1992

The Effects of Ethanol on Cultured Serotonergic Neurons by Denise Kay Lokhorst.

Denise Kay Lokhorst
Loyola University Chicago

THE EFFECTS OF ETHANOL ON CULTURED SEROTONERGIC NEURONS

by

Denise Kay Lokhorst

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University Chicago in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May

1992

To Pete

Thanks for being there

ACKNOWLEDGMENTS

In my struggle to obtain a Ph.D., I have encountered numerous obstacles. In helping to overcome them, I am grateful to many indivduals. I would like to thank my advisor Dr. Manteuffel, for her encouragement over the last few years. I would also like to thank my committee members, Drs. Collins, Emanuele, and Handa for their advice, and especially Dr. Van de Kar for his many helpful discussions and support. Members of my laboratory, Allison Kuo, Roberta Gillespie, Jim Woods and Nuzhath Tajuddin have provided a constructive environment for research. Thank-you Bob Cobuzzi for taking the time to teach me cell culture. I would like to extend my appreciation to the Arthur J. Schmitt Foundation for their generosity and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
DIV	days in vitro
5,7-DHT	5,7-dihydroxytryptamine
FAS	fetal alcohol syndrome
FCS	fetal calf serum
G	gestational day
GFAP	glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography
HS	horse serum
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
MAO	monoamine oxidase
MEM	minimal essential medium
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
PBS	phosphate buffered saline
PCPA	parachlorophenylalanine
PN	postnatal day
TH	tryptophan hydroxylase

CHAPTER ONE

INTRODUCTION

The leading cause of central nervous system birth defects in the Western world is attributed to women drinking ethanol during pregnancy. One child out of 3000 births is diagnosed with fetal alcohol syndrome (FAS) (Abel and Sokol, 1991). FAS describes a set of symptoms that occur in children of alcoholic women. FAS associated abnormalities include the following: decreased growth; characteristic head and face deformities such as a flattened face, drooping eyelids and a large space between the lip and nose; major organ malfunctions; and central nervous system dysfunction causing mental retardation. Most FAS children are mildly retarded; their average I.Q. is 65 (Streissguth et al., 1978). Mental handicaps are probably the most debilitating aspect of fetal alcohol syndrome.

In an attempt to determine the underlying causes of these central nervous system problems, considerable attention has been given to the effects of ethanol on the development of major CNS neurotransmitter systems in animal models of FAS. The affected systems include dopaminergic, noradrenergic,

cholinergic and glutaminergic neurons (reviewed by Druse, 1992). In addition, the development of the serotonergic neurotransmitter system is abnormal in rats that were prenatally exposed to ethanol (Rathbun and Druse, 1985; Tajuddin and Druse, 1989a, 1989b; Druse and Paul, 1989; Druse et al., 1990). The results of these studies suggest that the offspring of ethanol-fed rats have fewer serotonergic projections to cortical areas.

In addition, a deficiency of serotonin (5-HT) was found in embryonic/neonatal rats as well as in older rats. 5-HT is important during embryonic development, because it functions as a differentiation signal to target areas of the serotonergic system, a regulator of neurite outgrowth and possibly as a growth factor which autoregulates its own development (Lauder and Krebs, 1978; Goldberg et al., 1991; Goldberg and Kater, 1989; Whitaker-Azmitia and Azmitia, 1986). Consequently, fetal deficiencies of a trophic factor such as serotonin may augment the abnormal brain development seen in ethanol exposed offspring.

The mechanisms by which <u>in utero</u> ethanol exposure produce FAS are unknown. Several potential mechanisms include the following: 1) fetal hypoxia; 2) dysfunctional placental transport of nutrients, resulting in malnutrition; 3) excess formation of prostaglandin; and 4) direct toxicity of ethanol (Schenker et al., 1990). It is also not known whether ethanol exerts its negative effects on the development of CNS neurons

by altering the levels of another factor (i.e. hormone or second messenger) essential for normal growth. The goal of this dissertation was to investigate the hypothesis that in utero ethanol exposure directly inhibits the development of 5-HT containing neurons.

To investigate my hypothesis, primary cultures of fetal serotonergic neurons were generated and grown in the absence or presence of ethanol (50, 150 and 300 mg/dl). After a 4 day ethanol exposure, 5-HT uptake, 5-HT content, and the number of 5-HT immunopositive neurons were measured to assess serotonergic function. Protein and DNA content were evaluated as a general assessment of total neurons, including both serotonergic and non-serotonergic cells. Primary cultures were used because they have been shown to be useful for investigating developmental phenomena and they permit the study of direct effects of drugs on neurons and astrocytes in a controlled environment.

The effects of ethanol on 5-HT uptake sites in primary astroglia cultures were evaluated. Astrocytes play an important role in guiding neurons to their destination and providing growth factors for neuronal development. Astrocytes also possess 5-HT uptake sites which may be important during development (Katz and Kimelberg, 1985).

The proposed studies will further our understanding of the mechanisms underlying the serotonergic deficiencies seen in rats prenatally exposed to ethanol.

CHAPTER TWO

REVIEW OF THE RELATED LITERATURE

The Serotonergic System

Serotonin Synthesis

The synthesis of 5-HT begins with the hydroxylation of the essential amino acid tryptophan to 5-hydroxytryptophan, by the action of tryptophan hydroxylase. 5-Hydroxytryptophan is then decarboxylated by aromatic L-amino acid decarboxylase to produce 5-HT. 5-HT does not enter the brain from the circulation, so the brain is not influenced by peripheral 5-HT synthesis (Undenfriend et al., 1957). Neurons synthesize their own 5-HT from the tryptophan that is transported across the blood-brain barrier (Grahame-Smith, 1971).

Tryptophan hydroxylase (TH) is the rate limiting enzyme in the synthesis of serotonin. In brain tissue, the concentration of tryptophan is lower than its Km, so the enzyme is not saturated (Tong and Kaufman, 1975). Therefore, changes in tryptophan concentration can influence the rate of synthesis of brain 5-HT (Fernstrom and Wurtman, 1971). 5-HT does not exert negative feedback control over TH activity (Grahame-Smith, 1964).

Two full length cDNA clones that encode TH have been isolated from a cDNA library of rat pineal gland (Darmon et al., 1988). These two clones recognize a 1.8 and 4-kilobase mRNA species that differ in the length of their 3' untranslated region, the importance of which is unknown. Both clones encode a 51,000 dalton protein that has TH activity.

Demonstrating that raphe TH is encoded by the same mRNA transcripts found in the pineal has been difficult (Dumas et al., 1989; Kim et al., 1991). There appears to be a discrepancy between the expression of the TH transcript and the enzyme activity. TH activity is high in the raphe, but the mRNA level is very low; the opposite situation occurs in the pineal (Kim et al., 1991). This disparity can, in part, be explained by a difference in translation efficiencies (Kim et al., 1991). Post-translational modifications of TH may explain the different biochemical properties of TH obtained from these two regions (Lovenburg, 1977; Kim et al., 1991).

5-HT uptake sites are located presynaptically on the terminals of 5-HT containing neurons. They function to remove serotonin from the synaptic cleft, and thus terminate the response generated by 5-HT release. Once inside the terminal, 5-HT is deaminated to 5-hydroxyindoleacetaldehyde by mitochondrial monoamine oxidase (Tyce, 1990). 5-hydroxyindoleacetaldehyde is then oxidized by aldehyde dehydrogenase to produce 5-hydroxyindoleacetic acid.

Serotonin Anatomy

The majority of 5-HT containing cells are located outside of the nervous system. Roughly 95% of 5-HT is found in the enterochromaffin cells of the gastrointestinal tract. 5-HT is also present in low amounts in other tissues such as heart, kidney, spleen, thyroid, adrenal, and platelets (Tyce, 1990).

Neuronal serotonergic cell bodies are, for the most part, located within the boundaries of the brainstem raphe nuclei (Molliver, 1987; Tork, 1990) . However, some 5-HT cells lie outside these boundaries (in the reticular formation) and not all raphe cells are serotonergic. The 5-HT system can be divided into two subdivisions: an ascending rostral division, which projects primarily to the forebrain via the medial forebrain bundle; and a descending caudal division which projects to the spinal cord (Molliver, 1987; Tork, 1990). Both subdivisions innervate the cerebellum and target areas within the brainstem. According to the system of Dahlstrom and Fuxe (1964), serotonergic cell clusters are coded B1-B9, with B9 being the most rostral cluster.

The rostral division consists of the dorsal (B7) and median raphe (B8) and a ventrolateral (B9) cell group not affiliated with raphe nuclei. The dorsal raphe are the most prominent brainstem serotonergic nuclei and projects heavily to the striatum. The median raphe projects predominantly to the hippocampus and septum. Both nuclei have different but overlapping projections to the neocortex (Molliver, 1987;

Tork, 1990).

Ascending serotonergic neurons have two principal axonal types that form different synaptic contacts. Thin varicose axons, arising from the dorsal raphe, are diffuse and branch extensively in their target areas. These axons rarely make "traditional" synaptic contacts with their targets. Axon terminals arising from the median raphe are thick, non-varicose, with short thin beaded branches. This system makes well defined synapses with target cells (Kosofsky and Molliver, 1987). The functional significance of this dual projection system has not been determined. It is not known whether these two axonal systems synapse with specific post-synaptic neurons or receptors. However, these two classes of serotonergic axons appear to have different pharmacologic properties. The fine fibers arising form the dorsal raphe are selectively vulnerable to amphetamines, while the beaded axons from the median raphe are resistant (Mamounas et al., 1991).

Serotonin Receptors

Gaddum and Picarelli were the first to demonstrate that there are multiple 5-HT receptor sites in the periphery. They labelled these receptors D and M based on the ability of dibenzyline and morphine to block the contractile response of the guinea pig ileum to 5-HT (Gaddum and Picarelli, 1957). Almost twenty years later, Peroutka and Snyder (1979) showed that two different 5-HT receptor subtypes existed in the

brain. Based on their differential affinities for ^{3}H-5-HT, they designated these receptors as 5-HT_1 (high affinity) and 5-HT_2 (low affinity). The 5-HT_1 binding site can be further classified based on competition curves using spiperone. The component of the 5-HT_1 binding site in the rat which shows high affinity for spiperone is designated 5-HT_{1A}. A second component which shows low affinity for spiperone is designated 5-HT_{1B} (Pedigo et al., 1981). The 5-HT_{1A} receptor can be selectively labelled with ^{3}H-8-hydroxy-diproplyaminotetralin (reviewed by Frazer et al., 1990; Schmidt and Peroutka, 1989; Conn and Sanders-Bush, 1987). High densities of the 5-HT_{1A} receptor can be found in the hippocampus, septum, amygdala, entorhinal cortex and the raphe nuclei. 5-HT_{1A} sites are located both pre- and postsynaptically (Crino et al., 1990). The presynaptic 5-HT_{1A} receptor is a somatodendritic autoreceptor found in the dorsal and median raphe nuclei. This autoreceptor inhibits nerve impulse firing within serotonergic neurons (VanderMaelen et al., 1986; Adrien et al., 1989). The 5-HT_{1A} receptor is also located postsynaptically with respect to serotonergic projections (Hall et al., 1985; Crino et al., 1990). The 5-HT_{1A} receptor is linked to a G protein which inhibits adenylate cyclase (Frazer et al., 1990; Schmidt and Peroutka, 1989; Conn and Sanders-Bush, 1987).

The 5-HT_{1B} receptor is not present in all species. In the calf, guinea pig, pig and human the 5-HT_{1D} receptor appears to

be functionally equivalent to the 5-HT_{1B} receptor found in rats and mice (Hoyer and Middlemis, 1989). The 5-HT_{1B} receptor can be labelled using ^{125}I-iodocyanopindolol (Frazer et al., 1990; Schmidt and Peroutka, 1989; Conn and Sanders-Bush, 1987). This receptor is located with high density in the substantia nigra and globus pallidus in the rat. This receptor subtype is located both pre- and postsynaptically (Crino et al., 1990; Frazer et al., 1990; Schmidt and Peroutka, 1989; Conn and Sanders-Bush, 1987). The presynaptic autoreceptor controls 5-HT release from nerve terminals. As with the 5-HT_{1A} receptor, the 5-HT_{1B} receptor is linked to inhibition of adenylate cyclase.

A third 5-HT_1 receptor has been classified as 5-HT_{1C}, although this receptor appears to have more in common with the 5-HT_2 receptor. They have similar pharmacological profiles, are both linked to phosphatidylinositol turnover and have similar gene structures (Fargin et al., 1988; Julius et al.; 1988, Pritchett et al.; 1988, Albert et al., 1990). The 5-HT_{1C} receptor is probably misnamed and inappropriately placed in the 5-HT_1 receptor family. 5-HT_{1C} receptors are located in the substantia nigra, globus pallidus, layer III of the cerebral cortex and in the choroid plexus, where they play a role in the production of cerebrospinal fluid (Frazer et al., 1990; Schmidt and Peroutka, 1989; Conn and Sanders-Bush, 1987).

The 5-HT_2 receptor is the D receptor first discovered by

Gaddum and Picarelli (1957). The 5-HT_2 receptor is linked to phosphatidylinositol turnover through a G protein. These receptors are located postsynaptically and are in high density in layer IV of the cerebral cortex.

To date, there is one 5-HT receptor that is not linked to a G protein. The 5-HT_3 receptor is a ligand gated ion channel, and is found in high density in the dorsal hindbrain (Frazer et al., 1990; Schmidt and Peroutka, 1989; Conn and Sanders-Bush, 1987). This receptor was previously known as the M receptor (Bradley et al., 1986).

Recently several 5-HT receptors have been cloned including 5-HT_{1A}, 5-HT_{1C}, and 5-HT_2 (Fargin et al., 1988; Julius et al., 1988; Pritchett et al., 1988; Albert et al., 1990). As with other G protein linked receptors, these receptors are comprised of a single polypeptide with seven hydrophobic regions that span the membrane. The regions of greatest homology among members of the G protein linked receptor family correspond to the seven hydrophobic domains. The amino terminus is located outside the cell, and the carboxy terminus is located within the cytoplasm. 5-HT receptors may have derived from at least two gene families. The 5-HT_{1A} receptor gene is intronless, as are the adrenergic receptors. In contrast, the 5-HT_{1C} and the 5-HT_2 receptor genes contain multiple introns within the protein coding region (Hartig et al., 1990; Julius, 1991).

Function of Serotonin

Functionally, 5-HT has been implicated in diverse systems such as temperature regulation, pain, mental illness and tolerance to ethanol (Grahame-Smith, 1988; Smith et al., 1978; Tabakoff and Hoffman, 1987). 5-HT systems are involved in the promotion of sleep (Grahame-Smith, 1988). Inhibition of 5-HT synthesis by parachlorophenylalanine (PCPA) induces insomnia in the cat. 5-HT has also been implicated in appetite control (Grahame-Smith, 1988). Abnormalities in 5-HT function may be involved in anorexia nervosa and bulimia. Drugs which release 5-HT are used as anorectic agents in the treatment of obesity. Dysfunctional serotonergic neurons may also lead to the development of affective disorders including depression, schizophrenic, obsessive-compulsive disorder and panic attacks (Van de Kar, 1989). Several 5-HT receptors have been linked to regulation of hormone secretion (Van de Kar, 1989).

Development of Serotonin Neurons

The birthdate of a neuron is considered to be the day on which the precursor cell undergoes its last cell division. Differentiation of the neuron begins sometime after its birthdate. Neuronal genesis for serotonergic neurons is between gestational day (G) 11 and G15 (Lauder et al., 1982). The 5-HT nuclei develop as superior (rostral B7-9) and inferior (caudal B1-3) cell clusters. The superior cells develop as two bilateral groups connected by fibers that cross

the midline. On E18 these two groups fuse forming the dorsal and median raphe nuclei. Migration of serotonergic neuronal cell bodies occurs between G14 and G19, producing cell cluster shapes characteristic of adults. (Lauder et al., 1982; Lidov and Molliver, 1982; Wallace and Lauder, 1983; Liu et al., 1987).

Neurons in the superior (rostral) cell group are able to synthesize 5-HT shortly (24 hours) after they have been generated. 5-HT is first detected (via immunohistochemistry) in these cell groups on G12 (Lauder et al., 1982; Lidov and Molliver, 1982; Wallace and Lauder, 1983; Liu et al., 1987). Dendrites develop within hours of demonstrating immunoreactivity (Lidov and Molliver, 1982). Although the genesis of the inferior (caudal) nuclei begins on G11-G12, these nuclei do not express 5-HT immunoreactivity until G14.

5-HT remains low and constant until birth, when its levels are about 25-50% of the level found in the adult (Liu et al., 1987). By the third postnatal week the level of 5-HT reaches adult values. Increases in 5-HT are thought to reflect increases in TH activity. TH activity is low until postnatal day (PN) 12, at which time there is a dramatic increase in activity; adult values are reached by PN24 (Park et al., 1986). 5-HT uptake is about 10% of adult values at birth (Kirksey and Slotkin, 1979)

Both 5-HT_1 and 5-HT_2 receptors are measurable in the rat at birth (Smith and Gallager, 1989; Whitaker-Azmitia et al.,

1987; Bruinink et al., 1982). 5-HT_1 receptors show an interesting developmental pattern. Their density is high at the time of birth, but then decline in number during the first two weeks. Adult values are reached after PN30 (Whitaker-Azmitia et al., 1987). 5-HT_2 receptors in the forebrain, which are low at birth, show an age related increase in density to reach adult values by PN30 (Bruinink, 1982).

The onset of dendritic synaptogenesis in serotonergic neurons begins on G19. Somatic synaptogenesis does not begin until G20, which corresponds with the beginning of increased cell size. Only 15% of somatic synaptogenesis takes place before birth, the majority of this development occurs during the first two weeks of life (Lauder et al., 1982; Lauder and Bloom, 1975).

Serotonin as a Growth Factor/Growth Factors for Serotonergic Neurons

5-HT, in the rostral raphe, is produced early in the development of serotonergic neurons. Detectable levels of 5-HT are found shortly after these neurons begin to differentiate, before the functioning of neurotransmission or synaptogenesis (Liu et al., 1987). 5-HT cell bodies are generated 1-2 days before their known target areas (Lauder and Krebs, 1978). These observations led to the hypothesis that 5-HT may be functioning as a growth or regulatory factor during embryonic development.

Experimental alterations in fetal 5-HT levels should alter the development of target areas sensitive to 5-HT, if 5-HT has a regulatory function. To test this hypothesis Lauder (1978) treated pregnant rats with parachlorophenylalanine (PCPA), a TH inhibitor. This treatment, which caused a reduction in 5-HT, delayed the onset of neuronal differentiation in brain regions to which 5-HT neurons project. These studies provided evidence that 5-HT may be acting as a "differentiation signal" to target areas, by causing these neurons to cease cell division and to begin differentiation (Lauder et al., 1978).

In a similar experiment Goldberg and Kater (1989) injected 5,7-dihydroxytryptamine (5,7-DHT, a serotonergic neurotoxin) into the embryonic mollusk Helisoma trivolvia. These animals were then allowed to hatch and develop to sexual maturity. The morphology of neurons known to receive serotonergic innervation was abnormal. Furthermore, a transient decrease in 5-HT content may have stimulated neurite outgrowth as suggested by the enhanced synaptic coupling seen between adjacent the target neurons (Goldberg and Kater, 1989).

The response of Helisoma trivolvis neurites that are sensitive to 5-HT is complex. In a system using cultured Helisoma trivolvis neurons, 5-HT inhibited neurites in the process of elongating, while stable (non-elongating) neurites reinitiated outgrowth (Goldberg et al., 1991). 5-HT increases

intracellular Ca^{2+} levels, which then regulates growth cone behavior (Kater and Mills, 1991). 5-HT is stimulatory or inhibitory to neurite outgrowth depending on the level of intracellular Ca^{2+}. In neurites that are actively elongating, 5-HT increases the intracellular Ca^{2+} concentration to a greater level than is required for neurite outgrowth. The elevated Ca^{2+} inhibits neurite outgrowth. Neurites that are not actively elongating can be stimulated to do so if 5-HT raises their Ca^{2+} levels to optimal levels.

5-HT also regulates the development of corticosteroid receptors (Mitchell et al., 1990a; 1990b). Corticosterone, which is released from adrenal glands after stress-induced ACTH release, is concentrated in the hippocampus (McEwen et al., 1986). Corticosterone uptake in hippocampal cells is mediated by two different types of receptors: type I and type II. 5-HT levels in the hippocampus increase during the first two weeks of life. This development parallels that of the type II receptors (Mitchell et al., 1990a). Administration of 5,7-DHT (a 5-HT neurotoxin) or ketanserin (a 5-HT_2 antagonist) to neonates reduces type II receptor binding (Mitchell et al., 1990a). 5-HT and 5-HT_2 agonists increase type II receptor density in hippocampal cultures, suggesting that 5-HT acts directly on hippocampal cells, rather than an intermediary (Mitchell et al., 1990b).

Interestingly, corticosterone may be required for the normal development of tryptophan hydroxylase, the rate

limiting enzyme in 5-HT synthesis. (Sze, 1980). Adrenalectomy (which reduces circulating corticosteroids) prevents the developmental increase in tryptophan hydroxylase activity (Sze, 1976). Replacement injections of corticosterone reverse the effects of adrenalectomy. Corticosterone treatment in intact neonates also increases tryptophan hydroxylase activity. However, adrenalectomy does not reduce existing levels of TH, but blocks the development or stress-induced increases. Corticosterone is thought to play a "permissive" role, allowing the induction of tryptophan hydroxylase to occur (Sze, 1976; 1980; Meyer, 1985).

Studies of primary cell culture have shown that 5-HT may influence the maturation of serotonergic neurons by its actions on 5-HT autoreceptors (Whitaker-Azmitia and Azmitia, 1986; 1989). 5-HT or 5-methoxytryptamine, a 5-HT agonist, inhibit ^{3}H-5-HT uptake and neurite outgrowth of cultured serotonergic neurons (Whitaker-Azmitia and Azmitia, 1986). The neurites <u>in vitro</u> appear to encircle their cell body rather than extend forward. Because 5-HT neurons begin their development as paired bilateral groups, Whitaker-Azmitia and Azmitia (1986) have hypothesized that 5-HT may be inhibiting neurite outgrowth from 5-HT neurons to their target areas, while promoting collateral formation between the adjacent groups. 5-HT also activates serotonergic receptors on glial cells, causing them to release growth factors which stimulate serotonergic development (Whitaker-Azmitia and Azmitia, 1989).

Thus 5-HT actions on the development of serotonergic neurons may depend upon the concentration of 5-HT and on the presence of neuronal and glial receptors during different developmental periods.

Serotonergic Neurons in Culture

Neuronal cultures have been widely used as a model for development, and as an aid in identifying neurotrophic factors (Davila-Garcia and Azmitia, 1989; Azmitia and de Kloet, 1987; Azmitia and Whitaker-Azmitia, 1987; Azmitia et al., 1988). These cultures allow for the study of specific effects of drugs and hormones on neurons in a controlled environment. Basic assumptions about these cultures are that neurons grown in vitro express the same phenotypic characteristics as cells in vivo. It is also assumed that responses following manipulations of the environment in vitro mimic responses seen in vivo. One of the greatest advantages of primary cultures is that the impact of experimental changes can be measured directly.

Organ cultures used to study the serotonergic system were first performed in the early 1970's when Halgren and Varon (1972) cultured the raphe nucleus from neonatal rats. They demonstrated that these cultures could be maintained for up to three weeks. These cultures were also subjected to hormones and drugs known to affect 5-HT metabolism in vivo. Their results indicate that organ cultures can be manipulated in the

same manner as serotonergic cells *in vivo*.

In 1981 Yamamoto was the first to describe cultures of dissociated serotonergic neurons taken from embryonic day 13-15 rat brain stem. 5-HT immunoreactivity was seen in the cell bodies and processes as early as 24 hours after plating. The morphology of these stained cells were typical: they contained either pyramidal cells with multipolar processes or fusiform cells with bipolar processes. 5-HT immunoreactivity was demonstrated for as long as three weeks in culture. ^{3}H-5-HT uptake, routinely used as a measure of the maturation of serotonergic neuronal terminals, increased with time in culture (Yamamoto, 1981; Davila-Garcia and Azmitia, 1989; Azmitia and Whitaker-Azmitia, 1987). Thus, 5-HT neurons removed from embryonic brains can survive in a dissociated cell culture and retain characteristics attributed to serotonergic neurons (Yamamoto, 1981; Davila-Garcia and Azmitia, 1989; Azmitia and Whitaker-Azmitia, 1987). The synthesis of 5-HT by cultured serotonergic neurons can be inhibited by PCPA (Yamamoto, 1981).

Fetal Alcohol Syndrome

Effects of Ethanol on Neural Development

One of the main features of FAS is microcephaly. The effects of ethanol on brain development are varied and include the following: 1) decreased cell number; 2) decreased cell

size; 3) irregular axonal and dendritic morphology; and 4) abnormal organization of the brain (e.g. Miller 1986; 1987; 1988; 1989; 1990; West et al., 1986; Bonthius and West, 1990). Thus, prenatal ethanol exposure may affect cell proliferation, cell death, differentiation, growth and migration. The molecular and cellular mechanisms involved are for the large part unknown, and most of the work done so far is descriptive in nature.

All neurons are not equally susceptible to the effects of ethanol. The developmental state of a neuron may determine whether the neuron is sensitive to ethanol. For example, in the cortex and hippocampus, it has been suggested that the effects of ethanol are greatest during the period of neuronal generation. During this period, ethanol has been shown to inhibit or stimulate cell division (Miller, 1989). In contrast, cerebellar neurons are more sensitive to ethanol when they are differentiating, after they have been generated Bonthius and West, 1990). The hypothesis that ethanol affects neuronal populations depending on their stage of development can be tested by experimenting with the timing of the ethanol insult.

Cortical neurons are born during the last half of gestation and are generated from two proliferative zones. These two zones are the ventricular zone, which is active during the first half of cortical neurogenesis, and the subventricular zone, which is active during the second half

(Miller, 1989). Both zones are differentially affected by in utero ethanol exposure.

Ethanol depressed the proliferative activity of the ventricular zone, as shown by decreased ^{3}H-thymidine incorporation (Miller, 1989). In addition, ethanol increased the length of time a cell spends in phase G1 of the cell cycle immediately following cell division (Miller, 1990). The ventricular zone was thinner in ethanol-exposed animals, apparently due to a decrease in the number of cells.

In contrast, the proliferative activity of the subventricular zone was stimulated (Miller, 1989). The subventricular zone was thicker in ethanol exposed animals and the number of cells incorporating ^{3}H-thymidine was increased. Ethanol may be acting directly on cellular processes, stimulating cell division. Alternatively, this increase in proliferative activity may be compensating for the previous loss of cells. The sum of these effects in ethanol exposed animals is to delay the onset of neuronal generation in the cortex by 1 day and extend it by 2 days. The number of neurons born on each day is significantly lowered until G19, when an abnormally large number of neurons is generated (Miller, 1986; 1988). In the somatosensory cortex this leads to an overall reduction in the number of neurons and glia, as well as a decrease in neuronal cell size (Miller and Potempa, 1990).

Prenatal ethanol exposure also alters the distribution of neurons (Miller, 1986; 1987; 1988). Normally the cortex

follows an inside-to-outside pattern of development (Angevine and Sidman, 1961,). Neurons in deep layers such as V and VI, are generated first while those in the superficial layers are generated last. Consequently, neurons in layers II and III must migrate through already existing layers. Neurons migrate along radial glial fibers that extend from the proliferative zones to the pial surfaces (Hatten, 1990). After neuronal migration is complete, radial glial fibers transform into astroglia (Schmechel and Rakic, 1979). Misplaced neurons are found in all layers of the neocortex in ethanol exposed offspring (Miller, 1986; 1987; 1988). This is primarily true of late generated neurons, which normally reside in layers II and III but are found in layers V and VI. The presence of ectopic neurons suggests that ethanol affects neuronal migration in the developing cortex (Miller, 1986; 1987; 1988). Since the migration of neurons is dependent upon cell adhesion molecules located on both neurons and glia (Hatten, 1990), ethanol may alter the interaction between the neuron and the glia. Alternatively, ethanol may accelerate the transformation of the radial glial fibers into astrocytes (Miller et al., 1988). This latter hypothesis is particularly attractive and could explain why later-generated neurons are more likely to be misplaced.

In the hippocampal formation, the development of pyramidal and granule neurons are temporally separated; pyramidal neurons in the hippocampus are born prenatally,

granule cells in the dentate gyrus are generated postnatally (Angevine, 1965). While the number of pyramidal cells in the CA1 region of a mature rat is reduced by prenatal but not postnatal exposure to ethanol, CA3 pyramidal cells are unaffected (Barnes and Walker, 1981; West et al., 1986; Wigal and Amsel, 1990). Stem cell populations that generate granule cells also appear unaffected, because the number of granule neurons is unchanged by in utero ethanol exposure. Granule cell number has been found to either increase or remain unaffected by postnatal exposure to ethanol (West et al., 1986; Bonthius and West, 1990)

The cerebellum is particularly sensitive to ethanol. Cerebellar weights are typically reduced to a greater extent than total brain weight (Bonthius and West, 1990; Bauer-Moffett and Altman, 1977; Goodlett et al., 1990). The reduction in cerebellar weight is reflected by decreases in neurons that compose the cerebellum.

Like the hippocampus, the two main cell types of the cerebellum are generated at different times relative to birth, and are affected differently by ethanol exposure. In contrast to hippocampal pyramidal cells, the prenatally formed purkinje cells are more sensitive to ethanol after they have been generated. Purkinje cell numbers are significantly reduced with postnatal ethanol exposure, during differentiation when synaptic contacts are being established (Bauer-Moffett and Altman, 1977; Philips and Cragg, 1982; Bonthius and West,

1990). Interestingly, purkinje cells in early maturing cerebellar lobules are reduced to a greater extent than purkinje cells in later maturing lobules. It was concluded that purkinje cells in a more "mature state of differentiation" were more susceptible than those in a less mature state (Bonthius and West, 1990), which further supports the hypothesis that the developmental state of a neuron determines its susceptibility to ethanol.

The number of cerebellar granule cells, which are formed postnatally, is also reduced by postnatal ethanol exposure (Bauer-Moffett and Altman, 1977; Bonthius and West, 1990). This contrasts with the hippocampus, where granule cells are either unaffected or increased by ethanol exposure (Barnes and Walker, 1981; Bonthius and West, 1990). Consequently, it has been suggested that, in the cerebellum, ethanol initially acts on purkinje cells to reduce their numbers. Cerebellar granule cells, which project to and synapse with the purkinje cells are secondarily reduced because their target population is diminished. Hippocampal granule cells synapse with CA3 pyramidal cells, whose numbers are not affected by ethanol exposure (Wigal and Amsel, 1990).

Effects of Ethanol on Development of Serotonergic Neurons

Ethanol exposure during the embryonic period in the rat affects the normal development of the serotonergic system. 5-HT and 5-HIAA levels are decreased in the brainstem

(location of the raphe) of G15, G19 and PN5 ethanol-exposed rats (Druse et al., 1990). 5-HT_{1A} receptors in the frontal cortex are also decreased at PN5 (Druse et al., 1990). Early deficiencies of 5-HT may impede further development of serotonergic neurons, because 5-HT functions as a growth factor during embryonic development, by autoregulating the maturation of serotonergic neurons. Indeed, the 5-HT deficiency persists into late postnatal development. A 20-50% deficiency of 5-HT and 5-HIAA is detected in the motor and somatosensory cortex of 19- and 35- day-old offspring of rats fed an ethanol containing liquid diet on a chronic basis prior to parturition (Rathbun and Druse, 1985). 5-HT uptake in the motor cortex as well as 5-HT_1 binding sites on membranes from motor and somatosensory cortex are reduced 10-40% (Druse and Paul, 1989; Tajuddin and Druse, 1989a).

Neurochemical studies suggest that there is a deficiency in the projection of neurons from the raphe to the cortex since 5-HT, 5-HIAA, 5-HT uptake and some 5-HT_1 receptors are located presynaptically. 5-HT_2 receptors, located on postsynaptic neurons, appear not to be affected by in utero exposure to ethanol (Tajuddin and Druse, 1989b).

Ethanol in Cell Culture

Traditionally, FAS studies have been conducted on offspring from mothers that have been fed an ethanol-containing liquid diet. However, it is not easy to

distinguish the direct effects of ethanol from the indirect effects mediated via changes in the mother's status. As discussed below, there are many secondary ethanol-related changes in the mother, such as undernutrition, placental dysfunction, changes in maternal metabolism and endocrine imbalances which can also impair prenatal growth (Schenker, 1990; Snyder, 1986; Savoy-Moore et al., 1989). Direct cellular toxicity, independent of the mother, may be involved once ethanol reaches the embryo.

Tissue culture offers the benefit of examining the direct effects of known concentrations of ethanol on specific aspects of growth and differentiation. Advantages of primary cultures are that they are derived from normal tissue and probably closely resemble cells found in vivo. In contrast, established cell lines are either transformed or are obtained from cancerous sources.

One of the biggest disadvantages of cell culture is that growth conditions that simulate an in vivo environment are unknown. Conventional tissue culture "wisdom" dictates the use of serum, which supplies growth factors necessary for in vitro development. The exact components of serum have not been defined, and serum may vary from lot to lot. This uncertainty has lead some researchers to investigate alternate growth supporting media. There are several studies which implicate one or more serum factors with ethanol's effects on cultured cells. For example, ethanol significantly increased

adenylate cyclase activity in PC12 cells grown in serum-containing media, but not in chemically defined media (Rabin, 1990). Significant differences were also seen when the chemically defined media was supplemented with serum lipoproteins, implying that the observed effects of ethanol require the presence of a serum component. In addition, Smith et al. (1990) suggest that serum might provide a protective barrier to the toxic effects of ethanol. Survival of mouse dorsal root ganglion neurons was increased in serum-containing media treated with ethanol.

At one time, glial cells were considered "the glue" of the nervous system. While structurally important, few additional functions were attributed to non-neuronal cells. It is now thought that there are regionally specific glial cell populations which possess receptors, uptake sites and enzymes essential for neuronal functioning. Astrocytes possess 5-HT uptake sites, receptors and MAO (Katz and Kimelberg, 1985; Fillion et al., 1983; Fitzgerald et al., 1990). They also play a major role during development, in that they secrete neurotrophic factors and provide a scaffolding which guides neurons to their final destination (Hatten, 1990).

Considering the importance of glial cells, very little is known about how ethanol affects their development. It is conceivable that ethanol influences glial development, which in turn propagates the abnormal cytoarchitecture and neurochemical imbalances associated with FAS.

Decreases in cortical astroglia cell number can occur at an ethanol dose as low as 200 mg/dl (Davies and Cox, 1991). This could potentially happen if ethanol was either toxic to the glial cells or inhibited cell division. ^{3}H-Thymidine incorporation (measuring DNA synthesis) is decreased, suggesting that ethanol inhibits the generation of new cells (Geurri et al., 1990). The reductions seen in protein and RNA content (Kennedy and Mukerji, 1984) probably result from this reduction in cell number. Inconsistent with these previous reports are the findings of Guerri et al. (1990). They found that ^{3}H-leucine incorporation (measuring protein synthesis) is unaffected by in vitro ethanol exposure.

Differentiation refers to the process by which embryonic cells become specialized. In glial cells this is determined by measuring developmental increases seen in glial specific proteins. Ethanol causes a decrease in glutamine synthetase activity, a marker for astroglial differentiation, which parallels the loss of glial protein content (Kennedy and Mukerji, 1985).

Prenatal ethanol exposed astroglial cultured in the absence of ethanol, demonstrated a reduction in both ^{3}H-thymidine and ^{3}H-leucine incorporation (Guerri et al., 1990). This diminished proliferative activity suggests that in utero ethanol exposure may damage astroglial progenitor cells. Consequently, developmental processes occurring after the ethanol insult may still be in jeopardy of abnormal growth.

Cortical astroglia from embryonic chickens are relatively resistant to ethanol. DNA, protein content, and ^{3}H-leucine incorporation were reduced only at an extremely high concentration of 1000 mg/dl. Lower doses of 100 and 500 mg/dl had no effect on cell growth and proliferation (Davies and Vernadakis, 1984).

Neuronal cell cultures, obtained from a variety of sources, appear to be more resistant to a direct toxic effect of ethanol. The LD_{50} for mouse dorsal root ganglion neurons is 2000 mg/dl, while the LD_{50} for non-neuronal cells (presumably glia) in the same culture is 700 mg/dl (Scott et al., 1986). While ethanol caused a dose-dependent (0-250 mg/dl) inhibition of nerve growth factor induced neurite outgrowth in similar cultures, cell survival was not affected (Dow and Riopelle, 1985). Although the number of viable cells and protein content in cultured dopaminergic neurons were reduced at 550 mg/dl, lower doses of 125 and 200 mg/dl had no significant effect (Acosta et al., 1986). In addition, ethanol (230 mg/dl) had no effect on dopamine uptake in these cultures (Mytilineou et al., 1988; personal communication with Mytilineou). Exposure of rat cortical neurons to 920 mg/dl ethanol had no impact on the content of neuroactive amino acids (taurine, aspartic acid, glutamic acid, glycine and GABA) (Kuriyama et al., 1987). The activities of glutamic acid decarboxylase, GABA-transaminase, choline acetyltransferase and acetylcholinesterase were similarly

unaffected.

Possible Mechanisms

Many hypotheses have been put forward to explain how ethanol consumption damages the fetus, but in actuality, very little is known about the mechanisms of action. Clinical studies are complicated by the fact that alcoholic women frequently abuse other drugs. Cigarettes, cocaine and marijuana use may all contribute to growth retardation, which is also found in the fetal alcohol syndrome (Zuckerman et al., 1989; Meyer and Tonascia, 1977). Nevertheless, animal models have shown that ethanol has deleterious effects on the fetus in the absence of these other factors (e.g. Miller 1986; 1987; 1988; 1990).

Poor maternal nutrition may impair fetal growth and exacerbate the prognosis of the ethanol exposed infant. Defective placental transport of nutrients may also compromise fetal nutrition (Schenker et al., 1990). Placental nutrient transport serves as the primary source of the fetus' nutrient and energy supply. Normal fetal growth is dependent upon an adequate transport of essential amino acids, glucose and vitamins across the placenta. Several studies have shown impaired transport of amino acids and glucose in pregnant rats fed liquid diets containing ethanol (Henderson et al., 1982; Snyder et al., 1986). The human placenta is resistant to acute ethanol exposure (Schenker, 1990). However, pathologic

placental changes have been observed in chronic alcoholics (Schenker et al., 1989; Halmesmaki et al., 1987, Baldwin et al., 1982).

Despite the fact that there is considerable evidence that malnutrition can augment some of the abnormalities found in FAS, it is apparent that even in well-fed animal models of FAS significant ethanol associated abnormalities are found (e.g. Miller 1986; 1987; 1988; 1989; 1990; West et al., 1986; Bonthius and West, 1990). In animal models of FAS, both control and ethanol-fed animals receive an equal quantity of calories and major nutrients. However, impaired placental transport of nutrients cannot be excluded.

Hypoxic episodes resulting from ethanol consumption may also be damaging to the developing fetus. A reduction in blood flow in the umbilical vessels has been demonstrated in rats, monkeys, and humans (Jones et al., 1981; Mukherjee and Hodgen 1982; Savoy-Moore et al., 1989). In the human fetus, hypoxia primarily affects subcortical regions of the brain, especially those in the periventricular tissues (Jorgensen and Diemer, 1982). Interestingly, Miller has found that the proliferative zones which line the lateral ventricles are affected by <u>in utero</u> ethanol exposure (Miller, 1989). In addition, hypoxia produces cell loss of pyramidal neurons in the CA1 region of the hippocampus and purkinje cells in the cerebellum (Jorgensen and Diemer, 1982). This selective cell loss is similar to that observed by Barnes and Walker (1981)

and Bonthius and West (1990) following prenatal ethanol exposure. The areas that are most susceptible to hypoxic damage use aminoacids as excitatory neurotransmitters. Hypoxia may produce cell death in these regions by causing a release of excess glutamate resulting in overexcitation and cell death (Jorgensen and Diemer, 1982)

Ethanol exposure may also produce changes in factors that are necessary for normal development of the fetus. Prostaglandins are essential for growth and development of the fetus, as well as the maintenance of pregnancy (Challis and Patrick, 1980; Goldberg and Ramwell, 1975). Ethanol induced increases in PGE_2 have been correlated with suppression of fetal breathing movements in the near term fetus (Brien and Smith, 1990). Aspirin and indomethacin, which inhibit prostaglandin synthesis, reduce some of the deleterious effects of ethanol (Schenker et al., 1990; Randall et al., 1990; Brien and Smith, 1990). Ethanol consumption increases thromboxane and prostacylin, two vasoactive prostanoids important for the regulation of umbilical and placental blood flow (Schenker et al., 1990; Randall et al., 1990). Inhibition of placental blood flow may produce hypoxia in the fetus.

Since in utero ethanol exposure produces a wide range of abnormalities in the development of CNS abnormalities, it is possible that ethanol alters the function of growth factors that control cell survival, differentiation and migration.

Growth factors such as growth hormone-releasing hormone, nerve growth factor and epidermal growth factor prevent the ethanol induced decreases in choline acetyltransferase activity (Kentroti and Vernadakis, 1990; Brodie et al., 1991). In addition, Dow and Riopelle (1990) have demonstrated that in vitro ethanol exposure inhibits the production of heparin sulphate proteoglycans, which have neurite-promoting activity. Unfortunately, it is not known whether in utero ethanol exposure reduces the concentration of any of these factors.

The teratogenic effects of ethanol consumption could be due to direct effects of ethanol, since ethanol readily crosses the placenta (Rosett and Weiner, 1984). The most common consequence of gestational ethanol abuse is growth impairment. Normal growth is dependent on the ability of the organism to replicate and synthesize proteins. Reduced brain size may be due to the interference of nucleotide and protein synthesis by ethanol.

Under a variety of conditions, ethanol can inhibit protein, RNA and DNA synthesis (Dreosti et al., 1981; Garro et al., 1991; Tewari et al., 1987; Rawat, 1985; Sharma and Rawat, 1989). Ethanol inhibits the activity of aminoacyl tRNA synthetase and the transfer of amino acids to the growing polypeptide chains (Lamar, 1972; Fleming et al., 1975). Ethanol also disrupts the transportation of RNA across the nuclear membrane and reduces ribosomal binding to available mRNA (Tewari et al., 1975; 1980). Ethanol consumption by

pregnant mice suppresses DNA methyltransferase activity, resulting in hypomethylation of fetal DNA (Garro et al., 1991). The methylation state of DNA affects gene expression. DNA of inactive genes is more heavily methylated than that of active genes (Alberts et al., 1989).

The processes involved with gene transcription and translation are essential for cellular growth. Handicaps to any of these mechanisms during critical periods may lead to the development of certain features of the fetal alcohol syndrome. During development, each subsequent phase may depend on the normal development of the preceding stage. Ethanol-related changes in the protein synthetic machinery at any point could potentially affect the final cytoarchitecture and circuitry of the brain.

How much ethanol is too much? This apparently simple question is difficult to answer because of the difficulty in obtaining accurate reports of a woman's ethanol intake during pregnancy. Self-reports are highly inaccurate for two reasons. 1) Women must depend upon their memory of how much ethanol they drank. 2) Because there is a stigma associated with drinking while pregnant, the reported consumption may be lower than the actual consumption (Rosett and Weiner, 1984). For these and other reasons, it is not known if there is a "safe" lower limit for ethanol intake. Nonetheless, growth deficits have been observed in children of mothers who reportedly drank as little as two drinks per day (Hanson et

al., 1978). No information is available regarding the blood ethanol level of these women. This is potentially important because blood alcohol content (BAC) is a more accurate indicator of intoxication, than the amount of ethanol ingested.

Proposed Studies

Prenatal exposure to ethanol impedes the development of 5-HT neurons. Although several potential causes of ethanol-associated abnormalities have been discussed, the mechanisms producing abnormal development of serotonergic neurons is unknown. The purpose of this dissertation research was to investigate the hypothesis that *in utero* ethanol directly inhibits the development of serotonergic neurons. Primary cultures of neurons and astroglia were exposed to a range of clinically relevant doses (50, 150 and 300 mg/dl) of ethanol. The function of serotonergic neurons and astrocytes were assessed after 4 days of ethanol exposure. Astrocytes were investigated because of their important role in guiding neurons to their destination and providing growth factors for neuronal development.

CHAPTER THREE

METHODS

In Vitro Experiments

Ethanol Determination

Ethanol levels were measured in culture media and in blood samples using an enzymatic kit (Sigma #330-1; St. Louis, MO). This kit measures ethanol by measuring the nicotinamide adenine dinucleotide (NADH) formed when ethanol is converted to acetaldehyde via alcohol dehydrogenase (ADH) and when nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. An increase in absorbance at 340 nm occurs when NAD^+ is converted to NADH.

The ethanol concentration of culture media was analyzed 3, 6, 12, or 24 hours after the addition of ethanol (50, 150 and 300 mg/dl). Blood ethanol levels were determined in animals that consumed an ethanol-containing liquid diet for 5 days prior to sampling. These animals were not used in any subsequent experiments. Tail vein blood was removed 2 hours after the addition of fresh diet to animals that had been fasted for 12 hours. Deproteinization of the blood samples was performed by the addition of trichloroacetic acid (6.25%

w/v). Each sample was tightly capped to prevent evaporation of the ethanol and allowed to stand at room temperature for 5 minutes, followed by a slow speed centrifugation (International Clinical Centrifuge, Model CL; International Equipment Co., Boston, MA). One hundred microliters of this supernatant or 100 μl of culture media were added to a NAD-ADH assay vial containing 2.9 ml glycine buffer (0.5 M) and incubated at 37^{0}C for 10 minutes. The sample was transferred to a cuvette and the absorbance was read at 340 nm (Gilford Response spectrophotometer).

Neuronal Primary Cell Culture

Timed pregnant Sprague-Dawley rats were purchased from Holtzman (Madison, WI.). Dissociated cell cultures were prepared from rat embryos on Gestational day 14 (day of insemination = 0). Gestational day 14 was chosen because by this age most of the serotonergic cells located in the rhombencephalon have been generated (Konig et al., 1989; Lauder et al., 1989).

Uterine horns were removed from anesthetized rats (60 mg/kg sodium pentobarbital; Anthony Products Co., Arcadia, CA) and placed in a sterile petri dish containing cold Hank's Balanced Salt Solution (HBSS: 5.4 mM potassium chloride, 0.4 mM potassium phosphate monobasic anhydrous, 4.2 mM sodium bicarbonate, 0.14 M sodium chloride, 0.34 mM sodium phosphate dibasic anhydrous, 5.6 mM glucose). The embryos were removed

from the uterus and transferred to a second sterile petri dish containing cold HBSS.

All dissections were performed under a Zeiss dissecting microscope. The portion of the head containing the mesencephalon and the rhombencephalon were separated from the fetus. All of the surrounding meninges and blood vessels were carefully removed. Rhombencephalic cells were obtained by cuts made at the rhombencephalic isthmus and at the cervical flexure. The dissected tissues were collected in sterile HBSS and stored on ice until all fetuses were dissected.

The dissected brain regions were transferred to 1 ml of a solution containing a 0.05% (w/v) trypsin/0.02% (w/v) EDTA (Sigma, St. Louis, MO), 0.1% (w/v) DNAse (Sigma, St. Louis, MO) solution in HBSS, and incubated at 37^0C for 35 minutes to allow for dissociation of the tissue. The tissue was then triturated with a 10 ml sterile pipette and a sterile Pasteur pipette until the tissue had dissociated. Pieces that could not be dissociated were allowed to settle to the bottom of the tube and were removed by aspiration. Cells were then resuspended in Eagles minimal essential medium (MEM, Sigma, St. Louis, MO) with 1% (v/v) penicillin-streptomycin (Sigma, St. Louis, MO), 20 mM HEPES (JRH Biosciences, Lenexa, KS) and 10% (v/v) NuSerum (Collaborative, Bedford, MA). Except where noted, all neuronal cell cultures were grown in this media. Cell viability was determined by trypan blue exclusion. For all studies except immunohistochemistry, cells were plated at

an approximate density of 1.5 x 10^6 viable cells on 35mm wells (6 well plates, CoStar, Cambridge, MA) or 4.0 x 10^6 cells on 25 cm^2 flasks. Cells used for immunohistochemistry were plated on 22 mm wells (12 well plates) at a density of 0.5 x 10^6 cells per well. Both wells and flasks had been previously coated with polylysine (MW>70,000, Sigma, St.Louis, MO, 33 μg/ml). The cultures were maintained in an atmosphere of 5% CO_2, 95% air at 37^0C.

Twenty-four hours after initial culturing the media was replaced with fresh media. The media was changed again forty-eight hours after initial culturing and thereafter the cells were cultured in the absence or presence of ethanol (50, 150, 300, or 450 mg/dl) for four days. To prevent ethanol evaporation culture plates were wrapped in parafilm and media was replaced daily for the duration of the experiment. The caps on the flasks were tightly sealed. For experiments in which the cells were acutely exposed to ethanol, the cultures were grown in the absence of ethanol until the time of analysis of 5-HT uptake.

Glial Primary Culture

The protocol used to generate rhombencephalic neuronal cultures was also chosen to start the rhombencephalic glial cultures except that the latter cells were seeded onto plates that were not coated with polylysine (Lim and Miller, 1989). (Astrocytes attach directly to the plastic surface whereas

neurons can only adhere to coated plates or to flattened astrocytes.) Glial cells proliferate in media with high serum content, thus these cells were cultured in Eagle's MEM with 10% (v/v) HS, 5% (v/v) FCS, 1% (v/v) penicillin - streptomycin (Sigma, St. Louis, MO) and 20 mM HEPES (JRH Biosciences, Lenexa, KS).

After initial culturing the cells were incubated at 37^0C for 2 days without disturbance to discourage aggregation of cells. Forty-eight hours after initial culturing the media was changed. Thereafter, the media was changed every 3-4 days. The cells were confluent in 1 week. The cultures were represented by a heterogeneous population of cells consisting of mainly neurons and astrocytes. On the seventh day after culturing the cells were dislodged by incubating the cells with 0.05% (w/v) trypsin/0.02% (w/v) EDTA, 0.1% (w/v) DNAse in HBSS for 5 minutes. Neurons typically do not survive this procedure. The cells were collected by low speed centrifugation and subcultured with a 1:4 dilution. The media was changed 24 hours later. Thereafter, the astrocytes were cultured in the presence or absence of ethanol (50, 150, 300 mg/dl) for 4 days. The culture plates were wrapped in parafilm to prevent ethanol evaporation and the media was replaced daily for the duration of the experiment.

5-HT and GFAP Immunohistochemistry

5-HT and glial fibrillary acidic protein (GFAP) (both

antibodies from IncStar, Stillwater, MN) immunohistochemistry was performed using the peroxidase vectastain ABC kit from Vector Laboratories Inc (Burlingame, CA). Glial filaments are composed of GFAP and the presence of this protein is a marker for astroglia (Banker and Goslin, 1991). Cultures that were used for 5-HT immunohistochemistry were incubated with 100 μM L-tryptophan (Sigma, St. Louis, MO) and 10 μM pargyline (Sigma, St. Louis, MO) for 24 hours before processing. All cultures were washed 3 times with warm phosphate buffered saline (PBS: 2.7 mM potassium chloride, 1.5 mM potassium phosphate monobasic anhydrous, 0.14 mM sodium chloride, 8 mM sodium phosphate dibasic anhydrous) before fixing with cold 4% (w/v) paraformaldehyde for 1 hour. After fixation and between all subsequent steps the cultures were washed 3 times with cold PBS. To block non-specific biotin sites, cultures were first incubated with a 50% (v/v) avidin blocking solution (in PBS with 1.5% (v/v) normal goat serum) for 30 minutes followed by a 30 minute incubation in a 50% (v/v) biotin solution (in PBS with 1.5% (v/v) normal goat serum). Cultures were then exposed overnight to either anti-5-HT (1:2000 in PBS with 1.5% (v/v) normal goat serum) or anti-GFAP (as supplied from IncStar). On the following day cultures were incubated first with a biotinylated secondary antisera (0.5% (v/v) goat anti-rabbit IgG in PBS with 1.5% (v/v) normal goat serum) for 30 minutes, then with the avidin-biotin peroxidase complex (as supplied from Vector Labs). Visualization of bound antibody

was performed in a 0.03% (v/v) H_2O_2 (Sigma, St. Louis, MO), 0.01% (w/v) diaminobenzidine (Sigma, St. Louis, MO), 0.1 M tris solution.

The percentage of 5-HT neurons in the cultures was determined by counting the number of positively stained and unstained neurons, using a Nikon inverted microscope at a magnification of 400x. Six random fields were counted for each experiment and these numbers were averaged to produce an n=1. Six separate experiments were performed.

5-HT Uptake

Uptake was determined by measuring the accumulation of ^{3}H-5-HT (specific activity 25 Ci/mmol, New England Nuclear, Boston, MA) by the neuronal and glial cultures (Lauder, 1989). The cultures were first washed 3 times with warm (37°C) HBSS. The cultures were then incubated for 20 minutes at 37°C with 60 nM ^{3}H-5-HT in HBSS containing 0.1 mM L-cysteine (Sigma, St. Louis, MO) and 1 x 10^{-5} M pargyline (Sigma, St. Louis, MO). Nonspecific uptake, determined using 1 x 10^{-5} M fluoxetine (Lily, Indianapolis, IN) was 7% to 15% for all experiments. For cultures exposed to ethanol for 4 days, the 20 minute incubation with ^{3}H-5-HT took place in the absence of ethanol. For studies of the effects of an acute ethanol exposure on control neurons, the 20 minute incubation with ^{3}H-5-HT took place in the presence of either 0, 50, 150, or 300 mg/dl ethanol.

After the 20 minute incubation period, the ^{3}H-5-HT containing solution was removed and the cultures were washed 3 times with ice-cold HBSS. Cultures were allowed to air dry and were then extracted with 1 ml of 0.1 N NaOH for 1 hour. Culture wells were then rinsed with 0.5 ml of 0.1 N HCl. The two extracts were combined prior to liquid scintillation counting. An aliquot was saved for protein determination.

5-HT Content

5-HT content in neuronal cultures was determined by using an immunoassay kit from AMAC Inc. (Westbrook, ME). This kit makes use of an antibody that is fixed on the inner surface of the wells in a microtiter plate and has a high affinity for acylated bioamines. Acylated 5-HT, in the sample or standard, competes for antibody binding with acetylcholinesterase coupled to 5-HT. The absorbance of the standards (0 - 200 nM) and samples is read at 410 nm.

The cell cultures were washed 3 times with HBSS. The cells from six wells were pooled into a microcentrifuge tube and collected by a low speed centrifugation. The supernatant was aspirated and the cells were homogenized in 100 μl of ice cold 0.2 N perchloric acid (Mallinckrodt, Paris, KY). An aliquot was taken for protein determination. The cells were then centrifuged for 5 minutes at 10,000g at 4^{0}C. The supernatant was removed and neutralized with an equal volume of 1M potassium borate, pH 9.25 (EM Sciences, Gibbstown, NJ).

The samples were centrifuged again for 5 minutes at 10,000g at 4^0C. The supernatant was then transferred to a new microcentrifuge tube and stored at -70^0C for less than 2 months until the day of the assay.

On the day of assay the 5-HT standards (0 - 200 nM) were prepared by adding 1 ml of distilled water to the vial containing the lyophilized 5-HT (concentration after reconstitution is 1×10^{-5} M). A serial dilution of the reconstituted 5-HT was performed to obtain the desired 5-HT concentrations for the standards. Equal volumes of 0.2 N perchloric acid and 1 M potassium borate were used as diluents. The standards were centrifuged at 10,000g for 1 minute at 4^0C and the supernatant, which contained the 5-HT standards, was transferred to new tubes.

In the vials containing the acylating reagent, 100 μl of standard or sample were added and then left in the dark for 30 minutes at room temperature. Following this incubation, 20 μl of the acylated standard or sample and 200 μl of the 5-HT-acetylcholinesterase conjugate (as supplied from AMAC Inc., Westbrook, ME) was added to the wells in the microtiter plate. The microtiter plate was shaken in the dark for 3 hours at room temperature. The well contents were aspirated and the wells were washed 3 times with 300 μl of wash solution (as supplied from AMAC Inc., Westbrook, ME). Two hundred microliters of substrate (as supplied from AMAC Inc., Westbrook, ME) was added to each well and shaken at room

temperature for 20 minutes in the dark. Fifty microliters of stopping reagent (as supplied for AMAC Inc., Westbrook, ME) was then added to all wells and the absorbance was read at 410 nm on a microplate reader (Dynatech, Chantilly, VA).

DNA Content

DNA content was assessed using a colorimetric assay developed by Burton (1956). All cultures were rinsed 3 times with HBSS. To the neuronal cultures 750 μl of 1 N NaOH was added directly to each well. The glial cultures were combined prior to the start of the assay due to low DNA content. The glial cells from six wells were pooled into a microcentrifuge tube and collected by low speed centrifugation. The supernatant was aspirated and the cells were dissolved in 750 μl of 1N NaOH.

Two 250 μl aliquots (duplicates) were taken from each sample and transferred to new test tubes. The remaining 250 μl were saved for protein determination. To each sample and standard (0 - 150 μg DNA dissolved in 1 N NaOH) 250 μl of 20% (v/v) perchloric acid was added. Both samples and standards were hydrolyzed at room temperature for 30 minutes. After the incubation period, 500 μl diphenylamine (40 mg/ml in glacial acetic acid) and 25 μl acetaldehyde (stored as a 16 mg/ml stock solution in distilled water and diluted 1:10 prior to use) were added and vortexed. The test tubes were then capped and shaken overnight in a 30^0C waterbath. On the following day

the tubes were centrifuged at 800g (Sorvall RT6000) for 10 minutes. The optical density of the standards and samples were read at 595 nm on a Gilford Response spectrophotometer.

Protein Determination

Protein levels were assayed by a micromodification of the method of Lowry et al. (1951). Samples were previously collected and dissolved in 1N NaOH following the protocol for determining DNA content. Samples and standards (0-50 μg human serum albumin) were incubated in 100 μl of 1 N NaOH for 30 minutes. To each sample and standard 1 ml of a solution (0.1:0.1:10, v/v/v) containing 1% (w/v) cupric sulfate, 2% (w/v) K^+-Na^+ tartrate, and 2% (w/v) sodium carbonate. After a 10 minute incubation, 100 μl of 1.0 N Folin phenol reagent was added. After a 30 minute incubation, the optical density of the standards and samples was determined at 700 nm on a Gilford Response spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio).

SDS Page Gels

Cells were washed 3 times with cold HBSS. Lysis buffer (0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 0.2 U/ml aprotinin in TSA (0.01 M tris, 0.14 M sodium chloride, and 0.025% (w/v) sodium azide, pH 8.0) was added directly to the culture dishes and allowed to incubate on ice for 20 minutes. The cells were then

scraped from the bottom of the plate and transfered to a chilled microfuge tube. The lysate was centrifuged at 12,000g for 2 minutes at 4^0C (MC15; Hill Scientific, Derby, CT). The supernatent was transferred to a fresh tube and stored at -70^0C for less than 3 months until the day of the assay. An aliquot was removed for protein determination (Lowry et al., 1951).

On the day of the assay, the samples were thawed on ice. An aliquot equivalent to 25 μg protein (neuronal cultures) or 10 μg protein (glia cultures) was taken from each sample and was diluted with an equal volume of twice concentrated loading buffer (40 mM tris, 4% (v/v) mercaptoethanol, 1% (w/v) SDS, 40% (v/v) glycerol, and Bromophenol Blue). The samples, along with low molecular weight markers (Bio-Rad, Richmond, CA), were boiled for 5 minutes before loading onto a 12% (w/v) denaturing polyacrylamide gel (30% (w/v) acrylamide, 8% (w/v) N,N'-methylene-bisacrylamide, 1.5 M tris-HCl, 10% (w/v) SDS, 10% ammonium persulfate, and TEMED). The gel was run at 100 volts (constant voltage) in an electrophoresis buffer (0.125 M tris base, 0.950 M glycine, and 0.05% (w/v) SDS. The proteins were stained with 0.1% (w/v) Coomassie blue, 40% (v/v) methanol, 10% (v/v) acetic acid and destained with 40% (v/v) methanol and 10% (v/v) acetic aced. A densitometric scan was performed using a MicroScan 1000 Gel Analyzer (Technology Resources, Inc., Nashville, TN) Two peaks that could be identified as being the similiar protein on separate

gels were chosen and the numbers constituting the % area were subjected to a Students' t-test (neuronal cultures) or a one-way ANOVA (glial cultures).

Statistical Analysis

The fetuses from two mothers were combined for each experiment. The number of viable cells obtained from these fetuses was enough to plate four 6 well plates. These four plates were then divided into 4 groups: control; 50; 150; and 300 mg/dl ethanol. Each plate constituted an "n" of one. Thus in those experiments measuring 5-HT uptake, protein, or DNA content, the values from each well on a plate were averaged to produce n=1. The experiment was then repeated 3-9 times (n=3-9). Because of the low 5-HT content in neuronal cultures, and low DNA content in glial cultures, all 6 wells were combined prior to being assayed.

All experiments in which the cultures were subjected to multiple ethanol doses were analysized by a one-way ANOVA and a post-hoc Newman Keul's test. Student's 't' test was used to determine statistical significance in those experiments had only control and one ethanol dose. Each value represents the mean $\pm$ the standard error of the mean. P values of <0.05 represent significant differences.

In Vivo Experiments

Animal Model

Timed pregnant Sprague-Dawley rats were purchased from Holtzman (Madison, WI.). The animals were separated into four experimental groups: 1a) Animals which were fed a 3.3% (v/v) ethanol liquid diet between G9-G10, followed by a 6.6% (v/v) ethanol liquid diet between G11-G15; 1b) animals which were pair-fed a control liquid diet between G9-G15. 2a) Animals which received a 3.3% (v/v) ethanol liquid diet between G14-G15 followed by a 6.6% (v/v) ethanol liquid diet between G16-G20; 2b) animals which were pair-fed a control liquid diet between G14-G20. The 3.3% (v/v) ethanol liquid diet was given two days prior to initiation of the 6.6% ethanol liquid diet to acclimate the animals to drinking a high ethanol content liquid diet. The ages were chosen because G11-G15 corresponds to the generation of 5-HT neurons (Lauder et al., 1982) and G16-G20 corresponds to the developmental time points used in the *in vitro* experiments. The caloric content of the control diet consisted of 21% protein, 29% fat, and 50% carbohydrate, while the caloric content of the ethanol diet was 21% protein, 29% fat, 15% carbohydrate, and 35% ethanol (Noronha and Druse, 1982). Animals were maintained on a 12:12 hour light/dark cycle.

Dissection

The animals were decapitated within two hours after the lights were turned on. The fetuses were removed and placed into a chilled petri dish. The brains were removed and the brainstem and cortex were dissected and frozen immediately.

High Performance Liquid Chromatography

5-HT and 5-hydroxyindoleacetic acid levels were measured in control and ethanol-treated animals by high performance liquid chromatography (HPLC) with electrochemical detection (set at 0.8+ volts). The tissues were homogenized (Tekmar Tissumizer, Cinncinnati, OH) in 0.1 M perchloric acid and centrifuged at 21,800 x g (16,000 RPM) for 20 minutes at 4^0C (SE12 rotor, Sorvall RC-5B). Samples were separated by reverse phase HPLC using a C-18 microbondapak column (3.9 mm x 30 cm; Waters, Milford, MA). The mobile phase consisted of 0.1 M sodium phosphate-citric acid (pH 4.0), 1.0 mM disodium EDTA, 1 mM heptanesulfonic acid, and 7% (v/v) acetonitrile. The internal standard isoproteronol (40 ng) was included in each sample. The flow rate was 0.7 ml/minute and the sample run time was approximately 35 minutes. The HPLC system consisted of a Bio-Rad pump (Richmond, CA), amperometric detector LC-4B (Bioanayltic Systems, West Lafayette, IN) and a Hewlett Packard (Avondale, PA) 3390A integrator.

Statistical Analysis

The values reported for 5-HT and 5-HIAA content represent the mean value ± the standard error of the mean of six samples. Student's 't' test was used to determine statistical significance.

CHAPTER FOUR

RESULTS

Normal Development of Cultured Rhombencephalic Neurons

5-HT neurons are generated in the rhombencephalon, or hindbrain, between gestational days 11-15. Rhombencephalic neurons were cultured on G14 when most of the 5-HT neurons have been generated, but before neuronal processes have become extensive. The presence of 5-HT neurons in these cultures was verified by immunohistochemical staining using an antibody against 5-HT. The number of glia cells were kept to a minimum by growing the neuronal cultures in media containing 10% (v/v) NuSerum (Collaborative, Bedford, MA). NuSerum is a "semi-defined" serum replacement consisting of growth factors plus 25% (v/v) newborn calf serum. Thus the final serum concentration in the media was 2.5% (v/v) newborn calf serum. Glia cells proliferate in medium containing higher concentrations of serum, usually 10% (v/v) horse serum and 5% (v/v) fetal calf serum (Pettmann et al., 1979; Kaufman and Barrett, 1983; Borg et al., 1985).

When first cultured, the neurons were rounded. However, within 48 hours they began to develop processes (Figure 1). Considerable outgrowth of these processes occurred within 6

days of being cultured, producing a network of intermingled neurons and glia.

^{3}H-5-HT uptake has been used by several workers to monitor neuronal maturation (Yamamoto, 1981; Whitaker-Azmitia et al., 1986; 1987; 1989). Between 2 days in vitro (DIV) and 6 days in vitro there was a 400% increase in ^{3}H-5-HT uptake indicating a robust growth in 5-HT nerve terminal development (Figure 2, top graph). Protein content also increases 85% during this time (Figure 2, bottom graph).

Effects of Ethanol Exposure on Cultured Rhombencephalic Neurons

5-HT uptake by serotonergic neurons was determined by measuring the amount of ^{3}H-5-HT accumulated in 20 minutes. The acute effects of ethanol were determined in cultures that were grown in the absence of ethanol for 6 DIV. Ethanol was added to the assay buffer only during the 20 minutes in which the uptake experiment was taking place. Figure 3 shows that an acute ethanol exposure had no significant effect on ^{3}H-5-HT uptake [$F_{(3,22)}$=.21, $p>0.05$].

Neuronal cultures were also evaluated after a 4 day ethanol exposure between 2 and 6 days in vitro. Because the concentration of ethanol declined 40% after a 24 hour period (Figure 4), the media was changed daily for the duration of the experiment. Qualitatively, these neurons appear to develop in the same way as control neurons. 5-HT

immunoreactive neurons had either bipolar or multipolar processes. The gross morphology of 5-HT neurons does not appear to be influenced by in vitro ethanol exposure (Figure 5).

Ethanol exposure, which took place during the time 5-HT nerve terminals are developing (see Figure 2), does not affect ^{3}H-5-HT uptake (Figure 6). Although there was a 15% decrease in ^{3}H-5-HT uptake at the highest dose (300 mg/dl), this difference was not significant [$F_{(3,19)}$=.15, p>0.05]. Additional measures of serotonergic neurons were also unaffected by a 4 day ethanol exposure. 5-HT content (Figure 7) was unaffected [$F_{(3,26)}$=1.25, p>0.05] and there was no significant change in the percentage of 5-HT immunopositive neurons in cultured rhombencephalic neurons (Figure 8, [$F_{(3,20)}$=.08, p>0.05]). 5-HT immunopositive neurons account for approximately 1% of the cultured rhombencephalic cells; the remaining neuronal cell types have not been characterized.

Protein content was reduced 15% at 300 mg/dl ethanol, although this was not significant (Figure 9, $F_{(3,20)}$=.68 p>0.05). Total proteins, isolated from control and 300 mg/dl ethanol treated cultures, were separated on 12% SDS polyacrylamide gels (PAGE) (Figure 10) to determine if ethanol altered the concentration of specific, major neuronal proteins. Figure 11 shows a representative densitometric scan. Two major proteins with estimated molecular weights of 40,000 kDa and 35,000 kDa were chosen for quantitation because

these were major proteins, which were routinely recognized by the scanning densitometer. The relative percentage areas of these two proteins were compared in control and 300 mg/dl treated cultures. There were no significant differences in either the 40,000 kDa protein [$t_{(DF=4)}$=.445, p>0.05] or the 35,000 kDa protein [$t_{(DF=4)}$=.375, p>0.05]. However, this technique has many drawbacks. Due to the limitations of the densitometer, only major protein peaks that could be identified from gel to gel could be analyzed. Thus several other proteins could not be analyzed because the densitometer did not consistently identify less abundant proteins.

DNA content, an estimate of total cell number, was unaffected by ethanol exposure (Figure 12, $F_{(3,20)}$=.06, p>0.05).

The next experiments were performed to determine if higher concentrations of ethanol have any impact on serotonergic cultures. The neurons used for these experiments were cultured on 25 cm^2 flasks. The relevance of this change in procedure is that there is little ethanol evaporation in tightly sealed flasks after 24 hours. Thus, these cultures are exposed to 450 mg/dl ethanol continuously for 4 days. Figure 13 shows that 450 mg/dl ethanol had no significant effect on ^{3}H-5-HT uptake [$t_{(DF=10)}$=1.483, p> 0.05], protein [$t_{(DF=10)}$=1.762, p>0.05] or DNA content [$t_{DF=10)}$=1.783, p>0.05].

Glial cells were grown in different media than neuronal cells. To confirm that the media in which the glial cells were cultured did not influence ^{3}H-5-HT uptake, an experiment

was performed in which rhombencepahlic cells were cultured in the same type of media that had been used for glial cells. $^{3}5$-HT uptake in neuronal cells after a 4 day ethanol exposure was compared using the two different media. Figure 14 shows that both media produced comparable effects on ^{3}H-5-HT uptake [$F_{(1,27)}$=1.024, p>0.05, two-way ANOVA].

Effects of Ethanol on Cultured Glial cells

Glial cells were generated by plating dissociated rhombencephalic cells onto wells that were not coated with poly-l-lysine. Glial cells can attach directly to the well, but neurons need a substrate (such as poly-l-lysine or glial cells) for attachment to occur. The top panel in Figure 15 shows rhombencephalic cells (7 DIV) that were plated onto wells coated with poly-l-lysine and cultured in 10% NuSerum. The open arrow is pointing to a neuronal cell that has attached directly to the coated well. The shaded arrow is pointing to a glial cell. The middle panel in Figure 15 shows rhombencephalic cells (7 DIV) that were plated onto uncoated wells and cultured in 10% HS, 5% FCS. The open arrow is pointing to a neuronal cell that has adhered to a glial cell (shaded arrow). Note that the neuronal process did not venture onto an area of the dish where there were no glial cells. Plating rhombencephalic cells onto wells not coated with poly-l-lysine reduces the number of neurons initially adhering. Cultures such as those in the middle panel were

trypsinized, subcultured and replated. Neurons typically do not survive this procedure. The bottom panel in Figure 15 shows GFAP positive cells, devoid of neurons. The cells divide relatively rapidly, forming a confluent monolayer within a week. Prior to reaching confluence these cells were pleomorphic. Once the cultures reached confluence they exhibited a uniform cobblestone-like appearance.

Acute ethanol exposure did not affect ^{3}H-5-HT uptake in glial cells [Figure 16, $F_{(3,19)}$= 2.06, $p>0.05$]). However, the top graph in Figure 17 shows a dose-dependent decrease in ^{3}H-5-HT uptake per well after a 4 day ethanol exposure. ^{3}H-5-HT uptake per well was significantly reduced at 300 mg/dl [$F_{(3,20)}$=3.51, $p<0.05$]. However there were no significant differences in uptake when expressed per mg protein (Figure 17, bottom graph [$F_{(3,20)}$=.16, $p>0.05$]). This was attributed to the finding that there was a concurrent loss of protein per well. Protein/well was significantly reduced at 150 and 300 mg/dl (Figure 18 [$F_{(3,20)}$=6.91, $p<0.05$]). In contrast to proteins, DNA content was unaffected by a 4 day ethanol exposure (Figure 19 [$F_{(3,20)}$=.34]).

Proteins isolated from control and ethanol treated cultures were separated on 12% SDS PAGE gels (Figure 20) to determine if the decreased protein content could be attributed to a general reduction in astroglial proteins. A representative densitometric scan is shown in Figure 21. Two major proteins with estimated molecular weights of 40,000 kDa

and 45,000 kDa were quantitated because they are major proteins and their density was consistently integrated by the scanning densitometer. The relative percentage areas of these two proteins were compared in control and ethanol treated cultures. There were no significant differences in either the 40,000 kDa protein [$F_{(3,12)}=.1529$, $p>0.05$] or the 45,000 kDa protein [$F_{(3,12)}=.811$, $p>0.05$].

In Vivo Experiments

Ethanol consumption in pregnant rats produced a blood ethanol level of 145 mg/dl 3 hours after introduction of fresh diet following 12 hours of deprivation. 5-HT and 5-HIAA content were measured in the brainstem (location of the raphe) and in the cortex on G20. There was no significant change in 5-HT or 5-HIAA content in these areas after *in utero* ethanol exposure between G14-G20 (Figure 22) or G9-G15 (Figure 23).

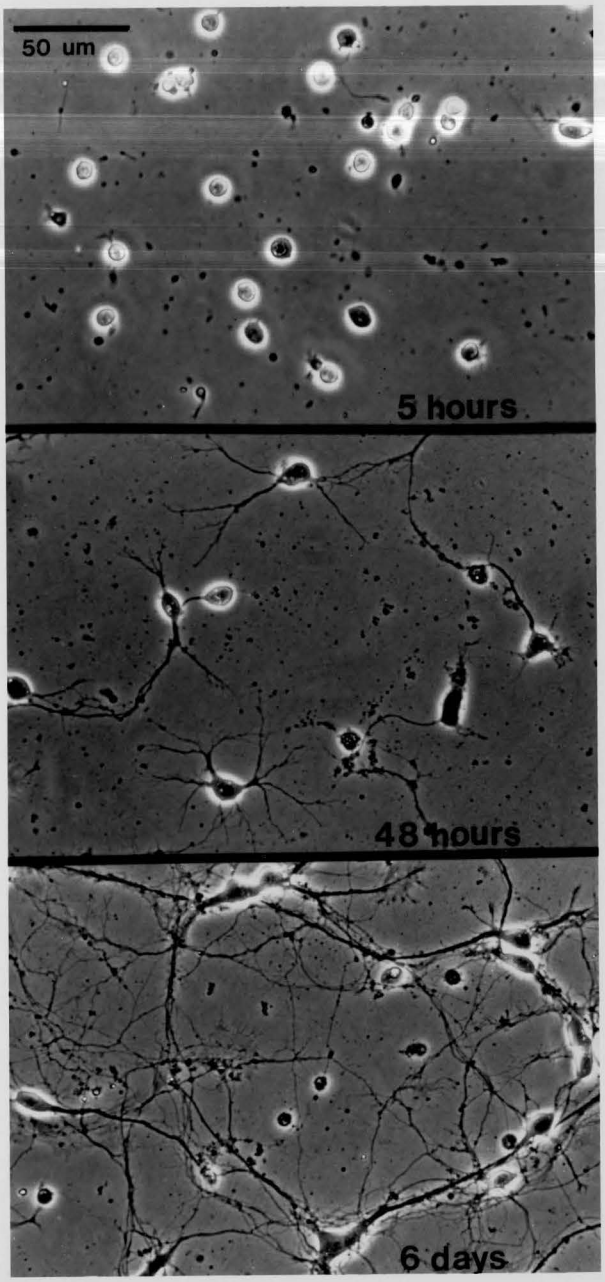

Figure 1. PHASE CONTRAST PHOTOMICROGRAPH OF REPRESENTATIVE FIELDS DEMONSTRATING THE DEVELOPMENT OF NEURONAL CULTURES OBTAINED FROM G14 RHOMBENCEPHALON. Top panel 5 hours after initial plating. Middle panel 48 hours in vitro. Bottom panel 6 days in vitro. Bar represents 50 μm.

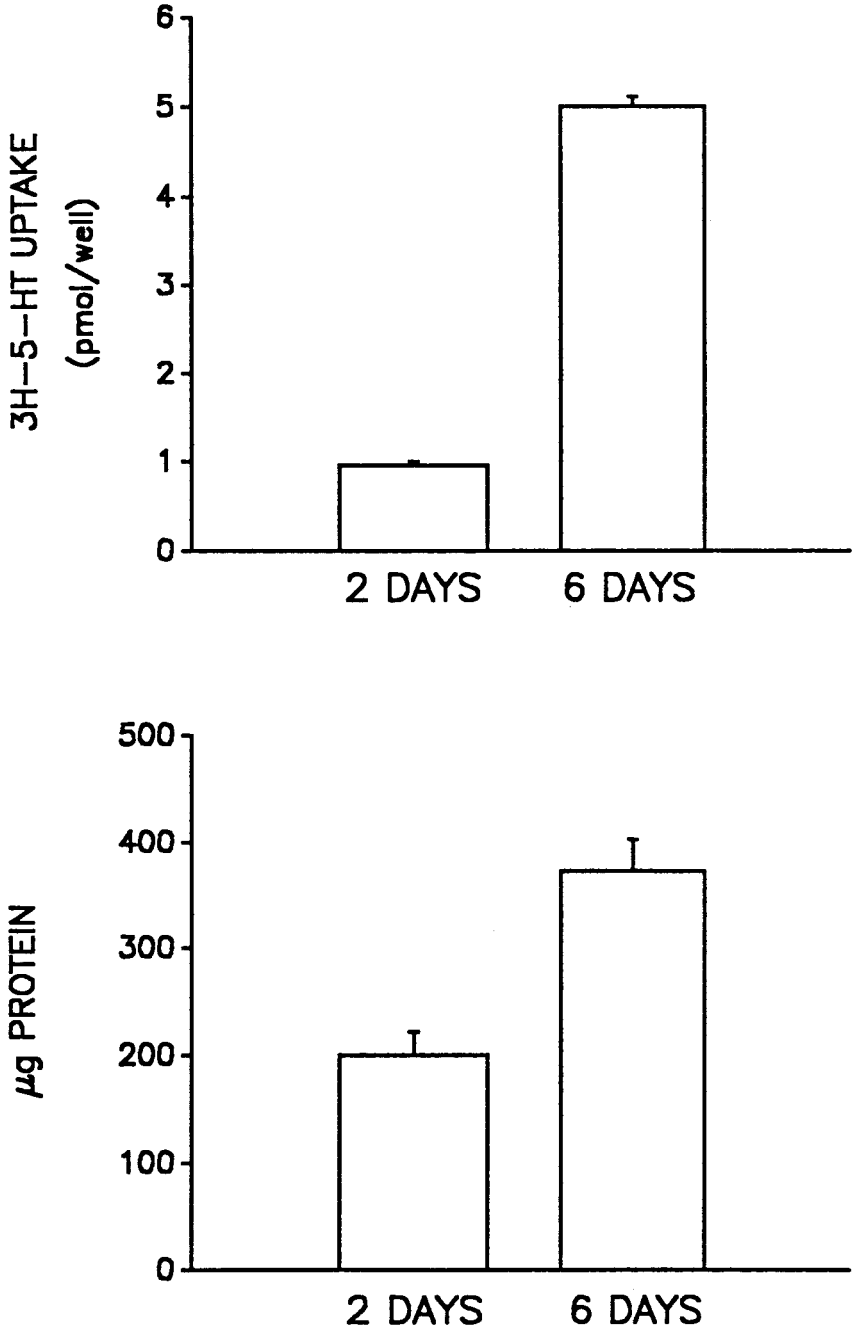

Figure 2. DEVELOPMENT OF ^{3}5-HT UPTAKE IN NEURONAL CULTURES AFTER 2 AND 6 DAYS IN VITRO. Top graph Uptake was determined by measuring the amount of ^{3}H-5-HT accumulated in 20 minutes as described in Methods. Non-specific uptake was determined by adding 10 μM fluoxetine in the assay buffer and was subtracted from total uptake. The Bottom graph depicts the protein content of cultures. Each value represents the mean ± the SEM of values obtained from 4 experiments.

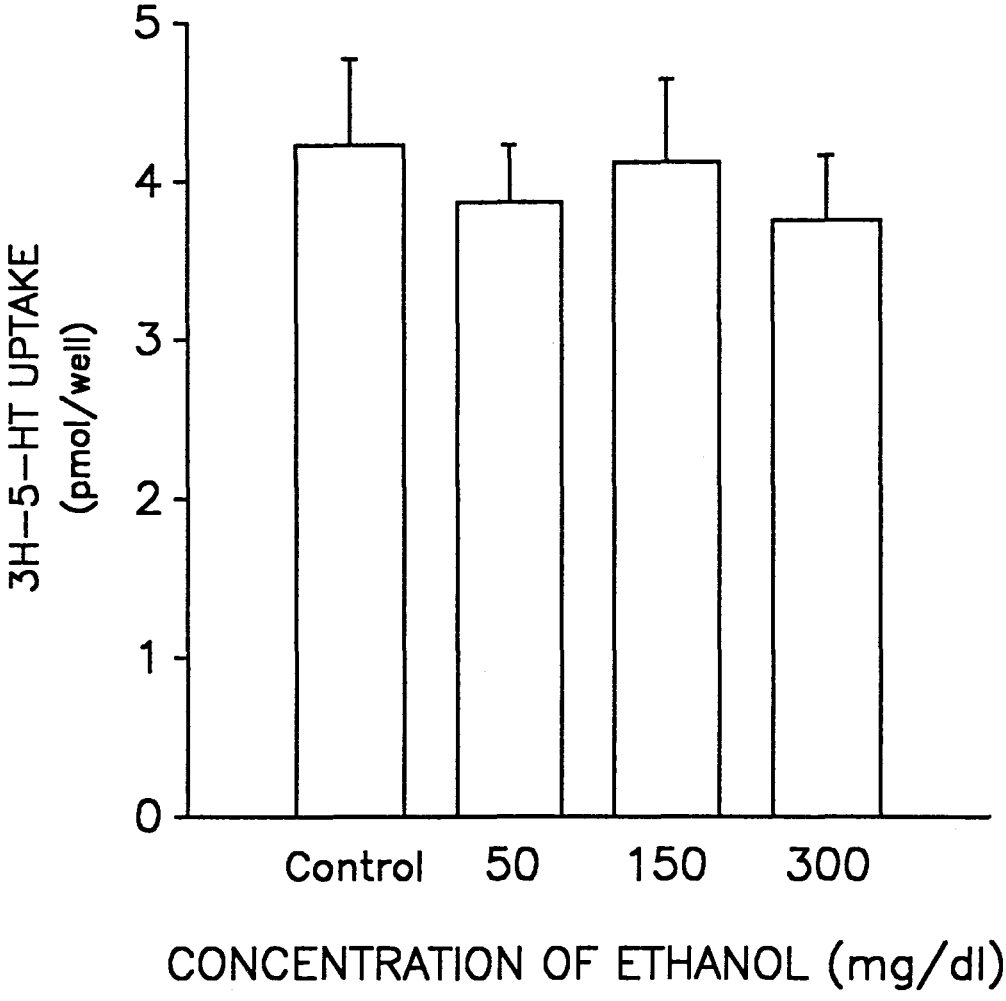

Figure 3. ^{3}H-5-HT UPTAKE IN NEURONAL CULTURES AFTER ACUTE ETHANOL EXPOSURE. Uptake was measured on the 6th day in vitro. Uptake was determined by measuring the amount of ^{3}H-5-HT accumulated in 20 minutes in the presence of ethanol. Non-specific uptake was determined by adding 10 μM fluoxetine in the assay buffer and was subtracted from total uptake. Each value represents the mean $\pm$ the SEM of values obtained from 6-7 experiments.

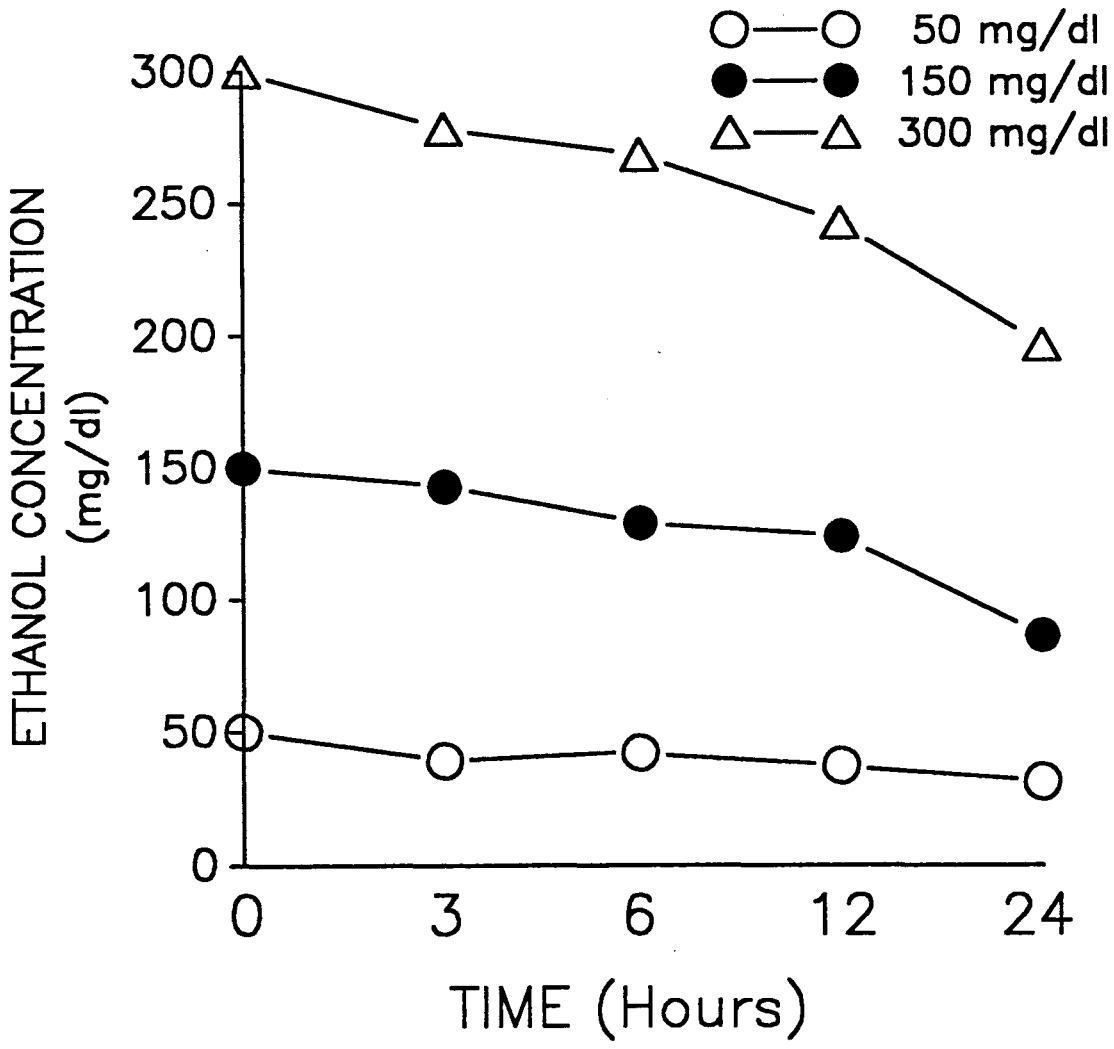

Figure 4. CONCENTRATION OF ETHANOL IN MEDIA DURINGA 24 HOUR PERIOD. Ethanol was incubated in MEM containing 10% NuSerum. The culture plates were wrapped in parafilm to prevent ethanol evaporation.

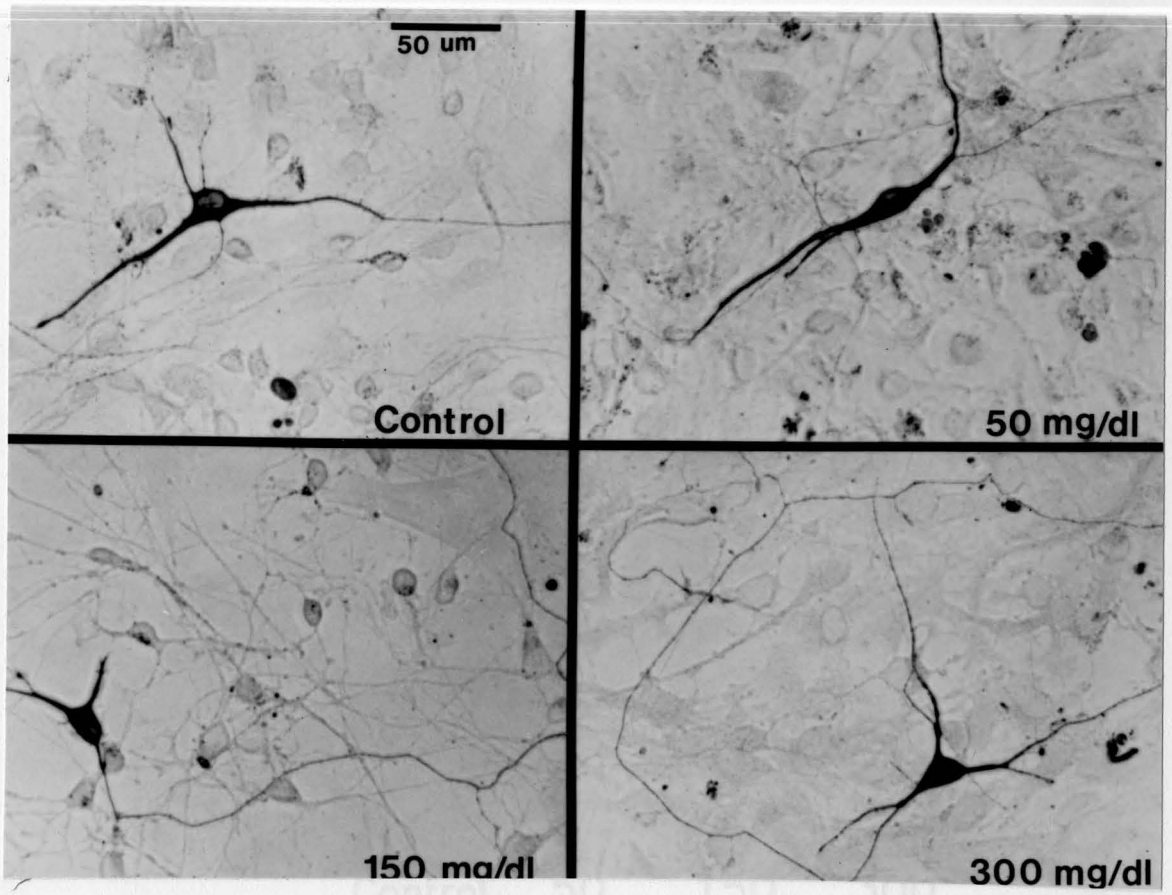

Figure 5. PHOTOMICROGRAPH OF REPRESENTATIVE FIELDS DEMONSTRATING 5-HT POSITIVE NEURONS IN CULTURE AFTER 4 DAYS OF ETHANOL EXPOSURE. Doses of ethanol are provided in the lower right corner of each picture. Bar represents 50 μm.

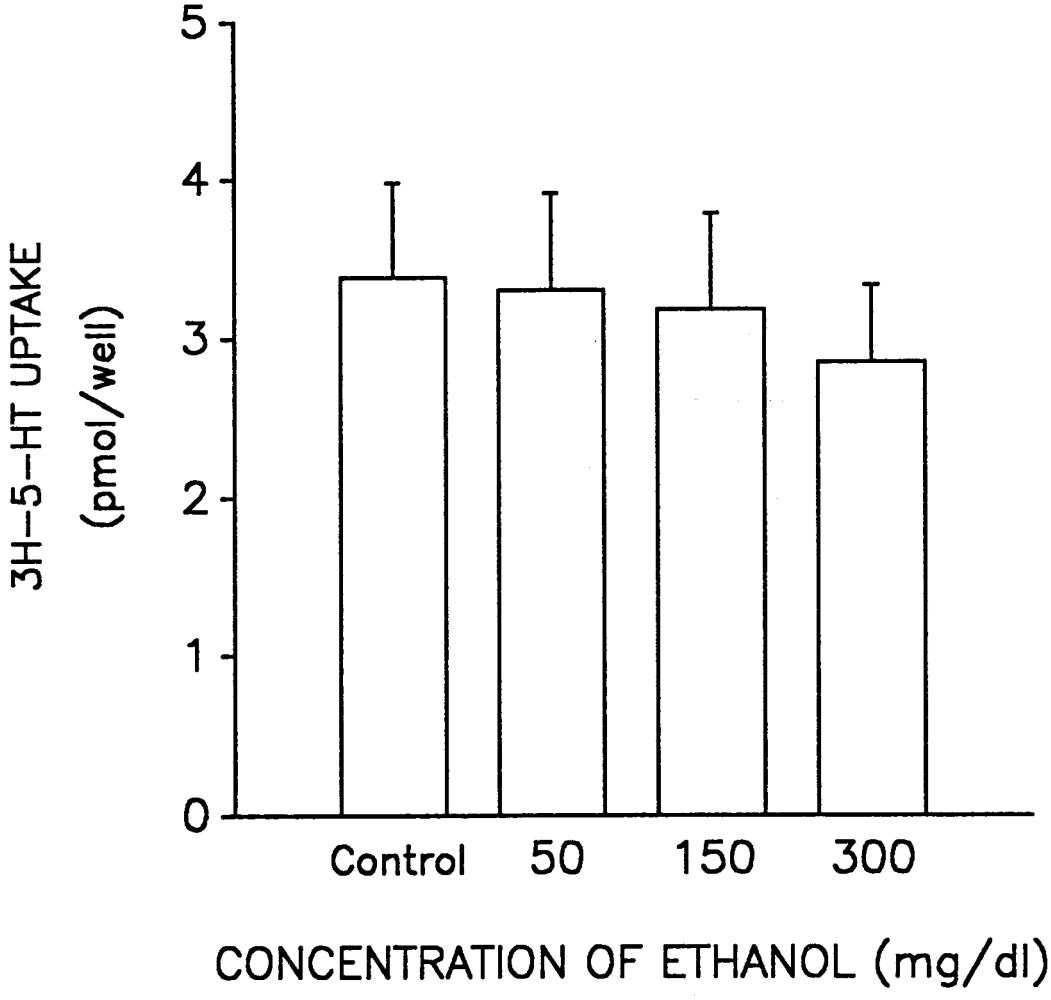

Figure 6. ^{3}H-5-HT UPTAKE IN NEURONAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. Uptake was measured on the 6th day in vitro. Uptake was determined by measuring the amount of ^{3}H-5-HT accumulated in 20 minutes. Non-specific uptake was determined by adding 10 μM fluoxetine in the assay buffer and was subtracted from total uptake. Each value represents the mean $\pm$ the SEM of values obtained from 5-6 experiments.

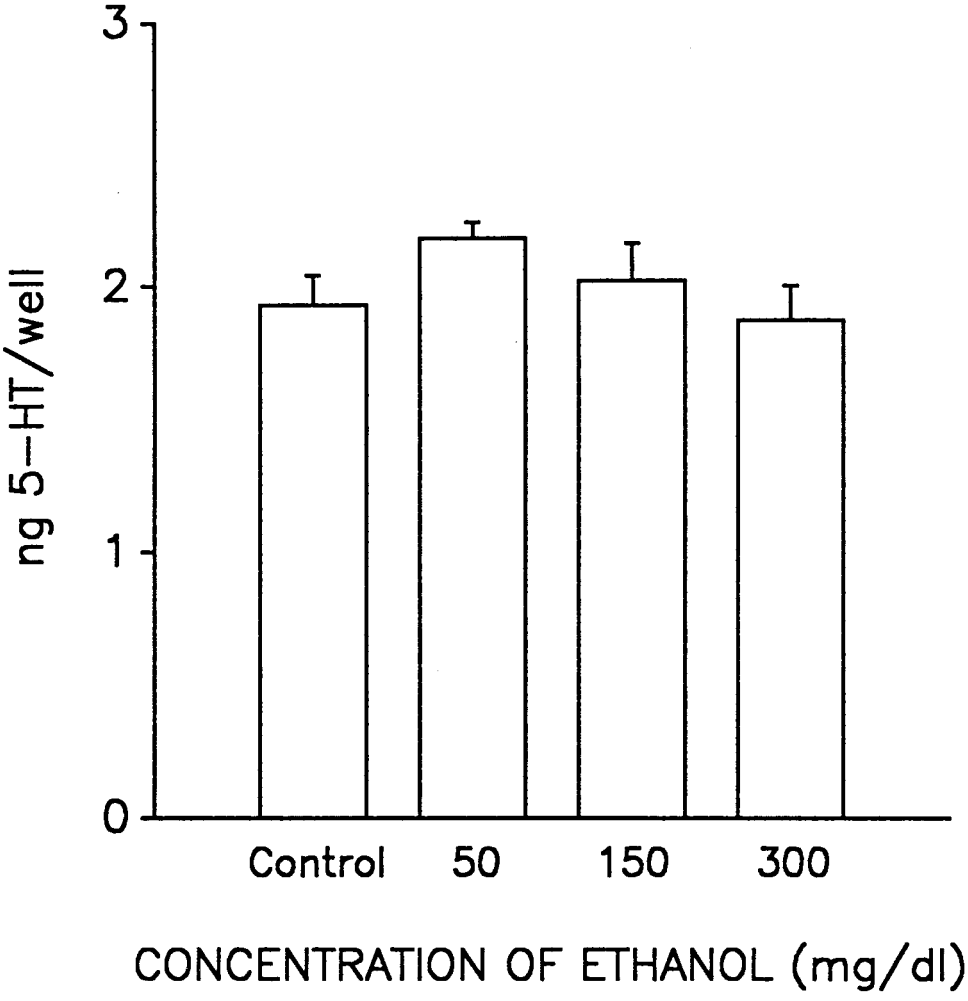

Figure 7. 5-HT CONTENT IN NEURONAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. 5-HT content was measured on the 6th day <u>in vitro</u> using an immunoassay kit as described in Methods. Each value represents the mean ± the SEM of values obtained from 6-9 experiments.

CULTURE TREATMENT	PERCENTAGE OF 5-HT POSITIVE NEURONS
Control	1.18±.11
50 mg/dl	1.22±.11
150 mg/dl	1.16±.13
300 mg/dl	1.14±.14

Figure 8. THE PERCENTAGE OF 5-HT POSITIVE NEURONS AFTER A 4 DAY ETHANOL EXPOSURE. The percentage of 5-HT neurons in the cultures was determined by counting the number of positively stained and unstained neurons, using a Nikon inverted microscope at a magnification of 400x. Six random fields were counted for each experiment and these numbers were averaged to produce an n=1. Values from six separate experiments were counted used to obtain the mean ± the SEM.

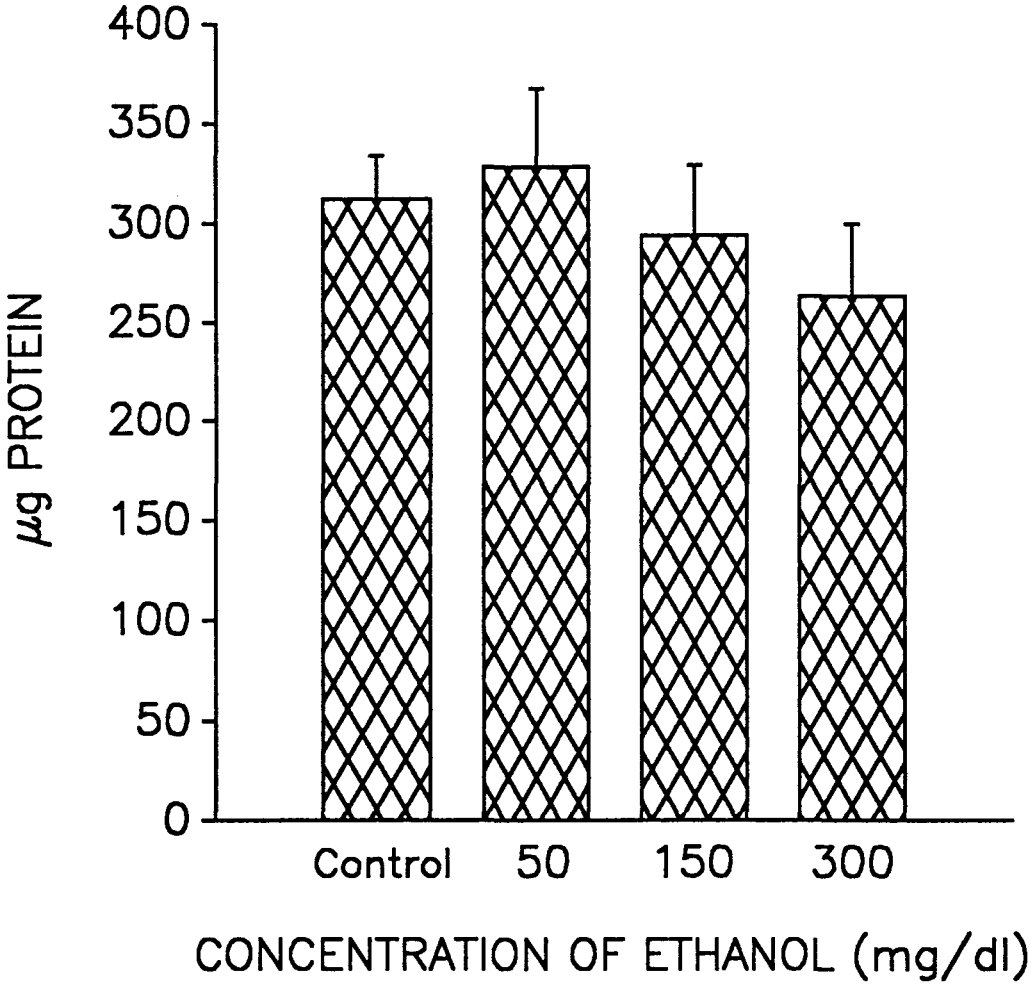

Figure 9. PROTEIN CONTENT IN NEURONAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. Protein content was measured on the 6th day in vitro. Each value represents the mean ± the SEM of values obtained from 6 experiments.

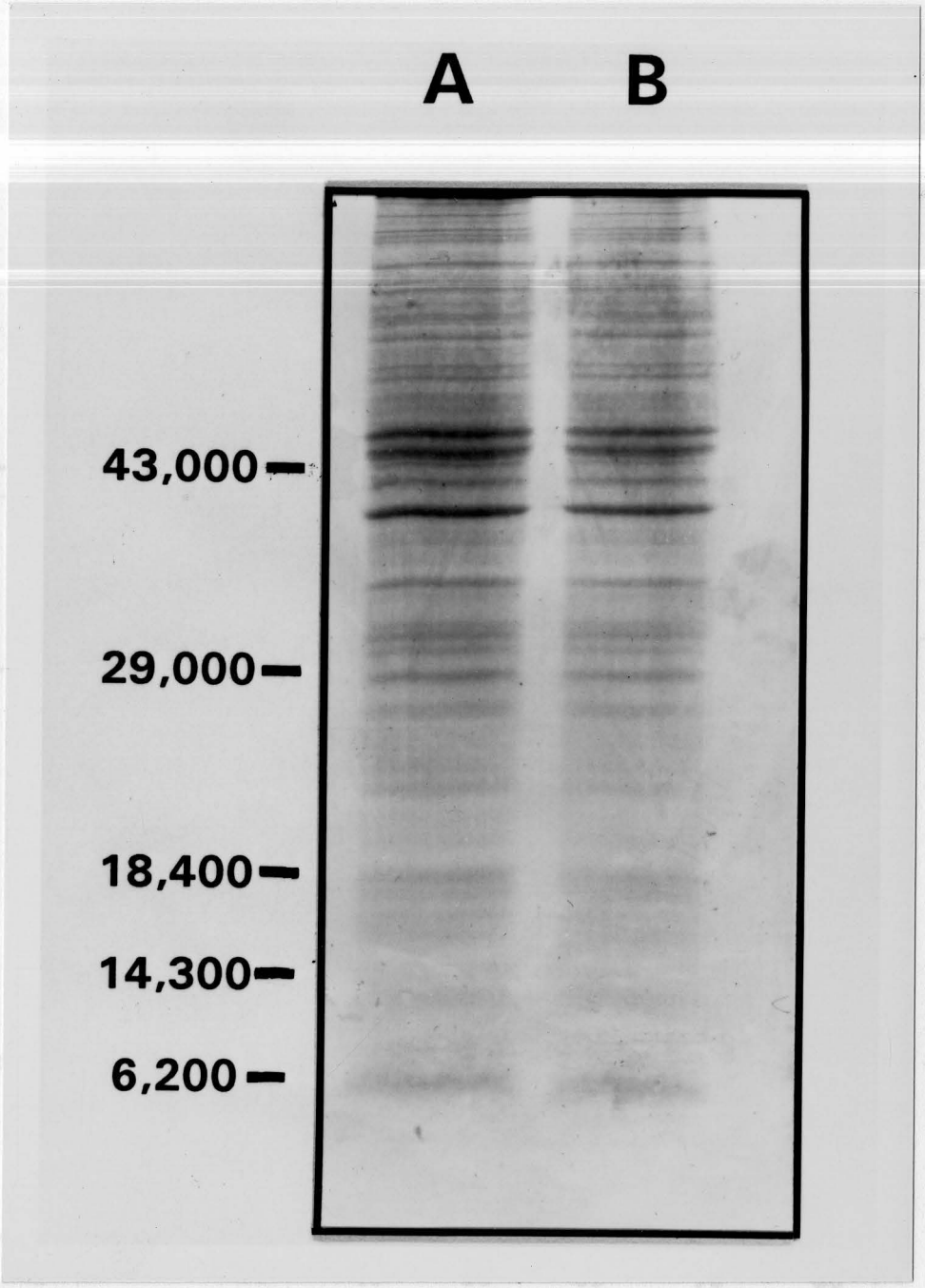

Figure 10. PHOTOGRAPH OF COOMASSIE BLUE STAINED NEURONAL PROTEINS THAT WERE SEPARATED ON A 12% SDS PAGE GEL. The proteins on lanes A and B were obtained from control and 300 mg/dl ethanol exposed cultures. Numbers on left represent molecular weight markers.

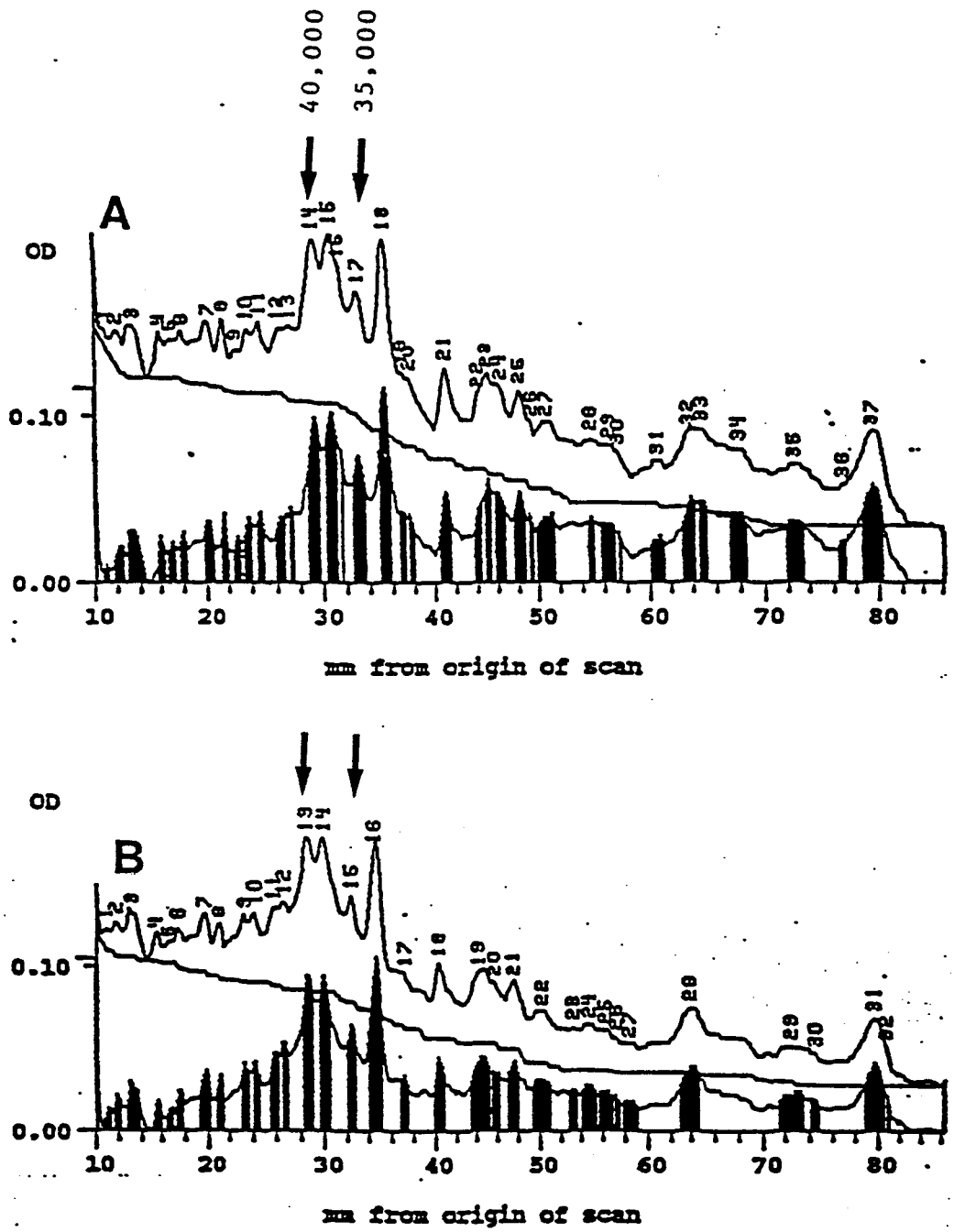

Figure 11. REPRESENTATIVE DENSITOMETER SCAN OF COOMASSIE BLUE STAINED NEURONAL PROTEINS. A) Control B) Ethanol (300 mg/dl)

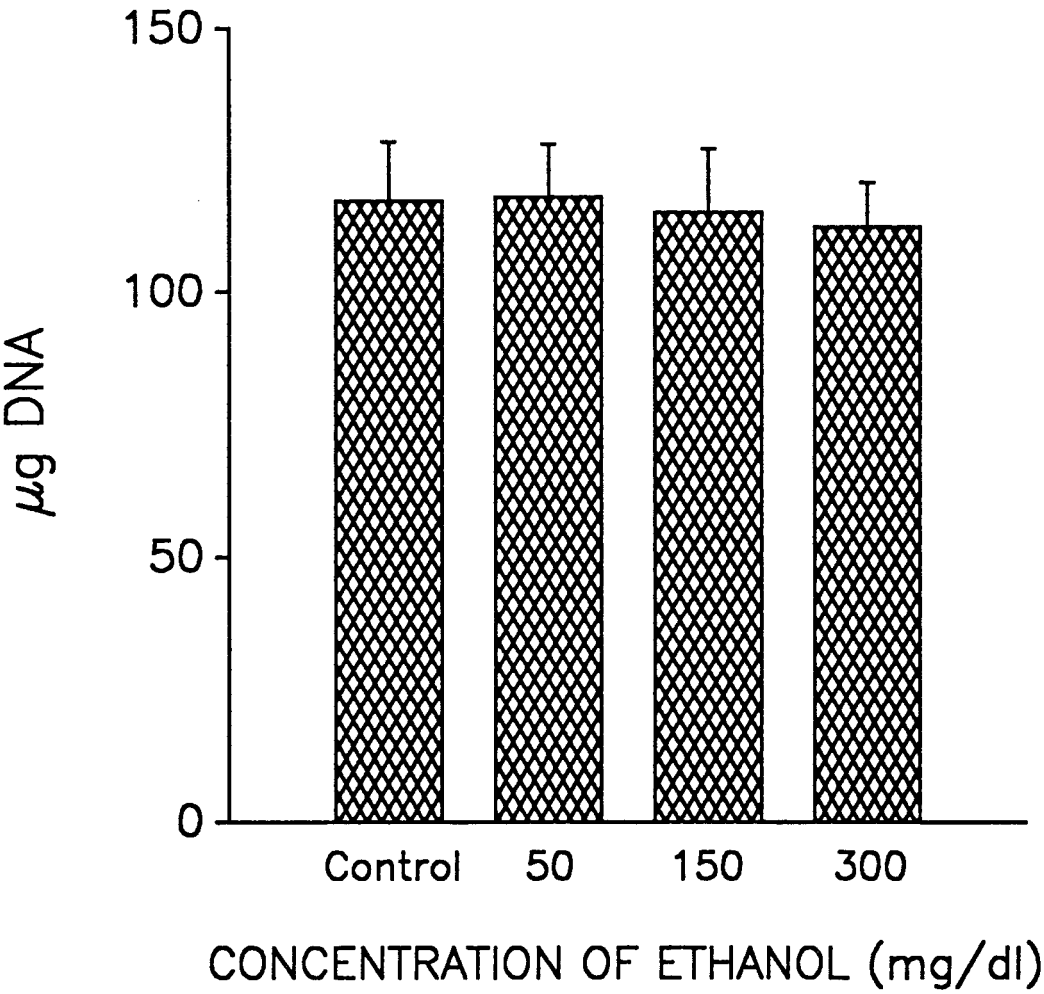

Figure 12. DNA CONTENT IN NEURONAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. DNA content was measured on the 6th day in vitro using a colorimetric assay as described in Methods. Each value represents the mean ± the SEM of values obtained from 6 experiments.

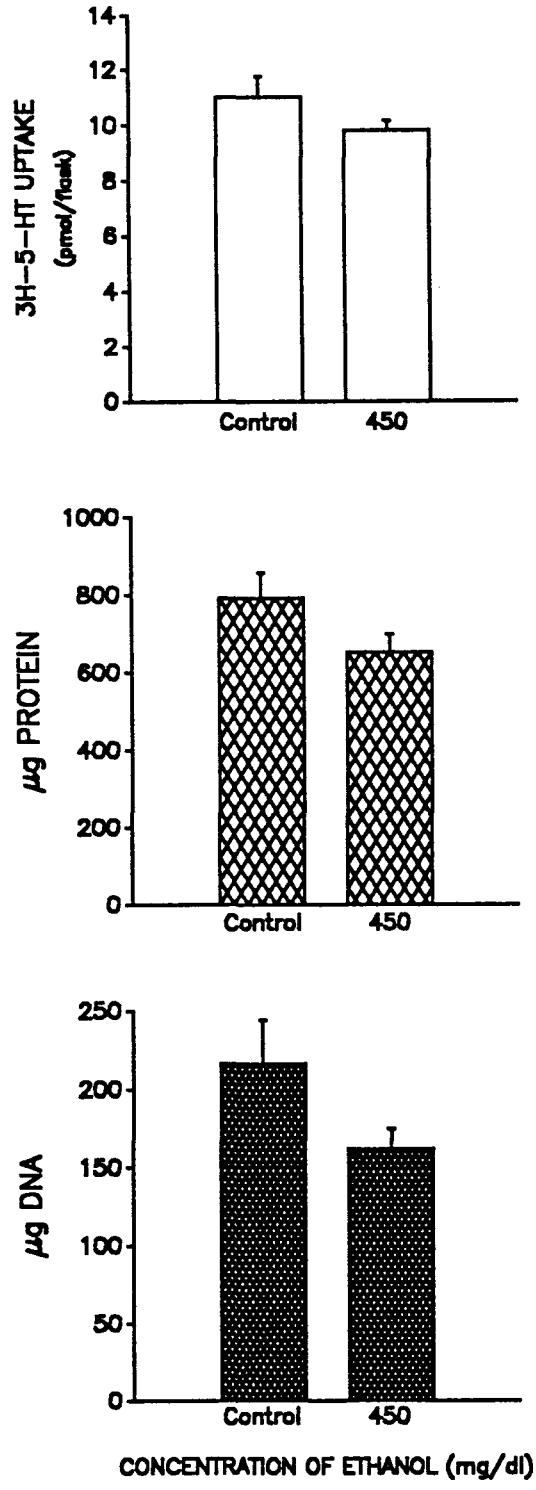

Figure 13. ^{3}H-5-HT UPTAKE, PROTEIN AND DNA CONTENT AFTER 4 DAYS EXPOSURE TO 450 MG/DL ETHANOL. Each value represents the mean ± the SEM of values obtained from six experiments. Top graph ^{3}H-5-HT uptake/flask. Middle graph µg protein/flask. Bottom graph µg DNA/flask.

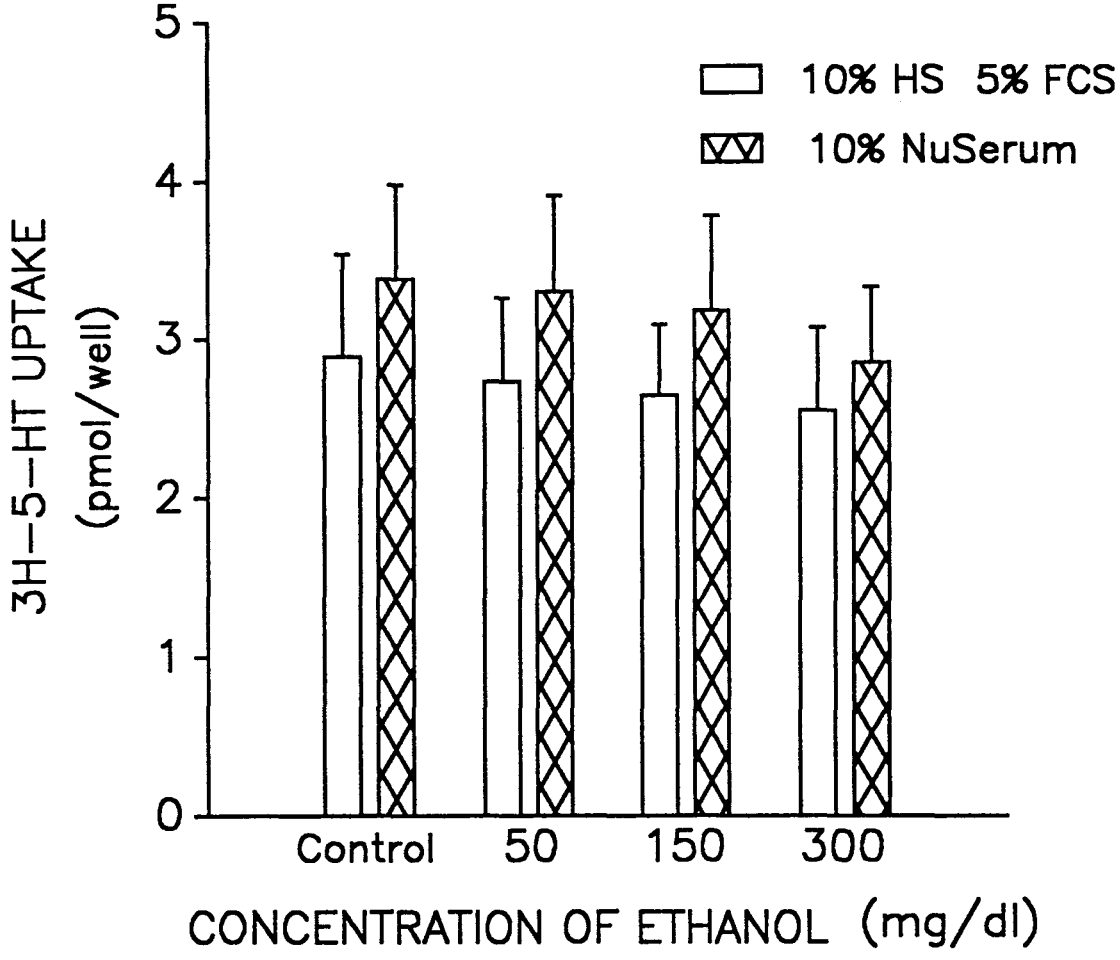

Figure 14. EFFECT OF CULTURE MEDIA ON ^{3}H-5-HT UPTAKE IN NEURONAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. Uptake was measured on the 6th day *in vitro*. Uptake was determined by measuring the amount of ^{3}H-5-HT accumulated in 20 minutes. Nonspecific uptake was determined by adding 10 μM fluoxetine in the assay buffer and was subtracted from total uptake. Each value represents the mean $\pm$ the SEM of values obtained from 3-6 experiments.

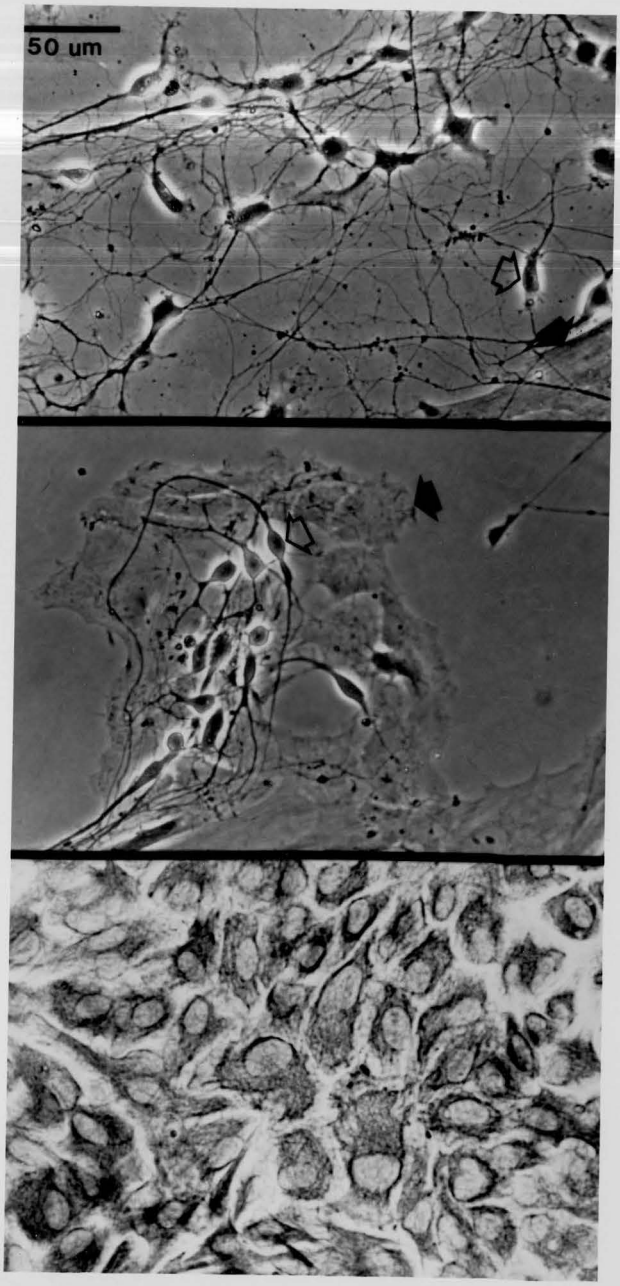

Figure 15. PHOTOMICROGRAPH DEMONSTRATING THE GENERATION OF GLIAL CULTURES. Top panel contains neurons cultured in 10% Nuserum, plated onto wells coated with poly-l-lysine. The open arrow is pointing to a neuron, the closed arrow is pointing to a glial cell. Middle panel contains neurons cultured in 10% HS/5% FCS, plated onto uncoated wells. These cultures were subcultured to produce the GFAP positive astroglia shown in the Bottom panel. Bar represents 50 μm.

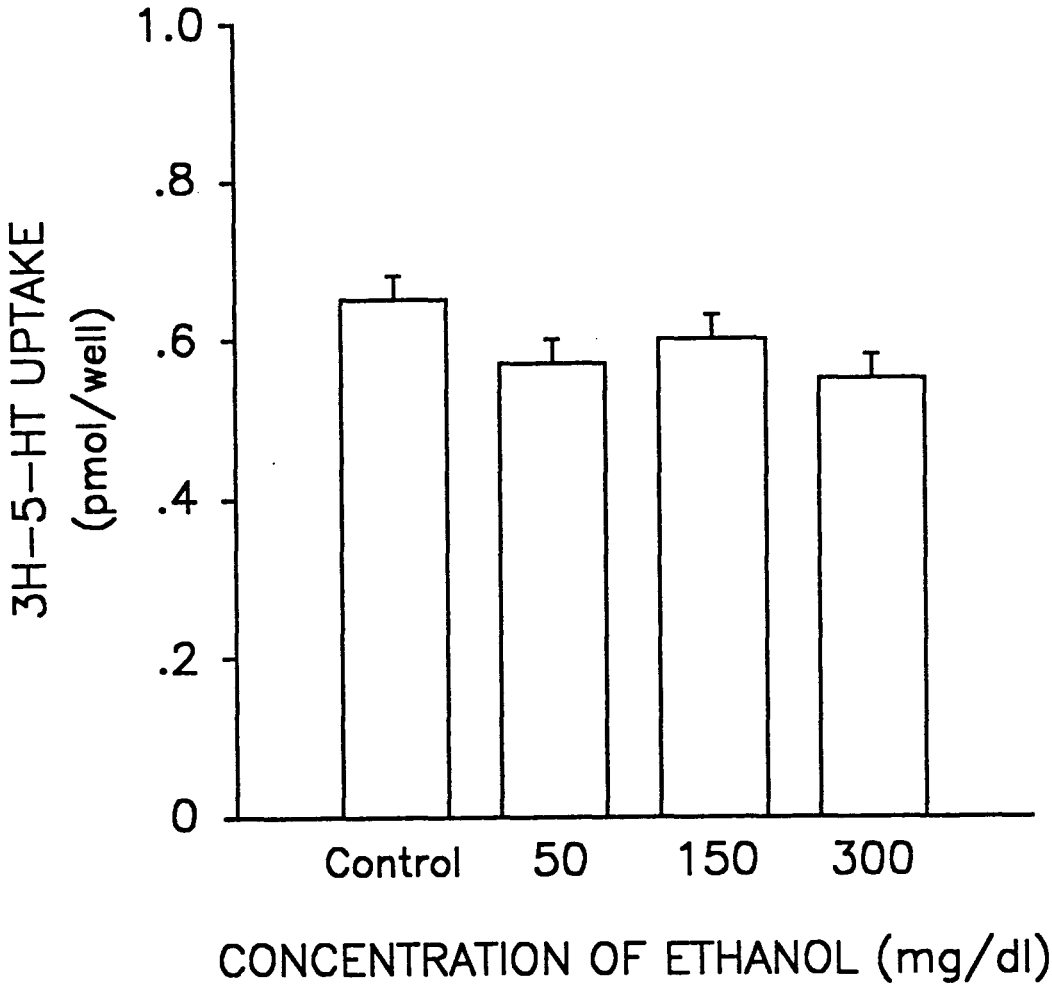

Figure 16. ^{3}H-5-HT UPTAKE IN ASTROGLIAL CULTURES AFTER ACUTE ETHANOL EXPOSURE. Uptake was measured on the 6th day in vitro. Uptake was determined by measuring the amount of 60 nm ^{3}H-5-HT accumulated in 20 minutes in the presence of ethanol. Nonspecific uptake was determined by adding 10 μM fluoxetine in the assay buffer and was subtracted from total uptake. Each value represents the mean $\pm$ the SEM of values obtained from 5-6 experiments.

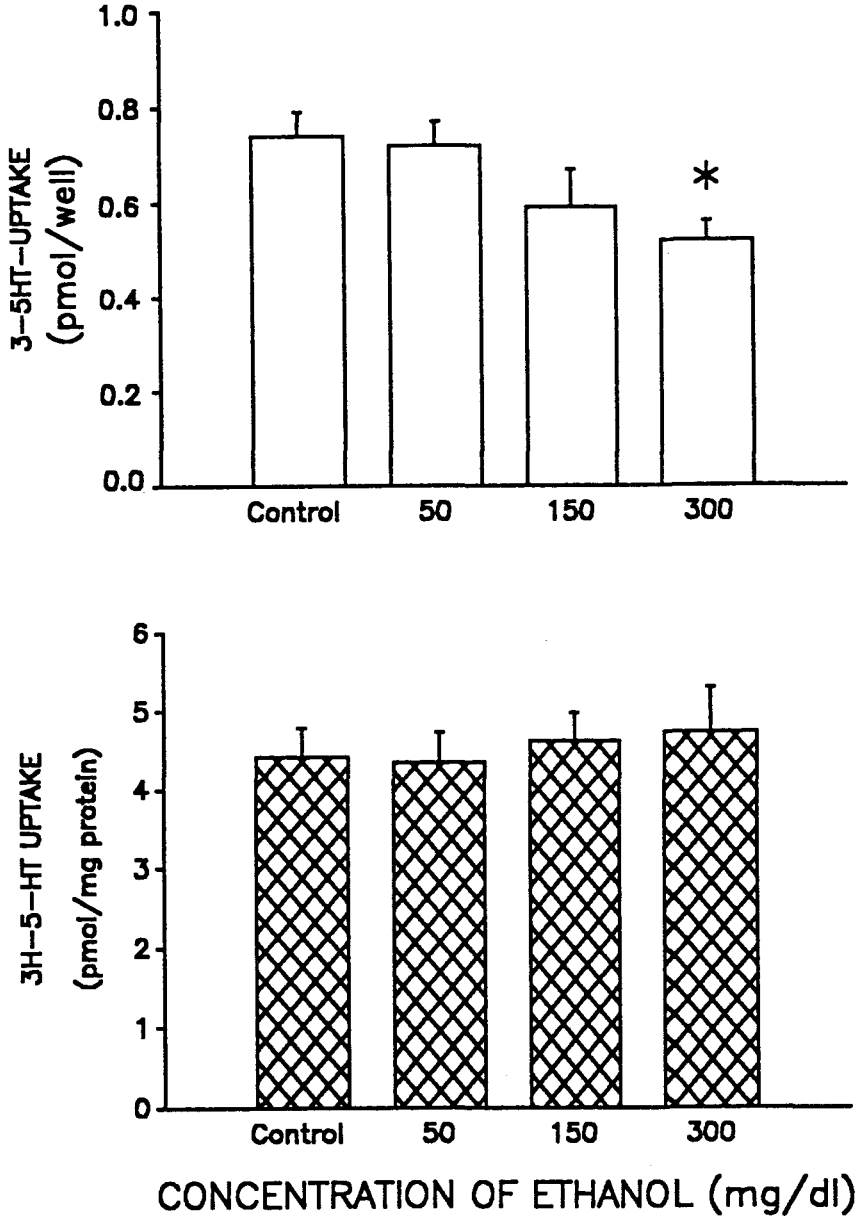

Figure 17. ^{3}H-5-HT UPTAKE IN ASTROGLIAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. Top graph depicts 5-HT uptake per well measured on the 6th day in vitro. Each value represents the mean ± the SEM of values obtained from six experiments. The symbol * indicates that ^{3}H-5-HT uptake in cultures exposed to 300 mg/dl ethanol for 4 days was significantly different from that of control cultures at $p<0.05$ (one-way ANOVA and Newman Keul's). Bottom graph depicts 5-HT uptake per mg protein. When uptake was expressed per mg protein there were no significant differences in control and ethanol-treated cultures.

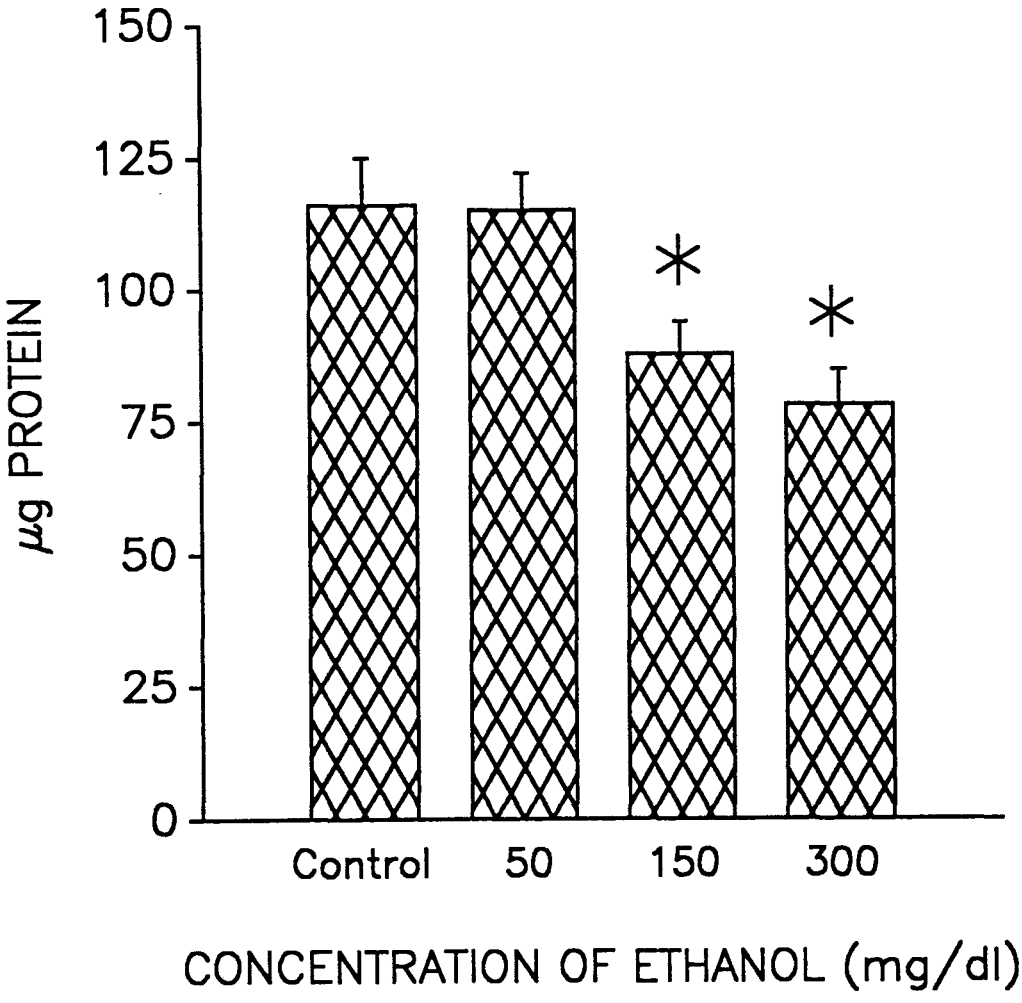

Figure 18. PROTEIN CONTENT DECREASES IN ASTROGLIAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. Protein content was measured on the 6th day in vitro. Each value represents the mean ± the SEM of values obtained from six experiments. The symbol * indicates that values from 150 and 300 mg/dl ethanol treated cultures differ significantly from those of control cultures ($p<0.05$, one-way ANOVA and Newman Keul's).

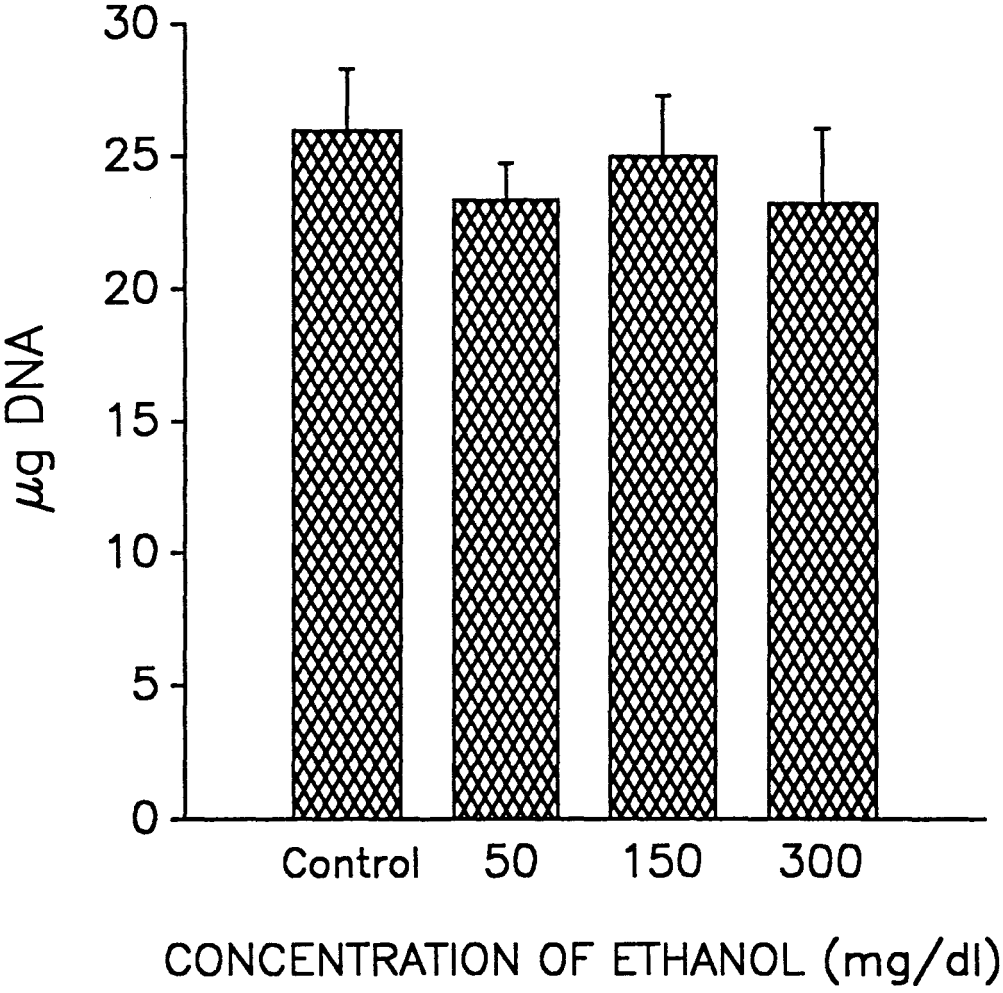

Figure 19. DNA CONTENT IN ASTROGLIAL CULTURES AFTER 4 DAYS OF ETHANOL EXPOSURE. DNA content was measured on the 6th day in vitro using a colorimetric assay as described in Methods. Each value represents the mean ± the SEM of values obtained from 6 experiments.

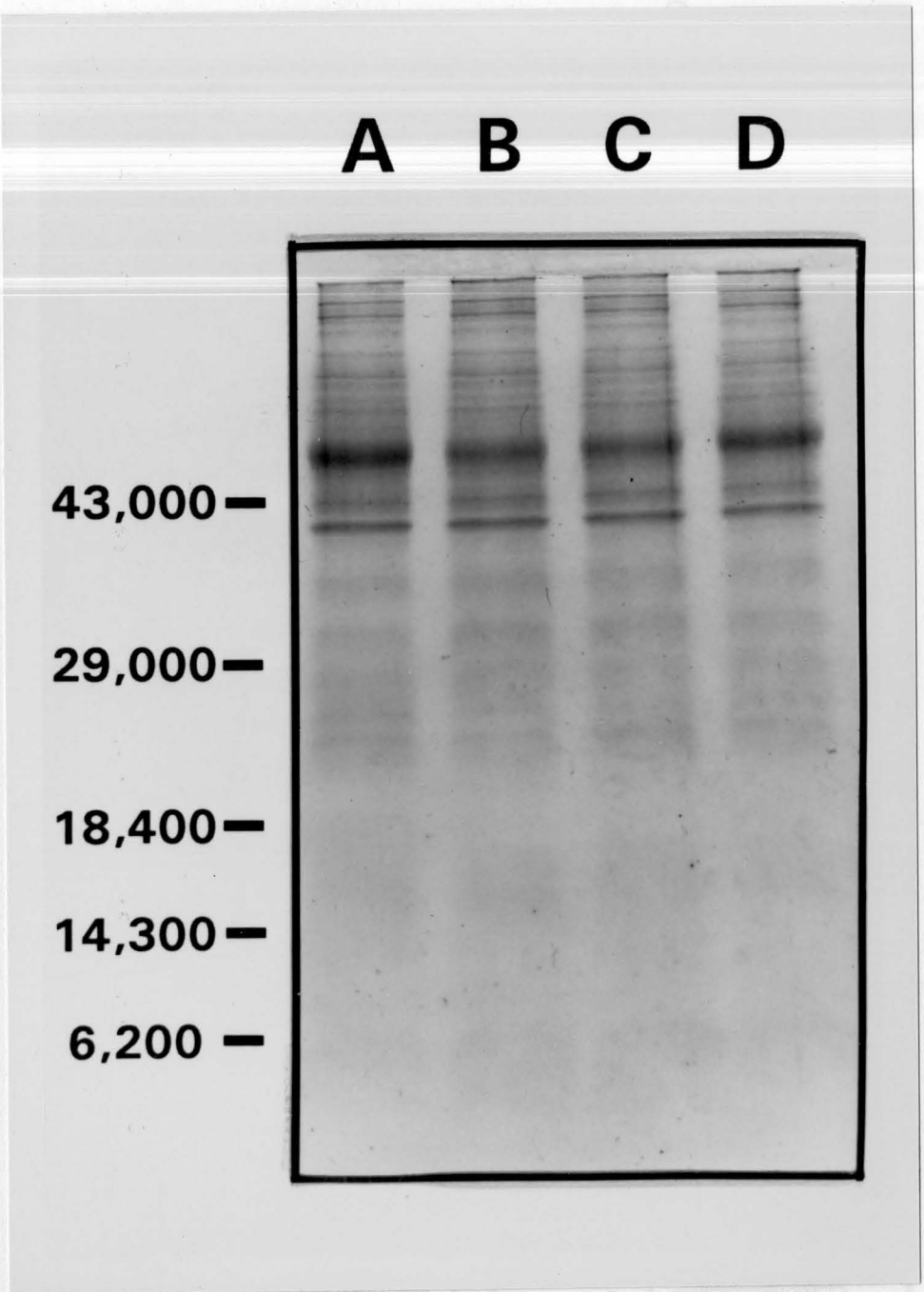

Figure 20. PHOTOGRAPH OF COOMASSIE-BLUE STAINED GLIAL PROTEINS THAT WERE SEPARATED ON A 12% SDS PAGE GEL. The proteins on gels A-D were obtained from A) control cultures or cultures that were exposed to B) 50 C) 150 D) 300 mg/dl ethanol for 4 days. Numbers on left represent molecular weight markers.

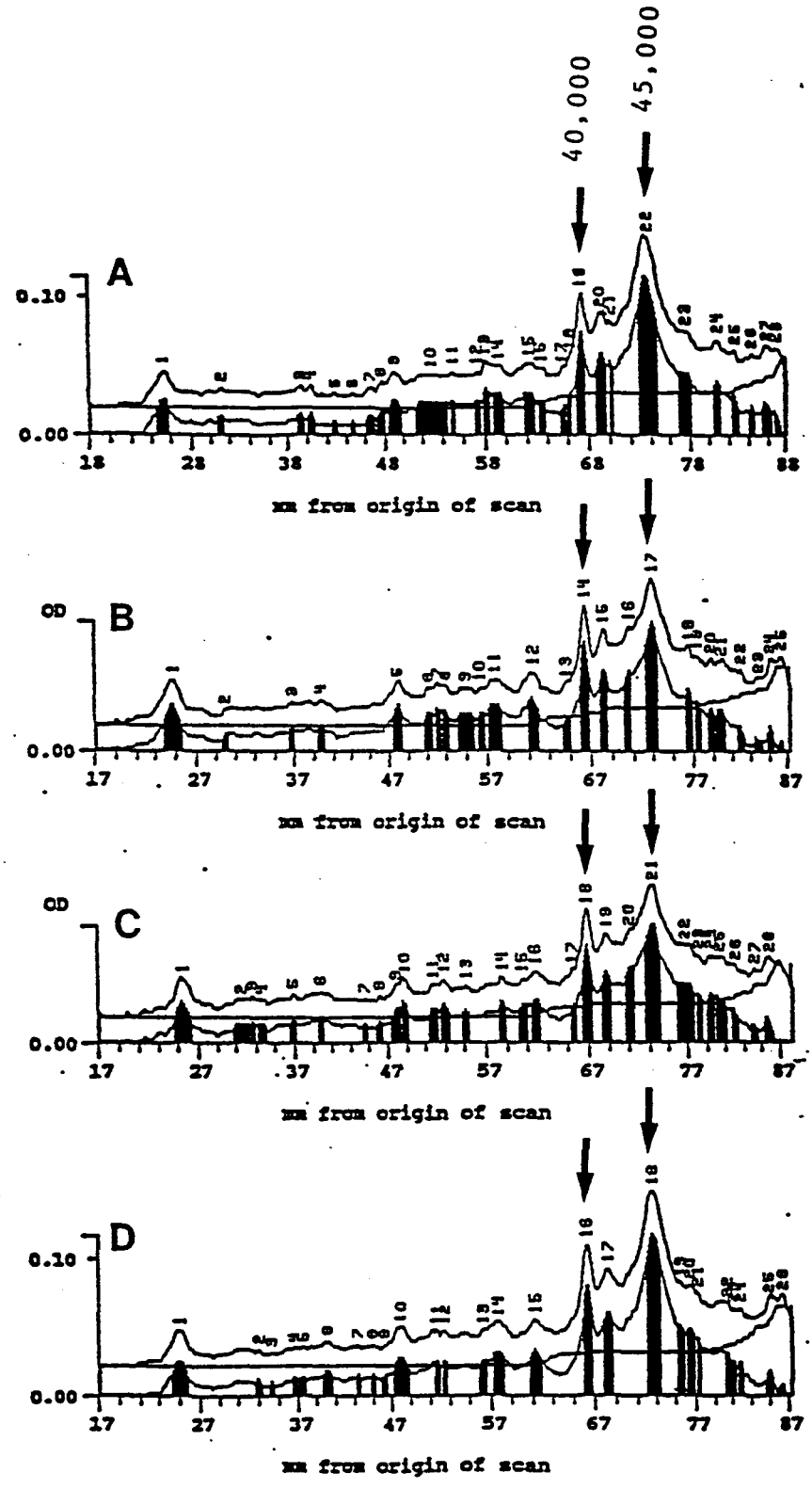

Figure 21. REPRESENTATIVE DENSITOMETER SCAN OF COOMASSIE BLUE-STAINED GELS OF GLIAL PROTEINS. The proteins that were separated on gels A-D were obtained from A) control cultures or cultures exposed to B) 50 C) 150 D) 300 mg/dl ethanol for 4 days.

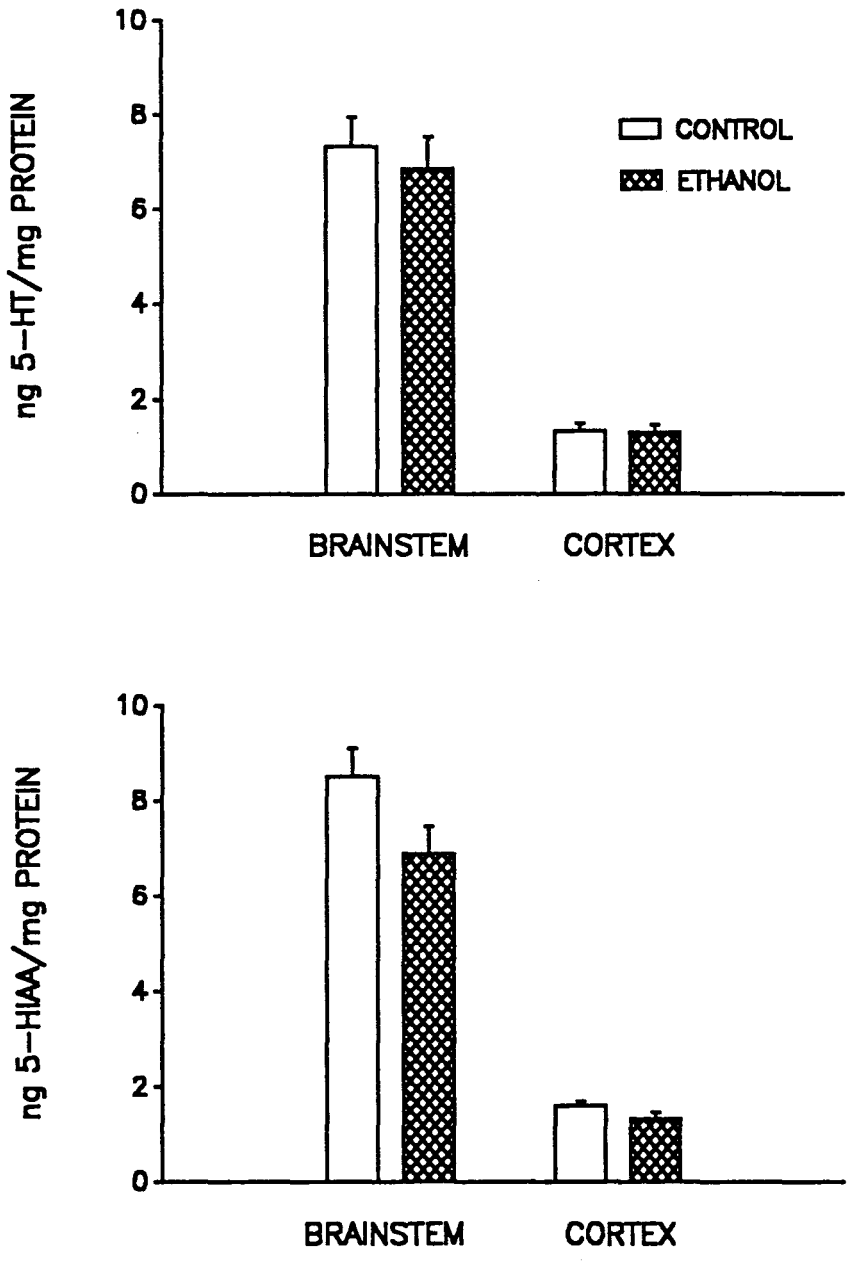

Figure 22. 5-HT AND 5-HIAA CONTENT ON G20 OF BRAINSTEM AND CORTEX OF RATS EXPOSED TO ETHANOL IN UTERO BETWEEN G14-G20. Each value represents the mean ± the SEM of values obtained from 6 animals.

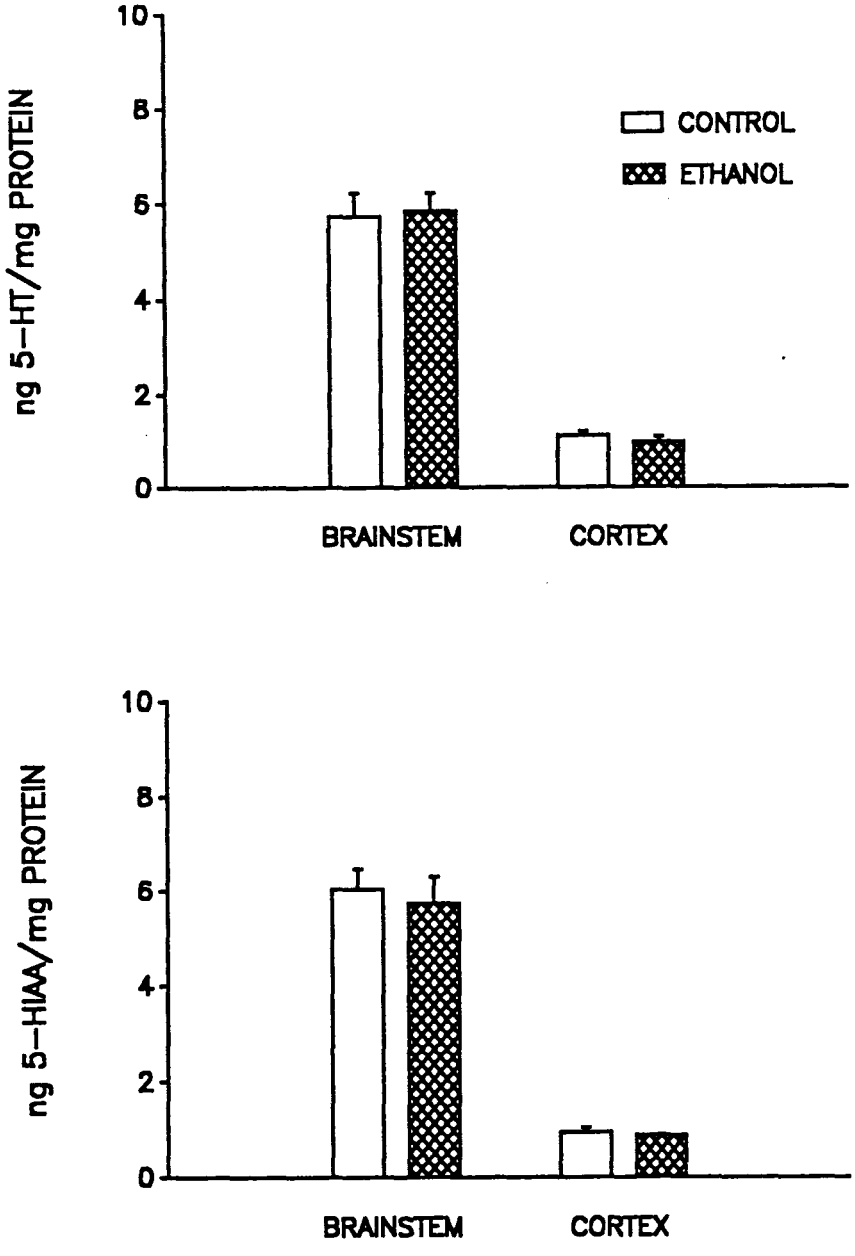

Figure 23. 5-HT AND 5-HIAA CONTENT ON G20 OF BRAINSTEM AND CORTEX OF RATS EXPOSED TO ETHANOL IN UTERO BETWEEN G9-G15. Each value represents the mean ± the SEM of values obtained from six animals.

CHAPTER FIVE

DISCUSSION

The objective of this research was to examine the effect of ethanol exposure on cultured serotonergic neurons. These studies were performed to elucidate the mechanism by which *in utero* ethanol produces abnormal development of the serotonergic system. It was hypothesized that ethanol would inhibit the *in vitro* development of serotonergic neurons. Several parameters were measured to assess the function of serotonergic neurons. These included ^{3}H-5-HT uptake, 5-HT content, and the percentage of 5-HT immunoreactive neurons. Protein and DNA content were assessed as a general evaluation of both serotonergic and non-serotonergic cells.

The neurons used for these cultures were obtained from G14 rhombencephalon, when most of the 5-HT neurons have been generated (Lauder et al., 1982). 5-HT neurons, like other neurons, are able to survive the dissociation step if they are cultured before developing extensive axonal and dendritic arbors. Shortly after the cells attached to the plate, minor process formation occurred. Within 2 days of being cultured, axonal and dendritic development was apparent. After 6 days *in vitro* the axonal and dendritic network was very dense and

elaborate. Ethanol was added to the media between 2 and 6 days *in vitro*, corresponding to an *in vivo* time point of G16-G20. During this time 5-HT nerve terminals are maturing, as demonstrated by the increase in ^{3}H-5-HT uptake between 2 and 6 days *in vitro*.

A four day ethanol exposure at a dose of 50, 150, 300, or 450 mg/dl does not inhibit the development of cultured serotonergic neurons. ^{3}H-5-HT uptake was only slightly reduced at the highest doses. Concurrent with this loss of uptake, was a reduction in protein content, suggesting that if ethanol was affecting protein synthesis it was not specific to serotonergic neurons. Mytilineou et al (1988) found that a 4 day exposure to 230 mg/dl ethanol also did not affect dopamine uptake in cultured dopaminergic neurons.

There was no evidence of ethanol-induced neurotoxicity to serotonergic or non-serotonergic neurons because neither the percentage of 5-HT neurons nor DNA content were changed. This is in agreement with other researchers who have found that neurons in cell culture are resistant to a direct toxic effect of ethanol (Scott et al., 1986; Dow and Riopelle, 1985; Acosta et al., 1986). Only at a dose of 550 mg/dl ethanol does cell viability begin to decrease in dopaminergic cultures (Acosta et al., 1986). It is interesting that *in vivo*, loss of neurons is well established with *in utero* ethanol exposure (e.g. Miller and Potempa, 1990; West et al., 1986).

Rodents metabolize ethanol faster than humans, so *in vivo*

substantial quantities of ethanol have to be administered to reach significant BAC. However, it is the resulting BAC that is correlated with amount of damage, not the amount of ethanol ingested (Pierce and West, 1986; Bonthius and West, 1990). With in vitro neuronal models, ethanol is added directly to the media to reach experimental ethanol levels. Ethanol is not metabolized; all loss of ethanol is presumably due to evaporation. The fluctuations in ethanol concentration seen in the media over a 24 hour period mimic those seen in vivo. For example, after drinking there is a peak in blood ethanol levels, which declines over time (Goodlett et al., 1990).

The concentration of ethanol in the media was in the range of clinically relevant doses and in the range of levels shown to alter serotonergic development in rats (Rathbun and Druse, 1985; Tajuddin and Druse, 1989a; 1989b; Druse and Paul, 1989; Druse et al., 1990). The animals used in the later experiments had BACs of about 120 mg/dl.

The neuronal cultures were grown in media containing 10% NuSerum (Collaborative, Bedford, MA). NuSerum is rich in growth factors, thus the possibility exists that an abundance of growth factors compensated for any potential adverse effects of ethanol. Rabin (1990) and Smith et al (1990) have shown that culture conditions influence the response of cultured cells to ethanol. The effects of two different culture conditions (10% NuSerum and 10% HS, 5% FCS) were compared. No change was observed in ^{3}H-5-HT uptake by

serotonergic neurons following a 4 day ethanol exposure in either media. Nonetheless, rich culture conditions did not prevent the development of ethanol-related abnormalities in astroglia. ^{3}H-5-HT uptake in astroglia is significantly reduced when cultured in media containing 10% HS, 5% FCS.

Even if suitable culture conditions for neuronal cells were established that demonstrate a significant ethanol-related effect, the results would not necessarily confirm in vivo experiments. These experiments, in which a 3.3% (v/v) ethanol-containing liquid diet was administered to pregnant rats during G14-G15, followed by a 6.6% (v/v) ethanol-containing liquid between G16-G20, did not demonstrate a decrease in 5-HT or 5-HIAA content in the brainstem or cortex on G20, two regions consistently shown to be affected by chronic ethanol exposure (Druse et al., 1990; Rathbun and Druse, 1985). Thus the in vitro experiments in this study may be an accurate reflection of the in vivo environment. During the time of ethanol exposure, maturation of the serotonergic system is occurring in both models. In vivo, 5-HT neuronal cell bodies are migrating to their final position, in addition to the ascending axonal projections from the rostral nuclei (B7-9 complex) are beginning to reach their target areas (Lauder et al., 1983; Wallace and Lauder, 1983). In vitro, 5-HT nerve terminals are developing.

Therefore, in the present studies, ethanol does not inhibit the maturation of 5-HT neurons in either in vitro or

in vivo models. Although the precise mechanism(s) by which *in utero* ethanol exposure is able to adversely affect the development of serotonergic system is unknown, there are several potential explanations.

1) The present studies do not rule out the possibility that ethanol affects the generation and differentiation of 5-HT neurons *in vivo*. Neurons, in culture, may go through one more cell division, if so programmed, although they typically do not divide *in vitro* (Banker and Goslin, 1991). Even if tissue is removed at a time of active neurogenesis, it is rare to observe cells that divide in culture and subsequently acquire a neuronal phenotype. Thus the possibility that ethanol may affect the generation of 5-HT neurons *in vivo* is difficult to detect using an *in vitro* model. *In utero* ethanol exposure between G9-G15 [G9-G10 at 3.3% (v/v) followed by a 6.6% (v/v) ethanol-containing liquid G11-G15] when 5-HT neurons are being generated, does not reduce 5-HT or 5-HIAA content on G20 in either the brainstem or cortex. Although these results suggest that ethanol does not interfere with generation of 5-HT neurons, precursor cells that give rise to 5-HT neurons may be able to compensate for any ethanol-induced cell loss occurring between G9-G15.

2) The concentration of tryptophan in brain influences the synthesis of 5-HT (Fernstrom and Wurtman, 1971). Thus a reduction in fetal tryptophan could be a potential mechanism by which *in utero* exposure results in lower 5-HT content. Lin

et al. (1990) have demonstrated reduced tryptophan content in the brain, plasma, and liver of fetal rats chronically fed an ethanol-containing diet. In the present study, pregnant rats were fed an ethanol-containing liquid diet for 6 days between either G9-G15 or G14-G20. This relatively short ethanol exposure may not have been sufficient to impair placental nutrient transfer of tryptophan. Alternatively, the tryptophan content in the fetus prior to the ethanol exposure may have been adequate for the normal synthesis of 5-HT to occur. The animals used in previous studies from this laboratory were exposed to ethanol for 4 weeks prior to conception and all during gestation (Rathbun and Druse, 1985; Tajuddin and Druse, 1989a; 1989b; Druse and Paul, 1989; Druse et al., 1990). Thus the placenta was chronically exposed to ethanol.

3) The present studies have demonstrated that astroglial cells in vitro appear to be more sensitive to ethanol than neurons. Abnormalities in astroglia may secondarily produce some of the neurochemical imbalances seen in ethanol exposed offspring. Protein content is consistently reduced in cortical astroglia following a chronic in vivo or in vitro ethanol exposure (Kennedy and Mukerji, 1984; Guerri et al., 1990). Although other researchers have found that 200 mg/dl ethanol decreases cell number in cortical astroglial cultures (Davies and Cox, 1991), in the present studies DNA content was unaffected by ethanol exposure. These results suggest that,

contrary to cortical astroglia, ethanol did not inhibit rhombencephalic astroglia cell division.

Astroglia possess several components which may influence 5-HT function such as 5-HT uptake sites, receptors, and monoamine oxidase (Fillion et al., 1983; Katz and Kimelberg, 1985; Fitzgerald et al., 1990). Chronic exposure of primary astroglia cultures to ethanol produced a significant decrease in ^{3}H-5-HT uptake per well. The number of uptake sites were most likely decreased per cell because there was no change in DNA content. A comparable decrease in protein per well was seen. Therefore ^{3}H-5-HT uptake per mg protein was unchanged by ethanol.

Although it appears that astroglia 5-HT uptake sites are not selectively lost during ethanol exposure, even a general deficiency of astroglial 5-HT uptake could have a marked, adverse effect on neuronal development. If the 5-HT uptake sites on astroglia cells function to remove 5-HT (Katz and Kimmelberg, 1985) a reduction in these sites would result in a transient increase in 5-HT. Because 5-HT is required for the normal development of serotonergic neurons, an increase could result in the abnormal development of 5-HT neurons. Interestingly, addition of 5-HT or 5-HT agonists inhibits ^{3}H-5-HT uptake and 5-HT neurite outgrowth in cultured serotonergic neurons (Whitaker-Azmitia and Azmitia (1986). In addition, astroglia synthesize and secrete a number of growth factors which are essential for normal neuronal development.

5-HT stimulates the release of S100b from astroglia, which regulates the growth of serotonergic neurons (Whitaker-Azmitia et al., 1990; Whitaker-Azmitia and Azmitia, 1989). Since ethanol nonspecifically reduces protein content in astroglia cells, it is likely that the density of this receptor is also reduced. A reduction of 5-HT receptors on astroglia could potentially lead to a reduction in the release of serotonergic growth factors.

If ethanol acts non-specifically to reduce all proteins then one would also expect a reduction in glutamate uptake sites which are present on astroglia (Hansson, 1986). A reduction in glutamate uptake may lead to increased extracellular glutamate which is neurotoxic (Jorgensen and Diemer, 1982). If this hypothesis is correct, then non-serotonergic as well as serotonergic neurons would be affected. Other researchers have shown that astroglial components associated with glutamate metabolism are inhibited with <u>in vitro</u> ethanol exposure. They have observed a reduction in glutamine synthetase, the enzyme which converts glutamate to glutamine (Kennedy and Mukerji, 1985).

4) The role of acetaldehyde as a teratogen is controversial. Although it is known that ethanol can cross the placenta (Rosett and Weiner, 1984), it is improbable that the fetus can oxidize the alcohol into acetaldehyde because alcohol dehydrogenase activity is very low in the fetus (Raiha et al., 1967). Thus, acetaldehyde is generated primarily in

the mother. It was once thought that the placenta prevented the diffusion of acetaldehyde into the fetal circulation by metabolizing the acetaldehyde (Kesaniemi and Sippel, 1975). However there are studies reporting detectable acetaldehyde levels in the fetus (Guerri and Sanchis, 1985). Aetaldehyde toxicity has been shown to occur in primary cell culture (Kuriyama et al., 1987), thus the possibility exists that acetaldehyde is teratogenic. In the in vivo studies, the ethanol exposure may have been too short to produce significant developmental abnormalities in the fetus.

The results of the present studies have demonstrated that the maturation of serotonergic neurons is not inhibited by either 4 days of in vitro or 6 days of in vivo ethanol exposure. Thus the mechanism(s) by which abnormal development of the serotonergic system occurs in the offspring of rats fed an ethanol-containing liquid diet on a chronic basis prior to parturition remain unidentified.

CHAPTER SIX

REFERENCES CITED

Abel EL, Sokol RJ (1991): A revised conservative estimate of the incidence of FAS and its economic impact. Alcoholism: Clin Exp Res 15:514-524.

Acosta D, Stege TE, Erickson CK (1986): Cytotoxicity of ethanol in primary cultures of rat mid-brain neurons. Toxicol Lett 30:231-235.

Adrien J, Lanfumey L, Gozlan H, Fattaccini CM, Hamon M (1989): Biochemical and electrophysical evidence for agonist action of CM 57493 at pre- and post synaptic 5-HT_{1A} receptors in brain. J Pharmacol Exp Ther 248:1222-1230.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J Control of gene expression In Molecular Biology of the Cell. Second Edition. Garland Publishing Inc. New York and London, pp 584-585.

Albert PR, Zhou Q, Van Tol HHM, Bunzow JR, Civelli O (1990): Cloning, functional expression and mRNA tissue distribution of the rat 5-hydroxytryptamine $_{1A}$ receptor gene. J Biol Chem 265:5825-5832.

Angevine JB, Sidman RL (1961): Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. Nature 192:766-768.

Angevine JB (1965): Time of origin in the hippocampal region: An autoradiographic study in the mouse. Exp Neurol 2[suppl]:1-70.

Azmitia EC, de Kloet RE (1987): ACTH neuropeptide stimulation of serotonergic neuronal maturation in tissue culture: modulation by hippocampal cells. Prog Brain Res 72:311-318.

Azmitia EC, Whitaker-Azmitia PM (1987): Target cell stimulation of dissociated serotonergic neurons in culture. Neuroscience 20(1):47-63.

Azmitia EC, Whitaker-Azmitia PM, Bartus R (1988): Use of

tissue culture models to study neuronal regulatory trophic and toxic factors in the aged brain. Neurobiol Aging 9:743-758.

Baldwin VJ, MacLeod, PM, Benirschke K (1982): Placental findings in alcohol abuse. Birth Defects 18:89-94.

Banker G, Goslin K (1991): Types of nerve cell cultures, their advantages and limitations. In Banker G and Goslin K (eds): Culturing Nerve Cells. Cambridge, MA:MIT press, pp 14-18.

Barnes DE, Walker DW (1981): Prenatal ethanol exposure permanently reduces the number of pyramidal neurons in the rat hippocampus. Dev Brain Res 1:333-340.

Bauer-Moffett C, Altman J (1977): The effect of ethanol chronically administered to preweanling rats on cerebellar development: A morphological study. Brain Res 119:249-268.

Bonthius DJ, West JR (1990): Alcohol-induced neuronal loss in developing rats: Increased brain damage with binge exposure. Alcoholism: Clin Exp Res 14:107-118.

Borg J, Spitz B, Hamel G, Mark J (1985): Selective culture of neurons from rat cerebral cortex: morphological characterization, glutamate uptake and related enzymes during maturation in various culture media. Dev Brain Res 18:37-49.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PP (1986): Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 25:563-576.

Brien JF, Smith GN (1990): Effects of alcohol (ethanol) on the fetus. J Dev Physiol 15:21-32.

Brodie C, Kentroti S, Vernadakis A (1991): Growth factors attenuate the cholinotoxic effects of ethanol during early neuroembryogenesis in the chick embryo. Int J Dev Neurosci 9:203-213.

Bruinink A, Lichtensteiger W, Schlumpf M (1982): Pre- and postnatal ontogeny and characterization of dopaminergic D_2, serotonergic S_2, and spirodecanone binding sites in rat forebrain. J Neurochem 40:1227-1236.

Burton K (1956): A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem J 62:315-323.

Challis JRG, Patrick JE (1980): The production of prostaglandins and thromboxane in the fetoplacental unit and their effects of the developing fetus. Semin Perinatol 11:53-63.

Conn PJ, Sanders-Bush E (1987): Central serotonin receptors: effector systems, physiological roles and regulation. Psychopharmacology 92:267-277.

Crino PB, Brent AV, Vokicer L, Wiley RG (1990): Cellular localization of serotonin 1A, 1B and uptake sites in cingulate cortex of the rat. J Pharmacol Exp Ther 252:651-656.

Dahlstrom A, Fuxe K (1964): Evidence for the existence of monoamine - containing neurons in the central nervous system I. Demonstration of monoamines in cell bodies of brainstem neurons. Acta Physiol Scand [Suppl] 232:1-55.

Darmon MC, Guibert, B, Leviel V, Ehret M, Maitre M, Mallet J (1988): Sequence of two mRNAs encoding active rat tryptophan hydroxylase. J Neurochem 51:312-316.

Davies DL, Cox WE (1991): Delayed growth and maturation of astrocytic cultures following exposure to ethanol: electron microscopic observation. Brain Res 547:53-61.

Davies DL, Vernadakis A (1984): Effects of ethanol on cultured glial cells: proliferation and glutamine synthetase activity. Dev Brain Res 16:27-35.

Davila-Garcia MI, Azmitia EC (1989): Effects of acute and chronic administration of leu-enkephalin on cultured serotonergic neurons: evidence for opioids as inhibitory neuronal growth factors. Dev Brain Res 49:97-103.

Dow KE, Riopelle RJ (1985): Ethanol neurotoxicity: Effects on neurite formation and neurotrophic factor production *in vitro*. Science 228:591-593.

Dow KE, Riopelle RJ (1990): Specific effects of ethanol on neurite-promoting proteoglycans of neuronal origin. Brain Res 508:40-45.

Dreosti IE, Ballard G, Belling B, Record IR, Manuel SJ, and Hetzel BS (1981): The effect of ethanol and acetaldehyde on DNA synthesis in growing cells and on fetal development in the rat. Alcoholism: Clin Exp Res 5:357-362.

Druse MJ (1992): Effects of *in utero,* ethanol exposure on the development of neurotransmitter systems. In Development

of the Central Nervous System: Effects of Alcohol and Opiates. Wiley-Liss, Inc., pp 139-167.

Druse MJ, Paul LH (1989): Effects of in utero ethanol exposure on serotonin uptake in cortical regions. Alcohol 5:455-459.

Druse MJ, Kuo A, Tajuddin N (1990): Effects of in utero ethanol exposure on the developing serotonergic system. Alcoholism: Clin Exp Res 15:678-684.

Dumas S, Darmon MC, Delort J, Mallet J (1989): Differential control of tryptophan hydroxylase expression in raphe and in pineal gland: evidence for a role of translation efficiency. J Neurosci Res 24:537-547.

Fargin A, Raymond JR, Lohse MJ, Kobilka BK, Caron MG, Lefkowitz RJ (1988): The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. Nature 335:358-360.

Fernstrom JD, Wurtman RJ (1971): Brain serotonin content: physiological dependence on plasma tryptophan levels. Science 173:149-142.

Fillion G, Beaudoin D, Fillion MP, Rousselle JC. Robaut C, Netter Y (1983): 5-Hydroxytryptamine receptors in neurones and glia. J Neural Transm [Suppl] 18:307-317.

Fitzgerald LW, Kaplinsky L, Kimelberg HK (1990): Serotonin metabolism by monoamine oxidase in rat primary astrocyte cultures. J Neurochem 55:2008-2014.

Fleming EW, Tewari S and Noble EP (1975): Effects of chronic ethanol ingestion on brain aminoacyl-tRNA synthetases and tRNA. J Neurochem 24:533-560.

Frazer A, Maayani S, Wolfe BB (1990): Subtypes of receptors for serotonin. Annu Rev Pharmacol Toxicol 30:307-348.

Gaddum JH, Picarelli ZP (1957): Two kinds of tryptamine receptors. Br J Pharmacol 12:323-328.

Garro AJ, McBeth DL, Lima V, Lieber CS (1991): Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. Alcoholism: Clin Exp Res 15:395-398.

Goldberg VJ, Ramwell PW (1975): Role of prostaglandins in reproduction. Physiol Rev 55:325-351.

Goldberg JI, Mills LR, Kater SB (1991): Novel effects of

serotonin on neurite outgrowth in neurons cultured from embryos of Helisoma trivolvis. J Neurobiol 22:182-194.

Goldberg JI, Kater SB (1989): Expression and function of the neurotransmitter serotonin during development of the Helisoma nervous system. Dev Biol 131:483-495.

Goldstein A, Aronow L, Kalman S (1974): Drug tolerance and physical dependence In Principles of Drug Action: The Basis of Pharmacology. Wiley:New York. pp 569-586.

Goodlett CR, Marcussen BL, and West JR (1990): A single day of alcohol exposure during the brain growth spurt induces brain weight restriction and cerebellar purkinje cell loss. Alcohol 7:1-8.

Goodman, Gilman (1990): Goodman and Gilman's The Pharmacological Basis of Therapeutics (eighth edition). Gilman AG, Rall TW, Nies AS, Taylor P eds. Pergamon Press:New York. pp 376-377.

Grahame-Smith DG (1964): Tryptophan hydroxylation in brain. Biochem Biophys Res Commun 16:586-592.

Grahame-Smith DG (1971): Studies in vivo on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. J Neurochem 18:1053-1066.

Grahame-Smith DG (1988): Serotonin (5-Hydroxytryptamine, 5-HT). Quarterly Journal of Medicine, New Series 67 254:459-466.

Guerri C, Saez R, Sancho-Tello M, Martin de Aquilera and Renau-Piqueras J. (1990) Ethanol alters astrocyte development: a study of critical periods using primary cultures. Neurochem Res 15(5) 559-565.

Guerri C, Sanchis R (1985): Acetaldehyde and alcohol levels in pregnant rats and their fetuses. Alcohol 2:267-270.

Halgren E, Varon S (1972): Serotonin turnover in cultured raphe nuclei from newborn rat: in vitro development and drug effects. Brain Res 48:438-442.

Halmesmaki E, Raivio KO, Ylikorkala O (1987): Patterns of alcohol consumption during pregnancy. Obstet Gynecol 69:594-597.

Hanson JW, Streissguth AP, Smith DW (1978): The effects of moderate alcohol consumption during pregnancy on fetal growth and morphogenesis. J Pediatr 92:457-460.

Hansson E (1986): Primary cultures from defined brain areas. III. effects of seeding time on [^{3}H]L-glutamate transport and glutamine synthetase activity. Dev Brain Res 24:203-209.

Hartig P, Kao HT, Macchi M, Adham N, Zgombick J, Weinshank R, Branchek T (1990): The molecular biology of serotonin receptors: an overview. Neuropsychopharmacology 3:335-347.

Hatten ME (1990): Riding the glial monorail: a common mechanism for glial-guided neuronal migration in different regions of the developing mammalian brain. Trends Neurosci 13:179-184.

Henderson GI, Patwardhan RV, McLeroy S, Schenker S (1982): Inhibition of placental amino acid uptake in rats following acute and chronic ethanol exposure. Alcoholism:Clin Exp Res 6:495-505.

Hicks SP, D'Amato CJ (1968): Cell migration to the isocortex in the rat. Anat Rec 160:619-634.

Hoyer D, Middlemiss DN (1989): Species difference in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. Trends Pharmacol 101:130-132.

Jones PJH, Leichter J, Lee M (1981): Placental blood frow in rats fed alcohol before and during gestation. Life Sci 29:1153-1159.

Jorgensen MB, Diemer NH (1982): Selective neuron loss after cerebral ischemia in the rat: Possible role of transmitter glutamate. Acta Neurol Scand 66:536-546.

Julius D (1991): Molecular biology of serotonin receptors. Ann Rev Neurosci 14:335-360.

Julius D, MacDermott AB, Axel R, Jessell TM (1988): Molecular characterization of a functional cDNA encoding the serotonin 1C receptor. Science 241:558-564.

Kater SB, Mills LR (1991): Regulation of growth cone behavior by calcium. J Neurosci 11:891-899.

Katz DM, Kimelberg HK (1985): Kinetics and autoradiography of high affinity uptake of serotonin by primary astrocyte cultures. J Neurosci 5:1901-1908.

Kaufman LM, Barrett JN (1983): Serum factor supports long-term survival of rat cortical neurons in culture. Science 220:1394-1396.

Kennedy LA, Mukerji S (1986): Ethanol neurotoxicity. 1. Direct effects on replicating astrocytes. Neurobehav Toxicol Teratol 8:11-15.

Kennedy LA, Mukerji S (1986): Ethanol neurotoxicity. 2. Direct effects on differentiating astrocytes. Neurobehav Toxicol Teratol 8:17-21.

Kentroti S, Vernadakis A (1990): Neuronal plasticity in the developing chick brain: interaction of ethanol and neuropeptides. Dev Brain Res 56:205-210.

Kesaniemi YA, Sippel HW (1975): Placental foetal metabolism and acetaldehyde in the rat. I. content of ethanol acetaldehyde in placenta and foetus of the pregnant rat during ethanol oxidation. Acta Pharmacol Toxicol 47:43-48.

Kim KS, Wessel TC, Stone DM, Carver CH, Joh TH, Park DH (1991): Molecular cloning and characterization of cDNA encoding tryptophan hydroxylase from rat central serotonergic neurons. Mol Brain Res 9:277-283.

Kirksey DF, Slotkin TA (1979): Concomitant development of dopamine and 5-hydroxytryptamine uptake systems in rat brain regions. Br J Pharmacol 67:387-391.

Konig N, Wilkie MB, Lauder JM (1989): Dissection of monoaminergic neuronal groups from embryonic rat brain. In Shahar A, de Vellis J, Vernadakis A, and Haber B (Eds): A Dissection and Tissue Culture Manual of the Nervous System. Alan R. Liss, Inc., New York.

Kosofsky BE, Molliver ME (1987): The serotonergic innervation of cerebral cortex: different classes of axon terminals arising from the dorsal and median raphe nuclei. Synapse 1:153-168.

Kuriyama K, Ohkuma S, Tomono S, Hirouche M (1987): Effects of alcohol and acetaldehyde on metabolism and function of neurotransmitter systems in cerebral cortical neurons in primary culture. Alcohol Alcohol 1[suppl]:685-689.

Lamar C (1972): The effects of acute ethanol administration on an *in vitro* protein synthesizing system from rat brain. Brain Res 44:698-700.

Lauder JM, Bloom FE (1975): Ontogeny of monoamine neurons in the locus coeruleus, raphe nuclei and substantia nigra of the rat II. synaptogenesis. J Comp Neurol 163:251-264.

Lauder JM, Krebs H (1978): Serotonin as a differentiation

signal in early neurogenesis. Dev Neurosci 1:15-30.

Lauder JM, Wallace JA, Krebs H, Petrusz P, McCarthy (1982): In vivo and in vitro development of serotonergic neurons. Brain Res Bull 9:605-625.

Lauder JM, Wilkie MB, Lieth E, Han V (1989): Dissociated cell culture and morphochemistry of monoamine neurons. In Shahar A, de Vellis J, Vernadakis A, and Haber B (Eds): A Dissection and Tissue Culture Manual of the Nervous System. Alan R. Liss, Inc., New York.

Lidov HGW, Molliver ME (1982): Immunohistochemical study of the development of serotonergic neurons in the rat CNS. Brain Res Bull 9:559-604.

Lim R, Miller JF (1989): Isolation of rat astroblasts In: A Dissection and Tissue Culture Manual of the Nervous System. Shahar A, de Vellis J, Vernadakis A, and Haber B editors. Alan R. Liss, Inc., New York. pp 88-89.

Lin GWJ, Lin TY, Jin L (1990): Gestational ethanol consumption on tissue amino acid levels: decreased free histidine and tryptophan in fetal tissues with concomitant increase in urinary histamine excretion. Alcoholism: Clin Exp Res 14:430-437.

Liu KP, Tamir H, Hsiung S, Adlersberg M, Gersham MD (1987): Prenatal development of serotonin binding protein in relation to other transmitter characteristics. Brain Res 429:31-41.

Lovenburg W (1977): The enzymology of tryptophan hydroxylase. In E Usdin, N Weiner, and MBH Youdim (Eds): Structure and Function of Monoamine Enzymes, Dekker, New York. pp 43-58.

Lowry OH, Rosebrough NH, Farr AL, Randall RH (1951): Protein measurement with folin phenol reagent. J Biol Chem 193:265-275.

Lubbert H, Hoggman BJ, Snutch TP, Van Dyke T, Levine AJ, Hartig PR, Lester HA, Davidson N (1987): cDNA cloning of a serotonin 5-HT_{1C} receptor by electrophysiological assays of mRNA-injected Xenopus oocytes. Proc Natl Acad Sci 84:4332-4336.

Mamounas LA, Mullen CA, O'Hearn E, Molliver ME (1991): Dual serotonergic projections to forebrain in the rat: morphologicaly distinct 5-HT axon terminals exhibit differential vulnerability to neurotoxic amphetamine derivatives. J Comp Neurol 314:558-586.

McEwen BS, de Kloet ER, Rostene WH (1986): Adrenal steroid receptors and actions in the nervous system. Physiol Rev 66:1121-1150.

Meyer JS (1985): Biochemical effects of corticosteroids on neural tissues. Physiol Rev 65:946-1020.

Meyer MB, Tenascia JA (1977): Maternal smoking, pregnancy complications and perinatal mortality. Am J Obstet Gynecol 128:494-502.

Miller MW (1986): Effects of alcohol on the generation and migration of cerebral cortical neurons. Science 233:1308-11.

Miller MW (1987): Effect of prenatal exposure to alcohol on the distribution and time of origin of corticospinal neurons in the rat. J Comp Neurol 257:372-382.

Miller MW (1988): Effect of prenatal exposure to ethanol on the development of cerebral cortex: 1. Neuronal generation. Alcoholism: Clin Exp Res 12:440-449.

Miller MW (1989): Effect of prenatal exposure to ethanol on the development of cerebral cortex. II. Cell proliferation in the ventricular and subventricular zones of the rat. J Comp Neurol 287:326-338.

Miller MW (1990): Effect of prenatal exposure to ethanol on the rate and schedule of neuronal migration to rat somatosensory cortex. Alcoholism: Exp Clin Res

Miller MW and Potempa G (1990): Numbers of neuronal and glia in mature somatosensory cortex: Effects of prenatal exposure to ethanol. J Comp Neurol 293:92-102.

Mitchell JB, Iny LJ, Meany MJ (1990a): The role of serotonin in the development and environmental regulation of type II corticosteroid receptor binding in rat hippocampus. Dev Brain Res 55:231-235.

Mitchell JB, Rowe W, Boksa P, Meany MJ (1990b): Serotonin regulates type II corticosteroid receptor binding in hippocampal cell cultures. J Neurosci 10:1745-1752.

Molliver ME (1987): Serotonergic neuronal systems: what their anatomic organization tells us about function. J Clin Psychopharmacol 7:3-23.

Mukherjee AB, Hodgen GD (1982): Maternal ethanol exposure induces transient impairment of umbilical circulation and fetal hypoxia in monkeys. Science 218:700-702.

Mytilineou C, Friedman LK, Danias P (1988): Studies on the toxicity of MPTP to dopamine neurons in tissue and cell cultures. In Franz Hefti and William J. Weiner, Progress in Parkinson Research. Plenum Publishing Corporation, pp 127-136.

Noronha AB, Druse MJ (1982): Maternal ethanol consumption and synaptic membrane glycoproteins in offspring. J Neurosci Res 8:83-97.

Park DH, Snyder DW, Joh TH (1986): Postnatal development changes of tryptophan hydroxylase activity in serotonergic cell bodies and terminals of rat brain. Brain Res 378:183-185.

Pazos A, Hoyer D, Palacios JM (1984): The binding of serotonergic ligands to the porcine choroid plexus: characterization of a new type of serotonin recognition site. Eur J Pharmacol 106:539-546.

Pedigo NW, Yamamura HI, Nelson DL (1981): Discrimination of multiple ^{3}H-5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. J Neurochem 36:220-226.

Peroutka SJ, Snyder SH (1979): Multiple serotonin receptors: differential binding of ^{3}H-5-hydroxytryptamine, ^{3}H-lysergic acid diethylamide and ^{3}H-spiroperidol. Mol Pharmacol 16:687-699.

Pettmann B, Louis JC, Sensenbrenner M (1979): Morphological and biochemical maturation of neurones cultured in the absence of glial cells. Nature 281:378-380.

Phillips SC, Cragg BG (1982): A change in susceptibility of rat cerebellar purkinje cells to damage by alcohol during fetal, neonatal and adult life. Neuropathol Appl Neurobiol 8:441-454.

Pierce DR, West JR (1986): Blood alcohol concentration: a critical factor for producing fetal alcohol effects. Alcohol 3:269-272.

Pritchett DB, Bach AWJ, Wozny M, Talib O, Dal Toso R, Shih J, Seeburg PH (1988): Structure and functional expression of cloned rat serotonin 5-HT_2 receptor. EMBO 73:4235-4241.

Rabin RA (1990): Chronic ethanol exposure of PC12 cells alters adenylate cyclase activity and intracellular cyclic AMP content. J Pharmacol Exp Ther 252:1021-1027.

Raiha NCR, Koskinen M, Pikkarainen P (1967): Developmental

changes in alcohol-dehydrogenase activity in rat and guinea-pig liver. Biochem J 103:623-626.

Randall CL, Ekblad U and Anton FR (1990) Perspectives on the pathophysiology of fetal alcohol syndrome. Alcoholism: Clin Exp Res 14:807-812.

Rathbun W, Druse MJ (1985): Dopamine, serotonin and acid metabolites in brain regions from the developing offspring of ethanol treated rats. J Neurochem 44:57-62.

Rawat AK (1985): Nucleic acid and protein synthesis inhibition in developing brain by ethanol in the absence of hypothermia. Neurobehav Toxicol Teratol 7:161-166.

Rosett HL, Weiner L (1984): Alcohol and the fetus: a clinical perspective. Oxford University Press.

Savoy-Moore RT, Dombrowski MP, Cheng A, Abel EA, Sokol RJ (1989): Low dose alcohol contracts the human umbilical artery in vitro. Alcoholism: Clin Exp Res 13:40-42.

Schenker S, Dicke JM, Johnson FR, Hays SE, Henderson GI (1989): Effect of ethanol on human placental transport of model amino acid and glucose. Alcoholism: Clin Exp Res 13:112-119.

Schenker S, Becker HC, Randall CL, Phillips DK, Baskin GS and Henderson GI (1990): Fetal alcohol syndrome: current status of pathogenesis. Alcoholism: Clin Exp Res 14:635-647.

Schmechel D, Rakic P (1979): Golgi study of radial glial cells in developing monkey telencephalon - morphogenesis and transformation into astrocytes. Anat Embryol 156:115-152.

Schmidt AW, Peroutka SJ (1989): 5-Hydroxytryptamine receptor "families". FASEB 3:2242-2249.

Scott B, Petit TL, Lew J (1986): Differential survival of fetal and adult neurons and non-neuronal cells exposed chronically to ethanol in cell culture. Neurotoxicol 7:81-90.

Sharma A, Rawat AK (1989): Toxicological consequences of chloroquine and ethanol on the developing fetus. Pharmacol Biochem Behav 34:77-82.

Sjoblom M, Pilstrom L, Morland J (1978): Activity of alcohol dehydrogenase and acetaldehyde dehydrogenases in the liver and placenta during the development of the rat.

Enzyme 23:108-115.

Smith BH, Sweet WH (1978): Monoaminergic regulation of central nervous system function. II. Serotonergic systems. Neurosurgery. 3:257-272.

Smith DA, Gallager DW (1989): Electrophysiological and biochemical characterization of the development of α_1 - adrenergic and 5-HT_1 receptors associated with dorsal raphe neurons. Dev Brain Res 46:173-186.

Smith RA, Orr DJ, Haitzman ML, MacPherson N, Storey ND (1990): The response of primary cultured adult mouse sensory neurons to ethanol, propanol, acetaldehyde and acrolein treatments. Virchows Archives [Cell Pathol] 58:323-330.

Snyder AK, Singh SP, Pullen GL (1986): Ethanol-induced intrauterine growth retardation: Correlation with placental glucose transfer. Alcohol: Clin Exp Res 16:167-170.

Streissguth AP, Herman CS, Smith DW (1978): Intelligence, behavior, and dysmorphogenesis in the fetal alcohol syndrome: a report on 20 patients. J Pediatr 92:363-367.

Sze PY (1976): Glucocorticoid regulation of the serotonergic system of the brain. Adv Biochem Psychopharmacol 15:251-265.

Sze PY (1980): Glucocorticoids as a regulatory factor for brain tryptophan hydroxylase during development. Dev Neurosci 3:217-223.

Tabakoff B, Hoffman PL (1987): Biochemical pharmacology of alcohol In HY Meltzer ed. Psychopharmacology, The Third Generation of Progress. Raven Press NY, pp 1521-1526.

Tajuddin N, Druse MJ (1989a): Chronic maternal ethanol consumption results in decreased serotonergic 5HT_1 sites in cerebral cortical regions from offspring. Alcohol 5:465-470.

Tajuddin N, Druse MJ (1989b): Effects of <u>in</u> <u>utero</u> ethanol exposure on cortical 5HT_2 binding sites. Alcohol 5:461-464.

Tewari S, Fleming EW, Noble EP (1975): Alterations in brain RNA metabolism following chronic ethanol ingestion. J Neurochem 24:561-569.

Tewari S, Sweeney FM, Fleming EW (1980): Ethanol-induced

changes in properties of rat brain ribosomes. Neurochem Res 5:1025-1035.

Tewari S, Greenberg SA, Do K, Grey PA (1987): The response of rat brain protein synthesis to ethanol and sodium barbital. Alcohol Drug Res 7:243-258.

Tyce GM (1990): Origin and metabolism of serotonin. J Cardiovasc Pharmacol 16[suppl 3]:S1-S7.

Tong JH, Kaufman S (1975): Tryptophan hydroxylase: purification and some properties of the enzyme from rabbit hindbrain. J Biol Chem 250:4152-43158.

Tork I (1990): Anatomy of the serotonergic system. Ann NY Acad Sci 600:9-36.

Undenfriend S, Weissbach H, Bogdanski DF (1957): Increase in tissue serotonin following admisistration of its precursor, 5-hydroxytryptophan. J Biol Chem 224:803-810.

Van de Kar (1989): Neuroendocrine aspects of the serotonergic hypothesis of depression. Neurosci Biobehav Rev 13:1-10.

Vandermaelen CP, Matheson GK, Wilderman RC, Paterson LA (1986): Inhibition of serotonergic dorsal raphe neurons by systemic and iontophoretic administration of buspirone, a non-benzo-diazepine anxiolytic drug. Eur J Pharmacol 129:123-130.

Wallace JA, Lauder JM (1983): Development of the serotonergic system in the rat embryo: an immunocytochemical study. Brain Res Bull 10:459-479.

West JR, Hamre KM, Cassell MD (1986): Effects of ethanol exposure during the third trimester equivalent on neuron number in the rat hippocampus and dentate gyrus. Alcoholism: Clin Exp Res 10:190-197.

Whitaker-Azmitia PM, Azmitia EC (1986): Autoregulation of fetal serotonergic neuronal development: role of high affinity serotonin receptors. Neurosci Lett 67:307-312.

Whitaker-Azmitia PM, Lauder JM, Shemmer A, Azmitia EC (1987): Postnatal changes in serotonin receptors following prenatal alterations in serotonin levels: further evidence for functional fetal serotonin$_1$ receptors. Dev Brain Res 33:285-289.

Whitaker-Azmitia PM, Azmitia EC (1989): Stimulation of astroglial serotonin receptors produces culture media which regulated growth of serotonergic neurons. Brain

Res 497:80-85.

Whitaker-Azmitia PM, Murphy R, Azmitia EC (1990): Stimulation of astroglial 5-HT_{1A} receptors releases the serotonergic growth factor, protein S-100, and alters astroglial morphology. Brain Res 528:155-158.

Yamamoto M, Steinbusch HWM, Jessell TM (1981): Differentiated properties of identified serotonin neurons in dissociated cultures of embryonic rat brainstem. J Cell Biol 91:142-152.

Zuckerman B, Frank DA, Hingsin R, Amaro H, Levenson SM, Kayne H, Parker S, Vina R, Aboagye K, Fried LE, Cabral H, Timperi R, Bauchner H (1989): Effects of maternal marijuana and cocaine use on fetal growth. N Engl J Med 320:762-768.

APPROVAL SHEET

The dissertation submitted by Denise K. Lokhorst has been read and approved by the following committee:

Mary Druse Manteuffel, Ph.D.
Professor
Department of Molecular and Cellular Biochemistry
Loyola University Stritch School of Medicine

Michael A. Collins, Ph.D.
Professor
Department of Molecular and Cellular Biochemistry
Loyola University of Chicago

Mary Ann Emanuele, M.D.
Associate Professor
Department of Medicine
Section of Endocrinology
Loyola University of Chicago

Robert Handa, Ph.D.
Assistant Professor
Department of Cell Biology, Neurobiology and Anatomy
Loyola University of Chicago

Louis D. Van de Kar, Ph.D.
Professor
Department of Pharmacology
Loyola University of Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 20, 1992
Date

Mary Druse Manteuffel
Director's Signature