Ethanol Affects Differentiation-Related Pathways and Suppresses Wnt Signaling Protein Expression in Human Neural Stem Cells

Sharada D. Vangipuram and William D. Lyman

**Background:** Prenatal exposure of the fetus to ethanol (EtOH) can be teratogenic. We previously showed that EtOH alters the cell fate of human neural stem cells (NSC). As Wnt signaling plays an important role in fetal brain development, we hypothesized that EtOH suppresses Wnt signaling protein expression in differentiating NSC and thereby contributes to fetal alcohol spectrum disorder.

**Methods:** NSC isolated from fetal human brains were cultured in mitogenic media to induce neurospheres, which were dissociated into single-cell suspensions and used for all experiments. Equal numbers of NSC were cultured on lysine/laminin-coated plates for 96 hours in differentiating media containing 0, 20, or 100 mM EtOH. Total mRNA was isolated from samples containing 0 or 100 mM EtOH and changes in expression of 263 genes associated with neurogenesis and NSC differentiation were determined by Oligo GEArray technology. The biological impact of gene changes was estimated using a systems biology approach with pathway express software and KEGG database. Based on the pathways identified, expression of Wnt proteins (Wnt3a and Wnt5a), Wnt-receptor complex proteins (p-LRP6, LRP6, DVL2, and DVL3), Wnt antagonist Naked-2 (NKD-2), and downstream Wnt proteins (β-catenin, Tyr-p-GSK3β, Ser-p-GSK3β) were analyzed by Western blot.

**Results:** Of the 263 genes examined, the expressions of 22 genes in differentiating NSC were either upwardly or downwardly affected by EtOH. These genes are associated with 5 pathways/cellular processes: axon guidance; hedgehog signaling; TGF-β signaling; cell adhesion molecules; and Wnt signaling. When compared to controls, EtOH, at both 20 and 100 mM concentrations, suppressed the expression of Wnt3a and Wnt5a, receptor complex proteins p-LRP6, LRP6 and DVL2, and cytoplasmic proteins Ser-p-GSK3β and β-catenin. Expression of NKD-2 and DVL3 remained unchanged and the expression of active Tyr-p-GSK3β increased significantly.

**Conclusions:** EtOH can significantly alter neural differentiation pathway-related gene expression and suppress Wnt signaling proteins in differentiating human NSC.

**Key Words:** Fetal Brain, Signaling Pathways, Ethanol.
shown that EtOH alters the cell fate and cell adhesion molecule (CAM) expression in human fetal brain derived NSC without increasing apoptosis (Vangipuram and Lyman, 2010; Vangipuram et al., 2008). Considering the number of possible mechanisms that could be involved in such an effect, we conducted an Oligo GEArray Microarray analysis to determine the differences in transcript levels between control and EtOH-treated human fetal brain derived NSC. For this study, we used a microarray that profiled the expression of 263 genes related to regulation of key neurogenesis processes such as regulators of cell proliferation and differentiation, synaptic transmission, synaptogenesis, and other key cellular functions.

Wnt signaling pathway plays an important role in embryogenesis and neurogenesis (Castelo-Branco and Arenas, 2006; Cerpa et al., 2009; Freese et al., 2010; Hur and Zhou, 2010; Smalley and Dale, 1999; Toledo et al., 2008). Wnts are shown to be essential at various stages of neural development including stem cell proliferation and maintenance of progenitor pool as well as in differentiation and lineage determination. Wnts are also known to be involved in cell migration, axon guidance, and neurite outgrowth as reviewed by Ille and Sommer (2005). Glycogen synthase kinase3β (GSK3β), a key component of the Wnt signaling pathway has been shown to be involved in EtOH-induced neurotoxicity (Liu et al., 2009; Luo, 2009). Based on our Oligo GEArray analysis and on the existing data on the possible role of GSK3β in EtOH-induced neurotoxicity, we hypothesized that EtOH suppresses Wnt signaling pathway protein expression in fetal human brain derived NSC under differentiating conditions.

Cell surface markers CD133/prominin and nestin expression has been used to define NSC by several groups including ours (Hockfield and McKay, 1985; Lendahl et al., 1990; Tateeno et al., 2004; Uchida et al., 2000; Vangipuram et al., 2008). To test our hypothesis we utilized CD133/nestin expression to select NSC from second trimester fetal human brain. Data obtained as a part of this study show that EtOH significantly suppresses endogenous levels of Wnt3α and Wnt5α and related receptor complex and cytoplasmic Wnt pathway proteins. As expected, EtOH increased Tyr phosphorylation of GSK3β, which promotes degradation of β-catenin, a key downstream Wnt signaling pathway protein.

**MATERIALS AND METHODS**

**Isolation of Neural Progenitor Cells**

CD133+/nestin+ NSC were isolated from fetal human brain tissue (14 to 17 weeks of gestation) obtained from the Albert Einstein College of Medicine, Bronx, NY as previously described (Vangipuram and Lyman, 2010; Vangipuram et al., 2008). This study is part of an ongoing research protocol approved by the human investigation committee, Wayne State University. Fetal brain tissue was obtained from voluntary terminations of normal pregnancies and the tissue was washed with Hank’s balanced salt solution and confirmed to be equivalent to the concentrations of alcohol in the blood of social drinkers to chronic alcoholics (Adachi et al., 2004; Uchida et al., 2000; Vangipuram et al., 2008). This study is part of an ongoing research protocol approved by the human investigation committee, Wayne State University. Fetal brain tissue was obtained from voluntary terminations of normal pregnancies and the tissue was washed with Hank’s balanced salt solution and confirmed to be equivalent to the concentrations of alcohol in the blood of social drinkers to chronic alcoholics (Adachi et al., 2004; Uchida et al., 2000; Vangipuram et al., 2008). This study is part of an ongoing research protocol approved by the human investigation committee, Wayne State University. Fetal brain tissue was obtained from voluntary terminations of normal pregnancies and the tissue was washed with Hank’s balanced salt solution and confirmed to be equivalent to the concentrations of alcohol in the blood of social drinkers to chronic alcoholics (Adachi et al., 2004; Uchida et al., 2000; Vangipuram et al., 2008).

**Total RNA Isolation**

After 96 hours, total RNA was extracted from control and 100 mM EtOH-treated NSC using RNeasy mini kit (Qiagen Inc., Valencia, CA) as per the kit manufacturer’s protocol. The quality and quantity of RNA was assessed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA with RNA Integrity Number (RIN) > 9 was used for Oligo GEArray DNA Microarray analysis using human neurogenesis and NSC array (SuperArray Biosciences, Frederick, MD).

**Oligo GEArray DNA Microarray Analysis**

Human neurogenesis and NSC Oligo GEArray microarray was used to determine differential transcript levels between control and 100 mM EtOH-treated samples (n = 3). The microarray profiled the expression of 263 genes related to regulation of key
neurogenesis processes. Standard Hyb tube protocol was followed for the analysis as per the kit manufacturer’s directions. Briefly, 3 μg of high quality total RNA (RIN > 9) was used to synthesize complementary C-DNA. True labeling-AMP 2.0 Oligo GEArray starter kit was used for c-RNA synthesis, amplification and biotinylation. C-RNA was purified using SuperArray ArrayGrade™ cRNA Cleanup Kit. A Nanodrop 1000 spectrophotometer (Thermo Scientific Inc., Rockford, IL) was used to assess the c-RNA yield and quality.

Oligo GEArray Hybridization and Detection

Oligo GEArray membranes were hybridized with 4 μg of biotinylated C-RNA from control and EtOH-treated samples (n = 3) as per kit manufacturer’s protocol. Briefly, the membranes were prehybridized in hybridization solution for 2 hours at 60°C in an oven with continuous, but slow agitation, followed by hybridization with C-RNA at 60°C, overnight with gentle agitation. The membranes were then washed and the signals detected using the chemiluminescent detection kit (SuperArray Biosciences) and digitized using Carestream CCD camera system (Carestream Health Inc., New Haven, CT). Fold change (1-fold = 100%) in the expression of genes from EtOH-treated and control samples were compared using integrated GEArray expression analysis software. Oligo GEArray® Human Neurogenesis and Neural Stem Cell Microarray from SuperArray Biosciences contained 263 genes which included positive and negative regulators of cell proliferation, negative regulators of cell cycle, cell cycle arrest genes, regulators of cell differentiation, synaptic transmission, synaptogenesis, apoptosis, and cell adhesion. Further, the array also included genes involved in neurogenesis such as molecules of notch signaling, Wnt receptor and G protein-coupled receptor pathways. Fold change (1-fold = 100%) in the expression of these genes from EtOH-treated and control samples were compared using integrated GEArray expression analysis software. All data sets were corrected using the lowest density reading of a spot on the array for background subtraction. After background subtraction, interquartile value was used for normalization. The interquartile normalization option normalizes each spot brightness to the mean intensity value of all the spots in the middle half (50%) of the range that remains after ignoring the most and least intense quarter (25%) of the entire data set. Only genes that showed up or down-regulation of at least 1.5-fold or more were considered to have been altered by EtOH.

Pathway Analysis

The potential biological impact of changes in gene expression was estimated using a systems biology approach that employs an impact analysis method of pathway express software. Pathway express and OntoExpress analysis software (Draghici et al., 2003; Khatri et al., 2005) was utilized to estimate the significance of changes in relation to biological pathways. Using this method, an impact factor is calculated for each pathway by incorporating multiple factors such as the normalized fold change of differentially expressed genes, the statistical significance of the set of pathway genes, and the topology of the signaling pathway (from KEGG database). Pathways which showed an impact factor higher than 5 along with a p-value <0.05 were considered to be significantly altered.

Western Blot Analysis

Western blot analysis was performed on control and EtOH-treated samples as previously described (Vangipuram and Lyman, 2010). CD133+ NSC differentiating in laminin/lysoleic-acidated 6 well plates were lysed with NP-40 lysis buffer containing 0.15 M NaCl; 1 mM EGTA; 1 mg/ml BSA; 10 mM Tris; 1% NP-40; 0.2 mM phenyl-methylsulfonyl fluoride; 5 mg/ml aprotinin, leupeptin, and pepstatin, pH 7.4 after washing with ice-cold PBS. Unlysed cells were pelleted by centrifuging at 13,000×g for 15 minutes at 4°C in Beckman microcentrifuge (Beckman Coulter, Fullerton, CA). The protein concentrations of the lysates were determined using the Pierce protein estimation kit (Thermo Fisher Scientific Inc.). The expression of Wnt proteins (Wnt3a and Wnt5a), Wnt-receptor complex proteins (p-LRP6, LRP6, DVL2, and DVL3), Wnt antagonist Naked-2 (NKD-2), and downstream Wnt proteins (β-catenin, Tyr-p-GSK3β, Ser-p-GSK3β) from the Wnt canonical pathway were analyzed by Western blot. Briefly, 25 to 30 μg/lane of protein lysates of all samples was loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, separated by gel electrophoresis, and then transferred to PVDF membranes. The membranes were washed with PBS-T and nonspecific binding was blocked with 5% nonfat dry milk in PBS-T for 1 hour with shaking. The membranes were then incubated with primary antibodies (Cell Signaling Technology, Danvers, MA) overnight with shaking at 4°C. Subsequent to washing, the membranes were incubated with secondary goat anti-rabbit peroxidase-conjugated antibody for 1 hour at 25°C. The membranes were then developed and visualized with the Pierce ECL Western blotting substrate (Pierce, Rockford, IL) followed by apposition of the membranes with autoradiographic Kodak X-OMAT AR film (Rochester, NY). The protein expression was quantified by digitizing the ECL signals using Carestream molecular imaging software 5.0. The bands corresponding to the expected molecular weight were analyzed for each of the proteins where more than 1 band was seen (Tyr-p-GSK3β—47 kDa, SER-p-GSK3β—46 kDa, Wnt3a—42 kDa, β-actin—45 kDa, and NKD-2—59 kDa). In all cases, the darkest band corresponded to the expected molecular weight.

Statistical Analysis

To examine mean differences in band densities of various proteins a parametric analysis of variance (ANOVA) procedure was employed. Assumptions of normality and/or homogeneity of variance were checked and verified. Normality was checked using stem and leaf plots and histograms. Homogeneity of variance was formally checked using Levene’s statistic. Post hoc tests were conducted using Bonferroni’s correction to balance multiple tests of hypotheses (pair-wise comparisons) and Type I error. SPSS Version 19.0 (IBM Inc., Chicago, IL) was used to perform all statistical procedures. Statistically significant differences were considered achieved with a p-value < 0.05.

RESULTS

Generation of Neurospheres from NSC Isolated from Second Trimester Fetal Human Brain

NSC were isolated from second trimester fetal human brain by positive immuno-selection. CD 133 and nestin expression was used to confirm the differentiation state of the cells (Fig. 1). Neurospheres were generated from these cells in serum-free mitogenic media in the presence of hEGF and bFGF as previously described (Vangipuram and Lyman, 2010; Vangipuram et al., 2008) and dissociated for experiments.

Oligo GEArray Analysis of Control and EtOH-Treated NSC

Oligo GEArray® Human Neurogenesis and Neural Stem Cell Microarray containing 263 genes related to neurogenesis and NSC differentiation including 12 housekeeping genes,
was used to study the effect of EtOH on NSC. The transcript signals were detected on the membranes by chemiluminiscent detection system and visualized on Carestream CCD camera. Using integrated GEArray expression analysis software, 22 genes were identified to be altered by at least 1.5-fold compared with controls. Of these, 6 genes were up-regulated and 16 genes were down-regulated (Table 1). Of the 263 genes on the array, 138 genes were absent in the samples (Table S1).

Pathway Express Analysis

The pathway express and OntoExpress analysis software was used to assign altered genes from the Oligo GEArray results to specific biological pathways/cellular processes with highest impact as described (Draghici et al., 2003; Khatri et al., 2005). Five pathways/cellular processes were identified to have an impact factor greater than 5 and a p-value <0.05, namely, axon guidance, hedgehog signaling, TGF-β signaling, CAMs, and Wnt signaling (Table 2). Of the pathways identified, Wnt signaling pathway was chosen for in-depth study due to the role played by Wnt signaling in neural development and neurogenesis (Cadigan and Nusse, 1997; Toledo et al., 2008) and in EtOH-induced neurotoxicity (Liu et al., 2009; Luo, 2009).

Western Blot Analysis of Wnt Signaling Pathway Proteins

Endogenous levels of Wnt proteins Wnt3a and Wnt5a were first estimated by Western blot analysis and band intensities estimated by densitometry. Expressions levels of both Wnts studied were significantly suppressed in differentiating NSC with exposure to both 20 and 100 mM concentrations of

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold expressed relative to control</th>
<th>Function in fetal brain development</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC4</td>
<td>Histone deacetylase4</td>
<td>1.78</td>
<td>Involved in TGF-β induced differentiation</td>
</tr>
<tr>
<td>HEY1</td>
<td>Hairy/ enhancer-of-split related with YRPW motif protein 1</td>
<td>1.54</td>
<td>Implicated in neurogenesis and somitogenesis</td>
</tr>
<tr>
<td>MDK</td>
<td>Midkine-neurite growth promoting factor 2</td>
<td>1.84</td>
<td>Cell proliferation, cell migration, angiogenesis, and fibrinolysis</td>
</tr>
<tr>
<td>PBX4</td>
<td>Pre-B cell leukemia homobox-4</td>
<td>1.99</td>
<td>Transcription factor involved in translocations</td>
</tr>
<tr>
<td>PCDH1</td>
<td>Protocadherin</td>
<td>3.14</td>
<td>Involved in neural adhesion and development</td>
</tr>
<tr>
<td>SPOCK1</td>
<td>Testican 1</td>
<td>3.10</td>
<td>Unknown—possible protease inhibition</td>
</tr>
<tr>
<td>AHE</td>
<td>Acetylcholinesterase</td>
<td>0.31</td>
<td>Apoptosis related</td>
</tr>
<tr>
<td>APAF1</td>
<td>Apoptotic protease activating factor 1</td>
<td>0.63</td>
<td>Part of apoptosis regulatory network</td>
</tr>
<tr>
<td>APBA1</td>
<td>Adapter protein x 11alpha</td>
<td>0.57</td>
<td>Essential component of synaptic vesicles</td>
</tr>
<tr>
<td>APBB2</td>
<td>Amyloid beta A4 precursor protein-binding family B member 2</td>
<td>0.47</td>
<td>Involved in signal transduction</td>
</tr>
<tr>
<td>APLP1</td>
<td>Amyloid beta (A4) precursor-like protein</td>
<td>0.32</td>
<td>Role in postsynaptic function</td>
</tr>
<tr>
<td>EP300</td>
<td>E1A binding protein p300</td>
<td>0.61</td>
<td>Important role in cell proliferation and differentiation</td>
</tr>
<tr>
<td>MT3</td>
<td>Metallothionein-3</td>
<td>0.62</td>
<td>Survival and neurite formation of cortical neurons</td>
</tr>
<tr>
<td>NCKAP1</td>
<td>NCK-associated protein 1</td>
<td>0.52</td>
<td>Membrane associated protein</td>
</tr>
<tr>
<td>NDP</td>
<td>Norrie disease (pseudoglioma)</td>
<td>0.64</td>
<td>Mutations in Norrie Disease</td>
</tr>
<tr>
<td>NRCAM</td>
<td>Neuronal cell adhesion molecule</td>
<td>0.64</td>
<td>Involved in neuron–neuron adhesion and directional growth</td>
</tr>
<tr>
<td>POU6F1</td>
<td>POU class 6 homeobox</td>
<td>0.65</td>
<td>Neurite growth promoting factor</td>
</tr>
<tr>
<td>PTN</td>
<td>Pleiotrophin</td>
<td>0.66</td>
<td>Axon guidance receptor and cell adhesion receptor</td>
</tr>
<tr>
<td>ROBO1</td>
<td>Roundabout homolog 1</td>
<td>0.55</td>
<td>Neutralizes supercharged oxygen molecules</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
<td>0.63</td>
<td>Cell fate determination</td>
</tr>
<tr>
<td>SOX3</td>
<td>SRY-related HMG-box</td>
<td>0.64</td>
<td>Stimulates cell proliferation and suppresses terminal differentiation</td>
</tr>
</tbody>
</table>

References for gene functions are available in Data S1. Values showed in bold represent up-regulated genes.
EtOH (Fig. 2). Subsequently, expression of Wnt receptor complex proteins, phosphorylated and total LRP6, DVL2, DVL3, and Wnt antagonist NKD-2 were studied. Although there was no significant change in the expression of NKD-2 and DVL3, expression of phosphorylated LRP6 and DVL2 was significantly suppressed by 20 and 100 mM EtOH and total LRP6 was significantly suppressed by 100 mM EtOH (Fig. 3). Finally, expression of cytoplasmic Wnt proteins, β-catenin, and GSK3β were studied. As expected, EtOH significantly suppressed the expression levels of β-catenin and Ser phosphorylated and total GSK3β. Expression of active Tyr phosphorylated GSK3β which phosphorylates cytoplasmic β-catenin leading to its ubiquitination and degradation, was significantly increased with EtOH exposure (Fig. 2).

DISCUSSION

The major finding of this study is that exposure of differentiating human NSC to EtOH significantly alters the levels of gene transcripts that could potentially impact 5 major cellular pathways as computed by pathway express software analysis. These are: axon guidance, hedgehog signaling, TGF-β signaling, cell adhesion molecules, and Wnt signaling (Table 2). Further, this study also shows that the key components of the Wnt signaling pathway are suppressed by EtOH in a dose-related manner suggesting that Wnt signal suppression could be a significant part of the mechanism of action of EtOH on NSC.

CD133, a 5-trans-membrane glycoprotein (Miraglia et al., 1997) and nestin, have been used as markers to confirm the undifferentiated state of NSC and to isolate NSC from a heterogeneous population of cells by our group and others (Tamaiki et al., 2002; Uchida et al., 2000; Vangipuram and Lyman, 2010; Vangipuram et al., 2008). In this study, we utilized the expression of CD133 to positively select NSC from second trimester fetal human brain and study the effects of EtOH on those cells while differentiating on laminin/lysine coated plates.

Although the teratogenic potential of EtOH has been known for more than 30 years (Jones et al., 1973, 1974a,b), neither the most vulnerable cell population in the developing brain nor the exact mechanism of action of EtOH on the developing fetus have been identified.

Several mechanisms have been proposed to explain the teratogenic action of EtOH. A number of groups, including ours, have suggested that EtOH alters CAM expression. Miñana and colleagues (2000) have shown that EtOH alters the pattern of NCAM expression during brain development, whereas Bearer and colleagues (1999) have suggested a possible L1-mediated inhibition of neurite outgrowth by EtOH. TGF-β induced NCAM expression has been shown to be inhibited by EtOH (Luo and Miller, 1999). Alcohol induced oxidative stress leading to cell death and damage has been proposed as another possible mechanism of action (Guerri, 1998; Henderson et al., 1995; Kotch et al., 1995). Alterations to DNA metabolism (Dreosti et al., 1981; Pennington et al., 1983; Shibley and Pennington, 1997) has been suggested as yet another possible mechanism. Others include, changes in energy and glucose metabolism (Mann et al., 1975; Miller and Dow-Edwards, 1993; Vingan et al., 1986) and alterations in growth factor mediated pathways as shown by several groups including those of Jones, Guerri and others.

Table 2. Pathways Significantly Impacted by Ethanol in Differentiating Neural Stem Cells Determined by Pathway Analysis Software

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genes involved</th>
<th>Impact factor</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon guidance</td>
<td>ROBO1, NRCAM, PTN, SOD1, PTN, HDAC4, HEY1, PBX4, ROBO1, PTN</td>
<td>15.99, 10.44, 6.57</td>
<td>5.35E-07, 1.2E-04, 0.010</td>
</tr>
<tr>
<td>Hedgehog signaling</td>
<td>HDAC4, HEY1, PBX4, ROBO1, PTN</td>
<td>5.48, 9.12, 7.07</td>
<td>0.0078, 1.9E-04, 8.4E-04</td>
</tr>
<tr>
<td>TGF-β signaling</td>
<td>HDAC4, SOX3, NRCAM, MT3, EP300</td>
<td>4.99, 11.67, 3.48</td>
<td>0.017, 2.3E-05, 0.07</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>PCDH1, NCKP1, NRCAM, HDAC4, EP300</td>
<td>8.1, 10.44, 3.01</td>
<td>0.038, 0.028, 0.01</td>
</tr>
<tr>
<td>Wnt signaling</td>
<td>HEY1, PBX4, PCDH1, ACHE, EP300</td>
<td>9.45, 10.12, 3.84</td>
<td>7.88E-05, 2.1E-04, 0.012</td>
</tr>
</tbody>
</table>
We exposed NSC to 100 mM EtOH for 96 hours under differentiating conditions as has been done previously in cell culture experiments (Prock and Miranda, 2007; Tateno et al., 2005; Vangipuram and Lyman, 2010; Vangipuram et al., 2008). A lower 20 mM dose of EtOH was also used in subsequent experiments to determine Wnt protein expression by Western blot.

Oligo GEArray Human Neurogenesis and Neural Stem Cell Microarrays have previously been used by other groups to study the changes in gene expressions in differentiating stem cells (Greco and Rameshwar, 2007). Our microarray data show that 22 genes were either up- or down-regulated by EtOH in differentiating NSC (Table 1) which were determined to have a high impact on 5 crucial pathways/cellular processes (Table 2). It is interesting that each of the 5 pathways/cellular processes affected by EtOH is independently essential for normal development of fetal brain. A similar
observation has been made by Hashimoto-Torii and colleagues (2011) who have recently shown that multiple pathways are altered by alcohol exposure in fetal human cerebral cortex in vitro including the Wnt signaling and TGF-β signaling pathways.

**Axon Guidance.** Axon guidance is an important stage in neuronal network formation. Several factors are involved in guidance of axons such as netrins, ephrins, slits, and semaphorins (Yu and Bargmann, 2001). These cues are read by growth cone receptors, and subsequently the signal transduction pathways downstream of these receptors bring changes in cytoskeletal organization that determines the direction of growth. It has been suggested prenatal EtOH disrupts the outgrowth of axons and dendrites in the development of rat hippocampus. Furthermore, Lindsay et al. (2003, 2006) have shown that prenatal EtOH retards initial axon outgrowth, but accelerates subsequent axon growth suggesting that EtOH may disrupt the axonal response to guidance cues. In our study with human NSC, we find that EtOH significantly alters the axon guidance pathway. It is possible that these early effects of EtOH on NSC could be responsible for the subsequent disruption in the axonal guidance (Table 2).

**Hedgehog Pathway.** The hedgehog proteins play an important role in the vertebrate embryogenesis and fetal development. Of the 3 hedgehog morphogens, sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh), Shh is known to play an important role not only in ventral patterning of the neural tube but also in determining the dopaminergic and serotonergic cell fates of neural plate (Charytoniuk et al., 2002). Several studies have shown that EtOH directly alters the Shh pathway in developing fetal brain in vivo. Ahlgren and colleagues (2002) have shown that EtOH induced cell death in neural crest cells is rescued by Shh in chick embryos. Other studies in mice have shown that fetal alcohol exposure impairs Shh signaling (Chrisman et al., 2004; Li et al., 2007; Loucks and Ahlgren, 2009; Yamada et al., 2005). Our microarray analysis showed that EtOH exposure to differentiating human NSC significantly disrupts Shh signaling (Table 2). It is possible that the early disruption of Shh pathway could lead to the neural crest cell death seen in FASD.

**TGF-β Signaling.** The transforming growth family members include several cytokines such as TGF-βs, activins, and bone morphogenetic proteins. Several functions have been attributed to the TGFs including, cell proliferation, differentiation, and migration. Yi and colleagues (2010) have shown that TGF-β signaling specifies axons during brain development in mice in vivo and that TGF-β receptors are expressed during development. Recently, Hicks and Miller (2011) have shown that EtOH-induced apoptosis in stem cells is mediated by both TGF-β dependent and independent mechanisms. Kuhn and Sarkar (2008) have shown that EtOH induces apoptotic death of endorphin neurons in rat by a TGF-β dependent mechanism. A disruption of TGF-β signaling is seen in our study with human NSC and EtOH which could be a part of the mechanism of action of EtOH.

**Cell Adhesion Molecules.** CAMs are cell surface glycoproteins which play a vital role in several cellular processes including embryogenesis and fetal neural development. Several studies have shown the importance of CAMs in neural development and that alcohol affects the expression of several CAMs (Parashar et al., 2010; Sakata-Haga et al., 2003; Siegenthaler and Miller, 2004). We suggest based on our microarray study that the changes in CAMs begin to occur at the NSC level in human fetal brain exposed to EtOH. Although all the above pathways appear to be affected by EtOH in human NSC, an in-depth study of the various molecules in each pathway is warranted to elucidate the exact mechanism of action of EtOH. In this study, we chose to investigate the effects of EtOH on the Wnt signaling pathway proteins in differentiating human NSC.

**Wnt Signaling Pathway.** Several functions have been attributed to Wnt signaling in neuronal development as reviewed by Ille and Sommer (2005). Wnts are secreted palmitoylated glycoproteins, which besides playing a key role in progenitor and stem cell expansion and maintenance also play a major role in differentiation processes leading to lineage decisions and in migration, axon guidance, and neurite out growth. Several studies have implicated Wnt signaling in the normal fetal brain development and neurogenesis. McMahon and Bradley (1990) have shown that ablation of Wnt1 protein caused deficiencies in the mid-hind brain formation in mice. Lee and colleagues suggest that Wnt3a is crucial in the development of the hippocampus and Wnt3, -3a, -7b, and -8b participate in the normal development of forebrain (Lee et al., 2000; Toledo et al., 2008). Severe murine phenotypes were observed with ablation or overexpression of Wnt proteins in mice (van Amerongen and Berns, 2006). Haegele and colleagues (2003) have shown that inhibition of Wnt signaling proteins impaired the differentiation of stem cells. Singh and colleagues (2009) have shown involvement of Wnt proteins in the impaired neurogenesis seen in pups when pregnant rats were administered EtOH (Singh et al., 2009). Neznanova and colleagues (2009) have shown that EtOH increased GSK3β and AKT phosphorylation in the tissue from medial prefrontal cortex suggesting a role for the Wnt signaling pathway in the action of EtOH.

The Wnts bind to cell surface receptors activating signaling cascades that follow 3 major pathways: (i) the Wnt/b-catenin pathway, the canonical Wnt signaling pathway, in which β-catenin is a crucial member, (ii) the Frizzled/planar cell polarity pathway, and (iii) the Ca2+ pathway (Kühn et al., 2000). In the Wnt canonical pathway, secreted extra cellular Wnts bind to a 7 transmembrane FZ proteins and lipoprotein receptor proteins (LRP) to form a complex. The phosphorylation of LRPCs leads to phosphorylation of Dsh proteins in the cytoplasm. Dsh proteins when activated by the Wnts inhibit
GSK3 phosphorylation by forming an axin/GSK3β/APC complex. This complex when uninhibited promotes proteolytic degradation of β-catenin. In the presence of Wnt, axin/GSK3β/APC complex is inhibited promoting a cytoplasmic pool of β-catenin which enters the nucleus to interact with TCF/LEF transcription factors to promote specific gene transcription. In our study, EtOH significantly suppressed the expression of 2 Wnt family proteins, Wnt3α and Wnt5a, both of which are involved in neurogenesis. Further, Wnt receptor complex proteins LRP6 and DVL2 were also significantly suppressed by EtOH in a dose-related manner. However, the Wnt antagonist NKD-2 and the receptor protein DVL3 were not affected by EtOH (Fig. 2). This suggests that action of EtOH is specific to only some of the pathway proteins. Next, we studied the expression of inactive Ser phosphorylated GSK3β and active Tyr phosphorylated GSK3β. GSK3β is a multifunctional Ser-thr enzyme and plays a key role in several pathways including the Wnt signaling pathway. It plays an important role in the development of the central nervous system, regulating diverse early events, including neurogenesis, neuronal migration, cell adhesion, synapse formation, neuronal survival, and neurite outgrowth. GSK3β is particularly abundant in the developing nervous system and has been implicated in EtOH-induced neurotoxicity in vivo (Luo, 2009). In this study, we find that EtOH suppressed the Ser phosphorylation of GSK3β in both doses tested and simultaneously significantly increased Tyr phosphorylation leading to increased β-catenin phosphorylation (Fig. 3). Phosphorylated β-catenin undergoes immediate proteosomal degradation and ubiquitination. As expected, the levels unphosphorylated β-catenin were significantly suppressed by EtOH (Fig. 3). A similar reduction in β-catenin levels in EtOH exposed neural crest progenitors has recently been reported by Flentke and colleagues (2011) who have further shown that normalizing β-catenin levels prevents neural crest loss. Lange and colleagues (2011) suggest that small molecule GSK3 inhibitors increase neurogenesis of human neural progenitor cells. Recent studies show that lithium, a GSK3β inhibitor, protects against EtOH neurotoxicity (Luo, 2009). As we show that EtOH has a direct effect on the Ser/Tyr phosphorylation of GSK3β in human cells, in vivo studies to reverse FASD in animal models using small molecule GSK3 inhibitors could shed light on possible future therapies.

We infer from our current data, that EtOH significantly alters at least 5 major pathways/cellular processes involved in neuronal development in differentiating NSC. Further, EtOH suppresses Wnt signaling pathway proteins that are involved in specific gene transcription in brain development. These findings could be a significant step in elucidation of the mechanism of action of EtOH on the developing brain. Future in vivo and in vitro studies using GSK3β inhibitors could lead to possible therapeutic interventions.

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REFERENCES


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes on the array that were absent/ present in samples.

Data S1. References for gene functions in Table 1.

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