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Embryonic Cerebral Cortical Progenitors Are Resistant to Apoptosis, but Increase Expression of Suicide Receptor DISC-complex Genes and Suppress Autophagy Following Ethanol Exposure

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Abstract

Background—*In utero* exposure to ethanol can result in severe fetal brain defects. Previous studies showed that ethanol induces apoptosis in differentiated cortical neurons. However, we know little about ethanol's effects on proliferating embryonic cortical progenitors. This study investigated the impact of ethanol exposure on the Fas/Apo-1/CD95 suicide receptor pathway, and on the survival of proliferating cortical neuroepithelial progenitors.

Methods—Murine embryonic-derived primary cortical neuroepithelial cells were maintained as neurosphere cultures and exposed to a dose-range of ethanol for periods ranging from 1 to 5 days. Programmed cell death was measured by four independent means (Annexin-V staining, caspase activation, DNA fragmentation and autophagic vacuole formation). Surface Fas/Apo-1 suicide receptor expression was measured by flow cytometry. Expression of Fas/Apo-1-associated DISC-complex genes was measured by quantitative polymerase chain reaction.

Results—Ethanol exposure did not substantially increase apoptosis, necrosis, or surface Fas/Apo-1 expression. Moreover, ethanol significantly decreased caspase activation and autophagic activity. Finally, ethanol exposure induced mRNA expression of genes that constitute the death receptor complex.

Conclusions—This study provides surprising evidence that ethanol does not induce either programmed cell death or necrosis of immature progenitors during neurogenesis, though ethanol may render neural progenitors susceptible to future apoptotic insults. Furthermore, our novel observation that ethanol suppresses autophagy is consistent with a hypothesis that ethanol promotes premature neural progenitor maturation. Taken together with our previous data regarding the role of the Fas/Apo-1 receptor in neural development, we conclude that ethanol disrupts basic proliferation and differentiation machinery rather than initiating cell death *per se*.

Keywords

Fetal Alcohol Syndrome; Neurogenesis; Apoptosis; Fas/Apo-1/CD95; Differentiation

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INTRODUCTION

Heavy prenatal ethanol exposure can result in a spectrum of physical abnormalities and growth deficiencies, collectively known as the Fetal Alcohol Spectrum Disorder (FASD). Defects associated with FASD include mental retardation, developmental delay and attention-deficit hyperactivity disorder (Mattson et al., 1996; Coles et al., 1997; Mattson and Riley, 1998; Roebuck et al., 1998; Thomas et al., 1998b; Kodituwakku et al., 2001; Schonfeld et al., 2001). The developing cortex is an important target of ethanol-induced damage. For example, it has been demonstrated that prenatal ethanol exposure results in a disproportionate reduction in frontal cortex size in the absence of global microcephaly (Wass et al., 2001).

Much of the research on gestational exposure to ethanol has focused on cell loss as a mechanism of ethanol-induced damage. Prenatal ethanol exposure during a crucial developmental period in the rat (gestational day [GD] 6 to birth) leads to a significant decrease in the number of neurons generated during the peak period of neurogenesis (GD 12–19) (Miller, 1988), and consequently, a decreased neuron number in the mature cortex (Miller and Potempa, 1990). This cell loss has largely been attributed to the ability of ethanol to induce apoptosis (Bhave and Hoffman, 1997; Cartwright et al., 1998; Bhave et al., 1999; Cheema et al., 1999; McAlhany et al., 2000; Ramachandran et al., 2003; Young et al., 2003; Takadera and Ohyashiki, 2004; Dikranian et al., 2005). Although we know that ethanol can induce apoptosis of fetal cortical neurons in late gestation (Ramachandran et al., 2003), relatively few studies (Kentroti and Vernadakis, 1991; Hao et al., 2003; Santillano et al., 2005) have focused on the apoptotic effects of ethanol on embryonic neural progenitors during early neurogenesis. Paradoxically, the onset of neurogenesis is accompanied by a wave of apoptotic cell death, similar to the death observed later in development as neurons seek appropriate synaptic targets (Blaschke et al.,1996; Thomaidou et al., 1997; Blaschke et al., 1998; Cheema et al., 1999; Bähr, 2000; De la Rosa and De Pablo, 2000). Indeed, apoptosis occurring during neurogenesis is as crucial to normal development as is the proliferation competency of progenitors. In fact, disruption of apoptosis via overexpression of *Bcl-2* results in abnormal neurogenesis and neuronal determination in *Xenopus* embryos (Yeo and Gautier, 2003).

Our laboratory has been interested in the role of apoptosis during the period of neurogenesis, and in particular the extent to which the suicide receptor Fas/Apo [Apoptosis]-1/CD95 is involved in the deletion of neural precursor cells. We have previously shown the transient expression of this receptor in cells of the developing cortex during the peak period of naturally occurring apoptosis, in close proximity to FasL expressing cells (Cheema et al., 1999). In this same study, we illustrated that administration of FasL or anti-Fas/Apo-1 antibody induced caspase-dependent cell death in primary embryonic cortical neuroblast cultures, indicating that a functional Fas/Apo-1 death pathway is present in these neural progenitors. We demonstrated that p53 activation is followed by the induction of Fas/Apo-1 expression (Cheema et al., 2004). Ethanol, in turn, induces p53 expression (Kuhn and Miller, 1998; de-la-Monte et al., 2000) and p53-dependent suicide factors including Bax (Moore et al., 1999). Furthermore, we observed an increase in Fas/Apo-1 mRNA expression in postnatal organotypic cortical explants treated with ethanol, coupled with a dose-dependent increase in apoptosis (Cheema et al., 2000). This led us to hypothesize that the Fas/Apo-1 receptor might be involved in mediating the apoptotic consequences of ethanol exposure in immature progenitors as well.

In the present study we examined the vulnerability of neural progenitors to ethanol-induced apoptosis during the early period of neurogenesis in the mouse, an intensely proliferative phase and a time when crucial cell fate decisions are being made. Given the evidence

illustrating that ethanol causes apoptosis of cortical neurons in both late gestation and the early postnatal period, and also considering the effects of this teratogen in ultimately decreasing the size of the cortex, we hypothesized that ethanol would result in a dose-dependent increase in apoptosis of proliferating cortical progenitors. Our previous data suggested that the Fas/Apo-1 receptor was a candidate ethanol target (Cheema et al., 2000), therefore we also hypothesized that ethanol would cause an increase in surface Fas/Apo-1 expression in neural progenitor cells along with an increase in downstream adaptor proteins of the extrinsic apoptotic pathway.

Contrary to our hypothesis, our data show that murine neural progenitors obtained during the early period of neurogenesis are relatively resistant to the apoptosis-inducing effects of ethanol. Interestingly, ethanol dramatically decreases both caspase activation and the formation of autophagic vacuoles. Ethanol exposure failed to cause an overall induction of surface Fas/Apo-1 expression. However, downstream adaptor proteins containing death effector domains were up-regulated following ethanol treatment. New evidence regarding the role of the DISC-complex adaptor proteins and autophagy in differentiation suggests that ethanol exposure during early neurogenesis may alter cortical progenitor maturation. These data suggest that ethanol exposure early in cortical development may be detrimental in ways unrelated to apoptosis---perhaps involving non-apoptotic functions of the extrinsic suicide pathway.

METHODS

Isolation and Culture of Cortical Progenitors

Timed pregnant C57BL6 mice were bred in-house, from breeder pairs initially purchased from Harlan (Harlan, TX). Gestational day (G.D.) 0.5 was the day that a vaginal mucus plug was detected. Pregnant females (G.D. 12.5) were anesthetized with a mixture of ketamine (0.09mg/gram body weight) and xylazine (0.106mg/gram body weight) by intramuscular injection. All animal procedures were approved by the Texas A&M University Laboratory Animal Care Committee. Fetuses were removed under aseptic conditions and rinsed in chilled phosphate buffered saline (PBS). Fetal brains were dissected, meningeal tissue removed and the structural precursor of the neocortex isolated. Fetal cortical tissue from 3–4 litters per experiment was combined into a single collection tube, dispersed by trypsinization (0.05%, Invitrogen #25300-054) and cells counted using a hemocytometer. Cells were grown in serum free mitogenic media (DMEM-F12 Invitrogen #11039-24) supplemented with 20ng/mL human recombinant Basic Fibroblast Growth Factor (bFGF, BD Biosciences #354060), 20ng/mL human Epidermal Growth Factor (hEGF, BD Biosciences #356052), ITS (Insulin, Transferrin, Selenium)-X supplement (Invitrogen #51500-056), 5ug/mL heparin (Sigma #4784), 0.15ng/mL Leukemia Inhibitory Factor (LIF, Alomone #L-200) and 20nM progesterone (Sigma #P7556). Cells were allowed to proliferate as neurospheres until cultures achieved a density of 2×10^6 cells per T25 flask (approximately 6–8 passages), and then used for experiments.

Ethanol Treatment

Neurosphere cultures at a density of 2×10^6 cells per T25 flask were randomly assigned to control or ethanol treatment groups representing low (60–95mg/dL (~13mM–21mM) based on gas chromatographic analyses), moderate (130–218mg/dL (~28mM–47mM)), and high doses (270–350mg/dL (~58–76mM)) of ethanol. Ethanol containing medium was prepared freshly before use, from 95% ethanol (Sigma), and ethanol concentrations in fresh and culture-exposed medium were measured for each experiment, by gas chromatography. Each flask was defined as a single sample. Culture medium was changed every 2 days. Control, and ethanol-treated flasks were capped tightly with phenolic caps, and sealed with parafilm

to limit the loss of ethanol, and ethanol concentrations in the culture medium remained stable through the course of the experiment. These measured doses range in equivalence to consumption levels that can be attained by social drinkers to those attained by chronic alcoholics (Perper et al., 1986; Adachi et al., 1991). All values reported on graphs and tables are averages of actual gas chromatography measures of ethanol content taken from six experimental samples per dosage condition.

Flow Cytometry

Measurement of surface antigen levels was conducted according to our previously published protocols (Santillano et al., 2005) on a flow-cytometer (FACSCalibur, Beckton Dickinson). Excitation wavelength was set at 488nm (argon laser) and emission spectra for fluorescein and propidium iodide were 525nm and 630 nm respectively. Analyses were generated using Cell Quest software for Macintosh, (V. 3.5).

Labeling for Surface Fas/Apo-1/CD95 Receptor Expression

Cells were harvested, washed twice in PBS and triturated to dissociate neurosphere structures into individual cells. Cells were then immediately fixed in 1% paraformaldehyde buffered in PBS for either 30 minutes or overnight at 4ºC. Subsequently cells were washed twice in PBS and 10^6 cells were labeled with a fluorescein-conjugated antibody against the surface Fas/Apo-1/CD95 antigen (Pharmingen #554257). An equal amount of fluorescein-conjugated isotype-matched IgG antibody was added to samples not receiving Fas/Apo-1/CD95 antibody to account for nonspecific staining. Fixed, unlabeled cells served to measure background fluorescence. The proportion of labeled cells was analyzed via flow cytometry from each sample ($N=10^4–10^5$ cells per sample).

Annexin-V Staining for Apoptosis Detection

Annexin-V labeling was conducted as per the Annexin-V-Fluorescein Staining Kit (Roche #1858777) protocol, and per our previously published protocols (Cheema et al., 1999). Briefly, 10^6 cells were washed in PBS, resuspended in labeling solution containing Annexin-V-Fluorescein and propidium iodide and incubated for 10–15 minutes at room temperature. The proportion of labeled cells was analyzed via flow cytometry from each sample ($N=10^4–10^5$ cells per sample).

ELISA for Apoptosis Detection

We utilized a photometric enzyme-immunoassay for the *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (Cell Death Detection ELISA^{PLUS} Roche #1774425). Briefly, 10^4 cells were incubated in lysis buffer for 30 minutes at room temperature. The lysate was centrifuged at 200× g for 10 minutes, and 20μL from the supernatant was added to a streptavidin coated microplate for analysis. This solution was incubated for 2 hours at room temperature with an antibody mixture of anti-histone-biotin (which binds the histone component of the nucleosomes and captures the immuno-complex via biotin to the coated microplate) and anti-DNA-peroxidase (which binds the DNA components of the nucleosomes and is conjugated to horseradish peroxidase to provide a color reaction). After incubation the plate is washed three times with incubation buffer and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) solution is added to provide a color reaction. The color product was measured by photometric analysis at a wavelength of 405nm, with a reference wavelength of 490nm. All samples were analyzed in triplicate, and in order to account for possible differences in the amounts of cells lysed per sample, all values were normalized to total protein levels.

Homogenous Caspase Assay

We utilized a fluorimetric assay for the quantification of activated caspases (Homogenous Caspase Assay, Roche #3005372). This assay is sensitive for caspases 2, 3, 6, 7, 8, 9, and 10. Briefly, 10^4 cells were simultaneously lysed and incubated in caspase substrate (DEVD-Rho110 [Asp-Glu-Val-Asp-Rhodamine 110]) at 37ºC for 2 hours. Activated caspases recognize and cleave at DEVD sites, and free R110 is determined fluorimetrically at 521nm. The developed fluorochrome is proportional to the concentration of activated caspases.

Fluorimetric Assay for Detection of Autophagic Vacuoles

The presence of autophagic vacuoles was detected with the fluorescent dye monodansylcadaverine (MDC, Sigma #30432) according to a previously published report (Biederbick et al., 1995). Cells were treated with ethanol for 72 hours, then harvested by centrifugation and resuspended in a .05mM solution of monodansylcadaverine dye in warm serum free mitogenic media (from 0.5M stock solution in DMSO), and incubated at 37ºC for 1 hour. Intracellular MDC fluorescence levels were measured by fluorescence photometry in a FLX-800 microplate reader (excitation wavelength 380nm, emission wavelength 525nm). An aliquot of cells was harvested and starved in warm Hank's Balanced Salt Solution (Invitrogen) for one hour to induce autophagy and serve as a positive control. All samples were analyzed in triplicate, and in order to account for possible differences in the amounts of cells lysed per sample, all values were normalized to total protein levels.

BCA Protein Assay

Protein concentrations of each lysate utilized for the Cell Death Detection ELISA and autophagy assay were obtained using a BCA Protein Assay Kit (Pierce #23225).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from ethanol-treated cultures using TRIZOL^{TM} reagent (Invitrogen #15596), followed by DNAse treatment and column purification using the SV Total RNA Isolation kit (Promega, #23100). The quality and quantity of RNA was assessed by micro-capillary electrophoresis on an Agilent 2100 Bioanalyzer. Five micrograms of total RNA per ethanol-treated sample was reverse transcribed with Superscript III (Invitrogen #18080-093), utilizing the manufacturer's protocol.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reactions were conducted on a Biorad MyIQ^{TM} ICycler Real-time machine using 96-well pcr plates (iCycler 96 well, Biorad #2239441). Reactions utilized iQ SYBR Green Supermix (Biorad #170-8880), and a starting primer concentration of 10μM. Standard curves were created for each primer set using various concentrations (ranging from 200ng/μL to 20ng/μL) of whole embryonic RNA from untreated GD 12.5 cultures. All target genes examined were compared to β-actin, a standard housekeeping reference gene. No-template controls were included for each primer set. We used a modification of the method outlined by M.W. Pfaffl (Pfaffl, 2001) to quantify gene expression (see Table 1 for primer characteristics), which takes into account the primer efficiency and the mean cycle threshold value for the control group as per the equation below.

$$\text{Ratio}=\frac{\text{Efficiency}_{\text{target}}\wedge(\text{Mean CT for control group of target gene}-\text{CT target gene})}{\text{Efficiency}_{\text{reference}}\wedge(\text{Mean CT for control group of reference gene}-\text{CT reference gene})}$$

CT = cycle threshold

target = gene of interest

reference gene = β-Actin

Data Analysis

Analysis of Variance (ANOVA), Multivariate Analysis of Variance (MANOVA) and post-hoc Fischer's Least Significant Difference (Fisher's LSD) tests were computed using a standard statistical package, SPSS (V. 11). Statistical significance was set at $p<0.05$. When necessary single outlying values greater than 3 standard deviations from the mean were eliminated from analysis.

RESULTS

Cortical Progenitors are Resistant to Ethanol Induced Apoptosis

In the present study, we sought to determine if immature neural progenitors, obtained from the second trimester equivalent period of gestation, were vulnerable to ethanol-induced apoptosis. We chose to assess levels of apoptosis after ethanol exposure by measuring Annexin-V staining (phosphatidylserine translocation to the outer leaflet of the cell membrane) by flow cytometry. Ethanol treatment did not result in a statistically significant difference in the percentage of cells gated in the apoptotic (Figure 1A, B, C) or in the necrotic fraction (Figure 2A, B, C) regardless of dosage or temporal exposure. However, the possibility remained that by measuring an early event in apoptosis, we failed to detect apoptosis that had already progressed to a later stage. Therefore, we measured DNA fragmentation (cytoplasmic oligonucleosomal fragments), a late event in the apoptotic cascade and one upon which a variety of caspase-dependent and independent pathways converge (Slagsvold et al., 2003; Chu et al., 2005; Culmsee et al., 2005). An overall ANOVA indicated a statistically significant interaction between ethanol concentration and time in culture ($p<0.027$). This interaction was mainly due to exposure to low and moderate doses of ethanol. Exposure to a low dose of ethanol (average ethanol concentration, 63mg/dL) led to a small decrease in DNA fragmentation as measured by the detection of (Figure 3, overall Fisher's LSD $p<0.004$). This was due to a statistically significant reduction in apoptosis for cells exposed to ethanol for 1 or 2 days ($p<0.027$ and 0.001 respectively), but not 5 days. The moderate dosage (average ethanol concentration, 167mg/dL) in contrast, resulted in a small but significant increase in DNA fragmentation (overall Fisher's LSD $p<0.002$). This was due to either a return to baseline control levels (at 1 day treatment) or a slight, but statistically significant increase in DNA fragmentation after 2 and 5-day exposures (all $p<0.05$). Overall, the highest dose (average ethanol concentration, 306mg/dL) did not result in a significant change in DNA fragmentation. In the aggregate, a modest 0.2-fold increase in apoptosis, as measured by DNA fragmentation, was observed only at the moderate ethanol dose. Caspases mediate both the extrinsic and intrinsic apoptotic pathways, and therefore we next assessed overall caspase activation in response to ethanol treatment as a measure of participation of either arm of the apoptotic program. We found that ethanol administration resulted in a dramatic and statistically significant (ANOVA, $p=0.013$) decrease in caspase activation that was observable even at the lowest dosage of ethanol (Figure 4).

Ethanol Suppresses Autophagy in Proliferating Cortical Neuroepithelial Precursors

Autophagy is classified as a type II cell death mechanism characterized by the engulfment of cytoplasmic contents and organelles into membrane-bound autophagic vesicles, which are targeted for destruction to lysosomes within the same cell (Clarke, 1990). Unlike apoptosis,

autophagic death does not result in pronounced DNA degradation (Gozuacik and Kimchi, 2004). Thus, we chose to examine whether or not ethanol might induce cell death via autophagy, using the uptake of monodansylcadaverine (MDC), a fluorescent dye that specifically marks the acidic autophagosomes/autolysosomes and not other endosomal structures (Biederbick et al., 1995). We exposed GD 12.5-derived neural progenitor cultures to ethanol for 72 hours and then measured the incorporation of MDC dye (Figure 5). Interestingly, we observed a statistically significant decrease in autophagic vacuole formation as indicated by a decrease in fluorescence after treatment with moderate and high doses of ethanol (ANOVA, p=0.021).

Ethanol Treatment Does Not Alter Surface Fas/Apo-1 CD95 Receptor Expression

The developing brain expresses and is sensitive to the Fas/Apo-1 suicide receptor (Cheema et al., 1999; Le-Niculescu et al., 1999; Cheema et al., 2000; Felderhoff-Mueser et al., 2000; Cheema et al., 2004). Even though ethanol did not induce substantial apoptosis, ethanol may increase sensitivity to apoptosis signals from the environment by up-regulating Fas/Apo-1 receptor expression. Neurosphere cultures were labeled with FITC-conjugated antibodies to the Fas/Apo-1 receptor and analyzed via flow cytometry. After subtraction of background fluorescence, cells expressing above 10^1 units of fluorescence were defined as Fas/Apo-1 expressing cells (Fas^{Total}, Figure 6A), and cells gated in the fraction above 10^2 as expressing a high level of the surface receptor (Fas^{Hi}, Figure 6A) as per our previously published protocols (Cheema et al., 2004). Ethanol exposure did not result in a change in the percentage of cells gated in the Fas^{Total} or Fas^{Hi} (Figure 6B and C) populations. However, we did observe an inherent heterogeneity of Fas/Apo-1 expression amongst cortical progenitors that was unrelated to ethanol exposure. By day 2, approximately 40–50% of cells sampled expressed some level of Fas/Apo-1 (Fas^{Total}), and of these 1–3% express a high amount of surface Fas/Apo-1 (Fas^{Hi}). Moreover, as the time spent in culture increases there is a pronounced and statistically significant (ANOVA, p= 0.001) decrease in overall expression levels (Figure 6B,C), so that expression was close to the limit of detection for control, low and moderate alcohol doses, and undetectable at the high dose.

The Expression of DISC-complex Genes Is Upregulated By Ethanol Exposure

We examined the expression of genes that code for proteins involved in the extrinsic apoptotic pathway, namely *Fadd* (Fas/Apo-1/CD95 associated adaptor protein), *Dedd* (Death effector domain-containing protein), *Madd* (Map-kinase activating death domain) and *Cradd*/*Raidd* (caspase and RIP adaptor with death domain). These specific adaptor proteins associate with ligand-bound receptor complexes, recruiting procaspases and other components to form the DISC complex (Alcivar et al., 2003; Peter and Krammer, 2003; Jabado et al., 2004; Wang et al., 2006). Multivariate analysis of variance (MANOVA) resulted in a Wilks' Lambda value of 0.034, indicating that overall a strong relationship exists between ethanol administration and expression of this group of death-receptor-associated genes. After two days of exposure to ethanol, we observed a general (though not statistically significant) upward dose-related trend in *Fadd* mRNA expression (Figure 7A). We did however observe a significant dose-related increase in the expression of *Dedd* (Figure 7B, * indicates ANOVA p=0.001) and *Cradd/Raidd* (Figure 7C, ** indicates ANOVA p=0.038) mRNA expression when compared to controls. *Madd* expression was virtually undetectable in our population, with control group cycle threshold (CT) values outside of the range of reliable detection (CT>37).

DISCUSSION

The ventricular neuroepithelium of the anterior neural tube expands rapidly during the second trimester-equivalent period of human gestation to generate telencephalic structures

like the cerebral cortex. Deletion of relatively few neural progenitors from the neuroepithelium during the critical developmental period of neuroepithelial proliferation is likely to lead to a disproportionate reduction in the growth of telencephalic structures. We isolated neuroepithelial cells from gestational day 12.5 mouse cerebral cortex to capture neuroepithelial progenitors that would give rise to the earliest (i.e., deepest) laminae of the developing cerebral cortical plate (Angevine and Sidman, 1961; Caviness, 1982; Takahashi et al., 1995) as well as progenitors that give rise to later-developing laminae, since evidence from several laboratories suggests that initial fate specification occurs within the ventricular zone itself (Donoghue and Rakic, 1999; Desai and McConnell, 2000).

At the start of the reported experiments, we hypothesized that ethanol would induce apoptosis in cortical progenitors similar to our observed induction of apoptosis in a more differentiated neural tissue (Cheema et al., 2000). We measured a variety of features characteristic of the apoptotic process, and our data show that cortical progenitors maintained as neurosphere cultures are remarkably resistant to ethanol-induced apoptosis, even at doses typically attained only by chronic alcoholics. Interestingly, total caspase activity was suppressed by ethanol. The lack of caspase activation, together with the fact that ethanol did not also induce necrosis, indicates that the alternate calpain pathway (Neumar et al., 2003) is also unlikely to be a target of ethanol. We therefore investigated the effect of ethanol on autophagy, a lysozome-mediated cellular mechanism that can result in programmed cell death. To the best of our knowledge this is the first study examining the effect of ethanol on autophagy, and surprisingly, we found that ethanol exposure significantly reduced the formation of autophagic lysozomes. The suppression of autophagy indicates that ethanol also did not lead to a significant destruction of cellular organelles. These experiments confirm initial studies in our laboratory demonstrating that ventricular zone progenitors isolated from a somewhat later developmental stage (i.e., the peak period of cortical plate neurogenesis) exhibited a similar resistance to apoptosis (Santillano et al., 2005).

Despite some evidence suggesting that ethanol can kill immature neuroblasts under specific circumstances (Kentroti and Vernadakis, 1991; Hao et al., 2003), ethanol-induced apoptosis may be a differentiation state-specific phenomenon. For example, the pro-apoptotic protein Bax, a necessary mediator of ethanol-induced apoptosis in differentiating neurons (Young et al., 2003), is not expressed in proliferating cortical neuroepithelial cells, and its expression is induced only following neuronal differentiation (Wade et al., 1999). The concept of developmental stages conferring either vulnerability or resistance to ethanol is certainly a documented phenomenon in the post-natal period, and in other brain regions such as the cerebellum (Thomas et al., 1998a; Heaton et al., 2003). Cerebellar granule cells for example, exhibit delayed apoptosis in response to ethanol exposure that follows a decline in their proliferation rate (Li et al., 2001), suggesting that cell cycle status may be linked to ethanol sensitivity. Given the inverse relationship between the expression of pro-apoptotic factors like p53 and cell cycle (Wade et al., 1999), along with our observations that ethanol induces cell cycle (Santillano et al., 2005), it is likely that the early proliferative stage of neurogenesis is a period of relative resistance to ethanol-induced apoptosis. It is also possible that sensitivity to ethanol-induced cell death is model system-dependent rather than differentiation state-dependent, i.e., that neural progenitors are more resistant to apoptosis simply because of the specific local milieu that emerges with an aggregate of cells maintained as neurospheres. However, the neurosphere model more closely mimics the natural, cell-dense environment of the second trimester neuroepithelium, consisting of high-density aggregates of stem and progenitor cells within ventricular and emerging sub-ventricular zones, than do more typically used, dissociated culture models. Nevertheless, apoptosis-resistance is not absolute, since recent work in our laboratory (unpublished

observations) indicates that disruption of c-kit signaling, or exit from cell cycle, does render progenitor cells vulnerable to ethanol-induced apoptosis.

Dis-regulation of the Fas/Apo-1 Receptor System

Our previous work showed that the Fas/Apo-1 is expressed in the developing cerebral cortex during the perinatal period, both by immature ventricular zone progenitors, and later, by maturing cortical plate neurons (Cheema et al., 1999). Additionally, our lab showed that ethanol induced a dose-dependent increase in Fas/Apo-1 mRNA in differentiated postnatal cortical explant cultures (Cheema et al., 2000). We subsequently found that high levels of cell-surface Fas/Apo-1 expression are associated with entry into G2 and M phases of the cell cycle (Cheema et al., 2004), suggesting that this suicide receptor may modulate progression through cell cycle checkpoints. Furthermore, the expression of Fas/Apo-1 is associated with the activation of cell cycle regulatory proteins like p53, further supporting a role for the Fas/Apo-1 pathway in cell cycle regulation. Cortical progenitors maintained in neurosphere cultures exhibit increased cell cycle activity in response to ethanol exposure (Santillano et al., 2005). We therefore hypothesized that if ethanol induced cell cycle activity, it would also increase the expression of the Fas/Apo-1 in proliferating neural progenitors. The present study demonstrates that neuroepithelial progenitors do exhibit a nascent heterogeneity with respect to cell surface Fas/Apo-1 expression. However, ethanol did not alter the numbers of cells expressing either moderate or high levels of surface Fas/Apo-1. Because cell-surface Fas/Apo-1 expression in embryonic cortical progenitors is tied to cell cycle and because ethanol induced cell cycle, our data may be interpreted to suggest that ethanol induces dissociation between cell cycle and sensitivity to receptor-mediated apoptosis. This dissociation may permit the survival of cell populations that would otherwise be eliminated. One prediction that arises from these data is that ethanol exposure may permit cells with defects in DNA replication to proceed through cell cycle checkpoints, leading to an increased accumulation of defective neural progenitors, and ultimately, the accumulation of aberrant neurons within the cortical plate. These neurons may not behave appropriately during development, and may contribute to lamination defects and the formation of heterotopias (Komatsu et al., 2001; Mooney et al., 2004).

Though ethanol did not alter cell-surface Fas/Apo-1 expression, multivariate analyses indicate that ethanol has a general dose-related inductive effect on the expression of mRNA transcripts for several DED (death-effector-domain)-containing genes that constitute part of the DISC (death-inducing signaling complex), the intracellular initiating component of the extrinsic apoptotic cascade. These data predict that while ethanol does not directly induce apoptosis in immature neuroepithelial cells, exposure to ethanol during the mitogenic period may render daughter neuroblasts susceptible to apoptotic stimuli as they differentiate into neurons.

Evidence for an Alternate Hypothesis of Ethanol-Induced Neuroepithelial Maturation

An alternate hypothesis, also collectively supported by our data is that ethanol promotes premature neuroepithelial differentiation. While autophagy is often associated with cell death, it also represents a major strategy for cellular adaptation. Autophagic degradation of long-lived proteins and organelles enables a cell to adapt to stressful conditions like amino acid starvation (Gozuacik and Kimchi, 2004). Autophagy is also associated with cellular differentiation. For example, undifferentiated colon adenocarcinoma cells express high levels of autophagic activity, and this activity is repressed following differentiation (Houri et al., 1995). Similarly, while Fas/Apo-1 was originally characterized as an obligatory cell-death receptor (Nagata and Goldstein, 1995), more recent evidence from a variety of laboratories including ours, have shown that this receptor also regulates cell cycle (Cheema et al., 2004) and differentiation (Cheema et al., 1999; Desbarats et al., 2003; Ceccatelli et al.,

2004; Tamm et al., 2004) signaling cascades. Additionally, DISC-complex adaptor proteins also exhibit non-apoptotic functions (Park et al., 2005). Consequently, in the absence of ethanol-induced apoptosis, the observed suppression of autophagy and induction of DISC mRNA transcripts are consistent with a model of ethanol-induced neuroepithelial maturation.

Other data from our group also support this 'premature differentiation' hypothesis. For example, we have shown that ethanol suppresses the expression of stem cell markers like Sca-1, ABCG2, CD117 and CD133 and induces asymmetric cell division (Santillano et al., 2005). Asymmetric division, resulting in two dissimilar daughter cells, promotes the emergence of neuronal lineage-committed precursors from the cortical ventricular zone that then proceed to populate the sub-ventricular zone (Noctor et al., 2004), before differentiating into cortical plate neurons. Collectively, we interpret these data to indicate that ethanol promotes stem-cell to blast-cell differentiation in embryonic cortical neuroepithelial-derived cells, and our current data are certainly compatible with that hypothesis.

Conclusion

Our data collectively support an unexpected and surprising conclusion---that ethanol is not obviously or immediately cytotoxic to cerebral cortical neuroepithelial-derived progenitors. Rather, the effects of ethanol appear to be more complex and pervasive. Ethanol appears to influence cortical neuroepithelial maturation programs, and collectively our data predict that episodes of maternal ethanol consumption during the period of fetal neuroepithelial proliferation are likely to have long-term consequences for subsequent neuronal differentiation. Furthermore, disrupting the timing of neuroepithelial maturation during the second trimester-equivalent period of brain development can lead to significant disorganization of the laminar pattern of the mature cortical plate, without the need to invoke cyto-toxicity as ethanol's mechanism of action.

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List of Abbreviations

Caspase	cysteine, aspartic-acid proteases
CRADD/RAIDD	caspase and RIP adaptor with death domain
DEDD	Death effector domain-containing protein
DISC	Death Inducing Signaling Complex
FADD	Fas/Apo-1/CD95 associated adaptor protein
Fas/Apo-1	Fas/Apo(Apoptosis)-1, CD95 suicide receptor
FASD	Fetal Alcohol Spectrum Disorder
FasL	Fas/Apo-1 ligand
GD	Gestational day
MADD	MAPk (Mitogen-activated protein kinase) activating death domain

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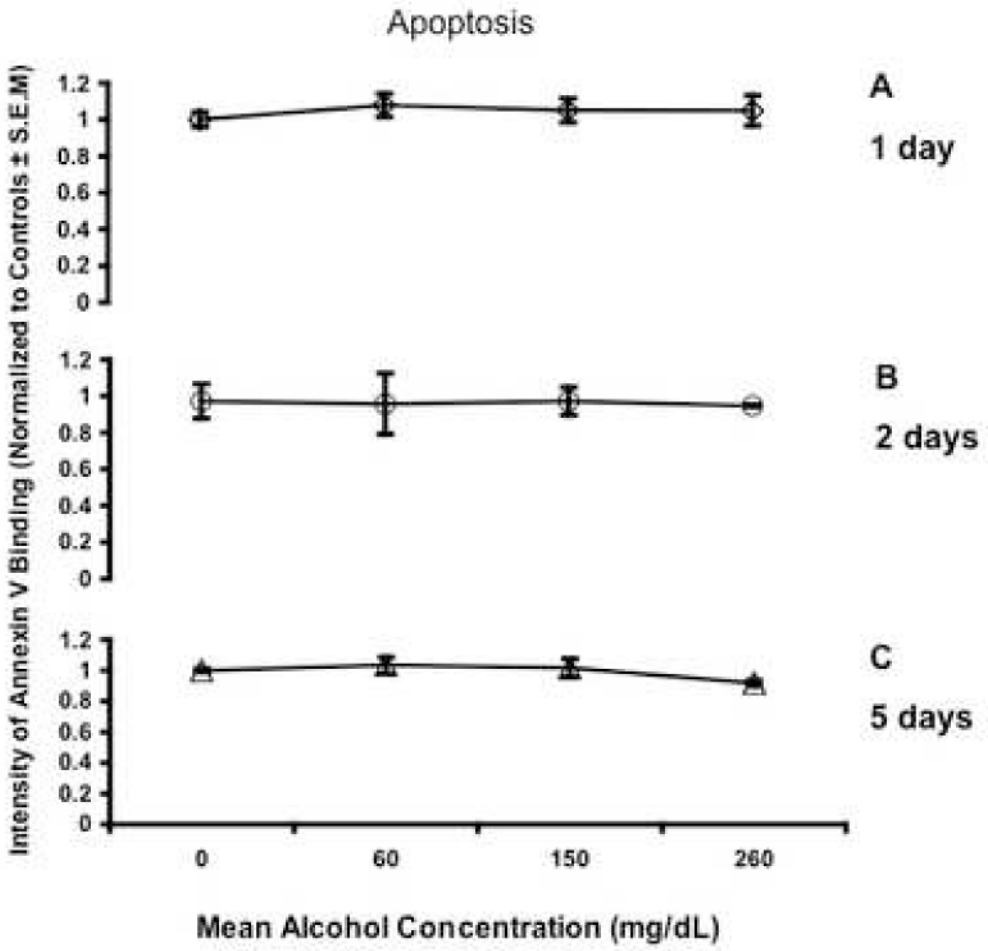

Fig. 1.

Line graphs depict intensity of Annexin V binding normalized to control cultures. There was no significant change in apoptosis, as detected by Annexin-V staining after a 1, 2 or 5 days of ethanol exposure. Apoptotic cells were defined as those that expressed Annexin-V but not propidium iodide binding. All error bars represent S.E.M.

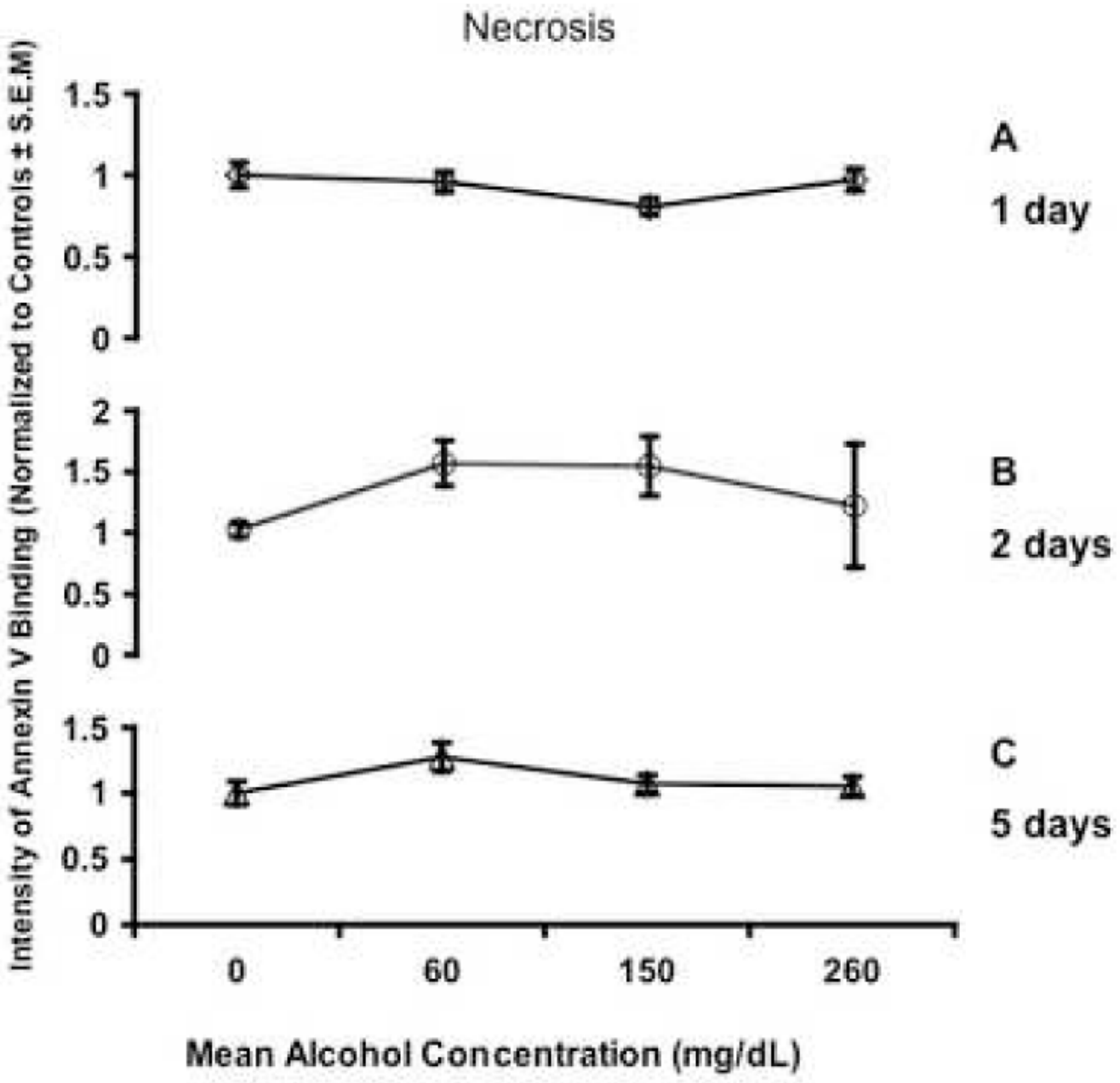

Fig. 2.

Line graphs depict intensity of Annexin V binding normalized to control cultures. There was no significant change in necrosis as detected by Annexin-V staining after a 1, 2, or 5 days of ethanol exposure. Necrotic cells were defined as expressing both Annexin-V and propidium iodide binding. All error bars represent S.E.M.

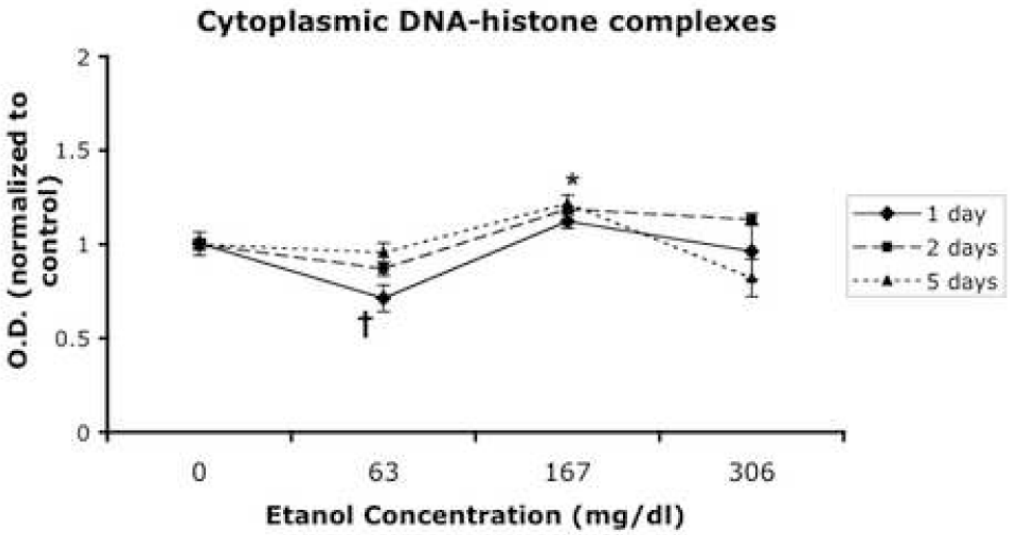

Fig. 3.

DNA fragmentation as measured by enzyme-linked immunosorbent assay (ELISA) observed after a 1, 2, and 5 days exposure. The y axis represents optical density normalized to controls. The † symbol indicates an overall statistically significant suppression compared to controls (LSD post-hoc $p<0.004$) at the low dose of ethanol, while * indicates an overall statistically significant induction compared to controls (LSD post-hoc $p<0.002$) at the moderate ethanol dose. The high ethanol dose was not statistically different from controls (LSD post-hoc $p<0.581$). All error bars represent S.E.M.

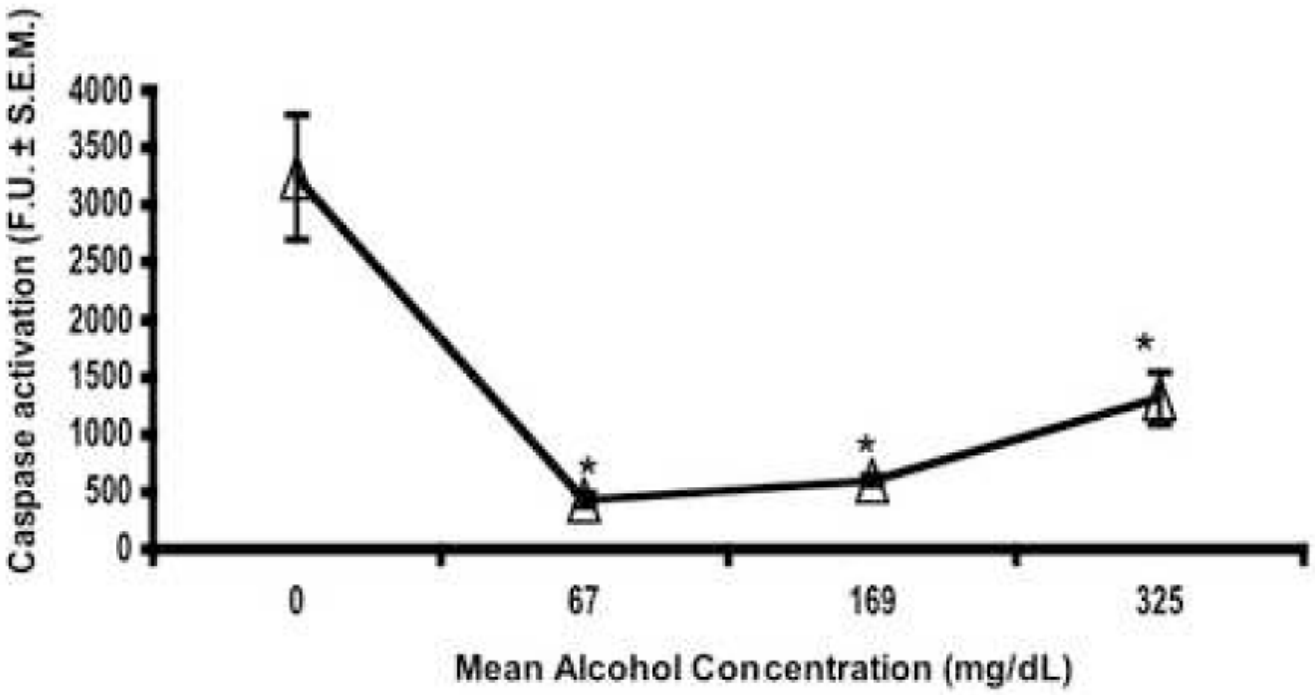

Fig. 4.

Ethanol decreased total caspase activation as measured by a fluorometric enzyme assay. F.U. indicates fluorescence units normalized to total protein. * indicates a significant difference when compared to controls (ANOVA $p<0.013$). All error bars represent S.E.M.

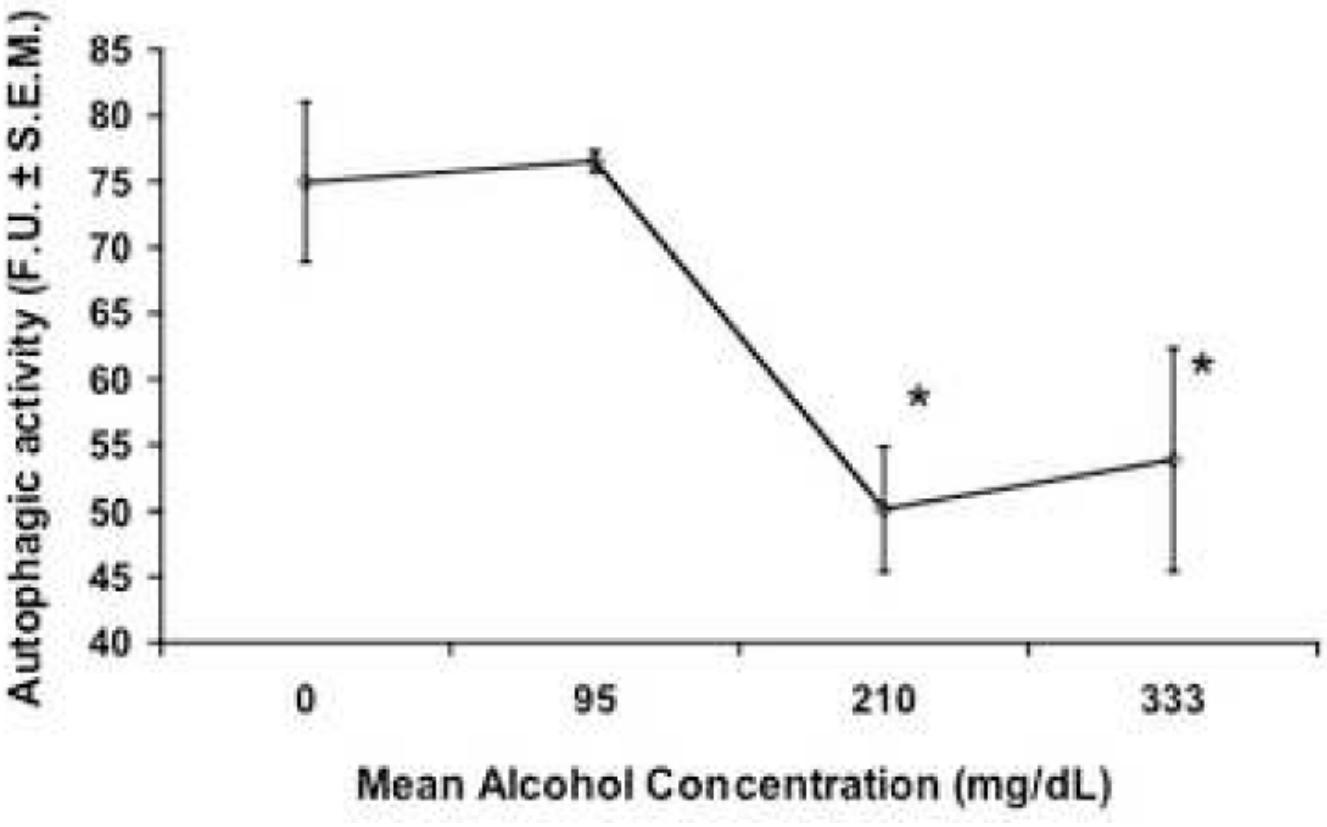

Fig. 5.

Ethanol decreased autophagy, as measured by MDC (monodansylcadaverine) fluorescent dye incorporation, after a 3 day exposure. F.U. is fluorescence units normalized to total protein. * indicates a significant difference when compared to controls (ANOVA $p<0.021$) All error bars represent S.E.M.

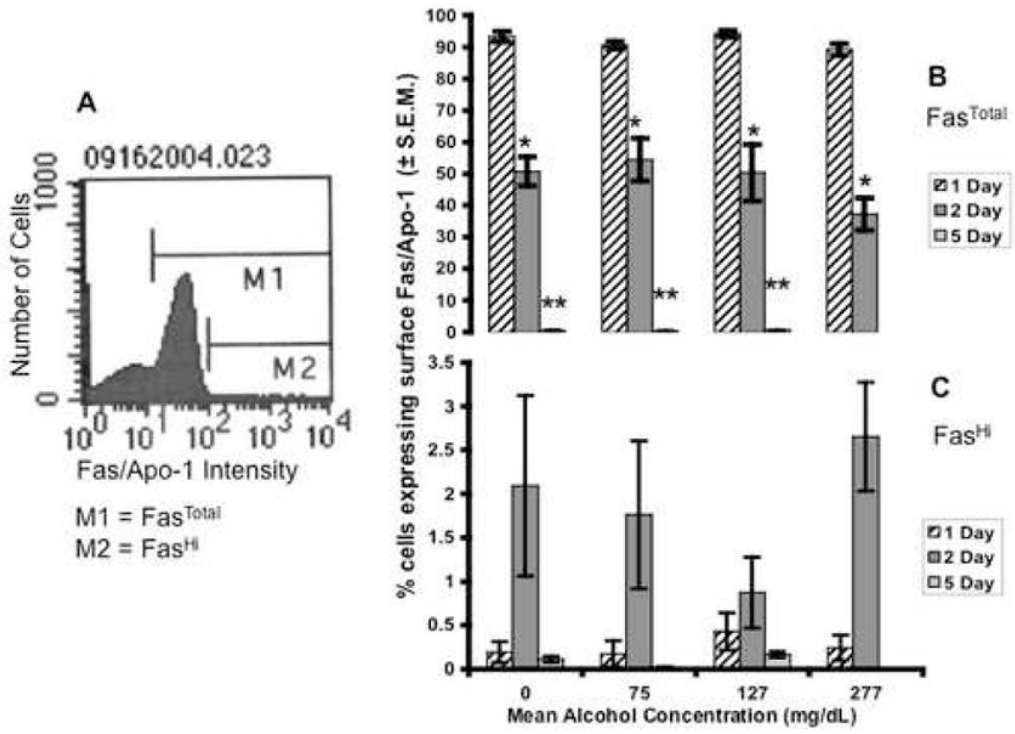

Fig. 6.

(A) Flow cytometry frequency histogram depicting the number of cells (Y axis) expressing cell-surface immunofluorescence for the Fas/Apo-1 suicide receptor (X-axis). M1 denotes all cells expressing cell-surface Fas/Apo-1 above isotype background control (Fas^{Total}). M2 denotes cells expressing high levels of Fas/Apo-1 on their cell surface (Fas^{Hi}). (B) Ethanol did not significantly change Fas^{Total} at either 1, 2 or 5 days of exposure. A decrease in surface Fas receptor expression was observed across time, * indicates significance with respect to 1 day of ethanol, ** indicates significance with respect to 2 days of ethanol (ANOVA, $p<0.001$ for both). (B) Ethanol did not change the proportion of cells in the Fas^{Hi} population, although at this developmental stage cortical progenitors maintained *in vitro* for 2 days have a higher basal level of Fas expression. All error bars represent S.E.M.

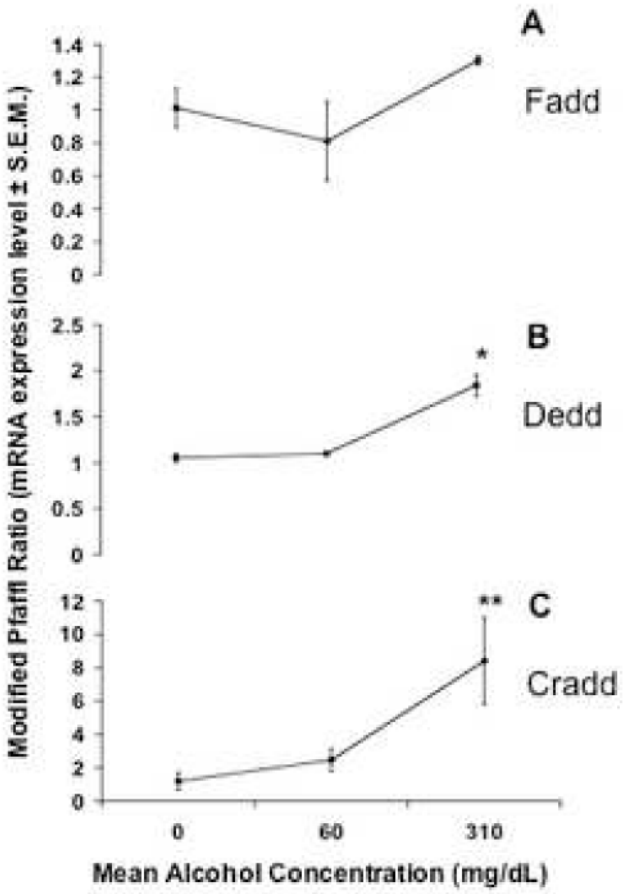

Fig. 7.

mRNA expression levels for death-receptor-associated genes *Fadd*, *Dedd* and *Cradd* after exposure to ethanol for 48 hours. The y axis represents mRNA expression levels as a modified Pfaffl ratio (see Methods). * indicates significant difference (ANOVA, p=0.000) for *Dedd*, ** indicates significant difference for *Cradd* (ANOVA, p=0.038) when compared to controls. All error bars represent S.E.M.

Table 1

PCR Primers

Name	Accession #	E%	C.C.	Forward Primer Sequence (5′--3′)	Reverse Primer Sequence (5′--3′)
Dedd	**NM_011615**	102	.99	ATC TGG AGG AAA CAT CAA TTC G	GCA GCA CAC CAC AGG ATA G
Fadd	**NM_010175**	90	.97	CGC CGA CAC GAT CTA CTG	TTC TTC TTC TCA GCA TTC TTC C
Madd	**BC003255**	131	.99	TTC AAC TCT GCT AAC G	GGC CTT GTC ACC AAT AAG G
Cradd	**NM_009950**	167	.98	CTT TAT GGT ACA GGT TCC C	TGT CCA GCA ACA GCA TTG TC
β-Actin	**XO3672**	84	.99	CAT CCG TAA AGA CCT CTA TGC	ACT CCT GCT TGC TGA TCC

E%= Primer Efficiency (%)

C.C.= Correlation Coefficient for Primer Amplification