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Effects of ethanol on cellular composition and network excitability of human pluripotent stem cell-derived neurons

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Abstract

Background—Prenatal alcohol exposure (PAE) in animal models results in excitatory-inhibitory (E/I) imbalance in neocortex due to alterations in the GABAergic interneuron (IN) differentiation and migration. Thus, E/I imbalance is a potential cause for intellectual disability in individuals with fetal alcohol spectrum disorder (FASD), but whether ethanol (EtOH) changes glutamatergic and GABAergic IN specification during human development remains unknown. Here we created a human cellular model of PAE/FASD and tested the hypothesis that EtOH exposure during differentiation of human pluripotent stem cell-derived neurons (hPSNs) would cause aberrant production of glutamatergic and GABAergic neurons, resulting in E/I imbalance.

Methods—We applied 50mM EtOH daily to differentiating hPSNs for 50 days to model chronic first trimester exposure. We used quantitative PCR, immunocytochemical, and electrophysiological analysis to examine the effects of EtOH on hPSN specification and functional E/I balance.

Results—We found that EtOH did not alter neural induction nor general forebrain patterning, and had no effect on the expression of markers of excitatory cortical pyramidal neurons. In contrast, our data revealed highly significant changes to levels of transcripts involved with IN precursor development (e.g. *GSX2*, *DLX1/2/5/6*, *NR2F2*) as well as mature IN specification (e.g. *SST*, *NPY*). Interestingly, EtOH did not affect the number of GABAergic neurons generated nor the frequency or amplitude of miniature excitatory and inhibitory postsynaptic currents.

Conclusions—Similar to *in vivo* rodent studies, EtOH significantly and specifically altered the expression of genes involved with IN specification from hPSNs but did not cause imbalances of synaptic excitation-inhibition. Thus, our findings corroborate previous studies pointing to aberrant neuronal differentiation as an underlying mechanism of intellectual disability in FASD. However,

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in contrast to rodent binge models, our chronic exposure model suggests possible compensatory mechanisms that may cause more subtle defects of network processing rather than gross alterations in total E/I balance.

Keywords

Fetal alcohol spectrum disorder (FASD); cortical neurons; excitatory-inhibitory balance; interneurons; somatostatin

Introduction

Fetal Alcohol Spectrum Disorder (FASD) describes the range of adverse developmental outcomes that occur as a consequence of alcohol exposure to the developing fetus (Sokol et al., 2003, Chudley et al., 2005). Animal models of prenatal alcohol exposure (PAE) have greatly facilitated our understanding of the underlying mechanisms of alcohol on the developing nervous system under controlled conditions. Critically, animals exposed to various doses of ethanol at different points during gestation closely mimic findings in FASD patients. For instance, high dose exposure during the first trimester causes physical malformations and neurobehavioral defects characteristic of full-blown fetal alcohol syndrome (FAS; (Patten et al., 2014)). In contrast, exposure to more moderate doses or at later developmental time points causes intellectual and/or cognitive dysfunction (Marquardt et al., 2014) similar to patients diagnosed with alcohol-related birth defects (ARBD) and neurological disorder (ARND) (O'Leary et al., 2010, Riley et al., 2011). Despite these advances in FASD model systems, the neurological basis of cognitive deficits across the FASD spectrum remains unknown.

Recent studies point to changes in excitatory-inhibitory (E/I) balance as a potential underlying mechanism for deficits observed in several developmental disorders that result in intellectual disability, including FASD. For a subset of individuals with disorders such as autism and schizophrenia, where specific genetic mutations are known, E/I imbalance appears to result from aberrant synapse formation, maintenance, and/or plasticity (Yizhar et al., 2011, Pak et al., 2015). Similarly, chronic third trimester-equivalent EtOH exposure can lead to changes in activity-dependent plasticity at excitatory and inhibitory synapses (Puglia and Valenzuela, 2010, Zucca and Valenzuela, 2010). In contrast, binge-type exposure during multiple developmental periods has also been shown to significantly alter cellular differentiation and migration (Smiley et al., 2015), specifically of GABAergic interneurons (INs). For instance, Cuzon and colleagues found significant increases in GABAergic INs occupying multiple layers of developing cortex following chronic EtOH exposure during relatively early fetal periods (Cuzon et al., 2008). More recently, Yeh and colleagues demonstrated that EtOH induced significant increases in parvalbumin (PV) INs in medial prefrontal cortex that resulted in decreased excitatory transmission and sustained cognitive deficits in adult animals (Skorput et al., 2015). In contrast, single binge episodes during human third-trimester equivalent (P7) show highly significant reductions in PV and calretinin (CR) INs in cortex that lead to long-lasting behavioral deficits (Sadrian et al., 2014, Smiley et al., 2015). Thus, in animal models of FASD, changes to E/I balance can occur due to influences on neuronal survival and specification as well as at the synaptic

level. However, it remains unclear whether similar effects can be observed in human neurons.

Neuronal differentiation from human pluripotent stem cells (hPSCs) has emerged as an experimentally accessible system to examine the developmental impact of a range of genetic and environmental factors. Differentiation of hPSC-derived neurons (hPSNs) has been shown to recapitulate many morphological and biochemical hallmarks of early neural induction and specification during a similar time course as *in vivo* development (Keller, 2005). hPSNs differentiate by “default” mechanisms to excitatory and inhibitory neurons (Johnson et al., 2007) that resemble populations that occupy mammalian cortex. To determine whether alterations in E/I balance may underlie the etiology of FASD, we exposed human neural progenitors to ethanol using two standard differentiation strategies. We found that EtOH specifically affected genes involved with specification and differentiation of INs, without coincident effects on glutamatergic neuron development. Interestingly, these changes were not reflected in the total number of GABAergic neurons nor gross disruption of E/I balance at the synaptic level, suggesting potential compensatory mechanisms that regulate network function. Our results are the first to demonstrate EtOH-induced alterations in human neuron subtype specification, and suggest that EtOH has complex effects on neural network regulation.

Materials and Methods

hPSC maintenance and differentiation

hPSCs (line WA09 passage 25-35) were maintained in feeder free conditions in mTeSR1 (Stem cell technologies, Vancouver, BC, Canada) on Matrigel (BD biosciences, San Jose, CA) and passaged as previously described (Zhang et al., 2001). Figure 1A illustrates the two different methods used for hPSN generation along with representative images at various time points. For Serum Free Embryoid Body (SFEB) differentiation we used previously established methods (Zhang et al., 2001, Weick et al., 2011). For differentiation using dual-SMAD inhibition (Chambers et al., 2009), cells were passaged using 1mg/ml dispase (Sigma, St. Louis, MO) and plated for differentiation in mTeSR1 containing the SMAD inhibitors SB431542 (10 μ M) and LDN193189 (100nM; Tocris, Bristol, UK), with daily media changes occurring until cells were 80% confluent. Over the first 6 days of differentiation the cells were slowly transitioned into Neural Induction Media (NIM; DMEM/F12 (Invitrogen, San Diego, CA) supplemented with 1x N2 (Invitrogen), 2 μ g/ml heparin (Sigma) with smad inhibitors). On day 0, cells were transferred to a 3:1 ratio of mTeSR1:NIM, 1:1 on day 2, 1:3 on day 4 and complete NIM on day 6. At this stage, neuroepithelial aggregates (neurospheres) were lifted using 1mg/ml dispase and grown in suspension until day 21 in NIM without addition of SB431542. At day 21 neurospheres were then plated onto polyornithine-laminin-coated coverslips (100 μ g/ml polyornithine, 10 μ g/ml laminin; Sigma) in a 24-well plate and fed every other day with Neural Differentiation Media (NDM) containing Neurobasal (Invitrogen) supplemented with BDNF and GDNF (10ng/ml; Peprotech, Rocky Hill, NJ), cAMP (1 μ M; Sigma), ascorbic acid (200 μ M; Sigma). For dual SMAD inhibition protocol, day 0 is defined as the first day of SMAD inhibitor exposure. For SFEB differentiation, day 0 is defined as the day of embryoid body formation

(lifting and aggregation of hPSCs). For ethanol treatment, 50mM ethanol was added daily to cells from day 0 until cells were assayed.

Ethanol Concentration

Media ethanol concentrations were determined using an ethanol assay kit as described by manufacturer (Biovision, Milpitas, CA). Samples (10 μ l) were collected from wells treated with EtOH for a specific number of days and collected between 18-24 hours following last EtOH addition. The samples were incubated with 50 μ l of reaction mix (prepared according to manufacturer) for 1 hr. in the dark at room temperature. The optical densities of the samples were measured at 570 nm with a fluorometric micro-plate reader (Tecan). Sample ethanol values were determined by regression analysis. Duplicates of samples for each number of treatment days were used for the assay.

Immunocytochemistry

Immunocytochemistry was as previously described (Weick et al., 2013). Briefly, coverslips were fixed with 4% paraformaldehyde for fifteen minutes, rinsed three times with phosphate-buffered saline (PBS) and permeabilized using 0.2% Triton for five minutes. Cells were blocked with 10% donkey serum in PBS for 30 minutes, followed by an overnight incubation of primary antibody in 5% donkey serum at 4°C. Primary antibodies consisted of monoclonal Oct4 (1:100, Thermo Fisher Scientific, Waltham, MA), polyclonal Pax6 (1:500, developmental studies hybridoma bank), monoclonal β III-Tubulin (1:2000, Sigma), and polyclonal GABA (1:100, Sigma). Cells were then washed with 1x PBS and incubated for one hour at 4°C with secondary antibody in 5% donkey serum. Secondary antibodies (anti-mouse DyLight 488 and anti-rabbit DyLight 550 (1:500; Thermo Fisher Scientific). Cells were washed with 1x PBS and then treated with DAPI at 1:10,000 (Thermo Fisher Scientific), followed by three washes with 1x PBS, and mounted on glass cover slides using Fluoromount-G (SouthernBiotech, Birmingham, AL).

Fluorescence-activated cell sorting (FACS) analysis

FACS analysis was carried out essentially as described in (Wang et al., 2014) with the following modifications. hPSCs or neuroepithelia (day10) were harvested and dissociated into a single cell suspension by using 0.25% Trypsin (Thermo Fisher Scientific), washed with FACS buffer containing 0.1% NaN₃ (Sigma) and 2% donkey serum (EMD Millipore, Billerica, MA) in PBS, and filtered through a nylon mesh filter with a pore size of 40 μ m (Corning, Corning, NY). Cells were fixed in ice-cold 0.1% paraformaldehyde for 10 min and permeabilized with ice-cold 90% methanol for 30 min. Approximately 1×10^6 cells were incubated overnight with either mouse anti-Pax6 (1:10; DSHB), rabbit anti-Oct4 (1:2500; Pierce), or normal mouse or rabbit IgG which were used as negative controls. Following three washes in ice-cold FACS buffer, cells were incubated with either donkey anti-mouse DyLight 488 or donkey anti-rabbit DyLight 550 (1:500; Pierce) for one hour. 30,000 labeled cells were analyzed by FACS on an LSR Fortessa II (BD Biosciences), and the ratios and mean fluorescence intensities of Pax6⁺ and Oct4⁺ cells were calculated using the FlowJo software (FlowJo, LLC, Ashland, OR).

Quantitative polymerase chain reaction (qPCR)

At day 50 of differentiation 1ml of Trizol (Sigma) was applied to 8-10 coverslips from each of three biological replicates from four conditions (Control and 50mM ethanol x SFEB and dual-SMAD inhibition; 12 samples total). RNA was isolated according to manufacturer's specifications, including addition of 4% glycogen as an RNA carrier during the precipitation step. RNA (1µg) was reverse transcribed using the iscript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. qPCR reactions containing 1x SYBR Green Master Mix (Thermo Fisher Scientific), 1 µM forward and reverse primers, 5 ng cDNA, and water were amplified using an Applied Biosystems 7300 real-time PCR system. GAPDH was used as an internal reference. Analysis of results was performed using the comparative C_T method to determine fold-change (Schmittgen and Livak, 2008). qPCR primers for all amplicons are listed in supplementary table 1.

Electrophysiology

Whole cell patch clamp recordings were performed as described (Marszalec et al., 1998) with minor modifications. The extracellular solution was a modified Hanks Balanced Salt Solution (HBSS) that contained (in mM): 150 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 23 glucose, pH 7.4, 300 mOsm. Recording pipettes with resistances of 3–5 MΩ were filled with an intracellular recording solution containing the following (in mM): 140 K-gluconate, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES acid, 2 Mg²⁺-ATP, 0.2 Na⁺-GTP, pH 7.2, 290 mOsm. Each experiment consisted of 8–12 cells per group (Control vs. EtOH x dual SMAD and SFEB) with a total of 122 cells included for analysis. Neurons were visualized using an Olympus Optical BX51WI microscope (Olympus Corp., Tokyo, Japan) with differential interference contrast optics at 40x. Recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 4 kHz and sampled at 100 kHz using a Digidata 1322A analog-to-digital converter (Molecular Devices). Whole-cell capacitance was fully compensated, and series resistance was compensated >70% except during PSC recordings, where it was not compensated. Access resistance was monitored before and after recordings, and cells with resistances greater than 25 MΩ at either point were discarded from analyses. Na⁺ and K⁺ currents were stimulated using 200-ms voltage steps that varied from –50 to +50 mV in 10-mV increments, with a holding potential of –70mV between steps. Peak inward currents generated by voltage steps were calculated by subtracting baseline (taken at 5ms prior to stimulus onset) from local minimum. Peak outward currents were measured at the local maximum between 1–30ms following stimulus onset while sustained outward currents were measured by averaging the last 20ms; both were calculated by subtracting the same baseline used for inward currents. All data were stored on a computer hard disk and analyzed with Clampfit v9.2 (Molecular Devices). Post-synaptic currents were measured using a holding potential of –40mV. All data were stored on a computer hard disk; voltage-gated currents were analyzed with pClamp 10.2 (Molecular Devices), and post-synaptic currents (PSCs) were analyzed using MiniAnalysis software (Synaptosoft, Fort Lee, NJ). All PSCs were verified visually and >20,000 synaptic events were examined. The liquid junction potential was adjusted according to previously published methods (Neher, 1992). All recordings were performed at 32°C.

Statistical Analyses

Student's t-tests were used to determine whether mean differences between groups (e.g. untreated vs. EtOH) were significant and were considered significant *a priori* if $p < 0.05$. The number of samples used for statistical analyses (n) refer to the number of biological replicates (independent trials of hPSN differentiation for each method (SFEB or dual-SMAD inhibition). Data are reported as mean $\pm$ standard error of the mean.

Results

EtOH does not affect neural progenitor differentiation

Figure 1A illustrates the timeline and critical developmental stages for two established cellular differentiation paradigms we used to drive neuroectoderm differentiation from hPSCs. The serum-free embryoid body (SFEB) (Zhang et al., 2001) and dual-SMAD inhibition (Chambers et al., 2009) methods allow neural specification to proceed by “default” patterning, without application of exogenous morphogens to drive specification of particular regional and transmitter phenotypes. Both methods have been shown to lead to forebrain patterning, including a highly uniform population of neural progenitors (Figure 1A, “rosettes”) that strongly express the ventricular zone marker Pax6 during early stages (Zhang et al., 2010). These progenitors then give rise to mixed populations of glutamatergic and GABAergic neurons (Johnson et al., 2007) similar to those found in mammalian cortex.

PAE in the first trimester is a common pattern of *in utero* exposure as a significant proportion (~25%) of pregnant mothers are not aware of the pregnancy until many weeks post-conception (Ayoola et al., 2009). Here we used a daily application of EtOH throughout the 50 day differentiation period at a concentration of 50mM, a dose previously shown to have multiple effects on progenitors and neurons *in vitro* (Hoffman et al., 2008, Talens-Visconti et al., 2011). This dose corresponds to blood concentrations near 200mg/dl, which have been reported in clinical studies of mothers whose children develop full-blown FAS (Kvigne et al., 2003), as well as preclinical studies of rodent models (Patten et al., 2014). As shown in Figure 1A, Pax6⁺ neural progenitors are determined by day 10 (d10) of differentiation, begin to become β III-tubulin⁺ post-mitotic hPSNs at approximately d21, and show robust functional properties by *in vitro* d50 (Johnson et al., 2007, Weick et al., 2013), as illustrated by robust synapsin-1 (green) staining (rightmost image). Thus, we chose d10 to analyze the effects of EtOH on neural induction and d50 as an endpoint for our analyses of neuronal subtype specification and function (see below). To verify exposure, EtOH concentrations were measured at various timepoints during differentiation by the oxidation product H₂O₂ (see methods). At all time points EtOH concentrations were similar to those predicted with a mean concentration of 44.5 $\pm$ 3.8mM.

In previous stem cell studies, EtOH significantly reduced proliferation and affected the expression of the pluripotency marker Oct4 and the early neuroectodermal transcription factor Pax6, but only at concentrations above 50mM (Mo et al., 2012, Ogony et al., 2013). To determine whether 50mM EtOH causes reductions in hPSC-derived progenitor proliferation and/or neuronal specification in our hands, we performed immunocytochemistry as well as fluorescence-activated cell sorting (FACS; (Zhang et al.,

2010)). Figure 1B (upper panels) shows that hPSCs at d0 of differentiation stained robustly for the pluripotency marker Oct4 (Nichols et al., 1998) with no Pax6⁺ cells evident (note that no cells at this time point received EtOH treatment). In contrast, neural rosettes at d10 of differentiation showed a complementary pattern of staining, with nearly all cells displaying the definitive human neuroectoderm marker Pax6 (Zhang et al., 2010) but lacking Oct4 expression (Figure 1B, lower panels). FACS data confirmed these results, where the vast majority of hPSCs-derived neuroepithelia were positive for Pax6 by d10 and negative for Oct4 (Figure 1C). No significant differences were observed for control and EtOH-treated cells (Figure 1B, C; n=6; p>0.05), regardless of differentiation method. Thus, EtOH-treated hPSC-derived progenitors were able to produce robust populations of neuroepithelia similar to control cells using both differentiation methods.

Ethanol does not affect neural specification and forebrain patterning

As mentioned, dual-SMAD inhibition and SFEB-mediated neural induction result in telencephalic patterning of neural progenitors, with multiple markers of cortical and subcortical layers showing strong expression (Espuny-Camacho et al., 2013). Using immunocytochemistry of cultures at d50 we first determined that EtOH did not significantly affect the proportion of β III-Tubulin⁺ neurons compared to untreated cells (Figure 2A-B; n=3, p>0.05). We then used quantitative PCR (qPCR) analysis to determine whether EtOH exposure affected the expression of transcripts related to general neural differentiation and forebrain patterning. Figure 2C shows transcript expression levels for EtOH-treated neurons that have been normalized to control cells (dashed line at a value of “1”). Analysis of genes involved with neural induction including *NES* (Nestin), *PAX6*, and *MEIS2* were not significantly different between groups (Figure 2C; n=3, p>0.05). Thus, consistent with our protein analysis of Pax6 at d10, EtOH-treated cells express multiple markers of NE cells to the same extent as control populations at d50. Furthermore, pan-forebrain markers such as *FOXG1* and *COUPTF1* were also not significantly altered upon EtOH treatment (Figure 2C; n=3, p>0.05). We also analyzed markers of deep-layer (*CTIP2*, *DKK3*) and superficial-layer cortical neurons (*FOXP2*, *CUX1*, and *TLE1*; Molyneaux et al., 2007), and found no significant differences between EtOH-treated and control cells using either differentiation protocol (Figure 2D; n=3, p>0.05). Thus, exposure of differentiating hPSCs to 50mM EtOH did not grossly affect regionalization of neural progenitor cells and neuronal differentiation.

Ethanol significantly affects GABAergic IN gene expression but spares glutamatergic specification

Although we did not observe changes in cortical layer marker expression, we examined whether EtOH could affect E/I balance via changes to markers for glutamatergic neurotransmission. Expression analysis of the primary subunits composing post-synaptic AMPA (*GRIA1-3*) and NMDA (*GRIN1*, *2B*) receptors showed no significant differences between groups (Figure 3A; n=3, p>0.05). We also found no change in the levels of mRNA for the post-synaptic scaffolding protein PSD-95 (*DLG4*) nor the pre-synaptic protein vGlut2 (*SLC17A6*; Figure 3A; n=3, p>0.05). Thus, we concluded that EtOH had no effect on excitatory neuron differentiation from hPSCs.

In contrast, EtOH treatment significantly altered the expression of multiple markers involved in specification of GABAergic INs. For instance, newly differentiated INs express high levels of multiple members of the distal-less homeobox (Dlx) family of transcription factors (Nord et al., 2015). We found that levels of *DLX1*, 2, 5, and 6 were all significantly reduced in EtOH-treated cultures (Figure 3B; $n=3$, $p<0.05$). While previous studies using default patterning of hPSCs have not elucidated the molecular identity of INs, they do not express *NKX2.1*, which is required for specification of INs derived from the medial ganglionic eminence (MGE). In contrast, *GSX2* is a transcription factor thought to lie upstream of a genetic regulatory cascade involved in the specification of INs derived from caudal ganglionic eminence (CGE), the other primary source of cortical INs (Kepecs and Fishell, 2014). We found that hPSNs do express *GSX2*, and that EtOH significantly reduced transcript levels to a similar degree as *DLX* family members (Figure 3B; $n=3$, $p<0.05$). Relative to control samples, cultures treated with 50mM EtOH also showed highly significant reductions in markers of mature INs including *SST* and *NPY* (Figure 3C; $n=3$, $p<0.05$). Additionally, we found that the vesicular GABA transporter (*VGAT*; *SLC32A1*), a marker of inhibitory presynaptic terminals, was also significantly downregulated (Figure 3E; $n=3$, $p<0.05$). In contrast, no changes were observed in postsynaptic GABA receptor subunits (*GABRA2*, *GABRB2*, and *GABAG2*) that showed high expression in control samples (Figure 3E; $n=3$, $p>0.05$). To determine whether these reductions in IN genes were also manifest in the overall GABAergic neuron population we stained cultures for GABA. Surprisingly, we found that the proportion of GABA⁺ neurons that co-stained with β III-Tubulin was slightly lower, but not significantly different from controls (Figure 3C-D; $n=3$, $p>0.05$). As a possible mechanism to explain these findings, we found several other markers of IN progenitors that were significantly upregulated, such as *COUPTFII* (*NR2F2*) and *ID4* (Figure 3F; $n=3$, $p<0.05$). And, although it did not reach significance, the definitive IN marker Calbindin2 (*CALB2*; Calretinin) showed a trend toward an increase as well (Figure 3F; $n=3$, $p=0.08$). No changes in other IN markers including *PV* (Figure 3D; $n=3$, $p<0.05$), Cholecystokinin (*CCK*; data not shown), nor the serotonin receptor subunit 3A (*HTR3A*; data not shown) were observed.

EtOH has no effect on passive or active membrane properties

Due to the specific alterations in gene expression related to IN development, we performed electrophysiological recordings to examine whether 50mM EtOH affects excitation-inhibition balance in hPSN cultures. We first examined whether EtOH affects passive properties including resting membrane potential (RMP), capacitance (CAP), input resistance (R_{in}), measures of cellular complexity and functional maturation. Compared to controls, mean RMP, CAP, and R_{in} remained unchanged upon EtOH treatment regardless of differentiation method (Table 1; $n=3$, $p>0.05$ in all conditions). These data suggest that EtOH did not affect process extension nor expression and insertion of ion channels that control membrane polarity. To examine active ion conductances we used a voltage-step protocol to determine the effect of EtOH on voltage-gated sodium (Na^+) and potassium (K^+) currents. Figure 4A-C shows representative traces from control and EtOH-treated cells that were given a series of 200ms voltage steps from -50mV to +50mV to examine inward Na^+ (I_{Na}) and outward K^+ currents (I_K), respectively. Figure 4C shows expanded time courses of I_{Na} to illustrate current kinetics. Current-voltage (I/V) relationship curves for cells

differentiated using SFEB- or dual-SMAD methods illustrate no significant difference between control and EtOH-treated cells at any potential for peak I_{Na} (Figure 4D, table 1), peak I_K (Figure 4E, table 1; $n=6$, $p>0.05$), nor sustained I_K (Figure 4F; $n=6$, $p>0.05$).

EtOH does not alter iPSC:ePSC ratio

To directly determine the effects of EtOH on E/I balance we measured the amplitude and frequency of miniature excitatory and inhibitory post-synaptic currents (ePSCs and iPSCs). Using previously published methods (Marszalec et al., 1998) we used a holding potential of -40mV to simultaneously record ePSCs and iPSCs from control and EtOH-treated hPSNs at d50. Figure 5 shows representative traces from hPSNs that were untreated (Figure 5A) or received 50mM EtOH (Figure 5B) during differentiation. Upward current deflections represent iPSCs while downward deflections represent ePSCs due to the driving forces for Cl^- and Na^+ , respectively. Pooled data show that neither mean frequency (Figure 5C-D; $n=6$, $p>0.05$) nor amplitude (Figure 5E-F; $n=6$, $p>0.05$) of ePSCs or iPSCs were significantly altered by EtOH-treatment during neuronal differentiation.

Discussion

Findings from animal models of prenatal alcohol exposure (PAE), as well as from patients with FASD, suggest that alterations in E/I balance contribute to neurological symptoms in adolescents and adults. Compared with the population at large, individuals with FASD experience a significantly higher incidence of seizure disorders (Bell et al., 2010). Estimates of prevalence range as high as 21% for FASD individuals compared with only 3-4% for the general population (McHugh and Delanty, 2008). In addition, the most common comorbidity in FASD is attention deficit-hyperactivity disorder (ADHD; (Fryer et al., 2007)), and alterations in E/I balance have been associated with ADHD. Studies using edited magnetic resonance spectroscopy have revealed that GABA levels in the dorsolateral prefrontal cortex negatively correlate with impulsivity (Boy et al., 2010), and reduced levels of GABA are found in multiple brain regions of ADHD patients (Edden et al., 2012). Furthermore, disinhibition of medial prefrontal cortex in rodents caused by perfusion of a GABA_A antagonist results in inattention and hyperactivity (Pezze et al., 2014). Interestingly, these findings suggest that *reduced* inhibition, possibly by reductions of specific IN populations in the cortex (Smiley et al., 2015), are critical for the effects observed in FASD and ADHD patients.

In contrast, most reports of ethanol exposure in rodents have demonstrated that EtOH causes *increased* IN differentiation, especially in models using prenatal exposure. Using either BAC- or Nkx2.1-Cre transgenic animals, Yeh and colleagues have found significant elevations in the number of parvalbumin-expressing INs in multiple regions of cortex (Cuzon et al., 2008) including medial prefrontal cortex (Skorput et al., 2015). In addition, they find that PAE animals show enhanced inhibitory inputs to layer 5 pyramidal neurons as well as hyperactivity and impaired cognitive flexibility (Skorput et al., 2015). These studies employed a binge exposure model whereby EtOH was administered specifically during IN development in mice between embryonic days 13.5-16.5. Future studies are needed to demonstrate whether first-trimester exposure can also affect IN development. In addition,

Yeh and colleagues use relatively moderate doses (~80mg/dl), whereas our *in vitro* studies employ higher doses (~200mg/dl equivalent). It will be interesting to determine whether differences between these data are due to pattern of administration (dosage/timing) and/or represent contrasts between human and murine systems.

Regardless of direction (increased vs. decreased inhibition), the mechanism of EtOH-induced alterations of IN development may lie with the developing GABAergic system itself. Activation of GABA_A receptors on neural progenitors and newly-differentiated neurons can act in an excitatory fashion when the intracellular chloride ($[Cl^-]_i$) concentration is high due to predominant expression of the Na⁺-K⁺-2Cl⁻ transporter, NKCC1 (i.e., a Cl⁻ importer). At later time points, GABA becomes inhibitory due to the increased expression of the KCC2 Cl⁻ transporter which exports Cl⁻, lowering $[Cl^-]_i$ (Ben-Ari, 2002). Blocking GABA synthesis or GABA_A receptor activation leads to significant increases in the number of neurons expressing excitatory transmitters in *Xenopus* spinal neurons (Root et al., 2008). Early overexpression of KCC2 in progenitor cells augments the formation of GABAergic synapses in rat hippocampal cultures (Chudotvorova et al., 2005). Furthermore, in mouse cortex, inhibition or downregulation of the NKCC1 transporter blocks GABA-mediated depolarization and significantly affects the formation of excitatory synapses, primarily due to its effects on Ca²⁺ entry via NMDA receptors (Wang and Kriegstein, 2008). Livesey and colleagues found that intracellular Cl⁻ activity in hPSNs (and neural precursor cells) is reduced from around 25 mM to less than 7 mM in more mature hPSNs, similar to those used in the current study (Livesey et al., 2014). This change corresponded to a switch in expression of KCC2 and NKCC1, in good agreement with *in vivo* developmental mechanisms (Ben-Ari, 2002).

As it relates to FASD, EtOH is a potent stimulator of GABAergic signaling, and may alter neurotransmitter specification of progenitor cells via altered GABAergic tone during development. EtOH has been shown to enhance GABA release in hippocampus, cerebellum, substantia nigra, among other regions (Kelm et al., 2011). EtOH can also act post-synaptically to increase the amplitude of currents in response to GABA release (Wu et al., 2005, Lovinger and Homanics, 2007). In early postnatal hippocampal CA3 neurons, EtOH potently stimulates giant depolarizing potentials that coincide with intracellular calcium $[Ca^{2+}]_i$ transients, which are blocked by GABA_A receptor antagonism (Galindo et al., 2005). Neurotransmitter specification is regulated by the frequency of calcium (Ca²⁺) transients during progenitor cell differentiation to post-mitotic neurons. In multiple CNS regions, alterations in the frequency of Ca²⁺ transients changes the proportion of neurons expressing inhibitory and excitatory neurotransmitters (Spitzer, 2012). Thus, it is possible that EtOH can alter Ca²⁺-dependent signaling cascades that ultimately determine the transmitter phenotype of post-mitotic neurons via potentiation of GABA-mediated currents in progenitor cells (Borodinsky et al., 2004, Root et al., 2008).

Despite these intriguing mechanisms, it is difficult to reconcile the current findings that show significant alterations in genes involved with IN specification in the absence of a change in number of GABAergic neurons or network properties. One possible explanation is that compensatory upregulation of other IN populations can normalize E/I balance. We did find that some IN markers were increased in response to EtOH, and may contribute to an IN

population that is less well-characterized in hPSNs. Alternatively, although we did not find a corresponding upregulation in *PV* or *HTR3A*, these markers are typically not expressed until postnatal periods in rodents (>P8; (de Lecea et al., 1995)), and may be underrepresented in hPSNs at relatively early timepoints (e.g. d50). Thus, there may be a population of EtOH-insensitive GABAergic neurons that is destined to express *PV*, *HTR3A*, or other mature IN marker, but has yet to acquire such expression at d50 of differentiation. Future experiments should examine later time points with more specific markers of IN subtypes to explore this possibility.

Lastly, it was surprising that significant reductions in VGAT mRNA did not manifest in altered iPSC frequency or amplitude. However, while previous reports of VGAT knock outs have shown dramatic reductions in both frequency and amplitude, heterozygous animals (i.e. showing 50% reductions in VGAT) did not display obvious changes in spontaneous inhibitory neurotransmission (Wojcik et al., 2006). Interestingly, homeostatic mechanisms have been shown *in vivo* to result in maintenance of basal E/I balance despite massive reductions in IN generation. Osten and colleagues utilized conditional deletion of all *PV* and *SST* neurons in layer 2-4 of mouse cortex to examine the subtype- and layer-specific roles of cortical INs (Takada et al., 2014). Surprisingly, while they found dramatic reductions in total INs populating layer 2-4, basal levels of synaptic E/I balance was maintained. However, KO animals did display deficits in the generation of high-frequency gamma oscillations, which are important rhythms thought to arise from synchronous activity between brain regions. These data suggest that homeostatic processes could scale up individual inhibitory inputs to pyramidal neurons to make up for the loss of PV/SST INs as measured by spontaneous iPSCs. However, the data revealed the specific role of PV/SST neurons in generating network behavior as a whole. Thus, future experiments are needed to determine whether inhibitory synaptic transmission is compromised during periods of heightened excitation, where reduced levels of VGAT may compromise refilling of presynaptic vesicles.

Conclusion

In conclusion, the data presented here represent the first examination of the effects of EtOH on neuronal subtype-specific lineage differentiation in a human system. Critically, our findings implicate the involvement of disrupted IN development similar to animal models. However, our data suggest novel mechanisms by which neural circuit function may be disrupted in individuals with FASD due to IN dysfunction that may be independent of basal E/I imbalance.

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Figure 1. Ethanol does not affect neural induction

(A) Diagram illustrating the methods and time course of neuronal differentiation used in the study. Representative images show cellular morphology at various time points during differentiation; image labeled “immature neurons” was taken from neurons at day 30 and shows β_{III} -Tubulin while the image labeled “functional neurons” was taken at day 50 and shows β_{III} -Tubulin (red) and Synapsin-1 (green). Scale bars indicate 100 μ m. (B) Paired confocal images of hPSCs at day 0 (upper panels) and neuroepithelia that were untreated (middle panels) or treated with 50mM EtOH (lower panels). Representative images illustrate the presence of Oct4 (red) and absence of Pax6 (green) at day 0. Cells at day 10 showed robust Pax6 (green) with little Oct4 (red) present. Nuclei are labeled with DAPI (blue). Scale bars indicate 50 μ m. (C) Fluorescence-activated cell sorting data confirmed that treated and untreated cells at day 10 lost Oct4 expression (red; lower left panels) but showed strong Pax6 expression (green; lower right panels), while cells at day 0 showed the opposite pattern of expression (upper panels). Plots illustrated in black indicate independent IgG controls for each antibody.

Figure 2. Ethanol does not affect neuroepithelial nor forebrain cortical neuron differentiation (A) Representative confocal micrographs of control (left) and EtOH-treated (right) hPSN cultures labeled with the neuronal marker β_{III} -Tubulin (green) and DAPI (blue). Scale bar indicates 50 μ m. (B) Pooled data revealed no significant difference in the proportion of β_{III} -Tubulin⁺/DAPI⁺ cells between groups. N = 6; p>0.05. (C) Pooled qPCR data shows that mean expression of neuroepithelial markers (*NES*, *PAX6*, *MEIS2*) and forebrain markers (*FOXG1*, *COUPTF1*) in EtOH-treated cells did not significantly differ from controls (normalized expression illustrated by dashed line) using either SFEB- or Dual-SMAD inhibition methods. (D) Expression of superficial layer (*CTIP2*, *DKK3*, *FOXP2*), and deep layer (*CUX1*, *TLE1*) cortical markers was not significantly different between EtOH-treated and control cells at day 50. N = 3; p>0.05.

Figure 3. Ethanol specifically and significantly affects GABAergic gene expression without altering the proportion of GABAergic neurons

(A) Pooled qPCR data shows that mean expression levels of markers for post-synaptic NMDA receptors (*GRIN1*, *2B*), AMPA receptors (*GRIA1-3*), *VGLUT2* and *PSD95*, were not significantly altered in EtOH-treated cells compared to controls (normalized expression illustrated by dashed line). (B) In EtOH-treated cells, multiple transcripts involved with GABAergic IN specification were significantly reduced using either SFEB- or Dual-SMAD inhibition methods compared to untreated-controls (* indicates significance for all markers shown). (C) Representative confocal micrographs of control (left) and EtOH-treated (right) hPSN cultures labeled with GABA (green), β_{III} -Tubulin (red), and DAPI (blue). Arrows indicate GABA⁺/ β_{III} -Tubulin⁺ cells while arrowheads indicate GABA⁻/ β_{III} -Tubulin⁺ cells.

Scale bar indicates 50 μ m. (D) Pooled data revealed no significant difference in the proportion of GABA⁺/ β III-Tubulin⁺ cells between groups. N = 6; p>0.05. (E) Pooled data showing mRNA expression for postsynaptic GABA receptors (α 2, β 2, γ 2) remained unchanged, while EtOH significantly reduced expression of the presynaptic marker VGAT. N = 3; *p<0.05. (F) Pooled data indicating expression of IN progenitor markers *ID4* and *COUPTFII* (*NR2F2*) were significantly elevated by EtOH treatment (N = 3; *p<0.05), while *CALB2* and *PARV* were not significantly changed. N = 3; p>0.05.

Figure 4. Ethanol does not affect voltage-gated currents in hPSNs

(A-B) Representative traces from voltage-clamped neurons during voltage steps revealed the presence of robust transient inward and sustained outward currents in EtOH-exposed and controls. (C) Expanded timescale of inward currents. (D-F) Current-voltage relationships for peak inward (D), peak outward (E), and sustained outward (F) currents were not significantly different between untreated hPSNs and those that received 50mM EtOH. $N = 6$; $p > 0.05$.

Figure 5. Ethanol does not affect spontaneous post-synaptic currents in hPSNs

(A-B) Representative traces from neurons in voltage-clamp mode and held at -40mV revealed the presence of robust EPSCs (downward deflections) and IPSCs (upward deflections) in EtOH-exposed and control hPSNs. (C-D) Pooled data demonstrate that mean frequency of EPSCs (C) and IPSCs (D) were not significantly different between untreated hPSNs and those that received 50mM EtOH for the duration of differentiation. $N = 6$; $p > 0.05$. (E-F) Pooled data demonstrate no difference in amplitude of EPSCs (E) and IPSCs (F) between controls and hPSNs that received EtOH. $N = 6$; $p > 0.05$.

Table 1

Passive and active membrane properties of d50 hPSNs

Condition	Method	RMP (mV)	Cap (pF)	R _{in} (GΩ)	I _{na} (pA)	I _K (pA)
Control	SFEB	-48 ± 2.9	19.9 ± 1.5	0.80 ± 0.12	3414 ± 465	2755 ± 164
	Dual-SMAD	-52 ± 1.3	28.5 ± 0.3	0.87 ± 0.09	3304 ± 261	3327 ± 344
	Mean	-50 ± 1.7	24.2 ± 2.0	0.84 ± 0.07	3359 ± 240	3041 ± 213
EtOH	SFEB	-49 ± 2.9	19.4 ± 0.7	1.0 ± 0.08	3736 ± 354	3075 ± 384
	Dual-SMAD	-55 ± 1.7	25.39 ± 0.4	0.89 ± 0.20	3322 ± 742	3005 ± 169
	Mean	-52 ± 2.0	22.4 ± 1.4	0.95 ± 0.10	3529 ± 379	3040 ± 188

EtOH does not affect RMP, Cap, R_{in}, Na⁺ or K⁺ currents in hPSNs