Cell Death and Differentiation (2003) 10, 1148–1155

www.nature.com/cdd

Ethanol-induced neuronal apoptosis *in vivo* requires BAX in the developing mouse brain

C Young[1], BJ Klocke[2], T Tenkova[1], J Choi[3], J Labruyere[1], Y-Q Qin[1], DM Holtzman[3], KA Roth[2] and JW Olney*[,1,2]

[1] Department of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA
[2] Department of Pathology, Washington University School of Medicine, St. Louis, MO, USA
[3] Department of Neurology and Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO, USA
* Corresponding author: JW Olney, Department of Psychiatry, Washington University in St. Louis, 660, South Euclid Avenue, MO 63110, USA. Tel: 314-362-2476; Fax: 314-747-0346; E-mail: olneyj@psychiatry.wustl.edu

Received 16.9.02; revised 13.3.03; accepted 29.4.03
Edited by D Nicholson

Abstract

A single episode of ethanol intoxication triggers widespread apoptotic neurodegeneration in the infant rat or mouse brain. The cell death process occurs over a 6–16 h period following ethanol administration, is accompanied by a robust display of caspase-3 enzyme activation, and meets ultrastructural criteria for apoptosis. Two apoptotic pathways (intrinsic and extrinsic) have been described, either of which may culminate in the activation of caspase-3. The intrinsic pathway is regulated by Bax and Bcl-X_L and involves Bax-induced mitochondrial dysfunction and release of cytochrome *c* as antecedent events leading to caspase-3 activation. Activation of caspase-8 is a key event preceding caspase-3 activation in the extrinsic pathway. In the present study, following ethanol administration to infant mice, we found no change in activated caspase-8, which suggests that the extrinsic pathway is not involved in ethanol-induced apoptosis. We also found that ethanol triggers robust caspase-3 activation and apoptotic neurodegeneration in C57BL/6 wildtype mice, but induces neither phenomenon in homozygous Bax-deficient mice. Therefore, it appears that ethanol-induced neuroapoptosis is an intrinsic pathway-mediated phenomenon involving Bax-induced disruption of mitochondrial membranes and cytochrome *c* release as early events leading to caspase-3 activation.

Cell Death and Differentiation (2003) **10,** 1148–1155. doi:10.1038/sj.cdd.4401277

Keywords: alcohol; apoptosis; Bax knockout; caspase-3; neurons

Abbreviations: WT, wildtype; PCR, polymerase chain reaction; NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid; Apaf-1, apoptotic protease activating factor-1; CNS, central nervous system; H & E, hematoxylin and eosin; AD, anterodorsal nucleus of the thalamus; Smac/DIABLO, second mitochondria-derived activator of caspase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAB, 3, 3′-diaminobenzidine; PMSF, phenylmethylsulfonyl fluoride; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride

Introduction

A single episode of ethanol intoxication, during the developmental period of synaptogenesis, can trigger widespread apoptotic neurodegeneration in the infant rat or mouse brain.[1,2] The cell death process occurs over a period of 6–16 h following ethanol administration, is accompanied by an early display of caspase-3 enzyme activation,[3] is readily detected by DeOlmos cupric silver staining, and meets ultrastructural criteria for apoptosis.[1,3,4] The ethanol-intoxicated infant mouse, therefore, provides an excellent model for studying *in vivo* apoptotic neurodegeneration. Two properties of ethanol, both of which reduce neuronal activity, are believed to be instrumental in initiating the apoptotic process – blockade of NMDA receptors and hyperactivation of $GABA_A$ receptors.[1,5] Reduced activity resulting in dysfunctional synaptogenesis may in some way lead to the apoptotic outcome, but the specific gene-regulated mechanisms and pathways that mediate this example of apoptosis remain to be elucidated.

Two major apoptotic pathways (extrinsic and intrinsic) have been described. The extrinsic pathway involves the binding of cytokines to death receptors, activation of caspase-8, and cleavage and activation of effector caspase-3, -6 or -7.[6] Caspase-8 is a key factor uniquely associated with this pathway. The intrinsic pathway involves translocation of Bax protein from the cytosol to the outer mitochondrial membrane, where it increases membrane permeability and promotes release of cytochrome *c*, which binds with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, resulting in its cleavage to form activated caspase-9. The activated caspase-9, in turn, cleaves procaspase-3 to its active form.[7] While it is well documented that caspase-3 activation occurs in ethanol-induced apoptotic neurodegeneration, and this is not accompanied by activation of caspase-6 or -7, activation of caspase-3 could signify involvement of either the extrinsic or intrinsic pathway.[2]

The Bcl-2 family of proteins consists of several pro- and antiapoptotic factors; among them, Bax and Bcl-x_L may have counterbalancing roles in the intrinsic apoptotic pathway. Mitochondrial release of cytochrome *c*, which is triggered by an action of Bax on the outer mitochondrial membrane, is in some experimental models considered the death commitment point in the intrinsic apoptotic pathway.[8,9]

Caspase-3 activation, which is responsible for producing many of the morphological changes characteristic of apoptotic cell death, occurs downstream from Bax/Bcl-X_L interactions. It appears that Bax is a key regulator of neuronal apoptosis, in

that targeted disruption of the *bax* gene has been shown to significantly decrease neuronal death in several apoptosis paradigms, including trophic factor deprivation[10] and *bcl-x*$_L$ deficiency.[11] However, during early CNS development of embryo, *bax* expression is not an absolute requirement for neuronal death since in the absence of the *bax* gene, low levels of apoptotic neuronal death are still detected, indicating that Bax-independent pathways for neuronal apoptosis also exist.[11]

If ethanol-induced apoptosis is mediated by the extrinsic pathway, it should be accompanied by a display of caspase-8 activation. If it is regulated via the Bax-dependent intrinsic apoptotic pathway, disruption of *bax* may result in ethanol being unable to trigger either caspase-3 activation or apoptotic neurodegeneration.

In the present study, we tested these two hypotheses, using Western blot analysis to test the first, and homozygous Bax-deficient mice to test the second.

Results

Similar blood ethanol concentrations in wildtype, *bax* heterozygous and *bax*-deficient mice

In our previous study, we have determined that the blood ethanol concentration reaches its peak at 3 h after the first dose of ethanol.[1] We thus compared the peak blood concentrations of ethanol in all the three genotypes of mice. The peak level for wildtype (WT) mice was 503.9 ± 51.2 mg/dl (n=6) and those for bax heterozygous and bax-deficient mice were 501.8 ± 35.9 mg/dl (n=9) and 499.7 ± 64.8 mg/dl (n=4), respectively. All of them achieved similar toxic levels ($P > 0.05$, Kruskal–Wallis nonparametric ANOVA).

Ethanol triggers robust caspase-3 activation in WT and *bax* heterozygous mice, but not *bax*-deficient mice

In the brains of WT pups treated with two doses of ethanol, there was a very robust caspase-3 activation pattern throughout the forebrain, identical to the pattern recently illustrated in Olney *et al*.[2]. At 8 h after the first dose of ethanol was an optimal time for visualizing the widespread pattern of caspase-3 activation. The pattern and magnitude of caspase-3 activation in the *bax*$^{+/-}$ mice was similar to that observed in WT mice, but no evidence for caspase-3 activation was detected in the brains of ethanol-treated *bax*$^{-/-}$ mice (Figures 1 and 2). It is noteworthy that in WT mice treated with saline, there were scattered cells showing caspase-3 activation, as would be expected due to neurons undergoing physiological death[2] (as illustrated recently in Olney *et al.*[2]). However, in the *bax* knockout mice, we detected almost no evidence for caspase-3 activation (Figures 1 and 2), which suggests that physiological neuronal death was also significantly inhibited by Bax deficiency.

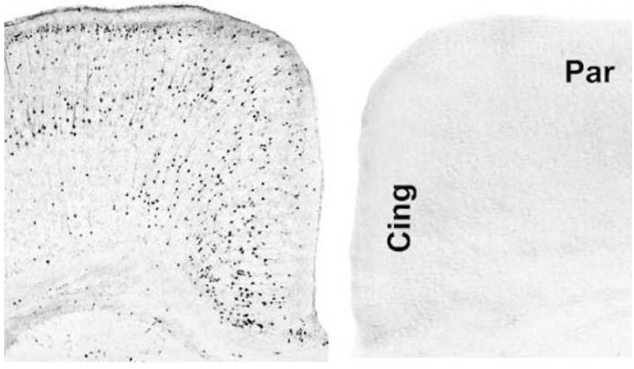

Figure 1 Coronal brain sections cut through the cingulate (cing) and parietal (par) cortices were immunohistochemically stained for cleaved caspase-3. The pattern of caspase-3 activation 8 h after ethanol treatment is similar in *bax*$^{+/-}$ mice (left side) to that illustrated previously[2] in ethanol-treated WT mice. In contrast, neurons displaying caspase-3 activation were not detected in *bax*$^{-/-}$ mice (right side)

Loss of cytochrome *c* immunoreactivity in neurons that showed activated caspase-3

In the brains of ethanol-exposed WT mice, dual detection of activated caspase-3 and cytochrome *c* showed a loss of granular, punctuate cytochrome *c* immunoreactivity in neurons exhibiting activated caspase-3 immunostaining (Figure 3, panels a–c). In contrast, Bax-deficient mice exposed to ethanol exhibited neither neuronal activated caspase-3 immunoreactivity nor a loss of punctuate cytochrome *c* immunostaining (Figure 3, panels d–f). These findings are consistent with ethanol-induced, Bax-dependent translocation of cytochrome *c* from its punctuate mitochondrial localization to the neuronal cytosol, where it contributes to apoptosome formation and caspase-3 activation.

Activation of caspase-3 is accompanied by cleavage of spectrin in ethanol-treated WT mice

In other apoptosis models, active caspase-3 cleaves a number of target proteins, including poly (-ADP-ribose) polymerase (PARP)[12] and nonerythroid alpha spectrin.[13,14.-] To clarify whether caspase-3 activation in our ethanol model leads to cleavage of cytoskeletal proteins, we performed immunohistochemical staining of activated caspase-3 and caspase-3-cleaved spectrin on serial sections of brains from WT mice that were treated with ethanol. At 8 h after ethanol treatment, the pattern of caspase-3-cleaved spectrin (Figure 4, panel b) was compatible with, though not as widespread as, the pattern of activated caspase-3 (Figure 4, panel a). The less widespread pattern may signify that spectrin is rapidly degraded following caspase-3 cleavage, thus providing only a narrow time window for detecting neurons positive for cleaved spectrin. Nevertheless, our results document that ethanol-induced caspase-3 activation is indeed coupled with degradation of downstream structural protein.

Ethanol causes acute neurodegeneration in WT mice but not in *bax* $^{-/-}$ mice

To evaluate the possibility that neurons in *bax*$^{-/-}$ mice undergo neurodegeneration through pathways other than

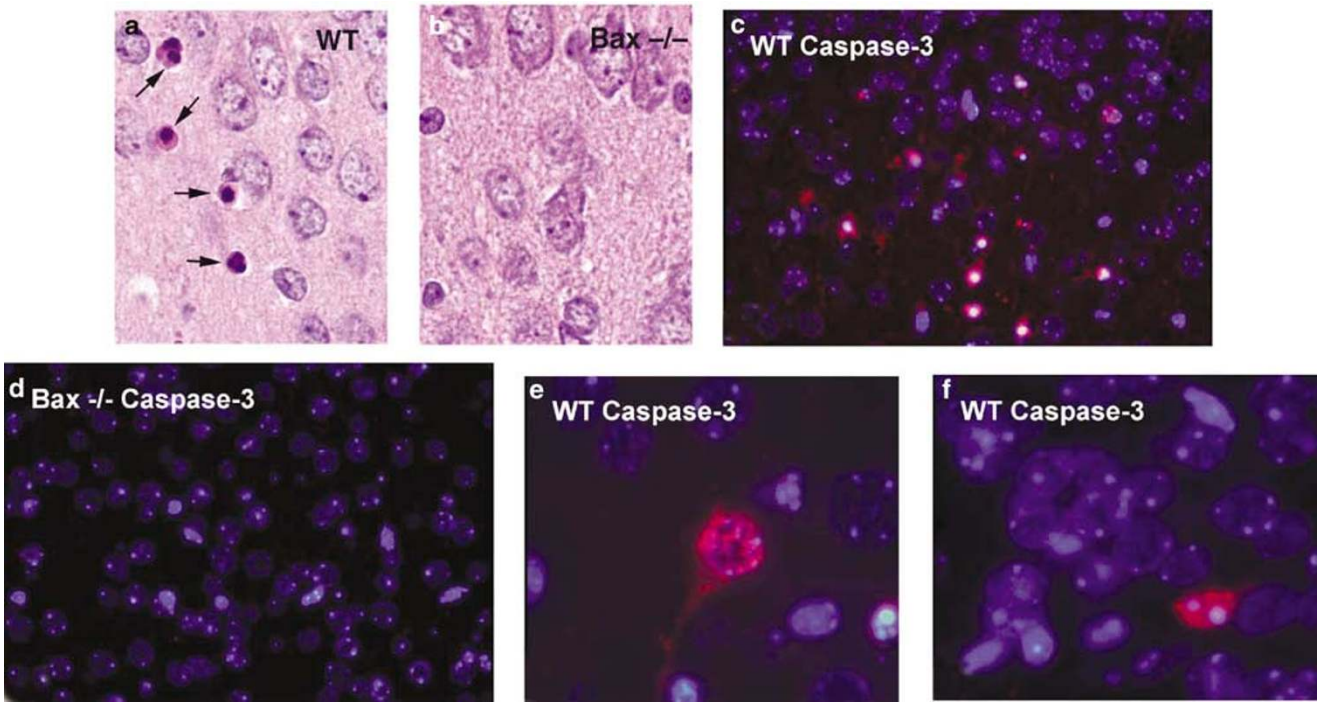

Figure 2 All panels are from the parietal cortex of 7-day-old mice 8 h after ethanol. Panels (**a**) and (**b**) are H & E-stained sections showing abundant apoptotic profiles (arrows) in wild type (WT) and none in *bax*$^{-/-}$ mice. Panels (**c**–**f**) are stained immunohistochemically, using CM1 antiactive caspase-3 primary antibody, tyramide signal amplification, cyanine-3 tyramide and bisbenzimide nuclear labeling. Note the abundant cells (stained red) with apoptotic morphology showing caspase-3 activation in WT controls (**c**) and absence of caspase-3 activation in *bax* $^{-/-}$ mice (**d**). In the WT brain, various types of neurons display caspase-3 activation in response to ethanol, including pyramidal cells (**e**) and smaller neurons (**f**)

caspase-3 activation, we examined the brains for evidence of neurodegeneration in paraffin-embedded H & E sections, semi-thin plastic sections, and vibratome sections stained with the DeOlmos cupric silver stain.

At 8 h after ethanol, H&E-stained sections revealed abundant apoptotic profiles in many brain regions of WT or *bax* $^{+/-}$ mice, but none were evident in the brains of *bax*$^{-/-}$ mice (Figure 2a and b).

At 20 h after ethanol treatment, plastic sections of the brains of WT pups displayed evidence of severe neurodegeneration throughout the forebrain (Figure 5a). There were many dying neurons containing spherical balls of clumped nuclear chromatin, and the neurophil surrounding these degenerating neurons was edematous and strewn with cellular debris, including many profiles having the appearance of apoptotic bodies. In contrast, no pathological changes were detected in the brains of the ethanol-treated *bax* $^{-/-}$ mice (Figure 5b).

Silver-stained sections demonstrating the cumulative pattern of neurodegeneration over a 24 h period following ethanol, exhibited strong and diffused silver staining throughout many brain regions in both WT and *bax* $^{+/-}$ mice. In contrast, silver staining was essentially absent from the brains of *bax* $^{-/-}$ mice (Figure 6).

Disruption of *bax* protects neurons from both acute and delayed degeneration

To rule out the possibility that ethanol might cause delayed degeneration of neurons that was not yet evident in plastic sections at 20 h or in silver-stained sections at 24 h, we examined plastic sections from brains 72 h after ethanol treatment. At 72 h postethanol, dead neurons and debris had been cleared almost completely from most brain regions of the WT pups, but a few regions that typically sustain the most severe damage, such as the anterodorsal nucleus (AD) of thalamus showed lingering signs of a late-stage degenerative process, including interstitial cystic changes, remnants of debris, and an accumulation of small dark cells presumed to be of glial origin (Figure 5c). In the brains of *bax* $^{-/-}$ mice at 72 h postethanol, just as at earlier post-treatment intervals, we could not detect any signs of a neurodegenerative process (Figure 5d). Neuronal counts performed on sections of the AD nucleus at its greatest areal dimension revealed that 72 h after ethanol treatment, the number of neurons remaining in the AD nucleus of heterozygous mice (n=4) was 34.7% of the number populating the AD nucleus of Bax homozygous mice (n=4) (Table 1). This is consistent with our previous observation that in normal 7-day-old C57BL/6 mice, ethanol treatment deletes approximately 68% of the neurons that naturally populate the AD nucleus of the thalamus.[3] It is noteworthy that the cell density in the AD nucleus of heterozygous mice was reduced to about half of that of the AD nucleus of homozygous mice. This signifies that the reduction in number of neurons in the heterozygotes was much greater than the reduction in space occupied by neurophil, which means that either the surviving neurons have expanded their dendritic processes to an exaggerated extent or there has been a marked increase in the ratio of glial to neuronal space. Electron microscopic evaluation is

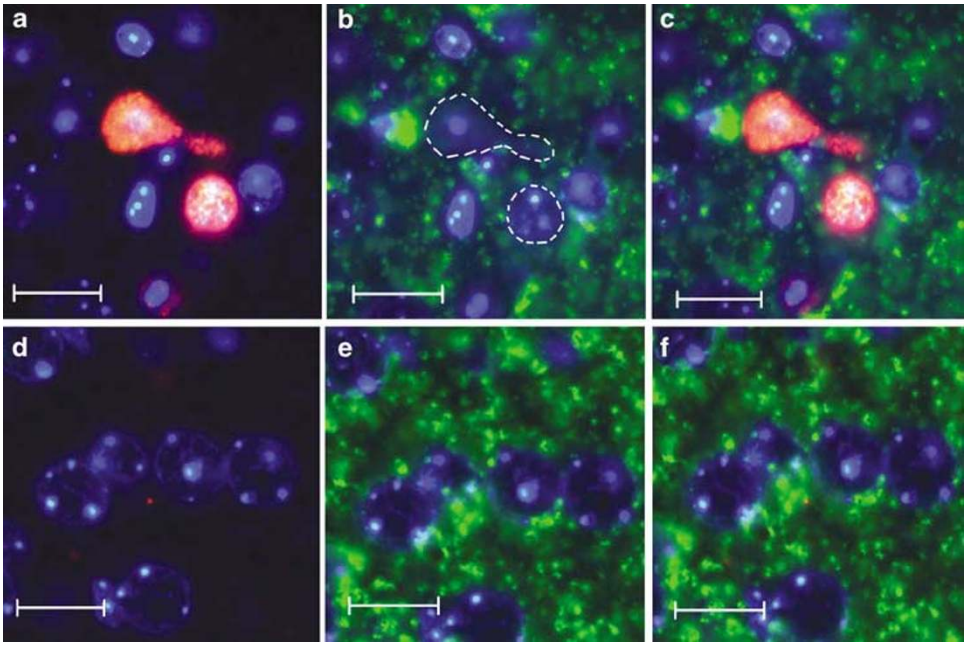

Figure 3 Dual immunocytochemical detection of activated caspase-3 and cytochrome *c* in ethanol-exposed neonatal mouse brain. Sections of WT (**a**–**c**) and Bax-deficient (**d**–**f**) brain were incubated with antibodies against activated caspase-3 and cytochrome *c* and detected with tyramide signal amplification using cyanine-3- (red) and fluorescein- (green) conjugated tyramine, respectively. Cell nuclei were stained with bisbenzimide (blue). Intense activated caspase-3 immunoreactivity was observed throughout the nucleus, soma, and neuritic projections of many neurons in the WT brain 8 h after ethanol exposure (**a**). Unlike neurons that lack activated caspase-3 immunoreactivity which exhibit punctuate cytochrome *c* immunoreactivity in their soma, neurons with activated caspase-3 immunoreactivity (outlined with dashes in panel (**b**)) display virtually no punctuate cytochrome *c* immunoreactivity (**b**). A composite image of the activated caspase-3, cytochrome *c*, and nuclear labeling is presented in panel (**c**). In the ethanol-exposed Bax-deficient brain, activated caspase-3 immunoreactivity was not observed (**d**) and cytochrome *c* immunoreactivity remained punctuate ((**e**); composite image in panel (**f**)). Scale bars equal 20 μm

currently being undertaken to determine which interpretation is correct. Brain regions other than AD nucleus, such as anterior cingulate cortex and CA1 region of hippocampus were also protected from ethanol toxicity in Bax-deficient mice (Table 1).

Absence of caspase-8 response to ethanol treatment

To clarify whether ethanol-induced neurodegeneration is mediated exclusively via the intrinsic apoptosis pathway or also utilizes the extrinsic pathway, we examined forebrain extracts from control and ethanol-exposed mice by Western blotting using antibodies that detect full length and cleaved (activated) caspase-8. There was no difference between control (n=5) and ethanol-treated mice in the amount of full length or cleaved caspase-8 immunoreactivity 8 h (n=5) after treatment (Figure 7). Similar results were obtained 10 h after treatment (data not shown). In contrast, as previously demonstrated, a marked increase in cleaved caspase-3 reactivity could be detected readily at 8 h and reached a peak at 12 h postethanol exposure.[2]

Discussion

Our findings indicate that in WT and heterozygous *bax*$^{+/-}$ mice, ethanol elicits robust caspase-3 activation and an equally robust neurodegenerative response, but in homozygous *bax* $^{-/-}$ mice there was no evidence for either phenomenon. Caspase-3 activation can occur either following activation of the intrinsic mitochondrial pathway – that is, release of cytochrome *c* from mitochondria and activation of caspase-9 – or following activation of caspase-8 from the death receptor pathway.[15] Since the proapoptotic action of Bax is mediated via the mitochondrial pathway,[8,9,16,17] the observation that Bax is required for ethanol-induced apoptotic neurodegeneration suggests that this apoptotic process involves not only Bax but also mitochondrial release of cytochrome *c* and activation of caspase-9. Our data confirmed ethanol-induced cytochrome *c* release and degradation in caspase-3-activated neurons in WT mice, but in *bax*$^{-/-}$ mice, ethanol did not induce either cytochrome *c* release or caspase-3 activation.

There is evidence for crosstalk between the Bax-regulated intrinsic mitochondrial pathway and the extrinsic death receptor pathway, in which activated caspase-8 is a key enzyme.[18] Specifically, it has been reported that activated caspase-8 can cleave Bid, which oligomerizes Bak or Bax into mitochondrial membrane pores that result in the release of cytochrome *c* and other mitochondria-derived proapoptotic molecules (e.g. Smac/DIABLO).[12,19] Noninvolvement of this caspase-8-dependent mechanism is suggested by our Western blot findings that in the postethanol treatment interval when apoptotic neurodegeneration is actively occurring, there is a robust caspase-3 activation response but no change in the level of activated caspase-8. Thus, we propose that ethanol-induced neuroapoptosis is an

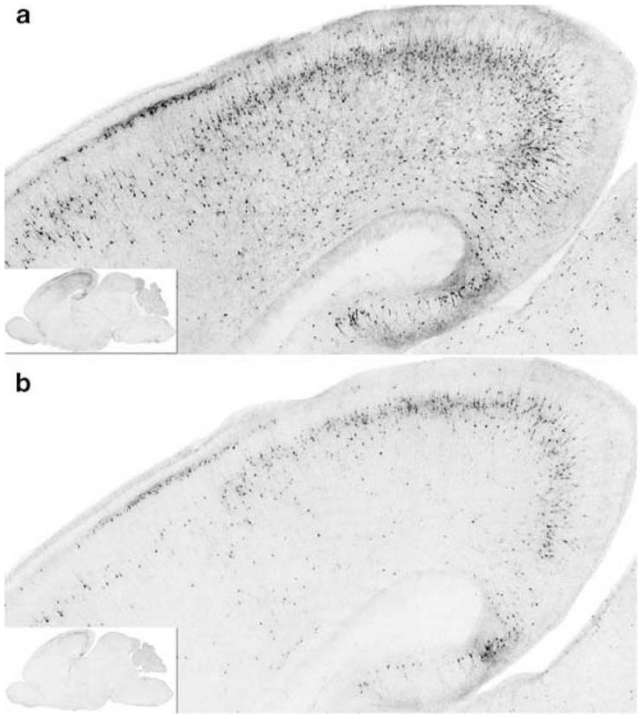

Figure 4 Serial sagittal sections (insets (**a**) and (**b**)) of WT mouse brains were immunohistochemically stained for activated caspase-3 (**a**) and caspase-3-produced spectrin fragments (**b**). The occipital cortex from each section is shown at higher magnification. In saline-treated animals, there were scattered active caspase-3 positive neurons, but no detectable caspase-3-cleaved spectrin-positive neurons (pictures not shown). Diffuse and robust activated caspase-3 staining was seen 8 h after ethanol treatment (panel (**a**)), with cortical layers II and IV showing the most abundant staining. A similar, but less widespread pattern was seen in sections stained for caspase-3-cleaved spectrin (panel (**b**)), suggesting that spectrin, after being cleaved by caspase-3, was rapidly degraded and, therefore, detectable in fewer neurons at any given time interval. Nevertheless, the similar staining pattern suggests that activated caspase-3 did function to cleave downstream proteins as an integral step in the cell death process

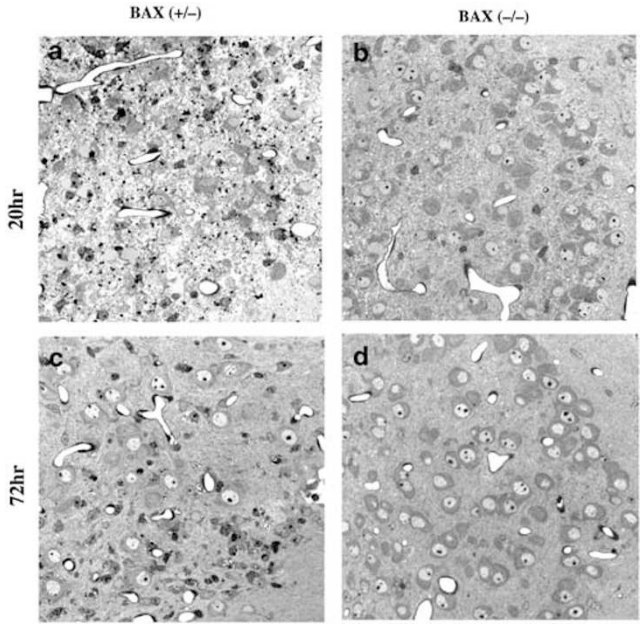

Figure 5 Semi-thin plastic sections from the AD nucleus of the thalamus of mouse brain either 20 or 72 h following ethanol treatment on the 7th postnatal day. (**a**) At 20 h after ethanol treatment, the AD nucleus of WT (not shown) and $bax^{+/-}$ mice displays many dark profiles, most of which are condensed and shrunken neurons in a late stage of degeneration, or apoptotic bodies given off by these degenerating neurons. There is a scarcity of normal appearing neurons and the neuropil throughout the AD region is markedly edematous. (**b**) The AD nucleus of the $bax^{-/-}$ mouse 20 h after ethanol is densely populated with normal appearing neurons and there are no signs of a pathological process. (**c**) At 72 h after ethanol, the pathological process in the AD nucleus of the $bax^{+/-}$ mouse has largely run its course, leaving a reduced number of normal appearing neurons and a residual accumulation of small dark-staining scavenger cells of uncertain origin. (**d**) At 72 h the AD nucleus of the $bax^{-/-}$ mouse is densely populated with normal appearing neurons and there are no signs of a pathological process. Comparing the number of normal neurons in C *versus* D (26 *versus* 77) reveals a three-fold difference, signifying that ethanol has deleted approximately 2/3 of the neurons from AD nucleus of the $bax^{+/-}$ brain, while leaving a normal number in the $bax^{-/-}$ brain

intrinsic pathway-mediated phenomenon, occurring in the absence of caspase-8 activation, and entailing Bax-induced release of mitochondrial cytochrome *c*, which leads, presumably via caspase-9 activation, to the formation of activated (cleaved) caspase-3.

Involvement of the mitochondrial pathway in the ethanol model of apoptotic neurodegeneration is consistent with ultrastructural evidence described recently by Dikranian *et al*.,[4] who found that early in the degenerative process induced by ethanol, neurons displayed distinctive mitochondrial changes, namely dissolution of the outer limiting membrane, but were otherwise free from changes in cytoplasmic organelles. Coincidental with these mitochondrial changes were more conspicuous changes in nuclear chromatin. It was not possible to determine whether the mitochondrial changes or nuclear chromatin changes occurred first, but both were detected early in the apoptotic neurodegenerative process, and this would be consistent with mitochondrial release of cytochrome *c* being an early event preceding changes in nuclear chromatin.

Our findings indicate that in the Bax-deficient condition, ethanol produced neither acute nor delayed neurodegeneration. This is in contrast to our recent finding that in caspase-3-deficient C57BL/6J mice, disruption of the *caspase-3* gene does not prevent ethanol-induced apoptosis, but rather causes it to occur on a delayed schedule with an altered morphological appearance.[20] In combination, these findings

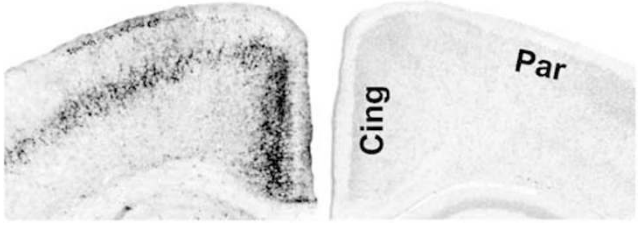

Figure 6 Silver-stained brain sections cut through the parietal (Par) and cingulate (Cing) cortex from 8-day-old $bax^{+/-}$ (left) and $bax^{-/-}$ mice (right), 24 h after ethanol treatment. The $bax^{+/-}$ brain shows a pattern of silver-positive neurodegenerative response, which is identical to that we have previously illustrated more extensively[2] in ethanol-treated WT C57BL/6 mice. In the brain of $bax^{-/-}$ mice, no evidence of silver-positive neurodegeneration was observed

Table 1 Neuron densities in three brain regions 72 h after ethanol treatment

Genotype	Ant. Cing (neurons/mm^2)	CA1 (neurons/mm^2)	AD (neurons/mm^2)
bax$^{-/-}$ (*n*=4)	1425.3±71.2**,†	4298.9±117.5*,†	1096.3±126.6*,††
bax$^{-/+}$ (*n*=4)	1069.6±112.7	2825.9±485.4††	597.0±96.9
bax$^{+/+}$ (*n*=4)	963.1±33.9	1809.6±76.6	707.3±37.3

Abbreviation and symbols: Ant. Cing, anterior cingulate cortex; CA1, CA1 region of hippocampus; AD, anterodorsal nucleus of thalamus; *$p<$ 0.01; **$p<$ 0.05 in comparison with bax$^{+/-}$; †$p<$ 0.01; ††$p<$ 0.05 in comparison with bax$^{+/+}$Statistics: ANOVA with *post hoc* test, Fisher's PLSD

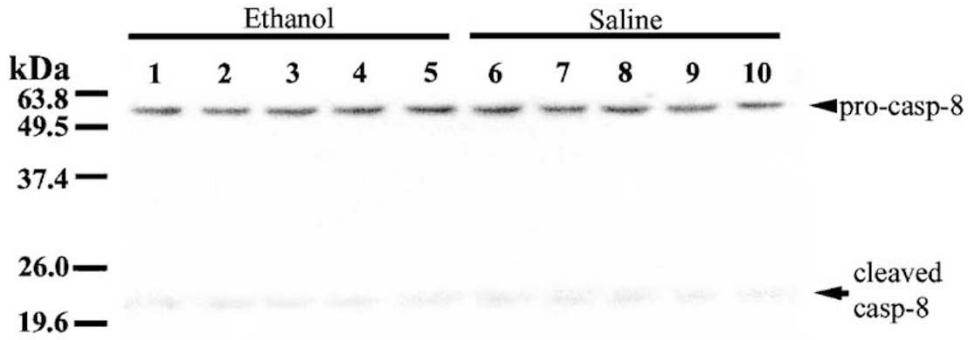

Figure 7 There is no change of caspase-8 activation after neonatal ethanol exposure in comparison with the saline-treated group. Protein extracts of ethanol-exposed (8 h post-treatment, lanes 1 – 5) and saline-treated (lanes 6 – 10) forebrains were prepared and analyzed for caspase-8 immunoreactivity on Western blot. The antibody recognizes both the proform of caspase-8 (55 kDa) as well as the cleaved (activated) forms which migrate at 40/36 (doublet, not detectable in this blot) and 23 kDa (arrow) in SDS-PAGE

suggest that the death commitment point in this system lies between Bax mitochondrial translocation and caspase-9-dependent caspase-3 activation.

Evidence that apoptotic neurodegeneration induced by ethanol, trophic factor deprivation,[10] axotomy[10] and neonatal hypoxia–ischemia[21] are all mediated via the mitochondrial pathway, makes it reasonable to propose that each of these three situations stresses the neuron in a similar manner. For example, the neuron is deprived in each case, deprived of a normal activity level, deprived of a vital trophic factor or deprived of a major limb through which it communicates with other neurons. Thus, in general, one might hypothesize that various types of deprivation stressors may serve as a trigger to initiate *bax*-dependent mitochondrial changes, from which apoptosis subsequently ensues.

Exposure of the developing human brain to ethanol causes a severe disruption in brain development and life-long neurobehavioral disturbances, a condition commonly referred to as the Fetal Alcohol Syndrome.[22–25] Recent research provides strong evidence that apoptotic deletion of neurons on a massive scale is the mechanism by which ethanol disrupts brain development in the rodent, and it seems likely that ethanol acts by the same mechanism to disrupt development of the human fetal brain.[1,4,26] The task now before us is to identify the signaling pathways and molecular events instrumental in mediating this apoptotic cell death process, and the ethanol-treated infant mouse provides an excellent model for this purpose. As an initial step toward fulfillment of this goal, we now present evidence that ethanol-induced neuroapoptosis in the developing mouse brain is mediated by the so-called intrinsic pathway, involving an action of Bax on mitochondrial membranes, causing mitochondrial release of cytochrome *c* and a series of subsequent events culminating in activation of caspase-3, which is instrumental in executing proteolytic events integral to the apoptotic cell death process.

Materials and methods

Animals and treatment

7 day old WT, *bax* $^{+/-}$ or *bax* $^{-/-}$, C57BL/6 mice were used in all experiments. The generation of *bax* $^{-/-}$ mice and the detection of endogenous and disrupted *bax* genes has been described previously.[11]

Ethanol was prepared as a 20% solution in normal saline and was administered subcutaneously to the mouse pups by a dosing regimen (2.5 g/kg at 0 h and again at 2 h) that we have found consistently produces a robust neurodegenerative response. At various intervals between 8 and 72 h following the first ethanol dose, the pups were deeply anesthetized with intraperitoneal injection of 10% chloral hydrate. Two small pieces of tail were cut from each pup for genotyping. The pups were then perfused through the left cardiac ventricle and ascending aorta with a fixative solution containing 4% paraformaldehyde in Tris or cacodylate buffer (for caspase-3 immunocytochemistry, DeOlmos silver staining or paraffin/H & E sections) or 4% paraformaldehyde and 2% glutaraldehyde in phosphate buffer (for plastic sections). The brains were cut into 50-μm-thick transverse sections on a Vibratome and processed for either silver staining or caspase-3 immunohistochemistry. For plastic sections, the brains were cut into 1 mm thick serial transverse slabs, postfixed in 1% osmium tetroxide, dehydrated in graded alcohol, cleared in toluene, and embedded in araldite. All the experiments included at least four pups in each group.

Genotyping mice

The endogenous and disrupted genes can be detected by PCR analysis of tail DNA extracts. Endogenous *bax* amplification using primers 5′-GCTATCCAGTT-CATCTCCAATTCGCC-3′ and 5′-GCTCTGAACAGAT-CATGAAGACAGGGG-3′ yields a 120 bp product, and the disrupted gene generated a 110 bp product with primers 5′-ATGGACGGGTCCGGG-GAGCAGCTT-3′ and 5′-GGGTGGGGTGGG- ATTAGATAAATG-3′. Cycling parameters were 10 min at 94°C, then 94°C for 1 min, 64°C for 1.5 min, and 72°C for 1.5 min for 30 cycles, followed by a 10 min extension at 72°C.

DeOlmos cupric silver-staining procedure

We have found that the DeOlmos cupric silver-staining method,[27] while not useful for distinguishing between apoptotic and nonapoptotic cell death processes, is an excellent method for marking dying neurons. For DeOlmos silver staining, the perfused brain is postfixed by immersion in the perfusate solution for 2 days before sectioning. It is then cut on a Vibratome into 50 μm thick sections. The free-floating sections are thoroughly washed in deionized distilled water and stained by the method of DeOlmos and Ingram.[27] In brief, the sections were incubated in cupric silver solution overnight, washed in acetone briefly, and stained with silver diammine solution for 35 min. The sections were reduced in citric acid solution for 2 min. After being washed with deionized distilled water, the sections were bleached with 0.3% $K_3Fe(CN)_6$, and stabilized with 0.1% $Na_2S_2O_3$.

Caspase-3, cytochrome *c* and spectrin immunohistochemistry

Vibratome sections, 50 μm thick, were removed from fixative, washed in 0.01 M PBS, quenched for 10 min in a solution of methanol containing 3% hydrogen peroxide, and then incubated for 1 h in blocking solution (2% BSA/0.2% milk/0.1% Triton X-100 in PBS), followed by incubation overnight in rabbit antiactive caspase-3 antiserum and antiserum specific to caspase-3-cleaved spectrin fragment (Cell Signaling Technology, Beverly, MA, USA) diluted 1 : 1500 in blocking solution. Following incubation with primary antiserum, the sections were incubated for 1 h in secondary antiserum (goat anti-rabbit 1 : 200 in blocking solution), then reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA), preincubated for 10 min in a filtered mixture containing 6 ml of 0.1 M Tris buffer, 2 mg DAB, and 400 mg imidazole, and then incubated for 15 min in 6 ml of the same DAB/imidazole/Tris mixture containing 1 μl H_2O_2. Alternatively, some sections were immunofluorescently stained, using CM1 antiactive caspase-3 primary antibody (BD Pharmingen, San Diego, CA, USA), tyramide signal amplification, cyanine-3 tyramide, and bisbenzimide nuclear labeling. Dual activated caspase-3 and cytochrome *c* immunostaining was performed as previously described.[28,29] Briefly, cytochrome *c* immunoreactivity was detected with mouse anticytochrome *c* antibodies (556433, Pharmingen, San Diego, CA, USA) and activated caspase-3 with rabbit antiactivated caspase-3 antiserum (D175, Cell Signaling Technology, Beverly, MA, USA). Primary antibodies were sequentially detected with horseradish peroxidase-conjugated secondary antibodies and direct tyramide signal amplification (TSA; Perkin-Elmer Life Science Products, Boston, MA, USA) with fluorescein and cyanine-3-conjugated tyramine. Fluorescently labeled sections were viewed with a Zeiss-Axioskop epifluorescence microscope and images were captured with a Zeiss Axiocam and Axiovision software.

Histopathology and cell counting

We used plastic sections or paraffin-embedded H & E sections for evaluation of the morphological appearance of neurons after ethanol treatment. Paraffin sections for H & E staining were cut 5 μm thick. Plastic sections, cut 1 μm thick, were heatdried onto microscopic slides and stained with a mixture of methylene blue (0.5%) and azure II (0.5%). For cell counts performed on the AD nucleus of the thalamus, we cut serial plastic sections through the entire extent of the AD nucleus and saved every fifth section. These sections were imaged and quantitatively evaluated with the help of a stereology system consisting of the following components: Stereo Investigator (MicroBrightField, Inc, Colchester, VT, USA) on a Pentium III PC, connected to a Prior Optiscan motorized stage (ES103 XYZ system, Prior Scientific Inc, Rockland, MA, USA) mounted on a Nikon Labophot-2 microscope. The contours of the AD nucleus were traced into the PC and from the tracings, Stereo Investigator calculated the area of AD nucleus in each section. Cell counts of surviving neurons were performed on sections cut through the AD nucleus at its greatest areal dimension. The population estimator function of Stereo Investigator was used to mark each neuron as it was counted to ensure that no neuron would be missed or counted twice. Only neurons with a visible nucleus were counted. A similar procedure was used to count survival neurons in the anterior cingulate cortex and CA1 region of hippocampus.

Western blot detection of cleaved caspase-3 and -8

Forebrain tissue was lysed by homogenizing in 400 μl of buffer A (10 mM HEPES, pH 7.4,. 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Chaps, 1 mM PMSF, and 1 μg/ml leupeptin) and was centrifuged at 12 000 $\times$ *g* for 10 min at 4°C, and the protein concentration of the lysates was determined with a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein samples (40 μg per lane) diluted in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) were boiled for 5 min, electrophoresed on a 15% SDS-PAGE and transferred to PVDF membrane (Immobilon P; Millipore, Burlington, MA, USA). The membrane was subsequently incubated with antiactive caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA) or anticaspase-8 polyclonal antibody (1 : 2000, BD PharMingen, San Diego, CA, USA), which recognizes both procaspase-8 (55 kDa) and the cleaved forms of caspase-8 (40 and 23 kDa). The membrane was then exposed to appropriate horseradish peroxidase-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) using a previously described procedure.[30]

Acknowledgments

This research was supported by NIH grants AG 11355, DA 05072, HD 37100, NS 35107. JWO is a NARSAD 2000 Toulmin Distinguished Investigator Awardee.

References

1. Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K and Olney JW (2000) Ethanol-induced apoptotic neurodegeneration and the fetal alcohol syndrome. Science 287: 1056–1060

2. Olney JW, Tenkova T, Dikranian K, Muglia LJ, Jermakowicz WJ, D'Sa C and Roth KA (2002) Ethanol-induced caspase-3 activation in the *in vivo* developing mouse brain. Neurobiol. Dis. 9: 205–219
3. Olney JW, Tenkova T, Dikranian K, Labruyere J, Qin YQ and Ikonomidou C (2002) Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. Dev. Brain Res. 133: 115–126
4. Dikranian K, Ishimaru MJ, Tenkova T, Labruyere J, Qin YQ, Ikonomidou C and Olney JW (2001) Apoptosis in the *in vivo* mammalian forebrain. Neurobiol. Dis. 8: 359–379
5. Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova T, Stefovska V, Turski L and Olney JW (1999) Blockade of glutamate receptors triggers apoptotic neurodegeneration in the developing brain. Science 283: 70–74
6. Ashkenazi A and Dixit VM (1998) Death receptors: signaling and modulation. Science 281: 1305–1308
7. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1312
8. Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D and Reed JC (1998) Bax directly induces release of cytochrome *c* from isolated mitochondria. Proc. Natl. Acad. Sci. USA 95: 4997–5002
9. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD (1997) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132–1136
10. Deckwerth TL, Elliott JL, Knudson CM, Johnson Jr. EM, Snider WD and Korsmeyer SJ (1996) Bax is required for neuronal death after trophic factor deprivation and during development. Neuron 17: 401–411
11. Shindler KS, Latham CB and Roth KA (1997) Bax deficiency prevents the increased cell death of immature neurons in bcl-x-deficient mice. J. Neurosci. 17: 3112–3119
12. Lazebnik YA, Kaufman SH, Desnoyers S, Poirier GG and Earnshaw WC (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371: 346–347
13. Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubia T, Saido TC and Green D (1995) Proteolysis of fodrin during apoptosis. J. Biol. Chem. 270: 6425–6428
14. Nath R, Huggins M, Glantz SB, Morrow JS, McGinnis K, Nadimpalli R and Wang KKW (2000) Development and characterization of antibodies specific to caspase-3-produced alpha II-spectrin 120 kDa breakdown product: marker for neuronal apoptosis. Neurochem. Int. 37: 351–361
15. Green DR (2000) Apoptotic pathways: paper wraps stone blunts scissors. Cell 102: 1–4
16. Brenner C and Kroemer G (2000) Apoptosis. Mitochondria – the death signal integrators. Science 289: 1150–1151
17. Wei MD, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, Macgregor GR, Thompson CB and Korsmeyer SJ (2001) Proapoptotic Bax and Bak: a requisite gateway to mitochondrial dysfunction and death. Science 292: 727–730
18. Li H, Zhu H, Xu CJ and Yuan J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491–501
19. Korsmeyer SJ, Wei MC, Saito M, Weller S, Oh KJ and Schlesinger PH (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. Cell Death Differ. 7: 1166–1173
20. Young C, Olney JW, Klocke BJ, Labruyere J, Qin YQ and Roth KA (2002) Absence of caspase-3 gene does not prevent but alters time course and morphological expression of ethanol-induced apoptotic neurodegeneration. Soc. Neurosci. Abstr. 28, Abst. #303.6
21. Gibson ME, Han BH, Choi J, Knudson M, Korsmeyer SJ, Parsadanian M and Holtzman DM (2001) BAX contributes to apoptotic-like death following neonatal hypoxia–ischemia: evidence for distinct apoptosis pathways. Mol. Med. 7: 644–655
22. Jones KL and Smith DW (1973) Recognition of the fetal alcohol syndrome in early infancy. Lancet ii: 999–1001
23. Jones KL and Smith DW (1975) The fetal alcohol syndrome. Teratology 12: 1–10
24. Stratton K, Howe C and Battaglia F (1996). *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention and Treatment*. (Washington, DC: National Academy Press)
25. Streissguth AP and O'Malley K (2000) Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders. Semin. Clin. Neuropsych. 5: 177–190
26. Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber N, Bittigau P and Ikonomidou C (2002) Brain Pathol 12: 488–498
27. DeOlmos JS and Ingram WR (1971) An improved cupric-silver method for impregnation of axonal and terminal degeneration. Brain Res. 33: 532–529
28. Roth KA (2002) Apoptosis techniques and protocols. In *Neuromethods* Vol. 3, LeBlanc AC, ed. (Totowa, NJ: Humana Press, Inc.), pp. 205–223
29. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA and Korsmeyer SJ (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400: 886–891
30. Deshmuk M, Vasilakos J, Deckwerth TL, Lampe PA, Shivers BD and Johnson Jr. EM (1996) Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE-family proteases. J. Cell. Biol. 135: 1341–1345