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VULNERABILITY OF MACAQUE CRANIAL NERVE NEURONS TO ETHANOL IS TIME AND SITE-DEPENDENT

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Abstract

The present study tested the hypotheses that vulnerability to ethanol depends upon (1) population-based characteristics of the neuronal progenitors and (2) the maturation of that population by examining the effects of prenatal exposure to ethanol on brainstem nuclei derived from different rhombomeres and from the alar and basal plates. *Macaca nemestrina* received an ethanol-containing solution one day per week during the first six (Et6) or 24 (Et24) weeks of gestation. Control animals received an equivalent volume of saline. The treatment regime for some animals included early gastrulation (gestational day (G) 19 or G20), whereas others were treated later (on G21 or G24). Brainstems were cryosectioned and stained with cresyl violet. Stereological methods were used to determine the numbers of neurons in six different nuclei: the abducens (VI), vagal (X), and hypoglossal (XII) motor nuclei and sensory components of the trigeminal brainstem nuclear complex (the principal (PSN), oral (SpVo), and interpolar (SpVi) subnuclei). There were no differences in the numbers of neurons in any of the nuclei between controls or Et6- and Et24-treated monkeys. In contrast, the number of trigeminal sensory neurons was significantly ($p<0.05$) lower in animals treated on G19/G20 than in control. No differences between controls and monkeys treated on G21/G24 were detected. No motor nuclei exhibited an ethanol-induced change. These data together with data on the trigeminal motor nucleus show that vulnerability to ethanol (1) is greater in sensory nuclei than in motor nuclei and (2) is temporally restricted to the time of gastrulation.

Keywords

alar plate; autism; brainstem; fetal alcohol spectrum disorder; fetal alcohol syndrome; gastrulation; rhombomere

INTRODUCTION

Developmental exposure to ethanol can reduce the final number of neurons in a particular structure. This results from a reduction in cell proliferation and an increase in neuronal death

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(Miller, 1995a; 1999;2006;Mooney et al., 2006). It has been hypothesized that the vulnerability to teratogen-induced damage is defined by the developmental derivation of the population (Rodier, 1996). Two major contributors to this vulnerability are (1) population-based characteristics including position, or location, of the structure and (2) the timing of the insult. The effect of position is apparent in cerebral cortex wherein populations of cells in different parts of the cortex respond differently to ethanol insult. For example, the pattern of cortical neuronogenesis is differentially disrupted in rostral and caudal cortex by prenatal exposure to ethanol (Miller, 1988). Moreover, the complement of neurons in early developing somatosensory cortex is unaffected by exposure to ethanol during the first two postnatal weeks, whereas the effect in the later developing visual cortex is Dtransient (Mooney and Napper, 2005).

A second critical factor defining ethanol vulnerability is the timing of the exposure. In the cerebral cortex, exposure during early gastrulation (Ashwell and Zhang, 1996; Miller, 2007) or the period of neuronal generation (Miller, 1995; 1996; 1997) affects neuronal number and projections. The effects of timing are also evident in the cerebellum. The number of Purkinje neurons is lower in animals exposed to ethanol on postnatal day (P) 4 or P5 than in control animals or in animals exposed to ethanol before P3 or on P8 or P9 (Goodlett et al., 1990; Pierce et al., 1999; Light et al., 2002). At least part of the response is shaped by the maturational state of the neurons. Earlier developing lobules (e.g., I-V, IX, and X) are more affected by exposure to ethanol on P4 than are later developing lobules (VI and VII) (Goodlett et al., 1990; Light et al., 2002).

The brainstem is uniquely suited to examine issues of regional and temporal vulnerability to ethanol. This is because during early development the brainstem is transiently compartmentalized into segments that are arranged orthogonal to the longitudinal neuraxis. Each segment, called a rhombomere, has a unique pattern of gene expression (Lumsden and Keynes, 1989; Murphy et al., 1989; Wilkinson et al., 1989; Lumsden, 1990). In addition, the developing brainstem can be subdivided into the alar and basal plates that give rise to sensory and motor components, respectively (Nieuwenhuys et al., 1998; Ju et al., 2004). These plates are oriented orthogonal to the rhombomeres and parallel to the neuraxis.

A key time for brainstem development appears to be gastrulation. The present study tested the hypothesis that the timing of the exposure to ethanol relative to gastrulation has different effects on brainstem nuclei derived from (a) different rhombomeres and (b) alar or basal plates. Six cranial nerve nuclei were examined (Fig. 1). Three were sensory nuclei derived from the alar plate across three different segments (rhombomere (r) 2, r4, and r6). Three motor nuclei were also examined. These were basal plate derivatives from r6 and r8.

MATERIALS AND METHODS

Animals

Fifteen pregnant *Macaca nemestrina* were housed in the Primate Center at the University of Washington, Seattle WA. All procedures were approved by the local Institutional Animal Care and Use Committee. The husbandry and care of these animals is described in detail elsewhere (Clarren and Astley, 1992). Briefly, animals received a solution by nasogastic intubation one day per week, i.e., every seventh day, for six or 24 weeks of gestation. Nine animals received an ethanol-containing solution (1.8 g/kg; Table 1). Of these, five were given ethanol during the first six (Et6) weeks of gestation and four received ethanol in all 24 (Et24) weeks of gestation. The remaining six animals received an equivalent volume of saline (Ct).

The timing of the ethanol dosing was critical. The feeding regime was set so that for four animals, the third feeding occurred at the time of gastrulation, on gestational day (G) 19 or

G20. These animals selectively exhibited the features of cranial dysmorphia associated with fetal alcohol syndrome (Astley et al., 1999), as well as neocortical changes (Miller, 2007). The remaining five ethanol-treated animals did not get dosed at the time of gastrulation, instead they received ethanol before (e.g., their second or third feedings on G14 or G17, respectively) and after gastrulation (e.g., their third or fourth feedings on G21 or G24, respectively). Venous blood was collected from mothers 2 hr after ethanol administration for determination of blood ethanol concentration (BEC) using gas chromatography.

Animals were allowed to give birth and offspring were reared normally. At four to five years of age, offspring were anesthetized (2.0 mg/kg xylazine and 10 mg/kg ketamine) and perfused intracardially with 200−300 ml saline, followed by 2−3 liter of 4.0% paraformaldehyde in 0.10 M phosphate buffer (PB; pH 7.4), and finally 1 liter of 10% sucrose in PB. Brains were removed and stored in a solution of 30% sucrose in PB for approximately 24 months.

Brainstems were separated by cutting through the rostral superior colliculus in the coronal plane and were hemisected (Mooney and Miller, 2001). The right half of each brainstem was frozen and cut into 40 μm thick sections in the horizontal plane. A series of every fifth section was collected for staining with cresyl violet.

Stereological methods

Neuronal counts were made using stereological methods (Mooney and Miller, 2001). The total numbers of neurons (N) in six brainstem nuclei (Table 2; Fig. 1) were calculated as the product of the volume of the nucleus (V) and the density of constituent neurons (Nv).

The Cavalieri estimator was used to determine V (Gundersen and Jensen, 1987; Miller and Muller, 1989). Identification of each brainstem nucleus relied on cytoarchitectonic features (Paxinos et al., 2000). In every section that contained a profile of the nucleus, the cross-sectional area (a) of that nucleus was measured using the Bioquant Image Analysis System (R&M Biometrics, Nashville TN). Volume of a nucleus (V) was calculated:

$$V = Ea \times t/f$$

wherein Ea was the sum of all cross-sectional areas for each nucleus, t was the section thickness, and f was the frequency of sections used in the analysis (Gundersen and Jensen, 1987; Miller and Muller, 1989; Mooney and Miller, 2001). The section thickness used in this calculation was initial section thickness, i.e., the thickness prior to processing. Thus, V represents the actual volume of each nucleus in the tissue and is independent of any processing-induced shrinkage.

The optical disector method was applied to determine neuronal density (N_V; Gundersen et al., 1988; Mooney and Miller, 2001). Images of the section were projected to a monitor and a sampling frame of known area (a_{frame}) was overlaid. Moving the microscope stage through a known depth (d) in the z-axis turned the two-dimensional frame into a three-dimensional box. The depth of the box used for counting was 20 μm, however, tissue processing resulted in 25% reduction of section thickness (from 40 μm to 30 μm). Thus, d in the calculation was adjusted for this shrinkage and was 26.67 μm. Guard zones of approximately 5 μm above and below the counting box were used for verification of cell identity. Three planes of the box were designated inclusion planes, and all neuronal nuclei completely within the box, or intercepting one of the inclusion planes, were counted (Q). Thus, the neuronal packing density was calculated by the following formula:

$$N_V = \Sigma Q / (\#frames \times a_{frame} \times d)$$

Statistical analysis

Data for the ethanol-exposed animals were divided into groups based upon the duration of ethanol exposure (Et6, n = 5 or Et24, n = 4) or by the timing of the exposure (G19/G20, n = 4 or G21/G24, n = 5). A mean (± the standard error of the mean) was determined for each value (e.g., nuclear volume, packing density, and neuronal number) for each group of animals. Multivariate one-way analyses of variance (MANOVA) were used to determine the effect of treatment. This was followed by individual one-way analyses of variance (ANOVAs) for each cranial nerve nucleus. In cases where an ANOVA showed a significant difference ($p<0.05$), *post-hoc* Tukey B tests were performed.

RESULTS

Blood ethanol concentrations

Mean BEC (± standard error of the mean) for all nine ethanol-treated macaques was 231 ± 20 mg/dl (Clarren and Astley, 1992). Parsing the data based on the duration or timing of the ethanol exposure did not reveal any differences in BEC (Mooney and Miller, 2001; Miller, 2007).

Brainstem nuclei

Exposure to ethanol did not affect the size or weight of the brainstems (Mooney and Miller, 2001). All six brainstem nuclei were identifiable in both Ct- and Et-treated macaques (Fig. 2). Motor nuclei were identified by their large polygonal cell bodies. This contrasted with the sensory nuclei which tended to be populated by smaller, rounder neuronal somata.

The data were parsed for analysis in two different ways: (1) according to the duration of the exposure to ethanol and (2) based on the timing of the exposure during the third week of gestation. The number of neurons in each nucleus was calculated as the product of the nuclear volume and the cell packing density. None of these parameters were significantly affected by the duration of the ethanol exposure, i.e., whether the treatment was confined to the first six weeks of gestation or occurred during all 24 weeks of gestation.

There was an effect of treatment at different times relative to gastrulation. Based on a MANOVA, both nuclear volume and neuronal number were significantly ($p<0.05$) affected by the timing of the ethanol exposure. Cell packing density was not significantly different among the groups in this data set.

Sensory nuclei

PSN. In the PSN, the ANOVA showed a significant ($F_{2,11} = 4.800$; $p = 0.032$) effect of treatment when data on the number of neurons was parceled based on timing of exposure (Fig. 3). *Post-hoc* comparison with the Student-Newman-Keuls test showed that number of neurons was significantly lower in monkeys treated with ethanol during early gastrulation compared with Ct-treated animals ($p<0.05$). This effect was largely due to a significant ($F_{2,11} = 5.914$; $p = 0.018$) change in the volume of the PSN. *Post-hoc* analysis showed that it was significantly ($p<0.05$) smaller in macaques treated on G19/G20 than in controls or in animals treated with ethanol on G21/24. There was no difference between Ct-treated monkeys and those exposed to ethanol on G21/24. Neuronal density was also significantly ($F_{2,11} = 6.844$; $p = 0.012$) lower in animals exposed to ethanol, however, in contrast with N and V, the effect on density was seen in animals treated on G21/24 compared with either controls or macaques that received ethanol on G19/G20.

SpVo. Neither volume nor neuronal packing density was significantly affected by exposure to ethanol (Fig. 3). Despite this, the SpVo showed a significant ($F_{2,11} = 5.647$; $p = 0.026$) effect of exposure to ethanol on neuron number in macaques exposed during early gastrulation.

Neuronal number was lower in animals exposed to ethanol on G19/G20 than Ct-treated animals ($p = 0.033$).

SpVi. There was no significant effect of exposure to ethanol on volume or neuronal packing density in the interpolar nucleus (Fig. 3). As seen in the SpVo, the number of neurons in the SpVi was significantly ($F_{2,11} = 4.529$; $p = 0.037$) affected by treatment. *Post-hoc* Tukey B tests showed that the number of neurons was significantly ($p<0.05$) lower in monkeys treated during early gastrulation than in Ct-treated animals.

Motor nuclei

The numbers of neurons in the three motor nuclei, abducens, vagus and hypoglossal, were not affected by exposure to ethanol, regardless of the duration or the timing of the exposure (Fig. 4). There was, similarly, no effect of ethanol on the volume or on the density of neurons within any nucleus.

DISCUSSION

Population-based ethanol vulnerability

Including the nuclei examined in a previous study (Mooney and Miller, 2001), the effects of ethanol on nine cranial nerve nuclei have been examined. Five of the nuclei are derivatives of the basal plate (motor nuclei) and four are alar plate derivatives (sensory nuclei; Fig. 1). The studies quantify the number of constituent neurons. Only one motor structure, the trigeminal motor nucleus, is affected by exposure to ethanol (Mooney and Miller, 2001). In contrast, the numbers of neurons in three of the four sensory nuclei (the PSN, SpVo, and SpVi, but not the medial superior olivary nucleus (MSO)), are affected in a time-dependent manner. A critical determinant of neuronal number in select brainstem nuclei is the timing of the exposure, i.e., the number of neurons is lower when the ethanol exposure includes early gastrulation. Thus, the alar plate is more vulnerable to the effects of ethanol exposure during gastrulation.

Vulnerability of cranial nerve nuclei to teratogenic agents, including ethanol, may be defined by the rhombomeric derivation of the population and timing of the insult (Rodier et al., 1996). This hypothesis is based on data from human children showing that r2 is especially vulnerable to teratogens such as valproic acid and thalidomide, but only when the exposure includes gastrulation. The present data on non-human primates are consistent with the concept that gastrulation is a critical window, but it does not support the rhombomere-specific vulnerability. Though both motor and sensory nuclei in r2 are affected, in more caudal rhombomeres, e.g., r4 and r6, only the sensory nuclei are altered.

It is noteworthy that though the SpVo in the r4 is affected by ethanol exposure on G19/G20, another sensory nucleus in r4 (the MSO) is not. There are two potential explanations for this apparent discrepancy. (1) Ethanol targets nuclei subserving general somatic afferents. The MSO does not transmit such information. (2) Susceptibility is defined by the timing of neuronal generation as well as position. In the rat, the peak production of MSO neurons is about a day earlier than the peak production of neurons in the SpVo (Altman and Bayer, 1980a; 1980b). Thus, it is possible that in the macaque the difference in maturational state of MSO neurons is protective.

The trigeminal-somatosensory system is preferentially targeted by ethanol. Interestingly, this includes motor and sensory components. It is uncertain whether (a) the primary effect is on both basal and alar plate derivatives within this system or (b) the change in the motor nucleus is secondary to a primary insult to the sensory nuclei. That there is no change in the facial motor nucleus (MoVII) and the two motor nuclei are linked such that a change in one can cause a change in the other (Byrd, 1988) argues against the latter possibility.

There is one caveat to this data, and that is that the sample sizes are small. The low "n" may result in a type II error, i.e., reporting a "not significant" finding when there is a difference.

Timing of ethanol vulnerability

A second contributor to ethanol vulnerability is the timing of the ethanol exposure. Damage to the trigeminal system occurs when the ethanol exposure includes early gastrulation. The implications of such targeting are (a) that system specification occurs at the time of gastrulation and (b) that a unique feature of trigeminal progenitors renders them vulnerable to ethanol exposure during gastrulation. It could be argued that the trigeminal specific effects follow the time-dependent effects of ethanol on craniofacial morphology. This argument is countered by the lack of a time-dependent effect on the MoVII which provides motor innervation to most of the face.

Interpretation of data from the present study of macaques is somewhat compromised because the subjects received ethanol doses over the course of weeks. This makes it difficult to ascribe exposure at a particular time to the outcome. Despite this impediment, the same animals as those used in the present study show that when the exposure includes G19 or G20, the offspring exhibit cranial dysmorphia (Astley et al., 1999). This concurs with data from mouse studies showing that exposure only at the time of gastrulation is sufficient to produce the cranial dysmorphia (Sulik et al., 1981; 1988; Dunty et al., 2002). Moreover, rats exposed to ethanol at the time of gastrulation show similar changes to brainstem cranial nerve nuclei (Mooney and Miller, 2007).

The greater sensitivity of the trigeminal system to ethanol-induced insult than the facial nucleus agrees with findings from other toxins, e.g., mercury chloride, and anti-epileptics, e.g., valproic acid (Gofflot et al., 1996; van Maele-Fabry et al.,1995; 1996). Interestingly, gestational exposure to another anti-epileptic, hydantoin, produces a syndrome with craniofacial features that are remarkably similar to those characteristic of fetal alcohol syndrome (cf. Jones and Smith, 1973; Hanson and Smith, 1975).

Underlying developmental events

Two contributors to the final number of neurons in a nucleus are cell proliferation and neuronal death. Ethanol can affect the number of neurons by causing a decrease in proliferation and/or by increasing neuronal death (Miller, 1995; 2006; Mooney et al., 2006; Mooney and Miller, 2007). Neural stem cells *in vivo* (Miller, 1996) and *in vitro* (Santillano et al., 2005; Prock and Miranda, 2007) appear to be refractory to ethanol-induced cell death. Thus, the reductions in the number of neurons in cranial nerve nuclei likely result from decreases in cell proliferation.

Cells with more restricted lineage are more susceptible to ethanol neurotoxicity, i.e., cell death is not uncommon among these cells (Miller, 1995; 1999; Jacobs and Miller, 2001; Miller et al., 2003). One alternative mechanism is that exposure to ethanol during gastrulation restricts or shifts cell fate (Kentroti and Vernadakis, 1992; Tateno et al., 2005; Miller and Hu, submitted). Accordingly, proportionally more non-neuronal cells are generated at the expense of neurons or more or one type of neuron is produced at the expense of others. Another possibility is that ethanol accelerates the process of cell definition (Miller and Robertson, 1993). The final consequence of any of these individually, or in combination, is that there are fewer cells in the mature structure.

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ABBREVIATIONS

ANOVA, analysis of variance
BEC, blood ethanol concentration
FAS, fetal alcohol syndrome
FASD, fetal alcohol spectrum disorder
G, gestational day
MANOVA, multivariate analysis of variance
MoV, motor nucleus of the trigeminal nerve
MoVI, motor nucleus of the abducens nerve
MoVII, motor nucleus of the facial nerve
MoX, motor nucleus of the vagal nerve
MoXII, motor nucleus of the hypoglossal nerve
MSO, medial superior Dolivary nucleus
N, total number
Nv, cell packing density
P, Epostnatal Pday
PSN, principal sensory nucleus of the trigeminal nerve
r, rhombomere
SpVi, interpolar portion of the spinal trigeminal nucleus
SpVo, oral portion of the spinal trigeminal nucleus
V, volume

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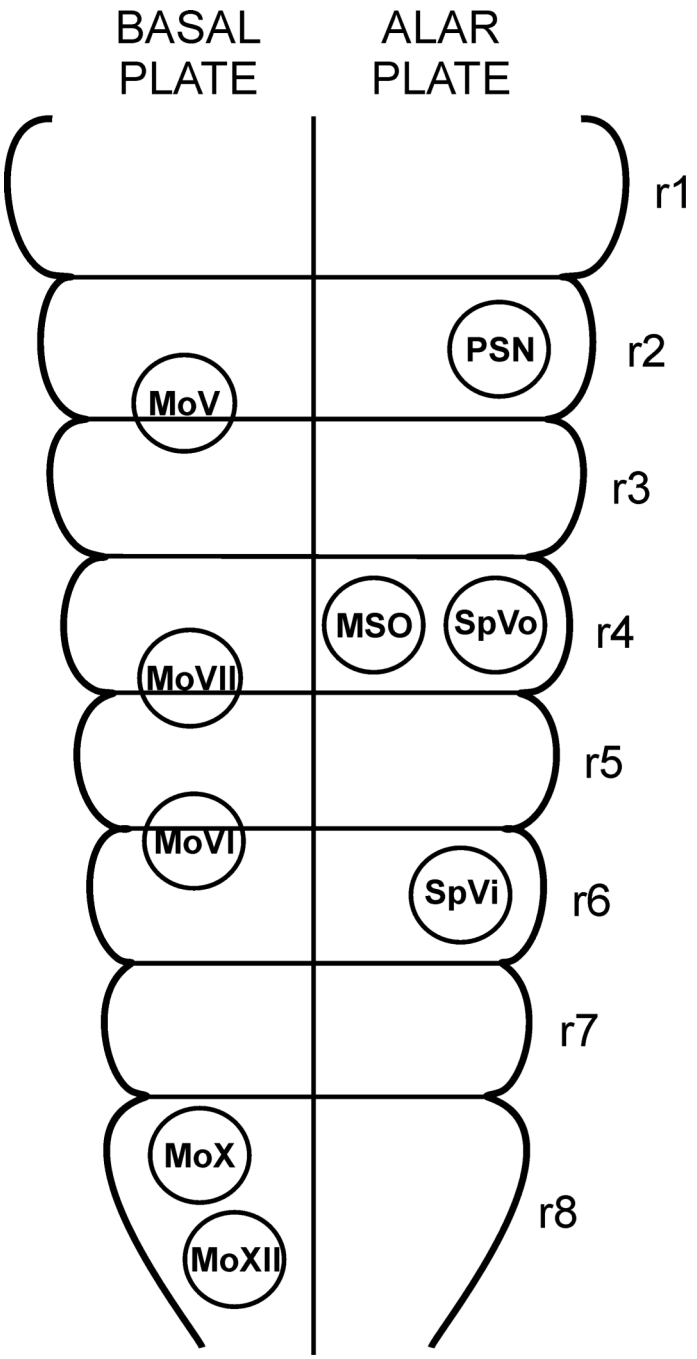

Figure 1. Schematic diagram of developing brainstem

During development the brainstem transiently forms at least eight rhomobomeres (r1-r8). Sensory neurons are derived from the alar, or roof, plate (right). Motor neurons are derived from the basal, or floor, plate (left).

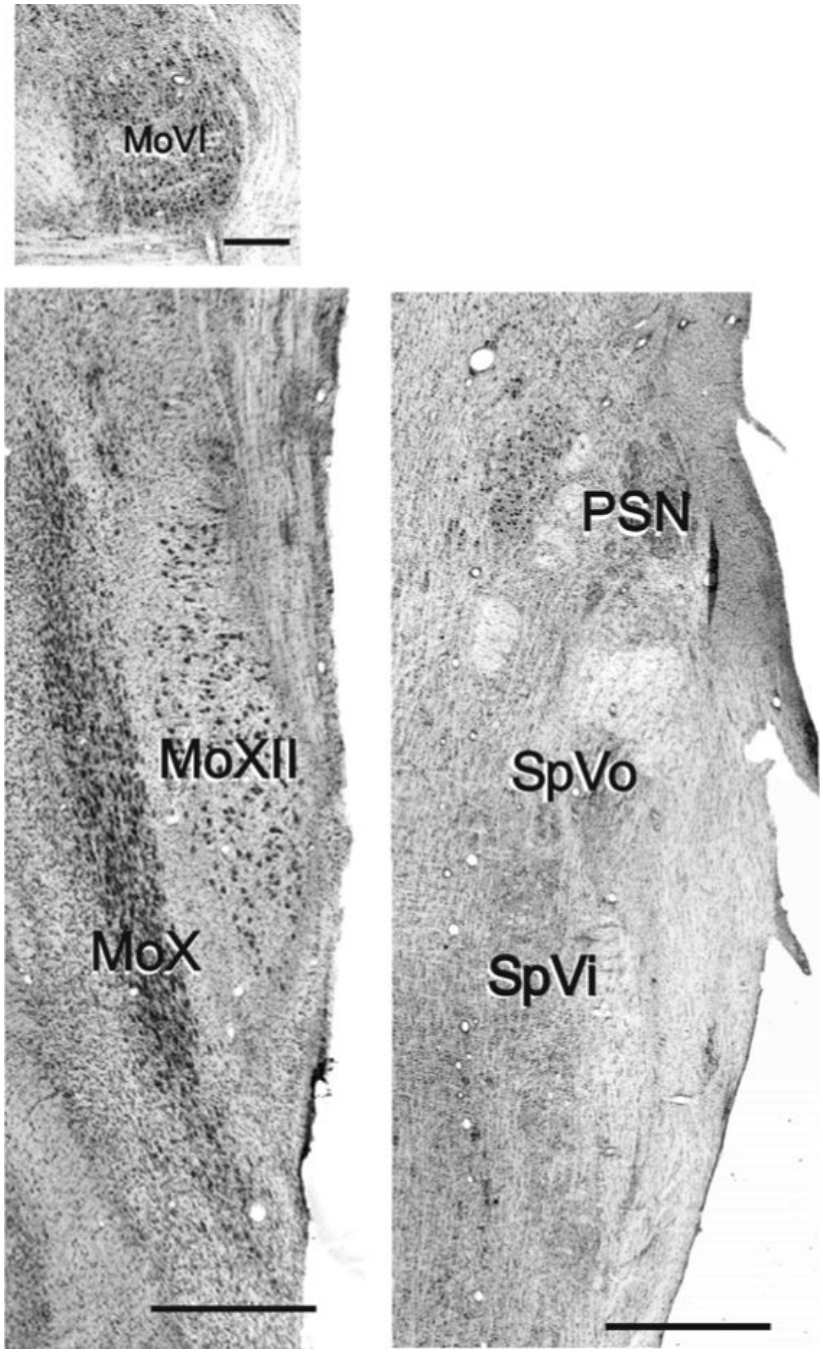

Figure 2. Appearance of the macaque brainstem

All six nuclei were readily identifiable in horizontal sections stained with cresyl violet. Three sensory nuclei of the trigeminal system are apparent within the same section; the principal sensory nucleus (PSN), and two parts of the spinal trigeminal nucleus - the oralis (SpVo) and the interpolaris (SpVi).

The motor nuclei are shown separately. MoVI motor nucleus of the abducens nerve. MoX motor nucleus of the vagus. MoXII motor nucleus of the hypoglossal. Scale bar is 500μm for all images.

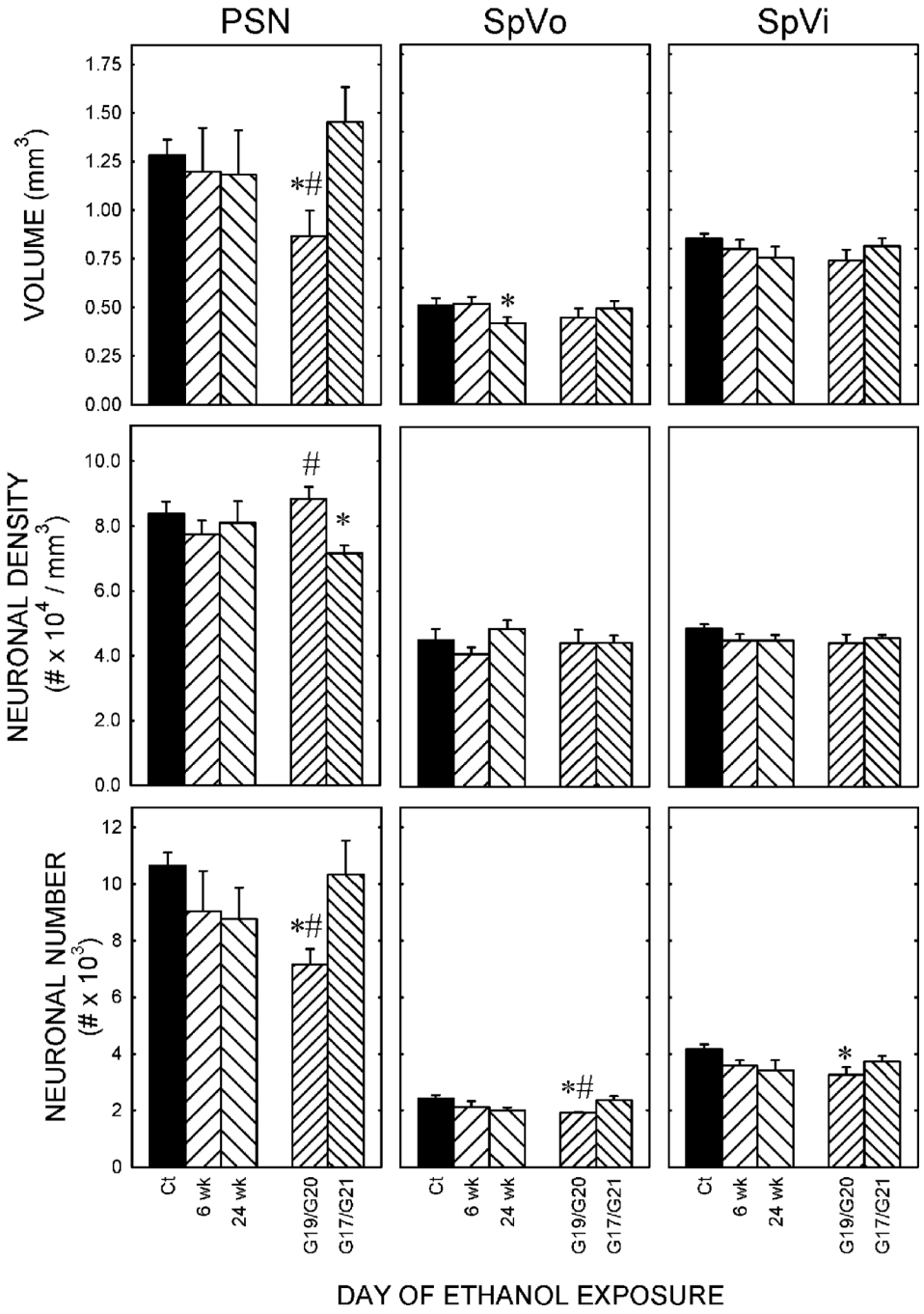

Figure 3. The effect of ethanol on the number of sensory neurons

Stereological methods were used to determine volume and cell packing density in each of three motor nuclei. These two parameters were multiplied to generate an estimate of the total number of neurons in each nucleus. Data on the left within each graph are organized according to the duration of exposure; animals were given ethanol on day per week for the first six or all 24 weeks of gestation. On the right within each graph, data are organized based on the day of exposure during the critical time of gastrulation; early gastrulation (gestational day (G)19 or G20) vs. late gastrulation (G21 or G24).

Left. The number of neurons in the PSN was significantly ($p<0.05$) lower in animals exposed to ethanol on G19 or G20 than in control animals. There were no significant differences among control animals and those exposed to ethanol for six or 24 weeks of gestation.
Middle. The number of neurons in the spinal trigeminal nucleus oralis was significantly lower in animals exposed to ethanol during early gastrulation compared with control-treated animals. This was a time-dependent effect, and was not seen in animals treated on G21 or G24. There was no significant effect of duration of exposure to ethanol.
Right. The time-dependent effect on the number of neurons in the spinal trigeminal nucleus interpolaris was also apparent. Exposure to ethanol during early gastrulation significantly reduced the number of neurons in this nucleus
* significantly different to control, $p<0.05$; # significantly different to animals exposed to ethanol on G21 or G24.

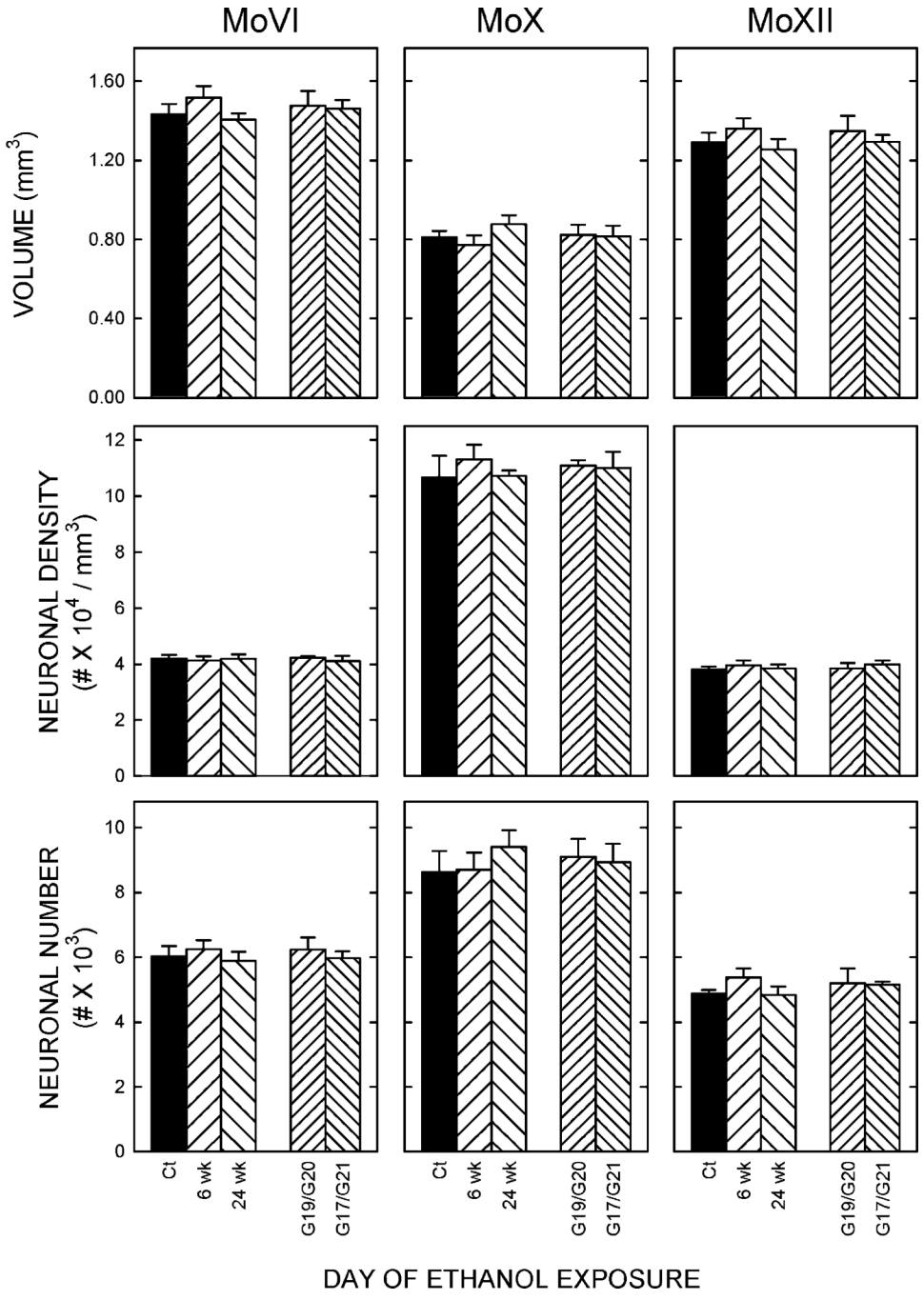

Figure 4. Quantification of the effect of ethanol on motor nuclei

Data were generated and organized as per Figure 3. Exposure to ethanol did not significantly affect the number of neurons in MoVI (left), MoX (middle), or MoXII (right).

Table 1

Ethanol exposure

Timing of Exposure	Duration of Exposure		n
	6 weeks	24 weeks	
G19/G20	2	2	4
G21/G24	3	2	5
n	5	4	

Table 2

Derivation of each brainstem nucleus.

Name	Abbreviation	Plate Derivation	Rhombomeric Derivation*
principal sensory nucleus of the trigeminal nerve	PSN	Alar	r2
spinal trigeminal nucleus, oral portion	SpVo	Alar	r4
spinal trigeminal nucleus, interpolar portion	SpVi	Alar	r6
motor nucleus of the abducens nerve	MoVI	Basal	r5−6
motor nucleus of the vagal nerve	MoX	Basal	r8
motor nucleus of the hypoglossal nerve	MoXII	Basal	r8

* Data from Cambronero and Puelles (2000).