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.

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Robert Clayton Burrows

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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.

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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 in utero 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).

<u>In vivo</u> 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 (± 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

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[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

Research Inc. St. Catherines Ontario, Canada). Cells from the nucleus, as defined for the counts per unit area, were digitized by projection of the image from an Olympus BH-2 microscope with a 20X objective into an interfaced video camera. The digitized image was projected to a video screen at a magnification of 1000X. Only those cells displaying a distinct nucleus and nucleolus were analyzed. The areas of the cell nucleus and nucleolus were determined using densiometric grey scale analysis. When measuring the area of the cell nucleus, the lightest part of the nucleus was chosen as the target value, while the darkest part of the adjacent cytoplasm was chosen as the background value. The value for the nucleus did not include the area of the nucleolus, which was determined separately, with the target value being the darkest part of the nucleolus and the background value being the lightest part of the adjacent cytoplasm. In addition to area, the perimeters of the neuronal nucleus and nucleolus were determined. Cells were selected on the basis of a nucleus and prominent nucleolus. Because the sections were two microns thick, and the equivalent circle diameter of the nucleolus of the neuron was approximately 3.7 microns and the nucleus is approximately spherical it is doubtful that a prominent section of the nucleolus would appear in the same cell in two adjacent sections. Fifty neurons were analyzed for each

of 6 experimental and 6 control animals, and the values averaged for each animal.

Golgi - Cox Analysis

Camera lucida drawings were made at 400X, (378X projected image) from Golgi - Cox impregnated multipolar neurons from the oculomotor nucleus, contained within the middle third of the depth of the section, using an Olympus BH-2 microscope with a drawing tube. The oculomotor nucleus was defined according to its relationship to the cerebral aqueduct and the red nucleus. Neurons whose processes were truncated near the cell body, or those exhibiting punctate staining, or neurons whose processes trailed off as a series of dots were excluded, as well as bipolar neurons.

Four neurons were analyzed in each of 5 animals from the experimental and control groups. The camera lucida drawings were then analyzed using the concentric ring method (Sholl, 1953) using 20 micron increments (from 20 to 200 microns from the center of the soma) to analyze the richness of the dendritic arbor relative to the distance from the soma. The branching order of the dendrites was tabulated for each cell. Those processes arising from the cell body were classified as primary dendrites, and the processes arising from the bifurcation point of the primary dendrite were classified as secondary dendrites and so on.

The area of each soma was then measured from the drawings using a bitpad interfaced with a computer software program (Sigmascan, Jandel Scientific, Corte Madera, CA). Cell processes were arbitrarily truncated at the point where they arose from the soma. Each neuron was traced five times and the values averaged.

Statistical Analysis

Data with one value per animal were analyzed using a Student's T-test or Mann Whitney non-parametric T-test using the Instat software Program (GraphPAD Software, San Diego, CA) when the standard deviations between the groups was significantly different. In circumstances where more than one value was obtained per animal a nested analysis of variance (ANOVA) (SAS Program) was also used to determine a p-value. In cases where the p value was substantially different with the two statistical tests, the different values are noted, otherwise the reported values are those of a two tailed Student's T-test.

RESULTS

Two Trimester Equivalency Exposure

The mean volume of diet consumed for the animals in the experimental group was 69.9 ± 7.9 ml per day, equivalent to an ethanol intake of 12.57 ± 0.98 gm/kg per day. The volume of daily diet intake for the control animals was identical since they were pair fed to the experimental animals. The mean blood alcohol concentrations (BAC), taken on GD 16 was 139.8 ± 43.7 mg/dl (n=6). Percent weight gain for the ethanol exposed dams (48.6%) averaged more than for the control dams (38.6%), (from G5 to G20), but the difference was not statistically significant ($p<0.36$). The brain weights (1.01 ± grams; 1.06 ± grams, control and experimental animals respectively) were not significantly different on the day of sacrifice, nor were the body weights, (27.62 ± grams; 32.7 ± grams control and experimental animals respectively) different. The brain/body weight ratio was significantly reduced in the ethanol exposed pups (3.26% ± 0.13) as compared to the control pups (3.66% ± 0.26, $p<0.003$).

Plastic Section Analysis

Qualitative observations of the two micron plastic sections (Figure 1) suggest that the entire oculomotor nucleus is less well organized in the ethanol exposed

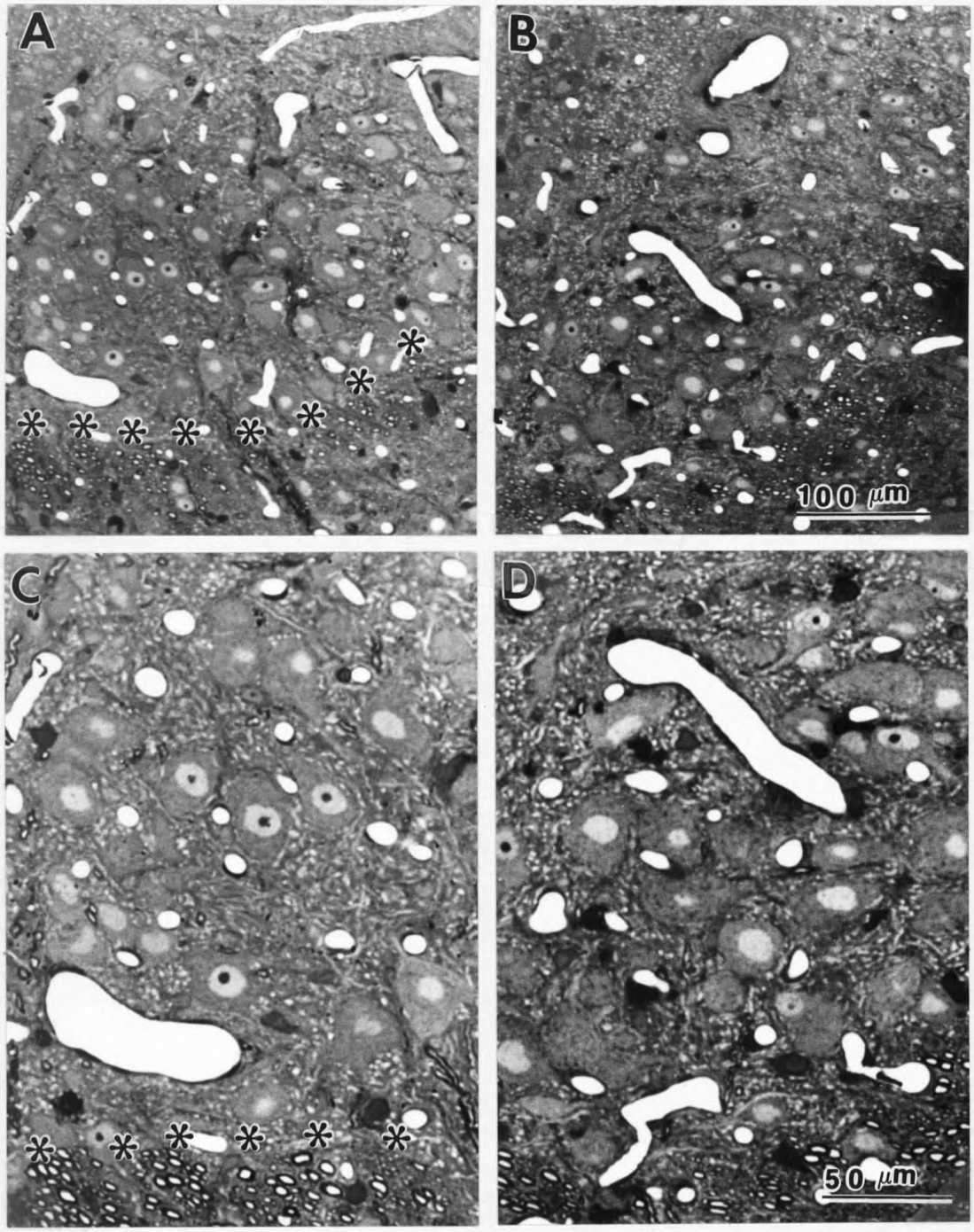

Figure 1. Light micrographs from the oculomotor nucleus of control (A,C) and ethanol exposed animals (B,D). The asterisks separate the oculomotor nucleus, above, from the medial longitudinal fasciculus, below. Magnifications are 187X (A,B) and 347X (C,D).

animals, and that the neurons in the ethanol exposed group are smaller and less basophilic than in the control group.

Cells per Defined Unit Area

Cell counts per unit area (Table 3 and Figure 2) showed a 19.4% decrease in the neuronal density in the ethanol exposed animals compared to the control animals, while the astrocyte density was increased 44.6% in the ethanol exposed animals compared to the control animals.

Table 3

Number of Cells/mm^2 for Defined Unit Area 2TE*

	NEURONS	ASTROCYTES	OLIGO-DENDROCYTE	TRANSITIONAL GLIA
CONTROL GROUP	588.70 ± 30.95	296.00 ± 19.70	72.90 ± 5.93	76.86 ± 8.57
ETOH GROUP	474.72 ± 34.18	428.23 ± 26.23	76.21 ± 6.94	112.56 ± 4.85
p-value	0.03	0.002	0.63	0.002

* mean values ± S.E.M.

The number of oligodendrocytes/mm^2 was not significantly different between the ethanol exposed group and the control group, but the number of transitional glial cells/mm^2 was increased 46.4% in the ethanol exposed animals as compared to the control animals. The total number of glial cells/mm^2 (Figure 3) was increased by 38.5% in the ethanol exposed animals (617.06 ± 27.75) compared to the control animals,

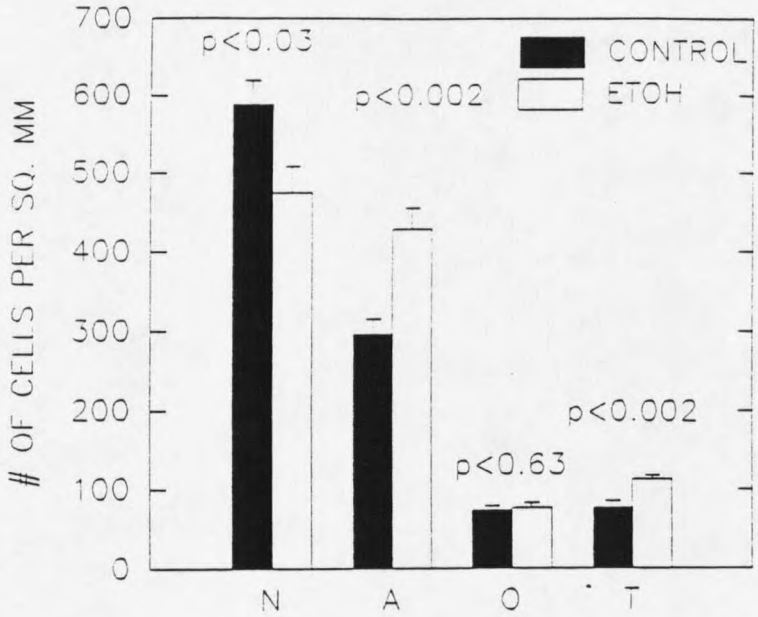

Figure 2. Cell densities determined from a unit area in the two trimester equivalency exposure on the 15th postnatal day. Bars represent, from left to right, Neurons, Astrocytes, Oligodendrocytes and Transitional glia (S.E.M.)

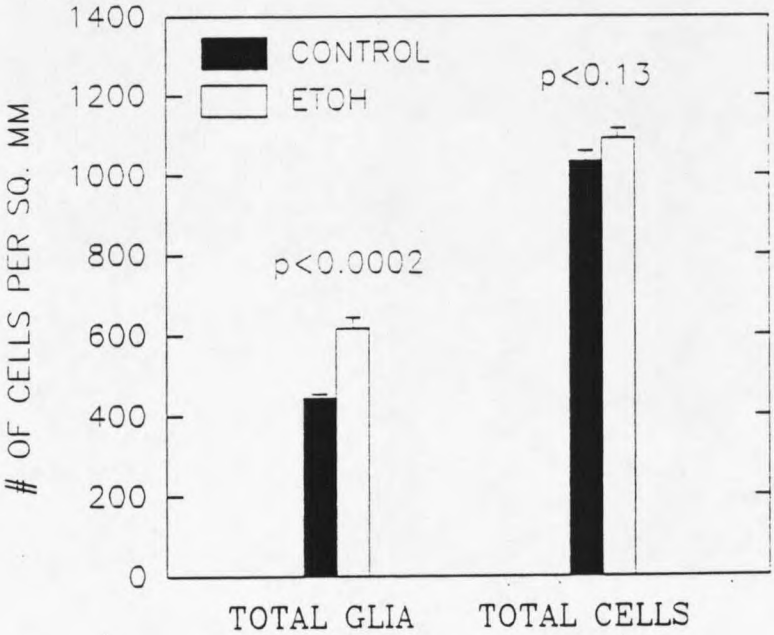

Figure 3. Total cell densities from a unit area for the two trimester exposure on the 15th postnatal day. Error bars are (S.E.M.)

(445.66 ± 10.03), while the total number of cells/mm^2 (neurons and glia) was similar in the ethanol exposed (1091.8 ±24) and control (1034.4 ±25.41) animals (Figure 3).

Cells Per Defined Oculomotor Region

The counts from the defined oculomotor region (Table 4) show that the absolute number of neurons was reduced 17% in the ethanol exposed as compared to control animals.

Table 4

Neuronal Counts and Area From Defined Oculomotor Region 2TE*

ANIMAL	RAW COUNTS	AREA in mm^2	CELLS/mm^2
#1 CONTROL	41.25 ± 2.2	.143 ± 0.004	291.88 ±34.5
#2 CONTROL	48.5 ± .86	.108 ± 0.003	449.49 ±17.8
#3 CONTROL	39.75 ± 2.37	.113 ± 0.002	350.53 ±46.7
#4 CONTROL	43.5 ± 2.24	.098 ± 0.006	443.9 ±51.1
#5 CONTROL	44.5 ± 4.2	.107 ± 0.004	415.88 ±87.8
MEAN FOR CONTROL GROUP	43.5 ± 1.5	.114 ± 0.007	390.33 ±30.2
#1 ETOH	35.25 ± 1.56	.096 ± 0.003	365.66 ±36.2
#2 ETOH	33.75 ± 3.25	.100 ± 0.002	336.15 ±72.4
#3 ETOH	37 ± 1.15	.086 ± 0.003	429.2 ±29.8
#4 ETOH	41 ± 3.63	.095 ±0.006	431.57 ±85.4
#5 ETOH	33.5 ± 2.02	.086 ±0.001	386.8 ±52.1
MEAN FOR ETOH GROUP	36.1 ± 1.37	.093 ±0.003	389.8 ±18.4
p-values	$p<0.0007$	$p<0.03$	$p<0.99$

* means ± S.E.M.

The mean proportional area of the oculomotor region was correspondingly decreased 18.5% in the ethanol exposed group as compared to the control group.

There was no difference then in the number of cells per mm^2 between the ethanol or control animals when the defined oculomotor region was used for cell counts.

Image Analysis

There were no marked or consistent differences evident in the area of the cell nucleus or nucleolus, or in their perimeters as measured by gray scale image analysis (Table 5).

Table 5

Nuclear and Nucleolar Area Measured by Image Analysis 2TE*

	NUCLEAR AREA	NUCLEAR PERIMETER	NUCLEOLAR AREA	NUCLEOLAR PERIMETER
CONTROL	87.13 ± 2.04	40.46 ± 0.37	11.11 ± 0.33	11.24 ± 0.18
ETOH	83.66± 2.38	39.71 ± 0.92	11.08 ± 0.62	11.2 ± 0.36
p-value	$p<0.29$	$p<0.46$	$p<0.97$	$p<0.91$

* Area is in um^2, perimeter is in um (± S.E.M.)

Golgi - Cox Analysis

Qualitatively, the Golgi impregnated multipolar oculomotor neuron cell bodies (Figure 4 and 5) appeared smaller and the complexity of the dendritic arbor, as well

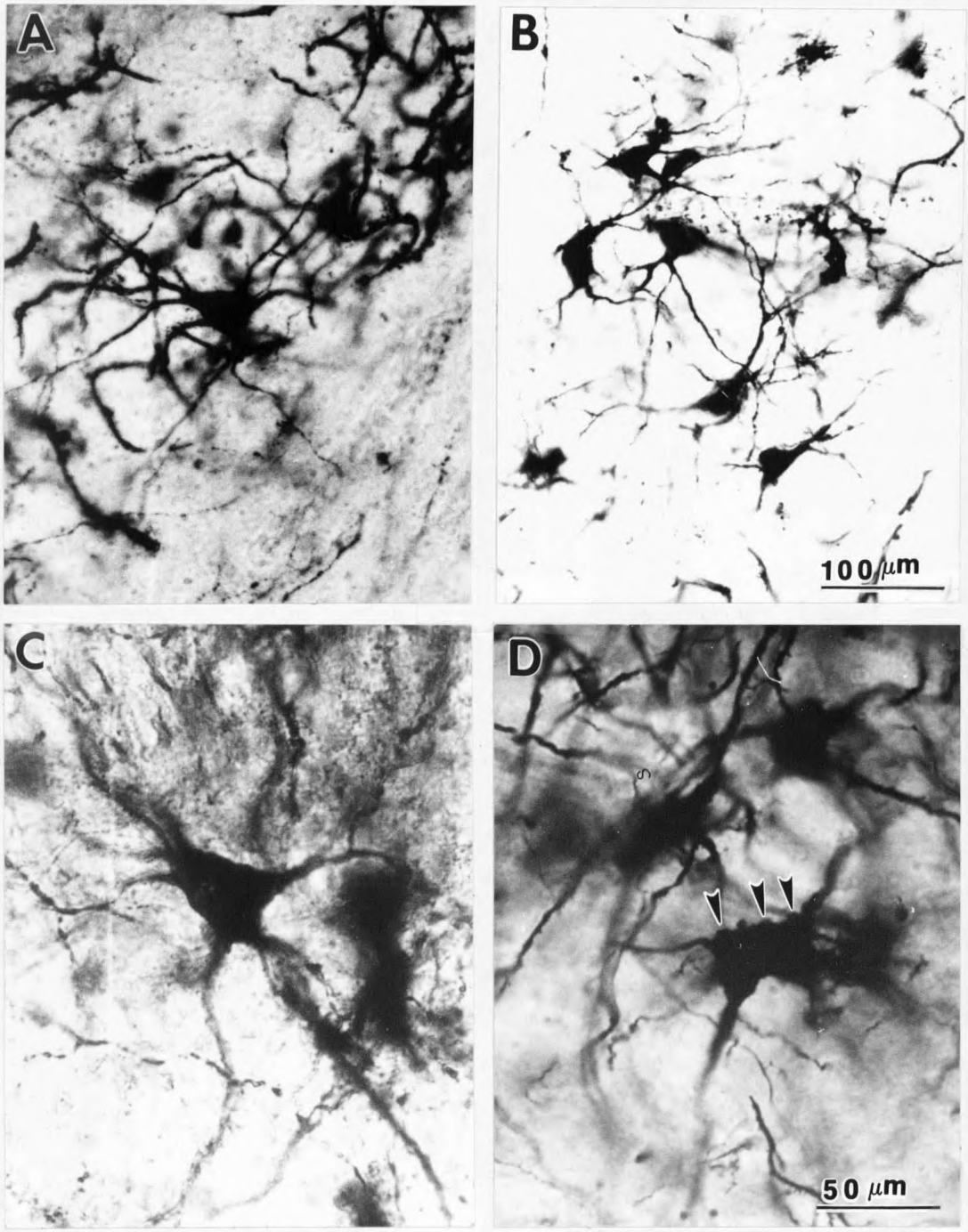

Figure 4. Golgi impregnated multipolar oculomotor neurons from the control animals (A,C) and ethanol exposed animals (B,D) in the 2TE experiment. Note the serrations on the cell body of the ethanol exposed neuron. Magnifications are 187X (A,B) and 347X (C,D).

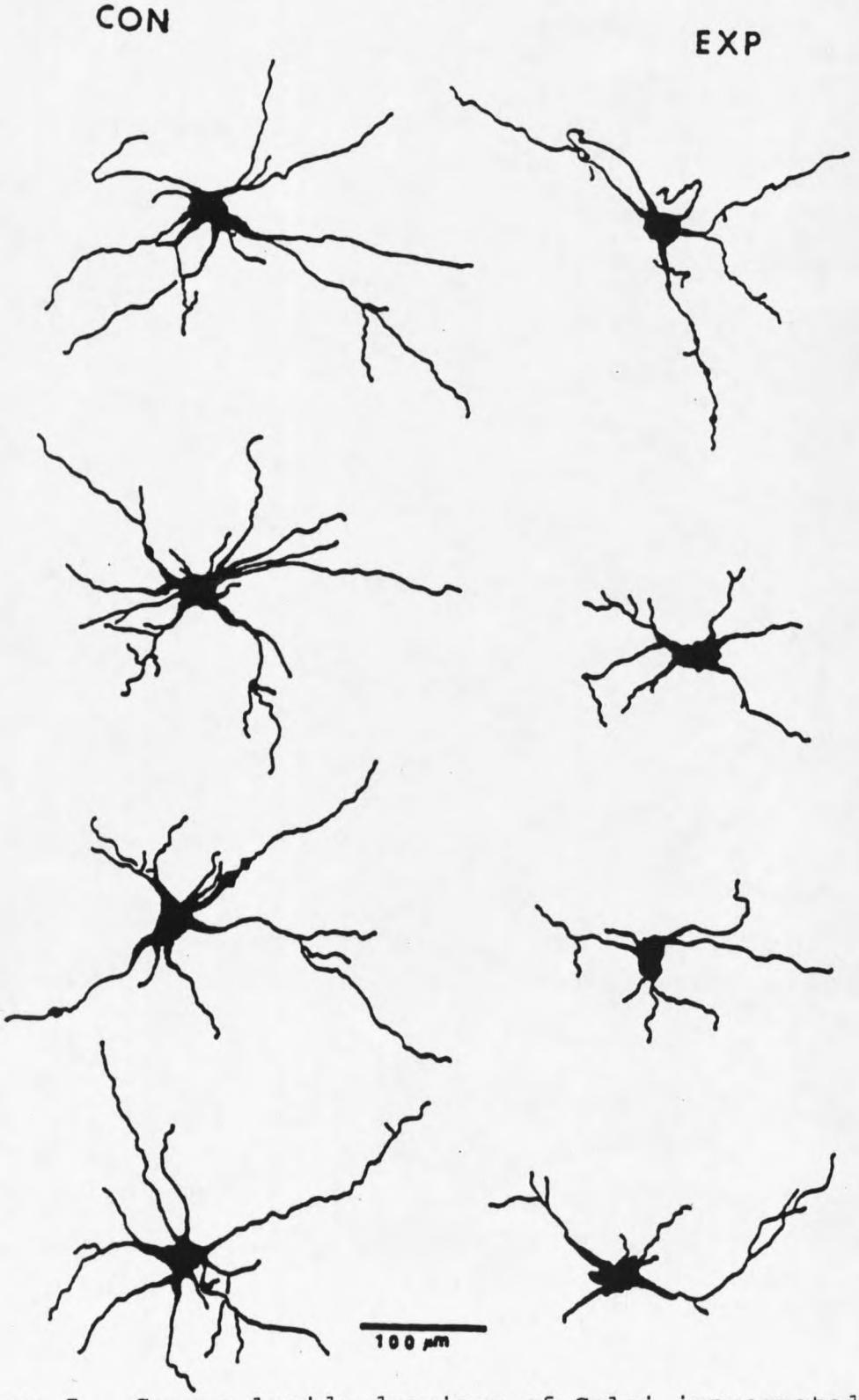

Figure 5. Camera lucida drawings of Golgi impregnated neurons from the two trimester equivalency exposure.

as the overall length of the dendrites appeared to be decreased in the ethanol exposed animals. Not all neurons were equally affected, some appeared similar to controls, while others were severely affected. Occasional neurons from the ethanol exposed animals showed serrations of the outline of the neuronal soma, perhaps indicative of a pathological condition. The mean area of the soma was decreased in the ethanol exposed animals, (507.92 + 32.1 um^2), by 29.3% as compared to the control group (707.77 ± 67.35 um^2) (Figure 8). Overall complexity of the dendritic branching was decreased in the ethanol exposed animals as compared to the control animals (Table 6, Figures 6 and 7).

Table 6

Dendritic Branching Analysis *

Branches	**Control**	**ETOH**	**p-value**
first order	6.50 ± 0.38	5.60 ± 0.54	0.07 0.22(ANOVA)
second order	8.15 ± 0.81	6.70 ± 0.98	0.22
third order	5.9 ± 0.61	4.05 ± 0.84	0.06
fourth order	3.00 ± 0.24	1.70 ± 0.64	0.03
fifth order	1.20 ± 0.28	0.40 ± 0.28	0.02
total # of dendrites	24.75 ± 1.38	18.40 ± 2.60	0.02

* mean ± S.E.M.

Table 7

Concentric Ring Analysis of Sholl *

Distance	Control	ETOH	p-value
20 microns	6.8 ± 0.53	5.6 ± 0.59	0.18
40 microns	9.85 ± 0.51	8.55 ± 0.36	0.08
60 microns	11.25 ± 0.29	8.65 ± 0.44	0.001
80 microns	10.35 ± 0.63	7.25 ± 0.52	0.009
100 microns	8.85 ± 0.87	6.75 ± 0.88	0.14
120 microns	6.65 ± 0.71	5.3 ± 0.69	0.27
140 microns	4.8 ± 0.36	3.75 ± 0.57	0.17
160 microns	3.55 ± 0.5	2.85 ± 0.33	0.29
180 microns	2.85 ± 0.48	2.1 ± 0.24	0.21
200 microns	1.7 ± 0.34	1.1 ± 0.17	0.17

* mean ± S.E.M.

The ethanol exposed animals had fewer dendritic segments at all levels of branching (Figure 6) compared to control animals, yet the difference was only significant ($p < 0.05$) for the fourth, and fifth order, and for the total number of dendrites as defined by the sum of all orders of branching (Figure 7).

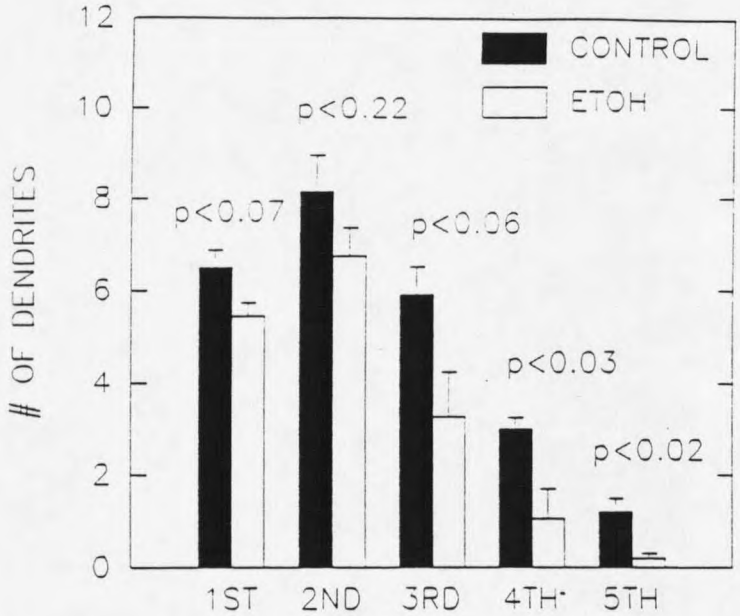

Figure 6. The number of first, second, third, fourth, and fifth order dendrites from Golgi impregnated multipolar oculomotor neurons from the two trimester equivalency exposure. Error bars are (S.E.M.) (n=4).

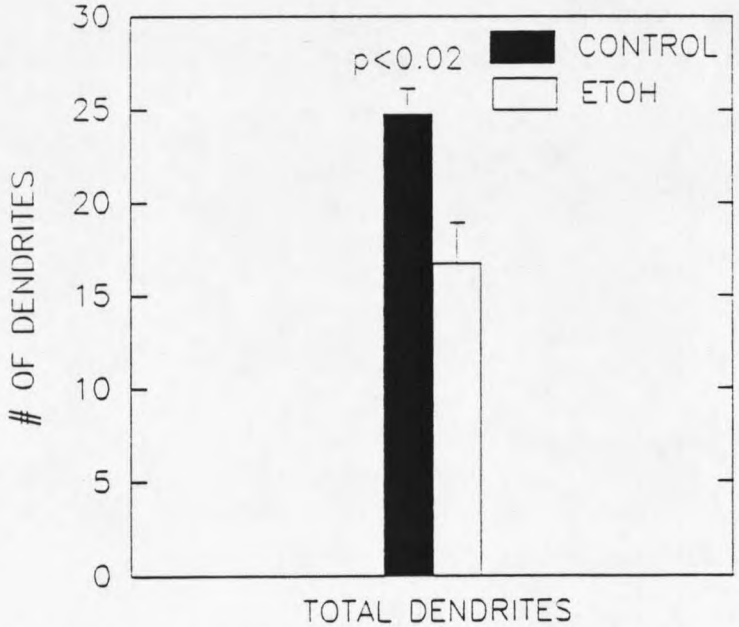

Figure 7. Total number of mean dendritic segments per neuron from the Golgi impregnated oculomotor neurons, in the two trimester equivalency exposure. Error bars are (S.E.M.) (n=4).

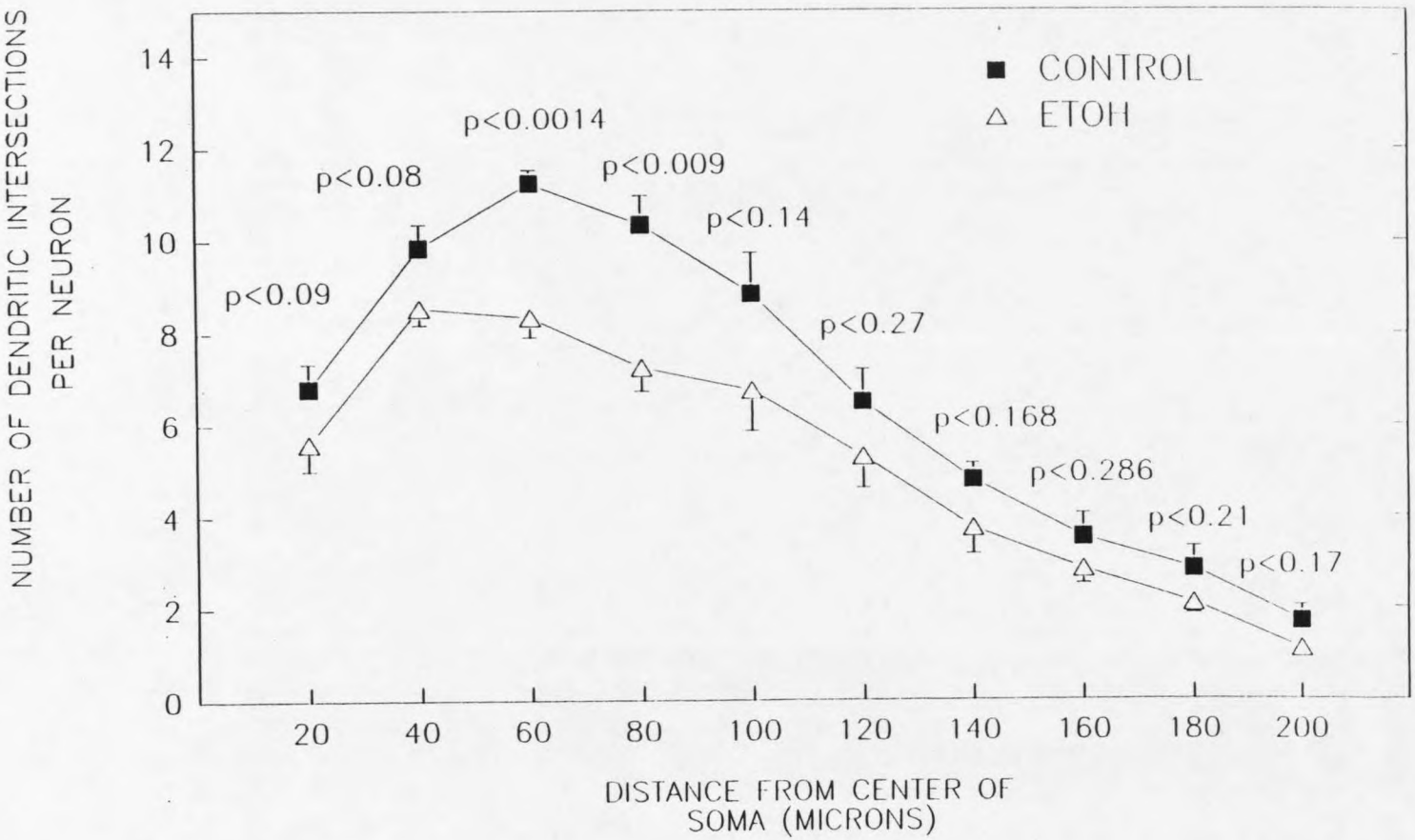

Figure 8. Sholl's concentric ring analysis comparing the branching pattern of dendrites from Golgi impregnated multipolar oculomotor neurons in the two trimester equivalency exposure. Points indicate the mean number (and S.E.M.) of dendrites intersecting with concentric circles at radius intervals of 20 um (n=4).

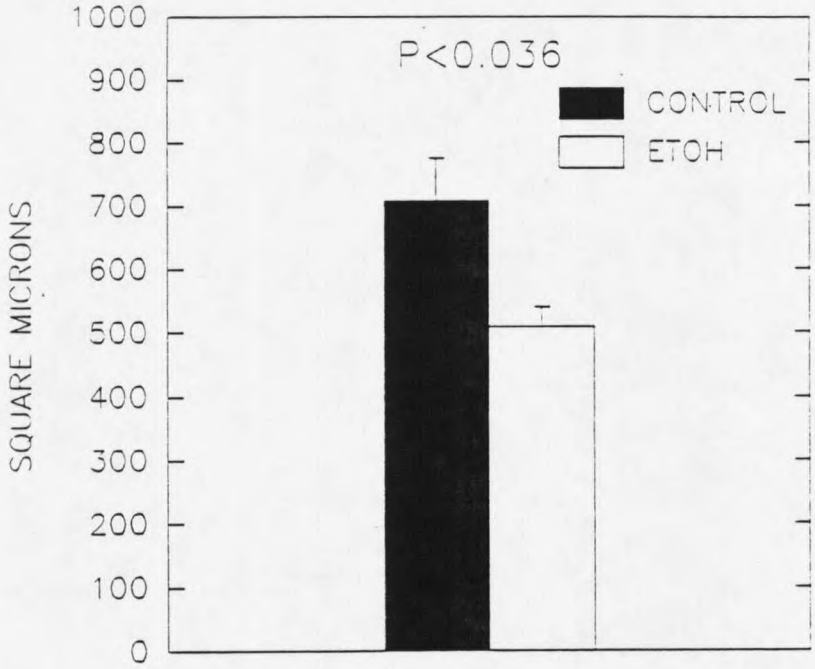

Figure 9. Area of the soma in Golgi - Cox impregnated multipolar neurons of the oculomotor nucleus from the two trimester equivalency exposure. Error bars are (S.E.M.) (n=4).

The concentric ring analysis showed a similar decrease in dendritic complexity in the ethanol exposed animals (Table 7 and Figure 8). There were fewer mean dendritic segments at every distance measured from the center of the soma in the ethanol exposed animals compared to control animals but the difference was statistically significant only at 60 and 80 microns from the soma. A nested ANOVA comparing the overall dendritic complexity in the ethanol exposed animals as compared to the control animals showed a significant decrease ($p<0.03$).

Three Trimester Equivalency

The percent weight gain was similar for the ethanol exposed (38.51%) and control (32.79%) dams (G5 through G20), and the difference was not statistically significant ($p<0.11$). The daily volume of liquid diet consumed per animal in the experimental group averaged 64.73 $\pm$ 7.45 ml/day, equivalent to an ethanol intake of 12.69 $\pm$ 2.36 gm/kg/day. The mean volume of liquid diet consumed for the control group was identical to that for the experimental group since they were pair fed. Blood alcohol concentrations were not determined for the dams, but were considered to be similar to those for the two trimester equivalency dams, and for previous groups in the lab (approximately 140mg/dl).

The pattern of weight gain in the ethanol exposed and control pups was virtually identical (Figure 10). On the day of sacrifice, PND 15, neither the brain weights (0.82 ± grams; 0.81 ± grams control and experimental animals respectively) nor the body weights (20.78 ± grams; 22.77 ± grams control and experimental animals respectively) were significantly different. The brain /body weight ratio was decreased 8.5% in the ethanol exposed pups (3.62%) as compared to the control pups (3.96%) but the difference was not statistically significant ($p<0.09$) with a two-tailed T-test.

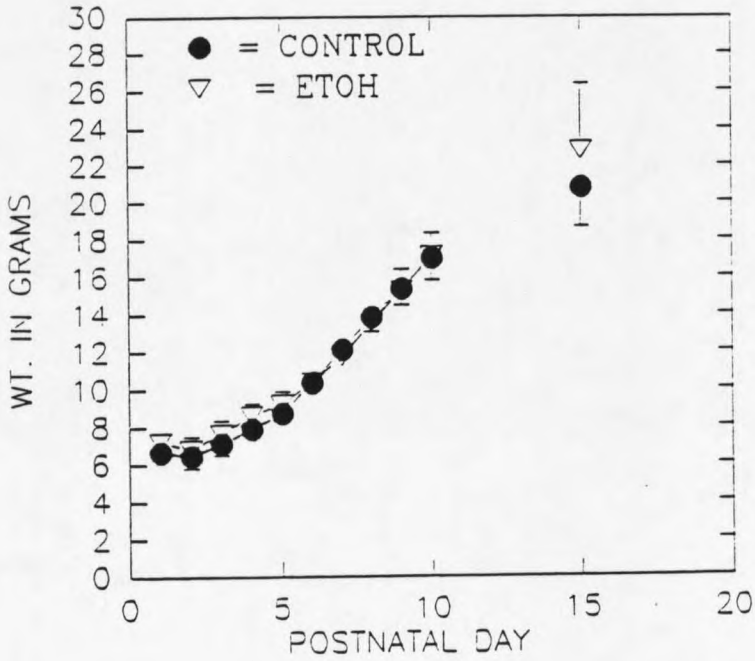

Figure 10. The weight in grams of the artificially reared pups from the 3TE series. Error bars are S.E.M. (not shown when compromised within the symbol).

Plastic Section Analysis

Qualitative observations of the two micron plastic sections of the oculomotor nucleus (Figure 11) suggested that the neurons were smaller and less basophilic in the ethanol exposed animals compared to the control animals. The oculomotor nucleus also appeared to be less organized in the ethanol exposed group.

Cells per Defined Unit Area

Cell counts per unit area (Table 8 and Figure 12) indicated that there was a 26.1% reduction in the neuronal density in the ethanol exposed animals as compared to control animals, while the mean astrocytic density was 16.5% higher in the ethanol exposed animals compared to the control animals, although the difference was not statistically significant.

Table 8

Number of Cells/mm^2 for Defined Unit Area 3TE *

	NEURONS	ASTROCYTES	OLIGO-DENDROCYTES	TRANSITIONAL GLIA
CONTROL	618.8 ± 54.68	353.37 ± 13.98	68.93 ± 8.69	78.9 ± 8.68
ETOH	457.27 ± 18.98	411.57 ± 28.04	69.47 ± 9.12	91.67 ± 10.1
p-value	0.01	0.09	0.97	0.36

* mean ± S.E.M.

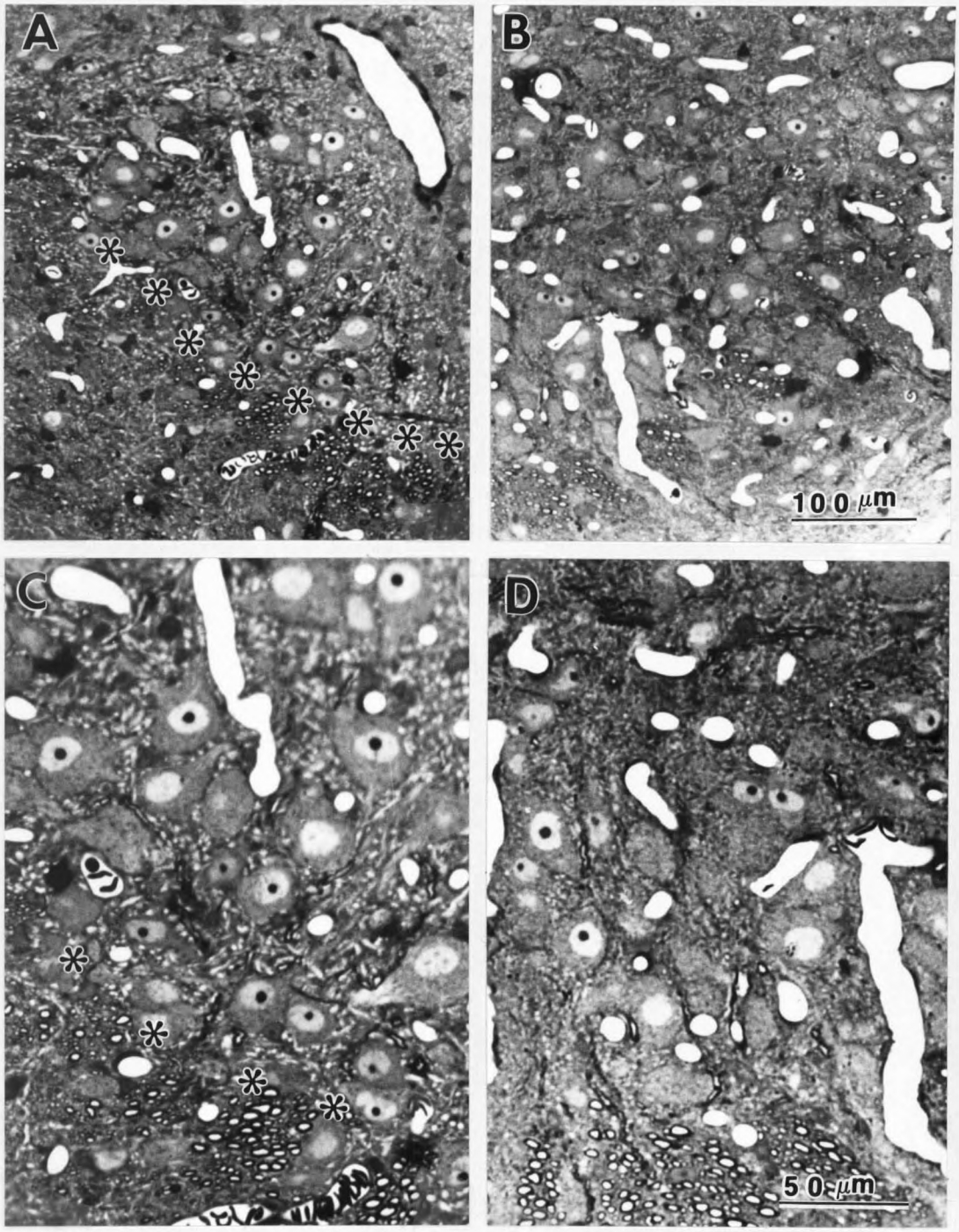

Figure 11. Light micrographs from the oculomotor nucleus of control (A,C) and ethanol exposed animals (B,D). The asterisks separate the oculomotor nucleus, above, from the medial longitudinal fasciculus, below. Magnifications are 187X (A,B) and 347X (C,D).

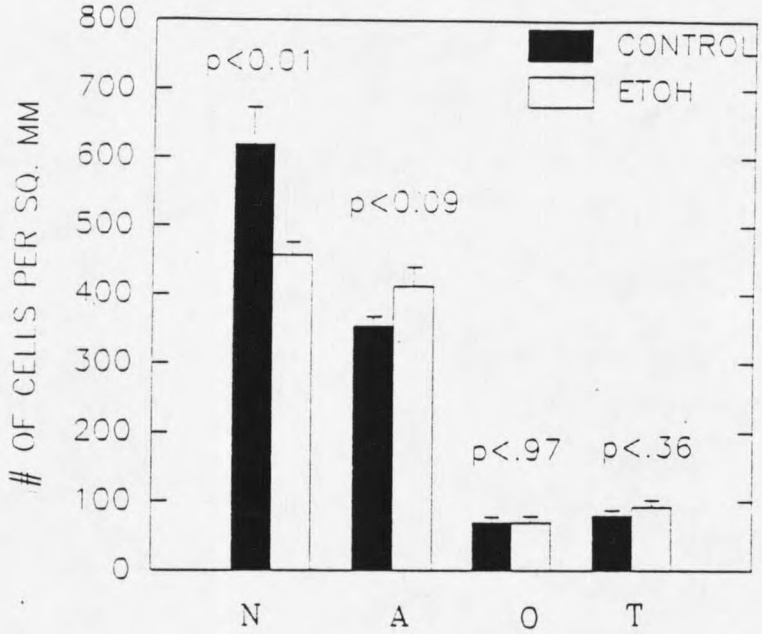

Figure 12. Cell densities determined from a unit area in the three trimester equivalency exposure, on the 15th postnatal day. Bars, from left to right, represent Neurons, Astrocytes, Oligodendrocytes, Transitional glia (S.E.M.)

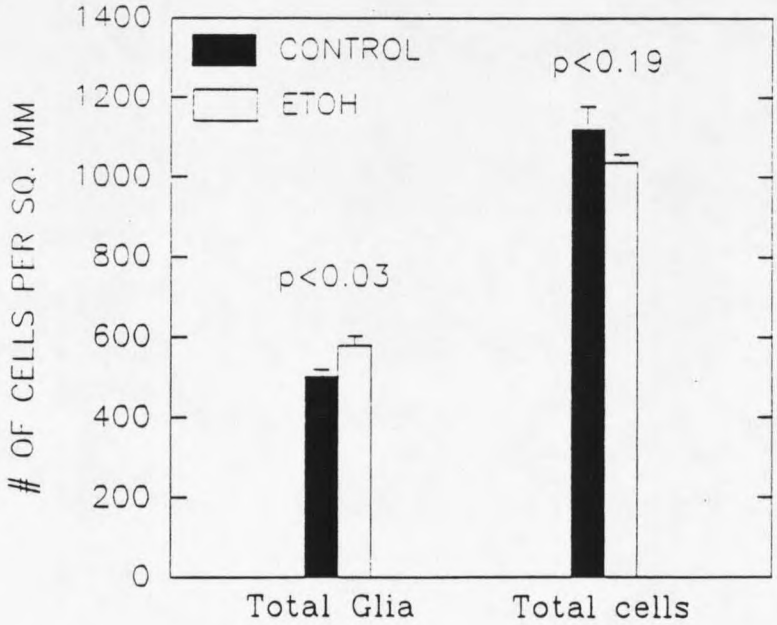

Figure 13. Total cell densities from a unit area for the three trimester equivalency exposure.
Error bars are (S.E.M.).

The number of oligodendrocytes/mm^2 was virtually identical in the control and ethanol exposed animals and the mean transitional glial cell density, while increased 16.2% in the ethanol exposed animals compared to the control animals, the difference was not statistically significant. The total number of glial cells/mm^2 (Figure 13) was significantly increased (15%) in the experimental animals (578.35 $\pm$ 25.13) over that of the control animals (501.2 $\pm$ 18.37), while the total number of cells/mm^2 (neurons and glia) was very similar in ethanol exposed (1035.6 $\pm$ 20.42) and the control animals (1120 $\pm$ 57.65) (E/C=.92).

Cells per Defined Oculomotor Region

The results from the neuronal counts in the defined oculomotor region are given in Table 9. The mean absolute number of neurons was slightly reduced, with the ratio of the values for the ethanol exposed and the control animals (E/C) equal to .93. The mean area of the defined oculomotor region was reduced a similar amount (E/C=.92), while the number of neurons per mm^2 was similar (E/C=.98) in both groups. None of the parameters measured were found to be statistically different.

Nuclear and Nucleolar Area and Perimeter

The area of the neuronal nucleus (Table 10) was reduced by 10.8% in the ethanol exposed pups as compared to the

control animals ($p<0.03$), while the perimeter of the nuclear membrane was similar in the two groups (E/C=.98, $p<0.5$). The area of the nucleolus was reduced by 15.5% in the ethanol exposed animals as compared to the controls, and the perimeter of the nucleolus was also reduced, but by a lesser amount (8.9%).

Table 9

Neuronal Counts and Area From Defined Oculomotor Region 3TE*

ANIMAL	RAW COUNTS	AREA	NEURONS/mm^2
#1 CONTROL	42.5 ± .93	.114 ± 0.002	373.5 ± 18.3
#2 CONTROL	42.25 ± 1.23	.115 ± 0.002	367.4 ± 23.9
#3 CONTROL	50.25 ± 7.4	.108 ± 0.006	467 ± 153.8
#4 CONTROL	34 ± 2.83	.092 ± 0.003	368.8 ± 68.6
#5 CONTROL	38 ± 4.48	.091 ± 0.004	419 ± 110.5
MEAN FOR CONTROL GROUP	41.4 ± 2.71	.104 ± 0.005	399.1 ± 19.5
#1 ETOH	36.25 ± 2.17	.108 ± 0.003	336.6 ± 40.3
#2 ETOH	42.25 ± 3.8	.093 ± 0.001	456.3 ± 82.1
#3 ETOH	37.75 ± 4.4	.084 ± 0.005	447 ± 104.3
#4 ETOH	38 ± 3.63	.098 ± 0.003	388.1 ± 74.1
MEAN FOR ETOH GROUP	38.6 ± 1.29	.096 ± 0.005	407 ± 27.9
p-value	$p<0.41$	$p<0.3$	$p<0.82$

* mean ± S.E.M.

Table 10

Nuclear and Nucleolar Areas Measured by Image Analysis* 3TE

	NUCLEAR AREA	NUCLEAR PERIMETER	NUCLEOLAR AREA	NUCLEOLAR PERIMETER
CONTROL	96.68 ± 3.64	41.11 ± 0.93	13.02 ± 0.43	12.29 ± 0.22
ETOH	86.29 ± 1.74	40.29 ± 0.69	11.01 ± 0.26	11.2 ± 0.16
p-value	$p<0.03$	$p<0.5$	$p<0.003$	$p<0.003$

* Area is in um^2, perimeter is in um (mean ± S.E.M.)

DISCUSSION

The present study showed that ethanol has the following effects on the development of the oculomotor nucleus in the rat (summary, Table 11) following a 2TE exposure: a decrease in the number of neurons per unit area and predefined oculomotor region, an increase in the number of astrocytes per unit area, an increase in the number of transitional glial cells, a decrease in the cross-sectional area of the defined region of the oculomotor nucleus; a decrease in the number of neurons in the oculomotor region, a decrease in the dendritic branching of the oculomotor multipolar neurons, a reduction in the complexity of the dendritic arbor, and a decrease in the area of the neuronal soma. The alcohol induced changes following a 3TE exposure included: a decrease in the number of neurons per unit area, an increase in the number of astrocytes per unit area, and an increase in the number of transitional glial cells.

Although counts from the defined oculomotor region showed no difference in the number of neurons/mm^2, the fixed area counts from the central region of the nucleus showed a significant decrease in the number of neurons/mm^2 as well as an increase in the number of astrocytes/mm^2 in both the 2TE and 3TE exposures in the ethanol exposed animals as compared to control animals. The area of the fixed counts, 0.05 mm^2, was approximately 1/2 of the area from the proportional

counts as determined from measurements of the camera lucida drawings of plastic sections.

Table 11.

Summary of Results from 2TE and 3TE Exposures

	Two Trimester Equivalency		Three Trimester Equivalency	
PARAMETER	E/C	p-value	E/C	p-value
neuron/area per mm^2	.81	$p<0.03$	.74	$p<0.01$
astrocyte/area per mm^2	1.45	$p<0.002$	1.16	$p<0.09$
oligodendrocyte/ area per mm^2	1.04	$p<0.63$	1.01	$p<0.97$
transitional glia/area/mm^2	1.46	$p<0.002$	1.16	$p<0.36$
neurons/region (raw counts)	.83	$p<0.0007$	.93	$p<0.41$
area of region in mm^2	.82	$p<0.03$	.92	$p<0.3$
neurons/region per mm^2	1.00	$p<0.99$	1.02	$p<0.82$
nuclear area	.96	$p<0.3$	.89	$p<0.03$
nuclear perimeter	.98	$p<0.47$	.98	$p<0.5$
nucleolar area	1.00	$p<0.97$	.84	$p<0.003$
nucleolar perimeter	.99	$p<0.91$	.91	$p<0.003$
dendritic order (range)	.33-.86	$p<0.02$-0.22	N/A	N/A
Sholl's analysis (range)	.65-.87	$p<0.001$ 0.29	N/A	N/A

E/C = Ethanol Exposed/Control values

The difference in the number of neurons/area in the fixed area and defined region counts possibly indicates an altered migration of neurons into the nuclear grouping from the place of origin in the embryonic ventricular zone. A ratio of the number of cells/mm^2 in the fixed area to that of the number of cells/mm^2 in the defined region should equal one if the distribution of neurons were homogenous throughout the oculomotor region. The ratio is 1.51 for the control animals in the 2TE exposure, and .1.55 for the control animals in the 3TE exposure, indicating a highly heterogenous distribution of neurons throughout the oculomotor region with the cells concentrated in the nucleus. The ratio is 1.22 in the experimental animals in the 2TE series, indicating a more homogenous distribution of neurons throughout the region. The ratio is 1.12 in the experimental animals from the 3TE series, indicating an even more homogenous distribution of neurons than in any of the other three groups. Both the higher ratios in the control animals and the appearance of the oculomotor nucleus in plastic sections indicate that a successful migration of neurons has taken place, which is typically completed by E17 (Puelles and Privat, 1977). Since the migration is normally completed in the late prenatal period, it is possible that the oculomotor neurons in the 2TE experimental animals continued their migration into the early postnatal period, a

time when no alcohol was present. However, the migration of neurons in the 3TE experimental animals would have also been delayed in the early postnatal period, beyond the delay that occurred in the 2TE animals thus a more altered migration. Further studies using either retrograde labeling techniques to localize oculomotor neurons or counts of neurons at varying distances from the center of the nuclear region (using concentric rings, similar to that used for the dendritic arbor analysis) need to be done to confirm this. Such adverse effects of ethanol on the migration of neurons are consistent with reports of alcohol induced delays of neuronal migration in the cerebellum (Bauer-Moffett and Altman, 1977; Shetty and Phillips, 1992), hippocampus (Davies and Smith, 1981; West et al., 1981) and the neocortex (Miller and Dow-Edwards, 1988; Miller and Potempa, 1990).

The mechanism by which neuronal migration could be impaired is unclear, but it could involve radial glial cells since they are known to play a role in guiding migration of developing neurons (Rakic, 1971; Rakic, 1972; Rakic and Sidman, 1973). Alterations in radial glial maturation have been hinted at in the cerebral cortex (Robertson and Miller, 1990), and shown in the Bergmann glial fibers of the cerebellar cortex (Shetty and Phillips, 1992). The exact mechanism by which alcohol would affect the interaction

between radial glia and neurons is unknown. However, it has been postulated that since ectopic neurons are found in the deeper layers of the cortex in ethanol exposed animals, the alcohol may act to cause the radial glial fibers to prematurely lose contact with the external limiting membrane surface, being transformed prematurely in to astrocytes (Miller, 1986). In the case of the cerebellum however, such loss of contact seems unlikely since the effect on Bergmann glia seems to primarily be a simple delay in cerebellar maturation (Shetty and Phillips, 1992). The molecules that control cell adhesion between the migrating neurons and the astrocytes could also be affected. Biochemical studies have indicated that ethanol affects other membrane bound molecules (Druse, 1986), but as yet no studies have been done to specifically address the effect of developmental ethanol exposures on cell adhesion molecules. It is also possible that ethanol may exert some of its effects on the elements of the neuronal cytoskeleton that are crucial for neuronal migration (Hassler and Moran, 1986; Maciejewski-Lenoir and Milner, 1989). Neural crest cells exhibit a highly disorganized contractile system with actin filaments appearing matted and thickened after exposure to ethanol (Hassler and Moran, 1986). The expression of mRNA for alpha-tubulin, a microtubular component, is significantly depressed during the first postnatal week in

the somatosensory cortex of the rat after ethanol exposure, at a time characterized by extensive neuronal migration in the neocortex (Maciejewski-Lenoir and Milner, 1989).

In addition to the reduction in the area of the oculomotor region, the number of neurons within the oculomotor region was also reduced in the ethanol exposed animals in both the 2TE (17.1%) and 3TE (6.8%) exposure, although the difference was only statistically significant in the 2TE exposure. If the raw counts are converted to the number of cells/mm^2, then there was no difference between the experimental and control animals of either the 2TE or the 3TE exposures. The fact that the number of neurons are not additionally decreased by the addition of the postnatal ethanol exposure suggests that ethanol exerts its effects on the neurons of the oculomotor system in the prenatal period, rather than in the third trimester equivalent.

The reduction in the overall brain region and the number of neurons in that region could be due to a preferential toxic effect of ethanol on neuronal stem cells early in development (Kotkoskie and Norton, 1990), or to a decrease in proliferation of neuronal stem cells. Previous studies tend to indicate that effects on proliferation are more likely since animals prenatally exposed to ethanol have shown a one to two day delay in the generation of neurons in the somatosensory cortex (Miller, 1986). It is also

possible that the ethanol acts to increase the normal neuronal cell death that occurs in the nucleus, causing a higher percentage of the cells to die (Phillips and Cragg, 1982). Finally ethanol could have a preferential effect on the target cells for the oculomotor neurons, the extraocular eye muscles. A situation analogous to this occurs in the microphthalmic rat (Tanaka et al., 1987a; Tanaka et al., 1987b), where the extraocular eye muscles are reduced in size to approximately 74% of normal, and so are the number of neurons within the brainstem nuclei controlling the muscles involved (Tanaka et al., 1987a; Tanaka et al., 1987b). If this were the case in the current study, one would expect the number of cells per unit area to remain the same, as the present data suggest.

There was a significant reduction in the area of the oculomotor region in the ethanol exposed and control animals of the 2TE series, but not in the 3TE series. The area of the region in the ethanol exposed animals in both the 2TE and 3TE series was very similar, while the area of the oculomotor region in the 3TE control group was less than that for the 2TE control animals, being approximately half way between the values for the experimental groups for the two exposure regimes and the value for the 2TE control animals. This difference, between the two control groups, can be attributed to the effects of the artificial rearing

procedure or it could simply indicate random variation in animals within the groups since it was not statistically significant. Slight but not statistically significant delays in brain maturation have been consistently demonstrated in pair fed artificially reared control animals compared to normally reared rat pups (West et al., 1984b). The 15 day 3TE control animals may be undergoing a rebound effect to compensate for the growth retardation induced by the artificial rearing procedure on postnatal days 1-10. This idea is supported by the results of the image analysis in which the area of the cell nucleus and nucleolus was measured. The area of the nucleolus was very similar in the experimental animals of the two exposure regimes as well as in the control animals of the 2TE exposure, while the area of the nucleolus in the control animals in the 3TE exposure was significantly greater. The area of the nucleolus has been used as a useful indicator for the metabolic state of the cell (Watson, 1988; McNeill and Koek, 1990), thus indicating a higher level of metabolic activity in the 3TE control animals which is consistent with the idea of a rebound effect.

The ethanol exposed neurons from both exposure regimes appear to be less basophilic than the control neurons in plastic sections, possibly indicating a decrease in the protein metabolism of the cell. Decreased protein synthesis

has been shown in the cells of the cerebellum following prenatal ethanol exposure (Rawat, 1975). Ultrastructural studies tend to confirm the possibility of alcohol induced changes in cell metabolism. Disruptions in the organization of the granular endoplasmic reticulum and increases in the volume fraction of Golgi complexes and lysosomes were observed in the pyramidal cells of the somatosensory cortex of ethanol treated animals (Al-Rabiai and Miller, 1989). Similar effects were reported in the Purkinje cells of the cerebellum (Nathaniel et al., 1986) in rats prenatally exposed to ethanol. No ultrastructural studies have been completed on the effects of prenatal exposure to ethanol on somatic motorneurons as found in the oculomotor nucleus. However, a preliminary examination is currently underway in the laboratory.

The increase in the number of astrocytes in the tissue of the ethanol exposed animals is consistent with reports in the literature that show an alcohol induced increase in the GFAP staining in the cerebral cortex (West et al., 1990; Leo et al., 1991), and in the central white matter of the cerebellum (Shetty and Phillips, 1992). An increase in the relative area occupied by astrocytic cell bodies has been shown in paraffin sections (Miller and Potempa, 1990) and an increase in the area occupied by astrocytic processes has been shown in ultrastructural studies of the cerebral cortex

(Robertson and Miller, 1990). Optic nerve studies have shown an increase in the number of astrocytes per unit area after developmental alcohol exposures (Phillips and Krueger, 1990; Phillips and Krueger, 1992). It is not clear in the present study whether the astrocytic hyperplasia is directly related to the altered migration of neurons, to a toxic effect of the ethanol itself, a mitogenic effect on the astrocytes, or to an exaggerated response of astrocytes growing to fill areas that normally are occupied by neurons. Since one of the functions of astrocytes is detoxification (Aschner and Kimelberg, 1991) the increased number of astrocytes may represent a detoxification response to something in the extracellular environment not normally present in the control animals. Another possibility is that since astrocytes act in supporting the metabolic activity of neurons (Kimelberg and Norenberg, 1989), and the neurons appear to be more dispersed in the ethanol animals, more astrocytes may be required to support the neurons.

One of the characteristic features of developmental ethanol exposures appears to be a developmental delay, and thus a delay in the formation of the blood brain barrier may also be present. If such a delay in the formation of the blood brain barrier exists it could produce an increase in the number of astrocytes since circulating mitogenic factors in the blood that do not normally cross the blood-brain

barrier may be present. Such factors known to be mitogenic for astrocytes include platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) (Jacobson, 1991). If the formation of the blood-brain barrier were delayed these circulating factors would have access to the brain for a longer period of time thus resulting in a gliotic hyperplasia. Studies on the effects of alcohol on the development of the blood-brain barrier are underway in the laboratory and could shed some light on this. Gliosis has also been shown to occur in brain regions having undergone developmental alterations such as sensory deprivation (Martinez Garcia et al., 1991). Whether or not a gliotic hypertrophy occurred in the present study is not known, but the presence of hypertrophy would not be surprising in light of other studies (West et al., 1990; Leo et al., 1991). The change in the density of astrocytes might also be a consequence of the sample area examined, as was the case for the neurons in the fixed area and defined region counts, but this cannot be determined because astrocytic density was not counted in the defined region study. Other studies describing alcohol induced gliosis (West et al., 1990; Leo et al., 1991; Shetty and Phillips, 1992) used GFAP immunocytochemistry, thus making it difficult to distinguish between gliotic hypertrophy, which may be a more temporary phenomena, and gliotic

hyperplasia, possibly reflecting a more permanent alteration. Later time points need to be examined with both GFAP immunostaining and cell counts to confirm whether the increase in astrocytic density represents hypertrophy, hyperplasia, or cell migration from another area.

Glial cells classified as transitional glia in the plastic section analysis have a morphology consistent with immature glial cells described previously (Skoff et al., 1976). Both the morphology and increase in number of the transitional glia reported here is consistent with reports showing an increase of immature cells in animals exposed developmentally to ethanol (Phillips and Krueger, 1990; Phillips and Krueger, 1992). Such increases are believed to represent delays in glial maturation (Phillips and Krueger, 1990; Phillips and Krueger, 1992). The presence of immature cells is consistent with the idea that ethanol exposure results in a developmental delay as previously indicated in both <u>in vitro</u> (Kennedy and Mukerji, 1986b; Kennedy and Mukerji, 1986a; Davies and Cox, 1991) and <u>in vivo</u> (Phillips and Krueger, 1990; Phillips and Krueger, 1992) studies of glial cell maturation after ethanol exposures. Because a later time point was not examined in the present study it is not possible to say if the observed changes represent a delay or a permanent alteration.

The fact that only a few oligodendrocytes were found in

the oculomotor region in the present study was not surprising since the oculomotor nucleus is a grey matter region and such regions normally contains few oligodendrocytes (Peters et al., 1991). Thus if there were any effects of alcohol on oligodendrocyte maturation it would not be as obvious as in other studies that examined white matter regions (Phillips and Krueger, 1990; Phillips and Krueger, 1992).

The neurons in the ethanol exposed animals in the 2TE series had consistently fewer dendritic branches than did control animals. This decrease in complexity is consistent with previous reports in the literature of decreased dendritic complexity in neurons of the cerebral cortex (Hammer and Scheibel, 1981; Smith et al., 1986), and from this laboratory, in the substantia nigra (Shetty et al., 1992).

The Sholl's ring analysis measures the richness of the dendritic arbor at various distances from the neuronal soma. The overall pattern in terms of dendritic richness relative to distance from the soma was similar in both ethanol exposed and control animals, yet the dendritic richness was consistently reduced in the ethanol exposed animals. As with the dendritic order the overall complexity of the dendritic arbor was significantly less in ethanol exposed animals as compared to the controls yet individual points

were only found to be significant at 60 and 80 microns from the center of the soma, at the richest part of the dendritic tree.

The decreased dendritic branching of the multipolar neurons within the oculomotor nucleus may be a secondary effect of alcohol acting on the serotonergic neurons whose terminals synapse in the oculomotor nucleus. Ethanol has been shown to have a preferential effect on serotonergic neurons (Rathbun and Druse, 1985), and since serotonergic terminals are distributed throughout the oculomotor nucleus (Takeuchi et al., 1983) it seems possible that by reducing afferent input from the afferent serotonergic cell population the alcohol could cause decreased dendritic branching and complexity.

Although several reports in the literature indicate that developmental ethanol exposures can produce decreases in the complexity of dendrites, similar to these (Hammer and Scheibel, 1981; Stoltenburg-Didinger and Spohr, 1983; Shetty et al., 1992), there are some reports that the dendritic arbor of corticospinal neurons prenatally exposed to alcohol (Miller et al., 1990) and of cerebellar granule cells in adult animals chronically exposed to ethanol (Durand et al., 1989) are more complex than that of control animals. Both of these studies examined animals at a later time than this study, so it is possible that the effect could be a rebound

sprouting phenomena produced after cessation of the alcohol. Whether this same situation occurs in somatic motor neurons is unknown, but if true it supports the idea of delayed maturation, with possible rebound sprouting.

There are several possible relationships between these findings and the oculomotor problems associated with FAS. Among the possibilities is that a delay in the migration of the oculomotor neurons results in altered synaptic contacts, possibly with the axons of the medial longitudinal fasciculus from the vestibular nuclei, or with abducens internuclear neurons. The majority of the fibers from the medial longitudinal fasciculus terminate in the subpopulation of the oculomotor nucleus containing neurons innervating the medial rectus muscle. This could contribute to the reported strabismus in FAS. However, if this were the case one might expect a certain degree of nystagmus, since many of the fibers in the MLF originate in the vestibular nuclei. Yet, nystagmus is not commonly reported in the literature. Another possibility concerns cerebellar efferent pathways associated with oculomotor function. Granule cells in the cerebellum receive mossy fiber afferents from the vestibular system and, in turn, synapse on the distal dendrites of Purkinje cells that provide efferent output from the cerebellum to the oculomotor system either directly or indirectly (Evinger, 1988). Purkinje

cells are reduced in number in animals exposed to ethanol during development (Bauer-Moffett and Altman, 1977; West et al., 1989; Marcussen et al., 1989), and there is also a delayed migration of the granule cells from the external granule layer (Quesada et al., 1990a; Shetty and Phillips, 1992). The delayed granule cell migration could result in inappropriate connections of the granule cells with the mossy fibers or, if the Purkinje cells are less developed, inappropriate connections from the parallel fibers to the distal dendrites on the Purkinje cells might occur. Either of these possibilities could result in changes in the cerebellar efferents associated with oculomotor neurons.

It is also possible that the myelination of the oculomotor nerve is impaired by alcohol, thus altering the conduction of impulses to the muscles. Such delays in myelin acquisition due to developmental alcohol exposures have been shown in both the CNS (Jacobson et al., 1979; Samorajaski 1986; Phillips et al., 1991b) and the PNS (Baruah and Kinder, 1989). Permanent reductions in thickness in CNS myelin have also been shown (Phillips et al., 1991a). If such effects on myelin were significant in this system one would expect all of the extraocular eye muscles to be equally affected, and reports of vertical oculomotor disturbances are not as severe or as common as horizontal disturbances. The effects could also involve

the eye muscles themselves, since the innervation patterns within the different eye muscles are different. The medial rectus contain the highest percentage of singly innervated muscle fibers (Evinger, 1988), and if these are more susceptible to ethanol than the multiply innervated fibers, then fewer neurons should be present in the subpopulation of the oculomotor nucleus controlling the medial rectus muscle. This could then account at least partially for the strabismus reported in the literature, but it would not explain the ptosis, which could be related to sympathetic innervation of the levator palpebrae muscle. Based on the present literature concerning the effects of ethanol on development it is clear that no single mode of action accounts for all of the symptoms of FAS, and that at least one if not all of the possibilities discussed above could be at least partially involved.

In conclusion these studies demonstrate that ethanol in either a 2TE or 3TE exposure results in a decrease in the area of the oculomotor region of the brain, a reduction in density of neurons, an increase in the density of astrocytes, and an increase in the density of transitional glial cells. Golgi - Cox studies indicated that prenatal exposure causes decreases in soma size, the overall complexity of the dendritic arbor, and the total number of dendrites (as evidenced as the sum of all dendritic branches

examined). It would be interesting in future studies to determine if the effects noted on the oculomotor neurons are permanent and if they also occur in the trochlear and abducens nuclei, and if so, whether the oculomotor system effects are from the action of alcohol on the neurons directly, or from the result of an altered afferent input.

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