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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).
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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, 1988; Dunty et al., 2002; Da Lee et al., 2004) exhibit craniofacial malformations 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,b, 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 from the alar plate and motor neurons are derived from the basal plate (e.g., Darnell, 2005). In

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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 a.m. 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 h of birth (postnatal day (P) 0), all litters were culled to ten. On P31, one animal from each litter was anesthetized (10 mg/kg ketamine and 10 mg/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 4 h 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-horizontal sections. 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 was 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 and Muller, 1989; Mooney and 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

$$V_T = \sum A_S \cdot t/f$$

in which $\sum A_S$ is 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 µm × 100 µm) 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

Table 1
Description of cranial nerve nuclei examined

Name	Abbreviation	Plate derivation	Rhombomeric derivation	Days of peak neuronal generation[a]
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

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

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