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Ethanol Exposure During Neurogenesis Induces Persistent Effects on Neural Maturation: Evidence From an Ex Vivo Model of Fetal Cerebral Cortical Neuroepithelial Progenitor Maturation

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Ethanol is a significant neuroteratogen. We previously used fetal cortical-derived neurosphere cultures as an ex vivo model of the second trimester ventricular neuroepithelium, and showed that ethanol directly induced fetal stem and progenitor cell proliferation and maturation without inducing death. However, ethanol is defined as a teratogen because of its capacity to persistently disrupt neural maturation beyond a specific exposure period. We therefore utilized a simplified neuronal maturation paradigm to examine the immediate and persistent changes in neuronal migration following ethanol exposure during the phase of neuroepithelial proliferation. Our data indicate that mRNA transcripts for migration-associated genes RhoA, Paxillin (Pxn), and CDC42 were immediately induced following ethanol exposure, whereas dynein light chain, LC8-type 1 (DYNLL1), and growth-associated protein (Gap)-43 were suppressed. With the exception of Gap43, ethanol did not induce persistent changes in the other mRNAs, suggesting that ethanol had an activational, rather than organizational, impact on migration-associated mRNAs. However, despite this lack of persistent effects on these mRNAs, ethanol exposure during the proliferation period significantly increased subsequent neuronal migration. Moreover, differentiating neurons, pretreated with ethanol during the proliferation phase, exhibited reduced neurite branching and an increased length of primary neurites, indicating a persistent destabilization of neuronal maturation. Collectively, our data indicate that ethanol-exposed proliferating neuroepithelial precursors exhibit subsequent differentiation-associated increases in migratory behavior, independent of mRNA transcript levels. These data help explain the increased incidence of cerebral cortical neuronal heterotopias associated with the fetal alcohol syndrome.

Key words: Fetal alcohol syndrome; Neural stem cells; Gap43; RhoA; Paxillin; CDC42; Dynein light chain; LC8-type 1 (DYNLL1); Migration

INTRODUCTION

Ethanol consumption during pregnancy is the leading cause of mental retardation associated with craniofacial and cardiovascular defects. Collectively this constellation of symptoms is termed "Fetal Alcohol Spectrum Disorders" or FASD (20,43). Fetal alcohol syndrome (FAS), represents the severe end of the FASD continuum, and is characterized by the presence of several neuroanatomical malformations including microencephaly, lissencephaly, heterotopias, and loss of inter-hemispheric fiber tracks (9,38).

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The severity and types of behavioral and anatomical deficits depend on the timing of ethanol exposure, and reflect underlying periods of susceptibility of critical developmental programs (19). Significant research has identified the third trimester as a particular period of neural vulnerability to ethanol (5,13,14, 48,49). Ethanol exposure during the third trimester results in the loss of trophic support mechanisms (10,21–23,25,27) and, consequently, death (8,26,29, 30) in many brain regions. In comparison, the effects of ethanol exposure during the second trimester are poorly understood. However, the second trimester is an equally important period of brain growth, because, during this period, germinative neuroepithelial zones of the neural tube give birth to millions of new neurons that populate emerging brain regions like the cerebral cortical plate (7,31,44). A teratogen that alters the fate of even a few neuroepithelial stem cells is likely to disproportionately influence subsequent brain development.

We previously used an ex vivo model of the rodent fetal neuroepithelium to show that ethanol does not kill undifferentiated neuroepithelial cells, despite inducing the expression of suicide receptor complex genes (35). Rather, ethanol-induced cell proliferation is accompanied by depletion of ABCG2, Sca-1, c-kit, and CD133-positive stem cells, without alteration in the expression of nestin (39). Furthermore, isolated ethanol-treated neural progenitors exhibit evidence for morphological transformation into radial glial-like cells. These data suggested that ethanol induced maturation and, consequently, depletion of neural stem cells. We also recently showed that a class of ethanol-sensitive micro-RNAs mediated apoptosis resistance and increased cell proliferation, following ethanol exposure (40). These data demonstrate that ethanol has immediate, activational effects on neural stem cell populations. However, an important question, pertinent to ethanol's status as a teratogen, is whether ethanol exposure during the period of stem cell maturation also has persistent, organizational effects on subsequent neuronal maturation.

The migration of neuroblasts away from the ventricular zone (VZ) towards the subventricular zone (SVZ) and cortical plate (CP) constitutes a key component of the early cortical neuronal differentiation program (31). It is possible that ethanol exposure during the period of neuroepithelial proliferation may have significant organizational effects on the subsequent neuronal migration. To begin to address the potential for ethanol exposure during neuronogenesis, to have a persistent effect on subsequent differentiation events, we treated neurosphere cultures derived from gestational day (GD) 12.5 mouse cerebral cortex with ethanol, at a level attainable in chronic alcoholics, to mimic ethanol exposure during the second trimester period of VZ proliferation. We then used a simplified mitogen withdrawal paradigm (6) to transform these proliferating progenitors into migratory, bipolar neurons or multipolar neurons. Using this combination of paradigms, we examined the immediate (activational) and persistent (organizational) effect of ethanol exposure during the proliferation period on several migration-associated genes. We selected genes based on their classification into specific gene ontologies (based on the Gene Ontology Consortium, http://www.geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) pathways related to cell motility (Table 1). We also examined the effects of ethanol exposure during the period of cell proliferation on cell survival, neurite outgrowth, and migration during the transformation of these neural progenitors into migratory neurons. We observed that ethanol generally induced immediate, but not persistent, effects on cell migration-associated genes. However, ethanol exposure during the proliferation period induced a significant increase in cell migration and neurite extension following neuronal differentiation.

MATERIALS AND METHODS

Cell Culture Model and Differentiation Paradigm

The TAMU animal care committee approved all animal protocols. Fetal cerebral cortical neuroepithelial cells were obtained from GD12.5 mouse dorsal telencephalic vesicle (corresponding to the structural precursor of the mouse iso-cortex) according to previously published protocols. Neuroepithelial cells were dispersed into defined culture medium [DMEM/F12 (#11330-032 Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; 13256-029 Invitrogen), 20 ng/ml human recombinant epidermal growth factor (EGF; #53003-018 Invitrogen), 0.15 ng/ml recombinant human leukemia inhibitory factor (LIF; #L200 Alomone Labs), ITS-X supplement (#51500-056 Invitrogen), 0.85 U/ml heparin (#15077-019 Invitrogen), and 20 nM progesterone (#P6149, Sigma)], and maintained as nonadherent neurosphere aggregates. Neurosphere cultures were randomly assigned to a control group (without ethanol) or to ethanol-treated groups [120 mg/dl (26 mM) or 320 mg/dl (70 mM) ethanol, prepared from 95% ethanol]. The final ethanol concentration in the culture medium was verified by gas chromatography, from samples of mitogenic medium collected at the beginning of the treatment, at 3 days, and at 5 days. The concentration of ethanol was chosen based on blood alcohol concentrations attainable

TABLE 1
GENE ONTOLOGY AND KEGG CLASSIFICATIONS OF CELL MIGRATION ASSOCIATED mRNA TRANSCRIPTS

Gene	GO ID#	GO: Description	KEGG ID#	KEGG Pathway Description
Pin/DYNLL1 (dynein, light chain, LC8-type 1)	GO:0006809	nitric oxide biosynthesis		
	GO:0007015	actin filament organization		
	GO:0007017	microtubule-based process		
	GO:0007018	microtubule-based movement		
	GO:0008360	regulation of cell shape		
Paxillin/Pxn	GO:0006928	cell motility	hsa04370	VEGF signaling pathway
	GO:0007155	cell adhesion	hsa04510	Focal adhesion
	GO:0007160	cell-matrix adhesion	hsa04810	Regulation of actin cytoskeleton
	GO:0007165	signal transduction		
Gap43	GO:0001558	regulation of cell growth		
	GO:0007205	protein kinase C activation		
	GO:0007399	nervous system development		
	GO:0010001	glial cell differentiation		
RhoA	GO:0007264	small GTPase mediated signal transduction	hsa04310	Wnt signaling pathway
	GO:0007266	Rho protein signal transduction	hsa04350	TGF-β signaling pathway
	GO:0030036	actin cytoskeleton organization and biogenesis	hsa04360	Axon guidance
	GO:0042346	tpositive regulation of NF-κB import into nucleus	hsa04510	Focal adhesion
	GO:0043123	positive regulation of I-κB kinase/NF-κB cascade	hsa04520	Adherens junction
			hsa04530	Tight junction
CDC42	GO:0000074	regulation of progression through cell cycle	hsa04010	MAPK signaling pathway
	GO:0001558	regulation of cell growth	hsa04360	Axon guidance
	GO:0007049	cell cycle	hsa04370	VEGF signaling pathway
	GO:0007163	establishment and/or maintenance of cell polarity	hsa04510	Focal adhesion
	GO:0007264	small GTPase mediated signal transduction	hsa04520	Adherens junction
			hsa04810	Regulation of actin cytoskeleton
Stmn1	GO:0007019	microtubule depolymerization	hsa04010	MAPK signaling pathway
	GO:0007052	mitotic spindle organization and biogenesis		
	GO:0007242	intracellular signaling cascade		

either during episodic binge drinking (120 mg/dl), or by chronic alcoholics (320 mg/dl).

We wanted to assay both the immediate effects of ethanol on the proliferating neuroepithelium and the longer term, persistent effects of ethanol exposure during the period of neurogenesis on subsequent neuronal differentiation. Consequently, at the end of the 5-day treatment period (Fig. 1i), neurospheres cultured in control mitogenic medium or mitogenic medium supplemented with ethanol were either processed for analysis or alternatively transferred to fresh, laminin-coated flasks [to activate integrin signaling (12), which promotes neuronal migration and differentiation (50)] containing differentiating media without ethanol. Neurosphere cultures were differentiated on these laminin-coated flasks for between 24 and 72 h, by the removal of EGF and LIF from the culture medium.

Real-Time PCR

For real-time PCR, total RNA was isolated from control and ethanol-treated neurospheres, and from early differentiated cells and ethanol-treated early differentiated cells using the Trizol method (#15596-026 Invitrogen). Total RNA from each sample was then purified with Qiagen's RNeasy MiniKit (#74104) and quantified by micro-fluidic separation (Agilent BioAnalyzer). Total RNA (1 μg) was reverse-transcribed with the First-Strand cDNA Synthesis system for RT-PCR (#12371-09 Invitrogen) and SuperScript II Rnase H-Reverse Transcriptase (#18064-014 Invitrogen). Prior to real-time PCR analysis, forward and reverse primers for selected genes were analyzed using the Beacon Designer v.3 software (Premier Bio Soft International). Primers (CDC42, NM_009861, forward 5′-TTGGTGATGGTGCTGTTGG-3′ and reverse 5′- GGGTAGA AATCCACATACTTGAC-3′; Gap43, NM_008083, forward 5′-GCTACCACTGA TAACTCC-3′ and reverse 5′-GTCTTCTTTACCCT CATCC-3′; PIN/DYNLL, NM_019682, forward 5′-CCTCTGCTCCACGGTAAC-3′ and reverse 5′-GA AGTAGATGAAGTGTTTGGTTTC-3′; Pxn, NM_01 1223, forward 5′-CGAGCAGGCGAGGAAGAG-3′ and reverse 5′-AGGGAGTGTTATTTTCTGGGA TG-3′; RhoA, NM_016802, forward 5′-ACTGGT

GATTGTTGGTGATGG-3′ and reverse 5′-CTCGT GTGCTCGTCATTCC-3′; Stmn1, NM_019641, forward 5′-ATGAGGCG GAAGTCTTGAAG-3′ and reverse 5′-GGGGTCTTTGGATTCTTTGTTC-3′) were then selected from a list of suggested primers only if the primer sequences crossed intron–exon boundaries to eliminate secondary structure formation and amplification of genomic DNA. Selected primers were then optimized by a standard curve using serial dilutions of the template to determine the amplification efficiency of the PCR reaction. The amplification of a single transcript was verified by thermal stability analysis. For real-time PCR, cDNA, forward and reverse primers, and BioRad's iQ Syber Green Supermix (#170-8882) were combined and analyzed with the iCycler MyiQ system from BioRad. Relative gene expression was calculated by the Pfaffl method (33), and cyclophilin-A served as the reference gene (35). The control proliferation condition served as the normalization control sample.

Cell Migration Assay

Control or ethanol pretreated neurospheres were differentiated in the early (+FGF/–EGF/–LIF) or late (–FGF/–EGF/–LIF) differentiation conditions on laminin-coated membrane inserts with 8-μm pores, for migration analysis (based on a modification of kit #NS200, Becton Dickinson). The proportion of cells that migrated from one side of the culture well insert membrane through the 8-μm pores to the other side was quantified by an adaptation of the manufacturer's protocol. Briefly, at 3 days, inserts were fixed with ethanol, washed, and stained with 0.09% cresyl violet. The cells that had migrated through the pores were scraped off from the membrane and the stain was extracted with extraction buffer [0.2 M acetate, pH 4.5, 50% alcohol (42)]. Optical density was measured spectrophotometrically at a wavelength of 595 nm, and data were normalized to control cells cultured on a BSA-coated membrane insert.

Neurite Outgrowth Analysis

For the neurite outgrowth quantification assay, control or ethanol-treated neurospheres differentiated with early or late differentiating media were cultured on laminin-coated, six-well plates. At the end of 3 days, cells were washed with PBS and fixed with ethanol at −20°C overnight. Fixed cells were washed with PBS and stained with 0.09% cresyl violet followed by several washes with water. At the end of the last wash, wells were loaded with 50% glycerol, sealed, and stored at 4°C. For neurite elongation quantification, cells were visualized with the Olympus BX60 microscope using a 40× objective and captured with Q-capture software (Lukas Microscope Service Inc). The length of the primary neurite was measured by calculating the number of pixels from the cell's soma to the end of the neurite using an imaging software package (Visilog 6 Viewer imaging software, Noesis). For second-order neurites, the length was measured from its branch point from the primary neurite to the tip of the second-order neurite.

Immunofluorescence Analyses

Immunofluorescence analysis was conducted according to previously published protocols (6,35,39, 40,47). Neurospheres (proliferation condition) and early or late differentiated cultures were washed with phosphate-buffered saline (PBS) and fixed with PBS and 80% ethanol. Fixed cells were washed with PBS and blocked with 2% normal serum, 0.1% BSA, 0.2% Triton X-100 in PBS for 1 h at room temperature. Cells were incubated with primary antibody (all from Chemicon International) for the neuroepithelial stem cell marker, nestin (1:200, MAB353), and the neuron-specific intermediate filament, neurofilament (NF; 1:500, MAB1592) overnight at 4°C in staining solution (PBS and 0.1% BSA). Cells were then washed three times with PBS and incubated with a fluorescein-conjugated secondary antibody (1:500; Molecular Probes) in staining solution for 2 h followed by three washes with PBS. Cells were then mounted with fluorescence mounting media containing DAPI (Vector Laboratories). Antibody binding was visualized with an Olympus BX60 microscope. Fluorescence images were captured with Q-capture software (Lukas Microscope Service Inc).

Data Analyses

Multivariate analyses of variance (MANOVAs) and analysis of variance (ANOVA), followed by "Least Significance Differences" post hoc tests were used to identify statistically significant differences between control and treatment groups, using the standard statistical software, SPSS version 13. The statistical significance criteria was set at $p < 0.05$.

RESULTS

Cerebral cortical progenitors, cultured as neurospheres, are nonadherent and express the intermediate filament protein, nestin (Fig. 1a–c), but not mature neuronal markers like neurofilament protein or NeuN. However, following withdrawal of EGF and LIF, and in the presence of laminin (+FGF/–EGF/–LIF/+laminin), neurosphere-derived cells become adherent to the culture dish, migrate away from the

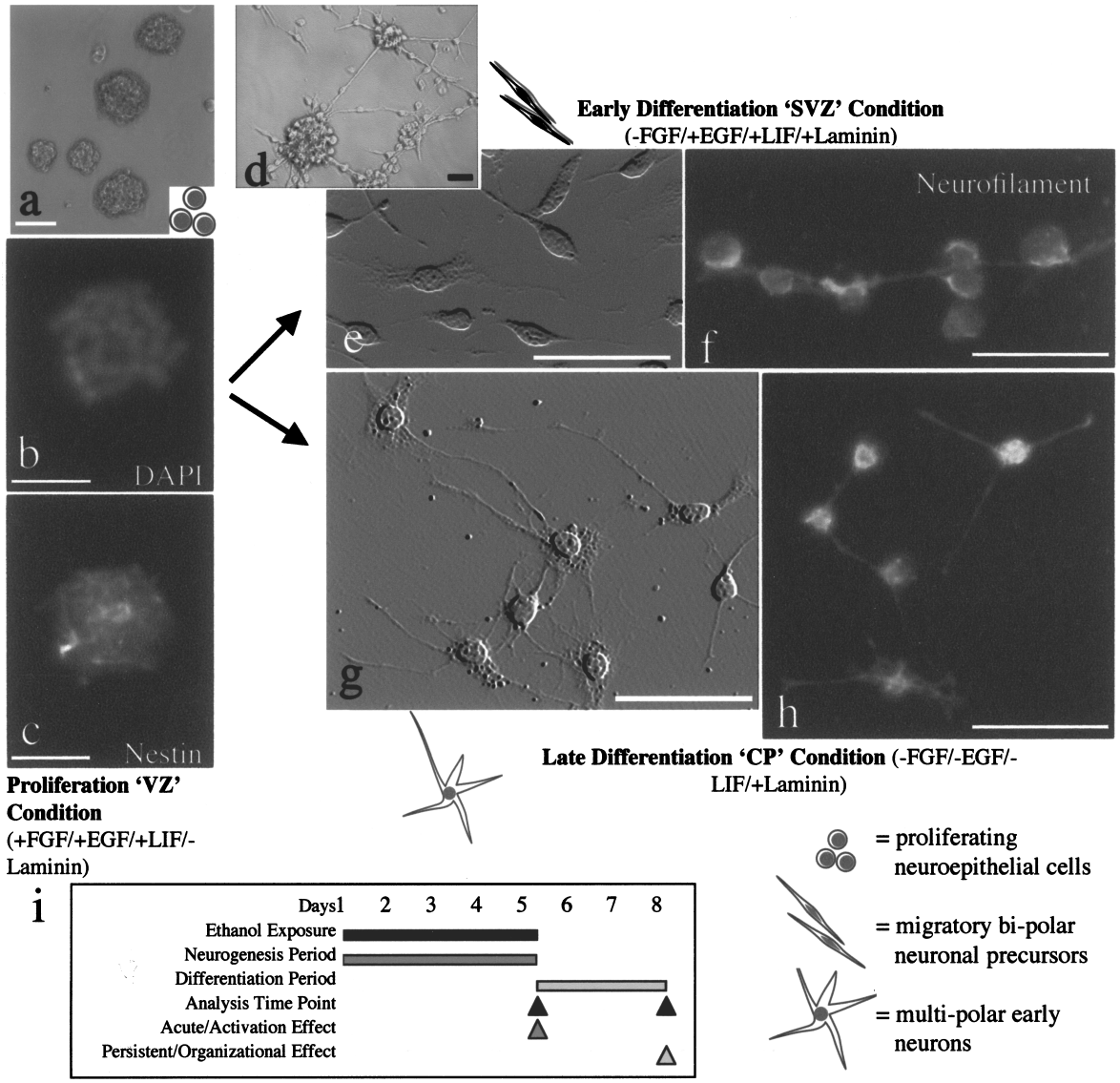

Figure 1. Neurosphere model of the fetal ventricular neuroepithelium and mitogen-withdrawal model of neuronal differentiation. (a–c) In the presence of mitogenic factors, FGF, EGF, and LIF, neuroepithelial cells proliferate to form neurospheres [the proliferation/VZ (ventricular zone) condition]. (a) Phase contrast image of neurospheres derived from gestational day 12.5 mouse cortex. (b–c) Sample neurosphere stained with DAPI (b) to visualize nuclei and immunolabeled for nestin (c) showing that neurospheres cells are immature. (d–f) Partial withdrawal of mitogenic factors, EGF and LIF, and addition of laminin cause neurosphere-derived neuroepithelial cells to become adherent and migrate away from the parental neurosphere (d and e, phase contrast image), assume a bipolar morphology (e) and express immunoreactivity for neurofilament protein (f), the early differentiation or SVZ (subventricular zone) condition. (g–h) Complete withdrawal of mitogenic factors, EGF, FGF, and LIF, results in the appearance of multipolar cells (g) that express immunoreactivity for neurofilament protein (h), the late differentiation or CP (cortical plate) condition. (i) Schematic of the experimental paradigm to examine the acute and persistent effects of ethanol exposure during the neurogenesis period. Scale bars: 50 μm.

parent neurosphere (Fig. 1d), and assume a bipolar morphology (Fig. 1e). This cellular transformation is accompanied by an expression of neuronal marker, neurofilament protein (Fig. 1f). We refer to this condition as the "early differentiation" or SVZ condition. Early differentiating cells expressed faint immunofluorescence for GFAP within the perikaryon, but not within the neurites, suggesting that astrocytic differentiation was not a significant aspect of the early neuronal differentiation program. Following complete withdrawal of mitogenic factors, and in the presence of laminin (–EGF/–FGF/–LIF/+laminin), neural progenitors transform into multipolar neurons (Fig. 1g), expressing neurofilament protein (Fig. 1h). We refer

to this condition as the "late differentiation" or cortical plate (CP) condition.

Ethanol Induces Immediate but not Persistent Alterations in the Expression of Cell Migration Genes

To determine whether ethanol exposure during the period of cell proliferation induced immediate or persistent changes in the expression of mRNA transcripts for cell migration-associated genes (defined according to Gene Ontology and KEEG pathway classifications, Table 1), neurosphere cultures were exposed to ethanol for 5 days. The expression of mRNAs for cell migration-associated genes, CDC42, RhoA, DYNLL1, Pxn, and Gap43 were examined immediately following ethanol exposure, or following an additional 72-h of exposure to the early differentiation condition (see Fig. 1i for schematic of the experimental paradigm). Multivariate (MANOVA) analyses indicated that there was an overall statistically significant interaction between ethanol preexposure history and differentiation state (Pillai's Trace statistic of 9.675, $df = 6,3$, $p < 0.032$). Post hoc analyses of variance (ANOVA) indicated that there was a statistically significant interaction effect between ethanol exposure history and differentiation state for Pxn ($p < 0.0001$), RhoA ($p < 0.005$), and Gap43 ($p < 0.009$). Additionally, there was a main effect of ethanol exposure for CDC42 ($p < 0.001$) and DYNLL1 ($p < 0.013$), and a main effect of differentiation state on the expression of CDC42 transcripts ($p < 0.005$). RhoA, Pxn, and CDC42 were all induced by immediate exposure to ethanol during the proliferation period, whereas DYNLL1 was suppressed during the same time period (Fig. 2). None of these mRNAs were persistently regulated during early neuronal differentiation, by preexposure during the proliferation period. In contrast to the above four transcripts, Gap43 was suppressed during the proliferation period, as an immediate consequence of ethanol exposure (Fig. 3). However, the differentiation-associated decline in Gap43 mRNA in control cultures was not observed in the ethanol pretreated cultures. Therefore, Gap43 levels during the differentiation period were higher in ethanol-pretreated cells compare to control cells. Though Stmn1 was not significantly regulated by ethanol exposure history or by differentiation state (Fig. 3), further analysis indicated a moderate but statistically significant correlation between the expression of Stmn1 and Gap43 mRNA transcripts (Pearson's $r = 0.646$, $p < 0.044$). We did not observe a statistically significant correlation between the expression of Stmn1 transcripts and any other migration-associated gene, suggesting that Gap43 and Stmn1 transcripts are preferentially coregulated. These data indicate that for a majority of the cell migration-associated genes, ethanol had an immediate, rather than persistent, effect on transcript levels.

Ethanol Preexposure During Neuroepithelial Proliferation Increases Neuronal Migration During Periods of Differentiation

Though ethanol generally did not induce persistent changes in the expression of migration-associated genes, we nevertheless sought to determine whether ethanol preexposure during the period of neuroepithelial proliferation would alter neuron migration during subsequent periods of differentiation. Neurosphere cultures were treated with ethanol or control medium for 5 days, and then transferred to 24-well microplates containing 6.5-mm transwell inserts with 8-μm pores, and cultured in either the early (SVZ) or late (CP) differentiation conditions. The number of cells migrating from one side of the porous membrane to the other was assessed quantitatively by a histochemical assay. Preexposure to ethanol induced a significant increase in migration in both early ($p < 0.001$) (Fig. 4a, b) and late differentiation ($p < 0.0001$) (Fig. 4c, d) conditions. Preexposure to the 320 mg/dl dose as well as a lower dose, 120 mg/dl, attainable during binge drinking, induced statistically significant increases in cell migration across a permeable barrier, suggesting that ethanol exposure during neuroepithelial proliferation promotes cell migration during differentiation.

Ethanol Preexposure During Neuroepithelial Proliferation Alters Neurite Length During Periods of Differentiation

We next quantified the length and number of neurites in differentiating neurons obtained from control or ethanol-pretreated conditions. In the early differentiation condition, the mean number of primary neuritis per cell was 2, while on average there were 3.5 primary neurites per cell in the late differentiation condition (Fig. 5a). Ethanol preexposure did not alter the number of primary neurites per cell in either condition (Fig. 5a), nor was the overall length of the primary neurites altered in the early differentiation condition (Fig. 5b). However, ethanol preexposure during neuroepithelial proliferation induced a significant increase ($p < 0.002$) in the length of primary neurites in the late differentiation condition (Fig. 5c). We observed a slight but not statistically significant decrease in the length of secondary neurites in the late differentiation condition (Fig. 5d). However, ethanol preexposure produced a threefold decline in the num-

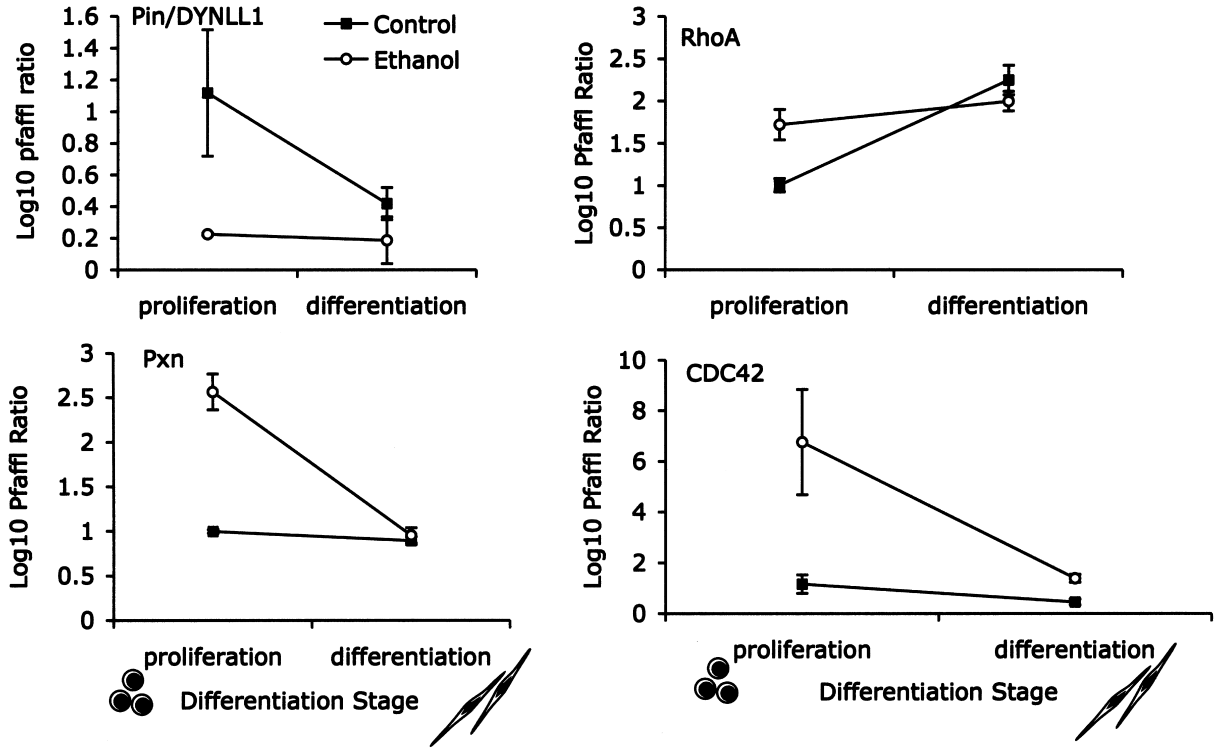

Figure 2. Real-time RT-PCR analyses of the expression of DYNLL1/Pin, RhoA, Pxn, and CDC42 mRNA transcripts during the proliferation phase or early differentiation phase in control cultures, or following ethanol exposure for 5 days, at 320 mg/dl during the proliferation period. Data are expressed as mean of the $\log_{10}$ Pfaffl ratio ± SEM based on three independent replicate experiments.

bers of cells in the late differentiation condition that expressed second-order neurite branches (Fig. 5e).

DISCUSSION

In utero ethanol exposure can induce persistent defects in regions of the brain like the developing cerebral cortex. However, the contributions of specific critical developmental periods to ethanol's teratogenecity have not been well defined. The second trimester is an important, and potentially vulnerable, period for brain development because, during this period, the cerebral cortical ventricular neuroepithelium proliferates rapidly to create nearly all of the neurons of the cerebral cortex. Committed neuronal precursors then migrate away from the germinal zone, to populate the laminating cortical plate in an inside-out gradient. The brains of children diagnosed with FAS can exhibit heterotopias, or collections of displaced differentiated neurons, which suggests that ethanol alters neuronal migration. Animal models of in utero ethanol exposure also lend support to the hypothesis that ethanol alters neuronal migration (28) and the development of long axonal projections (36). However, in utero ethanol exposure paradigms cannot separate the "activational" from the "organizational" effects of ethanol because during the exposure period, cohorts of neuroepithelial cells continue to undergo mitosis, while others simultaneously initiate migration and neuronal differentiation. The distinction between organizational and activational effects is important because the organizational effects of a teratogen represent persistent, potentially permanent, changes in the future growth and plastic responses of surviving neurons and neural stem cells. This persistent imprint of in utero ethanol exposure on these cell populations can have adverse implications for the development of therapeutic approaches. Recent research indicates that ethanol does have immediate effects on neurite extension and neuronal migration processes (18,45). However, the organizational effects of ethanol on these initial stages of neuronal differentiation remain to be determined.

To begin to distinguish between activational and organizational effects, we recently developed a paradigm where fetal cerebral cortical-derived neurosphere cultures are treated with ethanol to mimic exposure during the second trimester period of neuroepithelial proliferation (6,35,39,40). Ethanol-treated and control neurosphere cultures are then exposed to a simplified mitogen-withdrawal, extracellular matrix adhesion paradigm, mimicking the early migration and differ-

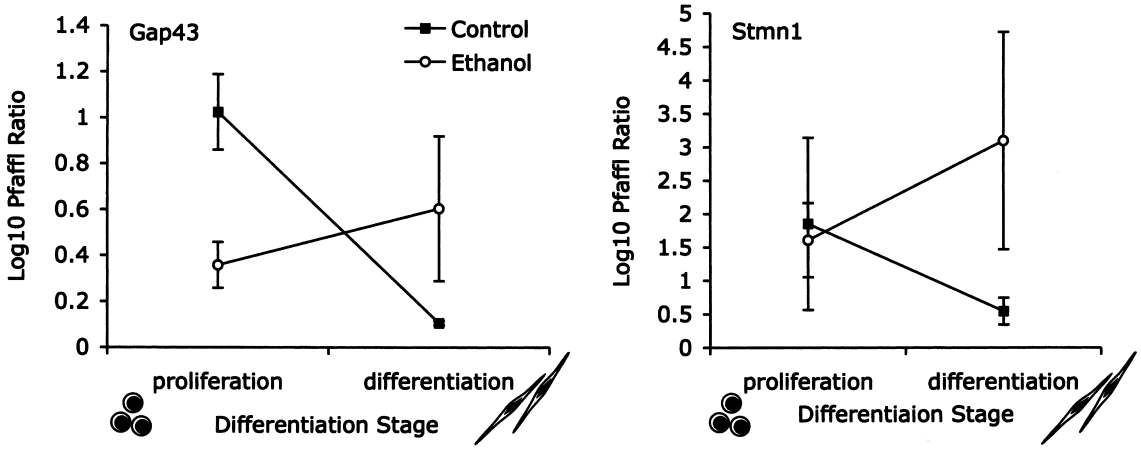

Figure 3. Real-time RT-PCR analyses of the expression of Gap43 and Stmn1 mRNA transcripts during the proliferation phase or early differentiation phase in control neurosphere cultures, or following ethanol exposure for 5 days, at 320 mg/dl during the proliferation period. Data are expressed as mean of the $\log_{10}$ Pfaffl ratio ± SEM based on three independent replicate experiments.

entiation events that accompany the formation of this cortical plate (6). Utilizing this combination of paradigms, we previously showed that ethanol exposure during the proliferation period could immediately alter the secretion of specific cytokines (activational effects). More importantly, ethanol exposure during the neural proliferation period could persistently repattern the subsequent developmental stage-specific secretion of vasculogenic cytokines and chemokines (organizational effects). In this study we asked whether ethanol exposure during the proliferation period could alter subsequent neuronal migration and maturation.

In general, our data show that ethanol induces activational, but not organizational, changes in the expression of genes associated with cell migration and early differentiation. Following differentiation in the absence of ethanol, levels of most genes returned towards levels expressed in controls. We observed that RhoA, Pxn, and CDC42 were immediately induced, whereas DYNLL1 (Pin) was suppressed by ethanol. Interestingly, KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/keeg/, Table 1) indicates that all of the upregulated genes belong uniquely to the focal adhesion kinase pathway (FAK, ko04510). Paxillin (Pxn) couples cell-matrix adhesion signals to intracellular signaling cascades by acting as a scaffold for other signaling molecules. Pxn therefore plays a central integrative role in initiating neurite extension (15), and cell motility (16). Additionally, Pxn also plays an important role in tumor metastasis (2), a process that bears some similarity to the formation of heterotopias in ethanol-exposed brains. The Rho family of GTPases, including Cdc42 and RhoA, are important regulators of actin filament organization during cell polarization, and play a key role in neurite elongation and cell motility. While Cdc42 controls cell polarity at the leading edge, RhoA controls the trailing edge of the cell (37). These data suggest that ethanol activates key migration-associated genes during the period of mitogenesis. These activational influences on cell matrix interactions are important for radial glia-independent migration, which forms a significant component of component of the migratory behavior of early postmitotic neurons (31).

We observed that Gap43 mRNA and protein were highly expressed in neurosphere cultures, and expression decreased as these precursor cells terminally differentiated into neurons. These data reflect an alternate role for Gap43 in immature neural precursors, and is consistent with reports in the literature showing that Gap43 is expressed at high levels by proliferating neural precursors in the fetal ventricular zone (24). However, the role of Gap43 in cell proliferation appears complex. Gap43 appears to promote cell proliferation in some subpopulations of neural precursors, while loss of Gap43 expression can lead to increased proliferation of other neural precursors, resulting in the accumulation of radial glia (41). Radial glia can, in turn, serve as precursors to neurons (1,11); therefore, the loss of Gap43 expression in our model is consistent with the process of neuronal maturation. However, ethanol exhibited a complex pattern of regulation of the Gap43 transcript. The direct, activational impact of ethanol exposure was a significant suppression of Gap43 mRNA expression, supporting our contention that ethanol promotes stem cell maturation, and consistent with our previous observations

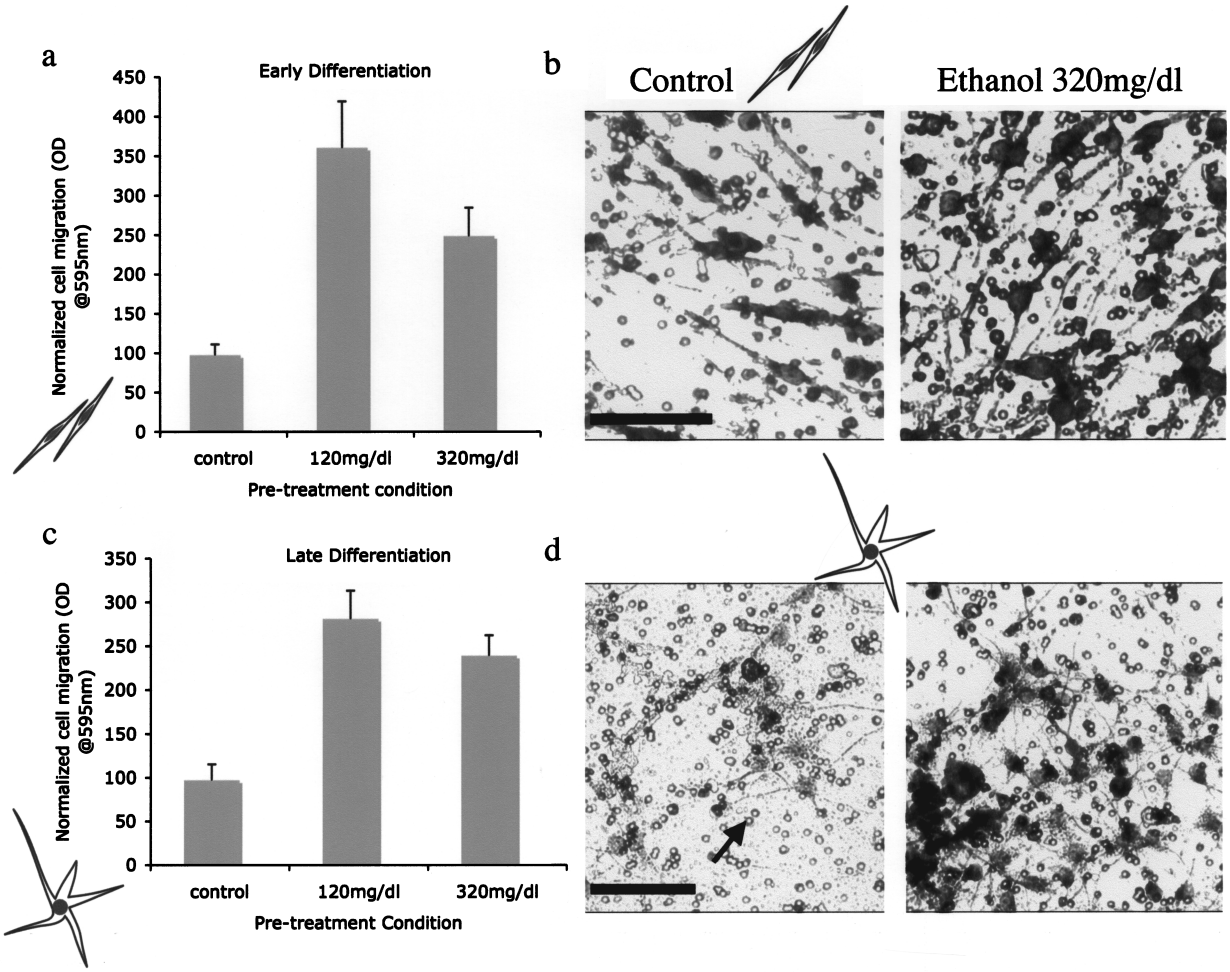

Figure 4. Assessment of neuronal migration following ethanol exposure, at 320 mg/dl for 5 days, during the proliferation period. (a, c) Quantitative graphical representation of shows that ethanol preexposure during the proliferation period significantly induces neuronal migration during both early (a) and late (c) differentiation phases. (b, d) Representative photomicrographs showing increased migration in ethanol-pretreated cultures, compared to control cultures. Control and ethanol-treated neurospheres were cultured in tissue culture inserts containing 8-μm pores (arrow in d). Scale bar: 50 μm.

that ethanol promotes the appearance of radial glia (39). However, ethanol also blunted the decline in Gap43 mRNA expression accompanying the process of neuronal differentiation, suggesting that ethanol might induce a persistent dysregulation of the neuronal maturation process.

Contrary to our expectation, DYNLL1 was downregulated by ethanol. However, aside from its role as a cell growth-associated molecule, DYNLL1 is also known to be an inhibitor of neuronal nitric oxide synthase (nNos) (17). It is possible that the suppression of DYNLL1 represents an adaptive and protective response to chronic ethanol that, as in other tissues (34), leads to an increase in nitric oxide. Nitric oxide in turn is protective against ethanol exposure (3,4,32), consistent with our observations that neuroepithelial cells are resistant to ethanol-induced apoptosis (35,39).

Despite the fact that ethanol's stimulatory effect on most migration-associated genes did not persist into neuronal differentiation, we observed that ethanol exposure during the proliferation period did persistently induce cell migration during differentiation. Furthermore, significant persistent increases in cell migration were observed even at lower doses of ethanol that can be attained by nonalcoholics during episodes of binge drinking. Interestingly, we observed increased migration in the late differentiation phase, which is ordinarily accompanied by the appearance of multipolar neurons. However, while neurons preexposed to ethanol during the proliferation period did exhibit a significant increase in the overall length of the primary neurites during the late differentiation period, they also exhibited a fourfold decline in the total frequency of second-order neurite branches. The loss of

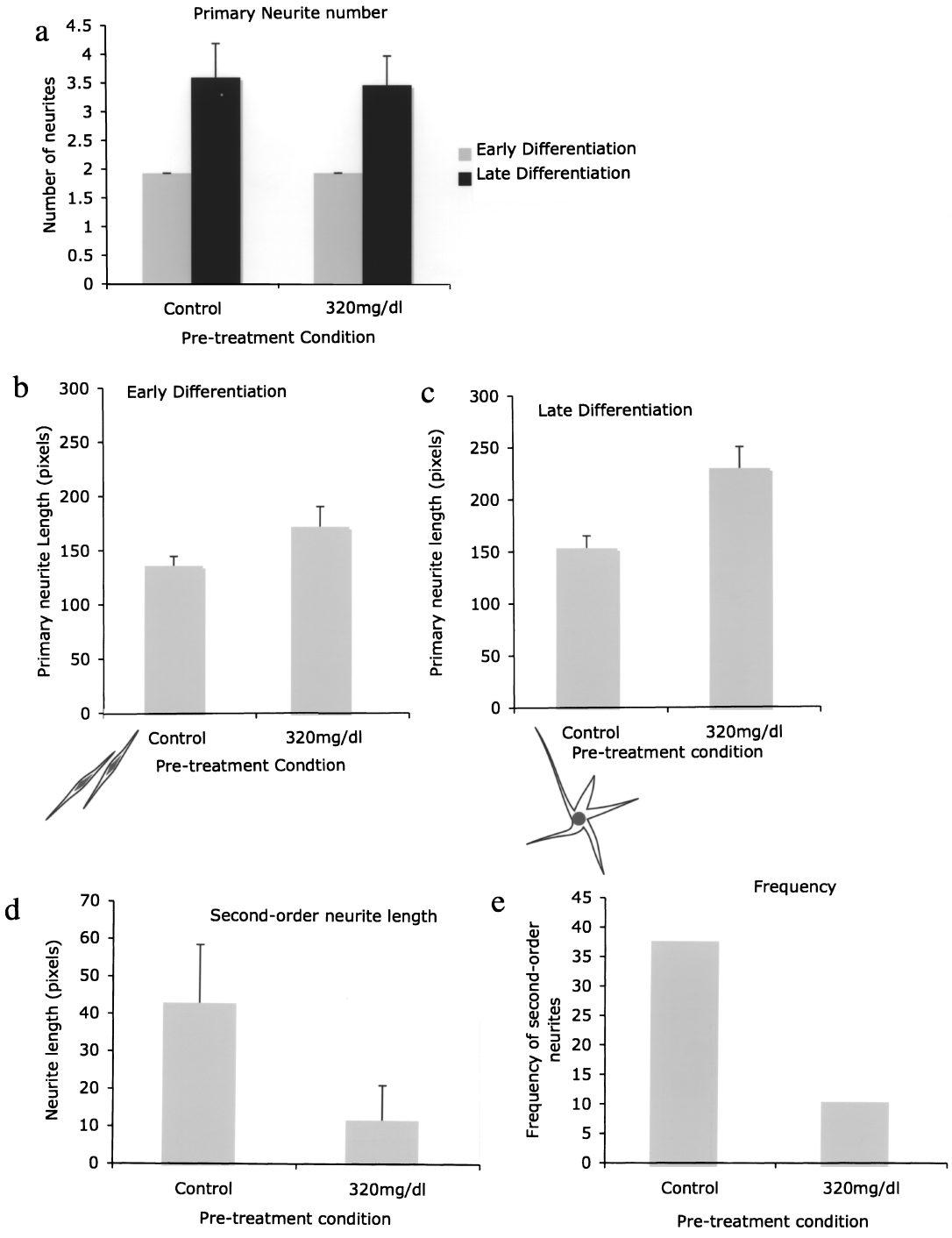

Figure 5. Assessment of neurite growth following ethanol exposure at 320 mg/dl for 5 days, during the proliferation period. (a) The number of primary neurites was regulated by differentiation state, but not by ethanol preexposure. (b–c) Ethanol preexposure during the proliferation phase did not significantly increase primary neurite length in the early differentiation phase (b), but induced a significant increase in primary neurite length in the late differentiation condition (c). (d) Ethanol preexposure during the proliferation phase slightly, but not statistically significantly, decreased the length of second-order neurites in the late differentiation condition. However, (e) ethanol preexposure decreased the proportion of cells that expressed second-order neurite branches in the late differentiation condition.

second-order branches in ethanol-pretreated neurons may indicate a delay in neuronal maturation, consistent with our observed dysregulation of Gap43 mRNA expression. The lack of second-order neurites further suggests that the primary neurite itself may be unstable, and may exhibit an enhanced capacity for remodeling. Such a possibility is consistent with our observation of increased neuronal migration. It remains to be seen whether other ethanol-sensitive signaling mechanisms such as those mediated by alternative adhesion substrates, L1 for example (45), underlie the persistent effects of ethanol on neuronal maturation. While these data were based on a continuous ethanol exposure paradigm, designed to mimic ethanol consumption by a chronic alcoholic, binge patterns of ethanol consumption are increasingly common among the general population of social drinkers (46). It would be important to determine whether cycles of ethanol exposure and withdrawal lead to similar persistent effects in fetal neuroepithelial cells.

CONCLUSIONS

Our data collectively indicate that ethanol exhibits a primarily activational effect on the expression of genes that are ontologically related to cell migration. Nevertheless, exposing proliferating neuroepithelial cells to ethanol for a period equivalent to the in utero phase of cortical plate neurogenesis results in persistent enhancement of migration in differentiating neuroepithelial cells. These data suggest that the second trimester period of stem cell expansion and maturation is a critical period of development, and that a drug with abuse potential such as ethanol can induce permanent alterations in neural stem and progenitor cells that influence their subsequent maturation behavior.

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