NEURONAL SUSCEPTIBILITY IN RAT MODELS OF DEVELOPMENTAL ETHANOL EXPOSURE: DESCRIPTIONS OF CELLULAR AND MOLECULAR ALTERATIONS

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For Terri and my Mentors, who nurtured my interest in Biology

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Developmental disorders arising from maternal consumption of ethanol during pregnancy are collectively termed the fetal alcohol syndrome. Ethanol exposure during development induces abnormalities in particular brain regions, and is known to alter the expression of particular genes and their protein products. The present body of work sought to further document neuronal populations in the brain which display vulnerability to developmental ethanol exposure. A further goal of this work was to investigate cell death gene expression shortly after ethanol insult in the cerebellum in order to test a specific hypothesis about the cellular mechanism of ethanol neurotoxicity.

A prenatal exposure model was used to examine long term changes in protein expression patterns of parvalbumin (a marker for gamma-aminobutyric acid- (GABA) expressing neurons) in the rat brain. Deficiencies in the mean number of immuno-positive cells per section were noted in the medial septum (in a sexually dimorphic

manner) and anterior cingulate cortex of ethanol-treated rats. This represents the first documentation of GABAergic neuronal susceptibility to ethanol in either brain region. A neonatal exposure model was used to examine long-term changes in the expression pattern of choline acetyl-transferase (ChAT, a marker for cholinergic neurons) in the medial septum; no significant ethanol-induced changes in the mean number of immuno-positive cells per section were noted.

A similar neonatal exposure paradigm was used to document Purkinje and granule cell numbers in the cerebellar vermis during known periods of ethanol sensitivity and insensitivity, and to investigate mRNA levels of the bcl-2 family of cell death molecules. First postnatal week ethanol treatment significantly reduced Purkinje and granule cell number, while second week exposure did not. bcl-2 family gene expression was measured in the vermis shortly after ethanol treatment to determine whether alterations in these genes might correlate with the noted cell death. Transcripts encoding the pro-apoptotic molecules bax and bcl-xs were up-regulated following both first and second week exposure. Thus, a positive correlation between altered bcl-2 expression and cerebellar cell death was not found. Suppression of these pro-apoptotic processes may be the critical determinant of cerebellar susceptibility. These findings suggest new avenues of research on the intracellular consequences of such expression changes.

CHAPTER 1
INTRODUCTION

Ethanol Exposure During Development and Resulting Nervous System Alterations

Ethanol's teratogenic actions have been recognized throughout recorded history and considerable research has defined its deleterious actions (West et al., 1994). With the recognition of the fetal alcohol syndrome (FAS), and the linkage of ethanol to malformations in children of alcoholic mothers, Jones and Smith (1973) spurred a plethora of studies on alcohol-induced fetal abnormalities (West et al., 1994). Consequently, prenatal ethanol exposure has been shown to result in serious developmental alterations, including intrauterine growth deficiencies, facial dysmorphias, mental retardation (Abel, 1984), attention deficiencies and autistic-like syndromes (Aronson et al., 1997), and lowered IQ (Mattson et al., 1997). Ethanol readily crosses the placental and blood-brain barriers, diffuses into all aqueous components of the developing fetus where it can interact with membrane proteins and lipids (Zajac and Abel, 1992), and has been shown to affect protein synthesis, placental nutrient transport, fetal glucose availability, fetal oxygen levels, generation of reactive oxygen radicals, and neurotrophic factor activity (Abel and Hannigan, 1995; Bonthius and West, 1990; Heaton and Bradley, 1995; Henderson et al., 1995; Mukherjee and Hodgen, 1982; West et al., 1994; Zajac and Abel, 1992).

Zajac and Abel (1992) have characterized fetal alcohol exposure as "the leading known cause of mental retardation in the Western world." Indeed, despite this recognition, the incidence of FAS in the United States has increased six-fold between 1979 and 1993 (Prevention, 1995) to 1.95/1000 live births, and 43.1/1000 live births among heavy drinkers (Abel and Hannigan, 1995). Sampson et al. (1997) recently estimated the incidence of FAS and alcohol-related neurodevelopmental disorders between 1975-1981 in Seattle as 9.1/1000 live births. As such, FAS remains a significant health problem in the United States. Further research describing the neurodevelopmental changes induced by ethanol and the cellular and molecular changes induced by ethanol is needed, especially since the mechanism of ethanol teratogenicity remains unknown.

The toll that ethanol exacts on the development of the central nervous system (CNS) represents its most potent danger. Children exposed prenatally to ethanol show, among other abnormalities, reduction in corpus callosum area (Riley et al., 1995) and malformations in cerebellar structure (Clarren et al., 1978; Sowell et al., 1996; Wisniewski et al., 1983). Animal models have been developed which recapitulate many of the neurodevelopmental alterations and behavioral outcomes seen in humans with FAS, and these models have allowed for the characterization of various nervous system changes resulting from developmental exposure to ethanol (Hannigan, 1996). While various model systems have been employed, including the chick (Bradley et al., 1997) and mouse (Schambra et al., 1990) the most common choice for investigators is the rat.

Rats exposed during embryonic development show long-lived learning impairment (Clausing et al., 1995), behavioral alterations (Riley, 1990), microencephaly (West and Pierce, 1986), changes in neuronal proliferation and migration (Miller, 1986;

Miller, 1995b; Miller, 1996), reductions of neuronal number (Barnes and Walker, 1981; Miller, 1995a; Napper and West, 1995b), alterations in neuronal circuitry (West et al., 1981), delays in synaptogenesis (Hoff, 1988), permutations in neuromorphological development (Burrows et al., 1995; Davies and Smith, 1981; Kotkoskie and Norton, 1989), changes in neurochemistry (Black et al., 1995; Swanson et al., 1995), and alterations in the levels of specific mRNA species (Lee et al., 1997) and specific receptor molecules such as the high affinity trkA nerve growth factor receptor and the low affinity neurotrophin receptor p75 (Dohrman et al., 1997).

The present body of work sought to further document neuronal populations in the brain which display vulnerability to developmental ethanol exposure. Another goal of this work was to investigate cell death gene expression shortly after ethanol insult in the cerebellum in order to test a specific hypothesis about the cellular mechanism of ethanol neurotoxicity. To accomplish these goals the following studies were performed: first, a prenatal rat exposure model was used to examine long term changes in protein expression patterns of parvalbumin (a marker for GABAergic neurons) in the rat medial septum and cingulate cortex by counting parvalbumin-immunoreactive neurons in these structures; second, a neonatal rat exposure model was used to examine long-term changes in the expression pattern of choline acetyltransferase (ChAT, a marker for cholinergic neurons) in the medial septum by counting ChAT-immunoreactive neurons in this region; and third, a similar neonatal rat exposure paradigm was used to document Purkinje and granule cell numbers in the cerebellar vermis during known periods of ethanol sensitivity and insensitivity, and to investigate mRNA levels of the bcl-2 family of cell death molecules.

Differential Temporal and Regional Vulnerability

An interesting property of ethanol and its effect on the developing CNS is the fact that particular brain regions are differentially affected in both human FAS and rodent models of FAS (West and Pierce, 1986). Fortunately, animal models of FAS provide for rigorously controlled studies of both regional and temporal susceptibilities, and allow for the identification of the neuroanatomical substrates that underlie FAS. Although the brain develops throughout the entire prenatal and early postnatal period in humans and rats, there are both regional and temporal susceptibilities to ethanol. In terms of regional vulnerability, the cerebral cortex (Miller, 1986), and hippocampus (Barnes and Walker, 1981) have been demonstrated to be severely affected with chronic prenatal ethanol exposure. In terms of temporal vulnerability, CNS development during the human third-trimester equivalent is especially tenuous. During this period rapid, global brain development is occurring and this dynamic phase, which occurs during the human third trimester, is often termed the brain growth spurt. Although all mammals develop in a similar manner, the timing of this intensified growth is different across species. The rat, for example, from which most animal data regarding FAS are derived, undergoes its brain growth spurt during the first two postnatal weeks, with a peak at postnatal day 4-10 (P4-10) (Dobbing and Sands, 1979). Ethanol exposure during this vigorous period of brain development affects both mature and proliferating neurons (West and Pierce, 1986). The principal neurons of the cerebellar cortex provide a good example, as neonatal exposure reduces the number of differentiating Purkinje cells as well as proliferating granule cells (Bonthius and West, 1990), albeit in a temporally restricted manner (see below).

Prenatal Ethanol Exposure and Neuroanatomical Alterations

Experimental studies, utilizing rodent models of FAS, have been successful in reproducing many of the behavioral and morphological changes found in FAS, and have described numerous CNS alterations resulting from in utero ethanol exposure. Some of these include alterations in a variety of developmental processes in the CNS, including perturbed neuronal generation and migration (Miller, 1986; Miller, 1995b; Miller, 1996), permutations in neuromorphological development (Burrows et al., 1995; Davies and Smith, 1981; Kotkoskie and Norton, 1989), changes in neurochemistry (Black et al., 1995; Swanson et al., 1995), delayed synapse turnover in the hippocampus (Hoff, 1988), alterations in neuronal number in hippocampus (Barnes and Walker, 1981), and long-lasting detrimental effects on learning and behavior (Clausing et al., 1995; Riley, 1990).

Septohippocampal system

The septohippocampal (SH) system of basal forebrain afferents and hippocampal targets has been shown to be sensitive to prenatal ethanol exposure. Reductions in CA1 pyramidal neuronal number (Barnes and Walker, 1981; Wigal and Amsel, 1990), alterations in hippocampal mossy fiber organization (West et al., 1981) and dendritic arborization (Davies and Smith, 1981; Smith and Davies, 1990), delays in synaptogenesis (Hoff, 1988), changes in neurochemistry (Black et al., 1995; Swanson et al., 1995), and deficits in hippocampal synaptic plasticity (Sutherland et al., 1997) have all been demonstrated in the rodent SH system following exposure to ethanol in utero. Given the importance of the hippocampus in learning and memory and the role of the SH system in generating and maintaining electrical activity in the hippocampus (Dutar et al., 1995), the

possibility exists that ethanol-induced changes in this system can have detrimental effects on offspring exposed to ethanol prenatally.

The SH system is a pathway of cholinergic and GABAergic fibers originating from the medial septal (MS) nucleus and the horizontal and vertical limbs of the nucleus of the diagonal band of Broca (DBB) which synapse on pyramidal neurons, granule cells and interneurons of the hippocampus (Dutar et al., 1995; Freund and Antal, 1988). Evidence exists for an ethanol-induced alteration of the cholinergic component of the SH pathway. Severe abnormalities were noted in the basal forebrain of fetal mice following an acute ethanol dose at gestational day 7 (G7) (Sulik et al., 1984), and Schambra et al. (1990) found a reduction in the number of ChAT+ neurons in fetal mice following an acute ethanol administration on G7. Arendt et al. (1988) reported similar findings in adult rats following chronic ethanol treatment, although their experimental design suffers from lack of pair-fed controls.

Studies from our laboratory utilizing a rat model of chronic prenatal ethanol treatment have shown an ethanol-induced delay in the normal ontogeny of ChAT enzyme activity in the SH pathway, but have not revealed an effect on ChAT-immunoreactive neuronal number (Swanson et al., 1995; Swanson et al., 1996). It was a goal of the present work to determine if the other major population of neurons in the medial septum, the GABAergic neuronal population, is affected by developmental ethanol exposure. Thus, studies were performed to test the hypothesis that GABAergic neurons in the SH pathway are susceptible to chronic prenatal ethanol treatment, in order to determine whether this subset of neurons in the medial septum is affected by ethanol. To achieve this aim, an antibody which recognizes parvalbumin (PA), a calcium binding protein

commonly found in GABAergic neurons, was used and PA-immunoreactive neurons were counted.

PA and the SH system

Although the GABAergic component of the SH projection has not been as extensively characterized as the cholinergic component, it is an important part of this system. Approximately 33% of the neurons in the MS/DBB region display immunoreactivity to PA, an 11.8 kDa member of a Ca^{2+} binding superfamily of proteins (Kiss et al., 1990a; Kiss et al., 1990b; McPhalen et al., 1994) commonly found in rapidly-firing GABAergic neurons where it influences the activity of Ca^{2+}-dependent K^{+} channels (Plogmann and Celio, 1993). In rats, PA ontogeny begins in the MS at G21 and coincides with the beginning of physiological activity such as spontaneous firing and excitatory synaptic input (Lauder et al., 1986; Solbach and Celio, 1991). The PA-expressing MS neurons innervate inhibitory interneurons in the hippocampus (Freund and Antal, 1988). Although CNS regions differ in the extent to which PA and GABA co-localize (Alonso et al., 1990; Brauer et al., 1991; Kiss et al., 1990a), within the MS nucleus most, if not all, of the hippocampal-projecting GABAergic neurons are parvalbumin-immunoreactive (Freund, 1989; Krzywkowski et al., 1995). Thus, PA-immunoreactivity serves as a reliable marker for hippocampal-projecting GABAergic neurons in the MS nucleus and identifies a subpopulation of the total GABAergic neuronal pool within the basal forebrain.

PA and cingulate cortex

Quantitative analyses of neuroanatomical changes in the cerebral cortex following prenatal ethanol exposure have been conducted and have suggested ethanol-induced

neuroanatomical alterations. Specifically, alterations in the generation and proliferation of neurons have been noted (Miller, 1986; Miller, 1996). Another goal of the present work was to determine whether prenatal ethanol exposure alters the neuroanatomy of limbic cortex by testing the hypothesis that the number of GABAergic interneurons expressing PA are altered in the cingulate cortex following prenatal ethanol exposure. Although little is known about the cingulate cortex, it is clear that the cingulate is a relay center of the limbic lobe and is important for emotion and memory (Kupfermann, 1991). PA expression in the cingulate cortex begins during the first postnatal week in rats and coincides with the functional maturation of cerebral interneurons (de-Lecea et al., 1995).

The cingulate cortex was chosen to extend the earlier observations in the cerebral cortex following prenatal ethanol exposure because of the observed behavioral problems in children with FAS, including poor judgment, distractibility, and difficulty perceiving social cues (Streissguth et al., 1991). Additionally, many alcoholics who develop Korsakoff's syndrome have deficiencies in glucose utilization within the cingulate cortex, potentially contributing to learning and memory defects due to interruption of Papez' circuitry (Joyce et al., 1994). It is conceivable that alterations in PA expression patterns might contribute to behavioral anomalies and/or learning and memory deficiencies.

Postnatal Ethanol Exposure and Neuroanatomical Alterations

SH system

The SH system of basal forebrain afferents and hippocampal targets also exhibits susceptibility to ethanol during neonatal development. While numerous studies have described the effects of neonatal ethanol exposure on neurons within the hippocampus

(Bonthius and West, 1990; Bonthius and West, 1991; Greene et al., 1992; Pierce and West, 1987; West and Pierce, 1986) and have documented deficits in spatial learning following neonatal ethanol exposure (Goodlett and Peterson, 1995; Kelly et al., 1988), the effect of ethanol exposure during the brain growth spurt on the cholinergic neurons of the medial septum is unknown. A previous study from our laboratory documented an ethanol-induced delay in the normal ontogeny of ChAT enzyme activity in the SH pathway, but did not reveal an effect on ChAT-immunoreactive neuronal number following chronic prenatal ethanol treatment (Swanson et al., 1995; Swanson et al., 1996). It was a goal of the present work to determine the long-term effects of ethanol exposure on the number of ChAT+ neurons in the rat MS when ethanol is delivered during the brain growth spurt to determine whether this neuronal population is sensitive to neonatal ethanol exposure.

Cerebellum

Evidence of cerebellar vulnerability to developmental ethanol exposure comes from human studies demonstrating size reduction in the cerebellar vermis of children exposed prenatally to ethanol (Sowell et al., 1996). In rodent studies, the cerebellum displays a pattern of differential temporal susceptibility to ethanol in the brain growth spurt. This effect has been demonstrated in multiple laboratories utilizing a variety of ethanol-exposure techniques. Purkinje cell number is reduced following exposure to ethanol postnatally, during differentiation, but not following exposure to ethanol prenatally during neurogenesis (Marcussen et al., 1994). Within the postnatal period, Purkinje cells have been shown to be particularly vulnerable to ethanol in the first postnatal week (Bauer-Moffett and Altman, 1977; Bonthius and West, 1991; Goodlett

and Eilers, 1997; Pauli et al., 1995; Pierce et al., 1993). Purkinje cells are generated in the rat cerebellum between embryonic day 14 and 17; the period of Purkinje cell death in the cerebellum begins late in gestation and peaks in the first postnatal week (Cragg and Phillips, 1985). Purkinje cell susceptibility to ethanol within the cell death period has been well documented, with ethanol-accelerated Purkinje cell loss found as early as 12 hours following a postnatal day 3 ethanol insult (Cragg and Phillips, 1985).

Exposure to comparable levels of ethanol in the second postnatal week, however, has been shown to have no effect on Purkinje cells (Goodlett et al., 1997; Hamre and West, 1993; Pauli et al., 1995) or very little effect (Thomas et al., 1998). In contrast to Purkinje cells, granule cells are generated during the rat brain growth spurt (Altman, 1969). However, like Purkinje cells, granule cells display a differential pattern of susceptibility to ethanol, with loss occurring following ethanol exposure in the first postnatal week but not in the second postnatal week (Hamre and West, 1993). One aim of the present work was to determine the pattern of cerebellar neuronal susceptibility in our laboratory following first and second postnatal ethanol treatment, in order to validate the model system for use in our laboratory. The main objective, however, was to determine whether neonatal ethanol treatment altered mRNA levels of members of the bcl-2 family of cell death regulators in the cerebellar vermis (and thereby influenced the survival or death of neurons in this region). Experiments were performed in order to test the specific hypothesis that altered bcl-2 family gene expression ensues following ethanol exposure.

A role in maintaining cerebellar neurons has previously been demonstrated for the bcl-2 family. Gillardon et al. (1995) investigated bcl-2 and bax gene expression in the

cerebella of Purkinje-cell-degeneration mice (mutants that lose nearly all of their Purkinje cells between P22-28 following otherwise normal development). They found that bcl-2 mRNA levels decreased while bax mRNA levels remained unchanged beginning on P22. In addition, thyroid hormone-induced upregulation of bcl-2 protects early-differentiating cerebellar granule cells from apoptosis in vitro (Muller et al., 1995), and transgenic mice overexpressing bcl-2 contain more cerebellar Purkinje and granule cells than controls (Zanjani et al., 1996; Zanjani et al., 1997). The following section provides background on the bcl-2 family and its involvement in cell death regulation.

Programmed Cell Death (PCD) and the bcl-2 Family

PCD, a developmental form of apoptotic cell death, is a common process in the animal kingdom (Ellis et al., 1991). In the vertebrate nervous system the regulation of neuronal survival is essential for the correct formation of synapses and for the survival of the appropriate number of neurons (Oppenheim, 1991). Only the most appropriate connections are maintained, making axon-target interactions maximally efficient and simultaneously ensuring that cells which are generated in excess, develop poorly, are functionally inadequate, or are harmful, do not endure in adult organisms (Ellis et al., 1991). Competition for a limited supply of target-derived neurotrophic factors is thought to determine which neurons survive the period of naturally occurring PCD (Davies, 1994). The mechanism by which non-essential neurons are eliminated is consistent with the apoptotic form of cell death (Johnson and Deckwerth, 1993), with morphological characteristics consisting of chromatin condensation, cell shrinkage, cleavage of DNA into oligonucleosomal fragments (Edwards et al., 1991), and phagocytosis of dead cells

(Ellis et al., 1991) without induction of the inflammatory response (Columbano, 1995). Most mammalian cells constituitivly express the proteins essential for the cell death program (Davies, 1995; Raff et al., 1993).

Cytotoxicity due to ethanol in vitro has been shown to be apoptotic in fetal hypothalamic neurons (De et al., 1994) and in thymocytes (Ewald and Shao, 1993). Additionally, ethanol-induced cell death in the cerebellum appears to proceed through an apoptotic mechanism. Cerebellar neurons undergo apoptosis in vitro and in vivo in response to ethanol (Bhave and Hoffman, 1997; Liesi, 1997; Renis et al., 1996; Singh et al., 1995) and ethanol induces nuclear DNA strand breaks in the cerebellum after chronic adult exposure (Renis et al., 1996).

Almost a decade ago, a gene, bcl-2, was discovered that appeared to modulate apoptosis. The name bcl-2 is an acronym for B-cell lymphoma/leukemia-2 gene (Reed, 1994) and the identification of this gene family resulted from studies examining the t(14;18) chromosomal translocation in human follicular non-Hodgkin's B-cell lymphomas (Tsujimoto et al., 1985). The protein product encoded by the bcl-2 gene, Bcl-2, is a 25 kDa protein found predominately in mitochondrial membranes (Hockenbery et al., 1990) but it is also found in endoplasmic reticula and outer nuclear membranes (Akao et al., 1994). In recent years, a number of new genes similar to bcl-2 have been characterized and added to the diverse bcl-2 family of genes. These include bcl-xl (Boise et al., 1993), bcl-xs (Boise et al., 1993), bax (Oltvai et al., 1993), bad (Yang et al., 1995) a1 (Lin et al., 1993), mcl-1 (Kozopas et al., 1993), bak (Chittenden et al., 1995), bcl-w (Gibson et al., 1996), brag-1 (Das et al., 1996), bok (Hsu et al., 1997), and bim (O'connor et al., 1998). Some members of the bcl-2 gene family serve to inhibit cell death (e.g. bcl-

2, bcl-xl, mcl-1, a1) and others have been found to promote cell death (e.g. bcl-xs, bax, bad, bak, bok).

Bcl-2 (and the similar anti-apoptotic protein Bcl-xl) functionally blocks apoptotic death in neurons (Allsopp et al., 1993; Garcia et al., 1992) by inhibiting caspase activation (Shimizu et al., 1996), regulating mitochondrial membrane potential, proton flux across mitochondrial membranes (Shimizu et al., 1998), and by preserving mitochondrial outer membrane integrity (Vanderheiden et al., 1997). The pro-apoptotic molecules of the bcl-2 family (such as the proteins Bad and Bak) function by inhibiting the ability of the anti-apoptotic molecules of the bcl-2 family to function. They do so by preventing the necessary homodimerization of the protective molecules through direct competition for binding to the ligand binding regions of these proteins (Ottilie et al., 1997). The intracellular mechanisms of bcl-2 family function are described in detail below.

Expression patterns

Given the identification of these molecules and the fact that apoptosis is known to occur in the developing nervous system, the possibility that bcl-2 family members and their protein products might modulate PCD in the nervous system has been explored. Castren et al. (1994) have shown that bcl-2 mRNA is expressed in high levels in the prenatal rat neuroepithelium and cortical plate, with a late-prenatal peak and expression reaching lower adult levels during postnatal development. In situ hybridization also revealed that bcl-2 expression is retained in the olfactory bulb, hippocampus, pons, cerebellum, and ependymal cells of the adult rat brain (Castren et al., 1994). Bcl-2 protein is also widely expressed in the developing nervous system of mice, rhesus

monkeys, and humans, especially during embryonic development, but also during the period of PCD (Merry et al., 1994). Neuroepithelial cells of the ventricular zone, postmitotic cells of the cortical plate, Purkinje and granule cells of the cerebellum, hippocampus, and spinal cord all express Bcl-2 (Gleichmann et al., 1998; Merry et al., 1994).

Expression of bcl-xl follows a similar pattern, with mRNA levels increasing at the beginning of the PCD period. In the brain, in contrast to Bcl-2, Bcl-xl expression increases after birth in neurons of the cortex and olfactory bulb, as well as in Purkinje cells, to reach a high level in the adult brain, suggesting a role for Bcl-xl in the adult CNS (Frankowski et al., 1995; Sohma et al., 1996). Although previous reports have suggested that bcl-xs is not expressed in the rat CNS, newer studies using more sensitive techniques have detected bcl-xs mRNA in the adult rat brain (Dixon et al., 1997; Rouayrenc et al., 1995). Although the protein product of bcl-xs is able to weakly bind Bcl-2 and Bcl-xl, it appears that Bcl-xs affects apoptosis by a distinct mechanism that, unlike other family members, does not involve direct protein interactions with cell death repressor proteins (Minn et al., 1996). Both long and short forms of bcl-x are also found in cerebellar granule cells during development (Gleichmann et al., 1998).

bax is a gene in the bcl-2 family whose protein product, Bax, is a 21 kDa protein with amino acid homology (21%) with Bcl-2 (Oltvai et al., 1993). Bax forms heterodimers with Bcl-2, Bcl-xl, Mcl-1, and A1 (Sedlak et al., 1995), and Bax can counteract the protective actions of Bcl-2 when overexpressed (Oltvai et al., 1993). It has been suggested that the ratio of Bcl-2 to Bax may determine survival or death from apoptosis by serving as a sort of rheostat: when Bcl-2 is in excess, cells survive, but

when Bax is in excess, cells die (Oltvai et al., 1993). bax mRNA has been found in the developing and adult rat brain. Similarly, in the adult mouse, the Purkinje and granule cells of the cerebellum, cerebral cortical neurons, and sympathetic neurons all express Bax protein (Gleichmann et al., 1998; Krajewski et al., 1994; Oltvai et al., 1993; Vekrellis et al., 1997). While Bax protein expression is high in the neonatal cerebral cortex and cerebellum, protein levels drop off dramatically after the PCD period, suggesting that neurons regulate their sensitivity to apoptosis during development by regulating expression of Bax (Vekrellis et al., 1997).

Consistent with the rheostat hypothesis, the ratio of Bcl-2:Bax correlates with cell survival in the mature rat hippocampus following global ischemia. bax mRNA and protein are both constitutively expressed in the ischemia-sensitive CA1 hippocampal neurons, whereas Bcl-2 is not expressed in these neurons (Chen et al., 1996). In hippocampal region CA3, a population more resistant to ischemia than cells in region CA1, Bcl-2 protein expression, but not that of Bax, is high (Chen et al., 1996). Furthermore, high levels of Bax and concomitant low levels of Bcl-2 have been found in other populations of neurons that are sensitive to cell death induced by ischemia, such as Purkinje cells (Krajewski et al., 1995). Additional evidence supporting the bcl-2 rheostat hypothesis comes from studies in mouse brain that have demonstrated up-regulation of Bax and down-regulation of Bcl-2 associated with kainate-induced apoptosis (Gillardon et al., 1995), as well as studies from rat that have shown a decrease in the Bcl-2:Bax ratio in motoneurons following sciatic nerve transection (Gillardon et al., 1996) and ischemia (Isenmann et al., 1998). Additional evidence from Dixon et al. (1997) shows that the pro-apoptotic bcl-xs mRNA is upregulated shortly after global ischemia in rats. Thus, it is

clear that the balance between pro- and anti-apoptotic molecules (at the protein and mRNA levels) during development and following injury in adulthood determines the survival of the cell.

Genetically engineered mice

Studies using genetically engineered mice have been fruitful in describing a role for various members of the bcl-2 gene family in vivo. In transgenic mice overexpressing Bcl-2 protein in the nervous system, a reduction in developmental cell death of facial motoneurons and retinal ganglion cells, and a general hypertrophy of the nervous system are observed (Martinou et al., 1994). Neurons from these animals are more resistant to ischemia, neurotrophic factor withdrawal, and axotomy (Dubois-Dauphin et al., 1994; Farlie et al., 1995). Similarly, facial motoneurons from mice overexpressing bcl-xl are resistant to axotomy during the postnatal period, indicating a role for the bcl-xl gene in the survival of postnatal CNS neurons (Parsadanian et al., 1998). Studies using genetically engineered mice lacking the bcl-2 gene have been similarly instrumental in defining the normal actions of Bcl-2 in vivo. bcl-2 -/- mice live through gestation, display massive apoptosis in lymphoid organs, and produce gray hair follicles (Veis et al., 1993). The brains of these animals, however, appear grossly normal at birth although fine analyses of specific neuronal populations have not been performed. The lack of massive cell death in neonatal bcl-2 knockouts has been attributed to redundancy, as the bcl-2 family is so large (Motoyama et al., 1995). However, analysis of neuronal populations after the PCD period reveal loss relative to controls of motoneurons, sympathetic, and sensory neurons, demonstrating a role for bcl-2 in maintaining these neuronal populations (Michaelidis et al., 1996).

Mice lacking the bcl-x gene have also been generated, but die around E13. Upon examination, extensive apoptotic cell death is evident in neurons of the brain, spinal cord, and dorsal root ganglion (Motoyama et al., 1995), revealing the importance of bcl-x in embryonic life. bax-deficient mice have been generated and have helped define the actions of cell-death promoting molecules in particular neuronal populations (Deckwerth et al., 1996). Early postnatal sympathetic and facial motoneurons from bax knockouts survive growth factor deprivation and axotomy. Additionally, superior cervical ganglia and facial nuclei of bax knockouts possess more surviving neurons in vivo. Thus, a role for bax has been demonstrated in cell death associated with growth factor deprivation and axotomy (Deckwerth et al., 1996). Shindler et al. (1997) have generated mice deficient in both bcl-xl and bax. While bax deficiency does not prevent the embryonic lethality of bcl-xl deficient mice, the double knockout did demonstrate the interplay between these pro- and anti-apoptotic molecules in vivo. Specifically, bax and bcl-xl deletion produced less apoptosis in the brainstem and spinal cord when compared with bcl-xl knockouts.

It is important to note that while studies using genetically engineered mice are interesting and often quite suggestive, they suffer from several caveats. For instance, overexpression of a particular gene or the creation of a mutant lacking a particular gene may cause compensatory up- or down- regulation of other gene products which can lead to developmental alterations in attempts to compensate for the altered gene (Gerlai, 1996). Moreover, mutant mice may differ in non-targeted gene loci and certain behavioral alterations may be due to differences in genetic background (Gerlai, 1996). Thus, while suggestive of the role of particular genes, studies utilizing genetically engineered mice must be analyzed in light of these caveats.

Intracellular mechanisms

Recent evidence has shed light on the intracellular mechanism by which bcl-2 family members operate and has revealed a "double identity" for the family as both ion channel and adapter protein (Reed, 1997). Bcl-xl, Bcl-2, and Bax have all been shown to form functional ion channels in lipid membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). Also, Bax channel-forming activity is inhibited by Bcl-2 (Antonsson et al., 1997). These ion channels appear to influence cell survival by regulating the permeability of the intracellular membranes in which they are anchored. In particular, the Bcl-2 and Bcl-xl membrane complexes inhibit the release of cytochrome C from mitochondria (Yang et al., 1997), regulate mitochondrial membrane potential and proton flux across mitochondrial membranes (Shimizu et al., 1998), and help preserve mitochondrial outer membrane integrity (Vanderheiden et al., 1997). Bax channels, in contrast, are known to promote the release of cytochrome C from mitochondria (Rosse et al., 1998).

Cytochrome C is a known activator of the PCD effector molecules, the caspases (Yang et al., 1997). Permeability transition across mitochondrial membranes appears to be an early event in PCD, and is related to the activation of the release of cytochrome C and the resultant activation of the effector phase of PCD (Petit et al., 1996). This activation is brought about by cytochrome C-dependent cleavage and activation of caspases 3 (Li et al., 1997) and 9 (Zou et al., 1997). In keeping with the adapter protein role of the anti-apoptotic molecules, recent evidence shows that Bcl-xl binds to Apaf-1, the newly discovered mammalian homolog of the C. elegans CED-4 (Zou et al., 1997), and these two proteins, along with the uncleaved and inactive caspase 9, exist in a ternary

complex (Pan et al., 1998). Cytochrome C release is thought to promote activation of the caspase cascade by promoting the dissociation of this ternary complex through an undefined mechanism. Once caspases are activated, they act upon a variety of intracellular substrates, including inhibitors of caspase-activated deoxyribonuclease (ICAD). Caspase 3 is known to cleave ICAD and result in the elimination of its normally inactivating effect on caspase-activated deoxyribonuclease (CAD). CAD functions downstream in the proteolysis cascade and its reduced inhibition results in DNA degradation characteristic of apoptotic cell death (Sakahira et al., 1998).

Another piece to this intriguing puzzle has been provided by Yang et al. (1998), who have shown that CED-4 (and likely the mammalian counterpart Apaf-1) promotes CED-3 (the C. elegans caspase) processing and activation by promoting the aggregation of unprocessed CED-3. This "induced proximity" is thought to sequester inactive caspase proenzymes to increase their local concentration and promote conformational changes which will increase the likelihood of their activation (Hengartner, 1998), and is brought about by oligomerization of the CED-4:CED-3 complex (Yang et al., 1998). Thus, an important role of Bcl-xl and Bcl-2 appears to be to prevent the association of these proenzymes by preventing CED-4 (and by association, Apaf-1) oligomerization (Hengartner, 1998). While a similar role for Apaf-1 has not been definitively demonstrated, this will surely be one of the important future discoveries in this blossoming field.

Newly uncovered evidence also indicates that the anti-apoptotic molecules of the bcl-2 family can contribute to the cell's demise under certain intracellular conditions. For example, if caspase activation proceeds to a critical point, caspases will act on the Bcl-2

and Bcl-xl proteins as substrates (Cheng et al., 1997; Clem et al., 1998). Indeed, caspase cleavage of these protective molecules converts them into Bax-like death-promoting molecules. This cleavage is thought to act as a feed-forward mechanism for further caspase activation, and should ensure cell death.

Besides these important discoveries, several others have demonstrated that apoptosis interacts with signal transduction intracellularly and that this interaction is mediated, in part, by the bcl-2 family (Gajewski and Thompson, 1996). The application of IL-3 to the FL5.12 cell line leads to phosphorylation and inactivation of the pro-apoptotic molecule Bad. The phosphorylation of Bad leads to its association with the cytosolic protein 14-3-3, prevents Bad:Bcl-xl heterodimerization, and promotes Bcl-xl homodimerization, which results in cell survival (Zha et al., 1996). Additionally, Wang et al. (1996) showed that Bcl-2 could target Raf-1 to mitochondrial membranes which results in the phosphorylation of Bad.

Two groups independently determined that the protein kinase Akt is also responsible for growth factor-mediated Bad phosphorylation (Datta et al., 1997; Delpeso et al., 1997). Akt (also known as protein kinase B, or PKB) is an important intracellular molecule and its phosphorylation (and activation) is brought about by growth factor-mediated phosphorylation of PI3 kinase, followed by PI3 kinase phosphorylation of Akt (Kahn, 1998; Zhou et al., 1997). Thus, it is apparent that a complex interaction between extracellular growth and survival signals integrates with the complex intracellular biology of individual neurons to determine whether a particular cell will survive a developmental process or injury.

Hypotheses Tested

As mentioned previously, neuronal populations are not uniform in their vulnerability to ethanol, nor are developmental stages uniform in susceptibility to ethanol treatment. And while a variety of CNS populations have been demonstrated to be adversely affected by prenatal ethanol exposure, the full extent of nervous system vulnerability to ethanol is unknown. Likewise, the cellular effects of ethanol exposure, such as changes in gene expression, are poorly understood. It is with this in mind that the research reported in this document was undertaken.

Because of the observed learning and memory deficits in children exposed in utero to ethanol (Streissguth et al., 1991), the SH system is a natural region in which to extend studies in rodents of neuroanatomy following ethanol treatment. While cell loss in the hippocampus has been documented following prenatal exposure (Barnes and Walker, 1981), cellular changes in the cholinergic basal forebrain component of the SH system have not been found after a similar pattern of exposure (Swanson et al., 1996). Thus, it was an aim of the present work to determine whether the GABAergic component of the SH pathway is susceptible to prenatal ethanol treatment. Additionally, the cingulate cortex was analyzed because of the observed behavioral problems in children with FAS (Streissguth et al., 1991) and the decreased glucose utilization in the cingulate cortex of alcoholics (Joyce et al., 1994). *Thus, the following hypothesis was explored: chronic prenatal ethanol exposure will lead to alterations in the number of neurons expressing PA in the adult rat medial septum and cingulate cortex.*

Rat studies have also identified the early postnatal period as a developmental time during which particular CNS structures are sensitive to ethanol's toxic effects. For example, the Purkinje cells of the cerebellar cortex are reduced in number when ethanol is delivered during the early neonatal period, but not when ethanol is given in utero (Marcussen et al., 1994). The SH system is a region which exhibits susceptibility to ethanol during both the prenatal and the neonatal periods. Neonatal ethanol exposure disrupts the normal development of the SH system in rodent models of FAS, and much research has documented neuroanatomical changes in the hippocampus (Bonthius and West, 1990; Bonthius and West, 1991; Greene et al., 1992; Pierce and West, 1987; West and Pierce, 1986). However, the effect of neonatal ethanol exposure on the MS has not been investigated. Because the number of cholinergic neurons of the medial septum are not altered by prenatal ethanol treatment, it was an objective of the present work to determine the long-term effects of neonatal ethanol exposure on the cholinergic neurons in the rat. *The following hypothesis was investigated: early postnatal ethanol exposure will lead to alterations in the number of neurons expressing ChAT in the adult rat CNS.*

While descriptions of susceptible neuronal populations following developmental ethanol exposure has been, and continues to be, a fruitful avenue of research, further investigation into the molecular consequences of ethanol treatment (e.g. gene expression changes resulting from ethanol) are warranted. Given the previously described temporal pattern of Purkinje and granule cell loss in the postnatal cerebellum, a survey of the bcl-2 literature raises the intriguing possibility that the differential temporal teratogenicity of ethanol on cerebellar cells may be related to changes in the levels of expression of PCD repressor and inducer genes. It is significant that proteins translated from bcl-2 and bax

mRNA dimerize, and as noted above, cell death or survival depends on the relative amounts (ratio) of these proteins (Oltvai et al., 1993). Similar cellular survival outcomes have been linked to the relative ratio of bcl-2 to bax gene expression (Basile et al., 1997; Chen et al., 1996).

The observation that cerebellar neurons are susceptible to ethanol neurotoxicity as a function of the timing of the ethanol insult suggests that ethanol may act in vivo to modulate (upregulate or downregulate) the expression of certain mRNAs of the bcl-2 family and thereby alter susceptibility to ethanol neurotoxicity. Thus, any ethanol-induced change in bcl-2 family gene expression might disrupt the normal balance of these proteins in developing neurons and decrease or increase their chance of cell death. Recent evidence, mentioned above, in addition to the aforementioned expression patterns, implicates bcl-2 family members in maintaining cerebellar neurons. Purkinje-cell-degeneration mouse mutants lose nearly all of their Purkinje cells between P22-28. Gillardon et al. (1995) investigated bcl-2 and bax expression in the cerebella of these mice and found that bcl-2 mRNA levels decreased while bax mRNA levels remained unchanged beginning on P22. There was a concomitant reduction of Bcl-2 expressing Purkinje cells in the mutants compared with wild-types, suggesting a down-regulation of bcl-2 in Purkinje cells destined to die (Gillardon et al., 1995).

In addition, thyroid hormone-induced upregulation of bcl-2 protects early-differentiating cerebellar granule cells from apoptosis in vitro (Muller et al., 1995). Moreover, transgenic mice overexpressing bcl-2 contain more cerebellar Purkinje and granule cells than controls whether transgene expression was induced in the embryonic or postnatal periods (Zanjani et al., 1996; Zanjani et al., 1997). *Therefore, another goal of*

the present work was to test following hypothesis: ethanol-induced alterations in the expression levels of bcl-2 family PCD genes in the cerebellum contribute to the cerebellum's relative temporal susceptibility to ethanol neurotoxicity.

The chapters that follow provide a detailed description of methods utilized to test these hypotheses, and the data collected are presented and fully discussed. Following these chapters is a concluding chapter that presents interpretations derived from these data and discusses specific methodological considerations and future directions which the current data suggest.

CHAPTER 2
EFFECTS OF PRENATAL ETHANOL EXPOSURE ON PARVALBUMIN-IMMUNOREACTIVE GABAERGIC NEURONS IN THE ADULT RAT MEDIAL SEPTUM AND ANTERIOR CINGULATE CORTEX

Summary

Exposure of human fetuses to ethanol often results in the fetal alcohol syndrome (FAS). Animal models of FAS have been developed and have been utilized to examine the consequences of prenatal ethanol exposure on the central nervous system. While cell loss in the hippocampus has been documented following prenatal ethanol exposure (Barnes and Walker, 1981), cellular changes in the cholinergic basal forebrain component of the septohippocampal (SH) system have not been found after a similar pattern of ethanol exposure (Swanson et al., 1996). The objective of this study was to determine the long-term effects of prenatal ethanol exposure on parvalbumin-expressing (PA+) GABAergic neurons of the rat medial septum and anterior cingulate cortex.

Pregnant Long-Evans rats were maintained on one of three diets throughout gestation: an ethanol-containing liquid diet in which ethanol accounted for 35% of the total calories, a similar diet with the isocaloric substitution of sucrose for ethanol, or a lab chow control diet. Offspring were sacrificed at postnatal-day 60 and their brains were prepared for parvalbumin immunocytochemistry. Female rats exposed to the ethanol-containing diet during gestation had 42 % fewer total PA+ neurons in the medial septum and reduced PA+ cell density when compared to female rats exposed to the sucrose diet.

Ethanol-exposed females also had fewer PA+ neurons per section than sucrose-control females. Male rats exposed to ethanol did not display a similar change in PA+ neurons or density. No effect of prenatal diet was found on the area or volume of the medial septum, or the size of the PA+ neurons therein. As such, prenatal exposure to ethanol appears to permanently reduce the number of PA+ neurons in the female rat medial septum without affecting the size of the structure or the size of the neurons.

Due to the observed behavioral problems in children with FAS (Streissguth et al., 1991) and the fact that alcoholics show decreased glucose utilization in the cingulate cortex (Joyce et al., 1994), the effect of prenatal ethanol exposure on the number of PA+ GABAergic neurons in the adult rat anterior cingulate cortex was also determined. This represents the first attempt to document developmental alterations in the cingulate cortex following ethanol exposure. Rats exposed to the ethanol-containing diet contained 45% fewer total PA+ neurons in the anterior cingulate cortex, and fewer PA+ neurons per section compared with sucrose and chow controls. No gender differences were found in measures of the anterior cingulate. The reduction in PA+ neurons occurred in the absence of changes in structure area or volume, and occurred in the absence of changes in PA+ neuronal size. Functional implications and possible relations to the fetal alcohol syndrome are discussed.

Introduction

Exposure of human fetuses to ethanol often results in a constellation of developmental anomalies which make up FAS. Such developmental perturbations include pre- and postnatal growth deficiencies, morphological (e.g. craniofacial)

abnormalities and central nervous system (CNS) deficits (Abel, 1995) and these typically persist into adulthood (Streissguth et al., 1991). The incidence of FAS in the general obstetric population of the United States is estimated at 1.95 per 1,000 live births (Abel and Hannigan, 1995) and FAS is thought to be the leading nongenetic cause of mental retardation (Abel and Sokol, 1986). Experimental studies, utilizing rodent models of FAS, have been successful in reproducing many of the behavioral and morphological changes found in FAS, and have described numerous CNS alterations resulting from in utero ethanol exposure. Alterations in CNS development include changes in neuronal proliferation and migration (Miller, 1986; Miller, 1995b; Miller, 1996), altered neuronal and cortical morphology (Burrows et al., 1995; Davies and Smith, 1981; Kotkoskie and Norton, 1989), changes in receptor density and enzyme ontogeny (Black et al., 1995; Swanson et al., 1995), delays in synaptogenesis (Hoff, 1988), alterations in neuronal number (Barnes and Walker, 1981), and long-lasting deficits in learning (Clausing et al., 1995) and behavior (Riley, 1990). The present study sought to determine the vulnerability of GABAergic interneurons expressing parvalbumin in the medial septum and anterior cingulate cortex following prenatal ethanol exposure.

Medial Septum

Numerous studies investigating the effects of prenatal ethanol exposure on rodents have determined that the CNS is not uniform in its susceptibility to ethanol. In addition to differential temporal susceptibility, the CNS exhibits differential regional vulnerability. The SH system of basal forebrain afferents and hippocampal targets is one region that has been shown to be sensitive to developmental ethanol exposure. Permanent reductions of CA1 pyramidal neurons (Barnes and Walker, 1981; Wigal and Amsel, 1990), alterations

in hippocampal neuronal circuitry (West et al., 1981) and dendritic arborization (Davies and Smith, 1981; Smith and Davies, 1990), delayed synaptogenesis in the hippocampal dentate gyrus (Hoff, 1988), changes in hippocampal muscarinic receptors (Black et al., 1995), and delayed choline acetyltransferase (ChAT) enzyme ontogeny in the basal forebrain (Swanson et al., 1995) have all been demonstrated in the rodent SH system following exposure to ethanol in utero. Because of the SH system's role in generating and maintaining electrical activity in the hippocampus (Dutar et al., 1995), and the importance of the hippocampus in learning and memory, the possibility exists that ethanol-induced changes in this system can have detrimental effects on offspring exposed to ethanol prenatally.

Interneurons, pyramidal neurons, and granule cells of the hippocampus all receive synapses from cholinergic and GABAergic fibers originating in the medial septum (MS) and the horizontal and vertical limbs of the nucleus of the diagonal band of Broca (DBB) (Dutar et al., 1995; Freund and Antal, 1988). Evidence exists for an ethanol-induced alteration of the cholinergic component of the SH pathway. Sulik et al. (1984), for example, noted severe abnormalities in the basal forebrain of fetal mice following an acute ethanol dose at gestational day 7 (G7). Moreover, Schambra et al. (1990) found a reduction in the number of ChAT-immunoreactive neurons in fetal mice following an acute ethanol administration on G7. Similar findings in adult rats were reported following chronic ethanol treatment (Arendt et al., 1988).

In contrast, studies from our laboratory utilizing a rat model of chronic prenatal ethanol treatment (CPET) have shown an ethanol-induced disruption of the normal ChAT ontogeny in the SH pathway, but have not revealed an effect on ChAT-immunoreactive

neuronal number (Swanson et al., 1995; Swanson et al., 1996). The present study sought to determine whether the GABAergic component of the SH pathway was susceptible to CPET.

Although not as extensively characterized as the cholinergic component, the GABAergic component of the SH projection is an important part of this system. Parvalbumin (PA), an 11.8 kDa member of a Ca^{2+} binding superfamily of proteins (Kiss et al., 1990a; Kiss et al., 1990b; McPhalen et al., 1994), is expressed in approximately 33% of the neurons in the MS/DBB region. PA is commonly found in fast-firing GABAergic neurons where it influences the activity of Ca^{2+}-dependent K^{+} channels (Plogmann and Celio, 1993). PA ontogeny begins in the MS of rats at G21 and coincides with the beginning of physiological activity such as spontaneous firing and excitatory synaptic input (Lauder et al., 1986; Solbach and Celio, 1991), and the PA-expressing MS neurons innervate inhibitory interneurons in the hippocampus (Freund and Antal, 1988). CNS regions differ in the extent to which PA and GABA co-localize (Alonso et al., 1990; Brauer et al., 1991; Kiss et al., 1990a), but within the MS nucleus most, if not all, of the hippocampal-projecting GABAergic neurons are PA+ (Freund, 1989; Krzywkowski et al., 1995). The present study sought to determine the long-term effects of prenatal exposure to ethanol on a PA+ subpopulation of GABAergic projection neurons in the SH pathway. For this determination we performed counts of PA+ neurons in the adult rat MS nucleus following CPET.

Cingulate Cortex

The present study also sought to determine the vulnerability of GABAergic interneurons expressing parvalbumin in the anterior cingulate cortex following CPET.

The ontogeny of PA mRNA and protein in the rat cingulate cortex, and its functional role, is similar to that noted previously for the medial septum. PA expression begins in the first postnatal week, and coincides with the functional maturation of cerebral interneurons in the cingulate (Alcantara et al., 1993; de-Lecea et al., 1995). The cingulate cortex was chosen for analysis because it is a major relay center of the limbic lobe and is involved in motor control, attention, emotion, and memory (Kupfermann, 1991; Muir et al., 1996; Paus et al., 1993; Picard and Strick, 1996). The well documented cognitive and behavioral impairments in children with FAS, including poor judgment, distractibility, and hyperkinetic and emotional disorders (Steinhausen et al., 1993; Streissguth et al., 1991), led us to investigate whether anatomical alterations in the cingulate cortex may underlie these behavioral defects. Thus, the current study also sought to determine the long-term effects of prenatal exposure to ethanol on PA+ interneurons of the cingulate cortex.

Materials and Methods

Subjects and ethanol treatment

Long-Evans hooded rats, purchased from Charles River Co. (Portage, MI), were housed individually, or in pairs, under controlled temperature and humidity conditions, and were maintained on a 07:00-19:00 hour light cycle. Nulliparous females were placed individually with a male overnight until vaginal smear the following morning was indicative of insemination. This was defined as G0. At this time, females were matched according to age and weight and assigned to one of three treatment groups from G0-G21: ethanol, sucrose, or chow. During this time the ethanol group was given free access to an

ethanol-containing liquid diet in which ethanol comprised 35% of the total calories. The sucrose group was pair-fed the same volume of a similar liquid diet which lacked ethanol and contained an isocaloric substitution of sucrose for ethanol. The liquid diet was prepared by mixing a stock ethanol or sucrose solution with Sustacal (Mead Johnson). Diets were additionally enriched with Vitamin Diet Fortification Mixture and Salt Mixture (ICN Nutritional Biochemicals). The diets contained 1.3 kcal/ml and have been shown to provide several times the daily requirement of all essential vitamins and nutrients (Walker and Freund, 1971). The chow group was given Purina Rodent Chow and water ad libitum and served as a control for non-specific effects of the liquid diet.

Upon birth (postnatal-day 0 [P0]) pups from an ethanol or sucrose dam were fostered to chow dams which had given birth at the same time. Litters were then randomly culled to ten pups, with approximately equal numbers of males and females. Pups were weaned at P21 and individually housed until perfusion at P60. In order to avoid litter bias, animals used in this study are representatives from at least eight different litters, and no more than one individual from each gender was used from a single litter. A total of 39 animals was used in this study, with approximately equal numbers of males and females in each diet treatment group (Ethanol, N=13; Sucrose, N=12; Chow, N=14).

Immunocytochemical procedures

Animals were randomly selected for immunocytochemical staining at P60, an age which represents adulthood in rats. This age was selected in order to examine the long term effects of chronic prenatal ethanol treatment on parvalbumin expressing neurons in the medial septum and cingulate cortex and corresponds to an age used in previous analyses of prenatal ethanol influences on cholinergic neurons in the medial septum

(Swanson et al., 1996). At P60, animals were euthanized by pentobarbital overdose prior to transcardial perfusion with phosphate buffered saline (PBS; 0.1 M, with 0.9% sodium chloride) followed by 10% formalin (in the same PBS). Brains were removed and equilibrated overnight in a cryoprotectant solution (PBS with 30% sucrose and 15% ethylene glycol) and frozen at -70°C until processing.

Animals of each gender from each diet group were processed for immunocytochemistry at a given time. This insured against staining differences between groups resulting from slight procedural differences. Brains were thawed and equilibrated in a 30% sucrose-PBS solution, and mounted on the frozen stage of a sliding microtome. Serial coronal sections were cut throughout the basal forebrain at a thickness of 40 μm. Free-floating sections were immunostained for PA using a monoclonal antibody (Accurate # 6092). Immunoreactivity was visualized as a blue-black reaction product using an avidin-biotin conjugate/nickel intensified staining (see below).

Primary incubation with the monoclonal PA antibody (1:2500 in PBS, 0.1% normal goat serum [NGS], 0.1% Triton X-100, and 0.005% sodium azide) was carried out at 4°C overnight. Sections were then washed with PBS and incubated with biotinylated anti-mouse IgG (Sigma, B0529; 1:10000 in PBS, 0.1% NGS, 0.1% Triton X-100, and 0.005% sodium azide) overnight at 4°C. Sections were then washed and incubated with Extravidin-horseradish peroxidase conjugate (Sigma; 1:1000 in PBS) overnight at 4°C. Sections were washed with 0.1 M sodium acetate (pH 7.2) to eliminate phosphate which can precipitate divalent cations. Sections were then reacted for 3 minutes at room temperature using a developing buffer with 0.8 M sodium acetate, 8 mM imidazole, 0.5% nickel (II) sulfate, 0.04% 3,3 diaminobenzidine tetrahydrochloride, and

0.005% hydrogen peroxide. Following development, sections were again washed in sodium acetate buffer, mounted onto slides in PBS, air dried, dehydrated and coverslipped. Control sections omitting the primary antibody were routinely developed to ensure that any observed staining was due to PA. Slides from animals of each gender from the three groups were randomized and coded such that all subsequent analyses were carried out blind.

MS cell count, area, and volume analysis

PA+ cell counts were conducted bilaterally on alternate sections throughout the entire rostral-caudal extent of the MS nucleus. The packing density of the DBB nucleus was too great for accurate cell counts to be made by the image software. Thus, for the purposes of this study the MS alone was examined and was defined rostrally by the ventral fusion of the hemispheres (at the level of the genu of the corpus callosum) and caudally by the decussation of the anterior commissure. The mean number of PA+ neurons per section was derived. Recent data have demonstrated the utility of manual cell counts (Clarke and Oppenheim, 1995) and show a direct correlation between cell counts performed manually and cell counts performed with the optical dissector (Hagg et al., 1997).

Images of PA immunostained sections were captured and digitized using a RasterOps 24STV video capture board and software on a Macintosh IIvx computer. Low magnification images (2.5x objective; effective scale 1.59 μm/pixel) were captured in order to obtain the entire MS region in one image. When capturing each image, the lighting and contrast enhancement were optimized for identification of individual cells. Images were digitally processed using the image analysis program NIH Image (freeware

from NIH). To reduce background variation across the image, a digitally defocused image was created by passing the primary image through a mean filter. The resultant image was then subtracted from the primary digitized image. This processed image was then passed through a Laplace filter to enhance edges and separation between cells.

Analysis of individual images was initiated by outlining the region of the MS nucleus. The area of a section outlined varied depending on the rostral-caudal location of the individual section. The MS nucleus in rostral sections was defined as the mediodorsal group of neurons which were separated from the ventrally-located DBB nucleus. In intermediate sections, where the demarcation between the MS and DBB nuclei is ambiguous, the MS was defined ventrally by a line perpendicular to midline at the level of the anterior commissure. In caudal sections, the MS was defined as the medially located cells dorsal to the anterior commissure. Cells were highlighted interactively by adjusting the grayscale threshold level to include only objects which were considered cells. A previous study from our lab has demonstrated that there is a close correlation (r^2= 0.956) between computer-automated and manual counts performed with a microscope and a drawing tube (Swanson et al., 1996). Given the thickness of each section (40 μm), the fact that alternate sections were analyzed, and the fact that PA+ neurons in the MS nucleus range from 6-26 μm in diameter (see below), it was not deemed necessary to perform a split-cell correction on these counts. The program counted the highlighted objects and measured the area of the outlined region (mm^2). Tissue volume (mm^3) was calculated using a modification of the Cavelieri method (Michel and Cruz, 1988). These measurements were taken in order to determine whether ethanol treatment changed the size of the structure of interest, and whether changes in the

number of PA+ neurons per section were due to concomitant changes in the size of the area examined (Peterson et al., 1997). This was done by multiplying the number of 40 μm sections analyzed for a given animal by the mean area per section for that animal and section thickness.

Anterior cingulate cell count, area, and volume analysis

The packing density of PA+ neurons in the anterior cingulate cortex was too great for accurate cell counts to be made by automated imaging software at the magnification necessary to include the entire region. Therefore, manual counts of PA+ neurons were performed on every sixth section through the left side from the genu of the corpus callosum caudally until decussation of the anterior commissure. The anterior cingulate gyrus was bordered laterally by the cingulum, and ventrally by the corpus callosum (Paxinos and Watson, 1982). The mean number of PA+ neurons per section was derived through manual counting of neurons at 400x.

Because our cell count data are expressed as the mean number of cells per section, it is necessary to demonstrate that any noted change in cells per section is not due to changes in the volume of the structure being examined (Peterson et al., 1997). Therefore, the volume of the anterior cingulate gyrus was calculated by a modification of the Cavaleri method (Michel and Cruz, 1988) to ensure that changes in mean number of PA+ neurons per section as a result of ethanol treatment were not due to changes in the size of the gyrus. For the volume determination, the mean area of the anterior cingulate gyrus was determined for each animal by measuring the distance from midline to the cingulum and the distance from the corpus callosum to the dorsal brain surface at 25x on three anatomically matched sections with an eyepiece micrometer. These distances were

multiplied and mean area per section was calculated. This area was then multiplied by the number of sections through the anterior cingulate gyrus and the section thickness to determine mean cingulate gyrus volume.

Other analyses

The blood ethanol concentration (BEC) in the ethanol group was determined between 00:00 and 02:00 hours on G18 utilizing the Sigma 333-UV kit. PA+ cell diameters were determined at 400x utilizing an eyepiece micrometer. Thirty cells from identical anatomical locations (in the respective structures) for each gender in all groups were measured. Density of PA+ neurons was determined by dividing the mean number of cells per section by the mean area (mm^2) per section for the respective structures.

Statistical analysis

Statistical analysis was performed with the program StatView (Abacus Concepts, Berkeley Ca). For each parameter examined, one-way Analysis of Variance (ANOVA) was used to test for the main effects of diet and gender separately. When appropriate, the data were further analyzed with the Fisher's Protected Least Significant Difference (PLSD) post hoc test to determine individual group differences. Because gender differences were noted for MS measures (following an initial two-way ANOVA for effects of diet and gender), males and females were analyzed separately. No gender differences were noted in cingulate measures (following an initial two-way ANOVA for effects of diet and gender), and so these data were not split by gender for analysis, and one-way ANOVA was used to test for effects of diet, followed by the PLSD when appropriate.

Results

BEC, body and brain weight, and brain to body weight ratio measures

BECs in the ethanol group ranged from 150-175 mg/dl (mean = 161 $\pm$ 18 mg/dl). This BEC represents a peak or near-peak level, as the nocturnal rats consumed the majority of the diet after the twelve hour light cycle and blood samples were taken between 00:00 and 02:00 hours on G18. P60 body weights were taken prior to perfusion and P60 brain weights were taken following perfusion and are presented in table 2-1, along with brain to body weight ratios (all tables are located at the end of the chapter). The ANOVA for P60 body weight revealed no significant effects of diet on P60 body weight of males or females. A gender difference was noted in P60 body weight for ethanol [$F(1,11) = 24.7$; $p < 0.0001$], sucrose [$F(1,10) = 31.4$; $p < 0.0001$], and chow [$F(1,12) = 39.6$; $p < 0.0001$] animals which was expected given the larger size of males at P60.

The ANOVA for P60 brain weight demonstrated no effect of diet treatment in males or in females. Likewise, no significant gender differences were noted for brain weight within ethanol, sucrose, or chow animals.

The ANOVA for brain to body weight ratio showed no effect of diet in males or females. Gender differences were noted in P60 brain to body weight ratio for ethanol [$F(1,11) = 37.5$; $p < 0.0001$], sucrose [$F(1,10) = 41.2$; $p < 0.0001$], and chow [$F(1,12) = 38.5$; $p < 0.0001$] animals, which was not surprising given the larger size of male animals at this age.

Number of sections, MS area per section, and PA+ neuronal density in the adult rat medial septum

Table 2-2 presents the number of sections, MS area per section, and PA+ neuronal density of the P60 MS. The number of alternate 40 μm sections through the MS was determined by counting the number of sections analyzed between the ventral fusion of the hemispheres (at the level of the genu of the corpus callosum) and the anterior commissure. The ANOVA showed no effect of diet for either males or females on the mean number of alternate sections through the MS, nor were there gender differences for ethanol or chow animals. The sucrose group, however, did show a gender effect on the mean number of sections with females containing significantly fewer than males ($F[1,10]= 4.511$; $p< 0.05$).

The area of the MS outlined on a given section was computed by the image software. No significant differences were noted between any group for males or females in mean area per section nor were there gender differences for ethanol, sucrose, or chow animals. Density of PA+ neurons (mean number of cells per section/mean area [mm^2] per section) was also calculated, and the ANOVA showed an effect of diet on cell density in females ($F[2,16]= 7.347$; $p< 0.01$) but not in males. The PLSD post-hoc test determined that cell density in ethanol females was significantly reduced compared to that of sucrose females ($p< 0.01$). Neuronal density in chow females was also significantly reduced compared to sucrose females ($p< 0.05$). The greater density of PA+ neurons noted in sucrose females is perhaps due to a non-specific effect of liquid diet (see below for discussion). A gender difference in the ethanol group was also noted for neuronal density: neuronal density of females was reduced from that of males ($F[1,11]= 5.075$; $p<$

0.05). This difference is best described by the observed reduction in total PA+ cell number, and not by differences in MS area. No gender differences in neuronal density were noted for the sucrose or chow groups.

Total PA+ neurons in the MS

Figure 2-1 (all figures are located at the end of chapter) presents the mean total number of PA+ cells counted on alternate sections throughout the P60 MS for animals of both genders from each diet group. The ANOVA for total number of PA+ cells in the MS determined a significant effect of diet treatment in females ($F[2,16]= 4.351$; $p< 0.05$) but not in males. The PLSD post-hoc test further revealed that ethanol females had 42 % fewer total PA+ cells than sucrose females ($p< 0.01$). A qualitative reduction in cell number in the MS of ethanol females as compared to sucrose females can be observed in Figure 2-2. Ethanol females were not different from chow females, nor were chow females different from sucrose females. Gender differences were noted in the ethanol group and the ANOVA for total number of PA+ cells in the MS indicated a reduction in the number of PA+ cells in ethanol females compared to ethanol males ($F[1,11]= 8.421$; $p< 0.05$). Gender differences were not noted in sucrose or chow groups.

Number of PA+ neurons per section in the MS

Figure 2-3 displays the mean number of PA+ neurons detected per section for animals of both genders from each group in the P60 MS. The ANOVA for mean number of cells per section revealed a significant effect of diet treatment in females ($F[2,16]= 7.342$; $p< 0.01$) but not males. The PLSD post-hoc test further determined that ethanol females had 44 % fewer PA+ cells per section than sucrose females ($p< 0.01$). Chow females also displayed a significant reduction in mean PA+ cells per section compared

with sucrose females ($p< 0.05$), while ethanol and chow females were not different from each other. The difference between ethanol females and sucrose females is due to a reduction in total cell number and not to a difference in tissue volume. The difference noted between chow females and sucrose females is perhaps due to a non-specific effect of liquid diet and may be related to the aforementioned increase in PA+ neuronal density noted in sucrose females (see below for discussion). Gender differences were also noted. The ANOVA showed a reduced mean number of PA+ cells per section in ethanol females, compared to ethanol males ($F[1,11]= 5.063$; $p< 0.05$). This difference was again due to a reduction in total PA+ cell number, and not to a difference in volume. No gender differences were noted for the sucrose or chow groups.

Diameter of PA+ MS neurons

PA+ cell diameters were measured for both genders in all groups and are presented in figure 2-4. No differences were noted between any treatment group for males or females; nor were gender differences noted for the ethanol, sucrose, or chow groups.

MS volume determination

Figure 2-5 presents MS volume (mm^3) for the analyzed sections. Volume was calculated by multiplying the number of 40 μm sections analyzed for a given animal by the mean area per section for each animal and the section thickness. The resulting volume was then compared across groups for each gender and between both genders for each diet treatment. No significant differences were found between diet groups for males or females. Gender specific effects of tissue volume, however, were noted in sucrose animals. The ANOVA showed a significant reduction of tissue volume in the sucrose

females compared to sucrose males ($F[1,10]= 6.102$; $p< 0.05$). This was likely due to a decrease in the number of sections for female sucrose animals compared to male sucrose animals (see table 2-2; $p< 0.05$). No gender differences in MS volume were noted for the ethanol or chow groups.

Number of sections, mean area per section, and PA+ neuronal density in the adult rat anterior cingulate cortex

Table 2-3 presents the number of sections, mean area per section, and PA+ neuronal density in the adult rat cingulate cortex. The number of 40 μm sections through the anterior cingulate gyrus was determined by counting the number of sections between the genu of the corpus callosum and the decussation of the anterior commissure. The ANOVA showed no effect of diet on the mean number of sections through this region.

The area of the gyrus on a given section was determined by measuring the distance from midline to the cingulum and the distance from the corpus callosum to the dorsal brain surface at a magnification of 25x on three anatomically matched sections with an eyepiece micrometer. No significant differences were noted in mean area per section. The Density of PA+ neurons in the anterior cingulate (mean number of cells per section/mean area [mm^2] per section) was also calculated, and the ANOVA showed an effect of diet on cell density ($F[2,30]= 7.041$; $p< 0.01$). The PLSD post-hoc test revealed that cell density in the ethanol group was significantly reduced compared to that of the sucrose ($p< 0.01$) and chow groups ($p< 0.01$).

Total PA+ neurons in anterior cingulate

Figure 2-6 presents the total number of PA+ neurons counted throughout the anterior cingulate cortex. The ANOVA for total number of PA+ neurons in the anterior

cingulate showed a significant effect of diet treatment ($F[2,30]= 12.314$; $p< 0.0001$). The PLSD post hoc test further revealed that ethanol animals had 45% fewer PA+ neurons than sucrose ($p< 0.0001$) or chow ($p< 0.0001$). Figure 2-7 demonstrates a qualitative reduction of PA+ neurons in the anterior cingulate cortex of adult rats exposed to ethanol prenatally when compared with sucrose controls.

Number of PA+ neurons per section in the anterior cingulate

Figure 2-8 presents the mean number of PA+ neurons per section in the anterior cingulate. The ANOVA revealed a significant effect of diet treatment on the mean number of PA+ neurons per section ($F[2,30]= 12.80$; $p< 0.0001$). The PLSD further determined that ethanol treatment reduced the mean number of PA+ neurons per section when compared with sucrose ($p< 0.001$) and chow ($p< 0.0001$) controls.

PA+ neuronal diameter in the anterior cingulate cortex

PA+ cell diameters were determined with an eyepiece micrometer at 400x. Ten whole cells from identical anatomical location for each gender in all groups were measured on three sections for each brain region. The mean PA+ neuronal diameter for each group was determined and is presented in figure 2-9. No significant differences in the mean PA+ neuronal diameter were noted.

Anterior cingulate gyrus volume

The mean gyrus volume for each group was determined, and is presented in figure 2-10. No significant effect of treatment on mean volume was noted.

Discussion

The long-term effects of chronic prenatal ethanol exposure on PA+ neurons were examined in the medial septum and anterior cingulate cortex of adult rats. This pattern of exposure produced no significant alterations in P60 body weight (although males were larger than females for all groups examined). Similarly, no significant differences were noted in P60 brain weight, or brain to body weight ratio as a function of ethanol treatment (although brain to body weight ratios were higher in females due to the larger size of males at P60). The lack of effect of chronic prenatal ethanol treatment (CPET) on the long-term growth of these animals is consistent with other reports from our laboratory (Swanson et al., 1995; Swanson et al., 1996). This exposure paradigm did, however, produce significant alterations in PA+ neurons in both regions examined, although a sexually dimorphic effect was noted for the medial septum. The results for each brain region are discussed separately below.

Medial Septum

A major conclusion drawn by the current study is that CPET affects the MS in a sexually dimorphic manner. This study has demonstrated anatomical changes in the expression of PA in the female rat MS following prenatal ethanol exposure. The evidence from this study suggests that a reduction in the number of MS neurons expressing PA occurs in the absence of area, volume, or PA+ cell size effects. Whether this reduction is due to lower PA levels in existing cells or to a loss of PA+ neurons is impossible to conclude. Regardless, the data suggest that alterations in PA expression

occur in female rats following CPET and that PA expression in female rats may be particularly susceptible to the long-term consequences of CPET.

Although male offspring exposed to ethanol in utero were unaffected as adults, a number of differences were noted in female ethanol-treated animals when compared to females in the sucrose group. The mean total number of PA+ neurons in the MS nucleus of adult females was reduced by 42 % following prenatal exposure to ethanol, PA+ neuronal density was reduced in ethanol females, and ethanol females had fewer PA+ neurons per section when compared to sucrose females. There were also gender differences in the ethanol groups for a number of measures. Female rats exposed to ethanol in utero contained fewer total PA+ neurons, reduced density of MS PA+ neurons, and fewer PA+ neurons per section when compared to males. Gender differences in MS volume in the sucrose group were also noted, with the MS of females significantly reduced compared to that of sucrose males. As mentioned previously, this difference was due to a lower number of 40 μm sections from the MS of female sucrose animals when compared to sucrose males. This is not surprising since P60 female rats are smaller than males. This difference was apparently enhanced in the sucrose animals, since neither the ethanol nor the chow groups displayed MS volume differences.

Differential gender susceptibility of PA+ neurons in the MS following CPET

The finding that PA expression was affected by prenatal ethanol exposure only in female animals suggests that differences in the hormonal environment of the males and females influenced PA-immunoreactivity and susceptibility to ethanol. In fact, the SH system itself is highly sexually dimorphic (Loy, 1986). Specifically, morphological differences in hippocampal asymmetry (Diamond et al., 1982) and differences in binding

capacity of hippocampal glucocorticoid receptors (Turner and Weaver, 1985) have been noted in rats. Additionally, Loy and Milner (Loy and Milner, 1980) have found differences in lesion-induced hippocampal sprouting between male and female rats. Whereas sprouting in female rats is uniform in the hippocampus following injury, sprouting in males occurs predominately in the dentate molecular layer. These results suggest that the CNS response to damage may be dictated by hormonal environment, and that this response is sexually dimorphic. Perhaps similar differences in hormonal environment determine sensitivity to ethanol in this region and account for the noted sexual dimorphism.

Gender differences have been reported in the alcohol literature as well: female rats are more affected than males in measures of radial arm maze performance following chronic adult ethanol treatment (Maier and Pohorecky , 1986); Witt et al. (1986) found an increase in muscarinic receptor binding sites in the hippocampus of ethanol-treated females but not ethanol-treated males following chronic adult ethanol treatment; Kelly et al. (Kelly et al., 1988) reported impaired spatial navigation in adult female rats but not adult male rats following neonatal ethanol exposure. As in the current study, these reports suggest an increased susceptibility to ethanol's effects in female animals and it has been suggested that females may be more sensitive than males to ethanol in animal models (West et al., 1989). However, not all studies have agreed with an increased susceptibility for females. Goodlett and Peterson (Goodlett and Peterson, 1995), for example, found increased susceptibility for spatial learning deficits in male rats following time-limited binge ethanol exposure. Sexually dimorphic effects of prenatal ethanol treatment have also been noted on daily water consumption, with males (but not females)

consuming more water than pair-fed controls as adults (McGivern et al., 1998). Clearly a greater focus on potential gender differences in animal models of FAS is warranted in order to clarify sexual dimorphism in relation to ethanol exposure. Moreover, an investigation into gender differences in human FAS is warranted, as no sexual dimorphisms have been reported in human offspring of mothers who drink during pregnancy.

What specific differences in hormonal environment might account for the observed sexually dimorphic effect of CPET on PA+ neurons in the female medial septum? Estradiol, for example, is known to influence the functioning of the cholinergic component of the SH system through its influence on choline re-uptake in the hippocampus (Singh et al., 1994). Indeed, removal of estradiol and other ovarian steroids by ovariectomy decreases cholinergic neurotransmission in the female hippocampus, and this is reversible by estradiol treatment (Singh et al., 1994). Moreover, estradiol infusion ameliorates fimbria-fornix lesion-induced decline of ChAT+ neurons in the rat medial septum (Rabbani et al., 1997). While estradiol is not known to influence the functioning of the GABAergic component of the SH system, estrogen is known to increase glutamic acid decarboxylase (the rate limiting enzyme in the synthesis of GABA) mRNA levels in the female rat brain (McCarthy et al., 1995). Thus, it is possible that an ethanol-induced decrease in ovarian steroids might account for some of the effects noted in the present study on GABAergic neurons. Effects of CPET on ovarian steroids would be a useful avenue of future research and may shed light on this interesting sexually dimorphic effect.

Liquid diet effects on the MS

In addition to the effects of ethanol on the MS of female rats, a non-specific effect of the liquid diet on females was noted for some measurements. Previous studies utilizing rodent models of prenatal ethanol exposure have noted an effect of liquid diet treatment. For example, Swanson et al. (1995) found a stimulatory effect of liquid diet on ChAT enzymatic activity during the first postnatal week. Additionally, studies quantifying ChAT+ cell number in the P14 rat MS found a liquid diet-induced increase in ChAT+ neuronal number for female sucrose animals, but not ethanol females or male sucrose animals (Swanson et al., 1996). It was speculated that a possible sucrose diet-induced stimulation of MS cholinergic development occurred in female rat pups at P14. The seemingly protective and perhaps stimulatory nature of the liquid diet is probably due to its high vitamin and mineral content.

The present study indicates similar diet effects for sucrose females. There seems to be a liquid diet-induced increase in both the density of PA+ neurons in the MS and the number of PA+ neurons per section in sucrose females. We speculate that a liquid diet-induced increase in cell density for the sucrose group, similar to the increase in ChAT+ neurons noted by Swanson et al. (Swanson et al., 1996), raised the base level of PA+ neurons in the liquid diet animals to a level greater than that seen in chows. Although there was not also a significant increase in cell number, the possibility exists that the period of naturally occurring cell death served to reduce this overall number while density remained high. The ethanol-treated group may also display this effect of liquid diet, but, nonetheless, a significant ethanol effect was noted as ethanol females had fewer PA+ neurons. Apparently the effect of prenatal ethanol treatment is sufficient to produce a

significant cellular reduction in ethanol females. The raised baseline due to the liquid diet, however, may mask a difference between ethanol and chow females.

Functional considerations for the MS

Although CNS regions differ in the extent to which PA and GABA co-localize, the MS is a region where most, if not all, GABAergic neurons are PA+. This statement is supported by studies of Freund (Freund, 1989) utilizing anterograde transport from the septum to the hippocampus. Of the projection neurons identified by the anterograde transport, all of the axons shown to be immunoreactive for GABA were immunoreactive for PA. Thus, PA-immunoreactivity serves as a reliable marker for GABAergic hippocampal projection neurons in the MS nucleus and identifies a subpopulation of the total GABAergic neuronal pool within the basal forebrain.

As noted previously, in the MS nucleus PA expression begins at G21, after the appearance of GABAergic neurons at G16 (Lauder et al., 1986; Solbach and Celio, 1991). The ethanol exposure regimen utilized in the present study encompasses the entire prenatal period, and thus ethanol was present in CNS tissue during the initiation of PA expression in MS neurons and the physiological maturation of these cells. Exposure of MS neurons to ethanol resulted in a long-term reduction in the number of PA+ neurons in adult females. How might these neurons have been affected by changes in the normal expression of PA?

The intracellular concentration of PA in neurons (6-45 μM) and the fact that it is commonly found in fast-firing neurons (Plogmann and Celio, 1993) implicate PA in buffering excess Ca^{2+} at presynaptic nerve terminals following rapid trains of action potentials (Heizmann, 1984). Since excessively high levels of intracellular Ca^{2+} are

known to initiate cell death, the possibility exists that an ethanol-induced reduction of PA expression resulted in fewer cells in the females by reducing those neurons' ability to buffer calcium and suppress the process of cell death. Though neurons expressing normal levels of PA can survive experimental cerebral ischemia (Nitsch et al., 1989) and fimbria-fornix transection (Kermer et al., 1995), any reduction in PA expression due to ethanol may impede a protective effect. Solodkin et al. (1996) have shown a decrease in PA immunostaining in parts of the entorhinal cortex which showed Alzheimer's pathologies. Since PA expression is reduced in the basal forebrain of aged rats (Krzywkowski et al., 1995) the possibility exists that the vulnerability of the septohippocampal pathway to degenerative diseases such as Alzheimer's is due to a reduction in PA expression. The observed reduction in PA+ neurons found following CPET in the current study may be due to direct loss of neurons by the process of cell death or a reduction in the level of PA in MS neurons which would preclude detection by immunostaining due to subthreshold amounts of antigen/epitope. In fact, Kermer et al. (1995) have detected reduced PA immunoreactivity in the medial septum following fimbria-fornix transection in the absence of PA+ neuronal cell death. However, whether cells are lost in our paradigm or not, the ability of these neurons to function adequately would likely be diminished in affected animals.

Altered MS function and FAS

A loss of effective GABAergic MS neurons, whether by a reduction in cell number or a reduction of PA expression, may impede the normal function of the septohippocampal system in learning and memory. GABAergic neurons in the basal forebrain are known to control activity of cholinergic neurons in this region (Dudchenko

and Sarter, 1991) and working memory in the rat is disrupted by antagonism of GABAergic transmission in the septum (Chrobak and Napier, 1992). Smythe et al. (1992) have shown that both the cholinergic and GABAergic components of the MS are necessary to influence the electrical activity in the hippocampus. Mechanistically, the influence of the basal forebrain on hippocampal electrical activity is known to be on theta cell rhythm (Bland and Bland, 1986) and the GABAergic component of the MS plays a part in this influence (Allen and Crawford, 1984; Smythe et al., 1992). PA+ MS neurons are known to depress the activity of inhibitory interneurons in the hippocampus (Freund and Antal, 1988). This would serve to increase the excitability of the hippocampus by removing local inhibitory potentials; theta rhythm would be initiated and the excitability of the principal hippocampal cells involved in long-term potentiation would be increased (Freund and Antal, 1988). The current study has demonstrated that CPET permanently reduces the number of PA+ GABAergic neurons in the female rat MS by 42 % in the absence of area or volume effects. Such a reduction in the number of PA+ GABAergic neurons could potentially affect the functioning of the septum and hippocampus. Indeed, some of the observed phenotypes of FAS, including mental retardation and spatial learning deficits (see Kelly et al., (1988) Goodlett and Peterson (1995) for animal studies), may be partially explained by a reduced septohippocampal efficacy. This possibility is supported by the findings of Miettinen et al. (1993) that the decline in aged rats in spatial learning correlates with a reduction in PA-containing neurons in the entorhinal cortex. A similar decline in learning in FAS children may also relate to PA expression in the SH system.

Cingulate Cortex

This investigation represents, to our knowledge, the first demonstration of neuroanatomical alterations in the cingulate cortex as a result of developmental ethanol exposure. In fact, this appears to be the first examination of teratogen-induced alterations in cingulate cortex neuroanatomy, and is a significant contribution to the literature since there exists a relative paucity of data on the cingulate cortex in normal or pathological states.

A conclusion of the present study is that CPET permanently reduced the number of PA+ neurons in the anterior cingulate cortex without altering size of the structure. These data are similar in their significance to those reported previously for the MS. One critical difference, however, between the effect of CPET on the anterior cingulate and the effect of CPET on the MS is the lack of sexually dimorphic effects in the former. The factors that are responsible for the lack of gender specific effects of CPET on the anterior cingulate remain unknown. It is possible that the medial septum and anterior cingulate respond differentially to different hormonal environments. While the medial septum is known as a sexually dimorphic region (see above), no data exist which point to a similar dimorphism for the anterior cingulate. Regardless, the overall pattern of ethanol-induced abnormalities is similar, with the volume of the anterior cingulate unaffected by prenatal ethanol exposure even though mean total PA+ neuronal number and mean PA+ neurons per section was affected. Additionally, the size of the PA+ neurons was determined, and was found to be unaltered by this pattern of in utero exposure. Our results are similar to those of Kril and Homewood (1993), who found that PA+ neuronal number was

decreased in the frontal cortex of adult rats following chronic ethanol treatment and thiamin deficiency.

Since the volume of the anterior cingulate was unaffected by treatment, and because the cells examined showed no decrease in size, it is unlikely that the decline in mean PA+ neurons per section is a result of an ethanol effect on the size of this area. Because our cell counts are derived from PA immunostained sections, we cannot definitively conclude that the reduced number of PA+ neurons is a result of ethanol-induced cell death. While this is a possibility, it is equally likely that the reduction of PA+ neurons noted after prenatal ethanol treatment is due to a decreased expression of PA in living neurons (perhaps as a result of ethanol interfering with PA ontogeny) which would preclude detection by immunostaining. Krzywkowski et al. (1995) examined PA immunoreactivity in the septum of aged rats and found a decrease in the number of PA+ neurons without loss of GABAergic neurons, suggesting that PA+ expression levels were decreased without cell loss. Similar findings have been reported in patients with Parkinson's disease (Hardman et al., 1996).

Yet another possibility is that ethanol interfered with the generation of neurons within the anterior cingulate cortex. Evidence from Miller (1986) shows that prenatal ethanol exposure delays and extends the period of cortical neuronal generation while reducing the number of neurons and altering the distribution of neurons in the mature cerebral cortex. It is conceivable that a similar reduction in the generation of cortical neurons is responsible for our observed decrease in PA+ neuronal number as a result of ethanol treatment.

Functional considerations for the cingulate

As mentioned previously, the present study examined PA+ neuronal number in the cingulate cortex following prenatal ethanol treatment because of earlier observations related to cerebral cortex functioning following ethanol exposure. These include severe behavioral problems in children with FAS, including poor judgment, distractibility, and difficulty perceiving social cues (Streissguth et al., 1991). Additionally, many alcoholics who develop Korsakoff's syndrome have deficiencies in glucose utilization within the cingulate cortex, potentially contributing to learning and memory defects due to interruption of Papez' circuitry (Joyce et al., 1994). Papez' circuit is a neuroanatomical pathway which originates in the hippocampus, proceeds through the fornix to the mammillary bodies, and then to the anterior thalamic nucleus, cingulate gyrus, entorhinal cortex, and then back to the hippocampus (Nolte, 1993). Therefore, it is conceivable that alterations in PA expression patterns might contribute to behavioral anomalies and/or learning and memory deficiencies.

Because the intracellular concentration of PA in neurons is in the range necessary for calcium buffering (6-45 μM) and the fact that it is commonly found in fast-firing neurons (Plogmann and Celio, 1993), PA appears to be involved in buffering excess Ca^{2+} at presynaptic nerve terminals following rapid trains of action potentials (Heizmann, 1984). Since excessively high levels of intracellular Ca^{2+} are known to initiate cell death, the possibility exists that an ethanol-induced reduction of PA expression resulted in fewer cells in the ethanol animals by reducing those neurons' ability to buffer calcium and suppress the process of cell death. Though neurons expressing normal levels of PA can survive experimental cerebral ischemia (Nitsch et al., 1989) and fimbria-fornix

transection (Kermer et al., 1995) any reduction in PA expression due to ethanol may impede a protective effect. Solodkin et al. (1996) have shown a decrease in PA immunostaining in parts of the entorhinal cortex which showed Alzheimer's pathologies. Similarly, the number of PA+ neurons is reduced in the prefrontal cortex of schizophrenics (Beasley and Reynolds, 1997).

While we cannot determine from the present study whether cell death or reduced PA antigen is responsible for the noted reduction in PA+ neurons, it should be noted that whatever the mechanism of the ethanol-induced reduction of PA+ neurons, inhibitory neurotransmission in the cingulate cortex is likely to be altered. For example, PA+ neurons are lost at epileptic foci in animal models of epilepsy (De Felipe et al., 1993). Moreover, Jacobs et al. (1996) showed that decreased PA immunoreactivity in cortical freeze lesions of neonatal rats leads to hyperexcitability in adjacent cortex. Such alterations in inhibitory and excitatory processes have clear functional consequences to the organism, and likely result in an imbalance in excitation and inhibition in these cortical regions (Wang et al., 1996). The current findings of decreased number of PA+ neurons in the anterior cingulate cortex of adult rats as a result of prenatal ethanol exposure may provide a neuroanatomical basis for the well described cognitive and behavioral impairments in offspring exposed to ethanol in utero.

Conclusions

The major findings of this study are that PA+ neurons in the medial septum and anterior cingulate cortex are susceptible to prenatal ethanol exposure. In the medial septum, a sexually dimorphic effect of ethanol was noted, with females more susceptible than males. No gender specific effects were noted for the anterior cingulate. These

reductions in PA+ neurons occurred in the absence of significant ethanol-induced alterations in the size of the structures examined, the size of the PA+ neurons within these structures, or the growth of these animals. This represents the first description of ethanol-induced alterations in PA+ GABAergic neurons in animal models of FAS. Further research will help to further define developmental windows of GABAergic vulnerability (for example, neonatal exposure models).

Table 2-1. Postnatal day 60 (P60) body and brain weight and brain to body weight ratio (br/bd)*

Diet group	N	P60 body weight (g)	P60 brain weight (g)	P60 br/bd (%)
Females				
Ethanol	8	220 ± 2.92	1.32 ± 0.02	0.600 ± 0.010†
Sucrose	6	216 ± 5.58	1.31 ± 0.02	0.606 ± 0.021†
Chow	6	224 ± 5.06	1.36 ± 0.02	0.607 ± 0.022†
Males				
Ethanol	5	341 ± 13.5†	1.34 ± 0.05	0.392 ± 0.032
Sucrose	6	351 ± 5.81†	1.35 ± 0.06	0.384 ± 0.019
Chow	8	348 ± 13.6†	1.39 ± 0.02	0.399 ± 0.030

*All measures are expressed as mean ± S.E.M. No significant differences were noted.
†Significantly increased, compared with other gender of same group ($p < 0.0001$).

Table 2-2. Number of sections, medial septum (MS) area per section, and parvalbumin-immunoreactive (PA+) neuronal density in the adult rat MS*

Diet group	N	Number of 40 μm sections	MS area/section (mm^2)	Density (PA+ cells/mm^2)
Females				
Ethanol	8	15.75 ± 0.453	0.722 ± 0.066	49.55 ± 6.716†,‡
Sucrose	6	15.50 ± 0.428¶	0.655 ± 0.061	89.38 ± 9.700
Chow	6	17.67 ± 1.054	0.670 ± 0.065	59.87 ± 6.237§
Males				
Ethanol	5	16.20 ± 0.374	0.733 ± 0.103	78.031 ± 12.01
Sucrose	6	17.50 ± 0.428	0.778 ± 0.061	66.16 ± 10.98
Chow	8	16.87 ± 0.811	0.733 ± 0.049	63.49 ± 5.957

*All measures are expressed as mean ± S.E.M. Area and volume measures are representative of approximately 1/2 of the total MS (alternate sections were analyzed; see materials and methods section for detail).
†Significantly reduced from sucrose females ($p < 0.01$).
‡Significantly reduced from ethanol males ($p < 0.05$).
§Significantly reduced from sucrose females ($p < 0.05$).
¶Significantly reduced from sucrose males ($p < 0.05$).

Table 2-3. Number of sections, mean area per section, and parvalbumin-immunoreactive (PA+) neuronal density in the adult rat anterior cingulate cortex*

Diet group	N	Number of 40 μm sections	Mean area/section (mm^2)	Density (PA+ cells/mm^2)
Ethanol	13	41.5 ± 2.56	2.135 ± 0.069	395.5 ± 67.85†
Sucrose	12	39.8 ± 2.56	2.262 ± 0.060	669.6 ± 54.56
Chow	14	35.8 ± 1.34	2.385 ± 0.080	697.4 ± 61.88

*All measures are expressed as mean ± S.E.M.

†Significantly reduced compared with sucrose ($p < 0.01$) and chow ($p < 0.01$).

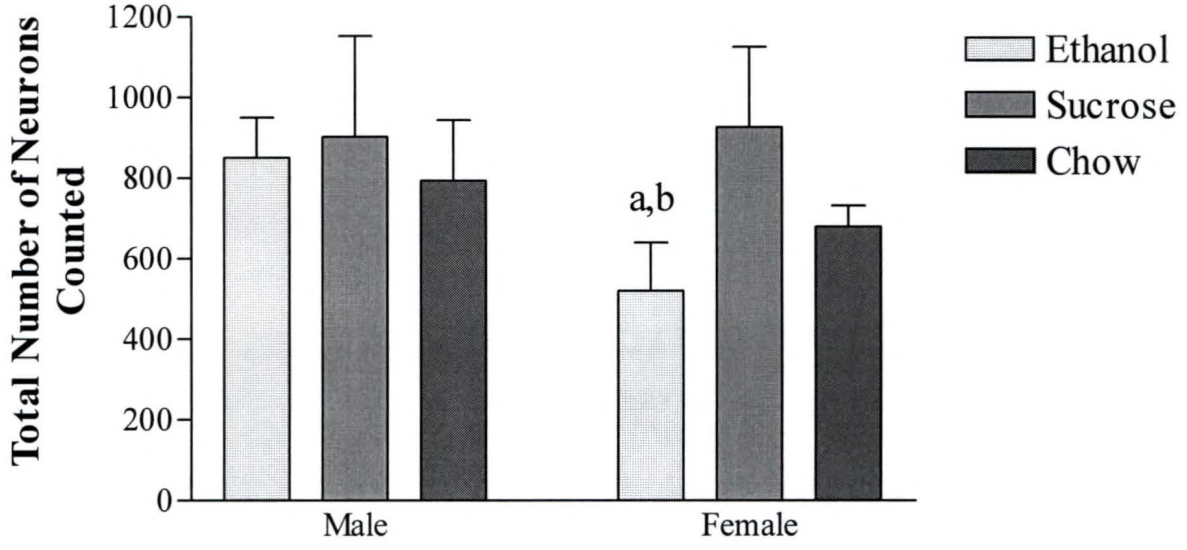
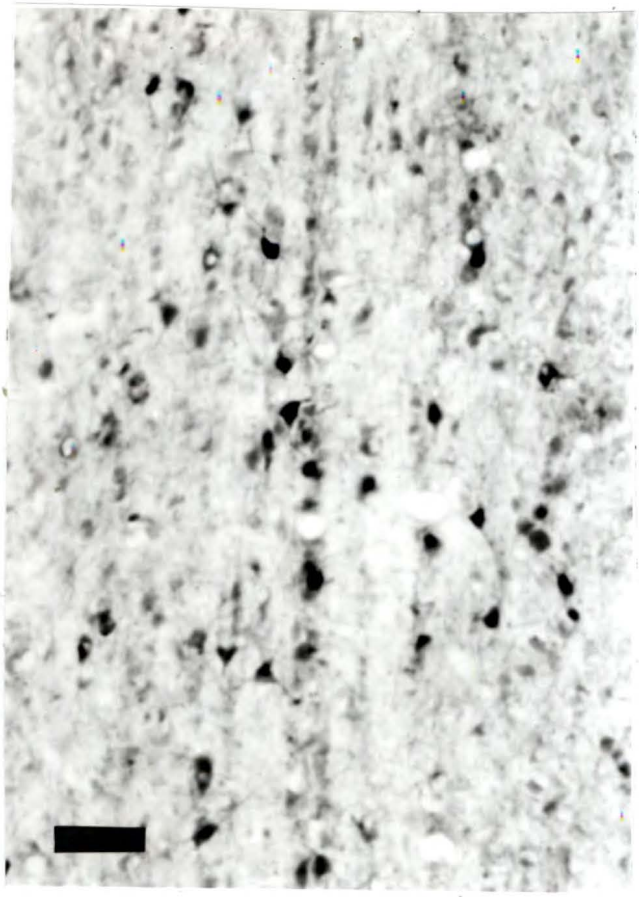


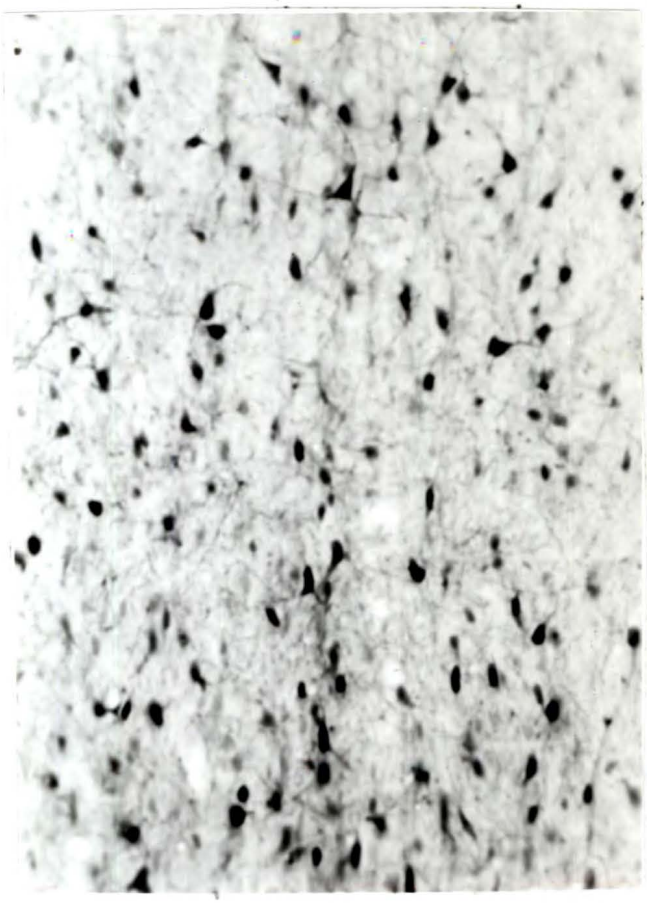
Figure 2-1. The mean total number of parvalbumin-expressing neurons detected on alternate sections through the medial septum of postnatal-day 60 rats exposed in utero to one of three diets, ethanol, sucrose, or chow (see materials and methods section for detail). Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with sucrose females ($p < 0.01$). b: significantly reduced compared with ethanol males ($p < 0.05$).

Figure 2-2. Photomicrographs of 40 μm coronal sections through the medial septum of ethanol-treated (A) and sucrose-treated (B) postnatal-day 60 female rats. Sections are matched for anatomical location and are representative of their respective group. A qualitative reduction in cell number for the ethanol females can be noted, as can an increase in cell density for sucrose females. Scale bar equals 125 μm.

A



B



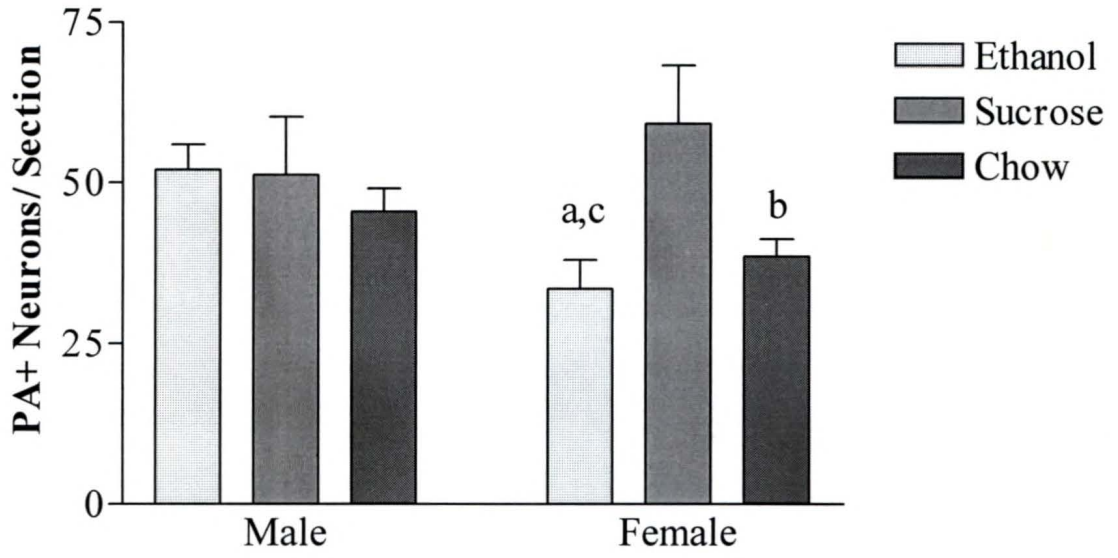


Figure 2-3. The mean number of parvalbumin-expressing neurons per section in alternate sections through the medial septum of postnatal-day 60 rats exposed in utero to one of three diets, ethanol, sucrose, or chow (see materials and methods section for detail). Data are expressed as mean $\pm$ S.E.M. a: significantly reduced compared with sucrose females ($p < 0.01$). b: significantly reduced compared with sucrose females ($p < 0.05$). c: significantly reduced compared with ethanol males ($p < 0.05$).

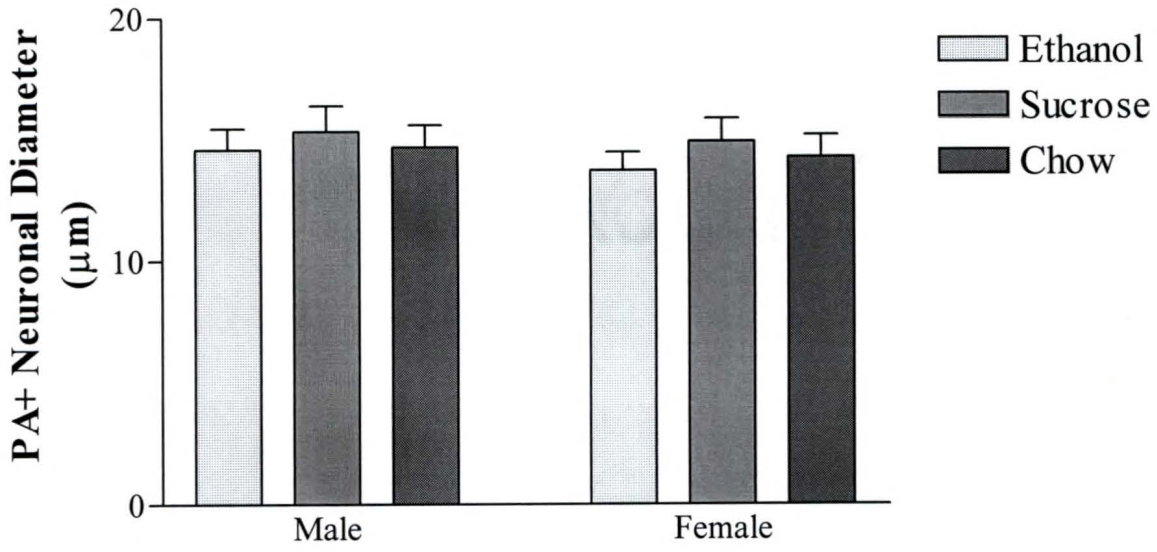


Figure 2-4. The mean diameter of parvalbumin-expressing neurons on alternate sections through the medial septum of postnatal-day 60 rats exposed in utero to one of three diets, ethanol, sucrose, or chow (see materials and methods section for detail). Data are expressed as mean $\pm$ S.E.M. No significant differences were noted.

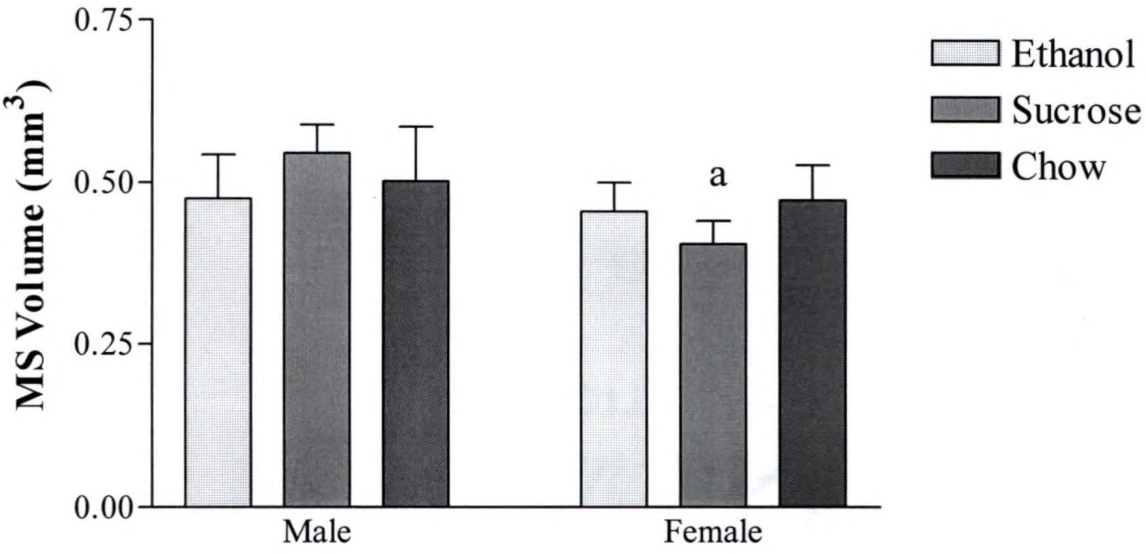


Figure 2-5. The mean medial septum (MS) volume of postnatal-day 60 rats exposed throughout gestation to an ethanol-containing liquid diet, sucrose-containing liquid diet, or lab chow and water. Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with sucrose males ($p < 0.05$).

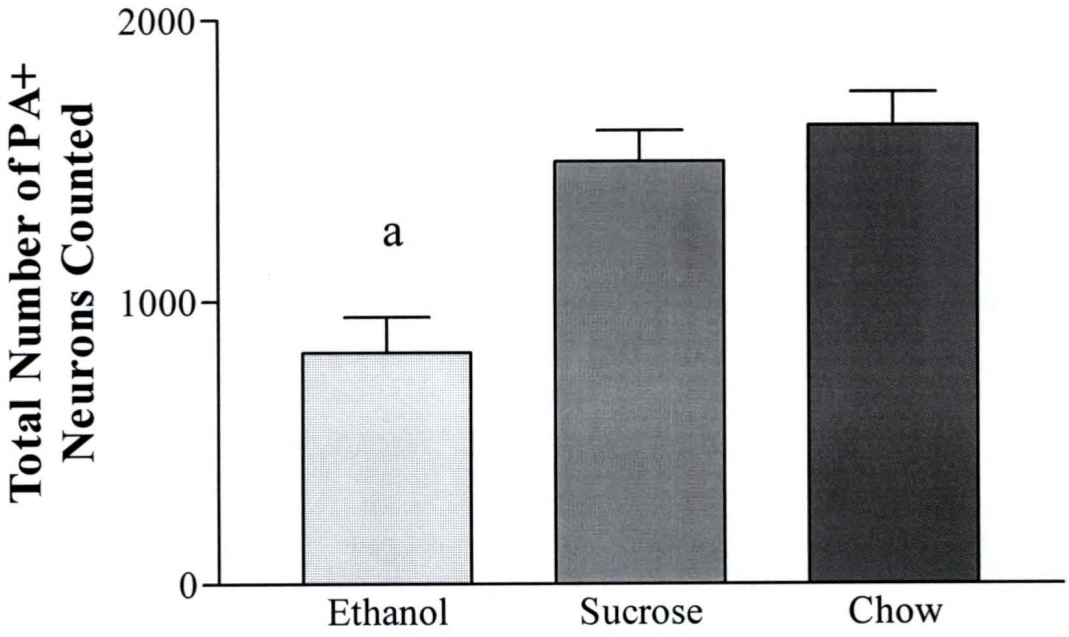
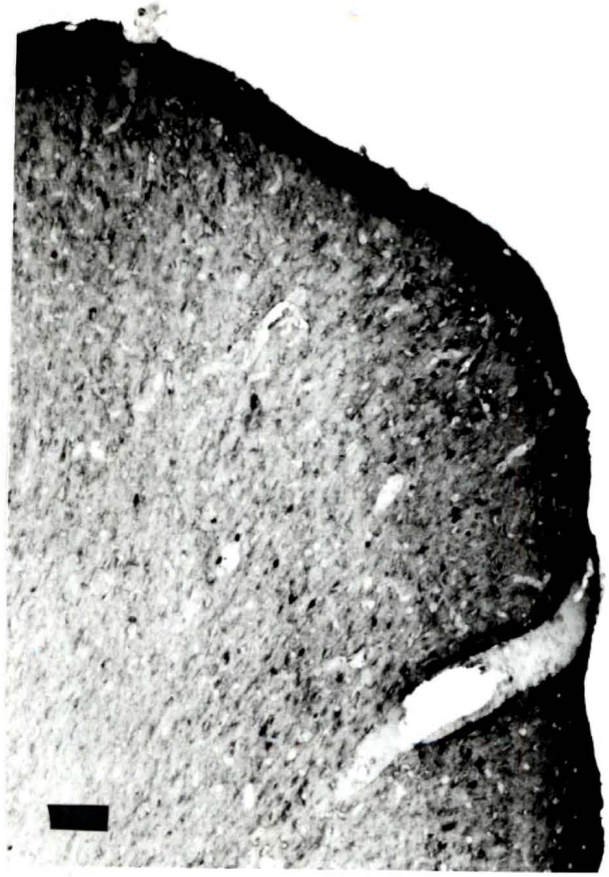


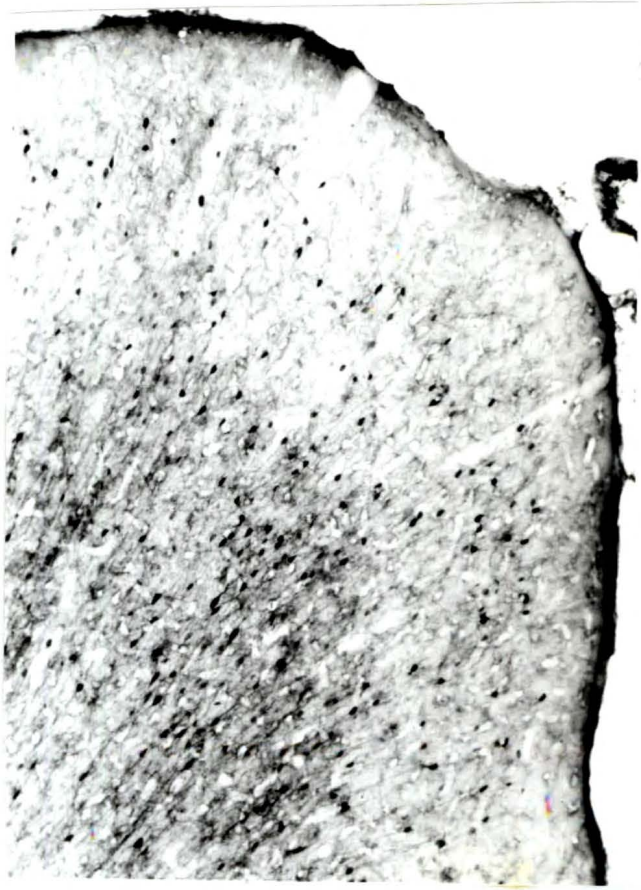
Figure 2-6. The mean total number of parvalbumin-expressing (PA+) neurons detected on alternate sections through the anterior cingulate cortex of postnatal-day 60 rats exposed in utero to one of three diets, ethanol, sucrose, or chow (see materials and methods section for detail). Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with sucrose ($p < 0.0001$) and chow ($p < 0.0001$).

Figure 2-7. Photomicrographs of 40 μm coronal sections through the anterior cingulate cortex of ethanol-treated (A) and sucrose-treated (B) postnatal-day 60 rats. Sections are matched for anatomical location and are representative of their respective group. A qualitative reduction in parvalbumin-immunoreactive neuronal number is seen in the ethanol-treated animal compared with the sucrose control. Scale bar equals 110 μm.

A



B



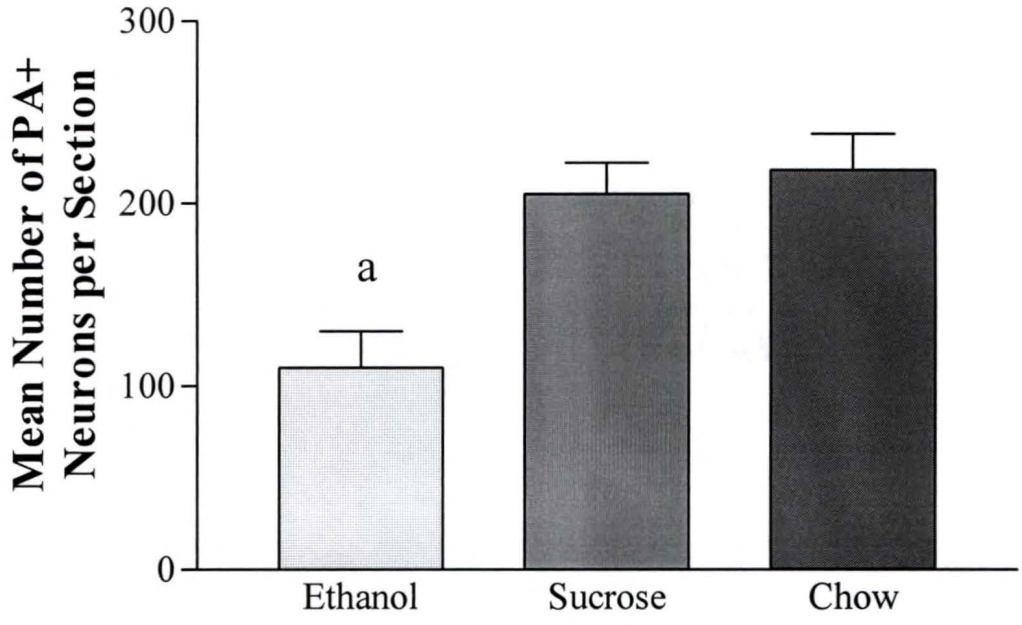


Figure 2-8. The mean number of parvalbumin-immunoreactive (PA+) neurons per section counted in the anterior cingulate cortex of postnatal-day 60 rats exposed throughout gestation to an ethanol-containing liquid diet, sucrose-containing liquid diet, or lab chow and water. Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with sucrose ($p < 0.001$) and chow animals ($p < 0.0001$).

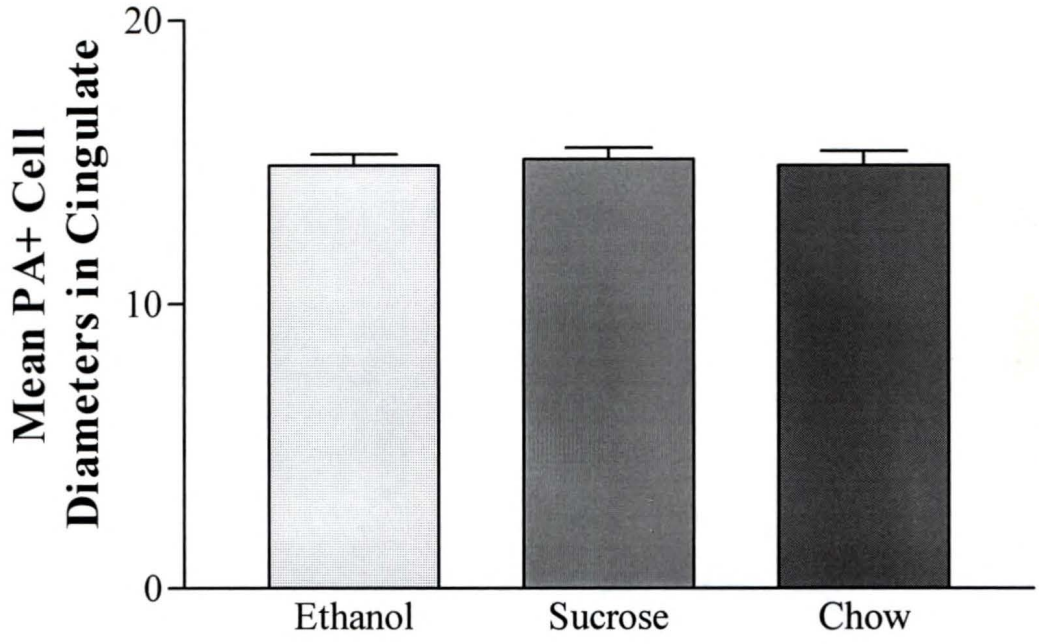


Figure 2-9. The mean diameter of parvalbumin-immunoreactive (PA+) neurons in the anterior cingulate cortex of postnatal-day 60 rats exposed throughout gestation to an ethanol-containing liquid diet, sucrose-containing liquid diet, or lab chow and water. Data are expressed as mean $\pm$ SEM. No significant differences were noted.

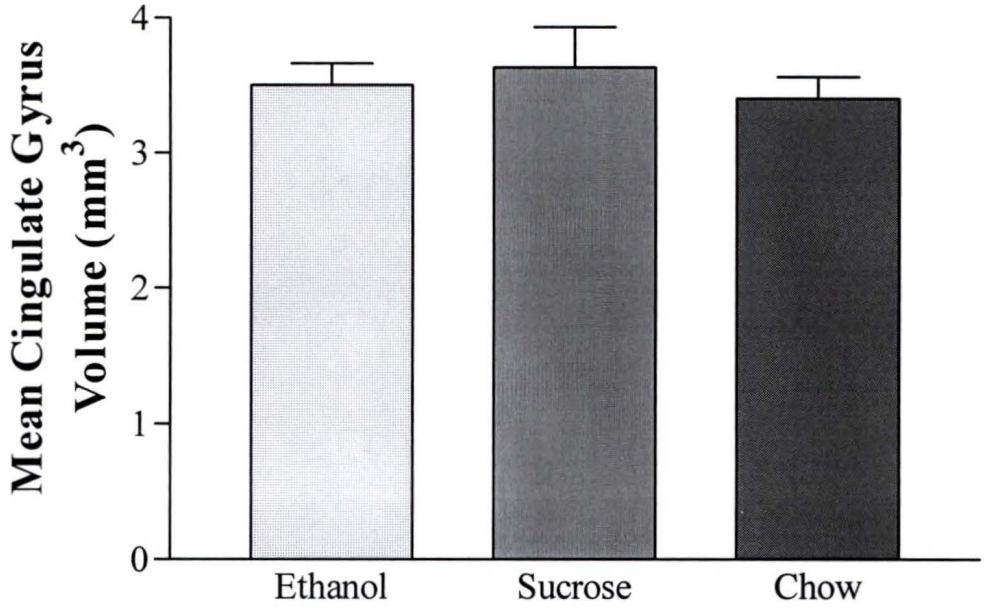


Figure 2-10. The mean cingulate gyrus volume of postnatal-day 60 rats exposed throughout gestation to an ethanol-containing liquid diet, sucrose-containing liquid diet, or lab chow and water. Data are expressed as mean $\pm$ SEM. No significant differences were noted.

CHAPTER 3
EFFECTS OF NEONATAL ETHANOL EXPOSURE ON CHOLINERGIC NEURONS OF THE RAT MEDIAL SEPTUM

Summary

Developmental ethanol exposure has been known to affect the normal development of the central nervous system. Studies in animal models have determined that chronic prenatal ethanol exposure has no effect on the number of cholinergic neurons in the rat medial septum (Swanson et al., 1996). Since many brain regions exhibit tight temporal windows of vulnerability to ethanol, the objective of this study was to determine the long-term effects of chronic neonatal ethanol exposure on the cholinergic neurons in the medial septum (MS) of the rat. On postnatal day 4 (P4) pups were assigned to one of three groups: an ethanol-receiving, gastrostomized group (EtOH); a pair-fed, gastrostomized control group (GC); and a dam-reared suckle control group (SC). Gastrostomized pups were infused with ethanol-containing or control diet as a 9.1% v/v solution for two feedings on each day from P4-10. Choline acetyltransferase (ChAT) immunocytochemistry was analyzed at P60.

Ethanol treatment resulted in long-lasting microencephaly in P60 animals. Ethanol exposure did not directly reduce mean total ChAT-expressing (ChAT+) neuronal number, or the mean number of ChAT+ neurons per section. Neither were changes noted in MS volume, mean area section, or cell density as a result of ethanol treatment.

However, ethanol exposure significantly reduced ChAT+ neuronal size in males compared with GC males but not SC males. No differences in ChAT+ neuronal size were noted in females. Thus neonatal ethanol exposure, while producing long-lived microencephaly and small changes in ChAT+ neuronal size, has no effect on the number of cholinergic neurons in the adult rat MS, and has no effect on the size of the MS.

Introduction

Fetal alcohol syndrome (FAS) has been well characterized since its description in 1973 (Jones and Smith, 1973). Perinatal growth deficiencies, craniofacial abnormalities, as well as central nervous system (CNS) dysfunction have been noted in human offspring exposed to ethanol prenatally (West et al., 1994). In attempts to thoroughly examine and accurately define the consequences of developmental ethanol exposure, rodent models of FAS have been developed and extensively utilized. These animal models have identified susceptible populations of neurons within the CNS and have described periods of development during which particular populations exhibit heightened vulnerability to the effects of ethanol (West et al., 1994). Studies in rats have demonstrated that the early postnatal period, the so-called brain growth spurt and the equivalent of the human third trimester (Dobbing and Sands, 1979), is a time of vulnerability for particular CNS regions.

The septohippocampal (SH) system of basal forebrain afferents and hippocampal targets is one such region in rats that exhibits susceptibility to ethanol during neonatal development. The SH system is a pathway of cholinergic and GABAergic fibers originating from the MS nucleus and the horizontal and vertical limbs of the diagonal

band of Broca (DBB) that influences electrical activity in the hippocampus (Dutar et al., 1995). These fibers originate from cell bodies in the basal forebrain and, beginning at embryonic day 20, innervate the hippocampus where they form synapses on hippocampal pyramidal neurons, granule cells, and interneurons (Freund and Antal, 1988; Milner et al., 1983). Cholinergic neurons comprise the major projection of the SH system and first express the catalytic enzyme choline acetyltransferase (ChAT) on embryonic day 17 (Armstrong et al., 1987; Dutar et al., 1995).

While numerous studies have described the effects of neonatal ethanol exposure on neurons within the hippocampus (Bonthius and West, 1990; Bonthius and West, 1991; Greene et al., 1992; Pierce and West, 1987; West and Pierce, 1986) and have documented deficits in spatial learning following neonatal ethanol exposure (Goodlett and Peterson, 1995; Kelly et al., 1988), the effect of ethanol exposure during the brain growth spurt on the cholinergic component of the SH system has yet to be characterized. However, studies examining the effect of chronic adult ethanol exposure on the neurons of the SH system have been reported and have suggested a decrease in the number of cholinergic neurons in the MS, though pair-fed controls were not examined (Arendt et al., 1995; Arendt et al., 1988). Moreover, studies examining the effect of prenatal ethanol exposure on the SH system in the rat (Swanson et al., 1995; Swanson et al., 1996) and mouse (Schambra et al., 1990; Sulik et al., 1984) have been completed. When rats were exposed to ethanol throughout gestation, a reduction in ChAT enzymatic activity was noted in the MS during the second postnatal week compared with pair-fed controls (Swanson et al., 1995).

In contrast, no change in the number or morphology of ChAT+ neurons in the MS was found (Swanson et al., 1996), though earlier experiments in mice had shown the cholinergic neurons of the basal forebrain to be particularly sensitive to acute prenatal ethanol exposure (Schambra et al., 1990; Sulik et al., 1984). The objective of the present study was to determine the long-term effects of ethanol exposure on the cholinergic neurons in the rat MS when ethanol was delivered during the brain growth spurt, a time when the cholinergic neurons are more mature and potentially more susceptible than at the time points examined in the previous prenatal studies (Bonthius and West, 1991). Ethanol was delivered from P4-10, a period of differentiation and synaptogenesis in the SH system (Dutar et al., 1995). ChAT+ neuronal number and morphology was examined in the adult MS of artificially reared ethanol-treated and pair-fed controls as well as in dam-raised suckle controls.

Methods

Subjects and artificial rearing

Long-Evans hooded rat pups were obtained from nine timed pregnant dams ordered from Charles River Co. (Portage, MI). Animals were housed with a 07:00-19:00 light cycle under controlled temperature and humidity conditions. At the time of birth (P0), litters were culled to 12 pups and the pups were randomly assigned to one of three groups: ethanol-receiving gastrostomized pups (EtOH); pair-fed gastrostomy controls (GC); and dam-reared suckle controls (SC). Artificial rearing was performed after West et al. (West et al., 1984). On P4 a gastrostomy feeding tube was surgically implanted into the EtOH and GC pups. Pups were placed under methoxyfluorane anesthetic while

gastrostomy tubes were inserted down the esophagus into the stomach. The tube was pulled through a small hole in the stomach and abdominal wall and secured on the outside by a small plastic washer. Pups were reared individually in plastic cups filled with bedding and a fur-like material. Cups were floated in a covered, heated aquarium (40°C) and pups were maintained on a 07:00-19:00 light cycle. SC pups were weighed daily from P4-10. Dams with SC pups always had a total of eight pups to minimize weight differences between groups.

Gastrostomized pups were infused with a liquid diet containing evaporated milk, sterile water, soy protein, L-methionine, L-tryptophan, calcium phosphate, deoxycholic acid, a vitamin mixture, and a mineral mixture (West et al., 1984). Pups received the milk formula from P4-10 in 12 feeding periods of 20 minutes each. EtOH pups received ethanol-supplemented formula as a 9.1% v/v solution for the first two feedings on each day for a total of 4 g/kg/day. GC pups received an isocaloric amount of maltose-dextrin-supplemented formula for the first two feedings on each day. The remaining 10 daily feedings consisted of milk formula alone. Gastrostomized pups were weighed daily and the daily diet consumption volume (in mls) was equivalent to 33% of the mean litter body weight. The liquid diet was administered to the EtOH and GC pups by connecting the gastrostomy tubes to a feeding line connected to diet-filled syringes held within a Stoelting (Wood Dale, IL) programmable infusion pump. On the morning of P11, pups were disconnected from the pump, feeding tubes were sealed, and they were returned to their original dam. EtOH and GC pups were accepted by the dams and began nursing soon after re-introduction to the home cage. At P21, pups were weaned and housed

individually until sacrifice and perfusion at P60. A total of 38 animals were used in this analysis (EtOH, n = 12; GC, n = 10; SC, n = 16).

Tissue preparation and immunocytochemistry

Animals were selected for immunocytochemical staining at P60, an age which represents adulthood in rats. This age was selected in order to examine the long-term effects of neonatal ethanol treatment on ChAT expression in cholinergic neurons of the medial septum and corresponds to an age used in previous analyses of prenatal ethanol influences on cholinergic and GABAergic neurons in the medial septum (Moore et al., 1997; Swanson et al., 1996). On P60, rats were anesthetized by pentobarbital overdose just prior to perfusion. Animals were perfused transcardially with phosphate buffered saline (PBS; 0.1M containing 0.9% sodium chloride) followed by 10% formalin (in the same PBS). The brains were removed and post-fixed overnight in the same fixative. The brain tissue was equilibrated overnight (or until it sank) in a cryoprotectant solution (PBS containing 30% sucrose and 15% ethylene glycol) and stored frozen at -70°C until processed for immunocytochemistry.

Brains were prepared for frozen sectioning by thawing and equilibrating with a 30% sucrose-PBS solution. Frozen serial coronal sections throughout the forebrain were cut at a thickness of 40 μm and processed free-floating. Alternate sections were immunostained for choline acetyltransferase using a polyclonal antibody (rabbit anti-human placental ChAT, Chemicon, Temecula, CA). Immunoreactivity was visualized as a blue-black reaction product using an avidin-biotin conjugate/nickel intensified staining (see below). Animals from each diet group were processed for immunocytochemistry at a

given time. This ensured against staining differences between groups resulting from procedural differences.

Endogenous peroxidase activity was quenched by initially treating tissue sections for 60 min at room temperature (RT) with PBS containing 3% hydrogen peroxide (H_2O_2) and 10% methanol. Sections were pretreated with 0.4% Triton-X100 (T-X) in PBS for 30 min at RT, then blocked for 60 min with PBS containing 3% normal goat serum (NGS) and 0.1% T-X. Primary incubation with the polyclonal ChAT antibody (1:750 in PBS + 3% NGS and 0.1% T-X) was carried out for 48 hr at 4°C. Sections were then washed in PBS containing 1% bovine serum albumin (BSA) and incubated with biotinylated goat anti-rabbit IgG (1:1000 in PBS +1% BSA) overnight at 4°C. Tissues were washed and incubated with extravidin-horseradish peroxidase conjugate (Sigma, St. Louis, MO; 1:1000 in PBS + 1% BSA) overnight at 4°C. Tissues were washed with 0.1M sodium acetate (pH 7.2) to eliminate phosphate which can precipitate divalent cations. Sections were then reacted for 3 min at RT using a developing buffer, pH 7.2, containing 0.8M sodium acetate, 8 mM imidazole, 0.5% nickel (II) sulfate, 0.04% 3,3 diaminobenzidine tetrahydrochloride, and 0.005% H_2O_2. The chromogen precipitation reaction was stopped with 0.1M acetate buffer (pH 7.2) containing 0.1% sodium azide. Sections were mounted in the same acetate buffer, air dried, dehydrated, and coverslipped. Control for nonspecific staining was carried out in parallel with stained tissues with omission of the primary antibody. Slides from individual animals were randomized and coded so that all subsequent analyses were carried out blind with regard to the diet treatment group and gender.

MS cell count, volume, and area analysis

ChAT-positive cell counts were conducted on alternate sections throughout the rostral-caudal extent of the MS nucleus. The packing density of the ChAT+ neurons in the DBB nucleus was too great for accurate cell counts to be made. Thus, for the purposes of this study the MS alone was examined and was defined rostrally by the ventral fusion of the hemispheres (at the level of the genu of the corpus callosum) and caudally by the decussation of the anterior commissure

Images of ChAT immunostained sections were captured and digitized using a RasterOps 24STV video capture board and software on a Macintosh computer. Low magnification images (4x objective; effective scale 1.59 μm/pixel) were captured in order to obtain the entire MS region in one image. When capturing each image, the lighting and contrast enhancement were optimized for identification of individual cells. Images were digitally processed using the image analysis program NIH Image (freeware from NIH, Bethesda MD). To reduce background variation across the image, a digitally defocused image (created by passing the primary image through a mean filter) was subtracted from the primary digitized image. This processed image was then passed through a Laplace filter to enhance edges and separation between cells.

Analysis of individual images was initiated by outlining the region of the MS nucleus. The area of a section outlined varied depending on the rostral-caudal location of the individual section. The MS nucleus in rostral sections was defined as the mediodorsal group of neurons which were separated from the ventrally-located DBB nucleus. In intermediate sections, where the demarcation between the MS and DBB nuclei is ambiguous, the MS was defined ventrally by a line perpendicular to midline at the level

of the anterior commissure. In caudal sections, the MS was defined as the medially located cells dorsal to the anterior commissure. Cells were highlighted interactively by adjusting the grayscale threshold level to include only objects which were considered cells. Given the thickness of each section (40 μm), the fact that alternate sections were analyzed, the fact that ChAT+ neurons in the MS nucleus range from 10-25 μm in diameter, and the fact that an estimation of the total population was not desired, it was not deemed necessary to perform a split-cell correction on these counts.

The program counted the highlighted objects and measured the area of the outlined region (mm^2). Because the MS is defined by clusters of ChAT+ cell bodies, the area outlined was determined by the pattern of ChAT staining on each individual section. MS volume (mm^3) was calculated using a modification of the Cavelieri method (Michel and Cruz, 1988) to ensure that changes in mean number of ChAT+ neurons per section as a result of ethanol treatment were not due to changes in the size of the structure of interest (Peterson et al., 1997). This was done by multiplying the number of 40 μm sections analyzed for a given animal by the mean area per section for that animal and the section thickness.

ChAT+ cell size determination

Because a previous study from our laboratory (Swanson et al., 1996) had analyzed ChAT+ neuronal size using the computer instead of simple measurements of cell diameters, we also chose to use the computer for these purposes so that comparisons could be made to the previous work. For morphometric analysis, moderate magnification images (10x objective; effective scale 0.978 μm/pixel) were captured in order to maximize the resolution yet minimize the loss of cells out of the focal plane of each

image. Primary images were not processed further prior to morphometric analysis. Cell size measurements were performed on ChAT+ neurons in the MS nucleus. Three sections through the MS were regionally matched in rostro-caudal extent for all animals. 25 non-overlapping cells per section were selected at random and measured. The cross sectional somatic area of individual cells was highlighted interactively by adjusting the grayscale threshold and was automatically measured by the computer. The program computed cross-sectional area for each cell and mean somatic area was determined for each animal.

Other analyses

The peak blood ethanol concentration (BEC) in the ethanol group was determined on P7 two hours after the second ethanol infusion (Goodlett et al., 1990) utilizing the Sigma 333-UV kit. Body and brain weights were recorded for individual animals at P60 following perfusion. Density of ChAT+ neurons was determined by dividing the mean number of cells per section by the mean area (mm^2) per section.

Statistical analyses

Statistical analysis was performed with the program StatView (Abacus Concepts, Berkeley Ca). For each parameter examined, one-way Analysis of Variance (ANOVA) was used to test for the main effects of diet and gender separately. When appropriate, the data were further analyzed with the Fisher's Protected Least Significant Difference (PLSD) post hoc test to determine individual group differences. Because gender differences were noted for many measures (following an initial two-way ANOVA for effects of diet and gender), males and females were analyzed separately.

Results

BEC, body and brain weights, and brain/body weight ratios

The mean BEC in EtOH pups two hours following the last ethanol treatment on P7 was 269 $\pm$ 23 mg/dl (n=5). Table 3-1 (all tables are located at the end of the chapter) presents the mean P60 body and brain weights, and brain/body weight ratios (br/bd) for all diet groups separated by gender. The ANOVA for P60 body weight revealed no significant effects of diet on P60 body weight of males or females. A gender difference was noted in P60 body weight for EtOH [$F(1,22) = 45.2$; $p < 0.0001$], GC [$F(1,14) = 38.5$; $p < 0.0001$], and SC [$F(1,16) = 40.8$; $p < 0.0001$] animals which was expected given the larger size of males at P60.

The ANOVA for P60 brain weight demonstrated an effect of diet treatment in males [$F(2,18) = 12.3$; $p < 0.001$] and in females [$F(2,25) = 6.69$; $p < 0.01$]. The PLSD revealed that EtOH male brains weighed less than GC male ($p < 0.01$), and SC male ($p < 0.01$) brains. EtOH female brains also weighed less than GC female ($p < 0.05$), and SC female ($p < 0.01$) brains. No significant gender differences were noted for brain weight within EtOH, GC, or SC animals.

The ANOVA for br/bd showed an effect of diet in males [$F(2,18) = 5.16$; $p < 0.05$] and females [$F(2,25) = 11.3$; $p < 0.001$]. The PLSD demonstrated that EtOH males had reduced br/bd compared with GC males ($p < 0.01$) while EtOH females had reduced br/bd compared with both GC females ($p < 0.001$) and SC females ($p < 0.001$). Gender differences were also noted in P60 br/bd for EtOH [$F(1,22) = 37.5$; $p < 0.0001$], GC

[$F(1,14) = 41.2$; $p < 0.0001$], and SC [$F(1,16) = 38.5$; $p < 0.0001$] animals, which was not surprising given the larger size of male animals at this age.

Number of sections, MS area per section, and ChAT+ neuronal density

Table 3-2 presents number of sections, MS area per section, and ChAT+ neuronal density measurements for each diet group for either gender. The number of alternate 40 μm sections through the MS was determined by counting the number of sections analyzed between the ventral fusion of the hemispheres (at the level of the genu of the corpus callosum) and the anterior commissure. The ANOVA showed no effect of diet for either males or females on the mean number of alternate sections through the MS. Additionally, no significant gender differences were noted in the number of alternate sections for EtOH, GC, or SC animals.

As mentioned above, the computer program determined the area of the outlined MS on each individual section. The mean MS area per section was determined for each animal. The ANOVA revealed no significant effect of diet in males or females on the mean MS area per section. Similarly, no significant gender differences were noted in MS area per section for EtOH, GC, or SC animals.

ChAT+ neuronal density was calculated by dividing the mean number of ChAT+ neurons per section by the mean area per section. The ANOVA demonstrated no significant effect of diet in males or females on ChAT+ neuronal density. Also, no significant gender differences were noted in ChAT+ neuronal density for EtOH, GC, or SC animals.

Cholinergic cell counts in the MS

The total number of ChAT+ neurons counted on alternate sections through the P60 MS was determined and is presented in figure 3-1 (all figures are located at the end of the chapter). The ANOVA for total number of ChAT+ cells demonstrated an effect of diet in males [$F(2,18) = 4.45$; $p < 0.05$] but not females. The PLSD further revealed that EtOH males contained 24 % fewer MS ChAT+ neurons than SC males ($p < 0.05$). No significant gender differences were noted in mean total number of ChAT+ neurons for EtOH, GC, or SC animals.

In order to normalize for volume, the average number of ChAT+ neurons per section was determined. Figure 3-2 presents the mean number of ChAT+ neurons per section. The ANOVA for mean number of ChAT+ neurons per section revealed an effect of diet on males [$F(2,18) = 3.34$; $p < 0.05$] but not females. The PLSD further demonstrated that EtOH males contained 19 % fewer ChAT+ neurons per section than SC males ($p < 0.05$), while the 14 % difference between GC males and SC males was not significant. No significant gender differences were noted in mean ChAT+ cells per section for EtOH, GC, or SC animals.

Cholinergic neuronal size

Figure 3-3 presents the mean somatic cross sectional area for ChAT+ neurons throughout the P60 MS. The ANOVA for mean somatic cross sectional area determined an effect of diet in males [$F(2,18) = 5.76$; $p < 0.05$] but not females. The PLSD further revealed that GC males contained an increased somatic cross sectional area compared with SC males ($p < 0.05$), while EtOH males contained a reduced somatic cross sectional

area compared with GC males ($p < 0.01$). No significant gender differences were noted in ChAT+ neuronal size for EtOH, GC, or SC animals.

MS volume

MS volume was determined by multiplying the number of 40 μm sections analyzed for a given animal by the mean area per section for that animal. Figure 3-4 presents the mean MS volume of P60 rats. The ANOVA demonstrated no effect of diet in males or females on the mean number of alternate sections through the MS. No significant gender differences in MS volume were noted for EtOH, GC, or SC animals.

Discussion

The major conclusion of the present work is that small reductions in the size of ChAT+ neurons of the medial septum are produced by neonatal ethanol exposure while changes in the number of cholinergic neurons or size of the MS are not noted. This study has also determined that long-lasting microencephaly is found in male and female P60 rats exposed to ethanol via artificial rearing from P4-10.

Because EtOH animals showed no difference when compared with GC animals in total ChAT+ neuronal number or mean number of cholinergic neurons per section, ethanol exposure did not directly reduce ChAT+ neuronal number. However, since EtOH males contained fewer cholinergic neurons when compared with SC males it appears that ethanol in combination with artificial rearing does affect these neurons. No changes were noted in mean area per section, cell density, or MS volume as a result of ethanol treatment in males. Female animals similarly exposed to ethanol did not contain a reduced number of ChAT+ MS neurons and no changes were noted in MS volume, mean

area per section, or cell density. A permanent reduction in the size of ChAT+ neurons was noted in EtOH males compared with GC males. However, this was concurrent with an increase in size of cholinergic neurons in GC compared with SC males. Moreover, no differences in cell size were found in female rats, making it difficult to determine whether the reduction in ChAT+ cell size in EtOH males is an effect of ethanol on neuronal size or merely an effect of diet and artificial rearing on GC males. Thus neonatal ethanol exposure, while producing long-lived microencephaly, has little effect on the cholinergic neurons of the adult rat MS, as measured by ChAT+ neuronal number and morphology.

Long-term effects of ethanol on brain growth

Microcephaly and microencephaly are hallmarks of FAS as well as neonatal animal models of FAS (West and Pierce, 1986). Reduced brain size represents one of the most reproducible features of ethanol-induced alterations in the brain, and the current study is no exception. Artificial rearing from P4-10 produced no long-lasting deficits in body weight and by P60 EtOH and GC pups weighed the same as SC pups. Despite the lack of effect on body weight, EtOH animals of both genders showed reduced brain weight at P60 compared with GC and SC animals. Brain to body weight ratio (br/bd) was similarly reduced in both males and females exposed to ethanol. Taken together, the reduced brain weight of EtOH animals and the reduced br/bd in EtOH animals demonstrate that neonatal ethanol exposure produced long-lasting microencephaly. Goodlett et al. (Goodlett et al., 1991) exposed rats from P4-9 in two of the 12 daily feedings as a 10.2% solution (an exposure paradigm similar to the current experiments). Exposure to this concentration of ethanol resulted in a peak BEC of 361 mg/dl (compared with 269 mg/dl in the current study) and produced microencephaly in both genders in

adult animals. The current study demonstrates that even with a lower peak BEC, brain growth is severely compromised.

The degree of ethanol-induced reduction in brain weight depends directly on the peak BEC produced. Bonthius and West (1991) provided an illustration of this by comparing the effects of a 6.6 g/kg/day ethanol treatment spread out over 12 feedings from P4-11 with the effects of 4.5 g/kg/day ethanol treatment given in just two feedings. Animals were sacrificed at P90 to observe long-term effects of neonatal ethanol delivery on brain weight. Interestingly, they found that the lower 4.5 g/kg/day dose produced profound brain weight and brain to body weight ratio deficiencies, while the 6.6 g/kg/day group exhibited no such microencephaly. This differential response to varying ethanol delivery patterns was directly related to peak BEC as the higher dose spread out over 12 feedings produced a peak BEC of only 43 mg/dl while the lower dose condensed into two feedings produced a peak BEC of 318 mg/dl (Bonthius and West, 1991). Thus, the pattern of exposure plays a critical role in the severity of ethanol-induced teratogenicity.

Long-term effects of ethanol and artificial rearing on ChAT+ neurons in the MS

Artificial rearing and ethanol exposure produced a decrease in the mean ChAT+ neuronal number per section in EtOH males compared with SC males in the absence of volume, area, or density changes. Female animals displayed no differences in mean cholinergic neuronal number per section, volume, area, or density measurements and, thus, the reduction occurs in a sexually dimorphic manner. Because the reduction in ChAT+ neuronal number noted in males was between the gastrostomized EtOH group and the dam-reared SC group (and not the pair-fed GC group) the reduction appears to be the result of an interaction between ethanol treatment and some characteristic of the

artificial rearing technique rather than the result of ethanol alone. Kelly (1997) has noted an effect of artificial rearing on the concentration of neurotransmitters in the SH system of male rats: neurotransmitter concentration was reduced in artificially reared males independent of ethanol exposure. While the current results do not implicate artificial rearing in reducing cholinergic neuronal number (the difference between GC and SC males in mean ChAT+ neurons per section is not significant) they do suggest that ethanol in combination with artificial rearing can reduce ChAT+ neuronal number. Perhaps an interaction between ethanol and the stress of the procedure (from invasiveness or maternal separation) accounts for the reduced cholinergic neuronal number in artificially reared and ethanol exposed pups.

Developmental ethanol exposure and ChAT+ neuronal number

The current finding that cholinergic neuronal number in the MS is unaffected by ethanol alone is consistent with previous experiments from our laboratory. Swanson et al. (1995; 1996) demonstrated reduced ChAT activity in P14 rats chronically exposed to ethanol in utero. However, analysis of ChAT+ neuronal number in the MS of P14 and P60 rats revealed no effect of diet treatment on cholinergic cell number. Similarly, evidence from Heaton et al. (1996) showed that ChAT+ neuronal number is not significantly affected in the striatum of male or female rats following CPET. A transient increase in cholinergic neuronal number was noted in the striatum on P14, but this was reduced to control levels by P60.

While studies from our laboratory (see above) have not indicated an effect of developmental ethanol exposure on cholinergic neuronal number in the rat MS or striatum, studies from other groups have demonstrated ethanol-induced alterations in

ChAT+ number, particularly in the MS. For example data suggest that acute prenatal exposure of mice to ethanol resulted in severe midline anomalies at embryonic day 18, including loss of basal forebrain neurons (Schambra et al., 1990) as well as loss of septal neurons and reduced volume at P15 (Ashwell and Zhang, 1996). However, it should be noted that these experiments produced high acute BECs (600-700 mg/dl) which were the result of one or two ethanol exposures early in embryonic development. Thus, differences in timing and dosage may account for the apparent discrepancy (Swanson et al., 1996).

Exposure of adult rats to ethanol for 12 or 28 weeks has been reported to reduce cholinergic neuronal number in the MS (Arendt et al., 1995; Arendt et al., 1988). However, it should be noted that comparisons were made between rats exposed to ethanol as a 20% solution of drinking water and rats fed chow and water ad libitum; no pair-fed controls were examined. Thus, it is possible that the reduction of cholinergic neurons in the MS is not due to the influence of ethanol alone. For instance, an interaction between ethanol and malnutrition may explain the noted differences. However, it is conceivable that chronic ethanol exposure during mature stages may reduce the number of MS cholinergic neurons, as found by Arendt et al. (1995; 1988). Regardless, the current data in combination with the Swanson et al. (1996) and Heaton et al. (1996) studies suggests that cholinergic neuronal number in the MS is not susceptible to moderately-high or moderate doses of ethanol during early postnatal or prenatal development, respectively. A future study examining ChAT+ neuronal number after exposure to a higher peak BEC would determine whether the apparent lack of effect of ethanol on cholinergic neurons is due to the moderate doses used in these studies.

Developmental ethanol exposure and ChAT+ neuronal size in the MS

Although ChAT+ neuronal counts were not directly affected by ethanol, analysis of the mean somatic cross sectional area of cholinergic neurons in the P60 MS demonstrated a diet effect in male animals. Specifically, neuronal size was reduced in EtOH males compared with GC males. However, GC males contained larger neurons compared with SC males and no differences were found in females, making it difficult to determine whether this is an effect of ethanol on cholinergic neuronal size or merely an effect of diet and artificial rearing on GC males. It is unlikely that the smaller size of cholinergic neurons in EtOH males is simply because of the noted microencephaly since the brains of EtOH and SC males were different in size but the mean size of ChAT+ neurons was similar.

It is possible, however, that GC males experienced an increase in somal size in response to either the stress of the artificial rearing procedure (such as maternal separation, isolation, or the invasive surgery) or to some component of the maltose-dextrin containing milk diet. Factors such as nerve growth factor (NGF) can induce hypertrophy of ChAT+ basal forebrain neurons (Higgins et al., 1989). NGF is also known to increase the size of cholinergic neurons in cultures of rat septal neurons (Markova and Isaev, 1992), and is able to reverse axotmy-induced decreases in MS cholinergic neuronal cell bodies (Hagg et al., 1989). It is conceivable that male GC animals increased the expression of NGF, as is known to occur following stress (Smith, 1996), resulting in larger cholinergic MS neurons. However, female GC animals were similarly stressed and did not display hypertrophy of ChAT+ neurons. Perhaps a more likely explanation is that a constituent of the liquid diet acted in a trophic manner on these

neurons. Whatever the explanation, these data are consistent with data from other investigators who have reported sexually dimorphic effects on the size of cholinergic neurons of the MS. For example, neonatal hyperthyroidism is capable of increasing ChAT+ neuronal size in the MS of male animals while simultaneously decreasing the size of ChAT+ neurons in females (Westlind-Danielsson et al., 1991).

One other report from our laboratory has examined cholinergic neuronal size at P60 following chronic prenatal ethanol exposure (Swanson et al., 1996). While the exposure times were different in the current study, it is useful to compare the present data with the Swanson et al data. The size of the ChAT+ neurons at P60 is quite similar. For example, the range of neuronal size in the Swanson et al. study was 90-100 μm^2, which is comparable to the range noted in the current study (80-110 μm^2). However, in contrast to the present data, Swanson et al. (1996) found no ethanol-induced alterations in ChAT+ neuronal size, and found no gender differences.

ChAT+ neuronal insusceptibility: possible role of neurotrophic factors

Why is ChAT+ neuronal number apparently unaltered following developmental ethanol exposure? One possible explanation is the importance of neurotrophic factors in regulating the development and maintenance of the SH system. The hippocampus is known to express a rich variety of neurotrophic factors, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and basic fibroblast growth factor (Ernfors et al., 1990; Maisonpierre et al., 1990). The high affinity receptors for the various neurotrophins found in the hippocampus, including trk A, trk B, and trk C, are also present in the developing septum, including the cholinergic neurons of the medial septum (Ringstedt et al., 1993). Evidence suggests a role for neurotrophins in regulating the

expression of ChAT in the basal forebrain. Intraventricular injection of NGF antibodies into rat neonates reduces ChAT immunostaining in the septum (Vantini et al., 1989). Moreover, mice which have been genetically engineered to lack the various neurotrophin receptors have been generated, and have further defined a role for neurotrophins in sustaining septal cholinergic neurons in vivo. For example, mice which lack trk A show reduced acetylcholinesterase activity in septal projection fibers (Smeyne et al., 1994), and mice which lack BDNF show reduced density of cholinergic neurons in the MS (Jones et al., 1994).

In vitro studies have also demonstrated a role for neurotrophins in sustaining septal neurons in normal conditions and in the presence of ethanol. For example, NGF stimulates ChAT activity, neuritic complexity, increased fiber length, and increased fiber outgrowth in cultures of septal cholinergic neurons (Hartikka and Hefti, 1988). Moreover, studies from our laboratory have demonstrated that NGF protects both cultured dorsal root ganglion neurons and septal neurons from ethanol neurotoxicity (Heaton et al., 1993; Heaton et al., 1994). It is conceivable that neurotrophic interactions are preserved in the current study, and that ethanol is unable to produce toxic effects on cholinergic neurons in the medial septum because these normal interactions are maintained. A consequence of this maintenance may be growth factor-mediated induction of protective molecules of the bcl-2 family of cell death molecules which would prevent ethanol-induced toxicity in this population. Similar mechanisms are known to operate in cerebellar granule cells during development. For example, Muller et al. (1997) demonstrated that NGF induces Bcl-2 protein expression and that this induction resulted in cell survival.

Conclusions

The current study and several others from our laboratory have demonstrated little effect of developmental ethanol exposure on cholinergic neurons in the MS. Prenatal ethanol exposure does not significantly reduce ChAT+ neuronal number in the basal forebrain (Swanson et al., 1996) or striatum (Heaton et al., 1996). If neither prenatal nor postnatal ethanol exposure in rats affects the cholinergic neurons in the MS, then what accounts for the impairment of spatial learning tasks in rats exposed to ethanol neonatally (Goodlett and Peterson, 1995; Kelly et al., 1988)? A possible explanation for these previously described spatial learning deficits is that a reduction of GABAergic neurons in the basal forebrain following neonatal ethanol exposure may alter normal learning of spatial tasks. Although the GABAergic component of the SH system has not been as extensively characterized as the cholinergic component, it is known to control activity of cholinergic neurons in the basal forebrain (Dudchenko and Sarter, 1991) and is necessary to influence hippocampal electrical activity (Smythe et al., 1992). A recent study from our laboratory showed a 42% reduction in parvalbumin immunoreactive neurons of the female MS following prenatal ethanol exposure (Moore et al., 1997). Parvalbumin is located within the GABAergic neurons of the MS (Freund, 1989; Krzywkowski et al., 1995). Additionally, parvalbumin immunoreactive GABAergic neurons of the anterior cingulate cortex are reduced in male and female rats following prenatal ethanol treatment (Moore et al., 1998b). Future investigations will determine whether these newly discovered neuroanatomical alterations resulting from developmental ethanol exposure can explain the noted cognitive impairments of FAS children.

Table 3-1. Mean body weight, brain weight, and brain weight to body weight ratio (br/bd) of postnatal-day 60 (P60) animals exposed to ethanol or control conditions from P4-10*

Diet group	N	P60 body weight (g)	P60 brain weight (g)	P60 br/bd (%)
Males				
EtOH	15	270 ± 6.780	1.47 ± 0.026‡	0.544 ± 0.010§†
GC	8	258 ± 7.610	1.61 ± 0.035	0.626 ± 0.024†
SC	8	289 ± 14.60	1.65 ± 0.019	0.578 ± 0.030†
Females				
EtOH	9	203 ± 8.450†	1.44 ± 0.032‡	0.712 ± 0.020¶
GC	8	182 ± 5.770†	1.53 ± 0.033	0.842 ± 0.018
SC	10	191 ± 7.000†	1.59 ± 0.029	0.841 ± 0.026

* All measures are expressed as mean ± SEM and are representative of nine artificially reared litters.

† Significantly reduced, compared with other gender of same group ($p < 0.0001$).

‡ Significantly reduced, compared with GC and SC animals of same gender ($p < 0.05$).

§ Significantly reduced, compared with GC males ($p < 0.01$).

¶ Significantly reduced, compared with GC and SC females ($p < 0.01$).

Table 3-2. Number of sections, medial septum (MS) area per section, and choline acetyltransferase-immunoreactive (ChAT+) neuronal density in the adult rat MS of postnatal-day 60 (P60) animals exposed to ethanol or control conditions from P4-10*

Diet group	N	Number of 40 μm sections	MS area/section (mm^2)	Density (ChAT+ neurons/mm^2)
Males				
EtOH	8	16.4 ± 0.625	0.319 ± 0.015	216 ± 13.2
GC	5	16.8 ± 1.020	0.313 ± 0.011	230 ± 19.8
SC	8	17.6 ± 0.460	0.353 ± 0.009	237 ± 14.5
Females				
EtOH	4	16.8 ± 0.479	0.345 ± 0.018	213 ± 7.79
GC	5	16.6 ± 0.678	0.358 ± 0.017	220 ± 13.7
SC	8	16.6 ± 0.625	0.382 ± 0.023	220 ± 18.9

*All measures are expressed as mean ± SEM and are representative of approximately one-half of the total MS (alternate sections were analyzed). No significant differences were noted. Animals are a subset of nine artificially reared litters.

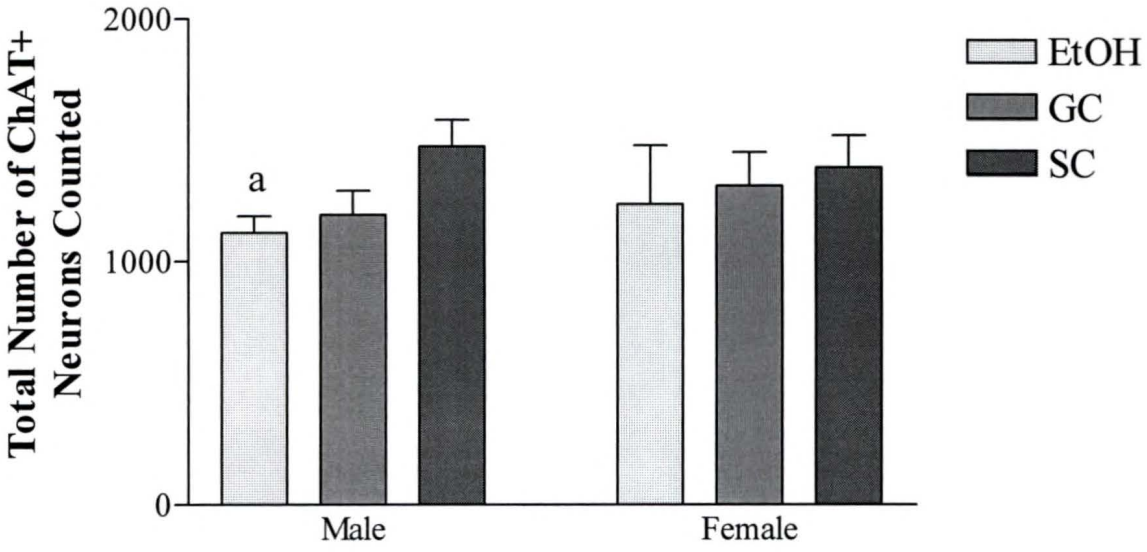


Figure 3-1. Mean total number of choline acetyltransferase-immunoreactive (ChAT+) neurons in the medial septum of postnatal day 60 (P60) rats. Rats were artificially reared from P4-10 and exposed to ethanol (EtOH), artificially reared and pair-fed (GC), or dam-reared (SC). Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with SC males ($p < 0.05$).

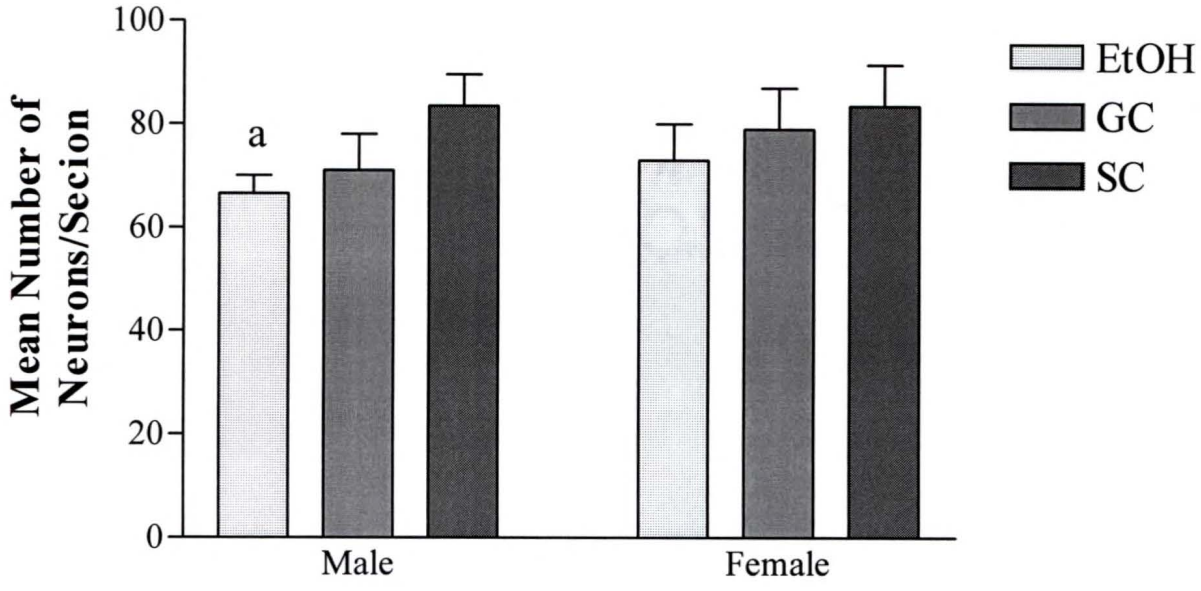


Figure 3-2. Mean number of choline acetyltransferase-immunoreactive neurons per section detected on alternate 40 μm sections through the medial septum of postnatal day 60 (P60) rats. Rats were artificially reared from P4-10 and exposed to ethanol (EtOH), artificially reared and pair-fed (GC), or dam-reared (SC). Data are expressed as mean ± SEM. a: significantly reduced compared with SC males ($p < 0.05$).

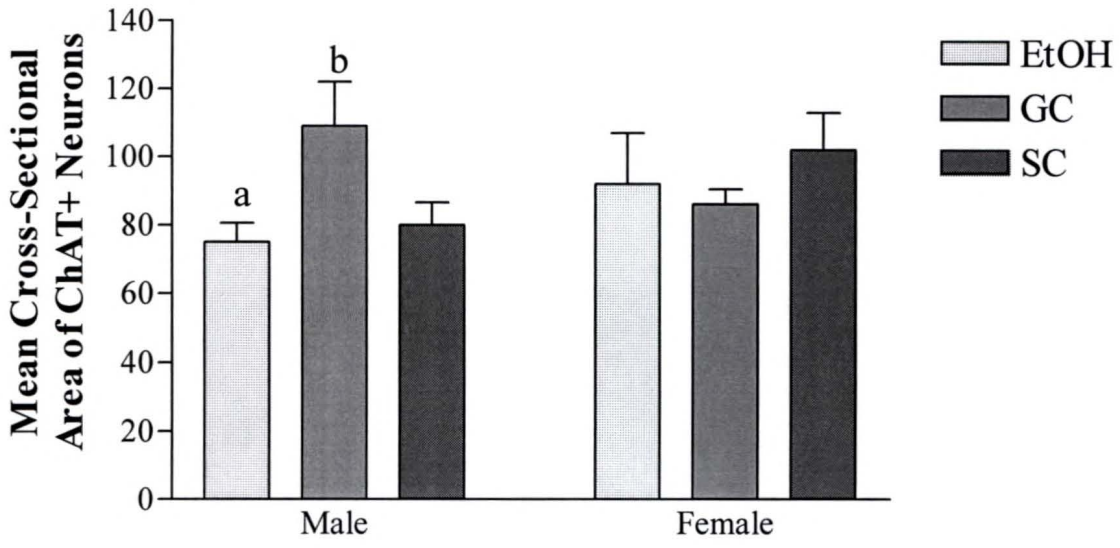


Figure 3-3. Mean somatic cross sectional area (μm^2) of choline acetyltransferase-immunoreactive (ChAT+) neurons in the medial septum of postnatal day 60 (P60) rats. Rats were artificially reared from P4-10 and exposed to ethanol (EtOH), artificially reared and pair-fed (GC), or dam-reared (SC). Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with GC males ($p < 0.01$). b: significantly increased compared with SC males ($p < 0.05$).

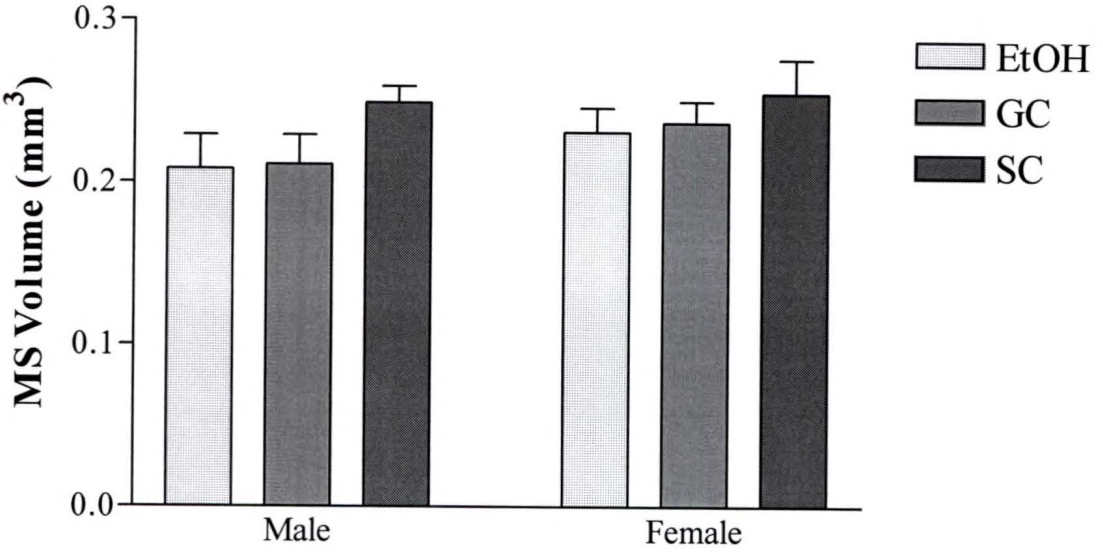


Figure 3-4. The mean medial septum (MS) volume of postnatal-day 60 (P60) rats. Rats were artificially reared from P4-10 and exposed to ethanol (EtOH), artificially reared and pair-fed (GC), or dam-reared (SC). Data are expressed as mean $\pm$ SEM. No significant differences were noted.

CHAPTER 4
EFFECTS OF NEONATAL ETHANOL EXPOSURE ON PURKINJE AND GRANULE CELLS AND BCL-2 FAMILY MRNA LEVELS IN THE RAT CEREBELLAR VERMIS

Summary

While many neuronal populations exhibit vulnerability to ethanol delivered during critical developmental stages, the cellular mechanism of ethanol's toxicity remains unknown. Often neuronal susceptibility within the brain is determined by the timing of the ethanol exposure. The principal neurons of the cerebellum (the Purkinje and granule cells) provide a good example of this differential temporal vulnerability to ethanol. It has been documented in studies by a number of investigators that first postnatal week ethanol exposure results in substantial Purkinje and granule cell loss while second postnatal week exposure does not (Goodlett and Eilers, 1997; Hamre and West, 1993). The objective of the present work was to test the hypothesis that differential ethanol-induced cerebellar cell death during development is related to ethanol-induced alterations in the expression of bcl-2 family of cell survival and death genes.

Rats were exposed to ethanol or control conditions during the neonatal period and transcript levels of bcl-2 family members relative to cyclophilin were determined. Pups exposed in parallel were taken for cerebellar cell counts. Ethanol exposure during the first postnatal week significantly reduced Purkinje and granule cell numbers by postnatal day 21 (P21). Acute first postnatal week ethanol exposure up-regulated mRNA

transcripts encoding the cell death-promoting molecules bax and bcl-xs as measured on P4. An additional day of exposure on P5 resulted in no further alterations in bcl-2 family transcripts, likely because Purkinje cell death was detectable as early as P5.

To determine whether pro-apoptotic gene expression changes were specific to first postnatal week ethanol neurotoxicity, we examined bcl-2 family mRNA levels in rats exposed to ethanol during a developmental period of cerebellar insusceptibility, the second postnatal week. Exposure on P7-8 produced no cerebellar cell death, but also resulted in increased levels of bax, though only after two-day ethanol exposure and not after acute exposure on P7. These data implicate altered expression of pro-apoptotic members of the bcl-2 gene family in acute ethanol-mediated cerebellar cell death during the first postnatal week. They also suggest that the differential survival of cerebellar neurons following ethanol exposure during more mature developmental stages may be related to more successful suppression of pro-apoptotic processes.

Introduction

Evidence of cerebellar vulnerability to developmental ethanol exposure comes from human studies demonstrating size reduction in the cerebellar vermis of children exposed prenatally to ethanol (Sowell et al., 1996). Moreover, in rat models of fetal alcohol syndrome, ethanol exposure during the first postnatal week results in decreased numbers of Purkinje and granule cells in the cerebellum, with the cerebellar vermis particularly affected (Goodlett and Eilers, 1997; Hamre and West, 1993). An interesting property of the cerebellar vermis is that it is differentially susceptible to ethanol. That is, ethanol exposure to more mature neonates, during the second postnatal week, does not

result in profound cerebellar neurotoxicity (Goodlett and Eilers, 1997; Hamre and West, 1993).

While cell loss following ethanol exposure during critical developmental periods is well documented, no molecular mechanisms of ethanol-mediated cell death are known. There is reason to hypothesize that the bcl-2 family of cell death molecules is involved in ethanol neurotoxicity. The bcl-2 family of genes is comprised of cell death regulators, and bcl-2 was the first of this family to be discovered. In recent years, a number of genes similar to bcl-2 have been found. These include bax and bad as well as bcl-x, whose mRNA is alternatively spliced into long (bcl-xl) and short (bcl-xs) forms (Reed, 1994). Some members of the bcl-2 gene family serve to inhibit cell death (e.g. bcl-2, bcl-xl, a1, mcl-1) and others promote cell death (e.g. bcl-xs, bax, bad, bak). It is the intracellular ratio of cell death repressor to cell death effector molecules which determines whether a cell will undergo apoptosis (Oltvai et al., 1993).

The suggestion that the bcl-2 family might be involved in ethanol-induced cell death in the cerebellum is supported by the literature. Cell death following ethanol exposure appears to proceed through an apoptotic mechanism, and cerebellar neurons undergo apoptosis in vitro and in vivo in response to ethanol (Bhave and Hoffman, 1997; Liesi, 1997; Renis et al., 1996; Singh et al., 1995). Furthermore, a role in regulating the death and survival of cerebellar neurons has been demonstrated for the bcl-2 family. Gillardon et al. (1995) investigated bcl-2 and bax gene expression in the cerebella of Purkinje-cell-degeneration mice (mutants that lose nearly all of their Purkinje cells between P22-28 following otherwise normal development). They found that bcl-2 mRNA levels decreased while bax mRNA levels remained unchanged beginning on P22.

In addition, thyroid hormone-induced upregulation of bcl-2 protects early-differentiating cerebellar granule cells from apoptosis in vitro (Muller et al., 1995), and transgenic mice overexpressing bcl-2 contain more cerebellar Purkinje and granule cells than controls whether transgene expression begins embryonically or neonatally (Zanjani et al., 1996; Zanjani et al., 1997). Additionally, data from our laboratory have indicated a role for bcl-2 in protecting cerebellar neurons in vivo. Transgenic mice overexpressing bcl-2 in nervous tissue are resistant to neonatal ethanol-induced Purkinje cell death (Heaton et al., unpublished observation).

Although the cerebellar expression patterns of some bcl-2 family genes, including bad and bak, are unknown, many bcl-2 family members, including bax, bcl-xs, bcl-xl, and bcl-2, are expressed throughout the brain during development, and their distribution includes cerebellar Purkinje and granule cells (Castren et al., 1994; Frankowski et al., 1995; Krajewski et al., 1994; Rouayrenc et al., 1995). Purkinje cells express high levels of both forms of the bcl-x gene in development and adulthood (Dixon et al., 1997; Frankowski et al., 1995), high levels of bax early in development, which is down-regulated in adults (Krajewski et al., 1995; Vekrellis et al., 1997), and low levels of bcl-2 (Hara et al., 1996). Granule cells express high levels of bcl-2 initially in development, but down-regulate it during adulthood (Castren et al., 1994). The bax gene is known to be expressed in granule cells during development (Gleichmann et al., 1998; Miller et al., 1997), as are both forms of the bcl-x gene (Gleichmann et al., 1998).

Studies utilizing genetically engineered mice have described roles for various members of the bcl-2 gene family in vivo. For instance, transgenic mice overexpressing bcl-2 in the nervous system show reduced developmental cell death in certain neuronal

populations, and show a general hypertrophy of the nervous system (Martinou et al., 1994). Neurons from these animals are better able to withstand ischemic episodes, removal of growth factor support, and axotomy (Dubois-Dauphin et al., 1994; Farlie et al., 1995). Additionally, mice overexpressing bcl-xl contain facial motoneurons that are resistant to axotomy during the postnatal period (Parsadanian et al., 1998).

Mice lacking the bcl-2 gene have been instrumental in defining the normal actions of this gene in vivo. A role for bcl-2 maintenance of motoneurons, sympathetic, and sensory neurons was indicated through analysis of cell numbers in these regions in bcl-2 knockout mice. After the PCD period, bcl-2 knockouts contained fewer neurons in each of these areas relative to controls (Michaelidis et al., 1996). Inactivation of the bcl-x gene is lethal, as mice lacking this gene die around E13. Investigation of fetuses revealed extensive apoptotic cell death in neuronal populations of the brain, spinal cord, and dorsal root ganglion (Motoyama et al., 1995). This indicates the importance of bcl-x in embryonic life.

Mice lacking the bax gene have also been generated and have determined a role for cell-death promoting molecules in sculpting neuronal populations (Deckwerth et al., 1996). For example, sympathetic and facial motoneurons from bax knockouts survive early postnatal growth factor deprivation and axotomy. Moreover, the normal process of cell death is disrupted in the superior cervical ganglia and facial nuclei of bax knockouts since these structures possess more surviving neurons after the period of apoptosis (Deckwerth et al., 1996). Given these demonstrated roles for bcl-2 family members in vivo, it was speculated that ethanol-induced alterations in the levels of these molecules might influence cell death in the cerebellar vermis.

The objective of the present study was to test the hypothesis that ethanol-induced cerebellar neurotoxicity during development is related to alterations in the expression of bcl-2 family genes. To accomplish this, rats were artificially reared and exposed to ethanol or control conditions for one or two days during the neonatal period. Ethanol was delivered during periods of vermis susceptibility (first postnatal week) and, for comparison, during a period of vermis insusceptibility (second postnatal week). mRNA levels of four bcl-2 family members (with known cerebellar expression) and the internal standard cyclophilin were determined. Pups exposed in parallel were sacrificed for cerebellar cell counts.

Materials and Methods

Experimental design

First and second postnatal week ethanol neurotoxicity and its relation to bcl-2 gene expression was examined in the rat cerebellar vermis since this region is particularly vulnerable to ethanol (Goodlett and Eilers, 1997; Hamre and West, 1993). First postnatal week exposure occurred on P4 only, or on P4 and P5. Second postnatal week exposure occurred on P7 only, or on P7-8. The relative levels of bcl-2 family mRNAs were determined two hours after ethanol exposure on each exposure day. For example, some pups were killed two hours after exposure on P4, while others were killed on P5 following two days of exposure on P4-5. The same is true for pups used for the second postnatal week experiments. We examined these acute timepoints because we anticipated that gene expression changes would occur rapidly following the ethanol insult. Cerebellar cell counts were performed at a common time, P21, following first or second

week exposure to ensure that differences in exposure times did not contribute to any noted differences in cell death. In order to determine the timecourse of first postnatal week cell death, some animals were sacrificed on P5 following ethanol treatment on P4-5.

Subjects and ethanol treatment

Sprague Dawley rat pups were obtained from timed pregnant dams from Harlan Sprague Dawley (Indianapolis, IN). Dams were housed with a 07:00-19:00 light cycle under controlled temperature and humidity conditions. On the morning that ethanol treatment began (P4 or P7), male pups were randomly assigned to one of three groups: ethanol receiving gastrostomized pups (EtOH); pair-fed gastrostomy controls (GC); and dam-reared suckle controls (SC), used to determine whether non-specific effects of artificial rearing are produced. Because our aim was to establish this model system in our hands, and since earlier investigations used only male pups, we decided to utilize male, and not female, animals in this study. Also on the morning that ethanol treatment began, a gastrostomy feeding tube was surgically implanted into the stomach of EtOH and GC pups under methoxyfluorane anesthetic and secured on the outside by a small plastic washer. Pups were reared individually in plastic cups filled with bedding and a fur-like material. Cups were floated in a covered, heated aquarium (40°C) and pups were maintained on a 07:00-19:00 light cycle.

Gastrostomized pups were infused with a liquid diet containing evaporated milk, sterile water, soy protein, L-methionine, L-tryptophan, calcium phosphate, deoxycholic acid, a vitamin mixture, and a mineral mixture (West et al., 1984). Pups received the

milk formula in feeding periods of 20 minutes each. EtOH pups received ethanol-supplemented formula as a 15.0 % v/v solution for two feedings on each exposure day for a total of 6.6 g/kg/day (the remaining 10 feedings were of milk alone). This pattern of exposure produced a mean blood ethanol concentration (BEC) of 335 $\pm$ 41 mg/dl dl as determined 2 hours after the final ethanol treatment on the first day of exposure. These days and this timepoint were chosen to measure BEC because they are known to represent the peak BEC for this pattern of exposure (Goodlett et al., 1990). GC pups received an isocaloric amount of maltose-dextrin-supplemented formula for two feedings on each day (the remaining 10 feedings were of milk alone). Gastrostomized pups were weighed daily and the daily diet consumption volume (in mls) was equivalent to 33 % of the mean litter body weight. The liquid diet was administered to the EtOH and GC pups by connecting the gastrostomy tubes to a feeding line connected to diet-filled syringes held within a Stoelting (Wood Dale, IL) programmable infusion pump.

Pups acutely exposed to ethanol (one-day treatment on P4 only or P7 only) received ethanol infusions only on the day of surgery. Pups exposed for two days received milk diet alone for the remaining 10 feedings on the first exposure day, and received two additional ethanol feedings on the morning of the second exposure day (P5 or P8). Pups used for mRNA analysis were sacrificed two hours after the last ethanol feeding on the final exposure day (the day of sacrifice; P4, P5, P7, or P8), as were pups taken for cell counts and anatomical analysis on P5. Pups exposed in parallel for P21 cell counts and anatomical analysis were returned to the dam after one- or two-day ethanol treatment, and raised by the dam until sacrifice at P21.

Histological procedures

On the day of sacrifice pups were anesthetized and decapitated, and the cerebellum was dissected away from the cerebral hemispheres and placed in Bouin's fixative. Following fixation, specimens were dehydrated, cleared, and embedded in paraffin. Serial sagittal 6 μm sections were then cut through the midline of the vermis, mounted onto glass slides and stained with hematoxylin and eosin (H&E).

Cerebellar cell counts

Manual cell counts of Purkinje and granule cells were performed and expressed as mean cells per section. While stereological cell enumeration has become a common method of cell counting, manual counting remains a viable alternative for cell number quantitation (Guillery and Herrup, 1997). In fact, recent data have demonstrated the utility of manual cell counts (Clarke and Oppenheim, 1995) and show a direct correlation between cell counts performed manually and cell counts performed with the optical dissector (Hagg et al., 1997). Criticisms of manual cell counting methods (and data expressed as mean cells per section) are that overprojection and truncation can result from incorrectly identifying cells within a focal plane, and that changes in reference volume can bias cell counts (Peterson et al., 1997). The choice of manual cell counting in the present study is appropriate for three reasons: first, thin sections were counted and the three chosen sections which were counted were separated by 60 μm. Second, vermis reference volume and lobule area were measured to determine whether ethanol treatment altered vermis size. Third we only wished to compare the mean number of cells per section between groups and did not intend to estimate total cell number (Hagg et al., 1997). An additional justification comes from recent reports that have utilized

stereological techniques to count cerebellar neurons following developmental ethanol treatment (Goodlett and Johnson, 1997; Napper and West, 1995a). These data are consistent with previous findings using manual cell counting and, in fact, have documented very similar Purkinje and granule cell losses following ethanol exposure (Hamre and West, 1993).

Prior to counting of Purkinje and granule cells, slides were coded and randomized with an identifying number. Cells were counted only on sections from the midline vermis, where lobule I and X are closest to each other, and deep cerebellar nuclei are absent. Cell counts were performed in the manner of Hamre and West (1993) on three sections separated by 60 μm to ensure that the same cell is not counted twice. Purkinje cells in lobule I were counted if they contained a well-delineated nuclear membrane, distinct nucleolus, darkly stained cytoplasm, and were clearly Purkinje cells based on location and size. The average number of Purkinje cells per section in lobule I was determined for each animal and means recorded for each group. Counts were performed in lobule I because, although all cerebellar lobules are susceptible to ethanol, the more anterior lobules display heightened vulnerability (Hamre and West, 1993). Granule cells were counted within a grid measuring 2.14 mm^2 that was placed in the granule layer of lobule I. Counts were performed on three coded midline sections. For cell counts on P5, both the external and internal granule cell layers were counted.

Anatomical measurements

Anatomical measurements were taken in order to determine whether ethanol treatment significantly altered the size of the vermis. Vermis volume was estimated by counting the number of sections where lobules I and X are both visible. Lobule I length

was also measured with an eyepiece micrometer to find whether treatment altered the length of the lobule. The diameter of Purkinje and granule cell nuclei was also measured with an eyepiece micrometer to determine whether treatment altered the size of Purkinje or granule cells. Cerebellar cell counts and anatomical measures were performed on 3-6 pups per group for every timepoint investigated.

Preparation of antisense RNA probes

cDNAs for bcl-2, bcl-xl, bcl-xs, and bax were cloned from an adult Sprague-Dawley cerebellum. Total RNA was extracted and reverse transcribed into first strand cDNA using oligo(dt)-primer and reverse transcriptase (Invitrogen cDNA cycle kit, Carlsbad, CA). Gene-specific PCR primers were designed for regions of low homology based on known rat cDNA sequences for bcl-2, bcl-xl, bcl-xs, and bax (Tilly et al., 1995). First strand cDNA was taken through 30 cycles of PCR amplification and then ligated into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA). E. coli competent cells (JM109) were transformed with the recombinant plasmid and plated. DNA sequencing (both strands) was performed by the DNA Sequencing Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida, to verify clones. Basic local alignment search tool analysis of the four cDNA clones revealed at least 98% nucleotide sequence homology with bcl-2, bcl-xl, bcl-xs, and bax previously cloned in rat (Tilly et al., 1995).

Antisense RNA probes complimentary to bcl-2, bcl-xl, bcl-xs, and bax mRNA coding sequences were synthesized in vitro from linearized plasmid templates. Only bcl-2 family members with a demonstrated expression in the cerebellum were examined. In vitro transcription was carried out at 37°C for 40 minutes, and was followed by treatment

with DNase for 15 minutes (also at 37°C). Two phenol-chloroform extractions were performed followed by precipitation with ammonium acetate and ethanol. An aliquot of probe was measured with a scintillation counter to determine counts per minute (CPM). ^{32}P-labeled cyclophilin probe (template from Ambion, Austin, TX), transcribed as described above, was included in each hybridization reaction along with individual bcl-2, bcl-xl, bcl-xs, and bax probes and served as a standard. The use of cyclophilin was appropriate because its transcription was unaltered in previous studies of mRNA expression following chronic ethanol treatment in adult male rats and embryonic rats (MacLennan et al., 1995; Maier et al., 1996).

Solution hybridization and RNase protection

The use of a gross technique such as the RNase protection assay to measure relative mRNA levels is appropriate for this study since expression of bcl-2 and other family members is known to be at low to undetectable levels in glia (Frankowski et al., 1995; Vyas et al., 1997). Thus, any observed changes in relative transcript levels are unlikely to be due to expression changes in non-neuronal cell types. Total RNA was extracted from the cerebellar vermis with the RNA STAT-60 extraction kit (Tel-Test Inc, Friendswood, TX), coded, and randomized with an identifying number, and stored at -80°C until hybridization. Samples were prepared for hybridization by precipitating 2 μg total RNA and 18 μg E. coli tRNA (to aid in pelleting) at -20°C with ammonium acetate and ethanol for 30 minutes, followed by centrifugation for 15 minutes. After allowing the pellet to dry, it was dissolved in 10 μl hybridization buffer containing the radiolabled probes of interest diluted at the appropriate CPM (about 100,000 for bcl-2 family probes and 20,000 for cyclophilin). Each individual probe of the bcl-2 family was hybridized to

sample RNA along with cyclophilin probe. Following incubation for 10 minutes at 80°C, hybridization was carried out at 45°C overnight.

The following day, each hybridization product was treated with a mixture of RNases A and T1 at 37°C for 60 minutes. This served to destroy any incomplete hybrids or single-stranded RNA in the reaction, leaving only double-stranded probe:mRNA hybrids. Proteinase K was used to hydrolyze the RNases. Hybrids were extracted with phenol and chloroform, were precipitated at -20°C for 30 minutes, and were then centrifuged for 15 minutes at 4°C. Pellets were dissolved in formamide loading buffer, denatured by boiling, quickly placed on ice for 30 seconds, and were then separated by polyacrylamide gel electrophoresis. After drying, gels were exposed to a phosphor imaging screen overnight. Band intensity was read as an optical density (OD) by imaging software. The OD of the bcl-2 family bands was normalized by dividing the OD of the cyclophilin band from each animal. Normalized OD from each probe (bcl-2, bcl-xl, bcl-xs, and bax) was averaged from 6-8 animals from each treatment group (EtOH, GC, and SC).

Other analyses

Blood ethanol concentration, determined from trunk blood on the day of sacrifice, was assayed with the 333-UV microenzymatic assay (Sigma, St. Louis, MO). Statistics were performed with the program Statview (Abacus Concepts, Berkeley, CA). One-way analysis of variance (ANOVA) was used to determine significant treatment effects. Fisher's protected least significant difference (PLSD) post-hoc test was used to determine specific group differences.

Results

Body and brain weight measures

Body weight, brain weight, and brain to body weight ratios were determined at the time of sacrifice at all timepoints (P4, P5, P7, P8, and P21) following the various exposure paradigms (see figure 4-1; all figures are located at the end of the chapter). The ANOVA showed that exposure on P4 produced body weight differences at P21 ($F[2,7]= 21.594$; $p< 0.001$). The PLSD revealed that SC pups weighed significantly more than EtOH ($p< 0.01$) and GC pups ($p< 0.01$), but that GC and EtOH pups (the most appropriate comparison) were not different. Similarly, exposure on P4 produced brain weight differences at P21 ($F[2,7]= 15.115$; $p< 0.01$). The brains of SC pups weighed significantly more than EtOH ($p< 0.01$) and GC pups ($p< 0.01$), but GC and EtOH pups were not different. An effect of treatment on brain to body weight ratio was noted at P21 following P4 exposure ($F[2,7]= 4.363$; $p< 0.05$). The brain to body weight ratio of SC pups was significantly greater than EtOH ($p< 0.05$) and GC pups ($p< 0.05$), but GC and EtOH pups were not different.

Figure 4-2 presents body weight, brain weight, and brain to body weight ratios taken at P4, P5, and P21 following treatment on P4-5. The ANOVA determined no effect of treatment on body weight at P4, P5, or P21. Brain weight was affected by treatment on P21 ($F[2,24]= 8.752$; $p< 0.05$), but not on P4 and P5. The post-hoc test found that P21 brain weight was reduced in EtOH pups compared with both GC ($p< 0.01$) and SC pups ($p< 0.01$). Brain to body weight ratios were changed by treatment on P5 ($F[2,39]= 10.284$; $p< 0.05$), but not on P4 and P21. The brain to body weight ratio of SC pups on

P5 was greater than that of both GC ($p< 0.01$) and EtOH ($p< 0.01$) pups, but EtOH and GC pups were not different.

Figure 4-3 presents body weight, brain weight, and brain to body weight ratios taken at P7, P8, and P21 following treatment on P7-8. The ANOVA showed no effect of treatment on body weight at P7, P8, or P21. Brain weight was influenced by treatment on P8 ($F[2,18]= 4.174$; $p< 0.05$) and P21 ($F[2,13]= 4.392$; $p< 0.05$) but not on P7. The PLSD determined that P8 brain weight was reduced in GC pups compared with SC pups ($p< 0.05$), but that EtOH and GC pups were not different. P21 brain weight was reduced in EtOH pups compared with SC pups ($p< 0.05$), although EtOH and GC pups were not different. Brain to body weight ratios were altered by treatment on P8 ($F[2,18]= 12.159$; $p< 0.001$), but not on P7 and P21. The brain to body weight ratio of SC pups was greater than that of both GC ($p< 0.001$) and EtOH ($p< 0.001$) pups at P8, but EtOH and GC pups were not different.

Measurements of vermis size: section number, lobule length and cerebellar cell nuclear diameters

Anatomical measurements were taken in order to determine whether ethanol treatment significantly altered the size of lobule I (see tables 4-1 through 4-4 located at the end of the chapter). The ANOVA found that acute ethanol treatment on P4 (table 4-1) did not alter the mean number of sections through the vermis, lobule length, Purkinje cell nuclear diameter, or granule cell nuclear diameter as measured on P21. Two day ethanol treatment from P4-5 produced the only significant volume difference noted at P21 (see table 4-2). A significant effect of ethanol treatment was noted on the mean number of sections through the vermis ($F[2,10]= 12.103$; $p< 0.01$). The post-hoc test indicated that

EtOH pups had significantly fewer 6 μm sections than GC ($p < 0.01$) and SC ($p < 0.01$) pups. Lobule length and Purkinje and granule cell nuclear size were unaffected. Treatment on P7-8 produced no significant size alterations as measured on P21 (see table 4-3). No effect of treatment was found on section number, lobule length, Purkinje or granule cell nuclear diameters. Ethanol treatment on P4-5 (see table 4-4) also did not produce significant differences as measured on P5, and no effect of treatment was seen on section number, lobule length, or Purkinje cell nuclear diameter. Granule cell nuclear diameters were not determined at this age because the small size of the cells made it impossible to measure with the eyepiece micrometer, even at a magnification of 600x. Because no ubiquitous volume alterations were noted (only the number of vermal sections, and not lobule length, was altered following P4-5 exposure; no other treatment pattern produced size changes) it is unlikely that our cell counts are biased due to changes in the size of lobule I. For example, ethanol exposure on P4-5 produced vermis size alterations and neurotoxicity as measured on P21, while exposure only on P4 produced no size alterations and yet also produced cerebellar cell death.

P21 cerebellar cell counts following first postnatal week ethanol treatment

Figure 4-4 shows cerebellar cell counts at P21 in ethanol-treated and control animals following first postnatal week exposure. The ANOVA showed a significant effect of treatment on the number of Purkinje cells per section in lobule I whether ethanol was delivered on P4 ($F[2,7] = 6.737$; $p < 0.05$), or P4-5 ($F[2,10] = 46.04$; $p < 0.0001$). The PLSD post-hoc test determined that ethanol treatment on P4 reduced the mean number of Purkinje cells per section in EtOH pups (by approximately 50%) compared with both GC ($p < 0.05$) and SC ($p < 0.05$) pups. Similarly, ethanol treatment on P4-5 reduced the mean

number of Purkinje cells per section in EtOH pups (by approximately 65%) compared with both GC ($p < 0.0001$) and SC ($p < 0.0001$) pups.

The mean number of granule cells per section was also altered whether ethanol was delivered on P4 ($F[2,7] = 7.477$; $p < 0.05$), or P4-5 ($F[2,10] = 5.877$; $p < 0.05$). Ethanol treatment on P4 reduced the mean number of granule cells per section in EtOH pups compared with both GC ($p < 0.05$) and SC ($p < 0.05$) pups. Similarly ethanol treatment on P4-5 reduced the mean number of granule cells per section in EtOH pups compared with both GC ($p < 0.05$) and SC ($p < 0.05$) pups. Therefore, one- or two-day ethanol treatment during the first postnatal week reduces the mean number of Purkinje and granule cells per section.

P21 cerebellar cell counts following second postnatal week ethanol treatment

Figure 4-5 shows cerebellar cell counts at P21 in ethanol-treated and control animals following second postnatal week exposure, a developmental time during which the cerebellar vermis has been shown to be less susceptible to ethanol-mediated neurotoxicity (Hamre and West, 1993). The ANOVA showed no significant effect of ethanol treatment on the mean number of Purkinje cells per section in lobule I following ethanol delivery on P7-8. Similarly, no significant effect of ethanol treatment was noted on the mean number of granule cells per section in lobule I following ethanol delivery on P7-8. Thus, ethanol delivered during the second postnatal week did not reduce the mean number of Purkinje cells per section or granule cells per section in lobule I.

P5 cerebellar cell counts following first postnatal week ethanol treatment

In order to resolve the timecourse of ethanol-induced neurotoxicity for cerebellar neurons exposed during the first postnatal week, we performed cell counts at P5

following ethanol delivery on P4-5 (figure 4-6). The ANOVA determined a significant effect of treatment on the number of Purkinje cells per section in lobule I at P5 ($F[2,10] = 5.80$; $p< 0.05$). The PLSD post hoc test found that ethanol treatment from P4-5 reduced the mean number of Purkinje cells per section in EtOH pups compared with both GC ($p< 0.05$) and SC ($p< 0.05$) pups. Granule cells were also counted in the external and internal granule layer on P5. No effect of treatment was found on the number of granule cells per section at P5 in the external or internal granule layer (see figure 4-6). Therefore, there is a significant loss of Purkinje cells as early as P5 following two-day exposure during the first postnatal week. The lack of reduction in granule cell number following ethanol treatment is likely due to the proliferative potential of granule cells during the first postnatal week (Altman, 1969).

bcl-2 family mRNA levels following first postnatal week ethanol exposure

Figure 4-7 shows bcl-2 family mRNA levels in the vermis, normalized to cyclophilin, following acute ethanol treatment on P4 (pups were sacrificed on P4 two hours after ethanol treatment). The ANOVA found a significant effect of treatment on bax ($F[2,21]= 3.929$; $p< 0.05$) and bcl-xs ($F[2,21]= 10.001$; $p< 0.001$) mRNA levels, but not bcl-xl or bcl-2. The post-hoc test determined that bax was significantly up-regulated in EtOH pups compared with GC ($p< 0.05$) and SC ($p< 0.05$) pups. bcl-xs was also significantly up-regulated in EtOH pups compared with GC ($p< 0.001$) and SC pups ($p< 0.001$). Therefore, acute ethanol exposure on P4 significantly up-regulated transcripts encoding pro-apoptotic members of the bcl-2 family, specifically bax and bcl-xs, without altering transcripts encoding anti-apoptotic members of the bcl-2 family (bcl-xl and bcl-2).

Figure 4-8 shows bcl-2 family mRNA levels, normalized to cyclophilin, following two-day ethanol treatment on P4-5 (pups were sacrificed on P5 two hours after ethanol treatment). No significant effect of treatment on bax, bcl-xs, bcl-xl, or bcl-2 transcripts was noted. Therefore, two-day ethanol treatment did not produce further alterations in mRNA levels of members of the bcl-2 family, probably because the susceptible Purkinje cells are already dying by P5.

bcl-2 family mRNA levels following second postnatal week ethanol exposure

For comparison, we also examined bcl-2 family gene expression following ethanol treatment during the second postnatal week. This corresponds to a developmental time during which the cerebellar vermis has been shown to be insusceptible to ethanol-mediated neurotoxicity (Hamre and West, 1993). Figure 4-9 shows bcl-2 family mRNA levels, normalized to cyclophilin, following acute ethanol treatment on P7 (pups were sacrificed on P7 two hours after ethanol treatment). The ANOVA showed a significant effect of treatment on bcl-xs mRNA levels ($F[2,20]= 3.966$; $p< 0.05$). The PLSD indicated that EtOH pups contained higher levels of transcripts encoding bcl-xs compared with SC pups ($p< 0.05$), but not GC pups (the more appropriate comparison for EtOH pups since both GC and EtOH pups are gastrostomized and reared artificially). However, there was no significant effect of treatment on bax, bcl-xl, or bcl-2. Thus, it does not appear that acute exposure during the second postnatal week up-regulates transcripts encoding pro-apoptotic members of the bcl-2 family to the same degree that acute exposure during the first postnatal week (P4) does.

Figure 4-10 shows bcl-2 family mRNA levels, normalized to cyclophilin, following two-day ethanol treatment on P7-8 (pups were sacrificed on P8 two hours after

ethanol treatment). The ANOVA determined that bax ($F[2,18]= 12.34$; $p< 0.001$) and bcl-xs ($F[2,17]= 11.085$; $p< 0.01$) mRNAs were altered by ethanol treatment. The PLSD showed that EtOH pups contained higher levels of transcripts encoding bax compared with GC ($p< 0.001$) and SC ($p< 0.001$) pups. The vermis of EtOH pups also contained higher levels of transcripts encoding bcl-xs compared with SC ($p< 0.01$) pups. EtOH and GC animals (the most critical comparison) were not different, but GC pups did contain higher vermal levels of transcripts encoding bcl-xs compared with SC ($p< 0.01$), indicating an effect of artificial rearing. However, no significant effect of ethanol treatment on bcl-xl or bcl-2 mRNAs was found. Therefore, two days of ethanol exposure during the second postnatal week can induce pro-apoptotic processes, just as acute exposure during the first postnatal week can.

Discussion

This study has shown that mRNAs encoding certain pro-apoptotic members of the bcl-2 family (bax and bcl-xs) are up-regulated in the cerebellar vermis of ethanol-exposed neonatal rats. Acute ethanol treatment on P4 induces pro-apoptotic, but not anti-apoptotic, gene expression, and also results in decreased Purkinje and granule cell numbers. An additional exposure on P5 does not produce further alterations in this gene expression. Ethanol administered during the second postnatal week does not produce cerebellar cell loss and results in the induction of pro-apoptotic (bax) gene expression, but *only* after two days of ethanol treatment and not after acute, one day exposure, as during the earlier period. Therefore, the differential susceptibility of the developing vermis to ethanol-induced cell death is not directly related to changes in the up-regulation of

mRNAs encoding pro-apoptotic members of the bcl-2 family. Rather, the major difference between first and second postnatal weeks may be the differential ability of cerebellar neurons to respond to ethanol-induced up-regulation of pro-apoptotic gene expression. What follows is a discussion of the effects of ethanol exposure during these stages on brain morphology, measurements of vermis size, cerebellar cell counts, and finally, bcl-2 family mRNA expression.

Effect of neonatal ethanol treatment on body weight, brain weight, and brain to body weight ratio

Acute neonatal ethanol exposure on P4 did not significantly affect the growth of the pups in the present study since P21 body weight in EtOH and GC animals was not different. However, a non-specific effect of artificial rearing was noted on P21 body weight following acute P4 exposure, as both EtOH and GC animals weighed less than the SC animals. Regardless, the fact that EtOH and GC animals did not differ in body weight on P21 demonstrates that this pattern of neonatal ethanol exposure does not, by itself, decrease growth. This is due to the fact that EtOH and GC animals are the most appropriate comparison in this neonatal exposure paradigm, and these groups were not different.

Similar effects were noted for P21 brain weight, with the brains of SC animals weighing more than both gastrostomized groups following acute P4 exposure. No differences existed between EtOH and GC animals for P21 brain weight following this pattern of exposure. Not surprisingly, brain to body weight ratios followed a similar trend, with SC animals reduced compared to EtOH and GC due to the increased body weight previously noted. The brain to body weight ratio of EtOH and GC pups was not

different, demonstrating that this pattern of acute P4 ethanol exposure does not significantly alter the growth of the brain.

Ethanol treatment from P4-5 also did not significantly alter the growth of the pups in this study. Body weight was not significantly different in any group at P4, P5, or P21. Brain weight was similarly unaffected at P4 and P5, but by P21 a significant decrease was noted in the EtOH group compared with the GC and SC group. Thus, two days of ethanol treatment resulted in microencephaly, while only one day of exposure did not. Brain to body weight ratios were unaffected by ethanol treatment as measured on P4 and P21, indicating that the observed microencephaly at P21 was due to the smaller (but not significantly smaller) size of EtOH animals at P21. The brain to body weight ratio was increased in SC animals on P5. Since EtOH and GC animals did not differ, however, it appears that ethanol treatment, per se, did not alter brain to body weight ratio. Rather, a non-specific effect of gastrostomy was noted. Nonetheless, the data taken together demonstrate no direct ethanol effect on relative brain growth at any age examined.

Ethanol exposure during the second postnatal week, from P7-8, also did not alter the growth of either gastrostomized group or the suckle control group. Body weight was unaffected by treatment on P7, P8, or P21. Brain weight was unaffected at P7, but on P8 the brains of GC animals weighed significantly less than the SC animals, indicating a non-specific effect of gastrostomy on brain weight in these pups. Ethanol treatment did not alter brain weight on P8, as EtOH and GC pups were not different, but by P21 the brains of EtOH animals weighed significantly less than SC pups but not GC pups. Brain to body weight ratios were unaffected by second postnatal week exposure as measured on

P7 and P21. However, this ratio was increased in SC animals on P8 compared with both EtOH and GC animals; EtOH and GC pups were not different.

The results presented here for animal growth (body weight) are similar to those reported in Hamre and West (1993). In both studies, utilizing a similar neonatal ethanol exposure paradigm, no significant differences in body weight were noted between the EtOH and GC group, even though the growth of the gastrostomized pups lagged behind that of the SC pups following return to the dam. Unfortunately, Hamre and West did not report brain weight or brain to body weight ratios following the one- or two-day pattern of exposure, making comparisons to the current data impossible.

Goodlett et al. (1990), however, did report brain weight following a single ethanol exposure on P4 using the same dose as used in the current study. They reported significant brain weight declines in EtOH pups as measured on P10. These data are in contrast to the data presented here, as no significant decline was noted in our pups as measured on P21 following similar one-day ethanol exposure. Indeed, in our hands, brain weight differences were only noted following two-day ethanol treatment from P4-5. It is possible that pups used in the current study exhibited "catch up" growth following return to the dam which would account for the discrepancy.

Neonatal ethanol and the size of the cerebellar vermis and diameter of cell nuclei within the vermis

The only significant volume measure that was affected by any pattern of neonatal ethanol exposure (P4 only, P4-5, or P7-8), was the mean number of sections through the vermis on P21 following ethanol exposure on P4-5 (lobule length and cell sizes were unaffected). While vermal section number is reduced by P21, measurements on P5

indicate that these size reductions do not occur until after the P5 timepoint. For all other exposure paradigms, the size of the vermis was unaffected by treatment. Likewise, for all other exposures lobule length and cell size were unaffected. Because we found no universal volume reductions, it is unlikely that our cell counts are biased because of changes in the size of the cerebellar vermis. Exposure on P4-5, for instance, produced both size alterations and neurotoxicity, while exposure only on P4 produced no size alterations but also resulted in cerebellar cell death. Similarly, P4-5 exposure produces cell number reductions at both the P5 and P21 timepoints, and the size of the vermis is unchanged at P5 but not P21.

This trend of first postnatal week reduction in the size of the vermis is consistent with the trends noted in brain weight, namely that ethanol exposure on P4 only produces neither vermis nor brain weight reductions, while two day treatment on P4-5 is capable of inducing these declines. It should be noted, however, that the cell loss noted in this study is independent of changes in either the size of the brain or the size of the vermis in these animals.

Unfortunately, recent reports which have utilized single day (e.g. P4 only) neonatal ethanol exposure have not reported data on cerebellar size (Goodlett and Eilers, 1997), and comparisons to our data are impossible. Hamre and West (1993), however, did report Purkinje cell layer length and found that P4-5 ethanol exposure significantly reduced the length of the Purkinje cell layer at P21. These results are in contrast to our own data regarding lobule length, which was unaffected by this same pattern of exposure. Our data do indicate, though, a change in the size of the vermis following P4-5 exposure based on the reduced mean number of sections through the vermis (a measure that Hamre

and West did not report). In agreement with our data, Hamre and West (1993) did not find any size alterations following P7-8 exposure.

Other reports have also examined the size of the nuclei of cerebellar neurons following neonatal ethanol exposure, notably Hamre and West (1993). In agreement with our data, no ethanol-induced alterations in the size of granule cell nuclei were found following ethanol exposure on P4-5 or P7-8. They did, however, report an increase in Purkinje cell nuclear diameter following P4-5 or P7-8 exposure. It should be noted, however, that these diameters were not measured directly but instead were extrapolated from Purkinje cell area measures. Moreover, it is difficult to make a convincing case that Purkinje cell diameters are directly altered by ethanol treatment when other studies from this group have not found an effect of similar patterns of ethanol exposure on cerebellar cell diameter (Pierce et al., 1989).

The measures for P21 vermis size and cell nuclei size were generally consistent across experiments utilizing the different ethanol exposure times. While some litter and individual variation was noted, it appears that period of exposure had little or no effect on these parameters. The mean number of sections, and cell nuclear diameters were most similar across experiments, while lobule length showed more variability. For example, the animals used for the P4-5 treatment groups displayed larger lobules than either of the other groups measured on P21 (note that no group differences were found in these animals), while Purkinje and granule cell nuclear size measurements and section number displayed less variability. Not surprisingly, the data demonstrate that the vermis size and cell nuclear size measurements were smaller for P5 animals than for P21 animals.

Differential temporal vermis susceptibility to developmental ethanol

The differential temporal pattern of vermis susceptibility to developmental ethanol exposure noted here is similar to that seen by other investigators (Goodlett and Eilers, 1997; Hamre and West, 1993; Pauli et al., 1995). The present study demonstrates that one- or two-day ethanol treatment during the first postnatal week reduced the mean number of Purkinje and granule cells per section in lobule I of the vermis as determined on P21. Also, we have documented that two-day exposure produced significant Purkinje (but not granule) cell loss as early as P5. In contrast, two days of ethanol treatment during the second postnatal week (from P7-8) did not reduce Purkinje or granule cells per section as measured on P21.

Goodlett and colleagues have used similar exposure methods and similar patterns of ethanol exposure to document the tight window of vulnerability of the cerebellar vermis. These investigators have determined that acute P4 exposure produces cerebellar cell loss, while exposure on P9 does not (Goodlett and Eilers, 1997). Interestingly, in a recent report (Thomas et al., 1998) a slight (approximately 15%) reduction in Purkinje cells following P8-9 exposure was noted. These results stand in contrast to results from second postnatal week exposure in the current study, which found no significant reduction in Purkinje cells following P7-8 exposure. They also differ from an earlier report which showed no Purkinje cell loss following P7-8 exposure (Hamre and West, 1993). Regardless, it is obvious that the first postnatal week is a period of heightened vulnerability, while exposure in the second postnatal week produces little or no cell loss.

The timecourse of Purkinje cell death noted in the present study is consistent with that noted in another report demonstrating Purkinje cell loss as early as 12 hours

following a P3 ethanol insult (Cragg and Phillips, 1985). While our data show that granule cells do not follow this rapid timecourse of cell death, this is not surprising. The lack of effect on granule cell numbers at early timepoints may be because cerebellar granule cells maintain their proliferative potential during the neonatal period (Altman, 1969). Therefore, direct ethanol-induced granule cell loss may be masked by compensatory proliferation. Another possibility is that fewer granule cells are dying following ethanol exposure at this time before the period of cell death. Alternatively, granule cells may be experiencing a secondary (and more prolonged) loss due to lost Purkinje cell targets, as hypothesized in other studies (Hamre and West, 1993). Regardless, we find a similar degree of long-term (P21) Purkinje and granule cell loss following first postnatal week ethanol treatment as other groups (Hamre and West, 1993), and we provide another demonstration of the developing cerebellum's tight window of vulnerability.

Because the loss of granule cells appears to be secondary to Purkinje cell death (Hamre and West, 1993), the key factor in cerebellar susceptibility is whether Purkinje cells are initially killed by the ethanol insult. Investigators have hypothesized that some property of Purkinje cells in the early stages of differentiation (or those in less mature stages, for example during generation and proliferation) makes them more susceptible to ethanol than more mature neurons (Goodlett and Eilers, 1997). Because differentiating Purkinje cells are also in the peak phase of naturally occurring cell death (Cragg and Phillips, 1985), we hypothesized that the differential susceptibility might be related to ethanol-induced alterations in bcl-2 family gene expression. We chose to investigate transcription of bcl-2 family genes with demonstrated Purkinje and granule cell

expression during neonatal rodent development, specifically bcl-2, bcl-xl, bcl-xs, and bax (Castren et al., 1994; Frankowski et al., 1995; Krajewski et al., 1994; Rouayrenc et al., 1995; Vekrellis et al., 1997).

Neonatal ethanol and bcl-2 family gene expression during the first postnatal week

Ethanol exposure to neonatal rats during the first postnatal week, a developmental timepoint corresponding with cerebellar neuronal susceptibility to ethanol, produced an up-regulation of mRNA transcripts encoding pro-apoptotic members of the bcl-2 gene family. Specifically, acute ethanol exposure on P4 significantly up-regulated transcripts encoding bax and bcl-xs two hours following the ethanol insult, without altering transcripts encoding anti-apoptotic members of the bcl-2 family (bcl-xl and bcl-2).

We also examined bcl-2 family transcripts on P5 after two-day ethanol treatment from P4-5. This pattern of exposure did not produce further alterations in mRNA levels of any members of the bcl-2 family examined (bax, bcl-xs, bcl-xl, or bcl-2). The lack of significant alterations in bcl-2 family mRNAs on P5 following ethanol exposure on P4-5 is presumably because the susceptible Purkinje cells undergo cell death as early as P5 (see above). We do not, therefore, detect any significant alterations in bcl-2 family mRNAs on P5 because we are measuring the transcription of bcl-2 family mRNAs in the surviving cells, which appear to be able to withstand the ethanol insult without further changes in the expression of bcl-2 family genes.

One important point to be considered is that the use of the RNase protection assay does not provide for identification of the cell type-specific changes in gene expression. Because the bcl-2 family expression in the vermis includes Purkinje and granule cells, future investigations utilizing in situ hybridization will be necessary to determine neuron-

specific changes in gene expression. Thus, an alternative explanation for the P5 data is that granule cell expression is masking Purkinje cell expression due to the greater numbers of granule cells. In other words, Purkinje cell expression could still be altered, but granule cell expression is masking these changes.

Another important consideration comes from the fact that ethanol-induced alterations in bax and bcl-xs are noted on P4, but granule cell death is not detectable until well after this age. While Purkinje cell death was evident as early as P5 following P4 or P4-5 exposure, concomitant reductions in granule cells were not noted until P21. Thus, granule cell numbers are reduced by one- or two-day ethanol exposure in the first postnatal week, but they undergo a prolonged loss, suggesting that the noted changes in pro-apoptotic gene expression on P4 do not occur in granule cells, and do not positively correlate with granule cell death. What factors, then, are responsible for the eventual death of granule cells?

As discussed above, other investigators have noted that granule cell death appears to be secondary to Purkinje cell death following ethanol treatment (Hamre and West, 1993). We speculate that granule cell death in our hands is due to the devastating reductions in the Purkinje cell population. As granule cells begin to exit the proliferative stage and enter the differentiation stage, fewer Purkinje cell targets are available for synapse formation, resulting in increased granule cell death due to lack of target-derived support. Of course, it is possible that bax and bcl-xs expression is elevated in granule cells, but that they are better able to survive the elevation, perhaps because of elevated anti-apoptotic expression of bcl-2 family members not investigated in this study. Future investigations utilizing in situ hybridization could definitively determine the anatomical

localization of the noted expression changes in pro-apoptotic genes, and would be useful for determining whether compensatory changes in anti-apoptotic genes occurred in granule cells. A lack of induction of anti-apoptotic genes in granule cells would suggest that loss of targets is responsible for the prolonged loss of granule cells.

Our data are consistent with reports of altered bcl-2 family expression from other groups working in different models of brain injury. For example, studies in mouse brain have shown up-regulation of Bax protein and down-regulation of Bcl-2 protein associated with kainate-induced apoptosis (Gillardon et al., 1995). Similar protein expression changes in Bax and Bcl-2 have been seen in hippocampus and cerebellum following ischemia (Krajewski et al., 1995). Ischemia also up-regulates bcl-xs mRNA in the hippocampus (Dixon et al., 1997). Additionally, the Bcl-2:Bax ratio is decreased in rat motoneurons following sciatic nerve transection (Gillardon et al., 1996), and experiments in Purkinje-cell-degeneration mice have determined that a decreased bcl-2:bax ratio (at the mRNA and protein levels) immediately precedes the gradual loss of Purkinje cells (Gillardon et al., 1995). Therefore, it is clear that alterations in bcl-2 expression, both at the message and protein levels, correlates positively with cell death in various model systems of brain injury.

In order to determine whether the alteration in bcl-2 family gene expression noted in the present study was specific to first postnatal week ethanol neurotoxicity (and positively correlated with ethanol-induced cell death), ethanol was delivered during a developmental period of cerebellar insusceptibility, the second postnatal week. Acute exposure on P7 did not produce the same dramatic induction of pro-apoptotic gene

expression that was noted two hours after treatment on P4. In fact, EtOH and GC pups did not differ in mRNA levels for any bcl-2 family member tested.

Two-day ethanol treatment during the second postnatal week on days 7-8, however, induced pro-apoptotic gene expression as measured on P8. Transcripts encoding bax were significantly up-regulated following this pattern of exposure. bcl-xs was induced by artificial rearing (both gastrostomized groups had elevated bcl-xs compared with SC pups) but ethanol treatment, per se, did not significantly alter bcl-xs expression. Nonetheless, the up-regulation of bax after two-day ethanol treatment suggests that an additional ethanol exposure during the second postnatal week induces pro-apoptotic gene expression. Therefore, it is not simply a difference in the induction of pro-apoptotic processes following second postnatal week ethanol exposure that is responsible for the difference in cerebellar neuronal loss.

Differential cerebellar susceptibility and the bcl-2 family

The differential susceptibility of the cerebellar vermis to ethanol during the first and second postnatal week is possibly due to differences in the timecourse of the induction of pro-apoptotic mRNA. Indeed, the present study documents that mRNAs encoding pro-apoptotic members of the bcl-2 family are up-regulated by only one day of ethanol treatment during the first postnatal week, whereas two exposures are necessary to induce pro-apoptotic mRNAs during the second week. A more likely difference, however, may be the ability of cerebellar neurons to respond to the increased transcription of pro-apoptotic genes. We speculate that the critical difference may be better suppression of pro-apoptotic processes, possibly by growth factors, in more mature cerebellar neurons following ethanol exposure in the second postnatal week.

Several neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), have distinct influences on the survival and differentiation of cerebellar Purkinje and granule cells during development. Neurotrophin receptors are expressed at high levels in rat Purkinje and granule cells during development and both ligands and receptors are regulated in a spatio-temporal manner (Lindholm et al., 1997). For example, only differentiating granule cells (and not proliferating granule cells) express Trk B receptors and respond to BDNF and NT-3 (Gao et al., 1995). Furthermore, Purkinje cell expression of trkA mRNA is detectable at P4, but only attains peak levels during later differentiation, around P10 (Wanaka and Johnson, 1990). This spatio-temporal pattern of trkA expression correlates with periods of ethanol-induced cerebellar cell death. Notably, during the early ontogeny of trkA (before the peak of its expression) Purkinje cells are susceptible to ethanol. However, during the peak period of trkA mRNA expression, Purkinje cells are not vulnerable to ethanol. Better established neurotrophic processes in the more mature cerebellum may explain the ability of Purkinje cells to withstand an ethanol insult.

Growth factor-mediated post-translational modulation of bcl-2 family members has been demonstrated, particularly with regard to the phosphorylation of the pro-apoptotic molecule Bad (Datta et al., 1997; Gajewski and Thompson, 1996; Zha et al., 1996). Bad phosphorylation inactivates its death-promoting properties by inhibiting its association with (and inactivation of) anti-apoptotic molecules. Growth factor-mediated activation of PI3 kinase, followed by PI3 kinase phosporylation and activation of Akt (Kahn, 1998), leads to Akt-mediated Bad phosphorylation (Datta et al., 1997; Delpeso et al., 1997; Zhou et al., 1997). Furthermore, endogenous bcl-2 expression plays a role in

the survival of BDNF-dependent neurons (Allsopp et al., 1995) and similar expression is induced by NGF in developing cerebellar granule cells (Muller et al., 1997). The main influence which may mediate differential cerebellar ethanol susceptibility is the ability of target-derived growth factors to suppress pro-apoptotic, or possibly enhance anti-apoptotic, mechanisms following ethanol exposure.

Therefore, an important hypothesis to test is that the key factor governing cerebellar neuronal susceptibility to ethanol is the presence of a target-derived growth factor source to suppress pro-apoptotic processes. For example, the present study indicates that cerebellar neurons exposed to ethanol during the second postnatal week experience an induction of pro-apoptotic processes but exhibit no cell death. It is possible that growth factor-induced phosphorylation of Bad compensates for the concomitant increase in pro-apoptotic mRNAs by enabling sufficient Bcl-2 function. Bcl-2 homodimerization (and functionality) would be favored in the presence of phosphorylated Bad because of cytosolic sequestering of Bad. While we presume from the present study that increased Bax and Bcl-xs will be competing with Bcl-2 for dimerization, it is possible that growth factor modulation of Bad enables sufficient homodimerization of anti-apoptotic members of the bcl-2 family to continue to regulate mitochondrial membrane potential and stave off cell death. Perhaps the lack of growth factor-induced Bad phosphorylation in the first postnatal week, along with the ethanol-induced induction of pro-death genes, would lead to inadequate Bcl-2 function, resulting in lost mitochondrial membrane potential, release of cytochrome C, and caspase activation. The intracellular mechanisms of bcl-2 family function are discussed in detail below.

Intracellular function of bcl-2 family members

While the complex intracellular mechanisms of various bcl-2 family members is still being worked out, recent evidence has revealed that certain members of the family function as both ion channel and adapter protein (Reed, 1997). Anti-apoptotic (Bcl-2 and Bcl-xl) and pro-apoptotic (Bax) members of the bcl-2 family are known to form functional ion channels in lipid membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). In addition, it is clear that the ability of Bax homodimers to form channels is inhibited by Bcl-2 (Antonsson et al., 1997), indicating that competition for dimerization is an important determinant of cell survival. These ion channels influence cell survival by regulating the permeability of the membranes in which they are anchored, and, in particular, by influencing the release of cytochrome C from mitochondria (Yang et al., 1997).

Cytochrome C is a known co-factor for caspase activation (Yang et al., 1997). Disruption of mitochondrial membranes, an early event in PCD, results in the release of cytochrome C and the activation of the effector phase of PCD (Petit et al., 1996) through cytochrome C-dependent cleavage and activation of caspases 3 (Li et al., 1997) and 9 (Zou et al., 1997). Interestingly, recent evidence shows that Bcl-xl (and presumably Bcl-2) binds to Apaf-1, the newly discovered mammalian homolog of the C. elegans CED-4 (Zou et al., 1997). These two molecules, along with the uncleaved and inactive caspase 9, exist in a ternary complex (Pan et al., 1998). How cytochrome C release helps to activate this complex remains unknown.

Another important role of Bcl-xl and Bcl-2 has been described by Yang et al. (1998). They have shown that CED-4 (and likely Apaf-1) promotes the processing and

activation of CED-3 by promoting the aggregation of unprocessed CED-3. Increasing the local concentration of CED-3 through "induced proximity" is thought to sequester inactive caspase proenzymes and promote conformational changes which will increase the likelihood of their activation (Hengartner, 1998). This is brought about by oligomerization of the CED-4:CED-3 complex (Yang et al., 1998). Thus another function of anti-apoptotic members of the bcl-2 family appears to be to prevent the activation of these proenzymes by preventing CED-4 oligomerization (Hengartner, 1998).

Yet another wrinkle in the complicated intracellular process of cell death is the fact that anti-apoptotic molecules of the bcl-2 family can contribute to the cell's demise under certain pathological conditions. If caspase activation proceeds to a critical point, they begin to act on the Bcl-2 and Bcl-xl proteins as substrates (Cheng et al., 1997; Clem et al., 1998). Caspase cleavage of these protective molecules converts them into Bax-like death-promoting molecules. Thus, cleavage of anti-apoptotic molecules appears to act as a feed-forward mechanism for further caspase activation, ensuring cell death.

Future investigations should attempt to characterize these intracellular processes following developmental ethanol exposure, and the data presented here offer many new avenues of research. For instance, is Bax channel formation enhanced by ethanol? Our data indicate increased bax gene expression, and suggest that ethanol would increase Bax homodimerization and channel formation. Other questions include is cytochrome C release increased by ethanol? Are caspases activated by ethanol, and are anti-apoptotic molecules cleaved by active caspases?

Conclusion and significance

While other reports have demonstrated bcl-2 expression changes in certain brain injury paradigms, this study is the first to document such changes following ethanol treatment. The significance of this work is that it suggests new avenues of research on ethanol's teratogenic actions, and suggests that suppression of pro-apoptotic processes might be of therapeutic benefit. Recent data from our lab indicate that mice over-expressing a bcl-2 transgene in nervous tissue (including cerebellar Purkinje cells) are resistant to ethanol-mediated Purkinje cell death (Heaton et al., unpublished observation). In conclusion, the present data indicate that the modulation of pro-apoptotic processes may have important implications for neuronal death in animal models of developmental ethanol exposure.

Table 4-1. Lobule I cerebellar vermis volume, area, and cell nuclei diameter data at P21 following exposure on P4 only*

Diet group	N	Section number	Lobule length (mm)	Purkinje nuclear diameter (μm)	Granule nuclear diameter (μm)
EtOH	3	260.3 ± 3.667	4.167 ± 0.434	27.07 ± 0.758	7.37 ± 0.315
GC	3	279.0 ± 15.00	4.933 ± 0.067	28.54 ± 1.120	7.16 ± 0.467
SC	4	283.3 ± 10.41	4.782 ± 0.309	28.28 ± 1.166	7.75± 0.185

*All measures are expressed as mean ± SEM. No significant differences were noted.

Table 4-2. Lobule I cerebellar vermis volume, area, and cell nuclei diameter data at P21 following exposure on P4-5*

Diet group	N	Section number	Lobule length (mm)	Purkinje nuclear diameter (μm)	Granule nuclear diameter (μm)
EtOH	6	247.6 ± 12.30†	6.445 ± 0.672	22.79 ± 0.625	7.026 ± 0.187
GC	4	338.0 ± 15.27	6.792 ± 0.330	21.80 ± 0.834	6.702 ± 0.304
SC	3	326.7 ± 16.76	7.000 ± 0.995	23.46 ± 1.155	6.793± 0.396

*All measures are expressed as mean ± SEM. †Significantly reduced compared with GC and SC animals ($p < 0.01$).

Table 4-3. Lobule I cerebellar vermis volume, area, and cell nuclei diameter data at P21 following exposure on P7-8*

Diet group	N	Section number	Lobule length (mm)	Purkinje nuclear diameter (μm)	Granule nuclear diameter (μm)
EtOH	5	297.4 ± 17.08	4.638 ± 0.434	21.80 ± 0.474	7.270 ± 0.218
GC	6	284.2 ± 12.65	5.675 ± 0.449	22.39 ± 0.729	7.330 ± 0.257
SC	4	312.0 ± 18.86	5.070 ± 0.458	23.63 ± 0.834	6.700 ± 0.294

*All measures are expressed as mean ± SEM. No significant differences were noted.

Table 4-4. Lobule I cerebellar vermis volume, area, and cell nuclei diameter data at P5 following exposure on P4-5*

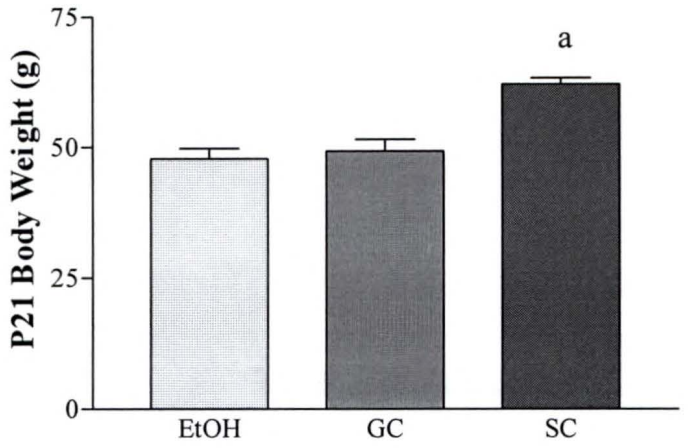
Diet group	N	Section number	Lobule length (mm)	Purkinje nuclear diameter (μm)
EtOH	6	192.6 ± 14.83	3.380 ± 0.900	14.75 ± 0.375
GC	4	158.0 ± 22.90	2.700 ± 1.250	15.00 ± 0.350
SC	3	185.0 ± 6.430	2.690 ± 1.350	15.25 ± 0.400

All measures are expressed as mean ± SEM. No significant differences were noted.

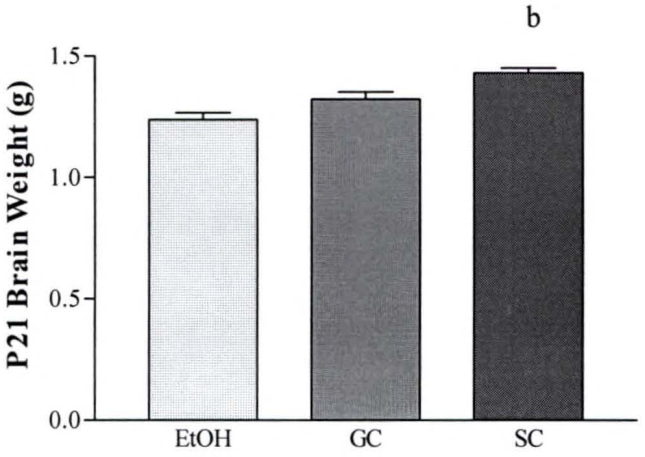
Figure 4-1. Effects of ethanol delivered on P4 only on P21 body weight (panel A), P21 brain weight (panel B) and P21 brain to body weight ratio (panel C). Data are expressed as mean $\pm$ SEM. a: significantly increased compared with EtOH and GC pups ($p < 0.01$); b: significantly increased compared with EtOH and GC pups ($p < 0.01$). c: significantly decreased compared with EtOH and GC pups ($p < 0.01$).

Ethanol Exposure on P4 Only

A Body Weight



B Brain Weight



C Brain to Body Weight Ratio

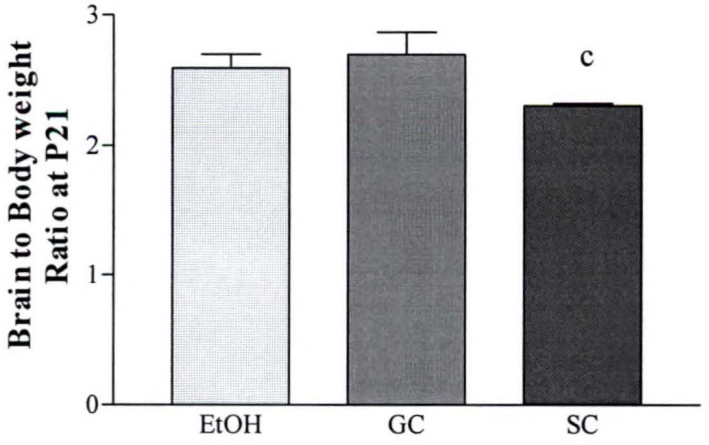
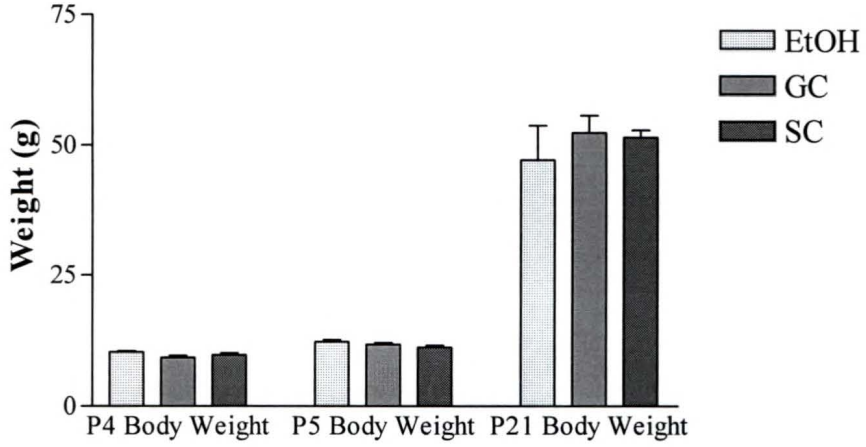


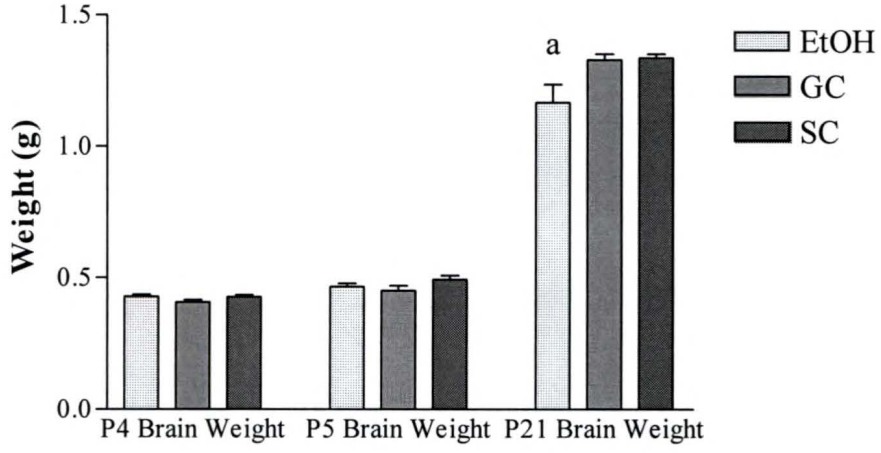
Figure 4-2. Effects of ethanol delivered on P4-5 on P4, P5, and P21 body weight (panel A), brain weight (panel B) and brain to body weight ratio (panel C). Data are expressed as mean $\pm$ SEM. a: significantly decreased compared with EtOH and GC pups ($p < 0.01$). b: significantly increased compared with EtOH and GC pups ($p < 0.01$).

Ethanol Exposure on P4-5

A Body Weight



B Brain Weight



C Brain to Body Weight Ratio

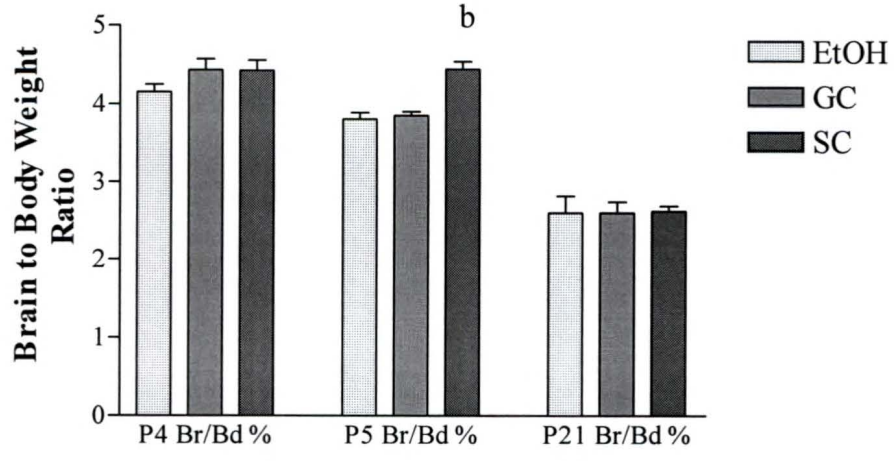
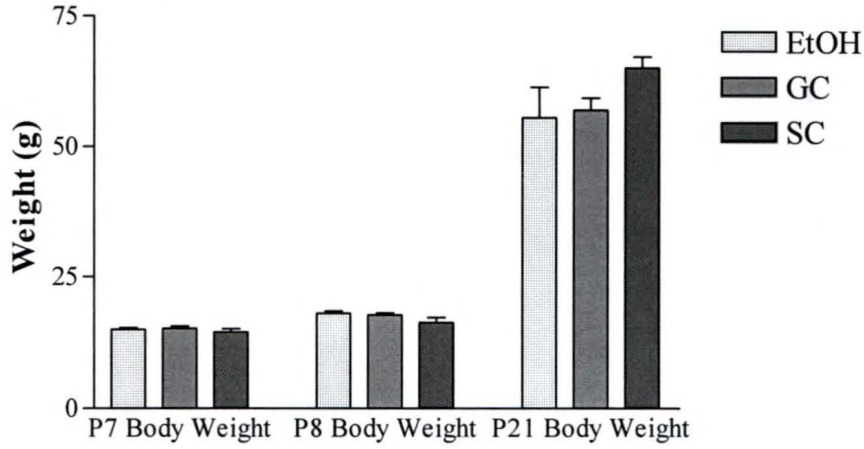


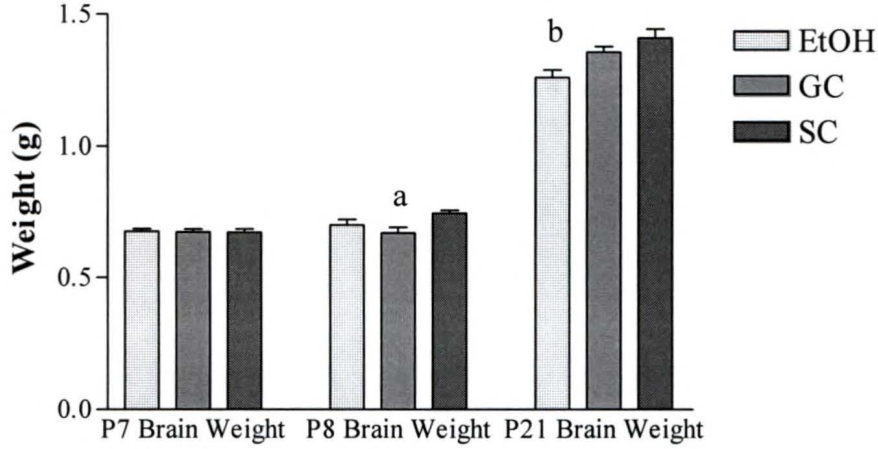
Figure 4-3. Effects of ethanol delivered on P7-8 on P7, P8, and P21 body weight (panel A), brain weight (panel B) and brain to body weight ratio (panel C). Data are expressed as mean $\pm$ SEM. a: significantly decreased compared with SC pups ($p < 0.05$). b: significantly decreased compared with SC pups ($p < 0.05$). c: significantly increased compared with EtOH and GC pups ($p < 0.001$).

Ethanol Exposure on P7-8

A Body Weight



B Brain Weight



C Brain to Body Weight Ratio

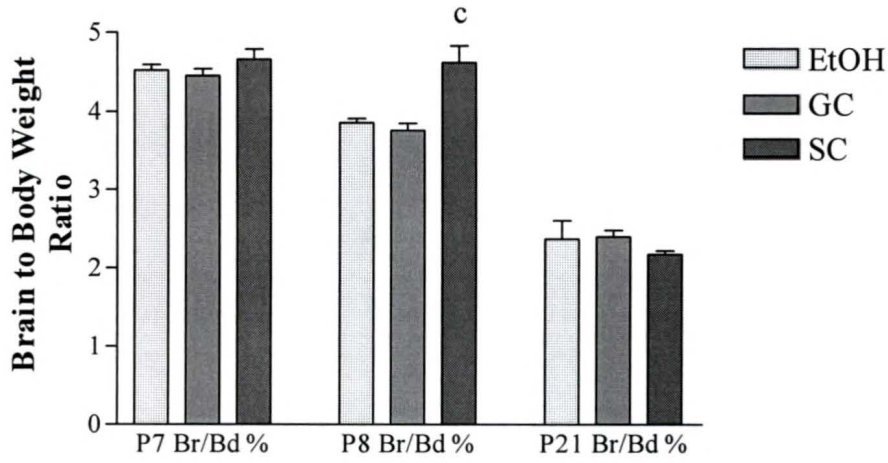
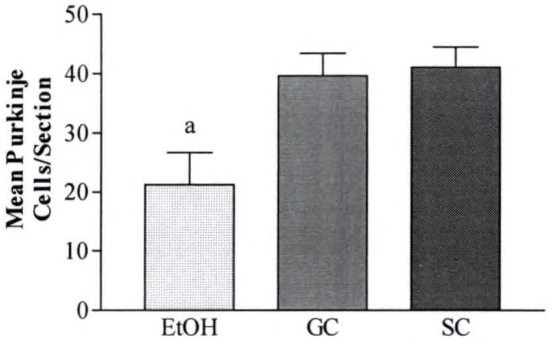


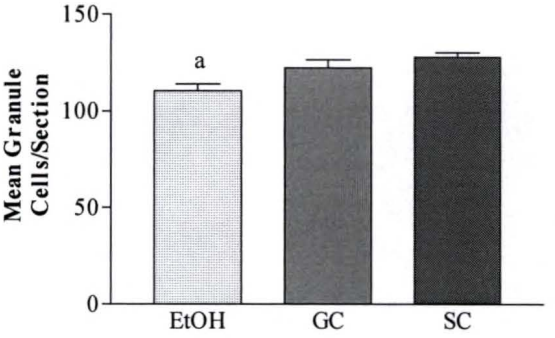
Figure 4-4. Ethanol delivered during the first postnatal week reduces mean Purkinje and granule cell number per section in lobule I of the cerebellar vermis. The mean number of Purkinje and granule cells per section in lobule I of the cerebellar vermis was determined at postnatal day 21 (P21) in 6 μm H&E-stained sections following ethanol or control exposure via artificial rearing on P4 (panels A and B) or P4-5 (panels C and D). Exposure to ethanol for one (P4) or two (P4-5) days during the first postnatal week significantly reduced Purkinje and granule cell number in ethanol (EtOH) pups compared with gastrostomized control (GC) and suckle control (SC) pups. Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with GC and SC ($p < 0.05$). b: significantly reduced compared with GC and SC ($p < 0.0001$).

Ethanol Exposure on P4 Only

A Purkinje Cells

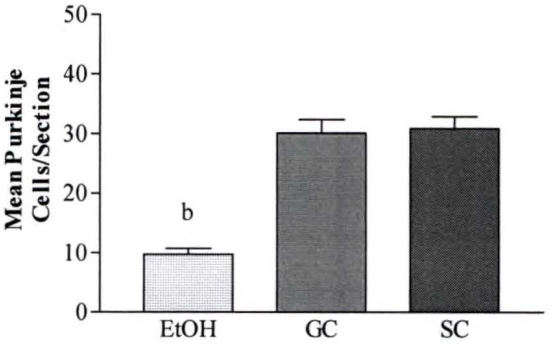


B Granule Cells

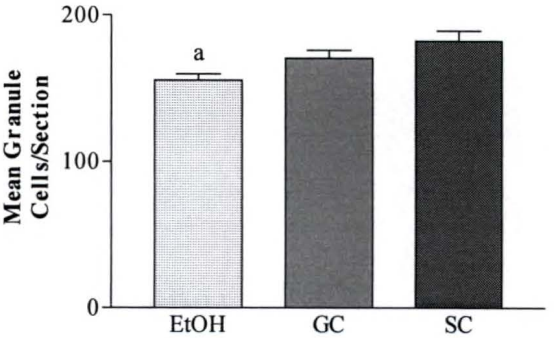


Ethanol Exposure on P4-5

C Purkinje Cells



D Granule Cells



Ethanol Exposure on P7-8

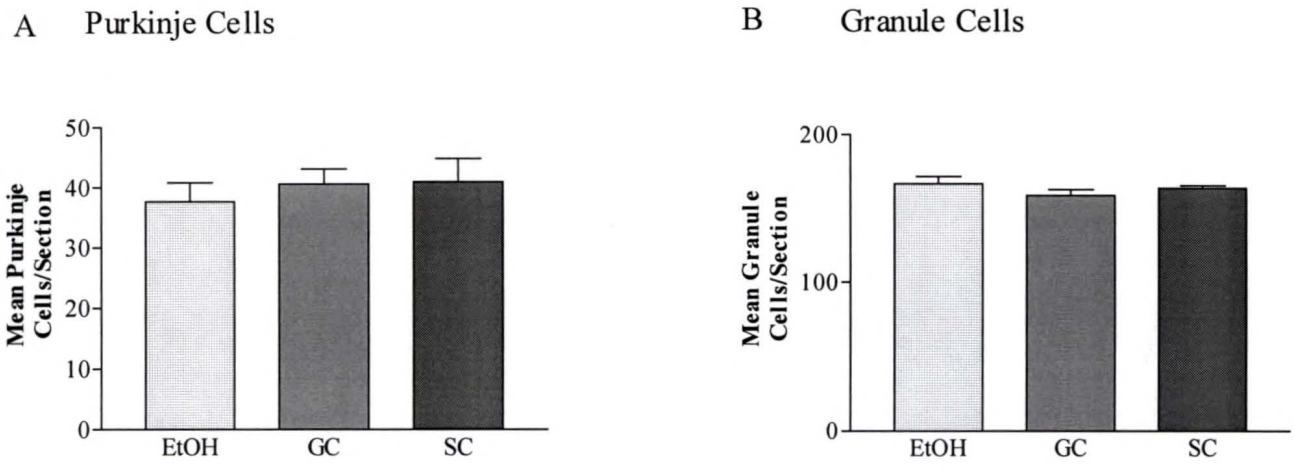
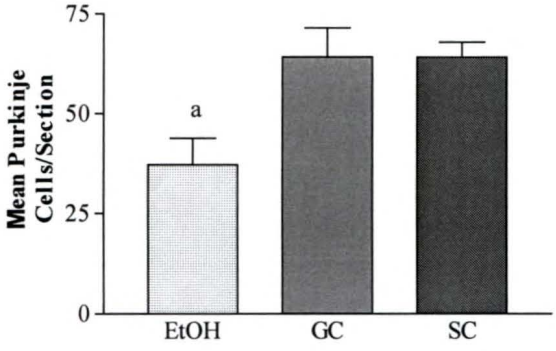


Figure 4-5. Ethanol delivered during the second postnatal week does not reduce mean Purkinje or granule cell number per section in lobule I of the cerebellar vermis. The mean number of Purkinje (panel A) and granule (panel B) cells per section in lobule I of the cerebellar vermis was determined at postnatal day 21 in 6 μm H&E-stained sections following ethanol or control exposure via artificial rearing on P7-8. Exposure to ethanol during the second postnatal week did not alter mean number of Purkinje or granule cells per section. Ethanol exposed (EtOH), gastrostomized control (GC), suckle control (SC). Data are expressed as mean $\pm$ SEM. No significant differences were noted.

Ethanol Exposure on P4-5

A Purkinje Cells



B Granule Cells

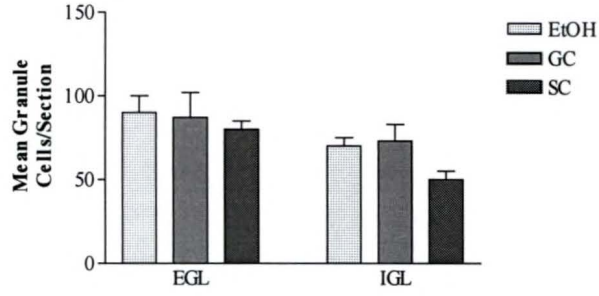
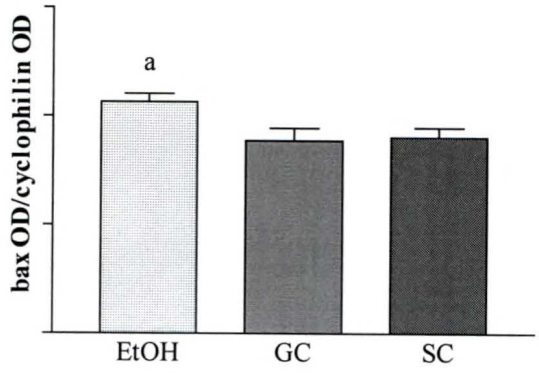


Figure 4-6. Ethanol delivered during the first postnatal week on days 4-5 reduces mean Purkinje but not granule cell number per section in lobule I of the cerebellar vermis as determined on postnatal day 5 (P5). The mean number of Purkinje (panel A) and granule (panel B) cells per section in lobule I of the cerebellar vermis was determined at P5 in 6 μm H&E-stained sections following ethanol or control exposure via artificial rearing on P4-5. Exposure to ethanol from P4-5 significantly reduced Purkinje cell number in ethanol (EtOH) pups compared with gastrostomized control (GC) and suckle control (SC) pups while granule cells were not affected. Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with GC and SC ($p < 0.05$).

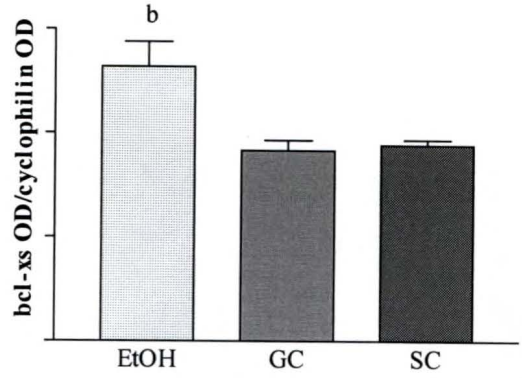
Figure 4-7. mRNAs encoding pro-apoptotic molecules of the bcl-2 family are upregulated following acute ethanol delivered on postnatal day 4. mRNA levels of bax (panel A), bcl-xs (panel B), bcl-xl (panel C), and bcl-2 (panel D), relative to the internal standard cyclophilin, were determined in the cerebellar vermis following ethanol or control exposure via artificial rearing on P4. Pups were sacrificed two hours after the final ethanol or control infusion on P4, and the relative mRNA levels determined via the RNase protection assay. Significant increases in mRNAs encoding the pro-apoptotic molecules bax and bcl-xs were observed after ethanol exposure (EtOH) on P4, when compared with gastrostomized control (GC) and suckle control (SC) pups. Data are expressed as mean $\pm$ SEM. a: significantly increased compared with GC and SC ($p < 0.05$). b: significantly increased compared with GC and SC ($p < 0.001$).

mRNA levels on P4 Following Acute P4 Ethanol Exposure

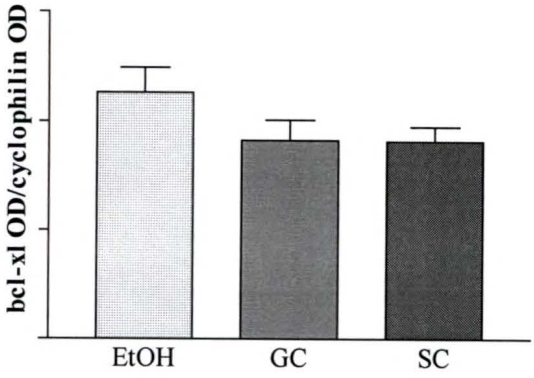
A bax



B bcl-xs



C bcl-xl



D bcl-2

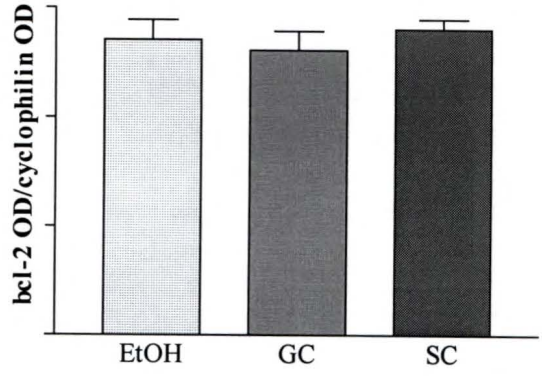
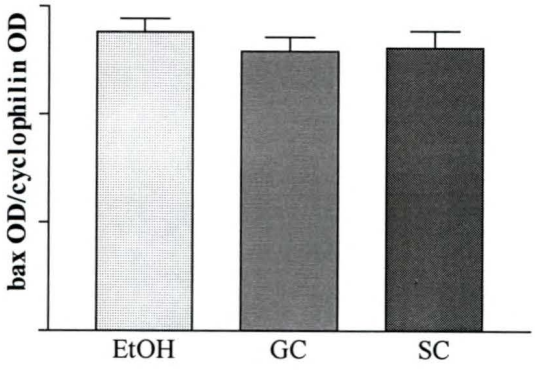


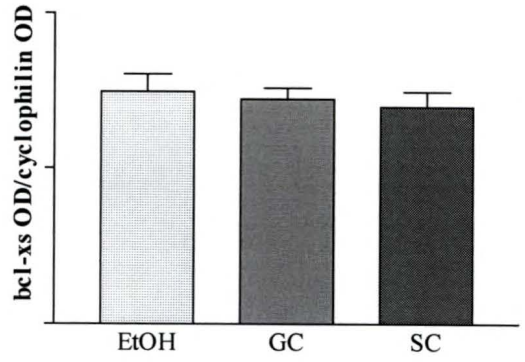
Figure 4-8. A further ethanol exposure on P5 does not significantly alter the expression of bcl-2 family mRNAs. mRNA levels of bax (panel A), bcl-xs (panel B), bcl-xl (panel C), and bcl-2 (panel D), relative to the internal standard cyclophilin, were determined in the cerebellar vermis following ethanol or control exposure via artificial rearing on P4-5. Pups were sacrificed two hours after the final ethanol or control infusion on P5, and the relative mRNA levels determined via the RNase protection assay. Ethanol exposed (EtOH), gastrostomized control (GC), suckle control (SC). Data are expressed as mean $\pm$ SEM. No significant differences were noted.

mRNA levels on P5 Following P4-5 Ethanol Exposure

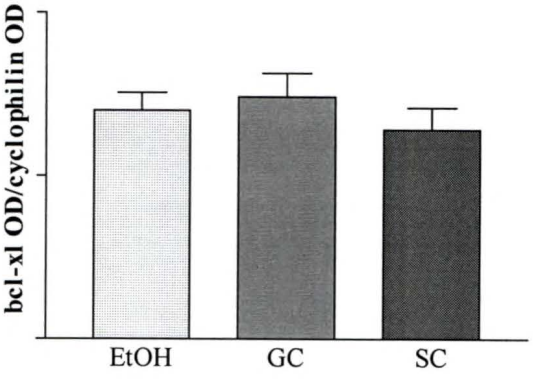
A bax



B bcl-xs



C bcl-xl



D bcl-2

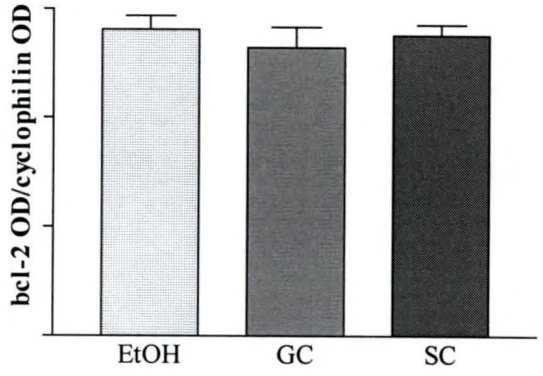
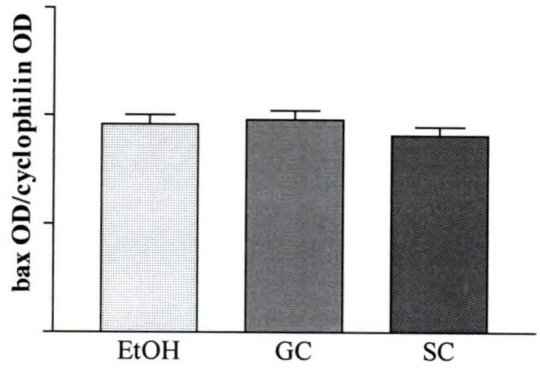


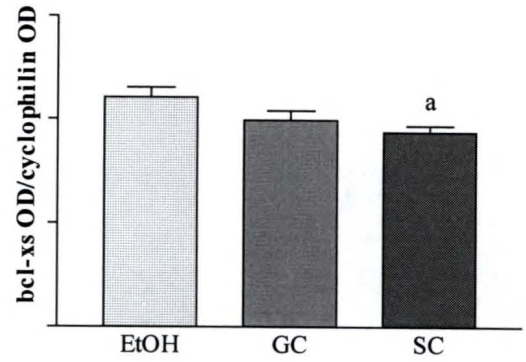
Figure 4-9. Effects of acute ethanol delivered on postnatal day 7 on bcl-2 family gene expression. mRNA levels of bax (panel A), bcl-xs (panel B), bcl-xl (panel C), and bcl-2 (panel D),relative to the internal standard cyclophilin, were determined in the cerebellar vermis following ethanol or control exposure via artificial rearing on P7. Pups were sacrificed two hours after the final ethanol or control infusion on P7, and the relative mRNA levels determined via the RNase protection assay. No significant alterations were noted for any transcript between ethanol (EtOH) and gastrostomized control (GC) pups, although EtOH and suckle control (SC) pups were significantly different in bcl-xs expression. Data are expressed as mean $\pm$ SEM. a: significantly reduced compared with EtOH ($p < 0.05$).

mRNA levels on P7 Following Acute P7 Ethanol Exposure

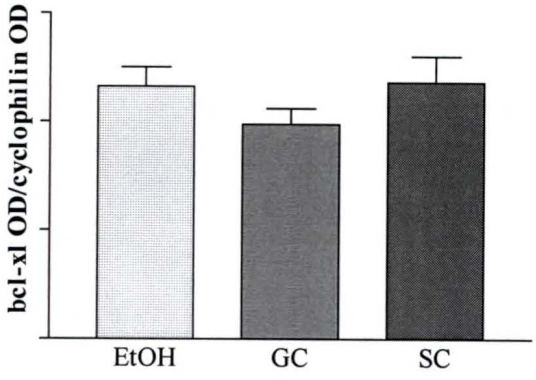
A bax



B bcl-xs



C bcl-xl



D bcl-2

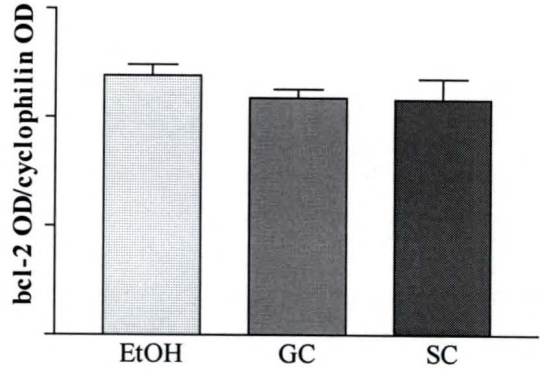
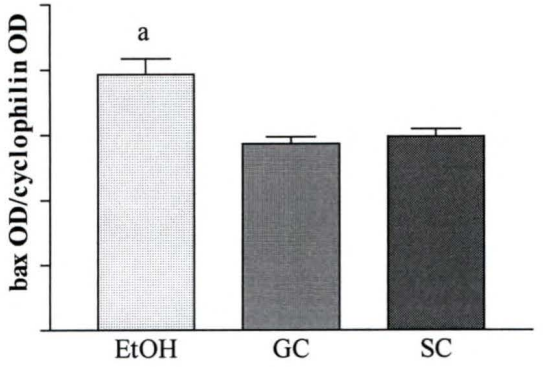


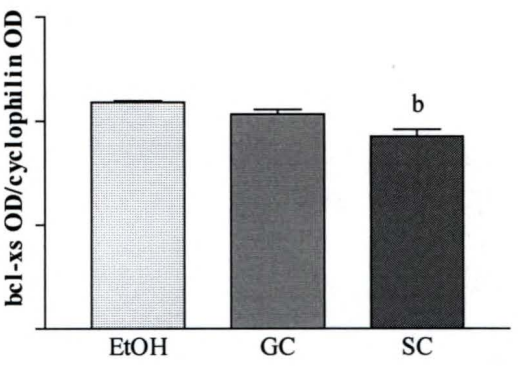
Figure 4-10. An additional ethanol exposure on postnatal day 8 increases mRNAs encoding the pro-apoptotic molecule bax. mRNA levels of bax (panel A), bcl-xs (panel B), bcl-xl (panel C), and bcl-2 (panel D), relative to the internal standard cyclophilin, were determined in the cerebellar vermis following ethanol or control exposure via artificial rearing on P7-8. Pups were sacrificed two hours after the final ethanol or control infusion on P8, and the relative mRNA levels determined via the RNase protection assay. Significant increases in mRNAs encoding the pro-apoptotic molecule bax were observed after ethanol exposure (EtOH) on P7-8, when compared with gastrostomized control (GC) and suckle control (SC) pups. SC pups contained significantly lower levels of mRNAs encoding bcl-xs, when compared with EtOH and GC pups. Data are expressed as mean $\pm$ SEM. a: significantly increased compared with GC and SC ($p < 0.001$). b: significantly reduced compared with EtOH ($p < 0.05$).

mRNA levels on P8 Following P7-8 Ethanol Exposure

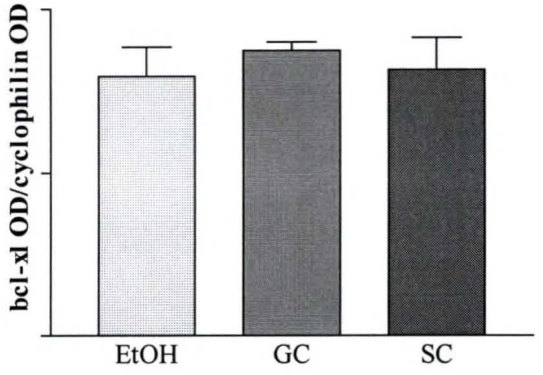
A bax



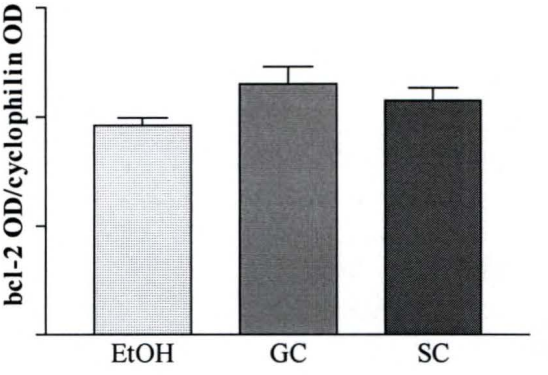
B bcl-xs



C bcl-xl



D bcl-2



CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Recapitulation of Results and Hypotheses Tested

Developmental disorders arising from maternal consumption of ethanol during pregnancy have been described in the clinical literature. An expanding body of work in animals is helping clinicians and basic scientists to better understand these developmental disorders so that prevention and treatment of the debilitating fetal alcohol syndrome may be pursued. Because ethanol exposure during development induces abnormal development in particular brain regions, and is known to alter the expression of particular genes and their protein products, the present body of work sought to further document neuronal populations in the brain which display vulnerability to developmental ethanol exposure. Another goal of the present work was to investigate cell death gene expression shortly after ethanol insult in the cerebellum in order to test a specific hypothesis about the cellular mechanism of differential ethanol neurotoxicity in the developing cerebellum. The results presented in the preceding chapters are summarized below, along with a recapitulation of the hypotheses that were tested, and their relation to the collected data.

Chapter 2: parvalbumin (PA) immunoreactivity in the medial septum and cingulate cortex following prenatal ethanol exposure

The objective of this study was to determine the long-term effects of prenatal ethanol exposure on parvalbumin-expressing (PA+) GABAergic neurons of the rat medial septum and anterior cingulate cortex. The hypothesis tested was that chronic prenatal ethanol exposure would lead to alterations in the number of neurons expressing PA in the adult rat medial septum and cingulate cortex. To test this, we fed an ethanol-containing liquid diet, a similar diet with the substitution of sucrose for ethanol, or a lab chow control diet to pregnant rat dams. In order to examine the long-term effects of this pattern of exposure, we sacrificed offspring of these dams on postnatal-day 60 and prepared their brains for parvalbumin immunocytochemistry.

While this exposure paradigm did not produce alterations in the size of the medial septum, or the size of the PA+ neurons therein, sexually dimorphic results were found for PA+ neuronal number in this region. Female rats, and not males, exposed to the ethanol-containing diet during gestation had 42 % fewer total PA+ neurons in the medial septum compared with sucrose controls. Females also had reduced PA+ cell density and fewer PA+ neurons per section when compared to female rats exposed to the sucrose diet. Male rats exposed to ethanol did not display a similar reduction in PA+ density or neurons per section.

The effect of prenatal ethanol exposure on the number of PA+ GABAergic neurons in the adult rat anterior cingulate cortex was also determined. No sexually dimorphic results were found. Male and female rats exposed to the ethanol-containing diet contained 45% fewer total PA+ neurons in the anterior cingulate cortex. Fewer PA+

neurons per section were also found in ethanol-treated rats compared with sucrose and chow controls. As with the MS, this reduction in PA+ neurons occurred in the absence of changes in the size of the region of interest or the size of PA+ neurons.

Thus, our hypothesis was supported by the data, namely that alterations in the number of neurons expressing PA in the adult rat medial septum and cingulate cortex were noted after chronic prenatal ethanol treatment. One interesting aspect of these data was that the medial septum was differentially affected by prenatal ethanol in male and females, with female offspring susceptible to reductions in PA+ neurons, and male offspring insusceptible. The anterior cingulate displayed no sexual dimorphism, and each gender was uniformly vulnerable to prenatal ethanol.

Chapter 3: choline acetyltransferase (ChAT) immunoreactivity in the medial septum following neonatal ethanol exposure

The objective of this study was to determine the long-term effects of neonatal ethanol exposure on the cholinergic neurons in the medial septum (MS) of the rat. The hypothesis tested was that early postnatal ethanol exposure would lead to alterations in the number of neurons expressing ChAT in the adult rat MS. To test this we utilized a neonatal ethanol exposure paradigm known as artificial rearing to infuse ethanol-containing or control diet from P4-10; gastrostomized pups and dam-reared pups were used as controls.

ChAT immunocytochemistry performed and the number of immuno-positive cholinergic neurons was determined at P60. Neonatal exposure did not directly reduce cholinergic neuronal number or the mean number of neurons per section. Similarly, no changes were noted in MS volume, mean area section, or cell density as a result of this

pattern of exposure. However, the size of cholinergic neurons was reduced in ethanol-treated males compared with gastrostomized controls, but not suckle control, males. No differences in ChAT+ neuronal size were noted for females. Ethanol treatment did result in long-lasting microencephaly in P60 animals, although cholinergic neurons in the MS were unaffected. Thus neonatal ethanol exposure produces long-lived microencephaly and small changes in ChAT+ neuronal size, but does not affect the number of cholinergic neurons or the size of the adult rat MS.

Therefore, our hypothesis was not supported by the data. Cholinergic neurons were not vulnerable to ethanol delivered during neonatal development. We did detect a small, but statistically significant, reduction in ChAT+ neuronal size, but this was only found in male animals. The whole of data from our laboratory regarding the effects of developmental ethanol exposure on the number of cholinergic neurons in the rat brain are consistent and point towards a lack of effect of ethanol on the number of cholinergic neurons (Swanson et al., 1996; Heaton et al., 1996; Moore et al., 1998a).

Chapter 4: differential cerebellar neuronal susceptibility and bcl-2 family gene expression following one- or two-day neonatal ethanol treatment

The objective of this study was to determine whether ethanol-induced cerebellar cell death during development is related to alterations in the expression of bcl-2 family genes. The hypothesis tested was that alterations in the expression levels of bcl-2 family PCD genes in the cerebellum contribute to the cerebellum's relative temporal susceptibility to ethanol neurotoxicity. To test this, a neonatal exposure paradigm similar to that used above was utilized. Transcript levels of bcl-2 family members relative to cyclophilin were measured to determine whether ethanol changed the expression of cell

death genes. To establish this method in our hands, pups exposed in parallel were taken for cerebellar cell counts to document the pattern of cerebellar cell loss following treatment. Exposure to ethanol during the first postnatal week resulted in significantly reduced Purkinje and granule cell numbers on postnatal day 21 (P21). Two hours after ethanol exposure on P4, transcripts encoding the cell death-promoting molecules bax and bcl-xs were up-regulated. Exposure for an additional day (on P5) resulted in no further alterations in bcl-2 family transcripts. This is probably because Purkinje cell death was found as early as P5.

To determine whether pro-apoptotic gene expression changes were specific to first postnatal week ethanol neurotoxicity, we examined bcl-2 family mRNA levels following ethanol treatment during the second postnatal week. This is a period of development in rats when the cerebellum does not exhibit profound toxicity in the presence of ethanol. While exposure on P7-8 produced no cerebellar cell death as measured on P21, this pattern of exposure did result in increased levels of bax mRNA. This up-regulation was found only after two-day ethanol exposure and not after acute exposure on P7. These data document increased expression bax and bcl-xs after acute ethanol exposure in first postnatal week. They also suggest that key factor influencing the differential survival of cerebellar neurons following ethanol exposure during more mature developmental stages may be related to better suppression of pro-apoptotic processes.

Thus, our hypothesis regarding differential cerebellar vulnerability and altered bcl-2 family gene expression was not supported by the data because neonatal ethanol exposure during both the first *and* second postnatal weeks produced altered bcl-2 gene expression. Thus, the situation is much more complicated that was initially predicted.

What likely accounts for the differential susceptibility, then, may be better suppression of pro-apoptotic processes in cerebellar neurons following ethanol exposure during the second postnatal week. These data are highly useful for the fetal alcohol field in that they provide a new avenue for future investigation, and provide the first demonstrations of altered bcl-2 family gene expression following ethanol insult.

In fact, it will be useful to consider the results from chapter 4 when follow-up investigations are made into neuroanatomical alterations resulting from developmental ethanol in regions such as the medial septum and cingulate cortex. For example, changes in the expression of bcl-2 family genes and proteins may occur in these regions following ethanol treatment. Perhaps a similar induction in pro-apoptotic genes underlies the noted reduction in PA+ neurons of the septum and cingulate. Perhaps the lack of effect of ethanol on cholinergic neurons is related to induction of anti-apoptotic genes, or, alternatively, better suppression of pro-apoptotic gene expression may occur, as seems to be the case with the cerebellum after second postnatal week exposure. Investigations at the protein level, using Western blot, will determine whether the noted changes in gene expression also occur at the protein level. The finding of increased expression of bax and bcl-xs can be extended to other pro-apoptotic bcl-2 family members, such as bad and bak. Anatomical techniques, such as in situ hybridization and immunohistochemistry, will determine whether both Purkinje and granule cells increase transcription of pro-apoptotic genes, and will determine whether changes occur at the same time, or whether induction happens first in Purkinje cells, and then granule cells. A more elaborate discussion of future directions is found below.

Choice of Animal Models

Two rat models of FAS have been utilized to examine the hypotheses put forth in this document. The first, used for chapter 2, involves the use of a pair-fed liquid diet to examine the effects of chronic prenatal ethanol treatment on PA+ neurons of the medial septum and anterior cingulate cortex. A pair-fed liquid diet was first utilized in rat models by Walker and Freund (1971), and has several advantages over other widely used prenatal exposure models, such as intraperitoneal injection or intubation. The most obvious is the elimination of nutrition as a confounding variable in the interpretation of results. Because the pair-fed animals in this paradigm are fed an isocaloric diet substituting sucrose for ethanol, and they consume the identical amount of diet as ethanol-treated counterparts, it is impossible that any observations result from reduced nutrition. Offspring from chow-fed dams are also included in our analyses to control for non-specific effects of the highly nutritive liquid diet. The importance of utilizing a pair-fed liquid diet in ethanol studies is driven home by an examination of data from other groups who have not utilized this technique. The most salient example is the choice of exposure method employed by Arendt et al. (1988) who simply added ethanol to the drinking water of adult rats and used chow-fed animals as controls. Clearly, nutrition cannot be ruled out in the interpretation of their data. Thus, in our prenatal experiments, every effort has been made to eliminate the possibility that nutritional deficiencies have contributed to the observed effects.

One drawback of the highly nutritive liquid diet is that it may lead to non-specific effects (see chapter 2). For example, Swanson et al. (1995) found a stimulatory effect of

liquid diet on ChAT enzymatic activity during the first postnatal week. Additionally, studies quantifying ChAT+ cell number in the P14 rat MS found a liquid diet-induced increase in ChAT+ neuronal number for female sucrose animals, but not ethanol females or male sucrose animals (Swanson et al., 1996). It was speculated that a possible sucrose diet-induced stimulation of MS cholinergic development occurred in female rat pups at P14. The seemingly protective and perhaps stimulatory nature of the liquid diet is probably due to its high vitamin and mineral content.

Similar effects were described in chapter 2, and we speculate that a liquid diet-induced increase in cell density for the sucrose group raised the base level of PA+ neurons in the liquid diet animals to a level greater than that seen in chows. The ethanol-treated group would presumably be exposed to the same factors in the liquid diet. However, a significant ethanol effect was noted since ethanol females had fewer PA+ neurons in the MS. Apparently prenatal ethanol treatment was damaging enough to produce a significant cellular reduction in ethanol females, even in the presence of the highly nutritive liquid diet. Even with these caveats, the use of the liquid diet is necessary to eliminate the possibility that altered nutrition contributes to any observed developmental alterations following prenatal ethanol exposure.

The second rat model utilized in this body of work (chapters 3 and 4) is the neonatal exposure model known as artificial rearing. This method of ethanol exposure is used when investigators want to mimic ethanol exposure during the third trimester equivalent in humans (Dobbing and Sands, 1979), and has advantages over other neonatal exposure techniques such as vapor inhalation and intubation. Precise delivery of diet by a syringe infusion pump (as with artificial rearing) is especially advantageous when longer

exposure times are utilized because the infusion pump can be programmed to deliver feedings when the investigator is away. With intubation, the investigator must administer each feeding, a laborious process when multiple feedings are performed daily. Experiments utilizing inhalation are often undermined by the inability of ethanol-exposed pups to nurse effectively after treatment (Ryabinin et al., 1995). With artificial rearing, post-ethanol feedings of milk alone are administered by the infusion pump, and thus no lapses in nutrition occur.

Artificial rearing was first introduced by Messer et al. (1969), modified by Hall (1975), and later modified by Samson and Diaz to include studies on ethanol and brain development (Diaz and Samson, 1980). West and colleagues have further refined the technique and have developed the most widely used variation of neonatal exposure paradigms (West et al., 1984). Even though this technique is complicated due to surgical implantation of gastric cannulas, as well as time consuming and expensive, it allows for precise nutritional control and ethanol delivery. Experimental design includes suckle-control pups, raised normally by the mother, gastrostomy-control pups, which are artificially reared, and fed a milk-based liquid diet, and ethanol pups, artificially reared and exposed to a similar diet including ethanol. Since both gastrostomy-control and ethanol groups are fed identical amounts of an isocaloric diet, ethanol effects can be examined without the confounding variable of nutritional deficits.

Disadvantages of artificial rearing do exist, such as labor-intensive surgery and pup care, expensive equipment and supplies, stress, and prolonged isolation from the dam and siblings. Another drawback is that the procedure itself can lead to direct effects or can interact with ethanol to produce alterations. For example, artificial rearing effects

have been noted on conditioned emotional response in female rats (Kelly et al., 1991), similar effects were and were noted in the present work (see chapter 3 and 4). We found that artificially reared control pups had increased ChAT+ somatic area compared with ethanol-exposed pups and suckle control pups. We also noted an increase in mRNA levels for bcl-xs in gastrostomized controls compared with suckle controls. These effects are likely because of the stress induced by the invasive technique in combination with littermate and maternal separation. Despite these shortcomings, the ability to exactly control the volume of diet delivered and the timing of delivery outweighs these drawbacks, and in many experiments artificial rearing effects are not found.

Ethanol inhalation, another method for neonatal ethanol delivery was first used by Goldstein (1972), and enables the non-invasive exposure of neonates. Pups are separated from the mother for an exposure period, and returned to maternal care following treatment. Alternatively, some newer techniques do not separate pups from the dam. In these protocols, the entire home cage is placed in the vapor chamber (Pal and Alkana, 1997). Ethanol exposure is carried out in a sealed chamber with an ethanol vapor source. Control groups consist of an unseparated group, and a control-separated group, which is taken from the mother for the same amount of time as the ethanol group, but inhales only air. The technique is advantageous since it allows for normal maternal interaction with pups, is inexpensive and easy to do, and requires no intensive animal care. However, the technique suffers from the need to cull ethanol exposed litters to three or more pups less than that of control-separated litters in an effort to eliminate nutritional differences. Moreover, the possibility of hypoxia exists, due to both reduced oxygen in the inhalation chamber (Ryabinin et al., 1995). Intubation, while not extensively used in the past, is

gaining in popularity, especially when experimental design includes short exposure periods (Goodlett et al., 1997). However, a drawback of this technique is that it requires much handling of pups, whereas with artificial rearing, minimal handling is used and pups are fed automatically by the syringe infusion pump. Stress is not significantly reduced by intubation since pups must be separated from the dam for the infusion period, and the method of administration still involves inserting a feeding tube down the esophagus of the pup. Thus, for the purposes of the experiments described herein, artificial rearing was the best choice of neonatal exposure method.

Choice of Cell Counting Methods

The recent development of stereological cell enumeration methods has created a controversy over the most appropriate and quantitative method for counting neurons. For example, the Journal of Comparative Neurology published a commentary stating that non-stereological cell estimation methods were assumption based and inappropriate for cell counting (Coggenshall and Lekan, 1996), leading Guillery and Herrup (1997) to conclude that journal policy is placing a "methodological straitjacket" on investigators. Criticisms of non-stereological cell counting methods (and data expressed as mean cells per section) are that overprojection and truncation can result from incorrectly identifying cells within a focal plane, and that changes in reference volume can bias cell counts (Peterson et al., 1997).

The use of manual and computer-automated cell counting in the present body of work is suitable because the use of relatively thin sections separated a distance which minimized the possibility of overprojection. Also, the size of the structures of interest

and the cells therein were always measured to determine whether ethanol treatment affected this parameter. Finally, we did not intend to estimate total cell number, and only wished to compare the mean number of cells per section between groups (Hagg et al., 1997). Moreover, Clarke and Oppenheim (1995) have demonstrated that the non-stereological cell counting methodology employed in the present work is as accurate and reproducible as stereological methods for a variety of neuronal populations.

Other Methodological Considerations

Immunohistochemistry was used in chapters 2 and 3 to examine the pattern of expression of parvalbumin and choline acetyltransferase after ethanol exposure during development. This is the best technique for localizing a specific protein in tissues, so that the anatomical localization of a particular molecule can be determined. Thus, for our purposes of identifying susceptible neuronal populations in structures such as the medial septum and cingulate cortex, where many different neuronal populations co-exist, the method of immunohistochemistry was most advantageous. This was one way to definitively examine only a subset of the entire neuronal populations within these structures (by utilizing specific antibodies rather than a gross staining technique such as a Nissl stain) and thus allowed for the testing of specific hypotheses regarding ethanol's effects on those subsets of neurons. Thus, it is important to selectively mark these populations for examination separately because many structures, for example, the medial septum, display differential neuronal vulnerability to ethanol. For our cerebellar cell counts in chapter 4, the use of a more general stain such as hematoxylin and eosin was

proper, because in that instance we were concerned with documenting direct ethanol-induced cell death in a structure without differential neuronal susceptibility.

The choice of the ribonuclease protection (RPA) assay for measurements of relative mRNA levels in chapter 4 is also a suitable choice, especially because of the highly quantitative nature and sensitivity of this technique. This is an important consideration when measuring transcripts of the bcl-2 family since the intracellular ratio of various molecules is an essential determinant of cell survival or death (Oltvai et al., 1993). The RPA is useful in this sense because it allows for the detection of small changes in expression that can have a significant influence on this ratio. Moreover, the fact that glial cells do not express high levels of these molecules means that there is little chance any observed changes in expression are due to non-neuronal cells (Frankowski et al., 1995; Vyas et al., 1997). Drawbacks for the use of RPA in this investigation include the lack of anatomical localization of the mRNA changes, and the lack of information at the protein level. However, the ease of quantitation and the ability to detect subtle gene expression changes provided by the RPA make it an appropriate choice for this study, which desired to determine relative levels of these transcripts following ethanol treatment.

Future Directions for Developmental Ethanol Research

The data presented herein are useful in that they add to the current knowledge of the range of CNS defects resulting from developmental ethanol exposure and suggest many future studies. What follows is an attempt to extend the discussion of the data reported here by listing future directions that are suggested from the data.

Further documentation of susceptible neuronal populations

Because the nervous system is not uniform in its vulnerability to developmental ethanol exposure an important aspect of fetal alcohol research has been, and will continue to be, to identify susceptible neuronal populations. This is necessary because the neuroanatomical substrates which underlie FAS must be identified before the syndrome can be adequately understood. The present body of work has demonstrated that PA+ neuronal number is reduced in the medial septum (in females but not males) and anterior cingulate cortex (in both genders). ChAT+ neurons were not affected in the medial septum following neonatal ethanol exposure, although cell size alterations were noted. Furthermore, we have replicated the observations of others that Purkinje and granule cells of the cerebellar vermis are decreased in number by neonatal ethanol treatment in the first, but not second, postnatal week.

One experiment suggested by the current data is to examine PA+ neuronal number in the anterior cingulate cortex of animals exposed to ethanol during neonatal development. This study would nicely complement the examination of PA+ neuronal number in the medial septum following chronic prenatal ethanol treatment, and would lend insight into whether these GABAergic neurons exhibit susceptibility only during the prenatal period, or if they are also vulnerable during the neonatal period. Another informative study would be to examine PA+ neuronal number in the hippocampus and cerebellum (after prenatal and neonatal ethanol exposure) since both structures contain many GABAergic neurons. It is conceivable that similar ethanol-induced alterations in PA+ neuronal number in these CNS areas might underlie the neuropathology of developmental ethanol disorders. Because no investigation has determined whether the

major excitatory neurons of the CNS are affected by ethanol, analysis of glutamatergic neurons (when specific markers are developed), especially in neocortical regions, would be useful following ethanol exposure during various developmental periods. All of these experiments could be completed with the use of the previously described techniques of immunohistochemistry followed by cell counts.

Functional studies

Because our data indicate that a subpopulation of GABAergic neurons expressing parvalbumin is reduced in number following developmental ethanol exposure, investigators should look for the functional consequences of this reduction. For example, investigators should record synaptic potentials from inhibitory interneurons in the rat hippocampus to determine whether their activity is altered following ethanol exposure. The GABAergic projections neurons from the medial septum depress the activity of these hippocampal interneurons normally, thereby exciting the pyramidal neurons involved with long-term potentiation (Freund and Antal, 1988). Ethanol exposure may depress the excitability of the hippocampus, and this might give insight into the mechanism of ethanol-induced learning and memory dysfunction.

Examining the excitation and inhibition of the anterior cingulate with extracellular recording could monitor functional alterations in this cortical region. Cortical lesions are known to result in decreased numbers of parvalbumin immunoreactive neurons leading to hyperexcitability of adjacent cortical areas (Jacobs et al., 1996). Similar release of inhibition may underlie the reductions noted in the present work. Similarly, the input of the cingulate into Papez' circuitry could be monitored by recording in the entorhinal cortex. Investigations such as this could help to further define the neuroanatomical

changes which underlie the learning and memory problems in children with fetal alcohol syndrome.

A variety of imaging studies are suggested by the data presented in this body of work. For example positron emission tomography with radio-labeled glucose would be useful to determine whether glucose utilization is altered in the basal forebrain, cingulate cortex, hippocampus, or entorhinal cortex of children with FAS. The noted reduction in parvalbumin immunoreactive neurons in the present study suggests that normal glucose utilization might be changed. This is especially possible because of the role of inhibitory interneurons and projection neurons in regulating the excitation of other regions. For example, hippocampal excitability (and glucose utilization) might be depressed in ethanol-exposed children due to a decline in inhibitory input from the medial septum on inhibitory hippocampal neurons.

Investigation into the involvement of bcl-2 family in PA+ neuronal susceptibility and ChAT+ neuronal insusceptibility

The results from chapter 4 on increased expression of pro-apoptotic mRNAs of the bcl-2 family raises the possibility that similar changes occur in the medial septum and/or cingulate cortex following developmental ethanol exposure. For example, induction of pro-apoptotic genes might precede the noted reduction in PA+ neurons in the medial septum and cingulate cortex. Other pro-apoptotic bcl-2 family members should be investigated, besides bax and bcl-xs, such as bad and bak, as different brain regions may utilize different cell death molecules. The lack of effect of developmental ethanol exposure on cholinergic neurons might be related to decreased expression of death genes, increased expression of survival genes, or successful post-translational squelching of cell

death processes (see below). Double-labeling with antibodies to both PA or ChAT and various bcl-2 family members, followed by fluorescence microscopy, would allow investigators to examine bcl-2 family expression in subpopulations of neurons.

Further investigation into the cellular and molecular mechanism of ethanol-induced neurotoxicity

While the descriptive studies outlined in chapters 2 and 3 (and the future experiments suggested above) are useful for identifying novel populations of neurons that exhibit vulnerability to developmental ethanol exposure, further investigation into the cellular and molecular mechanisms of ethanol-induced neurotoxicity is warranted. Data presented in chapter 4 attempted to address this issue by investigating bcl-2 family gene expression following neonatal ethanol exposure. The noted induction of pro-apoptotic gene expression following both first postnatal week and second postnatal week ethanol exposure suggested that the differential survival of cerebellar neurons following neonatal exposure may be related to better suppression of these pro-apoptotic processes. The following are suggestions to increase our understanding of how these pro-apoptotic processes are induced by ethanol, to identify key downstream players in ethanol-induced cell death, and to attempt to identify the key differences in more mature cerebellar neurons which decrease their sensitivity to ethanol. Many of the suggestions utilize the cerebellum due to the fact that the principal neuronal types, the Purkinje and granule cells, are susceptible to ethanol, the fact that it has been clearly demonstrated to be a structure which displays considerable vulnerability to developmental ethanol exposure, and the fact that it is useful for both in vivo and in vitro studies. Still other experiments involve the use of transfected cell lines and the use of dominant-negative mutations to

determine a molecule's involvement in ethanol-induced cell death. Further investigations into the cellular and molecular mechanisms of ethanol neurotoxicity will benefit the fetal alcohol field by identifying molecular targets for therapeutic intervention in ethanol neurotoxicity. The following are lines of investigation that the data presented in chapter 4 suggest.

Pro-apoptotic gene expression and p53

The present investigation documented increased expression of the pro-apoptotic genes bax and bcl-xs following neonatal ethanol exposure. One of the first experiments which should be done is to extend these investigations to other pro-apoptotic members of the bcl-2 gene family, such as bad and bak. This could be accomplished at the mRNA level with the RNase protection assay (for comparison to the present study), and at the protein level with Western blot. Another future direction is to determine what intracellular factors are responsible for the noted induction in pro-apoptotic mRNAs. Induction and expression of the tumor suppressor gene p53 is known to up-regulate bax gene expression (Mitry et al., 1997; Xiang et al., 1998), leading to apoptotic cell death. Additionally, phorbol ester-mediated activation of protein kinase C (PKC) blocks nitric oxide-induced apoptosis through suppression of p53 activation and decreased levels of Bax protein (Messmer and Brune, 1997). Intriguingly, preliminary data from our laboratory indicate that PKC activity is decreased in the cerebellar vermis two hours after ethanol treatment in vivo (Davis et al., unpublished observation). These data suggest that a similar role for reduced PKC activity may lead to p53 activation and increased bax/bcl-xs gene expression following ethanol exposure. This could be investigated in vivo with

phospho-specific antibodies for p53 activation and analysis of bax/bcl-xs gene expression following ethanol treatment.

It would be of interest to determine whether an induction of p53 following reduced PKC activity is sufficient to increase bax and/or bcl-xs mRNAs. An in vitro cerebellar model system utilizing primary cultures of cerebellar neurons could be used for this purpose. For example, can phorbol esters block ethanol-induced apoptosis of cultured cerebellar cells and does this lead to decreased p53 and Bax accumulation? p53 activation could be examined by the use of antibodies specific to phosphorylated p53 with standard Western blot techniques and extraction with protease and phosphatase inhibitors. Cell lines could be transfected with dominant negative mutants of p53, and bax and bcl-xs mRNA production monitored to directly determine a role for p53 in ethanol-induced apoptosis. A role for p53 would be indicated by amelioration of ethanol-induced cell death in cells transfected with the mutant (inactive) constructs. If cell death still ensued upon ethanol treatment, then other transcription factors would be implicated.

Many members of the bcl-2 family contain consensus sequences for NF-kB, so it is possible that this transcription factor is involved in bax and bcl-xs mRNA upregulation (Dixon et al., 1997). For example, does activation and nuclear localization of NF-kB result from ethanol treatment? If so, does ethanol-induced activation of NF-kB lead to increased mRNA levels of bax and bcl-xs? Do dominant negative mutants of NF-kB inhibit ethanol-induced cell death? Evidence from Dixon et al. (Dixon et al., 1997) indicates that the induction of bcl-xs gene expression following ischemia occurs along with increased NF-kB activation and nuclear translocation. A similar correlation may be found with ethanol-induced increases in bax and bcl-xs gene expression in our in vivo

cerebellar model system. These questions could be answered with phosph-specific antibodies to investigate NF-kB activation with standard Western techniques and sample homogenization in the presence of protease and phosphatase inhibitors. Immunoflorescence and confocal microscopy could be used to investigate nuclear translocation of NF-kB. An in vitro model system with cell lines stably transfected with dominant-negative constructs of NF-kB could be used to definitively demonstrate its involvement in ethnaol-induced up-regulation of bax and bcl-xs genes. mRNA levels of varioius bcl-2 family members can be investigated with the described methods (see chapter 4) for the RNase protection assay.

Downstream of the bcl-2 family: caspases and cytochrome C

Another direction suggested by the noted increase in bax and bcl-xs gene expression is to look at caspase induction and activation following developmental ethanol exposure. This could be done in vivo, for example, by examining caspase 3 protein levels and proteolytic activity in the cerebellar vermis in the acute phases following neonatal ethanol exposure. The fact that we saw significant gene expression changes two hours after the end of the ethanol insult suggests that this would be the timepoint to start looking for increased levels of caspases and increased activation. One could confirm caspase involvement by determining whether particular caspase substrates are cleaved, such as poly (ADP-ribose) polymerase (PARP), and whether ethanol treatment in vitro or in vivo results in the increased production of the 89 kDa fragment characteristic of PARP cleavage (Duriez and Shah, 1997). Another experiment would be to determine whether caspase inhibitors such as YVAD and DEVD can inhibit ethanol-induced cell death in cultures of cerebellar granule cells. It is conceivable that the difference between first and

second postnatal week suppression of pro-apoptotic processes occurs at the level of caspase activation. Thus, investigations should compare the cellular changes in caspases after first or second postnatal week exposure. Because release of cytochrome C is an important early event in the activation of caspases (Li et al., 1997), measurements of the cytoplasmic concentration of cytochrome C in cerebellar granule cell cultures would be useful following ethanol treatment.

bcl-2 family dimerization and post-translational modification

Members of the bcl-2 family are capable of homo- and hetero-dimerization via BH3 domains (bcl-2 homology domain 3) present on all family members (Zha et al., 1996; Zha et al., 1997). Homodimerization of anti-apoptotic members promotes cell survival while homodimerization of pro-apoptotic molecules promotes cell death (Reed, 1997). Because the data presented in the present work only address the induction of pro-apoptotic processes (as measured by gene expression) and do not address translational or post-translational issues, it would be important to describe the pattern of bcl-2 family protein levels and dimerization state, and phosphorylation status following ethanol treatment in the first and second postnatal week.

The subject of bcl-2 protein levels can be easily addressed with the Western blot technique. Levels of phosphorylated bcl-2 family members can be described with phospho-specific antibodies and the use of protease and phosphatase inhibitors during protein extraction. Dimerization can be investigated with co-immunoprecipitation followed by immunodetection with antibodies to proteins known to utilize the BH3 domain for dimerization. All of these experiments can be performed in vivo, and thus can utilize the in vivo cerebellar vermis following acute ethanol administration in the first

postnatal week. Comparisons of bcl-2 family protein levels, phosphorylation, and dimerization after second postnatal week ethanol treatment would also be enlightening, since this pattern of exposure was capable of inducing pro-apoptotic processes but not cell death. For example, is the phosphorylation or dimerization different following these patterns of exposure? Can post-translational modification counteract the up-regulation of these molecules and inhibit cell death?

JNKs and post-translational modification of anti-apoptotic molecules

Mitogen-acitvated protein kinases (MAPKs) are important intracellular mediators of extracellular survival, growth, differentiation, and stress signals (Elion, 1998). Multiple MAPK cascades exist in cells, and control such disparate processes as differentiation, growth, and stress response. The c-Jun N terminal kinase/stress activated protein kinase (JNK/SAPK) sub-family of MAPKs is one example that is important in intracellular processing of extracellular stress signals. There is reason to suspect that a stressor such as ethanol would activate this the JNK/SAPK pathway. Preliminary data from our laboratory indicate that ethanol delivered in vivo causes an induction of JNK activity (as indicated by increased phosphorylation of the p54 JNK isoform) two hours after ethanol treatment on P4 in the cerebellar vermis (Davis et al., unpublished observation). Because of the noted link between extracellular-derived factors and post-translational modification of bcl-2 family members (Kahn, 1998), it is of considerable importance for investigators of ethanol neurotoxicity to determine whether the intracellular effectors of these extracellular signals are altered by ethanol. For example, are neurons responding to stress signals initiated by the presence of high ethanol

concentrations by activating JNK/SAPK cascades? Moreover, are these activated kinases acting on bcl-2 family members as substrates to alter their functioning?

The noted induction of JNK activity following in vivo ethanol treatment might have profound implications for bcl-2 family members, as they are known substrates of activated JNK. In particular, the anti-apoptotic Bcl-2 protein is phosphorylated by p54 JNK, and MAPK specific phosphatases block Bcl-2 phosphorylation when given concurrently with JNK (Maundrell et al., 1997). The significance of increased Bcl-2 phosphorylation appears to be the inactivation of Bcl-2's anti-apoptotic function (Maundrell et al., 1997). Thus, an experiment of future importance would be to determine whether Bcl-2 and other bcl-2 family members are post-translationally modified by the noted p54 JNK activation. This could be accomplished with phospho-specific antibodies (serine and threonine residues) to bcl-2 family members on Western blots and could be done in vivo or in vitro. A follow-up experiment in vitro would be to inhibit p54 JNK activity with phosphatases to determine whether this blocks the ethanol-induced, (and possibly p54 dependent) bcl-2 family member phosphorylation. Comparisons of ethanol-induced changes in JNKs following exposure in the first and second postnatal week would be illuminating. It is possible that differential regulation of bcl-2 family members by JNKs is responsible for the differential suppression of pro-apoptotic processes following ethanol exposure.

Upstream mediators of bcl-2 family function: PI3K and Akt

As mentioned earlier, an important recent discovery has been that extracellular factors can influence the function of bcl-2 family members (Datta et al., 1997; Kahn, 1998; Zha et al., 1996). For example, it was known that growth factors could stimulate

PI3 kinase and its downstream target Akt (Zhou et al., 1997) but the mechanism of Akt-induced cell survival was unknown. Then two groups discovered that growth factor activation of PI3 kinase and Akt resulted in the phosphorylation of the pro-apoptotic bcl-2 member Bad (Datta et al., 1997; Zha et al., 1996). Bad phosphorylation inactivated its death-promoting activity and resulted in its accumulation in the cytosol, away from Bcl-2 or Bcl-xl (Zha et al., 1996). Therefore, it has been definitively demonstrated that extracellular signals influence the function of bcl-2 family members and that this influences the survival of the cell. Although similar modifications have not been described for Bax or Bcl-xs (molecules whose transcripts were up-regulated after neonatal ethanol exposure) it is possible that these mechanisms exist.

Another line of work would be to investigate the mediators of cell life and death which function upstream of the bcl-2 family to determine whether ethanol produces any alterations at these levels. For example, does ethanol treatment lead to decreased PI3 kinase and/or Akt activation and decreased Bad phosphorylation? Preliminary data from our laboratory indicate that Akt activation and Bad phosphorylation are decreased in the cerebellar vermis two hours after ethanol treatment in the first postnatal week (Davis et al., unpublished observations). Given that second postnatal week ethanol exposure does not produce cell death one question that arises is whether or not PI3 kinase activation is preserved following second postnatal week ethanol exposure, and is Bad phosphorylation similarly preserved? Can differences in the temporal availability of growth factors account for this differential toxicity? These questions can be answered with phospho-specific antibodies and standard Western blot techniques following protein extraction with protease and phosphatase inhibitors.

Growth-factor suppression of pro-apoptotic processes: extrinsic modulation of intracellular conditions favoring cell death

The most likely mediator of the differential toxicity noted in the cerebellar vermis seems to be better suppression of pro-apoptotic processes induced by ethanol by growth factors. This is an attractive hypothesis because neurotrophins are known influence the survival and differentiation of cerebellar Purkinje and granule cells developmentally. Additionally, the ontogeny of neurotrophins and their cognate receptors in the cerebellum is consistent with this hypothesis. Neurotrophin receptors are expressed at high levels in rat Purkinje and granule cells during development and the expression of neurotrophin ligands and receptors is regulated in a spatio-temporal manner (Lindholm et al., 1997). For instance, Trk B receptors are only found on differentiating granule cells (and not proliferating granule cells), and only granule cells in the process of differentiation respond to BDNF or NT-3 (Gao et al., 1995). Furthermore, although ontogeny begins on P4, the peak period of Purkinje cell expression of trkA mRNA does not occur untilP10, during the later stages of Purkinje cell differentiation (Wanaka and Johnson, 1990). Alternatively, other, as yet undiscovered, growth factor pathways may also interact with the bcl-2 family, and might play a role in differential ethanol-induced cell death. Whether PI3 kinase and Akt are the mediators of the suppression of ethanol-induced pro-apoptotic processes remains to be seen, and it is possible that other pathways are involved. However, the known influence of other growth factors on this pathway makes it an appropriate place to start. Activation of these pathways might result in post-translational modification of Bax or Bcl-xs protein, perhaps inactivating their death-promoting properties, or modification of Bad might be involved.

Therefore, an important hypothesis to test is that the activation of intracellular growth factor signaling processes, which would presumably decrease cell death by suppression of pro-apoptotic processes, is a key factor governing cerebellar neuronal susceptibility to ethanol. In our model system, second postnatal week ethanol exposure produces an induction of pro-apoptotic processes without concomitant cerebellar cell death. It is conceivable the increase in cell death mRNAs is counteracted by growth factor-derived phosphorylation of these or other molecules, such as Bad. This would presumably enable anti-apoptotic members of the bcl-2 family to function in their normal capacity to regulate mitochondrial membrane potential. The ethanol-induced induction of pro-death genes, in combination with a decline in growth factor-derived Bad phosphorylation in the first postnatal week, may lead to impaired anti-apoptotic processes, leading to lost mitochondrial membrane potential, release of cytochrome C, and caspase activation.

This hypothesis can be tested descriptively by documenting Akt activation and Bad phosphorylation following acute second postnatal week. Unchanged or increased levels of phosphorylated Bad, Bax, or Bcl-xs, along with unchanged or increased levels of active Akt, following second week exposure would provide support for this hypothesis. Comparisons should be made to the first postnatal week as well. As mentioned previously, Akt activation and Bad phosphorylation are both decreased in the vermis after first postnatal week ethanol exposure (Davis et al., unpublished observation). These studies should be extended to include Bax and Bcl-xs. Analysis of Akt activation Providing growth factors in vitro to early neonatal cerebellar neurons along with ethanol and examining cell death and pro-apoptotic molecule phosphorylation would determine

whether growth factor addition could protect early neurons from cell death induced by ethanol. Also, direct effects of growth factors on pro-apoptotic (and anti-apoptotic) gene expression could be examined in vitro.

Conclusion

Despite the fact the FAS has been recognized since 1973 (Jones and Smith, 1973; Jones et al., 1973) the incidence of ethanol-induced developmental abnormalities continues to increase (Prevention, 1995) and remains 20 times higher in the United States than in Europe (Abel, 1995). Thus, alcohol-related neurodevelopmental disorders constitute a serious health problem, and research on the neuroanatomical substrates underlying these disorders and the mechanisms through which ethanol acts as a teratogen is essential if treatments for these defects are to be discovered. With these goals in mind, the research described in this document was pursued.

The present work identified the population of GABAergic neurons expressing parvalbumin as one that is affected by developmental ethanol exposure. Parvalbumin-immunoreactive neurons were decreased in number in the medial septum (in a sexually dimorphic manner) and anterior cingulate cortex (without sexual dimorphism). This work also determined that cholinergic neurons are not susceptible to neonatal exposure during a more mature developmental timepoint. Another advance made by this body of work was to open up new lines of research on the molecular mechanisms of developmental ethanol neurotoxicity. This was accomplished by the determination that pro-apoptotic gene expression is up-regulated in the cerebellar vermis following neonatal ethanol exposure. New research on the consequences of this induction, and the potential

of growth factors to ameliorate this up-regulation, will potentially identify molecular targets for inhibiting ethanol-induced cell death. Therefore, the data generated in the current study are useful in that they not only identify a novel population of neurons affected by developmental ethanol treatment, but they also suggest new avenues of research on the molecular mechanism of ethanol teratogenicity. Investigators will certainly continue to pursue this, and other, lines of work in hopes of better understanding the developmental alterations induced by ethanol, and in hopes of developing treatments to prevent these disorders in alcoholic mothers.

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BIOGRAPHICAL SKETCH

David Blaine Moore, a Florida native, was born in Jacksonville on July 3, 1972. He attended Fletcher High School in Neptune Beach until 1990, where he developed an interest in science. He studied biology at the University of North Florida until 1993, and met his future wife, Terri Edwards, there. His graduate studies began in the Neuroscience Department at the University of Florida College of Medicine in 1994. His Ph.D. research on animal models of ethanol-induced brain injury during development was performed in the laboratory of Dr. Marieta Heaton and in collaboration with Dr. Don Walker. In graduate school, Blaine was the grateful recipient of an NIAAA predoctoral fellowship and an individual NRSA.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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