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Ethanol regulates angiogenic cytokines during neural development: Evidence from an *in vitro* model of mitogen-withdrawal induced cerebral cortical neuroepithelial differentiation

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Abstract

Background—Heavy alcohol consumption during pregnancy can cause significant mental retardation and brain damage. We recently showed that ethanol depletes reserve cerebral cortical stem cell capacity. Moreover, proliferating neuroepithelial cells exposed to ethanol were resistant to subsequent retinoic acid-induced differentiation. Emerging evidence suggests that cytokines play a crucial growth-promoting role in the developing neural tube.

Methods—We cultured murine cortical neurosphere cultures in control or ethanol-supplemented mitogenic medium, to mimic alcohol exposure during the period of neuroepithelial proliferation. Cultures were then treated with a step-wise mitogen-withdrawal, integrin-activation model to mimic subsequent phases of neuronal migration and early differentiation. We examined the impact of alcohol exposure during neurogenesis on the secretion of inflammatory and growth-promoting cytokines.

Results—Cortical neurosphere cultures exhibit increasingly complex differentiation phenotypes in response to step-wise mitogen-withdrawal and laminin exposure. Some inflammation modulating cytokines were secreted independent of differentiation state. However chemotactic cytokines specifically were secreted at high levels, as a function of differentiation stage. MCP-1, VEGF-A and IL-10 were coordinately decreased during differentiation compared to neuroepithelial proliferation, while GM-CSF was induced during differentiation, as compared to the neuroepithelial proliferation period. Ethanol exposure during the period of neuroepithelial proliferation prevented the early differentiation-induced increase in GM-CSF while inducing differentiation-associated increase in IL-12 secretion.

Conclusion—Embryonic cerebral cortical neuroepithelial-derived precursors secrete high levels of several angiogenic and neural-growth-promoting cytokines as they differentiate into neurons. Our data collectively suggest that ethanol exposure during the period of neuroepithelial proliferation significantly disrupts cytokine signals that are required for the support of emerging neurovascular networks, and the maintenance of neural stem cell beds.

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Keywords

Embryonic cerebral cortex; Granulocyte Macrophage-Colony Stimulating Factor; Monocyte Chemotactic Protein-1; Vascular Endothelial Growth Factor-A; IL12

Introduction

Heavy alcohol consumption during pregnancy can alter fetal brain and craniofacial development, resulting in a constellation of physical, cognitive and affective defects that are collectively termed 'Fetal Alcohol Spectrum Disorders' or FASD (Loock et al., 2005; Sokol et al., 2003). The 'Fetal Alcohol Syndrome', or 'FAS' (Jones and Smith, 1975) represents the severe end of the FASD continuum, and is characterized by microencephaly, malformations of gyri, diminution or loss of inter-hemispheric communicating fiber tracts like the corpus callosum (reviewed in (Clarren, 1986; Riley and McGee, 2005)), and the presence of 'brain warts' or heterotopias containing displaced neurons (Clarren, 1986; Clarren et al., 1978). The period of the brain growth spurt (the second to third trimester equivalent of human gestation, comparable to the latter half of gestation and the early post-natal period of rodent development (Dobbing and Sands, 1979)), characterized by a rapid neurogenesis (Bayer and Altman, 1995), and compensatory apoptosis mechanisms (Cheema et al., 2004; Cheema et al., 1999), appears to constitute a period of particular vulnerability to alcohol.

During the brain growth spurt, the ventricular zone (The Boulder Committee, 1970) neuroepithelium exhibits significant mitotic activity (Noctor et al., 2004; Walsh and Cepko, 1988; Walsh and Cepko, 1993), and gives rise to early differentiating neurons. These proto-neurons leave the ventricular zone to populate the cortical plate or the more superficial subventricular zone, where they may continue to proliferate (Noctor et al., 2004), before migrating into the developing cortical plate. The cortical plate is populated in an 'inside-out' gradient, by successive waves of migrating neurons from the ventricular and sub-ventricular zones, giving rise to the mature lamination pattern of the adult cerebral cortex.

Our laboratory has been interested in the impact of epigenetic influences like prenatal alcohol exposure on the development of the cerebral cortex. We, and others, have shown that, in differentiated neural tissue, some of these neurotoxic effects of alcohol may be explained by the induction of cell death mechanisms (Cheema et al., 2000; McAlhany et al., 2000; Mooney and Miller, 2001; Mooney and Miller, 2003), the loss of neurotrophic support (Dohrman et al., 1997; Luo and Miller, 1998; Luo et al., 1996; Maier et al., 1999; McAlhany et al., 1999; Miller et al., 2003), alterations in neuronal migration (Mooney et al., 2004), and in neurotransmitters systems (Hsiao et al., 2004), among others. However, we know little about the effects of alcohol exposure on the fate of neural stem cells, and on the early events that transform stem cells into neurons. To our surprise, we recently found that ethanol is not cytotoxic to fetal-derived neural stem cells (Santillano et al., 2005), in contrast to its pro-apoptotic effects on more differentiated neuronal cells (McAlhany et al., 2000). Rather, ethanol decreases the diversity and regenerative capacity of the neural stem cell pool. Furthermore, ethanol-exposed fetal-derived cortical stem cells are subsequently unable to respond to differentiation stimuli. These data support the hypothesis that ethanol exposure during the period of neuroepithelial proliferation has direct, immediate effects on the proliferating fetal neuroepithelium (i.e., activational effects), as well as longer-term, organizational effects on the subsequent differentiation of cortical neuroepithelial cells. The question that arises is what kinds of differentiation-relevant signals are targets for a teratogen like ethanol.

Substantial evidence from studies in tissues like bone marrow, shows that the transformation of stem cells to more fate-restricted blast progenitors, and subsequently, to differentiated progeny, is governed by the cytokine milieu (e.g., (Bernstein et al., 1991; Haylock et al., 1992; Makino et al., 1997)). The brain is a well-established source and target of cytokines, both during development and in the adult. Cytokines such as monocyte chemotactic factor (MCP-1)/CCL2 (Banisadr et al., 2005; Dzenko et al., 2005; Geppert, 2003; Stamatovic et al., 2003; Wiedera et al., 2004; Yamamoto et al., 2005), members of the vascular endothelial growth factor (VEGF) family (Hogan et al., 2004), and granulocyte macrophage-colony stimulating factor (GM-CSF, (Guo et al., 2003)), are expressed by cells of the developing brain, and in turn, shape the survival and proliferation of neural stem cells and the survival of more differentiated neurons (Kim et al., 2004; Ogunshola et al., 2002). We therefore set out to determine whether a teratogen like alcohol interfered with cytokine signals as part of its effects on neural stem cell diversity, proliferation, and differentiation fate.

In the following experiments, we treated embryonic murine cerebral cortical-derived neurosphere cultures with ethanol to model heavy alcohol exposure during the second trimester-equivalent period of ventricular zone (VZ) proliferation. We examined the release of chemotactic and inflammatory cytokines in control or ethanol-pretreated cultures, during the phase of neural progenitor proliferation, or following differentiation, induced by step-wise withdrawal of mitogenic factors, and the addition of extracellular matrix, to promote the activation of integrins (Gary and Mattson, 2001). We hypothesized that ethanol would induce an inflammatory-type response. However, inflammatory-type cytokines were not regulated by ethanol exposure. On the other hand, several chemotactic-type cytokines, like VEGF-A, MCP-1/CCL2, GM-CSF, IL-10 and IL-12 were regulated either during neuronal development, or by ethanol exposure. These data suggest that ethanol predominantly influences chemotactic, rather than inflammatory cytokine signals. Furthermore, our data also show that ethanol exposure during the proliferation period can have long-term, organizational effects on neuronal differentiation.

Materials and Methods

Isolation of Embryonic Neural Precursors

The University Laboratory Animal Care Committee approved all animal procedures. Timed-pregnant C57/Bl6 mice were generated by timed-mating and maintained in the animal housing facility at Texas A&M University System Health Sciences Center, College of Medicine for two days. At GD 12.5, fetuses were isolated from the gravid uterus, rinsed in chilled PBS, and brains were removed and placed in chilled Hanks's Balanced Salt Solution (#14175-095, Invitrogen) supplemented with glucose and magnesium chloride. Meningeal tissue was removed, regions of the mouse fetal brain corresponding to the structural precursor of the neocortex were isolated, and care was taken to exclude the structural precursors to the striatum and hippocampus. Individual cortical fragments were collected in sterile 15ml conical tubes and gently triturated in trypsin/EDTA. Trypsin was inactivated with DMEM containing 10% fetal bovine serum. The cell suspension was centrifuged for 5 minutes at 18°C, 1000 rpm (300xg). Cell pellets were resuspended in chilled PBS containing 0.5% BSA, Fraction-V, (#1526037, Invitrogen) and 2.0mM EDTA. Total cell counts were determined using a hemocytometer. Dispersed neuroepithelial precursors were established in culture at an initial density of 10^6 cells in T-25 flasks containing serum-free mitogenic media (DMEM/F12 (#11330-032 Invitrogen), 20ng/ml Basic Fibroblast Growth Factor (bFGF; 13256-029 Invitrogen), 20ng/ml human recombinant Epidermal Growth Factor (EGF; #53003-018 Invitrogen), 0.15ng/ml recombinant human Leukemia Inhibitory Factor (LIF; #L200 Alomone Labs) ITS-X supplement (#51500-056 Invitrogen), 0.85 Units/ml heparin (#15077-019 Invitrogen), and 20nM progesterone (# P6149 Sigma)). Cultures were incubated at 37°C, 5% CO₂ in a humidified environment to generate neurospheres.

Experimental Paradigm

Neurosphere cultures were assigned to a control group containing no ethanol, or a group exposed to ethanol at 320 mg/dl. Gas chromatographic analyses indicated that the ethanol treatment condition resulted in media ethanol levels of 244.68 $\pm$ 14.93 mg/dl (mean $\pm$ SEM; equivalent to 53.16 $\pm$ 3.24 mM). These concentrations are within the range that has been previously observed in chronic alcoholics (Adachi et al., 1991; Perper et al., 1986), and has previously been shown to induce apoptosis in animal models (Ikonomidou et al., 2000). Cultures were exposed to ethanol under mitogenic conditions (i.e., with bFGF, EGF and LIF) for a period of 5 days, to model gestational ethanol exposure during the period of neuroepithelial proliferation. Culture medium was changed on day 2 of the treatment protocol. At the end of the five-day ethanol exposure period, aliquots of culture-conditioned medium were aspirated, centrifuged to remove cells and cellular debris and stored at -80°C for further analysis.

We were interested in studying the impact of ethanol exposure during the period of neuroepithelial proliferation on the subsequent differentiation of cortical neural progenitors in terms of expression of cytokines. Therefore, control and ethanol-exposed cultures were transferred to fresh, laminin-coated flasks, with ethanol-free medium containing either a partial mitogenic signal (+bFGF/-EGF/-LIF) to mimic an early period of neural differentiation, or mitogen-free medium (-bFGF/-EGF/-LIF), to mimic a later period of neural differentiation. Laminin was provided as a substrate for activation of integrin signaling (Gary and Mattson, 2001), an important part of the neuronal migration and differentiation program. The combination of laminin and bFGF has previously been reported to prime progenitor cells to differentiate into glutaminergic and cholinergic and GABAergic neurons (Wu et al., 2002). We hypothesized that the +bFGF/-EGF/-LIF condition would mimic the environment of the cortical sub-ventricular zone, where neural progenitors are committed to a neuronal lineage, but may yet continue to divide (Noctor et al., 2004), whereas the removal of all mitogenic signals may mimic a later phase of neuronal differentiation, characteristic of post-mitotic neurons. Variations of this step-wise mitogen-exposure and removal paradigm have been previously used to model stem cell proliferation and differentiation in other neuronal populations as well (James et al., 2004; Pitman et al., 2004; Sakaguchi et al., 1997). Cultures were maintained for 3 days on laminin substrate, under either +bFGF/-EGF/-LIF or -bFGF/-EGF/-LIF conditions. At the end of this period, culture medium was again aspirated, cleared by centrifugation, and stored at -80°C for further analysis.

Measurement of Apoptosis—Flow cytometric analysis of apoptosis was measured according to our previously published protocols (Cheema et al., 2004; Santillano et al., 2005). Briefly, Cells were stained with propidium iodide (50ug/ml; #P4170 Sigma) in PBS containing 100ug/ml RNase A to measure cellular DNA content. Flow-cytometric measurements were conducted on a FACS Calibur Flow Cytometer (Becton Dickinson). An Argon laser was used for an excitation wavelength of 488nm and emission spectra of 630nm. A minimum of 30,000 cells from each sample and data were analyzed using the Cell Quest software. Cells with less than 2N (diploid) DNA content were defined as apoptotic.

Multiplexed Profiling of Mouse Cytokines—A panel of 18 mouse cytokines (Interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor), Interferon (IFN)- γ , KC (CXCL-1), Monocyte Chemotactic Protein (MCP)-1/CCl-2, RANTES/CCl-5 (Regulated on Activation, Normal T Expressed and Secreted; a member of the IL-8 superfamily), and Tumor Necrosis Factor (TNF)- α), were analyzed using the Beadlyte® multiplexed cytokine/chemokine assay service (by Upstate Cell Signaling Solutions/

Serologicals Corporation). Culture-conditioned medium from a total of 5 independent culture samples per experimental group, were analyzed for cytokine/chemokine expression, and each sample was assayed in duplicate. Briefly, 50 μ l of each culture-conditioned medium sample was loaded in duplicate onto a 96 well. Samples were exposed to a mixture of 18 beads, each labeled with an antibody to one of the 18 cytokines/chemokines (Beadlyte anti-mouse multi-cytokine beads). The binding of cytokines to the beads was detected by a second set of reporter antibodies conjugated to biotin, and labeled with Streptavidin-Phycoerythrin. Labeled beads were analyzed on a Luminex-100 instrument. The concentration of cytokines and chemokines in the conditioned-medium were quantified against standard concentration curves for each of the cytokines and chemokines. Standard cytokines and chemokines were diluted in culture medium that was used for the experiments. A separate standard curve with ethanol-containing medium was constructed for samples of conditioned medium that contained ethanol, to ensure that ethanol did not alter the data. Data were reported as concentrations of cytokines/chemokines (pg/ml) in culture conditioned medium.

VEGF-A ELISA assay—VEGF (Vascular Endothelial Growth Factor)-A levels in the neurosphere culture supernatant were measured separately using a quantitative sandwich enzyme immunoassay kit (R&D systems, MN). Samples and standards were loaded in duplicates onto a 96-well microplate, pre-coated with affinity-purified polyclonal antibody specific for mouse VEGF-A. Equal volume of assay diluent was pipetted to all wells containing samples and standards. After two hours of incubation at room temperature, the unbound antigens were washed away with wash buffer for five times. Thereafter, 100 μ l of HRP-conjugated polyclonal antibody (R&D systems, kit component) was added to each well and the plate was incubated again for 2 h. The unbound antibody-enzyme was removed by washes (5x). The presence of VEGF-A was detected by adding 100 μ l of chromogenic substrate for 30 min. The development of the color product was terminated by the addition of diluted hydrochloric acid. The colored reaction product was read at 450nm in an ELISA plate reader with a correction at 590nm. The concentration of VEGF-A present in the samples was interpolated from a linear standard curve. The sensitivity of this assay is 3 pg/ml.

Real-time PCR analysis—Primer pairs for three of the ethanol-regulated cytokines, GM-CSF, MCP-1 and the p35 subunit of IL-12 were designed (using Beacon Designer V3.0) to amplify a sequence of mRNA that crossed intron-exon boundaries (Intron-exon boundaries were identified using ELXR (Exon Locator & eXtractor for Resequencing), V2.0, (Schageman et al., 2004)). Standard curves were constructed for all primer sets. Correlation coefficients and PCR efficiency values were computed (sequence information, and primer characteristics are in Table 3), and the amplification of a single product was verified by melt curve analysis. Cyclophilin-A ($r=0.999$, efficiency = 93.9%) was used as a normalization control, according to previously published protocols (Santillano et al., 2005). 1 μ g total RNA was reverse transcribed with Superscript II. cDNA was used either immediately or stored at -80°C . Three samples from each experimental condition and all in triplicates was used to perform Quantitative real-time PCR (Q-rtPCR) using the iCycler iQ system (Bio-Rad) according to manufactures instructions. Background fluorescence was normalized by addition of fluorescein in the Syber mix. (iQ-SYBR Green Supermix, Bio-Rad). The relative quantity of gene expression was calculated using the Pfaffl mathematical model (Pfaffl, 2001) that takes into consideration the efficiency of each PCR primer in estimating relative expression ratios.

Immuno-fluorescence analysis—Cultures were assayed for the expression of the neuroepithelial marker Nestin, and neuronal markers NeuN (neuronal nuclear antigen) and

neurofilament protein, according to previously published protocols (Wade et al., 1999). Briefly, cultures were fixed in methanol, rinsed twice with PBS (#14190-144 Invitrogen), and incubated in blocking solution (2% normal serum, 0.1% BSA, 0.2% triton X-100, in TBS) for 1 hour at room temperature. Antibodies (all from Chemicon) against nestin 1:100 (MAB3353) and NeuN 1:100 (MAB377) or Neurofilament protein 1:500 (MAB1592), were diluted in staining solution (TBS 0.1% BSA), and incubated with cells overnight at 4°C. Following three washes in TBS, cells were labeled with rat-adsorbed, biotinylated secondary horse anti-mouse antibodies (1:250) (Vector Laboratories) in TBS for NeuN and nestin and Fluorescein-conjugated, goat-anti-mouse antibodies (1:500, #F2761 Molecular Probes) for neurofilament protein. NeuN and nestin antibody binding was visualized by conjugation with avidin-fluorescein (1:250, Vector Laboratories). Cells were mounted in Fluorescence Mounting Media containing DAPI (Vector Laboratories), and visualized with an Olympus BX60 microscope using a 40X objective.

Data Analysis—Data was analyzed using a standard statistical package (SPSS, v.13). Cytokine secretion and mRNA expression data were subjected to Multivariate Analysis of Variance (MANOVA), since multiple dependent variables were measured simultaneously. The effects of differentiation state, ethanol treatment and the interaction between differentiation state and ethanol treatment were analyzed by the Pillai's Trace Multivariate test (chosen for its robustness with small samples), followed by a corrected Analysis of Variance (ANOVA) model test of between-subject effects for each dependent variable. Following the ANOVA, we computed the Hochberg test for post-hoc comparisons. To determine the potential for co-regulation of the different cytokines, a Pearson product moment correlation matrix was computed. The statistical significance of the correlation was tested with a 2-tailed test of significance, with the alpha value set at 0.05. Graphs were constructed with Microsoft Excel. Data was reported in graphical form as Mean $\pm$ SEM (Standard Error of the Mean).

Results

Cerebral cortical neural progenitor cells maintained in neurosphere cultures, in the presence of the mitogenic factors bFGF, EGF and LIF, assume a spherical shape (Figure 1A) and express immunoreactivity for the intermediate filament protein nestin (Figure 1B), marking these cells as immature (Dahlstrand et al., 1995), and consequently, the *in vitro* equivalent of the cortical ventricular zone (VZ, Figure 1A). We refer to this stage as the 'neuroepithelial proliferation' stage. In the presence of laminin, and mitogenic factors, neurospheres do become adherent (Figure 1C) and individual cells exit the neurosphere (Figure 1C, arrow). However, migrating cells continue to exhibit epithelioid morphology (Figure 1C, inset), indicative of continued immaturity. In contrast, the step-wise removal of mitogenic stimuli, in the presence of laminin, resulted in the emergence of two uniquely identifiable differentiation neuronal phenotypes (that we term 'early' and 'late' differentiation phenotypes respectively). Following the removal of EGF and LIF, neural progenitors cultured on laminin, dis-aggregate from neurospheres within 24 hours, assume a bipolar morphology (Figure 1D), and migrate away from the neurosphere, along radial glial-like processes. Based on morphological and immunological characteristics, we modeled aggregates of these cells as the *in vitro* equivalent of the sub-ventricular zone (SVZ, Figure 1D, arrow). Over a period of 72 hours, a majority of these migratory cells assume a bi-polar appearance (Figure 1E), express NeuN in their nuclei (Figure 1G), and express the neuron-specific intermediate filament, neurofilament (Figure 1I), but not nestin (Figure 1K) suggesting that these cells had assumed a neuronal fate. Because of the 'bi-polar' phenotype, we refer to these cells as belonging to an 'early-differentiation stage'. Removal of bFGF, in addition to the removal of EGF and LIF, caused these neural cells to assume a stellate morphology (Figure 1F). These stellate-type cells continue to express nuclear NeuN (Figure

1H) and cytoplasmic neurofilament (Figure IJ), but not nestin (Figure 1L) and we refer to this phenotype as the 'late-differentiation stage'.

Cells in the neuroepithelial proliferation condition may be sequentially differentiated through the early and late differentiation phases (red arrows), or directly transferred to the late differentiation phase (blue arrow), producing in both cases, the same stellate-type phenotype. Finally, flow cytometric analysis of sub-G0 DNA-containing cells, using propidium iodide incorporation, indicates that there is no change in apoptosis as a function of transition from the proliferation to differentiation stages (Figure 1M).

Cytokine secretion during neuroepithelial proliferation and neuronal differentiation

Several cytokines and chemokines (e.g., IL-2, IL-3, IL-6, TNF- α , RANTES/CCL5 and KC/CxCL-1; see Table 1) were not detectable in cerebral cortical progenitor cells at any stage of differentiation. In contrast, others (e.g., IL-1 β , IL-5, and IFN- γ ; Table 1) were constitutively expressed by cerebral cortical progenitors, irrespective of differentiation state. We performed a two-way Multivariate Analysis of Variance (MANOVA) to determine the effect of differentiation state and ethanol pre-exposure on cytokine expression. The Pillai's trace multivariate statistic indicated that there was an overall significant effect of differentiation state on cytokine expression ($F_{(28,24)}=2.376$, $p<0.017$). Follow-up ANOVA tests indicated that four cytokines were significantly altered by differentiation state. These included IL-10, the p40 subunit component of the hetero-dimeric IL-12 complex, MCP-1/CCL2, and VEGF-A (for ANOVA p values, see Table 1).

Cortical neurosphere cultures secrete particularly high levels of VEGF-A and MCP-1. Though these levels decline significantly following differentiation (Figure 2), in terms of absolute levels, both VEGF-A and MCP-1 are the most highly secreted cytokines among those that were assayed, at any differentiation stage. Interestingly, we observed statistically significant positive correlations between levels of VEGF-A MCP-1 and IL-10 (see Table 2 for Pearson's product moment correlation and associated 'p' values associated with 2-tailed tests of significance). VEGF-A, MCP-1 and IL-10 are all suppressed during neurosphere differentiation, and the significant correlation suggests that these two cytokines may be co-regulated during the process of neuronal differentiation.

The chemokine GM-CSF, is also secreted at comparatively high levels by cortical neurosphere cultures, though the effect of differentiation state on GM-CSF expression reached marginal significance (ANOVA $p<0.058$), principally because of the significant interaction effect between ethanol treatment and differentiation state (see below). While VEGF-A, MCP-1 and IL-10 secretion is reduced, GM-CSF secretion is induced in control cultures during the differentiation of neurospheres (Figure 2), suggesting that GM-CSF may be co-regulated along with IL-10, VEGF-A, and MCP-1, as part of a neuronal differentiation program.

Effect of ethanol exposure on the expression of cytokines during neuroepithelial proliferation and neuronal differentiation

To determine the effect of ethanol on cytokine secretion, we treated proliferating cerebral cortical progenitors with ethanol for 5 days. Samples of culture-conditioned medium were analyzed immediately following this period of ethanol pre-treatment (neuroepithelial proliferation condition, to determine ethanol's direct activation effects) or following an additional period of three days, where ethanol pre-treated cultures were cultured on a laminin substrate with a step-wise removal of mitogens from the culture medium (to model organizational effects of ethanol). The Pillai's trace multivariate statistic indicated that, overall, while there was not a significant effect of ethanol by itself on the secretion of

cytokines ($F_{(14,11)}=2.234$, $p<0.093$), there was an overall trend towards significance. This analysis indicates that in general, ethanol does not have a global, consistent effect on cytokine and chemokine secretion, across all stages of differentiation. Two potential exceptions to this rule are VEGF-A ($p<0.042$) and MCP-1/CCL2 ($p<0.024$), in that both exhibited a significant effect of ethanol, but no significant interaction between ethanol treatment and differentiation state. However, even in the cases of VEGF-A and MCP-1, closer visual examination of the data (Figure 2) indicates that most of the ethanol-induced effects on secretion occurs in the neuroepithelial proliferation condition, and in terms of relative levels, the effects are modest.

The Pillai's trace multivariate statistic indicated that there was a statistically significant interaction between ethanol exposure and differentiation state ($F_{(28,24)}=2.019$, $p<0.04$), suggesting that ethanol's effect on cytokine expression was dependent on the differentiation state of the cerebral cortical progenitors. Multivariate-corrected ANOVAs indicated that two separate cytokines, IL-12 (both p40 and p70 iso-forms) and GM-CSF, were both regulated by ethanol in a differentiation stage-specific manner (Table 1, Figure 2 and 3). These ethanol-regulated cytokines (2 out of 18 unique cytokines) represent a small fraction (11%) of the cytokines assayed. Furthermore, ethanol exhibits divergent patterns of differentiation stage-specific regulation of cytokine secretion. In the case of GM-CSF, under control conditions, levels of GM-CSF are low when cerebral cortical progenitors were maintained in the neuroepithelial proliferation condition. GM-CSF levels are significantly induced in the early-stage differentiation condition (+bFGF/-EGF/-LIF), and the levels decrease somewhat following complete removal of mitogenic stimuli (-bFGF/-EGF/-LIF, i.e., the late differentiation condition). In contrast, ethanol pre-treated cultures exhibited a suppression of GM-CSF secretion in the early-stage differentiation condition, and GM-CSF levels were induced to reach control levels, only following removal of all mitogenic stimuli (the later differentiation condition).

The secretion of two isoforms of IL-12, the p40 subunit, and the p70 isoform (comprised of a hetero-dimer of the p40, and a p35 subunit) were also regulated by ethanol pre-exposure, in a differentiation-stage-specific manner (Figure 3). IL-12p40 secretion was significantly induced during the neuroepithelial proliferation phase, where as the mature hetero-dimeric isoform, IL-12p70, was only induced following complete removal of all mitogens (the late differentiation condition).

Effect of ethanol exposure on cytokine mRNA expression

We next determined the extent to which ethanol-induced changes in cytokine secretion reflected changes in mRNA expression. We therefore performed quantitative real-time PCR to assay for genes (GM-CSF, and the p35 subunit of IL-12 (the IL-12-specific subunit of the p70 heterodimer)), whose secretion exhibited statistically significant interaction effects between ethanol exposure and differentiation stage. In addition, we examined the expression of mRNA for MCP-1, since we observed a modest but statistically significant main effect of ethanol on MCP-1 secretion. Changes in gene expression were normalized to cyclophilin-A (Santillano et al., 2005), and expressed as the $\log_2$ (Pfaffl ratio) (Pfaffl, 2001). The Pillai's Trace multivariate statistic indicated no significant difference among any of the mRNAs due to differentiation state ($F_{(6,34)}=0.712$, $p<0.642$) or ethanol exposure ($F_{(3,16)}=1.948$, $p<0.163$), suggesting that, in general, cytokine mRNAs are not a significant target of ethanol. Despite the overall lack of significance in the multivariate analysis, further, post-MANOVA univariate analysis indicated a statistically significant effect of ethanol exposure on MCP-1 mRNA levels ($p<0.035$), due mainly to a large, 16-fold ethanol-induced reduction in MCP-1 mRNA levels in the neuroepithelial proliferation condition (Figure 4).

Discussion

The cytokine super-family members are important regulators of stem cell maturation in a variety of organs including hematopoietic tissues (Dai et al., 2000; Shih et al., 1999). The developing brain is also an important cytokine source and target (Banisadr et al., 2005; Dzenko et al., 2005; Geppert, 2003; Guo et al., 2003; Hogan et al., 2004; Stamatovic et al., 2003; Widera et al., 2004; Yamamoto et al., 2005). Since ethanol does not kill cerebral cortical stem cells, but rather, alters their proliferation and differentiation potential (Santillano et al., 2005), we hypothesized that ethanol would alter the secretion of diffusible factors like cytokines, that form part of the intrinsic signaling network within the cerebral cortical neuroepithelium.

Mitogen-withdrawal as a model for early cortical neuronal differentiation

We created a novel *in vitro* model for the early stages of neuronal differentiation within the cerebral cortical ventricular (VZ) and sub-ventricular (SVZ) zones, to identify important epigenetic targets of ethanol. We modeled neural progenitor proliferation within the cortical VZ using murine embryonic-derived neurospheres exposed to mitogens EGF, FGF and LIF, that have been shown to modulate stem cell proliferation in other neural tissue beds as well (James et al., 2004; Pitman et al., 2004; Sakaguchi et al., 1997). Differentiating neurons assume a bi-polar shape *in vivo*, and migrate initially from the VZ to the SVZ, with the help of integrins and other cell adhesion molecules. These early, 'proto-neurons' continue to be capable of mitosis in the SVZ, but can also differentiate into stellate-type neurons (Noctor et al., 2004). We modeled early SVZ proto-neuron differentiation by step-wise removal of mitogens, and addition of laminin as an extracellular-matrix molecule, to achieve either the migratory bi-polar or stellate neuronal phenotypes. Interestingly, the final stellate-type neuronal phenotype can be achieved either after the intermediate bi-polar phenotype, or directly from the mitogenic stage, by varying the composition of the mitogenic medium. It will be important to examine the general applicability of this paradigm to other aspects of cortical development as well.

Neural differentiation-state specific cytokines are sensitive to ethanol exposure

None of the inflammatory cytokines like IL1 β , TNF α and IFN γ were targets of ethanol. Instead, ethanol selectively targeted the release of chemotactic cytokines. These data were surprising, and contrary to our working hypothesis, that ethanol promotes an inflammatory cytokine response as part of its neurotoxicity. It is possible that cytokine sensitivity is developmental stage specific, and that while chemotactic cytokines are ethanol targets in the early embryonic neuroepithelium, inflammatory cytokines may yet be the preferred ethanol targets in the more mature brain.

Three major chemo-attractant cytokines, VEGF-A, MCP-1 and GM-CSF were expressed at high levels, but exhibited complementary differentiation-associated patterns of expression. In control cultures, VEGF-A and MCP-1 was secreted at high levels by cells in the proliferation phase. However, levels precipitously declined, in each case by greater than 4-fold, as progenitor cells were transformed into the early differentiation phenotype, and levels of secreted VEGF-A and MCP-1 continued to remain low in progenitor cells that were transformed into the late differentiation phenotype. GM-CSF secretion in contrast, was significantly increased by the transformation of neural progenitor cells into early-stage neurons. Interestingly, these cytokines were all targets of ethanol. Pre-exposure to ethanol resulted in a small but significant decline in MCP-1 secretion, and a small increase in VEGF-A secretion. Furthermore, unlike control cultures, ethanol pre-treated neural progenitors did not exhibit an increase in GM-CSF secretion during the 'early differentiation' condition. Levels of secreted GM-CSF in ethanol-pretreated cultures were

only increased following the treatment condition (+laminin/-bFGF/-hEGF/-LIF) used to mimic later stages of neuronal differentiation. Ethanol exposure during proliferation serves as a brake on the differentiation-associated expression of GM-CSF and may delay in the induction of biological processes that are controlled by this chemotactic cytokine.

Receptors for VEGF (Ogunshola et al., 2002), MCP-1 (Widera et al., 2004) and GM-CSF (Dame et al., 1999) are all expressed in neuronal and progenitor cells. VEGF for example, prevents apoptosis in embryonic neurons (Ogunshola et al., 2002). This anti-apoptotic function is important, since sub-populations of embryonic cortical precursors and fetal neurons express suicide receptors, and are sensitive to suicide signals present within their local milieu (Cheema et al., 2004; Cheema et al., 1999). The high level of VEGF-A expression during neurogenesis may also explain why proliferating embryonic cortical neuroblasts are generally resistant to apoptosis (Santillano et al., 2005).

MCP-1 induces neural cell migration, though this cytokine also prevents neurite sprouting in neurosphere cultures (Widera et al., 2004). In contrast, GM-CSF is reportedly required for neurite extension in mouse superior cervical ganglion cultures (Kannan et al., 1996) and basal forebrain neurons (Kamegai et al., 1990). The switch between MCP-1 and GM-CSF may therefore be necessary for neural progenitor cells to assume a bi-polar morphology and, at the same time, to migrate away from the parental neurosphere. Interestingly, while MCP-1 levels decrease precipitously, the absolute levels of MCP-1 secreted into the culture medium continues to be high under both differentiation conditions. It is possible that withdrawal of mitogenic stimuli may facilitate a decrease in MCP-1 secretion to levels that are just high enough to permit the emergence of a strong MCP-1/GM-CSF-dependent migration phenotype, without preventing process outgrowth and neuronal maturation. Furthermore, ethanol exposure, by decreasing MCP-1 levels and preventing the mitogen-withdrawal-dependent increase in GM-CSF secretion, may alter neuronal migration, leading to phenomena like heterotopias that have been observed in brains of FAS children (Clarren et al., 1978), and in animal and *in vitro* models of FAS (Kotkoskie and Norton, 1988; Mooney et al., 2004).

Discrepancy between cytokine secretion and mRNA levels suggest a post-transcriptional role for ethanol in cytokine regulation

Our analyses indicated that cytokine mRNAs are not a significant target of ethanol. These data are consistent with recent evidence showing that ethanol disrupts the Golgi complex and microtubule-transport mechanisms (Azorin et al., 2004; Tomas et al., 2005), so that protein processing and secretion, rather than gene transcription *per se*, is the immediate ethanol target. MCP-1 was an exception to the above observations, since the relative ethanol-induced suppression of MCP-1 mRNA levels was substantially greater than the observed suppression of secreted protein product, suggesting that neural precursors at least partly compensate for the loss of MCP-1 mRNA.

IL-12 as a candidate ethanol target: potential impact on vasculogenesis

The effect of ethanol on IL-12 secretion has particularly significant implications for the coordinate differentiation of neuronal and vascular compartments of the developing brain. The developing human fetal forebrain exhibits the first signs of vasculogenesis at the end of the second trimester (Kuban and Gilles, 1985), i.e., after the major period of cortical ventricular zone neurogenesis (Rakic, 1988) and migration (Sidman and Rakic, 1973). Increasing evidence suggests that it is the neural tube that signals this peri-neural vascular network to initiate vasculogenesis (Hogan et al., 2004). IL-12 (i.e., IL-12p70), a heterodimeric protein complex comprised of a 40kDa subunit (IL-12p40), and a 35kDa subunit, is a potent anti-angiogenic factor (Duda et al., 2000; Sunamura et al., 2000). Our data indicate that neural

cells ordinarily express very little IL-12 (either the p40 subunit, or the p70 hetero-dimeric complex), at any stage of development, especially when compared to pro-angiogenic factors VEGF-A, MCP-1 and GM-CSF. Ethanol leads to the preferential secretion of the p40 subunit during the period of neurogenesis, and pre-treatment with ethanol significantly increases expression of the p70 heterodimer during differentiation. The implication of the independent neural activation of the p40 subunit is not clear at this time, though accumulating evidence suggests that p40 is also part of the heterodimeric IL-23 complex, and can participate in a variety of auto-immune diseases, as part of the IL-23 complex (Hunter, 2005).

The delayed induction of the p70 heteromeric IL-12 complex, however, indicates that ethanol exposure during the period of neurogenesis causes differentiating neurons to produce factors that are likely to suppress angiogenesis during the period when blood vessels are first beginning to populate the brain. We have presented evidence that the secretion of pro-angiogenic factors like MCP-1 and GM-CSF are suppressed during this developmental period, either as part of the differentiation state, or ethanol exposure history. Evidence in support of an anti-angiogenic function for ethanol comes from studies of other vasculogenic beds, where ethanol has been shown to significantly suppress angiogenesis (Radek et al., 2005). This is important because, in addition to neural defects, some types of vascular malformations (Stromland and Sundelin, 1996) and cardiac defects (Daft et al., 1986) appear to be a common feature in FAS children and in animal models of FAS. In this study by Stromland (Stromland and Sundelin, 1996), retinal vasculature was described as 'tortuous' suggesting the possibility that blood vessels may have undergone cycles of regression. One uninvestigated possibility is that such retinal vascular anomalies are a sign that vasculogenesis in neural tissue is generally compromised, contributing to cognitive problems observed in FAS children. Though the relationship between cerebral blood flow and cognitive function is well established (e.g., (Fuster et al., 2005)), little work has been done on the impact of gestational ethanol exposure on vasculogenesis, and signals that shape interactions between brain and micro-vascular circulation.

Conclusion

In summary, we modeled the early stages of neuronal differentiation from mitosis in the VZ (neurosphere cultures), to the appearance of migratory SVZ-type bi-polar and stellate neurons, by the step-wise removal of the mitogenic factors EGF, LIF and bFGF, and the addition of laminin. Our data demonstrate that there are dramatic shifts in the types of cytokines that are secreted by neural stem cells as they progress through these phases of neuronal differentiation. Aside from generating neurons to populate the cortical plate, the neuroepithelium and its progeny also secrete important angiogenic cytokines VEGF-A, MCP-1 and GM-CSF. Ethanol exposure during the proliferation period had a significant impact on both immediate (activation) and longer-term (organization) cytokine expression profiles. It will be important to determine how ethanol-sensitive cytokines like GM-CSF and IL-12 interact with other ethanol-sensitive neurotrophic factors like GDNF (McAlhany et al., 2000), BDNF (Heaton et al., 2000; Maier et al., 1999) and TGF-beta (Chen et al., 2006), particularly since some of these factors (Donovan et al., 1995; Ishimura et al., 2006) themselves, are potent vasculogenic agents. Moreover, emerging evidence suggests that the vascular system in turn supports the growth and expansion of neural stem cells (Shen et al., 2004), and therefore, disrupting the cerebral cortical vasculogenic program is likely to reciprocally disrupt the formation and maintenance of neuronal stem cells.

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List of Abbreviations

bFGF	Basic Fibroblast Growth Factor	
EGF	Human Epidermal Growth Factor	
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor	
KC	Chemokine (C-X-C motif) ligand 1, CXCL-1	
LIF	Leukemia Inhibitory Factor	
MCP-1	Monocyte chemotactic protein-1	
RANTES	Regulated on Activation, Normal T Expressed and Secreted	CCI-5
SVZ	Sub-ventricular zone	
TNF- α	Tumor Necrosis Factor-alpha	
VEGF-A	Vascular endothelial growth factor-A	
VZ	Ventricular Zone	

References

- Adachi J, Mizoi Y, Fukunaga T, Ogawa Y, Ueno Y, Imamichi H. Degrees of alcohol intoxication in 117 hospitalized cases. *J Stud Alcohol* 1991;52(5):448–453. [PubMed: 1943100]
- Azorin I, Portoles M, Marin P, Lazaro-Dieguez F, Megias L, Egea G, Renau-Piqueras J. Prenatal ethanol exposure alters the cytoskeleton and induces glycoprotein microheterogeneity in rat newborn hepatocytes. *Alcohol Alcohol* 2004;39(3):203–12. [PubMed: 15082457]
- Banisadr G, Gosselin RD, Mechighel P, Kitabgi P, Rostene W, Parsadaniantz SM. Highly regionalized neuronal expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) in rat brain: Evidence for its colocalization with neurotransmitters and neuropeptides. *J Comp Neurol* 2005;489(3):275–92. [PubMed: 16025454]
- Bayer, S.; Altman, J. *The Rat Nervous System*. Second Edition. Academic Press; San Diego: 1995. Neurogenesis and neuronal migration.; p. 1041-1078.
- Bernstein ID, Andrews RG, Zsebo KM. Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+lin- cells, and the generation of colony-forming cell progeny from CD34+lin- cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. *Blood* 1991;77(11):2316–21. [PubMed: 1710148]
- Cheema ZF, Santillano DR, Wade SB, Newman JM, Miranda RC. The extracellular matrix, p53 and estrogen compete to regulate cell-surface Fas/Apo-1 suicide receptor expression in proliferating embryonic cerebral cortical precursors, and reciprocally, Fasligand modifies estrogen control of cell-cycle proteins. *BMC Neurosci* 2004;5(1):11. [PubMed: 15038834]
- Cheema ZF, Wade SB, Sata M, Walsh K, Sohrabji F, Miranda RC. Fas/Apo [apoptosis]-1 and associated proteins in the differentiating cerebral cortex: induction of caspase-dependent cell death and activation of NF-kappaB. *J Neurosci* 1999;19(5):1754–70. [PubMed: 10024361]
- Cheema ZF, West JR, Miranda RC. Ethanol induces Fas/Apo [apoptosis]-1 mRNA and cell suicide in the developing cerebral cortex. *Alcohol Clin Exp Res* 2000;24(4):535–543. [PubMed: 10798591]
- Chen CP, Kuhn P, Chaturvedi K, Boyadjieva N, Sarkar DK. Ethanol induces apoptotic death of developing beta-endorphin neurons via suppression of cyclic adenosine monophosphate production and activation of transforming growth factor-beta1-linked apoptotic signaling. *Mol Pharmacol* 2006;69(3):706–17. [PubMed: 16326933]

- Clarren, SK. Neuropathology in Fetal Alcohol Syndrome. In: West, JR., editor. Alcohol and Brain Development. Oxford University Press; New York: 1986. p. 158-166.
- Clarren SK, Alvord EC Jr, Sumi SM, Streissguth AP, Smith DW. Brain malformations related to prenatal exposure to ethanol. *J Pediatr* 1978;92(1):64-7. [PubMed: 619080]
- Daft PA, Johnston MC, Sulik KK. Abnormal heart and great vessel development following acute ethanol exposure in mice. *Teratology* 1986;33(1):93-104. [PubMed: 3738814]
- Dahlstrand J, Lardelli M, Lendahl U. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. *Developmental Brain Research* 1995;84(1):109-129. [PubMed: 7720210]
- Dai MS, Mantel CR, Xia ZB, Broxmeyer HE, Lu L. An expansion phase precedes terminal erythroid differentiation of hematopoietic progenitor cells from cord blood in vitro and is associated with up-regulation of cyclin E and cyclin-dependent kinase 2. *Blood* 2000;96(12):3985-7. [PubMed: 11090089]
- Dame JB, Christensen RD, Juul SE. The distribution of granulocyte-macrophage colony-stimulating factor and its receptor in the developing human fetus. *Pediatr Res* 1999;46(4):358-66. [PubMed: 10509354]
- Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev* 1979;3(1):79-83. [PubMed: 118862]
- Dohrman DP, West JR, Pantazis NJ. Ethanol reduces expression of the nerve growth factor receptor, but not nerve growth factor protein levels in the neonatal rat cerebellum. *Alcohol Clin Exp Res* 1997;21(5):882-893. [PubMed: 9267539]
- Donovan MJ, Miranda RC, Kraemer R, McCaffrey TA, Tessarollo L, Mahadeo D, Sharif S, Kaplan DR, Tsoulfas P, Parada L, et al. Neurotrophin and neurotrophin receptors in vascular smooth muscle cells. Regulation of expression in response to injury. *Am J Pathol* 1995;147(2):309-24. [PubMed: 7639328]
- Duda DG, Sunamura M, Lozonchi L, Kodama T, Egawa S, Matsumoto G, Shimamura H, Shibuya K, Takeda K, Matsuno S. Direct in vitro evidence and in vivo analysis of the antiangiogenesis effects of interleukin 12. *Cancer Res* 2000;60(4):1111-6. [PubMed: 10706132]
- Dzenko KA, Song L, Ge S, Kuziel WA, Pachter JS. CCR2 expression by brain microvascular endothelial cells is critical for macrophage transendothelial migration in response to CCL2. *Microvasc Res*. 2005
- Fuster J, Guiou M, Ardestani A, Cannestra A, Sheth S, Zhou YD, Toga A, Bodner M. Near-infrared spectroscopy (NIRS) in cognitive neuroscience of the primate brain. *Neuroimage* 2005;26(1):215-20. [PubMed: 15862221]
- Gary DS, Mattson MP. Integrin signaling via the PI3-kinase-Akt pathway increases neuronal resistance to glutamate-induced apoptosis. *J Neurochem* 2001;76(5):1485-96. [PubMed: 11238733]
- Geppert AM. Constitutive patterns of RANTES, MCP-1 and MIP-1 alpha expression at the mRNA and protein level during postnatal development of the rat brain. *Folia Neuropathol* 2003;41(2):79-88. [PubMed: 12899200]
- Guo CJ, Douglas SD, Lai JP, Pleasure DE, Li Y, Williams M, Bannerman P, Song L, Ho WZ. Interleukin-1beta stimulates macrophage inflammatory protein-1alpha and -1beta expression in human neuronal cells (NT2-N). *J Neurochem* 2003;84(5):997-1005. [PubMed: 12603824]
- Haylock DN, To LB, Dowse TL, Juttner CA, Simmons PJ. Ex vivo expansion and maturation of peripheral blood CD34+ cells into the myeloid lineage. *Blood* 1992;80(6):1405-12. [PubMed: 1381625]
- Heaton MB, Mitchell JJ, Paiva M, Walker DW. Ethanol-induced alterations in the expression of neurotrophic factors in the developing rat central nervous system. *Developmental Brain Research* 2000;121(1):97-107. [PubMed: 10837897]
- Hogan KA, Ambler CA, Chapman DL, Bautch VL. The neural tube patterns vessels developmentally using the VEGF signaling pathway. *Development* 2004;131(7):1503-13. [PubMed: 14998923]
- Hsiao SH, DuBois DW, Miranda RC, Frye GD. Critically timed ethanol exposure reduces GABAAR function on septal neurons developing in vivo but not in vitro. *Brain Res* 2004;1008(1):69-80. [PubMed: 15081384]

- Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 2005;5(7):521–31. [PubMed: 15999093]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskaya V, Horster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* 2000;287(5455):1056–60. [PubMed: 10669420]
- Ishimura A, Ng JK, Taira M, Young SG, Osada SI. Man1, an inner nuclear membrane protein, regulates vascular remodeling by modulating transforming growth factor {beta} signaling. *Development*. 2006
- James J, Das AV, Rahnenfuhrer J, Ahmad I. Cellular and molecular characterization of early and late retinal stem cells/progenitors: differential regulation of proliferation and context dependent role of Notch signaling. *J Neurobiol* 2004;61(3):359–76. [PubMed: 15452852]
- Jones KL, Smith DW. The fetal alcohol syndrome. *Teratology* 1975;12(1):1–10. [PubMed: 1162620]
- Kamegai M, Konishi Y, Tabira T. Trophic effect of granulocyte-macrophage colony-stimulating factor on central cholinergic neurons in vitro. *Brain Res* 1990;532(1-2):323–5. [PubMed: 2282525]
- Kannan Y, Bienenstock J, Ohta M, Stanisz AM, Stead RH. Nerve growth factor and cytokines mediate lymphoid tissue-induced neurite outgrowth from mouse superior cervical ganglia in vitro. *J Immunol* 1996;157(1):313–20. [PubMed: 8683132]
- Kim JK, Choi BH, Park HC, Park SR, Kim YS, Yoon SH, Park HS, Kim EY, Ha Y. Effects of GM-CSF on the neural progenitor cells. *Neuroreport* 2004;15(14):2161–5. [PubMed: 15371725]
- Kotkoskie LA, Norton S. Prenatal brain malformations following acute ethanol exposure in the rat. *Alcohol Clin Exp Res* 1988;12(6):831–836. [PubMed: 3064646]
- Kuban KC, Gilles FH. Human telencephalic angiogenesis. *Ann Neurol* 1985;17(6):539–48. [PubMed: 4026224]
- Loock C, Conry J, Cook JL, Chudley AE, Rosales T. Identifying fetal alcohol spectrum disorder in primary care. *CMAJ* 2005;172(5):628–30. [PubMed: 15738484]
- Luo J, Miller MW. Growth factor-mediated neural proliferation: target of ethanol toxicity. *Brain Research Reviews* 1998;27(2):157–167. [PubMed: 9622617]
- Luo J, West JR, Pantazis NJ. Ethanol exposure reduces the density of the low-affinity nerve growth factor receptor (p75) on pheochromocytoma (PC12) cells. *Brain Research* 1996;737(1-2):34–44. [PubMed: 8930347]
- Maier SE, Cramer JA, West JR, Sohrabji F. Alcohol exposure during the first two trimesters equivalent alters granule cell number and neurotrophin expression in the developing rat olfactory bulb. *J Neurobiol* 1999;41(3):414–23. [PubMed: 10526319]
- Makino S, Haylock DN, Dowse T, Trimboli S, Niutta S, To LB, Juttner CA, Simmons PJ. Ex vivo culture of peripheral blood CD34+ cells: effects of hematopoietic growth factors on production of neutrophilic precursors. *J Hematother* 1997;6(5):475–89. [PubMed: 9368184]
- McAlhane REJ, Miranda RC, Finnell RH, West JR. Ethanol decreases Glial-Derived Neurotrophic Factor (GDNF) protein release but not mRNA expression and increases GDNF-stimulated Shc phosphorylation in the developing cerebellum. *Alcohol Clin Exp Res* 1999;23(10):1691–1697. [PubMed: 10550003]
- McAlhane REJ, West JR, Miranda RC. Glial-derived neurotrophic factor (GDNF) prevents ethanol-induced apoptosis and JUN kinase phosphorylation. *Developmental Brain Research* 2000;119(2):209–216. [PubMed: 10675770]
- Miller M, Jacobs J, Yokoyama R. Neg, a nerve growth factor-stimulated gene expressed by fetal neocortical neurons that is downregulated by ethanol. *The Journal of Comparative Neurology* 2003;460(2):212–222. [PubMed: 12687686]
- Mooney SM, Miller MW. Effects of prenatal exposure to ethanol on the expression of bcl-2, bax and caspase 3 in the developing rat cerebral cortex and thalamus. *Brain Res* 2001;911(1):71–81. [PubMed: 11489446]
- Mooney SM, Miller MW. Ethanol-induced neuronal death in organotypic cultures of rat cerebral cortex. *Brain Res Dev Brain Res* 2003;147(1-2):135–41.
- Mooney SM, Siegenthaler JA, Miller MW. Ethanol Induces Heterotopias in Organotypic Cultures of Rat Cerebral Cortex. *Cereb Cortex* 2004;14(10):1071–80. [PubMed: 15166098]

- Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci* 2004;7(2):136–144. [PubMed: 14703572]
- Ogunshola OO, Antic A, Donoghue MJ, Fan SY, Kim H, Stewart WB, Madri JA, Ment LR. Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system. *J Biol Chem* 2002;277(13):11410–5. [PubMed: 11777931]
- Perper JA, Twerski A, Wienand JW. Tolerance at High Blood Alcohol Concentration: A study of 110 Cases and Review of the Literature. *J Forensic Sci* 1986;31(1):212–221. [PubMed: 3511175]
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29(9):e45. [PubMed: 11328886]
- Pitman M, Emery B, Binder M, Wang S, Butzkueven H, Kilpatrick TJ. LIF receptor signaling modulates neural stem cell renewal. *Mol Cell Neurosci* 2004;27(3):255–66. [PubMed: 15519241]
- Radek KA, Matthies AM, Burns AL, Heinrich SA, Kovacs EJ, Dipietro LA. Acute ethanol exposure impairs angiogenesis and the proliferative phase of wound healing. *Am J Physiol Heart Circ Physiol* 2005;289(3):H1084–90. [PubMed: 15863463]
- Rakic P. Specification of cerebral cortical areas. *Science* 1988;241(4862):170–6. [PubMed: 3291116]
- Riley EP, McGee CL. Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp Biol Med (Maywood)* 2005;230(6):357–65. [PubMed: 15956765]
- Sakaguchi DS, Janick LM, Reh TA. Basic fibroblast growth factor (FGF-2) induced transdifferentiation of retinal pigment epithelium: generation of retinal neurons and glia. *Dev Dyn* 1997;209(4):387–98. [PubMed: 9264262]
- Santillano DR, Kumar LS, Prock TL, Camarillo C, Tingling JD, Miranda RC. Ethanol induces cell-cycle activity and reduces stem cell diversity to alter both regenerative capacity and differentiation potential of cerebral cortical neuroepithelial precursors. *BMC Neurosci* 2005;6:59. [PubMed: 16159388]
- Schageman JJ, Horton CJ, Niu S, Garner HR, Pertsemlidis A. ELXR: a resource for rapid exon-directed sequence analysis. *Genome Biol* 2004;5(5):R36. [PubMed: 15128450]
- Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 2004;304(5675):1338–40. [PubMed: 15060285]
- Shih CC, Hu MC, Hu J, Medeiros J, Forman SJ. Long-term ex vivo maintenance and expansion of transplantable human hematopoietic stem cells. *Blood* 1999;94(5):1623–1636. [PubMed: 10477687]
- Sidman RL, Rakic P. Neuronal migration, with special reference to developing human brain: a review. *Brain Res* 1973;62(1):1–35. [PubMed: 4203033]
- Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. *JAMA* 2003;290(22):2996–9. [PubMed: 14665662]
- Stamatovic SM, Keep RF, Kunkel SL, Andjelkovic AV. Potential role of MCP-1 in endothelial cell tight junction ‘opening’: signaling via Rho and Rho kinase. *J Cell Sci* 2003;116(Pt 22):4615–28. [PubMed: 14576355]
- Stromland K, Sundelin K. Paediatric and ophthalmologic observations in offspring of alcohol abusing mothers. *Acta Paediatr* 1996;85(12):1463–8. [PubMed: 9001659]
- Sunamura M, Sun L, Lozonchi L, Duda DG, Kodama T, Matsumoto G, Shimamura H, Takeda K, Kobari M, Hamada H, Matsuno S. The antiangiogenesis effect of interleukin 12 during early growth of human pancreatic cancer in SCID mice. *Pancreas* 2000;20(3):227–33. [PubMed: 10766447]
- The Boulder Committee. Embryonic vertebrate central nervous system: revised terminology. The Boulder Committee. *Anat Rec* 1970;166(2):257–61. [PubMed: 5414696]
- Tomas M, Marin P, Megias L, Egea G, Renau-Piqueras J. Ethanol perturbs the secretory pathway in astrocytes. *Neurobiol Dis* 2005;20(3):773–84. [PubMed: 15953732]
- Wade S, Oommen P, Conner W, Earnest D, Miranda R. Overlapping and divergent actions of estrogen and the neurotrophins on cell fate and p-53 dependent signal transduction in conditionally immortalized cerebral cortical neuroblasts. *Journal of Neuroscience* 1999;15:6994–7006. [PubMed: 10436055]

- Walsh C, Cepko CL. Clonally related cortical cells show several migration patterns. *Science* 1988;241(4871):1342–5. [PubMed: 3137660]
- Walsh C, Cepko CL. Clonal dispersion in proliferative layers of developing cerebral cortex. *Nature* 1993;362(6421):632–5. [PubMed: 8464513]
- Widera D, Holtkamp W, Entschladen F, Niggemann B, Zanker K, Kaltschmidt B, Kaltschmidt C. MCP-1 induces migration of adult neural stem cells. *Eur J Cell Biol* 2004;83(8):381–7. [PubMed: 15506562]
- Wu P, Tarasenko YI, Gu Y, Huang LY, Coggeshall RE, Yu Y. Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. *Nat Neurosci* 2002;5(12):1271–8. [PubMed: 12426573]
- Yamamoto M, Horiba M, Buescher JL, Huang D, Gendelman HE, Ransohoff RM, Ikezu T. Overexpression of monocyte chemotactic protein-1/CCL2 in beta-amyloid precursor protein transgenic mice show accelerated diffuse beta-amyloid deposition. *Am J Pathol* 2005;166(5):1475–85. [PubMed: 15855647]

Figure 1.

Characterization of mouse cortical neurospheres and induction of neuronal differentiation by the step-wise removal of mitogenic factors. (A) Control cortical neurospheres cultured in mitogenic medium (+bFGF/+hEGF/+LIF). VZ = ventricular zone model (B) Confocal microscopy showing mouse cortical neurospheres immuno-positive for the intermediate filament protein, nestin (green), and counter-stained with DAPI (blue). (C) In the presence of mitogenic medium and laminin, neurosphere cultures become adherent, and cells migrate away from the neurosphere (arrow), but continue to maintain an epithelial morphology (inset). (D) Removal of the growth factors hEGF and LIF from the mitogenic medium and addition of a laminin substrate promotes the migration of neural cells from the neurosphere within 24 hours (SVZ equivalent), and (E) transformation into bi-polar cells resembling early differentiating neurons. (F) Additional removal of the growth factor, bFGF, from the medium promotes the differentiation of mouse cortical neurospheres into cells with a stellate morphology (arrows point to the extension of one of multiple processes terminating in a growth cone); -bFGF/-hEGF/-LIF. (G-H) Pseudo-color digital merge of NeuN immuno-fluorescence (red) with phase-contrast microscopy (green) shows that both early and late differentiating cells express the neuronal marker within the nucleus (I-J) Both bFGF positive and bFGF negative-differentiated cultures were immuno-positive for the neuronal intermediate filament, neurofilament (green) and counter-stained with DAPI (blue). (K-L) Both bFGF positive-differentiated cultures and bFGF negative-differentiated cultures lacked immuno-staining for nestin (green; cultures were counter-stained with DAPI, blue). Proliferating cells can be sequentially differentiated through early and late differentiation

stages (red arrows) or directly into late differentiation (blue arrow). (**M**) Flow cytometric analysis of propidium iodide incorporation into cells containing sub-G0 DNA content shows that there is no change in the proportion of apoptotic cells during differentiation compared to proliferation. Graphic insets depict stereotypic appearance of cells under neuroepithelial proliferation and differentiation conditions. Scale bars: 50um.

Figure 2.

Cytokine secretion (pg/ml culture medium), measured as a function of differentiation stage. Cerebral cortical neuroepithelial-derived neurosphere cultures were maintained in control or ethanol-supplemented (320mg/dl) mitogenic medium (+bFGF/+EGF/+LIF) for 5 days. Cytokines were measured directly in culture medium obtained from cells maintained as neurospheres in the presence of mitogenic medium, or following three additional days of exposure to conditions designed to mimic migration/early differentiation (+bFGF/-EGF/-LIF/+laminin) or later differentiation (-bFGF/-EGF/-LIF/+laminin). Data expressed as mean $\pm$ SEM.

Figure 3. Secretion of p40 and p70 subunits of IL-12 (pg/ml culture medium) as a function of ethanol exposure and differentiation stage. Data expressed as mean $\pm$ SEM.

Figure 4. Real-time PCR analysis of cytokine mRNA expression as a function of differentiation stage and ethanol exposure. Data is expressed as a Log2 Pfaffl ratio, where cytokine expression was normalized to cyclophilin-A expression. Data expressed as mean $\pm$ SEM

Table 1
Mitogen-Dependent Expression of cytokines in cerebral cortical-derived progenitors

Cytokine	FGF/EGF/LIF	FGF/-/-	-/-/-	P value * Differentiation state	P value ethanol	P value Ethanol × Differentiation
	Proliferation 	Differentiation 				
IL-1α	-	-	+	0.149	0.281	0.739
IL-1β	++	++	+	0.514	0.805	0.354
IL-2	-	-	-	**	**	**
IL-3	-	-	-	**	**	**
IL-4	-	+	+	0.093	0.246	0.285
IL-5	++	++	++	0.772	0.316	0.202
IL-6	-	-	-	**	**	**
IL-10	++	+	-	0.0001	0.087	0.083
IL-12p40	-	-	+	0.006	0.002	0.003
IL-12p70	+	+	-	0.062	0.147	0.013
IL-13	++	+++	+++	0.099	0.835	0.892
IL-17	-	+	+	0.333	0.442	0.853
TNF-α	-	-	-	**	**	**
Interferon-γ	++	++	++	0.862	0.163	0.96
KC/CXCL-1	-	-	-	**	**	**
RANTES/CCL5	-	-	-	0.238	0.851	0.419
GM-CSF/CSF2	++	+++	+++	0.058	0.115	0.019
MCP-1/CCL2	++++	++++	++++	0.0001	0.024	0.296
VEGF-A	+++++	+++++	+++++	0.0001	0.042	0.351

Code	-	+	++	+++	++++	+++++
Conc.	0	0.1-1.0pg/ml	1.0-5.0pg/ml	5-15pg/ml	15-50pg/ml	50-400pg/ml
						>400pg/ml

* Exact P value based on MANOVA

**
P value could not be calculated

Table 2

Pearson's product moment correlation (r) matrix of cytokines that are highly co-variant. Exact 'p' values (based on a 2-tailed test of statistical significance) are indicated in parentheses.

	VEGF	MCP-1	IL-10
VEGF	1.0	$r=0.848$ ($p<3.37e^{-009}$)	$r=0.769$ ($p<6.99e^{-007}$)
MCP-1		1.0	$r=0.693$ ($p<2.23e^{-005}$)
IL-10			1.0

Table 3

List of cytokine primers and their characteristics, used to determine levels of gene expression in differentiating neural cultures

Gene	Primers	Accession # and exon location	Correlation Coefficient	PCR efficiency
GM-CSF	Forward GAC CCG CCT GAA GAT ATT CG Reverse ATC CGC ATA GGT GGT AAC TTG	NM_009969.2 -3rd exon -4th exon	0.996	93.4%
MCP-1/CCL-2	Forward TCC TGT CAT GCT TCT GG Reverse ATC TTG CTG GTG AAT GAG TAG	NM_011333.1 -1st exon -2nd exon	0.995	93.2%
IL12-p35	Forward GCC AGG TGT CTT AGC CAG TCC Reverse TGG TTT GGT CCC GTG TGA TGT C	NM_008351.1 -3rd exon -4th exon	0.980	113.8%