Exposure to Ethanol during Gastrulation Alters Somatosensory–Motor Cortices and the Underlying White Matter in the Macaque

The present study tests the hypothesis that a critical window for cortical development coincides with the period of neural stem cell proliferation (during the first 6 weeks of gestation), specifically, gastrulation (on embryonic day [E] 19 and E20). Pregnant female macaques were exposed to ethanol 1 day/week for 6 or 24 weeks such that it included E19 or E20 or a time before or after the time of gastrulation. Total forebrain size was increased in macaques exposed to ethanol on E19 or E20. Thus, various features of the gray and white matter of the paracentral lobule of adolescent offspring were examined. Ethanol exposure affected the gray matter, for example, the 1.63 billion neurons in somatosensory cortex of controls (areas 3a and 3b) was 32% lower in ethanol-exposed monkeys, but neither duration nor timing of the episodic exposure had a differential effect. In contrast, the timing of the exposure during the third week critically affected the amount of white matter (the mass of myelopil, but not cell number). Therefore, fetal exposure to ethanol unveils a normal programming mechanism wherein neural stem cells appear to be a target and a critical window for forebrain development concurs with gastrulation.

Keywords: alcohol, autism, cell counts, fetal alcohol syndrome, fetal programming, hypertrophy, monkey, motor cortex, neural stem cell, somatosensory cortex, white matter

Introduction

Prenatal exposure to ethanol causes a variety of defects including changes to craniofacial structures and the brain (Miller, 2006a). The constellation of facial dysmorphology, intrauterine growth retardation/microcephaly, and mental dysfunction comprise the fetal alcohol syndrome (FAS). There appear to be critical windows for many of the features associated with FAS. For example, mice episodically exposed to ethanol on embryonic day (E) 7 or E8 (the time of gastrulation) exhibit the craniofacial malformations associated with FAS (e.g., Sulik et al. 1981; Sulik 2005). Similarly, monkeys exposed to ethanol during gastrulation (E19 or E20) also have FAS-like craniofacial malformations (Astley et al. 1999). This is particularly compelling because monkeys exposed to ethanol before and/or after this time have a normal facial appearance. Even if the monkey has been exposed at other times, the (narrow) window concurrent with gastrulation seems to be critical for producing the facial dysmorphia. This is likely because other developmental events have broader developmental windows and thereby allow the organism to mount compensatory responses, that is, recovery from the primary damage.

It is reasonable to predict that ethanol-induced changes in craniofacial structure are associated with altered brain stem structure. Indeed, brain stem development experiences critical windows of ethanol vulnerability. For the brain stem, these windows have been most thoroughly studied in rodents, and they coincide with gastrulation (Dunty et al. 2002; Sulik 2005; Mooney and Miller 2007), neuronogenesis (Miller and Muller 1989; Miller 1995), synaptogenesis (Pentney and Miller 1992; West et al. 1994; Miller 1995; Pierce et al. 1999; Lindsay 2006), and the period of naturally occurring neuronal death (Miller 1995; Pierce et al. 1999; Miller et al. 2006). In monkeys, too, exposure during gastrulation affects brain stem nuclei (Mooney and Miller 2001). Thus, the brain stem is vulnerable to ethanol over a broad period: from before neurons go through their final mitoses through the time when they are forming connections with other postmigratory neurons.

Whereas neurons in primate brain stem nuclei are among the first to be produced, neurons in the forebrain, for example, the cerebral cortex, develop later and over a more protracted period (Jacobson 1991). Presumably, episodic exposure to ethanol has less of an effect on forebrain neurons because there is time for compensation due to the longer time line of cortical development. The present study used the primate forebrain to test the hypothesis that challenge, for example, exposure to ethanol, during the time of gastrulation can alter forebrain structure. For ethanol to have such an effect requires that some facet of stem-cell definition occurs at the time that the basic plan for the nervous system is being specified (i.e., gastrulation) which is many days before neurons or glia in a particular structure are generated.

Methods

Subjects and Their Care

Fifteen Macaca nemestrina (6 controls and 9 exposed to ethanol) were reared and grown at the University of Washington Primate Center (e.g., Clarren and Astley 1992; Astley et al. 1999). They were mated at the peak time of fertility, that is, peak vulvar tumescence, according to strict mating procedures. Pregnancy was verified by laproscopic examination, and the first day of a successful pregnancy was designated as E0 (Heuser and Streeter 1941; Clarren et al. 1987).

Animals were exposed to ethanol 1 day/week (Clarren et al. 1987; Clarren and Astley 1992). Ethanol (1.8 mg/kg) was delivered by intra-gastric intubation in a single episode, every seventh day, for example, E7, E14, E21, E28, etc. This exposure varied in 2 important ways.

1. The duration of the period during which the monkeys were exposed was either 6 or 24 weeks (Table 1). The first 6 weeks is the time when neural stem cells destined to become cortical cells are generated. The next 18 weeks include the period when cortical neurons pass through their final mitotic division (Rakic 1974).

2. The timing of the exposure during the critical week of E19-E25 was assessed. Studies from the mouse and rat show that a window of vulnerability to ethanol exposure occurs during the process of gastrulation (e.g., Sulik et al. 1981; Ashwell and Zhang 1999; Dunty et al. 2002; Sulik 2005; Mooney and Miller 2007). In the embryonic...
monkey, the analogous time is Heuser and Streeter Stage 8, on E19 and E20 (Heuser and Streeter 1941; Hendrickx and Sawyer 1975). Some monkeys were exposed on E19 or E20, and other monkeys received their weekly dose of ethanol on E21 or E24. Note, as previous described by Astley et al. (1999), the macaques exposed to ethanol on E19 or E20 exhibited the facial dysmorphism characteristic of FAS. Monkeys exposed to ethanol on E21 or E24 did not have craniofacial malformations. It must be emphasized that the ethanol exposure was not restricted to just a single day during the week from E19 to E25. Macaques were exposed before and after this critical week. In summary, animals were described by 2 features, the period over which they received their ethanol treatment and the exposure during the week of E19-E25. This resulted in animals being designated into 1 of 4 groups (Table 1). For the purposes of the analysis, these groups were collapsed to determine the effect of the duration (Group A/C vs. Group B/D) and the timing (Group A/B vs. Group C/D) of the ethanol exposure.

Control monkeys (n = 6) were intubated 1 day/week with saline. Samples of blood were taken from the prospective mothers 2 h after the ethanol delivery and tested for ethanol content with gas chromatography (Baselt 1987). Plasma ethanol concentration peaked 100-120 min after dosing (Clarron and Astley 1992). Mean maternal blood ethanol concentrations were 231 ± 18 mg/dL and 234 ± 12 mg/dL for the Group A/B and Group C/D monkeys, respectively, and 231 ± 12 mg/dL for the Group A/B and Group C/D monkeys, respectively.

Each pregnancy resulted in a single offspring. No significant difference in birth weight was detected among ethanol-treated and control infants (Clarron et al. 1987; Clarron and Astley 1992). All offspring were surrogate-fostered by nursing mothers who had had no exposure to ethanol during their pregnancies. The young monkeys were weaned and group housed after their first birthdays.

### Tissue Preparation
When they reached adolescence (i.e. 5 years old), the animals were anesthetized (2.0 mg/kg xylazine and 10 mg/kg ketamine) and euthanized via intracardial perfusion with 4.0% paraformaldehyde (in 0.1 M phosphate-buffered saline, pH 7.4). The brain from each animal was removed from the cranium, postfixed in fresh fixative, and cryoprotected in phosphate-buffered 30% sucrose. Each brain was dissected into segments including the brain stem and forebrain. The forebrain, consisting of the cerebral hemispheres (cortex, hippocampus, and basal ganglia) and thalamus, was hemisected. The weight and volume (displacement in a graduated cylinder) of each hemisphere were determined. Blocks including the pre- and postcentral gyri were dissected from the drosomedia, central, and ventrolateral segments of the central sulcus, representations of the lower limb, hand, and face, respectively (Paul et al. 1972; Merzenich et al. 1978; Jones and Peters 1986). Blocks were frozen and cut into 40 μm sections perpendicular to the central sulcus. One series composed of every 10th section was stained with cresyl violet, and an alternate series was immunostained for NeuN using an avidin–biotin complex and diaminobenzidine as the chromogen (Miller, 2006b).

Blocks (1 mm × 1 mm × 2 mm) including the dorsolateral postcentral lobule from each animal were prepared for electron microscopy (Miller et al. 1999). After being dehydrated, osmicated, and embedded in Araldite, the blocks were cut into silver-gold ultrathin sections, which were stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 100CX transmission electron microscope. Five, nonoverlapping micrographs including a 15 μm × 15 μm box were taken from each block.

### Quantitative Analyses
#### Data Acquisition
Data were obtained from cortical areas 3a and 3b (primary somatosen- sory cortices) (Fig. 1). Some measures were also obtained for areas 2 and 4. Cortical areas were identified using standard cytoarchitectonic criteria (e.g., Jones et al. 1978; Huxley and Jones 1991; Darian-Smith and Darian-Smith 1993). Cytoarchitectonic areas were differentiated by their areal-specific laminar organization, and the size, shape, and packing density of neurons comprising each layer. Two layers that were particularly useful in identifying cytoarchitectonic areas were Layers IV and V. For example, a granular Layer IV distinguished koniocortical areas 3a and 3b. In contrast, area 4 had no Layer IV; it was characterized by the large Betz cells in Layer V.

The numbers of neurons in areas 3a and 3b were calculated as the product of the volume and the neuronal packing density of each area (Miller and Potempa 1990). Assessments were performed for each layer individually. The volume of each cortical layer was determined as the product of the mean area of each layer, and the length of the central sulcus was an estimate of the total volume of the layer (V_{L}).

### Table 1

<table>
<thead>
<tr>
<th>Duration of ethanol exposure</th>
<th>Total numbers</th>
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<tbody>
<tr>
<td>Occurred over the initial 6 weeks</td>
<td>Occurred over the initial 24 weeks</td>
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<tr>
<td>E19/E20</td>
<td>Group A</td>
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<tr>
<td>E21/E24</td>
<td>Group C</td>
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<tr>
<td>Total numbers</td>
<td>n = 5</td>
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### Laminar areas
Laminar areas (A_{L}) in a section of cytoarchitectonic areas 3a and 3b were determined at 3 locations in the postcentral gyrus: the dorsomedial, central, and ventrolateral sites. Measuring these areal spaces relied on the Bioquant Image Analysis System (R & M Biometrics, Nashville, TN). In addition, 2 linear measurements were taken. 1) The depth of a cortical area was the pia-to-white matter distance on a line perpendicular to the pial surface. 2) The length of the central sulcus (L_{CS}) from the medial surface to the lateral surface was determined. The total volume of the cytoarchitectonic area (V_{L}) was the sum of the laminar volumes.

The neuronal packing densities of each layer in areas 3a and 3b were estimated using the stereological method of Smolen et al. (1983; Miller and Potempa 1990). It is noteworthy that this method generated estimates consistent with those obtained with other stereological methods, namely, the "unbiased" optical dissector method (e.g., cf. Miller and Potempa 1990; Mooney and Napper 2005). Accordingly, the density of neurons was independently determined in randomly placed counting boxes of 10,000 μm^2 in each layer.

Neurons were identified in 2 ways (Miller 2006b). In the NeuN-immunostained sections, neurons were the labeled cells. In the cresyl violet-stained sections, neurons were identifiable as the large cells (somata with diameters > 8 μm) with readily discriminable perikarya, in contrast to the glia, which had small somata (diameters < 6 μm) and only a thin rim of cytoplasm. In the present study, the quantitative data relied on the cresyl violet-stained sections. This strategy was chosen for a number of reasons. 1) Direct comparisons between the gray and white matter were strengthened by using the same sections, obviating potential problems such as differential shrinkage. 2) Direct comparisons show that the packing density of NeuN-positive cells and cells identified as neurons in cresyl violet-stained sections was similar (Miller, 2006). 3) Examination of NeuN-immunostained sections counterstained with cresyl violet revealed that nearly all (> 99%) cresyl violet-stained cells that were identifiable as neurons were NeuN negative.

The numbers of neuronal nuclei in the counting box (N_{S}) were tallied. Cells included in the counts were those that did not intersect 2 adjoining exclusion planes or the surface of the section. This value was corrected for overestimations due to counting cut fragments with the following formula

\[
N_{V} = \left( N_{S} - t \right) / \left( t + D - 2k \right),
\]

in which N_{V} was the neuronal packing density, t was the section thickness, D was the mean maximal diameter of the counted nuclei, and...
Was the diameter of the smallest recognizable cap of a cut nucleus. The number of neurons in each layer \( (N_L) \) was calculated as the product of the laminar packing density and laminar volume:

\[
N_L = N_v \times V_L
\]

Parallel quantitative studies were performed on the subcortical white matter using the cresyl violet-stained sections. The depths of the white matter in the pre- and postcentral lobules were determined. The precentral lobule was delimited by the inferior arcuate sulcus (ias) rostrally and the central sulcus (cs) caudally. Hatching labels the region used to measure the size of postcentral white matter. This area was bounded by the gray matter below the ias and cs and the line parallel to the pial surface that passed through the base of the cs (dotted line). The precentral white matter was bordered by area 4 on the bank of the cs, the gray matter of area 6 in the inferior parietal sulcus (not shown), and a line parallel to the surface that intersects the base of the cs (dotted line and overlapping solid arrow). Orientation arrows are labeled with “l” and “r” for lateral and rostral directions, respectively. Images are from monkeys intubated with 1) saline (control, left) or ethanol on a 6-week sequence including E19 or E20 (Group A), or E21 or E24 (Group C). Scale bars are 1.0 mm. (Bottom) Micrographic strips depict cresyl violet-stained segments of cortical areas 4, 3b, and 2. The 3 micrographs at the bottom right (oriented horizontally) are from the core white matter within the postcentral lobule. Scale bars are 100 μm.

Figure 1. Cytoarchitecture of the pre- and postcentral lobule. (Top) The depths of 4 areas (somatosensory areas 2, 3a, and 3b and motor area 4) were measured using lines that were perpendicular to the pial surface (solid lines with T-ends). The depth of the white matter in the pre- and postcentral lobules (solid lines with arrows) was determined. The precentral lobule was delimited by the inferior arcuate sulcus (ias) rostrally and the central sulcus (cs) caudally. Hatching labels the region used to measure the size of postcentral white matter. This area was bounded by the gray matter below the ias and cs and the line parallel to the pial surface that passed through the base of the cs (dotted line). The precentral white matter was bordered by area 4 on the bank of the cs, the gray matter of area 6 in the inferior parietal sulcus (not shown), and a line parallel to the surface that intersects the base of the cs (dotted line and overlapping solid arrow). Orientation arrows are labeled with “l” and “r” for lateral and rostral directions, respectively. Images are from monkeys intubated with 1) saline (control, left) or ethanol on a 6-week sequence including E19 or E20 (Group A), or E21 or E24 (Group C). Scale bars are 1.00 mm. (Bottom) Micrographic strips depict cresyl violet-stained segments of cortical areas 4, 3b, and 2. The 3 micrographs at the bottom right (oriented horizontally) are from the core white matter within the postcentral lobule. Scale bars are 100 μm.

The area and density of axons in a 10 μm x 10 μm segment of postcentral white matter was determined on the electron micrographs (Miller et al. 1999). The areas were only determined on profiles cut in cross-section, that is, those that permitted delineation of the myelin wrappings. The number of all axons in the counting box, regardless of their orientation, was used to determine axonal density.
All measures relied on the Bioquant Image Analysis System.

Statistical Analysis
All quantitative data were obtained by investigators blind to the source of the material. Only after all of the data were obtained were they sorted and prepared for statistical analyses. A mean was calculated from 3 independent measures for each metric at each level of the central sulcus. For each animal, a grand mean for the data obtained at the 3 different levels of the central sulcus was calculated.

Two sets of analyses of variance (ANOVAs) were performed. The data based on the duration (Group A/C, Group B/D, or control animals) or timing (from the Group A/B, Group C/D, or control macaques) of the ethanol exposure were compared. In situations in which significant ($P < 0.05$) differences were detected, post hoc Student-Newman-Keuls tests were performed for comparisons relative 1) to the controls or 2) in the case exploring the effects of timing, additional comparisons were made relative to the Group C/D animals.

Results

Forebrain Size
The basic organization of the forebrain appeared unaffected by prenatal exposure to ethanol. That is, no heterotopias or blurring of laminar or gray matter–white matter interfaces were evident. The weight and volume of the forebrain were not significantly different in Group A/C, Group B/D, or control monkeys (Fig. 2). On the other hand, a 2-way ANOVA that took into account the timing of the ethanol exposure showed that ethanol significantly affected forebrain weight ($F_{2,12} = 4.592; P = 0.0331$) and volume ($F_{2,12} = 4.363; P = 0.0377$). No significant interaction between timing and duration of the exposure was detected. A post hoc Student-Newman-Keuls test revealed that the forebrain was significantly ($P < 0.05$) larger in Group A/B animals, than for Group C/D monkeys or controls. These differences were evident for both hemispheres. The quotient of the weight and volume, the specific gravity, was unaffected by the length or timing of the ethanol exposure.

Despite the increase in the size of the forebrain, the length of the central sulcus was not significantly affected by ethanol exposure. In control macaques the central sulcus was $36.0 \pm 2.2$ mm long as compared with $37.5 \pm 3.8$ mm and $35.8 \pm 3.1$ mm in Group A/B and Group C/D animals, respectively.

Cortical Gray Matter
The depth of the cortex was not significantly affected by ethanol treatment (Fig. 3). This lack of change was evident in the gray matter on both banks of the central sulcus, that is, primary somatosensory (areas 2, 3a, and 3b) and motor (area 4) cortices. Moreover, neither the duration nor the timing of the ethanol exposure induced a significant change in cortical depth. As shown by exemplary measures of cortical areas 3a and 3b, the lack of change in cortical depth was also evident in the cross-sectional area. The volume of each layer in areas 3a and 3b was not significantly affected by ethanol exposure. Likewise, the total areal volume was unaffected by ethanol exposure.

The composition (the size and density of neuronal somata) of each cortical layer in areas 3a and 3b was examined. Exemplary data for area 3b are provided in Figure 4 (comparable data for area 3a are not shown). Ethanol had no significant effect on the size of cortical neurons in each layer. On the other hand, ethanol-treated (Group A/C and Group B/D) macaques had 24.8–35.2% fewer neurons/mm$^3$ than control animals ($F_{2,12} = 6.499; P = 0.0122$). The only exception was Layer V where no changes were detected. Moreover, no significant differences between the Group A/B and Group C/D monkeys were detected i.e., there was no effect of timing.

Given the lack of changes in areal volume, the changes in neuronal packing densities translated into significant ($P < 0.05$) ethanol-induced changes in neuronal number. This change was evident throughout the cortex. The only exception was Layer V wherein no ethanol-induced differences, be it from the timing or the duration of the exposure, were detectable with an ANOVA or a Student-Newman-Keuls test. Ethanol-induced differences in neuronal packing density of the other layers ranged from 18.6% to 31.7%, the greatest difference being in Layer IV.

In total, areas 3a and 3b in control monkeys had 655 and 977 million neurons, respectively (Fig. 5). Ethanol-treated monkeys had ~26% fewer neurons in area 3a and ~37% fewer in area 3b. These numbers were statistically significant ($P < 0.01$) from those in the controls. No timing-dependent difference was detected. That is, the numbers of neurons in areas 3a and 3b were not significantly different in Group A/B monkeys versus Group C/D animals or controls.
Subcortical White Matter

The amount of white matter was affected by ethanol treatment. The depth of the white matter was measured in 2 places, in the pre- and postcentral lobules (Fig. 1). When the animals were exposed to ethanol once per week for 6 or 24 weeks, no differences were detected (Fig. 6). In contrast, the depths of both the pre- and postcentral lobules were significantly (pre-central lobule: \( F_{2,12} = 9.576; P = 0.00327 \); postcentral lobule: \( F_{2,12} = 4.609; P = 0.0327 \)) affected by the timing of the ethanol exposure. A post hoc test showed that Group A/B monkeys had significantly \((P < 0.05)\) deeper pre- and postcentral white matter relative to that in Group C/D animals and controls. Likewise, the cross-sectional area and volume of the postcentral lobule were significantly \((P < 0.05)\) greater in Group A/B animals than in monkeys exposed at another time or in controls.

The density of cells in the white matter of the postcentral lobule was significantly \((P < 0.05)\) deeper pre- and postcentral white matter relative to that in Group C/D animals and controls. Likewise, the cross-sectional area and volume of the postcentral lobule were significantly \((P < 0.05)\) greater in Group A/B animals than in monkeys exposed at another time or in controls.

The volume of the white matter comprising cell bodies was determined by multiplying the somatic size by the total number of cells. This did not vary significantly among the groups of monkeys; it ranged from 2.09 to 2.18 mm$^3$. The portion attributed to myelopil, elements other than cell bodies (ostensibly myelinated axons), was calculated as the difference between the total volume of the postcentral white matter and total somatic volume. This volume was unaffected by the duration of the ethanol exposure, that is, it was similar among the control, Group A/C, and Group B/D monkeys. On the other hand, the timing of the exposure had a significant \((F_{2,12} = 5.56; \) prenatal treatment (Fig. 7). It was significantly \((P < 0.05)\) lower in Group A/B animals. On the other hand, ethanol-induced changes in volume balanced changes in cell packing density so that the number of cells in the postcentral white matter, about 1.20 billion cells, was unaffected by prenatal treatment, regardless of the duration or timing of the ethanol exposure.

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This difference largely resulted from the dramatic and significant ($P < 0.05$) increase in the nonsomatic volume in the Group A/B monkeys relative to the Group C/D macaques (+37.8%) or to controls (+41.1%).

Electron microscopic analysis of the postcentral white matter was performed. The white matter was composed of cell bodies, presumably most of which were oligodendrocytes and astrocytes, and axons. Most of the axons were clustered in fascicles of myelinated axons, some of which are shown in Figure 8. The caliber of these axons varied from 0.2 to 1.0 μm or more. Some nonmyelinated axons were evident as well. Ethanol exposure did not affect the mean diameter of the axons, nor did it affect axonal density.

**Figure 4.** Laminar features. The mean somatic volume (top), neuronal packing density (middle), and neuronal number (bottom) for each cortical layer in area 36 are shown. Data are summarized for controls and for monkeys exposed to ethanol during the first 6 (Group A/C) or 24 (Group B/D) weeks of gestation, on E19/E20 (Group A/B) or E21/E24 (Group C/D). T-bars represent the standard errors of the mean. Significant ($P < 0.05$) differences relative to the controls are identified by asterisks. Notations are as in the caption of Figure 2.

**Figure 5.** Neuronal number. The effects of ethanol on the number of neurons in areas 3a and 3b were summed from the data for individual laminae (e.g., see Fig. 4). Notations are as in the caption of Figure 2.
Figure 6. White matter depth. The depths of the white matter within the precentral (top) and postcentral (middle) gyri were taken (see Fig. 1). The latter along with the data on the length of the central sulcus (data not shown) was used to estimate the volume of the postcentral white matter (bottom). Notations are as in the caption of Figure 2.

Figure 7. White matter composition. The mean cross-sectional area of the cell bodies in the white matter was determined. The packing density of these cells in the postcentral gyrus was determined. The number of cells in the white matter of the postcentral lobule was calculated as the product of the volume of the white matter (see Fig. 6 bottom) and the density of cells. The volume attributable to nonglial somata was calculated as the difference of the total white matter volume and the volume occupied by glial somata (the product of the somatic size and glial number). Notations are as in the caption of Figure 2.
Discussion

**Gastrulation and Forebrain**

The period of gastrulation coincides with a critical window for defining the size of the forebrain in the macaque. The size of the forebrain is greater in monkeys whose sequence of ethanol exposure included E19 or E20 (the time of gastrulation) relative 1) to animals exposed at other times or 2) to controls not exposed to ethanol. Given the current dosing regimen, it could be argued that another 2-day period weeks before or after E19/E20 is the critical period. This does not seem likely, however, in light of data from rodent studies wherein ethanol exposure is restricted to less than a single day during the period of gastrulation (Ashwell and Zhang 1999; Dunty et al. 2002; Sulik 2005; Mooney and Miller 2007).

Prenatal exposure to ethanol does not affect all areas of the cerebral cortex in a homogeneous manner. For example, in the rat, rostral cortex is affected by ethanol, whereas caudal segments are less affected (cf. Miller and Potempa 1990; Mooney and Napper 2005). Likewise, the present study shows that even within a specific area, the effects of ethanol can vary. The prime example is the macaque Layer V which is unaffected by ethanol insofar as neuronal number is concerned, whereas the number of neurons in other layers is lower in ethanol-treated animals. These data parallel ethanol-induced changes in the rat somatosensory cortex (Miller and Potempa 1990).

Though the composition of the gray matter in somatosensory and motor cortices is affected by ethanol, the size of the gray matter is unaffected. This lack of effect is evident regardless of the duration or timing of the ethanol exposure and implies that the increase in forebrain size in macaques exposed to ethanol on E19 or E20 results from a change in white matter. Quantitative analyses show that the volume of the postcentral white matter is greater in monkeys treated with ethanol on E19 or E20 than in monkeys exposed at other times or controls.

**Effects on Gray Matter**

The consensus is that ethanol treatment during gastrulation affects facial structure. For example, mice exposed to ethanol on E7 or E8 (Sulik et al. 1981; Sulik 2005) or macaques exposed to ethanol on E19 or E20 (Astley et al. 1999) exhibit FAS-associated facial dysmorphia. Facial dysmorphology is a salient feature of FAS (Lemoine et al. 1968; Jones and Smith 1973; Astley et al. 1999).
The window of vulnerability for facial dysmorphology also relates to brain development. Brain structures, notably the brain stem (Mooney and Miller 2001, 2007; Dunty et al. 2002; Sullik 2005) and forebrain (Ashwell and Zhang 1999; present study), are affected by exposure to ethanol at the time of gastrulation. Interestingly, the effects are specific; not all brain stem nuclei are affected. For example, the number of neurons in the trigeminal motor nucleus is lower in macaques exposed to ethanol on E19 or E20, whereas the number of neurons in the facial motor nucleus is not (Mooney and Miller 2001).

The macaque somatosensory cortex (the amalgam of areas 3a and 3b) comprises 1.63 billion neurons. This is 3 orders of magnitude more than that evident in the area 3 of the rat (2.72 million neurons; Miller and Potempa 1990). Despite this difference, prenatal exposure to ethanol has a similar effect on the rat and monkey somatosensory cortices. Monkeys exposed to ethanol have 32.0% fewer neurons than controls, and rats exposed to ethanol have 34.2% fewer neurons.

Cortical composition is also affected. Though the size of neuronal somata is unaffected by ethanol exposure, the density of cortical neurons in all laminae but Layer V is lower. Thus, the amount of cortical volume occupied by neuronal somata is lower, and inversely, the amount of neuropil is greater in monkeys exposed to ethanol. This parallels changes in the rat somatosensory cortex (Miller and Potempa 1990), wherein a contributor is dendritic hypertrophy (Miller et al. 1989).

So far as the gray matter is concerned, the timing of the ethanol exposure, that is, during the week of E19–E25, is not critical. If the data from the present study are parsed based on the number of weeks in which the monkeys were exposed to ethanol, that is, for 6 or 24 weeks, and compared to controls a significant decrease in neuronal number is evident. The amount of this decrease is similar in monkeys exposed to ethanol during the first 6 or first 24 weeks of gestation. The generation of the first cortical neurons (subplate neurons) commences on E13, after the monkeys exposed to ethanol only during the first 6 weeks had had their final ethanol doses. Therefore, it can be inferred that events occurring in the first 6 weeks of gestation (the time common to both exposure periods) are key to organizing cortical composition. Such events include the proliferation and lineage commitment of neural stem cells.

**White Matter Hypertrophy**

Timely exposure to ethanol induces compensating effects on the white matter. The total mass of the white matter increases, whereas the cell packing density decreases. The net result is that there is no change in total cell number in the subcortical white matter. That said, there is an increase in the amount of myelopil, the nonsomatic mass that is composed primarily of axons. In fact, the density of axons is unaffected by ethanol. Thus, in combination with the increase in white matter volume, this indicates that total axonal number is increased. Such a conclusion is consistent with data from a study of the monkey corpus callosum, showing that prenatal exposure to ethanol causes an increase in the number of callosal axons (Miller et al. 1999). This increase 1) is most noticeable in the rostral portion of the corpus callosum which carries frontal and parietal connections and 2) results from a hypertrophy of the callosum rather than a change in axonal caliber. The increase in axonal number likely results from an ethanol-induced interference in axonal pruning (e.g., Miller 1987; Miller and Al-Rabiai 1994; Clamp and Lindsley 1998).

Brain hypertrophy is not exclusive to ethanol teratogenicity. For example, children with autism also exhibit enlarged brains (Howard et al. 2000; Sparks et al. 2002; Schumann et al. 2004; Hazlett et al. 2005; Redcay and Courchesne 2005). Interestingly, FAS and autism can be comorbid disorders (Nanson 1992). An implication is that these syndromes have a common outcome and/or etiology. Indeed, it has been suggested that autism spectrum disorder can result from exposure to a toxin such as valproic acid or thalidomide at the time of gastrulation (Rodier 2000; Arndt et al. 2005).

Hypertrophy in autistic children appears to be age and structure related. It is quite evident in parietal and temporal cortex (Hardan et al. 2006). Data from a study of a putative murine model of autism show increases in dendritic arborization within forebrain structures (Kwon et al. 2006). In the hippocampus, the overgrowth appears to persist into adulthood. In contrast, in the amygdala, the early overgrowth normalizes before adolescence. Despite this, neuronal number remains significantly decreased (Schumann and Amaral 2006). Such differential effects on structural volume and cell number parallel the changes described in the cortices of ethanol-exposed macaques.

**Fetal Programing**

Ethanol affects neurons through large swathes of development, including the periods of neuronal generation, migration, and synaptogenesis (Pentney and Miller 1992; West et al. 1994; Lindsley 2006; Miller 2006c). For most structures in the monkey brain, these events take from a week to months. Thus, during the 6- and 24-week exposure regimen, developing brain structures may be subjected to multiple episodes of ethanol exposure. Brain development is plastic and allows for compensatory responses to a focal trauma such as a lesion (e.g., Hubel 1978; Wiesel 1982) and ethanol (e.g., Miller 1995, 2006a; Siegenthaler and Miller 2006). Thus, if exposure is short and the window is long, minimal damage may result. On the other hand, if the critical window is short, the ability of the brain to make corrections is profoundly limited. That is, the potential for compensation depends upon the duration of the trauma relative to the duration of the critical window. In the paradigm used in the present study, pulse exposure to ethanol on a single day (E19 or E20) takes up too much of the apparent 2-day critical period to allow the developing nervous system to recover from the focal damage.

Two conundrums are as follows: 1) how does exposure to ethanol on E19 or E20 (which is before the proto-nervous system appears) affect developmental events that occur during the third trimester, and 2) why are such events important for white matter development, for example, axonal pruning and oligodendrogiogenesis? The underlying mechanism likely involves changes that lie dormant (or hidden) and have long-term consequences. This “memory” process is referred to as fetal programing (Stewart et al. 1958; MacMahon 1962; McCance and Widdowson 1974; Śliwowska et al. 2006). Fetal programing may relate to the types of cells that are available, the range of activities that the cells can perform, and the numbers of cells in the network.
Fetal programing depends on various phenomena including decisions of cell fate, the number of founder cells, and neuronal generation and survival. Ethanol can affect each of these phenomena (e.g., Brodie and Vernadakis 1992; Kentroti and Vernadakis 1992; Miller 1986, 2006c; Gohlke et al. 2002; Yamada et al. 2005; Miller et al. 2006; Santillano et al. 2006; Zawada and Das 2006). The present study shows that a traumatic or toxic event during gastrulation, when the proto-nervous system is composed of neural stem cells, can have consequences that remain latent for weeks. Thus, fetal programing may be best appreciated following an experimental challenge.

Notes
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