ISSN- 0102-9010

EFFECTS OF ACUTE PRENATAL EXPOSURE TO ETHANOL ON THE POSTNATAL MORPHOLOGY OF THE PREFRONTAL CORTEX IN WISTAR RATS

Tales Alexandre Aversi Ferreira[1], Nadia Rubia Ferreira[2], José Oscar Rodrigues Morais[1] and Nilson Penha-Silva[2]

[1]Department of Morphology, Institute of Biological Sciences, Federal University of Goiás, Goiânia, GO,
[2]Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

ABSTRACT

Prenatal exposure to ethanol is frequently associated with microencephaly, hypomyelinization, delayed cell migration, and impaired neuronal and glial maturation in the offspring. The mechanism by which ethanol induces its effects on the development of the nervous system is still not fully understood. In this study, the influence of acute prenatal exposure to ethanol on the prefrontal cortex cells of rats were examined. Three doses of ethanol (3g/kg of body weight) were administered intraperitoneally to pregnant female rats on the 12th day of pregnancy, at 8 h intervals. Control rats received the same treatment but with a saline solution. Cells in the synthesis phase (S) of the cell cycle were labeled with bromodeoxyuridine. Six controls and 12 ethanol-treated neonates were sacrificed on the 8th day of postnatal life. The distance between nuclear cores in immunohistochemically labeled cells was determined by image analysis. Control rats had a normal neocortex, with six layers in the prefrontal region. Rats treated with ethanol showed ectopia of pyramidal neurons in layers I and II, heterotopia in the basal area of the prefrontal fissure, and a decrease in cellular density in layers II and VI of the cerebral prefrontal cortex. These alterations could help to explain some of the dysfunctions reported in patients with fetal alcohol syndrome.

Key words: Cortex, ectopia, ethanol toxicity, fetal alcohol syndrome, heterotopia, neuronal density, neuronal migration

INTRODUCTION

The development of the central nervous system involves a series of programmed cellular and molecular events, including patterned neuronal migrations and axonal growth. The generation of neurons and glial cells from the neocortex begins in the ventricular pseudostratified epithelium close to the ventricular cavity. Most neurons originate in the ventricular and subventricular zones. From these proliferative regions, the cells migrate to their targets where they differentiate into neurons. Thus, most neurons in the developing vertebrate nervous system are generated at sites that are significantly different from those where they reside in the adult brain [22]. In the ventricular and subventricular zones, neuronal precursors form a secondary proliferative population of pseudostratified epithelium. These cells migrate in the interval from embryonic stage E11 to E17, during which they complete 11 cell cycles in rats [26]. From those two proliferative zones, the cells migrate to reach their target site, where differentiation occurs.

Migration involves the displacement of a neuronal cell body from the proliferative zone to its final destination in the mature brain [20]. These events are regulated in such a way as to produce a neuronal density that is approximately the same from an area to another in the same brain and in the brains of several mammalian species [26]. The regulation of neuronal migration involves different cell populations, including Cajal-Retzius neurons, subplate neurons, neuronal precursors or radial glia, and multiple molecular mechanisms, such as cell cycle control, cell-cell interactions (usually mediated through cell adhesion molecules, CAMs, that include cadherins, selectins, integrins, mucins, and members of the IgG superfamily), neurotransmitter release, growth factor availability, platelet-activating factor degradation and signal transduction pathways [6-8]. Studies of the developing cerebral cortex have shown that its six layers are generated in an orderly sequence. The firsts neurons to migrate form the deep cortex layers,

Correspondence to: Dr. Nilson Penha-Silva
Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia (UFU), Av. Pará, 1720, Campus Umuarama, CEP 38400-902, Uberlândia, MG, Brasil. Tel: (55) (34) 3218-2203, ext. 23, Fax: (55) (34) 3218-2203, ext. 24, E-mail: nspenha@ufu.br

This work is part of a master's dissertation by Tales Alexandre Aversi Ferreira. TAAF present address: Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, MG.

whereas the last neurons to migrate form the most superficial layers [4,23]. Horizontal stratification of the brain cortex is very important for the normal physiology of the brain.

Disorders of neuronal migration lead to a variety of congenital malformations. The complexity and multiplicity of the mechanisms involved probably accounts for the clinical, radiological and genetic heterogeneity seen in human disorders of neuronal migration [8]. Prenatal exposure to ethanol is a cause of several abnormalities in the development of the brain cortex, and abnormal development can result in functional disturbances [5,9,14,18,27].

The aim of this study was to determine the effects of acute prenatal exposure to ethanol on the prefrontal cortex of rats treated with ethanol on the 12th day of intrauterine life (E12), when the neuroepithelia differentiate into neuroblasts [26], and neuronal migration begins. To our knowledge, this is the first report to describe the acute effects of ethanol on neuronal migration in the prefrontal cortex at the moment the first neurons are formed.

MATERIAL AND METHODS

Female Wistar rats (180-230 g), were housed overnight with male rats. The presence of a vaginal plug and sperm in the vagina confirmed successful mating and indicated the first day of gestation (E_0). Eighteen-day pregnant female rats were housed in cages at 22 ± 0.4°C, on a 12 h light/dark cycle, with free access to food and water. On the 12th day of pregnancy (E_{12}), 12 rats received three intraperitoneal injections of a 20% ethanol solution (3 g of ethanol/kg of body weight), at 8 h intervals. Ethanol was administered intraperitoneally because this route ensured that all rats received an equal volume and amount of drug and there was almost 100% absorption. Also at E_{12}, six control pregnant rats received saline injections of 0.9% at the same intervals as the ethanol-treated rats**.** Proliferating cells were labeled as described elsewhere [26]. All rats received a single intraperitoneal injection of BrdU (5 mg/mL in 0.9% NaCl, containing 70 mM NaOH) at a dose of 60 mg/kg 2 h after the last injection of ethanol or saline solution.

On the 8th day of postnatal life (P_8), all 18 litters were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and perfused with saline followed by 70% ethanol. The brains were removed and processed as described elsewhere [18]. Briefly, the brains were embedded in paraffin, sectioned saggitally (7 µm thick sections), and mounted on gelatin-coated slides. The sections were deparaffinized, hydrated in a graded ethanol series and treated with 1 N NaOH followed by 1 N sodium borate buffer (20 min each), prior to washing with phosphate buffer. After blocking non-specific sites with serum, the sections were incubated for 2 h with a monoclonal anti-BrdU antibody (diluted 1:500; Sigma, St. Louis, MO, USA) followed by incubation for 1 h with a goat anti-rat secondary antibody (diluted 1:200; Vector, Burlingame, CA, USA) and then treated with an avidin-biotin complex (Vector) and incubated with 3,3'-diaminobenzidine (Sigma). Sections were cover slipped with Entellan.

The presence of neuron clusters different from those normally seen in the prefrontal area was indicative of heterotopia, and the presence of unusual solitary neurons in the cerebral cortical layers was indicative of ectopia.

The distances among the nuclear cores of immunohistochemically labeled cells in each layer of the prefrontal cortex were determined by image analysis (ImageLab, version 2.3) and were measured perpendicularly to the major plane of the cortical layer. Thirty-six distances were measured per layer. Data were analyzed statistically using ANOVA and Student's t-test, with a value of $p<0.05$ indicating significance. All statistical analyses were done using the software Origin 6.0 (Microcal Software Inc.).

The experimental protocols described here were approved by the institutional ethics committee at the Federal University of Goiás.

RESULTS

Figure 1 shows the mean intercellular distances in layers I to VI of the cerebral prefrontal cortex. The differences between control and ethanol-treated rats were significant only for cortical layers II and VI. Ethanol-treated rats had lower neuronal densities in layers II and VI when compared with control rats. The most conspicuous change was the decrease in the neuronal population in the deepest layer (VI) of the prefrontal cortex in alcohol-treated rats.

Cellular ectopia was seen in all ethanol-treated rats (Fig. 2). Typical pyramidal neurons were seen invading the molecular and external granular layers. Ectopic neurons were observed underlying the pial surface above the molecular layer. Heterotopia was

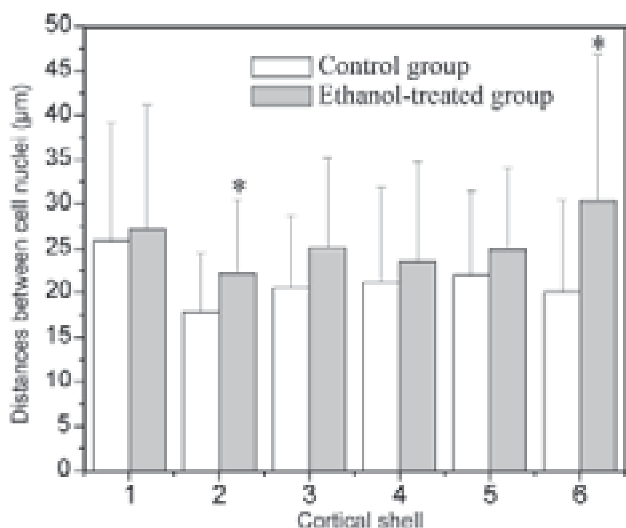

Figure 1. Distances between cell nuclei in the six cortical layers. The columns are the mean ± S.D. of 36 slides; *p<0.05 compared to the corresponding control.

observed in 16.7% of the rats that had been treated with ethanol, with a cluster of labeled heterotopic cells in the inferior area of the prefrontal fissure (Fig. 3). Control rats showed a homogenous pattern of cell distribution in the six layers of the prefrontal cortex, with no heterotopia or ectopia, and very similar distances among cells in each layer (Fig. 2A).

DISCUSSION

Human fetal alcohol syndrome (FAS) is characterized by retardation, microencephaly, neurologic abnormalities, facial dysmorphology, and other congenital anomalies [24]. Although several studies have demonstrated the negative effects of ethanol on the generation, migration, and concentration of neurons in the cerebral cortex of animals, the vulnerability of specific neuronal populations remains unclear [17,25,29].

The mammalian prefrontal cortex consists of six distinct thin layers that are rich in granular neurons and poor in pyramidal neurons [3]. These layers are characterized by distinct connections, neuro-transmitters and functions that vary with the age of the brain.

The effect of ethanol on neuronal migration can account for the presence of heterotopic clusters of neurons in the cerebral cortex [17]. Prenatal exposure of embryos to ethanol delays neuronal migration and inhibits the development of specific neuronal pathways *in vivo* and *in vitro* [17,28]. Ectopic pyramidal neurons were seen in all ethanol-treated rats examined here.

Defective neuronal migration is the major cause of gross brain malformations and more subtle abnormalities, such as abnormal neuronal positioning or altered patterns and sequence of synaptic circuits [1,21].

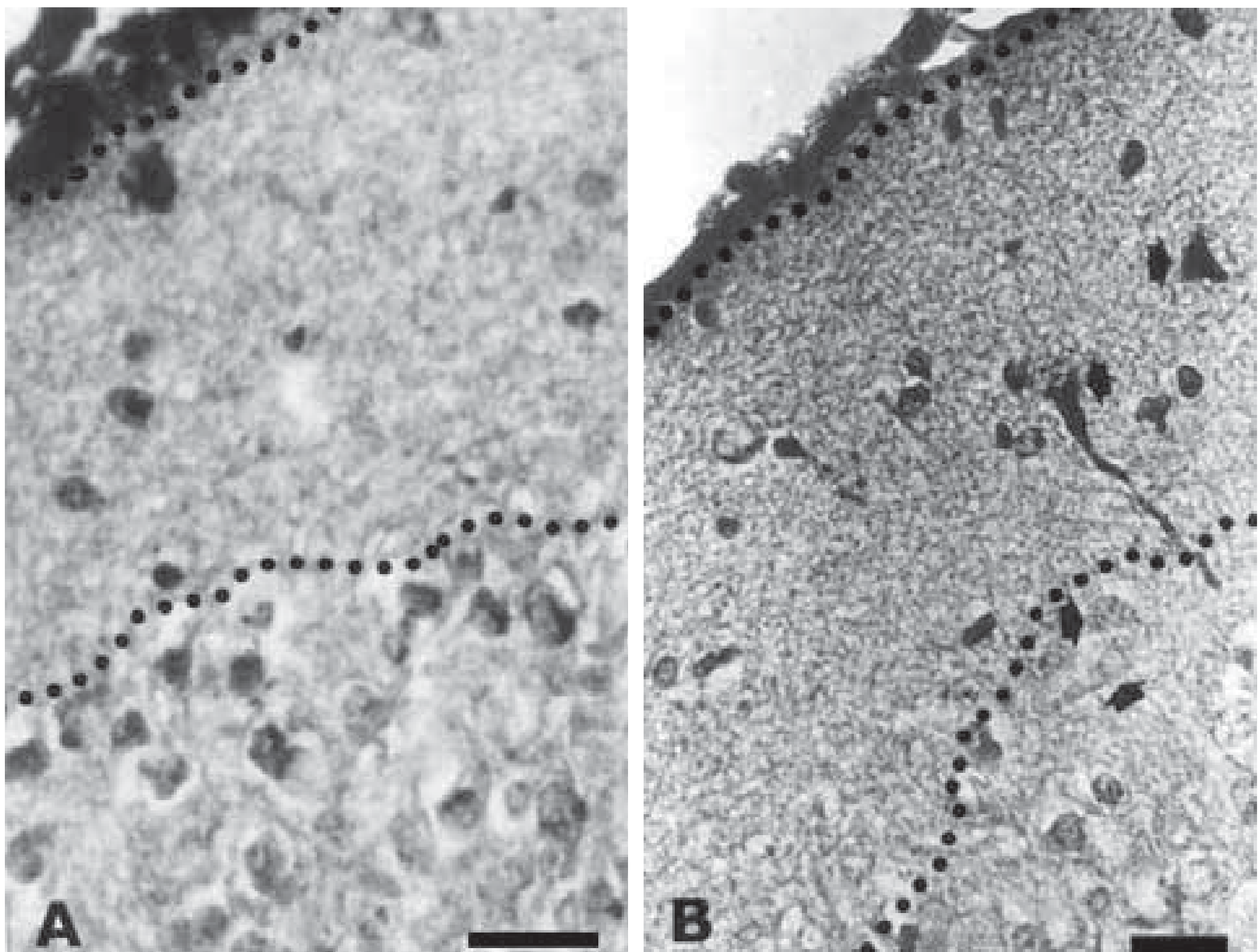

Figure 2. Pia matter and layers I (between the dotted lines) and II of the cerebral prefrontal cortex of control (**A**) and ethanol-treated (**B**) rats. S-phase cells labeled with BrdU have darkly stained nuclei. Neurons with pyramidal shapes (**arrows**) are present in layers I and II of the prefrontal cortex only in ethanol-treated rats (**B**). Bar = 20 μm.

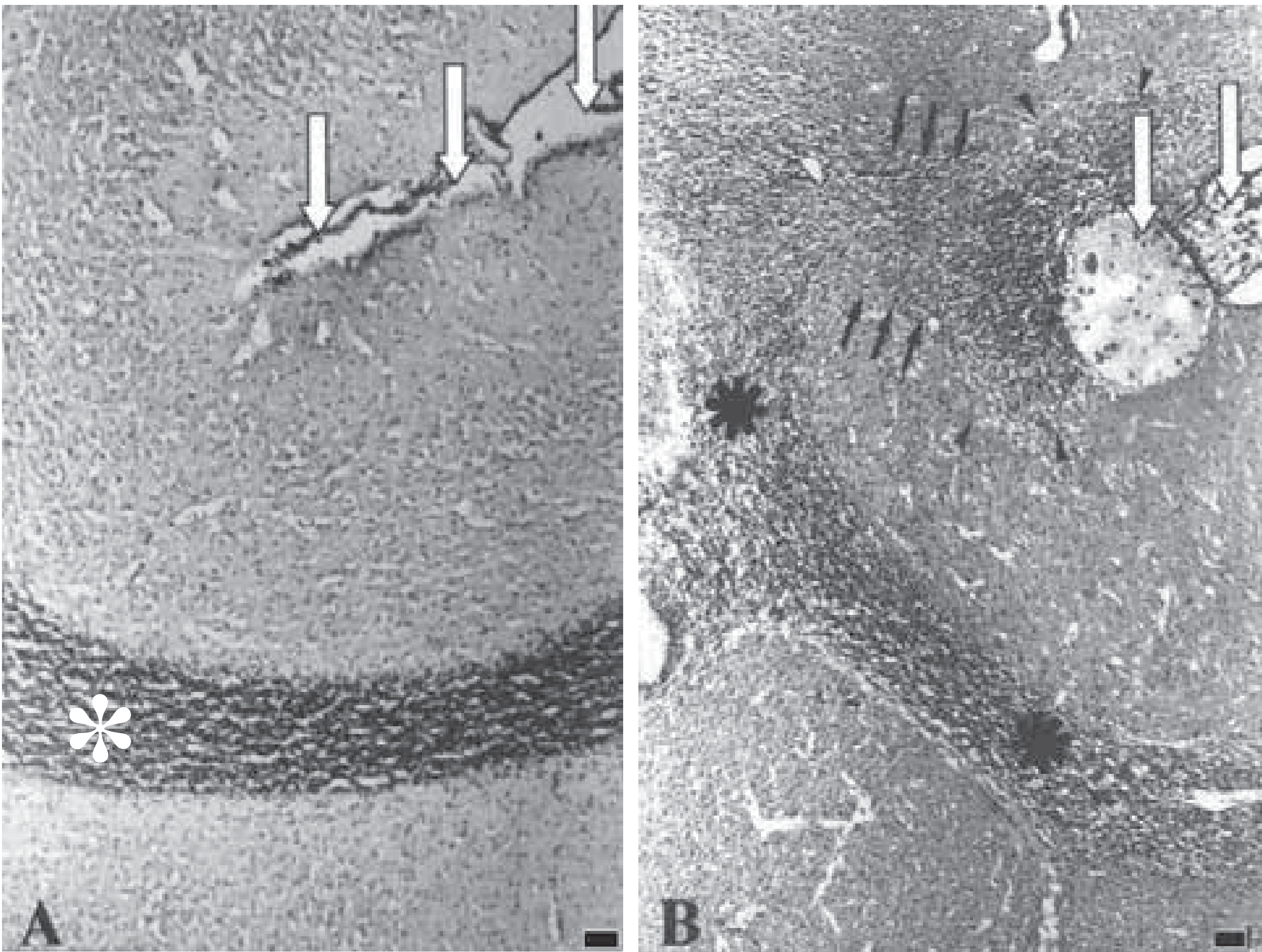

Figure 3. Normal neuronal migration (**asterisks**) to the olfactory bulb in control (**A**) and ethanol-treated (**B**) rats. The prefrontal fissure (**white arrows**) is evident in both groups. Abnormal neuronal migration to the olfactory bulb (**black arrows**) and heterotopic neurons (**arrowheads**) are present in ethanol-treated rats (**B**). Bar = 20 µm.

The time of exposure markedly influences the degree of injury since the teratogenic effects of ethanol appear to be greater when exposure occurs during neurogenesis. Little is known about the actual mechanism for the movement of neurons along glial fibers, although have been suggested to be involved in neuronal migration [10]. Ethanol consumption during pregnancy affects the phospholipid composition of the brain, liver and plasma of animals, with alterations in the polyunsaturated fatty acid content of brain phospholipids [2]. Exposure to ethanol also affects the phospholipid composition of *Xenopus* embryos [13]. Alterations in the lipid composition of the neuronal membrane will change the membrane fluidity and electrical behavior of the cell, and will probably also affect the physical and chemical forces that guide neuron migration. Such changes could be responsible for the neuronal ectopia reported in the literature [15,16] and also seen here. Neuronal migration also depends on an intact peroxisomal function in brain and in extraneuronal tissues. Indeed, a deficiency in peroxisome activity can lead to the accumulation of very long chain fatty acids (VLCFA) in brain and a specific impairment of neuronal migration, as seen in Zellweger's's syndrome [11]. Another possible cause of a change in neuronal migration may be related to the cell adhesion pattern since several acquired neurodevelopmental disorders have been correlated with defects in cell adhesion [7,30].

Acute exposure to ethanol also decreased the neuronal density in layers II and VI of the cerebral cortex, particularly during the period of cell proliferation, as shown by the increased distances among cell nuclei (Fig. 1). This effect could be caused by a decrease in the production of pyramidal and granular neurons. Another plausible explanation for the observed increase in the distance among cells could be an extensive loss of neuronal populations

[19,29]. Ethanol enhances cell death in the primitive neuroepithelium [9], which would explain the widespread cell death seen in the brain after intoxication [31]. Acute exposure of cultured neurons to ethanol results in single-strand breaks in DNA [12]. Ultimately, the deleterious effects of ethanol are probably related to the marked decrease in glucose metabolism throughout the brain.

In conclusion, the acute exposure of pregnant female rats to ethanol changes the structure of the cerebral prefrontal cortex of the offspring by altering the migration of neurons towards the deepest layers of the prefrontal cortex, by decreasing the neuronal density, and by producing ectopia and heterotopia. These changes could explain some of the dysfunctions reported in fetal alcohol syndrome in humans.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

1. Barth PG (1987) Disorders of neuronal migration. *Can. J. Neurol. Sci.* **14**, 1-16.
2. Burdge GC, Postle, AD (1995) Effect of maternal ethanol consumption during pregnancy on the phospholipid molecular species composition of fetal guinea-pig brain, liver and plasma. *Biochim. Biophys. Acta* **1256,** 346-352.
3. Caviness Jr VS (1975) Architectonic map of neocortex of the normal mouse. *J. Comp. Neurol.* **164,** 247-263.
4. Caviness Jr VS (1982) Neocortical histogenesis in normal and reeler mice: a developmental study based upon [^{3}H]thymidine autoradiography. *Brain Res.* **256,** 293-302.
5. Chevassus-au-Louis N, Congar P, Represa A, Ben-Ari Y, Gaiarsa JL (1998) Neuronal migration disorders: heterotopic neocortical neurons in CA1 provide a bridge between the hippocampus and the neocortex. *Proc. Natl. Acad. Sci. USA* **95**, 10263-10268.
6. Edelman GM, Jones FS (1998) Gene regulation of cell adhesion: a key step in neural morphogenesis. *Brain Res. Brain Res. Rev.* **26,** 337-352.
7. Greenberg DA (2003) Linking acquired neurodevelopmental disorders to defects in cell adhesion. *Proc. Natl. Acad. Sci. USA* **100**, 8043-8044.
8. Gressens *P* (2000) Mechanisms and disturbances of neuronal migration. *Pediatr. Res.* **48**, 725-730.
9. Gressens P, Lammens M, Picard JJ, Evrard P (1992) Ethanol-induced disturbances of gliogenesis and neuronogenesis in the developing murine brain: an *in vitro* and *in vivo* immuno-histochemical and ultrastructural study. *Alcohol Alcohol.* **27**, 219-226.
10. Haten ME (1993) Riding the glial monorail: a common mechanism for glial-guided neuronal migration in different regions of the developing mammalian brain. *TINS* **13**, 179-184.
11. Janssen A, Gressens P, Grabenbauer M, Baumgart E, Schad A, Vanhorebeek I, Brouwers A, Declercq PE, Fahimi D, Evrard P, Schoonjans L, Collen D, Carmeliet P, Mannaerts G, Van Veldhoven P, Baes M (2003) Neuronal migration depends on intact peroxisomal function in brain and in extraneuronal tissues. *J. Neurosci.* **23**, 9732-9741.
12. Lamarche F, Gonthier B, Signorini N, Eysseric H, Barret L (2003) Acute exposure of cultured neurons to ethanol results in ireversible DNA single-strand breaks, whereas chronic exposure causes loss of cell viability. *Alcohol Alcohol.* **38**, 550-558.
13. Lindi C, Montorfano G, Rossi F, Gornati R, Rizzo, AM (2001) Effect of ethanol exposure on *Xenopus* embryo lipid composition. *Alcohol Alcohol.* **36**, 388-392.
14. Luhmann HJ, Karpuk N, Qu M, Zilles K (1998) Characterization of neuronal migration disorders in neocortical structures. II. Intracellular *in vitro* recordings. *J. Neurophysiol.* **80**, 92-102.
15. Miller MW (1986a) Effects of alcohol on the generation and migration of cerebral cortical neurons. *Science* **233**, 1308-1311.
16. Miller MW (1986b) Effect of prenatal exposure to ethanol on the development of cerebral cortex. I. Neuronal generation**.** *Alcohol Clin. Exp. Res.* **12**, 440-449.
17. Miller MW (1992) Effects of prenatal exposure of ethanol on cell proliferation and neuronal migration. In: *Development of the Central Nervous System: Effects of Alcohol and Opiates* (Miller MW, ed). pp. 47-69. Wiley-Liss, Inc.: New York.
18. Miller MW (1993) Migration of cortical neurons is altered by gestational exposure to ethanol. *Alcohol Clin. Exp. Res.* **17**, 304-314.
19. Pierce DR, Goodlett CR, West JR (1989) Differential neuronal loss following early postnatal alcohol exposure**.** *Teratology* **40**, 113-126.
20. Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* **145,** 61-83.
21. Rakic P (1988) Defects of neuronal migration and the pathogenesis of cortical malformations. *Prog. Brain Res.* **73**, 15-37.
22. Rakic P (1990) Principles of neural cell migration. *Experientia* **46**, 882-891.
23. Sidman RL, Rakic P (1973) Neuronal migration with special reference to developing human brain: a review. *Brain Res.* **62**, 1-35.
24. Streissguth AP, Landesman-Dwyer S, Martin JC, Smith DW (1980) Teratogenic effects of alcohol in humans and laboratory animals. *Science* **209**, 353-361.
25. Strömland K, Pinazo-Durán MD *(2002)* Ophthalmic involvement in the fetal alcohol syndrome: clinical and animal model studies. *Alcohol Alcohol.* **37**, 2-8.
26. Takahashi T, Nowakowski RS, Caviness VS (1996) Interkinetic and migratory behavior of a cohort of neocortical neurons arising in the early embryonic murine cerebral wall. *J. Neurosci.* **16**, 5762-5776.
27. Velez-Domingues LC (1998) Neuronal migration disorders. *Gac. Med. Mex*. **134**, 207-215.
28. Ward G, West J (1992) Effects of ethanol during developing on neuronal survival and plasticity. In: *Development of the Central Nervous System: Effects of Alcohol and Opiates* (Miller MW, ed). pp.109-138. Wiley-Liss, Inc.: New York.
29. West JR, Hamre KM, Pierce DR (1984) Delay in brain growth induced by alcohol in artificially reared pups. *Alcohol* **1,** 213-222.
30. Wilkemeyer MF, Chen SY, Menkari CE, Brenneman DE, Sulik KK, Charness ME (2003) Differential effects of ethanol antagonism on neuroprotection in peptide fragment NAPVSIPQ prevention of ethanol-induced developmental toxicity. *Proc. Natl. Acad. Sci. USA* **100,** 854 3-8548.
31. Zhu W, Volkow ND, Ma Y, Fowler JS, Wang GK (2004) Relationship between ethanol-induced changes in brain regional metabolism and its motor, behavioural and cognitive effects. *Alcohol Alcohol.* **39**, 53-58.

Received: January 29, 2004
Accepted: May 11, 2004