholesterol modification and signaling. *Lab. Invest.* 87, 231–240. doi: 10.1038/labinvest.3700516

Lipinski, R. J., Hammond, P., O'Leary-Moore, S. K., Ament, J. J., Pecevich, S. J., Jiang, Y., et al. (2012). Ethanol-induced face-brain dysmorphology patterns are correlative and exposure-stage dependent. *PLoS ONE* 7:e43067. doi: 10.1371/journal.pone.0043067

Loucks, E., and Carvan, M. J. III. (2004). Strain-dependent effects of developmental ethanol exposure in zebrafish. *Neurotoxicol. Teratol.* 26, 745–755. doi: 10.1016/j.ntt.2004.06.017

MacDonald, B. T., Tamai, K., and He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17, 9–26. doi: 10.1016/j.devcel.2009.06.016

McCarthy, N., Wetherill, L., Lovely, C. B., Swartz, M. E., Foroud, T. M., and Eberhart, J. K. (2013). Pdgfra protects against ethanol-induced craniofacial defects in a zebrafish model of FASD. *Development* 140, 3254–3265. doi: 10.1242/dev.094938

Narla, A., and Ebert, B. L. (2010). Ribosomopathies: human disorders of ribosome dysfunction. *Blood* 115, 3196–3205. doi: 10.1182/blood-2009-10-178129

Oyedele, O. O., and Kramer, B. (2013). Nuanced but significant: how ethanol perturbs avian cranial neural crest cell actin cytoskeleton, migration and proliferation. *Alcohol* 47, 417–426. doi: 10.1016/j.alcohol.2013.04.001

Rahman, S., and Miles, M. F. (2001). Identification of novel ethanol-sensitive genes by expression profiling. *Pharmacol. Ther.* 92, 123–134. doi: 10.1016/S0163-7258(01)00163-2

Reimers, M. J., La Du, J. K., Periera, C. B., Giovanini, J., and Tanguay, R. L. (2006). Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. *Neurotoxicol. Teratol.* 28, 497–508. doi: 10.1016/j.ntt.2006.05.007

Rovasio, R. A., and Battiato, N. L. (2002). Ethanol induces morphological and dynamic changes on in vivo and in vitro neural crest cells. *Alcohol. Clin. Exp. Res.* 26, 1286–1298. doi: 10.1111/j.1530-0277.2002.tb02669.x

Sauka-Spengler, T., and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* 9, 557–568. doi: 10.1038/nrm2428

Schmidt, E. V. (1999). The role of c-myc in cellular growth control. *Oncogene* 18, 2988–2996. doi: 10.1038/sj.onc.1202751

Shimobayashi, M., and Hall, M. N. (2014). Making new contacts: the mTOR network in metabolism and signaling crosstalk. *Nat. Rev. Mol. Cell Biol.* 15, 155–162. doi: 10.1038/nrm3757

Smith, S. M., Garic, A., Flentke, G. R., and Berres, M. E. (2014). Neural crest development in fetal alcohol syndrome. *Birth Defects Res. C* (in press).

Streissguth, A. P., and Dehaene, P. (1993). Fetal alcohol syndrome in twins of alcoholic mothers: concordance of diagnosis and IQ. *Am. J. Med. Genet.* 47, 857–861. doi: 10.1002/ajmg.1320470612

Su, B., Debelak, K. A., Tessmer, L. L., Cartwright, M. M., and Smith, S. M. (2001). Genetic influences on craniofacial outcome in an avian model of prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 25, 60–69. doi: 10.1111/j.1530-0277.2001.tb02128.x

Sulik, K. K. (1984). "Critical periods for alcohol teratogenesis in mice, with special reference to the gastrulation stage of embryogenesis," in *Mechanisms of Alcohol Damage in Utero. Ciba Foundation Symposium* 105, eds Ruth Porter, Maeve O'Connor, and Julie Whelan (London: Pitman), 124–141.

Sulik, K. K., Johnston, M. C., and Webb, M. A. (1981). Fetal alcohol syndrome: embryogenesis in a mouse model. *Science* 214, 936–938. doi: 10.1126/science.6795717

Swartz, M. E., Wells, M. B., Griffin, M., McCarthy, N., Lovely, C. B., McGurk, P., et al. (2014). A screen of zebrafish mutants identifies ethanol-sensitive genetic loci. *Alcohol. Clin. Exp. Res.* 38, 694–703. doi: 10.1111/acer.12286

Trainor, P. A. (2010). Craniofacial birth defects: the role of neural crest cells in the etiology and pathogenesis of Treacher Collins syndrome and the potential for prevention. *Am. J. Med. Genet. Part A* 152A, 2984–2994. doi: 10.1002/ajmg.a.33454

Warren, K. R., and Li, T. K. (2005). Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Res. Part A* 73, 195–203. doi: 10.1002/bdra.20125

Wentzel, P., and Eriksson, U. J. (2008). Genetic influences on dysmorphogenesis in embryos from different rat strains exposed to ethanol in vivo and in vitro. *Alcohol. Clin. Exp. Res.* 32, 874–887. doi: 10.1111/j.1530-0277.2008.00647.x

Winston, N. J., and Maro, B. (1995). Calmodulin-dependent protein kinase II is activated transiently in ethanol-stimulated mouse oocytes. *Dev. Biol.* 170, 350–352. doi: 10.1006/dbio.1995.1220

Yan, D., Dong, J., Sulik, K. K., and Chen, S. Y. (2010). Induction of the Nrf2-driven antioxidant response by tert-butylhydroquinone prevents ethanol-induced apoptosis in cranial neural crest cells. *Biochem. Pharmacol.* 80, 144–149. doi: 10.1016/j.bcp.2010.03.004

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Reduced DNA methylation at the *PEG3 DMR* and *KvDMR1* loci in children exposed to alcohol *in utero*: a South African Fetal Alcohol Syndrome cohort study

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Fetal alcohol syndrome (FAS) is a devastating developmental disorder resulting from alcohol exposure during fetal development. It is a considerable public health problem worldwide and is characterized by central nervous system abnormalities, dysmorphic facial features, and growth retardation. Imprinted genes are known to play an important role in growth and development and therefore four imprinting control regions (ICRs), *H19 ICR*, *IG-DMR*, *KvDMR1* and *PEG3 DMR* were examined. It is proposed that DNA methylation changes may contribute to developmental abnormalities seen in FAS and which persist into adulthood. The participants included FAS children and controls from the Western and Northern Cape Provinces. DNA samples extracted from blood and buccal cells were bisulfite modified, the ICRs were amplified by PCR and pyrosequencing was used to derive a quantitative estimate of methylation at selected CpG dinucleotides: *H19 ICR* (six CpG sites; 50 controls and 73 cases); *KvDMR1* (7, 55, and 86); *IG-DMR* (10, 56, and 84); and *PEG3 DMR* (7, 50, and 79). The most profound effects of alcohol exposure are on neuronal development. In this study we report on epigenetic effects observed in blood which may not directly reflect tissue-specific alterations in the developing brain. After adjusting for age and sex (known confounders for DNA methylation), there was a significant difference at *KvDMR1* and *PEG3 DMR*, but not the *H19 ICR*, with only a small effect (0.84% lower in cases; $p = 0.035$) at *IG-DMR*. The two maternally imprinted loci, *KvDMR1* and *PEG3 DMR*, showed lower average locus-wide methylation in the FAS cases (1.49%; $p < 0.001$ and 7.09%; $p < 0.001$, respectively). The largest effect was at the *PEG3 DMR* though the functional impact is uncertain. This study supports the role of epigenetic modulation as a mechanism for the teratogenic effects of alcohol by altering the methylation profiles of imprinted loci in a locus-specific manner.

Keywords: fetal alcohol syndrome, imprinted genes, epigenetics, *PEG3, KvDMR1, H19 ICR, IG-DMR*

INTRODUCTION

Alcohol is a potent teratogen with devastating effects on the developing fetus. The most profound effects of prenatal alcohol exposure are on neuronal development, resulting in adverse cognitive and behavioral outcomes with lifelong implications, distinct dysmorphic features (shortened palpebral fissures, smooth philtrum, and thin vermilion border to the upper lip), and pre- and postnatal growth retardation (Stratton et al., 1996; Riley and McGee, 2005; Floyd et al., 2006). The outcomes are collectively referred to as fetal alcohol spectrum disorders (FASD) and range in severity with fetal alcohol syndrome (FAS) at the most severe end of the spectrum (Sokol et al., 2003). FAS is the leading cause of preventable mental retardation and developmental disability in the world. It is an international problem that shows no racial boundaries (Clarren and Smith, 1978; Masotti et al., 2006) and the consequences of prenatal alcohol exposure represent a major public health problem worldwide (May and Gossage, 2001; Sokol et al., 2003; Riley et al., 2011).

The worldwide average prevalence of FAS is estimated at 0.97 per 1000 live births, yet in some communities it is much higher (Abel and Hannigan, 1995; May and Gossage, 2001; McKinstry, 2005). Notably, the prevalence of FAS in South Africa is one of the highest reported in the world, at 40.5–46.4 per 1000 children of school going age in the Western Cape Province (May et al., 2000),

confirmed in two follow up studies from the same area reporting an increasing prevalence of 65.2–74.2 (Viljoen et al., 2005) and 68–89.2 per 1000 (May et al., 2007). In addition a study in the Northern Cape Province reported a similar prevalence of 67.2 per 1000 (Urban et al., 2008).

Fetal alcohol syndrome is a complex multifactorial condition and although prenatal alcohol exposure is the primary trigger, twin concordance studies and animal models suggest a significant genetic susceptibility for the development of FAS (Streissguth and Dehaene, 1993; Becker et al., 1996). Recent studies have proposed an epigenetic etiology and supporting evidence for such a mechanism is accumulating (Garro et al., 1991; Haycock, 2009; Ungerer et al., 2013). Gene expression disturbances can be caused by changes in DNA methylation, molecular modification of histones and through RNA interference. These mechanisms work together to produce a unique, and reversible epigenetic signature that regulates gene expression through chromatin remodeling. DNA methylation has been investigated extensively as a mechanism of alcohol teratogenesis.

Genomic imprinting is an epigenetic phenomenon resulting in mono-allelic gene expression according to the parent of origin in a locus-specific manner. It is mediated by differential DNA methylation and imprinted loci play an important role during normal development (Jirtle et al., 2000; Rodenhiser and Mann, 2006). The DNA methylation status can be influenced by the environment leading to a functional impact mediated by changes in the epigenome (Jirtle and Skinner, 2007). Imprinted genes are therefore suitable candidates for investigating the effects of teratogens on disease etiology. Almost all imprinted genes contain differentially methylated regions (DMRs) which serve as a mark that differentiates the paternal allele from the maternal allele. Some DMRs which regulate the methylation patterns of a cluster of imprinted genes are referred to as primary DMRs or imprinting control regions (ICRs). The CpG methylation at ICRs is established in the gametes and maintained in somatic tissues of offspring throughout development (Smallwood and Kelsey, 2012). Despite this trend, they may still be subject to tissue-specific effects and extrapolation from the tissue under investigation should be done with care. On the other hand, the imprinting of secondary DMRs is established after fertilization (Geuns et al., 2007). Individual loci may be hyper- or hypomethylated following alcohol exposure. A study by Kaminen-Ahola et al. (2010) reported that maternal alcohol exposure tended to induce hypermethylation at the A^{vy} locus, while Haycock and Ramsay (2009) reported hypomethylation at the *H19 ICR* in mouse placenta following *in utero* alcohol exposure and Stouder et al. (2011) also showed hypomethylation at the *H19 ICR* in the brain and sperm of *in utero* exposed offspring (Stouder et al., 2011). A study by Liu et al. (2009) has demonstrated that alcohol exposure during early neurulation can induce aberrant changes in DNA methylation with associated changes in gene expression in mice. These studies support an epigenetic mechanism as a contributing factor for the development of features observed in FASD. It is widely suggested that the effect is mediated through the interruption of the one carbon pathway that is critical in production of the methyl groups in the maintenance of DNA methylation (Halsted et al., 2002; Liu et al., 2009). Alcohol exposure is correlated with reduced DNA methylation through several plausible mechanisms. Firstly acetaldehyde, a metabolite of alcohol metabolism, inhibits methyltransferase activity, and secondly, folate deficiency as a result of alcohol consumption and poor nutrition, reduces the pool of methyl donors.

In this study we examined quantitative changes in DNA methylation in blood and buccal cells from individuals with FAS, compared to unaffected controls, at four ICRs that regulate gene expression at loci that are important during fetal growth and development: *H19 ICR*, *KvDMR1, IG-DMR,* and *PEG3 DMR*.

MATERIALS AND METHODS

STUDY PARTICIPANTS AND SAMPLE COLLECTION

The study participants included 87 individuals with a diagnosis of FAS and 58 controls. All participants were of mixed ancestry, referred to as "Coloreds" in the South African context, and were resident in the Western Cape and Northern Cape provinces of South Africa. The FAS cases were recruited from Wellington in the Western Cape and De Aar and Upington in the Northern Cape. They were diagnosed by a team of trained clinicians from the Division of Human Genetics, NHLS, Braamfontein, Johannesburg, and also the Foundation for Alcohol Related Research (FARR; http://www.farr-sa.co.za), led by Denis Viljoen. The control participants were recruited from the Northern Cape and no phenotype data were collected. The cases and controls were not age matched. The FAS cases has a median age of 9 years (range 1–16 years) and the control participants were 17–26 years of age (median age 20 years). Adult participants provided informed consent and the parents or guardians of minor participants provided informed consent on their behalf. Ethics approval for the study was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Medical) (Protocol numbers M02/10/41, M03/10/20 and M080548). Venous blood samples were collected into EDTA by qualified phlebotomists and buccal swabs were collected by the research staff.

DNA EXTRACTION FROM BLOOD AND BUCCAL TISSUES

DNA was extracted from whole blood using a manual salting out method according to a modified protocol from Miller et al. (1988). The buccal tissue DNA was extracted using the Gentra Puregene buccal cell kit (Qiagen, Valencia, CA, USA).

DNA BISULFITE MODIFICATION AND PCR AMPLIFICATION

Genomic DNA was bisulfite modified using the EZ-DNA Methylation Gold Kit ™ (Zymo Research, Orange, CA, USA). Published primer sets and custom designed primer sets were used to amplify specific regions within the ICRs of four imprinted loci: *H19 ICR; IG-DMR*; *KvDMR1*; and *PEG3 DMR*. Each locus is described briefly and the details of the PCR and sequencing primers are shown in **Table 1**.

The pre-pyrosequencing PCR step requires that one of the primers is 5′ biotin labeled. In this study we used a universal biotin labeled primer (5′-biotin-GACGGGACACCGCTGATCGTTTA-3′) which was included in the PCR cocktail to generate labeled DNA fragments (Colella et al., 2003). The sequences of primers

Table 1 | Locus specific information for PCR amplification and pyrosequencing.

Locus (contig)	No. CpG sites	PCR primers (5′–3′)	Amplicon length (bp)	Annealing Temp (°C)	Sequencing primer (5′–3′)	Reference
H19 ICR (AF087017)	6	Outer reverse CTTAAATCCCAAACCATAACACTA Outer forward GTATATGGGTATTTTTTGGAGGT Inner forward GTATATGGGTATTTTTTGGAGGT	217	61.5	TGGTTGTAGTTGTGGAAT	Present study
		Inner reverse Tag-ATATCCTATTCCCAAATAA		53		
IG-DMR (A117190)	7	Forward Tag-TTTATTGGGTTGGGTTTTGTTAG	267	58	Primer 1 CAATTACAATACCACAAAAT	Present study
	3	Reverse AACCAATTACAATACCACAAAATT			Primer 2 CCATAAACAACTATAAACCT	Present study
KvDMR1 (U90095)	7	Forward TTAGTTTTTTGYGTGATGTGTTTATTA Reverse Tag-CCCACAAACCTCCACACC	101	55	TTGYGTGATGTGTTTATTA	Bourque et al. (2010)
PEG3 DMR (AC006115)	7	Reverse Tag-CCTATAAACAACCCCACACCTATAC Forward TAATGAAAGTGTTTGAGATTTGTTG	272	62	GGGGGTAGTTGAGGTT	Boissonnas et al. (2010)

Tag-5′-biotin-GACGGGACACCGCTGATCGTTTA-3′—universal biotin labeled tag.

that were designed to be biotin labeled therefore had a 23 bp complementary tag sequence added to their 5′ ends for the priming of the universal biotin labeled primer. These primers are shown in **Table 1** as "tag" primers. Unless specified to the contrary, primer sets were designed using the PSQ assay design software (Biotage, Uppsala country, Sweden).

The *H19 ICR* contains seven CTCF binding sites, of which the sixth is differentially methylated. The sixth CTCF binding site was the target region in this study and contains five CpGs, but the amplified region included one extra CpG which was also included in the analysis. For the *H19 ICR* amplification, nested PCR was used with an outer and an inner PCR primer sets. The PCR reactions for this region were performed in triplicate.

The amplified *IG-DMR* region contains 15 CpGs, but only 10 CpGs were analyzed using two different sequencing primers (1 and 2), where one analyzed three and the other analyzed seven CpG sites. PCR primers used for amplification of the *KvDMR1* are published primers and the amplicon contains seven CpGs, including a differentially methylated *NotI* site (Bourque et al., 2010). The PCR forward primer and pyrosequencing sequencing primer had a wobble introduced to accommodate an unavoidable CpG site in the sequence template that could either be methylated or unmethylated. The *PEG3 DMR* amplified region contains 14 CpGs but only seven CpGs were analyzed. The PCR assays for *IG-DMR*, *KvDMR1*, and *PEG3 DMR* were run in duplicate.

PYROSEQUENCING FOR QUANTIFICATION OF DNA METHYLATION ANALYSIS

DNA methylation of the different amplified ICRs was quantified by pyrosequencing using the PSQ 96MA system with the PyroGold SQA reagent kit (Biotage, Uppsala, Uppsala country, Sweden). Pyrosequencing assays and sequencing primers (**Table 1**) were designed using PSQ Assay Design Software and the sequencing was done in triplicate (*H19 ICR*) or duplicate (*IG-DMR*, *KvDMR1*, and *PEG3 DMR*). The percentage methylation for each of the CpG sites within the target region was calculated using Pyro Q-CpG software (Biotage, Uppsala, Uppsala country, Sweden). Two non-CpG cytosine bases were included in all the pyrosequencing assays as internal controls to assess successful bisulfite conversion. Samples containing >5% unsuccessfully converted non-CpG cytosines were discarded.

STATISTICAL ANALYSIS

We analyzed methylation data for 145 individuals, 87 FAS cases, 58 controls. Not every individual provided complete data. There was no age overlap due to the cases being of primary school age (younger than 17 years old, mean age 9 years) and the controls being 17 years or older. This means that the age effect (difference between young and older) cannot be distinguished from the fetal alcohol (case-control) effect in this study. However, the effect per additional year of age could be estimated within each group. Both groups had similar gender distributions, as summarized in **Table 2**.

Linear mixed-effects models were used to generate all the results reported here. These analyses are based on joint models, where all the original methylation observations (individual replicates) are put into a single model to simultaneously do the tests. One advantage is that it avoids some false positive results, because all the results are adjusted for each other. These models also enabled us to adjust for different kinds of random variation as random effects: that between sites, and that between individuals, and that within individuals (between replicates). Adjusting for

Table 2 | Summary table for number of samples, sex and age distribution for the different loci tested in the control and case groups.

	Controls				Cases			
		Age	Sex			Age	Sex	
Locus	***N***	**Mean (years) (min:max)**	**Male**	**Female**	***N***	**Mean (years) (min:max)**	**Male**	**Female**
H19 ICR	50	21 (17:26)	27	23	73	8.7 (1:16)	41	32
KvDMR1	55	21 (17:26)	27	28	86	8.4 (1:16)	46	40
IG-DMR	56	21 (17:26)	29	27	84	8.5 (1:16)	45	39
PEG3 DMR	50	21 (17:26)	25	25	79	8.7 (1:16)	46	33

the variation between individuals is a different way of saying we adjusted for the correlation between replicates on the same individual. After confirming, using linear mixed-effects models, that age and sex were confounders, all further models were adjusted for them, as fixed effects. All *p*-values, effects sizes and standard errors (SE) come from interaction terms in the models. All results corresponding to *p*-values below 0.05 are described as significant, below 0.01 as highly significant and below 0.001 as very highly significant.

The observed methylation data are summarized with box plots. Each box extends from the first quartile to the third quartile (interquartile range), the line inside the box is at the median, and the whiskers extend to the non-outlying minimum and maximum, respectively. Outliers are shown as open circles. The freely available environment for statistical computing and graphics, R (R Core Team, 2014), and R package (Pinheiro et al., 2015), were used for these analyses.

RESULTS

The 87 FAS cases were recruited from several areas of the Western Cape and the Northern Cape, whereas the 58 control participants were mainly recruited from the Northern Cape. There are differences in the numbers of individuals tested per locus, due to failure to amplify in specific samples for specific loci. Similar percentages per sex were tested, 30 (52%) males and 28 (48%) females in the controls and 47 (54%) males and 40 (46%) females in the cases. The control participants ($N = 58$) all donated blood samples and of the 87 FAS cases, eight donated buccal samples and the remainder donated blood. A summary for the number of samples, sex and age distribution at the different loci in the case and control groups is shown in **Table 2**.

To address tissue specificity of DNA methylation at an imprinted locus, we showed that there was no significant difference in percentage methylation at the *H19 ICR* locus CpG sites between buccal and blood samples from 50 random participants from another study (data not shown). Methylation status between the two tissues was not assessed at *KvDMR1*, *IG-DMR*, and *PEG3 DMR*. Based on two previous studies, we concluded that methylation profiles at these ICRs are unlikely to differ between the two tissues. Bourque et al. (2010) compared average methylation profiles at *KvDMR1* between blood and saliva tissues in healthy adults and reported that their methylation patterns were similar. In addition Woodfine et al. (2011) examined the methylation patterns of 17 germline DMRs (including *H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*) amongst several somatic tissues (including brain, breast, colon, heart, kidney, and liver) and reported that the average methylation did not vary amongst the tissues. It is therefore unlikely that the origin of the tissue for the DNA methylation studies is a significant confounder in this study.

Figure 1 contains box plots summarizing the observed percentage methylation at individual CpG sites at all loci (*H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*), in controls (CON) and cases (FAS). **Figure 2** contains box plots summarizing the observed percentage average methylation at each locus: *H19 ICR*, *KvDMR1*, *IG DMR*, and *PEG3 DMR*, in controls (CON) and cases (FAS). It is not possible to visualize the data after correction for age and sex.

AGE AND SEX AS POTENTIAL CONFOUNDERS IN DNA METHYLATION STUDIES ON IMPRINTED LOCI

Age and sex are reported confounders in DNA methylation studies and their effects were investigated in the present study. The results for sex are summarized in **Table 3** and for age in **Table 4**. The sex effect was highly significant at *PEG3 DMR* in FAS cases, where males had an estimated 1.11% more methylation than females. In contrast, in controls at *PEG3 DMR*, males had a significant estimated 0.84% lower methylation compared to that in females. However estimated methylation did not differ by sex in control nor in FAS cases at any of *H19 ICR*, *IG-DMR.A*, *IG-DMR.B*, and *KvDMR1*. Since there was a significant difference at one locus, sex was adjusted for in downstream analyses. It was observed that *IG-DMR* has a wide variability in methylation at the different CpG sites analyzed. Most of the individuals had methylation of above 70% at CpG 1–5 while CpG site 6–10 have methylation of about 50%. Therefore *IG-DMR* was split into two regions for this analysis: sites 1–5 called *IG-DMR.A* and sites 6–10 called *IG-DMR.B*.

Due to ethical considerations in the selection of control participants, the study design was sub-optimal in terms of age. All cases were below 17 years of age and all controls were 17 years and above, where the latter were able to give individual informed consent, but the parents or guardians consented to the participation of the cases. This means that age is strongly confounded and that it is not possible to tell whether any differences between cases and controls are caused by the age difference or not. However, the effect of age inside each of the groups could and was investigated.

Table 4 shows estimates of the difference in methylation percentage over 1 year of age, together with its SE and *p*-values in cases and controls.

The largest effects are seen at *IG-DMR.A* and *IG-DMR.B* in FAS cases, where the estimated methylation percentage decreased

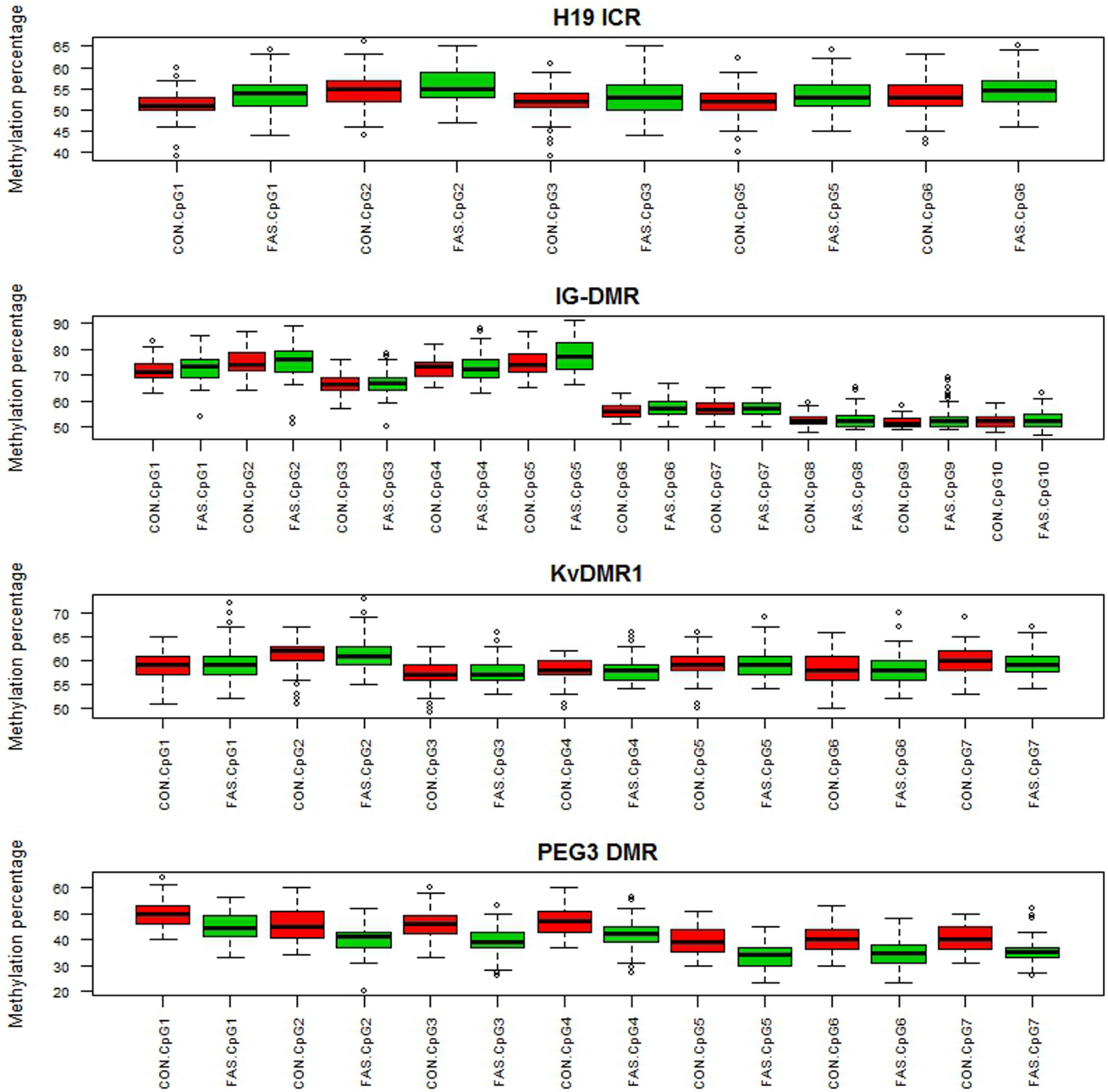

FIGURE 1 | Boxplots summarizing the observed percentage methylation at the CpG sites in *H19 ICR, IG-DMR, KvDMR1,* and *PEG3 DMR* in controls (CON) and cases (FAS).

by 0.43 and 0.38% respectively, for a 1 year increase in age. At *KvDMR1*, for every year increase in age, there is a significant estimated methylation increase of 0.19% in controls but in FAS cases there is a significant decrease by 0.11%. Again the highly significant effect is seen at *PEG3 DMR* in the control group, where estimated methylation percentage decreases by 0.22% for every year increase in age. No age effect was observed at *H19 ICR* (either in cases or controls), nor at *IG-DMR.A* and *IG-DMR.B* (in controls) nor at *PEG3 DMR* (in cases). **Table 5** summarizes, for each CpG site, the effect of 1 year of age on methylation, separately for controls and FAS cases, as well as the estimated difference between cases and controls in that effect. There are five CpG sites in *IG-DMR*, one in *KvDMR1*, where the effect of age on methylation is significantly lower in FAS cases and controls. At *IG-DMR* sites 2, 5, 6, 8, and 9, as well as at *KvDMR1* site 6, methylation decreased highly significantly with age in FAS cases but no significant effect was detected in controls. In *PEG3 DMR* site 2, the effect was significantly higher in FAS cases than controls.

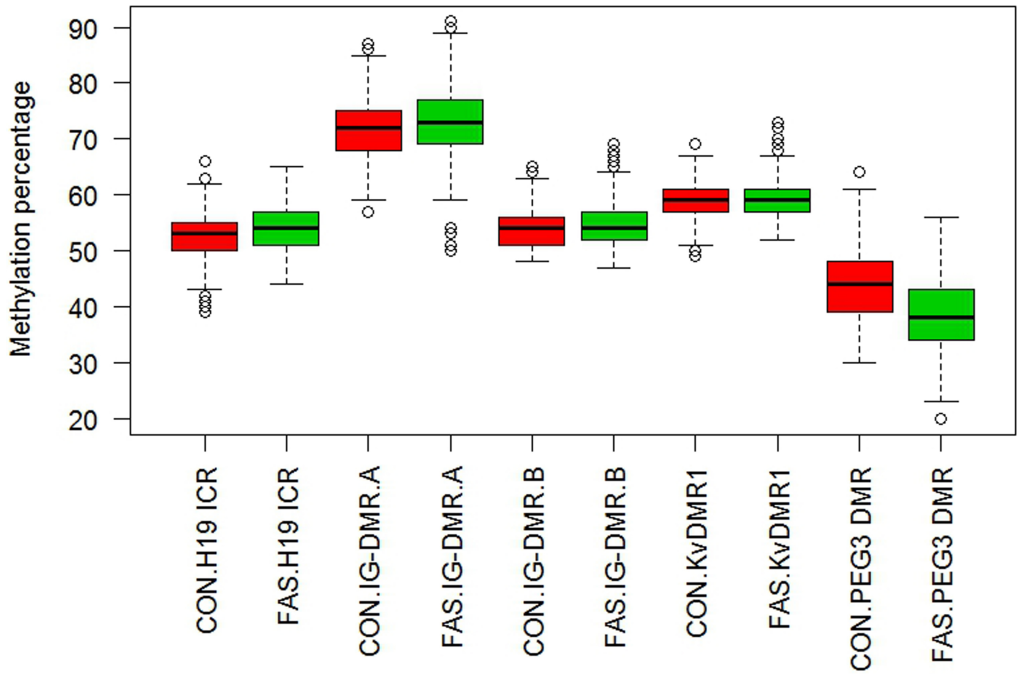

FIGURE 2 | Boxplot of observed percentage methylation per locus, *H19 ICR, IG-DMR, KvDMR1,* and *PEG3 DMR,* in controls (CON) and cases (FAS). A significant difference was detected for *H19 ICR* between cases and controls ($p = 0.024$), but after adjustment for sex and age this was no longer significant. The estimated methylation percentage difference between cases and controls at *PEG3 DMR* was highly significant ($p < 0.001$) and remained so after adjustment for age and sex.

Table 3 | Comparison of methylation within a locus between sexes, separately in FAS and controls.

Locus	Group	Effect	SE	*P*-value
H19 ICR	CON	0.33	0.48	0.495
H19 ICR	FAS	−0.16	0.40	0.687
IG-DMR1.A	CON	0.37	0.46	0.428
IG-DMR1.A	FAS	0.18	0.38	0.626
IG-DMR1.B	CON	−0.45	0.46	0.329
IG-DMR1.B	FAS	−0.60	0.38	0.112
KvDMR1	CON	0.10	0.39	0.795
KvDMR1	FAS	−0.22	0.32	0.490
PEG3 DMR	CON	−0.84	0.41	0.042
PEG3 DMR	FAS	1.11	0.33	0.001

CON, controls; FAS, FAS case; Effect, the estimated percentage difference in methylation between males and females in the specific group at the specific locus, using linear mixed-effects models, as described in methods section; SE, standard error of the effect estimate. Significant: $p < 0.05$. Analyses are adjusted for variation between sites and also for variation between individuals and within individuals as random effects.

Table 4 | The estimated effect of 1 year of age on % methylation per locus per group.

Locus	Group	Effect	SE	*P*-value
H19 ICR	CON	−0.05	0.10	0.634
H19 ICR	FAS	0.02	0.06	0.749
IG-DMR.A	CON	0.02	0.10	0.861
IG-DMR.A	FAS	−0.43	0.06	<0.001
IG-DMR.B	CON	0.01	0.10	0.920
IG-DMR.B	FAS	−0.38	0.06	<0.001
KvDMR1	CON	0.19	0.08	0.016
KvDMR1	FAS	−0.11	0.05	0.021
PEG3 DMR	CON	−0.22	0.08	0.008
PEG3 DMR	FAS	0.00	0.05	0.948

CON, controls; FAS, FAS case; Effect, estimated percentage difference in methylation between patients of a specific age and those 1 year younger, in the specific group at the specific locus, using linear mixed-effects models, as described in methods section; SE, standard error of the effect. Significant: $p < 0.05$. Analysis is adjusted for sex (fixed), CpG sites, individuals and replicates (random effects).

In light of these differences, sex and age were adjusted for in the subsequent analyses to assess differences between FAS cases and controls.

THE EFFECT OF ALCOHOL ON DNA METHYLATION AT DIFFERENT LOCI (FAS CASES COMPARED TO UNAFFECTED CONTROLS)

Unadjusted and adjusted results are presented to assess potential differences in methylation percentages at different CpG sites and also across loci, between controls and FAS cases. The random variation between sites, individuals and replicates per individual was adjusted for in all analyses.

Table 6 gives a summary of the estimated differences in CpG methylation between FAS cases and controls (FAS-CON), per CpG site, unadjusted and adjusted for age and sex. Both models were adjusted for random variation between and within individuals.

Table 5 | The estimated effect of 1 year of age on % methylation per CpG site per locus.

		Control group			FAS cases			Estimated difference in age effect on methylation between FAS and CON		
Locus	**Site**	**Age effect**	**SE**	***P*-value**	**Age effect**	**SE**	***P*-value**	**FAS-CON**	**SE**	***P*-value**
H19 ICR	CpG1	0.02	0.16	0.890	0.03	0.10	0.758	0.01	0.19	0.962
H19 ICR	CpG2	0.04	0.16	0.811	0.06	0.10	0.577	0.02	0.19	0.924
H19 ICR	CpG3	−0.16	0.16	0.298	0.02	0.10	0.851	0.18	0.19	0.326
H19 ICR	CpG5	0.10	0.16	0.522	−0.01	0.10	0.896	−0.11	0.19	0.541
H19 ICR	CpG6	−0.09	0.16	0.546	0.08	0.10	0.448	0.17	0.19	0.360
IG-DMR	CpG1	−0.05	0.17	0.789	−0.43	0.10	<0.001	−0.38	0.20	0.052
IG-DMR	CpG2	−0.19	0.17	0.273	−0.72	0.10	<0.001	−0.53	0.20	0.007
IG-DMR	CpG3	0.19	0.17	0.256	−0.02	0.10	0.811	−0.22	0.20	0.273
IG-DMR	CpG4	0.17	0.17	0.311	−0.17	0.10	0.089	−0.35	0.20	0.081
IG-DMR	CpG5	−0.11	0.17	0.520	−0.95	0.10	<0.001	−0.84	0.20	<0.001
IG-DMR	CpG6	−0.02	0.17	0.922	−0.45	0.10	<0.001	−0.43	0.20	0.030
IG-DMR	CpG7	0.05	0.17	0.774	−0.25	0.10	0.016	−0.30	0.20	0.134
IG-DMR	CpG8	0.03	0.17	0.878	−0.44	0.10	<0.001	−0.47	0.20	0.019
IG-DMR	CpG9	−0.04	0.17	0.812	−0.52	0.10	<0.001	−0.48	0.20	0.016
IG-DMR	CpG10	−0.03	0.17	0.863	−0.40	0.10	<0.001	−0.37	0.20	0.059
KvdMR	CpG1	0.26	0.17	0.122	−0.11	0.10	0.255	−0.37	0.20	0.056
KvdMR	CpG2	0.11	0.17	0.513	−0.04	0.10	0.687	−0.15	0.20	0.443
KvdMR	CpG3	0.10	0.17	0.562	−0.14	0.10	0.148	−0.24	0.20	0.218
KvdMR	CpG4	0.14	0.17	0.412	−0.09	0.10	0.363	−0.23	0.20	0.243
KvdMR	CpG5	0.26	0.17	0.126	−0.03	0.10	0.769	−0.29	0.20	0.142
KvdMR	CpG6	0.18	0.17	0.284	−0.22	0.10	0.023	−0.40	0.20	0.038
KvdMR	CpG7	0.15	0.17	0.380	−0.12	0.10	0.225	−0.27	0.20	0.170
PEG3 DMR	CpG1	−0.19	0.17	0.272	0.02	0.10	0.818	0.22	0.20	0.289
PEG3 DMR	CpG2	−0.28	0.17	0.115	0.25	0.10	0.017	0.53	0.20	0.010
PEG3 DMR	CpG3	−0.03	0.17	0.858	0.09	0.10	0.376	0.12	0.20	0.543
PEG3 DMR	CpG4	−0.11	0.17	0.523	0.03	0.10	0.793	0.14	0.20	0.495
PEG3 DMR	CpG5	−0.22	0.17	0.201	−0.14	0.10	0.194	0.09	0.20	0.665
PEG3 DMR	CpG6	−0.19	0.17	0.284	−0.16	0.10	0.125	0.03	0.20	0.895
PEG3 DMR	CpG7	−0.37	0.17	0.033	0.03	0.10	0.799	0.40	0.20	0.050

Effect is the estimated percentage difference in methylation between individuals of a specific age and those 1 year younger, in the specific group at the specific locus, using linear mixed-effects models, as described in methods section.

At *H19 ICR*, all six sites, and at *IG-DMR* sites 2, 5, 6, and 9, the case group had significantly higher methylation than the control group. However after adjusting for age and sex there was no longer a significant difference between controls and cases. The only significant effects detected at *KvDMR1*, were at sites 4 and 7, where methylation was significantly lower in FAS cases than controls, after adjustment for age and sex. At *PEG3 DMR*, across all CpG sites, estimated methylation was very highly significantly lower (all p-values < 0.001) in FAS than in controls, with and without adjustment for age and sex.

The estimated methylation percentage difference between controls and cases across each locus is summarized in **Table 7** and the observed percentage methylation is shown in **Figure 2**. At the *H19 ICR* locus, cases showed a highly significant increased average methylation compared to the controls, but this was no longer significant after adjusting for age and sex. At *KvDMR1* locus showed a significant lower average methylation after age and sex were adjusted. In the unadjusted analysis, the average methylation was significantly higher (1.15 and 0.75% respectively) in cases than controls, however after adjusting for age and sex the direction of the effect had changed but the reduced methylation was only significant at region B. The *PEG3 DMR* also showed a highly significant difference between cases and controls and the unadjusted ($p < 0.001$) and adjusted ($p < 0.001$) effect sizes were similar (5.47% lower in cases before adjustment and 7.09% lower in cases after adjustment).

DISCUSSION

Epigenetic modulation is increasingly studied as an important mechanism to explain fetal outcome based on environmental exposures during *in utero* development, with some effects lasting into adulthood. This includes maternal diet and exposure to teratogens, like alcohol, but may also include factors like stress. Since imprinted loci play an important role in fetal development, cellular differentiation and growth, we decided to investigate the levels of CpG methylation at four primary DMRs in children with FAS compared to methylation in unaffected controls. Our understanding of the relationship between DNA methylation with regard to sex, age and cell type remains incomplete, but in addition to inter-individual variation, it is clear that there are locus-specific effects. It is therefore expected that teratogens would also display locus-specific effects explaining their impact on fetal outcome. In addition, tissue-specific DNA methylation and tissue-specific epigenetic responses to prenatal alcohol exposure could potentially confound the interpretation of our study as we examined blood and buccal

Table 6 | Summary of estimated differential CpG methylation between FAS cases and controls (FAS-CON), per CpG site.

		Unadjusted			Adjusted for age and sex		
Locus	**Site**	**FAS-CON**	**SE**	***P*-value**	**FAS-CON**	**SE**	***P*-value**
H19 ICR	CpG1	1.80	0.5	<0.001	0.23	0.79	0.767
H19 ICR	CpG2	1.06	0.5	0.035	−0.49	0.79	0.537
H19 ICR	CpG3	1.17	0.5	0.019	−0.42	0.79	0.594
H19 ICR	CpG5	1.48	0.5	0.003	−0.05	0.79	0.950
H19 ICR	CpG6	1.35	0.5	0.007	−0.16	0.79	0.835
IG-DMR.A	CpG1	1.02	0.53	0.054	−0.50	0.81	0.540
IG-DMR.A	CpG2	1.07	0.53	0.043	−0.52	0.81	0.520
IG-DMR.A	CpG3	0.48	0.53	0.364	−1.01	0.81	0.216
IG-DMR.A	CpG4	0.19	0.53	0.724	−1.27	0.81	0.117
IG-DMR.A	CpG5	3.05	0.53	<0.001	1.50	0.81	0.065
IG-DMR.B	CpG6	1.34	0.53	0.012	−0.21	0.81	0.792
IG-DMR.B	CpG7	0.22	0.53	0.679	−1.31	0.81	0.106
IG-DMR.B	CpG8	0.68	0.53	0.202	−0.92	0.81	0.258
IG-DMR.B	CpG9	1.36	0.53	0.010	−0.22	0.81	0.782
IG-DMR.B	CpG10	0.24	0.53	0.646	−1.33	0.81	0.101
KvDMR1	CpG1	0.96	0.53	0.072	−0.53	0.81	0.512
KvDMR1	CpG2	0.28	0.53	0.596	−1.21	0.81	0.138
KvDMR1	CpG3	0.28	0.53	0.595	−1.20	0.81	0.141
KvDMR1	CpG4	−0.17	0.53	0.752	−1.67	0.81	0.040
KvDMR1	CpG5	−0.01	0.53	0.986	−1.43	0.81	0.079
KvDMR1	CpG6	−0.10	0.53	0.851	−1.55	0.81	0.057
KvDMR1	CpG7	−0.60	0.53	0.262	−2.12	0.81	0.009
PEG3 DMR	CpG1	−5.34	0.55	<0.001	−6.98	0.83	<0.001
PEG3 DMR	CpG2	−5.08	0.55	<0.001	−6.69	0.83	<0.001
PEG3 DMR	CpG3	−6.14	0.55	<0.001	−7.73	0.83	<0.001
PEG3 DMR	CpG4	−4.74	0.55	<0.001	−6.43	0.83	<0.001
PEG3 DMR	CpG5	−5.51	0.55	<0.001	−7.07	0.83	<0.001
PEG3 DMR	CpG6	−5.11	0.55	<0.001	−6.67	0.83	<0.001
PEG3 DMR	CpG7	−5.35	0.55	<0.001	−6.96	0.83	<0.001

The analysis was unadjusted and adjusted for age and sex. Both models were adjusted for random variation between and within individuals.

Table 7 | Estimated differences in percentage methylation between cases and controls at each locus.

	Unadjusted			Adjusted for age and sex		
Locus	**Effect**	**SE**	***P*-value**	**Effect**	**SE**	***P*-value**
H19 ICR	1.36	0.31	<0.001	−0.17	0.41	0.674
IG-DMR1.A	1.15	0.30	<0.001	−0.40	0.40	0.315
IG-DMR1.B	0.75	0.30	0.012	−0.84	0.40	0.035
KvDMR1	0.01	0.25	0.967	−1.49	0.37	<0.001
PEG3 DMR	−5.47	0.26	<0.001	−7.09	0.37	<0.001

SE, standard error. Significant: $p < 0.05$. Analyses were adjusted for variation between sites and variation between individuals, with and without adjustment for age and sex.

DNA from the participants, rather than neuronal tissue derived DNA.

SEX AND AGE DEMONSTRATE LOCUS SPECIFIC METHYLATION EFFECTS ON SELECTED ICRs

The effect of sex on global DNA methylation and locus-specific methylation has been reported. Global DNA methylation has a tendency toward higher methylation levels in males (Fuke et al., 2004; Shimabukuro et al., 2007). Studies on the effect of sex on locus-specific methylation have shown both increases and decreases in DNA methylation (Sandovici et al., 2005; Sarter et al., 2005; Eckhardt et al., 2006; El-Maarri et al., 2007).

In this study, the effect of sex on methylation was shown to be significant at only one locus, *PEG3 DMR*. Interestingly the effects are modest, but opposite in FAS cases and controls, with the former showing increased methylation (1.11%) in males and the latter a decrease of 0.84% in males. It is not clear why the sex effect on methylation is different in the two groups, but it may be due to the fact that the data were not adjusted for age when the analysis was done because it was done as a baseline comparison to decide if sex needed to be adjusted for in the main analysis. *PEG3 DMR* average methylation was shown to decrease in controls for every 1 year increase in age, suggesting that there may be an age sex interaction at this locus. There was no effect of sex on average methylation at *H19 ICR*, *KvDMR1,* and *IG-DMR*.

Age is reported to cause a reduction in global DNA methylation and causes dramatic changes in the distribution of 5-methylcytosine across the genome (Liu et al., 2011). With respect to specific genes, methylation can either be increased or decreased depending on the gene investigated (as reviewed by Liu et al., 2003). Issa et al. (1996) reported that the *IGF2 P2-P4* promoter-associated CpG island is methylated on the silenced maternal allele in young individuals, however with age this methylation also appears on the paternal allele resulting in biallelic methylation (indicating an overall increase in methylation with age). The promoter regions of many genes tend to switch from an unmethylated to a methylated state resulting in gene silencing

in an age dependent manner. This includes the promoters of several tumor and aging related genes (Wilson and Jones, 1983; Fuke et al., 2004; Liu et al., 2011). The mechanism contributing to the age dependent changes in global methylation includes a decrease in the expression of *DMNT1* (Lopatina et al., 2002; Liu et al., 2003). Longitudinal research on age effects that study the same individuals at several time points is rare (Florath et al., 2014; Flanagan et al., 2015). In two studies DNA methylation of participants was examined at two ages only, one where they were sampled 6 years apart and the other 8 years apart. It is therefore not yet clear whether age-related changes in methylation at CpG loci associated with age effects occur linearly with age.

We examined the effect of age on the different CpG sites and average methylation across each locus, separately in FAS cases and controls. In the control group, with the exception of *PEG3 DMR* CpG7, there was no CpG site specific age effect. In the FAS cases however, eight out of the 10 *IG-DMR* CpG sites, one *KvDMR1* site and one *PEG3 DMR* site showed a significant age effect. With a single exception, methylation in the FAS group decreased by a modest amount for every additional year of age. When examining the locus-averaged methylation and the effect of age, there was a small but significant effect for *KvDMR1*, but a larger effect in the FAS cases for *IG-DMR* (for both region A and B). This effect was not observed in controls. In contrast, the controls showed an age effect at the *PEG3 DMR*. The measure for an age effect is "difference in methylation per additional year of age"; however there was no overlap in absolute age between cases and controls. From our results, it would appear that age effects are more significant at younger ages (1–16 years) than in older age groups (17–26), in a locus-specific manner.

In this study age was shown to influence methylation at three of the four loci investigated. In alignment with our findings, a study on periconceptional famine exposure (Heijmans et al., 2008) found that within the age group of 43–70 years, the DNA methylation at the *IGF2 DMR* of a 10 year older group was associated with a 3.6% lower methylation ($p = 0.015$) in controls. The magnitude (0.36% per annum) of the effect in their study was greater than that observed in our study.

Since both sex and age showed some effect on DNA methylation at one or more of the imprinted loci in this study, we present sex and age adjusted analyses when comparing DNA CpG methylation between FAS cases and unaffected controls.

THE EFFECT OF *IN UTERO* ALCOHOL EXPOSURE ON DNA METHYLATION AT FOUR IMPRINTED LOCI

We assessed the possible effect of maternal alcohol consumption on DNA methylation at *H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*, by comparing methylation levels between FAS cases and unaffected controls. After adjustment for sex and age there was no observed correlation with *in utero* alcohol exposure at the CpG site level at two of the imprinted loci, *H19 ICR* and *IG-DMR*. Interestingly, a modest effect ($p = 0.035$) of decreased methylation (0.84%) for *IG-DMR* Region B was observed in FAS cases. The *IG-DMR* Region B shows roughly 50% methylation, in line with a parent of origin allelic effect whereas Region A had an overall higher methylation percentage.

The *IG-DMR* is a good candidate in terms of its potential biological impact, in line with the features of FAS. The paternally methylated *IG-DMR* is the primary ICR at the *DLK1/GTL2* (*MEG3*) imprinting domain on human chromosome 14q32, where it plays an essential role in regulating the monoallelic expression of several imprinted genes including the paternally expressed *DLK1* and maternally expressed *GTL2* genes (Lin et al., 2003). The methylation on the paternal allele is essential in maintaining the expression of imprinted genes, because failure to maintain the paternal methylation has been shown to result in considerable *Dlk* repression while *Gtl2* expression is increased (Schmidt et al., 2000).

The *DLK1/GTL2* (*MEG3*) imprinting cluster is a critical region for the phenotypes associated with both maternal and paternal uniparental disomy (UPD) of chromosome 14 (Coveler et al., 2002; Kagami et al., 2005; Temple et al., 2007; Buiting et al., 2008). Maternal uniparental disomy 14 [Upd(14)mat] and hypomethylation at the paternally imprinted *IG-DMR* (Ogata et al., 2008) are characterized by pre- and postnatal growth retardation, developmental delays, mild to moderate mental retardation, muscular hypotonia, small hands and feet, premature puberty and truncal obesity. The locus-averaged methylation of the *IG-DMR* was modestly reduced in FAS cases, tending toward hypomethylation and which may potentially contribute to the growth and neuronal deficits in affected individuals. The magnitude of alcohol effects may be tissue specific and may play a more important role in neurogenesis. These findings merit further study and validation.

After adjustment of sex and age, two *KvDMR1* CpG sites (4 and 7) showed significantly decreased DNA methylation in FAS cases which contributed to a locus-averaged decrease of 1.49% methylation in the *KvDMR1*. The functional impact of this difference is not clear. The biggest effect (a decrease of 7.09% methylation in FAS cases) was observed at the *PEG3 DMR*. Interestingly, it is the two maternally imprinted loci, *KvDMR1* and *PEG3 DMR*, which are significantly affected by *in utero* alcohol exposure and both show a decrease in methylation following alcohol exposure.

One of the key features of FAS is pre- and post-natal growth retardation and dysregulation of imprinting at *H19 ICR* has been associated with growth disorders (Reik et al., 1995; Gicquel et al., 2005; Ideraabdullah et al., 2008). The findings of our study are, however, in agreement with a study done in a mouse model by Haycock and Ramsay (2009) where they reported no difference in methylation at the *H19 ICR* of mouse embryos exposed to alcohol during the preimplantation period, when compared to unexposed control embryos. Interestingly *H19 ICR* hypomethylation was observed in the mouse placentas suggesting a localized effect on the extra-embryonic tissue, which could explain the effect on fetal growth. In two other related studies subtle differential DNA methylation was observed. Knezovich and Ramsay (2012) reported a significant decrease in methylation at the *H19 ICR* in mouse offspring following preconception paternal alcohol exposure and Downing et al. (2011) reported a subtle decrease in methylation at the mouse *Igf2 DMR1* locus in embryos following *in utero* alcohol exposure.

The hypomethylation at *KvDMR1* and *PEG3 DMR* is aligned to our original hypothesis suggesting that alcohol reduces DNA

methylation through the one carbon metabolism pathway and its effect on reducing folate levels. In the next sections the potential implications of hypomethylation at these loci are explored.

THE FUNCTIONAL IMPACT OF REDUCED *KvDMR1* METHYLATION IN FAS CASES IS UNCLEAR

KvDMR1 CpG site-specific and average locus-wide hypomethylation in response to *in utero* alcohol exposure would suggest a loss of methylation on the maternally methylated ICR which regulates the monoallelic expression of several imprinted genes located in the *CDKN1C/KCNQ1OT1* imprinting domain cluster. This imprinting domain harbors the paternally expressed non-coding antisense transcript to *KCNQ1* called *KCNQ1OT1*, and other maternally expressed protein coding genes including *KCNQ1* and *CDKNIC1*. Loss of imprinting, or hypomethylation, at the *KvDMR1* has been widely implicated in the Beckwith–Wiedemann syndrome (BWS; Gaston et al., 2001; Diaz-Meyer et al., 2003; Azzi et al., 2009), a congenital disorder characterized by pre- and postnatal overgrowth, organomegaly, and a high risk of childhood tumors (Weksberg et al., 2010). Paradoxically, the FAS cases showed significant hypomethylation at CpG sites 4 and 7 (1.67 and 2.1%, respectively), yet FAS affected individuals are growth restricted. It is unclear whether hypomethylation of only two of the seven CpG sites in this ICR will affect the levels of expression of the imprinted genes in the cluster and what the functional effect may be.

To gain further insight into gene regulation at this locus will require both gene expression and DNA methylation studies to more fully understand the impact of altered methylation at the *KvDMR1*. This is the first study to show the effect of alcohol on methylation status at *KvDMR1* and the findings are counter intuitive given that hypomethylation is associated with an overgrowth phenotype (BWS).

UNDERSTANDING THE ROLE OF ALCOHOL INDUCED HYPOMETHYLATION AT THE *PEG3* IMPRINTED GENE CLUSTER IN THE PATHOGENESIS OF FAS REQUIRES FURTHER KNOWLEDGE OF THE ICR CONTROLLED GENE EXPRESSION IN THIS REGION

The *PEG3* imprinting cluster is located on human chromosome 19q13.4 and is regulated by a maternally methylated ICR, the *PEG3 DMR*. The cluster includes several imprinted genes including the paternally expressed 3 gene (*PEG3*), the imprinted zinc-finger gene 2 (*ZIM2*) gene and the *USP29* gene, all of which are paternally expressed. Although these loci are syntenic in mouse and human, there are some interesting differences regarding their regulation, their tissue specific expression, and their exon structure and genomic arrangement. The *PEG3* gene is expressed in embryonic tissues, including the hypothalamus and brain, and in adult mouse and human brain, but most highly in human ovary, but not mouse ovary. *PEG3* encodes a DNA binding protein based on its multiple zing finger motifs (Relaix et al., 1996; Iuchi, 2001) and is an imprinted transcription factor that has multiple target genes (Thiaville et al., 2013). It has a proposed tumor suppressive function (Nye et al., 2013) and has been shown to induce *p53*-mediated apoptosis in multiple cell types (Yamaguchi et al., 2002). A mouse knockout model targeting the *Peg3* gene has shown that it is responsible for a variety of phenotypic outcomes including altered maternal offspring rearing behavior, low birth weight, alteration in fat tissue storage and synthesis, and lower metabolic activity (Li et al., 1999; Curley et al., 2004).

We observed that maternal alcohol consumption is correlated with a significant reduction of ∼7% methylation at the *PEG3 DMR* in FAS cases. The highly significant decrease in methylation was observed for all the CpG sites analyzed for this locus and also for the average methylation across this locus. It is possible that this change in the *PEG3 ICR* may affect multiple imprinted genes in the region. *PEG3* is expressed from the paternal allele and is reciprocally repressed on the maternal allele, suggesting that alcohol induced demethylation likely affects the maternal allele thus leading to derepression of the *PEG3* gene on the maternal allele, and therefore biallelic expression of *PEG3*. This would lead to an overall increase in *PEG3* expression. Several studies have focussed on the effects of reduced *Peg3*, but none has explored the phenotypic outcome of over expression of *PEG3*.

Gene expression studies, without correlation to their imprinting status, have demonstrated upregulation of *PEG3* (as well as several other genes) in intrauterine growth restriction (IUGR) placentas (reviewed in Ishida and Moore, 2013). Since IUGR is a cause of reduced fetal growth, this study supports our finding that the proposed increase in *PEG3* expression could be associated with a growth restriction phenotype. The role of the *PEG3 DMR* in regulating the imprinted gene cluster in humans requires further investigation.

CONCLUSION

Despite limitations in the study design, including the lack of age matching between cases and controls, the relatively small sample size, and the inaccessibility of neuronal tissue, significant differences in DNA methylation were observed at two primary DMRs when comparing FAS cases with unaffected controls. The observed hypomethylation at the *KvDMR1* has uncertain functional impact on gene expression and the FAS phenotype. The largest epigenetic effect among the loci investigated, was a locus-averaged 7% reduction in DNA methylation at the *PEG3 DMR* which was observed across all seven CpG sites. This ICR orchestrates a complex pattern of gene expression across the region with reported differences in mouse models compared to humans. It is proposed that hypomethylation of the *PEG3 DMR* would result in an increase in the paternally expressed *PEG3* gene. *PEG3* has a DNA binding motif and is considered an imprinted transcription factor, and therefore its function is most likely mediated by altered expression of its targets. Although there is some spatiotemporal congruence of gene expression in line with the developmental origins of the FAS related phenotype, the effect and mechanism of altered expression of *PEG3* and the other imprinted genes controlled by the *PEG3 DMR* remains unclear. Despite the uncertainty of the functional biological mechanism of the locus-specific hypomethylation of important ICRs in the blood of FAS cases, these findings support the role of an epigenetic mechanism in the development of FAS.

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REFERENCES

Abel, E. L., and Hannigan, J. H. (1995). Maternal risk factors in fetal alcohol syndrome: provocative and permissive influences. *Neurotoxicol. Teratol.* 17, 445–462. doi: 10.1016/0892-0362(95)98055-6

Azzi, S., Rossignol, S., Steunou, V., Sas, T., Thibaud, N., Danton, F., et al. (2009). Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. *Hum. Mol. Genet.* 18, 4724–4733. doi: 10.1093/hmg/ddp435

Becker, H. C., Diaz-Granados, J. L., and Randall, C. L. (1996). Teratogenic actions of ethanol in the mouse: a minireview. *Pharmacol. Biochem. Behav.* 55, 501–513. doi: 10.1016/S0091-3057(96)00255-9

Boissonnas, C. C., Abdalaoui, H. E. L., Haelewyn, V., Fauque, P., Dupont, J. M., Gut, I., et al. (2010). Specific epigenetic alterations of IGF2-H19 locus in spermatozoa from infertile men. *Eur. J. Hum. Genet.* 18, 73–80. doi: 10.1038/ejhg.2009.117

Bourque, D. K., Avila, L., Penaherrera, M., von Dadelszen, P., and Robinson, W. P. (2010). Decreased placental methylation at the H19/IGF2 imprinting control region is associated with normotensive intrauterine growth restriction but not preeclampsia. *Placenta* 31, 197–202. doi: 10.1016/j.placenta.2009.12.003

Buiting, K., Kanber, D., Martin-Subero, J. I., Lieb, W., Terhal, P., Albrecht, B., et al. (2008). Clinical features of maternal uniparental disomy 14 in patients with an epimutation and a deletion of the imprinted DLK1/GTL2 gene cluster. *Hum. Mutat.* 29, 1141–1146. doi: 10.1002/humu.20771

Clarren, S. K., and Smith, D. W. (1978). The fetal alcohol syndrome. *N. Engl. J. Med.* 298, 1063–1067. doi: 10.1056/NEJM197805112981906

Colella, S., Shen, L., Baggerly, K. A., Issa, J. P., and Krahe, R. (2003). Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. *Biotechniques* 35, 146–150.

Coveler, K. J., Yang, S. P., Sutton, R., Milstein, J. M., Wu, Y. Q., Bois, K. D., et al. (2002). A case of segmental paternal isodisomy of chromosome 14. *Hum. Genet.* 110, 251–256.

Curley, J. P., Barton, S., Surani, A., and Keverne, E. B. (2004). Coadaptation in mother and infant regulated by a paternally expressed imprinted gene. *Proc. Biol. Sci.* 271, 1303–1309. doi: 10.1098/rspb.2004.2725

Diaz-Meyer, N., Day, C. D., Khatod, K., Maher, E. R., Cooper, W., Reik, W., et al. (2003). Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith–Wiedemann syndrome. *J. Med. Genet.* 40, 797–801. doi: 10.1136/jmg.40.11.797

Downing, C., Johnson, T. E., Larson, C., Leakey, T. I., Siegfried, R. N., Rafferty, T. M., et al. (2011). Subtle decreases in DNA methylation and gene expression at the mouse Igf2 locus following prenatal alcohol exposure: effects of a methyl-supplemented diet. *Alcohol* 45, 65–71. doi: 10.1016/j.alcohol.2010.07.006

Eckhardt, F., Lewin, J., Cortese, R., Rakyan, V. K., Attwood, J., Burger, M., et al. (2006). DNA methylation profiling of human chromosomes 6, 20 and 22. *Nat. Genet.* 38, 1378–1385. doi: 10.1038/ng1909

El-Maarri, O., Becker, T., Junen, J., Manzoor, S. S., Diaz-Lacava, A., Schwaab, R., et al. (2007). Gender specific differences in levels of DNA methylation at selected loci from human total blood: a tendency toward higher methylation levels in males. *Hum. Genet.* 122, 505–514. doi: 10.1007/s00439-007-0430-3

Flanagan, J. M., Brook, M. N., Orr, N., Tomczyk, K., Coulson, P., Fletcher, O., et al. (2015). Temporal stability and determinants of white blood cell DNA methylation in the breakthrough generations study. *Cancer Epidemiol. Biomarkers Prev.* 24, 221–229. doi: 10.1158/1055-9965.EPI-14-0767

Florath, I., Butterbach, K., Müller, H., Bewerunge-Hudler, M., and Brenner, H. (2014). Cross-sectional and longitudinal changes in DNA methylation with age: an epigenome-wide analysis revealing over 60 novel age-associated CpG sites. *Hum. Mol. Genet.* 23, 1186–1201. doi: 10.1093/hmg/ddt531

Floyd, R. L., Ebrahim, S., Tsai, J., O'Connor, M., and Sokol, R. (2006). Strategies to reduce alcohol-exposed pregnancies. *Matern. Child Health J.* 10(Suppl. 5), S149–S151. doi: 10.1007/s10995-006-0116-9

Fuke, C., Shimabukuro, M., Petronis, A., Sugimoto, J., Oda, T., Miura, K., et al. (2004). Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. *Ann. Hum. Genet.* 68(Pt 3), 196–204. doi: 10.1046/j.1529-8817.2004.00081.x

Garro, A. J., McBeth, D. L., Lima, V., and Lieber, C. S. (1991). Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol. Clin. Exp. Res.* 15, 395–398. doi: 10.1111/j.1530-0277.1991.tb00536.x

Gaston, V., Le Bouc, Y., Soupre, V., Burglen, L., Donadieu, J., Oro, H., et al. (2001). Analysis of the methylation status of the KCNQ1OT and H19 genes in leukocyte DNA for the diagnosis and prognosis of Beckwith–Wiedemann syndrome. *Eur. J. Hum. Genet.* 9, 409–418. doi: 10.1038/sj.ejhg.5200649

Geuns, E., De Temmerman, N., Hilven, P., Van Steirteghem, A., Liebaers, I., and De Rycke, M. (2007). Methylation analysis of the intergenic differentially methylated region of DLK1-GTL2 in human. *Eur. J. Hum. Genet.* 15, 352–361. doi: 10.1038/sj.ejhg.5201759

Gicquel, C., Rossignol, S., Cabrol, S., Houang, M., Steunou, V., Barbu, V., et al. (2005). Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver–Russell syndrome. *Nat. Genet.* 37, 1003–1007. doi: 10.1038/ng1629

Halsted, C. H., Villanueva, J. A., Devlin, A. M., Niemela, O., Parkkila, S., Garrow, T. A., et al. (2002). Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10072–10077. doi: 10.1073/pnas.112336399

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod.* 81, 607–617. doi: 10.1095/biolreprod.108.074690

Haycock, P. C., and Ramsay, M. (2009). Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol. Reprod.* 81, 618–627. doi: 10.1095/biolreprod.108.074682

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., et al. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17046–17049. doi: 10.1073/pnas.0806560105

Ideraabdullah, F. Y., Vigneau, S., and Bartolomei, M. S. (2008). Genomic imprinting mechanisms in mammals. *Mutat. Res.* 647, 77–85. doi: 10.1016/j.mrfmmm.2008.08.008

Ishida, M., and Moore, G. E. (2013). The role of imprinted genes in humans. *Mol. Aspects Med.* 34, 826–840. doi: 10.1016/j.mam.2012.06.009

Issa, J. P., Vertino, P. M., Boehm, C. D., Newsham, I. F., and Baylin, S. B. (1996). Switch from monoallelic to biallelic human IGF2 promoter methylation during aging and carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11757–11762. doi: 10.1073/pnas.93.21.11757

Iuchi, S. (2001). Three classes of C2H2 zinc finger proteins. *Cell. Mol. Life Sci.* 58, 625–635. doi: 10.1007/PL00000885

Jirtle, R. L., Sander, M., and Barrett, J. C. (2000). Genomic imprinting and environmental disease susceptibility. *Environ. Health Perspect.* 108, 271–278. doi: 10.1289/ehp.00108271

Jirtle, R. L., and Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nat. Rev. Genet.* 8, 253–262. doi: 10.1038/nrg2045

Kagami, M., Nishimura, G., Okuyama, T., Hayashidani, M., Takeuchi, T., Tanaka, S., et al. (2005). Segmental and full paternal isodisomy for chromosome 14 in three patients: narrowing the critical region and implication for the clinical features. *Am. J. Med. Genet. A* 138A, 127–132. doi: 10.1002/ajmg.a.30941

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K. A., Fahey, P., Cox, T. C., et al. (2010). Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet.* 6:e1000811. doi: 10.1371/journal.pgen.1000811

Knezovich, J. G., and Ramsay, M. (2012). The effect of preconception paternal alcohol exposure on epigenetic remodeling of the h19 and rasgrf1 imprinting control regions in mouse offspring. *Front. Genet.* 3:10. doi: 10.3389/fgene.2012.00010

Li, L., Keverne, E. B., Aparicio, S. A., Ishino, F., Barton, S. C., and Surani, M. A. (1999). Regulation of maternal behavior and offspring growth by paternally expressed Peg3. *Science* 284, 330–333. doi: 10.1126/science.284.5412.330

Lin, S. P., Youngson, N., Takada, S., Seitz, H., Reik, W., Paulsen, M., et al. (2003). Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. *Nat. Genet.* 35, 97–102. doi: 10.1038/ng1233

Liu, L., van Groen, T., Kadish, I., Li, Y., Wang, D., James, S. R., et al. (2011). Insufficient DNA methylation affects healthy aging and promotes age-related health problems. *Clin. Epigenetics* 2, 349–360. doi: 10.1007/s13148-011-0042-6

Liu, L., Wylie, R. C., Andrews, L. G., and Tollefsbol, T. O. (2003). Aging, cancer and nutrition: the DNA methylation connection. *Mech. Ageing Dev.* 124, 989–998. doi: 10.1016/j.mad.2003.08.001

Liu, Y., Balaraman, Y., Wang, G., Nephew, K. P., and Zhou, F. C. (2009). Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4, 500–511. doi: 10.4161/epi.4.7.9925

Lopatina, N., Haskell, J. F., Andrews, L. G., Poole, J. C., Saldanha, S., and Tollefsbol, T. (2002). Differential maintenance and *de novo* methylating activity by three DNA methyltransferases in aging and immortalized fibroblasts. *J. Cell. Biochem.* 84, 324–334. doi: 10.1002/jcb.10015

Masotti, P., George, M. A., Szala-Meneok, K., Morton, A. M., Loock, C., Van Bibber, M., et al. (2006). Preventing fetal alcohol spectrum disorder in Aboriginal communities: a methods development project. *PLoS Med* 3:e8. doi: 10.1371/journal.pmed.0030008

May, P. A., Brooke, L., Gossage, J. P., Croxford, J., Adnams, C., Jones, K. L., et al. (2000). Epidemiology of fetal alcohol syndrome in a South African community in the Western Cape Province. *Am. J. Public Health* 90, 1905–1912. doi: 10.2105/AJPH.90.12.1905

May, P. A., and Gossage, J. P. (2001). Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Res. Health* 25, 159–167.

May, P. A., Gossage, J. P., Marais, A. S., Adnams, C. M., Hoyme, H. E., Jones, K. L., et al. (2007). The epidemiology of fetal alcohol syndrome and partial FAS in a South African community. *Drug Alcohol Depend.* 88, 259–271. doi: 10.1016/j.drugalcdep.2006.11.007

McKinstry, J. (2005). Using the past to step forward: fetal alcohol syndrome in the Western Cape Province of South Africa. *Am. J. Public Health* 95, 1097–1099. doi: 10.2105/AJPH.2004.056366

Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16, 1215.

Nye, M. D., Hoyo, C., Huang, Z., Vidal, A. C., Wang, F., Overcash, F., et al. (2013). Associations between methylation of paternally expressed gene 3 (PEG3), cervical intraepithelial neoplasia and invasive cervical cancer. *PLoS ONE* 8:e56325. doi: 10.1371/journal.pone.0056325

Ogata, T., Kagami, M., and Ferguson-Smith, A. C. (2008). Molecular mechanisms regulating phenotypic outcome in paternal and maternal uniparental disomy for chromosome 14. *Epigenetics* 3, 181–187. doi: 10.4161/epi.3.4.6550

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Core Team. (2015). *nlme: Linear and Nonlinear Mixed Effects Models*. R Package Version 3.1-119. Available at: http://CRAN.R-project.org/package=nlme

R Core Team. (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org/

Reik, W., Brown, K. W., Schneid, H., Le Bouc, Y., Bickmore, W., and Maher, E. R. (1995). Imprinting mutations in the Beckwith–Wiedemann syndrome suggested by altered imprinting pattern in the IGF2-H19 domain. *Hum. Mol. Genet.* 4, 2379–2385. doi: 10.1093/hmg/4.12.2379

Relaix, F., Weng, X., Marazzi, G., Yang, E., Copeland, N., Jenkins, N., et al. (1996). Pw1, a novel zinc finger gene implicated in the myogenic and neuronal lineages. *Dev. Biol.* 177, 383–396. doi: 10.1006/dbio.1996.0172

Riley, E. P., Infante, M. A., and Warren, K. R. (2011). Fetal alcohol spectrum disorders: an overview. *Neuropsychol. Rev.* 21, 73–80. doi: 10.1007/s11065-011-9166-x

Riley, E. P., and McGee, C. L. (2005). Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp. Biol. Med. (Maywood)* 230, 357–365.

Rodenhiser, D., and Mann, M. (2006). Epigenetics and human disease: translating basic biology into clinical applications. *CMAJ* 174, 341–348. doi: 10.1503/cmaj.050774

Sandovici, I., Kassovska-Bratinova, S., Loredo-Osti, J. C., Leppert, M., Suarez, A., Stewart, R., et al. (2005). Interindividual variability and parent of origin DNA methylation differences at specific human Alu elements. *Hum. Mol. Genet.* 14, 2135–2143. doi: 10.1093/hmg/ddi218

Sarter, B., Long, T. I., Tsong, W. H., Koh, W. P., Yu, M. C., and Laird, P. W. (2005). Sex differential in methylation patterns of selected genes in Singapore Chinese. *Hum. Genet.* 117, 402–403. doi: 10.1007/s00439-005-1317-9

Schmidt, J. V., Matteson, P. G., Jones, B. K., Guan, X. J., and Tilghman, S. M. (2000). The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. *Genes Dev.* 14, 1997–2002.

Shimabukuro, M., Sasaki, T., Imamura, A., Tsujita, T., Fuke, C., Umekage, T., et al. (2007). Global hypomethylation of peripheral leukocyte DNA in male patients with schizophrenia: a potential link between epigenetics and schizophrenia. *J. Psychiatr. Res.* 41, 1042–1046. doi: 10.1016/j.jpsychires.2006.08.006

Smallwood, S. A., and Kelsey, G. (2012). *De novo* DNA methylation: a germ cell perspective. *Trends Genet.* 28, 33–42. doi: 10.1016/j.tig.2011.09.004

Sokol, R. J., Delaney-Black, V., and Nordstrom, B. (2003). Fetal alcohol spectrum disorder. *JAMA* 290, 2996–2999. doi: 10.1001/jama.290.22.2996

Stouder, C., Somm, E., and Paoloni-Giacobino, A. (2011). Prenatal exposure to ethanol: a specific effect on the H19 gene in sperm. *Reprod. Toxicol.* 31, 507–512. doi: 10.1016/j.reprotox.2011.02.009

Stratton, K., Howe, C., and Battaglia, F. (1996). *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention and Treatment*. Washington, DC: National Academy Press.

Streissguth, A. P., and Dehaene, P. (1993). Fetal alcohol syndrome in twins of alcoholic mothers: concordance of diagnosis and, I. Q. *Am. J. Med. Genet.* 47, 857–861. doi: 10.1002/ajmg.1320470612

Temple, I. K., Shrubb, V., Lever, M., Bullman, H., and Mackay, D. J. (2007). Isolated imprinting mutation of the DLK1/GTL2 locus associated with a clinical presentation of maternal uniparental disomy of chromosome 14. *J. Med. Genet.* 44, 637–640. doi: 10.1136/jmg.2007.050807

Thiaville, M. M., Huang, J. M., Kim, H., Ekram, M. B., Roh, T. Y., and Kim, J. (2013). DNA-binding motif and target genes of the imprinted transcription factor PEG3. *Gene* 512, 314–320. doi: 10.1016/j.gene.2012.10.005

Ungerer, M., Knezovich, J., and Ramsay, M. (2013). *In utero* alcohol exposure, epigenetic changes, and their consequences. *Alcohol Res.* 35, 37–46.

Urban, M., Chersich, M. F., Fourie, L. A., Chetty, C., Olivier, L., and Viljoen, D. (2008). Fetal alcohol syndrome among grade 1 schoolchildren in Northern Cape Province: prevalence and risk factors. *S. Afr. Med. J.* 98, 877–882.

Viljoen, D. L., Gossage, J. P., Brooke, L., Adnams, C. M., Jones, K. L., Robinson, L. K., et al. (2005). Fetal alcohol syndrome epidemiology in a South African community: a second study of a very high prevalence area. *J. Stud. Alcohol* 66, 593–604.

Weksberg, R., Shuman, C., and Beckwith, J. B. (2010). Beckwith–Wiedemann syndrome. *Eur. J. Hum. Genet.* 18, 8–14. doi: 10.1038/ejhg.2009.106

Wilson, V. L., and Jones, P. A. (1983). DNA methylation decreases in aging but not in immortal cells. *Science* 220, 1055–1057. doi: 10.1126/science.6844925

Woodfine, K., Huddleston, J. E., and Murrell, A. (2011). Quantitative analysis of DNA methylation at all human imprinted regions reveals preservation of epigenetic stability in adult somatic tissue. *Epigenetics Chromatin* 4, 1. doi: 10.1186/1756-8935-4-1

Yamaguchi, A., Taniguchi, M., Hori, O., Ogawa, S., Tojo, N., Matsuoka, N., et al. (2002). Peg3/Pw1 is involved in p53-mediated cell death pathway in brain ischemia/hypoxia. *J. Biol. Chem.* 277, 623–629. doi: 10.1074/jbc.M107435200

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Molecular pathways underpinning ethanol-induced neurodegeneration

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While genetics impacts the type and severity of damage following developmental ethanol exposure, little is currently known about the molecular pathways that mediate these effects. Traditionally, research in this area has used a candidate gene approach and evaluated effects on a gene-by-gene basis. Recent studies, however, have begun to use unbiased approaches and genetic reference populations to evaluate the roles of genotype and epigenetic modifications in phenotypic changes following developmental ethanol exposure, similar to studies that evaluated numerous alcohol-related phenotypes in adults. Here, we present work assessing the role of genetics and chromatin-based alterations in mediating ethanol-induced apoptosis in the developing nervous system. Utilizing the expanded family of BXD recombinant inbred mice, animals were exposed to ethanol at postnatal day 7 via subcutaneous injection (5.0 g/kg in 2 doses). Tissue was collected 7 h after the initial ethanol treatment and analyzed by activated caspase-3 immunostaining to visualize dying cells in the cerebral cortex and hippocampus. In parallel, the levels of two histone modifications relevant to apoptosis, γH2AX and H3K14 acetylation, were examined in the cerebral cortex using protein blot analysis. Activated caspase-3 staining identified marked differences in cell death across brain regions between different mouse strains. Genetic analysis of ethanol susceptibility in the hippocampus led to the identification of a quantitative trait locus on chromosome 12, which mediates, at least in part, strain-specific differential vulnerability to ethanol-induced apoptosis. Furthermore, analysis of chromatin modifications in the cerebral cortex revealed a global increase in γH2AX levels following ethanol exposure, but did not show any change in H3K14 acetylation levels. Together, these findings provide new insights into the molecular mechanisms and genetic contributions underlying ethanol-induced neurodegeneration.

Keywords: QTL, apoptosis, hippocampus, cerebral cortex, chromosome modifications, histone marks

INTRODUCTION

Alcohol exposure during development induces a number of lasting physiological changes that result in a host of abnormalities in brain function. It has been consistently shown that the type and severity of ethanol-induced changes can be modulated by the genetics of the individual as shown in studies in both animals (Goodlett et al., 1989; Gilliam and Irtenkauf, 1990; Boehm et al., 1997; Gilliam et al., 1997; Ogawa et al., 2005; Downing et al., 2009) and humans (Christoffel and Salafsky, 1975; Chasnoff, 1985; Streissguth and Dehaene, 1993; Riikonen, 1994). As the mechanisms underlying this phenomenon remain unknown, rigorous examination of the relationship between ethanol exposure, its key physiological targets, and genetic variation will enable the identification of the molecular underpinnings of the resulting damage.

The central nervous system (CNS) is especially susceptible to developmental defects following alcohol exposure, with ethanol causing aberrant mitosis and cell migration, as well as alterations in neuronal process outgrowth and connectivity (as reviewed in Sulik et al., 1988; Kumada et al., 2007; Sadrian et al., 2013). However, one of the most common ethanol-induced alterations is cell death including apoptotic cell death. Cell death occurs in a time and dose-dependent fashion although there are windows of time when specific cell populations are particularly vulnerable to ethanol-induced cell death. For example, the work of Sulik and colleagues demonstrates that developing neuroblasts are particularly vulnerable to ethanol-induced cell death shortly after neural tube closure (Dunty et al., 2001, 2002) while Olney et al. (2002a,b), Dikranian et al. (2005) demonstrated that the more mature neurons in the developing cerebral cortex and other forebrain structures are particularly vulnerable to alcohol during the early postnatal period in mice. In the present study, ethanol was administered to mice postnatally during the time of the brain growth spurt (Dobbing, 1974). During the brain growth spurt, neurons are completing migration and actively establishing connections (Dobbing, 1974). The equivalent stage of brain growth in humans begins during

the third trimester and continues the first 1–2 years after birth (Dobbing and Sands, 1979).

The genetic contributions to a phenotype are often explored using knockout animals (e.g., de Licona et al., 2009; Noel et al., 2011). While this strategy is effective in establishing the function of a specific gene, it does not reflect the spectrum and complexity of variation observed across a population. To circumvent this drawback, we have harnessed the natural variation present within mice through the use of BXD recombinant inbred strains, generated by crossing the C57BL/6J and DBA/2J strains (Morse et al., 1979; Peirce et al., 2004).

Furthermore, genetic background also contributes to the distribution of epigenetic patterns established during early development (Weng et al., 1995; Padjen et al., 2005; Schilling et al., 2009). As histone modifications and DNA methylation also respond to various environmental and cellular cues, epigenetic marks may provide a link between genetic variation and susceptibility to ethanol-induced cell death (Meaney, 2010; Kobor and Weinberg, 2011). Previous studies have investigated the effect of different exposure paradigms on histone modifications in the brain, finding that chromatin structure responds to various teratogens during development (Cronican et al., 2013; Jordi et al., 2013; Luo et al., 2014). Recent evidence also shows that acute ethanol exposure alters dimethylation levels on lysine 9 and 27 of histone 3, which partially mediate ethanol's teratogenic effects in the brain (Subbanna et al., 2013).

In the present study, a high dose of ethanol was administered to mice during the brain growth spurt at postnatal day 7 (P7) (Dobbing, 1974). Previously, strain differences in levels of ethanol-induced cell death were observed following early prenatal ethanol exposure (Chen et al., 2011) and the current study expanded this to neonatal exposure. In order to identify the contribution of the genetic background to variable susceptibility to ethanol-induced cell death, activated caspase-3 immunostaining was performed on the hippocampus and different layers of the cerebral cortex in BXD strains. Quantitative trait locus (QTL) analysis was performed to identify chromosomal locations involved in the observed differences. Finally, as an initial analysis of chromatin-based modifications in ethanol-induced apoptosis, the effect of alcohol exposure on the levels of two histone modifications, phosphorylated H2A.X and acetylated lysine 14 of histone H3, was investigated using protein blots.

MATERIALS AND METHODS

ANIMALS

Figure 1 illustrates the experimental flow from the generation of mice through to the analysis. All animals were maintained at the University of Tennessee Health Science Center (UTHSC). Mice were maintained on a 12:12 light:dark cycle and given food and water ad libitum. The BXD strains, generated by crossing the C57BL/6J (B6) and DBA/2J (D2) parental strains and inbreeding the resulting offspring for over 20 generations (Morse et al., 1979; Peirce et al., 2004), as well as the B6 and D2 parental strains, were used in the current analyses. All experiments were conducted with approval of the Institutional Animal Care and Use Committee at UTHSC. All adult mice used to generate the neonates were a minimum of 90 days of age.

Timed matings were used to generate offspring used in this study. Males and females were mated for 4 h starting between 9 and 10 a.m. daily. After the 4 h, the females were removed and checked for the presence of a vaginal plug. The presence of a vaginal plug was termed day 0 of gestation. For the neonatal mice, the day of birth was recorded and animals were exposed to ethanol or control solution on postnatal day 7 (P7).

ETHANOL EXPOSURE AND TISSUE COLLECTION

The protocol of Olney et al. (2002a) was followed. Postnatal day 7 (P7) mice were given ethanol (20% v/v in saline) via subcutaneous injection. The total dose of ethanol was 5.0 g/kg given in two injections of 2.5 g/kg separated by 2 h. Controls were given isovolumetric saline. A maximum of one male and one female in each group from each litter were used and multiple litters were evaluated from each strain. Animals were sacrificed 7 h after the initial injection.

The P7 neonates used for cell count analysis were lightly anesthetized on ice and sacrificed by decapitation. Each brain was dissected from the skull and placed into a tissue chopper where 1 mm slabs were cut. Each slab was fixed overnight in 4% PFA and changed to buffer for tissue processing. For epigenetic analyses, the brain was dissected from the skull and microdissected into various brain regions including the hippocampus and cortex. Each region was placed into a separate microfuge tube, frozen in liquid nitrogen and stored at –80°C until processing.

TISSUE PROCESSING

One slab from each brain, corresponding approximately to the region Bregma –1.955 to –2.48 mm, was picked for sectioning in a cryostat and cryoprotected using 30% sucrose in PBS and embedded in OCT (Sakura). Coronal sections were cut at 16 μm thickness in a cryostat and directly mounted on glass slides (SuperFrost Plus, Fisher Scientific).

DETECTION OF CELL DEATH

For detection of apoptotic cells in P7 brains, we performed immunohistochemistry using an antibody specific for activated caspase-3 (Abcam). Tissue sections were put in boiling 10 mM citrate buffer for 6 min and then treated with 0.3% hydrogen peroxide to quench endogenous peroxidases. Sections were incubated in blocking solution, containing 30% bovine serum albumin (1:100, Sigma-Aldrich), normal goat serum (1:10, Bethyl Laboratories), and triton X-100 (1:100, Fisher Scientific) in PBS, for 20 min, after which they were incubated in primary antibody at a 1:1000 dilution at room temperature overnight. The sections were rinsed and incubated for 1 h with biotinylated goat anti-rabbit IgG (ABC Elite Kit, Vector Laboratories). Sections were next incubated with Avidin and Biotinylated horseradish peroxidase (Vector Laboratories) for 30 min at room temperature. Immunostaining was visualized using Diaminobenzidine and tissues were counterstained with methyl green.

QUANTIFICATION OF CELL DEATH

The tissues were examined with a Zeiss fluorescence microscope and photomicrographs were taken with Axio Vision software

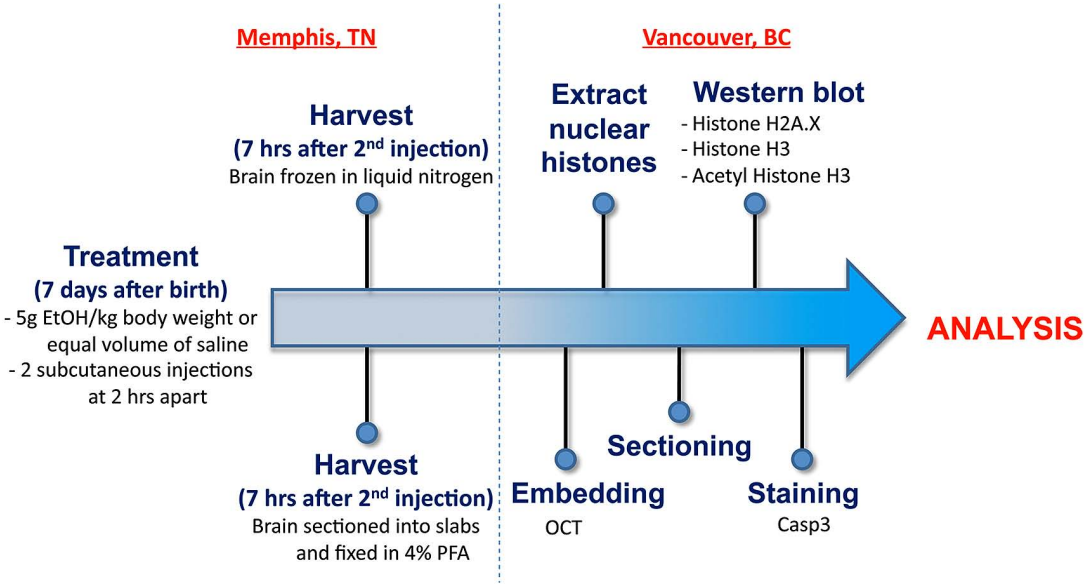

FIGURE 1 | An overview of the workflow from generation of postnatal mice through to staining and analysis.

(version 4.6). Area measurements were performed using ImageJ software (National Institutes of Health, version 1.43s). To quantify cell death, three sections from each sectioned slab were chosen, each separated by five sections. A 200 μm wide band of the CA1 region that was just distal to the CA1/2 border of the hippocampus and overlying cortex were chosen for quantification. The areas of CA1 or cortex that fell within the band were measured for caspase 3-positive cells and total cell number. Cortical layers were determined by visual assessment of cell morphology. To obtain a measure of cell death, the total number of cells within the region of interest in each section was estimated by obtaining a count of the number of cells within a 50 μm × 50 μm bin and extrapolating over the total area. The total number of stained cells within the 200 μm wide band was counted to determine a percentage of activated caspase 3-positive cells.

QTL ANALYSIS

Cell death data were registered in GeneNetwork, an open access online database containing BXD genomic information (http://www.genenetwork.org). Genome-wide interval mapping of QTLs regulating cell death was performed using WebQTL, a module of GeneNetwork. The likelihood ratio statistic (LRS) was computed to assess the strength of genotype–phenotype association of the genomic scans. Permutation test of 2000 permutations was computed to establish the significant and suggestive thresholds where the LRS values corresponded to a genome-wide p-value of 0.05 and 0.63, respectively. A significant QTL is referred to as a chromosomal region with LRS score equal to or above the genome-wide significant level ($p = 0.05$). A suggestive QTL is a region of the chromosome with LRS score equal or above the genome-wide suggestive level ($p = 0.63$).

PROTEIN BLOT ANALYSIS OF HISTONE MARKS

Nuclear histones were extracted from the cerebral cortex of male P7 mice (three control and four ethanol-treated) using previously described methods (Rumbaugh and Miller, 2011). Histones were loaded onto 15% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred onto nitrocellulose membranes and blocked with 5% milk for 2 h at room temperature. Membranes were incubated for 2 h with rabbit primary antibody at room temperature, followed by 16 h incubation at 4°C with mouse primary antibody. They were then incubated with secondary antibodies against mouse and rabbit (1/15,000) for 1 h at room temperature. Membranes were washed for 3 × 5 min between incubations with 0.1% Tween-20 Tris-buffered saline (TBST). Bands were imaged using the Li-Cor Odyssey scanner.

The antibodies used were as follows: 1/1000 rabbit polyclonal to histone H2A.X (ab10475, Abcam), 1/1000 mouse monoclonal to H2A.X (phospho-S139) (ab18311, Abcam), 1/2000 mouse monoclonal to histone H3 (ab10799, Abcam), 1/2000 rabbit polyclonal antibody to acetyl-histone H3 (Lys14) (06-911, Millipore), IRDye® 800CW conjugated Goat (polyclonal) anti-mouse IgG (926-32210, Li-Cor Biosciences), IRDye® 680 conjugated Goat (polyclonal) anti-rabbit IgG (926-32221, Li-Cor Biosciences).

QUANTIFICATION OF PROTEIN BLOTS

Using ImageStudioLite software (LiCor, Lincoln, NE), boxes were placed around each band of interest, which returned values of raw intensity. Background was removed from raw values using the median correction function to obtain the signal intensity for each protein band. γH2A.X signal intensity was normalized to total H2AX to obtain the relative ratio of γH2A.X/H2A.X for each sample and acetylated H3 (Lys14) signal was normalized to total H3 to obtain the relative ratio of H3K14ace/H3. Statistically significant

differences ($p < 0.05$) were identified using Student's t-test in Graphpad Prism 6.

RESULTS

The following study was designed to identify genetic differences in susceptibility to ethanol-induced cell death, as well as chromatin-based mechanisms that could modulate alcohol's teratogenic effect. As shown in **Figure 1**, mice from BXD strains were exposed to a high ethanol dose at P7 by subcutaneous injection. Brains were harvested and specific regions of the hippocampus and cerebral cortex analyzed for the level of ethanol-induced cell death. In parallel, whole brain regions were dissected from other samples for histone extractions and subsequent analyses of salient chromatin modifications.

GENETIC BACKGROUND ALTERED LEVELS OF ETHANOL-INDUCED CELL DEATH

In spite of the similarity observed between B6 and D2 progenitor strains, considerable differences in susceptibility to apoptosis were observed between various BXD recombinant inbred strains following ethanol treatment at P7.

In the CA1 region of the hippocampus, mean levels of caspase-3 positive cells following alcohol exposure varied between 2 and 6% of total cells within the analyzed area (**Figure 2**). Out of fourteen different lines, four exhibited cell death levels greater than 4% (BXD 1, 2, 96, 100), which were flagged as higher susceptibility backgrounds for subsequent analyses. In contrast, three strains (BXD 20, 60, 71) only displayed 2% or less mean apoptosis and were thus labeled as low vulnerability strains. Caspase-3 immunostaining of the hippocampal CA1 region in high (BXD96) and low (BXD20) susceptibility strains is illustrated in **Figure 2**.

In the cerebral cortex, ethanol-induced apoptosis was mainly localized to Layers 2/3, and 5 (**Figure 3**), with the highest levels of neurodegeneration occuring in Layer 2/3. Cell death occurred mainly in the superficial portion of Layers 2/3, while Layer 5 displayed a more homogeneous pattern of apoptosis. This specific localization was maintained across strains showing differential cell death levels. However, mean levels of apoptosis differed between genetic backgrounds (**Figure 3B**), ranging from below 5–20% of all cells within the analyzed area. Out of fourteen different strains, three (BXD 71, 80, 100) exhibited mean levels of cell death greater than 15% in Layer 2/3, and were identified as high vulnerability strains. Alternatively, three lines (BXD 32, 39, 51) showed 5% or less caspase-3 positive cells, and were flagged as low susceptibility backgrounds. In almost all cases, the percentage of cell death in Layer 5 was lower than Layer 2/3. However, the trend was similar between strains, where higher or lower cell death in a given line was observed in both regions. Caspase-3 immunostaining of the cerebral cortex in high (BXD80) and low (BXD20) vulnerability strains is illustrated in **Figure 3A**.

IDENTIFICATION OF AN ETHANOL-SUSCEPTIBILITY QUANTITATIVE TRAIT LOCUS

Previous studies have examined malformations in various strains of mice following ethanol exposure, subsequently identifying the chromosomal locations that modulated these strain differences (Downing et al., 2012a). In order to identify potential genetic drivers of variable ethanol vulnerability, QTL analysis was performed to establish correlations between cell death levels and the genotypes across BXD lines. First, levels of caspase-3 positive cells in the hippocampus and cerebral cortex were compared in order to identify a relationship between region-specific apoptosis and genetic background. However, in the examined BXD lines, no significant correlation was observed between mean levels of ethanol-induced cell death in these regions (**Figure 4**). Thus, the hippocampus and cerebral cortex were treated as separate entities for subsequent QTL analyses.

Quantitative trait locus analysis of caspase-3 positive cells in the hippocampal CA1 region revealed a significant locus on chromosome 12 (91.0–92.3 Mb, $p = 0.05$, LRS > 26.13), which is implicated in susceptibility to ethanol-induced cell death in the hippocampus (**Figure 5A**). Genes located within the locus on chromosome 12 include Dio2 deiodinase and a number of RIKEN cDNAs.

In contrast, QTL analysis of cell death in the cerebral cortex revealed a number of suggestive loci (LRS > 12.16), though none were significant (**Figures 5B,C**). QTL analysis for Layer 5 was performed separately and only identified a suggestive locus on chromosome 3 and two loci on chromosome 15 (**Figure 5C**). No suggestive loci overlapped between the different layers of the cortex, and neither had similarities with the hippocampal CA1 region.

ETHANOL EXPOSURE ALTERED H2A.X PHOSPHORYLATION

Given that both ethanol exposure and apoptosis are linked to the chromatin structure, we next examined their intersection on two physiologically relevant histone marks. An initial examination was conducted to assess the contribution of chromatin-based changes to cell death by measuring histone modification levels in the cerebral cortex of male P7 C57/BL6 mice treated with ethanol or saline. Two different modifications were examined to ascertain whether or not ethanol-exposure ubiquitously affects histone marks and if it alters modifications related to apoptosis. The first, phosphorylation of serine 139 on H2A.X (γH2A.X), was chosen due to its direct correlation with DNA damage and apoptosis. This histone H2A variant becomes locally phosphorylated in response to DNA damage to produce γ-H2AX foci in the vicinity of double-stranded breaks (Rogakou et al., 1998). While it does not play an active role in apoptosis, the generation of γ-H2AX during DNA fragmentation is essential for subsequent apoptotic phosphorylation of H2B (Rogakou et al., 2000; Fernandez-Capetillo et al., 2004). Thus, this modification provides a quantitative measure of ethanol-induced DNA damage, which may subsequently lead to cell death. The second, acetylation of lysine 14 on histone H3 (H3K14ace), was selected due to its association with transcriptional activation and presence in active enhancers, which may be indicative of changes in apoptotic gene expression (Karmodiya et al., 2012). A more recent study also showed that ethanol exposure increases levels of this mark in exon 1 of G9a, a histone demethylase involved in alcohol-induced apoptosis (Subbanna et al., 2014).

Using protein blots, analysis of γH2A.X in the cerebral cortex revealed a stark difference between ethanol- and saline-treated

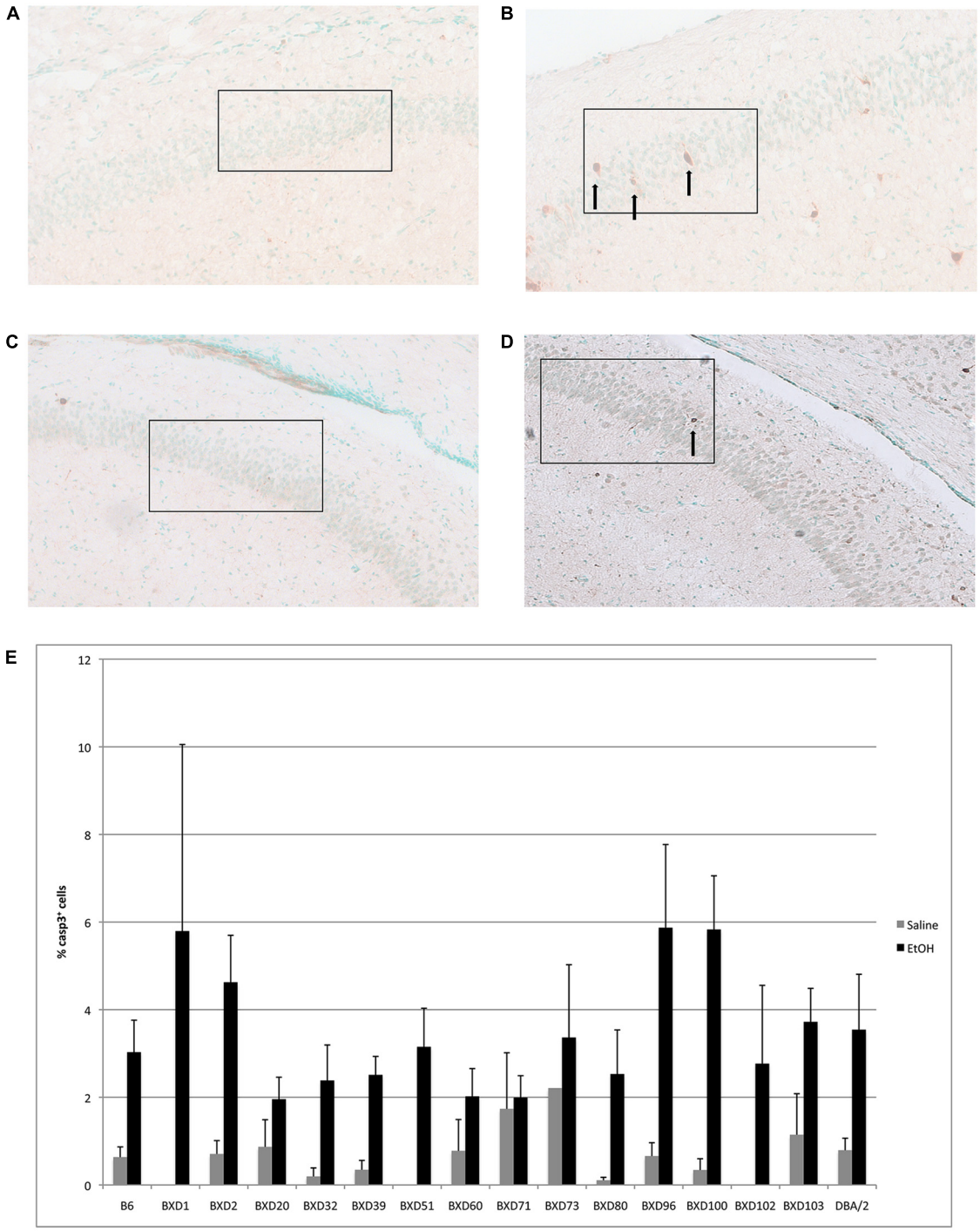

FIGURE 2 | (A) Cell death is shown in the CA1 region of resistant (BXD20) and **(B)** relatively susceptible (BXD96) strains. **(C,D)** Saline controls for BXD20 and BXD96, respectively. Boxes indicate the areas analyzed, arrows point to dying cells. **(E)** Cell death in CA1 is plotted as mean + SEM.

animals (**Figure 6A**). Alcohol exposure significantly increased the ratio of γH2A.X/H2A.X in the cerebral cortex when compared to saline-treated animals ($p = 0.04$; **Figure 6B**). This change in ethanol-treated animals was equivalent to a 1.54-fold increase compared to controls and was indicative of increased double-stranded breaks in DNA following ethanol exposure. Moreover, initial results also hinted at an increased γH2A.X/H2A.X ratio in both the cerebellum and hippocampus of P7 mice following acute ethanol exposure (data not shown).

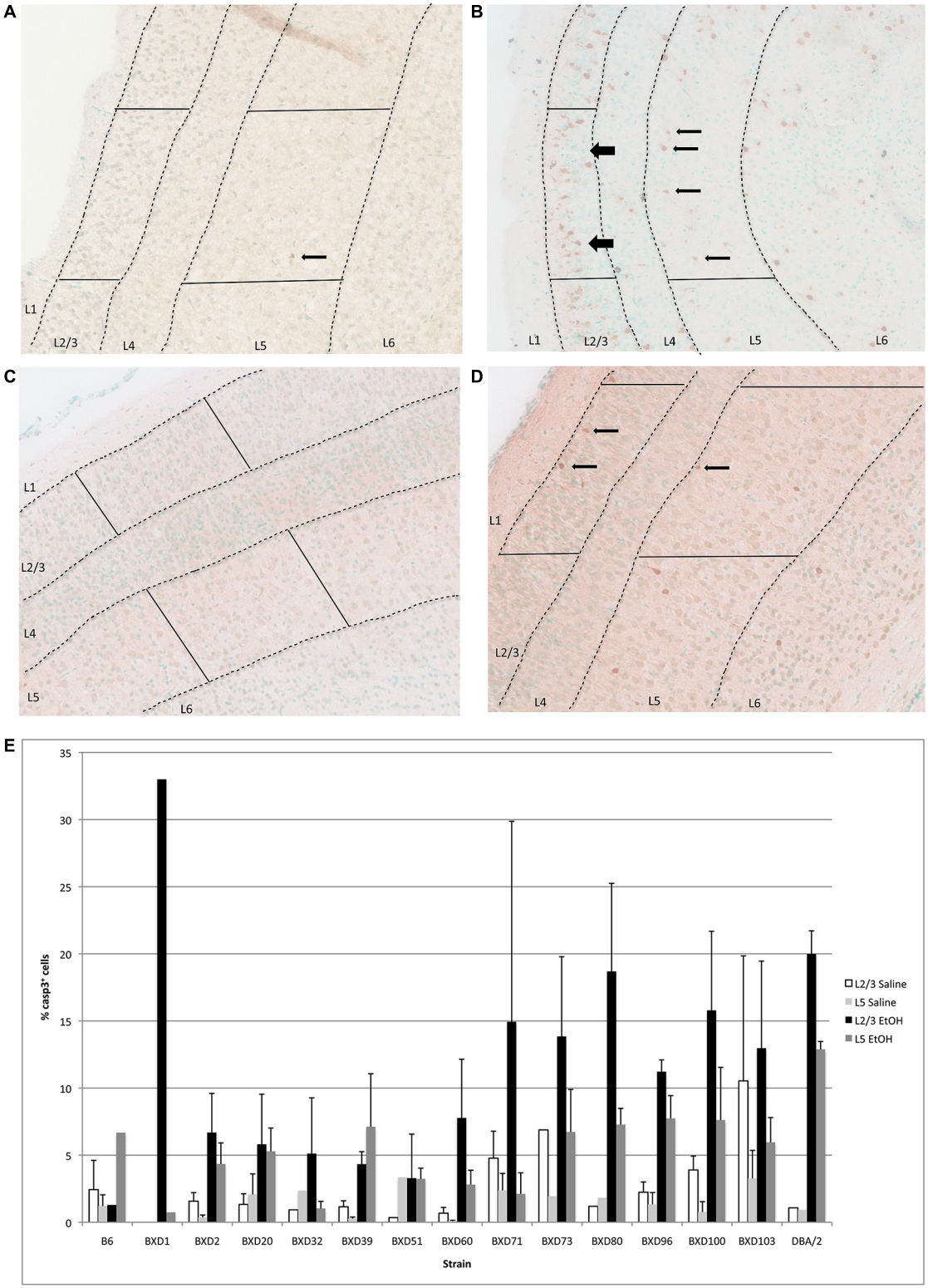

FIGURE 3 | (A) Cell death is shown in the cortex overlying the hippocampus of a region of a relatively resistant (BXD20) strain, and **(B)** a region of a relatively susceptible (BXD80) strain. **(C,D)** Saline controls for BXD20 and BXD80, respectively. Boxes highlight the area analyzed, dashed lines demarcate Layers 2/3 and 5, and arrows point to dying cells. **(E)** Cell death in cortex is plotted for Layers 2/3 and 5 as mean + SEM.

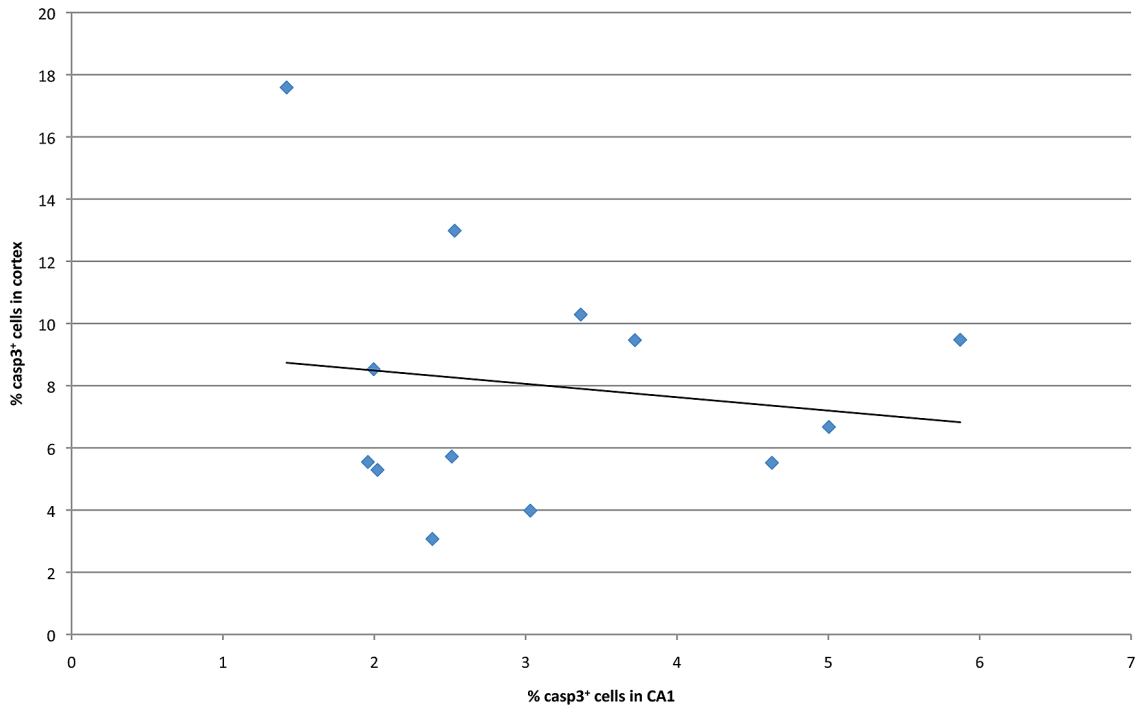

FIGURE 4 | Cell death in CA1 is plotted against averaged cell death in the cortex at P7 for all strains examined.

In contrast, total nuclear H3 and H3K14 acetylation were quantified to obtain the ratio of H3K14ace/H3, which is indicative of the relative amount of acetylated H3K14 (**Figure 7A**). However, this analysis did not reveal any differences between saline and ethanol-treated animal (**Figure 7A**), as the ratio of H3K14ace/H3 was almost identical for both treatment groups ($p = 0.32$, **Figure 7B**).

DISCUSSION

In the present study, lines of recombinant inbred mice were examined to determine if genetic variation influences the extent and localization of ethanol-induced cell death in the developing cerebral cortex and hippocampus at P7. Differential strain sensitivity to ethanol was observed, with hippocampal data translating into the identification of a QTL on chromosome 12, which mediates these strain-specific differences. To assess whether acute ethanol exposure also causes chromatin-based alterations in the developing brain, two histone modifications were examined in the cerebral cortex. Interestingly, acute ethanol exposure increased levels of γH2AX, a histone mark associated with DNA fragmentation, which is characteristic of apoptosis.

Several studies have previously examined cell death in the developing cerebral cortex and hippocampus following neonatal ethanol exposure in both rats and mice (Olney et al., 2002a,b; Wozniak et al., 2004; Young and Olney, 2006). Several of these studies utilized the same alcohol exposure paradigm and one of the same strains, B6, as the present study, which allows for direct comparisons between our results and published data. While Olney et al. (2002a,b) examined more global changes due to ethanol, rather than focusing on specific regions within structures, it is interesting to note that these papers also showed high levels of cell death within the CA1 region of the hippocampus and in much of the cerebral cortex. The type of cell death in the hippocampus has also been examined and all studies to date have shown evidence that these cells are dying via apoptotic mechanisms (e.g., Olney et al., 2002a; Wozniak et al., 2004; Young et al., 2005; Young and Olney, 2006; Ullah et al., 2011) consistent with the present study.

Additionally, examination of neuron number following neonatal ethanol exposure has been examined in the hippocampus. Because hippocampal neurons are generated prenatally, any decrease in neuronal number must result from cell death. These studies demonstrate that, within the hippocampus, the CA1 cells are highly susceptible to ethanol-induced cell death following exposure during the brain growth spurt (Livy et al., 2003; Tran and Kelly, 2003; de Licona et al., 2009). These studies are consistent with the results of the present study.

A caveat of the present experiment is that the type of cell that is undergoing apoptosis is unknown. Ethanol has been shown to result in cell death in both neurons and glia (e.g., Guerri et al., 2001; Dikranian et al., 2005) suggesting that either population could be the target. However, the location and morphology of the developing cells support the hypothesis that neurons are the vulnerable cell population in this ethanol exposure paradigm. The identification of the vulnerable population can provide insights to the identification of susceptibility genes that underlie the QTL and this will be examined in further studies using this model.

One of the issues in the present study is the small number of strains and how that may impact the ability to detect significant

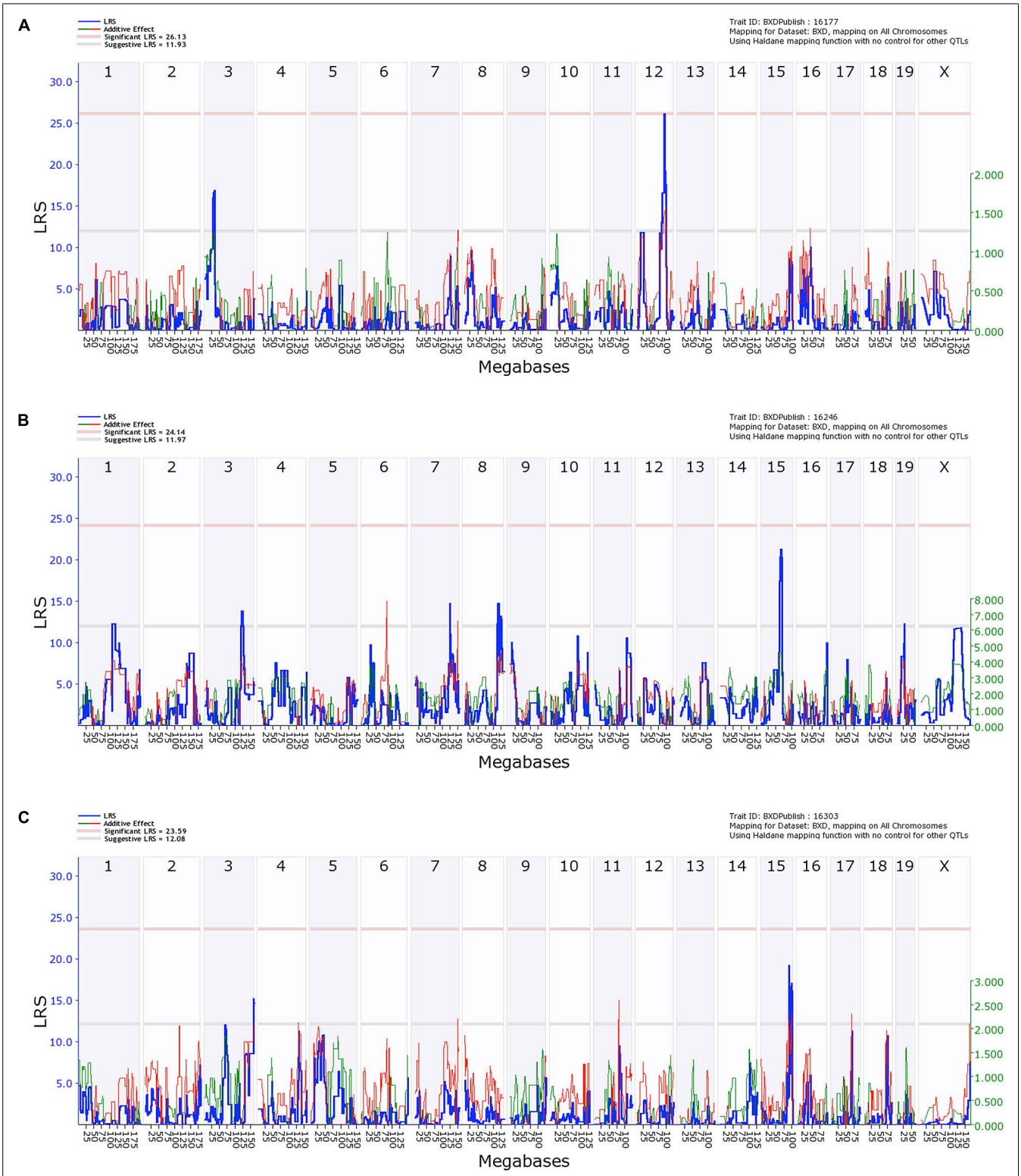

FIGURE 5 | Quantitative trait locus (QTL) analysis of cell death in the Hippocampus and Cortical Layers 2/3 and 5. On the X-axis are the chromosomes from 1 to 19 and the X. On the Y-axis are the LRS scores. The region bounded by the gray and red horizontal lines are suggestive QTLs while the region above the red line indicates significance. **(A)** Hippocampal cell death has a significant QTL on distal Chr 12 and a suggestive QTL on proximal Chr 2. **(B)** Cell death in Layers 2/3 of cortex has a series of suggestive QTLs as does Layer 5 **(C)**.

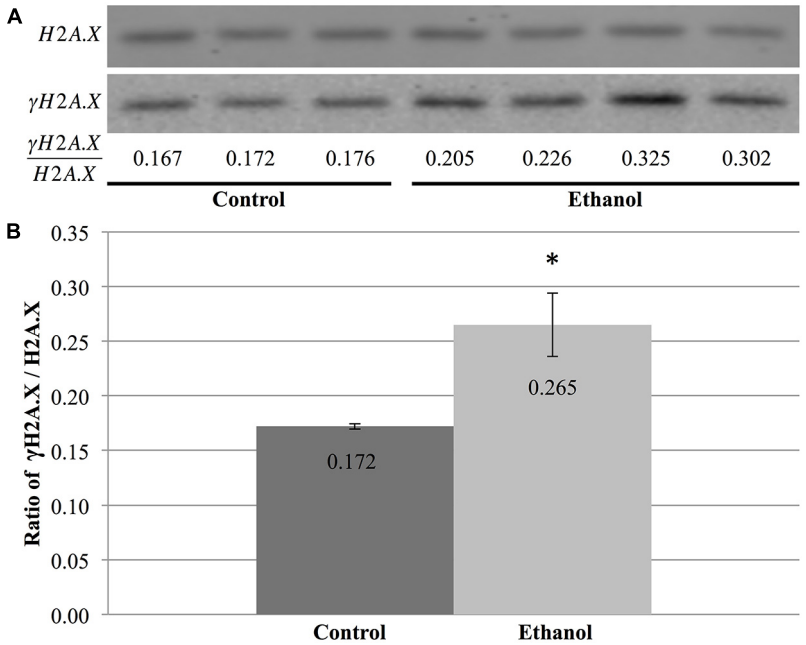

FIGURE 6 | Ethanol exposure increases levels of γH2A.X in the cerebral cortex. (A) Protein blot analysis was performed on nuclear histones isolated from the cerebral cortex of three control and four ethanol male P7 mice. H2A.X levels were used as a loading control to normalize γH2A.X levels between samples and obtain the ratio of γH2A.X/H2A.X. **(B)** The ratio of γH2A.X to H2A.X was higher in ethanol-treated animals than in control animals ($*p = 0.04$). The graph is plotted as mean +/− SEM.

QTLs as well as the reliability of the significant QTL that was detected. It is well known that the ability to detect QTLs is strongly influenced by the number of genes involved such that when there are a small number of genes with large effects on the phenotypic outcome, significant QTLs can be detected with a low number of strains (Flint et al., 2005). In the present experiment, a significant QTL was detected in the hippocampus suggesting that there is a gene within the QTL that has a large impact on genetic differences in ethanol-induced neurodegeneration in that brain region. In contrast, the small number of strains used in the present study translates into lower statistical power that can mean that significant QTLs are not detected. In the cerebral cortex in the present experiment, this is the case and if more strains had been examined, significant, rather than suggestive, QTLs may have been identified in the cortex as well. In regards to the issue of the reliability of significant QTLs, the presence of outliers is an important consideration. Confidence in the validity of a QTL is lessened if there are outliers that are strongly impacting the strain distribution in the phenotypic readout (Hayat et al., 2008; Yang et al., 2009). In the present experiment, the strains show a relatively even distribution and therefore, outliers are not influencing the current analyses providing support that this is a reliable QTL.

Quantitative trait locus analysis demonstrated that the strain-specific differences in ethanol-induced cell death in the hippocampus are modulated, at least in part, by a gene located on Chromosome 12 at approximately 90 Mb. This is a novel chromosomal location with no previously established relationship to ethanol's teratogenic actions (Downing et al., 2012a). This region is relatively narrow with only two genes, six RIKEN clones, and one EST located within the QTL interval. Cell death, and in particular apoptosis, can be caused by a number of mechanisms including loss of growth factors and excitotoxicity (e.g., Bhutta and Anand, 2002; Nikolić et al., 2013). It is of interest therefore, that proximal to the region of the QTL there are several genes that are related to growth factors including the latent transforming growth factor protein 2 (ltbp2), placental growth factor (pgf), and transforming growth factor beta (Tgf beta). However, while these are certainly interesting candidates that cannot be excluded at this time, the rapid nature of the cell death induction in the present analysis led to the hypothesis that the causal gene underlying the QTL is more likely to have a direct link to cell death and the analysis was focused on these genes.

This region of the genome contained several genes that were more directly linked to apoptosis and cell death. However, all the potential candidate genes have the following caveats: (1) none has been linked to cell death following any form of alcohol exposure, and (2) while present in the vicinity of the Ch 12 QTL, none are within the 1LOD interval of the QTL. The candidate gene with the strongest link to apoptosis is Rbm25, an RNA binding protein that has been shown to modulate the expression of isoforms of Bcl2 (Zhou et al., 2008). However, Rbm25 is located farthest from the QTL and its role in apoptosis within the CNS remains unknown. Additionally, both fos and the fos receptor (fosr) as well as jun dimerization protein (jdp2 or jund2) are located close to the QTL. While Fos and jdp2 have been linked to apoptosis (e.g., Lerdrup et al., 2005; Durchdewald et al., 2009), these intermediate early genes have well-documented functions in a number of processes

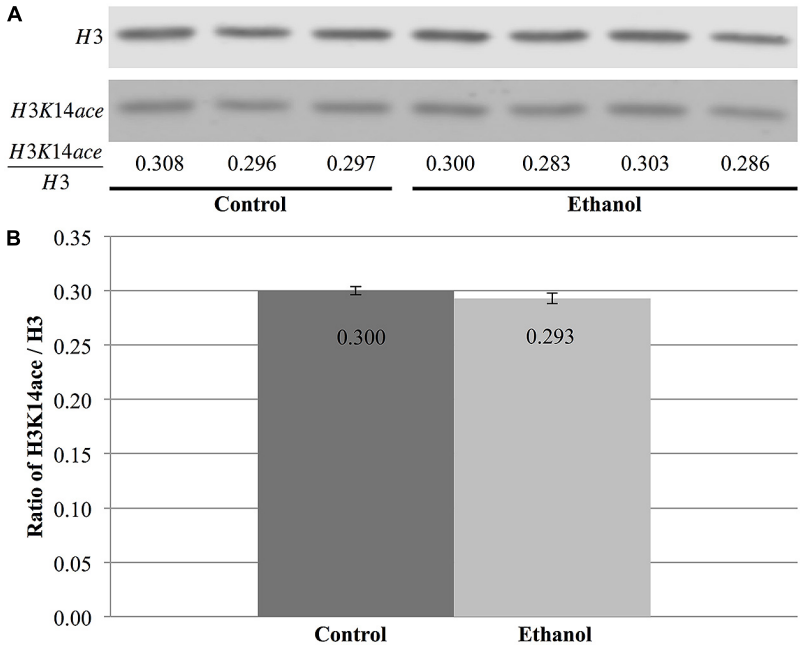

FIGURE 7 | Ethanol exposure does not affect levels of H3K14 acetylation in the cerebral cortex. (A) Protein blot analysis was performed on nuclear histones isolated from the cerebral cortex of three control and four ethanol male P7 mice. Total H3 levels were used as a loading control to normalize H3K14 acetylation levels between samples and obtain the ratio of H3K14ace/H3. **(B)** The ratio of H3K14ace to H3 was the same in ethanol-treated animals versus control animals ($p = 0.32$). The graph is plotted as mean +/− SEM.

and thus, the specificity of the effects to the apoptotic processes is currently unknown.

Based on the current data, the best candidate is neuroglobin (Ngb), a relatively recently described gene that encodes a protein that functions to provide oxygen to the CNS (Fiocchetti et al., 2013). Ngb sits close to the QTL region and, as shown on WebQTL, possesses several sequence polymorphisms between the B6 and D2 genome. Genes that lack sequence polymorphisms, and therefore are identical between the two strains, are less likely to mediate strain differences. Moreover, Ngb has been linked to apoptosis caused by a range of factors including oxidative stress (Li et al., 2008) and arsenic toxicity (Liu et al., 2013) while also playing a neuroprotective role following stroke (Yu et al., 2013). Given that hypoxia has been suggested to play a role in ethanol's teratogenic effects (Mukherjee and Hodgen, 1982; Mitchell et al., 1998; Parnell et al., 2007), neuroglobulin is an intriguing candidate for mediating strain differences in ethanol-induced cell death.

Differences in chromatin regulation may also be contributing to the strain-specific differences in cell death. Epigenetic marks are emerging as major regulators of gene-by-environment interactions and have been implicated in the etiology of ethanol-induced neurodegeneration. In fact, G9a-mediated increases of H3K9 and H3K27 dimethylation regulate proteolytic cleavage of histones by caspase-3 and subsequent neurodegeneration in the hippocampus and neocortex following acute, low-dose ethanol exposure (Subbanna et al., 2013). Furthermore, the same group recently showed H3K14 acetylation levels on exon1 of G9a increase following low-dose ethanol exposure (Subbanna et al., 2014).

Our results also support a role for chromatin-based marks in ethanol-induced apoptosis of cells in the CNS. However, similar to previous studies examining cocaine exposure (Jordi et al., 2013), this remains a global analysis of the genome, rather than a targeted gene approach, and does not identify changes in H3K14 acetylation or γH2A.X in specific regions of the genome. While the cerebral cortex did not show any differences in the levels of H3K14 acetylation following alcohol exposure, the ratio of γH2A.X to H2A.X was increased by an acute ethanol treatment. Although these two marks do not perform the same function, these results suggest that ethanol does not have a broad impact over different histone modifications, but, rather, acts in a specific fashion by altering at least a subset of chromatin-based mechanisms linked to apoptosis. Moreover, this effect seemed to occur across all analyzed brain regions (cortex; preliminary data: cerebellum, hippocampus), implying that this is a global response to ethanol exposure, rather than a region-specific event.

The observed difference of γH2A.X in the cerebral cortex following alcohol exposure is likely caused by increased rates of double-stranded breaks (Rogakou et al., 2000). This may be a direct effect of ethanol, where its exposure increases reactive oxygen species (ROS) levels in the cell, which, in turn, cause more DNA damage (Kotch et al., 1995). Widespread DNA damage might then cause the affected cells to undergo apoptosis. In this situation, γH2A.X might play a mechanistic role in ethanol-induced neuroapoptosis. However, the effect may also be indirect, where, instead, ethanol exposure activates apoptotic programming through other mechanisms, leading to DNA fragmentation and subsequent H2A.X phosphorylation (Rogakou et al., 2000).

Here, γH2A.X would simply be a passenger to neurodegeneration, a simple consequence of cell death. Though there is evidence in the field for both these possibilities, the current data does not allow for a distinction to be made between the two. However, further experiments investigating the link between ROS and γH2A.X following ethanol exposure and the activation patterns of apoptotic programs may provide additional insight into their plausibility.

Similar to the issue discussed above, protein blot analysis of the whole cerebral cortex does not allow for differentiation between the types of cells affected by ethanol. As the brain is composed of many different cell types, unaffected cells may dampen some signal in the event that a single cellular species is affected by ethanol in this fashion. In turn, this would explain the relatively low fold change of ethanol-treated animals compared to control (1.54). Higher resolution techniques, such as immunofluorescence will be better suited for this type of analysis in additional mouse strains.

Several studies have also begun to characterize DNA methylation changes following prenatal and postnatal ethanol exposure (Haycock, 2009; Haycock and Ramsay, 2009). In mouse models, ethanol exposure during embryonic days 9–11 was shown to cause global genomic hypomethylation and decreased DNA methyltransferase activity in the fetal genome (Garro et al., 1991). However, recent studies have shown that some regions become hypermethylated and others hypomethylated in embryonic cultures exposed to ethanol (Liu et al., 2009; Chen et al., 2013). In vivo animal studies have corroborated these findings, showing that fetal alcohol exposure results in long-lasting alteration to the brain's DNA methylome, notably in regulatory sequences and imprinted regions containing non-coding RNA (Laufer et al., 2013). Combined with data on histone modifications, these studies support a role for epigenetic changes in ethanol-induced developmental defects.

In summary, we demonstrate that acute neonatal ethanol exposure causes cell death in the developing cerebral cortex and hippocampus in a strain-specific manner. Moreover, we show that a region on chromosome 12 could, at least in part, mediate differential strain sensitivity to ethanol in the hippocampus and identify a number of putative candidate genes that may underlie this QTL. Further studies are required to pinpoint the gene(s) that mediates this vulnerability to ethanol-induced apoptosis in the hippocampus, as well as other brain regions. Identification will be facilitated by identifying whether any genes within the QTL exhibit strain-specific changes in expression following ethanol exposure similar to other studies examining stain-specific expression differences (Green et al., 2007; Downing et al., 2012b). This study also begins to examine whether chromatin-based modifications contribute to differential strain sensitivity, showing that acute ethanol exposure can indeed alter histone modifications. Additional studies into the level of epigenetic alteration between different genotypes will be required to identify their role in variable susceptibility to ethanol-induced neurodegeneration.

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REFERENCES

Bhutta, A. T., and Anand, K. J. (2002). Vulnerability of the developing brain. Neuronal mechanisms. *Clin. Perinatol.* 29, 357–372. doi: 10.1016/S0095-5108(02)00011-8

Boehm, S. L. II, Lundahl, K. R., Caldwell, J., and Gilliam, D. M. (1997). Ethanol teratogenesis in the C57BL/6J, DBA/2J, and A/J inbred mouse strains. *Alcohol* 14, 389–395. doi: 10.1016/S0741-8329(97)87950-5

Chasnoff, I. J. (1985). Fetal alcohol syndrome in twin pregnancy. *Acta Genet. Med. Gemellol. (Roma)* 34, 229–232.

Chen, Y., Ozturk, N. C., Ni, L., Goodlett, C., and Zhou, F. C. (2011). Strain differences in developmental vulnerability to alcohol exposure via embryo culture in mice. *Alcohol. Clin. Exp. Res.* 35, 1293–1304. doi: 10.1111/j.1530-0277.2011.01465.x

Chen, Y., Ozturk, N. C., and Zhou, F. C. (2013). DNA methylation program in developing hippocampus and its alteration by alcohol. *PLoS ONE* 8:e60503. doi: 10.1371/journal.pone.0060503

Christoffel, K. K., and Salafsky, I. (1975). Fetal Alcohol Syndrome in dizygotic twins. *J. Pediatr.* 87, 963–967. doi: 10.1016/S0022-3476(75)80919-X

Cronican, A. A., Fitz, N. F., Carter, A., Saleem, M., Shiva, S., Barchowsky, A., et al. (2013). Genome-wide alteration of histone H3K9 acetylation pattern in mouse offspring prenatally exposed to arsenic. *PLoS ONE* 8:e53478. doi: 10.1371/journal.pone.0053478

de Licona, H. K., Karacay, B., Mahoney, J., McDonald, E., Luang, T., and Bonthius, D. J. (2009). A single exposure to alcohol during brain development induces microencephaly and neuronal losses in genetically susceptible mice, but not in wild type mice. *Neurotoxicology* 30, 459–470. doi: 10.1016/j.neuro.2009.01.010

Dikranian, K., Qin, Y. Q., Labruyere, J., Nemmers, B., and Olney, J. W. (2005). Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Brain Res. Dev. Brain Res.* 155, 1–13. doi: 10.1016/j.devbrainres.2004.11.005

Dobbing, J. (1974). The later growth of the brain and its vulnerability. *Pediatrics* 53, 2–6.

Dobbing, J., and Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Hum. Dev.* 3, 79–83. doi: 10.1016/0378-3782(79)90022-7

Downing, C., Balderrama-Durbin, C., Broncucia, H., Gilliam, D., and Johnson, T. E. (2009). Ethanol teratogenesis in five inbred strains of mice. *Alcohol. Clin. Exp. Res.* 33, 1238–1245. doi: 10.1111/j.1530-0277.2009.00949.x

Downing, C., Balderrama-Durbin, C., Kimball, A., Biers, J., Wright, H., Gilliam, D., et al. (2012a). Quantitative trait locus mapping for ethanol teratogenesis in BXD recombinant inbred mice. *Alcohol. Clin. Exp. Res.* 36, 1340–1354. doi: 10.1111/j.1530-0277.2012.01754.x

Downing, C., Flink, S., Florez-McClure, M. L., Johnson, T. E., Tabakoff, B., and Kechris, K. J. (2012b). Gene expression changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 36, 1519–1529. doi: 10.1111/j.1530-0277.2012.01757.x

Dunty, W. C. Jr., Chen, S. Y., Zucker, R. M., Dehart, D. B., and Sulik, K. K. (2001). Selective vulnerability of embryonic cell populations to ethanol-induced apoptosis: implications for alcohol-related birth defects and neurodevelopmental disorder. *Alcohol. Clin. Exp. Res.* 25, 1523–1535. doi: 10.1111/j.1530-0277.2001.tb02156.x

Dunty, W. C. Jr., Zucker, R. M., and Sulik, K. K. (2002). Hindbrain and cranial nerve dysmorphogenesis result from acute maternal ethanol administration. *Dev. Neurosci.* 24, 328–342. doi: 10.1159/000066748

Durchdewald, M., Angel, P., and Hess, J. (2009). The transcription factor Fos: a Janus-type regulator in health and disease. *Histol. Histopathol.* 24, 1451–1461.

Fernandez-Capetillo, O., Allis, C. D., and Nussenzweig, A. (2004). Phosphorylation of histone H2B at DNA double-strand breaks. *J. Exp. Med.* 199, 1671–1677. doi: 10.1084/jem.20032247

Fiocchetti, M., De Marinis, E., Ascenzi, P., and Marino, M. (2013). Neuroglobin and neuronal cell survival. *Biochim. Biophys. Acta* 1834, 1744–1749. doi: 10.1016/j.bbapap.2013.01.015

Flint, J., Valdar, W., Shifman, S., and Mott, R. (2005). Strategies for mapping and cloning quantitative trait genes in rodents. *Nat. Rev. Genet.* 6, 271–286. doi: 10.1038/nrg1576

Garro, A. J., McBeth, D. L., Lima, V., and Lieber, C. S. (1991). Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol. Clin. Exp. Res.* 5, 395–398. doi: 10.1111/j.1530-0277.1991.tb00536.x

Gilliam, D. M., and Irtenkauf, K. T. (1990). Maternal genetic effects on ethanol teratogenesis and dominance of relative embryonic resistance to malformations. *Alcohol. Clin. Exp. Res.* 14, 539–545. doi: 10.1111/j.1530-0277.1990.tb01196.x

Gilliam, D. M., Mantle, M. A., Barkhausen, D. A., and Tweden, D. R. (1997). Effects of acute prenatal ethanol administration in a reciprocal cross of C57BL/6J and short-sleep mice: maternal effects and nonmaternal factors. *Alcohol. Clin. Exp. Res.* 21, 28–34.

Goodlett, C. R., Gilliam, D. M., Nichols, J. M., and West, J. R. (1989). Genetic influences on brain growth restriction induced by developmental exposure to alcohol. *Neurotoxicology* 10, 321–334.

Green, M. L., Singh, A. V., Zhang, Y., Nemeth, K. A., Sulik, K. K., and Knudsen, T. B. (2007). Reprogramming of genetic networks during initiation of the Fetal Alcohol Syndrome. *Dev. Dyn.* 236, 613–631. doi: 10.1002/dvdy.21048

Guerri, C., Pascual, M., and Renau-Piqueras, J. (2001). Glia and fetal alcohol syndrome. *Neurotoxicology* 22, 593–599. doi: 10.1016/S0161-813X(01)00037-7

Hayat Y., Yang, J., Xu, H. M., and Zhu, J. (2008). Influence of outliers on QTL mapping for complex traits. *J. Zheijiang Univ. Sci. B* 9, 931–937. doi: 10.1631/jzus.B0820045

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod.* 81, 607–617. doi: 10.1095/biolreprod.108.074690

Haycock, P. C., and Ramsay, M. (2009). Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol. Reprod.* 81, 618–627. doi: 10.1095/biolreprod.108.074682

Jordi, E., Heiman, M., Marion-Poll, L., Guermonprez, P., Cheng, S. K., Nairn, A. C., et al. (2013). Differential effects of cocaine on histone posttranslational modifications in identified populations of striatal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9511–9516. doi: 10.1073/pnas.1307116110

Karmodiya, K., Krebs, A. R., Oulad-Abdelghani, M., Kimura, H., and Tora, L. (2012). H3K9 and H3K14 acetylation co-occur at many gene regulatory elements, while H3K14ac marks a subset of inactive inducible promoters in mouse embryonic stem cells. *BMC Genomics* 13:424. doi: 10.1186/1471-2164-13-424

Kobor, M. S., and Weinberg, J. (2011). Focus on: epigenetics and fetal alcohol spectrum disorders. *Alcohol Res. Health* 34, 29–37.

Kotch, L. E., Chen, S. Y., and Sulik, K. K. (1995). Ethanol-induced teratogenesis: free radical damage as a possible mechanism. *Teratology* 52, 128–136. doi: 10.1002/tera.1420520304

Kumada, T., Jiang, Y., Cameron, D. B., and Komuro, H. (2007). How does alcohol impair neuronal migration? *J. Neurosci. Res.* 85, 465–470. doi: 10.1002/jnr.21149

Laufer, B. I., Mantha, K., Kleiber, M. L., Diehl, E. J., Addison, S. M., and Singh, S. M. (2013). Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis. Model. Mech.* 6, 977–992. doi: 10.1242/dmm.010975

Lerdrup, M., Holmberg, C., Dietrich, N., Shaulian, E., Herdegen, T., Jäättelä, M., et al. (2005). Depletion of the AP-1 repressor JDP2 induces cell death similar to apoptosis. *Biochim. Biophys. Acta* 1745, 29–37. doi: 10.1016/j.bbamcr.2005.06.008

Li, R. C., Morris, M. W., Lee, S. K., Pouranfar, F., Wang, Y., and Gozal, D. (2008). Neuroglobin protects PC12 cells against oxidative stress. *Brain Res.* 1190, 159–166. doi: 10.1016/j.brainres.2007.11.022

Liu, Y., Balaraman, Y., Wang, G., Nephew, K. P., and Zhou, F. C. S. (2009). Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4, 500–511. doi: 10.4161/epi.4.7.9925

Liu, X., Gao, Y., Yao, H., Zhou, L., Sun, D., and Wang, J. (2013). Neuroglobin involvement in the course of arsenic toxicity in rat cerebellar granule neurons. *Biol. Trace Elem. Res.* 155, 439–446. doi: 10.1007/s12011-013-9810-9

Livy, D. J., Miller E. K., Maier, S. E., and West, J. R. (2003). Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicol. Teratol.* 25, 447–458. doi: 10.1016/S0892-0362(03)00030-8

Luo, M., Xu, Y., Cai, R., Tang, Y., Ge, M. M., Liu, Z. H., et al. (2014). Epigenetic histone modification regulates developmental lead exposure induced hyperactivity in rats. *Toxicol. Lett.* 225, 78–85. doi: 10.1016/j.toxlet.2013.11.025

Meaney, M. J. (2010). Epigenetics and the biological definition of gene x environment interactions. *Child Dev.* 81, 41–79. doi: 10.1111/j.1467-8624.2009.01381.x

Mitchell, J. J., Paiva, M., Moore, D. B., Walker, D. W., and Heaton, M. B. (1998). A comparative study of ethanol, hypoglycemia, hypoxia and neurotrophic factor interactions with fetal rat hippocampal neurons: a multi-factor in vitro model of developmental ethanol effects. *Brain Res. Dev. Brain Res.* 105, 241–250. doi: 10.1016/S0165-3806(97)00182-X

Morse, H. C. III, Chused, T. M., Hartley, J. W., Mathieson, B. J., Sharrow, S. O., and Taylor, B. A. (1979). Expression of xenotropic murine leukemia viruses as cell-surface gp70 in genetic crosses between strains DBA/2 and C57BL/6. *J. Exp. Med.* 149, 1183–1196. doi: 10.1084/jem.149.5.1183

Mukherjee, A. B., and Hodgen, G. D. (1982). Maternal ethanol exposure induces transient impairment of umbilical circulation and fetal hypoxia in monkeys. *Science* 218, 700–702. doi: 10.1126/science.6890235

Nikolić, M., Gardner, H. A., and Tucker, K. L. (2013). Postnatal neuronal apoptosis in the cerebral cortex: physiological and pathophysiological mechanisms. *Neuroscience* 254, 369–378. doi: 10.1016/j.neuroscience.2013.09.035

Noel, M., Norris, E. H., and Strickland, S. (2011). Tissue plasminogen activator is required for the development of fetal alcohol syndrome in mice. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5069–5074. doi: 10.1073/pnas.1017608108

Ogawa, T., Kuwagata, M., Ruiz, J., and Zhou, F. C. (2005). Differential teratogenic effect of alcohol on embryonic development between C57BL/6 and DBA/2 mice: a new view. *Alcohol. Clin. Exp. Res.* 29, 855–863. doi: 10.1097/01.ALC.0000163495.71181.10

Olney, J. W., Tenkova, T., Dikranian, K., Muglia, L. J., Jermakowicz, W. J., D'Sa, C., et al. (2002a). Ethanol-induced caspase-3 activation in the in vivo developing mouse brain. *Neurobiol. Dis.* 9, 205–219. doi: 10.1006/nbdi.2001.0475

Olney, J. W., Tenkova, T., Dikranian, K., Qin, Y. Q., Labruyere, J., and Ikonomidou, C. (2002b). Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Brain Res. Dev. Brain Res.* 133, 115–126. doi: 10.1016/S0165-3806(02)00279-1

Padjen, K., Ratnam, S., and Storb, U. (2005). DNA methylation precedes chromatin modifications under the influence of the strain-specific modifier Ssm1. *Mol. Cell. Biol.* 25, 4782–4791. doi: 10.1128/MCB.25.11.4782-4791.2005

Parnell, S. E., Ramadoss, J., Delp, M. D., Ramsey, M. W., Chen, W. J., West, J. R., et al. (2007). Chronic ethanol increases fetal cerebral blood flow specific to the ethanol-sensitive cerebellum under normoxaemic, hypercapnic and acidaemic conditions: ovine model. *Exp. Physiol.* 92, 933–943. doi: 10.1113/expphysiol.2007.038091

Peirce, J. L., Lu, L., Gu, J., Silver, L. M., and Williams, R. W. (2004). A new set of BXD recombinant inbred lines from advanced intercross populations in mice. *BMC Genet.* 5:7. doi: 10.1186/1471-2156-5-7

Riikonen, R. S. (1994). Difference in susceptibility to teratogenic effects of alcohol in discordant twins exposed to alcohol during the second half of gestation. *Pediatr. Neurol.* 11, 332–336. doi: 10.1016/0887-8994(94)90012-4

Rogakou, E. P., Nieves-Neira, W., Boon, C., Pommier, Y., and Bonner, W. M. (2000). Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J. Biol. Chem.* 275, 9390–9395. doi: 10.1074/jbc.275.13.9390

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* 273, 5858–5868. doi: 10.1074/jbc.273.10.5858

Rumbaugh, G., and Miller, C. A. (2011). Epigenetic changes in the brain: measuring global histone modifications. *Methods Mol. Biol.* 670, 263–274. doi: 10.1007/978-1-60761-744-0_18

Sadrian, B., Wilson, D. A., and Saito, M. (2013). Long-lasting neural circuit dysfunction following developmental ethanol exposure. *Brain Sci.* 3, 704–727. doi: 10.3390/brainsci3020704

Schilling, E., El Chartouni, C., and Rehli, M. (2009). Allele-specific DNA methylation in mouse strains is mainly determined by cis-acting sequences. *Genome Res.* 19, 2028–2035. doi: 10.1101/gr.095562.109

Streissguth, A. P., and Dehaene, P. (1993). Fetal Alcohol Syndrome in twins of alcoholic mothers: concordance of diagnosis and IQ. *Am. J. Med. Genet.* 47, 857–861. doi: 10.1002/ajmg.1320470612

Subbanna, S., Nagre, N. N., Shivakumar, M., Umapathy, N. S., Psychoyos, D., and Basavarajappa, B. S. (2014). Ethanol induced acetylation of histone at G9a exon1 and G9a-mediated histone H3 dimethylation leads to neurodegeneration in neonatal mice. *Neuroscience* 258, 422–432. doi: 10.1016/j.neuroscience.2013.11.043

Subbanna, S., Shivakumar, M., Umapathy, N. S., Saito, M., Mohan, P. S., Kumar, A., et al. (2013). G9a-mediated histone methylation regulates ethanol-induced neurodegeneration in the neonatal mouse brain. *Neurobiol. Dis.* 54, 475–485. doi: 10.1016/j.nbd.2013.01.022

Sulik, K. K., Cook, C. S., and Webster, W. S. (1988). Teratogens and craniofacial malformations: relationships to cell death. *Development* 103(Suppl.), 213–231.

Tran, T. D., and Kelly, S. J. (2003). Critical periods for ethanol-induced cell loss in the hippocampal formation. *Neurotoxicol. Teratol.* 25, 519–528. doi: 10.1016/S0892-0362(03)00074-6

Ullah, N., Naseer, M. I., Ullah, I., Lee, H. Y., Koh, P. O., and Kim, M. O. (2011). Protective effect of pyruvate against ethanol-induced apoptotic neurodegeneration in the developing rat brain. *Neurophamacology* 61, 1248–1255. doi: 10.1016/j.neuropharm.2011.06.031

Weng, A., Magnuson, T., and Storb, U. (1995). Strain-specific transgene methylation occurs early in mouse development and can be recapitulated in embryonic stem cells. *Development* 121, 2853–2859.

Wozniak, D. F., Hartman, R. E., Boyle, M. P., Vogt, S. K., Brooks, A. R., Tenkova, T., et al. (2004). Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. *Neurobiol. Dis.* 17, 403–414. doi: 10.1016/j.nbd.2004.08.006

Yang, R., Wang, X., Li, J., and Deng, H. (2009). Bayesian robust analysis for genetic architecture of quantitative traits. *Bioinformatics* 25, 1033–1039. doi: 10.1093/bioinformatics/btn558

Young, C., and Olney, J. W. (2006). Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol. Dis.* 22, 548–554. doi: 10.1016/j.nbd.2005.12.015

Young, C., Roth, K. A., Klocke, B. J., West, T., Holtzman, D. M., Labruyere, J., et al. (2005). Role of caspase-3 in ethanol-induced developmental neurodegeneration. *Neurobiol. Dis.* 20, 608–614. doi: 10.1016/j.nbd.2005.04.014

Yu, Z., Poppe, J. L., and Wang, X. (2013). Mitochondrial mechanisms of neuroglobin's neuroprotection. *Oxid. Med. Cell. Longev.* 2013, 756989. doi: 10.1155/2013/756989

Zhou, A., Ou, A. C., Cho, A., Benz, E. J. Jr., and Huang, S. C. (2008). Novel splicing factor RBM25 modulates Bcl-x pre-mRNA 5' splice site selection. *Mol. Cell. Biol.* 28, 5924–5936. doi: 10.1128/MCB.00560-08

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Epigenetic regulation of the neural transcriptome and alcohol interference during development

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Alcohol intoxicated cells broadly alter their metabolites – among them methyl and acetic acid can alter the DNA and histone epigenetic codes. Together with the promiscuous effect of alcohol on enzyme activities (including DNA methyltransferases) and the downstream effect on microRNA and transposable elements, alcohol is well placed to affect intrinsic transcriptional programs of developing cells. Considering that the developmental consequences of early alcohol exposure so profoundly affect neural systems, it is not unfounded to reason that alcohol exploits transcriptional regulators to challenge canonical gene expression and in effect, intrinsic developmental pathways to achieve widespread damage in the developing nervous system. To fully evaluate the role of epigenetic regulation in alcohol-related developmental disease, it is important to first gather the targets of epigenetic players in neurodevelopmental models. Here, we attempt to review the cellular and genomic windows of opportunity for alcohol to act on intrinsic neurodevelopmental programs. We also discuss some established targets of fetal alcohol exposure and propose pathways for future study. Overall, this review hopes to illustrate the known epigenetic program and its alterations in normal neural stem cell development and further, aims to depict how alcohol, through neuroepigenetics, may lead to neurodevelopmental deficits observed in fetal alcohol spectrum disorders.

Keywords: neuroepigenetics, neural stem cells, DNA methylation, histone modification, miRNA, epigenomics, neural developmental pathway, gene–environment interaction

PART ONE: NORMAL EPIGENETIC PROGRAM IN DIFFERENTIATING NEURAL STEM CELLS (NSCs)

INTRODUCTION

When neural precursors begin their journey into specified, mature neurons they undergo much transcriptional re-programming. This involves the silencing of pluripotency genes that act to keep the cell in a primordial stage as well as the activation of neuron-specific genes that permit the morphological and functional capabilities of the mature cell. It comes as no surprise then that a host of chromatin remodeling proteins, including epigenetic machinery, undergo considerable transformation during this time. After all, to accommodate the transcriptional changes necessary for cellular specification, relevant DNA regions must undergo structural changes to either facilitate or hinder the accessibility of the loci to transcriptional machinery. In just the last decade, an unprecedented growth in our understanding of the molecular underpinnings of these structural changes has occurred. We have uncovered and expanded the investigation of several classes of epigenetic modifications from histone to DNA and more recently, non-coding elements of the genome which can also play a role in shaping which genes are expressed during the critical, developmental phases of neural maturation. Skepticism regarding the gravity of these epigenetic factors in normal mammalian development has been answered by the revelation that deleting critical enzymes, such as DNA methyltransferases, decreases the viability of offspring or results in embryonic lethality (Li et al., 1992; Okano et al., 1999). Likewise, mutations in the genes of other epigenetic machinery have been linked to developmental diseases such as the MeCP2 mutation in Rett syndrome, a disease which results in detrimental nervous system development (Amir et al., 1999; Guy et al., 2001). Together, this evidence suggests that epigenetic machinery not only plays a role but is required for the progression of normal neural development.

Much effort has been made to understand how epigenetic markers are altered during neuronal differentiation. Directed differentiation of neuronal fates from totipotent embryonic cells (*in vitro*) as well as live developmental study of mammalian animal models have shown that epigenetic transformation, in line with transcriptome reorganization, is robust and dynamic. Importantly, these changes occur in very cell (lineage)-specific ways and follow strict spatial and temporal cues. Altogether, we propose that an epigenetic program is necessary to drive the transcriptional profiles that differentiate a neural precursor. Here we present just a fraction of the hundreds of neural epigenetic targets that contribute to the development of a neuron. Particularly, we discuss these genes in the context of developmental signaling pathways known to be required for the specificity of mature neural cells-everything from cell cycle arrest to inhibition of neuronal apoptosis and the onset of neuron and glia differentiation. Throughout, we note that some genes are targets for multiple

epigenetic modifications and that one modification often begets another. Indeed, the epigenetic drivers of neurodevelopmental pathways are complex and highly integrated with one another thus allowing external influences a host of downstream opportunities from a single starting position. From this vantage point it is easier to understand why developmental time points are so much more sensitive to external stimuli and how these early exposures can drive lasting change in a neural system (more of which will be discussed in part two).

NEURAL STEM CELL DIFFERENTIATION: GLOBAL TRANSFORMATIONS

The earliest cellular commitment of a neural cell occurs when embryonic, totipotent stem cells become neural progenitors. During this time many investigators have noted global changes occurring in the epigenetic profile of these transformative cells. Histone acetylation increases among maturing neural progenitors *in vivo*, this is likely occurring to accommodate the increasing rates of RNA synthesis occurring in the cell (recall that acetylation of chromatin results in de-compression of DNA; Cho et al., 2011). Additionally, histone marks like H3K4me2 are predictably re-organized throughout the neural differentiation timeline on relevant genes. A high-throughput analysis revealed that H3K4me2 marks are acquired between the stages of pluripotent embryonic stem cells (ESCs) to neural progenitors and mature neurons on cell adhesion, synaptogenic, and neural transmitter signaling pathway genes (Zhang et al., 2012). The histone mark H3K27me3, on the other hand, has been found to decrease in the intergenic regions during neural progenitor cell (NPC) differentiation (Hahn et al., 2013). DNA methylation patterns have also been characterized in developing neural systems. Mainly, it has been shown that 5-methylcytosine (5 mC) is upregulated in neuroepithelial cells (NE) and rapidly downregulated during the specification of NE cells to mature neuronal populations (Chen et al., 2014). 5-Hydroxymethylcytosine (5 hmC; a derivative of 5 mC) patterns appear alternatively enriched at regions of active maturation compared to neuroprogenitor sites. Additionally, several high-throughput DNA methylation analyses of differentiating ESCs have reported that DNA methylation is altered on multiple genes on the path to neural progenitor conversion. These methylation shifts are bi-directional and include hypermethylation and hypomethylation (Singh et al., 2009; Cortese et al., 2011). Perhaps more important than cumulative levels of DNA methylation, however, are the recent findings that genomic landscapes undergo DNA methylation shifts such that regions previously methylated become hydroxymethylated while other un-methylated regions acquire methylation during this critical time of neural predisposition (Hirabayashi et al., 2013). The observation that neural gene clusters appear to acquire 5-hydroxymethylcytosine (5 hmC) during ESC to NPC differentiation led to the hypothesis that though 5 hmC does not directly up-regulate the genes that promote neural differentiation, the methylation intermediate may act as a "priming" mechanisms for the de-methylation which will eventually allow the expression of these genes (Tan et al., 2013). Many non-coding RNA transcripts are similarly altered during ESC conversion to neural progenitors (Iyengar et al., 2014), potentially impacting a host of complimentary mRNA. Briefly, microRNAs involved in provisioning self-renewal capacity to neural stem cells (NSCs; miR 134, 137, 25) are understandably reduced as NPCs undergo neural specification and lose their proliferative ability (Meza-Sosa et al., 2014). Conversely, miRs that support neurogenesis (miR 124, 9, let7) are upregulated during the developmental progression of NPCs to immature neurons. Finally, chromatin-remodeling proteins have been shown to undergo up to 30-fold changes during neural precursor specification (these can be entirely lineage specific; Juliandi et al., 2010; Weng et al., 2012). These protein complexes interact with and/or influence subsequent epigenetic modifications on the path to regulating neuronal transcriptomes. It is worth noting that studies of global epigenetic change during neural differentiation often come from two sources, cell populations analyzed in live developmental systems or cultured NSCs. The use of one versus the other can lead to contradictory conclusions regarding the nature of epigenetic change. As Cho et al. (2011) explains, this is not surprising given that epigenetic modifications are often products of external cues and *in vivo* extracellular environments have not yet been precisely recapitulated *in vitro*. Altogether, there is ample evidence to support that these epigenetic mechanisms (histone modification, DNA methylation, non-coding RNA elements, and chromatin remodeling proteins) contribute largely to the transcriptional reprograming that is required during stem cell commitment to neural lineages (**Figure 1**). Next we discuss particular gene targets and the integrative epigenetic modifications which guide them along the specification pathways that distinguish neural precursors.

NEURAL STEM CELL DIFFERENTIATION: GENE NETWORKS AND EPIGENETIC REGULATION

(A) Cell-cycle regulation and pluripotency

Cell cycle regulators play an important role in developing NSCs. Primarily, they allow the self-renewal of neural progenitors and are ultimately responsible for the cell cycle arrest that occurs when a progenitor becomes post-mitotic (no longer able to self-renew). Both symmetric (self-renewing) and asymmetric cell division are important for the development of cortical cells in the CNS. The number of cell divisions of neuroprogenitors will determine the number of mature neurons in the brain. The propagation of symmetric cell division past the normal developmental schedule or, conversely, the premature arrest of the cell cycle can drastically alter the structure and, ultimately, lead to functional aberrations. To make sure that cell cycles adhere to preset schedules, epigenetic mechanisms are utilized to regulate the expression of pro-mitotic and pro-pluripotent genes. One example of epigenetic regulation at the cell cycle level can be observed during the G2 to mitosis phase. For this mitotic progression to occur, cdk2/cyclinA and cdk1/cyclinB must sequentially phosphorylate FoxM1, an important transcription factor for pro-mitotic genes (*Cycb, Cenpf*). The transcription factor SC1, which has been shown to recruit the type II arginine methyltransferase PRMT5, works to repress the pro-mitotic genes *Cyclinb* and *Bub1b*, thereby keeping cells in a proliferative state (Chittka et al., 2012). When SC1 is deleted, premature differentiation of neural precursors is observed. This is just one small part of the cell cycle at which epigenetic interference can work to influence the developmental status of a neuroprogenitor. In fact many

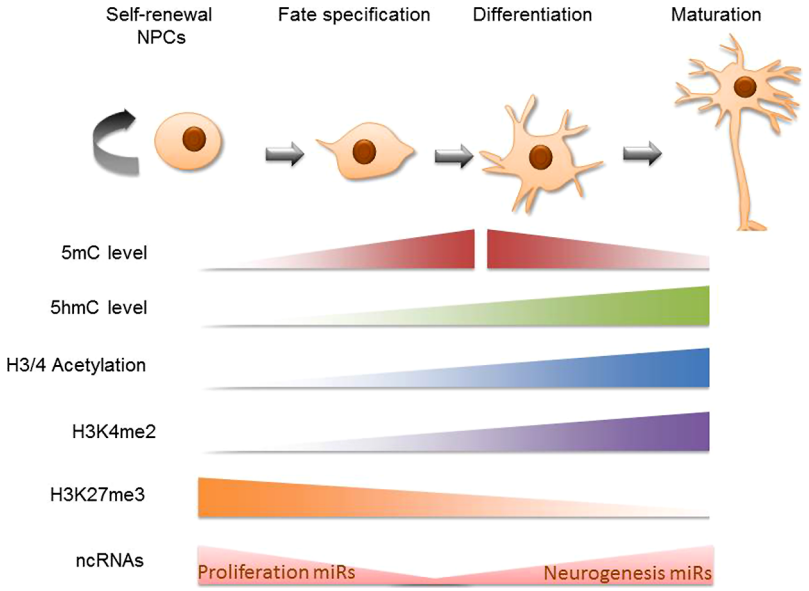

FIGURE 1 | Global epigenetic trends in neural stem cell differentiation. Schematic diagram of cellular epigenetic program during neurogenesis. The top panel shows cell states during neurogenesis, from self-renewing neural progenitor cells (NPCs) to fate-determined neuroblasts, to differentiating and maturating neurons. The epigenetic programing is mapped in the bottom panel accordingly: cells gain 5 mC at the beginning of cell specification and sequentially gain 5 hmC at the beginning of cell differentiation; both 5 mC and 5 hmC accumulate during neuronal differentiation and maturation while at later stages of neuronal maturation, 5 mC levels decrease (Chen et al., 2013). Global trends in H3 and H4 acetylation have been traced *in vivo* to find that mature cells such as those in the mouse cortical plate are richer for these markers than the neural progenitor cells that preceed them (Cho et al., 2011). H3K4me2 is primarily acquired in the neural progenitor cell stage and becomes pronounced in the matured brain cell stage (Zhang et al., 2012). H3K27me3 has been shown to be negatively correlated with 5 hmC on intergenic regions during NPC differentiation (Hahn et al., 2013). Finally, MicroRNAs that support proliferative gene expression are diminished as self-renewable NPCs become specified neural precursors while pro-neurogenesis non coding RNA are upregulated during NSC conversion to a mature neuronal state (Meza-Sosa et al., 2014).

more cell cycle regulatory genes have been ousted as epigenetic targets.

Another facet of neural development that aids in the "stemness" or the self-renewal property of a neural precursor involves a network of pluripotency-promoting transcription factors. Oct4 and Nanog share an overwhelming number of target genes, most of which promote the Inner Cell Mass (ICM) conditions from which ESCs are derived. They help maintain cells in a pluripotent state by either repressing or activating the expression of associated genes. They may also form a complex with Sox2 and together, regulate neighboring Sox elements involved in embryonic development. JARID1B, the H3K4me2 demethylase, has been shown to affect the expression of Oct4 and Nanog. Specifically, JARID1B plays a hand in suppressing the expression of these transcription factors as deletion allows Oct4 and Nanog expression to continue past their normal time course in an *in vivo* developmental model (Schmitz et al., 2011). The H3K9 demethylase JMJD1C has also shown a direct binding capacity to Oct4 (Wang et al., 2014a). Additionally, the Jarid family of proteins may not be acting alone as they have shown complex-forming capacity with polycomb repressor proteins (PcGs)-chromatin remodeling proteins that act to repress gene activation (Pasini et al., 2010). Histone demethylases therefore contribute to neural differentiation dynamically by inhibiting activating histone methylation marks and by recruiting proteins that catalyze repressive histone methyl marks. In cases where PcGs overlap on target genes with histone demethylases (reportedly 90%), it is unclear whether there exists competition between demethylases of repressive histone marks and PcGs conferring new repressive histone marks. Oct4, Nanog and Sox2 have also been identified in screens of differential methylation during neural differentiation, indicating that a DNA methylation reprogramming occurs in these genes at the onset of their quiescence (Kim et al., 2014). Nanog, for example, lost 5 hmC in the enhancer regions and gained 5 mC promoter methylation while displaying decreased expression (though it remains to be resolved whether gain of 5 hmC or loss of 5 mC is primarily responsible for the observed expression change). Finally, Sox2 has also been identified as a direct target of the long non-coding RNA RMST and the miRNA 200c (Peng et al., 2012). lncRMST misexpression can inhibit normal neural maturation by affecting the expression of pro-neural genes regulated by Sox2.

As stated earlier, these are only small fractions of the cellular and genomic cascades that govern the replication of a neural precursor. Many other genes and factors are at work beyond what is presented here. Also, it is likely that, as in the case of Sox2, genes governing cellular stemness are actually affected by a myriad of epigenetic factors, both direct and indirect. This suggests that the ultimate expression of the target cannot merely be attributed to one epigenetic mechanism, as is customarily investigated and described, but rather the sum of all their interactions. Such epigenetic–genetic mapping would be a welcome and useful undertaking toward a

more complete understanding of the epigenetic governance of an entire cellular property.

(B) Neurogenesis/gliogenesis and cell survival

Of course the path to neuronal maturation does not end upon exit of the cell cycle. For a neuronal precursor to mature to a final state it must follow a pathway of neural specification. Since there are many different mature neuronal fates, each with unique morphological and functional specificities, it only makes sense that there would exist many distinct neural pathways driving each neuronal subtype. Here we will only focus on a few of the many cascades of genetic profiles that ultimately drive a mature neuron into existence. These have been selected to showcase the interplay of pro-neural genes and epigenetic mechanisms.

The initiation of transcriptional drivers of neuronal maturation often comes from an escape from a repressive action. These inhibitory signals must first be lifted in order for pro-neural genes to activate the maturation schemes of the neural progenitor. A major inhibitory signaling cascade that exemplifies this is the Notch1 pathway. The Notch1 pathway plays a big role in CNS development and, depending on the timing of its activation, can heavily influence the fate of multipotent CNS precursors (Yoon and Gaiano, 2005). Notch1 activation signals the upregulation of Hes family genes. Hes1 and Hes5 specifically, can act to repress the pro-neural genes *Mash1 (Ascl1)* and *Ngn1/2*. These pro-neural factors typically form complexes and act as transcriptional activators of downstream genes important for neuronal specification (more of which will be covered later). While Notch1 signaling is active, neuronal precursors are pushed toward self-renewal (itself necessary to maintain an appropriate progenitor pool size). Meanwhile, cells that manage to become post-mitotic upregulate Notch1 ligands which, through lateral inhibition, restrict neighboring cells from undergoing their own post-mitotic specification (Kageyama et al., 2008). This negative feedback loop, however, is in competition with the negative regulator(s) of Notch signaling-at least one of which has also been found downstream of post-mitotic gene cascades (Kaltezioti et al., 2010). In other words, neuronal differentiation both inhibits and partly promotes Notch activation/silencing in neighboring cells.

Another factor in the neurogenesis/stemness equation is the presence of the transcription factor Pax6 – a regulator of NSC maintenance and neurogenesis genes. Pax6 interjects with Notch signaling by pairing with Ngn2 to promote neurogenesis. Together, they override the inhibitory action of Notch-mediated Hes1 (Sansom et al., 2009). Pax6 also plays a Notch-independent role in neurogenesis by inhibiting the expression of the pluripotency genes *Oct4* and *Nanog* (Zhang et al., 2010). Finally, several important neurodevelopmental transcription factors such as Sox2 and Pax6 exhibit ZEB-dependent expression (Du et al., 2013). ZEB family proteins act as transcriptional repressors for competing signaling cascades which seek to drive stem cells away from ectodermal (neural) lineages, namely BMP signaling (Postigo, 2003).

Some of the same pathways that direct neurogenesis play a hand in gliogenesis as well. Notch1 for example (likely through Hes1/5 and the downstream effector Dll1) interacts with the FGF-mediated Sox9 to promote astroglial fates (Grandbarbe et al., 2003; Wu et al., 2003; Bani-Yaghoub et al., 2006; Esain et al., 2010). The FGF signaling pathway, alternatively, can contribute to the formation of oligodendrocyte progenitors (OLP). This specification is probably due to the expression of the *Olig2* gene, which is expressed under the combined signaling of FGF and SHH (Esain et al., 2010). Additionally, FGF-regulated Sox9, when paired with Sox10, has been shown to aid in OLP survival and migration (Finzsch et al., 2008). Having presented just a faction of the many developmental signaling pathways that interact to regulate neuronal and glial fates, it becomes apparent that these signals are carefully poised to converge in space and time to drive a specific lineage. As such, deviations from these thresholds, no matter how small, can alter a signaling network enough to change the trajectory of a neural precursor. In other words, even small disturbances in these delicate signaling networks can produce a sort of "domino" effect by which lasting neurodevelopmental changes are propagated in an organism. In this vein, let us next consider the opportunities or "windows" that exist in these networks for epigenetic regulation and indirectly, for external input to propel developmental change.

Beginning with the Notch pathway, NSC differentiation analysis has identified both *Notch1 and Hes5* (along with a handful of other downstream Notch1 genes) as targets of differential DNA methylation (Kim et al., 2014). Specifically, the expression of these Notch-related genes displays some dependency on the methylation status of their promoter and/or gene body. Additionally, the histone modifiers SIRT1 and JMJD2B have been shown to affect the expression of Hes1 and Notch1, respectively, in models of neural progenitor differentiation (Hisahara et al., 2008; Das et al., 2013). At least in the case of Notch1, the histone demethylase JMJD2B acts on Notch1 expression by regulating the presence of the repressive histone mark H3K9me3 on the gene promoter. The pro-neural genes *Mash1* and *Ngn1* are not only repressed through active Notch signaling but also serve as direct targets of the Sox2-regulated miRNA let-7i (Cimadamore et al., 2013). This miRNA sequestration results in decreased neuroprogenitor proliferation and neurogenesis similar to that of Sox2-defficient precursors (recall that Mash1 and Ngn1 are downstream targets of Sox2). Ngn1 also serves as a target of the polycomb repressive complex (PRC) 1 and 2 (Hirabayashi et al., 2013). As discussed above, the transcription factor Sox2 is susceptible to both lncRNA and miRNA in addition to exhibiting differential methylation patterns during neural commitment. Additionally, the transcription factor has a promoter binding capacity for the histone 2B ubiquitinylase USP22, which in turn alters the recruitment of histone 3 methylation marks and ultimately leads to Sox2 repression, a function necessary for stem cell differentiation (Sussman et al., 2013). USP22 can also form a complex with the histone deacetylase SIRT1, which serves a similar repressive action on Sox2. There is evidence that some of the transcriptional regulators (inhibitors) of pluripotency factors also exist under the regulation of epigenetic machinery. The master transcription factor Pax6 which suppresses the stemness factors Oct4 and Nanog during ESC conversion to neural progenitor, is a target of the miRNA 96 family (Du et al., 2013). The repression of Pax6 by miR-26 members was experimentally confirmed and inhibited

the differentiation of stem cells into neural precursory lineages exclusively. Finally, the ZEB transcription factor family, which is critical for repression of the competitive epidermal BMP signaling, has been isolated as a target of the miRNA 200 family (Du et al., 2013). This miRNA-mediated repression of ZEB is likely poised at the commitment of ectodermal precursors to either neural or epidermal fates. BMP (epidermal) repression via this epigenetic mechanism indeed swayed ESC populations toward neuroectodermal fates. Interestingly, early attempts to reshape the epigenetic landscape of important fate-determining pathways like Notch1 with epigenetic modifiers have proved unsuccessful (Reichrath and Reichrath, 2012). It is likely that as further investigation with more targeted approaches and diverse cell populations will yield promising results that will strengthen our understanding of these signaling pathways and their vulnerability to epigenetic influence.

We have now covered some of the major pathways that allow neural precursors to both self-renew and differentiate into more committed cells. We have discussed that this process involves the precise activation of pro-neural transcriptional networks as well as the timely de-activation or suppression of competing influences. Some of these competing pathways are aimed at repressing the maturity of a cell while other pathways work to drive maturing cells toward non-neuronal trajectories. Interestingly, we have seen that many genes play roles in multiple pathways and that neurogenesis/gliogenesis and their specification are likely the overall effect of converging networks and multiple contributing factors. Also, epigenetic mechanisms are likely involved in the intrinsic schedule that directs normal neural development. For example, differentiation cues in a stem cell can trigger DNA methylation re-distribution/conversion, histone modification or non-coding RNA binding. Like the integrative nature of the neural differentiation transcriptome, epigenetic factors are likewise heavily intertwined. In other words, one gene can be affected by multiple epigenetic mechanisms and it is unsurprising that sometimes the onset of one modification can recruit other alterations both on the same locus and/or in nearby regions. Early neural commitment is not the terminal point of the neural differentiation program. Before we address the pathways that further specify and finalize mature neuronal attributes we will first address a small portion of the pathways that are utilized for the maintenance and survival of committed neural precursors.

During development, many neurons undergo programmed cell death. Neurons that are "engaged" with one another, however, are typically spared. Of particular importance in neuronal survival is the PI3K-Akt cascade of the BDNF pathway, which can induce the transcription of either pro-survival or pro-apoptotic genes in a BDNF-dependent manner (Brunet et al., 2001). BDNF is a neurotrophin which, through TrkB activation, can trigger a variety of downstream cascades ultimately resulting in the transcription of survival factors like NFK-B and CREB (Romashkova and Makarov, 1999). Conversely, in the absence of BDNF, genes like the pro-apoptotic members of the Bcl-2 family can be upregulated and promote apoptosis (Brunet et al., 2001). Typically, the channel-gated accumulation of intracellular calcium upon neuronal communication triggers the initial activation of the BDNF cascade (either through PLC-g, CaM kinases, or the PI3K-Akt pathway) each independently capable of driving the nuclear transcription of BDNF and other survival genes (Marini et al., 2004). BDNF produced from these initial reactions can thus come back as a ligand for further TrkB activation. TrkB activation by neurotrophins can recruit PI3K to the inner surface of the plasma membrane where they produce phospholipids that recruit the kinase Akt. PDK1-mediated phosphorylation of Akt serves as an activating event which further allows Akt to act on a variety of downstream targets. For example, the unphosphorylated BAD protein is bound to the pro-survival factor Bcl-xL inhibiting it from promoting cell-survival. Upon neurotrophic Akt activation, BAD is phosphorylated and unbound from Bcl-xL, freeing it to promote survival (Datta et al., 2000). Factors that regulate apoptotic machinery and promote cellular survival are important to ensure proper neural development. Deviations from the intrinsic neural schedule of the expression of these genes can thus be detrimental to the overall architecture of the brain.

Epigenetic regulation of the BDNF cascade that promotes neuronal survival can be achieved through the BDNF gene itself. A natural antisense transcript for BDNF has been reported to repress BDNF expression *in vivo* (Modarresi et al., 2012). BDNF expression has also been increasingly tied to promoter DNA methylation in various models of neurological disease, indicating that even under normal developmental conditions, BDNF promoter methylation may be significantly responsible for neurotrophic levels (Ikegame et al., 2013). Activity-dependent changes in promoter methylation of the BDNF gene (5 mC, CpG methylation) are also thought to mediate the release of a repressive chromatin remodeling protein (mSin3A) from the promoter thereby providing anther epigenetic mechanism of BDNF regulation (Martinowich et al., 2003). The Akt1 gene exhibits similar methylation-dependent transcriptional regulation. During ESC differentiation, the gene is upregulated and this increase is reportedly correlated with the acquisition of intragenic 5 hmC (Kim et al., 2014). Downstream of Akt, CREB has revealed sites in its activating region where the CREB-binding protein (CBP) can acetylate lysine residues and in so doing, modulate CREB-mediated gene expression (Lu et al., 2003). Finally, the anti-apoptotic gene Bcl2 which promotes neural cell survival has been identified as a target of the miRNA 497 in some studies of neural insult, indicating that externally regulated neuronal cell death is at least partially achieved through epigenetic regulation of pro-survival transcripts (Yadav et al., 2011). As in the case of pluripotency and neurogenesis, multiple levels of epigenetic regulation may converge on a single gene. The ultimate regulatory action is thereby dependent on the sum of all these influences, which may act in similar or contradicting directions. Additionally, the onset of one epigenetic modification can often trigger sequential acquisition of further changes. A thorough understanding of the factors that dictate epigenetic change in the developing nervous system are still far beyond reach though it is clear that external impacts make use of epigenetic machinery to induce transcriptional and phenotypic change in the brain. Next we address the latent stages of neural development. After neuronal/glial commitment cells of the nervous system undergo transcriptional changes to further direct the specialized cell they will become for the remainder of their lifespan. These changes include migration, neurite outgrowth, and a host of synaptic

preparations and refinements-some of which are never fully static and continue to evolve throughout adulthood.

(C) Late-stage neuronal specification and synaptic plasticity

After neurogenesis, maturing precursors continue to experience the fluctuation of a progressive trancriptome. This serves to accommodate the changing needs of a cell to acquire specific traits like proteins that would become receptors of electrical and chemical signals from neighboring cells. For example, cortical neuron specification can occur from radial glia precursors. The expression of the transcription factor Pax6 allows radial glia to produce both neuronal and glial precursors. The downstream upregulation of the transcription factor Tbr2 begins to negatively regulate the expression of Pax6 restricting radial glia production to only neuronal fates. Further, the onset of Tbr1 expression negatively regulates Tbr2, conferring corticogenesis (Englund et al., 2005). Further specification of mature cortical subtypes is controlled by distinct combinations of downstream genes. Subcortical projection neurons for example, have been linked to the expression of Fezf2 and Ctip2 sequentially (Leone et al., 2008). Alternatively, Satb2 likely regulates the fate of cortico-callosal projection neurons by repressing the aforementioned subcortical Ctip2 cell identity pathway (Alcamo et al., 2008). Many more specialized classes of cortical neurons exist and are regulated by a variety of genes. The cues directing the activation and inactivation of these key cell specification factors likely come from the microenvironment surrounding the precursor at a specific time point.

Another example of a highly specialized neuronal maturity cascade has been studied in dopaminergic neurons. Neural progenitors isolated from the ventral midbrain show a dependence on Ngn2 and Nurr1 (Nr4a2) for the production of morphologically mature and functional dopaminergic neurons *in vitro* (Andersson et al., 2007). Further, the transcription factor Pitx3 has been implicated in DA neuron survival and production of AHD2, an enzyme which produces retinoic acid and is present only in a subset of DA neurons (Chung et al., 2005). Retinoic acid binds and activates retinoic acid receptors which in turn may regulate the expression of tyrosine hydroxylase and influence the production of dopamine (Jeong et al., 2006). Corticogenesis and dopaminergic specification are both examples of the later-stages of neural development where neural progenitors have already been committed to neuronal fates but require lots of "fine-tuning." These types of specialized neurons can take a long time to fully mature as many genes (not discussed here) will continue to regulate phenomena such as the production and migration of synaptic receptors and the appropriation of morphological properties conducive to the functions of the cell.

Epigenetic mechanisms have demonstrated the ability to regulate the timely expression of these fate-determining genes. As previously mentioned, the neuron-conferring transcription factor Pax6 is targeted by microRNA and has also been shown to express differential DNA methylation in accordance with neuronal maturity (Kim et al., 2014). Further down the cortical specification cascade, Ctip2 has been shown to bind histone modification enzymes to aid in transcriptional repression (Marban et al., 2007; Tan et al., 2012). Finally, the *Satb2* gene, thought to play a role in specification of cortical-callosal projections, is downregulated in the absence of the histone methyltransferase ESET, implying that this histone enzyme is required for normal neural development, likely through the regulation of one or more corticogenesis pathways (Tan et al., 2012). The role of dopaminergic neuron late-stage specification has also been epigenetically investigated. Histone modification, for example, has been shown to regulate the expression of Nurr1 and its downstream targets Pitx3 and Dlk1 – all essential in the synthesis, metabolism, and transport of dopamine (van Heesbeen et al., 2013). Similarly, epigenetic elements have been discovered in the regulation of specification genes of other mature neuronal types (Boshnjaku et al., 2011; Banerjee et al., 2013).

Some properties of cellular structure and function are continually fluid, or plastic, in the brain. In fact, it is cellular plasticity which allows neurons the ability to aid in human learning, cognition, and memory (among others). Thus, the constant disposition of certain developmental factors allow for the adaptation of a cell to changing demands throughout life. An example of paramount importance is synaptic plasticity or the ability of the neuron to change the components of its neurotransmitters and/or receptors to adapt to the strength of incoming/outgoing signals. The activation of NMDA receptors in the hippocampus during long-term depression (LTD) and long-term potentiation (LTP), both of which contribute to learning and memory in the adult brain is just one of the many ways that lasting plasticity is achieved. NMDA Receptors are ionotropic receptors of the excitatory neurotransmitter glutamate. Activation of these receptors triggers the influx of extracellular Ca^{2+} such that they are able to bind to the calcium-dependent protein calmodulin at the cytoplasm. From here calmodulin is able to translocate calcium so as to reach intranuclear calcium-calmodulin dependent kinases. Specifically, CaMKIV have been shown to be critical for the phosphorylation of CBP and downstream CREB/CBP-dependent transcription (Impey et al., 2002). Interestingly, it has been reported that in the hippocampus, CREB phosphorylation (activation) is attainable via endogenous intranuclear calcium and CaM kinase stores, independent of calmodulin (Hardingham et al., 2001). Synaptic action potentials acting through NMDA receptor-induced calcium transients thus regulate genes capable of re-shaping the synaptic landscape. Furthermore, duration of calcium transients are thought to dictate the transcriptional response of CBP/CREB (Chawla and Bading, 2001) and in this manner determine whether LTP or LTD of synapses is achieved (Luscher and Malenka, 2012). Nr4a2 (Nurr1) is an important transcriptional regulator of dopaminergic lineages and a target of CREB/CBP (Vecsey et al., 2007). The neuronal survival neurotrophin BNDF has also been shown to be under the transcriptional regulation of CBP/CREB (West et al., 2001; Hardingham et al., 2002). Many other genes relevant to neuronal growth and maintenance have been implicated in CBP/CREB transcriptional control including immediate early genes (Cole et al., 1989).

Much remains unknown about the role of epigenetics in the late-stage refinement of a maturing neuron. Puckett and Lubin summarize various classes of known epigenetic modifications which may occur in fully mature, adult neurons such as those in the hippocampus involved in long term potentiation

and other experience-driven molecular responses. To point out just a few, the transcriptional repressor CREB2, an important player in memory-related synaptic plasticity, is targeted by a type of non-coding RNA (piwi-associated RNA; Rajasethupathy et al., 2012). The CBP itself has been characterized as a recruiter of histone acetyltransferases to gene promoters thereby stimulating transcription (Bannister and Kouzarides, 1996). Downstream targets of CBP/CREB also exhibit epigenetic modifications which together with CBP/CREB can account for expression levels during activity-dependent synaptic plasticity (Guan et al., 2009). Beyond individual gene modifications, large-scale DNA methylation and histone acetylation have also been shown to be critical for memory consolidation and synaptic activity in the hippocampus (Vecsey et al., 2007; Miller et al., 2008). HDACs in particular have been attributed to reduced dendritic spine number and synapse number (Guan et al., 2009). This disposition of fully mature neurons to epigenetic change is particularly important when considering environmentally acquired epigenetic modifications. This means that even fully mature cells can be susceptible to aberrant external stimuli and thus supports the role of epigenetic processes in mediating not only developmental but late-onset disease. Beyond intracellular calcium fluxes and CBP/CREB elements, not much is known about the mechanisms translating neuronal activity into epigenetic regulation. Progress continues to be made toward the understanding of epigenetic regulation of synaptic plasticity networks, such as the involvement of histone acetyltransferases on NFk-B targets during memory consolidation (Stilling et al., 2014) and the interplay of histone 3 acetylation and phosphorylated CREB on the promoter of the gene encoding PSD-95 during reward learning (Wang et al., 2014b). Still, it remains important to investigate the molecular basis of epigenetic response to altered neural stimuli (the other half of the equation), which surely spans beyond the current confines of calcium response elements. This will be essential to forward our understanding of the genes provisioning synaptic plasticity and our ability to intervene in activity-dependent disease.

CROSSTALK

While here we have primarily discussed epigenetic modifications that contribute to neural cell development as singular contributions, a more realistic scenario is that epigenetic modifications influence each other and that the ultimate transcriptional outcome is the sum of all these interactions. One example, established by Meissner et al. (2008) is the finding that histone methylation marks are strongly correlated with DNA methylation in a model of differentiating stem cells. Specifically, the activating acquisition of intragenic 5 hmC during neural differentiation is closely tied to loss of H3K27me3. Moreover, this epigenetic shift was concomitant with loss of promoter Polycomb marks which, when induced, drove cellular differentiation away from the neural fate (Hahn et al., 2013). These are just some of the many instances of epigenetic dependence and/or interaction. Jobe et al. (2012) outlines other experimental examples of such epigenetic interrelations which occur in NSC fate specification. Finally, **Figure 2** attempts to visually represent some of the studies reviewed herein and to offer a conceptual understanding of the many gene targets affected by epigenetic alteration during neural specification, development, and synaptic plasticity. The vulnerability of gene targets in the neurodevelopmental cascade to epigenetic change leave one very important implication. Though epigenetic mechanisms regulating neurodevelopmental genes may have an intrinsic component, it is also highly likely that epigenetic modification is a response to an environmental input (Jaenisch and Bird, 2003; Feil and Fraga, 2011). As such, genes critical to structural and functional neuronal specification serve as "targets" for external factors which may compromise the normal epigenetic developmental program. Some of these external signals have been identified as air contaminants, fetal nutritional components, and substances of abuse (Heijmans et al., 2008; Guerrero-Preston et al., 2010; Esposito et al., 2014).

PART TWO: ALCOHOL (ENVIRONMENTAL) INTERFERENCE OF THE NEURODEVELOPMENTAL EPIGENETIC PROGRAM

ALCOHOL DYSREGULATION OF NEURAL DEVELOPMENTAL PROGRAMS

Because the transcriptional programs of a maturing cell are under epigenetic control, there is a pathway for environmental regulation of cellular maturation as well. Alcohol, for example has been shown to inhibit the differentiation of NSCs in culture. Compromised cellular growth, migration, and cell viability have been reported in models such as these (Zhou et al., 2011; Campbell et al., 2014). Additionally, a host of genes have been shown to be deregulated in precursory neurons by alcohol exposure (Sanchez-Alvarez et al., 2013) **Figure 3** summarizes three important physiological processes known to be targeted by alcohol in a gene-specific manner.

First, the importance of a tightly controlled cell cycle transcriptome was described earlier. NSCs treated with ethanol exhibit cell cycle delays, reduced NSC proliferation and increased DNA fragmentation (Anthony et al., 2008; Hicks et al., 2010). Some of the genes involved in cell cycle progression are unsurprisingly transcriptionally altered by ethanol. Of these, a few concomitantly exhibit epigenetic alteration. For example, DNA hypermethylation was detected on *CcnB1, Cdc20, Bub1,* and *Plk1* in the presence of alcohol (Hicks et al., 2010). In another NSC model, the presence of ethanol blocked the intrinsic hypermethylation of the cell cycle genes *Adra1a, Tnf, Pik3r1,* and *Sh3bp2* that is observed during differentiation (Zhou et al., 2011). The cell cycle genes for cyclinD1 and cdk6 have also been identified as targets of the alcohol-induced miR 34a in lung cancer cells (Sun et al., 2008). It would be interesting to investigate whether miR 34a or other miRNA families similarly target cell cycle regulatory genes in neuronal models.

Another cellular pathway affected by early ethanol exposure is cell survival. Neural progenitors exposed to alcohol *in utero* exhibit marked increases in cellular loss and markers of cell death (Ikonomidou et al., 2000). There appear to be several ways that ethanol can interfere with normal neuronal survival cues. In cultured granule cells, ethanol suppresses the endogenous miR29b, thought to protect against apoptosis, or cell death via the SP1 cascade of PKR phosphorylation (Qi et al., 2014). Other pathways that have been investigated indicate that ethanol acts through Bcl-xL (Bax) as deletion of the gene inhibits the ethanol-mediated apoptotic response (Young et al., 2003). Like SP1, several genes involved in neuronal survival have demonstrated some degree of epigenetic regulation. The miRs 497 and 302b are both elevated in

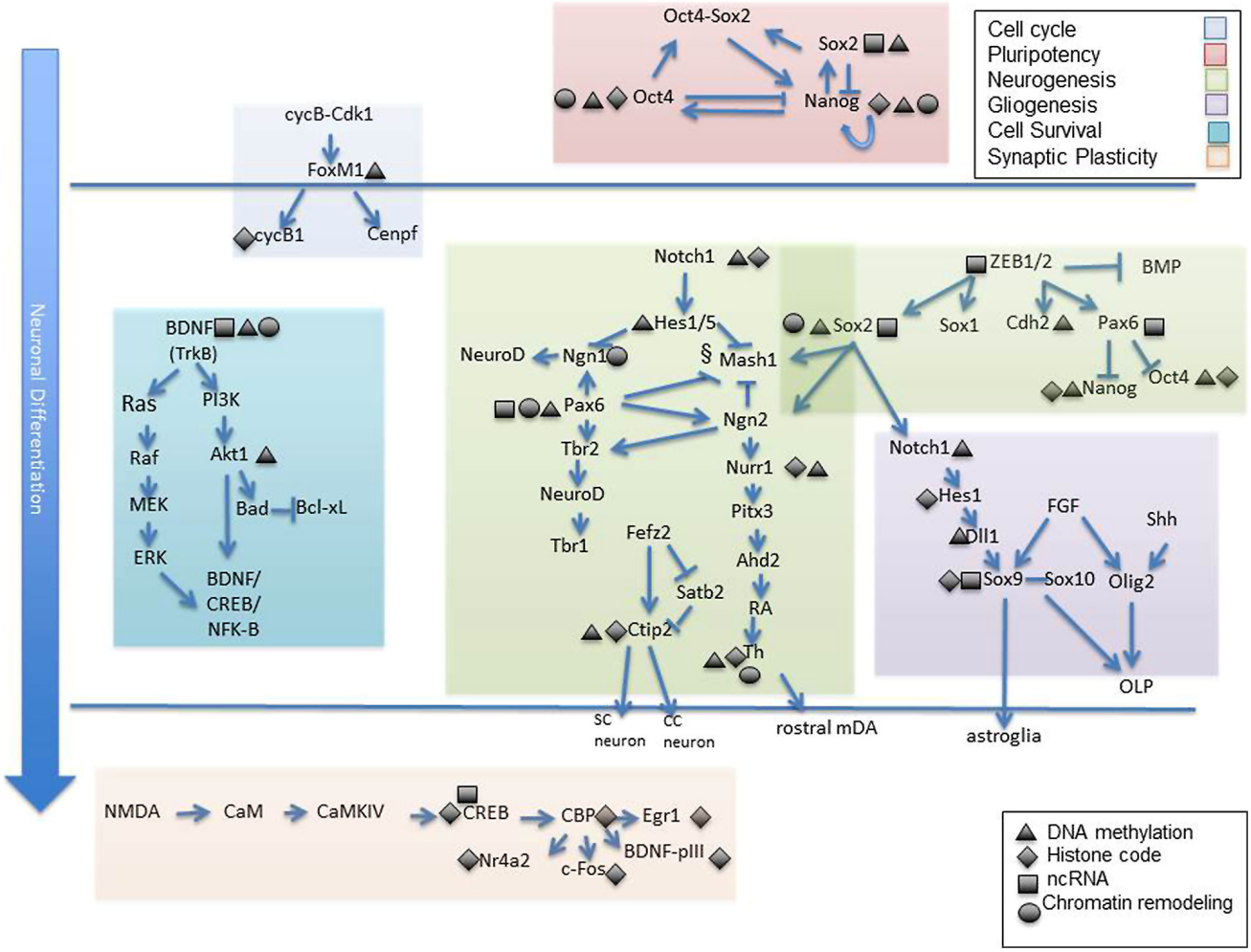

FIGURE 2 | Epigenetic targets of neuronal differentiation. Select gene cascades are presented which correspond to various biological processes known to mediate neuronal commitment and specification from early to late stages. Among these are genes regulating cell cycle progression (shaded blue), pluripotency (shaded pink), neurogenesis (shaded green), gliogenesis (shaded dark purple), cell survival (shaded turquoise) and synaptic plasticity (shaded orange). Gene targets know to exhibit epigenetic sensitivity are depicted as such via symbolic representation (black triangle, DNA methylation; black diamond, histone modification; black rectangle, non coding RNA, black cirlce; chromatin remodeling complex). Individual reference to each gene is made in the text. OLP, oligodendrocyte progenitors; SC, subcortical neuron; CC, corticocortical neuron.

the presence of ethanol and target the cell survival genes *Bcl2* and *Ccnd2* (Yadav et al., 2011). Interestingly, ethanol appears to exert a bidirectional effect on miRs-upregulating some miRs while suppressing others. Ultimately, it is proposed that alcohol initiates a physiological response like apoptosis in neural progenitors only if the miRs targeting antiapoptotic factors out-compete the miRs targeting apoptotic factors (Sathyan et al., 2007). Finally, we have previously reported that DNA methylation is altered by alcohol on the survival genes *E2f7* and *Tnf* (Hicks et al., 2010; Zhou et al., 2011). Reactive oxygen species (ROS) play a role in neuronal apoptotic pathways and are reportedly upregulated by ethanol exposure in a human neuronal cell line. Interestingly, treatment with the HDAC inhibitor Trichostatin A was neuroprotective and aided in the reduction of ROS (Agudelo et al., 2011). These results indicate that ethanol-mediated oxidative stress acts at least partially through histone modification enzymes.

In addition to the timely progression of cell cycle program and tightly regulated neuronal survival transcriptome, neuronal precursor maturation relies heavily on the appropriate differentiation signals we refer to as proneural cues. Several published reports have identified that exposure of ethanol to neural precursors delay or divert the intrinsic developmental trajectory (Chen et al., 2013; Sanchez-Alvarez et al., 2013; Veazey et al., 2013). Ethanol has even been shown to inhibit specific morphological aspects crucial to neuronal differentiation such as axon outgrowth and migration (Zhou et al., 2001; Chen and Charness, 2008). Part 1 of this chapter outlined some of the differentiation pathways which contain genes known to be targeted by epigenetic regulation, from early differentiation cues, to later-stage specification markers. Once again the question remains whether any of these genes are epigenetically altered in an alcohol-dependent manner. These are the genes that we will want to

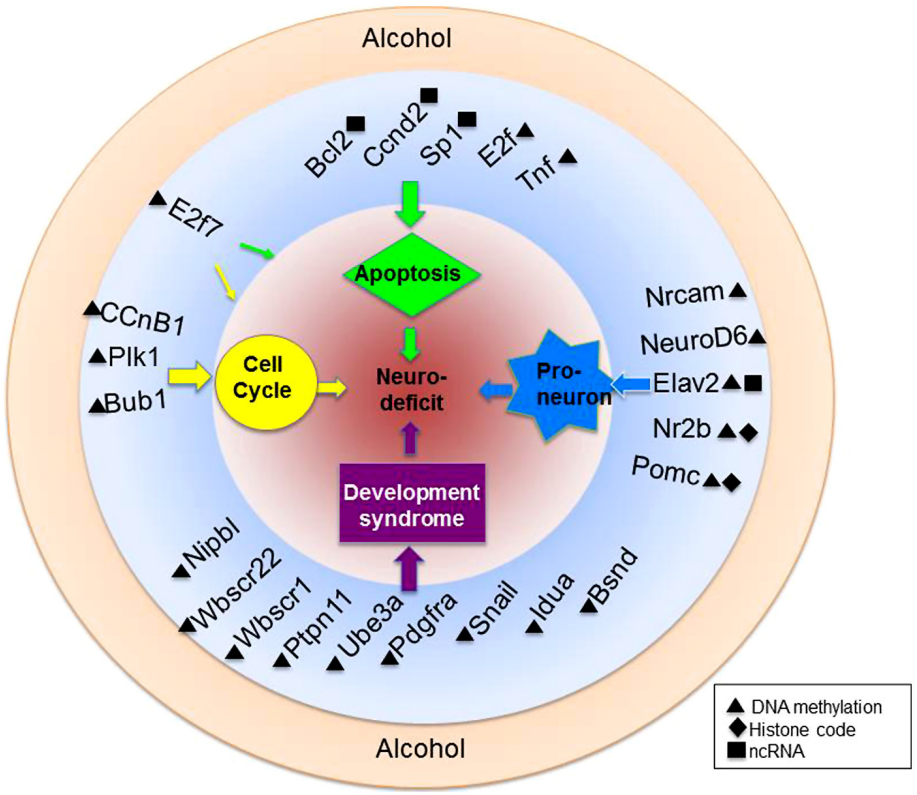

FIGURE 3 | Alcohol-mediated epigenetic targets of neural development. Alcohol is a teratogen with known capabilities to alter the epigenome. Highlighted here are just a fraction of genes within various biological pathways of known vulnerability to ethanol-mediated epigenetic alteration. Also depicted are genes associated with known developmental syndromes and their epigenetic alteration. Genes are more specifically discussed and referenced in the text.

probe for their role in alcohol-mediated developmental disease such as fetal alcohol syndrome-those known to dysregulate normal, neural developmental programs. The *Nr2b* gene, which encodes a protein subunit of the NMDA receptor, has exhibited alcohol-induced histone 3 lysine 9 acetylation in conjunction with increased expression (Qiang et al., 2011). Nr2b upregulation has been linked with alcohol dependence-related hyperexcitability though the epigenetic action of alcohol on this locus has not been thoroughly examined in development. Several other neurotransmitter receptor genes have been identified as epigenetic targets of alcohol exposure in NSCs including the AMPA3 gene *Gria3*, which undergoes promoter methylation alterations (Zhou et al., 2011) and the brain cannabinoid receptor 1 gene which is downregulated following the induction of the miR26b (Stringer et al., 2013). The prodynorphin promoter reportedly undergoes alcohol-mediated downregulation related to histone methylation and acetylation (D'Addario et al., 2011). Additionally, prodynorphin SNPs have exhibited differential methylation patterns in the post-mortem brains of alcohol-dependent patients (Taqi et al., 2011). Finally, the proopiomelanocortin (POMC) gene is genetically and functionally altered by fetal alcohol exposure and these changes are lasting into adulthood in beta-endorphin producing POMC neurons (Bekdash et al., 2013). Genetic alterations were accompanied by hypermethylation of the gene and more importantly, were normalized when fetal alcohol exposure was paired with gestational choline supplementation. Not only does a greater understanding of the epigenetic mechanisms of developmental gene regulation allow us to fully grasp intrinsic neurodevelopmental processes, it provides opportunities for therapeutic intervention of neurodevelopmental diseases with known or suspected epigenetic etiologies.

We have only provided a brief look at some of the canonical pathways and genes affecting neurodevelopment that are known targets of alcohol. Much remains to be uncovered about the role of alcohol in epigenetic dysregulation of other pathways critical to neuronal maturation. The pluripotency genes *Oct4 (Pou5f1)*, *Sox2*, and *Nanog* for example, have demonstrated an ethanol-specific delay of downregulation in NSC models (Ogony et al., 2013; Sanchez-Alvarez et al., 2013). Though we now know that each of these genes displays some degree of epigenetic sensitivity, it remains to be seen whether alcohol specifically acts on these transcripts through an epigenetic mechanism. It is likely that many other genes across a plethora of developmental cascades will exhibit association with epigenetic modification in the coming years. Some likely candidates which are dually but independently altered by ethanol and epigenetic modifiers can be found in **Table 1** and cross a variety of biological roles. These genes may serve as possible origins of the neurodevelopmental deficits observed in

Table 1 | Neurodevelopmental genes as dual targets of alcohol and epigenetic modifiers.

Gene	Class	Alcohol effect on gene expression	Epigenetic modifiers effect on gene expression
Oct3/4	Pluripotency	↑ (Arzumnayan et al., 2009; Ogony et al., 2013; Sanchez-Alvarez et al., 2013)	↑ by 5-azacytidine, TSA+5-aza-2′-deoxycytidine, LSD1 small inhibitors, VA/5-aza-2′-deoxycytidine + dTALEs (Tsuji-Takayama et al., 2004; Ruau et al., 2008; Wang et al., 2011; Bultmann et al., 2012)
Sox2	Pluripotency	↑ (Arzumnayan et al., 2009; Sanchez-Alvarez et al., 2013)	↑ by 5-azacytidine, LSD1 small inhibitors (Tsuji-Takayama et al., 2004; Wang et al., 2011)
Nanog	Pluripotency	↑ (Arzumnayan et al., 2009)	↑ by 5-azacytidine, TSA + 5-aza-2′-deoxycytidine (Tsuji-Takayama et al., 2004; Ruau et al., 2008)
SSEA-1	Pluripotency	↑ (Arzumnayan et al., 2009)	↑ by 5-azacytidine (Tsuji-Takayama et al., 2004)
Dlx2	Pro-neural	↑ (Veazey et al., 2013)	↓ by TSA (Ignatius et al., 2013)
Nestin	Pro-neural	↑ (Veazey et al., 2013)	↑ by 5-aza-2′-deoxycytidine/TSA/RA (Han et al., 2009)
CCnB1	Cell-cycle regulation	↓ (Hicks et al., 2010)	↓ by TSA + SAHA w/silibinin (Mateen et al., 2012)
CCnD1	Cell-cycle regulation	↓ (Hicks et al., 2010)	↓ by honokoil (Singh et al., 2013)
Pttg1	Cell-cycle regulation	↓ (Hicks et al., 2010)	↑ by p300 (Li et al., 2009)
hdac4	Neural maturation	↑ (Vangipuram and Lyman, 2012)	↑ by TSA (Chu et al., 2008)
Ache	Neural maturation	↓ (Vangipuram and Lyman, 2012)	↑ by 5-aza-2′-deoxycytidine (Bartolucci et al., 1989)

Genes involved in several biological pathways including neural differentiation exhibit dual sensitivity to alcohol and epigenetic modifiers. Some of these sensitivities have been tested in neural stem cells while others have been demonstrated in cancer cells. The disposition of these genes to expression changes by epigenetic modification and alcohol along with their biological relevance in cell development make them prime candidates for more thorough investigation in neurodevelopment and neurodevelopmental disease etiology. TSA, trichostatin A; VA, valproic acid; LSD1, lysine-specific demethylase 1; dTALE, designer transcription activator-like effector; RA, retinoic acid; SAHA, suberoylanilide hydroxamic acid.

fetal alcohol models which include craniofacial dysmorphology, growth deficits, and intellectual disability. By altering the epigenetic code of primitive neural cells, environmental affectors such as ethanol are capable of re-shaping the course of normal, neural development to drive lasting, structural and functional changes. While the mechanisms by which environmentally driven epigenetic modifications act on transcriptional machinery are still being worked out, it is important to strive for a deeper understanding of the genetic/epigenetic dynamic.

Lastly, there are genes which have been specifically identified in neurodevelopmental disease etiology (such as autism and fetal alcohol syndrome). These genes exhibit epigenetic sensitivity, though the causal nature of the epigenetic mechanisms remains to be scrutinized. Some of these genes have been previously outlined (Resendiz et al., 2013). Briefly, FASD models have identified Pten, Nmnat1, Slitrk2, and Otx2 as targets of ethanol-directed miRs. Additionally, the imprinted genes *Ube3a* and *Dlk1* have exhibited lasting differential methylation (Laufer et al., 2013). Other diseases with phenotypes overlapping with FAS, such as intellectual deficits rooted in neurodevelopmental aberration, have been investigated. An increase in 5 mC and reduction of 5 hmC at the A2AR receptor gene was identified and associated with transcript reduction in the putamen of Huntington's disease patients (Villar-Menendez et al., 2013). Rett syndrome and autism patient cohorts have both revealed mutations in the demethylase enzyme JARID1C, thought to regulate transcriptional repression (Adegbola et al., 2008; Wynder et al., 2010). The epigenetic importance of developmental genes mediating or involved in disease etiology has become particularly apparent as reports of the longevity and heritability of epigenetic marks continue to be published.

Environmental toxins such as alcohol can alter the epigenome and recent evidence has supported that these epigenetic changes can be inherited across multiple generations. For investigators seeking familial disease transmissibility mechanisms beyond the genome, the inheritance of parentally acquired epigenetic change (epigenetic inheritance) has provided that alternative. For example, it has long been known that paternal alcoholism can result in deleterious effects including reduced birth weight and impaired cognitive functioning in offspring (Hegedus et al., 1984; Little and Sing, 1987). The effect of paternal alcohol exposure on two paternally methylated imprinted control regions (H19 and Rasgrf1) in paternal sperm and somatic DNA of offspring has been studied (Knezovich and Ramsay, 2012). Significant reductions in methylation at the H19 binding sites were observed in the offspring of ethanol-treated sires, and correlated with reduced postnatal weight. Interestingly, no alteration of sperm DNA methylation was observed in the offspring. The authors suggest that other epigenetic factors such as ncRNA or chromatin remodeling may be responsible for paternal transmission of the phenotype. Additionally, other toxins, such as methoxychlor, bisphenol A and the fungicide vinclozolin, have been tied to transgenerational epigenetic reprogramming and function of the male germline across generations (Skinner et al., 2013).

Much translational epigenetic study has additionally come from alcohol studies in cell populations involved in modulating stress responses. Neurons containing *Pomc* gene products, located primarily in the anterior pituitary and hypothalamus, have

diverse neuroendocrine-immune and metabolic functions. These neurons have a diminished function in people with a family history of alcoholism, suggesting alcohol effects on the imprinted *Pomc* gene are transmissible across generations (Govorko et al., 2012). Alcohol-induced effects include *Pomc* hypermethylation, altered histone-modifying proteins and DNA methyltransferase levels with associated functional defects. Epigenetic modifications of *Pomc* genes are reportedly transmitted through F2 and F3 germlines, but not in female germlines.

Finally, multi-generational prenatal alcohol models have reported increased risk of gestational hyperglycemia and aberrant glucose and insulin responsiveness of offspring. The implication of an alcohol-associated hyperglycemic environment during development places subsequent generations at risk for metabolic disorders such as *diabetes mellitus*, even without subsequent fetal alcohol exposure. Specifically, a study of grandmaternal alcohol consumption in mice demonstrated transgenerational transfer of glucose intolerance (Harper et al., 2014). Sprague Dawley dams were fed ethanol liquid diets or control diets during gestational days 8–20. Dams consuming ethanol were hyperglycemic and their F1 offspring demonstrated altered glucose responsiveness, without additional alcohol exposure. A reciprocal breeding experiment using F1 Sprague–Dawley rats bred to naïve Brown Norway rats demonstrated persistent glucose intolerance in the F2 generation. This effect on glucose intolerance was normalized upon grandmaternal administration of thyroxine (T4), a thyroid hormone involved in the regulation of metabolism. This was the first experiment demonstrating that prenatal ethanol-induced alteration of glucose responsiveness can affect subsequent generations, possibly via epigenetic effects on the germ line. For all of its attractiveness as a potential mechanism of transgenerational disease, much is lacking from our understanding of epigenetic heritability-particularly in disease. As Heard and Martienssen (Heard and Martienssen, 2014) point out, much of what we may perceive as transgenerational epigenetics is confounded by the many co-factors which occur in tandem with epigenetic change and further regulate epigenetic factors. For example, there are strong correlations between differentially methylated regions and transposable elements such as LINE1 and Alu, particularly in studies of prenatal alcohol exposure (Wilhelm-Benartzi et al., 2012). Still another factor to consider is the sustainability of metabolic signatures across generations-metabolic elements which can in turn regulate the epigenetic enzymes which confer chromatin modification. These and other subtle sequence variations will make it difficult to distinguish epigenetic disease inheritance.

CONCLUSION

The slew of transcriptional fluctuations that allow for dynamic expression to fit the specific needs of a maturing neuron are made possible only by a precise and tightly controlled regulatory system. The precision of the neurodevelopmental transcriptome is thus likely achieved by a convergence of extracellular and intrinsic cues. The work reviewed herein provides for the large and meaningful role of epigenetic mechanisms as a molecular means of such transcriptional regulation. The importance of non-coding RNA, DNA methylation, and histone modification is made even clearer by the epigenomic alteration that is demonstrated in multiple disease models. Fetal alcohol exposure has identified multiple "suspect" genes by which epigenetic dysregulation can transmit the teratogenic action of alcohol exposure. One of the most striking descriptions of epigenetic mechanisms at work in a developmental disease model was told by Kaminen-Ahola et al. (2010). The gestational exposure of mice to ethanol affected the expression of the epigenetically sensitive allele of the Agouti gene (a dominant mutation) which confers mouse coat color. Ethanol induced hypermethylation in the promoter region and increased the transcriptional repression of the gene resulting in the outwardly observable phenotype of Agouti-colored mouse coats (Kaminen-Ahola et al., 2010). This example demonstrates that fetal alcohol exposure is fully capable of translating an environmental element into molecular consequences that can result in an observable phenotype. It will be interesting to see what other environmental exposures can do to the neurodevelopmental system through epigenetic alteration. Similarly, the elucidation of such epigenetically sensitive genes in FAS and other developmental diseases is highly anticipated.

The epigenetic regulation of neurodevelopmental gene networks offers a potent and diverse mechanism for how complex neural systems are achieved. It goes without saying that characterizing every single epigenetic influence on every neurodevelopmental gene will be a long and rigorous endeavor. Epigenomic high-throughput and epigenetic editing methods perceivably will continue to quicken the pace of such findings and expand upon our current understanding of genetic–epigenetic interactions. It will certainly become more clear as the field grows that epigenetic modifications are context specific, meaning that, the changes that occur in one gene cluster or cell population do not necessarily apply to the next. This fits with our current understanding that epigenetic modifications are substantially guided by external cues often provided by the biological microenvironment. As such, it is imperative that current investigators are aware of this as they attempt to understand whole systems and tissues. Moreover, it is likely that not all epigenetic modifications are created equal. Just as specific regulatory regions govern the activity of a gene, it appears that some genomic sites are more vulnerable/receptive to epigenetic change. It will be important to isolate these sites as our focus in the epigenetic community turns from a descriptive effort to targeted modulation. Knowing that environmental-linked developmental disease is largely translated via epigenetic mechanisms, the logical progression will be investigating thresholds of epigenetic change – modifications that are necessary or sufficient to enable transcriptional change. A big hurdle to targeted epigenetic modulation will be the aforementioned fact that epigenetic modifications are widely interconnected. When these can be accurately teased out and understood, only then will we unlock the opportunity to rewrite the epigenetic codes which convey disease. Surely, the task toward elucidating gene–epigenetic relationships to the point where targeted epigenetic therapy is a possibility will be as complex as the capacities of the neural system itself.

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REFERENCES

Adegbola, A., Gao, H., Sommer, S., and Browning, M. (2008). A novel mutation in JARID1C/SMCX in a patient with autism spectrum disorder (ASD). *Am. J. Med. Genet. A* 146A, 505–511. doi: 10.1002/ajmg.a.32142

Agudelo, M., Gandhi, N., Saiyed, Z., Pichili, V., Thangavel, S., Khatavkar, P., et al. (2011). Effects of alcohol on histone deacetylase 2 (HDAC2) and the neuroprotective role of trichostatin A (TSA). *Alcohol. Clin. Exp. Res.* 35, 1550–1556. doi: 10.1111/j.1530-0277.2011.01492.x

Alcamo, E. A., Chirivella, L., Dautzenberg, M., Dobreva, G., Farinas, I., Grosschedl, R., et al. (2008). Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 57, 364–377. doi: 10.1016/j.neuron.2007.12.012

Amir, R. E., Van Den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat. Genet.* 23, 185–188. doi: 10.1038/13810

Andersson, E. K., Irvin, D. K., Ahlsio, J., and Parmar, M. (2007). Ngn2 and Nurr1 act in synergy to induce midbrain dopaminergic neurons from expanded neural stem and progenitor cells. *Exp. Cell Res.* 313, 1172–1180. doi: 10.1016/j.yexcr.2006.12.014

Anthony, B., Zhou, F. C., Ogawa, T., Goodlett, C. R., and Ruiz, J. (2008). Alcohol exposure alters cell cycle and apoptotic events during early neurulation. *Alcohol Alcohol.* 43, 261–273. doi: 10.1093/alcalc/agm166

Arzumnayan, A., Anni, H., Rubin, R., and Rubin, E. (2009). Effects of ethanol on mouse embryonic stem cells. *Alcohol. Clin. Exp. Res.* 33, 2172–2179. doi: 10.1111/j.1530-0277.2009.01057.x

Banerjee, K., Akiba, Y., Baker, H., and Cave, J. W. (2013). Epigenetic control of neurotransmitter expression in olfactory bulb interneurons. *Int. J. Dev. Neurosci.* 31, 415–423. doi: 10.1016/j.ijdevneu.2012.11.009

Bani-Yaghoub, M., Tremblay, R. G., Lei, J. X., Zhang, D., Zurakowski, B., Sandhu, J. K., et al. (2006). Role of Sox2 in the development of the mouse neocortex. *Dev. Biol.* 295, 52–66. doi: 10.1016/j.ydbio.2006.03.007

Bannister, A. J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. *Nature* 384, 641–643. doi: 10.1038/384641a0

Bartolucci, S., Estenoz, M., Longo, A., Santoro, B., Momparler, R. L., Rossi, M., et al. (1989). 5-Aza-2′-deoxycytidine as inducer of differentiation and growth inhibition in mouse neuroblastoma cells. *Cell Differ. Dev.* 27, 47–55. doi: 10.1016/0922-3371(89)90043-9

Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2013). Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and proopiomelanocortin (POMC) gene expression in beta-endorphin-producing POMC neurons of the hypothalamus. *Alcohol. Clin. Exp. Res.* 37, 1133–1142. doi: 10.1111/acer.12082

Boshnjaku, V., Ichi, S., Shen, Y. W., Puranmalka, R., Mania-Farnell, B., Mclone, D. G., et al. (2011). Epigenetic regulation of sensory neurogenesis in the dorsal root ganglion cell line ND7 by folic acid. *Epigenetics* 6, 1207–1216. doi: 10.4161/epi.6.10.17592

Brunet, A., Datta, S.R., and Greenberg, M.E. (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K–Akt signaling pathway. *Curr. Opin. Neurobiol.* 11, 297–305. doi: 10.1016/S0959-4388(00)00211-7

Bultmann, S., Morbitzer, R., Schmidt, C. S., Thanisch, K., Spada, F., Elsaesser, J., et al. (2012). Targeted transcriptional activation of silent oct4 pluripotency gene by combining designer TALEs and inhibition of epigenetic modifiers. *Nucleic Acids Res.* 40, 5368–5377. doi: 10.1093/nar/gks199

Campbell, J. C., Stipcevic, T., Flores, R. E., Perry, C., and Kippin, T. E. (2014). Alcohol exposure inhibits adult neural stem cell proliferation. *Exp. Brain Res.* doi: 10.1007/s00221-014-3958-1 [Epub ahead of print].

Chawla, S., and Bading, H. (2001). CREB/CBP and SRE-interacting transcriptional regulators are fast on-off switches: duration of calcium transients specifies the magnitude of transcriptional responses. *J. Neurochem.* 79, 849–858. doi: 10.1046/j.1471-4159.2001.00645.x

Chen, S., and Charness, M. E. (2008). Ethanol inhibits neuronal differentiation by disrupting activity-dependent neuroprotective protein signaling. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19962–19967. doi: 10.1073/pnas.0807758105

Chen, Y., Damayanti, N., Irudayaraj, J., Dunn, K., and Zhou, F. C. (2014). Diversity of two forms of DNA methylation in the brain. *Front. Genet.* 5:46. doi: 10.3389/fgene.2014.00046

Chen, Y., Ozturk, N. C., and Zhou, F. C. (2013). DNA methylation program in developing hippocampus and its alteration by alcohol. *PLoS ONE* 8:e60503. doi: 10.1371/journal.pone.0060503

Chittka, A., Nitarska, J., Grazini, U., and Richardson, W. D. (2012). Transcription factor positive regulatory domain 4 (PRDM4) recruits protein arginine methyltransferase 5 (PRMT5) to mediate histone arginine methylation and control neural stem cell proliferation and differentiation. *J. Biol. Chem.* 287, 42995–43006. doi: 10.1074/jbc.M112.392746

Cho, B., Kim, H. J., Kim, H., and Sun, W. (2011). Changes in the histone acetylation patterns during the development of the nervous system. *Exp. Neurobiol.* 20, 81–84. doi: 10.5607/en.2011.20.2.81

Chu, F., Chou, P., Mirkin, B. L., Mousa, S. A., and Rebbaa, A. (2008). Cellular conditioning with trichostatin A enhances the anti-stress response through up-regulation of HDAC4 and down-regulation of the IGF/Akt pathway. *Aging Cell* 7, 516–525. doi: 10.1111/j.1474-9726.2008.00403.x

Chung, S., Hedlund, E., Hwang, M., Kim, D. W., Shin, B. S., Hwang, D. Y., et al. (2005). The homeodomain transcription factor Pitx3 facilitates differentiation of mouse embryonic stem cells into AHD2-expressing dopaminergic neurons. *Mol. Cell. Neurosci.* 28, 241–252. doi: 10.1016/j.mcn.2004.09.008

Cimadamore, F., Amador-Arjona, A., Chen, C., Huang, C. T., and Terskikh, A. V. (2013). SOX2-LIN28/let-7 pathway regulates proliferation and neurogenesis in neural precursors. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3017–E3026. doi: 10.1073/pnas.1220176110

Cole, A. J., Saffen, D. W., Baraban, J. M., and Worley, P. F. (1989). Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature* 340, 474–476. doi: 10.1038/340474a0

Cortese, R., Lewin, J., Bäckdahl, L., Krispin, M., Wasserkort, R., Eckhardt, F., et al. (2011). Genome-wide screen for differential DNA methylation associated with neural cell differentiation in mouse. *PLoS ONE* 6:e26002. doi: 10.1371/journal.pone.0026002

D'Addario, C., Johansson, S., Candeletti, S., Romualdi, P., Ogren, S. O., Terenius, L., et al. (2011). Ethanol and acetaldehyde exposure induces specific epigenetic modifications in the prodynorphin gene promoter in a human neuroblastoma cell line. *FASEB J.* 25, 1069–1075. doi: 10.1096/fj.10-168534

Das, N. D., Choi, M. R., Jung, K. H., Park, J. H., Lee, H. T., Das, A., et al. (2013). Functional analysis of histone demethylase Jmjd2b on lipopolysaccharide-treated murine neural stem cells (NSCs). *Neurotox. Res.* 23, 154–165. doi: 10.1007/s12640-012-9346–9343

Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., et al. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol. Cell* 6, 41–51. doi: 10.1016/S1097-2765(00)00006-X

Du, Z.-W., Ma, L.-X., Phillips, C., and Zhang, S.-C. (2013). miR-200 and miR-96 families repress neural induction from human embryonic stem cells. *Development* 140, 2611–2618. doi: 10.1242/dev.092809

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bulfone, A., et al. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.* 25, 247–251. doi: 10.1523/jneurosci.2899-04.2005

Esain, V., Postlethwait, J. H., Charnay, P., and Ghislain, J. (2010). FGF-receptor signalling controls neural cell diversity in the zebrafish hindbrain by regulating olig2 and sox9. *Development* 137, 33–42. doi: 10.1242/dev.038026

Esposito, S., Tenconi, R., Lelii, M., Preti, V., Nazzari, E., Consolo, S., et al. (2014). Possible molecular mechanisms linking air pollution and asthma in children. *BMC Pulm. Med.* 14:31. doi: 10.1186/1471-2466-14-31

Feil, R., and Fraga, M. F. (2011). Epigenetics and the environment: emerging patterns and implications. *Nat. Rev. Genet.* 13, 97–109. doi: 10.1038/nrg3142

Finzsch, M., Stolt, C. C., Lommes, P., and Wegner, M. (2008). Sox9 and Sox10 influence survival and migration of oligodendrocyte precursors in the spinal cord by regulating PDGF receptor alpha expression. *Development* 135, 637–646. doi: 10.1242/dev.010454

Govorko, D., Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2012). Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. *Biol. Psychiatry* 72, 378–388. doi: 10.1016/j.biopsych.2012.04.006s

Grandbarbe, L., Bouissac, J., Rand, M., Hrabe De Angelis, M., Artavanis-Tsakonas, S., and Mohier, E. (2003). Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. *Development* 130, 1391–1402. doi: 10.1242/dev.00374

Guan, J. S., Haggarty, S. J., Giacometti, E., Dannenberg, J. H., Joseph, N., Gao, J., et al. (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459, 55–60. doi: 10.1038/nature07925

Guerrero-Preston, R., Goldman, L. R., Brebi-Mieville, P., Ili-Gangas, C., Lebron, C., Witter, F. R., et al. (2010). Global DNA hypomethylation is associated with in utero exposure to cotinine and perfluorinated alkyl compounds. *Epigenetics* 5, 539–546. doi: 10.4161/epi.5.6.12378

Guy, J., Hendrich, B., Holmes, M., Martin, J. E., and Bird, A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat. Genet.* 27, 322–326. doi: 10.1038/85899

Hahn, M. A., Qiu, R., Wu, X., Li, A. X., Zhang, H., Wang, J., et al. (2013). Dynamics of 5-hydroxymethylcytosine and chromatin marks in mammalian neurogenesis. *Cell Rep.* 3, 291–300. doi: 10.1016/j.celrep.2013.01.011

Han, D. W., Do, J. T., Arauzo-Bravo, M. J., Lee, S. H., Meissner, A., Lee, H. T., et al. (2009). Epigenetic hierarchy governing Nestin expression. *Stem Cells* 27, 1088–1097. doi: 10.1002/stem.43

Hardingham, G. E., Arnold, F. J., and Bading, H. (2001). Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. *Nat. Neurosci.* 4, 261–267. doi: 10.1038/85109

Hardingham, G. E., Fukunaga, Y., and Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat. Neurosci.* 5, 405–414. doi: 10.1038/nn835

Harper, K. M., Tunc-Ozcan, E., Graf, E. N., and Redei, E. E. (2014). Intergenerational effects of prenatal ethanol on glucose tolerance and insulin response. *Physiol. Genomics* 46, 159–168. doi: 10.1152/physiolgenomics.00181.2013

Heard, E., and Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* 157, 95–109. doi: 10.1016/j.cell.2014.02.045

Hegedus, A. M., Alterman, A. I., and Tarter, R. E. (1984). Learning achievement in sons of alcoholics. *Alcohol. Clin. Exp. Res.* 8, 330–333. doi: 10.1111/j.1530-0277.1984.tb05522.x

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., et al. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17046–17049. doi: 10.1073/pnas.0806560105

Hicks, S. D., Middleton, F. A., and Miller, M. W. (2010). Ethanol-induced methylation of cell cycle genes in neural stem cells. *J. Neurochem.* 114, 1767–1780. doi: 10.1111/j.1471-4159.2010.06886.x

Hirabayashi, K., Shiota, K., and Yagi, S. (2013). DNA methylation profile dynamics of tissue-dependent and differentially methylated regions during mouse brain development. *BMC Genomics* 14:82. doi: 10.1186/1471-2164-14-82

Hisahara, S., Chiba, S., Matsumoto, H., Tanno, M., Yagi, H., Shimohama, S., et al. (2008). Histone deacetylase SIRT1 modulates neuronal differentiation by its nuclear translocation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15599–15604. doi: 10.1073/pnas.0800612105

Ignatius, M. S., Unal Eroglu, A., Malireddy, S., Gallagher, G., Nambiar, R. M., and Henion, P. D. (2013). Distinct functional and temporal requirements for zebrafish Hdac1 during neural crest-derived craniofacial and peripheral neuron development. *PLoS ONE* 8:e63218. doi: 10.1371/journal.pone.0063218

Ikegame, T., Bundo, M., Murata, Y., Kasai, K., Kato, T., and Iwamoto, K. (2013). DNA methylation of the BDNF gene and its relevance to psychiatric disorders. *J. Hum. Genet.* 58, 434–438. doi: 10.1038/jhg.2013.65

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, D. F., Koch, C., Genz, K., et al. (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* 287, 1056–1060. doi: 10.1126/science.287.5455.1056

Impey, S., Fong, A. L., Wang, Y., Cardinaux, J. R., Fass, D. M., Obrietan, K., et al. (2002). Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV. *Neuron* 34, 235–244. doi: 10.1016/S0896-6273(02)00654-2

Iyengar, B. R., Choudhary, A., Sarangdhar, M. A., Venkatesh, K. V., Gadgil, C. J., and Pillai, B. (2014). Non-coding RNA interact to regulate neuronal development and function. *Front. Cell. Neurosci.* 8:47. doi: 10.3389/fncel.2014.00047

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33(Suppl. 1), 245–254. doi: 10.1038/ng1089

Jeong, H., Kim, M. S., Kwon, J., Kim, K. S., and Seol, W. (2006). Regulation of the transcriptional activity of the tyrosine hydroxylase gene by androgen receptor. *Neurosci. Lett.* 396, 57–61. doi: 10.1016/j.neulet.2005.11.011

Jobe, E. M., Mcquate, A. L., and Zhao, X. (2012). Crosstalk among epigenetic pathways regulates neurogenesis. *Front. Neurosci.* 6:59. doi: 10.3389/fnins.2012.00059

Juliandi, B., Abematsu, M., and Nakashima, K. (2010). Chromatin remodeling in neural stem cell differentiation. *Curr. Opin. Neurobiol.* 20, 408–415. doi: 10.1016/j.conb.2010.04.001

Kageyama, R., Ohtsuka, T., Shimojo, H., and Imayoshi, I. (2008). Dynamic Notch signaling in neural progenitor cells and a revised view of lateral inhibition. *Nat. Neurosci.* 11, 1247–1251. doi: 10.1038/nn.2208

Kaltezioti, V., Kouroupi, G., Oikonomaki, M., Mantouvalou, E., Stergiopoulos, A., Charonis, A., et al. (2010). Prox1 regulates the notch1-mediated inhibition of neurogenesis. *PLoS Biol.* 8:e1000565. doi: 10.1371/journal.pbio.1000565

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K.-A., Fahey, P., Cox, T. C., et al. (2010). Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet.* 6:e1000811. doi: 10.1371/journal.pgen.1000811

Kim, M., Park, Y. K., Kang, T. W., Lee, S. H., Rhee, Y. H., Park, J. L., et al. (2014). Dynamic changes in DNA methylation and hydroxymethylation when hES cells undergo differentiation toward a neuronal lineage. *Hum. Mol. Genet.* 23, 657–667. doi: 10.1093/hmg/ddt453

Knezovich, J. G., and Ramsay, M. (2012). The effect of preconception paternal alcohol exposure on epigenetic remodeling of the h19 and rasgrf1 imprinting control regions in mouse offspring. *Front. Genet.* 3:10. doi: 10.3389/fgene.2012.00010

Laufer, B. I., Mantha, K., Kleiber, M. L., Diehl, E. J., Addison, S. M., and Singh, S. M. (2013). Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis. Model Mech.* 6, 977–992. doi: 10.1242/dmm.010975

Leone, D. P., Srinivasan, K., Chen, B., Alcamo, E., and Mcconnell, S. K. (2008). The determination of projection neuron identity in the developing cerebral cortex. *Curr. Opin. Neurobiol.* 18, 28–35. doi: 10.1016/j.conb.2008.05.006

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69, 915–926. doi: 10.1016/0092-8674(92)90611-F

Li, T., Huang, H., Huang, B., Huang, B., and Lu, J. (2009). Histone acetyltransferase p300 regulates the expression of human pituitary tumor transforming gene (hPTTG). *J. Genet. Genomics* 36, 335–342. doi: 10.1016/s1673-8527(08)60122-8

Little, R. E., and Sing, C. F. (1987). Father's drinking and infant birth weight: report of an association. *Teratology* 36, 59–65. doi: 10.1002/tera.1420360109

Lu, Q., Hutchins, A. E., Doyle, C. M., Lundblad, J. R., and Kwok, R. P. S. (2003). Acetylation of cAMP-responsive element-binding protein (CREB) by CREB-binding protein enhances CREB-dependent transcription. *J. Biol. Chem.* 278, 15727–15734. doi: 10.1074/jbc.M300546200

Luscher, C., and Malenka, R. C. (2012). NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). *Cold Spring Harb. Perspect. Biol.* 4. doi: 10.1101/cshperspect.a005710

Marban, C., Suzanne, S., Dequiedt, F., De Walque, S., Redel, L., Van Lint, C., et al. (2007). Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. *EMBO J.* 26, 412–423. doi: 10.1038/sj.emboj.7601516

Marini, A. M., Jiang, X., Wu, X., Tian, F., Zhu, D., Okagaki, P., et al. (2004). Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: from genes to phenotype. *Restor. Neurol. Neurosci.* 22, 121–130.

Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., et al. (2003). DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* 302, 890–893. doi: 10.1126/science.1090842

Mateen, S., Raina, K., Jain, A. K., Agarwal, C., Chan, D., and Agarwal, R. (2012). Epigenetic modifications and p21-cyclin B1 nexus in anticancer effect of histone deacetylase inhibitors in combination with silibinin on non-small cell lung cancer cells. *Epigenetics* 7, 1161–1172. doi: 10.4161/epi.22070

Meissner, A., Mikkelsen, T. S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454, 766–770. doi: 10.1038/nature07107

Meza-Sosa, K. F., Pedraza-Alva, G., and Pérez-Martínez, L. (2014). microRNAs: key triggers of neuronal cell fate. *Front. Cell. Neurosci.* 8:175. doi: 10.3389/fncel.2014.00175

Miller, C. A., Campbell, S. L., and Sweatt, J. D. (2008). DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiol. Learn. Mem.* 89, 599–603. doi: 10.1016/j.nlm.2007.07.016

Modarresi, F., Faghihi, M. A., Lopez-Toledano, M. A., Fatemi, R. P., Magistri, M., Brothers, S. P., et al. (2012). Inhibition of natural antisense transcripts in vivo

results in gene-specific transcriptional upregulation. *Nat. Biotechnol.* 30, 453–459. doi: 10.1038/nbt.2158

Ogony, J. W., Malahias, E., Vadigepalli, R., and Anni, H. (2013). Ethanol alters the balance of Sox2, Oct4, and Nanog expression in distinct subpopulations during differentiation of embryonic stem cells. *Stem Cells Dev.* 22, 2196–2210. doi: 10.1089/scd.2012.0513

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247–257. doi: 10.1016/S0092-8674(00)81656-6

Pasini, D., Cloos, P. A., Walfridsson, J., Olsson, L., Bukowski, J. P., Johansen, J. V., et al. (2010). JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. *Nature* 464, 306–310. doi: 10.1038/nature08788

Peng, C., Li, N., Ng, Y. K., Zhang, J., Meier, F., Theis, F. J., et al. (2012). A unilateral negative feedback loop between miR-200 microRNAs and Sox2/E2F3 controls neural progenitor cell-cycle exit and differentiation. *J. Neurosci.* 32, 13292–13308. doi: 10.1523/jneurosci.2124-12.2012

Postigo, A. A. (2003). Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. *EMBO J.* 22, 2443–2452. doi: 10.1093/emboj/cdg225

Qi, Y., Zhang, M., Li, H., Frank, J. A., Dai, L., Liu, H., et al. (2014). MicroRNA-29b regulates ethanol-induced neuronal apoptosis in the developing cerebellum through SP1/RAX/PKR cascade. *J. Biol. Chem.* 289, 10201–10210. doi: 10.1074/jbc.M113.535195

Qiang, M., Denny, A., Lieu, M., Carreon, S., and Li, J. (2011). Histone H3K9 modifications are a local chromatin event involved in ethanol-induced neuroadaptation of the NR2B gene. *Epigenetics* 6, 1095–1104. doi: 10.4161/epi.6.9.16924

Rajasethupathy, P., Antonov, I., Sheridan, R., Frey, S., Sander, C., Tuschl, T., et al. (2012). A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. *Cell* 149, 693–707. doi: 10.1016/j.cell.2012.02.057

Reichrath, S., and Reichrath, J. (2012). No evidence for induction of key components of the Notch signaling pathway (Notch-1, Jagged-1) by treatment with UV-B, 1,25(OH)(2)D(3), and/or epigenetic drugs (TSA, 5-Aza) in human keratinocytes in vitro. *Dermatoendocrinology* 4, 44–52. doi: 10.4161/derm.19027

Resendiz, M., Chen, Y., Ozturk, N. C., and Zhou, F. C. (2013). Epigenetic medicine and fetal alcohol spectrum disorders. *Epigenomics* 5, 73–86. doi: 10.2217/epi.12.80

Romashkova, J. A., and Makarov, S. S. (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401, 86–90. doi: 10.1038/43474

Ruau, D., Ensenat-Waser, R., Dinger, T. C., Vallabhapurapu, D. S., Rolletschek, A., Hacker, C., et al. (2008). Pluripotency associated genes are reactivated by chromatin-modifying agents in neurosphere cells. *Stem Cells* 26, 920–926. doi: 10.1634/stemcells.2007–2649

Sanchez-Alvarez, R., Gayen, S., Vadigepalli, R., and Anni, H. (2013). Ethanol diverts early neuronal differentiation trajectory of embryonic stem cells by disrupting the balance of lineage specifiers. *PLoS ONE* 8:e63794. doi: 10.1371/journal.pone.0063794

Sansom, S. N., Griffiths, D. S., Faedo, A., Kleinjan, D.-J., Ruan, Y., Smith, J., et al. (2009). The Level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. *PLoS Genet.* 5:e1000511. doi: 10.1371/journal.pgen.1000511

Sathyan, P., Golden, H. B., and Miranda, R. C. (2007). Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *J. Neurosci.* 27, 8546–8557. doi: 10.1523/jneurosci.1269-07.2007

Schmitz, S. U., Albert, M., Malatesta, M., Morey, L., Johansen, J. V., Bak, M., et al. (2011). Jarid1b targets genes regulating development and is involved in neural differentiation. *EMBO J.* 30, 4586–4600. doi: 10.1038/emboj.2011.383

Singh, R. P., Shiue, K., Schomberg, D., and Zhou, F. C. (2009). Cellular epigenetic modifications of neural stem cell differentiation. *Cell Transplant.* 18, 1197–1211. doi: 10.3727/096368909x12483162197204

Singh, T., Prasad, R., and Katiyar, S. K. (2013). Inhibition of class I histone deacetylases in non-small cell lung cancer by honokiol leads to suppression of cancer cell growth and induction of cell death in vitro and in vivo. *Epigenetics* 8, 54–65. doi: 10.4161/epi.23078

Skinner, M. K., Haque, C. G., Nilsson, E., Bhandari, R., and Mccarrey, J. R. (2013). Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. *PLoS ONE* 8:e66318. doi: 10.1371/journal.pone.0066318

Stilling, R. M., Ronicke, R., Benito, E., Urbanke, H., Capece, V., Burkhardt, S., et al. (2014). K-Lysine acetyltransferase 2a regulates a hippocampal gene expression network linked to memory formation. *EMBO J.* doi: 10.15252/embj.201487870 [Epub ahead of print].

Stringer, R. L., Laufer, B. I., Kleiber, M. L., and Singh, S. M. (2013). Reduced expression of brain cannabinoid receptor 1 (Cnr1) is coupled with an increased complementary micro-RNA (miR-26b) in a mouse model of fetal alcohol spectrum disorders. *Clin. Epigenetics* 5:14. doi: 10.1186/1868-7083-5-14

Sun, F., Fu, H., Liu, Q., Tie, Y., Zhu, J., Xing, R., et al. (2008). Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. *FEBS Lett.* 582, 1564–1568. doi: 10.1016/j.febslet.2008.03.057

Sussman, R. T., Stanek, T. J., Esteso, P., Gearhart, J. D., Knudsen, K. E., and Mcmahon, S. B. (2013). The epigenetic modifier ubiquitin-specific protease 22 (USP22) regulates embryonic stem cell differentiation via transcriptional repression of sex-determining region Y-box 2 (SOX2). *J. Biol. Chem.* 288, 24234–24246. doi: 10.1074/jbc.M113.469783

Tan, L., Xiong, L., Xu, W., Wu, F., Huang, N., Xu, Y., et al. (2013). Genome-wide comparison of DNA hydroxymethylation in mouse embryonic stem cells and neural progenitor cells by a new comparative hMeDIP-seq method. *Nucleic Acids Res.* 41:e84. doi: 10.1093/nar/gkt091

Tan, S. L., Nishi, M., Ohtsuka, T., Matsui, T., Takemoto, K., Kamio-Miura, A., et al. (2012). Essential roles of the histone methyltransferase ESET in the epigenetic control of neural progenitor cells during development. *Development* 139, 3806–3816. doi: 10.1242/dev.082198

Taqi, M. M., Bazov, I., Watanabe, H., Sheedy, D., Harper, C., Alkass, K., et al. (2011). Prodynorphin CpG-SNPs associated with alcohol dependence: elevated methylation in the brain of human alcoholics. *Addict. Biol.* 16, 499–509. doi: 10.1111/j.1369-1600.2011.00323.x

Tsuji-Takayama, K., Inoue, T., Ijiri, Y., Otani, T., Motoda, R., Nakamura, S., et al. (2004). Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells. *Biochem. Biophys. Res. Commun.* 323, 86–90. doi: 10.1016/j.bbrc.2004.08.052

Vangipuram, S. D., and Lyman, W. D. (2012). Ethanol affects differentiation-related pathways and suppresses Wnt signaling protein expression in human neural stem cells. *Alcohol. Clin. Exp. Res.* 36, 788–797. doi: 10.1111/j.1530-0277.2011.01682.x

van Heesbeen, H. J., Mesman, S., Veenvliet, J. V., and Smidt, M. P. (2013). Epigenetic mechanisms in the development and maintenance of dopaminergic neurons. *Development* 140, 1159–1169. doi: 10.1242/dev.089359

Veazey, K. J., Carnahan, M. N., Muller, D., Miranda, R. C., and Golding, M. C. (2013). Alcohol-induced epigenetic alterations to developmentally cruciasssl genes regulating neural stemness and differentiation. *Alcohol. Clin. Exp. Res.* 37, 1111–1122. doi: 10.1111/acer.12080

Vecsey, C. G., Hawk, J. D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., et al. (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J. Neurosci.* 27, 6128–6140. doi: 10.1523/jneurosci.0296-07.2007s

Villar-Menendez, I., Blanch, M., Tyebji, S., Pereira-Veiga, T., Albasanz, J. L., Martin, M., et al. (2013). Increased 5-methylcytosine and decreased 5-hydroxymethylcytosine levels are associated with reduced striatal A2AR levels in Huntington's disease. *Neuromolecular Med.* 15, 295–309. doi: 10.1007/s12017-013-8219-0

Wang, J., Lu, F., Ren, Q., Sun, H., Xu, Z., Lan, R., et al. (2011). Novel histone demethylase LSD1 inhibitors selectively target cancer cells with pluripotent stem cell properties. *Cancer Res.* 71, 7238–7249. doi: 10.1158/0008-5472.can-11–0896

Wang, J., Park, J. W., Drissi, H., Wang, X., and Xu, R. H. (2014a). Epigenetic regulation of miR-302 by JMJD1C inhibits neural differentiation of human embryonic stem cells. *J. Biol. Chem.* 289, 2384–2395. doi: 10.1074/jbc.M113.535799

Wang, Z., Yan, P., Hui, T., and Zhang, J. (2014b). Epigenetic upregulation of PSD-95 contributes to the rewarding behavior by morphine conditioning. *Eur. J. Pharmacol.* 732, 123–129. doi: 10.1016/j.ejphar.2014.03.040

Weng, M. K., Zimmer, B., Poltl, D., Broeg, M. P., Ivanova, V., Gaspar, J. A., et al. (2012). Extensive transcriptional regulation of chromatin modifiers during human neurodevelopment. *PLoS ONE* 7:e36708. doi: 10.1371/journal.pone.0036708

West, A. E., Chen, W. G., Dalva, M. B., Dolmetsch, R. E., Kornhauser, J. M., Shaywitz, A. J., et al. (2001). Calcium regulation of neuronal gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 98, 11024–11031. doi: 10.1073/pnas.191352298

Wilhelm-Benartzi, C. S., Houseman, E. A., Maccani, M. A., Poage, G. M., Koestler, D. C., Langevin, S. M., et al. (2012). In utero exposures, infant growth, and DNA methylation of repetitive elements and developmentally related genes in human placenta. *Environ. Health Perspect.* 120, 296–302. doi: 10.1289/ehp.1103927

Wu, Y., Liu, Y., Levine, E. M., and Rao, M. S. (2003). Hes1 but not Hes5 regulates an astrocyte versus oligodendrocyte fate choice in glial restricted precursors. *Dev. Dyn.* 226, 675–689. doi: 10.1002/dvdy.10278

Wynder, C., Stalker, L., and Doughty, M. L. (2010). Role of H3K4 demethylases in complex neurodevelopmental diseases. *Epigenomics* 2, 407–418. doi: 10.2217/epi.10.12

Yadav, S., Pandey, A., Shukla, A., Talwelkar, S. S., Kumar, A., Pant, A. B., et al. (2011). miR-497 and miR-302b regulate ethanol-induced neuronal cell death through BCL2 protein and cyclin D2. *J. Biol. Chem.* 286, 37347–37357. doi: 10.1074/jbc.M111.235531

Yoon, K., and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* 8, 709–715. doi: 10.1038/nn1475

Young, C., Klocke, B. J., Tenkova, T., Choi, J., Labruyere, J., Qin, Y. Q., et al. (2003). Ethanol-induced neuronal apoptosis in vivo requires BAX in the developing mouse brain. *Cell Death Differ.* 10, 1148–1155. doi: 10.1038/sj.cdd.4401277

Zhang, J., Parvin, J., and Huang, K. (2012). Redistribution of H3K4me2 on neural tissue specific genes during mouse brain development. *BMC Genomics* 13(Suppl. 8):S5. doi: 10.1186/1471-2164-13-s8-s5

Zhang, X., Huang, C.T., Chen, J., Pankratz, M.T., Xi, J., Li, J., et al. (2010). Pax6 is a human neuroectoderm cell fate determinant. *Cell Stem Cell* 7, 90–100. doi: 10.1016/j.stem.2010.04.017

Zhou, F. C., Balaraman, Y., Teng, M., Liu, Y., Singh, R. P., and Nephew, K. P. (2011). Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcohol. Clin. Exp. Res.* 35, 735–746. doi: 10.1111/j.1530-0277.2010.01391.x

Zhou, F. C., Sari, Y., Zhang, J. K., Goodlett, C. R., and Li, T. (2001). Prenatal alcohol exposure retards the migration and development of serotonin neurons in fetal C57BL mice. *Brain Res. Dev. Brain Res.* 126, 147–155. doi: 10.1016/S0165-3806(00)00144-9

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Long-term genomic and epigenomic dysregulation as a consequence of prenatal alcohol exposure: a model for fetal alcohol spectrum disorders

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There is abundant evidence that prenatal alcohol exposure leads to a range of behavioral and cognitive impairments, categorized under the term fetal alcohol spectrum disorders (FASDs). These disorders are pervasive in Western cultures and represent the most common preventable source of neurodevelopmental disabilities. The genetic and epigenetic etiology of these phenotypes, including those factors that may maintain these phenotypes throughout the lifetime of an affected individual, has become a recent topic of investigation. This review integrates recent data that has progressed our understanding FASD as a continuum of molecular events, beginning with cellular stress response and ending with a long-term "footprint" of epigenetic dysregulation across the genome. It reports on data from multiple ethanol-treatment paradigms in mouse models that identify changes in gene expression that occur with respect to neurodevelopmental timing of exposure and ethanol dose. These studies have identified patterns of genomic alteration that are dependent on the biological processes occurring at the time of ethanol exposure. This review also adds to evidence that epigenetic processes such as DNA methylation, histone modifications, and non-coding RNA regulation may underlie long-term changes to gene expression patterns. These may be initiated by ethanol-induced alterations to DNA and histone methylation, particularly in imprinted regions of the genome, affecting transcription which is further fine-tuned by altered microRNA expression. These processes are likely complex, genome-wide, and interrelated. The proposed model suggests a potential for intervention, given that epigenetic changes are malleable and may be altered by postnatal environment. This review accentuates the value of mouse models in deciphering the molecular etiology of FASD, including those processes that may provide a target for the ammelioration of this common yet entirely preventable disorder.

Keywords: epigenetics, neurodevelopment, mouse models, fetal alcohol spectrum disorders, DNA methylation, microRNA, histone modifications, gene expression

INTRODUCTION

The development of an organism from a single cell to a complex structure of distinct cell types that can interact, communicate, and respond to internal and external cues is an enigmatic process. What is known is that it is initiated by non-identical but complimentary maternal and paternal genetic contributions that comprise the diploid genome of the developing fetus, leading to the development of a complex organism comprised of differentiated organ systems. The development of the human brain is perhaps the most poorly-understood process, beginning early *in utero* and extending well into adolescence. Also, at each stage it is directed by precise spatial and temporal control of gene expression that may be influenced by external cues such as maternal gene products, micronutrient availability, and environmental molecules (Ellis et al., 2005). Decades of research have demonstrated that the fetal environment, particularly neurodevelopmental adversity, places individuals at higher risk for cognitive, behavioral, and social deficits (Shonkoff et al., 2009). At its extreme, an adverse developmental environment has been associated with later-life psychopathologies. Few disorders, however, have such a clear etiology as fetal alcohol spectrum disorders (FASD). This common yet entirely preventable set of cognitive and behavioral abnormalities are caused by alcohol's ability to pleotropically disrupt neurodevelopmental processes and resulting in altered brain function over the lifetime of an affected individual.

Despite overwhelming evidence that alcohol adversely affects the developing fetus, many women continue to drink during pregnancy. The reasons for this are varied: naïvité of potential consequences, increase in the prevalence of alcohol use among females of child-bearing age, addiction, or unawareness of pregnancy may all contribute to the growing number of children exhibiting FASD. Regardless, the pervasiveness of prenatal alcohol exposure (PAE) in North America is high, with an estimated rate of 1 in every 100 live births (Sampson et al., 1997; Chudley et al., 2005). This incurs staggering socio-economic costs (Stade et al.,

2009), burdening healthcare, affected individuals, and their families. Indeed, the cognitive and behavioral impairments associated with FASD often lead to poor social and academic performance, higher rates of mental illness, and increased risk for delinquent behavior (Streissguth and O'Malley, 2000; Fast and Conry, 2004).

Expecting women of childbearing age to abstain completely from alcohol in western cultures is unrealistic. Yet, understanding the molecular mechanisms that underlie the origin, manifestation, and maintenance of FASD phenotypes may assist in the development of preventive and amelioration strategies to improve the outcome of affected individuals. The current review examines work conducted in our laboratory and others that seek to evaluate how ethanol exposure at different stages of neurodevelopment can trigger immediate changes in the brain leading to FASD. The current data have allowed us to propose an epigenetic model of neurodevelopmental disruption that may account for the causes and consequences of this prevalent disorder.

MODELING FASD IN MICE

In order to understand how PAE may result in the epidemiological findings relating to FASD in human populations will require an understanding of how alcohol affects the molecular processes that guide neurodevelopment. This requires the development and use of effective animal models that can be reliably generated and that recapitulate the endophenotypes commonly observed in individuals with FASD. Much of our understanding of the genetic and epigenetic consequences of PAE has come from mouse models. These models generally fall into two categories. First, multiple groups have evaluated the effect of chronic voluntary maternal ethanol consumption throughout pregnancy by utilizing strains of mice with high ethanol preference or high vulnerability to the intoxicating effects of alcohol, such as C57BL6/J (B6) mice (Gilliam et al., 1989; Allan et al., 2003; Boehm et al., 2008; Kleiber et al., 2011). Pregnant B6 females continue to consume approximately 70% of their total liquid intake in the form of an ethanol solution, exposing the developing fetus to low-to-moderate levels of ethanol throughout gestation. The resulting offspring show subtle but consistent phenotypes relevant to FASD such as delays in the development of neuromuscular reflexes and coordination, increases in novelty-induced anxiety, and deficits in spatial learning (Kleiber et al., 2011). These models have face validity in that they likely represent a common pattern of alcohol consumption in humans that choose to drink while pregnant. However, they can make it difficult to ascertain the direct effects of ethanol on particular neurodevelopmental processes or at specific times. To address this, we and others have utilized a second type of exposure paradigm where a punctuated high dose ("binge"-like) treatment of ethanol is administered at a specific developmental time. These binge doses are administered typically during the mid-first, second, or third trimester human equivalent, with the first two given via injection or gavage to pregnant females and the latter given directly to neonate offspring due to the variation in human vs. mice neurodevelopmental timelines (Kleiber et al., 2013; Mantha et al., 2013). Such "binge" models have allowed us to evaluate how ethanol may disrupt the molecular processes active at each stage of brain development, and how these disruptions may translate to later-life phenotypic abnormalities.

THE INITIAL EFFECTS OF ETHANOL INDUCE CELLULAR STRESS LEADING TO APOPTOSIS OR ADAPTATION AND CELL SURVIVAL

Multiple studies have documented the initial effects of ethanol exposure on immature brain cells. Perhaps most consistent is the finding that ethanol, particularly at high doses, is toxic to cells actively undergoing developmental processes such as neurulation, differentiation, migration, and synaptogenesis. A binge-like exposure to such cells can cause mass apoptosis in susceptible cell types in the developing brain, which has been observed in multiple brain regions and at multiple developmental stages (Ikonomidou et al., 2000; Light et al., 2002; Dikranian et al., 2005; Young et al., 2005; Zhou et al., 2011). It is interesting to note that susceptibility to ethanol-induced neurodegeneration varies with developmental stage. Certain regions show vulnerability early in brain development (first trimester equivalent), such as derivatives of the vetromedial forebrain and gastrulating mesodermal cells (Sulik, 2005) and others displaying sensitivity much later (third trimester), such as the hippocampus, cerebellum, corpus callosum, and regions of the prefrontal cortex (Olney et al., 2002). High ethanol doses early in gestation may ultimately result in congenital abnormalities, preterm births, or fetal death and spontaneous abortion (Harlap and Shiono, 1980). The effects of binge-drinking later in gestation may not display as cranio-facial abnormalities such as those associated with FAS, but as more specific neuroanatomical differences. Neuroimaging studies have consistently identified abnormalities in brain structure in individuals prenatally exposed to alcohol, such as reduced cerebral and cerebellar volume, hypoplasia of the corpus callosum, reduced hippocampal volume, and reduction of the caudate nucleus and basal ganglia (reviewed in Norman et al., 2009). Further, regional increases in cortical thickness and gray matter, as well as decreased volume and disorganization in white matter have been reported in individuals lacking the cranio-facial abnormalities required for an FAS diagnosis (Sowell et al., 2008a). Diffusion tensor imaging findings indicate that inter- and intraregional connectivity is also reduced in PAE individuals, significantly affecting the corpus callosum and tracts innervating the frontal, occipital, and parietal lobes of the cortex, as well as the hypothalamic-pituitary-adrenal (HPA) axis (Lebel et al., 2008; Sowell et al., 2008b; Fryer et al., 2009). Whether these abnormalities represent apoptosis of particular cell types and subsequent reorganization of cells or alterations in neurodevelopmental synaptic pruning is uncertain, but it is likely the result of some combination of the two.

Apoptosis is a normal developmental process that eliminates abnormally overactive and underactive neurons from the total cell population through distinct molecular pathways. However, alcohol can inappropriately trigger this process in the developing brain via its ability to act as an NMDA receptor antagonist and a GABAergic agonist. This process is associated with the activation of *caspase-3*, a hallmark of the intrinsic apoptotic pathway (Ikonomidou et al., 2000), that is dependent on Bax, a pro-apoptotic member of the Bcl-2 family, suggesting that mitochondrial release of apoptotic signals is critical to ethanol-induced neurodegeneration (Nowoslawski et al., 2005). Data from our laboratory suggests that this may be initiated by the general up-regulation of genes and pathways that drive apoptosis, including

glutamate receptors (*Grin2a*, *Grin2b*), *Tgfb3*, *Foxo3*, and *Jun* (Kleiber et al., 2013). Gene ontology (GO) biological functions and pathways affected by ethanol (identified based on altered mRNA transcript profiling) are associated with the positive regulation of genes associated with apoptosis and cell membrane integrity, and down-regulation of genes associated with mitosis and biomolecule synthesis. These data corroborate that ethanol exposure initiates a strong stress response in developing cells that is designed to minimize energetically costly processes to maximize cell survival. It is clear that some developing neurons succumb to ethanol toxicity; however, certain cell types encounter terotagenic adversity and undergo molecular adaptation, and form the foundation of further development. These surviving cells may then undergo further mitosis, differentiation, and establish synaptic connectivity to eventually become part of the final functional network of the mature brain. Indeed, the molecular players that aid in these processes are expected, in part, to depend on the developmental timing of alcohol exposure.

ALCOHOL AND NEURODEVELOPMENTAL STRESS RESPONSE: SURVIVAL AND ADAPTATION

It is unclear why some cells succumb to apoptosis while others survive. But, those cells that survive represent a population that must reinitiate neurodevelopment and adjust their developmental trajectories to recoup, at least somewhat, the functionality of those cells that are lost. These cells must do so within a relatively limited amount of time, via alterations to gene expression patterns. We are only beginning to understand how this interruption to developmental cues leads to an altered pattern of gene expression and genomic regulation that results in a molecular "footprint" that, while established early during neurodevelopment, may be persistent throughout the lifetime of an alcohol-exposed individual.

Experimental evidence that ethanol effects include both short and long-term changes to gene expression is accumulating. Most of these studies, particularly within the last few years, have been concerned with the brain as a major target of ethanol and a driver of the long-term neurobehavioral and cognitive effects. Interestingly, the molecular changes that may occur following ethanol exposure seem to, in part, be dependent on the timing of the ethanol exposure.

EARLY-GESTATION (FIRST TRIMESTER) EXPOSURE: DISRUPTION OF NEURULATION, STRUCTURAL REMODELING, AND EPIGENETIC REPROGRAMMING

Most studies examining ethanol's effects on the human first trimester equivalent of brain development have focused on gestational days (GDs) 7–9 in mouse models as a representative model for early-gestational ethanol exposure. Ethanol-exposure during this stage of development may also lead to craniofacial abnormalities similar to human FAS (Sulik, 2005). Microarray studies, such as Hard et al. (2005) and Green et al. (2007), identified genes associated with ethanol exposure at GD 7 and 8, respectively. Hard et al. (2005) identified six annotated genes, all down regulated, involved in extracellular membrane remodeling, including *Timp4* and growth factor signaling gene *Bmp15*. Green et al. (2007) examined not only the gene expression changes that occur in the brain following ethanol exposure at GD 8, but also how genetic background may affect both physiological and genetic changes. C57BL6/J mice were found to be extremely susceptible to early-exposure craniofacial abnormalities as compared to C57BL6/N mice, but, interestingly, the infrequency of physiological abnormalities in the latter strain was not indicative of the strength of genetic response within the brain. These results may explain why individuals both with and without craniofacial abnormalities may be similarly cognitively affected by ethanol teratogenesis. Major pathways associated with early gestational exposure included down-regulation of ribosomal proteins and the up-regulation of glycolysis and the pentose phosphate pathway, alterations to cellular adhesion, integrity, and cytoskeletal pathways, including canonical Wnt signaling (Hard et al., 2005; Green et al., 2007). These findings are corroborated by a more recent study illustrating that ethanol exposure at GD 9 results in altered expression of genes associated with mRNA processing, protein synthesis ubiquitination, apoptosis (Downing et al., 2012). The results argue that early-gestation ethanol exposure disrupts cellular processes associated with cellular proliferation, survival, mitosis, and migration, which is consistent with the physiological phenotypes observed in these studies. Interestingly, early gestational exposure is also associated with alterations in a number of genes associated with epigenetic processes including methylation, chromatin organization, and remodeling, including *Ilf3*, a gene involved in chromatin remodeling, and *Hist3h2a* (Zhou et al., 2011; Downing et al., 2012). The disruption of these processes have long-lasting consequences on gene expression and, subsequently, neural function (Kleiber et al., 2013). Expression array analysis of adult (PD 60) mouse brain tissue following early-gestational ethanol exposure revealed the altered expression of genes involved in cellular assembly, proliferation, apoptosis, and tissue morphology. Many of these functions are associated with the altered expression of *Ntf3*, a canonical neuronal survival growth factor. Further, these long-term effects of trimester one-equivalent exposure include the decreased expression of genes that regulate endoplasmic stress response such as *Dnajjc3*, suggesting that the surviving population of cells may show reduced ability to navigate further environmental stressors and may be more vulnerable to future insults.

MID-GESTATION (SECOND TRIMESTER) EXPOSURE: DISRUPTION OF CELLULAR MIGRATION AND DIFFERENTIATION

At the end of the first trimester and throughout the second trimester, neural stem cells (NSC) produce a large proportion of what will become mature, adult neurons (Bystron et al., 2008). As such, the effects of ethanol exposure have the potential to be amplified by the high rate of cell proliferation and maturation that occurs during this period. Recent publications have examined the short and long-term effects of a binge-like exposure at GD 16 (Kleiber et al., 2013; Mantha et al., 2014), roughly equivalent to mid-gestation in humans in terms of active neurodevelopmental processes. Similar to early gestational exposure, the initial cellular response to ethanol at mid-to-late-gestation largely involves cellular stress response and apoptosis, and the altered expression of genes that regulate cell cycle. Interestingly, GD 16 exposure also triggers changes to genes involved in cell

assembly and organization such as *Pip5k1b*, involved in actin polymerization, and cellular movement, such as *Ccl3* and *Ccnt1*. During trimester two, newly-generated cortical NSC migrate from the ventricular zone (VZ) to the cortical plate following mitosis (Noctor et al., 2004). Given that trimester two is a period of cellular reorganization and migration, it is logical that cellular signaling that guide these processes are particularly responsive to intra- and extracellular cues. Alterations to these genes may result in decreased proliferation rate of NSC, increased migration out of the VZ and into the subventricular zone (SVZ), and subsequently, a decreased thickness of this region (Miller and Nowakowski, 1991) that has been observed in humans with FASD. Interestingly, these NSC are not readily susceptible to ethanol-induced apoptosis (Prock and Miranda, 2007) but rather show increased migration and inappropriate differentiation patterns (Camarillo and Miranda, 2008). These reports are consistent with findings that suggest that genes associated with cellular differentiation, migration, and morphology remain altered at PD 60. We identified altered expression of *Dlx1* and *Dlx2*, among the earliest genes to be expressed in the SVZ and critical to interneuron differentiation and migration (Eisenstat et al., 1999; Ghanem et al., 2012).

LATE-GESTATION (THIRD TRIMESTER) EXPOSURE: DISRUPTION OF CELLULAR COMMUNICATION AND SYNAPTIC CONNECTIVITY

The third trimester has been called the "brain growth spurt" due to the occurrence of a period of rapid synaptogenesis during which much of the basis of cell-to-cell communication that will form adult neural circuitry is established. This period is also extremely sensitive to the ability of ethanol to trigger neurodegeneration, with a large proportion of cells observed to undergo apoptosis in numerous regions such as the cortex, cerebellum, corpus callosum (Olney et al., 2000). This is, in part, attributed to ethanol's ability to disrupt glutamatergic and GABAergic signaling. In rodents, synaptogenesis occurs during the first 2 weeks of neonatal life, with the peak occurring at approximately PD 7 (Dobbing and Sands, 1979). Given this difference in neurodevelopmental timelines between mice and humans, ethanol exposure during the third trimester can be modeled by early neonatal ethanol treatment in mice. Similar to first trimester exposure, the initial response to ethanol at this developmental stage is characterized by cellular stress, including an up-regulation of genes associated with apoptosis and a down-regulation of genes involved in energetically costly processes such as protein synthesis and mitotic progression. This is also associated with reduced expression of a number of growth factors such as *E2f4*, *Egr3*, *Egr4*, and *Vegfa*. Aside from cellular stress, ethanol affects the expression of a number of genes relevant to synaptic formation and maintenance, including *Cpeb1*, *Gabra5*, *Grin2a*, and *Grin2b*. Given that the formation of functional neural circuits is dependent on the synchronous activity of glutamate and GABA signaling, alterations to these genes likely disrupt the establishment of normal synaptic connectivity (Kleiber et al., 2013).

Additionally, given that much of the brain has undergone substantial differentiation by this stage, it is likely that ethanol affects gene expression in a particularly region-specific and cell type-specific manner. In particular, the hippocampus and the HPA axis appears to be susceptible to third trimester exposure as evidenced by the impairments in cognitive and behavioral phenotypes consistently associated with late-gestation (in humans) and early neonatal (in mice) ethanol exposure. Studies have identified alterations in NMDA and GABA subunit receptor expression and function immediately following neonatal ethanol exposure as well as into adulthood (Mameli et al., 2005; Toso et al., 2006; Puglia and Valenzuela, 2010; Kleiber et al., 2013). This is associated with impairments in the formation of organized synaptic connections and persistent deficits in long-term potentiation, explaining the consistent observation of impaired learning and memory formation in mouse models of FASD as well as affected individuals.

Other consistently-identified gene pathways altered shortly after ethanol exposure that remain altered into adulthood include endocannabinoid and retinoic acid signaling (Kleiber et al., 2013; Subbanna et al., 2013a). Retinoic acid receptor signaling has also been implicated in ethanol's effects on HPA axis formation and reactivity. Specifically, ethanol has been shown to affect steroid hormone signaling, including the immediate and long term dysregulation of thyroid hormone/retinoid X receptor signaling, propiomelanocortin, and Period gene expression (Chen et al., 2006; Kleiber et al., 2013). Interestingly, this effect is most pronounced in animal models exposed during the brain growth spurt period (Earnest et al., 2001; Sakata-Haga et al., 2006). Phenotypically, this results in altered Circadian rhythm and gluccocorticoid signaling that is associated with increased stress reactivity and vulnerability to anxiety, depression, hyperactivity, and diminished cognitive function, all of which are consistently observed in individuals exposed to ethanol during neurodevelopment (Earnest et al., 2001; Girotti et al., 2007; Weinstock, 2010).

CONTINUOUS MODERATE EXPOSURE THROUGHOUT GESTATION: EVIDENCE FOR NO SAFE AMOUNT?

In our research we have not only modeled high-dose fetal alcohol exposures at specific neurodevelopmental times (via maternal or neonate injection) but also low-to-moderate chronic exposure by means of voluntary maternal consumption. Results from these studies are critical in our understanding of how alcohol affects the brain in ways that may not be obvious at an epidemiological or clinical level. There is evidence that specific neurodevelopmental times are particularly sensitive to ethanol teratogenesis and that significant neuroapoptosis can be triggered by a transient small increase in blood alcohol concentration (Young and Olney, 2006), leading to subtle but significant phenotypic consequences. Our group has contributed to these data by generating a mouse model of continuous gestational moderate alcohol exposure (Kleiber et al., 2011). The adult offspring exposed to ethanol using this paradigm exhibit subtle but consistent alterations to not only behavior, but transcriptomics and epigenetic patterning (Kleiber et al., 2011, 2012; Laufer et al., 2013). Such results imply that even low-to-moderate alcohol exposure can negatively affect neurodevelopment, leading to increased risk for behavioral and cognitive alterations. Interestingly, the effect of any prenatal alcohol exposure may be detectable by subtle but consistent transcriptomic and epigenetic changes. The results from these low-dose studies argue that neurodevelopment is highly

susceptible to ethanol and that ethanol exposure, even at low doses, may produce long-term effects. Further, reports in mice have also suggested a transgenerational inheritance of fetal alcohol effects in subsequent unexposed generations (Govorko et al., 2012). These findings, if established in human populations, will impose yet another layer of complexity in FASD as a public health issue.

EPIGENETIC MECHANISMS UNDERLYING MOLECULAR ADAPTATION TO NEURODEVELOPMENTAL ETHANOL EXPOSURE

Individuals born with FASD show phenotypes that persist throughout their lifetime. However, not all fetuses exposed to alcohol develop clinical manifestations of the disorder. We hypothesize that this variability may be related to the threshold of neurotranscriptomic changes that induce deficits in neurulation, cellular migration and differentiation, and synaptic development. Also, this may determine phenotypic severity, which ranges from fetal death to subtle or no obvious effects. Indeed, it has been suggested that the variety of molecular and cellular responses to neurodevelopmental ethanol exposure is likely to involve a "potentially bewildering array of heterogeneity" (Haycock, 2009). Further adding to this variability is the known effects of alcohol on epigenetic mechanisms (Shukla et al., 2008).

Studies have established that a fundamental change in the adult transcriptome persists beyond the cessation of alcohol exposure and developmental processes (Chang et al., 2012; Kleiber et al., 2012, 2013), and attention has been turned to the processes that may regulate and maintain these changes. Specifically, the impact of prenatal alcohol exposure on developmental epigenetic processes have generated a number of important recent reviews, dealing with the topic from molecular and clinical perspectives (Haycock, 2009; Resendiz et al., 2013; Ungerer et al., 2013). Alcohol-induced alterations to epigenetic processes may strongly impact normal developmental and adult brain gene expression. These processes can have transient or long-lasting effects, meaning that ethanol-induced disruption of the establishment of epigenetic patterning will also be long-lasting. On-going studies have implicated both DNA methylation, histone modifications, and non-coding regulatory RNAs (ncRNAs) in the effects of neurodevelopmental ethanol exposure.

DNA METHYLATION AND GENOMIC IMPRINTING

Development includes dynamic epigenetic changes, including genome-wide demethylation following oocyte fertilization prior to implantation and *de novo* genome methylation during gastrulation that continues to be established in a cell-specific, tissue-specific, or parent-of-origin manner (Reik et al., 2001). The alteration of DNA methylation patterning, occurring at CpG dinucleotides and within CpG islands to control gene activation, provides a potential target for ethanol to alter gene expression via epigenetic regulatory control, including within developmentally imprinted regions. Ethanol interferes with one-carbon metabolism, the primary methyl donor in the DNA-methyltransferase pathway, and it has been shown that one-carbon metabolism is indeed impaired by ethanol exposure in rodent models (Halsted et al., 2002; Fowler et al., 2012). This is accomplished, in part, by reducing folate availability. Folate is converted in a step-wise process to methionine, which is then converted to the active methyl donor S-adenosylmethionine (SAM). Reductions in SAM impair the ability of DNA methyltransferases (DNMTs) to maintain DNA methylation. Ethanol can also reduce SAM levels by reducing the activity of methionine synthase (Barak et al., 1996).

As early as 1991, Garro et al. (1991) demonstrated that gestational ethanol exposure resulted in genomic hypomethylation and reduced methylase activity. More recently, DNA methylation studies have shown that adult mice prenatally exposed to ethanol show alterations in known methylation-sensitive genes (Kaminen-Ahola et al., 2010) and show broad alterations when examined at the whole-genome scale, including within imprinted regions (Laufer et al., 2013). These results concur with other studies reporting DNA methylation changes in genes that are known to be genomically imprinted following prenatal alcohol exposure (Liu et al., 2009). Imprinted genes are expressed in a parent of origin-specific manner that is based on differential methylation of an imprinting control region (ICR). Imprinting is critical during neurodevelopment, as well as in the normal functioning of the brain (Davies et al., 2008). Further, approximately 30% of parentally-imprinted transcripts are hypothesized to be non-coding RNAs (ncRNA), meaning that ethanol-induced methylation changes can cause long-term changes in gene regulation at both the level of transcription and translation (Morison et al., 2005). Interestingly, many sequences vulnerable to ethanol-induced methylation changes possess CCCTC-binding factor (CTCF) sites, a transcription factor that is sensitive to the methylation status of its binding sequence. CTCF motifs control the parent-of-origin-based expression of many ICRs through the binding of CTCF, an insulator zinc-finger protein. Previous research has found that the CTCF binding sites in *H19/Igf2* ICRs show significantly altered methylation patterns in ethanol-exposed placental tissue (Haycock and Ramsay, 2009). Also, subtle changes to *Igf2* locus DNA methylation and expression following prenatal alcohol exposure have also been reported (Downing et al., 2011).

The *H19/Igf2* region was also identified by Laufer et al. (2013), which reported altered methylation in the adult brain of mice prenatally exposed to moderate chronic alcohol throughout gestation. In this study, analysis of the upstream sequences of 30 genes with altered expression within the adult brain of prenatally-exposed mice indicated that 12 (40%) showed sequences that were strongly predicted to be CTCF binding motifs. Among these were genes associated with canonical PTEN/AKT/mTOR signaling, with 57% of molecules involved in the *Pten* pathway showing significant differential methylation and a gain of methylation observed at a predicted CTCF-binding site within the promoter region of *Akt* (Laufer et al., 2013). This pathway regulates a number of neurodevelopmental processes such as morphogenesis, dendritic development, synapse formation, and synaptic plascticity (Yoshimura et al., 2006). This results are interesting in light of previous gene expression and protein activity studies that have suggested PTEN/AKT signaling as a potential initiation point for the actions of ethanol on the developing brain (Xu et al., 2003; Green et al., 2007). These data suggest

that this site, and consequently imprinted regions of the genome that utilize CTCF as an insulator, may be particularly vulnerable to methylation alterations following neurodevelopmental alcohol exposure. This would argue that ethanol has the ability to alter the expression of numerous genes via altered methylation patterning as well as via altered control of small ncRNAs present within imprinted genomic regions. These epigenetic changes may underlie the longevity of the gene expression changes observed by transcriptomic studies. Ultimately, these data suggest that changes in DNA methylation, particularly within imprinted regions that play critical roles in neurodevelopment and brain function, may have a role in the long-term maintenance of altered gene expression and cognitive endophenotypes associated with FASD.

HISTONE MODIFICATIONS

Studies evaluating the involvement of histone modifications in prenatal alcohol exposure phenotypes are rather preliminary, though the relevance of histone modifications as a molecular consequence of alcohol abuse has been established (Kim and Shukla, 2006; Pal-Bhadra et al., 2007; Shukla et al., 2008). Ethanol exposure during the human third trimester-equivalent has been shown to alter histone acetylation in the developing rat cerebellum (Guo et al., 2011). Also, ethanol-induced hippocampal neurodegeneration induced on PD 7 in mice is achieved in part by the enhanced activity of G9a (lysine dimethyltransferase) and increased levels of histone H3 lysine 9 (H3K9me2) and 27 (H3K27) dimethylation (Subbanna et al., 2013b). Work in NSC has also found that ethanol exposure leads to reductions in H3K27me3 and H3K4me3 at promoters of genes involved in neural precursor cell identity and differentiation (Veazey et al., 2013). Many of these genes also showed corresponding changes in gene expression. Further, HDAC mRNA levels (Kirpich et al., 2012), protein levels (Kirpich et al., 2013), and protein function (Choudhury and Shukla, 2008) have been shown to be affected by ethanol exposure, including within our own studies (Kleiber et al., 2013). Importantly, some results have relevance to FASD-specific behavioral phenotypes. For instance, a recent report by Bekdash et al. (2013) showed that prenatal ethanol exposure resulted in decreased histone activation marks (H3K4me3, Set7/9, acetylated H3K9, phosphorylated H3S10) and increased repressive marks (H3K9me2, G9a, Setdb1) associated with hypothalamic *pro-opiomelanocortin* (*Pomc*) regulation, resulting in decreased *Pomc* expression and a heightened cortisol response. These results suggest an association between prenatal alcohol exposure, histone modifications, and HPA-associated phenotypes relevant to FASD.

MicroRNAs

There is substantial evidence that microRNAs (miRNAs) are heavily involved in mammalian neurodevelopmental processes including cell proliferation, apoptosis, differentiation, synapse formation, and remodeling (Coolen et al., 2013; Nowak and Michlewski, 2013; Hu et al., 2014). In 2007, Sathyan et al. (2007) first explored of the role of regulatory miRNAs in the teratogenic effects of ethanol on the developing brain. This study reported the potential interplay of miR-9, miR-21, miR-153, and miR-335 miRNAs and their mRNA, illustrating the delicate yet sensitive balance between antagonistic biological cues that may ultimately determine cellular apoptosis or survival and adaptation following ethanol insult. Importantly, this study identified that miRNAs serve as an effective intermediary between a teratogen and cellular response as they are able to affect the regulation of numerous genes and developmental pathways in a complex and divergent manner.

We have employed a genome-wide strategy of interrogating miRNA: mRNA transcript relationships. Our results show that ethanol exposure during both trimester two and three-eqivalents results in the alteration of expression of a number of miRNAs (**Table 1**) (Mantha et al., 2014). A number of developmental processes, including cell maturation, are guided by miRNA-based control of transcript regulation. Of note is miR-335, found to be down-regulated in the adult brain following late-gestation ethanol exposure, and shown to be ethanol-sensitive in NSCs and regulates NSC differentiation (Sathyan et al., 2007; Mantha et al., 2014). Further, we also identified miR-10b to be down-regulated in the adult brain, which has been previously identified as ethanol-responsive (Wang et al., 2009). This miRNA is a regulator of the Hox gene family, which plays a key role in neuronal migration (Geisen et al., 2008). Similarly, the miR-302 family of miRNAs involved in cell cycle progression and the maintenance of embryonic stem-cell pluripotency, potentially through its interactions with WNT signaling (Groenendyk and Michalak, 2014).

Analysis of alcohol-induced miRNA expression changes following third trimester-equivalent exposure yielded a slightly larger list of altered miRNAs (**Table 1**). Five of these showed an inverse relationship to a number of putative gene targets, involved in a number of neurodevelopmental process including corticotrophin and retinoic acid signaling, both critical to HPA axis development and function, as well as PI3/AKT/mTOR signaling (Kleiber et al., 2013; Laufer et al., 2013). Specifically, PI3K/AKT/mTOR signaling may be altered by the up-regulation of miR-721 and the down-regulation of its target, the tumor suppressor protein *Tsc1*, which has been associated with impairments in the migration and developmental positioning of pyramidal neurons in the hippocampus leading to cognitive function, learning, and memory deficits (Orlova and Crino, 2010; Mejia et al., 2013). Other studies have implicated specific miRNAs depending on cell type or ethanol treatment paradigm and our results have replicated some of these same molecules, including miR-335 [identified by Sathyan et al. (2007)] and miR-10b [identified by Mantha et al. (2014) and Wang et al. (2009)]. These results provide insight into how ethanol may alter the expression of numerous genes through the altered regulation of a select group of miRNAs. Further, a given biological pathway or process may be affected from multiple vantages simultaneously via the alteration of a few miRNA species, acting in an antagonistic and/or synergistic manner. We are truly only beginning to understand the regulatory control of miRNAs within the brain, but these results support the role of miRNAs in the neurodevelopmental alterations that follow prenatal alcohol exposure.

Table 1 | miRNAs and predicted mRNA targets with inversely correlated alterations following neurodevelopmental ethanol exposure.

Treatment days	miRNA	Expression change[#]	Predicted mRNA target(s)	Expression change[#]
GD 8/11[†]	miR-1192	↑	Atf1, Gng4, Map3k1, Rpe, Setd2, Stxbp6, Zc3h6	↓
	miR-532-5p	↑	Atf1, Itpripl2, Stxbp6	↓
GD 14/16*	miR-10b	↓	Aak1	↑
	miR-184	↓	Myl9	↑
	miR-302c	↑	Ccdc6, Mfap3, Ptpro, Rnd3, Rpl36a/r, Sema3c, Stoml3, Supt3h	↓
	miR-342-5p	↓	Aak1, Cables2, Rhog	↑
	miR-343	↑	Asic4, Dcn, Gpr116, Ptpro, Stoml3	↓
	miR-449b	↓	Ina	↑
PD 4/7[†]	miR-26b	↑	Adam9, Chsy1, Cnr1, Exoc8, Hs6st1, Lingo1, Map3k7, Mras, Pfkfb3, Ppm1b, Rhou, Sema6d, Shank2, Tab3, Tdrd7, Ube2j1	↓
	miR-34b-5p	↓	Kitl	↑
	miR-184	↑	Ncor2, Prkcb	↓
	miR-721	↑	Akap11, B4galt, Cnr1, Efnb2, Fam20b, Ino80, Irf1, Lrrk2, Ncoa3, Pfkfb3, Ppargc1a, Rbm9, Shank2, Spen, Sphk2, Tsc1, Wdfy3	↓
	miR-1970	↓	Arhgap6	↑

[#] *Significance for expression change was 1.2-fold, $p < 0.05$.*

**detailed data published in Mantha et al. (2013).*

[†]data unpublished.

THE INTERPLAY OF EPIGENETIC FACTORS IN FASD-ASSOCIATED GENOMIC DYSREGULATION

Although these data point to a substantial role of epigenetic modifications in FASD etiology, research has not attempted to understand these changes in the larger context of the complete epigenetic landscape. Such an approach is important since epigenetic modifications do not operate in isolation; often, modification cross-talk is vital for function. Further, DNA methylation, histone modification, and ncRNA can co-regulate each other in complex regulatory networks (Sato et al., 2011). In order to address this, we characterized changes in four epigenetic processes and gene expression in the hippocampus of mice exposed to alcohol during the third trimester-equivalent. This analysis was performed in three stages: examination of DNA methylation at known promoters using methylation DNA immunoprecipitation followed by hybridization to genome arrays (MeDIP-chip); histone methylation analysis at H3K4me3 and H3K27me3 sites, which are, respectively, positively, and negatively correlated with gene expression (Barski et al., 2007); and miRNA and mRNA transcript profiling using expression arrays. Since the hippocampus is important for learning and memory, and its structure is affected by neonatal ethanol exposure, it is possible that ethanol may induce molecular changes in the hippocampus that are relevant to the learning and memory deficits observed in animal models and humans affected with FASD. Our MeDIP-chip results identified over 10,000 regions were differentially methylated (MEDME AMS algorithm, $p < 0.05$), with approximately 100–200 regions differentially enriched for H3K4me3 and H3K27me3. The results shown in **Figure 1** show the genomic position of these changes in association to 40 miRNA and 60 mRNA transcripts shown to be differentially expressed following ethanol exposure in the hippocampus. These data, while admittedly preliminary and needing further examination, suggest that widespread epigenetic changes occur across the genome following neurodevelopmental ethanol exposure, and that the molecular factors that underlie the changes to neural gene expression and function are multifaceted and complex. They suggest that epigenomic dysregulation represents an integral aspect of prenatal alcohol exposure response that contributes to the development of FASD.

THE ROLE OF POSTNATAL ENVIRONMENT IN PHENOTYPIC OUTCOMES ASSOCIATED WITH FASD

Children with PAE are often raised in suboptimal conditions, but the effect of this stress has not been adequately explored. Mammalian offspring are fully dependent on maternal care during the early postnatal period. In this way, the quality and quantity of maternal interaction or caregiving poses a strong environmental influence of stress-related gene expression (Korosi and Baram, 2009). Protective factors against FASD in humans include a stable home environment, infrequent changes in living arrangement and non-exposure to violence (Streissguth et al., 1994). Maternal separation models are often used to model chronic early life stress, whereby 3 h of separation per day from postnatal days 2–14 can result in anxiety-like behaviors in adult mice that affect epigenetic patterning (Franklin et al., 2010). Following maternal separation as a stressor, mice display increased anxiety-like behaviors on open-field testing (Romeo et al., 2003) similar to those observed in PAE models. We have determined that prenatal alcohol exposure, particularly during the third trimester equivalent, alters a number of genes and pathways associated with HPA axis

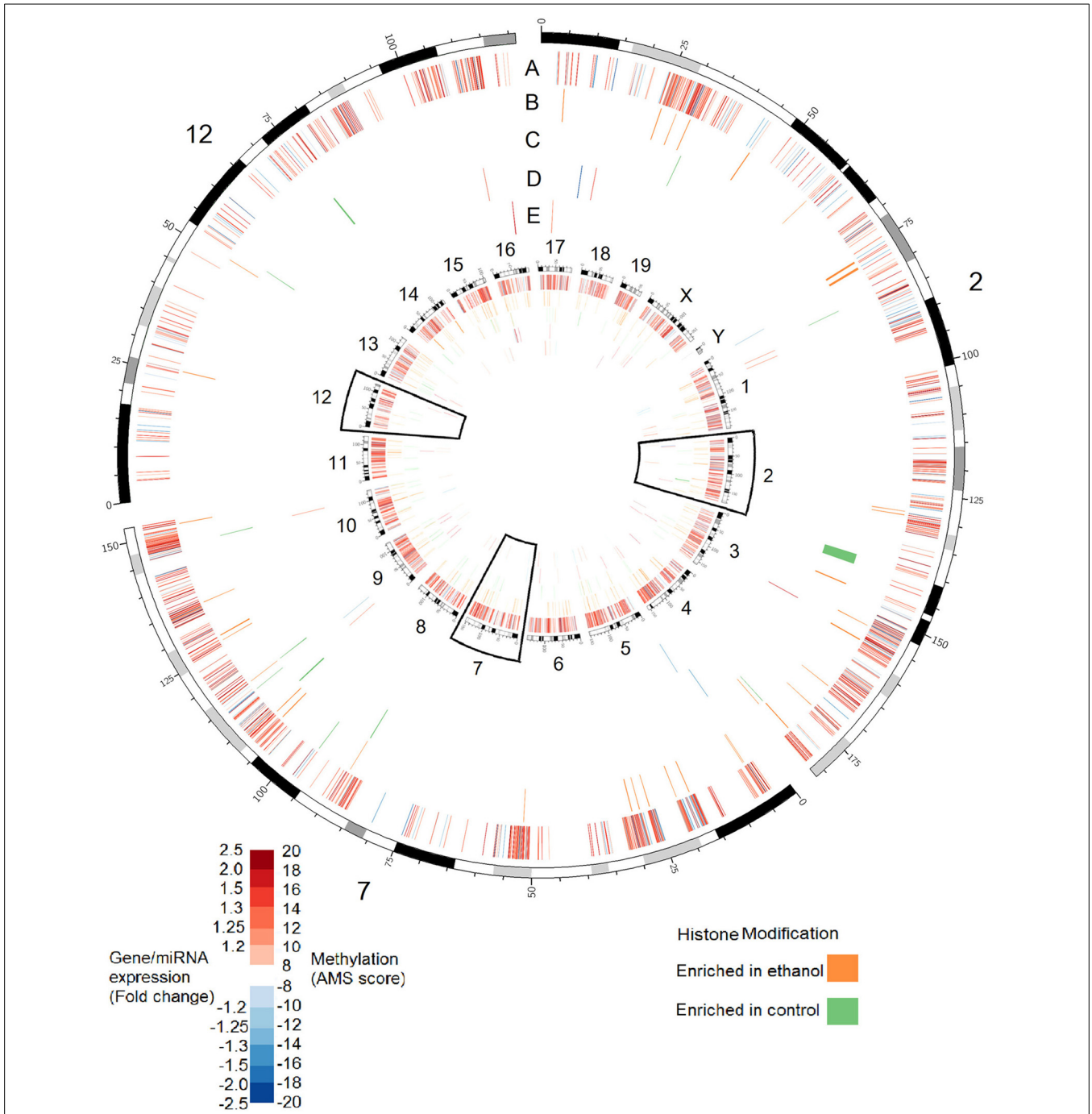

FIGURE 1 | Alterations in DNA methylation, histone modifications, miRNA expression, and gene expression in the hippocampus of adult (PD 60) mice exposed to ethanol during the trimester three equivalent (PD 7). Tracks show alterations in: (A) DNA methylation as measured by absolute methylation score (AMS); (B) Histone H3 lysine 27 trimethylation; (C) Histone H3 lysine 4 trimethylation; (D) miRNA expression; (E) Gene expression. Inner circle shows changes for all chromosomes. Outer circle shows an expanded view of chromosomes 2, 7, and 12, which contain major imprinting centers. DNA methylation significance was determined by the MEDME algorithm using an AMS *p*-value cutoff $p < 0.05$; miRNA and mRNA cutoff: $p < 0.05$, fold-change > 1.2.

development, and that these pathways are among the few that remain altered significantly into adulthood (Kleiber et al., 2013). These include altered regulation of *pro-piomelanocortin* (*Pomc*), *Nr4a1*, and genes associated with thyroid hormone/retinoid X receptor function. Alterations to these pathways have been associated with HPA axis reactivity and are associated with increased risk for depression, anxiety, and poor coping skills related to exposure to later-life stressors, which have been consistently

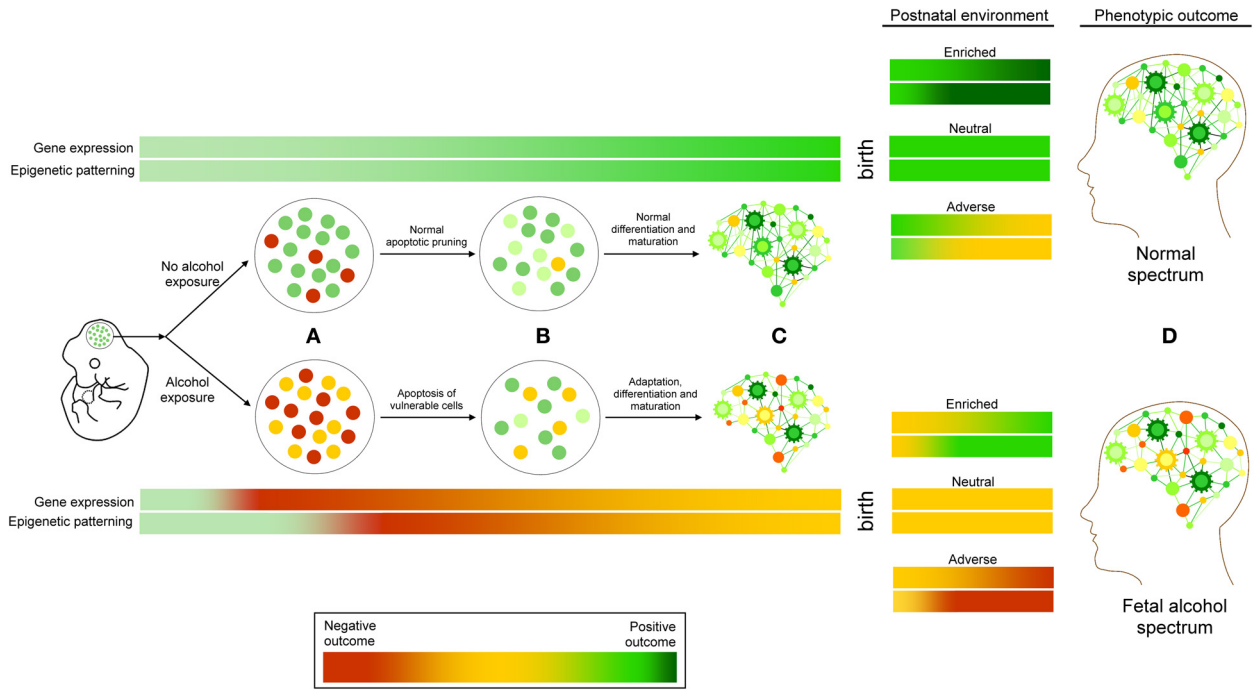

FIGURE 2 | A working model of FASD as a continuum of genetic and epigenetic events. (A) Ethanol exposure results in cellular stress response, leading to the apoptosis of vulnerable cell types. **(B)** These changes are followed by molecular adaptations in surviving cells that include changes to epigenetic programming, including DNA methylation, histone modifications, and ncRNA regulation, which are **(C)** Subsequently inherited and maintained through cellular differentiation and maturation. These changes may be exacerbated or ameliorated by postnatal environmental conditions. Adverse (red) or positive (green) outcomes may depend on the interaction of these factors, contributing to the etiology of fetal alcohol spectrum disorders **(D)**.

associated with prenatal alcohol exposure (Hellemans et al., 2008; Weinberg et al., 2008). Indeed, these studies have shown that PAE leads to later-life vulnerability to stress that is associated with changes to HPA axis function, with both hormonal and behavioral consequences.

The outcome of prenatal ethanol exposure appears to depend significantly on neonatal environment. A high-stress, unpredictable environment may increase the severity of manifestation of FASD-related phenotypes, while a stable, enriched environment may ameliorate them. Rehabilitative therapies in children with FASD currently aim to develop verbal, math and social skills concurrent with counseling sessions and specialized classes (Peadon et al., 2009; Kodituwakku, 2010). These effects may also be assessed in mouse models of FASD and typically include exposure to to physically and cognitively challenging or "enriched" postnatal environments. Rodents exposed to alcohol during neurodevelopment but postnatally reared in enriched environments show less susceptibility to novelty-induced stress and improved memory performance (Hannigan et al., 2007). Given how fetal alcohol exposure affects neurodevelopment, it is possible that the functional effectiveness of these enriched environments result from a targeted activation of specific molecular mechanisms that modify neural structure and function and are ultimately expressed as "rehabilitated" behaviors. Our lab has assessed the behavioral recovery of mice exposed to alcohol during neurodevelopment that are raised postnatally in either an enriched or neutral (standard) environment. The results suggest that at least some aspects of these FASD-specific alterations may be ammeliorated by engaging affected pups cognitively within an enriched environment, including decreased anxiety-related traits in the elevated plus maze assay and improved memory of novel and familiar objects. It will be valuable to assess if these phenotypic corrections have genetic and epigenetic correlates.

DEVELOPING A WORKING MODEL FOR FASD

Neurodevelopmental ethanol exposure results in a complex array of genetic and epigenetic changes in the brain. Currently, the results from studies examining these factors are varied, but consistent themes are emerging. They allow for the proposal of FASD as a continuum of molecular events (**Figure 2**). At the cellular level, it begins with neurotoxicity and ends with the selection and adaptation of those cells that comprise the adult neural population, resulting in life-long behavioral and cognitive changes. Ethanol exposure represents an interruption in normal neurodevelopmental processes. The surviving cells must adapt and acquire developmental trajectories that involve molecular adaptations that are detectable by genome-wide changes in gene expression and epigenetic patterning. These epigenetic alterations likely involve the interaction of DNA methylation, particularly in imprinted genomic regions, histone modification, and ncRNA regulation. These epigenetic changes are expected to be stably inherited following subsequent neurogenesis, differentiation, and maturation, and represent an enduring molecular "footprint" of neurodevelopmental ethanol exposure. This reprogramming

of neurogenomic patterning may be further compounded by subsequent ontogenetic factors such as postnatal environment, further exacerbating or ameliorating epigenetic signatures. These signatures may—if shared by peripheral tissue sources—offer a source of early diagnosis of prenatal alcohol exposure. Also, if these changes may be mitigated by postnatal environmental interventions is an intriguing avenue for further investigation. Given the unlikelihood of total abstinence from alcohol consumption during pregnancy, evidence that the effects of prenatal alcohol exposure may be amended or reversed, both at the phenotypic and molecular level, would represent a significant step toward in improving the prognosis of individuals with FASD.

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REFERENCES

Allan, A. M., Chynoweth, J., Tyler, L. A., and Caldwell, K. K. (2003). A mouse model of prenatal ethanol exposure using a voluntary drinking paradigm. *Alcohol. Clin. Exp. Res.* 27, 2009–2016. doi: 10.1097/01.ALC.0000100940.95053.72

Barak, A. J., Beckenhauer, H. C., and Tuma, D. J. (1996). Betaine effects on hepatic methionine metabolism elicited by short-term ethanol feeding. *Alcohol* 13, 483–486. doi: 10.1016/0741-8329(96)00040-7

Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., et al. (2007). High-resolution profiling of histone methylations in the human genome. *Cell* 129, 823–837. doi: 10.1016/j.cell.2007.05.009

Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2013). Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and proopiomelanocortin (POMC) gene expression in beta-endorphin-producing POMC neurons of the hypothalamus. *Alcohol. Clin. Exp. Res.* 37, 1133–1142. doi: 10.1111/acer.12082

Boehm, S. L. 2nd., Moore, E. M., Walsh, C. D., Gross, C. D., Cavelli, A. M., Gigante, E., et al. (2008). Using drinking in the dark to model prenatal binge-like exposure to ethanol in C57BL/6J mice. *Dev. Psychobiol.* 50, 566–578. doi: 10.1002/dev.20320

Bystron, I., Blakemore, C., and Rakic, P. (2008). Development of the human cerebral cortex: boulder committee revisited. *Nat. Rev. Neurosci.* 9, 110–122. doi: 10.1038/nrn2252

Camarillo, C., and Miranda, R. C. (2008). Ethanol exposure during neurogenesis induces persistent effects on neural maturation: evidence from an *ex vivo* model of fetal cerebral cortical neuroepithelial progenitor maturation. *Gene Expr.* 14, 159–171.

Chang, G. Q., Karatayev, O., Liang, S. C., Barson, J. R., and Leibowitz, S. F. (2012). Prenatal ethanol exposure stimulates neurogenesis in hypothalamic and limbic peptide systems: possible mechanism for offspring ethanol overconsumption. *Neuroscience* 222, 417–428. doi: 10.1016/j.neuroscience.2012.05.066

Chen, C. P., Kuhn, P., Advis, J. P., and Sarkar, D. K. (2006). Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. *J. Neurochem.* 97, 1026–1033. doi: 10.1111/j.1471-4159.2006.03839.x

Choudhury, M., and Shukla, S. D. (2008). Surrogate alcohols and their metabolites modify histone H3 acetylation: involvement of histone acetyl transferase and histone deacetylase. *Alcohol. Clin. Exp. Res.* 32, 829–839. doi: 10.1111/j.1530-0277.2008.00630.x

Chudley, A. E., Conry, J., Cook, J. L., Loock, C., Rosales, T., and LeBlanc, N. (2005). Fetal alcohol spectrum disorder: canadian guidelines for diagnosis. *CMAJ* 172, S1–S21. doi: 10.1503/cmaj.1040302

Coolen, M., Katz, S., and Bally-Cuif, L. (2013). miR-9: a versatile regulator of neurogenesis. *Front. Cell Neurosci.* 7:220. doi: 10.3389/fncel.2013.00220

Davies, W., Isles, A. R., Humby, T., and Wilkinson, L. S. (2008). What are imprinted genes doing in the brain? *Adv. Exp. Med. Biol.* 626, 62–70. doi: 10.1007/978-0-387-77576-0_5

Dikranian, K., Qin, Y. Q., Labruyere, J., Nemmers, B., and Olney, J. W. (2005). Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Brain Res. Dev. Brain Res.* 155, 1–13. doi: 10.1016/j.devbrainres.2004.11.005

Dobbing, J., and Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Hum. Dev.* 3, 79–83. doi: 10.1016/0378-3782(79)90022-7

Downing, C., Flink, S., Florez-McClure, M. L., Johnson, T. E., Tabakoff, B., and Kechris, K. J. (2012). Gene expression changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 36, 1519–1529. doi: 10.1111/j.1530-0277.2012.01757.x

Downing, C., Johnson, T. E., Larson, C., Leakey, T. I., Siegfried, R. N., Rafferty, T. M., et al. (2011). Subtle decreases in DNA methylation and gene expression at the mouse Igf2 locus following prenatal alcohol exposure: effects of a methyl-supplemented diet. *Alcohol* 45, 65–71. doi: 10.1016/j.alcohol.2010.07.006

Earnest, D. J., Chen, W. J., and West, J. R. (2001). Developmental alcohol and circadian clock function. *Alcohol. Res. Health* 25, 136–140.

Eisenstat, D. D., Liu, J. K., Mione, M., Zhong, W., Yu, G., Anderson, S. A., et al. (1999). DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. *J. Comp. Neurol.* 414, 217–237. doi: 10.1002/(SICI)1096-9861(19991115)414:2%3C217::AID-CNE6%3E3.0.CO;2-I

Ellis, B. J., Essex, M. J., and Boyce, W. T. (2005). Biological sensitivity to context: II. Empirical explorations of an evolutionary-developmental theory. *Dev. Psychopathol.* 17, 303–328. doi: 10.1017/S0954579405050157

Fast, D. K., and Conry, J. (2004). The challenge of fetal alcohol syndrome in the criminal legal system. *Addict. Biol.* 9, 161–166. discussion: 167–168. doi: 10.1080/13556210410001717042

Fowler, A. K., Hewetson, A., Agrawal, R. G., Dagda, M., Dagda, R., Moaddel, R., et al. (2012). Alcohol-induced one-carbon metabolism impairment promotes dysfunction of DNA base excision repair in adult brain. *J. Biol. Chem.* 287, 43533–43542. doi: 10.1074/jbc.M112.401497

Franklin, T. B., Russig, H., Weiss, I. C., Graff, J., Linder, N., Michalon, A., et al. (2010). Epigenetic transmission of the impact of early stress across generations. *Biol. Psychiatry* 68, 408–415. doi: 10.1016/j.biopsych.2010.05.036

Fryer, S. L., Schweinsburg, B. C., Bjorkquist, O. A., Frank, L. R., Mattson, S. N., Spadoni, A. D., et al. (2009). Characterization of white matter microstructure in fetal alcohol spectrum disorders. *Alcohol. Clin. Exp. Res.* 33, 514–521. doi: 10.1111/j.1530-0277.2008.00864.x

Garro, A. J., McBeth, D. L., Lima, V., and Lieber, C. S. (1991). Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol. Clin. Exp. Res.* 15, 395–398. doi: 10.1111/j.1530-0277.1991.tb00536.x

Geisen, M. J., Di Meglio, T., Pasqualetti, M., Ducret, S., Brunet, J. F., Chedotal, A., et al. (2008). Hox paralog group 2 genes control the migration of mouse pontine neurons through slit-robo signaling. *PLoS Biol.* 6:e142. doi: 10.1371/journal.pbio.0060142

Ghanem, N., Andrusiak, M. G., Svoboda, D., Al Lafi, S. M., Julian, L. M., McClellan, K. A., et al. (2012). The Rb/E2F pathway modulates neurogenesis through direct regulation of the Dlx1/Dlx2 bigene cluster. *J. Neurosci.* 32, 8219–8230. doi: 10.1523/JNEUROSCI.1344-12.2012

Gilliam, D. M., Kotch, L. E., Dudek, B. C., and Riley, E. P. (1989). Ethanol teratogenesis in selectivity bred long-sleep and short-sleep mice: a comparison to inbred C57BL/6J mice. *Alcohol. Clin. Exp. Res.* 13, 667–672. doi: 10.1111/j.1530-0277.1989.tb00402.x

Girotti, M., Weinberg, M. S., and Spencer, R. L. (2007). Differential responses of hypothalamus-pituitary-adrenal axis immediate early genes to corticosterone and circadian drive. *Endocrinology* 148, 2542–2552. doi: 10.1210/en.2006-1304

Govorko, D., Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2012). Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. *Biol. Psychiatry* 72, 378–388. doi: 10.1016/j.biopsych.2012.04.006

Green, M. L., Singh, A. V., Zhang, Y., Nemeth, K. A., Sulik, K. K., and Knudsen, T. B. (2007). Reprogramming of genetic networks during initiation of the Fetal Alcohol Syndrome. *Dev. Dynam.* 236, 613–631. doi: 10.1002/dvdy.21048

Groenendyk, J., and Michalak, M. (2014). Disrupted WNT signaling in mouse embryonic stem cells in the absence of calreticulin. *Stem Cell Rev*. 10, 191–206. doi: 10.1007/s12015-013-9488-6

Guo, W., Crossey, E. L., Zhang, L., Zucca, S., George, O. L., Valenzuela, C. F., et al. (2011). Alcohol exposure decreases CREB binding protein expression and histone acetylation in the developing cerebellum. *PLoS ONE* 6:e19351. doi: 10.1371/journal.pone.0019351

Halsted, C. H., Villanueva, J. A., Devlin, A. M., Niemela, O., Parkkila, S., Garrow, T. A., et al. (2002). Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10072–10077. doi: 10.1073/pnas.112336399

Hannigan, J. H., O'Leary-Moore, S. K., and Berman, R. F. (2007). Postnatal environmental or experiential amelioration of neurobehavioral effects of perinatal alcohol exposure in rats. *Neurosci. Biobehav. Rev.* 31, 202–211. doi: 10.1016/j.neubiorev.2006.06.019

Hard, M. L., Abdolell, M., Robinson, B. H., and Koren, G. (2005). Gene-expression analysis after alcohol exposure in the developing mouse. *J. Lab. Clin. Med.* 145, 47–54. doi: 10.1016/j.lab.2004.11.011

Harlap, S., and Shiono, P. H. (1980). Alcohol, smoking, and incidence of spontaneous abortions in the first and second trimester. *Lancet* 2, 173–176. doi: 10.1016/S0140-6736(80)90061-6

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod.* 81, 607–617. doi: 10.1095/biolreprod.108.074690

Haycock, P. C., and Ramsay, M. (2009). Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol. Reprod.* 81, 618–627. doi: 10.1095/biolreprod.108.074682

Hellemans, K. G., Verma, P., Yoon, E., Yu, W., and Weinberg, J. (2008). Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. *Ann. N.Y. Acad. Sci.* 1144, 154–175. doi: 10.1196/annals.1418.016

Hu, Z., Yu, D., Gu, Q. H., Yang, Y., Tu, K., Zhu, J., et al. (2014). miR-191 and miR-135 are required for long-lasting spine remodelling associated with synaptic long-term depression. *Nat. Commun.* 5, 3263. doi: 10.1038/ncomms4263

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, D. F., Koch, C., Genz, K., et al. (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* 287, 1056–1060. doi: 10.1126/science.287.5455.1056

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K. A., Fahey, P., Cox, T. C., et al. (2010). Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet.* 6:e1000811. doi: 10.1371/journal.pgen.1000811

Kim, J. S., and Shukla, S. D. (2006). Acute *in vivo* effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol Alcohol.* 41, 126–132. doi: 10.1093/alcalc/agh248

Kirpich, I., Ghare, S., Zhang, J., Gobejishvili, L., Kharebava, G., Barve, S. J., et al. (2012). Binge alcohol-induced microvesicular liver steatosis and injury are associated with down-regulation of hepatic Hdac 1, 7, 9, 10, 11 and up-regulation of Hdac 3. *Alcohol. Clin. Exp. Res.* 36, 1578–1586. doi: 10.1111/j.1530-0277.2012.01751.x

Kirpich, I., Zhang, J., Gobejishvili, L., Kharebava, G., Barker, D., Ghare, S., et al. (2013). Binge ethanol-induced HDAC3 down-regulates Cpt1alpha expression leading to hepatic steatosis and injury. *Alcohol. Clin. Exp. Res.* 37, 1920–1929. doi: 10.1111/acer.12172

Kleiber, M. L., Laufer, B. I., Wright, E., Diehl, E. J., and Singh, S. M. (2012). Long-term alterations to the brain transcriptome in a maternal voluntary consumption model of fetal alcohol spectrum disorders. *Brain Res.* 1458, 18–33. doi: 10.1016/j.brainres.2012.04.016

Kleiber, M. L., Mantha, K., Stringer, R. L., and Singh, S. M. (2013). Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure. *J. Neurodev. Disord.* 5:6. doi: 10.1186/1866-1955-5-6

Kleiber, M. L., Wright, E., and Singh, S. M. (2011). Maternal voluntary drinking in C57BL/6J mice: advancing a model for fetal alcohol spectrum disorders. *Behav. Brain Res.* 223, 376–387. doi: 10.1016/j.bbr.2011.05.005

Kodituwakku, P. W. (2010). A neurodevelopmental framework for the development of interventions for children with fetal alcohol spectrum disorders. *Alcohol* 44, 717–728. doi: 10.1016/j.alcohol.2009.10.009

Korosi, A., and Baram, T. Z. (2009). The pathways from mother's love to baby's future. *Front. Behav. Neurosci.* 3:27. doi: 10.3389/neuro.08.027.2009

Laufer, B. I., Mantha, K., Kleiber, M. L., Diehl, E. J., Addison, S. M., and Singh, S. M. (2013). Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis. Model. Mech.* 6, 977–992. doi: 10.1242/dmm.010975

Lebel, C., Rasmussen, C., Wyper, K., Walker, L., Andrew, G., Yager, J., et al. (2008). Brain diffusion abnormalities in children with fetal alcohol spectrum disorder. *Alcohol. Clin. Exp. Res.* 32, 1732–1740. doi: 10.1111/j.1530-0277.2008.00750.x

Light, K. E., Belcher, S. M., and Pierce, D. R. (2002). Time course and manner of Purkinje neuron death following a single ethanol exposure on postnatal day 4 in the developing rat. *Neuroscience* 114, 327–337. doi: 10.1016/S0306-4522(02)00344-5

Liu, Y., Balaraman, Y., Wang, G., Nephew, K. P., and Zhou, F. C. (2009). Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4, 500–511. doi: 10.4161/epi.4.7.9925

Mameli, M., Zamudio, P. A., Carta, M., and Valenzuela, C. F. (2005). Developmentally regulated actions of alcohol on hippocampal glutamatergic transmission. *J. Neurosci.* 25, 8027–8036. doi: 10.1523/JNEUROSCI.2434-05.2005

Mantha, K., Kleiber, M. L., and Singh, S. M. (2013). Neurodevelopmental timing of ethanol exposure may contribute to observed heterogeneity of behavioural deficits in a mouse model of Fetal Alcohol Spectrum Disorder (FASD). *J. Behav. Brain Sci.* 3, 85–99. doi: 10.4236/jbbs.2013.31009

Mantha, K., Laufer, B. I., and Singh, S. M. (2014). Molecular changes during neurodevelopment following second-trimester binge ethanol exposure in a mouse model of fetal alcohol spectrum disorders: from immediate effects to long-term adaptation. *Dev. Neurosci.* 36, 29–43. doi: 10.1159/000357496

Mejia, L. A., Litterman, N., Ikeuchi, Y., de la Torre-Ubieta, L., Bennett, E. J., Zhang, C., et al. (2013). A Novel Hap1-Tsc1 interaction regulates neuronal mTORC1 signaling and morphogenesis in the brain. *J. Neurosci.* 33, 18015–18021. doi: 10.1523/JNEUROSCI.2290-13.2013

Miller, M. W., and Nowakowski, R. S. (1991). Effect of prenatal exposure to ethanol on the cell cycle kinetics and growth fraction in the proliferative zones of fetal rat cerebral cortex. *Alcohol. Clin. Exp. Res.* 15, 229–232. doi: 10.1111/j.1530-0277.1991.tb01861.x

Morison, I. M., Ramsay, J. P., and Spencer, H. G. (2005). A census of mammalian imprinting. *Trends Genet.* 21, 457–465. doi: 10.1016/j.tig.2005.06.008

Noctor, S. C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7, 136–144. doi: 10.1038/nn1172

Norman, A. L., Crocker, N., Mattson, S. N., and Riley, E. P. (2009). Neuroimaging and fetal alcohol spectrum disorders. *Dev. Disabil. Res. Rev.* 15, 209–217. doi: 10.1002/ddrr.72

Nowak, J. S., and Michlewski, G. (2013). miRNAs in development and pathogenesis of the nervous system. *Biochem. Soc. Trans.* 41, 815–820. doi: 10.1042/BST20130044

Nowoslawski, L., Klocke, B. J., and Roth, K. A. (2005). Molecular regulation of acute ethanol-induced neuron apoptosis. *J. Neuropathol. Exp. Neurol.* 64, 490–497.

Olney, J. W., Ishimaru, M. J., Bittigau, P., and Ikonomidou, C. (2000). Ethanol-induced apoptotic neurodegeneration in the developing brain. *Apoptosis* 5, 515–521. doi: 10.1023/A:1009685428847

Olney, J. W., Tenkova, T., Dikranian, K., Qin, Y. Q., Labruyere, J., and Ikonomidou, C. (2002). Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Brain Res. Dev. Brain Res.* 133, 115–126. doi: 10.1016/S0165-3806(02)00279-1

Orlova, K. A., and Crino, P. B. (2010). The tuberous sclerosis complex. *Ann. N.Y. Acad. Sci.* 1184, 87–105. doi: 10.1111/j.1749-6632.2009.05117.x

Pal-Bhadra, M., Bhadra, U., Jackson, D. E., Mamatha, L., Park, P. H., and Shukla, S. D. (2007). Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. *Life Sci.* 81, 979–987. doi: 10.1016/j.lfs.2007.07.030

Peadon, E., Rhys-Jones, B., Bower, C., and Elliott, E. J. (2009). Systematic review of interventions for children with Fetal Alcohol Spectrum Disorders. *BMC Pediatr.* 9:35. doi: 10.1186/1471-2431-9-35

Prock, T. L., and Miranda, R. C. (2007). Embryonic cerebral cortical progenitors are resistant to apoptosis, but increase expression of suicide receptor DISC-complex genes and suppress autophagy following ethanol exposure. *Alcohol. Clin. Exp. Res.* 31, 694–703. doi: 10.1111/j.1530-0277.2007.00354.x

Puglia, M. P., and Valenzuela, C. F. (2010). Ethanol acutely inhibits ionotropic glutamate receptor-mediated responses and long-term potentiation in the

developing CA1 hippocampus. *Alcohol. Clin. Exp. Res.* 34, 594–606. doi: 10.1111/j.1530-0277.2009.01128.x

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. *Science* 293, 1089–1093. doi: 10.1126/science.1063443

Resendiz, M., Chen, Y., Ozturk, N. C., and Zhou, F. C. (2013). Epigenetic medicine and fetal alcohol spectrum disorders. *Epigenomics* 5, 73–86. doi: 10.2217/epi.12.80

Romeo, R. D., Mueller, A., Sisti, H. M., Ogawa, S., McEwen, B. S., and Brake, W. G. (2003). Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. *Horm. Behav.* 43, 561–567. doi: 10.1016/S0018-506X(03)00063-1

Sakata-Haga, H., Dominguez, H. D., Sei, H., Fukui, Y., Riley, E. P., and Thomas, J. D. (2006). Alterations in circadian rhythm phase shifting ability in rats following ethanol exposure during the third trimester brain growth spurt. *Alcohol. Clin. Exp. Res.* 30, 899–907. doi: 10.1111/j.1530-0277.2006.00105.x

Sampson, P. D., Streissguth, A. P., Bookstein, F. L., Little, R. E., Clarren, S. K., Dehaene, P., et al. (1997). Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology* 56, 317–326. doi: 10.1002/(SICI)1096-9926(199711)56:5<317::AID-TERA5>3.0.CO;2-U

Sathyan, P., Golden, H. B., and Miranda, R. C. (2007). Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an *ex vivo* model of the fetal cerebral cortical neuroepithelium. *J. Neurosci.* 27, 8546–8557. doi: 10.1523/JNEUROSCI.1269-07.2007

Sato, F., Tsuchiya, S., Meltzer, S. J., and Shimizu, K. (2011). MicroRNAs and epigenetics. *FEBS J.* 278, 1598–1609. doi: 10.1111/j.1742-4658.2011.08089.x

Shonkoff, J. P., Boyce, W. T., and McEwen, B. S. (2009). Neuroscience, molecular biology, and the childhood roots of health disparities: building a new framework for health promotion and disease prevention. *JAMA* 301, 2252–2259. doi: 10.1001/jama.2009.754

Shukla, S. D., Velazquez, J., French, S. W., Lu, S. C., Ticku, M. K., and Zakhari, S. (2008). Emerging role of epigenetics in the actions of alcohol. *Alcohol. Clin. Exp. Res.* 32, 1525–1534. doi: 10.1111/j.1530-0277.2008.00729.x

Sowell, E. R., Johnson, A., Kan, E., Lu, L. H., van Horn, J. D., Toga, A. W., et al. (2008a). Mapping white matter integrity and neurobehavioral correlates in children with fetal alcohol spectrum disorders. *J. Neurosci.* 28, 1313–1319. doi: 10.1523/JNEUROSCI.5067-07.2008

Sowell, E. R., Mattson, S. N., Kan, E., Thompson, P. M., Riley, E. P., and Toga, A. W. (2008b). Abnormal cortical thickness and brain-behavior correlation patterns in individuals with heavy prenatal alcohol exposure. *Cereb. Cortex* 18, 136–144. doi: 10.1093/cercor/bhm039

Stade, B., Ali, A., Bennett, D., Campbell, D., Johnston, M., Lens, C., et al. (2009). The burden of prenatal exposure to alcohol: revised measurement of cost. *Can. J. Clin. Pharmacol.* 16, e91–e102.

Streissguth, A. P., Barr, H. M., Sampson, P. D., and Bookstein, F. L. (1994). Prenatal alcohol and offspring development: the first fourteen years. *Drug Alcohol Depend.* 36, 89–99. doi: 10.1016/0376-8716(94)90090-6

Streissguth, A. P., and O'Malley, K. (2000). Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders. *Semin. Clin. Neuropsychiatry* 5, 177–190. doi: 10.1053/scnp.2000.6729

Subbanna, S., Shivakumar, M., Psychoyos, D., Xie, S., and Basavarajappa, B. S. (2013a). Anandamide-CB1 receptor signaling contributes to postnatal ethanol-induced neonatal neurodegeneration, adult synaptic, and memory deficits. *J. Neurosci.* 33, 6350–6366. doi: 10.1523/JNEUROSCI.3786-12.2013

Subbanna, S., Shivakumar, M., Umapathy, N. S., Saito, M., Mohan, P. S., Kumar, A., et al. (2013b). G9a-mediated histone methylation regulates ethanol-induced neurodegeneration in the neonatal mouse brain. *Neurobiol. Dis.* 54, 475–485. doi: 10.1016/j.nbd.2013.01.022

Sulik, K. K. (2005). Genesis of alcohol-induced craniofacial dysmorphism. *Exp. Biol. Med.* 230, 366–375.

Toso, L., Poggi, S. H., Roberson, R., Woodard, J., Park, J., Abebe, D., et al. (2006). Prevention of alcohol-induced learning deficits in fetal alcohol syndrome mediated through NMDA and GABA receptors. *Am. J. Obstet. Gynecol.* 194, 681–686. doi: 10.1016/j.ajog.2006.01.003

Ungerer, M., Knezovich, J., and Ramsay, M. (2013). *In utero* alcohol exposure, epigenetic changes, and their consequences. *Alcohol Res* 35, 37–46.

Veazey, K. J., Carnahan, M. N., Muller, D., Miranda, R. C., and Golding, M. C. (2013). Alcohol-induced epigenetic alterations to developmentally crucial genes regulating neural stemness and differentiation. *Alcohol. Clin. Exp. Res.* 37, 1111–1122. doi: 10.1111/acer.12080

Wang, L. L., Zhang, Z., Li, Q., Yang, R., Pei, X., Xu, Y., et al. (2009). Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Hum. Reprod.* 24, 562–579. doi: 10.1093/humrep/den439

Weinberg, J., Sliwowska, J. H., Lan, N., and Hellemans, K. G. (2008). Prenatal alcohol exposure: foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. *J. Neuroendocrinol.* 20, 470–488. doi: 10.1111/j.1365-2826.2008.01669.x

Weinstock, M. (2010). Intrauterine factors as determinants of depressive disorder. *Isr. J. Psychiatry Relat. Sci.* 47, 36–45.

Xu, J., Yeon, J. E., Chang, H., Tison, G., Chen, G. J., Wands, J., et al. (2003). Ethanol impairs insulin-stimulated neuronal survival in the developing brain: role of PTEN phosphatase. *J. Biol. Chem.* 278, 26929–26937. doi: 10.1074/jbc.M300401200

Yoshimura, T., Arimura, N., and Kaibuchi, K. (2006). Molecular mechanisms of axon specification and neuronal disorders. *Ann. N.Y. Acad. Sci.* 1086, 116–125. doi: 10.1196/annals.1377.013

Young, C., and Olney, J. W. (2006). Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol. Dis.* 22, 548–554. doi: 10.1016/j.nbd.2005.12.015

Young, C., Roth, K. A., Klocke, B. J., West, T., Holtzman, D. M., Labruyere, J., et al. (2005). Role of caspase-3 in ethanol-induced developmental neurodegeneration. *Neurobiol. Dis.* 20, 608–614. doi: 10.1016/j.nbd.2005.04.014

Zhou, F. C., Zhao, Q., Liu, Y., Goodlett, C. R., Liang, T., McClintick, J. N., et al. (2011). Alteration of gene expression by alcohol exposure at early neurulation. *BMC Genomics* 12:124. doi: 10.1186/1471-2164-12-124

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Fetal alcohol spectrum disorders and their transmission through genetic and epigenetic mechanisms

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Fetal alcohol spectrum disorders (FASD) are a group of related conditions that arise from prenatal exposure to maternal consumption of the teratogen, ethanol. It has been estimated that roughly 1% of children in the US suffer from FASD (Sampson et al., 1997), though in some world populations, such as inhabitants of some poorer regions of South Africa, the rate can climb to as high as 20% (May et al., 2013). FASD are the largest cause of mental retardation in U.S. neonates, and ironically, are entirely preventable. FASD have been linked to major changes in the hypothalamic-pituitary-adrenal (HPA) axis, resulting in lifelong impairments through mental disorders, retardation, and sensitivity to stress. FASD are linked to an impaired immune system which consequently leads to an elevated risk of cancer and other diseases. FASD arise from a complex interplay of genetic and epigenetic factors. Here, we review current literature on the topic to tease apart what is known in these areas particularly emphasizing HPA axis dysfunction and how this ties into new studies of transgenerational inheritance in FASD.

Keywords: fetal alcohol, FASD, HPA axis, proopiomelanocortin, transgenerational epigenetic

INTRODUCTION

A negative impact from alcohol consumption has been observed since ancient times, leading to cultural prohibitions on alcohol consumption, particularly among women of childbearing years. The ancient Greeks demonstrated some awareness of the risks of alcohol consumption – for example, Plato proposed limiting wine consumption in people under 40 years of age (Abel, 1984). In some ancient societies, such as the Carthaginian Empire, the prohibitions were even written into law (Jones et al., 1973; Abel, 1984). Biblical and Talmudic references suggest that possibly, the ancient Hebrews might have had some awareness that alcohol consumption among fathers and mothers prior to conception could be harmful to the offspring (Abel, 1984). Ancient Vedic writings list prohibitions for drinking by Brahmins (Sharma et al., 2010) suggesting recognition of some negative effects of alcohol, though it is not clear whether an impact on offspring was known. Many sources discussing parental alcohol consumption before modern times focused upon paternal consumption more than maternal (Abel, 1984). With the writings of Francis Bacon in 1627, we see definite concerns about the impact of maternal alcohol consumption on offspring, and in the early 18th century in England, the "gin epidemic" gave rise to commentary that drinking specifically during pregnancy harmed the developing child (Abel, 1984).

Studies in guinea pigs a century ago found that not only did paternal chronic alcohol consumption result in higher mortality rates of offspring, but that the mortality rate was elevated even in the grandchildren of the alcohol-consuming father (Stockard, 1913), giving the first known evidence of a potential multigenerational effect of alcohol exposure. Fetal alcohol syndrome (FAS), the more severe manifestations of the FASD, first began to appear in the medical literature in 1973 (May and Gossage, 2011), and research in the field of fetal alcohol exposure began to take off as the research community launched a coordinated effort into understanding FASD. This led to the recent discovery of a transgenerational impact of fetal alcohol exposure upon stress axis dysfunction. This impact was discovered to be mediated by epigenetic mechanisms carried specifically in the male germline of rats (Govorko et al., 2012).

DYSREGULATION OF THE HPA AXIS IN FETAL ALCOHOL EXPOSED OFFSPRING

The fetal stage of human life is arguably the most susceptible to harm from alcohol as the fundamental development of organs and pathways occurs in this stage. Not surprisingly, mothers who consume alcohol despite the risks may create severe developmental problems in their offspring. FASD, as its name implies, comprises a wide range of mental, emotional, craniofacial, physiological, and immune disorders which arise from maternal consumption of alcohol. In general, severity of the disorder correlates with the degree of maternal alcohol consumption, though some individuals are more resistant to the effects than others. Individuals with milder forms of FASD may not show any obvious deformities, but may be affected with hyperactivity, depression, anxiety or other disorders that impair quality of life (Schneider et al., 2002). At the other end are individuals with visible deformities, severe retardation, an impaired immune system, impaired metabolic function, and lifelong problems coping with stress, individuals who have FAS (Momino et al., 2012). At the molecular level, many intertwined causal factors contribute to FASD leading to the varied impacts seen among those who suffer. Among the factors, FASD is intimately tied to hyperstress-response and anxiety disorders that are

connected to the dysregulation of hypothalamic-pituitary-adrenal (HPA) axis functions.

The HPA axis is a complex neuroendocrine loop maintained by crosstalk between the hypothalamus, pituitary, and the peripheral adrenal glands. The paraventricular nucleus (PVN) of the hypothalamus generates corticotrophin-releasing hormone (CRH) and vasopressin, stimulating the production of the precursor polypeptide proopiomelanocortin (POMC) in the anterior lobe of the pituitary. The breakdown of POMC releases adrenocorticotropic hormone (ACTH) initiating the delivery of glucocorticoids (GCs) to peripheral circulation from the adrenal glands. The primary GC produced in the human adrenal cortex is cortisol (corticosterone in rats). Corticosterone can inhibit the production of POMC in the anterior lobe of the pituitary in rats, creating a feedback loop (Eberwine and Roberts, 1984). GCs reduce inflammatory responses through an immunosuppressive action, and stimulate the sympathetic "fight or flight" response giving a rapid, temporary boost to an organism responding to an environmental threat. The autonomic nervous system complements the stress response, either through "fight or flight" (mentioned above), or by "tend and mend," an opposing process through the parasympathetic nervous system. ACTH also stimulates the production of catecholamines (CATs) from the adrenal glands. Epinepherine, also known as adrenaline, and its counterpart, norepinephrine (noradrenaline) are CATs released by the adrenal medulla that activate the sympathetic stress response, leading to many of the common physiological symptoms of stress, such as sweating, dry mouth, and a rapid heartbeat. Though the sympathetic stress response increases survival, it comes at a significant cost in terms of metabolism, the immune system, digestion, and other physiological processes, and cannot be maintained indefinitely (reviewed in Rachdaoui and Sarkar, 2013; Wynne and Sarkar, 2013).

β-endorphin is another peptide product of POMC generated through the stress response. β-endorphin is an opioid that can regulate pain, but it also regulates ACTH (another peptide product of POMC; Fratta et al., 1981) and corticotrophin-releasing hormone (CRH; Plotsky, 1986). Hypothalamic β-endorphin is important to the homeostasis of the stress response. CRH and catecholamines stimulate β-endorphin release that suppresses the HPA axis response (Boyadjieva et al., 2009). δ and μ opioid receptors bind central β-endorphin, regulating the autonomic nervous system through the PVN. Levels of pituitary β-endorphin have a smaller role on the autonomic nervous system and are regulated by arginine vasopressin (AVP) and CRH (reviewed by Sarkar et al., 2012).

Proopiomelanocortin neurons in the arcuate nucleus of the hypothalamus play a critical function in regulation of the HPA axis as well as reward pathways and the immune system, through the neuropeptides melanocortin, ACTH, and β-endorphin derived from the POMC precursor polypeptide (Sarkar et al., 2012). POMC neuronal functions were found to be impaired in fetal alcohol-exposed (FAE) rats (Sarkar et al., 2007; Hellemans et al., 2008; Boyadjieva et al., 2009). Recent experiments showed that replacing β-endorphin/POMC-producing cells in FAE rats led to an improvement in stress and immune response in the animals, demonstrating a role for POMC in FASD (Boyadjieva et al., 2009).

VARIANT ALLELES OF GENES INVOLVED IN DEVELOPMENT, THE HPA AXIS, AND ALCOHOL METABOLISM PLAY A STRONG ROLE IN FASD SUSCEPTIBILITY AND SYMPTOMS

Though studies showing heritable damage from fetal alcohol exposure go back at least a century (Stockard, 1913), it was only with the use of molecular biology approaches during the 1980s that science began to unravel the genetic underpinnings of FASD. At the chromosomal level, structural damage was observed at a high frequency among prenatally exposed individuals in a clinical setting. In one study, 8.75% (7/80) of FASD patients in a genetic screening were determined to have chromosomal abnormalities, typically microduplications or microdeletions (Douzgou et al., 2012). Strong evidence for a genetic component to FASD includes a comparison between mono- and dizygotic twins. Monozygotic twins (having the same genome) show 100% concordance for FASD; that is, if 1 twin has FASD, the other will also have FASD. In comparison, dizygotic twins (having genomes that are moderately different) show only 63% concordance (Streissguth and Dehaene, 1993), showing that even the modest genetic differences of siblings sharing the same environment lead to a significantly different rate of susceptibility to FASD.

It is known that multiple genetic loci are affected by alcohol, with each variant allele interacting in a complex biological pathway with other genes (Johnson et al., 2006). In FASD, this complexity is multiplied as the genes of the mother can impact the fetal environment and expression of genes of the developing fetus, shaping its susceptibility toward fetal alcohol exposure. Maternal RNA and proteins are present in the oocyte to allow for development to occur in the early embryo until it has developed to the Maternal-to-Zygotic transition (MZT) at which point the embryonic cellular processes take over (Schier, 2007). Maternal hormones have also been shown to play an important role in development of an offspring. These examples show that maternal genes (and their products) can have a strong impact on the development of the fetus beyond contribution of genes alone, dubbed the "maternal effect." By contrast, transmission of a phenotype from the father passed through the father's sperm ("paternal effect") is more limited but has been demonstrated (Fitch et al., 1998). In short, altered parental expression of important genes could result in significant vulnerability or resistance of the offspring to ethanol-induced dysregulation in embryonic development even in the absence of those genes in the child, laying down a dysfunctional or more resistant foundation that lasts a lifetime.

A number of genes have also been identified as potentially playing a direct role in FASD. In a study of rhesus monkeys, it was demonstrated that fetal monkeys carrying a short serotonin transporter gene polymorphic region variation (rh5-HTTLPR), an allele with a functional analog in humans, were particularly susceptible to prenatal alcohol exposure during early gestation, leading to sensory disorders (Schneider et al., 2009). Prior studies indicated that this allele was linked to a greater incidence of irritability and stress-responsiveness in monkey offspring subjected to prenatal alcohol exposure (Kraemer et al., 2008).

Genes coding for alcohol dehydrogenase variants have been found to be particularly relevant for FASD, sometimes resulting in varied incidence or severity of FASD. In some cases, they

may alter maternal drinking patterns: ADH variants have been linked to alcohol addiction, while other variants may cause mothers to drink less. Alcohol dehydrogenases are found ubiquitously across the kingdoms of living organisms. The products of these genes are involved in converting alcohols (ROH) into aldehydes (R-CHO) and ketones (RCOR') through reduction of the coenzyme nicotinamide adenine dinucleotide (NAD^+) into NADH. Humans have six different ADHs, and alcohol dehydrogenase plays an important role in ethanol metabolism and alcohol addiction. Slower metabolizing variants of ADH2 and -3 have been linked to alcoholism in Asian populations (Osier et al., 1999). ADH1B has a strong link to alcoholism (Li et al., 2011). In FASD, it was discovered that maternal variant alleles of ADH1B, ADH1B*2, and ADH1B*3 (both relatively common in African-descended populations) which metabolize alcohol rapidly, led to reduced incidence of FASD in offspring, even those without the gene (Warren and Li, 2005; Jacobson et al., 2006). It is known that ADH1B*3 leads to reduced alcohol intake, so possibly it creates an unpleasant association with elevated aldehyde levels through rapid metabolization of alcohol, or the enzyme could help protect offspring by reducing the peak blood alcohol levels, a critical factor in determining the amount of damage done to fetuses (Jacobson et al., 2006), suggesting that these could be underlying mechanisms for reducing the incidence of FASD. In fetal mice exposed to alcohol, variant genotypes of *aldh2*, an alcohol dehydrogenase gene, have been linked to defects in brain development and vision. This was exacerbated in mice lacking *fancd2*, which plays a downstream role in processing aldehyde metabolites of alcohol (Langevin et al., 2011). In zebrafish, *mars* (a gene involved in alcohol metabolism) null fish were linked to developmental face and brain dysfunction during an embryonic alcohol study (McCarthy and Eberhart, 2014). Other genes impacted by ethanol in an embryonic alcohol study conducted in zebrafish include *hinfp, plk1, foxd1*, and *vangl2,* which have significance in cellular processes such as translation and the cell cycle, and in early development (McCarthy and Eberhart, 2014). Bioinformatic data mined from the results of published literature searches identified from a screen of over 10,000 candidate genes, a subset of 87 genes within the TGF-β, MAPK, and Hedgehog signaling pathways which were likely relevant for FASD. These include *gnas*, and *msx1*, important in apoptosis and cell signaling, *fgfr1-3*, important for embryonic bone development, and *bmp4*, important in myogenesis. Also included were *foxg1b*, *hoxa1*, and *pax6*, important in brain development (Lombard et al., 2007). These genes were examined through pathway, protein-protein and transcription binding analysis and are rich targets for studies into the genetics of FASD. They also fit well with observed phenotypic changes in FASD patients including facial deformities, cardiovascular irregularities, skeletal defects, and brain growth defects (Clarren et al., 1978; Clarren, 1986).

In summary, many genes, either related to alcohol metabolism, or to development and the HPA stress axis, have been identified in relation to FASD. Variants of some of these genes have been shown to contribute to the varied responses seen to fetal alcohol exposure, and it seems likely that other genes also contribute. This may explain in part why the percentage of women who drink during pregnancy is so much higher than the percentage of children who are born with FASD.

FETAL ALCOHOL EXPOSURE CAUSES EPIGENETIC CHANGES CRITICAL TO FASD

Epigenetics refers to changes in gene expression that do not arise from changes in the underlying DNA sequence. Environmental toxins such as ethanol may impact the expression of genes through altering DNA methylation patterns or modifying histone tails by methylation or acetylation. Epigenetic studies related to FASD are still an emerging field (Haycock, 2009) but have led to the discovery that many symptoms of FASD can be traced back at least in part to aberrant epigenetic marks laid down during gamete production, or during embryonic development under the influence of alcohol. Recent studies have found epigenetic changes due to ethanol that are permanent (Govorko et al., 2012), and they can act broadly across the genome (Kaminen-Ahola et al., 2010). Both DNA methylation and histone modifications, two of the most commonly studied epigenetic mechanisms, can alter the accessibility of DNA to the molecular transcriptional machinery, providing a powerful method for ethanol to create developmental havoc through changing the expression of genes (Renthal and Nestler, 2009).

DNA METHYLATION

The process of DNA methylation involves the transfer of a methyl group by a DNA methyltransferase (DNMT), utilizing S-adenosyl methionine (SAM), to the C^5 carbon of a cytosine residue, typically in regions containing strings of CpG dinucleotides (CpG islands; Bestor, 2000). CpG islands (CGIs) are traditionally defined by having an extended stretch of nucleotides (>200 bases), a C/G nucleotide composition above 50%, and an observed CpG dinucleotide content of 65+% (Gardiner-Garden and Frommer, 1987). Roughly 70% of annotated promoters show the presence of CGIs that either contain transcription start sites (TSS) or are near them (Saxonov et al., 2006). In vertebrates, CpGs are in low overall abundance and often methylated, however, CpGs in CGIs are frequently unmethylated (Deaton and Bird, 2011). Approximately half of CGIs in mice and humans are associated with TSS (Illingworth et al., 2010; Deaton and Bird, 2011). The remaining, dubbed "orphan CGIs" are themselves often associated with novel promoters (Illingworth et al., 2010; Maunakea et al., 2010).

Methylation of CpG islands is often regulated during development to control gene expression and the level of methylation correlates with the magnitude of gene inactivation (Doerfler, 1983).

Alcohol may affect DNA methylation through an impact on key methylating enzymes such as DNA methyltransferases (DNMTs). Studies have shown that a downstream metabolite of alcohol breakdown, acetaldehyde, can inhibit DNMT1 (Garro et al., 1991). DNA methylation changes can also occur by altering the interconnected choline, methionine and 5-methyltetrahydrofolate (folate) pathways, which provide key substrates for methylation (Hamid et al., 2009). Alcohol exposure may act through folate (Hidiroglou et al., 1994) and *S*-adenosylmethionine (SAM; Barak et al., 1993; Lu et al., 2000). Alcohol reduces the absorption of folate from the diet, a vitamin that is necessary for carbon-transfer reactions in

the methionine–homocysteine pathway, putting limits on DNA methylation (Halsted et al., 2002). Folate deficiency is linked to global DNA hypomethylation (Kim et al., 1997). Choline supplementation reduces FASD symptoms by providing sufficient methyl groups to the methinone–homocysteine pathway (Thomas and Tran, 2012; Bekdash et al., 2013; Wozniak et al., 2013).

Alcohol-mediated aberrant methylation has been documented in at least several developmental genes in alcohol-abusing humans (Bönsch et al., 2006; Hillemacher et al., 2009). In addition, alcohol can act through demethylation (Ponomarev, 2013). DNA methylation changes have been observed to occur during early development. Liu et al. (2009) found that extensive methylation occurred in developmental genes of alcohol-exposed mouse embryos (Liu et al., 2009). Kaminen-Ahola et al. (2010) found that the A^{vy} gene in mice was hypermethylated following alcohol exposure in early fetal development. Prenatal alcohol exposure studies in mice demonstrated DNA methylation changes resulting in neurofacial and growth defects analogous to FASD in humans (Liu et al., 2009). Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation and alters methylation of imprinted genes, known to play roles in the cell cycle, growth, apoptosis, cancer, and in a large number of genes associated with olfaction. These results indicate that gene specific and global changes in DNA methylation occur in response to alcohol during fetal development.

Exposures to ethanol and other toxicants during critical windows in development can be particularly damaging and can cause epigenetic changes in gene expression of the brain that impact the brain for the life of the organism (Kobor and Weinberg, 2011; Ungerer et al., 2013; Nestler, 2014). Alcohol is highly damaging before and during very early development, specifically at three events. When gametes are being produced or are maturing, they are vulnerable to alcohol abuse (Haycock, 2009) as demonstrated by several studies (Ouko et al., 2009; Govorko et al., 2012). This is likely because DNA is being actively repackaged during this period. Also germ cells persist for extended periods of time in the body, providing a longer window for the actions of repeated exposures to environmental toxins like ethanol. A second highly vulnerable period occurs after fertilization but before implantation, when the embryo is undergoing rapid developmental changes and is preparing for implantation. The third occurs at gastrulation, when the three germ layers are being defined. As a result, histone marks are being laid down to specify cell types (Shi and Wu, 2009), and the developing embryo is at its most vulnerable to alcohol at this stage (Armant and Saunders, 1996; Haycock, 2009).

Rat-based studies have found that POMC expression is affected by fetal alcohol exposure, leading to the loss of β-endorphin producing neurons and a reduction in POMC expression (Chen et al., 2006; Sarkar et al., 2007; Kuhn and Sarkar, 2008). It has been found that ethanol is able to increase DNA methylation of the promoter region of POMC in fetal pups, changing the epigenetic markings to downregulate POMC expression (Govorko et al., 2012). This is important as POMC neurons, located in the hypothalamus, are critical for bringing stress homeostasis. The perturbation of the HPA axis is a direct cause of many of the symptoms associated with FASD including deficient stress response, depression, anxiety, and impaired immunity (Sarkar, 1996; Pritchard et al., 2002; Raffin-sanson et al., 2003; Sarkar et al., 2012; Rachdaoui and Sarkar, 2013).

In short, DNA methylation has been found to be a strong factor in the incidence of FASD. Alcohol acts through several routes to affect DNA methylation, and seems to be particularly damaging at specific stages of embryonic development resulting in significant changes in methylation that can last a lifetime.

HISTONE MODIFICATION

Histone modifications are other major biological processes by which epigenetic modification of gene expression occurs. DNA is wound around histones, affecting its ability to interact with transcriptional machinery. Tight packaging results in less interaction. Covalent modifications to the tails of histones 3 and 4 (H3 and H4) are commonly studied. However, H3 and H4 are not the only histones that can be epigenetically modified. For H3 and H4, heterochromatin, the silenced state, is associated with hypoacetylation (de-acetylated state), and di- or trimethylation of the nineth lysine residue on H3 (H3K9me2 or H3K9me3). The open state, euchromatin, is associated with acetylated H3 and H4, and di- or tri-methylation of the fourth lysine residue of H3 (H3K4me2 or H3K4me3; Arney and Fisher, 2004). Thus, histone methylation, depending on the target of methylation, can result in a change in expression in either direction. There can be coordinated regulation between histone modifications and other epigenetic mechanisms, including DNA methylation (Jin et al., 2011).

Histone marks have been linked to alcohol consumption (Pal-Bhadra et al., 2007; Pandey et al., 2008). Alcohol causes histone marks to occur in specific genomic locations (Kim and Shukla, 2006). Usually in alcohol studies this has been H3K4me3 (Histone 3 Lysine 4 residue, trimethylated) and acetylation/deacetylation of H3 and H4 (Ponomarev, 2013). Govorko et al. (2012) found that histone deacetylation occurred in fetal rat pups exposed to ethanol, leading to decreased expression of POMC, which partly explains the subsequent disruption of the HPA axis and FASD symptoms observed. See **Figure 1** for a summary of current knowledge.

A number of studies into the impact of ethanol on development have been conducted using cell culture models. Cardiac development was affected by ethanol using cardiac progenitor cells. Under ethanol exposure, it was found that heart development was significantly affected, and H3 acetylation ensued. The effect was dose dependent, leading to greater gene expression changes in higher ethanol doses (Zhong et al., 2010). In fetal neuronal stem cell neurospheres, ethanol was observed to remove histone methylation marks from promoters (either H3K4me3 and H3K27me3, or both) across 20 candidate genes playing roles in processes such as the regulation of neural stem cell biology, and neural patterning (Veazey et al., 2013). Ethanol also impacted the ES-like bivalent signature of the cells (Veazey et al., 2013). Interestingly, for most genes transcriptional control was not changed by the change in histone modifications (Veazey et al., 2013).

By itself, studies of H3K4me3 methylation in relation to alcohol exposure have been found to not be a good predictor of expression (Ponomarev, 2013). Some recent findings have suggested that global chromatin modifications in relation to drug exposure tend

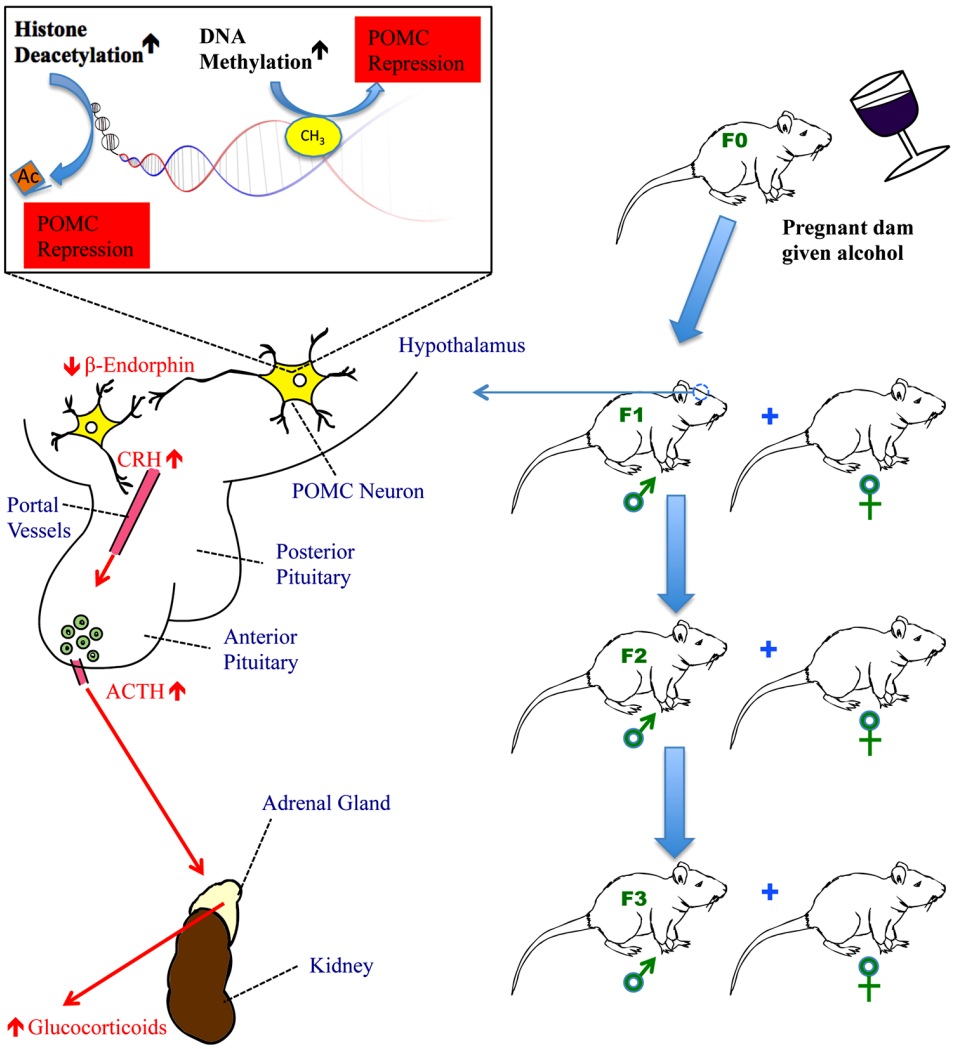

FIGURE 1 | The impact of fetal alcohol exposure on the hypothalamic-pituitary-adrenal (HPA) axis. Fetal alcohol exposure increases DNA methyltransferase activity to methylate CpGs in the promoter of the POMC gene in the hypothalamus and activates histone deacetylases to remove acetyl groups from histones in the proximity of the POMC gene. Both methylation and deacetylation lead to the repression of POMC gene transcription. This results in reduced production of β-endorphin, a peptide derived from POMC gene, in the hypothalamus. Reduced β-endorphin production disrupts the normal feedback regulation of the HPA axis causing hyper-response of CRH following stress and subsequently, increased secretion of ACTH from the anterior pituitary and glucocorticoids (corticosterone in rats and cortisol in humans) from the adrenal gland. In fetal alcohol-exposed rats, the HPA axis is dysregulated resulting in hyperstress-response, mental dysfunction, and an impaired immune system. POMC gene hypermethylation and its subsequent impact on the HPA axis persist for a minimum of three generations of progeny, carried through the male germline.

to be transient, and revert back to "normal" states within hours to days after the toxicant is removed suggesting that global changes may not be informative (Nestler, 2014). Though transient in some studies, if damage from aberrant methylation occurs at critical windows such as during fetal development when fundamental structures are being laid down, then at least some of the effects could be permanent.

Epigenetic modifications such as those described above have been linked to many diseases, including major mental disorders such as depression, schizophrenia, bipolar disorder ("manic-depression"), and addiction (Jaenisch and Bird, 2003; Eger et al., 2004; Hsieh and Gage, 2005; Sharma et al., 2008; Maze and Nestler, 2011). Haley et al. (2006) found that the HPA stress axis in infants was impacted from fetal alcohol exposure, likely due to epigenetic changes during gastrulation. Given the links between the HPA axis, FASD and mental disorders, understanding the dysregulation of POMC through epigenetic mechanisms could lead to a better understanding of these diseases and new avenues of treatment.

MicroRNAs

MicroRNAs (miRNAs) are a class of non-coding RNAs. They are roughly 22nt in length and function primarily by targeting the 3′ untranslated region (UTR) of transcripts, leading to their down-regulation. MiRNAs increasingly have been found to be important

post-transcriptional gene regulators involved in many biological processes widely across animals and most kingdoms (Reviewed in Berezikov, 2011). The effects of microRNAs in fetal alcohol exposure can occur both at the genetic level and at the level of epigenetics. MiRNA expression has been observed to vary between individuals, leading to changes in predisposition to, and severity of, diseases (Chu et al., 2012; Lukiw, 2013). In some cases, single nucleotide polymorphisms (SNPs) in the genes coding for miRNAs impact their expression and function (Sun et al., 2009). These individual differences could lead to significant differences in the consequences to offspring under maternal alcohol consumption. Alternatively, microRNA expression can be modified through epigenetic mechanisms by ethanol (Miranda, 2012), and, as microRNAs interact directly with epigenetic machinery at multiple levels (reviewed in Singh and Campbell, 2013), it seems likely that maternal ethanol consumption could also affect DNA methylation and histone acetylation/deacetylation via microRNAs. As miRNAs have been implicated in FASD models in rodent studies, particularly in neural development (Wang et al., 2009; Miranda, 2012), changes to miRNA expression likely have a significant role in FASD development in the children of women who abuse alcohol.

There is also evidence of miRNA interaction with the HPA stress axis. Recently, it was demonstrated in mice that paternal stress could impact the HPA stress axis, via epigenetic mechanisms acting on DNA in sperm to transmit heritable changes (Rodgers et al., 2013). Among the microRNAs in the list of those impacted by paternal stress which interact with the HPA stress axis, the expression of miR-29c and miR-204 were found to change in expression due to alcohol exposure in adult rat prefrontal cortex (PFC; Tapocik et al., 2013), whereas miR-29c and -30a were found to be altered in fetal mouse brains during exposure to maternal ethanol (Wang et al., 2009), suggesting that these microRNAs may impact HPA axis development during fetal alcohol exposure.

TRANSGENERATIONAL EPIGENETIC INHERITANCE OF FASD SYMPTOMS

HERITABLE EPIGENETIC MODIFICATIONS PLAY A ROLE IN MANY DISEASES

Transgenerational epigenetic inheritance, changes carried through the germline (Guerrero-Bosagna et al., 2010; Thornburg et al., 2010), provides an explanation for questions that molecular biologists have not been able to answer through genetics alone. Heritable epigenetic modifications play a key role in some human diseases. Several syndromes in the literature include Angelman, Prader-Willi, and Beckwith-Wiedemann syndrome. These are linked to heritable changes, such as deletions, in specific chromosome regions that undergo genomic imprinting (Adams, 2008). In imprinting, parental differences in epigenetic modifications of specific genes occur in sperm and ova, resulting in parent-specific gene expression. When the contribution to a gene from one parent is silenced through epigenetic mechanisms, deleting the copy from the other parent results in loss of the gene function.

TRANSGENERATIONAL EPIGENETIC MODIFICATIONS ARE RARE

Transgenerational epigenetic marks on genes are less common than transient marks as most epigenetic signatures are typically lost during gametogenesis. In some cases, certain marks may be retained due to a bias in removal (Morison and Reeve, 1998). Some genes continue to show parental methylation of promoter regions (Borgel et al., 2010). Many epigenetic marks are removed at meiosis (Bond and Finnegan, 2007), and the DNA is methylated again by during early development, often to mark cell type specificity (Shi and Wu, 2009), as there are over 200 different cell types in the body (Alberts et al., 2002).

Prior to fertilization, the male gamete carries the father's germline epigenetic signature. However, when fertilization occurs, most of the male's epigenetic marks are lost. Late in the production of sperm, protamines condense the paternal DNA, protecting it and replacing most histones. However, not all histones are replaced; some histones that remain will keep their prior epigenetic marks (Hammoud et al., 2009; Brykczynska et al., 2010). During the interaction of sperm and egg, after the nuclear membranes fuse and the sperm DNA is deposited, the protamines are lost, replaced with histones from the egg. The DNA from the male enters a more open state- most of the replacing histones are acetylated and the much of the methylation present in the DNA is removed (Oswald et al., 2000; Fulka et al., 2004). Interestingly, some regions seem to be protected from demethylation including imprinted genes (Li, 2002). Following fusion, maternal enzymes re-methylate significant regions of the sperm DNA.

Transgenerational epigenetic effects due to exposure to hazardous chemicals have been documented for 4 or more generations (Anway et al., 2005). To be considered a transgenerational epigenetic effect, the effect must persist for multiple generations. The number of generations required to demonstrate a transgenerational epigenetic effect differs, depending on whether the effect is maternally or paternally transmitted. For maternal transmission, three affected generations of offspring are required to demonstrate a transgenerational epigenetic effect. This is because the fetus (F1) inside the mother (F0) is developing gonads (which give rise to the F2 generation), so a purely environmental effect could in theory directly impact the grandchild through alterations to the gonads of F1, leading to a multigenerational effect, but not demonstrating heritable epigenetic transmission. Therefore, to demonstrate persistence of the effect, three generations (F3) of progeny must be impacted. For paternal transmission, the minimum number of impacted generations is two; that is the grandchild of the founder. Sperm could be directly impacted by an environmental agent, leading to an impact on the F1 offspring, but it would require an epigenetic mechanism to then transmit this effect to the F2 generation (Jirtle and Skinner, 2007).

TRANSGENERATIONAL EPIGENETIC MODIFICATIONS CAUSED BY MATERNAL ALCOHOL CONSUMPTION CAN PROCEED THROUGH THE MALE GERMLINE

There are a number of multigenerational demonstrations of the heritability of alcohol-related disorders in the literature. In a recent study of Native American women who abused alcohol, F2 generation offspring (that is the grandchildren) of an alcohol-abusing woman have a higher tendency to show FAS than those who do F2 progeny of control women (Kvigne et al., 2008). Recently, Govorko et al. (2012) showed that following *in utero*

exposure to ethanol, regulatory regions of POMC in the hypothalamus of rats undergo epigenetic modifications: altered histone marks and DNA methylation of the proximal promoter. In addition, histone modifying HDACs and DNA methyltransferases (DNMTs) were shown to be impacted, suggesting a causal relationship between alcohol and epigenetic changes. As a result, POMC neurons are impacted across at least three generations, perturbing the expression of key POMC-derived peptides such as β-endorphin, and affecting the production of its downstream messenger corticosterone leading to dysregulation of the HPA axis and an elevated response to stress in the adult offspring. This was the first demonstration of a true transgenerational epigenetic effect for prenatal alcohol exposure. Interestingly, Govorko et al. (2012) were able to reverse this effect through HDAC and DNA methylation inhibitors, providing additional support for their conclusion.

There is also evidence that hypomethylation occurs in the sperm of alcoholic men (Ouko et al., 2009). Transmission of the effects of alcohol through the male germline has precedents in the literature for induction of symptoms like those found in FASD. These include mental impairment, cardiac defects, low birth weight, and hyperactivity, compared to controls, as assessed in human epidemiological studies and backed by animal studies (Abel, 2004). This supports the findings of Govorko and colleagues that factors that impact POMC and subsequently affect the HPA axis and FASD, can be transmitted by males through the germline.

SUMMARY AND FUTURE DIRECTIONS

FASD is caused by a complex interaction of genes and environment, and is regulated by both parental and fetal genes. Some symptoms of FASD are caused by decreased expression of POMC, and it's peptide product, β-endorphin, important in the HPA stress axis regulation. Recent studies by Govorko et al. (2012) have elucidated that POMC epigenetic changes are transmitted through pups in the male germline descended from fetal alcohol exposed animals for several generations. It is currently unknown how this occurs, as direct changes to the enzymes involved in methylation and deacetylation should also impact female progeny. It is speculated that the non-pairing region of the Y chromosome, which is euchromatic, may be partly protected from demethylation and could carry the epigenetic modifications to future male progeny.

The reversibility of the POMC system defect that Govorko and colleagues demonstrated via the modulation of the components of the epigenetic machinery may have therapeutic potential. Histone deacetylase inhibitors have proven to be effective in reducing some symptoms of alcohol damage. These act by preventing HDACs from removing acetyl groups from the tails of histones, and ultimately maintain a potentially transcriptionally active state. Rat studies have shown that this can improve the symptoms of FASD (Govorko et al., 2012), and also can function to prevent tolerance and withdrawal in adult rats (Sakharkar et al., 2012).

Choline influences SAM levels, and choline deficiency during development phenotypically mimics folate deficiency (Zeisel, 2004, 2006). Choline chloride supplementation has been successful in reducing the impact of maternal alcohol consumption on developing fetuses (Thomas et al., 2007, 2010; Bekdash et al., 2013). The use of choline and HDAC or DNMT inhibiting supplements to mitigate FAS symptoms in rats suggests that supplementation could assist at-risk populations during pregnancy, though more studies need to be done in this area. In addition, developments in the understanding of epigenetic regulation of POMC may suggest additional treatment strategies to reduce symptoms of the illness related to the HPA stress axis in adults, and given the links to other disorders, such as cancer and mental disorders, the effects of this research could be far-reaching. The future is promising.

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REFERENCES

Abel, E. (1984). "Is fetal alcohol syndrome a new discovery?" in *Fetal Alcohol Syndrome and Fetal Alcohol Effects*, ed. E. Abel (New York, NY: Springer Press), 1–28. doi: 10.1007/978-1-4613-2669-4_1

Abel, E. (2004). Paternal contribution to fetal alcohol syndrome. *Addict. Biol.* 9, 127–133. doi: 10.1080/13556210410001716980

Adams, J. (2008). Imprinting and genetic disease: Angelman, Prader-Willi and Beckwith-Weidemann syndromes. *Nat. Educ.* 1, 129.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). *Molecular Biology of the Cell*, 4th Edn. New York: Garland Science.

Anway, M. D., Cupp, A. S., Uzumcu, M., and Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* 308, 1466–1469. doi: 10.1126/science.1108190

Armant, D. R., and Saunders, D. E. (1996). Exposure of embryonic cells to alcohol: contrasting effects during preimplantation and postimplantation development. *Semin. Perinatol.* 20, 127–139. doi: 10.1016/S0146-0005(96)80080-2

Arney, K. L., and Fisher, A. G. (2004). Epigenetic aspects of differentiation. *J. Cell. Sci.* 117, 4355–4363. doi: 10.1242/jcs.01390

Barak, A. J., Beckenhauer, H. C., Junnila, M., and Tuma, D. J. (1993). Dietary betaine promotes generation of hepatic S-adenosylmethionine and protects the liver from ethanol-induced fatty infiltration. *Alcohol Clin. Exp. Res.* 17, 552–555. doi: 10.1111/j.1530-0277.1993.tb00798.x

Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2013). Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and proopiomelanocortin (POMC) gene expression in ß-endorphin-producing POMC neurons of the hypothalamus. *Alcohol Clin. Exp. Res.* 37, 1133–1142. doi: 10.1111/acer.12082

Berezikov, E. (2011). Evolution of microRNA diversity and regulation in animals. *Nat. Rev. Genet.* 12, 846–860. doi: 10.1038/nrg3079

Bestor, T. H. (2000). The DNA methyltransferases of mammals. *Hum. Mol. Genet.* 9, 2395–2402. doi: 10.1093/hmg/9.16.2395

Bond, D. M., and Finnegan, E. J. (2007). Passing the message on: inheritance of epigenetic traits. *Trends Plant Sci.* 12, 211–216. doi: 10.1016/j.tplants.2007.03.010

Bönsch, D., Lenz, B., Fiszer, R., Frieling H., Kornhuber, J., and Bleich, S. (2006). Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. *J. Neural Transm.* 113, 1299–1304. doi: 10.1007/s00702-005-0413-2

Borgel, J., Guibert, S., Li, Y., Chiba, H., Schübeler, D., Sasaki, H., et al. (2010). Targets and dynamics of promoter DNA methylation during early mouse development. *Nat. Genet.* 42, 1093–1100. doi: 10.1038/ng.708

Boyadjieva, N. I., Ortigüela, M., Arjona, A., Cheng, X., and Sarkar, D. K. (2009). β-Endorphin neuronal cell transplant reduces corticotropin releasing hormone hyperresponse to lipopolysaccharide and eliminates natural killer cell functional deficiencies in fetal alcohol exposed rats. *Alcohol Clin. Exp. Res.* 33, 931–937. doi: 10.1111/j.1530-0277.2009.00911.x

Brykczynska, U., Hisano, M., Erkek, S., Ramos, L., Oakeley, E. J., Roloff, T. C., et al. (2010). Repressive and active histone methylation mark distinct promoters

in human and mouse spermatozoa. *Nat. Struct. Mol. Biol.* 17, 679–687. doi: 10.1038/nsmb.1821

Chen, C. P., Kuhn, P., Chaturvedi, K., Boyadjieva, N., and Sarkar, D. K. (2006). Ethanol induces apoptotic death of developing beta-endorphin neurons via suppression of cyclic adenosine monophosphate production and activation of transforming growth factor-beta1-linked apoptotic signaling. *Mol. Pharm.* 69, 706–717. doi: 10.1124/mol.105.017004

Chu, Y. H., Tzeng, S. L., Lin, C. W., Chien, M. H., Chen, M. K., and Yang, S. F. (2012). Impacts of MicroRNA gene polymorphisms on the susceptibility of environmental factors leading to carcinogenesis in oral cancer. *PLoS ONE* 7:e39777. doi: 10.1371/journal.pone.0039777

Clarren, S. K. (1986). "Neuropathology in fetal alcohol syndrome," in *Alcohol and Brain Development*, ed. J. R. West (New York: Oxford University Press), 158–166.

Clarren, S. K., Alvord, E. C. Jr., Sumi, S. M., Streissguth, A. P., and Smith, D. W. (1978). Brain malformations related to prenatal exposure to ethanol. *J. Pediatr.* 92, 64–67. doi: 10.1016/S0022-3476(78)80072-9

Deaton, A. M., and Bird, A. (2011). CpG islands and the regulation of transcription. *Genes Dev.* 25, 1010–1022. doi: 10.1101/gad.2037511

Doerfler, W. (1983). DNA methylation and gene activity. *Annu. Rev. Biochem.* 52, 93–124. doi: 10.1146/annurev.bi.52.070183.000521

Douzgou, S., Breen, C., Crow, Y. J., Chandler, K., Metcalfe, K., Jones, E., et al. (2012). Diagnosing fetal alcohol syndrome: new insights from newer genetic technologies. *Arch. Dis. Child.* 97, 812–817. doi: 10.1136/archdischild-2012-302125

Eberwine, J. H., and Roberts, J. L. (1984). Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. *J. Biol. Chem.* 259, 2166–2170.

Eger, G., Liang, G., Aparcio, A., and Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 429, 457–463. doi: 10.1038/nature02625

Fitch, K. R., Yasuda, G. K., Owens, K. N., and Wakimoto, B. T. (1998). Paternal effects in Drosophila: implications for mechanisms of early development. *Curr. Top. Dev. Biol.* 38, 1–34. doi: 10.1016/S0070-2153(08)60243-4

Fratta, W., Rossetti, Z. L., Poggioli, R., and Gessa, G. L. (1981). Reciprocal antagonism between ACTH1-24 and beta-endorphin in rats. *Neurosci. Lett.* 24, 71–74. doi: 10.1016/0304-3940(81)90361-X

Fulka, H., Mrazek, M., Tepla, O., and Fulka, J. (2004). DNA methylation pattern in human zygotes and developing embryos. *Reproduction* 128, 703–708. doi: 10.1530/rep.1.00217

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* 196, 261–282. doi: 10.1016/0022-2836(87)90689-9

Garro, A. J., McBeth, D. L., Lima, V., and Lieber, C. S. (1991). Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol Clin. Exp. Res.* 15, 395–398. doi: 10.1111/j.1530-0277.1991.tb00536.x

Govorko, D., Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2012). Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. *Biol. Psychiatry* 72, 378–388. doi: 10.1016/j.biopsych.2012.04.006

Guerrero-Bosagna, C., Settles, M., Lucker, B., and Skinner, M. K. (2010). Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. *PLoS ONE* 5:e13100. doi: 10.1371/journal.pone.0013100

Haley, D. W., Handmaker, N. S., and Lowe, J. (2006). Infant stress reactivity and prenatal alcohol exposure. *Alcohol Clin. Exp. Res.* 30, 2055–2064. doi: 10.1111/j.1530-0277.2006.00251.x

Halsted, C. H., Villanueva, J. A., Devlin, A. M., and Chandler, C. J. (2002). Metabolic interactions of alcohol and folate. *J. Nutrit.* 132(8 Suppl.), S2367–S2372.

Hamid, A., Wani, N. A., and Kaur, J. (2009). New perspectives on folate transport in relation to alcoholism-induced folate malabsorption—association with epigenome stability and cancer development. *FEBS J.* 276, 2175–2191. doi: 10.1111/j.1742-4658.2009.06959.x

Hammoud, S. S., Nix, D. A., Zhang, H., Purwar, J., Carrell, D. T., and Cairns, B. R. (2009). Distinctive chromatin in human sperm packages genes for embryo development. *Nature* 460, 473–478. doi: 10.1038/nature08162

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod.* 81, 607–617. doi: 10.1095/biolreprod.108.074690

Hellemans, K. G., Verma, P., Yoon, E., Yu, W., and Weinberg, J. (2008). Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. *Ann. N. Y. Acad. Sci.* 1144, 154–175. doi: 10.1196/annals.1418.016

Hidiroglou, N., Camilo, M. E., Beckenhauer, H. C., Tuma, D. J., Barak, A. J., Nixon, P. F., et al. (1994). Effect of chronic alcohol ingestion on hepatic folate distribution in the rat. *Biochem. Pharmacol.* 47, 1561–1566. doi: 10.1016/0006-2952(94)90532-0

Hillemacher, T., Frieling, H., Hartl, T., Wilhelm, J., Kornhuber, J., and Bleich, S. (2009). Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving. *J. Psychol. Res.* 43, 388–392. doi: 10.1016/j.jpsychires.2008.04.006

Hsieh, J., and Gage, F. H. (2005). Chromatin remodeling in neural development and plasticity. *Curr. Opin. Cell Biol.* 17, 664–671. doi: 10.1016/j.ceb.2005.09.002

Illingworth, R. S., Gruenewald-Schneider, U., Webb, S., Kerr, A. R. W., James, K. D., Turner, D. J., et al. (2010). Orphan CpG islands identify numerous conserved promoters in the mammalian genome. *PLoS Genet.* 6:e1001134. doi: 10.1371/journal.pgen.1001134

Jacobson, S. W., Carr, L. G., Croxford, J., Sokol, R. J., Li, T. K., and Jacobson, J. L. (2006). Protective effects of the alcohol dehydrogenase-ADH1B allele in children exposed to alcohol during pregnancy. *J. Pediatr.* 148, 30–37. doi: 10.1016/j.jpeds.2005.08.023

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33, 245–254. doi: 10.1038/ng1089

Jin, B., Li, Y., and Robertson, K. D. (2011). DNA methylation: superior or subordinate in the epigenetic hierarchy? *Genes Cancer* 2, 607–617. doi: 10.1177/1947601910393957

Jirtle, R., and Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nat. Rev.* 8, 253–262. doi: 10.1038/nrg2045

Johnson, C., Drgon, T., Liu, Q. R., Walther, D., Edenberg, H., Rice, J., et al. (2006). Pooled association genome scanning for alcohol dependence using 104,268 SNPs: validation and use to identify alcoholism vulnerability loci in unrelated individuals from the collaborative study on the genetics of alcoholism. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 141B, 844–853. doi: 10.1002/ajmg.b.30346

Jones, K. L., Smith, D. W., Ulleland, C. N., and Streissguth, A. P. (1973). Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1, 1267–1271. doi: 10.1016/S0140-6736(73)91291-9

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K. A., Fahey, P., Cox T. C., et al. (2010). Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet.* 6:e1000811. doi: 10.1371/journal.pgen.1000811

Kim, J. S., and Shukla, S. D. (2006). Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol Alcohol.* 41, 126–132. doi: 10.1093/alcalc/agh248

Kim, Y. I., Pogribny, I. P., Basnakian, A. G., Miller, J. W., Selhub, J., James, S. J., et al. (1997). Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am. J. Clin. Nutr.* 65, 46–52.

Kobor, M. S., and Weinberg, J. (2011). Epigenetics and fetal alcohol spectrum disorders. *Alcohol Res. Health* 34, 15–37.

Kraemer, G. W., Moore, C. F., Newman, T. K., Barr, C. S., and Schneider, M. L. (2008). Moderate level fetal alcohol exposure and serotonin transporter gene promoter polymorphism affect neonatal temperament and limbic-hypothalamic-pituitary-adrenal axis regulation in monkeys. *Biol. Psychiatry* 63, 317–324. doi: 10.1016/j.biopsych.2007.07.017

Kuhn, P., and Sarkar, D. K. (2008). Ethanol induces apoptotic death of β-endorphin neurons in the rat hypothalamus by a TGF-β1-dependent mechanism. *Alcohol Clin. Exp. Res.* 32, 706–714. doi: 10.1111/j.1530-0277.2008.00627.x

Kvigne, V. L., Leonardson, G. R., Borzelleca, J., and Welty, T. K. (2008). Characteristics of grandmothers who have grandchildren with fetal alcohol syndrome or incomplete fetal alcohol syndrome. *Matern. Child Health J.* 12, 760–765. doi: 10.1007/s10995-007-0308-y

Langevin, F., Crossan, G. P., Rosado, I. V., Arends, M. J., and Patel, K. J. (2011). Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. *Nature* 475, 53–58. doi: 10.1038/nature10192

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.* 3, 662–673. doi: 10.1038/nrg887

Li, H., Gu, S., Han, Y., Xu, Z., Pakstis, A. J., Jin, L., et al. (2011). Diversification of the ADH1B gene during expansion of modern humans. *Ann. Hum. Genet.* 75, 497–507. doi: 10.1111/j.1469-1809.2011.00651.x

Liu, Y., Balaraman, Y., Wang, G., Nephew, K. P., and Zhou, F. C. (2009). Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4, 500–511. doi: 10.4161/epi.4.7.9925

Lombard, Z., Tiffin, N., Hofmann, O., Bajic, V. B., Hide, W., and Ramsay, M. (2007). Computational selection and prioritization of candidate genes for fetal alcohol syndrome. *BMC Genomics* 8:389. doi: 10.1186/1471-2164-8-389

Lu, S. C., Huang, Z. Z., Yang, H., Mato, J. M., Avila, M. A., and Tsukamoto, H. (2000). Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 279, G178–G185.

Lukiw, W. J. (2013). Variability in micro RNA (miRNA) abundance, speciation and complexity amongst different human populations and potential relevance to Alzheimer's disease (AD). *Front. Cell. Neurosci.* 7:133. doi: 10.3389/fncel.2013.00133

Maunakea, A. K., Nagarajan, R. P., Bilenky, M., Ballinger, T. J., D'Souza, C., Fouse, S. D., et al. (2010). Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* 466, 253–257. doi: 10.1038/nature09165

May, P. A., and Gossage, J. P. (2011). Maternal risk factors for fetal alcohol spectrum disorders: not as simple as it might seem. *Alcohol Res. Health* 34, 15–26.

May, P. A., Blankenship, J., Marais, A. S., Gossage, J. P., Kalberg, W. O., Barnard, R., et al. (2013). Approaching the prevalence of the full spectrum of fetal alcohol spectrum disorders in a South African population-based study. *Alcohol Clin. Exp. Res.* 37, 818–830. doi: 10.1111/acer.12033

Maze, I., and Nestler, E. J. (2011). The epigenetic landscape of addiction. *Ann. N. Y. Acad. Sci.* 1216, 99–113. doi: 10.1111/j.1749-6632.2010.05893.x

McCarthy, N., and Eberhart, J. K. (2014). Gene-ethanol interactions underlying fetal alcohol spectrum disorders. *Cell Mol. Life Sci.* doi: 10.1007/s00018-014-1578-3 [Epub ahead of print].

Miranda, R. C. (2012). MicroRNAs and fetal brain development: implications for ethanol teratology during the second trimester period of neurogenesis. *Front. Genet.* 3:77. doi: 10.3389/fgene.2012.00077

Momino, W., Félix, T. M., Abeche, A. M., Zandoná, D. I., Scheibler, G. G., Chambers, C., et al. (2012). Maternal drinking behavior and fetal alcohol spectrum disorders in adolescents with criminal behavior in southern Brazil. *Genet. Mol. Biol.* 35(Suppl. 4), 960–965. doi: 10.1590/S1415-47572012000600011

Morison, I. M., and Reeve, A. E. (1998). A catalogue of imprinted genes and parent-of-origin effects in humans and animals. *Hum. Mol. Genet.* 7, 1599–1609. doi: 10.1093/hmg/7.10.1599

Nestler, E. (2014). Epigenetic mechanisms of drug addiction. *Neuropharmacology* 76B, 259–268. doi: 10.1016/j.neuropharm.2013.04.004

Osier, M. V., Pakstis, A. J., Kidd, J. R., Lee, J. F., Yin, S. J., Ko, H. C., et al. (1999). Linkage disequilibrium at the ADH2 and ADH3 loci and risk of alcoholism. *Am. J. Hum. Genet.* 64, 1147–1157. doi: 10.1086/302317

Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., et al. (2000). Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.* 10, 475–478. doi: 10.1016/S0960-9822(00)00448-6

Ouko, L. A., Shantikumar, K., Knezovich, J., Haycock, P., Schnugh, D. J., and Ramsay, M. (2009). Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: implications for fetal alcohol spectrum disorders. *Alcohol Clin. Exp. Res.* 33, 1615–1627. doi: 10.1111/j.1530-0277.2009.00993.x

Pal-Bhadra, M., Bhadra, U., Jackson, D. E., Mamatha, L., Park, P., and Shukla, S. D. (2007). Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- and down-regulation of genes by ethanol in hepatocytes. *Life Sci.* 81, 979–987. doi: 10.1016/j.lfs.2007.07.030

Pandey, S., Rajesh, U., Zhang, H., Tang, L., and Prakash, A. (2008). Brain chromatin remodeling: a novel mechanism of alcoholism. *J. Neurosci.* 28, 3729–3737. doi: 10.1523/JNEUROSCI.5731-07.2008

Plotsky, P. M. (1986). Opioid inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation of rats. *Reg. Peptides* 16, 235–242. doi: 10.1016/0167-0115(86)90022-4

Ponomarev, I. (2013). Epigenetic control of gene expression in the alcoholic brain. *Alcohol Res.* 35, 69–76.

Pritchard, L. E., Turnbull, A. V., and White, A. (2002). Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signaling and obesity. *J. Endocrinol.* 172, 411–421. doi: 10.1677/joe.0.1720411

Rachdaoui, N., and Sarkar, D. K. (2013). Effects of alcohol on the endocrine system. *Endocrinol. Metab. Clin. N. Am.* 42, 593–615. doi: 10.1016/j.ecl.2013.05.008

Raffin-sanson, M. L., de Keyser, Y., and Bertagna, X. (2003). Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *Euro J. Endocrinol.* 149, 79–90. doi: 10.1530/eje.0.1490079

Renthal, W., and Nestler, E. J. (2009). Chromatin regulation in drug addiction and depression. *Dialog Clin. Neurosci.* 11, 257–268.

Rodgers, A. B., Morgan, C. P., Bronson, S. L., Revello, S., and Bale, T. L. (2013). Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *J. Neurosci.* 33, 9003–9012. doi: 10.1523/JNEUROSCI.0914-13.2013

Sakharkar, A. J., Zhang, H., Tang, L., Shi G., and Pandey, S. C. (2012). Histone deacetylases (HDAC)-induced histone modifications in the amygdala: a role in rapid tolerance to the anxiolytic effects of ethanol. *Alcohol Clin. Exp. Res.* 36, 61–71. doi: 10.1111/j.1530-0277.2011.01581.x

Sampson, P. D., Streissguth, A. P., Bookstein, F. L., Little, R. E., Clarren, S. K., Dehaene, P., et al. (1997). Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology* 56, 317–326. doi: 10.1002/(SICI)1096-9926(199711)56:5<317::AID-TERA5>3.0.CO;2-U

Sarkar, D. K. (1996). Neuroendocrine – immune axis of alcoholics. Symposium. *Alcohol Clin. Exp. Res.* 20, 256A-259A.

Sarkar, D. K., Kuhn, P., Marano, J., Chen, C., and Boyadjieva, N. (2007). Alcohol exposure during the developmental period induces ß-endorphin neuronal death and causes alteration in the opioid control of stress axis function. *Endocrinology* 148, 2828–2834. doi: 10.1210/en.2006-1606

Sarkar, D. K., Murugan, S., Zhang, C., and Boyadjieva, N. (2012). Regulation of cancer progression by β-endorphin neuron. *Cancer Res.* 72, 836–840. doi: 10.1158/0008-5472.CAN-11-3292

Saxonov, S., Berg, P., and Brutlag, D. L. (2006). A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1412–1417. doi: 10.1073/pnas.0510310103

Schier, A. F. (2007). The maternal-zygotic transition: death and birth of RNAs. *Science* 316, 406–407. doi: 10.1126/science.1140693

Schneider, M. L., Moore, C. F., Kraemer, G. W., Roberts, A. D., and DeJesus, O. T. (2002). The impact of prenatal stress, fetal alcohol exposure, or both on development: perspectives from a primate model. *Psychoneuroendocrinology* 27, 285–298. doi: 10.1016/S0306-4530(01)00050-6

Schneider, M. L., Moore, C. F., Larson, J. A., Barr, C. S., DeJesus, O. T., and Roberts, A. D. (2009). Timing of moderate level prenatal alcohol exposure influences gene expression of sensory processing behavior in rhesus monkeys. *Front. Integr. Neurosci.* 3:30. doi: 10.3389/neuro.07.030.2009

Sharma, R. P., Grayson, D. R., and Gavin, D. P. (2008). Histone deacetylase 1 expression is increased in the prefrontal cortex of schizophrenia subjects: analysis of the National Databank microarray collection. *Schizophr. Res.* 98, 111–117. doi: 10.1016/j.schres.2007.09.020

Sharma, H. K., Tripathi, B. M., and Pelto, P. J. (2010). The evolution of alcohol use in India. *AIDS Behav.* 14(Suppl. 1), S8-S17.

Shi, L., and Wu, J. (2009). Epigenetic regulation in mammalian preimplantation embryo development. *Reprod. Biol. Endocrinol.* 7, 59. doi: 10.1186/1477-7827-7-59

Singh, P. K., and Campbell, M. J. (2013). The interactions of microRNA and epigenetic modifications in prostate cancer. *Cancers (Basel)* 5, 998–1019. doi: 10.3390/cancers5030998

Stockard, C. (1913). The effect on the offspring of intoxicating the male parent and the transmission of the defects to subsequent generations. *Am. Nat.* 47, 641–682. doi: 10.1086/279379

Streissguth, A. P., and Dehaene, P. (1993). Fetal alcohol syndrome in twins of alcoholic mothers: concordance of diagnosis and IQ. *Am. J. Med. Genet.* 47, 857–861. doi: 10.1002/ajmg.1320470612

Sun, G., Yan, J., Noltner, K., Feng, J., Li, H., Sarkis, D. A., et al. (2009). SNPs in human miRNA genes affect biogenesis and function. *RNA* 15, 1640–1651. doi: 10.1261/rna.1560209

Tapocik, J. D., Solomon, M., Flanigan, M., Meinhardt, M., Barbier, E., Schank, J. R., et al. (2013). Coordinated dysregulation of mRNAs and microRNAs in the rat medial prefrontal cortex following a history of alcohol dependence. *Pharmacogenomics J.* 13, 286–296. doi: 10.1038/tpj.2012.17

Thomas, J. D., Biane, J. S., O'Bryan, K. A., O'Neill, T. M., and Dominguez, H. D. (2007). Choline supplementation following third-trimester equivalent alcohol exposure attenuates behavioral alterations in rats. *Behav. Neurosci.* 121, 120–130. doi: 10.1037/0735-7044.121.1.120

Thomas, J. D., Idrus, N. M., Monk, B. R., and Dominquez, H. D. (2010). Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. *Birth Defect. Res. A Clin. Mol. Teratol.* 88, 827–837. doi: 10.1002/bdra.20713

Thomas, J. D., and Tran, T. D. (2012). Choline supplementation mitigates trace, but not delay, eyeblink conditioning deficits in rats exposed to alcohol during development. *Hippocampus* 22, 619–630. doi: 10.1002/hipo.20925

Thornburg, K. L., Shannon, J., Thuillier, P., and Turker, M. S. (2010). In utero life and epigenetic predisposition for disease. *Adv. Genet.* 71, 57–78. doi: 10.1016/B978-0-12-380864-6.00003-1

Ungerer, M., Knezovich, J., and Ramsay, M. (2013). In utero alcohol exposure, epigenetic changes, and their consequences. *Alcohol Res.* 35, 37–46.

Veazey, K. J., Carnahan, M. N., Muller, D., Miranda, R. C., and Golding, M. C. (2013). Alcohol-induced epigenetic alterations to developmentally crucial genes regulating neural stemness and differentiation. *Alcohol. Clin. Exp. Res.* 37, 1111–1122. doi: 10.1111/acer.12080

Wang, L. L., Zhang, Z., Li, Q., Yang, R., Pei, X., Xu, Y., et al. (2009). Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Hum. Reprod.* 24, 562–579. doi: 10.1093/humrep/den439

Warren, K. R., and Li, T. K. (2005). Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Res. A Clin. Mol. Teratol.* 73, 195–203. doi: 10.1002/bdra.20125

Wozniak, J. R., Fuglestad, A. J., Eckerle, J. K., Kroupina, M. G., Miller, N. C., Boys, C. J., et al. (2013). Choline supplementation in children with fetal alcohol spectrum disorders has high feasibility and tolerability. *Nutr. Res.* 33, 897–904. doi: 10.1016/j.nutres.2013.08.005

Wynne, O., and Sarkar, D. K. (2013). "Stress and neuroendocrine–immune interaction: a therapeutic role for β-endorphin," in *The Wiley-Blackwell Handbook of Psychoneuroimmunology*, eds A. W. Kusnecov and H. Anisman (Chichester: John Wiley & Sons Ltd), 198–211. doi: 10.1002/9781118314814.ch10

Zeisel, S. H. (2004). Nutritional importance of choline for brain development. *J. Am. Coll. Nutr.* 23, 621S–626S. doi: 10.1080/07315724.2004.10719433

Zeisel, S. H. (2006). Choline: critical role during fetal development and dietary requirements in adults. *Annu. Rev. Nutr.* 26, 229–250. doi: 10.1146/annurev.nutr.26.061505.111156

Zhong, L., Zhu, J., Lv, T., Chen, G., Sun, H., Yang, X., et al. (2010). Ethanol and its metabolites induce histone lysine 9 acetylation and an alteration of the expression of heart development-related genes in cardiac progenitor cells. *Cardiovasc. Toxicol.* 10, 268–274. doi: 10.1007/s12012-010-9081-z

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Hypothesis: genetic and epigenetic risk factors interact to modulate vulnerability and resilience to FASD

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Fetal alcohol spectrum disorder (FASD) presents a collection of symptoms representing physiological and behavioral phenotypes caused by maternal alcohol consumption. Symptom severity is modified by genetic differences in fetal susceptibility and resistance as well as maternal genetic factors such as maternal alcohol sensitivity. Animal models demonstrate that both maternal and paternal genetics contribute to the variation in the fetus' vulnerability to alcohol exposure. Maternal and paternal genetics define the variations in these phenotypes even without the effect of alcohol *in utero*, as most of these traits are polygenic, non-Mendelian, in their inheritance. In addition, the epigenetic alterations that instigate the alcohol induced neurodevelopmental deficits can interact with the polygenic inheritance of respective traits. Here, based on specific examples, we present the hypothesis that the principles of non-Mendelian inheritance, or "exceptions" to Mendelian genetics, can be the driving force behind the severity of the prenatal alcohol-exposed individual's symptomology. One such exception is when maternal alleles lead to an altered intrauterine hormonal environment and, therefore, produce variations in the long-term consequences on the development of the alcohol-exposed fetus. Another exception is when epigenetic regulation of allele-specific gene expression generates disequilibrium between the maternal vs. paternal genetic contributions, and thereby, modifies the effect of prenatal alcohol exposure on the fetus. We propose that these situations in which one parent has an exaggerated influence over the offspring's vulnerability to prenatal alcohol are major contributing mechanisms responsible for the variations in the symptomology of FASD in the exposed generation and beyond.

Keywords: prenatal ethanol, strain differences, allele specific expression, rat, thyroid hormones, second generation

INTRODUCTION

Alcohol consumption during pregnancy can result in fetal alcohol spectrum disorder (FASD), a constellation of disabilities including deficient pre- and postnatal growth, morphological malformations of the face and/or brain, and cognitive and behavioral deficits (Manning and Eugene Hoyme, 2007). These teratological outcomes vary significantly among individuals with respect to range and severity, even after allowing for the effects of timing, duration, and amount of alcohol exposure. This strongly suggests that genetic vulnerability may contribute to the etiology of FASD. We hypothesize that genetic sensitivity of the mother and the fetus to the direct and indirect effects of alcohol on epigenetically regulated genes leads to the individual variations observed in the severity of FASD symptoms.

It has been shown in mouse models that the same maternal alcohol exposure protocol in different inbred mouse strains results in a spectrum of affectedness from severe malformations to no teratogenesis, depending on the strain (Downing et al., 2009). Thus, vulnerability to fetal alcohol, as manifested in specific phenotypes, could be analyzed as a quantitative trait. Few studies have attempted this in mouse (Browman and Crabbe, 2000; Anthony et al., 2010; Chen et al., 2011; Downing et al., 2012), and we are unaware of any human studies trying to map loci for fetal alcohol vulnerability using a non-candidate gene approach. The difficulty lies in part in mapping disease genes for complex traits *per se*. In addition, the vulnerability to fetal alcohol can differ for each of the many different endophenotypes, ranging from gross morphological effects to subtle neurobehavioral changes, and these specific endophenotypes most likely have differing polygenic contributions. Another reason for the difficulty to identify genetic contribution to FASD is that genetic and epigenetic effects in FASD are highly interactive, defying simple associations (Rakyan et al., 2011; Liu et al., 2013). Therefore, animal models present important opportunities for discovering new candidate mechanisms and pathways toward the understanding of the etiology of FASD.

NON-CANONICAL MATERNAL AND PATERNAL GENETIC CONTRIBUTIONS

PROGRAMMING OF OFFSPRING'S HEALTH BY IN UTERO ENVIRONMENT: MATERNAL GENETIC EFFECTS

We argue that fetal programming influenced by the *in utero* environment can interact with genetic sources of vulnerability. Fetal programming is "epigenetic" rather than genetic since it affects F1 phenotypes via *in utero* programming rather than by inherited DNA sequence *per se*. In a series of classic studies conducted in the

early 1990s, Barker and his group observed the first "fetal origin of adult disease" phenomenon, whereby the prenatal environment influences the phenotype of adult offspring (Barker et al., 1989, 1990, 1993). The authors revealed a negative correlation between size at birth and future incidence of cardiovascular and metabolic disease, including hypertension (Barker et al., 1990), ischemic heart disease (Barker et al., 1989), and non-insulin dependent diabetes (Barker et al., 1993). The association between lowered fetal and infant weight and subsequent type 2 diabetes, hypertension, and hyperlipidemia was confirmed in two individual cohorts born during different time periods (Barker et al., 1993). They also hinted at the mechanisms, suggesting that in the face of poor early nutrition, the fetus undergoes endocrine and metabolic fetal adaptations.

Fetal programing by alcohol includes changes in maternal and fetal hormone levels that exert long-lasting consequences. For example, increased or decreased levels of glucocorticoids of the pregnant dams with or without *in utero* alcohol exposure can affect the neuroendocrine stress-response of the progeny (Mcgivern and Redei, 1994; Slone and Redei, 2002; Wilcoxon et al., 2003; Glavas et al., 2007; Hellemans et al., 2010; Brunton and Russell, 2011; Liang et al., 2011). Our work illustrates that maternal thyroid hormones can superimpose additional phenotypic consequences to the underlying genetic susceptibilities to prenatal alcohol inherent in specific strains of rats (Sittig et al., 2011b). We have shown that Sprague Dawley (S) rat dams have lower plasma triiodothyronine (T3) and higher thyroid stimulating hormone (TSH) levels than Brown Norway (B) dams (**Figure 1**) (Sittig and Redei, 2010). Moreover, the thyroid function of S dams is more labile, as shown by their increased T3/logTSH ratio after alcohol consumption, in contrast to the stable measures in the B dams. S dams on alcohol-containing diet also show significantly lower plasma free T4 levels compared to those of B dams (Sittig and Redei, 2010). This maternal genetic susceptibility makes their fetus exceedingly vulnerable by lowering *in utero* free thyroxine (T4) levels, which are relevant and critical for fetal brain development. Supplementation of the vulnerable S dam with T4 during alcohol consumption ameliorates the memory deficits observed in adult offspring (**Figure 2**) (Wilcoxon et al., 2005), supporting this premise. Thus, decreased levels of maternal T4, reduced by alcohol consumption in combination with the alleles for the *a priori* lower thyroid hormones in the S mother, are risk factors for the developing fetus. Although this vulnerability is the consequence of maternal genetic differences, it is epigenetic in terms of the mechanism by which it ultimately affects the fetus.

The interaction between *in utero* environmental challenges and maternal genetic effects can result in increased vulnerability or relative resilience to these challenges, as illustrated above. Studies of genetic vulnerability to alcohol exposure in mice have cited maternal genetic effects as playing an important role in the vulnerability of offspring (Gilliam and Irtenkauf, 1990; Gilliam et al., 1997; Downing et al., 2009). Such effects were seen when progeny of reciprocal crosses of two differentially susceptible strains showed differences in the consequences of exposure to prenatal alcohol, based on the maternal strain (Gilliam and Irtenkauf, 1990). Although these offspring are genetically identical, their maternal strain has a strong influence on fetal vulnerability to the environmental (alcohol exposure) insult. Similarly, we found that offspring of the vulnerable alcohol-consuming S dams show social behavioral deficits, while the genetically identical offspring of B dams do not (Sittig et al., 2011b).

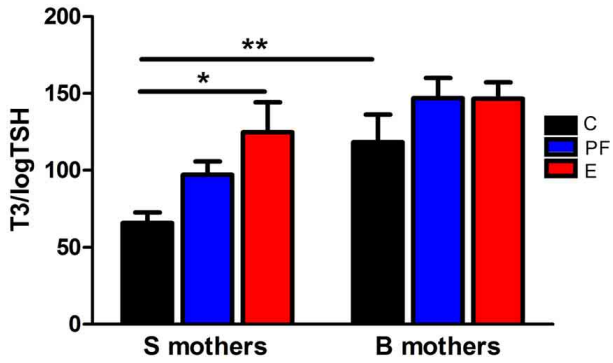

FIGURE 1 | Pregnant Sprague Dawley (S) and Brown Norway (B) dams differ in their thyroid function and thyroid hormonal response to alcohol. Diet administration and prenatal diet groups are routinely used in our lab and are the same throughout the following experiments. Female S or B dams were divided into 3 prenatal treatment groups: C, control; PF, pair-fed; and E, ethanol. Control dams were kept on conventional laboratory chow *ad libitum* throughout their pregnancy. The PF and E treatment groups received liquid diet (Lieber-DeCarli'82; Bio-Serv. Frenchtown, NJ, USA) starting at gestational day (GD) 4. Ethanol diet began at GD8, and from GD8 to 10 the percentage of E in the diet was increased until it reached 5% (w/v) and then was kept constant until GD20. Each individual PF dam received a liquid diet that was isocaloric to the amount consumed by an individual E dam on the previous GD. On GD21, plasma levels of total T3 and TSH were measured by radioimmunoassay as previously described (Sittig and Redei, 2010). The T3/logTSH ratio was derived as a measure of thyroid function. The T3/logTSH ratios were lower in S mothers than B mothers [strain $F_{(1,\ 51)} = 13.94$, $p < 0.01$] and alcohol consumption significantly altered this ratio [diet $F_{(2,\ 51)} = 5.04$, $p < 0.05$]. Data were analyzed by Two-Way ANOVA; Bonferroni *post-hoc* test results are shown. Values are mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$, N = 9–11/group. This figure is modified from Sittig and Redei (2010).

PATERNAL GENETIC EFFECTS

The ability to experimentally observe the paternal influence requires two different paternal strains, and that the two strains used in the experiment be phenotypically different for the trait being studied. An important paradigm for studying maternal vs. paternal genetic effects is the reciprocal F1 design, using one strain of rat as the alcohol-consuming mother (S or B) and varying the strain used as the father, B or S, respectively (**Figure 3**). Evidence from this paradigm has shown the extent of paternal influence on fetal vulnerability. Specifically, BS F1 fetuses (maternal strain is first) are more vulnerable than BB fetuses to the alcohol-induced decrease in fetal body weight (Sittig and Redei, 2010). Although the specific genes/alleles underlying fetal body weight deficit vulnerability remain to be elucidated, the above experiment provides proof that this particular vulnerability is subject to paternal genetic influence. Moreover, it is worth noting that since the paternal strain

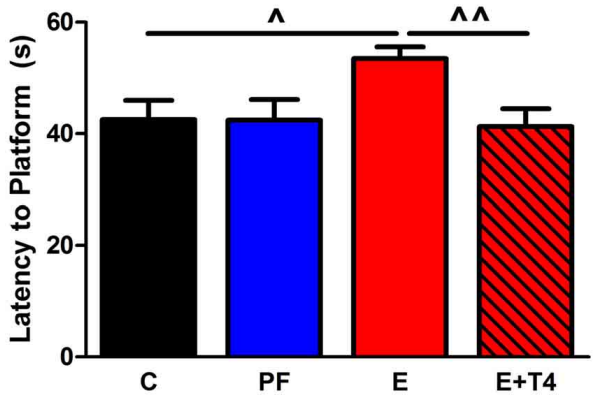

FIGURE 2 | Thyroxine (T4, 30 μg/ml) in the ethanol-containing liquid diet during gestation ameliorates spatial learning/memory deficits in adult offspring of Sprague Dawley (S) dams. Adult male and female SS offspring (80–90 days old) of C, PF, E and ethanol+thyroxine (E+T4) were trained in the Morris Water Maze to locate a hidden platform. The E+T4 group received 30 μg/ml T4 (Sigma-Aldrich Co, St Louis, MO, USA) in the E-containing liquid diet, which, based on the daily diet consumption, is equivalent to approximately 3 mg/100 gBW/day of T4. Training consisted of four trials daily for six consecutive days. No sex differences were found, so male and female data were combined. In the last day of the test, E adult offspring still showed higher latency to reach the platform, which was reversed by maternal T4 supplementation [$F_{(3,\ 23)} = 3.26$, $p < 0.05$]. Data were analyzed by One-Way ANOVA; hypothesis testing is by Student *t*-test shown in the figure. Values are mean ± s.e.m. ^$p < 0.05$, ^^$p < 0.01$, $N = 6$/group. This figure is modified from Wilcoxon et al. (2005).

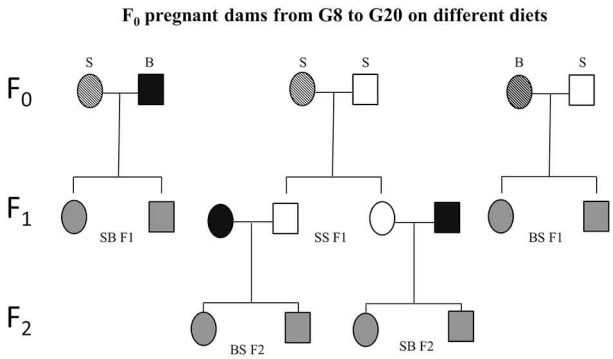

FIGURE 3 | Schematic experimental design. Sprague Dawley (S) or Brown Norway (B) females (F_0) were mated with either S or B males. From gestational day 8 (G8) through 20 (G20), these dams were exposed to one of four prenatal treatments C, PF, E, or E+0.3 μg/ml T4. Based on the daily diet consumption, dams receive the equivalent of 8 μg/100 gBW/day of T4 in the E+T4 group. The resulting SS, SB, and BS (maternal strain first) first generation (F1) offspring were employed in the different tests and/or mated. Experimentally naïve SS F1 offspring of all four treatment groups were mated with naïve male and female B mates. The dams used to generate the BS F2 and SB F2 offspring did not consume ethanol while pregnant. Circles designate females and squares designate males. Open fill are S, black fill are B rats, and gray fill indicate F1 or F2 crosses between B and S. A strip pattern indicates alcohol consumption during pregnancy.

significantly influences the body weight of offspring, a measure of vulnerability that was previously thought to depend mostly on how much the mother drinks and her investment of nutritional resources in the fetus, paternal genetic influence could be considerable in other FASD-related phenotypes as well.

Further effects of paternal genetic background can be seen in the differences in activity levels between SS and SB F1 males in the open field test (OFT). The activity levels, as measured by the OFT show that there is a paternal genetic effect. SB F1 males exposed to prenatal alcohol show hypoactivity in the OFT (as measured by distance moved), when compared to their genetically identical control, PF and T4 treated cohort. This decrease in activity is not observed in SS F1 cohort, as there are no treatment effects in this genetic background (**Figure 4A**). In contrast, SS F1 E females showed hyperactivity compared to their nutritional control, while SB F1 females did not show this phenotype (**Figure 4B**). Since the maternal *in utero* environment is the same for these two sets of F1s, their differential response to prenatal alcohol can be attributed to the vulnerability conferred by the paternal genetic influence. Interestingly, both of these alterations in activity induced by prenatal alcohol were reversed by administration of a low dose (0.3 μg/ml) T4 to the alcohol-consuming pregnant dam.

EPIGENETIC VULNERABILITY

Genomic imprinting is an epigenetic phenomenon that affects physiological and behavioral programming of the offspring. It is defined as the differential expression of the maternal and paternal alleles for particular gene(s) in certain genomic loci and thought to be controlled primarily by combinations of epigenetic modifications (i.e., DNA methylation, expression of various types of noncoding RNAs and histone tail modifications, such as acetylation, methylation etc.) (Delaval and Feil, 2004; Mazzio and Soliman, 2012; Seisenberger et al., 2013). The epigenetic marks that maintain the differential allelic expression can be sex-, developmental stage-, and tissue-specific. In addition to the placenta, the brain has been shown to exhibit enriched imprinting (Gregg et al., 2010). These paternal and maternal epigenetic "imprints" are created during gametogenesis and carried in sperm and oocytes, respectively. Immediately after conception, DNA methylation marks on the parental gametes are erased in two waves of de-methylation. First, the paternal pronucleus undergoes rapid de-methylation in the zygote followed by a passive loss of DNA methylation marks in the maternal genome. Subsequently a wave of global re-methylation occurs in the early embryo, whereby different cell lineages are re-methylated appropriately, but often differently. DNA methylation at the differentially methylated regions of imprinted genes are reset in primordial germ cells but are protected from reprogramming in the early embryo (Seisenberger et al., 2013; Skinner et al., 2013). The time frame of both de-methylation and re-methylation differs between male and female embryos, providing the possibility for sex-specific imprinting differences to occur (Reik et al., 2001; Seisenberger et al., 2013).

During re-methylation, the embryo is more sensitive to environmental perturbations that affect the methylation status at important regulatory loci (Feil and Fraga, 2011). For example, humans prenatally exposed to the Dutch "hunger winter" famine

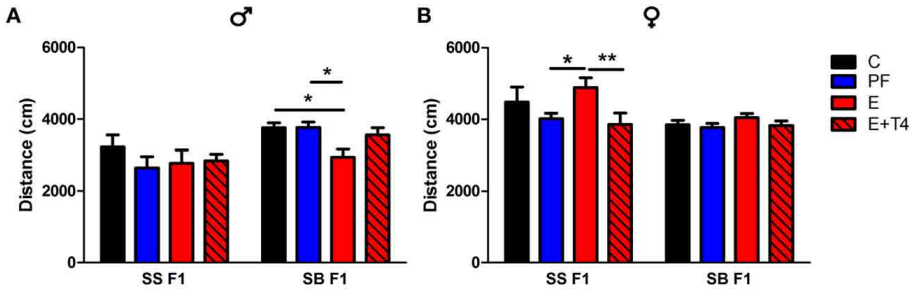

FIGURE 4 | Prenatal alcohol affects activity of adult offspring depending on paternal genetic background in the open field test. Adult male **(A)** and female **(B)** rats of the SS and SB genetic backgrounds (see **Figure 3**) were exposed to four prenatal treatments (C, PF, E, or E+0.3 μg/ml T4) as described previously (Tunc-Ozcan et al., 2013). They were tested in a novel, 82 cm diameter circular open field arena with a light intensity of 60 lux for 10 min. Distance traveled in cm was analyzed from recordings by TSE videomot 2 software (version 5.75, Bad Homburg, Germany). In males, total distance traveled was decreased by *in utero* ethanol exposure only if the paternal genetic background is B [$F_{(1,\ 80)} = 14.14$, $p < 0.01$], while in females, increased activity with *in utero* ethanol exposure was found only with the paternal genetic background of S [$F_{(1,\ 79)} = 9.38$, $p < 0.01$]. Data were analyzed by Two-Way ANOVA, Bonferroni *post-hoc* results are shown. Values are mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$, $N = 6$–18/group.

of 1944, showed decreased DNA methylation at the differentially methylated region associated with insulin-like growth factor 2 (*IGF2*). This altered methylation was detected approximately six decades after the original environmental insult (Heijmans et al., 2008), which directly illustrates the power of environmental insults to induce long-term, physiologically-relevant epigenetic changes. A growing body of literature implicates alcohol as a potent epigenetic modifier during prenatal development. Fetal alcohol exposure alters genomic imprinting at the *H19-Igf2* locus (Downing et al., 2011; Stouder et al., 2011; Knezovich and Ramsay, 2012) and at *Rasgrf1* (Knezovich and Ramsay, 2012). In addition, it is implicated in long-lasting alterations in DNA methylation in imprinted domains that harbor non-coding RNAs (Balaraman et al., 2013; Laufer et al., 2013). Together, these data support the "fetal origin of adult disease" hypothesis predicted by Barker (2004).

Here we will provide examples from our own work, which illustrate that small, brain-regional variations in complex gene expression patterns can influence the severity of outcome in an FASD model.

CHANGES IN ALLELIC GENE EXPRESSION: BRAIN REGION- AND SEX-SPECIFICITY

Since imprinted genes are epigenetically regulated, they are particularly vulnerable to disruptions induced by alcohol exposure during development (Haycock, 2009). Knowing this, we tracked maternal and paternal expression of the maternally imprinted gene that encodes the thyroid metabolizing enzyme deiodinase 3 (Dio3) in several brain regions of the SB F1 rat offspring (Sittig et al., 2011b). Our analysis shows a switch from primarily paternal expression in the fetal frontal cortex to slightly elevated maternal expression in the fetal hippocampus (**Figure 5A**) (Sittig et al., 2011a,b). Furthermore, transcript levels of *Dio3* are significantly influenced by maternal alcohol consumption, but in the opposite direction in these brain regions. Curiously, this brain region-specific difference in allelic expression of *Dio3* strengthens in adulthood (**Figure 5B**) (Sittig et al., 2011b). Specifically, while *Dio3* expression becomes mostly biallelic in the frontal cortex, it becomes clearly maternal in the hippocampus. Furthermore, fetal alcohol exposure exacerbates maternal-specific expression, thereby conferring a subsequent decrease in paternal contribution in the hippocampus. This allele-specific expression is accompanied by a corresponding decrease in *Dio3* enzyme levels and an increase in T3 levels. T3 is a substrate of *Dio3* (**Figure 5B**). These effects are sex-specific, occurring only in adult males, but not females (Sittig et al., 2011b). The patterns of changes in hippocampal thyroid hormone levels correspond to hippocampus-based deficits in social behavior and memory in males only (Sittig et al., 2011b).

Showing that relative allelic expression levels (maternal vs. paternal) of one gene can be tuned across brain regions leads to intriguing questions about the forces that shape differential evolution of gene expression control across brain regions. Should a certain brain region be favored by one parent or the other in control over expression of a specific imprinted gene, the consequences could include the biased inheritance of functions orchestrated by that brain region. This implies that parental bias can be transferred to the germ cells and that any epigenetic modification affecting the expressed allele could affect brain function of the progeny. If many genes are biased to maternal expression in the hippocampus, such as *Dio3*, then the hippocampus might be a site where maternal genetic influence trumps that of the father. In cases where the mother has known to have deleterious sequence variations, this could lead to predictions about hippocampus-specific functional deficits. In this way, these "exceptional" imprinted genes that give rise to highly tunable and therefore highly vulnerable gene expression patterns could be the ones that have the potential to influence vulnerability the most.

SECOND GENERATIONAL EFFECTS OF ALCOHOL: MATERNAL vs. PATERNAL TRANSMISSION

Evidence from both human and animal studies indicates that the second generation (F2) progeny incurs consequences of the first generation's exposure to alcohol (Rouleau et al., 2003; Kvigne et al., 2008; Govorko et al., 2012; Harper et al., 2014). These

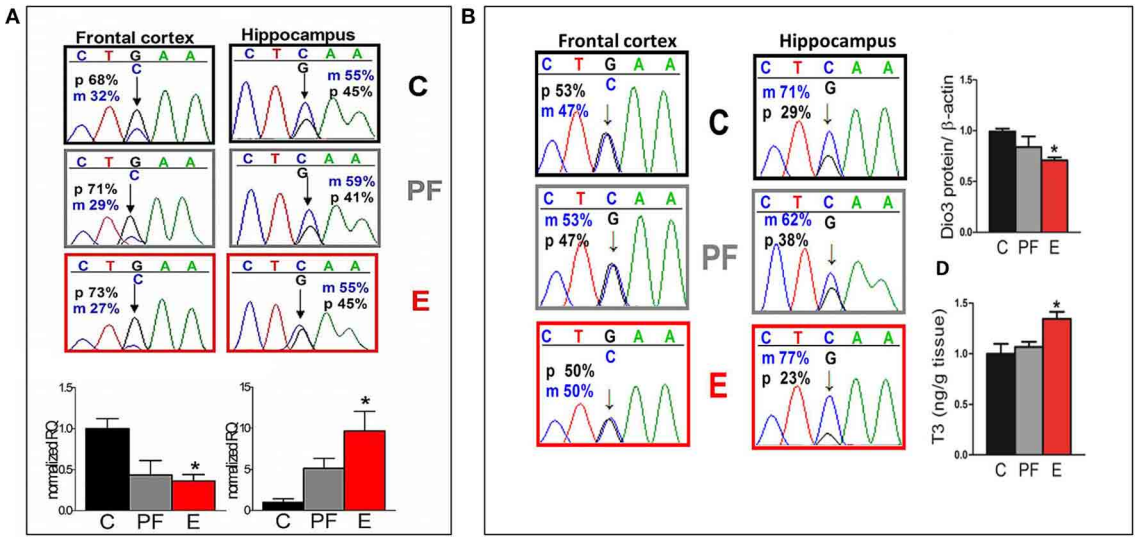

FIGURE 5 | (A) Allele-specific expression of *Dio3* is brain region specific, preferentially paternal in the fetal frontal cortex and slightly maternal in the fetal hippocampus; prenatal alcohol inversely affects expression of total *Dio3* in these regions. Representative sequence traces of *Dio3* transcripts containing the SNP between Sprague Dawley (S) ("C") and Brown Norway (B) ("G") strains in male fetal SB frontal cortices, and hippocampi from three prenatal treatment groups (C, PF, and E). Pyrosequencing means of paternal (p; black = G) and maternal (m; blue = C) allelic *Dio3* ratio are inset onto individual fetal sequence traces. Total *Dio3* mRNA levels were measured by quantitative real-time RT-PCR in male fetal frontal cortices, and hippocampi from the three prenatal treatment groups. Relative quantification values are normalized to the appropriate control group. $N = 3$–7/prenatal treatment/brain region. **(B)** The effects of prenatal alcohol on adult *Dio3* imprinting is hippocampus specific and leads to functional consequences. Representative sequence traces of *Dio3* in adult SB frontal cortices and hippocampi derived from individual male offspring from the three prenatal treatment groups (C, PF, and E). Dio3 protein levels were measured by Western blot and normalized to β-actin levels in the adult SB male hippocampus. Free T3 was measured by radioimmunoassay after extraction from individual samples and the T3/wet tissue weight values (ng/g) were normalized to controls in the adult SB male hippocampus. $N = 4$–6/prenatal treatment/brain region. Data were analyzed by One-Way ANOVA, Bonferroni *post-hoc* results are shown. Values are mean ± s.e.m., $^*p < 0.05$ C vs. E. This figure is modified and shown by permission from Sittig et al. (2011b).

deficits could be transmitted to the next generation through a variety of mechanisms. Inheritance of the phenotype could occur indirectly, through alcohol-induced changes in hormonal programming of the F1 generation that affect their progeny (Govorko et al., 2012; Mead and Sarkar, 2014). In addition, prenatal alcohol exposure could permanently change the epigenetic landscape of the F2 gametes as they are developing in the F1 generation (Mead and Sarkar, 2014), conferring the phenotype on the F2 generation through their reprogrammed gametes. Animal models are proving exceptionally powerful for evaluating intergenerational effects since they allow complete control over environmental exposure. For example, we tested the intergenerational effects of maternal alcohol consumption using a rat model, where S pregnant dams received alcohol-containing liquid diet as described previously (Revskoy et al., 1997; Wilcoxon et al., 2005; Sittig and Redei, 2010), then their SS F1 offspring were allowed to grow to adulthood with no further alcohol exposure. Males and females from the F1 generation were mated to alcohol naive male and female Brown Norway (B) rats to generate matrilinear SB F2 and patrilinear BS F2 progeny (**Figure 3**). Adult offspring of all generations and crosses were tested in a glucose tolerance test (GTT).

Dams consuming E during pregnancy were hyperglycemic and their F1 offspring showed insulin resistance (Harper et al., 2014). Both males and females exposed to alcohol prenatally had hyperglycemic and hyperinsulinemic responses to GTT (**Figures 6A, 7A,B**). However, F2 progeny's responses to GTT varied depending on the sex of the prenatal alcohol exposed parent. Female offspring of males exposed to alcohol or PF diet prenatally showed hyperinsulinemic responses to GTT (**Figure 7C**). As the E did not differ from the PF effect, there was no patrilinear effect of grandmaternal alcohol exposure during pregnancy. In contrast, both male and female SB F2 progeny whose *mother* was exposed to ethanol *in utero* displayed hypoglycemic GTT response patterns (**Figure 6C**). Furthermore, a sex difference was seen in their insulin responses to GTT: male offspring presented hyperinsulinemic responses even though both male and female SB F2 progeny showed a flattened insulin and glucose response to GTT. Therefore, prenatal alcohol-induced dysregulation of glucose metabolism affected the matrilinear next generation because of the SS F1 female offspring's impaired glucose tolerance, which can put their progeny at risk for developing their own metabolic problems due to their intrauterine environment (Eberle and Ament, 2012).

Alternatively, primordial germ cells of the F1 offspring, while *in utero*, undergo alcohol-induced epigenetic changes in genes or genomic loci that contribute to abnormalities of glucose regulation in the F2 generation. Since the BS F2 progeny of SS F1 males do not show specific dysregulation of the GTT responses (**Figures 6B, 7C**), we can conclude that epigenetic changes leading to deficits in the F2 generation seem to be specific to the female F1 fetus. The mechanism of this vulnerability is not

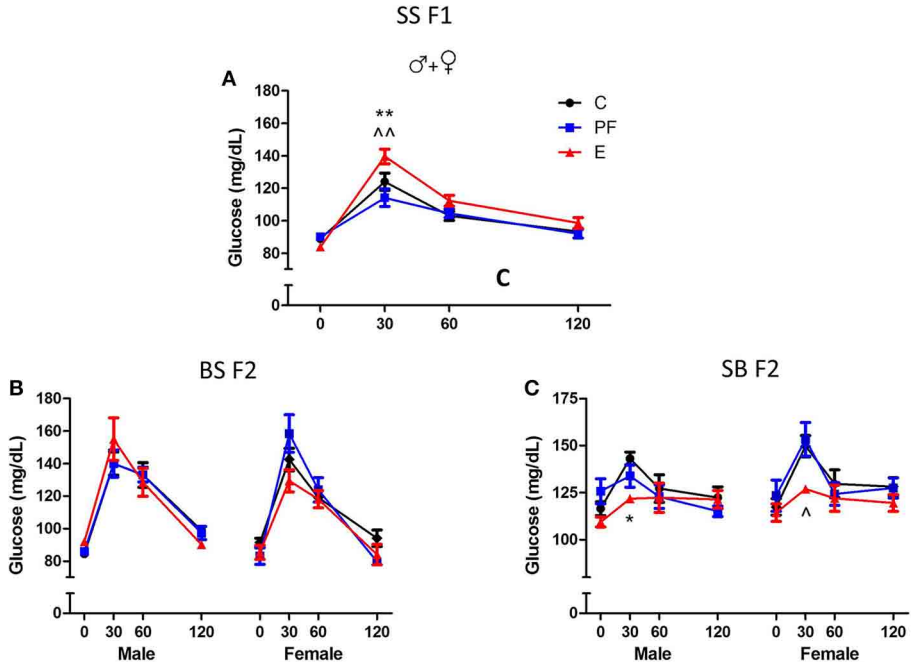

FIGURE 6 | Grandmaternal alcohol consumption during pregnancy affects serum glucose response to glucose tolerance test of grand-offspring via the maternal line. (A) Adult Sprague Dawley (SS F1) male and female rats exposed to alcohol *in utero* showed hyperglycemic glucose responses in the glucose tolerance test (GTT). **(B)** Prenatal alcohol does not affect the BS F2 offspring of naïve B female mated to SS F1 males. **(C)** Male and female SB F2 progeny of SS F1 females exposed to alcohol prenatally, mated to naïve B males, are hypoglycemic. Animals were fasted overnight and blood was collected in the morning before, and 30, 60, and 120 min after an intraperitoneal injection of 2 g/kg body weight dextrose as described previously (Harper et al., 2014). Glucose levels were measured in duplicates by Stanbio glucose liquicolor kit. There were no sex differences in the glucose response to GTT of SS F1 offspring; therefore, male and female data were combined. Statistical analyses were conducted by appropriate ANOVA followed by Bonferonni *post-hoc* tests. $p < 0.05$ *C vs. E, ^PF vs. E, $^{+}$C vs. PF. Data are presented as mean ± s.e.m.; $N = 4$–11/group. This figure includes data from Harper et al. (2014).

known, but is likely to include DNA methylation, since prenatal alcohol exposure induces changes in DNA methylation, and subsequently imprinting (Garro et al., 1991; Kaminen-Ahola et al., 2010; Downing et al., 2011). Future work is currently aimed at determining the epigenetic modifications responsible for the second generational effects of prenatal alcohol exposure, and whether they will transfer to the next, F3 generation.

GENETIC × EPIGENETIC VULNERABILITY

We presented evidence that highly individual and brain region-specific variability in allelic gene expression contributes significantly to the variable consequences of prenatal alcohol exposure observed in an FASD model. We illustrated above an additional layer of complexity, whereby genotype effects the epigenetic regulation of gene expression and the intergenerational transfer of the alcohol-induced endophenotype. These include not only the hippocampal strain-dependent and allele-specific changes in *Dio3* expression after prenatal alcohol exposure in the SB vs. BS F1 offspring, but also the altered glucose metabolism of SB vs. BS F2 progeny. One common denominator is that the SB cross results in a vulnerable offspring, while the BS cross seems to remain resistant to the maternal or grandmaternal effects of alcohol. For the intergenerational transfer of prenatal alcohol-induced deficit, the genetic × epigenetic vulnerability is complicated further by lineage effect due to prenatal alcohol exposure of the mother or the father. These data represents the first example of a genetic susceptibility and resilience based on parent-of-origin effects. It also shows a biological substrate for enhanced vulnerability to specific endophenotypes of FASD present in certain individuals.

POTENTIAL GENETIC BASIS FOR GENETIC × EPIGENETIC INTERACTIONS

What could be the mechanistic basis for such complex patterns of vulnerability to alcohol exposure? We hypothesized that the preferentially maternal expression of *Dio3* in the hippocampus of SB F1 animals vs. the preferentially paternal expression in the BS F1s (Sittig et al., 2011a) is due to sequence variations between the S and B strains at *Dio3* regulatory regions. Thus, alcohol may exaggerate this effect via epigenetic changes resulting in the differential effect of alcohol on hippocampal allelic *Dio3* expression in the SB vs. BS F1 offspring (Sittig et al., 2011b). Since S is the maternal strain in SB, but the paternal strain in BS, parent-specific hippocampal epigenetic marks could be affected by alcohol differently in these reciprocal crosses. To test the sequence variation hypothesis, we first mapped the hitherto unmapped *Dlk1-Dio3* imprinted region in the rat (**Figure 8A**) (Sittig and Redei, 2012). We identified four novel polymorphisms in the *Dio3* promoter region between these strains (**Figure 8A**). Furthermore, F1 offspring generated with another rat strain

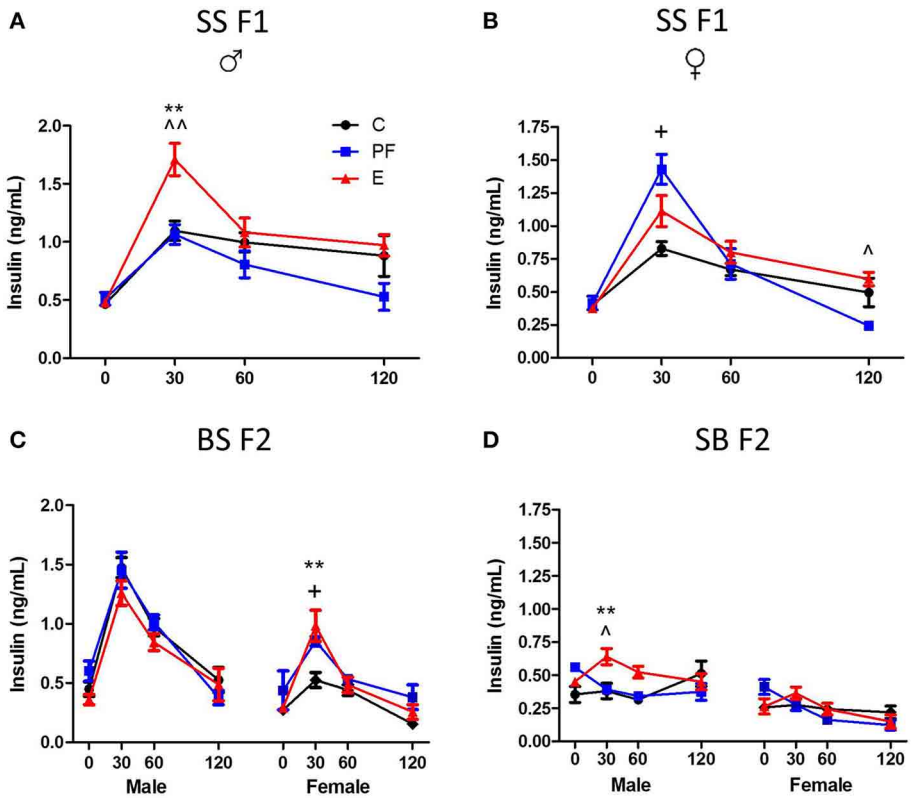

FIGURE 7 | Grandmaternal alcohol consumption during pregnancy affects serum insulin response to glucose tolerance test of grand-offspring via the maternal line. (A) Prenatally alcohol exposed adult SS F1 males have dramatically greater insulin response in the GTT. **(B)** Female SS F1 offspring of dams on pairfed diet, but not on alcohol, are hyperinsulinemic. **(C)** Male BS F2 progeny of grandmaternal treatment groups show no differences in insulin response, while both PF and alcohol diet of grandmothers during gestation leads to hyperinsulinemis GTT profile of females. **(D)** SB F2 male progeny of SS F1 females exposed to alcohol prenatally is also hyperinsulinemic. GTT protocol is as described in **Figure 6** and in Harper et al. (2014). Insulin levels were measured in duplicates by Ultra sensitive rat insulin ELISA kit. Statistical analyses were conducted by appropriate ANOVA followed by Bonferonni *post-hoc* tests. $p < 0.05$ *C vs. E, ^PF vs. E, $^{+}$C vs. PF; **.^^$p < 0.01$. Data are presented as mean ± s.e.m.; $N = 4$–11/group. This figure includes data from Harper et al. (2014).

sharing these polymorphisms with S (**Figure 9**) have the same pattern of exaggerated maternal contribution to hippocampal *Dio3* expression (Sittig and Redei, 2012). Thus, any or all of these polymorphisms within the *Dio3* promoter could contribute to the differential effects of alcohol on SB vs. BS offspring.

In addition to the sequence variation between the S and B strains used for the allele-specific pyrosequencing of *Dio3*, we identified a second C/A SNP in the 3′ untranslated region (UTR) (**Figure 8B**). The 3′ UTR of *Dio3* may bind miRNA differently based on this sequence variation leading to differential allelic expression. To determine whether this SNP could affect the binding of target miRNAs to the 3′UTR of *Dio3*, we input both the S and B alleles as sequences into MicroInspector miRNA binding prediction program. Interestingly, the S allele permitted a binding site for miR-93 whereas the B SNP abolished the putative site. Expression data from microRNA.org indicated that rno-miR-93 (rat miR-93) is expressed, among other tissues, in the embryonic and adult hippocampus. A schematic indicating how miR-93 binding could stabilize the *Dio3* maternal allele for S animals and the paternal allele for B animals in the hippocampus is shown in **Figure 8B**. Positively correlated miRNA-mRNA interactions have been reported previously in the brain (Nunez et al., 2013), and should a similar mechanism be at play in the regulation of *Dio3* allelic expression in the hippocampus, the maternal S allele of SB progeny would be induced over its paternal B allele.

These are some of the many possibilities that may explain the individual susceptibility to FASD that is described and observed in animal models. We argue that there are many more instances where complex, brain region-specific, epigenetically regulated changes in gene expression underlie the manifestation of endophenotypes in FASD on an individual basis. By using animal models to demonstrate individual examples of complex vulnerability, we not only gain a conceptual understanding, but we build a mechanistic understanding of FASD that can be mined for major patterns, alleles, and biological pathways.

WHAT IS NEXT?

One could imagine that a few pathways may emerge as common "hits" where epigenetic and genetic vulnerabilities converge with specific functional consequences that correspond to common

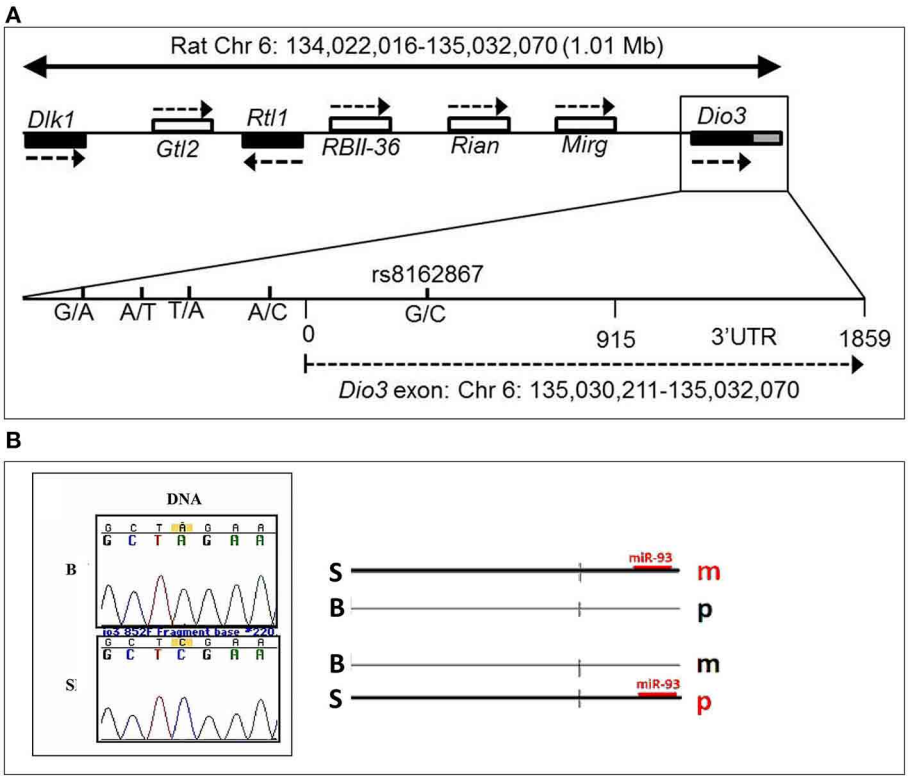

FIGURE 8 | (A) Genomic location of the rat *Dlk1-Dio3* imprinted locus and the *Dio3* gene. Relative position of the paternally expressed genes *Dlk1, Rtl1*, and *Dio3* (filled boxes), and maternally expressed non-coding transcripts *Gtl2*, RBII-36 C/D snoRNAs, *Rian* and *Mirg* (open boxes). *Dio3* is located at the distal end of the imprinted locus and usually exhibits preferential paternal expression. *Dio3* contains a single exon (black) and 3′ untranslated region (gray). Specific subregion that was sequenced for polymorphisms between Brown Norway vs. Sprague Dawley and Long-Evans strains. Four polymorphisms were found within the high-GC promoter region (−500 to 0) proximal to the *Dio3* start site (0). A synonymous G/C SNP in the *Dio3* exon (342) allows determination of paternal/maternal allelic expression. Chromosomal bp location of the *Dio3* transcript is given below. Location of genes and non-coding transcripts are not to scale. This figure is shown by permission from Sittig and Redei (2012). **(B)** A Brown Norway SNP in the *Dio3* 3′UTR abolishes a putative miR-93 binding site. We have previously identified a C/A SNP between Sprague Dawley (S) and Brown Norway (B) strains in the *Dio3* 3′UTR (Dr. Laura Herzing, unpublished data). MicroInspector miRNA binding prediction program predicted a miR-93 binding site within the S sequence of the 3′UTR of *Dio3*, but not in the B sequence. This suggests that regulation by miR-93 binding to the S 3′UTR is only possible on the S allele. A schematic hypothetical illustration of how miR-93 binding to S but not B alleles could stabilize parent-of-origin *Dio3* alleles (m, maternal; p, paternal) to result in preferentially maternal expression in SB and preferentially paternal expression in BS hippocampus. This figure is shown by permission from Sittig (2012).

FASD pathophysiology (i.e., thyroid hormone homeostasis in brain). Although there is no limit on potential human (or animal) genetic variation, the development of a useful panel of assays that look at pathways affecting multiple FASD endophenotypes is possible. For example, the data on thyroid hormone related vulnerability to FASD that we have presented here show specific examples within a literature where altered thyroid hormone levels are implicated in placenta, brain, maternal and fetal blood in both rodents and humans exposed to alcohol (Heinz et al., 1996; Cudd et al., 2002; Wilcoxon and Redei, 2004; Liappas et al., 2006; Sittig and Redei, 2010; Shukla et al., 2011). We have begun to illuminate the "why" and "how" of thyroid hormone involvement in FASD by examining the genetic basis of thyroid hormone homeostasis, such as the imprinted domain containing *Dio3*. The answers turn out not to be simple, but they lead to specific targets for treatment and diagnosis. For example, alterations in thyroid hormone related markers in placenta are a promising source of functional readout for alcohol exposure at birth (Shukla et al., 2011). Furthermore, administration of low dose T4 to the alcohol-consuming dam can reverse the social interaction and spatial memory deficits in the adult offspring (Tunc-Ozcan et al., 2013).

CONCLUSION

We argue for our hypothesis that aspects of genetic regulation that are considered exceptions to Mendelian genetics play an especially important role in FASD vulnerability. We point to hormonal changes in the maternal *in utero* environment, and parent of origin allelic gene expression differences as mechanisms that impact the first generation with direct intrauterine alcohol exposure, and which can potentially affect the second generation. Both mechanisms are based on non-Mendelian evolutionary systems that allow the parents to shape the offspring in preparation for the environment. We illustrate the complexity of such mechanisms

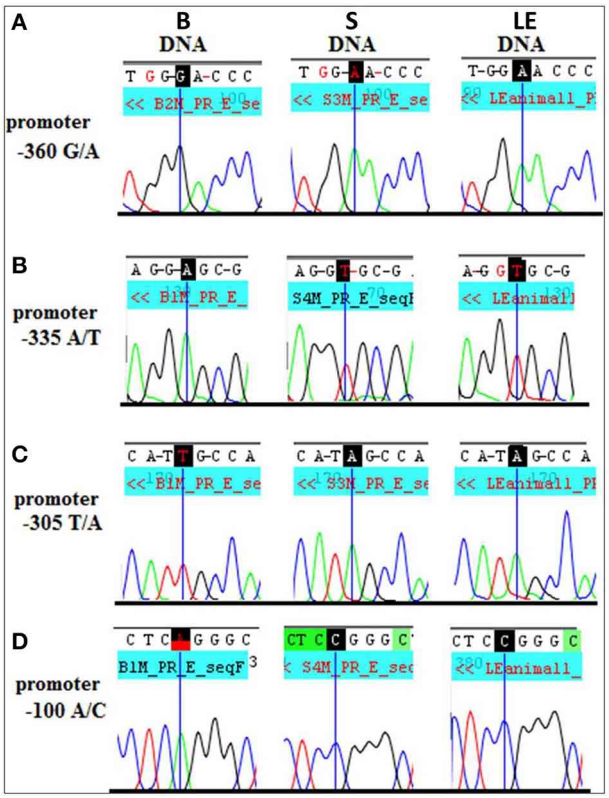

FIGURE 9 | Long Evans (LE) strain recapitulates the Sprague Dawley (S) genotype in the *Dio3* exon and its promoter. Standard sequencing traces of four novel polymorphisms within the *Dio3* promoter region and their location relative to the *Dio3* start site are shown. **(A)** G/A at −360 bp upstream of *Dio3*; **(B)** A/T at −335 bp; **(C)** T/A at −305 bp; and **(D)** A/C at −100 bp. Brown Norway (B) genotype is given first, followed by S and LE. B genotype differs from the matching S and LE genotype at each polymorphic site. This figure is shown by permission from Sittig and Redei (2012).

by focusing on examples of alcohol-induced changes in the F0, F1, and F2 generations in a rat model of FASD. Specifically, we show brain region-specific changes as a result of prenatal alcohol exposure in the expression of an imprinted gene, which changes differ by maternal and paternal genotypes. Additional examples illustrate that maternal genetic vulnerability to alcohol can affect both F1 and F2 generations via altered maternal hormone levels and the subsequent *in utero* hormonal re-programming of the offspring. Both of these types of effects result in differences in vulnerability or resilience of the individuals to prenatal alcohol effects. Finally, we argue that these complex influences probably converge on final common pathways of which thyroid hormone homeostasis is an example, where known epigenetic and genetic vulnerabilities could be evaluated to improve clinical intervention.

AUTHOR CONTRIBUTIONS

Experiments conceived and designed: Eva E. Redei and Laura J. Sittig. Performed: Laura J. Sittig, Elif Tunc-Ozcan, Kathryn M. Harper, and Evan N. Graf. Analyzed data: Eva E. Redei, Elif Tunc-Ozcan, Laura J. Sittig, Kathryn M. Harper, and Evan N. Graf. Wrote the manuscript: Laura J. Sittig, Eva E. Redei, and Elif Tunc-Ozcan. Edited and revised manuscript: Laura J. Sittig, Elif Tunc-Ozcan, Kathryn M. Harper, Evan N. Graf, and Eva E. Redei. Approved final version of manuscript: Laura J. Sittig, Elif Tunc-Ozcan, Kathryn M. Harper, Evan N. Graf, and Eva E. Redei.

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REFERENCES

Anthony, B., Vinci-Booher, S., Wetherill, L., Ward, R., Goodlett, C., and Zhou, F. C. (2010). Alcohol-induced facial dysmorphology in C57BL/6 mouse models of fetal alcohol spectrum disorder. *Alcohol* 44, 659–671. doi: 10.1016/j.alcohol.2010.04.002

Balaraman, S., Tingling, J. D., Tsai, P. C., and Miranda, R. C. (2013). Dysregulation of microRNA expression and function contributes to the etiology of fetal alcohol spectrum disorders. *Alcohol Res.* 35, 18–24.

Barker, D. J. (2004). The developmental origins of chronic adult disease. *Acta Paediatr.* 93, 26–33. doi: 10.1111/j.1651-2227.2004.tb00236.x

Barker, D. J., Bull, A. R., Osmond, C., and Simmonds, S. J. (1990). Fetal and placental size and risk of hypertension in adult life. *BMJ* 301, 259–262.

Barker, D. J., Hales, C. N., Fall, C. H., Osmond, C., Phipps, K., and Clark, P. M. (1993). Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 36, 62–67.

Barker, D. J., Winter, P. D., Osmond, C., Margetts, B., and Simmonds, S. J. (1989). Weight in infancy and death from ischaemic heart disease. *Lancet* 2, 577–580.

Browman, K. E., and Crabbe, J. C. (2000). Quantitative trait loci affecting ethanol sensitivity in BXD recombinant inbred mice. *Alcohol Clin. Exp. Res.* 24, 17–23. doi: 10.1111/j.1530-0277.2000.tb04547.x

Brunton, P. J., and Russell, J. A. (2011). Neuroendocrine control of maternal stress responses and fetal programming by stress in pregnancy. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1178–1191. doi: 10.1016/j.pnpbp.2010.12.023

Chen, Y., Ozturk, N. C., Ni, L., Goodlett, C., and Zhou, F. C. (2011). Strain differences in developmental vulnerability to alcohol exposure via embryo culture in mice. *Alcohol Clin. Exp. Res.* 35, 1293–1304. doi: 10.1111/j.1530-0277.2011.01465.x

Cudd, T. A., Chen, W. J., and West, J. R. (2002). Fetal and maternal thyroid hormone responses to ethanol exposure during the third trimester equivalent of gestation in sheep. *Alcohol Clin. Exp. Res.* 26, 53–58. doi: 10.1111/j.1530-0277.2002.tb02431.x

Delaval, K., and Feil, R. (2004). Epigenetic regulation of mammalian genomic imprinting. *Curr. Opin. Genet. Dev.* 14, 188–195. doi: 10.1016/j.gde.2004.01.005

Downing, C., Balderrama-Durbin, C., Broncucia, H., Gilliam, D., and Johnson, T. E. (2009). Ethanol teratogenesis in five inbred strains of mice. *Alcohol Clin. Exp. Res.* 33, 1238–1245. doi: 10.1111/j.1530-0277.2009.00949.x

Downing, C., Balderrama-Durbin, C., Kimball, A., Biers, J., Wright, H., Gilliam, D., et al. (2012). Quantitative trait locus mapping for ethanol teratogenesis in BXD recombinant inbred mice. *Alcohol Clin. Exp. Res.* 36, 1340–1354. doi: 10.1111/j.1530-0277.2012.01754.x

Downing, C., Johnson, T. E., Larson, C., Leakey, T. I., Siegfried, R. N., Rafferty, T. M., et al. (2011). Subtle decreases in DNA methylation and gene expression at the mouse Igf2 locus following prenatal alcohol exposure: effects of a methyl-supplemented diet. *Alcohol* 45, 65–71. doi: 10.1016/j.alcohol.2010.07.006

Eberle, C., and Ament, C. (2012). Diabetic and metabolic programming: mechanisms altering the intrauterine milieu. *ISRN Pediatr.* 2012:975685. doi: 10.5402/2012/975685

Feil, R., and Fraga, M. F. (2011). Epigenetics and the environment: emerging patterns and implications. *Nat. Rev. Genet.* 13, 97–109. doi: 10.1038/nrg3142

Garro, A. J., Mcbeth, D. L., Lima, V., and Lieber, C. S. (1991). Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal

alcohol syndrome. *Alcohol Clin. Exp. Res.* 15, 395–398. doi: 10.1111/j.1530-0277.1991.tb00536.x

Gilliam, D. M., and Irtenkauf, K. T. (1990). Maternal genetic effects on ethanol teratogenesis and dominance of relative embryonic resistance to malformations. *Alcohol Clin. Exp. Res.* 14, 539–545. doi: 10.1111/j.1530-0277.1990.tb01196.x

Gilliam, D. M., Mantle, M. A., Barkhausen, D. A., and Tweden, D. R. (1997). Effects of acute prenatal ethanol administration in a reciprocal cross of C57BL/6J and short-sleep mice: maternal effects and nonmaternal factors. *Alcohol Clin. Exp. Res.* 21, 28–34.

Glavas, M. M., Ellis, L., Yu, W. K., and Weinberg, J. (2007). Effects of prenatal ethanol exposure on basal limbic-hypothalamic-pituitary-adrenal regulation: role of corticosterone. *Alcohol Clin. Exp. Res.* 31, 1598–1610. doi: 10.1111/j.1530-0277.2007.00460.x

Govorko, D., Bekdash, R. A., Zhang, C., and Sarkar, D. K. (2012). Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. *Biol. Psychiatry* 72, 378–388. doi: 10.1016/j.biopsych.2012.04.006

Gregg, C., Zhang, J., Weissbourd, B., Luo, S., Schroth, G. P., Haig, D., et al. (2010). High-resolution analysis of parent-of-origin allelic expression in the mouse brain. *Science* 329, 643–648. doi: 10.1126/science.1190830

Harper, K. M., Tunc-Ozcan, E., Graf, E. N., and Redei, E. E. (2014). Intergenerational effects of prenatal ethanol on glucose tolerance and insulin response. *Physiol. Genomics* 46, 159–168. doi: 10.1152/physiolgenomics.00181.2013

Haycock, P. C. (2009). Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod.* 81, 607–617. doi: 10.1095/biolreprod.108.074690

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., et al. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17046–17049. doi: 10.1073/pnas.0806560105

Heinz, A., Bauer, M., Kuhn, S., Kruger, F., Graf, K. J., Rommelspacher, H., et al. (1996). Long-term observation of the hypothalamic-pituitary-thyroid (HPT) axis in alcohol-dependent patients. *Acta Psychiatr. Scand* 93, 470–476. doi: 10.1111/j.1600-0447.1996.tb10679.x

Hellemans, K. G., Sliwowska, J. H., Verma, P., and Weinberg, J. (2010). Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. *Neurosci. Biobehav. Rev.* 34, 791–807. doi: 10.1016/j.neubiorev.2009.06.004

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K. A., Fahey, P., Cox, T. C., et al. (2010). Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet.* 6:e1000811. doi: 10.1371/journal.pgen.1000811

Knezovich, J. G., and Ramsay, M. (2012). The effect of preconception paternal alcohol exposure on epigenetic remodeling of the h19 and rasgrf1 imprinting control regions in mouse offspring. *Front. Genet.* 3:10. doi: 10.3389/fgene.2012.00010

Kvigne, V. L., Leonardson, G. R., Borzelleca, J., and Welty, T. K. (2008). Characteristics of grandmothers who have grandchildren with fetal alcohol syndrome or incomplete fetal alcohol syndrome. *Matern Child Health J.* 12, 760–765. doi: 10.1007/s10995-007-0308-y

Laufer, B. I., Mantha, K., Kleiber, M. L., Diehl, E. J., Addison, S. M., and Singh, S. M. (2013). Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis. Model Mech.* 6, 977–992. doi: 10.1242/dmm.010975

Liang, G., Chen, M., Pan, X. L., Zheng, J., and Wang, H. (2011). Ethanol-induced inhibition of fetal hypothalamic-pituitary-adrenal axis due to prenatal overexposure to maternal glucocorticoid in mice. *Exp. Toxicol Pathol.* 63, 607–611. doi: 10.1016/j.etp.2010.04.015

Liappas, I., Piperi, C., Malitas, P. N., Tzavellas, E. O., Zisaki, A., Liappas, A. I., et al. (2006). Interrelationship of hepatic function, thyroid activity and mood status in alcohol-dependent individuals. *In Vivo* 20, 293–300.

Liu, Y., Aryee, M. J., Padyukov, L., Fallin, M. D., Hesselberg, E., Runarsson, A., et al. (2013). Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. *Nat. Biotechnol.* 31, 142–147. doi: 10.1038/nbt.2487

Manning, M. A., and Eugene Hoyme, H. (2007). Fetal alcohol spectrum disorders: a practical clinical approach to diagnosis. *Neurosci. Biobehav. Rev.* 31, 230–238. doi: 10.1016/j.neubiorev.2006.06.016

Mazzio, E. A., and Soliman, K. F. (2012). Basic concepts of epigenetics: impact of environmental signals on gene expression. *Epigenetics* 7, 119–130. doi: 10.4161/epi.7.2.18764

Mcgivern, R. F., and Redei, E. (1994). Adrenalectomy reverses stress-induced suppression of luteinizing hormone secretion in long-term ovariectomized rats. *Physiol. Behav.* 55, 1147–1150. doi: 10.1016/0031-9384(94)90402-2

Mead, E., and Sarkar, D. (2014). Fetal alcohol spectrum disorders and their transmission through genetic and epigenetic mechanisms. *Front. Genet.* 5:154. doi: 10.3389/fgene.2014.00154

Nunez, Y. O., Truitt, J. M., Gorini, G., Ponomareva, O. N., Blednov, Y. A., Harris, R. A., et al. (2013). Positively correlated miRNA-mRNA regulatory networks in mouse frontal cortex during early stages of alcohol dependence. *BMC Genomics* 14:725. doi: 10.1186/1471-2164-14-725

Rakyan, V. K., Down, T. A., Balding, D. J., and Beck, S. (2011). Epigenome-wide association studies for common human diseases. *Nat. Rev. Genet.* 12, 529–541. doi: 10.1038/nrg3000

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. *Science* 293, 1089–1093. doi: 10.1126/science.1063443

Revskoy, S., Halasz, I., and Redei, E. (1997). Corticotropin-releasing hormone and proopiomelanocortin gene expression is altered selectively in the male rat fetal thymus by maternal alcohol consumption. *Endocrinology* 138, 389–396. doi: 10.1210/endo.138.1.4838

Rouleau, M., Levichek, Z., and Koren, G. (2003). Are mothers who drink heavily in pregnancy victims of FAS. *J. FAS Int.* 1, 1–5.

Seisenberger, S., Peat, J. R., Hore, T. A., Santos, F., Dean, W., and Reik, W. (2013). Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368:20110330. doi: 10.1098/rstb.2011.0330

Shukla, P. K., Sittig, L. J., Ullmann, T. M., and Redei, E. E. (2011). Candidate placental biomarkers for intrauterine alcohol exposure. *Alcohol Clin. Exp. Res.* 35, 559–565. doi: 10.1111/j.1530-0277.2010.01373.x

Sittig, L. J. (2012). *Developmental Programming of Brain Regional Thyroid Status by the Deiodinase-III: Parent-of-Origin Genetic and Epigenetic Influences*. Chicago, IL: Northwestern University.

Sittig, L. J., Herzing, L. B., Shukla, P. K., and Redei, E. E. (2011a). Parent-of-origin allelic contributions to deiodinase-3 expression elicit localized hyperthyroid milieu in the hippocampus. *Mol. Psychiatry* 16, 786–787. doi: 10.1038/mp.2011.19

Sittig, L. J., and Redei, E. E. (2010). Paternal genetic contribution influences fetal vulnerability to maternal alcohol consumption in a rat model of fetal alcohol spectrum disorder. *PLoS ONE* 5:e10058. doi: 10.1371/journal.pone.0010058

Sittig, L. J., and Redei, E. E. (2012). Novel polymorphisms within the Dlk1-Dio3 imprinted locus in rat: a putative genetic basis for strain-specific allelic gene expression. *Front. Genet.* 3:296. doi: 10.3389/fgene.2012.00296

Sittig, L. J., Shukla, P. K., Herzing, L. B., and Redei, E. E. (2011b). Strain-specific vulnerability to alcohol exposure *in utero* via hippocampal parent-of-origin expression of deiodinase-III. *FASEB J.* 25, 2313–2324. doi: 10.1096/fj.10-179234

Skinner, M. K., Haque, C. G., Nilsson, E., Bhandari, R., and Mccarrey, J. R. (2013). Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. *PLoS ONE* 8:e66318. doi: 10.1371/journal.pone.0066318

Slone, J. L., and Redei, E. E. (2002). Maternal alcohol and adrenalectomy: asynchrony of stress response and forced swim behavior. *Neurotoxicol. Teratol* 24, 173–178. doi: 10.1016/S0892-0362(01)00186-6

Stouder, C., Somm, E., and Paoloni-Giacobino, A. (2011). Prenatal exposure to ethanol: a specific effect on the H19 gene in sperm. *Reprod. Toxicol* 31, 507–512. doi: 10.1016/j.reprotox.2011.02.009

Tunc-Ozcan, E., Ullmann, T. M., Shukla, P. K., and Redei, E. E. (2013). Low-dose thyroxine attenuates autism-associated adverse effects of fetal alcohol in male offspring's social behavior and hippocampal gene expression. *Alcohol Clin. Exp. Res.* 37, 1986–1995. doi: 10.1111/acer.12183

Wilcoxon, J. S., Kuo, A. G., Disterhoft, J. F., and Redei, E. E. (2005). Behavioral deficits associated with fetal alcohol exposure are reversed by prenatal thyroid hormone treatment: a role for maternal thyroid hormone deficiency in FAE. *Mol. Psychiatry* 10, 961–971. doi: 10.1038/sj.mp.4001694

Wilcoxon, J. S., and Redei, E. E. (2004). Prenatal programming of adult thyroid function by alcohol and thyroidhormones. *Am. J. Physiol. Endocrinol. Metab.* 287, E318–E326. doi: 10.1152/ajpendo.00022.2004

Wilcoxon, J. S., Schwartz, J., Aird, F., and Redei, E. E. (2003). Sexually dimorphic effects of maternal alcohol intake and adrenalectomy on left ventricular hypertrophy in rat offspring. *Am. J. Physiol. Endocrinol. Metab.* 285, E31–E39. doi: 10.1152/ajpendo.00552.2002

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