Ethanol-Induced Apoptosis in the Developing Visual System during Synaptogenesis

Tatyana Tenkova,1,2 Chainllie Young,1,2 Krikor Dikranian,1 Joann Labruyere,1 and John W. Olney1

PURPOSE. Ethanol is known to have deleterious effects on the human fetal nervous system (fetal alcohol syndrome), including components of the visual system, but only modest progress has been made in understanding these effects. The authors have recently demonstrated that, during the period of synaptogenesis, a single episode of ethanol intoxication lasting for several hours triggers a massive wave of apoptotic neurodegeneration in several regions of the developing rat or mouse forebrain. The present study was undertaken to determine to what extent the developing visual system is vulnerable to the apoptogenic effects of ethanol.

METHODS. Infant rats and mice at ages from birth to 21 days were treated subcutaneously with a single dose of ethanol or with two doses, 2 hours apart, on a single day. Blood alcohol levels were determined, and the retinas and visual centers in the brain were examined by light and electron microscopy at various times from 4 to 24 hours after treatment.

RESULTS. Retinal ganglion cells and neurons in the lateral geniculate nucleus, superior colliculus, and visual cortex were all highly susceptible to ethanol's apoptogenic action, the period of peak sensitivity being postnatal days 1 to 4 for ganglion cells and 4 to 7 for the other visual neurons. A transient elevation of blood alcohol to approximately 120 mg/dL was sufficient to activate the cell death program in visual neurons.

CONCLUSIONS. During synaptogenesis, a single ethanol intoxication episode triggers apoptotic cell death of neurons at all levels of the visual system from retina to the visual cortex. 

Invest Ophthalmol Vis Sci. 2003;44:2809–2817 DOI: 10.1167/iovs.02-0982

Intrauterine exposure of the human fetus to ethanol causes a dysmorphogenic neuropathological syndrome,1–4 including craniofacial malformations and reduced brain mass, which is associated with a variety of neurobehavioral disturbances, ranging from hyperactivity and attention deficit disorder and learning disabilities in childhood,7 to major depressive and psychotic disorders in adulthood.8 Although the distinctive multifaceted clinical picture, as originally described in its fully developed form,7 has come to be known as the fetal alcohol syndrome (FAS), it is now recognized that the fetotoxic effects of ethanol can manifest as a partial syndrome comprising largely neurobehavioral disturbances ranging from mild to severe, unaccompanied by craniofacial malformations. Alcohol-related neurodevelopmental disorder (ARND) is a term recently recommended for referring to such partial syndromes,8 and a new term currently emerging to represent all clinicopathological manifestations of ethanol's fetotoxic effects is fetal alcohol spectrum disorders (FASDs).9 Regardless of terminology, disruption of central nervous system (CNS) development and consequent neurobehavioral disturbances are the most debilitating effects of ethanol on the developing human fetus.

Since FAS was first described three decades ago, there have been many efforts to reproduce various aspects of the syndrome in laboratory animals, to identify the stage(s) in development when the immature CNS is most sensitive to ethanol's deleterious effects, and to gain insight into the underlying mechanism(s). At the turn of the century, and millennium, only limited success had been achieved in meeting these goals, and the mechanism(s) underlying ethanol's injurious effects on the developing CNS remained a complete mystery. However, findings reported recently10–12 are beginning to provide new insights into this enigma. These findings reveal that ethanol, by interfering with major neurotransmitter systems (glutamate and γ-aminobutyric acid [GABA]) during a critical period of development, can cause developing nerve cells en masse to die by apoptosis (i.e., commit suicide). The mode of cell death meets all morphologic, including ultrastructural, criteria for apoptosis,13 is dependent on Bax,14,15 is well known apoptosis initiator, and is accompanied in the early stages by a robust display of caspase-3 activation,14–17 an event associated with the execution stage of apoptosis. The window of vulnerability to ethanol's apoptogenic effects on the brain coincides with the period of synaptogenesis,18 also known as the brain growth-spurt period, a period that is confined to postnatal life in rodents, but in humans encompasses the last trimester of gestation and first several years after birth.18 Abnormalities in the visual system of immature rodents exposed subchronically to ethanol during development have been described, including dysmyelination and hypoplasia of the optic nerve,19–21 and loss of retinal ganglion cells.22 In human FAS victims, Stromland and Pinazo-Duran23,24 have described optic nerve hypoplasia and impaired vision, and have proposed that dysmyelination of the optic nerve may be the primary mechanism underlying ethanol's disruptive action on the developing visual system. There is very little information regarding the deleterious effects of ethanol on other neural...
components of the developing visual system, either in animals or humans.

The present study was undertaken to characterize the sensitivity of various neural components of the developing visual system to the apoptogenic effects of ethanol. Parameters examined include the pattern of neurodegeneration in various components of the visual system, developmental window of vulnerability, time course of the cell death process, and minimal exposure levels necessary to trigger apoptotic degeneration of visual neurons.

METHODS

Animals and Drug Treatments

All animal care procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Infant Sprague-Dawley rats at ages from postnatal day (PND) 1 to PND 21 were used in an initial series of experiments pertaining to both the retina and brain. Additional experiments, focusing primarily on the brain, were performed on 7-day-old C57BL/6 mice to confirm vulnerability of a second species. Half of the pups in each litter were treated with saline and the other half with ethanol (2.5 g/kg at 0 hours and again at 2 hours). Ethanol was prepared as a 20% solution in sterile normal saline and was administered subcutaneously in all experiments. At least four, and often six, experimental pups and an equal number of control pups per treatment condition were used. These were drawn from a total of 36 rat litters and 16 mouse litters. At the termination of each experiment, the pups were deeply anesthetized with halothane and perfused transcardially with aldehyde fixatives, as described previously.12,15,17

Histologic Procedures

To study the pattern of degeneration, sections of brain or retina were stained by the DeOlmos cupric silver method, as previously described,12,24 and examined 16 to 20 hours after initiation of ethanol or saline treatment. Because caspase-3 activation is considered an impor-

FIGURE 1. In the retina of 2-day-old rats, many neurons in the ganglion cell layer (GCL) showed caspase-3 activation (B) at 8 hours and silver staining (D) at 16 hours after ethanol, whereas in the saline control (A, C) ganglion cells showing either caspase-3 activation or silver staining were very scarce (totally absent in the examples shown). Cells displaying caspase-3 activation typically have a larger and more healthy appearance than do silver-stained neurons, because dying cells become silver positive at a later interval when they are in a more advanced stage of condensation and fragmentation.
tant step in the execution phase of apoptotic neuronal degeneration, and because we have found that neurons undergoing apoptotic degeneration after ethanol treatment display a robust caspase-3 activation response, we applied immunocytochemical methods, as previously described, to demonstrate caspase-3 activation patterns at various intervals after ethanol administration. For studying the time course of degeneration and sequence of changes in detail and to confirm the apoptotic nature of the cell death process, we examined the brains and retinas at various survival intervals (4, 8, 12, 16, 20, and 24 hours after the first ethanol dose), using a combined light and electron microscopy approach that has been described in detail.

In a separate experiment, 7-day-old C57BL/6 mice received a single subcutaneous injection of normal saline (n = 12) or ethanol (n = 15) at a reduced dose (1.25 g/kg, which is one quarter the dose used in the experiments described earlier), and their brains were examined 4 hours later to determine by quantitative methods whether, at this reduced dose and at this time interval, there was a significant increase in neuronal profiles showing caspase-3 activation. Quantitative data were gathered for many brain regions, and this evidence will be the subject of a separate report. A data subset pertaining to the superior colliculus will be reported herein. In addition, blood alcohol concentrations were measured at 30, 45, 60, 90, and 120 minutes after a single 1.25 g/kg dose of ethanol, and this information will also be reported herein.

Quantitative Cell Counts
Four hours after a low dose of ethanol or saline, we counted the number of cellular profiles in the superior colliculus that were positive for activated caspase-3. The counts were performed on 50-μm-thick sections cut through the superior colliculus at a level that also displayed the medial geniculate nucleus in its largest dimension. These

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933710/)  
**FIGURE 2.** (A) The mean (± SEM) number of cellular profiles per section in the ganglion cell (GC) layer of the 1-day-old rat retina that displayed immunocytochemical evidence of caspase-3 activation after saline treatment or 4, 6, or 8 hours after a high dose of ethanol (2.5 g/kg × 2). The small number of profiles showing caspase-3 activation in the saline control reflects the rate of spontaneous apoptosis (physiological cell death). At 4, 6, or 8 hours after ethanol the apoptosis rate was approximately 12, 16, or 40 times higher, respectively, than this baseline rate. Each experimental value is significantly different from the control (P < 0.001). (B) The number of cellular profiles per square millimeter (mean ± SEM) showing immunocytochemical evidence of caspase-3 activation in the superior colliculus 4 hours after saline (n = 12) or a low subcutaneous dose (1.25 g/kg) of ethanol (n = 15). The density of activated caspase-3-positive profiles was approximately three times greater after ethanol treatment, a difference that is statistically significant (P = 0.0012; unpaired t-test with Welch correction).

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933710/)  
**FIGURE 3.** These silver-stained brain sections are from 8-day-old mice, 20 hours after a large dose (2.5 g/kg × 2) of ethanol (right) or saline (left). Note the widespread pattern of silver staining after ethanol treatment, which included dense staining in the visual cortex and dorsolateral geniculate (DLG), and moderate staining in the ventrolateral geniculate (VLG). Each dark speck in the silver stain represents a dead neuron or a fragment thereof. When there is a high density of degenerating neurons it causes the affected brain region to become diffusely darkened. There were degenerating neurons in the saline control brain (due to physiological cell death) but they were so sparse and scattered in distribution that they were barely visible at low magnification.
FIGURE 4. This brain section, stained immunocytochemically for activated (cleaved) caspase-3, is from a 7-day-old mouse, 8 hours after a large dose of ethanol (2.5 g/kg × 2). This section is from the same rostrocaudal level as the sections in Figure 3 and displays the pattern of caspase-3 activation induced by ethanol in the visual cortex, dorsolateral geniculate (DLG), and ventrolateral geniculate (VLG). Note that the caspase-3 activation pattern in these brain regions of the ethanol-treated pup is the same as the pattern of silver staining in these regions illustrated in Figure 3. For illustrations documenting that very few neurons in saline control brains show caspase-3 activation, see Olney et al.12
sections were imaged and quantitatively evaluated with the help of a stereology system consisting of the following components: stereology mapping system software (Stereo Investigator; MicroBrightField, Inc., Colchester, VT) and a computer (with Pentium III microprocessor, Intel, Mountain View, CA) connected to a motorized stage (Prior Optiscan ES103 XYZ system, Prior Scientific, Inc., Rockland, MA) mounted on a microscope (Labophot-2; Nikon, Melville, NY). The contours of the superior colliculus were traced into the computer and from the tracings, the stereology computer program calculated the area of superior colliculus in each section. Caspase-3–positive neurons with dendritic processes visible were all counted. For those profiles without obvious dendritic processes, only those with a size larger than 8 μm were counted. The population estimator function of the stereology software was used to mark each profile as it was counted to ensure that no profile would be missed or counted twice. Statistical analysis was performed on computer (InStat 3.0; GraphPad, San Diego, CA).

To evaluate the time course and extent of neurodegeneration in the retina, we performed counts of cellular profiles in the ganglion cell layer of 1-day-old rats that were positive for activated caspase-3 after saline treatment or at 4, 6, or 8 hours after ethanol treatment. The counts were performed on complete 50-μm-thick retinal sections cut through the optic nerve head in a horizontal meridian from the nasal to temporal ora serrata. Five such retinal sections from each eye were counted, and the mean number of caspase-3–positive profiles per section for each eye was determined. From these counts the mean number of caspase-3–positive profiles per section for each treatment group (8 eyes per group) was determined.

Blood Ethanol Determination

After decapitation, the blood was collected in heparinized microhematocrit capillary tubes (VWR Scientific, Inc., Chicago, IL) and centrifuged at 5000 rpm for 20 minutes. Ethanol standard solutions of 300, 100, 80, 50 mg/dL were obtained from Sigma Diagnostics, Inc (St. Louis, MO). Ten microliters of plasma or standard solution was mixed with 3 mL nicotinamide adenine dinucleotide-alcohol dehydrogenase (NAD-ADH) reagent in glycine buffer (NAD, 9.6 micromoles, ADH, 800 U in 16 mL glycine buffer; Sigma Diagnostics, Inc.). Exactly 10 minutes after mixing, the absorbance of fluorescence at 540 nm was read on a spectrophotometer (Ultrospec 2100 pro UV/Visible; Biochrom Ltd., Cambridge, UK). All samples were run in triplicate and averaged. Serum from saline-treated mice was used as a blank. The absorbances of standard solutions were plotted and fitted for linear regression on computer (Axograph 4.2; Axon Instruments, Inc., Union City, CA). The
correlation coefficient was 0.999. The ethanol concentrations of the samples were determined fitting the absorbances to the standard curve.

RESULTS

In the retinas of rats treated on a single day with ethanol at various ages, there was a significant increase in retinal ganglion cell degeneration during a relatively narrow developmental time window from PND 1 to PND 4. This early response was observed only in the ganglion cell layer and was robustly evident in sections stained for activated caspase-3 within 4 to 8 hours, and in silver-stained sections, 10 to 16 hours after administration of ethanol (Fig. 1). The number of profiles in the ganglion cell layer showing caspase-3 activation 4, 6, or 8 hours after a high dose (2.5 mg/kg × 2) of ethanol was increased 12-, 16-, or 40-fold, respectively, compared with the number displaying caspase-3 activation (due to physiological cell death) in the saline control (Fig. 2A). The high rate of caspase-3 activation at the 8-hour interval is consistent with a prior observation that in many brain regions caspase-3 activation peaks at 8 hours after ethanol treatment.17 This high rate at 8 hours does not reflect the full magnitude of the degenerative response, because caspase-3 activation is an ephemeral phenomenon that is detectable in a given neuron for approximately 2 to 3 hours.17

Thus, many neurons showing caspase-3 positivity at 4 hours are not reflected in the count at 8 hours. Between PND 3 and PND 7, as the inner nuclear layer was undergoing formation, relatively large numbers of inner nuclear layer cells displayed caspase-3 activation in both the ethanol and saline control retinas. Whether this phenomenon occurred at a higher rate in ethanol-treated pups was not evaluated quantitatively.

In the brain, evidence for an increased rate of degeneration of visual neurons after ethanol treatment was first detected in the lateral geniculate nucleus and superior colliculus on approximately PND 3 and in the visual cortex on PND 4. In these regions the rate of neurodegeneration after ethanol treatment increased from PND 3 to 4 to PND 7 to 8 and then gradually declined, with little or no degenerative reaction being detected after approximately PND 12.

Degeneration of visual neurons in the brain was readily detected in sections stained by the DeOlmos silver method (Fig. 3), or stained for activated caspase-3 (Figs. 4, 5), but the timing for optimal staining and the information obtained was different with the two methods. As we have recently described,17 caspase-3 activation is a transient phenomenon detectable in a given neuron for approximately 2 to 3 hours, and it occurs in some neuronal populations earlier than in others. For example, superior collicular neurons respond within 3 to 4 hours after ethanol exposure, whereas lateral geniculate neurons become responsive in the 6- to 8-hour interval. Application of the caspase-3 stain after the optimal time window has transpired for a given group of neurons yields a negative result for those neurons, although other neurons may still display caspase-3 activation. However, if a Nissl counterstain is applied shortly after neurons have ceased showing caspase positivity, the counterstained sections reveal abundant small, dark structures in the regions that were previously caspase-3 positive (Fig. 5). These Nissl-positive dark structures are the fragmented remains of neurons that initially were caspase-3 positive and then progressed to a late, caspase-3-negative stage of apoptotic neurodegeneration. In contrast to caspase-3 staining, the silver stain yields best results if applied at approximately 12 to 20 hours after ethanol, after all neurons have progressed to a
relatively late stage of degeneration. In this time interval, the silver stain detects all neurons, or fragments of neurons, that have responded to the apoptogenic effects of ethanol. An additional difference between the two stains is that the caspase-3 activation stain, if applied at the optimal time, appears very robust, because it deeply stains the entire neuron, including its cell body and dendritic processes, and it is staining the entire neuron in a very early stage of apoptosis before shrinkage and fragmentation have occurred. In contrast, the silver stain marks a larger number of structures but most of them are in an advanced stage of shrinkage and fragmentation have occurred. In contrast, the silver stain marks a larger number of structures but most of them are in an advanced stage of shrinkage and fragmentation, so that the overall pattern is more diffuse and less striking in its display of individual neurons.

Electron microscopic examination of the degenerating neurons in the retina, lateral geniculate nucleus, superior colliculus, and visual cortex (Fig. 6) revealed that in all locations the degenerating neurons showed the kind and sequence of ultrastructural changes that are well recognized as being pathognomonic of apoptosis.13,26,27

In experiments testing the response of various neuronal populations to different doses of ethanol, we observed that superior colliculus neurons, regardless of dose, show a rapid (within 4 hours) caspase-3 activation response to ethanol (Fig. 7), which suggests a high degree of sensitivity to ethanol neurotoxicity. Therefore, we administered either saline or a low dose of ethanol (1.25 g/kg) to 7-day-old mice and counted neuronal profiles displaying caspase-3 activation in the superior colliculus 4 hours after treatment. As is shown in Figure 2B, the ethanol-exposed pups had a significantly (P = 0.0012) larger number of profiles showing caspase-3 activation than did the saline control. Blood ethanol concentrations after this dose of ethanol rose to a peak of 122 mg/dL at 30 minutes and declined to less than 100 mg/dL 60 minutes later.

**DISCUSSION**

In the current study in infant rats and mice, ethanol triggered degeneration of neurons in the ganglion cell layer of the retina and in the lateral geniculate nucleus, superior colliculus, and visual cortex of the brain. By electron microscopy it was determined that the nature of the cell death process was apoptotic. Retinal ganglion cells become sensitive earlier than neurons in the brain, and neurons in the lateral geniculate nucleus and superior colliculus become sensitive slightly before neurons in the visual cortex. However, the vulnerability period for each of these types of neurons is confined to a time segment within the first 2 weeks after birth, which in the rat or mouse corresponds to the synaptogenesis period, also known as the brain growth-spurt period. In humans the brain growth-spurt period spans the last trimester of gestation and continues into the first several years after birth.18 In the human retina,
synaptogenesis begins much earlier, at approximately the end of the first or beginning of the second trimester. Thus, exposure of the human fetus to ethanol at any time during the second and third trimesters of pregnancy entails risk of neurons being destroyed at some level of the visual system (retina, lateral geniculate nucleus, superior colliculus, or visual cortex).

It is thought that ethanol exerts its proapoptotic action in the developing brain by a dual mechanism (blockade of N-methyl-D-aspartate [NMDA] glutamate receptors and hyperactivation of GABA<sub>B</sub> receptors). This interpretation is based on evidence that ethanol has both NMDA antagonist<sup>29,30</sup> and GABAmimetic properties, and on the recent finding<sup>10–12</sup> that various other drugs that have either NMDA antagonist or GABAmimetic properties induce distinctive patterns of apoptotic neurodegeneration in the developing rat brain. Reinforcing this interpretation is evidence<sup>11</sup> that NMDA antagonists and GABAmimetics induce two different patterns of degeneration in the developing brain and the pattern induced by ethanol resembles a composite of these two patterns. Whether these mechanisms, in the final analysis, will be sufficient to explain all of ethanol’s deleterious effects on the CNS, remains to be determined.

Stromland and Pinazo-Duran<sup>22</sup> have reported loss of retinal ganglion cells after chronic exposure of developing rats to ethanol. Having also observed dysmyelination and hypoplasia of the optic nerve in ethanol-treated rats<sup>19</sup> and in humans affected by FAS,<sup>22,23</sup> they have proposed that dysmyelination of the optic nerve may be the primary mechanism underlying ethanol’s disruptive action on the developing visual system. In view of the present findings that a single dose of ethanol triggers acute apoptotic neurodegeneration of neurons at all levels of the visual system, including ganglion cells in the retina, a more likely interpretation would be that optic nerve changes are a secondary phenomenon that occurs as a result of primary degeneration of retinal ganglion cells and/or other visual neurons. That retinal ganglion cells show signs of apoptotic degeneration and caspase-3 activation within hours after ethanol administration is highly consistent with a primary impingement on the ganglion cell itself, whereas a more protracted course would be expected if ganglion cell degeneration occurred secondary to a disturbance in myelination. Moreover, optic nerve myelination does not begin in the rodent until after PND 5,<sup>34</sup> and dysmyelination therefore could not be the mechanism by which ethanol deletes retinal ganglion cells preferentially on PND 1 to 4, as we have described herein. A tenable alternate interpretation that our findings do not rule out would be that ethanol exerts a primary toxic impingement on the optic nerve as well as on neurons in the retina and brain. Regardless of whether optic nerve changes are primary or secondary, the findings we present—that ethanol can cause neurons to commit suicide at all levels of the visual system—provide a plausible explanation for the abnormalities of vision that have been reported in humans with FAS and suggest that ethanol may have significant cell-killing effects in some portions of the visual system that have previously gone unrecognized.

Using immunocytochemically detected caspase-3 activation as a marker of neurons undergoing ethanol-induced apoptosis, we found that a relatively low dose of ethanol (1.25 mg/g) was sufficient to cause a substantial and statistically significant degenerative response in superior collicular neurons within 4 hours after ethanol exposure. This dose of ethanol, in the 7-day-old infant mouse, produced blood ethanol elevations that slightly exceeded 100 mg/dL for approximately 1 hour. Blood levels in this range are not uncommonly encountered in a social drinking context. However, several caveats must be exercised in interpreting these findings: (1) Rodent-to-human extrapolations provide an imperfect basis for risk evaluation; (2) the rodent data are incomplete, in that doses lower than 1.25 mg/g remain to be tested, brain regions other than the superior colliculus have not been adequately evaluated, and developmental ages other than the seventh postnatal day were not studied; 3) it can be argued that a robust display of caspase-3 activation does not necessarily signify that the neuron is destined to die. However, the bulk of evidence contradicts this argument. For example, in brain regions severely damaged by ethanol, light and electron microscopic evaluation of neurons at sequential intervals as they undergo degeneration reveals that between 45% and 68% of the neurons populating certain brain regions do unequivocally die and are phagocytically deleted from the brain<sup>12</sup> and these are the same type, location and numbers of neurons that show caspase-3 activation in the early posttreatment intervals.

Placing these findings in a practical context, the legal definition of alcohol intoxication in the United States was originally set at 200 mg/dL, and more recently has been reduced to 100 or 80 mg/dL in most states. Thus, subject to the above caveats, the present data suggest that if a human mother in mid to late pregnancy drinks enough in a single setting to achieve a blood ethanol level that transiently exceeds the legal intoxication level, this may be sufficient to cause neurons to degenerate in the developing fetal CNS, including neurons that perform visual functions. Regardless of whether the threshold in humans is higher or lower than in rodents, an important observation that is likely to have relevance in humans is that only a single ethanol exposure is necessary for many neurons to be deleted from the developing retina and brain.

Identifying the second and third trimesters as the time window when ethanol’s deleterious effects on the developing CNS are most likely to occur, does not rule out vulnerability to deleterious effects during the first trimester. It has long been assumed that the craniofacial malformations that occur in some cases of FAS are due to an action of ethanol in the first trimester. Consistent with this assumption, Duntly et al.<sup>35</sup> have reported recently that a single exposure of the developing rat embryo to ethanol on gestation days 6.5 to 11 triggers apoptotic degeneration of certain progenitor cells destined to form craniofacial structures. This period of development in the rodent is comparable to the first trimester of pregnancy in the human. Therefore, it appears that there is no stage of pregnancy when alcohol can be liberally imbibed with impunity.

References

Ethanol-Induced Apoptosis during Synaptogenesis


