Time-Specific Effects of Ethanol Exposure on Cranial Nerve Nuclei: Gastrulation and Neuronogenesis

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Abstract

During the development of the central nervous system, neurons pass through critical periods or periods of vulnerability. We explored periods of vulnerability for cranial nerve nuclei by determining the effects of acute exposure to ethanol during development on the number of neurons in mature brainstem. Long-Evans rats were injected with 2.9 g ethanol/kg body weight on one day between gestational day (G) 7 and G13, inclusive. Two hours later, animals received a second injection of 1.45 g/kg. Controls were injected with equivalent volumes of saline. Brainstems of 31-day-old offspring were cryosectioned and stained with cresyl violet. Stereological methods were used to determine the volume and numerical density of neurons in three trigeminal sensory nuclei (the principal sensory nucleus of the trigeminal nerve, and the oral and interpolar subnuclei of the spinal trigeminal nuclear complex) and three motor nuclei (the trigeminal, facial, and hypoglossal nuclei). The numbers of neurons in most nuclei were lower following early (on G7 and/or G8) or later (on G12 and/or G13) exposure. Only the trigeminal interpolar nucleus was affected by neither early nor late ethanol exposure. Thus, prenatal exposure to ethanol affects the number of neurons in brainstem nuclei in a time-dependent manner. Windows of vulnerability coincide with gastrulation (G7/G8) and the period of neuronal generation (G12/G13).

Keywords

alcohol; facial dysmorphia; facial nucleus; fetal alcohol syndrome; hypoglossal; trigeminal; rhombomere

INTRODUCTION

During development of the central nervous system, neurons pass through discrete periods, or windows, of vulnerability to environmental factors, including naturally expressed substances (e.g., neurotrophins and growth factors) and xenobiotics, e.g., ethanol. One critical window of ethanol vulnerability coincides with the period of gastrulation. In mice, exposure to ethanol on gestational day (G) 7 or G8 increases the expression of markers for cell death (Dunty et al., 2002;Kilburn et al., 2006). More impressive is that mice exposed to ethanol during gastrulation (Sulik et al., 1981;Dunty et al., 2002;Da Lee et al., 2004) exhibit craniofacial malformations

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like those that characterize human children with fetal alcohol syndrome (FAS) (Lemoine et al., 1968;Jones and Smith, 1973). Similar time-dependent vulnerability is evident in the monkey (Astley et al., 1999). Another window of vulnerability to ethanol-induced teratogenesis coincides with the time of cell generation (Miller, 1995a;1995b; 1996). Ethanol alters neuronal generation (Miller, 1986;2006), at least in part, by lengthening the cell cycle (Miller and Nowakowski, 1991). Ethanol may also affect neuronal generation by altering the number of cells in the cycling population.

In light of ethanol-induced changes in craniofacial structures, the brainstem is a logical structure of interest. After all, the brainstem contains the cranial nerve nuclei that connect to and receive input from the facial musculoskeletal structures. Furthermore, the brainstem is a compelling model for examining teratogenicity because of its orderly development. Sensory and motor nuclei are generated in discrete regions; neurons in sensory nuclei are generated in the alar plate and motor neurons are derived from the basal plate (e.g., Darnell, 2005). In addition, the developing brainstem is transiently organized into segments known as rhombomeres. Each rhombomere can be identified by a unique set of spatiotemporally expressed patterning homeobox genes (e.g. Nieto et al., 1992). Studies of the consequences of prenatal exposure to the teratogen valproic acid indicate that timely prenatal trauma affects alar plate-derivatives in select rhombomeres (Rodier et al., 1996). The present study builds on this work and tests the hypothesis that ethanol affects brainstem development in a time- and site-specific manner. In the present study, six brainstem nuclei were examined. They were chosen to determine potential effects of ethanol on (a) different rhombomeres and (b) alar or basal plates (Table 1).

MATERIALS AND METHODS

Animals

Timed pregnant Long-Evans rats were purchased from Taconic Farms (Germantown, NY). The first day on which a sperm-positive plug was detected was designated G1. All procedures were performed with approval from the Committee for Humane Use of Animals at Upstate Medical University and the Institutional Animal Care and Use Committee at the Syracuse Veterans Affairs Medical Center. Animals were given an intraperitoneal (i.p.) injection of 2.90 g ethanol (20% v/v ethanol in saline) per kg body weight at 9:00 AM on one day between G7 and G13, inclusive. Two hours later, animals received a second i.p. injection of ethanol (1.45 g/kg). Each control animal received a pair of i.p. injections of equivalent volumes of saline on G8 or G10.

Within 24 hours of birth (postnatal day (P) 0), all litters were culled to ten. On postnatal day (P) 31, one animal from each litter was anesthetized (1.0 ml/kg ketamine and 0.10 ml/kg xylazine) and perfused transcardially with a solution of 4.0% paraformaldehyde in 0.10 M phosphate buffer (PB; pH 7.4). Each group of offspring was comprised of half males and half females. The brains were removed, post-fixed in fixative for four hours at room temperature, washed in PB, and stored in fresh PB at 4°C.

Brainstems were isolated by a coronal cut caudal to the superior colliculus and the cerebellum was removed by cutting through the peduncles. Samples were dehydrated through increasing concentrations of ethanol, cleared with butanol, and then infiltrated with Paraplast Plus paraffin (VWR, West Chester, PA). Paraffin embedded tissue was cut into 5.0 μm thick sections in the horizontal plane. Sections were de-paraffinized in xylene, rehydrated through decreasing concentrations of ethanol, stained with cresyl violet, dehydrated, and then coverslipped.

The concentration of ethanol in the blood (BEC) was regularly monitored in a second group of animals that were treated as described above. Samples of blood were obtained from clipped

tails at 60 min intervals after the first injection. BEC was determined using the Analox GM7 (Analox Instruments, Lunenburg MA).

Anatomic studies

Stereological methods were used to estimate the total number of neurons in six brainstem nuclei: the principal sensory nucleus of the trigeminal nerve (PSN), the oral and interpolar subnuclei of the spinal trigeminal nucleus (SpVo and SpVi, respectively), and the trigeminal, facial, and hypoglossal motor nuclei (MoV, MoVII, and MoXII, respectively). These nuclei and salient features of their development are described in Table 1. The total number of neurons in a nucleus (N_T) was calculated as the product of the volume of the nucleus (V_T) and the neuronal packing density (N_V).

The Cavalieri estimator of volume was used to determine the total volume of each nucleus (Gundersen and Jensen, 1987;Miller & Muller, 1989;Mooney & Miller, 2001b). The borders of each brainstem nucleus were identified using cytoarchitectonic criteria (see Paxinos and Watson, 1982). In every section that contained the profile of a cranial nerve nucleus, the cross-sectional area (A_S) of that nucleus was measured using the Bioquant Image Analysis System (R&M Biometrics, Nashville TN). The total volume was calculated from the formula

$$V_T = \Sigma A_S \bullet t \, / \, f$$

in which ΣA_S was the sum of all cross-sectional areas through each nucleus, t was the section thickness, and f was the frequency of sections in the series.

Neuronal packing density (N_V) was determined using Smolen's correction of Abercrombie's estimator (Abercrombie, 1946;Smolen et al., 1983;Miller and Muller, 1989). The numbers of neurons with nuclei in a box ($100 \, \mu m \times 100 \, \mu m$) were counted. The counting element, neuronal nuclei, tended to be round; their eccentricity was 0.820 ± 0.01. Only neurons with nuclei fully in the box or contacting one of two adjacent inclusion sides of the box were counted. Nuclei crossed by one of the other two sides of the box were excluded from the tallies. Diameters of the profiles of all counted nuclei were determined. N_V was determined using the formula:

$$N_V = n \bullet t \, / \, \left(t + \bar{D} - 2k \right)$$

in which n was the number of neurons counted in the counting box, t was the section thickness, $\bar{D}$ was the mean maximal diameter of the cell bodies, and k was the diameter of the smallest recognizable cap of a cut nucleus.

Statistical analysis

The mean ($\pm$ the standard error of the mean) was determined for each group of animals. As cannot be assumed that the data from any individual nucleus is independent from all other nuclei, multivariate analyses of variance (MANOVA) were used to determine the overall effect of treatment. In instances in which significant differences were detected with a MANOVA test, *post-hoc* Dunnett's tests were performed to compare ethanol-treated groups to the controls. Statistics were performed using StatView software (SAS Institute Inc, Cary NC).

RESULTS

Blood ethanol concentration

The temporal change in the BEC after the pair of injections is described in Figure 1. The BEC rose during the first three hours post-initial injection. Mean peak BEC (285 ± 4 mg/dl) was attained at this time (three hours after the first injection and one hour after the second injection). By nine hours after the initial injection, the BEC had returned to baseline (control) values.

General changes in the brainstem

All six nuclei were identifiable in horizontal sections stained with cresyl violet (Fig. 2 and 4). No gross differences in the appearance of the nuclei were apparent among the treatment groups.

The volume and cell packing density for each nucleus was determined and these data were used to estimate of the total number of neurons. Based on a MANOVA, a significant ($p<0.0001$) overall effect of treatment was apparent in all three data sets. Statistical analyses for these overall data as well as ANOVA for the data sets for specific features of each nuclei are provided in Tables 2-4.

Sensory nuclei

The PSN was affected by transient exposure to ethanol. *Post-hoc* Dunnett's tests show that the volume of the PSN was generally unaffected, but ethanol exposure on G12 or G13 did cause a significant ($p<0.05$) fall in PSN volume. Neuronal packing density was significantly ($p<0.05$) lower after exposure on G8 or on G13. Taken together, these data showed that the PSN of rats exposed to ethanol during gastrulation (on G8) or during neuronal generation (G12 or G13) had significantly ($p<0.05$) fewer neurons compared with controls.

Few changes were evident in the volumes or neuronal packing densities of spinal trigeminal subnuclei. Neuronal numbers in the SpVo were lower after ethanol exposure on G7 than in controls, however, neuronal numbers in SpVi were unaffected by treatment with ethanol at any time between G7 and G13.

Motor nuclei

The three motor nuclei were identifiable in horizontal sections stained with cresyl violet (Fig. 4). In contrast with the sensory nuclei, all motor nuclei were smaller after treatment with ethanol early (G7 or G8) or late (G12 or G13)(Fig. 5).

Only the MoV volume showed an effect of ethanol on all days. Interestingly, the packing density of neurons in the MoV was higher in animals treated on G10 or G12 than in controls. Thus, exposure to ethanol on during gastrulation (on G8) or during neuronal generation (G12 or G13) significantly ($p<0.05$) reduced the number of neurons in the MoV.

The packing densities in the MoVII and MoXII were largely unaffected by ethanol (Fig. 5). The number of neurons in the MoVII was lower in animals exposed to ethanol on G7, G8, G10, G12, or G13. The number of neurons in the MoXII was lower than in control animals after ethanol exposure during neuronogenesis, i.e., on G12 or G13.

DISCUSSION

Timing of the ethanol-induced vulnerability

The effect of ethanol on neuronal number is variable. Among the nuclei that are affected by ethanol, the number of neurons is 11 to 38% fewer in ethanol-treated rats than in controls. General patterns of the effects of ethanol are evident. Rostral rhombomeres (r2 and r4) are more susceptible to early administration of ethanol (on G7/G8) than are caudal rhombomeres (e.g., r6 and r8). Additionally, motor nuclei are more affected by later treatment with ethanol (on G12/G13). These data support the hypothesis that ethanol has site and time-dependent effects.

The developing brainstem experiences two periods of vulnerability to ethanol: gastrulation (on G7/G8) and neuronogenesis (G12/G13). Gastrulation is the time at which the embryo becomes organized into three germ layers, and the primary axes of the basic body plan are established

(Ladher and Schoenwolf, 2005). Neuronal generation is the process by which progenitors undergo their final mitosis. Four nuclei affected by ethanol exposure during gastrulation include three trigeminal nuclei (the PSN, SpVo, and MoV) and the MoVII. These changes in the brainstem occur after an exposure to ethanol that is associated with FAS-like craniofacial malformations (Sulik et al., 1981;Astley et al., 1999;Dunty et al., 2002;Da Lee et al., 2004). In addition, four nuclei (the MoV, MoVII, MoXII, and PSN) are affected by ethanol exposure during neuronal generation.

Unlike exposure during gastrulation, ethanol treatment during neuronogenesis is not associated with FAS-like craniofacial changes. One explanation is that exposure to ethanol during gastrulation induces changes in brainstem anatomy that are coordinate with craniofacial changes, whereas exposure during neuronogenesis has direct effects on the brainstem that are divorced from marked alterations in the face.

Mechanisms of ethanol toxicity

Fewer neurons in cranial nerve nuclei can result from four factors: a reduction in the size of the proliferative population, a change in the cycling activity of proliferating cells, a shift in the fates of post-mitotic cells, or selective survival of a subpopulation. These explanations for the ethanol-induced changes are not mutually-exclusive.

(1) A change in the size of the founder population can alter the potential number of progeny. The founder population is the group of seed cells available at the time that the neurons in a particular brain structure are being generated (Caviness et al., 1995). A small change in the size of the founder population has the potential to cause a large change in the number of cells produced. Currently, no data address the effects of ethanol on the size of a founder population. Nevertheless, it is reasonable to predict that exposure to ethanol at a pivotal time such as the time of neuronal generation would reduce the size of the founders. Presumably, the eight hour exposure to ethanol does not permit a recovery because the exposure duration is sufficient part of the period of vulnerability to eliminate any potential compensatory responses.

(2) The number of potential cycles a proliferating cell can pass through contributes to the number of eventual progeny. The potential number of cycles depends on the length of the cell cycle and the number of days (or hours) during which neurons are generated. In the cerebral cortex (Miller, 1986;1988;Siegenthaler and Miller, 2005) and in primary cultures of astrocytes (Guerri et al., 1990;Luo and Miller 1999) or neurons (Jacobs and Miller, 2001), ethanol increases the length of the cell cycle. On the other hand, at least in the PSN (Miller and Muller, 1989), ethanol does not increase the period of neuronal generation. Thus, the total number of cycles through which a proliferating cell likely passes is reduced by ethanol. The result is that the potential number of progeny is limited.

(3) Neural stem cells and progenitors are the cells that are present at the time of exposure. Based on *in vivo* studies, it is difficult to differentiate neural stem and progenitor cells. Published *in vivo* studies do show that ethanol can affect the proliferation of progenitor cells (e.g., Miller and Nowakowski, 1991;Miller and Kuhn, 1995; Miller, 1996) and fate decisions (e.g., Kentroti and Vernadakis, 1992). Data from *in vitro* studies show that the proliferation (Vemuri and Chetty, 2005; Santillano et al., 2006) and decisions of cell lineage (Vemuri and Chetty, 2005;Rubert et al., 2006) of neural stem cells are affected by ethanol.

(4) There are two potential targets of ethanol-induced death- proliferating cells and post-mitotic cells (Rehen and Chun, 2006). It appears that ethanol does not induce death of neural stem/ progenitor cells *in vitro* (Luo and Miller, 1997;Jacobs and Miller, 2001;Santillano et al., 2005). In contrast, ethanol can cause death of post-mitotic neurons (Miller, 1995;Jacobs and Miller, 2001;Mooney and Miller, 2001a;Heaton et al., 2003;Mooney et al., 2006).

Embryos exposed to ethanol during gastrulation display increased expression of markers associated with cell death (Dunty et al., 2001;2002). Such labeling is most apparent in the cranial neural crest, which may underlie the craniofacial changes, and the rostral hindbrain, i.e., rhombomeres 1-3, inclusive. The implication of the latter result is that structures that form within rhombomeres 1-3 are most susceptible to ethanol at this time. The findings of the present study do not support this prediction. The two nuclei in rhombomere 2, the PSN and MoV, are affected by ethanol exposure during gastrulation, but cranial nerves in more caudal rhombomeres (e.g., the SpV and MoVII) are also affected. Indeed, the amount of the ethanol-induced change is similar in derivatives of rhomobomeres 2 and 4.

An alternative mechanism of ethanol-induced changes may be through on pattterning genes. In mice, exposure to ethanol during gastrulation results in rapid (i.e., within hours) changes in gene expression in the neural tube (Du and Hamre, 2003) and expression of apoptosis-related markers (Dunty et al., 2001;2002). Treatment with another teratogen, valproic acid, affects the expression of *hox* genes (Stodgell et al., 2006). *Hox* gene expression begins around the time of gastrulation (Deschamps et al., 1999;Forlani et al., 2003), and is important for establishing patterns and nuclear boundaries in the developing brainstem (e.g., Nieto et al., 1992).

A refractory period

Interestingly, there is a period between gastrulation and neuronal generation during which exposure to ethanol has little effect on neuronal number; i.e., there is a period when the brainstem cranial nerve nuclei are refractory to the effects of ethanol. Two events ongoing during the windows of vulnerability are cell migration and proliferation. Migrating cells are also a target of ethanol (e.g., Miller, 1986;1993;Siegenthaler and Miller, 2004;Kumada et al., 2006). Between these two windows, the proliferating cell population is expanding. Exposure to ethanol may not change the fate of the cells during this refractory period and the length of the exposure relative to the total period of may permit a recovery.

The present study focuses on the number of neurons in the mature animal. Therefore, it is possible that the brainstem underwent a transient change that was followed by recovery. The lack of change in neuronal number following ethanol exposure has been reported for other brain regions such as somatosensory cortex (Mooney and Napper, 2005), hippocampus (Miller, 1995b), thalamus (Mooney and Miller, 1999;Livy et al., 2001), and cerebellum (Marcussen et al. 1994). The circumstances of the ethanol exposure (e.g., the peak ethanol concentration, the duration of the exposure, and ongoing developmental event) may define the response. In at least the case of the ventrobasal nucleus of the thalamus, a longitudinal study of neuronal number over time shows that neither neuronal generation nor death is altered by ethanol (Mooney et al., 2005).

In summary, brainstem cranial nerve nuclei are vulnerable to ethanol-induced alterations in neuronal number at two distinct times of development; gastrulation and neuronogenesis. During gastrulation, ethanol targets rostral rhombomeres, whereas the basal plate is more vulnerable during neuronal generation. The different effects of ethanol at these two times likely results from targeting different cell populations; stem cells and progenitor cells, respectively. Interestingly, in the period between these two windows of vulnerability the cranial nerve neurons are refractory to ethanol. Thus, ethanol targets specific populations in a time-specific manner.

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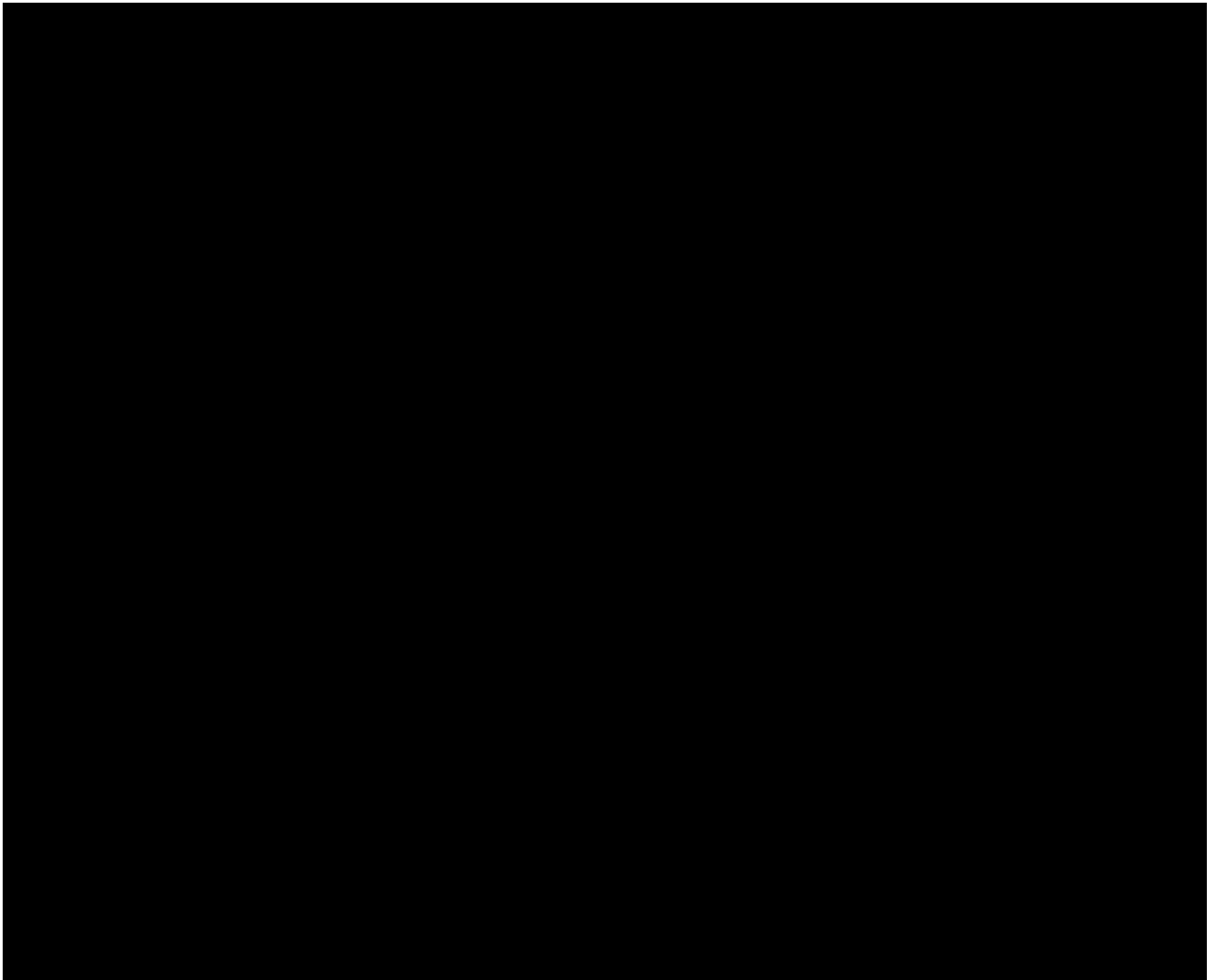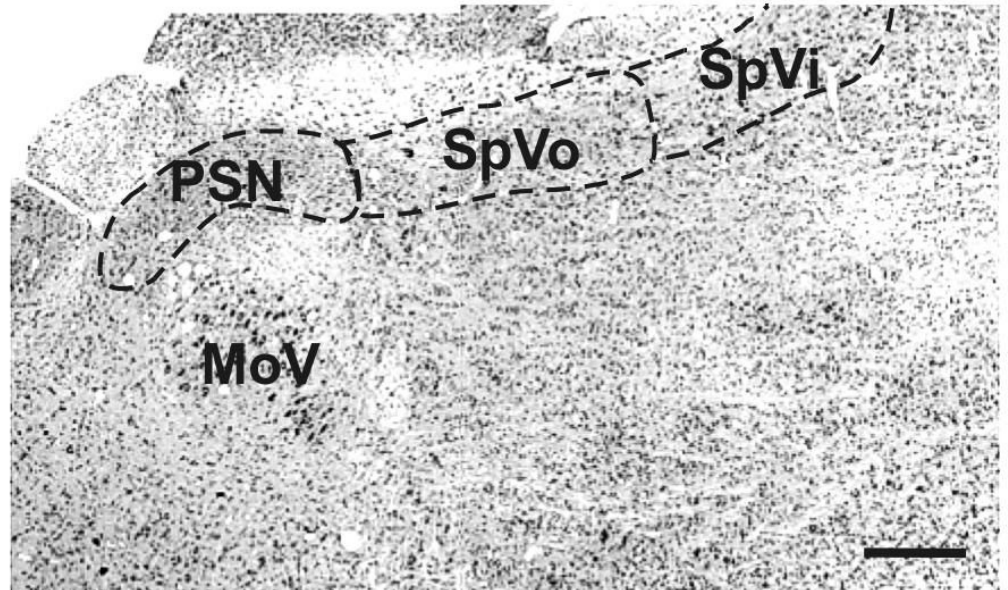
Figure 1.
Blood ethanol concentration (BEC).
The BEC rose precipitously to reach 233 mg/dl at 1.0 hour after the first injection. Peak BEC was reached two hours later. By eight hours post-injection, the BEC was 0. Arrows show the two injection times. Symbols represent the means of four animals (± standard errors of the means).
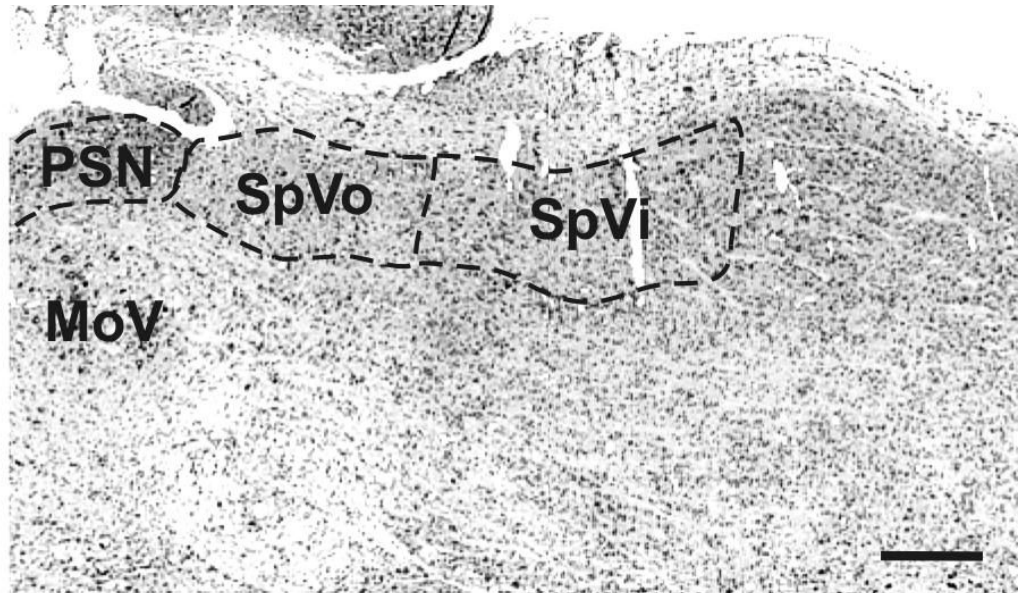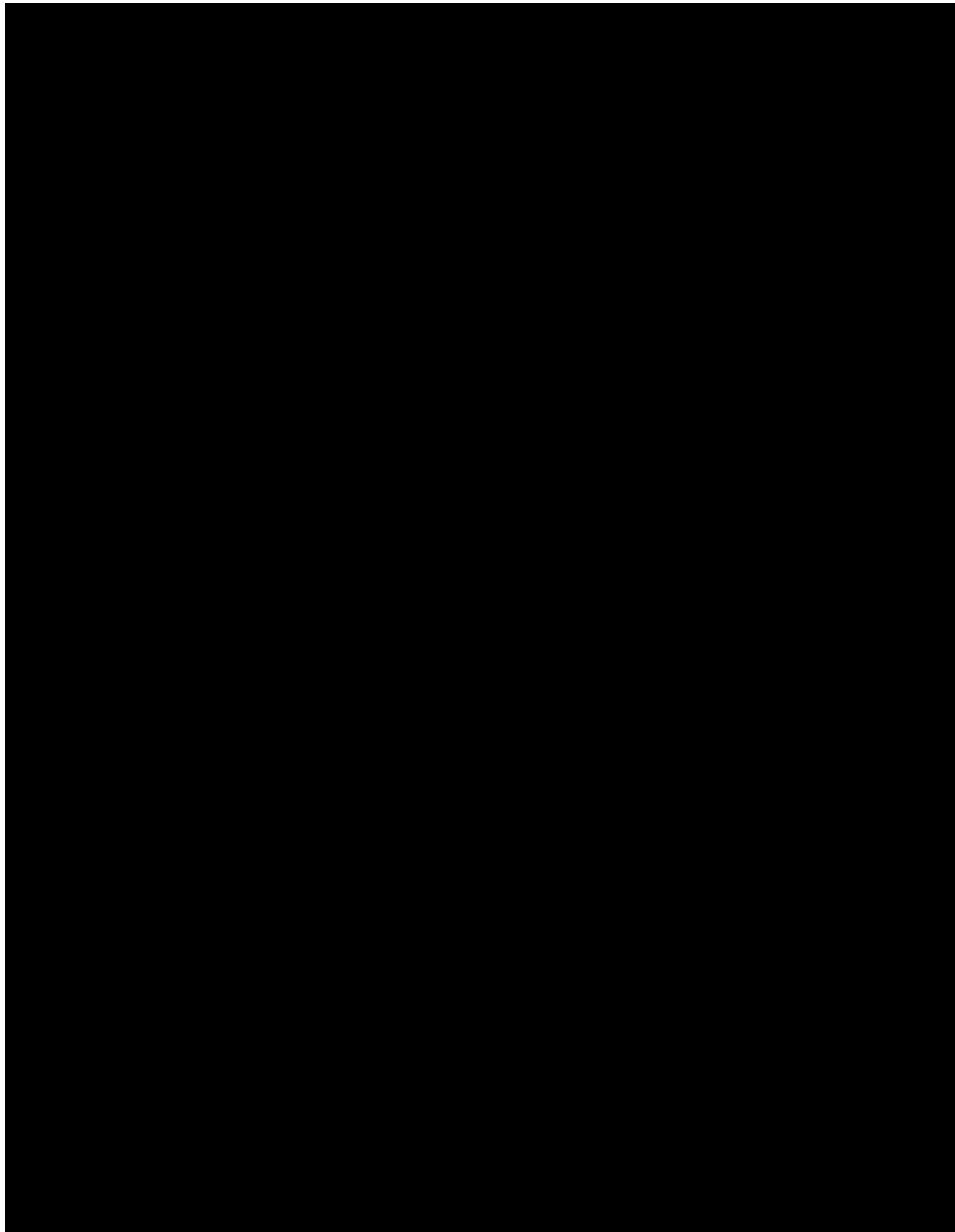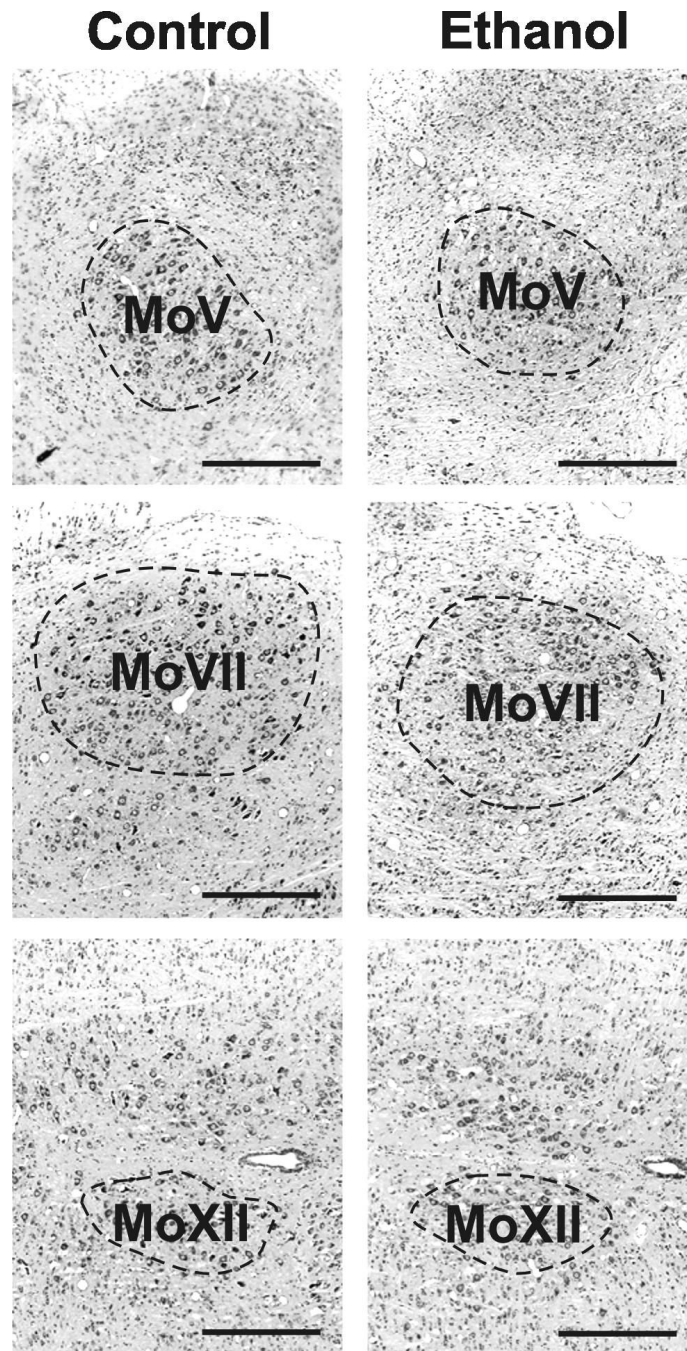
Figure 2.
Appearance of the sensory cranial nerve nuclei
Three sensory trigeminal nuclei were identifiable in horizontal sections stained with cresyl violet. Samples representing the animals treated with saline (controls) or ethanol were taken from cohorts dosed on gestational day 8. No gross differences were apparent between the groups. Rostral is oriented to the left and lateral to the top. Scale bars are 500 μm.

Figure 3.
Quantitative measures of the effects of ethanol on sensory nuclei
Stereological methods were used to estimate the volume (top) and the neuronal density of each nucleus (middle). Total neuronal number was estimated as the product of volume and density. Bars represent the means and T-bars signify the standard errors of the means. Each mean is based on five or six animals per group. Asterisks identify differences relative to the controls that were statistically significant (p<0.05).

Figure 4.
Appearance of the motor cranial nerve nuclei
Three motor nuclei (the motor nuclei of the trigeminal (MoV), facial (MoVII), and hypoglossal (MoXII) nerves were evident in horizontal sections. Images are oriented so that rostral is to the left and lateral to the top. Scale bars are 500 μm.
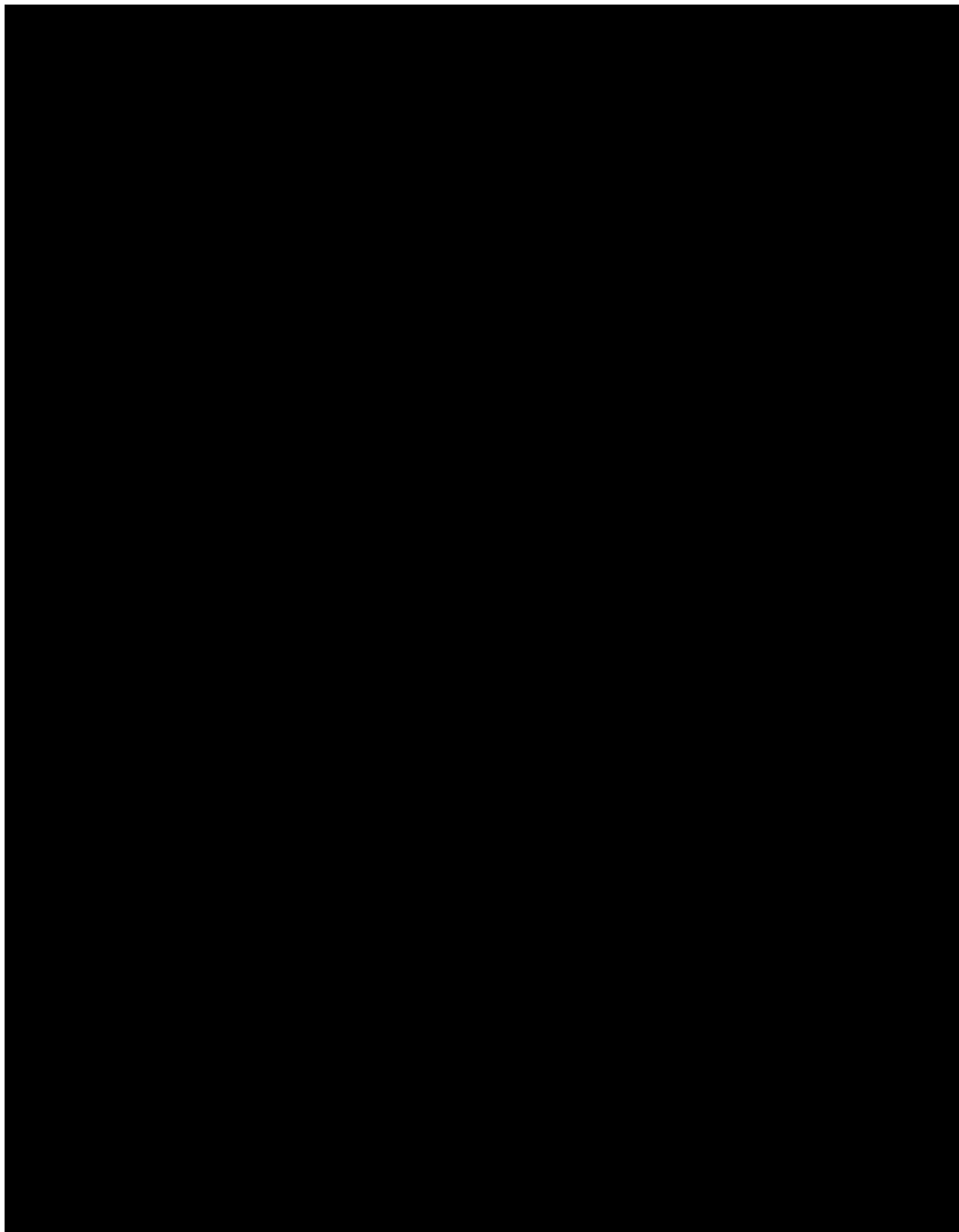
Figure 5.
Quantitative measures of the effects of ethanol on motor nuclei Notations as in Figure 3.

Table 1

Description of cranial nerve nuclei examined.

Name	Abbreviation	Plate derivation	Rhombomeric derivation	Days of peak neuronal generation [#]
principal sensory nucleus of the trigeminal nerve	PSN	alar	r2	G12 - 13
spinal trigeminal nucleus, oral subnucleus	SpVo	alar	r4	G13 - 14
spinal trigeminal nucleus, interpolar subnucleus	SpVi	alar	r6	G14 - 15
motor nucleus of the trigeminal nerve	MoV	basal	r2	G11 - 12
motor nucleus of the facial nerve	MoVII	basal	r4	G12 - 13
motor nucleus of the hypoglossal nerve	MoXII	basal	r8	G12 - 13

[#] Data from Altman and Bayer (1980a;1980b;1980c) and Miller and Muller (1989).

Table 2

Statistical analyses: nuclear volume.

Statistical test	Structure	F-statistic	Degrees of freedom numerator	Degrees of freedom denominator	P value
MANOVA	Overall	3.733	42	111	<0.0001[*]
ANOVA	PSN	9.324	7	28	<0.0001[*]
ANOVA	SpVo	2.672	7	28	0.0299[*]
ANOVA	SpVi	1.258	7	28	0.3063
ANOVA	MoV	12.822	7	28	<0.0001[*]
ANOVA	MoVII	6.678	7	28	0.0001[*]
ANOVA	MoXII	8.363	7	28	<0.0001[*]

[*] statistically significant differences

Table 3

Statistical analyses: neuronal density.

Statistical test	Structure	F-statistic	Degrees of freedom numerator	Degrees of freedom denominator	P value
MANOVA	Overall	3.563	42	111	<0.0001[*]
ANOVA	PSN	3.009	7	28	0.0173[*]
ANOVA	SpVo	6.604	7	28	0.0001[*]
ANOVA	SpVi	6.468	7	28	0.0001[*]
ANOVA	MoV	3.165	7	28	0.0135[*]
ANOVA	MoVII	2.463	7	28	0.0421[*]
ANOVA	MoXII	3.724	7	28	0.0057[*]

[*] statistically significant differences

Table 4

Statistical analyses: neuronal number.

Statistical test	Structure	F-statistic	Degrees of freedom numerator	Degrees of freedom denominator	P value
MANOVA	Overall	3.653	42	111	<0.0001[*]
ANOVA	PSN	13.136	7	28	<0.0001[*]
ANOVA	SpVo	5.861	7	28	0.0003[*]
ANOVA	SpVi	4.761	7	28	0.0013[*]
ANOVA	MoV	7.665	7	28	<0.0001[*]
ANOVA	MoVII	7.222	7	28	<0.0001[*]
ANOVA	MoXII	3.563	7	28	0.0073[*]

[*] statistically significant differences