The effects of ethanol exposure on the morphological development of the Oculomotor nucleus in the rat

by Robert Clayton Burrows

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

Abstract:
Morphological development of the Oculomotor nucleus was investigated under the influence of either a prenatal ethanol exposure or a prenatal and early postnatal exposure, through the first 9 days. These two types of exposure regimes represented either a two trimester or a three trimester equivalency exposure in humans. Plastic sections were analyzed with the light microscopic and the number of neurons per unit area was found to be decreased in the ethanol exposed animals in both the two and three trimester equivalency exposures. The ethanol exposed animals showed an increase in the number of astrocytes as well as the number of transitional glial cells per unit area in both exposure regimes. No change in the number of neurons/mm2 was found in counts done on the defined oculomotor region, yet the area of the defined oculomotor region was decreased in the ethanol exposed animals as compared to the control animals. Densiometric analysis on the area of the cell nucleus and nucleolus showed a significant reduction in the experimental animals in the three trimester equivalency.

Analysis of the Golgi - Cox impregnated multipolar neurons from the two trimester equivalency showed that the experimental neurons were decreased in soma size, had less complexity of dendritic branching, and were found to contain less complex dendritic arbors using a concentric ring analysis of Sholl. The results indicate that exposure to ethanol during development causes significant alterations in the morphology of the oculomotor nucleus in the rat and the timing of these deficits appears to occur in the prenatal period.

THE EFFECTS OF ETHANOL EXPOSURE ON THE MORPHOLOGICAL DEVELOPMENT OF THE OCULOMOTOR NUCLEUS IN THE RAT

by

Robert Clayton Burrows

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biological Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 1992

APPROVAL

of a thesis submitted by

Robert Clayton Burrows

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

July 29, 1992
Date

Dwight E. Phillips
Chairperson, Graduate Committee

Approved for the Major Department

July 29, 1992
Date

R E Moore by H Preston acting
Head, Biology Department

Approved for the College of Graduate Studies

8/4/92
Date

R L Brown
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made.

Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted by the copyright holder.

Signature Robert Clayton Burrows

Date July 29, 1992

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and deepest respect to Dr. Dwight E. Phillips, advisor, and Dr. Ashok K. Shetty for their encouragement, guidance, and friendship throughout this study. I would also like to thank Dr. Charles Paden, and Dr. Anne Rusoff for serving as my graduate committee.

I would also like to express a heartfelt gratitude to my parents James A. Burrows, Helen R. Burrows, and my brother Joseph A. Burrows for their support and encouragement.

TABLE OF CONTENTS

TABLE OF CONTENTS–Continued

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Morphological development of the Oculomotor nucleus was investigated under the influence of either a prenatal ethanol exposure or a prenatal and early postnatal exposure, through the first 9 days. These two types of exposure regimes represented either a two trimester or a three trimester equivalency exposure in humans. Plastic sections were analyzed with the light microscopic and the number of neurons per unit area was found to be decreased in the ethanol exposed animals in both the two and three trimester equivalency exposures. The ethanol exposed animals showed an increase in the number of astrocytes as well as the number of transitional glial cells per unit area in both exposure regimes. No change in the number of neurons/mm^2 was found in counts done on the defined oculomotor region, yet the area of the defined oculomotor region was decreased in the ethanol exposed animals as compared to the control animals. Densiometric analysis on the area of the cell nucleus and nucleolus showed a significant reduction in the experimental animals in the three trimester equivalency.

Analysis of the Golgi - Cox impregnated multipolar neurons from the two trimester equivalency showed that the experimental neurons were decreased in soma size, had less complexity of dendritic branching, and were found to contain less complex dendritic arbors using a concentric ring analysis of Sholl. The results indicate that exposure to ethanol during development causes significant alterations in the morphology of the oculomotor nucleus in the rat and the timing of these deficits appears to occur in the prenatal period.

INTRODUCTION

Alcohol's ability to act as a teratogen in utero has been well documented and characterized as Fetal Alcohol Syndrome (FAS) in humans (Jones and Smith, 1973; Jones and Smith, 1975). The clinical manifestations of FAS are craniofacial malformations; growth deficiencies; psychomotor retardation, hyperactivity, and other evidences indicative of CNS damage (Jones et al., 1973; Streissguth, 1986 Streissguth et al., 1986; Abel and Sokol, 1987). Of the clinical manifestations, CNS damage and its effects are the most devastating.

In an effort to understand this CNS damage in humans, numerous experimental studies using animals exposed to ethanol during development have shown a wide variety of effects on the developing nervous system. Among these are delays in the development of neurons (reviewed by West and Pierce, 1986), glial cells (Phillips and Krueger, 1990; Phillips and Krueger, 1992; reviewed by Phillips, 1992), and myelin (Samorajski et al., 1986; Phillips, 1989; Phillips et al., 1991a). Alterations of nerve and glial cell proliferation and maturation (Kennedy and Elliott, 1985; Miller, 1986), decreases in the complexity of the dendritic arbor (Hammer and Scheibel, 1981; Smith et al., 1986), as well as alterations in the structure and distribution of

neuronal cytoplasmic organelles (Al-Rabiai and Miller, 1989) are common. The number of neurons per unit area is decreased in the somatosensory cortex (Miller and Potempa, 1990), hippocampus (West and Pierce, 1986), and cerebellum (Goodlett et al., 1990; Bonthius and West, 1991) after developmental alcohol exposures. Neuronal alterations in midbrain and brainstem structures appear to be more variable. An ethanol induced increase in neuronal density has been shown during development in the superior and inferior colliculi (Zajac, 1987; Zajac et al., 1988) as well as in the rostral red nucleus, with no change in the caudal red nucleus (Zajac et al., 1989). Yet, a decrease in the neuronal density has been demonstrated in the principle sensory nucleus of the trigeminal nerve (Miller and Muller, 1989) following prenatal exposure to ethanol.

Despite the number of systems studied, one of the systems most clearly affected in humans has received little attention in animals. Visual system defects associated with FAS in humans include microphthalmia, cataracts, hypoplasia of the optic disc, increased tortuosity of the retinal vessels, and varying degrees of myopia, ptosis, and strabismus (Stromland, 1982; Stromland, 1985; Stromland, 1990; Stromland et al., 1991). Many of these latter defects potentially involve actions of the extraocular eye muscles, their nerve supply, or associated nuclei and

interconnections in the brainstem. Presently a void exists in the literature concerning the effects of alcohol on the development of the oculomotor system. Therefore, this study was designed to test the hypothesis that one of the effects of developmental alcohol exposure is an alteration of the development of the oculomotor nucleus, which innervates four of the six extraocular eye muscles.

In an effort to extrapolate from animals to the human, similar stages of brain growth must be examined. Thus, in similar studies periods of brain development are generally referred to by their human trimester equivalencies (West, 1987). At birth the rat's brain is at a stage of development equivalent to a human brain at the end of the second trimester. It isn't until about the end of the tenth postnatal day that the development of the rat brain is equivalent to that of a human brain at birth (Dobbing and Sands, 1979).

Two types of developmental exposures to alcohol are routinely used in this laboratory: a two trimester equivalency (2TE), in which the pregnant dams are given alcohol in their diet throughout gestation; and a three trimester equivalency (3TE) where, in addition to the prenatal exposure, the rats are artificially reared from postnatal day 1 (PND 1) through the morning of PND 10, and exposed to ethanol through a chronically implanted

gastrostomy tube (Samson and Diaz, 1982a; West et al., 1984b; Phillips et al., 1991a).

This study employed both a 2TE and a nearly full 3TE exposure to alcohol to examine the effects of alcohol on the development of the oculomotor nucleus on PND 15 in rats. Both plastic section light microscopy and Golgi - Cox staining were used to evaluate the nucleus for potential alterations in its development and in the development of dendritic arbors. A level approximating the middle third of the nucleus was used, an area that contains neurons primarily innervating the inferior rectus, medial rectus, superior rectus and levator palpebrae muscles (Glicksman, 1980; Labandeira Garcia and Gomez Segade, 1983).

LITERATURE REVIEW

Recognition of Fetal Alcohol Syndrome (FAS)

Mention has been made of the adverse effects of alcohol on the development of the human fetus since mythological times (Abel, 1984); however, it was not until 1968 that those effects were defined in the biomedical literature by Lemoine (as cited in Peiffer et al., 1979), and it was 1973 before Fetal Alcohol Syndrome (FAS) was defined clinically (Jones et al., 1973; Jones and Smith, 1973). The three most common characteristic features of FAS are prenatal and postnatal growth retardation; facial dysmorphology; and central nervous system (CNS) dysfunction (Jones et al., 1973; Jones and Smith, 1973; Jones and Smith, 1975; Clarren et al., 1978; Streissguth et al., 1978; Streissguth, 1986). The occurrence of one or two of the above features, but not all three combined, in the context of maternal alcohol abuse is defined as Fetal Alcohol Effects (FAE) (Abel and Sokol, 1987).

Of the above features of FAS, the most distressing are those that manifest themselves with CNS dysfunction, including motor dysfunction, intellectual deficits, and other behavioral and neurological manifestations of developmental brain damage (Streissguth et al., 1991). Measured intelligence quotients of FAS victims range from 15 to 105 with a mean of 65 and appear to change very little

with maturation (Streissguth et al., 1985; Streissguth, 1986; Streissguth et al., 1986; Streissguth et al., 1991). Fine motor dysfunction is evidenced by weak grasp, poor hand-eye coordination, tremulousness during infancy, and ocular disturbances involving the movement of the eyes (Jones and Smith, 1975; Stromland, 1981b; Stromland, 1982; Stromland, 1985; Stromland et al., 1991). The wide range of CNS dysfunction that can occur, combined with the fact that CNS pathology commonly occurs in the apparent absence of any external abnormalities (Clarren et al., 1978; Peiffer et al., 1979), the fact that the brain is one of the first organs to begin developing and the last to finish, and the incredibly intricate complexity of the developing brain make the CNS especially susceptible to <u>in utero</u> ethanol exposure.

The implications of FAS to society are enormous. The frequency of full-blown FAS in the U.S. has been estimated as high as 1.9/1000 live births, while the frequency of FAE has been estimated as being 3-10 times higher (Abel and Sokol, 1987; Abel and Sokol, 1991). FAS is the leading cause of mental retardation in the U.S. with an estimated annual cost of $321 million annually (calculating cost to 21 years of age) (Streissguth et al., 1991). This estimate does not include the cost of treating the milder cases of FAE, or the cost across the lifetime of FAS affected children, estimated at 1.4 million per case.

Such a tremendous economic impact on society makes it important to understand the specific and regional vulnerability of the nervous system to alcohol, and to understand the response of nervous tissue as a whole to such developmental insults. At a more basic level it is important and of interest to understand the effects of ethanol on the cellular components of the developing nervous system, including different neuronal populations, different classes of glial cells, and their elaborations that are important for normal neuronal function.

Experimental Models for Developmental Ethanol Exposure

Experimental models for the effects of ethanol on the developing CNS have utilized a variety of species, dating as far back as the 1880's, (reviewed by Abel, 1984) including fish, chicken, dogs, mice, sheep, and monkeys (Abel, 1982; Clarren and Bowden, 1982). The rat has probably been used the most extensively because it is relatively easy to maintain, has a short gestation period, and a vast literature is readily available on the normal development and organization of the brain (Abel, 1982; Abel, 1984).

Since one of the goals of such animal studies is to extrapolate the findings to humans, it becomes important to be able to relate the results in a similar developmental time frame, and trimester equivalencies have been used most frequently (West, 1987). All mammals pass through similar

stages of brain development; yet, their timing relevant to birth can differ considerably. The period of most rapid brain development is commonly referred to as the "brain growth spurt". It is characterized by a dramatic increase in glial cell proliferation, primarily oligodendroglia, followed by a period of rapid myelination as well as by extensive development of the dendrites and synapses in the neuropil (Dobbing and Sands, 1979; Dobbing, 1981). The timing of the brain growth spurt varies considerably between species (Dobbing and Sands, 1979; Dobbing, 1981). In humans the brain growth spurt begins at approximately 18 weeks of gestation, peaks around birth, and continues for 10-12 months postnatally. The stage of development of the brain of a rat at birth is considered equivalent to that of an 18 week gestational age human brain. At 8-10 days postnatally the development of the rat brain is considered equivalent to that of a human brain at birth. Therefore the entire gestational period in the rat is only equivalent to the first two trimesters in the human in terms of brain development.

Most of the studies concerning the effects of alcohol on brain development have focused on two trimester equivalencies (2TE), since such alcohol exposures are relatively easy to provide and control (Weinberg, 1984). A variety of techniques have been used to expose the fetal rat

to alcohol in 2TE exposure studies including: the addition of alcohol to a liquid diet (West and Hodges-Savola, 1983; Miller and Potempa, 1990; Phillips et al., 1991a), or to drinking water (Borges and Lewis, 1983), inhalation of alcohol vapors (Phillips and Cragg, 1982b), or gavage (West et al., 1981; Abel et al., 1983). The alcohol containing liquid diet is considered to provide the best controlled exposure to alcohol since it allows for pair fed animals to be included as a control for caloric intake. Methods other than adding alcohol to a commercially prepared liquid diet have been shown to be complicated by undernutrition. Intoxicated animals ingest less food and additionally the calories from alcohol provide little nutritional value (Abel, 1984; Weinberg, 1984; Testar et al., 1988). This is minimized (or at least controlled for) by delivering the alcohol in a standardized commercially prepared diet, and by pair feeding the control animals the same volume of isocaloric diet as weight matched counterparts in the ethanol exposed group.

In order to achieve a third trimester equivalency exposure in the rat, alcohol must be administered through at least the first 9 postnatal days. This has been accomplished using a variety of techniques, including dietary exposure in the milk of lactating dams given dietary alcohol (Borges and Lewis, 1983; Lancaster et al., 1986),

gastric intubation (Light et al., 1989), vapor inhalation (Bauer-Moffett and Altman, 1977), and artificial rearing procedures, in which the animals are reared in isolation and fed through a chronically implanted gastrostomy tube (Diaz and Samson, 1980; West et al., 1984b; Phillips, 1989). These methods of exposing the pups postnatally are not without their problems. Lactational exposures cannot be controlled for dose since ethanol has been shown to reduce the amount of oxytocin available, thus reducing the amount of milk available (Fuchs, 1969); and the ethanol concentration in the milk is always much lower than that ingested by the dam; and the feeding behavior of the pups is altered by the alcohol (Swiatek et al., 1986). Giving alcohol to the pups directly, such as by intubation, inhalation, or injection also has its problems, since there is no way to assure that the intoxicated pup will receive adequate nutrition through suckling. The artificial rearing procedure in which the pups are fed through a gastrostomy tube, typically on postnatal days 5 through 9 (West et al., 1982; West et al., 1984b; Phillips, 1989; Goodlett et al., 1991) is not without criticism. The procedure can be faulted because of its invasiveness, the isolation of the pups, the potential stress involved, and the possibility of malnourishment with an artificial diet.

Very few attempts have been made to provide an ethanol exposure that is the equivalent to a full three trimester exposure (3TE) in humans. In most of these studies exposure to the fetus during the first two trimester equivalents is usually accomplished by adding ethanol to a liquid diet fed to the dam. The postnatal, third trimester equivalent, exposure methods have generally utilized either lactation (Lancaster et al., 1984), with its inherent problems for control of nutrition; or artificial rearing with postnatal exposure of ethanol beginning on day 4 (Phillips, 1989; Wigal and Amsel, 1990), thus leaving over three days of uncontrolled exposure. A nearly "full" three trimester equivalency exposure has been developed in this lab, where the pups of gestationally exposed dams are implanted with a gastrostomy tube on postnatal day 1, and exposed to ethanol beginning on the morning of the second postnatal day (Phillips et al., 1991a; Phillips and Krueger, 1992), thus providing an almost continuous three trimester equivalency exposure.

Effects of Ethanol on CNS and Neuronal Development

Neuropathological findings from human FAS victims have revealed microencephaly, cortical disorganization, occasional agenesis of the corpus callosum or anterior commissure, neuroglial heterotopias throughout the leptomeninges indicative of abnormal glial migration, and a

variable degree of glial hypertrophy and gliosis (Clarren et al., 1978; Peiffer et al., 1979; Wisniewski et al., 1983; Clarren, 1986).

Results from experimental models using animals have shown developmental delays and gross brain abnormalities similar to those seen in humans. Developmental delay is one of the most common features of FAS, and grossly manifests itself in the CNS as microencephaly that appears early on as reduced brain weight and volume in animal studies. The degree of microencephaly appears to be highly variable with reductions in brain weight ranging from 0% to 26%, in studies achieving blood alcohol concentrations (BAC) between 160 mg/dl and 300 mg/dl (Kornguth et al., 1979; Phillips and Cragg, 1982a; West et al., 1984b). Exposure during the third trimester equivalent appears to be more harmful to gross brain growth than earlier exposures (Kornguth et al., 1979; West et al., 1984b), most likely due to effects on glial cell proliferation, myelin acquisition, or neuronal maturation during the brain growth spurt.

Most morphological studies of the effects of developmental ethanol exposure on brain development have examined either the hippocampus, cerebellum, or the cerebral cortex. In the rat hippocampus and cerebellum the pyramidal cells and Purkinje cells respectively are generated fairly early in the prenatal period, E12-E15, while the granule

cells of the dentate gyrus and those in the cerebellum are generated in the early postnatal period, during the brain growth spurt (reviewed in Jacobson, 1991). The number of pyramidal cells in the CA1 region of the hippocampus appear to be vulnerable to either a 2TE or 3TE exposure (Barnes and Walker, 1981; Wigal and Amsel, 1990), while an isolated third trimester equivalency exposure appears to have no effect (West et al., 1986; Pierce et al., 1989). Granule cells in the dentate gyrus are significantly reduced in number following either a 2TE or 3TE exposure (Barnes and Walker, 1981; Wigal and Amsel, 1990), and are either slightly increased in density (West et al., 1986) or are unaffected (Pierce et al., 1989) in an isolated third trimester exposure. In the cerebellum reduced numbers of Purkinje cells and granule cells have been found from either a 2TE (Phillips and Cragg, 1982b), 3TE (Volk, 1984), or an isolated third trimester equivalent exposure (Bauer-Moffett and Altman, 1977; Phillips and Cragg, 1982b; Bonthius et al., 1989; Quesada et al., 1990a). Granule cells in the hippocampus and the cerebellum appear to be less susceptible than either the Purkinje cells or the pyramidal cells to an ethanol insult in either a 2TE, 3TE, or isolated third trimester equivalent exposure.

Purkinje cells are reduced in all three types of exposures, however a gradient appears to exist in the

cerebellum where the more mature cells, either Purkinje cells or granule cells, are more vulnerable to an alcohol insult than the less mature cells, as determined by correlating the extent of damage in various lobules of the cerebellum with their time of maturation (Pierce et al., 1989; Bonthius and West, 1990). The degree of cell maturation during the time of ethanol exposure thus appears to be a key factor in determining the vulnerability to ethanol.

In addition to maturation, delays and regional vulnerability also appear to be key factors in determining susceptibility to ethanol. Prenatally administered ethanol appears to delay the proliferation of pyramidal cells, in the somatosensory cortex of the rat, as evidenced by tritiated thymidine studies (Miller, 1986). Even more dramatic in terms of regional vulnerability are the findings of Sulik et al., who demonstrated that a single intraperitoneal injection of alcohol on gestational day 7 in mice can reduce or prevent the development of the septal nucleus (Sulik et al., 1984).

The effect of alcohol on gross brain growth might also be a product of retarded neuronal growth. Prenatal alcohol exposure results in a temporary reduction in the nuclear diameter of Purkinje cells in the cerebellum (Volk et al., 1981; Mohamed et al., 1987a). The pyramidal neurons of the

somatosensory cortex in prenatally exposed rats have smaller soma size (Hammer and Scheibel, 1981), and the same is true for pyramidal and fusiform neurons in the substantia nigra (Shetty et al., 1992). However, not all neuronal systems appear to be affected similarly since the soma of granule cells in the dentate gyrus do not appear to be reduced in size after a limited postnatal ethanol exposure during their peak proliferative phase (West and Hamre, 1985). Therefore, even though ethanol does seem to cause a delay in the maturation as evidenced by a temporary reduction in the size of the neuronal somata, it does not affect all neurons equally. These differences appear to be related to variations in the timing of the maturation of the different cell types.

Reductions in soma size are not the only evidences indicative of developmental delays. Alterations in dendritic growth and complexity have been reported following developmental ethanol exposures. Evidence for the retardation of dendritic growth comes primarily from Golgi studies. Pyramidal cells in the CA1 region of the hippocampus exhibit stunted basal dendrites (Davies and Smith, 1981), and a less extensive dendritic arborization can be seen in the pyramidal cells of the rat somatosensory cortex (Hammer and Scheibel, 1981) following prenatal ethanol exposure. Pyramidal cells and fusiform cells in the

substantia nigra have less complex dendritic elaborations following prenatal ethanol exposure, as visualized with both tyrosine hydroxylase immunocytochemistry and Golgi stain (Shetty et al., 1992). Postnatal exposures can produce reduced area of dendrites in layer V of the somatosensory cortex as visualized in plastic sections examined by light microscopy (Phillips and Harper, 1987). Alterations in dendritic spines have also been observed in the cells of layer V of the parietal cortex (Stoltenburg-Didinger and Spohr, 1983).

Although most studies have shown alcohol-induced reductions in dendritic complexities, some regions of the nervous system show considerable dendritic growth, consistent with a sprouting phenomena, after ethanol exposure. Chronic ethanol consumption in adult rats results in an increase in the number of dendrites in the distal portion of the dendritic arbor of hippocampal granule cells after cessation of alcohol exposure (Durand et al., 1989). A similar phenomena occurs in rats prenatally exposed to ethanol, and examined 35 days postnatally (Miller et al., 1990). The complexity of the dendritic arbor is greater in the basal dendrites of corticospinal neurons of rats prenatally exposed to alcohol as compared to the control animals using a Sholl's concentric ring analysis of dendritic complexity. Infrapyramidal mossy fiber

projections show a dramatic hypertrophy in their terminal field distributions in midtemporal hippocampal levels in adult rats following heavy prenatal ethanol exposure (West et al., 1984a; Dewey and West, 1984; Dewey and West, 1985). In addition, 9 days of postnatal alcohol exposure results in a more aberrant mossy fiber terminal field than 20 days of prenatal ethanol exposure (West and Hamre, 1985). Since few mossy fibers are known to terminate at a distal infrapyramidal location, the increase in mossy fiber terminations in that area could be classified as a hyperdeveloped projection (West and Hodges-Savola, 1983).

Alterations in dendritic complexity may be the result of altered neuronal interconnectivity or reduced synaptic contact (Davies and Smith, 1981; Mohamed et al., 1987b). Gradual target loss has been shown to result in regression of the dendritic tree (Hughes and LaVelle, 1975; Oppenheim et al., 1978). Ultrastructural studies of synaptogenesis show that, in animals exposed to ethanol postnatally, the neuropil was generally similar in both the ethanol and control groups at 56 days. However, the dendritic profiles were enlarged, perforated and degenerating synapses were present in the experimental animals, suggestive of synaptic remodeling (Jones and Colangelo, 1985). Such alterations could have dramatic effects on brain function.

Alterations indicative of developmental delays in neuronal maturation are also evident in ultrastructure of neurons in ethanol exposed animals. Following prenatal ethanol exposure Purkinje cells of the cerebellum have a reduced nuclear diameter, and the cytoplasm contains disrupted cisternae of rough endoplasmic reticulum (Volk et al., 1981; Mohamed et al., 1987a). Pyramidal cells of the somatosensory cortex display similar alterations of nuclear diameter and of the granular endoplasmic reticulum. In addition, a higher volume percentage of the neuronal soma is occupied by Golgi apparatus and lysosomes (Al-Rabiai and Miller, 1989). These findings have been presumed to be related to altered protein synthesis.

Alterations of neuronal migration have also been described after developmental ethanol exposures. Aberrant neurons have been found in the deeper layers of the somatosensory cortex of the rat (Miller, 1986; Miller, 1988), indicative of altered neuronal migration. The migration of granule cells from the external granular layer inward to the internal granular layer are delayed in the cerebellum (Quesada et al., 1990b; Shetty and Phillips, 1992). The mechanism of such alcohol induced delays in neuronal migration is unknown. The time between the generation of a postmitotic neuron and the beginning of its migration from the proliferative zones in the rat

somatosensory cortex is significantly increased in ethanol treated animals (Miller, 1986).

Alterations of the neuronal cytoskeleton, such as microtubules, could be involved in the delays in migration. Alcohol is known to affect alpha-tubulin, a microtubular component and a main constituent of the neuronal cytoskeleton, causing it to appear matted and thickened in ethanol exposed cultures (Hassler and Moran, 1986). Following prenatal ethanol exposure, the mRNA expression for alpha-tubulin is decreased during the first postnatal week in the rat somatosensory cortex (Maciejewski-Lenoir and Milner, 1989), at a time corresponding to neuronal migration in the somatosensory cortex (Jacobson, 1991). Ethanol has also been shown to affect membrane glycolipids (Druse, 1986), on the surface of cells. Since cell adhesion molecules required for neuronal migration are also found on the surface of cells, it is possible that they too may also be affected as a result of the effect on glycolipids. Alterations of radial glia could also be involved since they are known to play a role in the migration of neurons in the cerebrum (Rakic, 1972; Sidman and Rakic, 1973; Rakic, 1981; Cameron and Rakic, 1991) and cerebellum (Rakic, 1971; Rakic and Sidman, 1973; Rakic, 1985). Thus it has been postulated that since ethanol delays the maturation of radial glia, neuronal migration could be altered (Shetty and Phillips,

1992), perhaps causing the radial glia to prematurely lose contact with the pial surface (Miller, 1986).

Effects of Ethanol on Glial Cell Development

Glial cells are numerically the most common cell type in the adult mammalian brain and are intimately involved in maintaining and establishing the microenvironment of neurons. Astrocytes have functional roles in detoxification, formation of glial scars, neuronal migration, and differentiation of neurons during development (for reviews see Privat and Fulcrand, 1978; Vernadakis, 1988; Kimelberg and Norenberg, 1989). Oligodendrocytes are responsible for the formation and maintenance of the myelin sheath of myelinated nerve fibers, while microglia are believed to primarily play a phagocytic role in the nervous system (Peters et al., 1991).

Many of the maturational and developmental delays that have been demonstrated in neurons of animals developmentally exposed to ethanol have also been found in developing astrocytes. Most studies concerning the effects of ethanol on developing astrocytes have been done in primary astrocytic cultures and show a decrease in RNA content, reduced protein synthesis and, to a lesser degree, reduced DNA content, suggesting that cell maturation is more dramatically affected than cell number, that is characteristic of a temporal delay in maturation (Davies and

Vernadakis, 1984; Kennedy and Mukerji, 1986a; Kennedy and Mukerji, 1986b; Renau-Piqueras et al., 1988;). The expression of glutamine synthase in astrocytes coincides with astrocytic maturation and has been shown to be delayed by analyzing the accumulation of glutamine synthase in primary astrocyte cultures exposed to alcohol (Kennedy and Mukerji, 1986b; Guerri et al., 1989). Delays in the accumulation of the intermediate filament protein, glial fibrillary acidic protein (GFAP) (Renau-Piqueras et al., 1989), and delays in morphological maturation also occur after ethanol exposure (Davies and Cox, 1991; Davies and Ross, 1991). Not all reports agree, and there are some reports that neither DNA synthesis (Bass and Volpe, 1988), GFAP accumulation (Lipsky et al., 1988), or GS activity is decreased (Chiappelli et al., 1991) in cultured astrocytes exposed to ethanol. In addition, cultured oligodendrocytes show delayed expression of galactocerebroside (GC), transferrin, and myelin basic protein (MBP) as revealed by immunocytochemical staining and chemical isolation (see review by Phillips, 1992).

In vivo studies concerning the effects of alcohol on glial cells are far more limited than those on neurons. GFAP immunohistochemistry has been used to demonstrate the temporal delay in radial glia maturation in the Bergmann glial fibers in the cerebellum (Shetty and Phillips, 1992),

as well as to demonstrate alcohol-induced astrocytic hypertrophy in the cerebral cortex (West et al., 1990; Leo et al., 1991) and cerebellar white matter (Shetty and Phillips, 1992). Ultrastructural studies have shown alcohol-induced increases in the number of astrocytes and reductions in the number of oligodendrocytes in rat optic nerve (Phillips and Krueger, 1990; Phillips and Krueger, 1992). The signals for these changes in cell number could be brought about by factors released from possibly injured cells (Vijayan et al., 1990), or circulating mitogens in the blood, such as platelet derived growth factor (PDGF), glial maturation factor (GMF), fibroblast growth factor (FGF), and interlukin-1 (IL-1) (Giulian et al., 1991).

Alcohol has also been shown to delay myelin acquisition, both biochemically (Druse and Hofteig, 1977; Hofteig and Druse, 1978; Lancaster et al., 1982), and morphologically (Phillips 89; Phillips et al., 1991a; Samorajski et al., 1986; see review by Phillips, 1992). Some reports indicate that such delays are reversible (Druse and Hoftig, 1977; Rosman and Malone, 1979). The delay in myelin acquisition appears to be related to a delay in the maturation of oligodendrocytes (Phillips and Krueger, 1990; Phillips and Krueger, 1992), which are responsible for the production of myelin. The delay in myelin maturation appears to occur as a delay in the initiation of myelin

development, not as delays in the process of enwrapment or compaction (Phillips, 1989; Phillips et al., 1991a). Full three trimester equivalency exposures result in an even more dramatic delay in myelin acquisition as well as a permanent reduction in myelin thickness relative to axon diameter (Phillips et al., 1991a).

Evidence of Involvement of the Oculomotor System in FAS

Ocular defects reported in FAS victims include microphthalmia (Jones et al., 1973; Jones and Smith, 1975) strabismus, ptosis, moderate to severe myopia (Stromland, 1981a; Stromland, 1981b), as well as hypoplastic optic discs and an increased tortuosity of retinal vessels (Stromland, 1981a; Stromland, 1981b; Sulik and Johnston, 1983). In animals studies, only microphthalmia and cataracts have been reported (Sulik et al., 1981; Cook et al., 1987). Between the two types of strabismus, esotropia, the deviation of one eye toward that of the other eye, occurs much more frequently than exotropia, the deviation of one eye away from that of the other eye (Stromland et al., 1991). This would imply a disorder involving either a weakness of the lateral rectus muscle, or an increase in the tonic excitation of the medial rectus muscle resulting in esotropia.

It is possible that the numerous anomalies associated with the oculomotor system could contribute significantly to

the visual problems related to the learning deficits characteristic of children afflicted with FAS. Consequently it is of interest to examine the oculomotor system. Such studies have not been done in animal models. The deficits could arise from any number of places within the oculomotor system, including reduced myelination of the peripheral nerves involved, altered circuitry within the oculomotor system, decreased neuronal numbers within the extraocular nuclei, or an effect on the extraocular eye muscles themselves. Of the three brainstem nuclei controlling the extraocular muscles, the oculomotor nucleus was chosen as the site of this study since it innervates four of the six extraocular eye muscles, some of which may be involved in esotropia, and the levator palpebrae muscle which is involved in ptosis.

Development of the Oculomotor Nucleus

The oculomotor system consists of six extraocular eye muscles controlled by three brain stem nuclei. The general organization of the oculomotor system is as follows: The abducens nucleus innervates the ipsilateral lateral rectus (LR) muscle; the trochlear nucleus innervates the contralateral superior oblique (SO) muscle; and the oculomotor nucleus innervates the ipsilateral medial rectus (MR) muscle, inferior rectus (IR) muscle, inferior oblique (IO) muscle, and the contralateral superior rectus (SR)

muscle and levator palpebrae (LP) muscles. Groups of neurons have been found in the oculomotor nucleus innervating the individual eye muscles supplied by the nucleus. The MR neurons are lateral and ventral in the nucleus adjacent to the medial longitudinal fasciculus, while the IR neurons tend to lie ventrally and medially to the MR neurons in the rostral two thirds of the nucleus. In the caudal part of the nucleus the SR and LP neurons assume a lateral location next to the medial longitudinal fasciculus, and the IO neurons are medial (Glicksman, 1980; Labandeira Garcia and Gomez Segade, 1983). Those neurons that infiltrate the medial longitudinal fasciculus (MLF) in rats are most often associated with the ipsilateral MR.

The early development of the oculomotor nucleus has been characterized in plastic section using light microscopy and electron microscopy by Puelles et al. (1977,1978) and autoradiographically by Altman and Bayer (1981). The mesencephalon on embryonic day 12 (E12) is a thin walled round vesicle in which neuronal differentiation has already begun. Development appears more advanced in the ventrolateral basal plates, where the oculomotor neuroblasts accumulate, than in the more dorsal region of the tectum. Immediately ventral to the oculomotor neuroblast primordium lies a well defined medial longitudinal fasciculus, separating the oculomotor primordia from the sparse tectal

fibers traversing the floor plate in the marginal zone. The floor plate of the ventricular zone extends nearly the entire distance ventrally from the ventricular surface to the external limiting membrane. The enlarged ventricular zone separates the oculomotor primordia from the midline. At this stage the neurons of the oculomotor primordia have round or oval light-staining nuclei and little cytoplasm as seen in plastic sections stained with toluidine blue (Puelles and Privat, 1977). Approximately 60% of the multipolar neurons in the oculomotor nucleus are generated on E12 and the rest on E13, as shown by tritiated thymidine studies (Altman and Bayer, 1981). The developmental gradient is from rostral (anteroventral) to caudal (posterodorsal) in the oculomotor nucleus, as described in coronal sections. In the sagittal plane, the gradient appears to be from proximal to distal in relation to the medial longitudinal fasciculus. The existence of these gradients was interpreted as evidence of the dispersal of neurons from a common neuroepithelial source, the embryonic aqueduct (Altman and Bayer, 1981). The dispersal occurs in two directions with the earliest generated neurons settling closest to the medial longitudinal fasciculus and the latter generated neurons piling on top of these (Altman and Bayer, 1981). By E14 the oculomotor nucleus can be clearly identified and consists of a homogenous population of

tightly packed cells with light-staining cytoplasm. The nucleus is separated into two to three subdivisions by the passage of blood vessels and radially oriented ventricular cell processes (Puelles and Privat, 1977; Puelles and Bendala, 1978). The light-staining oculomotor cells have their long axis oriented transversely whereas the ventricular cells are always oriented perpendicular to the floor plate. It is assumed that the light-staining cells are migrating oculomotor neuroblasts (Puelles and Privat, 1977). The ultrastructure of the presumed immature neuroblasts resembles that of other immature neurons, being fusiform in shape with an oval or elongated nucleus containing dispersed chromatin and clear nucleoplasm, and a high concentration of free ribosomes in the cytoplasm as well as rough endoplasmic reticulum, Golgi apparati and mitochondria (Puelles and Privat, 1977). The cells innervating the contralateral SR muscle migrate across the midline and have completed their migration by E16 with their axonal or trailing processes constituting the oculomotor commissure (Puelles and Privat, 1977). The migrated neuroblasts lose their fusiform shape and high neurotubular content and become indistinguishable from cells that did not participate in the migration (Puelles and Privat, 1977). The leading processes of the postmigratory cells are gradually transformed into dendrites, but no synapses are

detectable in the oculomotor nucleus at this stage. By E17 the nucleus has taken on an essentially adult configuration, with the oculomotor neurons being bigger and more rounded than before, with many of the cells having two or three dendrites, however no typical synaptic structures can be detected at this stage (Puelles, 1978; Puelles and Bendala, 1978).

As described above, the cells of the oculomotor nucleus in the rat are generated and have completed their migration by birth. Therefore a prenatal ethanol exposure should have a greater effect on the proliferation and migration of the neurons, while a postnatal exposure should exert its effect on the maturation of the neurons in the oculomotor nucleus.

Morphology of the Extraocular Motorneurons

Human oculomotor neurons are approximately 50 microns in diameter, and have 12 to 15 primary dendrites (Abdel-Maguid and Bowsher, 1979), while in the rat oculomotor neurons are approximately 30 microns in diameter with five to six primary dendrites (Durand, 1989). The dendrites of individual oculomotor neurons extend over approximately one third of the nucleus and into at least one adjacent population of motorneurons in mammals (Evinger, 1988).

Most ultrastructural studies of neurons innervating the extraocular eye muscles (EOM) thus far have focused primarily on the cat, and in particular on the abducens nucleus (Evinger, 1988). Neurons in this nucleus range from 13 to 60 microns in diameter, and have circular nuclei with a smooth or slightly invaginated nuclear membrane, and extensive cisternae of granular endoplasmic reticulum (Evinger, 1988). In contrast the internuclear neurons of the abducens nucleus have fluted or deeply invaginated nuclear membranes, and poorly developed cisternae of granular endoplasmic reticulum. Synaptically however these neurons are virtually identical, having between 4.2-4.6 synapses per 100 micron^2 of soma surface (internuclear neurons and motorneurons respectively) (Evinger, 1988). Both types of neurons have a higher percentage of synapses with flattened vesicles on the soma than on the dendrites. Synapses with round vesicles predominate on soma of oculomotor neurons (Evinger, 1988).

Afferents to the Oculomotor Nucleus

The oculomotor nucleus receives afferents from the vestibular, trochlear and abducens nuclei, the accessory oculomotor nuclei, the prepositus nucleus, and the reticular formation (Evinger, 1988). The medial longitudinal fasciculus is the major fiber tract by which afferents reach the oculomotor nucleus. The brachium conjuctivum has been

shown to act as an alternative pathway to the oculomotor nucleus for the neurons of the superior vestibular nucleus (Evinger, 1988). Abducens internuclear neurons (AbIN) are also a major source of afferents to the oculomotor nucleus, and appear to be the clearest anatomical way to control conjugate horizontal gaze (Buttner-Ennever and Akert, 1981), connecting the LR neurons of the abducens nucleus to the MR motorneurons of the contralateral eye.

Efferents from the Oculomotor Nucleus

The majority of the efferents from the oculomotor nucleus are to the extraocular eye muscles, through the oculomotor nerve. Other oculomotor neurons are internuclear and are known to terminate in the abducens nucleus, spinal cord, cerebellum, and the facial nucleus (Evinger, 1988). The physiological characteristics of the interneurons are virtually unknown. The high diversity of the internuclear projections outside of the nucleus suggests that only a small subset of the neurons may have a direct role in controlling eye movements, while most are involved in pathways controlling corresponding head and neck movement associated with eye movements (Evinger, 1988).

METHODS

Experimental Design

This study was designed to examine the effects of alcohol on the development of the oculomotor region of the brainstem in rats exposed to ethanol using two different exposure regimes. One was a prenatal exposure alone, where the dam was exposed to dietary ethanol throughout gestation, in an effort to provide an exposure to the fetus that corresponds to a first two trimester human equivalency. The second exposure regime was designed to represent the equivalency of a human three trimester exposure, in which the rat fetus was exposed prenatally, via maternal diet, plus exposure on postnatal day 2 (PND 2) through the morning of PND 10, via gastrostomy fed diet in an artificial rearing procedure.

All animals were sacrificed on PND 15 with the day of birth being counted as PND 1. Table 1 indicates the number of animals in each exposure regime and for each type of analysis performed. Light microscopic analyses were done on two micron plastic coronal sections of the rostral oculomotor region of the mesencephalon. Golgi analyses were done on multipolar neurons from the same region. Soma size was measured in camera lucida drawings, dendrites were counted for order of branching and analyzed

using the concentric ring method of Sholl (Sholl, 1953) for the richness of the dendritic arbor.

Table 1.

Summary of Animals Used for Light Microscopic and Golgi - Cox Analysis

	Light Microscopic Study		Golgi - Cox Study	
EXPOSURE	# CONTROL	# ETOH	# CONTROL	# ETOH
2-TRIMESTER	6	6	4	4
3-TRIMESTER	6*	6	-	-

* All animals were male except for 2 females in the three trimester equivalency control group

Animal Breeding

Nulliparous female Sprague/Dawley rats (Holtzman/Sasco), 80 to 100 days of age and weighing between 218 and 318 grams, were used for breeding. The animals were housed in the M.S.U. Animal Resource Center under conditions of constant temperature and humidity with a 12 hour light/dark cycle. Animals were gang caged in groups of 4-5 animals with ear punches used for individual identification.

The stage within the estrus cycle for each female was determined by examination of sloughed vaginal cells. Autoclaved Q-tips, moistened with sterile water, were inserted into the vaginal canal to collect a cell sample. The cells were then smeared onto a slide, coverslipped, and examined with an Olympus BH-2 compound microscope at either

100 or 200X. Smears from animals in the proestrus stage were characterized by an abundance of nucleated cuboidal epithelial cells and very few if any leukocytes or non-nucleated squamous epithelial cells. Prior to proestrus an abundance of leukocytes were common while following proestrus an abundance of non-nucleated epithelial cells were typical. The vaginal smears were repeated daily until 8 to 10 animals were identified in proestrus on the same day, at which time the animals were prepared for matings. Individual females were housed with individual males overnight in breeding cages, beginning at 4 p.m., just prior to the start of the dark cycle. The following morning, gestational day 0 (GD 0), 5-6 females with either visible vaginal plugs, or with sperm positive vaginal smears were selected as the experimental ethanol exposed group. The remaining impregnated 4-5 animals were used as surrogate foster dams, which were housed individually and given free access to standard lab chow and water. The same breeding procedure was repeated 2 to 3 days later to establish the pair fed control group. Each animal in the ethanol exposed group had a weight matched counterpart ($\pm$ 10 grams) in the pair fed control group that was fed the same volume of diet consumed by the matched experimental animal.

Prenatal Exposure

From GD 0 until parturition the pregnant dams were fed a commercial version of the high protein liquid diet perfected by Lieber and DeCarli (1982) for pregnant dams (#F1264, F1265 Bioserve Inc., Frenchtown NJ). In this diet 37.5% of the daily caloric intake (6.7% v/v) was derived from ethanol for the experimental group or from maltose-dextrin for the control group. The powdered form of the diet was prepared as liquid diet every other day in a blender and then stored in 1 liter plastic Nalgene containers at 4°C to allow time for the air bubbles to dissipate. On the morning of the feeding, diet was poured into calibrated glass feeding tubes (Bioserve Inc., Frenchtown, NJ). Only diet less than 24 hours old at the start of the feeding cycle was used. The ethanol exposed animals had free access to the diet, while each animal in the control group was pair fed by volume, and thus calories, to its weight matched counterpart in the experimental group. The diet was changed daily between 9 and 10 A.M. and the volume consumed was recorded. Pregnant dams were weighed on gestational days 0, 1, 5, 7, 10, 14, 15, 16, 20, and 21. On the day of birth (generally day 22) the litters were culled to 8 pups per litter, consisting of 4 males and 4 females. Those animals in the two trimester equivalency experiment were then cross fostered to surrogate dams to assure that no

offspring were reared postnatally by their natural mother. Those animals in the three trimester equivalency experiment were then prepared for the artificial rearing procedure.

Postnatal Exposure

Animals given the three trimester equivalency exposure were exposed to ethanol prenatally as described above, and postnatally until day 10. Beginning on G22 the pregnant dams were checked every 2 hours, except between midnight and 8 A.M., and the time of parturition recorded. Dams that gave birth between midnight and 8 A.M. were recorded as having given birth at 8 A.M.. Only those animals born within 12 hours of each other were used, and the males from these liters were pooled and weighed. Pups to be reared artificially were then selected from near the mean of the group, thus excluding extremely light or heavy pups. The gastrostomy tube was placed in all animals within 12 hours of birth, and the pups were then artificially reared through PND 10, with their diet fed through the gastrostomy tube by way of an infusion pump.

Gastrostomy

Pups to be implanted with a gastrostomy cannula were weighed and then anaesthetized by inhalation of methoxyflurane (Pitman-Moore). An adequate level of anaesthesia was assured by the absence of movement in

response to a tail pinch and by the presence of rapid, shallow breathing. When a desirable anaesthetic plane had been achieved, the animal was placed on a heating pad (39°C) for cannulation, as described previously (Messer et al. 1969; Samson and Diaz, 1982b; West et al., 1984b), and as done previously in this laboratory (Phillips and Krueger, 1990; Phillips, 1989).

The cannula[1] was implanted into the anaesthetized pup by using an 8 cm piece of 25 gauge wire covered with a 9 cm piece of silastic tubing that extended approximately 2 mm past the insertion end of the wire. The silastic covered wire was inserted into the mouth of the supinely positioned animal and carefully guided down the esophagus into the stomach, where the tip of the wire could be palpated. The tip of the wire was then positioned along the left border of the greater curvature of the stomach and the silastic sheath was pulled back, baring the tip of the wire. The wire was then pushed through the stomach and the abdominal wall, so that the exit wound was just anterior to the midaxillary line. The silastic sheath was then pulled off the wire at its superior end and a cannula lightly lubricated with corn

---

[1]The cannula consisted of a 25 cm piece of polyethylene tubing(Clay Adams, Parsippany, NJ), heat flared at one end. A plastic disk washer, 6mm in diameter made with a paper punch from 0.7 mil plastic was threaded onto the shaft of a 20 gauge needle. The end of the PE tubing opposite that of the heat flare was placed into the lumen of the needle, and the disk was then slid onto the tubing.

oil was friction fitted onto the superior end of the wire. The wire and cannula were then pulled through the exit wound so that the flared end of the cannula, and the intragastric washer, were pulled through the length of the esophagus and into the stomach. Once the cannula was in place in the stomach, the wire was removed. Two disks, made from tygon tubing (1.5mm thick wall) with a paper punch, were threaded onto the midlength of the cannula. The cannula was then re-fitted over the wire, and the wire was pushed through a skin fold over the upper back at the approximate level of the 2nd or 3rd thoracic vertebrae. The wire was then removed and a third tygon disk threaded onto the cannula. The first disk was positioned against the abdomen, while the second and third disks were positioned on each side of the skin fold on the upper back so that the cannula could not move more than 2-3 mm. Topical antibiotic (Nitrofurazone TechAmerica, Kansas City, MO), was then applied to all skin wounds and the animal was kept on a heating pad until it regained consciousness.

Postnatal Diet Preparation

The postnatal diet (Table 2) was a modified version of the diet used by Diaz (Diaz et al., 1982), and consisted of evaporated milk supplemented with protein, vitamins, and minerals. The experimental diet consisted of the stock diet with absolute ethanol (3%,v/v), while the control diet

consisted of the stock diet with an isocaloric amount of maltose- dextrin added in place of the ethanol. The stock solution was made in a 1 liter batch and separated into control and experimental portions, which were stored in 60 ml aliquots at -20°C. The control diet had maltose-dextrin added prior to storage. The experimental diet was stored as stock solution and had the ethanol added immediately prior to use. Oxytetracycline (Terramyacin, Pfizer, New York, NY) was added at a rate of .2mg/ml of diet). This was equal to a daily dietary intake of 55mg/kg per day and is less than 6% of a dose (1200 mg/kg per day) known to have no effect on the incidence of congenital malformations in rats (Morrissey et al., 1986; Phillips et al., 1991a; Phillips and Krueger, 1992).

Table 2

Postnatal Diet Formula, Stock Preparation

evaporated milk (Carnation)	750 mls
0.1% deoxycholic acid (Sigma)	170 mls
vitamin drops (Poly Vi-Sol without Iron)	10 mls
corn oil (Mazola)	60 grams
dl-methionine (Sigma)	0.4 grams
l-tryptophan (Sigma)	0.5 grams
riboflavin (Sigma)	0.01 grams
casein (Sigma)	30 grams
double distilled water	80 mls
mineral solution*10 mls	

* Composition/10 mls H_2O

FeII gluconate (Sigma)	0.0207 grams
Zn gluconate (Sigma)	0.016 grams
Cu gluconate (Sigma)	0.0105 grams

Artificial Rearing

After recovery from anaesthesia, the pups were placed in plastic cups 11 cm in diameter at the top, 9 cm in diameter at the bottom and 7.5 cm tall (grocery store liver cups). The cups contained autoclaved wood chip bedding and were covered with a plastic lid with holes in it. A square piece of artificial fur, folded back on itself, was attached to one side of the cup to provide additional warmth for the animal and to serve as a type of "surrogate mother". The cup was placed into another cup of identical dimensions but containing two large flat washers fastened to the bottom for ballast. The double cups were then floated in a large water bath kept initially at a temperature of 41°C (39°C in the cup) and then lowered to 39°C on PND 6 for the remainder of the artificial rearing. Twelve hours after the start of the first cannulation animals were "serviced" to aid in defecation and micturition by gently massaging the anal-genital area with a moistened Q-tip.

Each animal then received an amount of basic diet equivalent to 2.1% of the mean body weight of the cannulated pups that day. The diet was administered through the cannula, by placing the end of the cannula into the lumen of a short piece of PE-50 tubing, which was fitted to a 25 gauge needle and 3 cc syringe containing the stock diet.

Beginning on the second postnatal day, and continuing through PND 10, the animals were "serviced", weighed, their bedding changed, their cannulas rinsed with 0.1% deoxycholic acid, and their health assessed each morning. After determination of the mean weight of all surviving pups, the volume of daily diet to be delivered was calculated based on a volume to weight ratio. On PND 2 the pups received 29% in cc of the mean body weight in grams; 30% on PND 3; 31% on PND 4; 32% on PND 5; and 33% on PND 6 through PND 9.

Three separate custom built syringe pumps, capable of holding 10-12 syringes each, were used to deliver the diet. The pumps were calibrated daily and either 3cc or 10 cc syringes (Becton Dickinson, Rutherford NJ) to deliver the diet. The syringes were attached to 18 gauge needles inserted into the lumen of a 40 cm piece of PE-90 tubing. A 30 cm piece of PE-50 tubing was fitted into the distal end of the PE-90. The gastrostomy cannula was passed through a hole in the lid of the cup and fitted into the distal end of the PE-50. Ice packs were used to cool the syringes of two pumps, while the third had a self-contained water cooling apparatus. The feeding cycles were 20 minutes out of every 2 hours with 11 cycles per day. In addition to the morning servicing animals were serviced between the fifth and sixth daily infusion, at approximately 9 P.M., when the ice packs on the pumps were also changed. These artificial rearing

procedures were repeated daily through postnatal day 9, which was the last full day for feeding through the gastrostomy tubes.

On the morning of postnatal day 10 the animals were removed from the pump, and again serviced and weighed. An amount of stock diet, equivalent to one feeding cycle for that day, was infused manually by syringe. The cannulas were then removed by gently pulling the intragastric washer through the abdominal wall. The pups were tattooed on the shoulders and paws with India ink for individual identification. The artificially reared pups were placed in a cup together with the pups from the surrogate dams, and allowed to intermingle for 15-20 minutes prior to transferring the artificially reared pups to the surrogate dam. Only artificially reared pups were transferred to the surrogates, and each surrogate received 6-8 pups. The pups were watched closely for 20-30 minutes after cross fostering to assure that the surrogate had accepted the pups. The pups were then examined daily until the day of sacrifice on postnatal day 15 to insure that they were feeding normally.

Blood Alcohol Concentrations

Blood alcohol concentrations (BAC) of the ethanol consuming dams were determined at 10 P.M. on the evening of GD 16, 6 hours after the commencement of the dark cycle and

after the animals had consumed a large portion of their diet. The unanesthetized pregnant dam was weighed, placed in a restraining tube, and the circulation to the tail enhanced by immersion of the tail in warm water. A 25 gauge needle and tuberculin syringe was then used to collect the blood sample from the tail vein and to transfer the sample to a heparinized capillary tube. The sample was then separated using a centrifuge, and the serum used to determine the BAC using a Sigma diagnostic kit #330-1.

Light and Electron Microscope Tissue Preparation

On the morning of the 15th postnatal day,the animals were weighed, anaesthetized with ether, and tied in the supine position. A midline skin incision was made from the lower abdomen to the base of the neck, and the skin reflected laterally. The chest plate was then reflected superiorly after making bilateral cuts through the lateral border of the rib cage. The pericardial sac was opened, the right atrium cut, a 20 gauge needle inserted into the left ventricle, and the animal perfused at a pressure of 90 to 100 mm/Hg for 5 minutes with 4% paraformaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer (pH=7.4).

The cranial cavity was opened and the whole brain removed, with olfactory bulbs and cervical spinal cord attached. The brain was then further immersion fixed in the primary fixative for 8 hours at 4°C. The brains were then

weighed after uniform removal of the olfactory bulbs, cerebellar flocculi, and spinal cord. Prior to weighing, excess moisture was blotted from the brains to minimize water weight. The midbrain was then isolated with cuts made slightly rostral to the superior colliculus and another cut made caudal to the inferior colliculus. Vibratome (Energy Beam Sciences) sections (300 microns thick) were taken throughout the level of the superior colliculus and collected in 4% paraformaldehyde in 0.1M cacodylate buffer (pH=7.4). The sections were analyzed with reference to an atlas (Pellegrino and Cushman, 1967), and those sections corresponding with stereotaxic atlas coordinates between 4.2mm to 5.2mm posterior to the bregma were isolated and trimmed with a razor blade to remove the dorsal tectum, as well as the lateral and ventral sides of the tegmentum.

Dehydration and Embedding

Trimmed sections were stored in primary fixative overnight and then rinsed in 0.1M cacodylate buffer (4 changes 15 minutes each). The sections were then postfixed in 1% osmium tetroxide containing 1.5% potassium ferracyanide (Langford and Coggeshall, 1980) in 0.1M cacodylate buffer for 2.5 hours with constant, gentle agitation on a shaker. After four 15 minute buffer washes, the tissues were dehydrated through a graded series of ethanol (50%, 70%, 95%, 100%, 15 minutes each 4°C). Three

more washes in 100% ethanol and 2 washes in propylene oxide were done at room temperature. The tissue was then infiltrated for 1 hour at room temperature in a 1:1 mixture of Embed 812 resin (Electron Microscopy Services, Ft. Washington PA) and propylene oxide, followed by 20 hours in a 2:1 mixture. Tissue was embedded in flat bottom plastic Beem capsules using 100% Embed 812 resin, polymerized in an oven for 20 hours at 45°C, followed by 24 hours at 60°C.

Golgi - Cox Methods

Male offspring exposed prenatally to ethanol were prepared for Golgi - Cox study on PND 15 using methods as described by Millhouse (1981). The animals were deeply anaesthetized with ether, to a point very near death, then the cranium opened and the brains rapidly removed. The brain was then blocked using a razor blade to isolate the midbrain as described previously, and the tissue block placed in Golgi fixative, (1% potassium chromate, 1% potassium dichromate, and 0.8% mercuric chloride in H_2O), in a dark amber bottle containing 15-20 drops of DMSO to aid in the penetration of fixative into the tissue. Fixative was changed weekly for 6-8 weeks, after which time a thin slice of tissue (collected with a razor blade) was checked for staining by immersion of the tissue slice in concentrated ammonium hydroxide. When the desired degree of staining had been obtained the tissue blocks were dehydrated through a

graded series of ethanol and ether (50%, 70%, 90%, 100%, absolute ethanol and ether 1:1 for 24 hours). After dehydration the tissue was infiltrated with increasing concentrations of Pyroxylin (Polysciences Inc. Warrington, PA) in a 1:1 mixture of ethyl ether and absolute ethanol (3%, 6%, 10%, for 7-10 days each). The tissue was placed in paperboard wells filled with 10% Pyroxylin, and exposed to concentrated sulfuric acid fumes (70%) for 2 hours. Then the sulfuric acid was replaced with cotton balls soaked in chloroform for another four to five hours until the blocks were hard. Blocks were then stored in 70% ethanol until sectioned.

Sections, 120 microns thick, were cut on a sliding microtome and collected in 70% ethanol. The stain was precipitated on the sections by incubating the sections in concentrated ammonium hydroxide, followed by 20% sodium thiosulfate. Sections were then dehydrated (50%, 70%, 80%, 90%, 95%, 100% absolute 1-butanol, 100% xylene, 100% xylene), mounted in serial order on slides, and coverslipped with Permount.

Plastic Section Light Microscopy Analysis

Two micron thick plastic sections were cut on an Ultracut E ultramicrotome (Reichert-Jung) using glass knives, from each of four blocks per animal to determine which block contained the appropriate level of the

oculomotor nucleus for analysis. The appropriate level was determined by using three characteristic landmarks, the shape of the aqueduct, the position of the nucleus relative to the medial longitudinal fasciculus, and the relationship to the magnocellular portion of the red nucleus (when visible). Sections were collected on slides, stained with basic toluidine blue, and coverslipped with Permount.

Cell counts were done blind to tissue origin on coded slides. An Olympus BH-2 microscope, equipped with a standard 10 X 10 grid in a 10X ocular eyepiece, a 40X objective, and a drawing tube projected onto a bitpad interfaced with a computer using the Sigmascan software (Jandel Scientific Corte Madera, CA) was used for the analysis.

Counts Per Unit Area

The eyepiece grid, representing a tissue area 0.05mm^2, was used to identify a consistent region within the oculomotor nucleus. First the densest part of the nucleus was centered in the grid using a 20X objective, then the objective was switched to 40X and all cells containing a nucleus within the grid were counted. Any cell in which over 50% of the cell was outside of the grid was not counted. All neurons containing a nucleus were counted and all glial cells with a nucleus were classified and counted. Astrocytes were identified as having pale staining

cytoplasm, a pale staining nucleus with a band of condensed heterochromatin near the edge of the nuclear membrane, and a very non-distinct cell border. Oligodendrocytes were generally slightly smaller than astrocytes and had very dark-staining cytoplasm as well as a dark-staining nucleus and a very distinct cell border. Glial cells that could not be placed unambiguously into one of the above two categories were classified as transitional glia. It is known from electron microscopic studies on developing glial cells (Phillips and Krueger, 1990; 1992) that cells in this transitional group consist primarily of immature astrocytes and oligodendrocytes. This procedure was repeated on three separate cross sections of oculomotor nuclei per animal for each of 6 experimental and 6 control animals from both the two trimester and three trimester equivalency exposures.

Counts Per Defined Region

Camera lucida drawings were made at 100X (106X projected image) from two separate sections from each animal in both the two and three trimester equivalency exposures. Only sections which contained the entire aqueduct and the medial longitudinal fasciculus and both the right and left oculomotor nuclei were used. Five experimental and five control animals were examined from the two trimester equivalency exposure, while four experimental and five control animals were examined from the three trimester

equivalency exposure. In the drawings from the two trimester equivalency control animals a line was drawn which approximated the dorsal and medial borders of the oculomotor nuclei. The distance was then calculated from the center of the aqueduct to the dorsal border of the nucleus, and this was expressed as a percentage of the distance from the center of the aqueduct to the ventral border of the MLF. The medial border of the nucleus was expressed in the same manner using the median plane and the point at which the dorsal border of the nucleus intersected the lateral aspect of the MLF. In these animals the dorsal border of the nucleus was 66.7% of the distance from the center of the aqueduct to the ventral aspect of the MLF, and the medial border was 12% of the distance from the median plane to the lateral edge of the MLF at the level of the dorsal border of the nucleus. The slides of both the two and three trimester equivalency experiments were then coded and camera lucida drawings were made blind as to the source or origin of the tissue. Boundaries were then drawn for the oculomotor region as described, and all neurons within the boundaries were counted. The area of the region was then measured using a computerized bitpad.

Image Analysis

Measurements of the neuronal nuclei and nucleoli were made using a video image analyzer and software (MCID, Image