ELSEVIER

Neurobiology of Disease

www.elsevier.com/locate/ynbdi
Neurobiology of Disease 17 (2004) 403–414

Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults

David F. Wozniak,[a] Richard E. Hartman,[a] Maureen P. Boyle,[b,c,d] Sherri K. Vogt,[b,c,d] Ashley R. Brooks,[b,c,d] Tatyana Tenkova,[a] Chainllie Young,[a] John W. Olney,[a] and Louis J. Muglia[b,c,d,*]

[a]*Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110, USA*
[b]*Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110, USA*
[c]*Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA*
[d]*Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO 63110, USA*

Received 14 February 2004; revised 7 August 2004; accepted 25 August 2004

Administration of ethanol to rodents during the synaptogenesis period induces extensive apoptotic neurodegeneration in the developing brain. This neurotoxicity may explain the reduced brain mass and neurobehavioral disturbances in human Fetal Alcohol Syndrome (FAS). Here, we report binge-like exposure of infant mice to ethanol on a single postnatal day triggered apoptotic death of neurons from diencephalic structures that comprise an extended hippocampal circuit important for spatial learning and memory. The ethanol exposure paradigm yielding these neuronal losses caused profound impairments in spatial learning and memory at 1 month of age. This impairment was significantly attenuated during subsequent development, indicating recovery of function. Recovery was not associated with increased neurogenesis, suggesting plastic reorganization of neuronal networks compensated for early neuronal losses. We hypothesize that neuroapoptotic damage in homologous regions of human brain underlies cognitive deficits in FAS and the human brain of FAS victims has a similar capacity to effect functional recovery.

Keywords: Apoptosis; Behavior; Fetal Alcohol Syndrome; Hippocampus; Mice; Thalamus

Introduction

Exposure of in utero human fetuses to ethanol (EtOH) can cause a debilitating disorder termed the Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973). FAS is associated with reduced brain mass and a variety of neurobehavioral disturbances (Burd and Martsolf, 1989), ranging from attention-deficit hyperactivity disorder and mild to severe learning impairment in children, to a high incidence of major depression or psychosis in adults (Famy et al., 1998; Streissguth and O'Malley, 2000).

Transient exposure to EtOH during the synaptogenesis period, which occurs primarily postnatally in rodents, but both pre- and postnatally in humans, triggers a massive wave of apoptotic neurodegeneration in many different regions of the developing rodent brain that causes reduced brain mass (Ikondomidou et al., 1999; Olney et al., 2002a,b). The apoptotic potential of ethanol has been attributed to its ability to block NMDA glutamate receptors and activate $GABA_A$ receptors, since drugs that have either of these properties trigger EtOH-like neuroapoptosis in the neonatal rodent brain (Ikondomidou et al., 1999, 2000).

The ability of EtOH to induce widespread apoptotic neurodegeneration can explain many of the neuropathological findings and also the neurobehavioral impairments observed in FAS. However, because EtOH's potential to induce neuroapoptosis was discovered only recently, the relationship between apoptotic neuronal loss and ensuing cognitive dysfunction has not been adequately studied. Spatial learning deficits following neonatal EtOH administration have been reported in rats, but it is not clear whether learning impairments detected in juvenile animals are present to the same degree in adults. For example, "binge-like" EtOH exposure on one or more days from P4 to P10 in rats produces place learning (water maze) deficits when evaluated at P26–P61 (Goodlett and Johnson, 1997; Pauli et al., 1995;

* Corresponding author. Department of Pediatrics, Washington University School of Medicine, 660 S. Euclid Avenue, PO Box 8208, St. Louis, MO 63110. Fax: +1 314 286 2893.
E-mail address: Muglia_L@kids.wustl.edu (L.J. Muglia).
Available online on ScienceDirect (www.sciencedirect.com.)

0969-9961/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2004.08.006

Tomlinson et al., 1998), although data on whether these impairments remain into adulthood are much less consistent (Cronise et al., 2001; Kelly et al., 1988; Goodlett and Peterson, 1995). Thus, while evidence suggests that functional recovery from spatial learning deficits can occur in EtOH-treated rats, it is not clear whether differences in testing and exposure paradigms might explain at least some of the apparent functional recovery, nor is it clear how behavioral deficits and potential recovery relate to degenerative or regenerative changes in the brain.

In the present study, we exposed infant mice to EtOH on a single postnatal day (P7) in a manner simulating a binge-drinking episode and evaluated both the histopathological changes and subsequent neurobehavioral disturbances associated with such exposure. To clarify whether functional recovery from early learning deficits can occur in EtOH-treated mice, we used both longitudinal and cross-sectional research designs. In the histopathological analysis, special emphasis was focused on an "extended hippocampal circuit" comprised of the hippocampus, anterior thalamic nuclei (ATN), mammillary bodies, and retrosplenial cortex, which is believed to mediate allocentric (landmark-based) spatial learning and memory functions (Aggleton and Brown, 1999; Mitchell et al., 2002), such as those evaluated in rodents by using the Morris water and radial arm mazes. This memory model stresses the importance of interactions between the diencephalic components of this circuit (ATN and mammillary bodies) and the hippocampus as being critical for normal episodic memory. In accord with this notion, damage to individual components of the extended circuit has been shown to result in anterograde memory deficits (Aggleton and Brown, 1999) as evaluated by allocentric spatial learning/memory tests. We also explored the possibility that recovery of function might occur by regeneration of neurons to replace those that were deleted by EtOH-induced neuroapoptosis during development.

Materials and methods

Ethanol treatment

All mouse protocols were in accordance with NIH guidelines and were approved by the Animal Care and Use Committee of Washington University School of Medicine. C57BL/6 mice were weighed and received subcutaneous (sc) injections of EtOH (2.5 g/kg at 0 h and again at 2 h = 5.0 g/kg total) or normal saline on P7. The EtOH/saline treatment was distributed across sexes and litters as evenly as possible. In the first behavioral study, mice from six litters were sampled while eight litters were sampled in the second behavioral study. Subsets of mice used for the behavioral and histological studies (EtOH, $n = 16$; saline controls, $n = 17$) were weighed on a daily basis from P7 to P21 (weaning).

Blood EtOH concentrations

After decapitation, blood was collected in heparinized micro-hematocrit capillary tubes (VWR Scientific Inc. Chicago, IL) and centrifuged at 3000 rpm for 20 min. Ethanol standard solutions of 300, 100, 80, and 50 mg/dl were obtained from Sigma (St. Louis, MO). Ten microliters of plasma/standard solution was mixed with 3 ml NAD-ADH reagent in glycine buffer (NAD, 9.6 μmol and ADH, 800 U in 16 ml glycine buffer, Sigma). The absorbance of fluorescence at 340 nm was read on an Ultrospec 2100 pro UV/Visible Spectrophotometer (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 using Prism 3.0CX (GraphPad Software Inc., San Diego, CA). The correlation coefficient was 0.999. The ethanol concentrations of the samples were determined fitting the absorbances to the standard curve.

Histopathology

Acute effects

In acute studies, mice were dosed with the EtOH treatment described above on P7 and were killed at 8–24 h post-treatment. The brains were processed for cupric silver staining and activated caspase-3 immunohistochemistry (IHC) according to previously published methods (e.g., Olney et al., 2002a,b). Briefly, this involved subjecting the mice to intracardiac perfusion with a fixative composed of 4% paraformaldehyde in cacodylate buffer. After removal from the skull, brains were post-fixed by immersion in the perfusate solution for 2 days before the brain was cut into 50-μm sections using a vibratome. Free-floating sections were thoroughly washed in triple distilled water and stained using the cupric silver technique of de Olmos and Ingram (1971). Other sections were washed in 0.01 M phosphate-buffered saline (PBS), quenched for 10 min in a solution of methanol containing 3% hydrogen peroxide, then incubated for 1 h in blocking solution (2% bovine serum albumin (BSA)/0.2% milk/0.1% Triton X-100 in PBS), followed by incubation overnight in rabbit anti-active caspase-3 antiserum (D175, Cell Signaling Technology, Beverly, MA, USA) diluted 1:1500 in blocking solution. Following incubation with D175 primary antibody, the sections were incubated for 1 h in secondary antibody (goat anti-rabbit 1:200 in blocking solution) then reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit, Vector Labs., Burlingame, CA, USA), preincubated for 10 min in a filtered mixture containing 6 ml of 0.1 M Tris buffer, 2 mg diaminobenzidine (DAB), and 400 mg imidazole, and then for 15 min in 6 ml of the same DAB-imidazole-Tris mixture containing 3 μl H_2O_2.

Long-term effects

C57BL/6 mice were treated with saline or EtOH on P7 (as described above) and were killed on P14, P30, or P90 ($n = 5$–8 per treatment group per age). Mice sacrificed at the above three ages were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in PBS. Brains were postfixed in 4% paraformaldehyde for 1 day, cryopreserved in 30% sucrose, and cut into 50-μm coronal sections. All sections beginning rostrally at the lateral septal nuclei and continuing through the entire hippocampal formation were collected. Every other section beginning before and extending through the anterodorsal thalamic nuclei (ADT) was evaluated by light microscopy to determine the region of the ADT with the greatest cross-sectional area for demonstration of cellularity in Nissl-stained sections. For unbiased volume measurement, every 2nd section was collected for sampling and mounted onto glass slides, dried overnight, and mounted in medium containing a DAPI.

For nuclear visualization, we used an Olympus BX40 microscope equipped with a motor-driven stage and a permanently attached microcator to measure the z-axis position. A 4× objective lens was used to define the boundaries of the specific brain regions. For volume quantitation, we used StereoInvestigator software from

Micro Bright Field Inc (Colchester, VT). These protocols allow for bias-free sampling and provide automated control of the microscope stage movement to sampling sites and through the tissue section. Subregion volume was determined for the ADT and granular retrosplenial cortex using planimetry calculated by adding together the cross-sectional areas of all the referenced regions and multiplying by the distance between reference sections. Every fourth section was sampled for the granular retrosplenial cortex beginning at the start of the hippocampus and continuing until the first appearance of the posterior commissure. Every other section was sampled through the entire anterodorsal nucleus. Subregions were defined as previously described (Franklin and Paxinos, 1997).

BrdU labeling technique

C57BL/6 mice treated with normal saline or ethanol (n = 4–5 per group) on P7 underwent subcutaneous implantation of osmotic minipumps (Alza Corp.) on P21. Each pump delivered 10 μg/h of BrdU for a total of 7 days (20 mg/ml solution at 0.5 μl/h). On P54, mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in PBS. Brains were postfixed in 4% paraformaldehyde for 1 day, cryopreserved in 30% sucrose, and cut into 40-μm sections. For determination of total and neuron-specific BrdU-positive nuclei, three sections from each mouse spanning the ADT and including matched regions of the hippocampus were subjected to double label immunofluorescence. DNA in sections was denatured by incubation in 50% formamide/2× SSC at 65°C for 2 h, rinsed in 2× SSC, incubated 30 min in 2 N HCl at 37°C, then rinsed in 0.1 M borate buffer pH 8.5 for 10 min. After blocking in 3% normal horse serum/0.25% Triton X-100 in Tris-buffered saline, sections were incubated with mouse monoclonal anti-NeuN (1:200 dilution, Chemicon Intl., Temecula, CA) and rat monoclonal anti-BrdU (1:800 dilution, Abcam Ltd., Cambridge, UK)). Immunoreactivity was visualized by incubation with goat anti-mouse FITC and goat anti-rat Cy3 antibodies for NeuN and BrdU, respectively, each at a 1:200 dilution (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Matched regions of the hippocampus, granular retrosplenial cortex, and ADT had total BrdU-positive cells quantified, and differences between groups analyzed for statistical significance by unpaired t test.

Behavioral tests

One-hour locomotor activity and open-field behavior test

To evaluate general activity levels and possible alterations in emotionality, mice were evaluated over a 1-h period in transparent (47.6 × 25.4 × 20.6 cm high) polystyrene enclosures as previously described (Schaefer et al., 2000) before being first tested on the Morris water navigation test. In the first behavioral study, activity was evaluated during the early postweaning period while testing was conducted in early adulthood (P70) in the second behavioral study. Each cage was surrounded by a frame containing a 4 × 8 matrix of photocell pairs, the output of which was fed to an on-line computer (Hamilton-Kinder, LLC, Poway, CA). The system software (Hamilton-Kinder, LLC) was used to define a 33 × 11 cm central zone and a peripheral or surrounding zone that was 5.5 cm wide with the sides of the cage being the outermost boundary. This peripheral area extended along the entire perimeter of the cage. Variables that were analyzed included the total number of ambulations, as well as the number of entries, the time spent, and the distance traveled in the center area as well as the distance traveled in the periphery surrounding the center.

Sensorimotor battery

In order to evaluate possible effects induced by neonatal EtOH treatment on balance, strength, and coordination, the mice were tested on a sensorimotor battery shortly after being assessed on locomotor activity in the first and second behavioral studies (P25 and P72, respectively). The battery included inclined and inverted screen tests, as well as the platform, ledge, walking initiation, and pole tests performed according to previously published methods (Wang et al., 2002). The protocols are briefly described below for each test.

60° and 90° inclined screen and inverted screen tests. For the 60° and 90° inclined screen tests, each mouse was placed on top of an elevated (47 cm above the floor) wire mesh grid (16 squares per 10 cm) that was inclined to 60° or 90°. Each animal was placed in the middle of the screen with its head oriented down and was timed for how long it remained on the screen and how long it took to climb to the top of the screen. For the inverted screen test, mice were placed as above and then the screen was inverted to 180°. A maximum score of 60 s was given if an animal did not fall.

Platform test. Each mouse was timed for how long it remained on an elevated (47 cm above the floor) circular platform (1.0 cm thick; 3.0 cm in diameter). A maximum score of 60 s was assigned if the mouse remained on the platform for the maximum amount of time or if it could climb down on a very thin pole that supported the platform without falling.

Ledge test. Each mouse was timed for how long it could maintain its balance on a 0.75-cm-wide Plexiglas ledge without falling (60 s maximum). A score of 60 s was also assigned if the mouse traversed the entire length (51 cm) of the Plexiglas ledge and returned to the starting place in <60 s without falling.

Walking initiation test. Each mouse was placed in the middle of a square outlined by white cloth tape (21 × 21 cm) on a smooth black surface of a large table top. The time it took each mouse to leave the square (place all four paws outside of the tape) was recorded. The maximum time allowed was 60 s.

Pole test. A mouse was placed "head upward" on top of a vertical rod (diameter, 8 mm; height, 55 cm) that had a finely textured surface. A mouse was timed for how long it took it to turn downward 180° and how long it took it to climb down the pole and reach the floor. Each mouse was given two trials with each trial lasting a maximum of 120 s. If a mouse fell from the pole before reaching the floor it was given the maximum score of 120 s for that trial. A mean score was computed from the two trials given each animal.

Morris water navigation

Reference memory was evaluated using the Morris water navigation test utilizing procedures similar to previously published methods (Ho et al., 2000). Our protocol included cued (visible platform), place (submerged and not visible platform), and probe (platform removed) trials with both escape path length (distance traveled to platform) and latency (time taken to reach the platform) being collected for both cued and place trials and swimming speeds also being calculated for both types of trials. All trials were conducted in a round pool (100 cm inner diameter) of opaque water

and were videotaped, while swim paths were tracked and recorded by a computerized system (Polytrack, San Diego Instruments, San Diego, CA), which calculated escape path length and latency. Mice were first trained on the cued condition to determine if non-associative factors (e.g., sensorimotor or visual disturbances or alterations in motivation) were likely to affect acquisition performance during subsequent place trials. During cued trials, mice were trained to swim to a submerged platform, the location of which was marked (cued) by a rod protruding out of the water, and on top of which a red tennis ball was attached. Mice received four trials per day for two consecutive days of cued training. The platform was moved to a different location for each trial within a day and there were very few distal cues available during this time. An intertrial interval (ITI) of 60 s was used with a mouse being allowed to remain on the platform for 30 s before being removed. Movement of the platform on each trial, minimizing the number and salience of distal cues, and the brevity of training reduced the likelihood that significant spatial learning occurred during the cued trials. Three days later, the mice were trained in the "place" condition to learn the location of a submerged platform that was not apparent since the rod was removed. Multiple distinct spatial cues were positioned around the room for the place trials to facilitate association of cues with the submerged platform location. During place training, the mice were given four trials per day for 10 days (60-s maximum for a trial) with the platform remaining in the same location for all place trials using an ITI of 60 s. The daily protocol involved administering two blocks of two trials with each block being separated by approximately 2 h. A probe trial was administered approximately 1 h after completion of the place trials on the 5th and 10th days to evaluate retention of the platform location. During the 60-s probe trial, the escape platform was removed and a mouse was placed in the quadrant diagonally opposite from the previous platform location. Time spent and distance traveled in the quadrant where the platform had been, and the number of crossings made over the previous platform location (platform crossings), were recorded. In Study 1, testing began on P28 and mice were retested when they were 5–6 month of age while testing was initiated at 2.5 months of age in Study 2 and mice were retested at 8 months of age. Cued trials were not administered during the retests (second Morris water maze experiments) within Studies 1 or 2.

Radial arm maze

A win-shift spatial discrimination protocol was used to evaluate working memory capabilities according to previously published procedures in mice (Hartman et al., 2001; Wozniak et al., 1996). The maze was a standard 8-arm radial maze consisting of an octagonal central platform enclosed by a Plexiglas frame containing eight experimenter-controlled doors that blocked access to the eight arms. The mice were habituated to handling and the experimental procedures and were shaped to traverse the arms and retrieve and consume a food reward ('Frooty Pebble') placed in a cup at the end of each arm. Acquisition involved baiting each arm with a Frooty Pebble piece and training a mouse to visit each baited arm and consume the reinforcer (a correct response) and to remember the arms that it had been reinforced in so that it would not revisit those arms (commit a retracing error). Acquisition was defined by an a priori criterion of at least eight correct responses out of the first nine responses for four consecutive days. Mice from Study 2 were first evaluated at 4.5–5.5 months of age.

Statistical analyses

In general, ANOVA models were used to analyze behavioral data. Typically, the statistical models included two between-subjects variables, Treatment (EtOH vs. saline) and gender, and usually one within-subjects variable, such as Blocks of Trials. When ANOVAs with repeated measures were conducted, the Huynh-Feldt (H-F) adjustment of alpha levels was used for all within-subjects effects containing more than two levels in order to protect against violations of the sphericity/compound symmetry assumptions underlying this ANOVA model.

Results

Blood ethanol levels

The P7 EtOH treatment paradigm we employed resulted in sustained blood EtOH levels that have been noted previously to produce neurotoxic effects in neonatal rats (Ikonomidou et al., 2000). Specifically, the blood EtOH data show two peaks across post-treatment times (Fig. 1). The first peak (270 mg/dl) occurred 45 min after the first 2.5 g/kg dose while the second peak (510 mg/dl) occurred 1 h after the second 2.5 g/kg dose (3 h after the first dose). This blood EtOH curve is very similar to that observed for rats and shares the important property that blood EtOH levels remained above the toxic threshold (above 200 mg/dl for more than 2 h in the rat) for producing extensive apoptotic neurodegeneration.

Histopathology

The P7 EtOH exposure described above resulted in a widespread pattern of neurodegeneration throughout the forebrain when evaluated by the de Olmos method of cupric-silver staining and by activated caspase-3 IHC, consistent with our previously published results in C57BL/6 mice (Olney et al., 2002a,b). In the present work, we focused particular attention on specific brain regions that

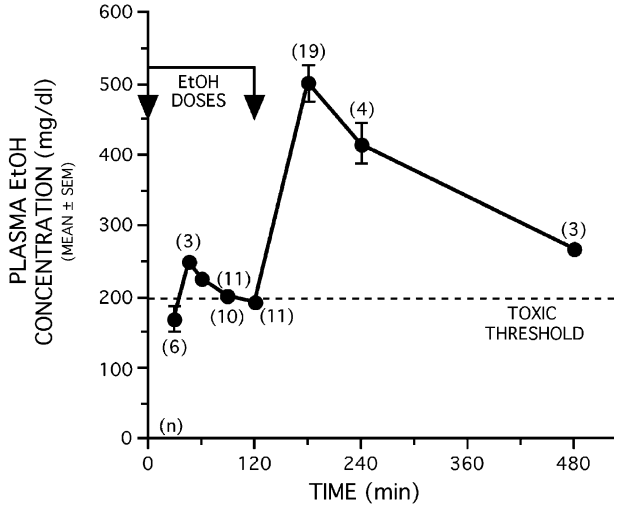

Fig. 1. Blood ethanol concentrations as a function of post-treatment time. Ethanol (EtOH) was administered to C57BL/6 mice on P7 as two doses of 2.5 g/kg that were spaced 2 h apart. The number of independent samples for each time point is indicated in parentheses.

are integral components of an "extended hippocampal" diencephalic circuit, which have been identified as important for mediating learning and memory functions. In addition to the hippocampal formation, the major brain regions comprising this circuit are the anterior thalamic nuclei (ATN), mammillary bodies, and retrosplenial cortex. Many of the regions damaged by P7 EtOH treatment are shown at two rostrocaudal levels in silver-stained sections in Fig. 2. The ATN and retrosplenial cortex are two structures that are severely damaged at these two levels. The magnitude of damage sustained in the ATN was greatest in the anterodorsal thalamic nucleus (ADT), which showed a 60% long-term reduction in total volume at P14, P30, and P90 as determined by stereological methods (Fig. 3). Similarly, an ANOVA on the volumes measured for the retrosplenial cortex at P30 and P90 yielded a significant main effect of Treatment [$F(1,8) = 8.74$, $P = 0.018$], indicating that the volumes of the retrosplenial cortex in the ethanol groups were significantly smaller than controls. Both P30 (Saline 0.099 $\pm$ 0.002 mm^3 vs. EtOH 0.089 $\pm$ 0.004 mm^3) and P90 (Saline 0.080 $\pm$ 0.005 mm^3 vs. EtOH 0.072 $\pm$ 0.003 mm^3) showed a 10% reduction in retrosplenial cortex volume in ethanol groups, which achieved statistical significance in pairwise comparison at P30 ($P = 0.037$) but not P90.

Activated caspase-3 IHC staining after ethanol administration demonstrated that the mechanism of cell death reflected in silver-stained neurons in Fig. 2 was apoptosis for the ATN and mammillary bodies (Fig. 4). Previously published studies have demonstrated activated caspase-3 immunoreactivity in the retrosplenial cortex and hippocampus (Olney et al., 2002b), as well, indicating apoptotic neuronal damage occurs to all areas of the extended hippocampal circuit.

General health and behavioral effects

General health

In our first study, mice were weighed and inspected on a daily basis following EtOH or saline injections on P7. No obvious developmental anomalies were observed in EtOH-treated mice. An analysis of body weight gains indicated that EtOH-treated mice showed a significantly lower weight gain than saline controls on P8 ($P < 0.0005$), but they rapidly resumed their normal daily weight gains such that no differences were found in weight gains after P8 (data not shown).

Behavioral effects—Study 1

In order to determine how behavior is altered by early neonatal EtOH treatment, we tested the mice on a 1-h locomotor activity/open-field behavior test and on a sensorimotor battery during the first week postweaning. No differences were found between EtOH-treated mice and controls on either the 1-h locomotor activity/open-field behavior test or on any of the measures of the sensorimotor battery (data not shown). We next assessed how EtOH exposure affected subsequent spatial learning and memory by evaluating the performance of the mice on the Morris water navigation test. In the first Morris water navigation experiment, the mice were tested on the cued (visible platform) condition on P28–29 to determine if any subtle behavioral changes (e.g., sensorimotor or visual disturbances or alterations in motivation) would likely affect subsequent performance on the place trials. Consistent with the findings from the 1-h activity test and sensorimotor battery, the groups did not differ in escape path length (Fig. 5A) or latency (not shown), nor were there differences in swimming speeds (not shown) during the cued trials.

Although the groups of mice performed similarly during the cued trials, EtOH mice were profoundly impaired during the place trials (Fig. 5B). An ANOVA on the path length data from the place trials showed a significant main effect of Treatment, ($F(1,16) = 33.999$, $P < 0.0005$), and a significant Treatment by Blocks of Trials interaction [$F(9,144) = 2.447$, $P = 0.025$ (H-F corrected)]. An ANOVA on the latency data yielded similar results (data not shown). Not only were there very large performance differences between the two groups during place trials, EtOH mice showed no improvement over time, suggesting that little or no learning had occurred in this group. Consistent with these results, EtOH mice were also significantly impaired in probe trial performance (Figs. 5C and D). EtOH mice spent significantly less time in the target quadrant compared to controls [$F(1,16) = 8.520$, $P = 0.010$] and made fewer platform crossings [$F(1,16) = 9.625$, $P = 0.007$]. The mice were retested on the water navigation task when they were 5–6 months of age to determine if any place learning deficits could still be detected at this time. No performance differences between

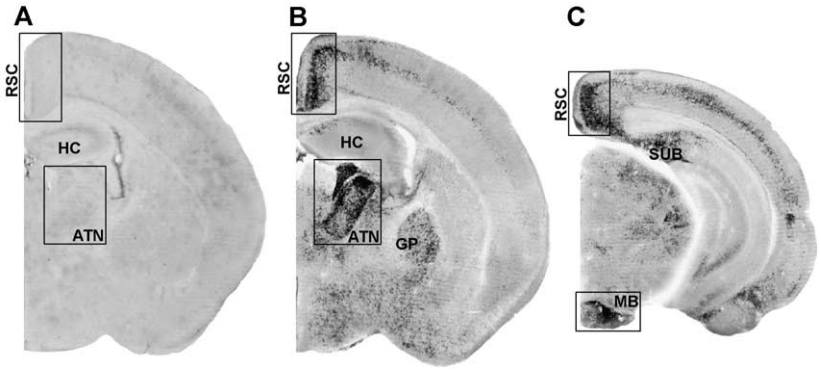

Fig. 2. The histopathological effects of P7 EtOH administration on brain regions comprising an "extended hippocampal circuit". The hippocampus (HC), retrosplenial cortex (RSC), anterior thalamic nuclei (ATN), and mamillary bodies (MB) are depicted in silver-stained coronal sections of a saline control brain (A) and a brain exposed to ethanol 24 h earlier (B and C). The silver stain detects all of the neuronal degeneration that has occurred over the 24 h posttreatment interval (Olney et al., 2002b). During this stage in development, apoptotic neurodegeneration occurs in normal brain, but the dying cells are sparse so that in the saline control brain they are barely detectable at low magnification. In contrast, following ethanol treatment, at two rostrocaudal levels, most brain regions show degenerative changes and some, including the ATN, RSC, and MB, are very severely affected. In the HC, a patchy pattern of degeneration was evident, and the degree of damage was in the mild to moderate range. Additional areas showing silver deposition are the globus pallidus (GP) and subiculum (SUB).

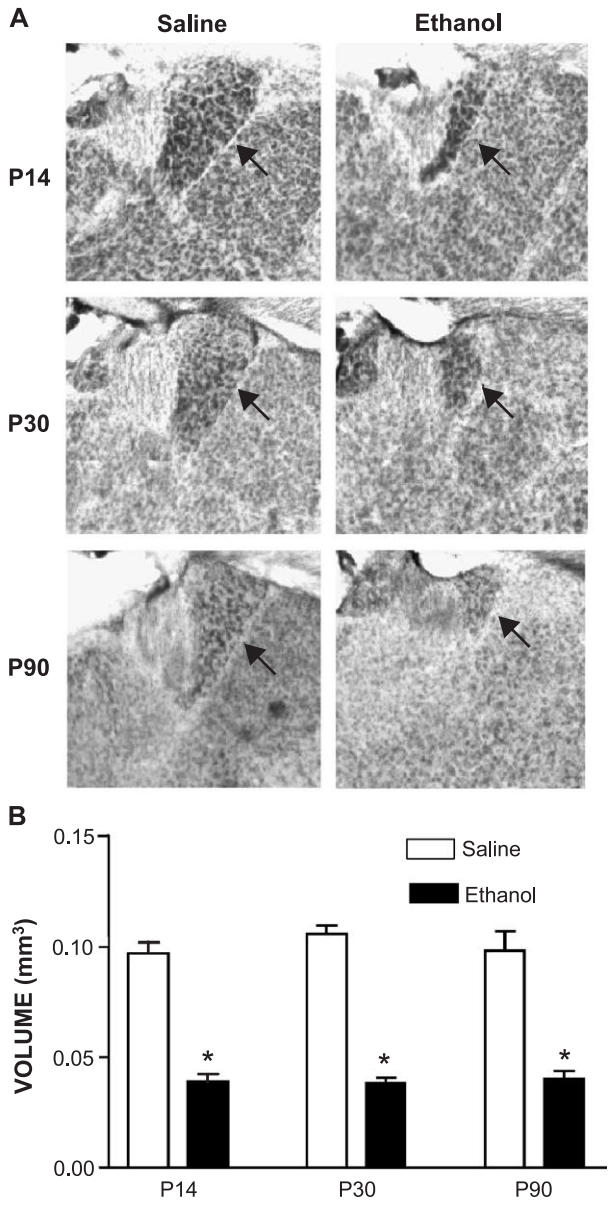

Fig. 3. Treatment with EtOH in P7 mice produces long-term damage in the anterodorsal nucleus of the thalamus (ADT). (A) Nissl-stained sections in saline controls (left panels) and EtOH-treated mice (right panels) that were sacrificed at P14, P30, or P90 (representative of n = 5–8 per group). Note the reduction in the mass of the ADT (arrows) across all ages in the EtOH-treated mice, suggesting substantial and permanent neuronal loss in this nucleus while the ADT remains relatively unchanged across the different-aged control mice. (B) Volume quantitation of the ADT using unbiased stereological methods. The graph demonstrates a significant main effect of Treatment ($P < 0.0005$), with subsequent pairwise comparisons showing significant differences between saline and ethanol groups at each postnatal day (*$P < 0.0005$). There was no significant effect of postnatal day or Treatment by postnatal day interaction.

groups were present at this time with regard to place (Fig. 5E) or probe conditions.

Behavioral effects—Study 2

In our second study (Study 2) involving larger sample sizes, mice that were treated with the same EtOH dosing regimen or normal saline on P7 were tested on an expanded version of the sensorimotor battery and on the 1-h locomotor activity/open-field behavior test before being evaluated on the water navigation task at 2.5 months of age, a time midway between the period during which EtOH-treated mice failed to learn and then later demonstrated learning in the initial study. Similar to the results from Study 1, the groups of mice did not differ on any of the measures within the sensorimotor battery. However, the EtOH mice in Study 2 (Fig. 6) were found to be hyperactive relative to controls for both total ambulations [$F(1,42) = 9.38$, $P = 0.004$) and total distance traveled [$F(1,42)$ 6.63, $P = 0.014$). EtOH mice also traveled a greater distance in the center area [$F(1,42) = 7.09$, $P = 0.011$] likely due to heightened general activity levels since the EtOH mice also traveled a greater distance in the periphery [$F(1,42) = 4.92$, $P = 0.032$]. The ANOVAs also yielded significant group by gender interactions for all of the above variables ($P < 0.05$) except for distance traveled in the periphery. As Fig. 6 shows, the hyperactivity of the EtOH mice was mostly due to the male EtOH mice being significantly more active relative to the male control mice ($P < 0.012$) on all four of the variables mentioned above while the female groups did not differ on any of the variables.

When the mice were evaluated on the first water maze test at 2.5 months of age, no differences were observed between the groups during the cued trials condition (Fig. 7A), although the EtOH mice were again impaired during the place trials (Fig. 7B). An ANOVA on the path length data from the place trials showed a significant main effect of Treatment [$F(1,42) = 7.724$, $P = 0.008$] and a significant Treatment by Blocks of Trials interaction [$F(9378) = 2.493$, $P = 0.016$ (H-F corrected)]. An ANOVA on the latency data yielded similar results (not shown). Both groups showed evidence of improved performance over time, suggesting that learning had occurred in both groups. Moreover, the EtOH mice eventually performed at the level of the control mice toward the end of the acquisition trials. The EtOH mice were not significantly impaired in probe trial performance compared to controls (not shown) for either time spent in the target quadrant or platform crossings. Retesting the mice on the water navigation task when they were 8 months of age (Fig. 7C) provided some evidence

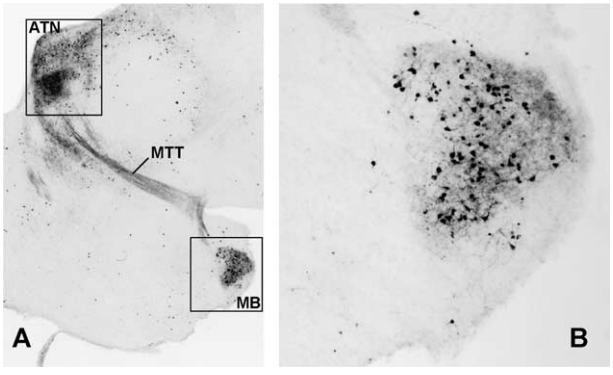

Fig. 4. Ethanol treatment in P7 mice induces robust activated caspse-3 staining in the anterior thalamic nuclei (ATN) and mamillary bodies (MB). (A) Sixteen hours after EtOH treatment, a prominent pattern of caspase-3 activation is shown in the ATN and MB, and in the mammillothalamic tract (MTT) through which these two structures are interconnected in this sagittal section. (B) A higher magnification of the MB that is enclosed within the "boxed" area in the lower right-hand corner of "A" is shown.

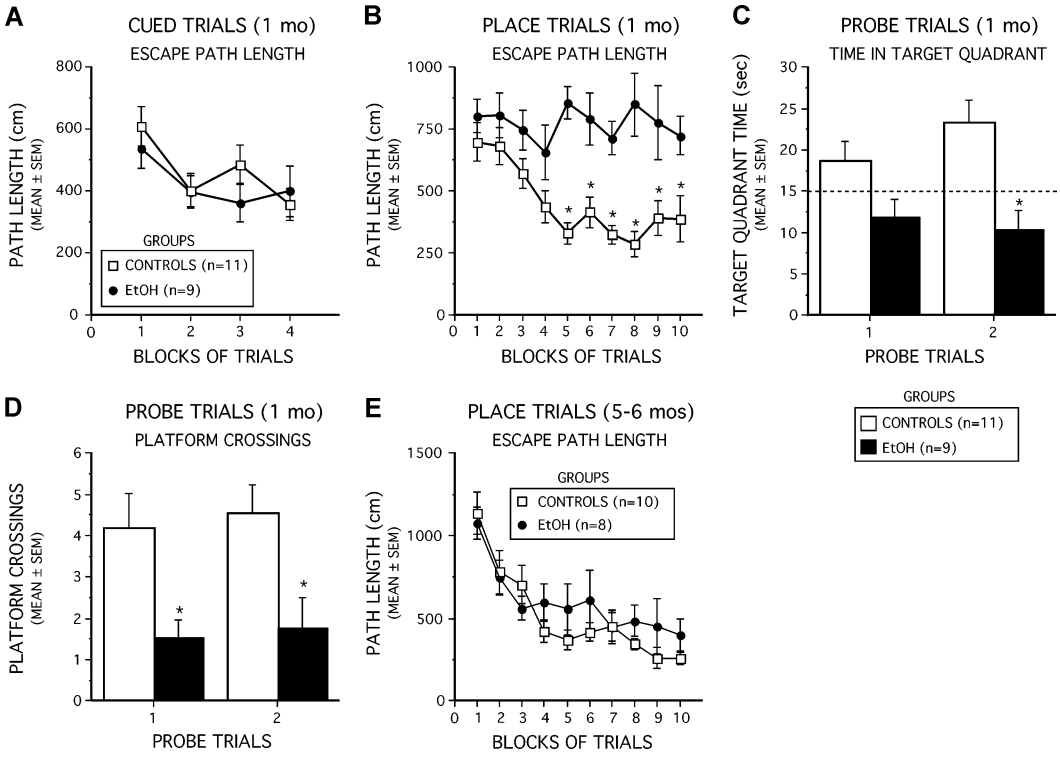

Fig. 5. Performance of EtOH-treated and saline control mice on the Morris water navigation task in Experiment 1. (A) EtOH-treated and control mice did not differ in performance during cued trials as indexed by escape path length. (B) In contrast, the EtOH mice were profoundly impaired during the place trials. Although the groups started out at similar levels, the control mice showed progressive improvement while the EtOH mice showed no evidence of learning. The control mice exhibited significantly shorter escape path lengths during the 5th through the 10th blocks of trials ($P < 0.003$ for blocks 5–8; $P < 0.028$ for blocks 9 and 10, see text for additional statistical details). The group differences in acquisition performance were also reflected in retention performance during probe trials in terms of time spent in the target quadrant (C) and platform crossings (D). A significant main effect of Treatment was found for time spent in the target quadrant ($P = 0.010$) where the platform had been and subsequent contrasts showed that the controls spent significantly more time in the target quadrant compared to the EtOH mice during the second trial ($P = 0.005$). The dotted line shows the expected time in the target quadrant based on chance alone. A significant main effect of Treatment was also found for platform crossings ($P = 0.007$) and subsequent comparisons showed that the control mice had significantly more platform crossings than the EtOH mice for both trials 1 and 2 ($P = 0.032$ and 0.024, respectively). (E) Different results were found compared to the P30 place trial data when mice were retested on the place condition as adults (5–6 months of age) using a different submerged platform location. As adults, the EtOH-treated mice not only showed evidence of place learning during acquisition, but they also performed as well as the saline controls. This finding was further reinforced by a lack of differences between groups on probe trial measures (data not shown).

that the EtOH mice still exhibited mildly impaired performance during the place trials. Specifically, an ANOVA on the path length data yielded a significant main effect of Treatment [$F(1,41) = 4.607$, $P = 0.038$]. However, analysis of the latency data showed a similar trend in group performances although the effect of Treatment did not reach significance ($P = 0.098$). In addition, similar to earlier findings, the groups did not differ with regard to probe trial performance for either time spent in the target quadrant or platform crossings.

In addition to the Morris water maze, these groups of mice were also tested on a working memory protocol in the radial arm maze. When tested in the radial arm maze at 4.5–5.5 months of age, the EtOH mice required significantly more days to reach the acquisition criterion compared to controls [$F(1,42) = 5.81$, $P = 0.020$] although differences in errors to criterion were not significant (Figs. 8A–C). Additional contrasts showed the presence of gender-dependent effects with significant performance differences between the groups of males for both days to criterion ($P = 0.048$) and errors to criterion ($P = 0.035$) while the performance of the female groups did not differ significantly on either variable (Figs. 8D and E).

Analysis of neurogenesis

The results from the two studies described above strongly suggest that the profound early place learning performance deficits in EtOH-treated mice diminish with age. This functional recovery from the cognitive deficits present in juvenile (P30) EtOH-treated mice could result from either augmented neurogenesis or plasticity of surviving neurons to compensate for early cell damage. Since evidence of improved learning was present in EtOH-treated mice tested at P75, we hypothesized that neurogenesis occurring before this period would result in either sustained maintenance or increased production of new neurons that could compensate for earlier losses. To evaluate neurogenesis, we implanted 7-day sustained-release mini-osmotic pumps containing BrdU into normal saline control or ethanol-treated mice at P21 and harvested the mice at P54 to allow an adequate interval for neuronal differentiation and assessment of survival. Quantitation of NeuN-positive neurons in sections encompassing the largest cross-section of the ADT revealed a 73% reduction in neuron number in EtOH-treated mice (saline 202 ± 33, $n = 5$; ethanol 54 ± 9, $n = 5$; $P = 0.002$) when analyzed at P54, consistent with the profound damage

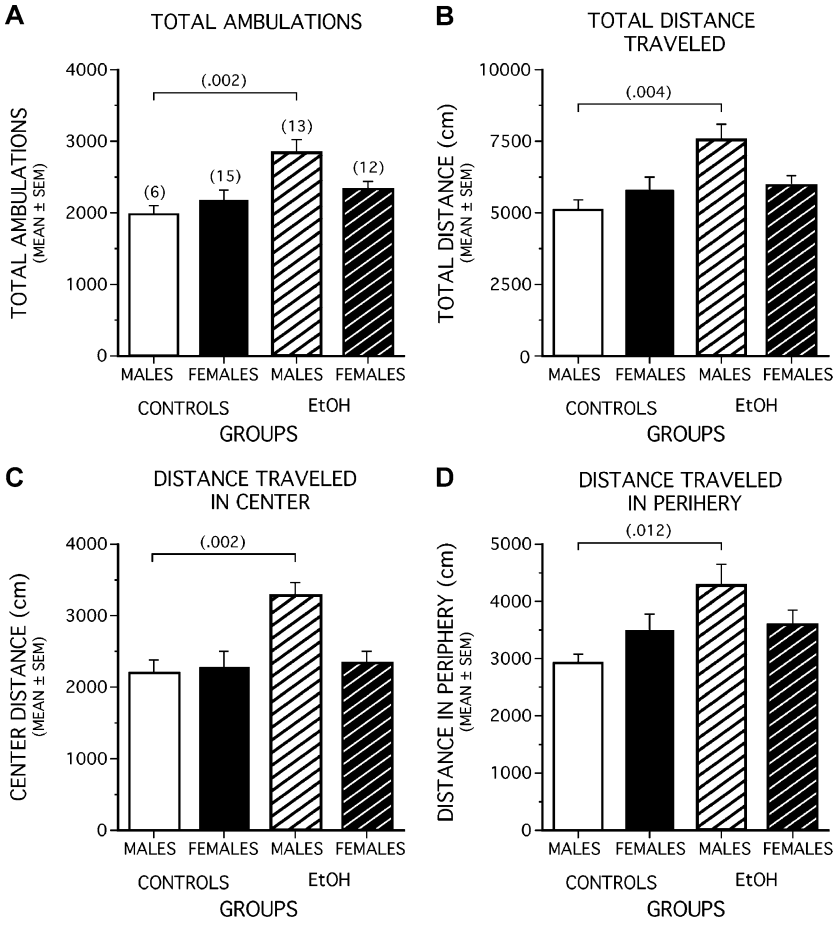

Fig. 6. The EtOH-treated mice in Study 2 were hyperactive relative to controls when evaluated in early adulthood (P70) and this was mostly due to gender-related effects involving the hyperactivity of the male EtOH mice. Specifically, the male EtOH mice exhibited significantly more total ambulations (A; $P = 0.002$), traveled a greater total distance (B; $P = 0.004$), and traveled greater distances in the center (C; $P = 0.002$) and periphery (D; $P = 0.012$), compared to the saline control mice.

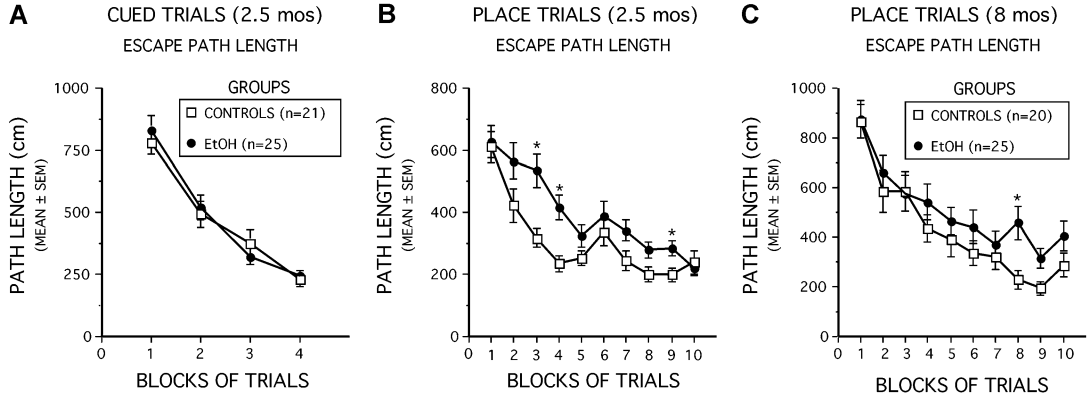

Fig. 7. Performance of the EtOH-treated and saline control mice on the water navigation task in Study 2 when first tested in early adulthood (2.5 months old) and later in life (8 months old). (A) The EtOH and control mice performed almost identically during the cued trials for escape path length when tested at 2.5 months of age. (B) However, the EtOH mice were significantly impaired for path length during the place trials compared to controls (see text for statistical details) when tested at this age. Although the two groups started the place trials at similar performance levels, the control mice improved more rapidly than the EtOH mice and exhibited significantly (*) shorter path lengths on the 3rd, 4th, and 9th blocks of trials ($P < 0.004$ for blocks 3 and 4; $P < 0.016$ for block 9). Note, however, the EtOH mice eventually performed as well as the control mice by the end of acquisition training. (C) When the mice were retested at 8 months of age, a significant main effect of Treatment ($P = 0.038$) indicated that the control mice still performed better than the EtOH mice in terms of path length. Subsequent contrasts showed that the control mice had significantly (*) shorter path lengths compared to the EtOH mice for block 8 ($P = 0.007$).

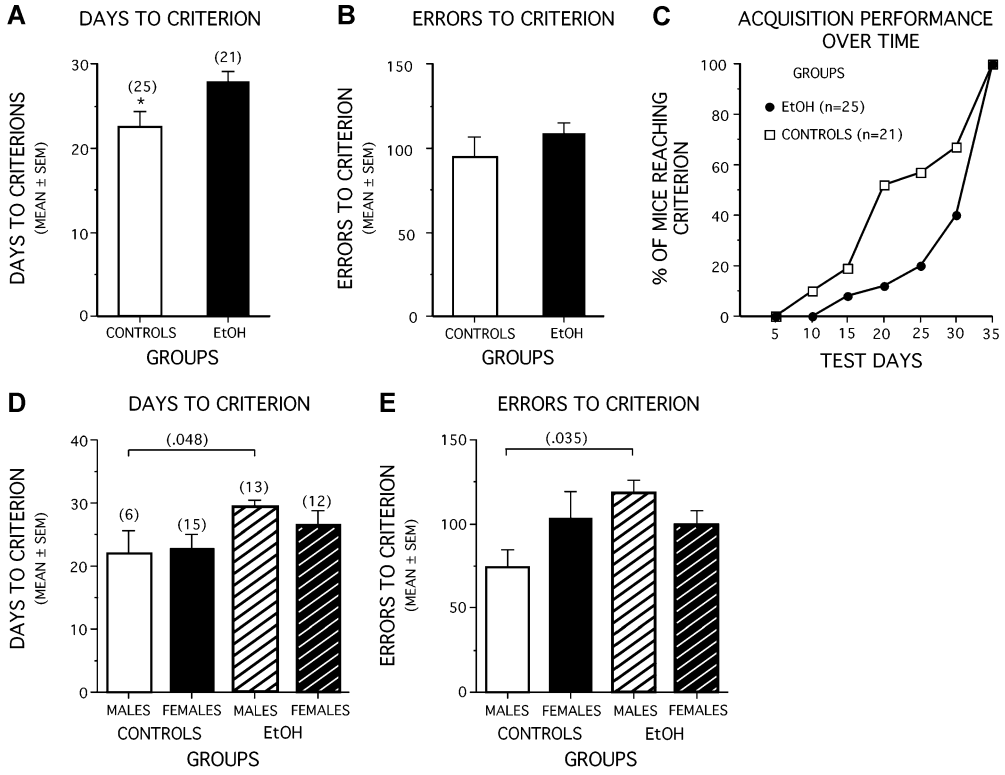

Fig. 8. Performance of the EtOH-treated and saline control mice on the working memory version (win-shift spatial discrimination) of the radial arm maze when tested at 4.5–5.5 months of age in Study 2. (A) The EtOH mice required significantly more days to criterion than the control mice although the two groups did not differ in errors to criterion as shown in "B". (C) The acquisition rate of the controls was faster than that of the EtOH mice. Note that the curves come together by day 35. This reflects terminating the experiment and assigning scores to animals that had not reached criterion. (D and E) These graphs show that much of the group differences in radial maze performances were due to gender-related effects in that the EtOH males required significantly more days (D) and errors (E) to reach criterion compared to male control mice, while the female groups did not differ on either of the performance variables.

we observed 1 day after ethanol treatment and the sustained reduction in ADT size we found in stereological volume analyses at P14, P30, and P90 (Fig. 9). We found no BrdU-labeled neurons in either the retrosplenial cortex or anterodorsal thalamic nucleus, while in these same mice we found robust neurogenesis in the dentate gyrus of the hippocampus, an area well known to support postnatal neuron birth (n = 4 per group; Fig. 9). The percent of BrdU-labeled cells that were NeuN-positive neurons (saline 81.4 ± 4.2%; ethanol 84.8 ± 3.2%) and the total number of BrdU-labeled cells (saline 170.8 ± 17.7; ethanol 190.5 ± 11.4) in matched sections of the dentate gyrus did not differ between normal saline and ethanol groups. Similar results were obtained in mice undergoing BrdU labeling at postnatal day 40 for 7 days (data not shown).

Discussion

Here, we demonstrate that exposure of infant mice to EtOH on a single postnatal day (P7) induces extensive apoptotic neurodegeneration in the developing brain, and subsequent spatial learning and memory impairments that are very severe at P30, less severe if testing is first performed at P75, and minimal in later adulthood. Longitudinal behavioral testing of the same animals at different ages, together with cross-sectional testing at different ages (thereby avoiding repeated measures effects), provided evidence favoring the interpretation that recovery of reference-memory-based functions occurred following the initial period of profound learning/memory impairment. In adulthood, working memory performance is also subtly compromised in EtOH-treated mice in a gender-dependent fashion, with the male EtOH mice being functionally impaired. Because food restriction is part of the radial arm maze procedure, we did not evaluate juvenile mice on this test to avoid adversely affecting somatic development. As a result, we cannot assess whether the limited differences in working memory in adult mice reflect recovery in this domain or whether the deficits were less profound initially. Additional studies are needed to determine the degree of impairment of EtOH-treated mice on working-memory-based tests during the juvenile period and whether there are gender-dependent effects at this age as well as during later adulthood.

Because neuronal degeneration was particularly severe in several of the specific brain regions that comprise the extended hippocampal (diencephalic) circuit believed to be important for spatial learning and memory, we propose that deletion of neurons from portions of this circuit contributed substantially to the profound learning and memory impairments measured at P30. We looked for, but did not find, evidence that neurons deleted from components of this circuit, or from other brain regions, were replaced by newly generated neurons. Therefore, recovery of function in the period between P30 and adulthood presumably occurred by a process of adaptation of surviving brain regions to the early neuronal loss. This adaptation could include remodeling of synaptic connections, changes in intrinsic physiological proper-

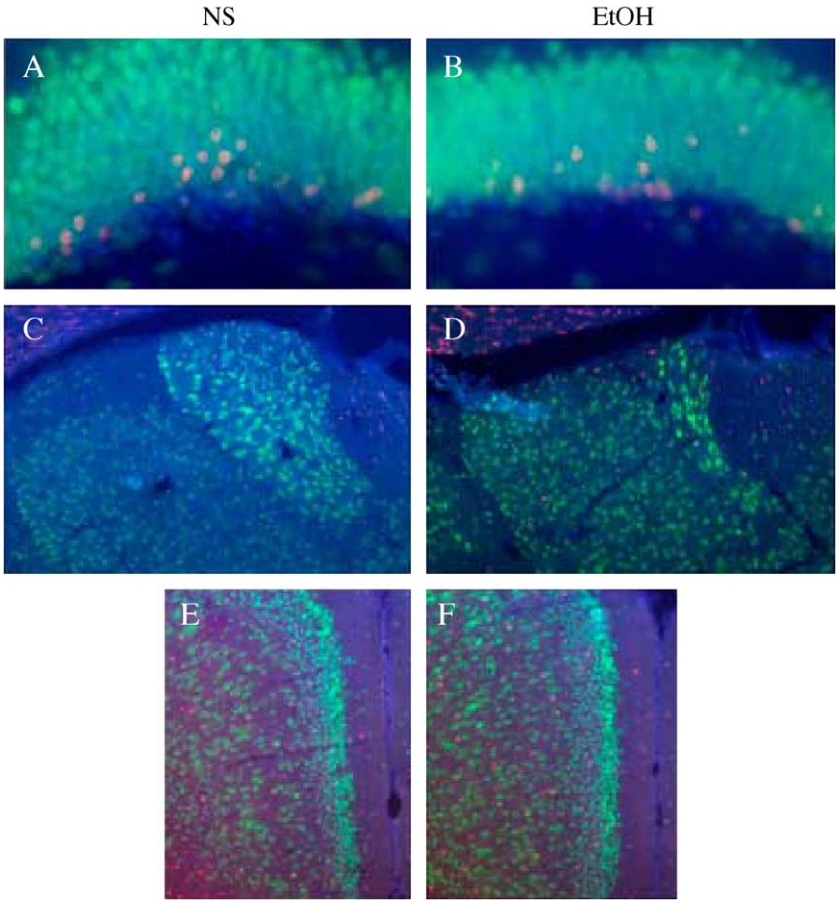

Fig. 9. Analysis of adult neurogenesis in ethanol-treated mice. Normal saline (NS, panels A, C, and E) or EtOH-treated (B, D, and F) mice underwent 7 days of treatment with BrdU beginning at P21 and were harvested for analysis at P54 (n = 4–5). Immunoreactive NeuN (green) and BrdU (red) appear yellow when co-localized. All sections are counterstained with DAPI (blue). Shown are representative matched regions of hippocampal dentate gyrus (A and B), ADT (C and D), and retrosplenial cortex (E and F). Double-labeled BrdU and NeuN-positive neurons were only found in the hippocampal dentate gyrus.

ties, or acquisition in other brain regions of new functions, of the surviving neurons. Using this mouse model, it may be possible to differentiate among these possible mechanisms using genetic and pharmacological methods in future studies and further support the hypothesis that similar plasticity and functional recovery could occur in human patients with FAS.

The research design we employed—examining the behavioral status of some animals longitudinally and others cross-sectionally—permits the conclusion that mice have the potential to recover from certain severe spatial learning deficits induced by EtOH during early development. These findings are supported by comparing across results of previous studies done on rodents of different ages. For example, following treatment of infant rats with EtOH on one or more days from P4 to P10, others have reported that performance during place trials in the Morris water maze was impaired when animals were tested at P26–P61 (Goodlett and Johnson, 1997; Pauli et al., 1995; Tomlinson et al., 1998), and when tested at later ages only males (Johnson and Goodlett, 2002) or only females (Kelly et al., 1988) were impaired during place trials. This suggests the possibility that the impairment was more robust and easily detected at the earlier ages and was beginning to resolve at later ages. The ability to regain learning capacity following severe apoptotic neuronal losses is of considerable interest because developmental neuroapoptosis is a newly discovered mechanism that may contribute to human neurodevelopmental delays, not only in FAS but following early exposure to other environmental agents, such as anesthetic (Jevtovic-Todorovic et al., 2003) and anticonvulsant (Bittigau et al., 2002) drugs. We propose that the nervous system may be able to overcome this type of insult more readily than certain other types, such as an ischemic infarct which, instead of subtly removing a percentage of neurons from many brain regions, totally destroys all neurons in certain specific regions and causes irreversible telltale neurological deficits, such as spastic paralysis. If, as we propose, surviving neurons have the plastic capacity to effect substantial recovery of function following this kind of developmental insult, the implications are not trivial. It would signify that substantial developmental neuroapoptosis can occur following exposure to EtOH or various other environmental neuroapoptogenic agents and go undetected, because recovery of function may remove the symptomatic traces efficiently enough such that only the most severe cases will come to medical attention and be diagnosable as a pathological deviation from the norm.

To our knowledge, this study is the first attempting to characterize extensive neuropathological changes that may underlie the behavioral outcomes following single-day exposure of infant rodents to EtOH. In examining the pattern of neuroapoptosis induced by EtOH in P7 mouse brain, we were struck by the observation that hippocampal damage was only moderately severe compared to the much more dramatic damage affecting certain other structures that have been described as components of an

extended hippocampal circuit that is believed to mediate spatial learning and memory functions (Aggleton and Brown, 1999). Based on this observation, we propose that it would be an oversimplification to attribute EtOH-induced developmental memory impairments to hippocampal damage per se, and more realistic to ascribe it, at least in large part, to pervasive damage riddling the extended hippocampal circuit. This is not to imply that damage in other CNS areas played no part, but rather that the severe damage affecting several extrahippocampal components of the extended hippocampal circuit makes these components, individually and collectively, prime candidates for explaining developmental delays. This hypothesis is consistent with the results of recent studies showing that the ATN (Aggleton et al., 1996; Mitchell et al., 2002; van Groen et al., 2002) and the mammillary bodies (Aggleton et al., 1995; Krazem et al., 1995; Sziklas and Petrides, 1993) may play significant roles in mediating learning and memory functions, and the extensive damage to these structures observed in the present study, particularly the long-term devastation of the ADT, supports this hypothesis. While long-term changes have been described in several brain regions in humans with severe in utero alcohol exposure (Mattson et al., 2001), one recent study evaluating humans with FAS and mild cognitive deficits by positron emission tomography demonstrated relatively restricted decreases in metabolism that prominently involved the thalamus (Clark et al., 2000). This finding suggests that the thalamic nuclei may also show heightened susceptibility to ethanol-induced damage in humans. Regarding the possibility that neuronal losses from other brain regions play a role, normal performance of EtOH-treated mice in sensorimotor tasks and during the cued trials in the Morris maze tend to rule out a major contribution of these other neuronal losses. It should be noted, however, that EtOH treatment during the neonatal period can delete neurons from all levels of the visual system (retina, lateral geniculate nucleus, superior colliculus, visual cortex) (Tenkova et al., 2003) and from the cerebellum (Bauer-Moffet and Altman, 1977; Dikranian et al., 2002; Goodlett and Eilers, 1997; Hamre and West, 1993). However, which neurons will be deleted depend on the timing of exposure. For example, cerebellar Purkinje neurons are selectively vulnerable in the P2 to P5 period and retinal ganglion cells in the P1 to P3 period. Therefore, motor or visual disturbances attributable to these neuronal losses would not be relevant to the present study in which EtOH exposure was limited to P7.

Our findings have potential significance for long-term outcomes in humans afflicted with FAS who often have severe learning disabilities and attention deficit disorder. Our data suggest that even in severely compromised children, early deficits may be at least partially overcome in later life. The mouse model used in the present study is a favorable model for studying early apoptotic neuronal losses and recovery therefrom, in that it lends itself to genetic as well as behavioral, pharmacological, biochemical, and histopathological investigations aimed at determining the mechanism(s) by which destructive and reparative processes take place and for identifying future targets of therapeutic intervention.

Acknowledgment

We thank Anna Sahrhage for assistance with the behavioral testing. This work was supported by NIH grants AA12957 (L. J. M) from the NIAAA, and DA05072 and HD37100 (J. W. O. and D. F. W.) from the NIDA and NICHD, respectively.

References

Aggleton, J.P., Brown, M.W., 1999. Episodic memory, amnesia, and the hippocampal-anterior thalamic axis. Behav. Brain Sci. 22, 425–489.

Aggleton, J.P., Neave, N., Nagle, S., Hunt, P.R., 1995. A comparison of the effects of anterior thalamic, mammillary body and fornix lesions on reinforced spatial alternation. Behav. Brain Res. 68, 91–101.

Aggleton, J.P., Hunt, P.R., Nagle, S., Neave, N., 1996. The effects of selective lesions within the anterior thalamic nuclei on spatial memory in the rat. Behav. Brain Res. 81, 189–198.

Bauer-Moffet, C., Altman, J., 1977. The effect of ethanol chronically administered to preweaning rats on cerebellar development. Brain Res. 119, 249–268.

Bittigau, P., Sifringer, M., Genz, K., Reith, E., Pospischil, D., Govindarajalu, S., Dzietko, M., Pesditschek, S., Mai, I., Dikranian, K., Olney, J.W., Ikonomidou, C., 2002. Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. Proc. Natl. Acad. Sci. U. S. A. 99, 15089–15094.

Burd, L., Martsolf, J.T., 1989. Fetal alcohol syndrome: diagnosis and syndromal variability. Physiol. Behav. 46, 39–43.

Clark, C.M., Li, D., Conry, J., Conry, R., Loock, C., 2000. Structural and functional brain integrity of fetal alcohol syndrome in nonretarded cases. Pediatrics 105, 1096–1099.

Cronise, K., Marion, M.D., Tran, T.D., Kelly, S.J., 2001. Critical periods for the effect of alcohol on learning in rats. Behav. Neurosci. 115, 138–145.

de Olmos, J.S., Ingram, W.R., 1971. An improved cupric-silver method for impregnation of axonal and terminal degeneration. Brain Res. 33, 523–529.

Dikranian, K.T., Labruyere, J., Qin, Y.Q., Olney, J.W., 2002. Ethanol-Induced Apoptotic Neurodegeneration in the Developing Rodent Cerebellum and Brain Stem. Program No. 105.8 2002 Abstract Viewer/Itinerary Planner. Society for Neuroscience, Washington, DC. Online.

Famy, C., Streissguth, A.P., Unis, A.S., 1998. Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. Am. J. Psychol. 155, 552–554.

Franklin, K.B.J., Paxinos, G., 1997. The Mouse Brain in Stereotaxic Coordinates. Academic Press, San Diego, CA.

Goodlett, C.R., Peterson, S.D., 1995. Sex differences in vulnerability to developmental spatial learning deficits induced by limited binge alcohol exposure in neonatal rats. Neurobiol. Learn. Mem. 64, 265–275.

Goodlett, C.R., Eilers, A.T., 1997. Alcohol-induced Purkinje cell loss with a single binge exposure in neonatal rats: a stereological study of temporal windows of vulnerability. Alcohol., Clin. Exp. Res. 21, 738–744.

Goodlett, C.R., Johnson, T.B., 1997. Neonatal binge ethanol exposure using intubation: timing and dose effects on place learning. Neurotoxicol. Teratol. 19, 435–446.

Hamre, K.M., West, J.R., 1993. The effects of the timing of ethanol exposure during the brain growth spurt on the number of cerebellar Purkinje and granule cell nuclear profiles. Alcohol., Clin. Exp. Res. 17, 610–622.

Hartman, R.E., Wozniak, D.F., Nardi, A., Olney, J.W., Sartorious, L., Holtzman, D.M., 2001. Behavioral phenotyping of GFAP-apoE3 and -apoE4 transgenic mice: ApoE4 mice show profound working memory impairments in the absence of Alzheimer's-like neuropathology. Exp. Neurol. 170, 326–344.

Ho, N., Liauw, J.A., Blaeser, F., Wei, F., Hanissian, S., Muglia, L.M., Wozniak, D.F., Nardi, A., Linden, D.J., Zhuo, M., Muglia, L.J., Chatila, T.A., 2000. Impaired synaptic plasticity and CREB activation in CaMKIV/Gr-deficient mice. J. Neurosci. 20, 6459–6472.

Ikondomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., Tenkova, T., Stevosk, V., Turski, L., Olney, J.W., 1999. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283, 70–74.

Ikonomidou, C., Bittigau, P., Ishimaru, M.J., Wozniak, D.F., Koch, C., Genz, K., Price, M.T., Stefovska, V., Hörster, F., Tenkova, T., Dikranian, K., Olney, J.W., 2000. Ethanol-induced apoptotic

neurodegeneration and fetal alcohol syndrome. Science 287, 1056–1060.

Jevtovic-Todorovic, V., Hartman, R.E., Izumi, Y., Benshoff, N.D., Dikranian, K., Zorumski, C.F., Olney, J.W., Wozniak, D.F., 2003. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J. Neurosci. 23, 876–882.

Johnson, T.B., Goodlett, C.R., 2002. Selective and enduring deficits in spatial learning after limited neonatal binge alcohol exposure in male rats. Alcohol. Clin. Exp. Res. 26, 83–93.

Jones, K.L., Smith, D.W., 1973. Recognition of the fetal alcohol syndrome in early infancy. Lancet. 2, 999–1001.

Kelly, S.J., Goodlett, C.R., Hulsether, S.A., West, J.R., 1988. Impaired spatial navigation in adult female but not adult male rats exposed to alcohol during the brain growth spurt. Behav. Brain Res. 27, 247–257.

Krazem, A., Beracochea, D., Jaffard, R., 1995. Effects of mammillary bodies and mediodorsal thalamic lesions on the acquisition and retention of a learning set in mice: paradoxical effect of the intersession interval. Behav. Brain Res. 67, 51–58.

Mattson, S.N., Schoenfeld, A.M., Riley, E.P., 2001. Teratogenic effects of alcohol on brain and behavior. Alcohol Res. Health 25, 185–191.

Mitchell, A.S., Dalrymple-Alford, J.C., Christie, M.A., 2002. Spatial working memory and the brainstem cholinergic innervation to the anterior thalamus. J. Neurosci. 22, 1922–1926.

Olney, J.W., Tenkova, T., Dikranian, K., Labruyere, J., Qin, Q.Y., Ikonomidou, C., 2002a. Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. Dev. Brain Res. 133, 115–126.

Olney, J.W., Tenkova, T., Dikranian, K., Muglia, L.J., Jermakowicz, W.J., D'Sa, C., Roth, K.A., 2002b. Ethanol-induced caspase-3 activation in the in vivo developing mouse brain. Neurobiol. Dis. 9, 205–219.

Pauli, J., Wilce, P., Bedi, K.S., 1995. Spatial learning ability of rats following acute exposure to alcohol during early postnatal life. Physiol. Behav. 58, 10013–11020.

Schaefer, M.L., Wong, S.T., Wozniak, D.F., Muglia, L.M., Liauw, J.A., Zhuo, M., Nardi, A., Hartman, R.E., Vogt, S.K., Luedke, C.E., Storm, D.R., Muglia, L.J., 2000. Altered stress-induced anxiety in adenylyl cyclase type VIII-deficient mice. J. Neurosci. 20, 4809–4820.

Streissguth, A.P., O'Malley, K., 2000. Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders. Semin. Clin. Neuropsychol. 5, 177–190.

Sziklas, V., Petrides, M., 1993. Memory impairments following lesions to the mammillary region of the rat. Eur. J. Neurosci. 5, 525–540.

Tenkova, T., Young, C., Dikranian, K., Labruyere, J., Olney, J.W., 2003. Ethanol-induced apoptosis in the developing visual system during synaptogenesis. Investig. Ophthalmol. Vis. Sci. 44, 2809–2817.

Tomlinson, D., Wilce, P., Bedi, K.S., 1998. Spatial learning ability of rats following differing levels of exposure to alcohol during early postnatal life. Physiol. Behav. 63, 205–211.

van Groen, T., Kadish, I., Wyss, J.M., 2002. Role of the anterodorsal and anteroventral nuclei of the thalamus in spatial memory in the rat. Behav. Brain Res. 132, 19–28.

Wang, Q., Bardgett, M.E., Wong, M., Wozniak, D.F., Lou, J., McNeil, B.D., Chen, C., Nardi, A., Reid, D.C., Yamada, K., Ornitz, D.M., 2002. Ataxia and paroxysmal dyskinesia in mice lacking axonally transported FGF14. Neuron 35, 25–38.

Wozniak, D.F., Brosnan-Watters, G., Nardi, A., McEwen, M., Corso, T.D., Olney, J.W., Fix, A.S., 1996. MK-801 neurotoxicity in male mice: histologic effects and chronic impairment in spatial learning. Brain Res. 707, 165–179.