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Brief communication

Morphologic and neurotoxic effects of ethanol vary with timing of exposure in vitro

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Abstract

Results of investigations with animal models of fetal alcohol syndrome (FAS) seem to indicate that neuronal vulnerability to ethanol-induced cell death may be correlated with specific developmental events. In the present study, we sought to test this observation in a cell culture model of neuronal development in which morphogenesis as well as survival could be assessed. Using embryonic rat hippocampal pyramidal neurons in primary cultures, we compared the sensitivity of neurons to ethanol added, at 400 mg/dl, to the medium at different times relative to the development of axons and dendrites. Quantitative morphometric analysis was performed by using phase contrast at 12 h (0.5 day) and 24 h (1 day), or fluorescence microscopy after microtubule-associated protein-2 (MAP2) immunostaining at 6 and 14 days. Survival was assessed by counting the number of neurons per unit area of the substrate at 14 days. Addition of ethanol 1 day after plating, when most neurons had developed an axon, had no effect on survival up to 14 days in vitro, but resulted in significantly shorter, less branched dendrites than observed when ethanol was added 2 h after plating. Despite the shorter duration of ethanol exposure, the addition of ethanol on day 6, after rapid growth of dendrites and synapses had begun, resulted in loss of all but about one third of the neurons by 14 days. This supports the suggestion that increased neuronal vulnerability to the morphoregulatory effects of ethanol is correlated with the establishment of polarity, but that the sensitivity of neurons to the cytotoxic effects of ethanol occurs later, when dendrites and synapses are rapidly forming.

Keywords: Hippocampus; Neuron development; Fetal alcohol syndrome; Dendrite; Alcohol; In vitro

1. Introduction

Neuronal differentiation is a complex phenomenon characterized by the outgrowth and molecular specialization of axons and dendrites, the timing of which is critical for the formation of normal neural circuitry. Key neuropathologic features of fetal alcohol syndrome (FAS) include reduced numbers of neurons and abnormal neuronal form and synaptic connectivity in the hippocampus and other brain regions [reviewed in Berman & Hannigan (2000); Pentney & Miller (1992); Stratton et al. (1996)]. However, not all neurons are equally susceptible to ethanol-induced damage, even within the same brain region. Results of several studies with rodent models of FAS support the long-held idea that there are transient periods during postmitotic development of particular neuronal populations when they may be especially sensitive to the cytotoxic or proapoptotic effects of ethanol. For example, Bonthius and West (1990) showed that Purkinje cells of the developing rat cerebellum that were most vulnerable to ethanol-induced loss were the most mature at the time of ethanol exposure. In contrast, cerebellar granule cells were relatively immature when ethanol exposure reduced their numbers (Pierce et al., 1989). Although there have been numerous studies whose findings have confirmed that ethanol is particularly damaging to proliferating neural cells [reviewed in Luo & Miller (1998)], relatively few studies have explored the determinants of postmitotic neuronal susceptibility to ethanol-induced death. In a recent study, Ikonomidou et al. (2000) showed that neurons from various brain regions of the developing rat may be more vulnerable to ethanol-induced damage at a stage of rapid synaptogenesis than neurons at earlier or later stages of development.

The possibility that there are similar "windows of vulnerability" for the effects of ethanol on the morphologic development of neurons has not been explored, but it seems likely in view of the differential expression of signaling systems and receptors involved in axonal and dendritic outgrowth and their sensitivity to disruption by ethanol. For example, in addition to their involvement in excitotoxicity, *N*-methyl-D-aspartate (NMDA) receptors influence neurite

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extension and branching in various neuronal cell cultures (Brewer & Cotman, 1989; Pearce et al., 1987), and ethanol inhibits NMDA-activated Ca^{2+} currents (Hoffman et al., 1989; Lovinger et al., 1989).

In vitro models of this stage-specific vulnerability of developing neurons to ethanol are needed to facilitate investigations focused on the mechanisms underlying these effects by overcoming some of the difficulties of cellular analyses in the complex milieu of the intact developing brain. To model the effects of ethanol on neuronal development in our laboratory, we use low-density cultures of fetal rat hippocampal pyramidal neurons in which the morphogenesis of axons and dendrites of individual neurons can be readily examined. Like their counterparts in vivo, neurons in these cultures develop axons and dendrites in a stereotyped sequence of events and form synapses with one another (Goslin et al., 1998). Neurons attach to the substrate within 1–2 h after plating. Within about 12 h, most of the neurons develop several short neurites lacking definitive axonal or dendritic characteristics, and by about 24 h one process extends rapidly and becomes the axon of the cell. Several days later, the remaining short neurites elongate and acquire the taper and molecular characteristics of dendrites, and synapses rapidly form at axodendritic and axosomatic contacts (Dotti et al., 1988; Fletcher et al., 1991).

We have previously shown that continuous exposure to ethanol at 200–400 mg/dl, in medium, beginning about 2 h after plating, differentially affects the development of axons and dendrites of pyramidal neurons in these cultures, increasing the proportion of neurons with axons 1 day after plating and inhibiting the growth of dendrites, without affecting their survival for up to 6 days (Clamp & Lindsley, 1998; Yanni & Lindsley, 2000). In the current study, we investigated whether the timing of ethanol exposure, relative to the development of axons and dendrites, would alter the effects of ethanol on axonal or dendritic outgrowth or on neuronal survival in this model system of neuronal development.

2. Materials and methods

2.1. Preparation of neuronal cultures

Hippocampal pyramidal neuron cultures were prepared from hippocampi of fetal Sprague–Dawley rats at gestational day 19, essentially as described by Goslin et al. (1998). Briefly, a pregnant dam was deeply anesthetized with halothane, and the uterine horns were removed by using a protocol approved by the Institutional Animal Care and Use Committee of the Animal Resources Facility of Albany Medical College. The Animal Resources Facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Fetuses were removed and decapitated. The hippocampi were dissected from the cerebral hemispheres and dissociated in 0.25% trypsin for 15 min at 37°C, triturated, and plated at 2,600 $cells/cm^2$ on poly-D-lysine–coated glass coverslips in minimum essential medium (MEM) with 10% heat-inactivated horse serum. After 2 h, neurons adherent to the coverslips were transferred into dishes containing a monolayer of rat cortical astrocytes in serum-free MEM with N2 supplements, 0.1% ovalbumin and 0.1 mM pyruvate, and oriented so that they faced the glia but did not contact them. Some dishes of astrocytes received ethanol just before transfer of the coverslips (see below). Cytosine arabinoside (ara-C, 5×10^{-6} M) was added 2 days after plating the neurons to inhibit the proliferation of nonneuronal cells on the coverslips, which otherwise interfere with morphologic analysis. Ara-C is not toxic to the nearly confluent monolayer of astrocytes, which proliferate very slowly, nor to the postmitotic neurons added at plating, both of which survive well for up to 4 weeks under control conditions after the addition of ara-C to the medium. Results of a pilot study, conducted to determine whether ara-C altered neuronal response to ethanol, showed no differences in the effect of ethanol, added 6 days after plating, on neuron survival at 14 days in cultures with ara-C compared with findings for cultures without ara-C. However, a full analysis of the effect of ara-C on neuronal development in medium containing ethanol has not been performed.

2.2. Ethanol treatments

Absolute *USP* ethanol was added directly to the medium in dishes containing neurons and astroglia at the time of transferring coverslips (0 days), or at 12 h (0.5 days), 24 h (1 day), or 6 days after plating, to achieve a final concentration of 400 mg/dl. This concentration of ethanol was used because it is commonly achieved in the blood of pregnant, alcohol-dependent women and their fetuses (Deitrich & Harris, 1996), and our previous study results have shown this concentration of ethanol alters axonal and dendritic growth in these cultures when added within 2 h of plating the neurons onto coverslips (Clamp & Lindsley, 1998; Yanni & Lindsley, 2000). Controls received no ethanol. Control and ethanol-treated cultures were maintained in modular incubator chambers at 37°C in 5% CO_2 saturated with water or water/ethanol at 400 mg/dl, which we have shown attenuates the evaporation of ethanol from the medium over time (Yanni & Lindsley, 2000).

2.3. Quantitative morphometric analysis of neurons 0.5 and 1 day after plating

For morphometric analysis of early developmental stages with the use of phase contrast microscopy, neurons were fixed either 0.5 or 1 day after plating on the coverslips for 15 min at 37°C with 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.12 M sucrose; rinsed in PBS; and mounted on slides in Aquamount (Shandon Lipshaw, Pittsburgh, PA). Coverslips were coded and subsequent analyses were performed blind to treatment. Cells for analysis were randomly selected by using a predetermined sampling pattern, and nonpyramidal neurons and neurons whose processes intermingled with those of neighboring cells were excluded. Pyramidal neurons account for about 90%–94% of the cells on the coverslips, the rest being nonpyramidal neurons and nonneuronal cells (Goslin et al., 1998). The criteria we used to distinguish pyramidal neurons from nonpyramidal neurons and nonneuronal cells have been previously described in detail (Clamp & Lindsley, 1998; Dotti et al., 1988) and can be summarized as follows. Pyramidal neurons had a phase-dark, oval or pyramidal cell body with a diameter of 15–20 μm, numerous vacuoles, and one or two prominent nucleoli. Nonpyramidal neurons had spindle-shaped cell bodies of 8–10 μm in diameter and two or three short, thick processes. Nonneuronal cells were flattened, had poor phase contrast, and lacked distinct processes. The following morphometric parameters were measured at 0.5 and 1 day in each treatment group: (1) number of minor processes per cell, (2) number of axons per cell, and (3) proportion of neurons in each stage of development (stages 1–3). As defined previously (Dotti et al., 1988), stage 1 pyramidal neurons had lamellipodia encircling the cell body and no processes. Stage 2 was defined by the presence of at least one minor process (typically about 15–20 μm long), with or without lamellipodia, and the absence of any process that exceeded 40 μm (the length equivalent of one grid unit superimposed on the field by a reticle in the eyepiece). Stage 3 was defined by the presence of at least one process that could be identified unequivocally as an axon by its length being ≥ 40 μm.

2.4. Quantitative morphometric analysis of microtubule-associated protein-2 (MAP2)–immunostained neurons 6 and 14 days after plating

For morphometric analysis at later stages of development, neurons were fixed 6 or 14 days after plating (as described above), subsequently permeabilized in 0.3% Triton X-100 for 4 min at room temperature, and, finally, rinsed in PBS. Cells were incubated with 5% fetal bovine serum and 5% normal goat serum in PBS (blocking solution) for 1 h at 37°C, followed by AP20, a mouse monoclonal anti-MAP2 antibody (1:250, Cat. No. MAB3418, Chemicon International, Temecula, CA) diluted in blocking solution, overnight at 4°C. After incubation with the primary antibody, the cells were rinsed in PBS, and an Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) was used to prevent nonspecific binding of Biotin-Avidin System reagents to biotin present in the blocking solution. The cells were incubated with Avidin D solution for 15 min at room temperature, rinsed with PBS, incubated with Biotin solution for 15 min at room temperature, and rinsed again with PBS. The cells were subsequently incubated with biotin-SP-conjugated affinity-purified goat anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) in 3% bovine serum albumin for 2 h at room temperature. After rinsing with PBS, the cells were incubated with rhodamine-conjugated Avidin D (1:200; Vector laboratories) for 30 min at room temperature, rinsed in PBS, and mounted on slides in Aquamount (Shandon Lipshaw, Pittsburgh, PA) containing 2.5% DABCO. Morphometric analysis was performed with the use of Image-Pro Plus software (MediaCybernetics, Des Moines, IA) and digital images of MAP2 immunofluorescent neurons.

Coverslips were coded so that the investigator was blind to the treatment group during all subsequent analyses. Between 60 and 70 cells per treatment group were selected at random for analysis by using a predetermined sampling pattern, and a digital image of the MAP2 fluorescence of each cell was captured with a CCD camera attached to the microscope. Nonpyramidal neurons and cells whose processes intermingled with those of neighboring cells were excluded. As previously described in detail (Benson et al., 1994; Goslin et al., 1998), pyramidal neurons at this stage of development have a phase-dark, oval or pyramidal cell body with a diameter of 15–20 μm, and between one and seven tapering dendrites. Nonpyramidal GABAergic neurons, which account for fewer than 7% of cells in the cultures, were also excluded from the analysis. These cells were readily identified by their 8- to 10-μm diameter, fusiform-shaped cell bodies, and two or three nonspiny dendrites. The very few nonneuronal cells in these cultures were flattened, had poor phase contrast, and lacked distinct processes.

From the MAP2 immunostaining we determined (1) length of each dendrite (including the length of any branches originating from it), (2) number of dendrites per cell, and (3) number of branches per cell. A dendrite was defined as a tapering, MAP2-stained process that was greater than 20 μm in length. This length criterion was chosen because axon hillocks and minor processes, which are generally less than 20 μm in length (Dotti et al., 1988; Goslin & Banker, 1989), can stain lightly with anti-MAP2. Dendritic length was measured from its emergence at the cell body to the tip of each dendritic segment, without retracing any portion, by using the freehand line tool in Image-Pro Plus software (MediaCybernetics, Des Moines, IA). The total length of the dendritic arbor per cell was also calculated as the sum of the cell's dendrite lengths.

2.5. Analysis of neuron survival

Because fewer than 1% of the neurons in these cultures divide (Dotti et al., 1988), changes in neuronal number over time are indicative only of cell loss, which is minimal under control conditions for up to 4 weeks. Therefore, the survival of neurons at 14 days in each treatment group was determined by comparing the mean number of neurons per unit area of substrate. Cells on coverslips from each treatment group were fixed on day 14, mounted on slides, and coded as described above. Neurons were visualized by phase contrast and counted in 20 microscope fields at predetermined stage coordinates on each of three coverslips per treatment. Field boundaries were defined by a grid superimposed on the image by an eyepiece reticle. Only pyramidal neurons whose cell bodies were entirely located within the grid were counted. Pyramidal neurons were distinguished from nonpyramidal neurons and nonneuronal cells as described above. Most dead or dying cells float off the coverslips into the medium or during fixation. The few that remained were readily identified by their pyknotic somata and varicose processes and were not counted.

2.6. Statistical analyses

Three separate experiments were performed and showed comparable results. All data were normally distributed. Data on cell survival, as well as comparisons between morphometric parameters in 6- and 14-day-old neurons in each treatment group, were analyzed by using a one-way analysis of variance (ANOVA) and Scheffé post hoc test. The chi-square test was used to compare the proportions of neurons in each of the three stages of early development at 0.5 and 1 day.

3. Results

3.1. Neuron survival at 14 days

To determine the effect of varying the timing and duration of ethanol exposure during development on the survival of neurons in culture, we compared the number of neurons per field on coverslips at 14 days after the addition of ethanol, at 400 mg/dl, at the times shown in Fig. 1. Continuous exposure to ethanol at 400 mg/dl, beginning 2 h after plating, had no significant effect on the mean number of neurons per field at 14 days compared with findings for control cultures without ethanol. This confirms our observation that this treatment is not overtly cytotoxic for up to 6 days (Yanni & Lindsley, 2000) and extends this observation to include continued development under these conditions for up to 2 weeks. Delay of the addition of ethanol—until either 0.5 days after plating, when most neurons have extended three to five short, undifferentiated neurites, or 1 day after plating, when most neurons have extended an axon—also had no effect on neuron survival. However, delay of the addition of ethanol to the cultures until 6 days after plating, when most neurons had developed dendrites and synapses, significantly decreased the number of neurons surviving at 14 days when compared with findings for controls or for addition of ethanol at the earlier time points. Only about one quarter of the number of neurons in control cultures were still present at 14 days after the addition of ethanol beginning at 6 days. The overall appearance of surviving neurons supported the suggestion that they were unhealthy, with numerous varicosities along their axons and irregularly shaped cell bodies (data not shown).

3.2. Effect of delayed addition of ethanol on early events in neuronal development

To determine whether the timing of ethanol exposure relative to initial outgrowth of the axon, which establishes polarity of the neuron, influenced the effect of ethanol on progress of the neurons through early stages of process outgrowth, we compared the morphologic characteristics of neurons 1 day after plating in control cultures with cultures to which ethanol was added either shortly after plating, before the emergence of the first neuritic processes, or after 0.5 days, when most of the neurons have extended three to five neurites but before elongation of an axon. As previously observed (Clamp & Lindsley, 1998), the addition of ethanol beginning soon after plating resulted in a significant increase in the proportion of neurons with axons at 1 day, and delay of the addition of ethanol until 0.5 days did not significantly alter this effect (data not shown). This seems to indicate that the effects of ethanol on these early events in the establishment of polarity can occur regardless of whether the neuron has initiated process outgrowth when it is first exposed to ethanol.

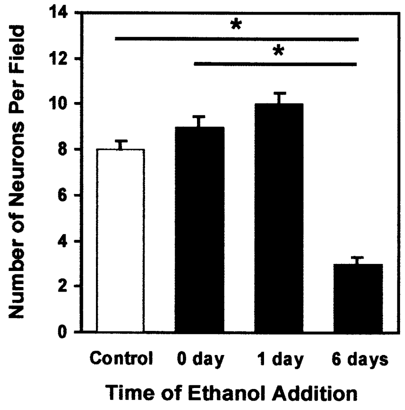

Fig. 1. Effect of timing of ethanol addition to medium of dissociated E19 rat hippocampal cultures on the survival of pyramidal neurons, as indicated by number per field at 14 days. Exposure to ethanol at 400 mg/dl, beginning either 2 h after plating (0 day) or 24 h later (1 day), did not significantly affect pyramidal neuron survival. However, delay of the addition of ethanol until 6 days after plating resulted in significantly fewer pyramidal neurons per field at 14 days, compared with findings for control cultures and compared with findings with addition of ethanol 2 h after plating. Data from one of three experiments with comparable results are presented as the mean and S.E.M. ($^*P < .001$, $n = 60$ fields per treatment.)

3.3. Effect of delayed addition of ethanol on dendritic development

Morphologic analyses were performed on 6-day-old neurons in each treatment group (Fig. 2). In agreement with our previous observations, the addition of ethanol at 400 mg/dl, 2 h after plating, significantly decreased the total dendrite length per cell at 6 days (Yanni & Lindsley, 2000). However, delay of the addition of ethanol until 1 day after plating, when cells have formed an axon and several dendritic precursor processes, resulted in significantly greater inhibition of total dendrite length per cell, despite the 1 day shorter duration of exposure to ethanol (Fig. 2A). Similarly, the number of dendritic branch points per cell at 6 days was decreased by the addition of ethanol at 400 mg/dl, 2 h after plating, and delay of the addition of ethanol until 1 day after plating resulted in significantly fewer dendritic branches (Fig. 2B). The number of dendrites per cell at 6 days was

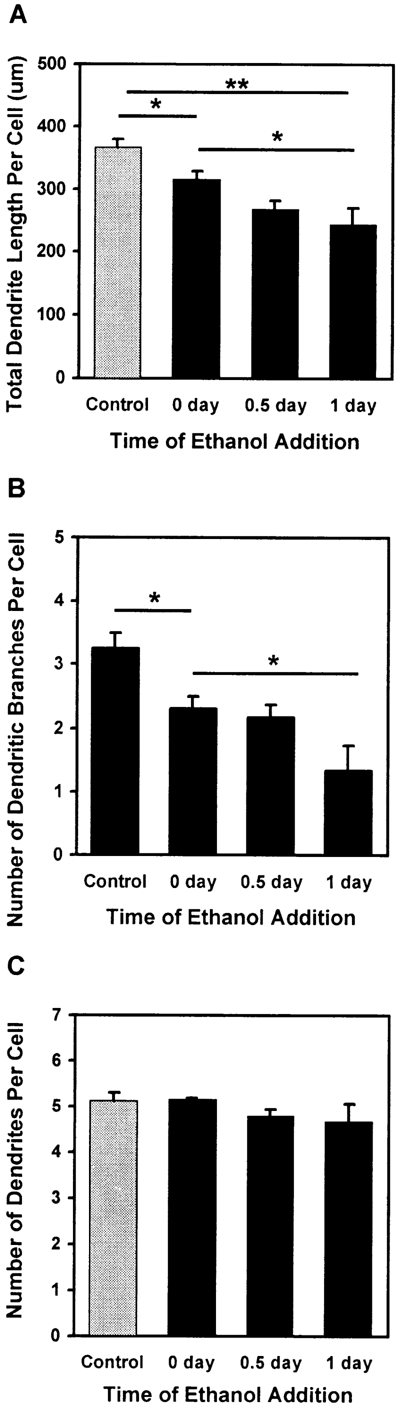

Fig. 2. Quantitative morphometric data on the dendrites of 6-day-old hippocampal pyramidal neurons that were maintained in control medium or exposed to ethanol at 400 mg/dl, beginning 2 h (0 day), 12 h (0.5 days), or 24 h (1 day) after plating. Dendrites were visualized by microtubule-associated protein-2 (MAP2) immunofluorescent staining and measured by using the free-hand line tool of Image-Pro Plus software (MediaCybernetics, Des Moines, IA). Total dendrite length per cell (A) and number of dendritic branches per cell (B) were significantly decreased by the addition of ethanol to the medium beginning 2 h after plating. Both were decreased significantly more by delay of the addition of ethanol until 1 day after plating. Ethanol had no effect on number of dendrites per cell, regardless of the timing of its addition to the medium (C). Data from one of three experiments with comparable results are presented as the mean and S.E.M. (*$P < .01$, **$P < .001$, $n = 70$ cells per treatment.)

not significantly different from the number in control cultures in which ethanol was added at 400 mg/dl just 2 h after plating, or after 0.5 or 1 day (Fig. 2C). Similar results were observed at 14 days. However, the extensive overlapping of dendrites in the cultures at this age in culture limited the sample numbers under all conditions except the one in which ethanol was added at 6 days, in which poor survival at 14 days prevented analysis.

These results confirmed our previous finding that ethanol at 400 mg/dl, added soon after plating before initial process outgrowth, inhibits the development of hippocampal pyramidal neuron dendrites observed at 6 days, without affecting survival (Clamp & Lindsley, 1998) and extended these findings by showing that dendritic branching is also decreased under these conditions. The effects of ethanol added soon after plating, or at 0.5 or 1 day after plating, on the morphologic characteristics of later-developing dendrites could not be accounted for by a selective loss of a subpopulation of larger neurons because there was no decrease in neuron survival for up to 14 days under any of these conditions. Taken together, these results support the suggestion that the dendrites of neurons exposed to ethanol after early events in process outgrowth have occurred, but before the elongation of dendritic precursors has begun, are more severely inhibited in their subsequent development in the presence of ethanol than when ethanol is present beginning before initial stages of process development.

4. Discussion

It has long been supposed that there is a relation between severity of ethanol-induced brain damage and the timing of exposure (Bauer-Moffett & Altman, 1977), but the molecular mechanisms underlying this heightened sensitivity have yet to be fully clarified. The results of the current study are the first to show that the timing of ethanol exposure relative to neuronal developmental events influences the effects of ethanol on morphologic development, as well as its cytotoxic effects. This observation, that "windows of vulnerability" to the effects of ethanol on both dendritic development and neuronal survival are different, seems to indicate that multiple mechanisms may be involved.

The sensitivity of rat pheochromocytoma cells (PC12) in vitro to the neurotoxic effects of ethanol differs depending on whether the cells are undifferentiated or differentiated by nerve growth factor (NGF) (Oberdoerster & Rabin, 1999). The novel finding of the current study was that increased neuronal vulnerability to the effects of ethanol on dendritic development was correlated with exposure beginning when neurons first became polarized by the outgrowth of a definitive axon compared with effects after exposure at earlier or later stages of development. The mechanisms by which ethanol exposure at this particular time may more severely inhibit subsequent growth and branching of dendrites are unknown, but they may involve developmentally regulated expression, function of certain receptors, or both. Calcium signaling is well known to communicate growth-related signals to cytoskeletal and vesicular apparatus responsible for controlling axon and dendrite morphogenesis (Clapham, 1995; Neely & Nicholls, 1995), and ethanol can alter voltage-dependent Ca^{2+} channel expression and function in neural cells (Bergamaschi et al., 1993; Leslie et al., 1990). Moreover, the effects of ethanol on L- versus N-type Ca^{2+} channels may not be constant during neuronal differentiation (Bergamaschi et al., 1995), supporting the suggestion of a mechanism for differential sensitivity to these effects during neuronal development.

In contrast to the vulnerability of neurons to the effects of ethanol on dendritic development, which correlated with establishment of polarity, their susceptibility to the cytotoxic effects of ethanol was correlated with when dendrites and synapses are forming rapidly. Although we did not attempt to determine whether neurons died from excitotoxic or apoptotic mechanisms in these studies, both processes have been implicated in developing neural cells exposed to ethanol (Cheema et al., 2000; McAlhany et al., 2000). A correlation between the period of rapid synaptogenesis and sensitivity to ethanol-induced neuronal damage has been reported in vivo in the mammalian brain (Ikonomidou et al., 2000). The findings of the current study are qualitatively consistent with this correlation, even though the relative number of cells lost was greater in our cultures. The unique advantages of this culture model of pyramidal neuron development make it a potentially useful system for directly testing certain hypotheses regarding the molecular basis for this stage-specific vulnerability to ethanol-induced damage. In particular, it is well established that numerous axonal and dendritic membrane proteins, including various glutamate and $GABA_A$ receptors and voltage-dependent calcium channels, are appropriately compartmentalized in neurons in these cultures [reviewed in Craig & Banker (1994)], and the timing of their expression and distribution has been described (Craig et al., 1993; Killisch et al., 1991; Shitaka et al., 1996; Verderio et al., 1994).

The correlation between ethanol-induced cell death and the developmental period of rapid synaptogenesis is in contrast with other reports that vulnerability of postmitotic neurons to cell depletion by ethanol in vitro is highest when they are relatively immature. For example, the number of postnatal rat cerebellar granule cells in primary culture is depleted if they are exposed to ethanol soon after plating, but not if ethanol is added at later times in culture (Pantazis et al., 1993). There are key differences between cerebellar granule cell cultures and hippocampal neuron cultures, other than the obvious fact that they are derived from distinctly different brain regions. Some of these differences may influence when neurons developing in these cultures are most sensitive to the cell-killing effects of ethanol. For example, in cultures of hippocampal neurons, synapses form between pyramidal neurons and other pyramidal neurons and between pyramidal neurons and interneurons (Fletcher et al., 1991; Goslin et al., 1998), and both are typical of synaptic interactions in the hippocampus in vivo (Swanson et al., 1987). In contrast, cerebellar granule cell cultures are devoid of normal afferent mossy fiber innervation under standard conditions (Hatten et al., 1998). This may be particularly important if, as Olney et al. (2001) have suggested, relative inactivity of the neurons when they are first establishing synaptic contacts is the trigger for ethanol-induced apoptosis.

The presence of nonneuronal cells in these cultures raises the possibility that the effects of ethanol on the neurons are indirect, resulting from the effects of ethanol on the astrocytes, which release factors that influence neuronal development and survival. We recently demonstrated that the inhibitory effects of ethanol on dendrites in these cultures do not require astrocyte response to ethanol (Yanni et al., 2002), although we have not yet tested the effects of delayed addition of ethanol on neuron–astrocyte signaling. Identifying the variables that influence neuronal vulnerability to morphoregulatory as well as cytotoxic effects of ethanol is likely to lead to better understanding of the mechanisms of ethanol-induced neuropathologic changes and to enhance efforts to prevent or reverse these effects in individuals with alcohol-related neurodevelopmental brain damage.

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References

Bauer-Moffett, C., & Altman, J. (1977). The effect of ethanol chronically administered to preweanling rats on cerebellar development: a morphological study. *Brain Res 119*, 249–268.

Benson, D. L., Watkins, F. H., Steward, O., & Banker, G. (1994). Characterization of GABAergic neurons in hippocampal cell cultures. *J Neurocytol 23*, 279–295.

Bergamaschi, S., Battaini, F., Trabucchi, M., Parenti, M., Lopez, C. M., & Govoni, S. (1995). Neuronal differentiation modifies the effect of ethanol exposure on voltage-dependent calcium channels in NG 108–15 cells. *Alcohol 12*, 497–503.

Bergamaschi, S., Govoni, S., Battaini, F., Parenti, M., & Trabucchi, M. (1993). Effect of ethanol exposure on voltage-dependent calcium channels in in vitro cellular models. *Alcohol Alcohol Suppl 2*, 209–212. Also in P. V. Taberner, & A. A. Badawy (Eds.). (1993). *Advances in Biomedical Alcohol Research*: *Proceedings of the Sixth ISBRA Congress, Bristol, UK, 21–26 June 1992*. New York: Pergamon Press.

Berman, R. F., & Hannigan, J. H. (2000). Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. *Hippocampus 10*, 94–110.

Bonthius, D. J., & West, J. R. (1990). Alcohol-induced neuronal loss in developing rats: increased brain damage with binge exposure. *Alcohol Clin Exp Res 14*, 107–118.

Brewer, G. J., & Cotman, C. W. (1989). NMDA receptor regulation of neuronal morphology in cultured hippocampal neurons. *Neurosci Lett 99*, 268–273.

Cheema, Z. F., West, J. R., & Miranda, R. C. (2000). Ethanol induces Fas/Apo [apoptosis]-1 mRNA and cell suicide in the developing cerebral cortex. *Alcohol Clin Exp Res 24*, 535–543.

Clamp, P. A., & Lindsley, T. A. (1998). Early events in the development of neuronal polarity in vitro are altered by ethanol. *Alcohol Clin Exp Res 22*, 1277–1284.

Clapham, D. E. (1995). Calcium signaling. *Cell 80*, 259–268.

Craig, A. M., & Banker, G. (1994). Neuronal polarity. *Annu Rev Neurosci 17*, 267–310.

Craig, A. M., Blackstone, C. D., Huganir, R. L., & Banker, G. (1993). The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. *Neuron 10*, 1055–1068.

Deitrich, J. S., & Harris, R. A. (1996). How much alcohol should I use in my experiments? *Alcohol Clin Exp Res 20*, 1–2.

Dotti, C. G., Sullivan, C. A., & Banker, G. A. (1988). The establishment of polarity by hippocampal neurons in culture. *J Neurosci 8*, 1454–1468.

Fletcher, T. L., Cameron, P., De Camilli, P., & Banker, G. (1991). The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J Neurosci 11*, 1617–1626.

Goslin, K., Asmussen, H., & Banker, G. (1998). Rat hippocampal neurons in low-density culture. In G. Banker, & K. Goslin (Eds.), *Culturing Nerve Cells, 2nd edition* (pp. 339–390). Cambridge, MA: MIT Press.

Goslin, K., & Banker, G. (1989). Experimental observations on the development of neuronal polarity by hippocampal neurons in culture. *J Cell Biol 108*, 1507–1516.

Hatten, M. B., Gao, W.-Q., Morrison, M. E., & Mason, C. A. (1998). The cerebellum: purification and coculture of identified cell populations. In G. Banker, & K. Goslin (Eds.), *Culturing Nerve Cells, 2nd edition* (pp. 419–459). Cambridge, MA: MIT Press.

Hoffman, P. L., Rabe, C. S., Moses, F., & Tabakoff, B. (1989). *N*-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J Neurochem 52*, 1937–1940.

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, D. F., Koch, C., Genz, K., Price, M. T., Stefovska, V., Horster, F., Tenkova, T., Dikranian, K., & Olney, J. W. (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science 287*, 1056–1060.

Killisch, I., Dotti, C. G., Laurie, D. J., Luddens, H., & Seeburg, P. H. (1991). Expression patterns of $GABA_A$ receptor subtypes in developing hippocampal neurons. *Neuron 7*, 927–936.

Leslie, S. W., Brown, L. M., Dildy, J. E., & Sims, J. S. (1990). Ethanol and neuronal calcium channels. *Alcohol 7*, 233–236.

Lovinger, D. M., White, G., & Weight, F. F. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science 243*, 1721–1724.

Luo, J., & Miller, M. W. (1998). Growth factor-medicated neural proliferation: target of ethanol toxicity. *Brain Res Brain Res Rev 27*, 157–167.

McAlhany, R. E. Jr., West, J. R., & Miranda, R. C. (2000). Glial-derived neurotrophic factor (GDNF) prevents ethanol-induced apoptosis and JUN kinase phosphorylation. *Brain Res Dev Brain Res 119*, 209–216.

Neely, M. D., & Nicholls, J. G. (1995). Electrical activity, growth cone motility and the cytoskeleton. *J Exp Biol 198*, 1433–1446.

Oberdoerster, J. & Rabin, R. A. (1999). NGF-differentiated and undifferentiated PC12 cells vary in induction of apoptosis by ethanol. *Life Sci 64*, 267–272.

Olney, J. W., Wozniak, D. F., Jevtovic-Todorovic, V., & Ikonomidou, C. (2001). Glutamate signaling and the fetal alcohol syndrome. *Ment Retard Dev Disabil Res Rev 7*, 267–275.

Pantazis, N. J., Dohrman, D. P., Goodlett, C. R., Cook, R. T., & West, J. R. (1993). Vulnerability of cerebellar granule cells to alcohol-induced cell death diminishes with time in culture. *Alcohol Clin Exp Res 17*, 1014–1021.

Pearce, I. A., Cambray-Deakin, M. A., & Burgoyne, R. D. (1987). Glutamate acting on NMDA receptors stimulates neurite outgrowth from cerebellar granule cells. *FEBS Lett 223*, 143–147.

Pentney, R. J., & Miller, M. W. (1992). Effects of ethanol on neuronal morphogenesis. In M. W. Miller (Ed.), *Development of the Central Nervous System: Effects of Alcohol and Opiates* (pp. 71–107). New York: Wiley-Liss.

Pierce, D. R., Goodlett, C. R., & West, J. R. (1989). Differential neuronal loss following early postnatal alcohol exposure. *Teratology 40*, 113–126.

Shitaka, Y., Matsuki, N., Saito, H., & Katsuki, H. (1996). Basic fibroblast growth factor increases functional L-type Ca^{2+} channels in fetal rat hippocampal neurons: implications for neurite morphogenesis in vitro. *J Neurosci 16*, 6476–6489.

Stratton, K., Howe, C., & Battaglia, F. (1996). *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment*. Washington, DC: National Academy Press.

Swanson, L. W., Kohler, C., & Bjorklund, A. (1987). The limbic region. I. The septohippocampal system. In A. Bjorklund, T. Hokfelt, & L. W. Swanson (Eds.), *Handbook of Chemical Neuroanatomy* (vol. 5, pp. 1256–1277). New York: Elsevier Science.

Verderio, C., Coco, S., Fumagalli, G., & Matteoli, M. (1994). Spatial changes in calcium signaling during the establishment of neuronal polarity and synaptogenesis. *J Cell Biol 126*, 1527–1536.

Yanni, P. A., & Lindsley, T. A. (2000). Ethanol inhibits development of dendrites and synapses in rat hippocampal pyramidal neuron cultures. *Brain Res Dev Brain Res 120*, 233–243.

Yanni, P. A., Rising, L. J., Ingraham, C. A., & Lindsley, T. A. (2002). Astrocyte-derived factors modulate the inhibitory effect of ethanol on dendritic development. *Glia 38*, 292–302.