Experimental Fetal Alcohol Syndrome:

The effects of prenatal ethanol exposure on reproductive behavior and neuronal expression of the proto-oncogene c-fos.

by

Regina Vivo

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ABSTRACT

Experimental Fetal Alcohol Syndrome:
The effects of prenatal ethanol exposure on reproductive behavior and neuronal expression of the proto-oncogene c-fos.

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Ethanol, the main ingredient in alcohol, is the cause of Fetal Alcohol Syndrome. Although it's mechanisms of actions remain unknown, prenatal exposure to ethanol has been known to produce severe effects on embryogenic development including CNS anomalies, hyperactivity, growth deficits, infant mortality and behavioral abnormalities. This experiment was designed to examine the effects of ETOH exposure in utero on the reproductive behavior and neuronal expression of c-fos in adult male rats. Pregnant rats were divided into three experimental groups and given diets of either 35% ETOH or liquid control or solid food control. Offspring from the ETOH treated group had higher infant mortality rates when compared to either the pair-fed or solid food control groups and significantly shorter body lengths on day 1. Results from copulatory tests showed no significant differences in IL, ML, and EL between groups for three test sessions. Moreover, the decreases in ML, IL, and EL seen between copulatory tests 1 and 2 denotes a pattern of learning behavior. Therefore, we suggest that ETOH

treatment had no significant effects on the sexual learning of the offspring.

The immunohistochemical analysis of fos revealed c-fos expression in the NAc, DBB, CPU, and MPOA in animals of all test groups. However, an overall increase in fos levels were found in ETOH treated animals suggesting that prenatal exposure to ETOH elevates the expression of fos in response to stimulation. A significant main effect of diet was seen in the NAc and DBB areas between test groups with a greater number of fos-stained nuclei in the ETOH group. There was also a significant interaction between diet and exposure to female pheromones in the levels of fos expressed in the MPOA. The decreased number of fos-stained nuclei in the ETOH treated animals was related to the ETOH exposure in utero. Exposure to female pheromones caused an increased fos expression in the CPU of solid food diet animals versus controls. Furthermore, fos levels were increased in the ETOH controls as compared to the solid controls. These findings suggest that prenatal exposure to ETOH has little effect on copulatory behavior and no effect on sexual learning. In contrast, a significant effect of ETOH on the neuronal expression of c-fos in select brain areas was observed.

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CHAPTER I

INTRODUCTION

Alcohol abuse is one of the major world wide health concerns. It is second only to caffeine, as the most commonly used psychoactive substance (Samson and Harris, 1992). North American Studies report alcohol addiction in 4% of women and 19% of men (Froster and Baird, 1992). In 1990, approximately $136 billion was spent on alcohol-related institutions and an increase to over $150 billion is estimated for 1995 unless changes in drinking habits and prevention approaches occur (Sampson and Harris, 1992).

Since alcohol produces both physiological and psychological effects and the mechanisms involved are still unknown, attempts to combat alcohol related problems are further complicated. These statistics put even greater significance on the need for alcohol research. Currently, alcohol is the most commonly researched behavioral teratogen and a more complete understanding of how alcohol effects embryogenesis, biochemical and physiological systems along with behavioral patterns will provide us with better prevention and defense (Streissguth et al., 1986).

Alcohol is a compound produced from the replacement of an H atom with a hydroxyl group in a hydrocarbon. Based on its structural diversities, actions are believed to be attributed to multiple receptors. Alcohol also belongs to the family of intoxicant anesthetics and displays both cross dependency and cross tolerance. Moreover, the pharmacological behavior of alcohol abuse

is based on a variety of determining factors such as type, dose, and route of administration. Conditioned environmental cues are also known to elicit alcohol dependant behaviors and are important in the relationship to human alcohol abuse and drinking patterns (Sampson and Harris, 1992).

Ethanol (ETOH) is the main alcohol ingredient in alcoholic beverages. ETOH is an easily diffusible, moderately polar mono hydrate substance that is infinitely water soluble and produced through sugar fermentation of a variety of vegetable sources. Once ingested, it is immediately absorbed and distributed throughout the body where it is oxidized into CO_2 and water in the liver (Van Theil et al., 1988). ETOH metabolism in the liver occurs in two separate pathways: 1) oxidatively to acetaldehyde via alcohol dehydrogenase or the microsomal ethanol oxidizing system (MEOS) and 2) nonoxidatively to fatty acid ethyl esters (FAEE). The primary oxidative metabolite of ethanol, acetaldehyde, is rapidly metabolized by embryonic tissue and suspected to contribute to the ethanol induced teratogenic effects seen with Fetal Alcohol Syndrome (FAS) (Bearer et al. 1992; Van Thiel et al. 1989). Interesting results however, show acetaldehyde to be a very strong embryotoxic agent, easily killing embryos at 20 ug/ml concentrations. The synergistic effects of ethanol and acetaldehyde as major factors in producing FAS merit further investigations (Giavini et al., 1991). The formation of the nonoxidative metabolite of ethanol in adults, FAEE, is catalyzed by the enzyme FAEE synthetase. Formation of these FAEE accumulate in

the major organs and may indicate an ethanol induced toxicity mechanism or a marker for FAS (Bearer et al., 1992).

Fetal Alcohol Syndrome

Among the many alcohol-related problems in humans, Fetal Alcohol Syndrome (FAS) is one of major concern. FAS is a collaboration of structural malformations, growth deficiencies and CNS abnormalities. Growth deficiencies are both prenatal and postnatal with retardation in height and weight (Streissguth et al., 1986). Manifestation of the CNS anomalies include structural anomalies, neurological abnormalities, developmental delays, hyperactivity and learning disabilities (Streissguth, 1992).

FAS was first documented by Jones et al, in 1973 which gave international awareness to its correlation with alcohol abuse (Giavini et al., 1992). Presently, one in 600 infants may be born with FAS as a result of maternal alcohol consumption during pregnancy (Streissguth, 1992). Recent reports show that about 40% of alcohol-related pregnancies result in severely teratogenically affected offspring (Froster and Baird, 1992). Furthermore, past reports have shown this syndrome to be dose related and vary in severity with other factors such as environment, drinking habits, overall health, and length of exposure during gestational growth and development (Streissguth et al., 1986). Ethanol is the teratogenic cause of FAS and although its mechanism of action is unknown, it is reported to produce severe effects on embryogenic development. An individual must have abnormalities that meet the

three requirements used as a guideline to positively diagnose a condition as FAS. The three criteria are as follows: 1. Alterations in the CNS (including abnormalities in neurological and brain development along with behavioral disorders); 2. Characteristic facial features (including craniofacial anomalies); and 3. Pre- and/or postnatal growth deficits in weight and/or length (Sokol and Clarren, 1989). In addition, FAS requires a specific diagnosis buy medical personnel with specialized training and experience in dysmorphologies and congenital abnormalities (Streissguth, 1992). Diagnosing FAS is difficult because of the subtleties involved and as a result most progeny most likely go undetected at birth. Only the most severely affected with obviously distinguishable characteristics can be diagnosed early. Most detections have been made by 8 years of age because the characteristic physical features become less distinct after puberty. Therfore, the high percentage of undiagnosed FAS cases are usually attributed to the lack of trained personnel (Streissguth, 1992).

Many malformations associated with FAS are craniofacial anomalies and include a thin upper lip, indistinct philtrum, flat midface, and short palpebral fissures as well as other less differentiating features such as flat nasal bridge, micrognathia, microcephaly, and epicanthial folds (Streissguth et al., 1986). Other related congenital defects include limb deformities, mainly terminal transverse defects, skeletal anomalies such as metacarpal fusions and radio-ulnar synostosis, and fusion of the cervical spine (Froster and Baird, 1992). Moreover, cardiac defects are

also commonly seen accompanying limb defects in patients with FAS (Kotch and Sulik, 1992). Several pathological deformities have been documented in the eyes as well. Various ocular anomalies include microcornea, asymmetrical microphthalmia and marked loss of retinal ganglion cells (Clarren et al., 1990).

More milder cases, not quite meeting all requirements but displaying similar characteristics have been termed Fetal Alcohol Effects (FAE) and are not considered to be reciprocal with alcohol teratogenicity (Streissguth et al., 1992). It has been suggested, however, that the term FEA not be used for diagnostic/prognostic use in the medical society because of it's ambiguous and non-uniform meaning (Sokol and Clarren, 1989).

Animal models have been particularly important because they demonstrate a wide variety of affects that are associated with FAS as well as provide an opportunity to control for the factors which are usually unaccounted for in human studies such as exposure time and dose of ETOH treatment (Streissguth et al.,1986 and 1980). Neurological defects, fetal growth retardation, and embryonic loss have been a few of the effects seen in laboratory rats as a result of ethanol exposure in utero (Fadel and Persaud, 1992). Studies done on the effects of prenatal ETOH exposure in rat embryogenesis report the most severe effects were seen in the placodes, branchial arch mesenchyme, and neuroepithelial thickness (Giavini, et al. 1991). There was also a significant delay in the closure of the post neuropore along with a considerable decrease in the number of somites (Fadel and Persaud, 1992). These authors also demonstrated

that exposure to ETOH during early neuroblast proliferations produced abnormal phenotypic patterns of expression, highly dysmorphic limb development, head anomalies, and defects in skeletal bone structure. Compared to controls, ETOH exposed embryos showed head lengths that were significantly decreased along with decreases in the degree of flexion ability and morphological scores (Fadel and Persuad, 1992).

In order to better understand the effects of ETOH exposure in utero, clinical studies of FAS diagnosed humans have been conducted as well as various animal studies. These studies have provided a wealth of information concerning the effects of prenatal ETOH exposure on a wide range of systems. For example, specific gender differences have been noted. Previous experiments have demonstrated that ETOH exposure in males caused a greater reduction in locomotor activity (Middaugh, et al., 1991) whereas females demonstrated delayed sexual maturity (Weinberg and Jerrells, 1991). The immune system, including immune cell function, has also been reported to be compromised as a result of ETOH exposure in utero (Weinberg and Jerrellis, 1991). In addition, glucose homeostasis as well as metabolic function have been reported to be altered by ETOH exposure in utero as well. Glucose, the primary brain energy substrate, is a crucial determinant in the maintenance of fetal tissue. The effects of ETOH exposure in utero have been reported to alter glucose metabolism and therefore, contribute to the fetal mortality, growth retardation, and congenital deformaties that are associated with FAS and the similar effects seen in laboratory

rodents (Murdoch and Simm, 1992). Furthermore, glucose uptake in the brain depends upon both the blood glucose concentrations and the transport mechanism of the blood brain barrier's plasma membrane (Singh, 1992). This transport mechanism is mediated by a glucose transport carrier (GT) which also serves to be a potential rate-limiting step in the utilization of glucose. Singh and Pullen (1992) reported maternal ETOH consumption decreased the net uptake of glucose into fetal brain, decreased the amount of GT gene expression and reduced fetal plasma glucose concentrations nearly 26% lower than controls (Singh, 1992).

Effects of ETOH on the Central Nervous System

FAS has been reported to be the major cause of mental retardation (Streissguth et al. 1986). Mental deficiencies vary greatly from severely retarded to normal intelligence in FAS patients. However, the average FAS level of intelligence in adults is mildly retarded with an average academic function ability of the 2nd to 4th grade (Streissguth 1992). One clinical report demonstrated the inverse relationship of IQ scores to the severity of diagnosis with the most severely diagnosed as having the lowest IQ. Average IQ score for a FAS child was 68 (Streissguth et al. 1986).

Of all academic challenges, math skills were reported to be the most difficult. This is probably attributed to the difficulty associated with abstract thought processing. FAS progeny also suffer from alterations in social interactions and abnormal

behavioral patterns. One case study by Streissguth (1992) showed that few FAS children remained in school to completion, an even smaller percentage were able to hold jobs, and none were able to live independently. Furthermore, because the effects of FAS are sustained throughout the life of an individual, many neurobehavioral adaptive characteristics are seen. Adolescents, for instance, usually fail to consider the consequence of their actions, are unresponsive to social cues and lack both reciprocal friendships and initiative. They are also more often highly distractable, inattentive, hyperactive and intolerant. Additionally, newborns with FAS are reported to be at a higher risk for feeding and sucking defects as well as have an increased susceptibility to seizures and sleep disorders (Streissguth 1992).

Effects of ETOH on Neurodevelopement

Neurochemical abnormalities along with CNS dysfunction are well known results of ethanol teratogenesis. Presently, little is known about the mechanism of action ethanol has on neural development.

The timing of ethanol exposure during gestation is a contributing factor in the outcome of the teratogenic effects (Streissguth et al. 1986). In humans, most brain development occurs during fetal growth and throughout the first 2 years of life whereas in the rodent, it is during fetal growth and the first few days after birth. Additional reports have stated that gestation (G) at approximately 20-30 days for humans is equivalent to G at 9.5 to 11.5 days for rats (Fadel and Persaud 1991).

Results from an experiment in rats showed ETOH exposure from G5 to G22 resulted in several changes in neuronal generations. Ethanol-induced disturbance therefore, may be reflected as changes in mature cortex neurons and the proliferation of neuronal precursors which occur in the rat during the latter half of gestation, G12-G21 (Miller and Nowakowski, 1991).

Additionally, alcohol abuse during the critical stages of embryo development has also been reported to alter the "laying down" of important neuronal structures resulting in dramatic neurological abnormalities (Duffy et al., 1991).

Furthermore, ETOH is also known to have profound effects on neuro and cellular physiologies. Specific alterations in cellular physiology include changes in protein kinase C, second messenger systems, and ligand or voltage-gated calcium flux. Since these systems modulate gene expression, speculations were made that gene expression may be responsible for cellular and neuronal adaptations to alcohol. Consequently, efforts are now being made to identify such ethanol-responsive genes (EtRGs) that might play a role in the adaptive process (Miles et al., 1991) . Results in various animal studies have shown that the cerebellum is very sensitive to ethanol exposure. Effects of ETOH exposure <u>in utero</u> on developing rat cerebellums showed an overall decrease in size and mass, delays in synaptic formation and neuronal differentiation, as well as decreases in the number of purkinje cells (Gruol, 1991). Purkinje fiber development also showed multiple physiological alterations in the presence of ethanol such as gross morphological changes in

structure, firing properties and reduction in amplitude of synaptic potentials. Thus, these results suggest cellular mechanisms may be a possible target site for the action of ethanol (Urrutia and Gruol, 1991).

Effects of ETOH on Behavior and Reproduction

Although it has been agreed that alcohol is a teratogen, several problems exist in determining it's manifestations on behavior. One reason may be lack of differentiating behavioral characteristics at birth when other effects appear to be more pronounced. Other reasons include the strong influence of genetic determinants and postnatal conditions that also effect behavior (Streissguth, 1986). Some clinical reports suggest the behavioral effects of ETOH can occur with or without the presence of physical disorders. However, the maladaptive behaviors that exist into adulthood are what present the greatest challenge to future management. Despite the amount of research done since 1973, the severity of the teratogenic effects of alcohol on behavior are still difficult to predict because unlike animal experiments, human alcohol exposure cannot be quantified precisely over the 9 months of gestation (Stressguth et al. 1986). Furthermore, because CNS development continues throughout gestation, the assessment of neuromotor development, activity and learning is also very unpredictable. One experiment in rats demonstrated delayed neuromotor development, especially coordination via latency to right and hyperactivity. No effects, however, were seen on

learning as assessed by the Morris water maze test (Torres and Zimmerberg, 1991). Human studies have also supported these results. For example, children exposed to alcohol throughout gestation showed attention deficits on computerized performance task assessments and were evaluated by their teachers to be more inattentive than children not prenatally exposed to alcohol (Brown et al., 1991).

Chronic ethanol consumption during pregnancy has lead to a number of anomalies in the reproductive systems of both males and females (Creighton-Taylor and Rudeen, 1991). Effects on sexual orientation in male rats include femininization, increased female sexual responses (lordosis), and lack of masculine traits. Absence of the peak testosterone (T) levels normally seen in the last trimester of male rat fetuses were also noted. Moreover, Dahlgren et al. (1990) also reported that male rats affected by ETOH exposure in utero displayed induction of feminine copulatory motor components along with a lack of sexual preferences toward mating pairs. These findings imply that prenatal exposure to ethanol attenuates the normal sexual tendencies of the male rat (Dahlgren et al. 1990).

Delays in the onset of puberty were also seen for ethanol affected females as compared to controls. Further results also showed a delay in the first estrus cycle and vaginal openings for females treated with ad libitum diet containing 6% ethanol (Creighton-Taylor and Ruden, 1991).

Since ovarian steroids control female sexual behavior in the

rat, several alterations in sexual receptivity could result from embryonic ethanol intoxication. Minette and Fulginiti (1990) also reported that ETOH exposure in utero during the gastrulation stage of embryogenesis resulted in an increased sexual receptivity in response to estrogen administration in adult ovariectomized females.

Sites of ETOH action in CNS

Relatively few studies have been designed to investigate the effects of ETOH exposure in specific brain areas. Reports from Yoshimoto et al. (1991) demonstrated an increase in dihydroxyphenyl acetic acid (DOPAC), dopamine (DA), and serotonin from the nucleus accumbens (NAc) as a result of high doses of systemic ethanol administration. Additional studies using brain dialysis techniques in the rat NAc and dorsal caudate also suggest that serotonin receptors may play a part in mediating alcohol activity in the accumbal DA system since DA release could be attenuated by administration of a serotonin antagonist (Yoshimoto et al., 1991). Anokhina et al. (1990) reported an increase in c-fos expression in the rat cerebral cortex after ETOH administration to offspring prenataly exposed to ETOH. Furthermore, ethanol has also been reported to have a direct effect on the sexual dimorphic nuclei of the preoptic area which is normally very extensive in males but appeared to diminish as a result of ethanol exposure in utero (Dahlgren et al., 1991; Norton and Kotkoskie, 1991).

ETOH: Mechanism of Action

Membranes as well as membrane-bound enzymes play a crucial role in normal CNS function. Alterations in fetal membrane stability (fluidity) as well as activity of fetal Na^{+}, K^{+}-ATPase, Ca^{2+}-ATPase, acetylcholinesterase and other membrane-bound enzymes are greatly affected by ethanol exposure in utero. Ethanol is considered to change these membrane capacities through it's pharmacological effects (Custo et al., 1987).

Other effects of ETOH may additionally result in changes in saturated/unsaturated fatty acid ratios or by increased fatty acid saturation. Composition and saturated/unsaturated fatty acid ratios are crucial determantants in the fluidicity of neuronal membranes. "Fluidity" refers to the physical structure of the fatty acyl 1 chains and has a direct relationship to the unsaturated fatty acid content. Experimental results of ethanol exposure during development of the fetal brain have demonstrated an increase in membrane fluidization, disruption in the metabolism of essential fatty acids, and a significant alteration in the lipid bilayer. These reports suggest that ETOH exposure during a critical point in development could interfere with the incorporation and formation of essential lipid constituents. Therefore, the pharmacological effects of ETOH are thought to be exerted in part by changing the cell membrane composition (Duffy et al., 1991).

Effects on Transmitters and Neurohormones

Ethanol has been reported to have a complex "psychopharmacodynamic profile" (Engel et al., 1992). The oxidative deamination of dopamine, serotonin, norepinephrine (NE), and other biogenic monoamines result in the production of aldehydes which are metabolized into acids via aldehyde dehydrogenase and into alcohols via aldehyde reductase. One hypothesis states that the increases in biogenic aldehydes is a result of ethanol metabolism which further suggested the possibility of their direct involvement with the actions of ethanol (Palmer et al., 1986).

Previous reports imply that increased catecholaminergic synaptogenesis along with serotonergic or dopaminergic dysfunctions may play play a collaborative role in the neurological dysfunctions associated with FAS. Nio et al., (1991) reported obvious alterations in the signal transduction system, protein kinase C levels, and number of D2 receptors and (^{3}H) IP_3 binding sites, particularly in the cerebellum. Furthermore, moderate changes were also reported in the muscarinic, cholinergic, and adenosine A_1 receptors (Nio et al., 1991).

The effects of ETOH have also been reported to increase the activity of adenylate cyclase and firing of ventral tegmental neurons as well as extracellular levels of dopamine in the nucleus accumbens. Increases in cholinergic expression were also noted (Sampson and Harris, 1992). Moreover, Norton and Kotkoskie (1991) reported reduced levels of postnatal acetylcholinesterase as a result of ethanol exposure in the last week of gestation suggesting

that ethanol may also cause developmental delays or permanent alterations in the cholinergic system. Experiments showing enhanced choline acetyltransferase activity suggest results may be due to effects of ethanol on membrane bound enzymes (Brodie and Verndakis, 1991). Further speculations were made stating the increase choline acetyltransferase activity may be a compensatory mechanism for the reduction in GABAergic expression (Brodie and Vernadakis, 1991).

Behavioral as well as neurochemical reports reveal alterations in GABA receptor activity as a result of alcohol exposure. Effects on $GABA_A$ function, however, differ with acute and chronic exposure displaying an increase and decrease respectively. The GABA receptor has also been linked to some genetic differences in the action of alcohol. Additionally, alterations in mRNA for specific subunits on the GABA receptor suggest chronic ethanol exposure may effect gene expression (Sampson and Harris, 1992). In further support, previous experiments demonstrated decreases in GABAergic phenotypic expression and neuronal viability in the presence of ethanol. (Brodie and Vernadakis, 1991).

The hypothalamo-pituitary-adrenal (HPA) axis is known to play a significant role in many aspects of development. It has also been stated that immune system defects may occur secondary to changes in the HPA axis (Norton and Kotkoskie, 1991). The effects of ETOH exposure <u>in utero</u> have also been known to disrupt the developing HPA axis resulting in pituitary-adrenal hyper-responsiveness. Weinberg (1988) reports that this hyper-

responsiveness may be caused by not only increased activation, but also by a longer recovery towards basal levels and decreased inhibition. Fetuses exposed prenatally to ETOH also show a variety of neuroendocrine changes related to the HPA axis such as alterations in the synthesis and secretion of hypothalamic corticotropin releasing factor or pituitary ACTH (Norton and Kotkoskie, 1991).

Neurohormones show extensive sensitivity to ethanol leading to severe alterations in synthesis and secretion. Results indicate FAS leads to a variety of changes in steroidogenesis as well as the gonadotrophin regulatory system (Creighton-Taylor and Rudeen, 1991). Both male and female hypothalamo-pituitary-gonadal (HPG) axis have been reported to be adversly affected by maternal ETOH intake. Weinberg and Jerrellis (1991) report reductions in testosterone (T) levels, testicular steroidogenic activity and number of Leydig cells for FAS affected males as well as alterations in sexual behavior into adulthood. Creighton-Taylor and Rudeen (1991) report ethanol exposure <u>in utero</u> resulted in considerable decreases in Luteinizing Hormone Release Hormone (LHRH) and Luteinizing Hormone (LH) in 30 to 40 day old females rats which may in turn influence the peptide expression or development of the LHRH neurons. It has been further implied that prenatal ethanol exposure retards the maintenance of LHRH neurons and that the pituitary gonadotropin responsiveness may be decreased in FAS animal models since LHRH is modified by gonadal steroids (Creighton-Taylor and Rudeen, 1991). It was suggested, however,

that a decrease in pituitary responsiveness may also contribute to reduced serum LH levels. Although little information has been obtained on the interaction between prolactin (Prl) and ethanol, Ho et al. (1992) reported that ethanol increases the amount of Prl released from the pituitary. The authors also report that increased Prl levels were the result of modified dopamine turnover which would then alter LH secretion. Suggestions were also made that ETOH may have an increasing effect on hypothalamic levels of Prl and LH and that a significant behavioral interaction may exist between the three (Ho, 1992).

Proto-oncogene C-fos

To further investigate the biochemical changes caused by FAS a more specific look at neurochemical physiology is needed.

Immunocytochemical staining for fos, the protein product of the proto-oncogene c-fos, has been used as a relatively new approach to mapping the patterns of postsynaptic stimulation in the CNS (Robertson et al., 1992). The proto-oncogene c-fos, the normal counterpart of the viral oncogene v-fos (Sagar et al., 1988), is a 380 amino acid (MW=62kDa) nuclear phosphoprotein which has recently opened up new avenues of research in neural development (Morgan and Curran, 1991). C-fos, along with another proto-oncogene, c-jun, belong to the class of immediate early genes (IEG) which can be activated independently by certain hormones and transmitters. IEG also function as transcriptions factors. They are practically undetectable in quiescent cells but increase rapidly to

transcription levels within minutes of stimulation, independent of any protein synthesis (Sheng and Greenberg, 1990). The fos and jun proto-oncogene families are the most commonly investigated transcription factors today. They both share the specific DNA AP-1 binding site as well as the leucine-zipper dimerization motif. It is believed that c-fos and c-jun synergistically interact in the transcription of genes having the AP-1 binding site (Kononen et al., 1992).

Immunohistochemical methods have been employed to detect c-fos in some parts of the brain as well as other regions of the CNS. C-fos expression, however, only marks the activated neuronal cell bodies which makes it an excellent marker to signify neuroactive substance targets (Sagar and Curran, 1988; Morgan and Curran, 1991). Furthermore, studies over the last decade have revealed the function of proto-oncogenes to be intracellular signal transducers which allow a cell's genetic material to respond to extracellular stimuli (Morgan and Curran, 1991).

Although nuclear levels of c-fos are normally very low, certain types of stimulation such as electrical activity, drug agents, stress and perhaps alcohol are known to cause detectable elevations throughout the nervous system (Kononen et al., 1992). Hoffman et al. (1990) states that a strong correlation exists between cellular stimulations and the levels of c-fos expression. Consequently, Bing et al. (1992) previously reported that stimulation of adrenergic receptors resulted in the increased production of fos in the cortex. In addition, Anoklina et al. (1990) also reported a

significant increase in c-fos in the cerebral cortex of offspring from rats chronically treated with ETOH before breeding when given ETOH administration as a stimulus. Fei et al. (1990), however, reported no alterations in c-fos as a result to acute ethanol treatment. Another report further suggested that ethanol withdrawl seizures were directly associated with increased levels of c-fos mRNA particularly in the hippocampus as well as the cerebellum and cerebral cortex (Dave et al., 1989). Speculations have also been made that suggest c-fos may act as a third messenger system able to integrate and encode a multitude of extracellular stimuli into long-term responses through alterations in gene expression (Arenander and de Vellis, 1992; Sagar and Curran, 1988). Furthermore, Morgan and Curran (1991) reported that the calcium-calmodulin, cyclic AMP and diacylglycerol-protein kinase C systems are 3 specific second messenger pathways that can be correlated with c-fos activation and IEG induction. Other studies have also provided evidence that c-fos possesses a variety of neurological and biochemical functions including cellular transformation, gene expression, transcription regulation and specific synergistic actions with c-jun (Gonzalez-Martin et al., 1992). Therefore, based on these and other findings, c-fos is suggested to be a possible marker for neuronal activity, function, and development (Robertson et al., 1992).

Although c-fos staining offers cellular resolution, it lacks quantitative measures as well as specific values in the amount of stimulus needed to produce a change in expression (Sagar and

Curran, 1988). Recent investigations have been made in attempt to determine the receptor system responsible for c-fos expression. Evidence has revealed that glutamate receptors appear to be a key mechanism. One study in particular, demonstrated elevated c-fos levels in the hippocampus due to a specific glutamate reaction at the N-methyl-D-aspartate (NMDA) form of the receptor (Morgan and Curran, 1991). To investigate this theory, Kaczmarek et al. (1987) reported that formation of long-term potentiation (LTP) in rat hippocampus depends on NMDA receptors and is considered to represent an electrophysiological model of memory. The authors concluded that c-fos can be nonspecifically induced in the brain, however, it was not considered to be crucial for hippocampus memory formation.

Neurons in the striatal region have varied c-fos expression due to differences in the location of the D1 and D2 dopamine regulating receptors. Results from in situ hybridization experiments confirmed this by demonstrating that D2 receptors are expressed by striatopallidal neurons while the D1 receptor expression is found in the striatonigral neurons (Robertson et al., 1992).
Robertson et al. (1991) also reported that D1 receptor agonists along with D2 receptor antagonists increase c-fos levels in the striatum. The effects on striatal c-fos as a result of D1 and D2 receptors are not necessarily mediated solely by the dopamine receptors located therein. This speculation was based on the presence of these two receptors being found in the nucleus accumbens, septum, thalamus, and cortex (Robertson et al. 1992).

Further reports have been made on the ability of L-DOPA (a dopamine precursor) to increase c-fos activity in the striatum as well (Dragunow et al., 1990).

Furthermore, recent progress in molecular biology and neuroendocrinology has suggested c-fos as a promising approach to investigate and analyze LHRH systems at the cellular level. Hoffman et al., (1990) demonstrated that hormonal administration of estrogen-progesterone in female rats did induce c-fos in LHRH neurons (100% induction seen in test animals) and that induction was consistent with simultaneous activation of the LHRH system. Other studies by Lee et al. (1992) reported a direct correlation between the number of positive fos stained nuclei expressed in LHRH neurons and the peak amplitude of the LHRH surge.

Moreover, c-fos has also been used as a neural activity marker in experiments investigating male sexual behavior. Experiments by Kollack and Neuman (1992) on syrian male hamsters reported the first demonstration of a dynamically selective pattern of neural activity in specific brain nuclei involved in regulating mating behavior. Immunohistochemical means were used to identify c-fos in the medial preoptic area (MPOA), medial nucleus of amygdala (Me), and the bed of the stria terminalis (BNST). Results demonstrated that mating behavior induced the selective expression of fos in certain nuclei of the chemosensory pathway. Activation of the vomeronasal pathway was also indicated by fos expression in the Me and posteromedial cortical nucleus as well as in the amygdala-hippocampal areas. Other results showed a considerable increase in

fos-stained nuclei when male hamsters were exposed to vaginal secretions alone (Kollack and Newman, 1992). Bialy et al. (1992) reported a significant c-fos accumulation in the sensory cortex in relation to sexual learning. His experiment examined fos levels in different regions of the brain in response to learning copulatory behavior. It was suggested that certain learning processes are evident during the first sexual experience. Additionally, activation of NMDA receptors along with protein biosynthesis, were required for copulatory learning. Results demonstrated that sensory cortices of sexually mature rats had increased c-fos mRNA levels, although this increase did not occur after the first intromission which suggests that fos expression increases after copulatory behavior is learned (Bialy et al., 1992). There has also been reports of increased fos in male MPOA and nucleus accumbens following sexual stimulation (Robertson et al., 1991). This is believed to be due partly by the dopaminergic influences on male sexual behavior since DA agonists are known to enhance sexual activity where as DA antagonists decrease it. It is possible that fos activation may depend on the dopamine release which is believed to facilitate male copulatory behavior (Robertson et al. 1991). Increases in neural expression of fos have also been reported in male rats in response to sexually relevant olfactory cues. Olson and Leipheimer (1993) and Olson (1994) reported significant increases in fos-stained nuclei in the NAc, diagonal band of broca (DBB), corpus striatum (CPU), and MPOA of intact male rats exposed to female pheromones. These results suggest that the increase in

neuronal activity in these brain areas may be related to the increase in sexual arousal following exposure to female pheromones.

Therefore, numerous investigations have demonstrated that prenatal ETOH exposure effects a number of different physiological systems such as the CNS, the endocrine system, and the reproductive system including effects on learning and behavior. The present experiments were designed to examine, in greater detail, the effects of <u>in utero</u> ETOH exposure on sexual behavior and on the neuronal expression of fos in select brain areas of male rats.

CHAPTER II

Materials and Methods

Animals

This experiment used Long-Evans rats, bred in the YSU colony from rats originally purchased from Charles River Laboratories. The rats were kept in reversed light cycles of 12 h light/12 h dark with lights off at 1000 h. Colony room temperature was 22^0 C. Animals were allowed free access to standard laboratory chow and water. Adult females were bred with sexually experienced males. Successful breeding was determined by the presence of a sperm plug on the bottom of the cage and/or by vaginal examination. Presence of a vaginal plug or spermatozoa in the vaginal smear signified the first day of gestation.

Fifteen females were successfully impregnated and divided into three experimental groups. Group (1) contained six females which received a diet of 35% ethanol-derived calories (EDC) incorporated into their liquid diet intake, Group (2) consisted of five females which were kept on a control liquid diet while the four females in group (3) maintained free access to solid food and water. Rats on ETOH diets also have reduced caloric intake because they drink less of the liquid diet containing ETOH. Therefore, liquid diet controls were necessary to distinguish effects due to ETOH from those that may have been caused by reduced caloric intake. Animals in the liquid control group were pair-fed with their respective partner in the ETOH group. Pairing was determined according to

body weights taken within the first week of pregnancy. Animals receiving liquid ETOH diets were originally given 120 ml of diet mixture daily. Amounts ingested per individual animal were determined by measuring the remaining volume the following morning. This determined the amount of liquid control diet given to the respective pair-fed partner in order to control for caloric intake.

The ethanol and control liquid diets, brands #F1265SP and #F1264SP respectively, were purchased from BIO-SERV, Frenchtown, NJ 08825. The offspring from females fed ETOH liquid, liquid control or solid food control diets are hereafter referred to as ETOH, LC and SC, respectively.

A total of 220 pups were born to the three groups; 87 to the ETOH treated females, 77 to the liquid control group, and 56 to the solid food control group. Litters from the ETOH treated groups were transferred to foster females the day of birth to assure proper care and feeding (Farr et al., 1987). Mothers were returned to regular solid rat chow and water after delivery. A mortality record for the first postnatal week was also kept for each group. All pups were weighed and measured in length on days 1, 5, and 12. Weights were also recorded on days 19 and 26, but not length measurements because pups were too active to be measure accurately. At 4 weeks of age rats were weaned and separated according to gender. Animals were allowed to mature in order to investigate the effects of ETOH exposure <u>in utero</u> on neuroendocrine function in the adult male rat.

Experimental Design

Experiment 1. The effects of ETOH treatment in utero on male sexual behavior were examined. Since repeated sexual experience is known to increase copulatory behavior in male rats and ETOH treatment has been reported to inhibit learning ability (Streissguth, 1992), this experiment was designed to test the effects of prenatal ETOH exposure on male copulatory behavior and learning ability associated with repeated testing. Therefore, at approximately 12 weeks of age, 8 naive males from each experimental group were ear-tagged for identification and tested for sexual behavior. This experiment consisted of three separate copulation tests for each male, with approximately three weeks between tests. During copulation tests the number of mounts, intromission patterns, and ejaculatory patterns were entered on an Esterline Angus minigraph event recorder. Calculations from the data recorded provided the mount latency: time from introduction of female to first mount; intromission and ejaculatory latencies: time from female introduction to first intromission and time from first intromission to ejaculation, respectively; postejaculatory interval: time from ejaculation to the next intromission; inter-intromission interval: ejaculation latency divided by total number of intromissions; and the copulatory efficiency: total number of intromissions divided by the sum of mounts plus intromissions as previously described (Sachs and Barfield, 1976). Males were placed in observation chambers for a 10 min acclimation period before the addition of an estrous female. Females were brought

into estrus with an injection of estrogen (50 ug/.1 ml) 48 h before testing and by an injection of progesterone (500 ug/.1 ml) 4 h before testing. Copulatory behavior was recorded and testing was concluded after the postejaculatory interval was reached.

Experiment 2. The effects of <u>in utero</u> ETOH exposure on neuronal expression of c-fos were examined immunohistochemically. A total of 36 niave males, 12 from each test group, were used for the immunohistochemical study. A total of 9 animals, consisting of representatives from each group, were tested on any given test day. These experiments were designed to examine the neuronal expression of fos protein in males exposed to female phermones as described previously (Olson and Leipheimer, 1993, Olson, 1994). Males were placed in clean observation cages (47.5 cm x 25 cm x 30 cm) with fresh bedding for a 10 minute acclimation period. Soiled bedding collected from either ovariectomized (control) or estrous (experimental) females was then placed with the male for a period of 1.5 hours in order to test for the effects of phermones on fos expression. At the end of this experimental period males were then immediately killed in order to assay for c-fos in the specific brain areas of interest.

<u>Perfusion</u>

Intracardial perfusion was performed in rats anesthetized with a combination of ketamine (50 mg/kg) and xylazine (4 mg/kg) administered by IM injection. Once the animal was under deep

anesthesia, determined by pinch reflex and/or cornea reflex, it was stabilized in a supine position to a wire perfusion rack. A blunt ended 20 guage needle was carefully introduced into the left ventricle, passed through the aortic valve and then into the aorta. Rats were perfused with 50 ml of normal saline followed by 100 ml of 10% phosphate buffered formalin. Signs of perfusate through the nasal membranes along with twitching reflexes were indicative of complete and thorough perfusion. Following perfusion the rats were immediately decapitated with the use of a small animal dispatcher (Harvard Bioscience). Brains were removed, trimmed, and stored for assaying purposes in the same 10% phosphate buffered formalin solution for 24 h at 4^0C.

Immunoperoxidase Staining for C-fos

Twenty frozen serial tissue sections, 90 um thick, were taken in an anterior to posterior direction through the nucleus accumbens, diagonal band of broca, corpus striatum, and medial preoptic area with the use of a cryostat microtome. Four to five serial sections, 90 um thick, were also taken through the olfactory bulbs. The immunohistochemical (IHC) determination of fos was measured by the immunoperoxidase staining method which uses the biotin-peroxidase-labeled antibody reaction to it's reciprocal tissue antigen. The technique employs a biotinylated second antibody (BAb) directed against a primary Ab which is in turn bound to the fos protein. The avidin binds to the biotinylated second Ab at one site and to the biotinylated peroxidase at the other 3 available

sites (Ashton, 1985).

Tissues were sectioned and placed in tris buffered saline (TBS) prepared from 8.10 g NaCl and 1.0 g bovine serum albumin in 900 ml deionized water and diluted to 1000 ml with 0.5 M tris buffered stock (pH 7.6). One ml triton-X 100 and 0.1 g merthiolate were then added. Sections were washed twice (15 min each) with the TBS solution. To block endogenous peroxidase activity, sections were incubated at room temperature for 30 min in a solution of 1.0% hydrogen peroxide in TBS and followed by two 15 min washings. Sections were then incubated for 48 hours with a primary polyclonal antibody originally developed in rabbits (c-fos-Ab-2, Oncogene Science, Inc. Uniondale, NY). The antibody (1 mg/ml) was diluted 1:1000 with TBS. This dilution was determined in a preliminary titration experiment. Two 15 min washes followed the antibody incubation period. This and subsequent wash solutions did not contain triton-X 100. Sections were then incubated with the biotinylated second antibody (Vectastain ABC kit, Vector Laboratories, Inc. Burlingame, CA.) for 1 hour at room temperature, washed twice and then incubated in ABC reagent for 1 hour. Another set of 15 min washings followed and then sections were incubated in 150 ul of diaminobenzidine stain (DAB, 3,3-diaminobenzidine tetrahydrocholoride, Sigma Chemicals) at a concentration of .126 mg/ml for about 5-6 min or until the sections turned to a light brown color. DAB is a chromogenic substrate that reacts with the peroxidase labeled biotin to cause a color change in order to visualize the reaction. The staining reaction was

stopped with .025 M tris buffer. The treated sections were then transferred onto albuminized slides, allowed to dry overnight and counterstained with methylene blue. The staining procedure was as follows: methylene blue (5 min), 70% ETOH (2 min), 95% ETOH (3 min), absolute ETOH (5 min) and xylene (5 min). The slides were then dried and coverslipped with permount medium. Slides were microscopically analyzed and the number of positively stained (dark brown) fos nuclei were counted by one individual using a grid. Stained nuclei were counted in three consecutive sections for the nucleus accumbens, corpus striatum, diagonal band of broca, and medial preoptic area. Antibody specificity was also determined by incubating several sections with a mixture of fos antigen (1 mg/ml, diluted 1:50) and fos antibody (1 mg/ml, diluted 1:500). The primary antibody binds to the excess antigen and is therefore prevented from binding to the fos protein in the tissue section. The sections incubated with this mixture showed no staining for fos protein in this experiment thus ensuring antibody specificity.

Data Analysis

Differences between treatment groups with respect to weight gain, growth of offspring, and sexual behavior were analyzed by repeated measures ANOVA (Sigma Stat). When significant differences were found between groups, they were further probed by the Student-Newman-Keuls test. Differences between treatment groups with respect to fos expression in specific brain nuclei were analyzed by 2-Way ANOVA (Sigma Stat). When significant differences were noted

between groups they were further probed by the Student-Newman-Keuls test. Differences between treatment groups in percent of pups that died within one week of birth were analyzed by chi-square (Sigma Stat, Jandel Scientific).

CHAPTER III

RESULTS

Analysis of pup viability revealed a mortality rate of 21.8% for the ETOH treated group compared to the solid and liquid control groups which were 0% and 5.2%, respectively (Fig. 1). No significant weight differences were seen between groups and all offspring showed progressive increases in weight over 26 days (Fig.2). Analysis of pup lengths (nose to rump) revealed that ETOH-exposed pups were shorter than the solid food controls on day 1 ($p < .05$). All offspring continued to grow over the 12 days that measurements were taken, however, the ETOH-exposed pups tended to be shorter than the solid food controls (Fig. 3).

Experiment 1. Effects of ETOH exposure on male sexual behavior.

Data recorded for copulatory testing showed all animals (n=8 in each group) demonstrated recognition of females and exhibited sexual arousal with no significant differences in mount latencies (ML, Fig. 4) or intromission latencies (IL, Fig. 5) between groups in three separate test sessions. There were, however, large variabilities seen in the ML and IL for each treatment group in test one. A decrease in both ML and IL between tests one and two were observed and is representative of learning behavior.

There were also no significant differences found between groups for ejaculation latencies, but again there were progressive decreases in latencies between tests one and three (Fig. 6).

Significant differences in the inter-intromission interval (III) for test one were found between groups. The ETOH group was significantly greater than the liquid control group, however, there were no differences between the ETOH and solid food controls (Fig. 7). The inter-intromission interval determined for tests two and three however, showed no significant differences between groups.

Figure 8 depicts the postejaculatory interval (PEI) for all tests. There were also no significant differences between groups although the postejaculatory interval in test one for the ETOH treated animals tended to be longer than that observed for the solid food controls, ($p=0.0668$, $n=8$). There were also no significant differences between groups for copulatory effiency or the number of mounts or intromissions (data not shown).

Experiment 2. Effects of ETOH exposure on neuronal fos expression.

Immunohistochemical analysis revealed fos immunoreactivity (fos-positive stained nuclei) in the CPU, DBB, NAc, and the MPOA of animals in all test groups. As expected for the solid controls, an increase in fos expression in the CPU was seen in the animals exposed to estrous bedding (female pheromones) when compared to controls ($p < .05$, $n=6$, Fig. 9). There were also significantly higher levels of fos stained nuclei recorded for the control animals in the ETOH group versus controls in the solid group ($p < .05$, Fig. 9). Results of fos expression in the DBB showed a significant main effect of diet when analyzed by 2-way ANOVA ($p < .002$, $n=6$, Fig. 10). ETOH control values were significantly

greater than both the solid control and experimental animals ($p < .05$). In addition, the number of fos-stained nuclei in the ETOH experimental animals was greater than the solid control group ($p < .05$, $n=6$).

A significant main effect for diet was also reported in fos analysis of the NAc as seen in figure 11. The ETOH group showed a significant elevation in fos expression when compared to the number of fos-stained nuclei in the solid control group ($p < .05$).

Two-way ANOVA determined a significant interaction between diet and exposure to female pheromones on the expression of fos-stained nuclei in the MPOA ($p < .05$, Fig. 12). In the MPOA, ETOH treatment was associated with a decrease in fos-stained nuclei after males were exposed to female pheromones. Intense staining was also observed in all sections of the olfactory bulbs with no apparent differences between groups (data not shown).

Figure 1. Percent of pups that died within one week after delivery from dams given ETOH-treated diets, liquid control diets or solid food.

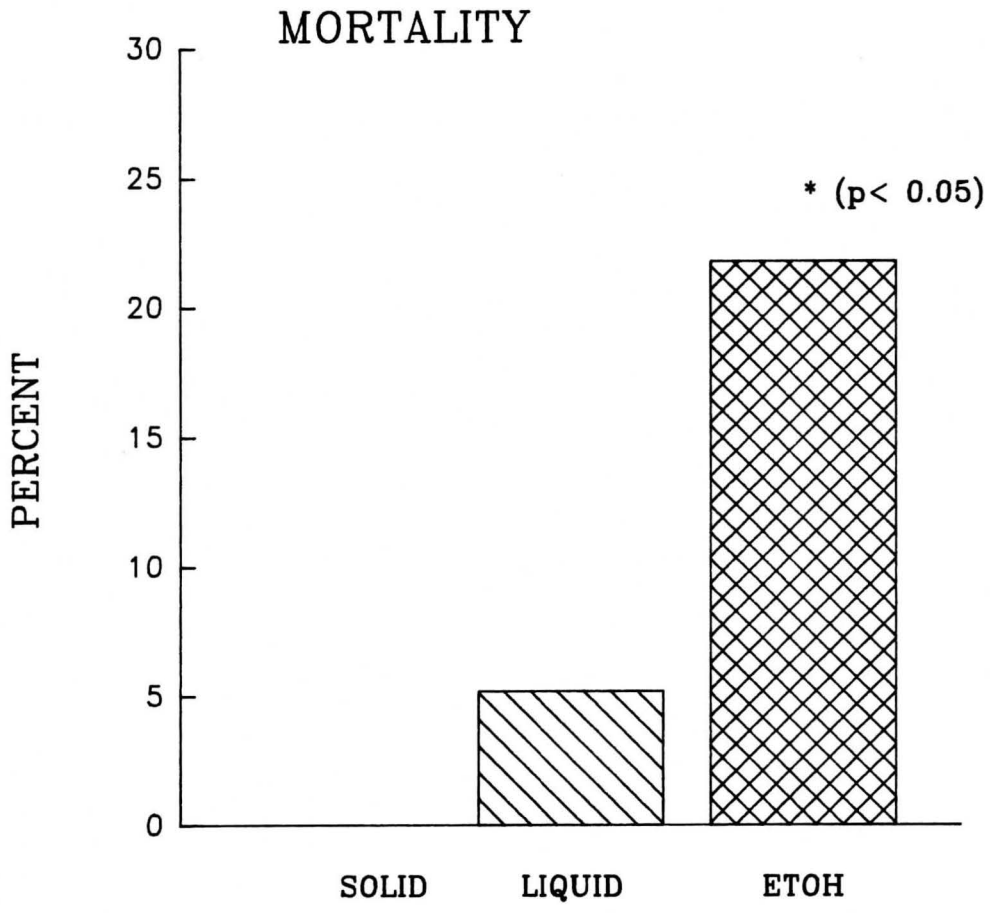

MORTALITY
30
25
20
15
10
5
0
PERCENT
* (p< 0.05)
SOLID
LIQUID
ETOH

Figure 2. Weight gain (expressed in grams) of offspring on day 1 (delivery) and on days 5, 12, 19, 26. Values are the mean +/- S.E.M. When not illustrated, the standard error bars are within the symbol.

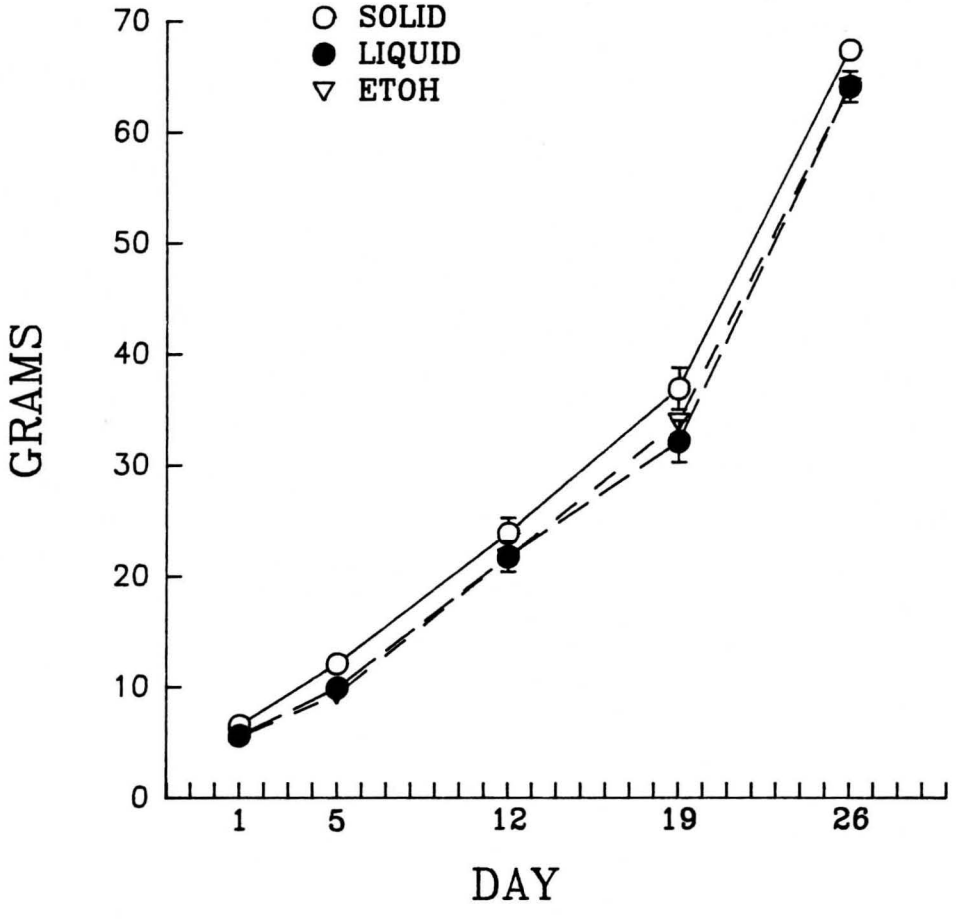

SOLID
LIQUID
ETOH
70
60
50
40
30
20
10
0
GRAMS
1
5
12
19
26
DAY

Figure 3. Growth of offspring (expressed in cm of body length) and day 1 (delivery) and on days 5 and 12. Values given are the mean +/- S.E.M. When not illustrated, the standard error bars are within the symbol. The body lengths of the ETOH offspring were significantly less than that of the solid control group on day 1. * $p < 0.05$.

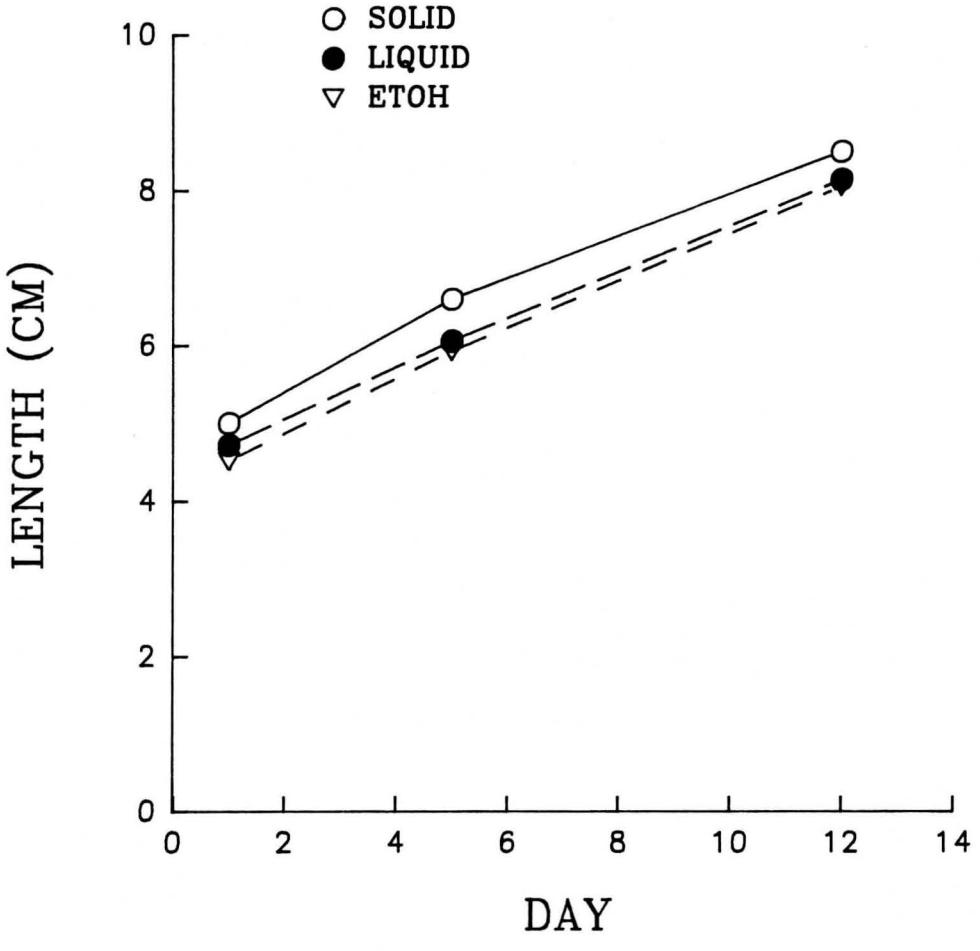

SOLID
LIQUID
ETOH
LENGTH (CM)
DAY
0
2
4
6
8
10
12
14

Figure 4. Latency to mount (ML) in 3 test sessions for males treated prenatally with ETOH or liquid control or solid food diets. Abreviations in this figure and figures 5-8: T1=first test session; T2= second test session; T3=third test session. Values reported are the mean +/- S.E.M. *p <0.05 vs T2 and T3 of same group.

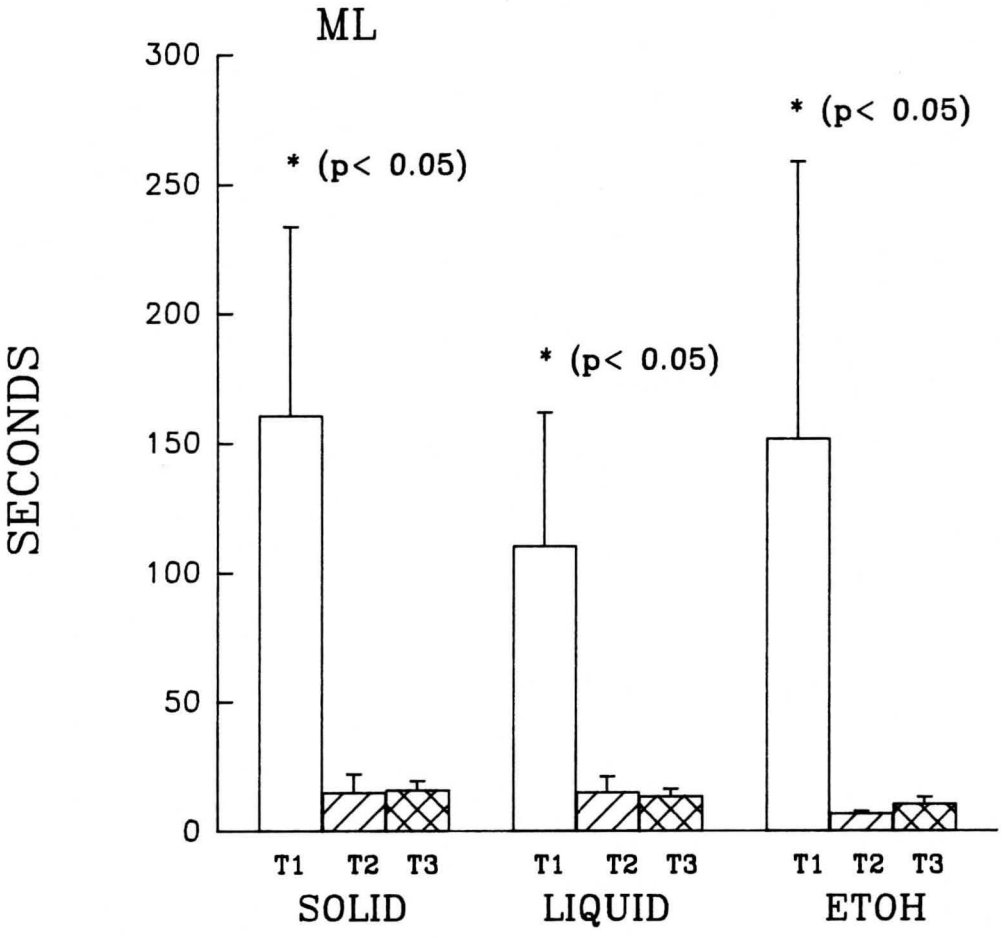

ML
SECONDS
300
250
200
150
100
50
0
* (p< 0.05)
* (p< 0.05)
* (p< 0.05)
T1 T2 T3
T1 T2 T3
T1 T2 T3
SOLID
LIQUID
ETOH

Figure 5. Intromission latencies (IL) in 3 tests sessions for males treated prenantally with ETOH or liquid control or solid food diets. Values reported are the mean +/- S.E.M. *p <0.05 vs T2 and T3 of same treatment groups.

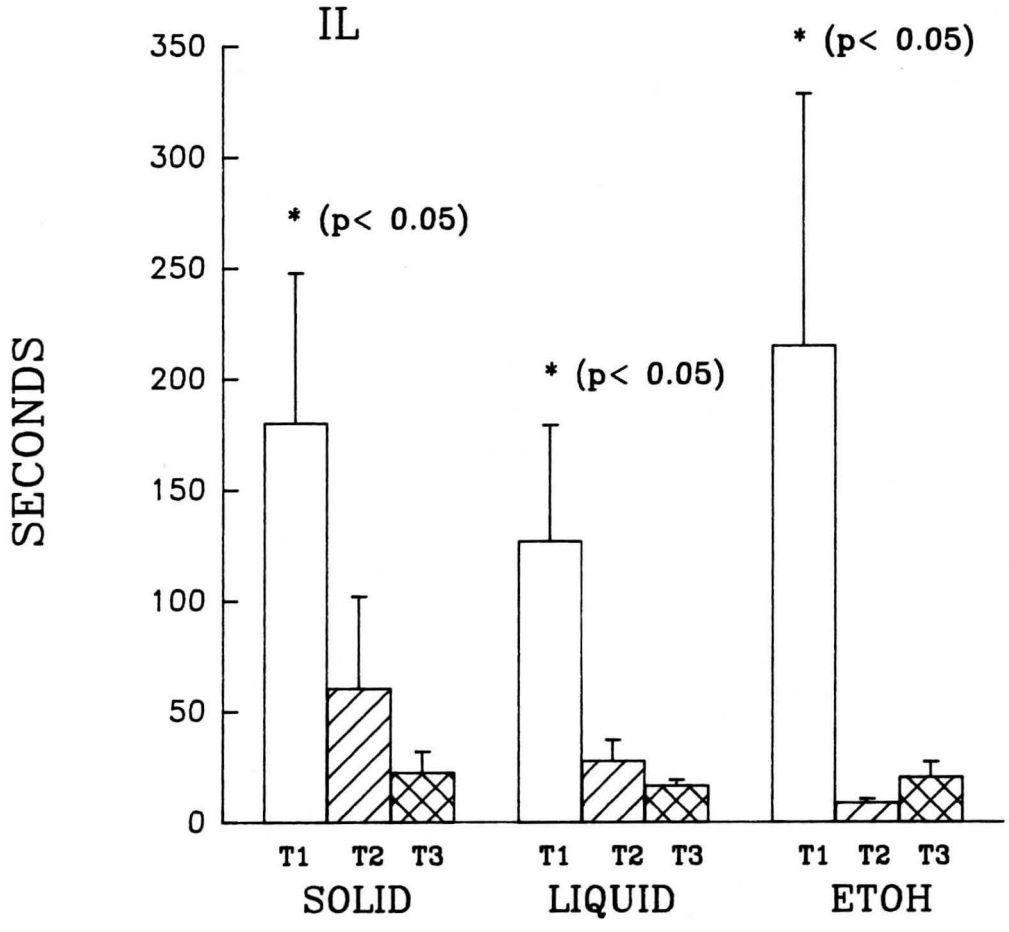

IL
SECONDS
350
300
250
200
150
100
50
0
* (p< 0.05)
* (p< 0.05)
* (p< 0.05)
T1 T2 T3
T1 T2 T3
T1 T2 T3
SOLID
LIQUID
ETOH

Figure 6. Ejaculation latencies (EL) in 3 test sessions for males treated prenatally with ETOH or liquid control or solid food diets. Values given are the mean +/- S.E.M. *p <0.05 vs T2 and T3 of same treatment group.

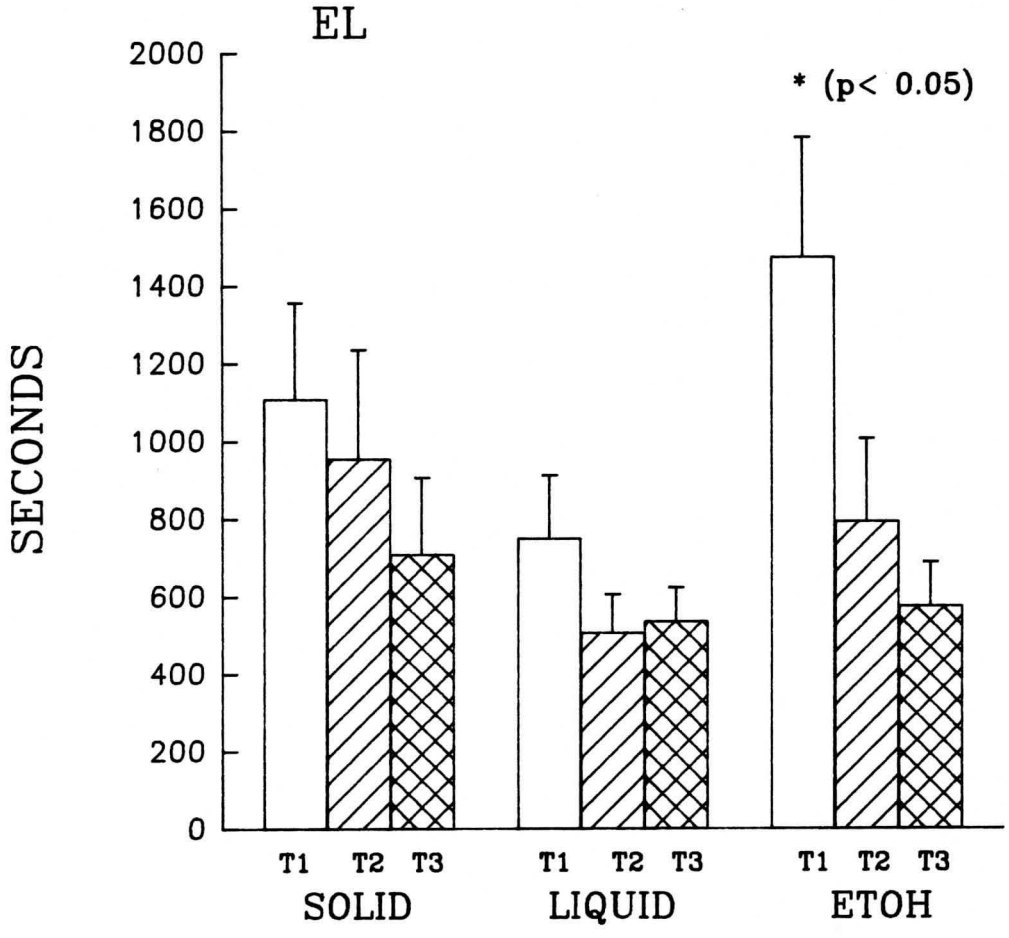

EL
* (p< 0.05)
SECONDS
2000
1800
1600
1400
1200
1000
800
600
400
200
0
T1 T2 T3
T1 T2 T3
T1 T2 T3
SOLID
LIQUID
ETOH

Figure 7. Inter-intromission interval (III) in 3 test sessions for males prenatally exposed to ETOH or liquid control or solid food diets. Values reported are the mean +/- S.E.M. *1 p <0.05 vs T3 of solid contol group; *2 p <0.05 vs T2 and T3 of ETOH group, and vs T1 of liquid control group.

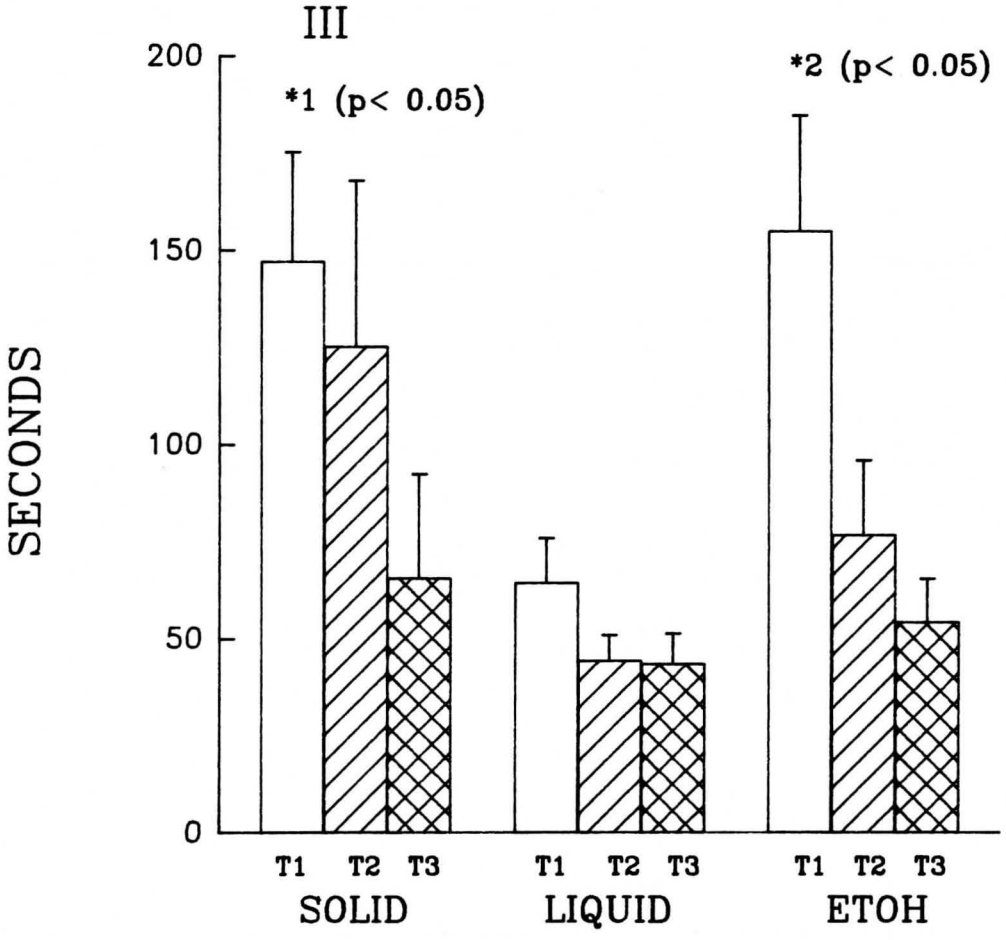

III
200
150
100
50
0
SECONDS
*1 (p< 0.05)
*2 (p< 0.05)
T1 T2 T3
T1 T2 T3
T1 T2 T3
SOLID
LIQUID
ETOH

Figure 8. Postejaculatory intervals (PEI) for male rats prenatally treated with ETOH or liquid control or solid food diets. Values given are the mean +/- S.E.M. 1. p =0.06 vs T1 solid control.

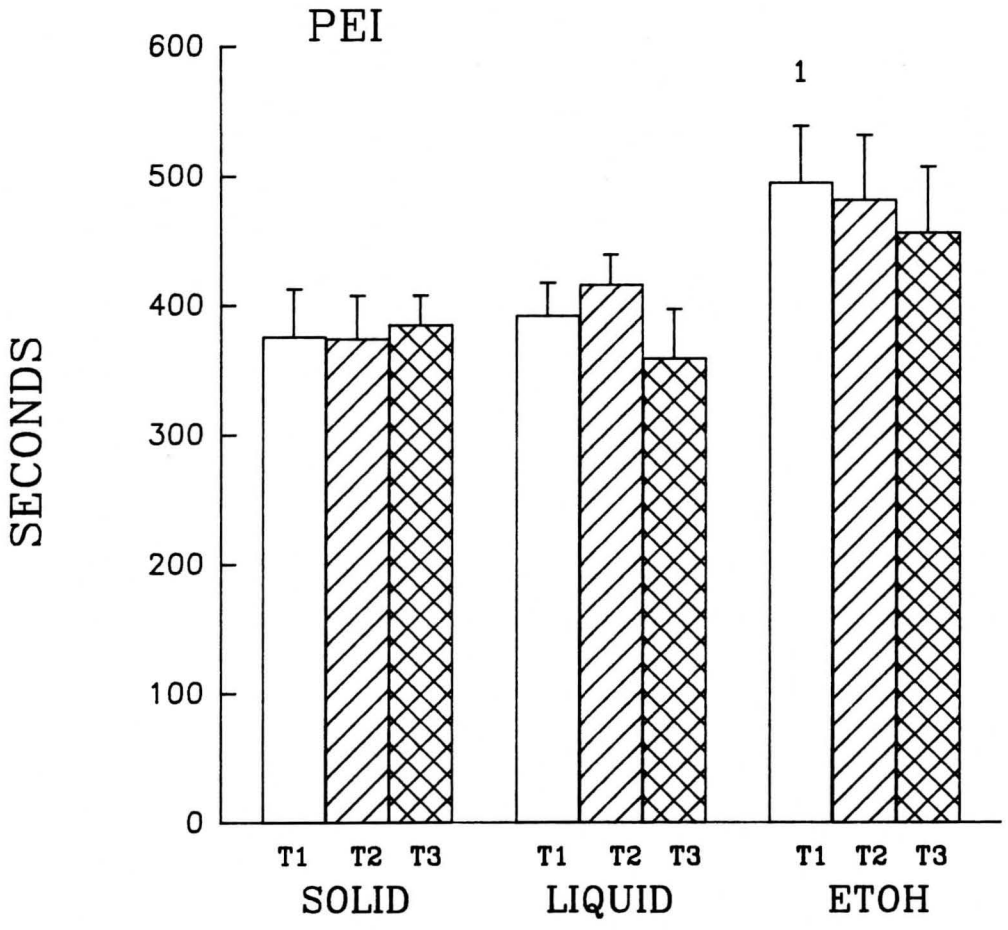

PEI
SECONDS
600
500
400
300
200
100
0
1
T1
T2
T3
T1
T2
T3
T1
T2
T3
SOLID
LIQUID
ETOH

Figure 9. Number of positively stained fos nuclei expressing fos immunoreactivity in the Corpus Striatum (CPU) of male rats prenatally exposed to ETOH or liquid control or solid food diets. Abreviations in this and subsequent figures: C = Control males exposed to ovariectomized female bedding; E = Experimental males exposed to pheromones from estrous female bedding.

* $p < 0.05$ vs solid control.

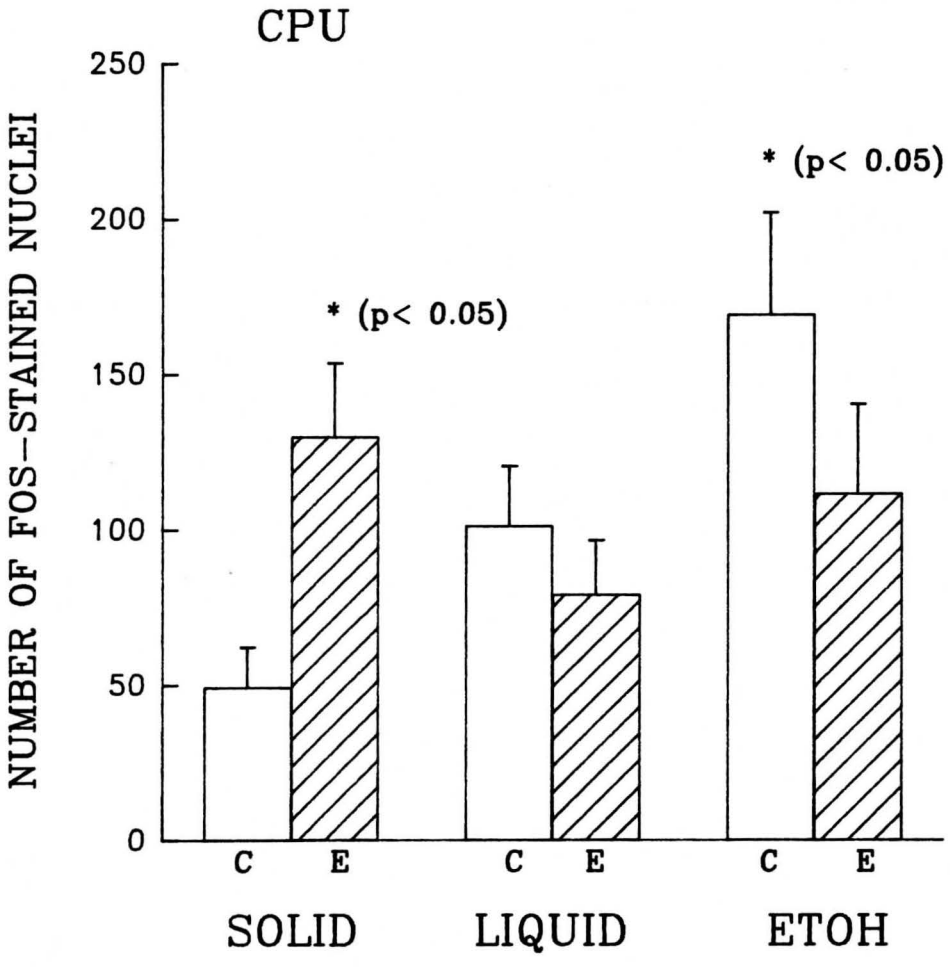

CPU
NUMBER OF FOS-STAINED NUCLEI
250
200
150
100
50
0
* (p< 0.05)
* (p< 0.05)
C
E
C
E
C
E
SOLID
LIQUID
ETOH

Figure 10. Number of stained nuclei in the Diagonal Band of Broca (DBB) of male rats prenatally exposed to ETOH or liquid control or solid food diets. Significant main effect of diet was also noted when analyzed by 2-way ANOVA ($p < .002$). * $p < 0.05$ vs solid control.

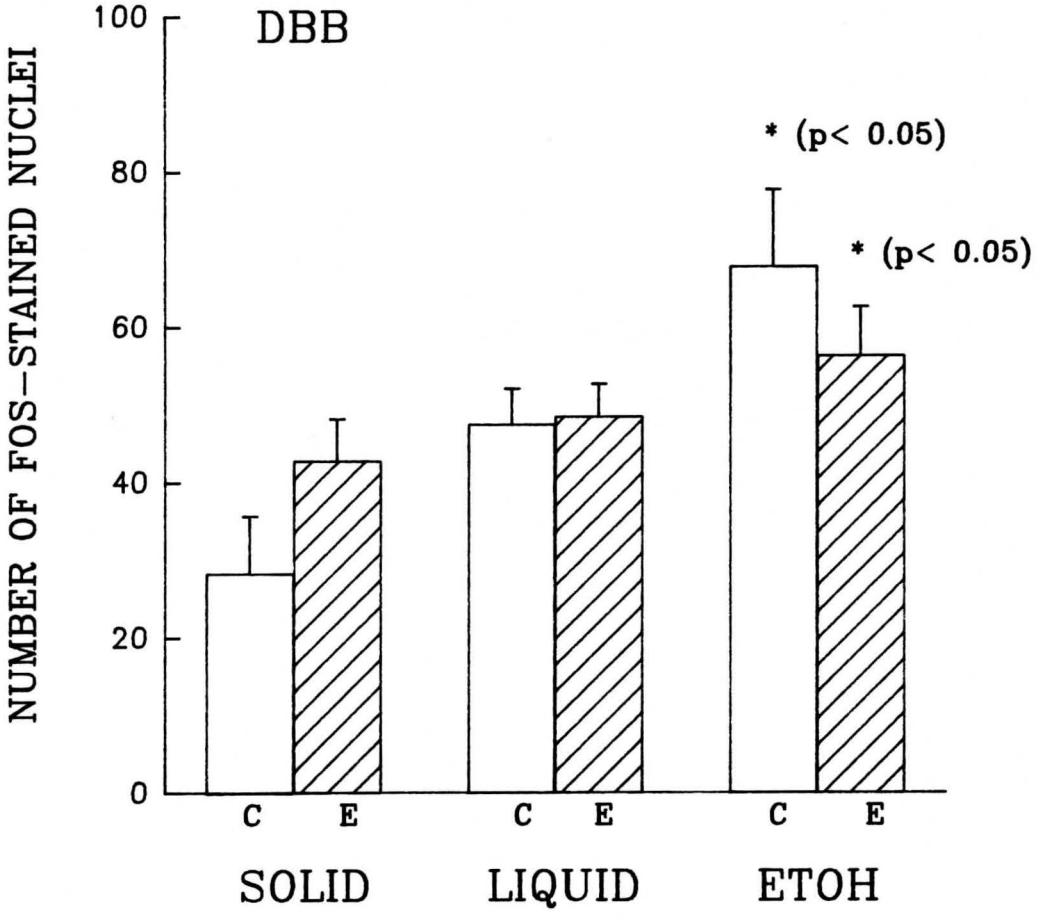

DBB
NUMBER OF FOS-STAINED NUCLEI
100
80
60
40
20
0
* (p< 0.05)
* (p< 0.05)
C
E
C
E
C
E
SOLID
LIQUID
ETOH

Figure 11. Number of fos stained nuclei in the Nucleus Accumbens (NAc) of male rats prenatally treated with ETOH or liquid control or solid food diets. A significant main effect of diet was noted when analyzed by 2-way ANOVA ($p = 0.03$). * $p < 0.05$ vs solid control.

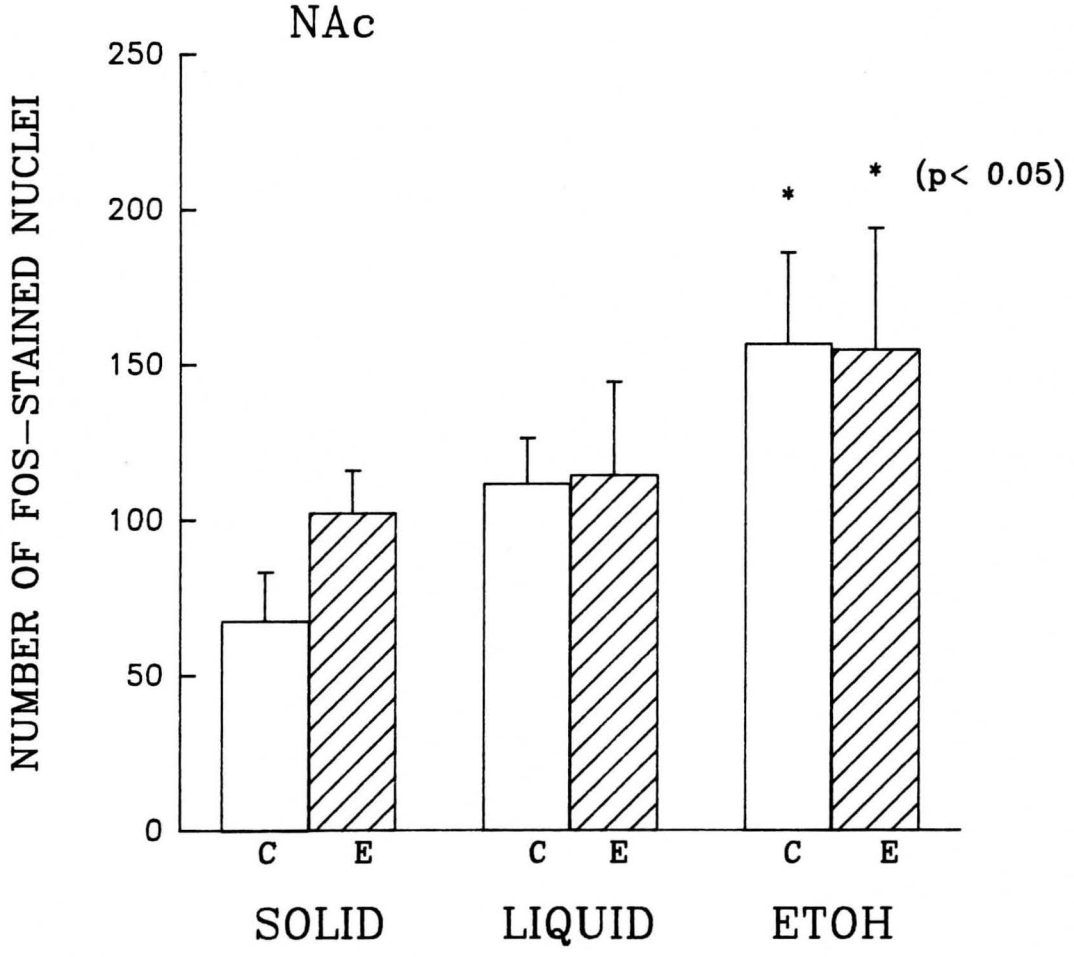
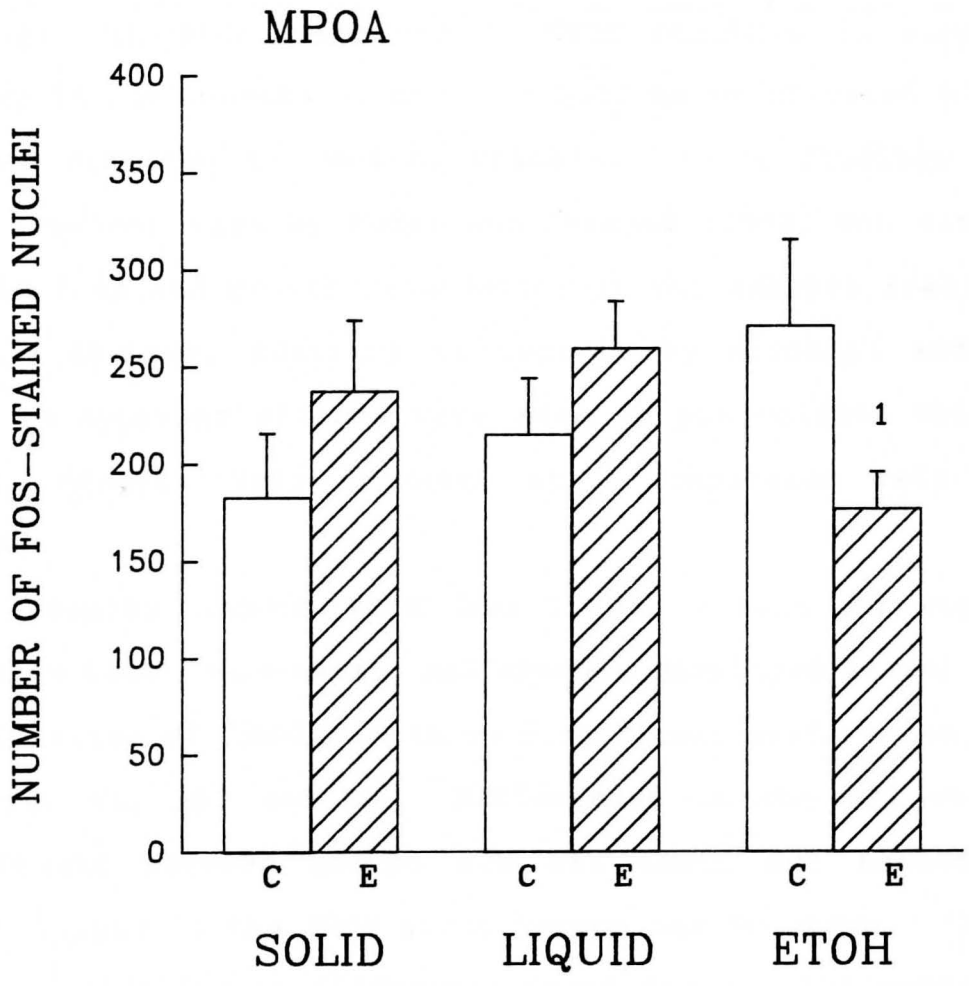

NAc
NUMBER OF FOS-STAINED NUCLEI
250
200
150
100
50
0
* (p< 0.05)
*
C E C E C E
SOLID LIQUID ETOH

Figure 12. Number of fos stained nuclei in the Medial Preoptic Area (MPOA) of male rats prenatally treated with ETOH or liquid control or solid food diets. 1. A significant interaction was seen between diet and exposure to female pheromones. The ETOH animals exposed to pheromones had a decrease in fos staining ($p < .05$).


MPOA
NUMBER OF FOS-STAINED NUCLEI
400
350
300
250
200
150
100
50
0
1
C
E
C
E
C
E
SOLID
LIQUID
ETOH

CHAPTER IV

DISCUSSION

Results from the present study demonstrate that prenatal exposure to ETOH has an effect on neuroendocrine function in the offspring. In utero exposure to ETOH resulted in significant decreases in pup lengths on day 1 as well as an elevated mortality rate when compared to control animals. These findings further support previous work by Fadel and Persuad (1992) who also noted embryonic loss and growth retardation in rat embryos treated with alcohol. However, contrary to reports by Middaugh and Boggan (1991), no apparent effects were seen on pup weights which were shown to progressively increase at a comparable rate in all offspring.

Test results showed ETOH had little effect on copulatory behavior in adult male rats. All animals displayed sexual arousal and recognition of females with no significant differences between groups for ML, IL, and EL. Differences in the PEI were also insignificant between groups for all tests but tended to be slightly longer in the ETOH group versus the SC group. There was however, a significant difference found for the III between ETOH treated animals and the LC group in test 1. The marked decreases observed in ML, IL, and EL between copulation tests one and two in all groups denotes learning behavior. In further support of these findings, Bialy et al. (1992) previously reported that decreases in EL and IL are indicative of learning behavior. It is important to

note that our results showed no effects of ETOH treatment on learning behavior in these animals. In contrast to various research done in FAS affected humans, which demonstrates a significant deficit in learning ability (Streissguth, 1992), other work by Torres and Zimmerberg (1992) reported no effect on learning behavior in offspring of rats treated with ETOH before pregnancy as assessed by the Morris water maze test. Other important alterations in male sexual behavior due to ETOH exposure in utero, as reported by Dahlgren et al. (1990), include feminization, lack of sexual preference, and increased female responses, ie. lordosis. These parameters, however, were not examined in this experiment.

The immunohistochemical analysis of fos was also conducted for all groups. In general, an overall elevation of fos levels were found in select brain areas of both control and experimental animals of the ETOH group. This suggests that ETOH treatment in utero increased c-fos expression in response to control or test stimulations. These findings are supported by those of Anokhina (1990) which demonstrated a distinct increase of c-fos gene expression in the cerebral cortices of offspring exposed prenatally to ETOH after receiving ETOH treatment as adults.

Moreover, since hyperactivity is a well known effect of FAS, perhaps ETOH exposure in utero causes a general increase in neuronal (c-fos) excitability. Sagar and Curran (1988) further suggested that the transient expression of fos results from neuronal stimulation. Therefore, it could be hypothysized that the hyperactivity seen in FAS patients may be linked to an elevated c-

fos responsiveness.

Differences in fos expression in the NAc showed a significant main effect of diet between test groups. A considerable elevation of fos-stained nuclei was seen in the ETOH animals versus the solid control animals signifying that ETOH exposure in utero disrupted normal c-fos gene expression. Although the mechanisms by which ETOH effects certain brain areas remain unknown, other studies have reported a variety of ETOH effects on the CNS. Yoshimoto et al. (1991) reported that a systemic dose of ETOH in rats resulted in an increase in the release of DA in the NAc. The authors further suggested that alcohol may be activating the DA pathways in the CNS. In addition, it has been previously reported that D1 receptor agonists and D2 receptor antagonists cause an increase in fos immunoreactivity. This is important to note since both D1 and D2 DA receptors are found in the NAc (Robertson et al., 1992). Therefore, we can only speculate that the effects of ETOH exposure in utero on fos expression in the NAc may be mediated via long term effects on the DA pathway.

A significant main effect of diet was also seen in the DBB showing significantly greater fos expression in the ETOH group as compared to both the experimental and control animals in the solid food control group. Moreover, a greater increase in fos-stained nuclei was found in the experimental animals of the ETOH group versus those in the solid group. Based on it's location, the DBB has been hypothesized to be a critical link between the olfactory

cortex and limbic system. Roman et al. (1993) reported that the DBB may function in memory storage of olfactory cues in rats.

As expected, exposure to female pheromones caused an increase in fos expression in the CPU of solid food experimental animals when compared to controls. There was also a greater number of fos stained nuclei recorded for the control animals in the ETOH group versus the solid and liquid controls. Again, prenatal exposure to ETOH was shown to increase the level of fos immunoreactivity in the CPU. Moreover, it has previously been demonstrated that D2 receptor antagonists increase fos expression in the striatum as well as in the NAc. The reason for this is because in the striatum, D2 receptors exert an inhibitory action on D1 receptors. Furthermore, when the actions of D2 receptors are blocked by antagonists, the activation of D1 receptors results in an increase in fos expression (Rogue and Vincendon, 1992). Thus, as hypothesized for the NAc, the increase in fos may be linked to activation of dopamine receptors.

Furthermore, there was a significant interaction between diet and exposure to female pheromones in the number of fos-stained nuclei in the MPOA. Therefore, the decrease in fos expression in the ETOH treated animals after exposure to female pheromones, was associated with the ETOH treatment <u>in utero</u>. In addition, others have further reported that alcohol exposure <u>in utero</u> diminishes the normally extensive sexually dimorphic nucleus of the preoptic area (Dahlgren et al., 1990). Baum and Everitt (1992) further reported that fos expression in the MPOA of males rats exposed to auditory,

visual, and olfactory cues of an estrous female was not effected. Increases in fos were normally seen, however, after copulation.

Results from Olson (1994) demonstrated an increase in fos expression for the NAc, DBB, and MPOA of control animals exposed to female pheromones. These results differ from the present findings in that exposure to female pheromones did not cause a significant increase in fos levels in these brain areas in the solid control animals. One possible reason for this difference may be in the sexual experience of the experimental animals. In the present study, all males were sexually naive, whereas sexually experienced males were used by Olson (1994). In further support of this speculation, Bialy et al. (1992) reported an increase in c-fos mRNA levels in the sensory cortex of sexually experienced males, however, this increase was not seen in naive males after the first copulatory experience.

In summary, our results demonstrate that prenatal ETOH exposure resulted in an altered expression of c-fos in select brain areas. Neuronal activity, as determined by the number of fos-stained nuclei, was increased by ETOH treatment <u>in utero</u> in the CPU, NAc and DBB. In the MPOA, however, experimental values were lower than controls. In contrast, prenatal ETOH exposure had little effect on male copulatory behavior. Furthermore, <u>in utero</u> ETOH exposure did not interfere with the mechanisms involved in the process of sexual learning that occurs with experience in male rats.

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