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The Effects of Chronic Ethanol Exposure of the Dopaminergic System of the Rat Brain

Susan Marie Pellegrino

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LOYOLA UNIVERSITY

THE EFFECTS OF CHRONIC ETHANOL EXPOSURE
ON THE DOPAMINERGIC SYSTEM OF THE RAT BRAIN

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL OF
LOYOLA UNIVERSITY OF CHICAGO
IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR AND CELLULAR BIOCHEMISTRY

BY
SUSAN MARIE PELLEGRINO

CHICAGO, ILLINOIS
JANUARY, 1991
ethanol-fed rats. The concentrations of dopamine and DOPAC were increased two-fold in the frontal cortex, ventral pallidum, and ventral tegmental area, while the levels were unchanged in the striatum and globus pallidus. In the substantia nigra, the dopamine level was decreased and the level of DOPAC was increased. Although the dopamine level in the nucleus accumbens was normal, the level of DOPAC was decreased. An analysis of the G proteins, Gs and Go, demonstrated minimal, if any, changes in either protein content and mRNA levels in brain regions analyzed. Concerning the inhibitory G proteins, the mRNA levels of Gi1 and Gi2 were unchanged in all brain regions examined. However, there was a trend towards an increase in the mRNA levels of Gi3 in all regions except the substantia nigra. In the frontal cortex and striatum, no change was seen in dopamine-sensitive adenylate cyclase activity. Adenylate cyclase activity in the presence of dopamine was significantly increased in the nucleus accumbens of ethanol-treated rats. In comparison to control animals, there was an increase in cholesterol levels, while the levels of phospholipids were not significantly different. There was also an increase in the ratio of cholesterol to phospholipid.

These results suggest a selective ethanol effect on several components of the dopamine system in three-month-old Fisher 344 rats following one month of ethanol consumption.
Those brain regions which are affected are associated with the mesolimbic dopamine pathway. The changes in the brain regions of the mesolimbic dopamine pathway are notable because this pathway is involved with the reward circuitry. It is possible that the mesolimbic dopamine neurons may be involved in the rewarding effects of ethanol. Alterations in the brain regions associated with this pathway may contribute to the reinforcing effects of ethanol.
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To my advisor, Dr. Mary Manteuffel, for her support and guidance.

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To Nuzhath Tajuddin, for her friendship and assistance in the laboratory.

To John Tentler, for his technical genius in molecular biology and slide-making.

To my parents, my sister, and my brothers for their encouragement.
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LIST OF ABBREVIATIONS

α
  alpha
αi
  inhibitory alpha subunit
αs
  stimulatory alpha subunit
ATP
  adenosine triphosphate
B
  beta
Bmax
  maximum binding
bp
  base pair
BSA
  bovine serum albumin
Ca++
  calcium ion
cAMP
  adenosine 3', 5'-monophosphate
cDNA
  complementary deoxyribonucleic acid
CNS
  central nervous system
COMT
  catechol-0-methyltransferase
COOH
  carboxyl group
CTP
  cytidine triphosphate
D1
  dopamine receptor subtype
D2
  dopamine receptor subtype
DARPP-32
  dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein
DEPC
  diethyl pyrocarbonate
DHBA
  3,4-dihydroxybenzylamine
dl
  deciliter
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<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DOPA</td>
<td>dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(B-aminoethyl ether) N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory postsynaptic potential</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>ferrous ion</td>
</tr>
<tr>
<td>t</td>
<td>gamma</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide binding protein</td>
</tr>
<tr>
<td>Gi</td>
<td>inhibitory guanine nucleotide binding protein</td>
</tr>
<tr>
<td>Gs</td>
<td>stimulatory guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
</tr>
<tr>
<td>GF/C</td>
<td>glass filter (1.2 micron)</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>5’-guanylylimidodiphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylyphperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>5-HT2</td>
<td>serotonin receptor subtype</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
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</table>
IBMX 3-isobutyl-1-methylxanthine
K⁺ potassium ion
kb kilobase
kD kilodalton
Kd dissociation constant
KH₂PO₄ potassium phosphate
LB broth Lurie-Bertani medium
MAO monoamine oxidase
mg milligram
MgCl₂ magnesium chloride
ml milliliter
mM millimolar
µCi microcurie
µg microgram
µl microliter
µM micromolar
mol/L moles/liter
MOPS 3-(N-morpholino)propanesulfonic acid
mRNA messenger ribonucleic acid
Na⁺ sodium ion
NaCl sodium chloride
NAD nicotinamide adenine dinucleotide (oxidized form)
NADH nicotinamide adenine dinucleotide (reduced form)
NaF sodium fluoride
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NaN3</td>
<td>sodium azide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>Na/PO4</td>
<td>sodium phosphate buffer</td>
</tr>
<tr>
<td>Na2PO4</td>
<td>sodium phosphate</td>
</tr>
<tr>
<td>Na2SO3</td>
<td>sodium sulfite</td>
</tr>
<tr>
<td>NaHSO3</td>
<td>sodium bisulfite</td>
</tr>
<tr>
<td>NH2</td>
<td>amino group</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>(NP)</td>
<td>nonpreferring</td>
</tr>
<tr>
<td>NPA</td>
<td>R(-)-10,11-dihydroxy-N-n-propylnorapomorphine hydrochloride</td>
</tr>
<tr>
<td>O2</td>
<td>molecular oxygen</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
</tr>
<tr>
<td>(P)</td>
<td>preferring</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPM</td>
<td>synaptic plasma membrane</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride/sodium citrate buffer</td>
</tr>
<tr>
<td>SSPE</td>
<td>sodium chloride/sodium phosphate/EDTA buffer</td>
</tr>
<tr>
<td>Tag I</td>
<td>Thermus aquaticus DNA polymerase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris/sodium chloride/Tween 20 buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tris-HCL</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>octyl phenoxy polyethoxyethanol</td>
</tr>
<tr>
<td>TSA</td>
<td>Tris/sodium chloride/sodium azide buffer</td>
</tr>
<tr>
<td>TTP</td>
<td>thymidine triphosphate</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>v/v</td>
<td>percent volume in volume</td>
</tr>
<tr>
<td>w/v</td>
<td>percent weight in volume</td>
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</tbody>
</table>
CHAPTER I
INTRODUCTION

Alcohol abuse and alcoholism represent serious health problems in the United States. Approximately 18 million adults exhibit some form of alcoholism, alcohol dependence, or alcohol abuse, and at least 3 out of 100 deaths in the United States can be attributed to alcohol use (Van Natta et al. 1984).

Several central nervous system (CNS) neurotransmitter systems appear to be sensitive to alcohol administration. These include: the gamma-aminobutyric acid (GABA) system, the glutamate system, the dopamine system, the norepinephrine system, and the serotonin system (Greenberg et al. 1986; Valverius et al. 1987; Celetano et al. 1988; Murphy et al. 1988; Lovinger et al. 1989). Of particular interest is the dopaminergic system because both the nigrostriatal dopamine pathway, which is involved with locomotor activity (Fibiger et al. 1974; Liljequist et al. 1981), and the mesolimbic dopamine pathway, which has been implicated in the reinforcement of several drugs of abuse (Lyness et al. 1979; Bozarth and Wise 1981; Matthews and German 1984), appear to be sensitive to the effects
of ethanol exposure (Mereu et al. 1984; Gessa et al. 1985). In light of ethanol's reported actions on locomotor activity and its apparent reinforcing properties, it was hypothesized that chronic ethanol consumption either alters the ability of the dopamine signal to effect cellular changes through signal transduction or alters the level of dopamine itself. In order to test this hypothesis, I proposed to examine the effects of chronic ethanol consumption on the level of dopamine as well as several membrane components involved with signal transduction. This is important because of the substantial amount of evidence for ethanol's effects on CNS membranes and membrane components (Hoffman and Tabakoff 1979; Rabin and Molinoff 1981; Sun and Sun 1985; Hruska 1988). The membrane components which were examined include the D1 and D2 dopamine receptors, the stimulatory (s) and inhibitory (i) guanine nucleotide binding proteins (G proteins) which couple the dopamine receptors to adenylate cyclase, and the activity of dopamine-stimulated and dopamine-inhibited adenylate cyclase. These components were measured in brain regions which are associated either with the nigrostriatal system or mesolimbic dopamine system. Changes in the level of one or more of the membrane components (receptors, G proteins, or adenylate cyclase activity) could result in alterations in cellular activity. Changes in dopamine or in these membrane components could contribute to ethanol-induced changes in motor activity and
to the reinforcing effects of ethanol.
CHAPTER II
LITERATURE REVIEW

The Dopaminergic System

In the central nervous system, dopamine has been identified in several brain regions including the striatum, the nucleus accumbens, and the cortex (Hokfelt et al. 1976; Arlison et al. 1984; Seguela et al. 1988). There are two major dopaminergic pathways: the nigrostriatal pathway and the mesolimbic dopamine pathway. The nigrostriatal pathway originates in the substantia nigra which sends projections to the striatum and collaterals to the globus pallidus (Fibiger et al. 1974; Lindvall and Bjorkland 1979). This pathway is a major component of the extrapyramidal motor system (Fibiger et al. 1974). The mesolimbic dopamine pathway consisting of projections from the ventral tegmental area to the nucleus accumbens (Lindvall et al. 1978; Nauta et al. 1978) is recognized as an important component of the reward circuitry (Phillips and Fibiger 1978).

Dopamine, a catecholamine, is synthesized from its amino acid precursor tyrosine by the action of the enzymes tyrosine hydroxylase and aromatic amino acid decarboxylase. Tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA).
by the enzyme tyrosine hydroxylase. This enzyme is stereospecific and requires molecular oxygen (O₂), ferrous ion (Fe⁡⁺²), and the cofactor tetrahydropteridine. It serves as the rate limiting step in the biosynthesis of dopamine. The second enzyme involved in catecholamine synthesis is aromatic amino acid decarboxylase. This enzyme catalyzes the conversion of DOPA to dopamine.

The major enzymes in metabolic degradation of the catecholamines are monoamine oxidase (MAO) and catechol-0-methyltransferase (COMT). Monoamine oxidase converts catecholamines to their corresponding aldehydes. Dopamine is converted to 3,4-dihydroxyphenylacetaldehyde. This aldehyde intermediate is rapidly oxidized by the enzyme aldehyde dehydrogenase. The aldehyde, 3,4-dihydroxyphenylacetaldehyde, is converted to 3,4-dihydroxyphenylacetic acid (DOPAC) which in turn is converted to homovanillic acid (HVA) by the enzyme catechol-0-methyltransferase (Cooper et al. 1986).

There are two types of dopamine receptors (Kebabian and Calne 1979), originally defined by either an ability to stimulate adenylate cyclase activity (the D1 receptor) (Iversen 1975) or an inability to stimulate adenylate cyclase activity (the D2 receptor). In addition to the stimulation of adenylate cyclase, the D1 receptor may be coupled to inositol phosphate production and calcium (Ca⁺²) mobilization (Mahan et al. 1990). The D2 receptors are
characterized functionally by their ability to inhibit adenylate cyclase activity (Enjalbert and Bockaert 1983). The D2 receptor appears to couple selectively with the Gi2 subtype (Jones and Reed 1987) of the G proteins (Senogles et al. 1990). Activation of D2 receptors also inhibits calcium channels, increases potassium conduction, and may inhibit accumulation of inositol phosphate (Simmonds and Strange 1985; Malgaroli et al. 1987; Lacey et al. 1987).

The D1 and D2 dopamine receptors exist in two forms: a high affinity state and a low affinity state with respect to agonists (Sibley et al. 1982; Leff et al. 1985). The state of these receptors is modulated by the guanine nucleotide binding proteins (G proteins) which couple the receptors to adenylate cyclase (Delean et al. 1982). The binding of an agonist to the receptor promotes a receptor-G protein interaction. The agonist is bound with higher affinity to the receptor of the resulting ternary complex (agonist-receptor-G protein) (Boeynaems and Dumont 1975). When guanosine triphosphate (GTP) binds to this complex, the receptor dissociates from the G protein and is converted to the low affinity state. The GTP-G protein complex is the active intermediate which interacts with adenylate cyclase (Katada et al. 1984).

In order to study the dopamine receptors, a variety of pharmacological ligands have been developed. Some examples of the D1 agonists include SKF 38393 and SKF 82526 (Setler
et al. 1978; Flaim et al. 1985). Examples of D2 agonists include LY 141865, RU 24213, and propylnorapomorphine (NPA) (Miller et al. 1976; Euvrard et al. 1980; Titus et al. 1983). Dopamine antagonists have also been developed. These include the D1 antagonists SCH 23390 and SKF 83566 (Hyttel 1983; O'Boyle and Waddington 1984) and the D2 antagonists spiperone, haloperidol, and YM 09151-2 (Hyttel 1978; Grewe et al. 1982).

Antagonist binding exhibits different characteristics than agonist binding. Combined use of agonist and antagonist binding exhibits competition curves that can be resolved into the high affinity component and the low affinity component. Most agonists bind to a receptor population representing the high affinity component. In the presence of guanine nucleotides (i.e. GTP and 5'-guanylylimidodiphosphate), agonist/antagonist curves represent a single affinity state similar to the low affinity component. Guanine nucleotides bind to the agonist-receptor-G protein complex resulting in the conversion of the receptor to its low affinity state. Agonist binding is decreased in the presence of guanine nucleotides, whereas antagonist binding which measures both the agonist high affinity component and the low affinity component is not altered. (Boeynaems and Dumont 1975; Jacobs and Cuatrecasas 1976; Limbird 1981; Hess and Creese 1987). Antagonist and agonist binding assays can be used to
Several different functions have been associated with the dopamine D1 receptor; these functions may vary with the tissue localization of the receptor. The prototype D1 receptor is located in the parathyroid gland and mediates the release of parathyroid hormone (Niznik et al. 1988). Peripheral D1 receptors promote renal vasodilatory responses (Kohli and Goldberg 1987) and appear to modulate gap junction permeability in the retina (Lasater and Dowling 1985). In the central nervous system, D1 receptors may exert a modulatory influence on the activity of the D2 receptor (Clark and White 1987). Presynaptic D1 receptors are located on GABAergic terminals in the substantia nigra, possibly regulating the release of GABA (Filloux et al. 1987). During development, central nervous system D1 receptor stimulation may be involved with regulation of neuron growth and differentiation (Lankford et al. 1988).

The neuronal D1 receptor is a glycoprotein (Niznik et al. 1986). A polypeptide of apparent Mr = 72,000/74,000 represents the major ligand binding subunit (Niznik et al. 1988). The polypeptide is sialylated and has terminal mannose residues. However, the neuronal receptor and the parathyroid receptor are differentially glycosylated. Upon complete deglycosylation, the D1 receptor (neuronal and parathyroid) is a single polypeptide Mr = 46,000. This polypeptide probably represents the protein backbone of
central and peripheral D1 receptors (Jarvie et al. 1989; Niznik et al. 1989).

Recently, four laboratories cloned a cDNA encoding the D1 dopamine receptor coupled to the stimulation of adenylate cyclase (Dearry et al. 1990; Monsma et al. 1990; Sunahara et al. 1990; Zhou et al. 1990). A D1 clone was used to isolate and characterize the gene encoding the human D1 dopamine receptor. This gene is located on an intronless region of the long arm of chromosome 5 and encodes a protein of 446 amino acids with a predicted molecular mass of 49,300. The protein sequence of this receptor indicates the presence of seven transmembrane segments which is characteristic of G protein-coupled receptors (Dixon et al. 1986; Kubo et al. 1986; Kobilka et al. 1987; Bonner et al. 1989). There are several potential sites for post-translational modification including a cysteine residue in the carboxy terminus that is conserved in most G protein-coupled receptors and two consensus sequences for cAMP-dependent protein kinase phosphorylation. The cloning of the gene for a D1 dopamine receptor will provide a valuable tool to study the regulation and function of this receptor.

DARPP-32 (dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein; molecular weight: 32 kD) is a cytosolic protein enriched in dopaminergic neurons of the brain (Hemmings and Greengard 1986). Its distribution correlates with the distribution of the D1 receptors.
coupled to adenylate cyclase (Schalling et al. 1990). Dopamine increases the state of phosphorylation of DARPP-32. Upon phosphorylation, DARPP-32 is converted to a very potent inhibitor of protein phosphatase-1 (Hemmings et al. 1984). Protein phosphatase-1 dephosphorylates phosphoproteins that are substrates for a variety of protein kinases. Thus, one mechanism by which dopamine may modulate the phosphorylation state of substrates is through the phosphorylation and activation of DARPP-32 (Hemmings et al. 1989).

The D2 dopamine receptor is also associated with a variety of functions. The prototype D2 receptor is located in the anterior pituitary and inhibits prolactin release (Weiner and Gangong 1978). Dopamine somatodendritic autoreceptors appear to be of the D2 receptor subtype (Lehmann et al. 1983; Brown et al. 1985). D2 receptors are also localized to the terminals of the cholinergic interneurons of the striatum. Stimulation of these D2 receptors leads to the decreased release of acetylcholine. (Joyce and Marshall 1985, 1987; Stoof et al. 1987).

The D2 receptor is a glycoprotein and the major ligand binding subunit is a polypeptide (Mr=94,000). This subunit contains acetylglucosamine chains and terminal sialic acid (Grigoriadis et al. 1988). After complete deglycosylation, the binding subunit is a polypeptide (Mr=44,000). It is not known whether this subunit represents the functional D2 receptor moiety (Jarvie et al. 1988).
A full-length cDNA clone of the rat D2 receptor has been isolated (Bunzow et al. 1988). This cDNA clone was used to clone a human genomic fragment (rhD2G1) which contains the last coding exon of the D2 receptor gene. The human D2 receptor is encoded by a gene located at the q22-q23 junction of chromosome 11. The D2 gene locus contains a frequent two allele TaqI RFLP (restriction fragment length polymorphism) (Grandy et al. 1989).

Two transcripts exist for the D2 receptor which differ only by the presence of an 87-bp insert (Rao et al. 1990). The D2 receptor is thought to be an integral membrane protein of seven transmembrane spanning regions similar to other G protein-coupled receptors (Lefkowitz and Caron 1988). The 87-nucleotide insert is within the region coding for the third cytoplasmic loop. It is suggested the two forms of the D2 receptor represent alternately spliced transcripts. This hypothesis is consistent with the evidence of introns in the rat D2 receptor gene (Bunzow et al. 1988).

O'Malley and colleagues (1990) suggest there must be at least two genes that produce D2 receptor subtypes designated D2A and D2B. The D2A gene contains 8 exons and spans at least 50 kb with exons 2-7 clustered in approximately 13 kb of the genome. The D2A gene undergoes alternate RNA processing within intron 5, resulting in an insertion of 29 amino acids to the predicted 415 amino acid sequence of the D2A protein. The authors suggest the presence of
tissue-specific factors that control not only $D_{2A}$ gene transcription but also alternate splicing of the pre-mRNA.

The dopamine D1 and D2 receptor sites are differentially distributed throughout the brain. The localization of these dopamine sites has been examined utilizing receptor autoradiography and in situ hybridization techniques (Boyson et al. 1986; Charuchinda et al. 1987; Mansour et al. 1990). The D1 sites are located in the basal ganglia, cortical regions, amygdala, and hippocampus and high concentrations of D2 sites are confined primarily to the olfactory bulb, basal ganglia, and pituitary. Lesion studies have demonstrated that both receptor subtypes are localized on presynaptic and postsynaptic membranes (Porceddu et al. 1986; Joyce and Marshall 1987; Filloux et al. 1987, 1988). In most brain regions, the D1 receptors, found predominately in the low affinity state, are present in greater density than the D2 receptors, found predominately in the high affinity agonist state (Huff and Molinoff 1984; Boyson et al. 1986; Richfield et al. 1989). Guanine nucleotide-sensitive and -insensitive D1 and D2 receptors have been identified in several brain regions (De Keyser et al. 1989). A regional heterogeneity based on this guanine nucleotide effect has been proposed. These unique distributions of the D1 and D2 receptors suggest complex interactions resulting in dopamine-mediated behaviors. A complex functional interaction exists between the D1 and
D2 receptors which is involved in the generation of some motor behaviors. The D1 receptor exerts its greatest behavioral effects when the D2 receptor is stimulated simultaneously. (Barone et al. 1986). Braun (1986) has suggested that D1 receptor activation provides a "tonic" background which allows the "phasic" component of the D2 receptor to be effective. Stimulation of the D2 receptor can initiate motor behavior optimally when there is a specific level of D1 receptor activation. The D1 receptor may control the qualitative changes in D2 stimulated behaviors.

Dopamine has two general classes of behavioral action: motor activation and reward-mediated learning (Beninger et al. 1989; Miller et al. 1990; Wickens 1990). Dopamine stimulates motor activity (Beninger 1983; Starr and Starr 1986; Johansson et al. 1987) and dopamine antagonists prevent locomotor stimulation by dopamine agonists (Hoffman and Beninger 1985; Molloy and Waddington 1985). Dopamine agonists facilitate, while dopamine antagonists impair, the effects of reward on learning (Zarevics and Setler 1979; Gallistel and Karras 1984; Mazurki and Beninger 1986).

The neuronal model for reward-mediated learning suggests this learning process consists of the selection and strengthening of specific synaptic connections (Miller et al. 1990; Wickens 1990). The paradigm for synaptic strengthening involves two sequential processes. First, there is the selection of synapses (possibly corticostriatal
synapses). These synapses would have been activated as a result of a specific stimuli. The synapse must be effective in firing a striatal neuron. When this has taken place, a temporary "state of readiness" is established and the synapses are eligible for modification. Second, if the behavior which follows the neuronal discharge has motivationally favorable consequences, reward pathways (i.e. the mesolimbic dopamine pathway) are activated and the efficacy of synaptic transmission is enhanced in synapses in the "state of readiness". Subsequent encounters with these stimuli result in easier activation of the neuron by these synapses (synaptic enhancement).

This synaptic enhancement may occur in dopamine-mediated motor activation as well as reward-mediated learning. Dopamine acting on D2 receptors of the striatal cholinergic terminals exerts an inhibitory effect on acetylcholine release (Stoof et al. 1987). Lower levels of acetylcholine reduce membrane resistance and the excitatory postsynaptic potential (EPSP) amplitude (Dodt and Misgeld 1986). This effect may lead to an enhancement of the synapses. Dopamine acting on the D1 receptors located on striatal projection neurons (Ariano 1987) may bring about the enhancement of selected corticostriatal synapses. These dopamine effects may in turn link dopamine-mediated motor activation to reward-mediated learning (Wickens 1990).

Modulation of motivational processes and organization
and initiation of goal-directed behaviors are associated with the ascending projections of the ventral tegmental area (Fibiger et al. 1987; Phillips et al. 1982, 1987). One of the largest projections is to the nucleus accumbens. Approximately 90% of nucleus accumbens dopamine derives from this projection (Nauta et al. 1978). The nucleus accumbens is a major component of the ventral striatum which provides a ventral pathway by which the limbic system and prefrontal areas can influence the initiation of voluntary motor behaviors (Nauta 1986). The ventral pallidum receives a dopamine projection from the ventral tegmental area (Nauta et al. 1978). This projection may modulate information related to motor function (Depue and Iacono 1989). It is also well established that the ventral tegmental area dopamine pathway mediates reward, specifically the incentive type of reward that activates goal acquisition (Stein 1983; Bozarth 1987; Fibiger and Phillips 1987). It has been suggested that the nucleus accumbens may serve as an interface between the limbic and motor systems. Dopamine activity in the nucleus accumbens appears to modulate the flow of motivational information from the limbic system to the motor system; thereby contributing to the processes of locomotor activity initiation, incentive, and goal-directed behaviors (Oades and Halliday 1987; Taghzouti et al. 1985; Depue and Iacono 1989).
**G Proteins**

G proteins are membrane-associated heterotrimeric proteins composed of $\alpha$, $\beta$, and $\gamma$ subunits. The $\alpha$ subunit has a guanine nucleotide binding site. In a nonactivated state, GDP is bound to the $\alpha$ subunit. A stimulated receptor activates the G protein by catalyzing the exchange of GDP for GTP which leads to the dissociation of the $\beta\gamma$ subunits from the $\alpha$ subunit. The GTP-bound $\alpha$ subunit is free to regulate the activity of various enzymes and ion channels (Breitweiser and Szabo 1985; Cockroft and Gompert 1985; Pfaffinger et al. 1985; Yatani et al. 1987, 1988).

Molecular cloning techniques have allowed the isolation and characterization of cDNA clones for several G protein subunits. Four $\alpha_1$ subunits have been identified which appear to arise from alternate splicing mechanisms (Bray et al. 1986). Other $\alpha$ subunits (i.e. $\alpha_{11}$, $\alpha_{12}$, $\alpha_B$, $\alpha_o$, $\alpha_z$, and $\alpha_i$) are products of unique genes (Lochrie et al. 1985; Medynski et al. 1985; Jones and Reed 1987; Itoh et al. 1988).

The guanine nucleotide binding proteins (G proteins) serve as signal transducers linking neurotransmitter and hormone receptors to membrane bound effectors (Chang and Bourne 1987; Stiles 1989; Birnbaumer 1990). The $G_i$ protein is linked to the activation of adenylate cyclase and to calcium ($Ca^{2+}$) channels (Gilman 1987; Yatani et al. 1987). The $G_i$ protein is linked to the inhibition of adenylate cyclase and the activation of potassium ($K^+$) channels.
(Yatani et al. 1988). The G₀ protein is the most abundant G protein in the brain and may be involved in the regulation of calcium (Ca^{2+}) and potassium (K^{+}) channels (Birnbaumer et al. 1987). The Gᵢ protein is coupled to rod and cone opsins and to the regulation of cGMP phosphodiesterase (Medynski et al. 1985).

The α subunit amino acid sequences are highly conserved. The greatest variability among the sequences occurs at the amino (NH₂) and carboxyl (COOH) terminals (Strathman et al. 1989). The αᵢ and α₀ subunits are post-translationally modified by the addition of myristic acid (Buss et al. 1987). In the proposed tertiary structure, the amino and carboxyl terminals are spatially close to each other and face the plasma membrane. The guanine nucleotide binding site is on the cytoplasmic face of the protein (Holbrook and Kim 1989). The amino terminus may regulate the interaction of the βᵣ subunit complex with the α subunit (Navon and Fung 1987). The carboxyl terminus may have a receptor contact site and regulate receptor binding and activation of the G protein (Deretic and Hamm 1987; Sullivan et al. 1987; Weiss et al. 1988). It is proposed that the proximity of the receptor binding domain (carboxyl terminus) and the βᵣ interaction site (amino terminus) would provide the structural basis for conformational changes within the subunits which induce guanine nucleotide exchange and subunit dissociation.
Several β and γ subunits have been identified and there is heterogeneity among these subunits (Gierschek et al. 1985; Hildebrandt et al. 1985; Gao et al. 1987). This heterogeneity may account for altered α subunit regulation and specific receptor recognition. The γ subunit of the Gt protein is particularly distinct and may account for this G protein being an extrinsic membrane protein whereas the other G proteins are intrinsic membrane proteins. It is hypothesized that the βγ complex is required for the efficient interaction of the α subunit with the receptor and activation of the guanine nucleotide exchange. This complex may also serve to anchor the G protein to the membrane (Weiss et al. 1988).

The primary sequence has been determined for many of the receptors which are coupled to G proteins (e.g. dopamine receptors, adrenergic receptors, serotonin receptors, and muscarinic receptors) (Dixon et al. 1986; Kubo et al. 1986; Kobilka et al. 1987; Bonner et al. 1989). These receptors are predicted to have a tertiary structure of seven membrane spanning regions, three cytoplasmic loops and the carboxyl terminus on the cytoplasmic side of the membrane. The receptor-G protein interaction occurs within the cytoplasmic domains. The amino acid sequences of cytoplasmic loops I-II and III-IV are conserved, but the sequence of cytoplasmic loop V-VI is highly variable (Dohlman et al. 1987). The V-VI loop may contain the recognition sites for G protein binding.
(Strader et al. 1987; Kobilka et al. 1988). The third cytoplasmic loop and the carboxyl terminal may influence receptor selectivity and G protein activation (O'Dowd et al. 1988).

Receptor activation of the G proteins is described as a catalytic exchange requiring the dissociation of GDP before the binding of GTP (Wessling-Resnick et al. 1987). The molecular interactions during activation are allosteric and display positive cooperation (Wessling-Resnick and Johnson 1987). It is proposed that agonist binding to the receptor induces conformational changes which increase binding affinity and efficiency in the interaction with the G protein (Wessling-Resnick et al. 1987).

**Adenylate cyclase**

Adenylate cyclase is one of the membrane effectors regulated by the G proteins. This enzyme is an integral membrane protein which catalyzes the synthesis of adenosine 3',5'-monophosphate (cAMP), a second messenger for many hormones and neurotransmitters (Johnson and Dhanasekaran 1989; Birnbaumer 1990). There are two species of this enzyme, and a cDNA encoding one form has been cloned (Graziano and Gilman 1987). The primary amino acid sequence consists of 1134 residues which represent two alternating sets of hydrophobic and hydrophilic regions. A proposed model suggests each hydrophobic domain contains six
transmembrane segments (Krupinski et al. 1989). The overall topology of adenylate cyclase resembles structures of various membrane channels and transporters (Gilman 1989). Gilman (1989) has suggested that adenylate cyclase may be a multifunctional molecule, serving as both an enzyme of cAMP synthesis and a transporter that exports cAMP from the cell.

In the nervous system, hormones and neurotransmitters affect the cellular content of cAMP. The cAMP levels are determined by the rate of synthesis from ATP by adenylate cyclase and the rate of hydrolysis to 5'-AMP by phosphodiesterase (Nathanson and Greengard 1981). The effects of cAMP are mediated by its activation of cAMP-dependent protein kinases. These protein kinases consist of two regulatory subunits and two catalytic subunits. The binding of cAMP to the regulatory subunits leads to the dissociation of the complex and the activated catalytic subunits are free to phosphorylate cellular proteins. This phosphorylation usually occurs on serine residues of the substrate, resulting in alterations in cellular function (Spiegel et al. 1985; Gilman 1989).

**Ethanol Effects on Dopamine Metabolism**

In animal studies, acute administration of ethanol has a biphasic effect on neural function. Low doses of ethanol cause behavioral stimulation whereas higher doses cause sedation (Hunt 1981; Majchrowicz 1981; Engel and Liljequist
1983; Di Chiara and Imperato 1985). Some of these behavioral changes may be attributed to the effects of ethanol on dopamine metabolism. Low doses result in increased dopamine synthesis, utilization, and release (Carlsson and Lindquist 1973; Darden and Hunt 1977; Fadda et al. 1980). However, at higher doses there is a decrease in dopamine utilization and release with a greater increase in synthesis (Carlsson and Lindquist 1973; Darden and Hunt 1977; Bacapoulous et al. 1978; Fadda et al. 1980). After chronic treatment with ethanol, dopamine synthesis and release are reduced (Hunt and Majchrowicz 1983) and the dopaminergic neurons exhibit a hyposensitivity to haloperidol and apomorphine (Tabakoff and Hoffman 1978; Tabakoff et al. 1978; Black et al. 1980).

The mesolimbic dopamine neurons which originate in the ventral tegmental area (VTA) and project to the nucleus accumbens (Lindvall et al. 1978) appear to be more sensitive to ethanol than those of the nigrostriatal pathway. Low doses of ethanol produced a dose-dependent increase in the firing rate of dopaminergic neurons in the VTA (Gessa et al. 1985; Brodie et al. 1990). Higher doses (approximately 5-fold) were required to produce comparable responses in the dopamine neurons of the substantia nigra (Mereu et al. 1984). Doses of ethanol which enhanced dopamine release in the nucleus accumbens of freely moving rats did not modify dopamine release in the striatum (Imperato and Di Chiara 1986; Di Chiara and Imperato 1988).
The mesolimbic dopamine neurons have been implicated in the mediation of the reinforcing properties of drugs of abuse such as cocaine (Lyness et al. 1979; Roberts and Koob 1983), heroin (Bozarth and Wise 1981), and morphine (Matthews and German 1984). It is possible these neurons may be involved in mediating the rewarding effects of ethanol (Koob and Weiss 1990; Lewis and June 1990; McBride et al. 1990; Samson et al. 1990).

The voluntary intake of ethanol by laboratory animals and humans may be influenced by genetic factors which in turn may be manifested in differences in specific neurotransmitter systems (Goodwin 1979; Deitrich and McClearn 1981; Schuckit et al. 1985). Animal models exhibiting alcohol preferring behaviors have been established to study the genetic and biological basis for alcohol abuse (Crabbe et al. 1985, 1987). Neurochemical differences have been found between alcohol-preferring (P) and alcohol-nonpreferring (NP) rats. The differences appear to be genetic because they exist even in animals never exposed to alcohol. The levels of dopamine and its metabolites are lower in the nucleus accumbens of alcohol-preferring rats as compared with alcohol-nonpreferring rats. In contrast, the dopamine content in the striatum and frontal cortex were not significantly different in these animal lines (Murphy et al. 1987, 1988; McBride et al. 1990).
Ethanol Effects on the Dopamine Receptors

When reviewing the literature related to the effects of alcohol on dopamine receptors, it is important to consider differences among each laboratory's experimental protocols. Ethanol dose, route of administration, duration of treatment, animal species, and assay conditions may account for discrepancies in results obtained from different laboratories. Furthermore, most earlier studies concentrated on ethanol's effect on the dopamine D2 receptor due to the unavailability of a specific dopamine D1 receptor ligand until recently and many of these studies were limited to the striatal receptors.

Ethanol treatment appears to affect dopamine receptors. However, the effects seem to vary with brain region analyzed and duration of treatment. Nevertheless, some generalizations can be made. 1) Short term ethanol exposure (7 days or less) does not alter the number or affinity of dopamine receptors (Hoffman and Tabakoff 1979). 2) Various studies report an increase, decrease, and no change in D1 and D2 receptor number in the mesolimbic area, striatum, and cortex after long term exposure in mice and rats (two weeks to thirty-two weeks) (Lai et al. 1980, Muller et al. 1980, Lucchi et al. 1983, 1988; Hruska 1988; Syvalahti et al. 1988; Hietala et al. 1990). 3) An ethanol-associated effect in one brain region may not be present in all brain regions. For example, Muller at al. (1980) reported a significant
decrease in D2 receptors in the mesolimbic area, while there was no difference in striatal D2 receptors after long term ethanol exposure. 4) Ethanol does not consistently alter the affinity of dopamine receptors. That is, the majority of studies report ethanol exposure has no effect on the affinity of D1 and D2 receptors (Lai et al. 1980; Muller et al. 1980; Hruska 1988; Lucchi et al. 1988; Syvalahti et al. 1988; Hietala et al. 1990).

A portion of ethanol's effects may relate to its action as a nonspecific membrane perturbant, altering the physical properties of the lipid components of cell membranes which may lead to changes in optimal membrane fluidity (Chin and Goldstein 1977; Sun and Sun 1985), for protein function. Neural membranes become more fluid with acute ethanol intoxication (Chin and Goldstein 1977). After chronic ethanol exposure, the membranes are resistant to the fluidizing effects of ethanol (Lyon and Goldstein 1983). This response may serve as an adaptive mechanism to compensate for the fluidizing effects of ethanol. Changes in membrane cholesterol content may be part of this adaptive mechanism because cholesterol can maintain the order of membrane phospholipids by hindering their movement (Stockton and Smith 1976; Crews et al. 1983; Yeagle 1985). Changes in membrane composition and fluidity may alter receptor-ligand interactions, receptor-effector coupling, and enzyme activity (Guerri and Grisola 1983; Yeagle 1985).
Guanine nucleotide binding proteins (G proteins) are important components in signal transduction, coupling hormone and neurotransmitter receptors to second messenger pathways (e.g. adenylate cyclase pathway, ion channels, phosphoinositide pathway) (Sternweis et al. 1981; Cockcroft 1987; Codina et al. 1987; Hescheler et al. 1987). The G proteins are heterotrimers, consisting of $\alpha$, $\beta$, and $\gamma$ subunits. The $\alpha$ subunit contains a guanine nucleotide binding site and displays GTPase activity (Northup et al. 1983).

In the adenylate cyclase system, an agonist interacts with a receptor resulting in the association of the agonist-receptor complex with the $G_s$ protein which has GDP bound to the $\alpha$ subunit. This interaction promotes the displacement of GDP allowing the binding of GTP to the $\alpha$ subunit. When GTP binds, the agonist-receptor-G protein complex dissociates. The $\alpha$ subunit bound to GTP dissociates from the $\beta\gamma$ subunits of the G protein and in turn interacts with adenylate cyclase. The activation of adenylate cyclase by the $\alpha$ subunit is terminated by hydrolysis of GTP and reassociation of the $\alpha$ subunit with the $\beta\gamma$ subunit (Gilman 1987, 1989; Freissmuth et al. 1989; Johnson and Dhanasekaran 1989).

Protein-protein interactions within the cell membrane, such as those involving G proteins, may be susceptible to
the actions of ethanol. Under acute conditions, some studies have demonstrated a selective effect of ethanol on Gs, the stimulatory guanine nucleotide binding protein, rather than on the adenylate cyclase enzyme or the receptor linked to the G protein. In studies with homogenates of mouse striatum, ethanol in vitro elicited an increase in basal adenylate cyclase activity. This effect was enhanced in the presence of dopamine and guanine nucleotides (Rabin and Molinoff 1981; Luthin and Tabakoff 1984). A similar effect was observed in cerebral cortex. Ethanol increased guanine nucleotide-stimulated and isoproterenol-stimulated adenylate cyclase activity (Saito et al. 1985).

Studies of ethanol-adenylate cyclase interactions in cultured cells have supported the findings in brain membrane preparations. In several cultured cell lines, including the N1E-115, NG 108-15, and S49 lymphoma lines, acute ethanol exposure increased basal, and to a greater extent, agonist-stimulated, guanine nucleotide-stimulated, and fluoride-stimulated adenylate cyclase activity (Rabin and Molinoff 1983; Gordon et al. 1986). It has been postulated that acutely, ethanol appears to alter the rate of activation of Gs and to enhance the interaction of α, with guanine nucleotides (Rabin and Molinoff 1981, 1983; Luthin and Tabakoff 1984).

Since ethanol increases the stimulation of adenylate cyclase under acute conditions, it is possible that there
would be an adaptive change in adenylate cyclase activity after chronic ethanol exposure. Dopamine-stimulated adenylate cyclase activity is reduced after chronic ethanol treatment in striatal membranes of mice and rats (Tabakoff and Hoffman 1979; Lucchi et al. 1983; Saffey et al. 1988). In vitro addition of ethanol to striatal membranes restored dopamine-stimulated adenylate cyclase activity to normal levels, indicating that membranes from ethanol-tolerant rats require the presence of ethanol for normal activity (Tabakoff and Hoffman 1979). In contrast, another study reported an increase in dopamine-stimulated, GTP-stimulated, and sodium fluoride-stimulated adenylate cyclase activities in the striatum of chronically ethanol-treated rats (Rabin 1987). The discrepancies may be related to different strains of rat, mode and duration of ethanol administration, or time of assessment relative to ethanol withdrawal.

Norepinephrine-stimulated adenylate cyclase activity in the cerebral cortex is affected by withdrawal from chronic ethanol exposure. During ethanol withdrawal, there was a decrease in this activity in the cerebral cortices of mice and rats (French et al. 1975; Saito et al. 1987). Also, at the time of withdrawal, the stimulation of adenylate cyclase by guanine nucleotides, isoproterenol, vasoactive intestinal peptide, and forskolin was decreased (Saito et al. 1987).

The effect of chronic ethanol exposure on adenylate
cyclase activity has also been examined in cultured cells. The neuroblastoma cell lines, N1E-115 and NG 108-15, displayed a decrease in the response of adenylate cyclase to prostaglandin E1 stimulation after chronic ethanol treatment. The addition of ethanol restored the response to control values (Richelson et al. 1986). Chronic ethanol treatment of primary cerebellar cultures resulted in a reduction in isoproterenol-stimulated and adenosine-stimulated adenylate cyclase activity (Rabin 1990).

It has been hypothesized that changes in adenylate cyclase activity after chronic ethanol treatment are indicative of an uncoupled receptor-adenylate cyclase system (Hoffman and Tabakoff 1990). Alternatively, the changes in adenylate cyclase activity following chronic ethanol exposure may also reflect qualitative or quantitative changes in the α subunit of the Gs protein. Mochly-Rosen and colleagues (1988) demonstrated a decrease in mRNA levels and protein content of the α subunit of the Gs protein after chronic ethanol treatment using the neuroblastoma cell line NG 108-15. Charness and colleagues (1988) also reported changes in G protein content in several ethanol-treated neuroblastoma cell lines. This group reported a decrease in the content of the Gs α subunit in the NG 108-15 and N1E-115 cell lines, but not in the N18TG2 cell line. They also found a
dose-dependent increase in the G\textsubscript{i} \(\alpha\) subunit in the N1E-115 cell line, with no change in the NG108-15 and N18TG2 cell lines. Although the content of G proteins is altered in neural cell lines, no significant alteration in the content of the G\textsubscript{s} \(\alpha\) subunit was found in cerebral cortical tissue from chronically treated mice (Hoffman and Tabakoff 1990). Nonetheless, it has been suggested that in vivo chronic ethanol exposure may cause posttranslational modification of the \(\alpha\) subunit which may alter its interaction with the receptor and adenylate cyclase (Hoffman and Tabakoff 1990).

Possible Genetic Basis of Alcoholism

The development of alcoholism may be determined by genetic as well as environmental factors (Tarter et al. 1985; Zucker and Gomberg 1986; Cloninger 1987). Studies, particularly twin and adoption studies, have demonstrated there is a likely genetic predisposition to alcoholism (Cotton 1979; Cloninger et al. 1981; Hrubec and Omenn 1981; Kaprio et al. 1987). Several genes are probably involved in determining susceptibility to alcoholism.

Two human studies suggest that alterations in platelet and lymphocyte adenylate cyclase may serve as markers for a genetic predisposition to alcoholism. 1) A reduction in the stimulation of adenylate cyclase activity by guanine nucleotides (36\%), fluoride (26\%), and prostaglandin E\textsubscript{1} (35\%) was observed in platelets from human alcoholic
patients (Tabakoff et al. 1988). Alcoholics with the greatest number of alcoholic first degree relatives had the largest reduction in stimulated adenylate cyclase activity. The reduced activity was also found in patients who had abstained for 1-4 years. It is possible the persistent reduction in platelet adenylate cyclase activity is an inherent characteristic of alcoholics (Tabakoff et al. 1988). 2) Lower cAMP levels (a 75% decrease) were observed in lymphocytes obtained from alcoholic patients (Diamond et al. 1987). These cells were maintained in culture in the absence of alcohol for 4-6 generations. The differences in cAMP levels were still observed and these cells were more sensitive to ethanol's effects (Nagy et al. 1988). Thus, genetic factors may influence ethanol's effect on adenylate cyclase activity.

Blum et al. (1990) recently reported an allelic association of the dopamine D2 receptor gene with alcoholism. In their patient population, the "A1" allele of the D2 receptor was found in 77% of the alcoholic patients and it was absent in 72% of the nonalcoholic patients. The authors suggest that an abnormality in the gene coding for the D2 receptor may cause susceptibility to a subtype of alcoholism. The authors also suggest that the gene for the D2 receptor may eventually be used as a diagnostic marker for the risk of alcoholism. In contrast to Blum's study of an association of the A1 allele of the D2 receptor in
alcohol-related disease in humans, a study comparing alcohol-sensitive and alcohol-insensitive rat lines, Korpi et al. (1987) find no association of the striatal dopaminergic D2 receptors with sensitivity to alcohol. Although, Blum and colleagues (1990) have provided a candidate probe with potential promise in the research on the genetics of alcoholism, future genetic studies must include a broader living human sample as well as extended families of alcoholics. Furthermore, the classification of alcohol abusers should be done by a single, cooperative group, rather than by reviewing medical records of deceased patients.

Proposed Studies

Alcoholism and alcohol abuse constitute a major health problem in the United States. However, the biochemical mechanisms by which this disease manifest itself have not been fully investigated. Among the studies which have attempted to delineate these mechanisms are those which have demonstrated that several central nervous system neurotransmitters are sensitive to alcohol consumption (Greenberg et al. 1986; Valverius et al. 1987; Celetano et al. 1988; Murphy et al. 1988; Lovinger et al. 1989). Among the neurotransmitter systems which are sensitive to ethanol is the dopamine system. The dopaminergic system is involved with locomotor activity and is recognized as a component of
the reward circuitry (Fibiger et al. 1974; Phillips and Fibiger 1978). Since ethanol does have an effect on locomotor activity and displays reinforcing properties, it is hypothesized that chronic ethanol consumption may alter dopamine signal transduction or dopamine levels. It is the objective of the research described in this dissertation to specifically examine the biochemical changes in the dopaminergic system.

This dissertation examined the effects of chronic ethanol exposure on several membrane components of the dopaminergic system, as well as the level of dopamine. The membrane components include the D1 and D2 receptors, the stimulatory and inhibitory guanine nucleotide binding proteins (G proteins), and adenylate cyclase activity. These components were measured in brain regions associated with the dopaminergic system. All studies were done on adult male Fisher 344 rats pair fed a control or 6.6% (v/v) ethanol liquid diet for one month prior to sacrifice at three months of age. This study is important because changes in dopamine levels or membrane components could result in alterations in cellular activity which could contribute to ethanol-induced changes in motor activity or to the reinforcing properties of ethanol.
CHAPTER III

METHODS

Animal Model

Male Fisher 344 rats (2 months) were used in these experiments. The animals were pair-fed using a 6.6% (v/v) ethanol liquid diet or control diet for 4-5 weeks (Noronha and Druse 1982). The caloric content of the control diet consisted of 21% protein, 29% fat, and 50% carbohydrate, the ethanol diet consisted of 21% protein, 29% fat, 15% carbohydrate, and 35% ethanol. Animals were maintained on a 12:12 hour light/dark cycle. The animals were weighed weekly to ensure equal weight gain.

Blood Ethanol Determination

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

The procedure required the deproteinization of blood samples by the addition of trichloroacetic acid (6.25%, w/v). The sample was 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) to obtain a clear supernatant. To each NAD-ADH assay vial, 2.9 ml glycine buffer (0.5 mol/L, pH 9.0) was added, and the vial was capped and inverted gently to dissolve contents. The sample (100 µl) was added to the vial, mixed by inversion, and incubated at 37°C for 10 minutes. The sample was transferred to a cuvette and covered to exclude contamination and evaporation from air. The absorbance was recorded at 340 nm. Blood samples were collected at the time of sacrifice after an overnight fast.

Dissection Technique

The animals were decapitated between 7 a.m. and 10 a.m.. The brains were quickly removed and dissected on a chilled glass plate. The frontal cortex, striatum, and nucleus accumbens were dissected. Other brain regions were dissected using the punch technique as described by Palkovits and Brownstein (1988). In the latter method brain samples were fixed to a specimen holder with the embedding medium Tissue-Tek (Miles; Elkart, IN).

The mounted brains were sectioned using a cryostat set
at -15°C. Sections of 300-500 µm thickness were sliced and placed on ice-cold slides for punching. Punching tools with 1.0 and 2.0 mm diameters were used to remove brain regions of interest. Punched areas included: globus pallidus, substantia nigra, ventral tegmental area, and ventral pallidum. Samples were stored at -80°C. All experiments were performed on frozen tissue.

**Dopamine Antagonist Binding Assay**

The dopamine (D1 and D2) binding sites in control and ethanol-treated animals were analyzed using a modified method of Hess and colleagues (1986). An antagonist binding assay measures total receptor number (high and low affinity state receptors). The D1 antagonist [3H]-SCH 23390 (New England Nuclear; Boston, MA) was used to measure total D1 sites and the D2 antagonist [3H]-spiperone (New England Nuclear; Boston, MA) was used to measure total D2 sites.

Brain tissue was homogenized in 40 volumes of cold 50 mM Tris-HCl buffer (pH 7.4) using a motorized teflon homogenizer (6 up and down strokes, setting 9) (Caframo RZR3; Warton, Ontario). The tissue was incubated for 15 minutes at 37°C to remove endogenous dopamine, followed by centrifugation (SS34 rotor, Sorvall RC-5B centrifuge) at 30,900 x g (16,000 rpm) for 20 minutes at 4°C. The pellet was resuspended in Tris-HCl buffer and centrifuged again as described above. This wash was repeated. The final pellet
was suspended in assay buffer containing: 50 mM Tris-HCl, 5 mM MgSO₄, 0.5 mM EDTA, and 0.02% (w/v) ascorbic acid (pH 7.4).

The D1 binding assay was performed using a saturation-competition assay containing 0.0-0.5 nM [³H]-SCH 23390 and 0.0-5.0 nM SCH 23390. Nonspecific binding was determined by the dopamine antagonist, (+)-butaclamol. The D2 binding assay was performed using 0-1.0 nM [³H]-spiperone in the presence of 40 nM ketanserin to block serotonin sites (5-HT₂ sites). Nonspecific binding was determined using 1 µM (+)-butaclamol.

The reaction was initiated with the addition of tissue homogenates (0.1 to 0.3 mg protein/assay tube) (triplicate samples) and incubated for 30 minutes at 37°C. The reaction was terminated by vacuum filtration using a Brandel Cell Harvester (Gaithersburg, MD) with Whatman GF/C filters followed by three 5 milliliter (ml) washes with cold Tris-HCl buffer. Filters were placed in scintillation vials containing 10 ml Bio-Safe II (RPI; Mt. Prospect, IL). The radioactivity on the filters was determined by liquid scintillation counting by the Beckman LS 7500 counter (Beckman, Fullerton, CA).

The kinetic parameters (Kd and Bmax) were calculated by computer-assisted Scatchard analysis developed in this laboratory. The Scatchard plot is a linear transformation of the saturation isotherm. The x-axis is represented by bound
values and the y-axis is represented by the bound/free values. From this plot, the Bmax (receptor number) is the x-intercept and the Kd (affinity) is -1/slope.

**Dopamine Agonist Binding Assay**

The agonist binding assays were performed using a modification of the methods of Hess et al. (1986) and Sibley et al. (1982). Agonist binding was used to measure the percentage of receptors in the high affinity state and the ability of guanine nucleotides to convert the receptors from the high affinity state to the low affinity state. The D2 receptors were measured using the D2 agonist, \([^3]H\)-N-n-propylnorapomorphine (NPA) (New England Nuclear; Boston, MA). The assay conditions were similar to that described previously for the antagonist binding assay, however, the assay buffer for the NPA studies also contained 120 mM NaCl to stabilize the high affinity state. In order to measure the conversion of the D2 receptor from the high affinity state to the low affinity state, assays were performed in the absence and presence of 300 µM GTP.

**Adenylate Cyclase Assay**

Basal and dopamine-sensitive adenylate cyclase activity of brain regions obtained from control and ethanol-treated animals were assessed by a modification of the methods of Tabakoff and Hoffman (1979) and Battaglia et al. (1986).
Adenylate cyclase activity was measured in the striatum, nucleus accumbens, and frontal cortex. Samples were homogenized in 50 volumes of ice-cold homogenization buffer using a Tekmar Tissumizer (Cincinnati, OH) (setting 6, 10 seconds). The homogenization buffer contained 10 mM Tris-maleate (pH 7.4) and 2.0 mM EGTA. The samples were centrifuged (SS34 rotor, Sorvall RC-5B centrifuge) at 30,900 x g (16,000 rpm) for 10 minutes (4°C). The pellet was washed with homogenization buffer and centrifuged as described above. The final pellet was resuspended in the homogenization buffer.

The assay buffer contained 80 mM Tris-maleate (pH 7.4), 0.4 mM EGTA, 4.0 mM MgSO₄, 1.0 mM isobutylmethylxanthine (IBMX), 5.0 mM phosphocreatine, 0.02% (w/v) ascorbic acid, and 50 U/ml creatine kinase. Triplicate samples were preincubated in assay buffer for 5 minutes at 37°C in the presence (dopamine-stimulated/dopamine-inhibited) or absence (basal) of 100 µM dopamine. To measure D₁-stimulated adenylate cyclase activity, 50 nM spiperone was added to block D₂ sites, while 100 nM SCH 23390 was used to block D₁ sites in assays of D₂-inhibited adenylate cyclase activity. Basal adenylate cyclase activity was also measured in the presence of 100 µM forskolin, 10 mM NaF (sodium fluoride), or 100 µM GTP to determine the site of any ethanol effect. The reaction was initiated with the addition of 1 mM ATP (adenosine triphosphate) and allowed to proceed for
5 minutes at 37°C. The reaction was terminated by the addition of 50 nM sodium acetate buffer (pH 6.2) and immersion in boiling water for 3 minutes. Precipitated protein was removed by centrifugation (SE12 rotor, Sorvall RC-5B centrifuge) at 21,800 x g (16,000 rpm) for 15 minutes (4°C). The supernatant was collected and stored at -80°C for measurement of cAMP levels by radioimmunoassay (RIA).

**cAMP Radioimmunoassay**

The cAMP radioimmunoassay was a modified method of Steiner (1972). Samples and standards were acetylated to resemble the antigen used to generate the antibody for cAMP and to increase the sensitivity of the assay. Samples and standards were acetylated with the addition of triethylamine (20 µl) and acetic anhydride (10 µl). Duplicate acetylated standards (0 to 500 fmoles of cAMP) and samples were transferred to 12 x 75 mm glass test tubes and [125I]-cAMP (0.01 µCi) in 50 mM sodium acetate (pH 6.2) was added to each sample. The reaction was initiated with the addition of anti-cAMP antibody in sodium acetate buffer. The samples were incubated overnight (20°C). The assay was terminated with the addition of 1% (w/v) gamma globulin (50 µl) and 95% (v/v) isopropanol (2 ml). The samples were centrifuged for 30 minutes at 1,623 x g (2800 rpm) in a Jouan GR4.11 centrifuge (4°C). The supernatant was decanted and the radioactivity was measured by a LKB Wallac 1272 CliniGamma
gamma counter.

**Transformation/Plasmid Preparation**

Lyophilized samples containing the cDNA probes for the \( \alpha \) subunits of the G proteins (in the pGEM-2 plasmid) were obtained from Dr. Randall Reed (Jones and Reed 1987). The samples were diluted in Tris-EDTA buffer (10 mM Tris, 2.0 mM EDTA) and incubated for 5 minutes at 65°C. The samples were diluted again to a 1:20 dilution and used for transformation.

The transformation procedure was the method of Chung et al. (1989). Microcentrifuge tubes were chilled on ice and 100 µl of cells [\( \text{E. coli HB101/F}^+; (\text{F}^+=\text{Tn 10:Kan recA}) \)] from Dr. Mark Kelley and 1 µl of cDNA were added. Samples were mixed gently and incubated for 30 minutes at 4°C. After the incubation, LB broth (5 g NaCl, 10 g tryptone, 5 g yeast extract/1L) was added and the cells were incubated for 30 minutes at 37°C. The cells were plated onto ampicilllin plates and incubated at 37°C overnight. A single colony from the transformation plate was streaked onto a new ampicilllin plate. The plate was incubated at 37°C overnight and served as the master plate. A single colony from the transformation plate was added to culture tubes containing LB broth and ampicillin. The cells were incubated at 37°C overnight. This preparation was used in the plasma preparation.

The plasmid preparation (Morelle 1989) extracts the
plasmid DNA from the bacterial cells. The cells in the LB broth were aliquoted into microcentrifuge tubes and centrifuged for 1 minute at 10,000 x g (10,000 rpm) in a tabletop centrifuge (mc15, Hill Scientific, Derby, CT). The supernatant was removed by aspiration. The pellet was suspended in 250 µl of Tris-EDTA buffer (50 mM Tris, 100 mM EDTA) and 250 µl of NaOH/SDS buffer (200 mM NaOH, 1% (w/v) SDS) was added. After incubation for 5 minutes at room temperature, 250 µl of potassium acetate (2.55 M) buffer was added and the sample was centrifuged for 15 minutes at 4°C. The supernatant was transferred to another microcentrifuge tube and 400 µl of isopropanol (60%, v/v) was added. After 5 minutes at room temperature, the sample was centrifuged for 15 minutes at 10,000 x g (10,000 rpm) in a tabletop centrifuge (mc15; Hill Scientific). The supernatant was removed by aspiration and the pellet was washed with 70% (v/v) ethanol by inversion. Following aspiration of ethanol, the pellet was incubated in a heat block (65°C) to evaporate the remaining ethanol. The pellet was suspended in Tris-EDTA (10 mM Tris, 1.0 mM EDTA) buffer. The cDNA probes were subjected to a DNA digest by Eco RI and separated on a 0.7% (w/v) agarose gel to ensure purity. These samples of the cDNA probes for the G proteins were used for hybridization.
RNA Extraction and Analysis

Total RNA was extracted from brain regions of control and ethanol-treated animals using the modified method of Chomczynski and Sacchi (1987). The tissue was homogenized in guanidine isothiocyanate buffer (4.0 M guanidine isothiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, and 0.5% (w/v) sarcosyl) and allowed to stand for 5 minutes. The samples were transferred to microcentrifuge tubes. To these samples, 50 µl of 2 M sodium acetate (pH 4.0), 500 µl phenol, and 100 µl chloroform:isoamyl alcohol (49:1, v/v) were added. Samples were placed on ice for 15 minutes and then centrifuged for 20 minutes at 10,000 x g (10,000 rpm) in a tabletop centrifuge (mc15, Hill Scientific). The aqueous layer was transferred to a new microcentrifuge tube and an equal volume of isopropanol was added. The sample was then placed in a dry ice/methanol bath (-70°C) for one hour. The sample was centrifuged as described above. Following aspiration of isopropanol, the pellet was resuspended in guanidine isothiocyanate buffer. The sample was incubated in a heat block (65°C) for 5 minutes. To this sample, 10 µl 3.0 M sodium acetate (pH 5.2) and 100 µl isopropanol were added, prior to cooling on dry ice for 10 minutes. The sample was centrifuged as described and the supernatant was removed by aspiration. The pellet was washed with 70% (v/v) ethanol and centrifuged for 10 minutes at 10,000 x g (10,000 rpm) in a
tabletop centrifuge (mc15, Hill Scientific). The pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated water and incubated in a heat block (65°C) for 5 minutes. Samples were stored at -20°C. The amount of RNA extracted was determined by a spectrophotometer (Spectronic 21D, Milton Roy Co.) reading (260 nm).

Equal amounts of RNA in DEPC-treated water were diluted to 1:10 and 1:1000 in microcentrifuge tubes. Formaldehyde (37%, v/v) in a 3.0 M sodium chloride/0.3 M sodium citrate (20X SSC) buffer (1:1) was added to each sample and the samples were incubated in a heat block (65°C) for 15 minutes. The samples were loaded into the wells of the Manifold II with Nytran filter (0.45 µm) (Scheicher & Schuell; Keene, NH) and vacuum filtered. The wells were washed with 10X SSC buffer to remove the formaldehyde. The RNA was cross-linked to the Nytran using the UV Stratalinker (Stratagene; La Jolla, CA).

Hybridization Procedure

The levels of G protein mRNA from control and ethanol-treated animals were measured using cDNA probes for the α subunits of the G proteins (Jones and Reed 1987). The Nytran filter was prehybridized for 2 hours under high stringency conditions in a buffer containing: 50% (v/v) formamide, 100x Denhardt's solution [2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin, and 2% (w/v) Ficoll 400], 20% (v/v)
salmon sperm DNA (10mg/ml), and a Na/PO₄ (5.0 M NaCl, 50 mM Na₂PO₄, and 0.5% (w/v) pyrophosphate) buffer. The cDNA probes isolated in the plasmid preparation were labeled with [³²P]-CTP by random priming (Feinberg and Vogelstein 1983). The cDNA (2 µl) was diluted in 28 µl sterile distilled water and denatured at 100°C for 3 minutes. The sample was chilled on ice for 5 minutes. To the sample, 10 µl labeling buffer (250 mM Tris, 25 mM MgCl₂, 10 mM DTT, and 1 mM HEPES), 2 µl nonlabeling dNTP's (1.5 mM each ATP, GTP, TTP), 2 µl acetylated BSA (1 mg/ml), 1 µl Klenow (5 U), and 5 µl α-[³²P]-dCTP were added and the sample was incubated at room temperature for one hour. The sample was heated to 100°C for 2 minutes, chilled on ice, and 2 µl 0.5 M EDTA and 48 µl sterile distilled water were added. The sample was added to a Sephadex G50 column and centrifuged for 5 minutes at 1000 x g (2000 rpm) (Beckman TJ-6 centrifuge). The ³²P-labeled cDNA was added to the prehybridization buffer and the filter was incubated overnight at 42°C on a rotator. After hybridization, the filter was washed three times (65°C) to remove excess labeled probe with a buffer containing 0.5% (v/v) SDS and 0.2x SSC (high stringency). The filter was blotted dry and exposed on film at -80°C (Kodak XAR-5; Eastman Kodak Co., Rochester, N.Y.). The filters were stripped to remove all labeled probe. The stripping buffer contained 3.0 M sodium chloride, 0.2 M sodium phosphate, and 0.02 M EDTA (20X SSPE) and SDS. The
filters were washed three times in boiling stripping buffer and exposed on film at -80°C. The filters were reprobed with cDNA probes for other G proteins and actin. Actin served as a control.

Western Analysis

G protein content in control and ethanol-treated animals was measured by Western analysis. Tissue samples were homogenized in a TSA (0.01 M Tris (pH 8.0), 0.14 M NaCl, and 0.025% (w/v) NaN₃) buffer containing: 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetamide, and 0.2 U/ml aprotinin. The samples were diluted 1:1 in a loading buffer (40 mM Tris, 4% (v/v) mercaptoethanol, 1% (w/v) SDS, 40% (v/v) glycerol, and Bromophenol Blue). The samples were boiled for 5 minutes and then centrifuged for 5 minutes in a tabletop centrifuge (mc15; Hill Scientific). The samples (20 µg protein) and molecular weight markers (Bio-Rad; Richmond, CA) were loaded onto a 10% (w/v) acrylamide gel (30% (w/v) acrylamide, 8% (w/v) N,N'-methylene-bisacrylamide, 4x Tris/SDS (pH 8.8), 10% (v/v) ammonium persulfate, and TEMED) using the Bio-Rad Protein II gel apparatus (Laemmli 1970). The gel was run at 150 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 either stained with Coomassie Blue (0.05% (w/v) Coomassie Blue, 20% (v/v) methanol, 10% (v/v) acetic
acid) or transferred to a nitrocellulose filter (0.2 µm) (Schleicher & Schuell; Keene, NH) by a Bio-Rad Mini Trans-Blot electrophoretic transfer cell. Prior to transfer, gels and nitrocellulose filters were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) for 20 minutes. Protein transfer was completed by 45 minutes at 150 mA (constant current). The filter was washed in TBST (100 mM Tris base, 1.5M NaCl, and 5 ml Tween 20) buffer for 5 minutes, followed by a one hour incubation in blot buffer (1% (v/v) TBST, 5% (w/v) powdered milk, 2 ml Nonidet P-40, 0.02% (w/v) SDS). After blot incubation, the primary antibody (anti-Гα8 or anti-Гα0, Dupont; Boston, MA), diluted 1:1000 in blot buffer, was added to the filter and the filter was incubated overnight on a rotator at 4°C. The filter was washed three times for 15 minutes in TBST buffer. The second antibody (125I]-anti-rabbit IgG, Amersham; Arlington Heights, IL), diluted to 0.1 µCi/ml in blot buffer, was added to the filter and the filter was incubated for 3 hours at room temperature on a rotator. The filter was washed three times for 15 minutes in TBST buffer. The filter was blotted dry and placed on film (Kodak XAR-5) and exposed at -80°C. Actin also served as the control in Western analysis. Actin monoclonal antibody (ICN; Costa Mesa, CA) was used as the primary antibody and [125I]-anti-mouse IgG (ICN; Costa Mesa, CA) was used as the second antibody.
High Performance Liquid Chromatography

Dopamine and DOPAC (3,4-dihydroxyphenylacetic acid) levels were measured in control and ethanol-treated animals by high performance liquid chromatography (HPLC). Tissue samples were extracted in 0.1 M perchloric acid. The larger brain regions were homogenized by the Tekmar Tissumizer (setting 6, 10 seconds) (Tekmar Co., Cincinnati, OH). The other brain regions were homogenized by a motorized teflon homogenizer (Deltaware, Kimble Science Products). The samples were centrifuged at 21,800 x g (16,000 rpm) for 20 minutes at 4°C (SE12 rotor, Sorvall RC-5B centrifuge). 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 contained 0.1 M sodium phosphate-citric acid (pH 4.0), 1.0 mM disodium EDTA, 1 mM heptanesulfonic acid, and 7% (v/v) acetonitrile (Hortnagal et al. 1989). The internal standard 3,4-dihydroxybenzylamine (DHBA) was included in each sample. The flow rate was 0.7 ml/min and the sample run time was approximately 35 minutes. Six to eight individual samples were measured for each brain region. The HPLC system consisted of a Bio-Rad (Richmond, CA) pump, BAS (West Lafayette, IN) Amperometric detector LC-4B, and a Hewlett Packard (Avondale, PA) 3390A integrator.

Synaptic Plasma Membrane Preparation

Synaptic plasma membranes (SPM) were prepared from
whole brain from control and ethanol-treated animals by the method of Cotman and Matthews (1971). The preparation was maintained at 4°C. The brains were removed and homogenized (20%, w/v) in ice cold 0.32 M sucrose, 5.0 mM Tris (pH 7.0) buffer (sucrose buffer) using a motorized teflon homogenizer (Caframo RZR3; Warton, Ontario) (6 up and down strokes, setting 9). The homogenate was diluted to 10% (w/v) and centrifuged at 1100 × g (3000 rpm) for 5 minutes (SS34 rotor, Sorvall RC-5B centrifuge). The supernatant was decanted into another centrifuge tube and pelleted at 17,000 × g (13,700 rpm) for 10 minutes. The pellet was resuspended in sucrose buffer (10%, w/v) using a round bottom glass homogenizer and centrifuged for 20 minutes at 11,000 × g (11,000 rpm). The pellet was suspended in 5 ml of sucrose buffer and layered onto a discontinuous Ficoll-sucrose gradient consisting of 13 ml of 7.5% (w/v) Ficoll 400 in sucrose buffer and 13 ml of 13.0% Ficoll 400 in sucrose buffer. The gradient was centrifuged at 63,581 × g (22,000 rpm) for 45 minutes (SW-28 rotor, Beckman L8-M ultracentrifuge). The synaptosomal material, found at the 7.5% /13.0% Ficoll-sucrose interface, was removed and diluted with 4 volumes of sucrose buffer, followed by centrifugation at 106,000 × g (24,000 rpm) for 30 minutes. This synaptosomal pellet was suspended in a small volume of sucrose buffer, osmotically shocked in 6 volumes of 6.0 mM Tris, pH 8.1 for 1.5 hours, and
centrifuged for 15 minutes at 54,500 x g (20,000 rpm). The resulting pellet was resuspended in 5 ml of sucrose buffer and layered onto a discontinuous sucrose gradient consisting of the following concentrations in ascending order: 38.0% (w/v) (7.5 ml), 35.0% (w/v) (7.5 ml), 32.5% (w/v) (7.5 ml), and 25% (w/v) (7.5 ml). This gradient was centrifuged for 1.5 hours at 106,000 x g (24,250 rpm). The synaptic plasma membranes (SPM) were found at the 25% /32.5% interface. This layer was removed, diluted with 4 volumes of sucrose buffer, and centrifuged at 106,000 x g (24,250 rpm) for 30 minutes. The final pellet was suspended in sucrose buffer and stored at -80°C. Cholesterol and phospholipid content was measured in the SPM preparation.

**Phosphorus Assay**

The SPM lipids were extracted with chloroform:methanol (2:1, v/v) and dried under nitrogen (Folch et al. 1957) before measuring the phosphorus content by a modification of the method of Fiske and Subbarrow (1925). To each triplicate sample, 100 µl of perchloric acid (70%, v/v) was added. The samples were heated by a heat block to 170°C until colorless. After cooling, 1 ml of distilled water was added to each sample. Standards (0 to 40 µg phosphorus) were prepared from a stock solution of 1.3 mM KH₂PO₄ (1 ml equals 40 µg phosphorus). Perchloric acid (100 µl) and distilled water (1 ml) were also added to each standard. To each
sample and standard were added 250 µl of 2.5% (w/v) ammonium molybdate, 100 µl of reducer (3 g Na₂SO₃·anhydrous, 60 g NaHSO₃, and 1 g 1-amino-2-naphthol-4-sulphonic acid in 500 ml distilled water), and distilled water (to a final volume of 2.5 ml). Samples and standards were allowed to stand at room temperature for 10 minutes. The O.D. of the samples and standards was determined at 625 nm.

Cholesterol Assay

Cholesterol levels in the SPM preparations were assayed using the Sigma Diagnostic Kit (#351, St. Louis, MO). The Sigma procedure involves a series of enzymatic reactions coupled with the p-hydroxybenzenesulfonate and 4-aminoantipyrine chromogenic system. In the last step of the reaction series, hydrogen peroxide reacts with 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase to yield a quinoneimine dye, which has a maximum absorbance at 500 nm.

The micro method (1 ml reaction volume) was used to measure total cholesterol in the synaptic plasma membrane (SPM) preparations from control and ethanol-treated animals. To each cuvette, 100 µl of SPM sample (in triplicate) and 1 ml of cholesterol assay solution were added. The cuvettes were covered immediately and mixed by inversion. The cuvettes were incubated for 10 minutes at 37°C. The absorbance at 500 nm was recorded immediately after the
incubation. Sample values were calculated based on the absorbance of the cholesterol standard (200 mg/dL).

**Protein Determination**

Protein levels were assayed following the method of Lowry et al. (1951). Human serum albumin (1 mg/ml) served as the protein standard. The standard curve consisted of concentrations of 0-50 µg protein. Standards and samples were incubated in 100 µl of 1.0 N NaOH for 30 minutes at room temperature to solubilize membrane bound proteins. 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 O.D. of the standards and samples was determined at 700 nm.

**Statistical Analysis**

The values reported for dopamine and DOPAC concentrations, adenylate cyclase activity, G protein content, and cholesterol and phospholipid levels represent the mean value ± the standard deviation of several samples. Student's 't' test was used to determine statistical significance. P values of <0.05 represent significant differences.

Certain types of experiments yield day to day
variability in control values. Nonetheless, a comparison of multiple studies of paired analyses of experimental and control animals may consistently show the same effect (e.g. an increase or decrease). To compensate for the day to day variability in control values, a pair of samples for control and ethanol-treated rats were assayed on the same day with the same assay conditions. Multiple paired samples were statistically analyzed utilizing the t-test for paired comparisons. This type of analysis was used for the dopamine D1 and D2 binding assays and for the slot blot analysis of G protein mRNA levels.
<table>
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SCH 23390
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Sodium bisulfate
Sodium carbonate
Sodium chloride
Sodium dodecyl sulfate
Sodium fluoride
Sodium hydroxide
Sodium phosphate
Sodium potassium tartrate
Sodium sulfite
Spiperone
[³H]-Spiperone
Sucrose
TEMED
Tetrasodium pyrophosphate
Triethylamine
Tris
Tris-HCL
Triton X-100
Trizma base
Tryptone
Tween-20
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CHAPTER IV

RESULTS

Animal Model

This study examined Fisher 344 male rats that were pair-fed a control or ethanol liquid diet for 4-5 weeks (chronic exposure) prior to sacrifice at three months. The animals consumed 80-100 ml/day which corresponds to 20-25 g ethanol/kg weight for the ethanol-treated animals. The initial weights of the two-month old rats were 172.2 ± 30.6 grams for the control animals and 174.4 ± 31.0 grams for the ethanol-fed animals. After diet consumption for 4-5 weeks, the weights of the control animals were 238.1 ± 31.0 grams and the weights of the ethanol-treated animals were 233.8 ± 31.3 grams, reflecting comparable weight gain (Figure 1). The final weights were significantly greater than the initial weights (p<0.05). Blood ethanol levels of the ethanol-fed animals ranged between 80-130 mg/dL following a 90 minute exposure to ethanol. Ethanol was undetectable in the control animals.

Dopamine D1 Receptors

The D1 receptors were assessed using the radioligand SCH 23390, a D1 antagonist which binds to both the high and
Figure 1. Weights of control and ethanol-treated animals.

Weights of control and ethanol-treated animals are shown. This data represents the mean weight ± the standard deviation obtained from 130 ethanol-treated animals and 120 control animals. The initial (two months of age) and final weights (12 to 13 weeks of age) of control and experimental animals were compared using Student's 't' test. There was no significant difference in the initial weights between control and ethanol-treated rats before diet consumption nor after 4-5 weeks of pair-feeding (p>0.05).
ANIMAL WEIGHTS

GRAMS

CONTROL
ETHANOL

INITIAL WEIGHTS
4–5 WEEKS DIET

FIGURE 1
low affinity sites of the D1 receptor, in several brain regions of control and ethanol-treated animals. The regions analyzed include the frontal cortex, the striatum, the nucleus accumbens, and the globus pallidus.

Typical binding assays for each brain region are depicted in Figures 2 to 5. The figures represent saturation curves. The x-axis represents ligand concentration (nM), while the y-axis indicates specific binding (pmol/mg protein). Scatchard plots are shown in the inset. In Scatchard plots, the x- and y-axes stand for bound and bound/free, respectively. Nonspecific binding of SCH 23390, determined in the presence of 1 μM (+)-butaclamol, accounted for 10-15% of the total bound radioactivity in the striatum and nucleus accumbens. Nonspecific binding was approximately 25% in the globus pallidus and 25-30% in the frontal cortex. Under the assay conditions established in this laboratory, the Kd values ranged from 0.3 nM in the globus pallidus to 2.0 nM in the frontal cortex. For a typical assay, an attempt was made to assess binding at ligand concentrations ranging from 1/8-1/10 Kd to 8-10 Kd. For the SCH 23390 binding assays, several ligand concentrations between 0.05 nM to 5.0 nM were selected. These concentrations were generally sufficient to establish saturation. In the frontal cortex, an apparent second site was detected at ligand concentrations greater than 6.0 nM. However, saturation was established at ligand concentrations of 4.0 to 5.5 nM. A
Figure 2. Binding of SCH 23390 to membranes from the nucleus accumbens of control and ethanol-treated animals.

This figure represents a typical study of SCH 23390 binding to D1 receptors in membranes from the nucleus accumbens. The saturation curve depicts the binding of 0.0 nM to 0.5 nM [³H]-SCH 23390 and the binding of 0.5 nM [³H]-SCH 23390 in the presence of several concentrations (0.0 nM to 5.0 nM) of unlabelled SCH 23390. Membranes from three control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 μM (+)-butaclamol, accounted for 10-15% of the total binding at each ligand concentration. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

\[
\begin{align*}
\text{Kd: } & \quad 0.802 \pm 0.088 \text{ nM (control)} \\
& \quad 0.601 \pm 0.055 \text{ nM (ethanol)} \\
\text{Bmax: } & \quad 0.788 \pm 0.051 \text{ pmol/mg protein (control)} \\
& \quad 0.476 \pm 0.024 \text{ pmol/mg protein (ethanol)}
\end{align*}
\]

The differences in the Kd and Bmax values obtained from six similar paired experiments were statistically analyzed using the t-test for paired comparisons. It was found that the Bmax for D1 binding sites was significantly decreased (p<0.05) in the nucleus accumbens of ethanol-treated animals. There were no significant differences in the Kd values (p>0.05).
NUCLEUS ACCUMBENS

Graph showing specific binding (pmol/mg protein) against SCH 23390 (nM) with two curves: one for ETHANOL (open circles) and one for CONTROL (solid circles). The inset on the right shows a plot of bound/free vs. bound for both groups.
Figure 3. Binding of SCH 23390 to cortical membranes from control and ethanol-treated animals.

This figure represents a typical study of SCH 23390 binding to D1 receptors in membranes of the frontal cortex. The saturation curve depicts the binding of 0.0 nM to 0.5 nM $[^3H]$-SCH 23390 and the binding of 0.5 nM $[^3H]$-SCH 23390 in the presence of several concentrations (0.0 nM to 5.0 nM) of unlabelled SCH 23390. Membranes from three control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 µM (+)-butaclamol, accounted for 25-30% of the total binding at each ligand concentration. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

Kd: $2.422 \pm .257$ nM (control)  
$2.282 \pm .461$ nM (ethanol)

Bmax: $0.251 \pm .017$ pmol/mg protein (control)  
$0.209 \pm .022$ pmol/mg protein (ethanol)

The differences in the Kd and Bmax values obtained from six similar paired experiments were statistically analyzed using the t-test for paired comparisons. It was found that the Bmax for D1 binding sites was significantly decreased (p<0.05) in the frontal cortex of ethanol-treated animals. There were no significant differences in the Kd values (p>0.05).
FRONTAL CORTEX

![Graph showing specific binding (pmol/mg protein) against SCH 23390 (nM). The graph compares data for ethanol (O) and control (●) conditions.](image)
Figure 4. Binding of SCH 23390 to striatal membranes of control and ethanol-treated animals.

This figure represents a typical study of SCH 23390 binding to D1 receptors in striatal membranes. The saturation curve depicts the specific binding of 0.0 nM to 0.5 nM $[^3]$H-SCH 23390 and the binding of 0.5 nM $[^3]$H-SCH 23390 in the presence of several concentrations (0.0 nM to 5.0 nM) of unlabelled SCH 23390. Membranes from three control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 uM (+)-butaclamol, accounted for 10-15% of the total binding at each ligand concentration. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

$$K_d: 0.681 \pm 0.029 \text{ nM (control)}$$
$$0.708 \pm 0.025 \text{ nM (ethanol)}$$

$$B_{max}: 0.420 \pm 0.009 \text{ pmol/mg protein (control)}$$
$$0.398 \pm 0.007 \text{ pmol/mg protein (ethanol)}$$

The differences in the Kd and Bmax values obtained from six similar paired experiments were statistically analyzed using the t-test for paired comparisons. It was found that neither the Kd nor the Bmax were significantly different in striatal membranes of ethanol-treated animals ($p>0.05$).
Figure 4

STRIATUM

- ETHANOL
- CONTROL

SP. BINDING (PMOL/MG PROTEIN)

SCH 23390 (nM)
Figure 5. Binding of SCH 23390 to membranes from the globus pallidus of control and ethanol-treated animals.

This figure represents a typical study of SCH 23390 binding to D1 receptors in membranes from the globus pallidus. The binding of SCH 23390 to membranes isolated from the globus pallidus was assayed in duplicate at each ligand concentration. The saturation curve depicts the binding of 0.0 nM to 1.0 nM of [3H]-SCH 23390. Membranes from five control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 µM (+)-butaclamol, accounted for 25% of the total binding at each ligand concentration. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

Kd: 0.277 ± 0.048 nM (control)
0.295 ± 0.043 nM (ethanol)

Bmax: 0.306 ± 0.028 pmol/mg protein (control)
0.318 ± 0.025 pmol/mg protein (ethanol)

The differences in Kd and Bmax values obtained from four similar paired studies were statistically analyzed using the t-test for paired comparisons. It was found that neither the Kd nor the Bmax were significantly different in membranes of ethanol-treated animals (p>0.05).
GLOBUS PALLIDUS

**FIGURE 5**

- **O** ETHANOL
- **○** CONTROL

**SP. BINDING (PMOL/µG PROTEIN)**

![Graph showing the relationship between SCH 23390 (nM) and specific binding](image)

**SCH 23390 (nM)**

- 0.0
- 0.2
- 0.4
- 0.6
- 0.8
- 1.0

**BOUND**

- 0.0
- 0.1
- 0.2
- 0.3
- 0.4

**BOUND PERCENT (µg)**

- 0.0
- 5.0
- 10.0
- 15.0
component of SCH 23390 binding in the frontal cortex may be the serotonin (5-HT₂) receptor (Hess et al. 1986). Binding assays for the frontal cortex were repeated in the presence of 40 nM ketanserin, a 5-HT₂ antagonist, to discriminate multiple binding sites (Figure 6). For all binding assays, the Kd values were calculated from the slope and Bmax values from the x-intercept of the Scatchard plot utilizing linear regression. The linear fit of the Scatchard plots represent correlation coefficients of at least 0.9.

Dopamine D1 binding sites were measured at multiple ligand concentrations when sufficient tissue was available. However, in quantitatively small regions, binding was initially examined using five three-point analyses. The three concentrations of [³H]-SCH 23390 represented approximately 1/5 Kd, Kd, and 5 Kd values. Four saturation analyses were performed to confirm the observations made using the three point analyses. The t-test for paired comparisons demonstrated that the Bmax was decreased approximately 40% in the nucleus accumbens and 25% in the frontal cortex (p<0.05). Figure 13 demonstrates that the ketanserin competition for [³H]-SCH 23390 binding in the frontal cortex represents a single binding site; therefore, the loss of D1 receptors in the frontal cortex appears to be only a loss of the D1 sites, not the serotonin sites. In contrast, there were no significant differences in either the Kd or Bmax for SCH 23390 binding to membranes from the
This figure represents the binding of SCH 23390 in the presence of 40 nM ketanserin, a 5-HT₂ antagonist, to the D₁ receptors in membranes from the frontal cortex. The saturation curve depicts the binding of 0.0 nM to 0.5 nM [³H]-SCH 23390 and the binding of 0.5 nM [³H]-SCH 23390 in the presence of several concentrations (0.0 nM to 5.0 nM) of unlabelled SCH 23390. The nonspecific binding, determined in the presence of 1 µM (+)-butaclamol, accounted for 35-40% of the total binding at each ligand concentration. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

Kd: 2.078 ± .140 nM (control)  
1.966 ± .164 nM (ethanol)

Bmax: 0.216 ± .010 pmol/mg protein (control)  
0.175 ± .010 pmol/mg protein (ethanol)

This experiment demonstrates that the ketanserin competition for SCH 23390 binding in the frontal cortex represents a single binding site. It was found that the Bmax for D₁ binding sites in the presence of ketanserin was decreased (20%) in the frontal cortex of ethanol-treated animals, as observed in previous experiments.
FRONTAL CORTEX

SP. BINDING (PMOL/MG PROTEIN)

0.00  0.05  0.10  0.15  0.20

0.0  2.0  4.0  6.0

SCH 23390 (nM)

ETHANOL

CONTROL

BOUND/FREE (x 10^-5)

0.0  0.5  1.0  1.5  2.0

BOUND

FIGURE 6
striatum or globus pallidus of three-month old ethanol-treated rats \( (p>0.05) \). In addition, there was a nonsignificant trend towards increased affinity (decreased \( K_d \)) in the nucleus accumbens in all experiments. Under the assay conditions described, binding sites for SCH 23390 were not detectable in the substantia nigra, ventral pallidum, and ventral tegmental area. Others have detected D1 sites in these areas utilizing quantitative autoradiography (Boyson et al. 1986; Mansour et al. 1990).

This analysis has demonstrated that three-month old Fisher 344 rats, fed an ethanol liquid diet on a chronic basis, had a significant decrease in the \( B_{\text{max}} \) for SCH 23390 binding in the nucleus accumbens and frontal cortex \( (p<0.05) \).

Dopamine D2 Binding Assay

The radioligand \( [^3\text{H}]\)-spiperone was used to measure total dopamine D2 sites in the striatum and nucleus accumbens of control and ethanol-treated animals. Figures 7 and 8 represent typical binding assays in which D2 binding sites were measured in the striatum and nucleus accumbens, respectively. The D2 sites were labeled with \( [^3\text{H}]\)-spiperone in the presence of 40 nM ketanserin to block 5-HT\(_2\) sites. Nonspecific binding, determined by 1 \( \mu \text{M} \) (+)-butaclamol, accounted for 15\% to 35\% of the total binding at the lowest to the highest concentration in the striatum and 25\% to 40\%
This figure represents a typical study of spiperone binding to D2 receptors in striatal membranes. The saturation curve depicts the binding of 0.0 nM to 0.9 nM [3H]-spiperone. Membranes from three control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 μM (+)-butaclamol, represented 20% of the total binding. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

Kd: 0.154 ± .012 nM (control)  
0.121 ± .014 nM (ethanol)

Bmax: 0.248 ± .009 pmol/mg protein (control)  
0.252 ± .016 pmol/mg protein (ethanol)

The differences in the Kd and Bmax values obtained from six similar paired experiments were statistically analyzed using the t-test for paired comparisons. It was found that neither the Kd nor the Bmax were significantly different in striatal membranes of three-month-old ethanol-treated animals (p>0.05).
Figure 8. Binding of spiperone to membranes from the nucleus accumbens of control and ethanol-treated animals.

This figure represents a typical study of spiperone binding to D2 receptors in membranes of the nucleus accumbens. The saturation curve depicts the binding of 0.0 nM to 0.8 nM $[^3H]$-spiperone. Membranes from three control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 µM (+)-butaclamol, represented 30% of the total binding. The Kd and Bmax ± values the standard deviation, derived from the linear regression of the data points, were:

\[
\begin{align*}
\text{Kd:} & \quad 0.083 \pm 0.012 \text{ nM (control)} \\
& \quad 0.082 \pm 0.006 \text{ nM (ethanol)} \\
\text{Bmax:} & \quad 0.129 \pm 0.009 \text{ pmol/mg protein (control)} \\
& \quad 0.112 \pm 0.004 \text{ pmol/mg protein (ethanol)}
\end{align*}
\]

The differences in the Kd and Bmax values obtained from six similar paired experiments were statistically analyzed using the t-test for paired comparisons. It was found that neither the Kd nor the Bmax were significantly different in membranes from the nucleus accumbens of three-month-old ethanol-treated animals (p>0.05).
NUCLEUS ACCUMBENS

○ ETHANOL
■ CONTROL

SP. BINDING (PMOL/MG PROTEIN)

3H-SPIPERONE (nM)

FIGURE 8
of that in the nucleus accumbens. The Kd values ranged from 0.08 nM in the nucleus accumbens to 0.15 nM in the striatum. For the kinetic assays, several ligand concentrations between 0.0 nM and 1.0 nM were selected to establish saturation. The Kd values were calculated from the slope and Bmax values from the x-intercept of the Scatchard plots utilizing linear regression. The linear fit of the Scatchard plots represent correlation coefficients of at least 0.9.

The t-test for paired comparisons demonstrated there were no significant differences in either the Kd or Bmax for [3H]-spiperone binding to membranes from the striatum or the nucleus accumbens. This analysis indicates that neither the number nor the affinity of spiperone binding sites are altered in these brain regions of three-month-old Fisher 344 rats fed an ethanol-containing liquid diet for one month prior to sacrifice.

Using the binding techniques described, [3H]-spiperone labeled D2 receptors were below the level of detectability in the frontal cortex and globus pallidus. The nonspecific binding was at least 90% at ligand concentrations greater than 0.2 nM in these regions. As described for the D1 binding assay, three point surveys were also performed on quantitatively small regions. Spiperone binding sites were not measurable in the substantia nigra, ventral pallidum, and ventral tegmental area.
**Dopamine D2 Agonist Binding Assay**

The dopamine receptors exist in two states: a high affinity state and a low affinity state. The radioligand propylnorapomorphine, a selective D2 agonist, was used to measure the high affinity state of the D2 receptor in brain regions of three-month-old control and ethanol-treated rats. The assay was also performed in the presence of GTP to measure the ability of these receptors to be converted to the low affinity state, which would indicate alterations in the functionality of the G proteins. The regions analyzed include the striatum and the nucleus accumbens, areas where spiperone binding was measurable using the described ligand binding technique.

Both three point surveys and saturation assays were performed to assess $[^3H]$-propylnorapomorphine binding and conversion of the D2 high affinity sites. In the three point assays, the concentrations of $[^3H]$-propylnorapomorphine represented approximately $1/5$ Kd, Kd, and 5 Kd values. Typical binding assays for each brain region are depicted in Figures 9 and 10. Binding assays reflecting conversion of the high affinity state to the low affinity state are shown in Figures 11 and 12. The figures represent saturation curves and Scatchard plots. Nonspecific binding, determined in the presence of 1 $\mu$M (+)-butaclamol, accounted for approximately 20% of the total radioactivity at the lowest ligand concentration and 50% of the total radioactivity at
Figure 9. Binding of propynorapomorphine to striatal membranes of control and ethanol-treated animals.

The binding of $[^{3}\text{H}]$-propynorapomorphine to striatal membranes was assayed in duplicate at each ligand concentration. The saturation curve depicts the binding of 0.0 nM to 1.6 nM $[^{3}\text{H}]$-propynorapomorphine. Membranes from four control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 µM (+)-butaclamol, ranged from 20% to 50% of the total binding. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

\[
\begin{align*}
\text{Kd: } & \quad 0.167 \pm 0.030 \text{ nM (control)} \\
& \quad 0.164 \pm 0.024 \text{ nM (ethanol)} \\
\text{Bmax: } & \quad 0.208 \pm 0.014 \text{ pmol/mg protein (control)} \\
& \quad 0.201 \pm 0.010 \text{ pmol/mg protein (ethanol)}
\end{align*}
\]

The differences in the Kd and Bmax values obtained from four similar paired experiments were statistically analyzed using the t-test for paired comparisons. It was found that neither the Kd nor the Bmax were significantly different in striatal membranes from three-month-old ethanol-treated Fisher 344 rats (p>0.05).
**STRIATUM**

![Graph showing SP. BINDING (PMOL/MG PROTEIN) vs 3H-NPA (nM)]

- **ETHANOL**
- **CONTROL**
Figure 10. Binding of propylnorapomorphine to membranes from the nucleus accumbens of control and ethanol-treated rats.

The binding of $[^3\text{H}]$-propylnorapomorphine to membranes from the nucleus accumbens was assayed in duplicate at each ligand concentration. The saturation curve depicts the binding of 0.0 nM to 1.7 nM $[^3\text{H}]$-propylnorapomorphine. Membranes from four control or ethanol-treated animals were pooled prior to analysis. The nonspecific binding, determined in the presence of 1 µM (+)-butaclamol, ranged from 20% to 50% of the total binding. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

$$K_d: 0.373 \pm 0.052 \text{ nM (control)}$$
$$0.494 \pm 0.090 \text{ nM (ethanol)}$$

$$B_{max}: 0.103 \pm 0.008 \text{ pmol/mg protein (control)}$$
$$0.109 \pm 0.012 \text{ pmol/mg protein (ethanol)}$$

The differences in the Kd and Bmax values obtained from four paired similar experiments were statistically analyzed using the t-test for paired comparisons. It was found that neither the Kd nor the Bmax were significantly different in membranes from the nucleus accumbens ethanol-treated Fisher 344 rats ($p>0.05$).
Figure 11. Binding of propylnorapomorphine in the presence of GTP to striatal membranes in control and ethanol-treated rats.

Figures a and b represent the conversion of the high affinity state of the D2 receptor to its low affinity state in ethanol-treated and control animals, respectively. The saturation curves depict the binding of 0.05 nM to 1.6 nM [3H]-propylnorapomorphine in the absence and presence of 300 µM GTP. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

**Ethanol-treated:**
- Kd: 0.164 ± 0.024 nM
- 0.234 ± 0.044 nM (300 µM GTP)
- Bmax: 0.201 ± 0.010 pmol/mg protein
- 0.111 ± 0.008 pmol/mg protein (300 µM GTP)

**Control:**
- Kd: 0.167 ± 0.030 nM
- 0.244 ± 0.019 nM (300 µM GTP)
- Bmax: 0.208 ± 0.014 pmol/mg protein
- 0.111 ± 0.004 pmol/mg protein (300 µM GTP)

In the ethanol-treated animals approximately 45% of the D2 high affinity sites were converted to the low affinity state and in the control animals, approximately 47% of the sites were converted. It was found that the proportion of striatal D2 sites converted from high affinity state to the low affinity state was not significantly altered by ethanol treatment (p>0.05).
Figure 12. Binding of propylnorapomorphine in the presence of GTP to the nucleus accumbens of control and ethanol-treated rats.

Figure a and b represent the conversion of the high affinity state of the D2 receptor to the low affinity state in ethanol-treated and control animals, respectively. The saturation curves depict the binding of 0.0 nM to 1.7 nM [3H]-propylnorapomorphine in the absence and presence of 300 µM GTP. The Kd and Bmax values ± the standard deviation, derived from the linear regression of the data points, were:

**Ethanol-treated:**
- \( K_d: 0.494 \pm 0.090 \text{ nM} \)
- \( 0.813 \pm 0.123 \text{ nM (300 µM GTP)} \)
- \( B_{max}: 0.109 \pm 0.012 \text{ pmol/mg protein} \)
- \( 0.075 \pm 0.008 \text{ pmol/mg protein (300 µM GTP)} \)

**Control:**
- \( K_d: 0.373 \pm 0.052 \text{ nM} \)
- \( 0.735 \pm 0.125 \text{ nM (300 µM GTP)} \)
- \( B_{max}: 0.103 \pm 0.008 \text{ pmol/mg protein} \)
- \( 0.066 \pm 0.007 \text{ pmol/mg protein (300 µM GTP)} \)

In the ethanol-treated animals approximately 31% of the D2 high affinity sites were converted to the low affinity state and in the control animals, approximately 36% of the sites were converted. It was found that the proportion of the D2 sites converted from the high affinity state to the low affinity state in the nucleus accumbens was not significantly altered by ethanol treatment (p>0.05).
FIGURE 12

a

NUCLEUS ACCUMBENS (ETHANOL)

(300 uM GTP)

b

NUCLEUS ACCUMBENS (CONTROL)

(300 uM GTP)
the highest ligand concentration in the striatum and the nucleus accumbens. The Kd values ranged from 0.15 nM in the striatum to 0.40 nM in the nucleus accumbens; with the addition of GTP, there was approximately a two-fold increase in the Kd values. Several ligand concentrations between 0.02 nM to 2.0 nM were selected to establish saturation. The Kd and Bmax values were calculated from the Scatchard plots utilizing linear regression analysis. The linear fit of the Scatchard plots represent correlation coefficients of at least 0.9.

In membranes from both control and ethanol-fed rats, 300 µM GTP stimulated the conversion of the high affinity binding state to the low affinity state. In the striatum, an average 50% of the high affinity sites were converted to the low affinity state, while in the nucleus accumbens, an average 35% of the high affinity sites were converted to the low affinity state.

The t-test for paired comparisons demonstrated that there were no significant differences in either the Kd or Bmax for propylnorapomorphine binding to membranes from the striatum or nucleus accumbens (p>0.05). In addition, in the presence of GTP, the conversion of the high affinity state to the low affinity state was similar in membranes from control and ethanol-treated animals (p>0.05). The percent conversion for all experiments was statistically analyzed by Student's 't' test. The results of these studies demonstrate
that consumption of an ethanol-containing diet for one month
does not alter the high affinity state of the D2 site or the
ability of this site to convert to the low affinity state in
three-month-old Fisher 344 rats.

**Determination of Dopamine and DOPAC Concentrations**

This study determined dopamine and
3,4-dihydroxyphenylacetic acid (DOPAC) concentrations (ng/mg
protein) in several brain regions of control and
ethanol-treated animals. The regions analyzed include: the
striatum, globus pallidus, and substantia nigra which are
components of the nigrostriatal pathway and the nucleus
accumbens, ventral pallidum, frontal cortex, and ventral
tegmentum which are components of the mesolimbic dopamine
pathway. The results of these studies, presented as the mean
± the standard deviation, were compared for statistical
significance using Student's 't' test.

The results of these studies are depicted in Figures 13 to 16. Six to eight individual samples from control rats and
six to eight samples from ethanol-treated rats were examined
for each brain region. In comparison to control animals,
there was a 2- to 3-fold increase in dopamine and DOPAC
concentrations in the frontal cortex, ventral tegmentum, and
ventral pallidum, a 2-fold increase in DOPAC concentration
in the substantia nigra (p<0.01), and approximately a 30%
decrease in dopamine concentration in the substantia nigra.
Figure 13. Concentration of dopamine and DOPAC in the frontal cortex and nucleus accumbens.

Figure a depicts the concentration (ng/mg protein) of dopamine and DOPAC in the frontal cortex.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>$5.33 \pm 1.87^{''}$</td>
<td>$2.23 \pm 1.16$</td>
</tr>
<tr>
<td>DOPAC</td>
<td>$4.14 \pm 1.08'$</td>
<td>$2.61 \pm 1.01$</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of values obtained from six to eight individual animals. The symbols ' and " indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.

Figure b depicts the concentration (ng/mg protein) of dopamine and DOPAC in the nucleus accumbens.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>$180.3 \pm 49.1$</td>
<td>$194.3 \pm 45.6$</td>
</tr>
<tr>
<td>DOPAC</td>
<td>$95.6 \pm 51.5'$</td>
<td>$233.5 \pm 94.3$</td>
</tr>
</tbody>
</table>
FIGURE 13

FRONTAL CORTEX

![Graph showing neurotransmitter levels in the frontal cortex with control (open bars) and ethanol (hatched bars).](image)

- **P < 0.01
- * P < 0.05

NUCLEUS ACCUMBENS

![Graph showing neurotransmitter levels in the nucleus accumbens with control (open bars) and ethanol (hatched bars).](image)

- **P < 0.01
Figure 14. Concentration of dopamine and DOPAC in the ventral pallidum and the ventral tegmental area.

Figure a depicts the concentration (ng/mg protein) of dopamine and DOPAC in the ventral pallidum.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>31.5 ± 10.0''</td>
<td>14.0 ± 7.9</td>
</tr>
<tr>
<td>DOPAC</td>
<td>55.5 ± 35.5&quot;</td>
<td>17.9 ± 12.9</td>
</tr>
</tbody>
</table>

Figure b depicts the concentration (ng/mg protein) of dopamine and DOPAC in the ventral tegmental area.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>50.3 ± 11.8''</td>
<td>19.1 ± 5.8</td>
</tr>
<tr>
<td>DOPAC</td>
<td>164.8 ± 60.8''</td>
<td>77.2 ± 16.9</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of values obtained from six to eight individual animals. The symbols ' and '' indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.
**FIGURE 14**

**a**

**VENTRAL PALLIDUM**

![Graph showing the comparison of dopamine and DOPAC levels in the ventral pallidum between control and ethanol groups. Significant differences are indicated with asterisks: **P<0.01 and *P<0.05.**]

**b**

**VENTRAL TEGMENTAL AREA**

![Graph showing the comparison of dopamine and DOPAC levels in the ventral tegmental area between control and ethanol groups. Significant differences are indicated with asterisks: **P<0.01.**]
Figure 15. Concentration of dopamine and DOPAC in the striatum and the globus pallidus.

Figure a depicts the concentration (ng/mg protein) of dopamine and DOPAC in the striatum.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>265.5 ± 137.6</td>
<td>278.4 ± 77.2</td>
</tr>
<tr>
<td>DOPAC</td>
<td>87.4 ± 51.0</td>
<td>125.7 ± 51.2</td>
</tr>
</tbody>
</table>

Figure b depicts the concentration (ng/mg protein) of dopamine and DOPAC in the globus pallidus.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>347.8 ± 96.2</td>
<td>241.9 ± 88.8</td>
</tr>
<tr>
<td>DOPAC</td>
<td>37.3 ± 8.7</td>
<td>31.4 ± 11.8</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of values obtained from six to eight individual animals. The symbols • and •• indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.
FIGURE 15

**STRIATUM**

- CONTROL
- ETHANOL

**GLOBUS PALLIDUS**

- CONTROL
- ETHANOL

**Figure a**

**Figure b**

Bar graphs showing the levels of dopamine and DOPAC in the striatum and globus pallidus under control and ethanol conditions.
Figure 16. Concentration (ng/mg protein) of dopamine and DOPAC in the substantia nigra.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>26.6 ± 4.9*</td>
<td>38.6 ± 9.7</td>
</tr>
<tr>
<td>DOPAC</td>
<td>21.2 ± 9.2**</td>
<td>8.2 ± 1.7</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of values obtained from six to eight individual animals. The symbols * and ** indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.
FIGURE 16

SUBSTANTIA NIGRA

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>ETHANOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPAMINE</td>
<td><img src="Dopamine_graph" alt="Graph" /></td>
<td><img src="Dopac_graph" alt="Graph" /></td>
</tr>
<tr>
<td>DOPAC</td>
<td><img src="Dopamine_graph" alt="Graph" /></td>
<td><img src="Dopac_graph" alt="Graph" /></td>
</tr>
</tbody>
</table>

**P < 0.01
* P < 0.05

ng/mg protein
Although DOPAC was 60% below control values in the nucleus accumbens (p<0.01), the dopamine concentration was not significantly altered (p>0.05). In the striatum and globus pallidus, dopamine and DOPAC concentrations were not significantly different (p>0.05). The findings of this study demonstrate that consumption of an ethanol liquid diet for one month results in significant changes in the concentration of dopamine and DOPAC in specific brain areas of three-month-old Fisher 344 rats.

**Adenylate Cyclase Activity**

Basal and dopamine-sensitive adenylate cyclase activity were measured in several brain regions of control and ethanol-treated animals. The brain regions analyzed include the striatum, nucleus accumbens, and frontal cortex. Adenylate cyclase activity also was assessed in the presence of forskolin, GTP, and sodium fluoride in order to identify the site of any observed change. Forskolin directly activates adenylate cyclase whereas GTP and sodium fluoride directly stimulate the G protein. The levels of cAMP (nmoles/mg protein/5 min) measured by radioimmunoassay were used as an assessment of adenylate cyclase activity.

Table 1 demonstrates the stimulation or inhibition of adenylate cyclase by these drugs. There was a significant increase in adenylate cyclase activity in the presence of forskolin, GTP, and sodium fluoride in all brain regions.
Table 1

Adenylate Cyclase Activity  
(nmoles cAMP/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Striatum</th>
<th>Accumbens</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.14 ± .03</td>
<td>0.08 ± .01</td>
<td>0.16 ± .02</td>
</tr>
<tr>
<td>+ Forskolin</td>
<td>42.5 ± 1.3&quot;</td>
<td>27.0 ± 1.4&quot;</td>
<td>6.72 ± .25&quot;</td>
</tr>
<tr>
<td>+ NaF</td>
<td>4.46 ± .43&quot;</td>
<td>2.24 ± .38&quot;</td>
<td>2.05 ± .33&quot;</td>
</tr>
<tr>
<td>+ GTP</td>
<td>1.12 ± .05&quot;</td>
<td>0.63 ± .21&quot;</td>
<td>0.88 ± .05&quot;</td>
</tr>
</tbody>
</table>

Dopamine

- stimulated | 1.94 ± .23' | 1.19 ± .27' | 1.07 ± .08' |
- inhibited  | 0.92 ± .09' | ND          | ND          |

Each value represents the mean ± the standard deviation of three individual experiments for each brain region. Basal adenylate cyclase activity was measured in the absence or presence of either 100 µM forskolin, 100 µM GTP, or 10 mM NaF. The data also depicts the mean ± the standard deviation of three individual experiments of the stimulation (+ 50 nM spiperone) or inhibition (100 nM SCH 23390) of adenylate cyclase activity by 100 µM dopamine. The symbols ' and " indicate the values that differ significantly at p<0.05 and p<0.01, respectively, as compared to basal values or basal values in the presence of GTP.
analyzed (p<0.01). Compared to basal adenylate cyclase activity in the presence of GTP, there was a significant increase in dopamine-stimulated adenylate cyclase activity (in the presence of 50 nM spiperone) in all regions and a significant decrease in dopamine-inhibited adenylate cyclase activity (in the presence of 100 nM SCH 23390) in the striatum (p<0.05). These results indicate that there was a true stimulation of the D1 receptors in the striatum, nucleus accumbens, and frontal cortex, and a true inhibition of the D2 receptors in the striatum.

The results of this study are depicted in Figures 17 to 19. Figure 17 represents the basal and dopamine-sensitive adenylate cyclase activity in the striatum and the effects of forskolin, GTP, and sodium fluoride on striatal adenylate cyclase activity. Similarly, Figures 18 and 19 depict the adenylate cyclase activity of the nucleus accumbens and the frontal cortex, respectively. The activity is defined as nmoles cAMP formed per mg protein per 5 minutes. The nonspecific binding determined by bovine serum albumin (BSA) was an average 3% of total binding and the cAMP antibody accounted for an average 30% of the total binding. Each value represents the mean ± the standard deviation of three individual experiments. Student's 't' test was used to determine significant differences.

The results of this study of adenylate cyclase activity demonstrate that consumption of an ethanol liquid diet for
Figure 17. The effect of chronic ethanol treatment on adenylate cyclase activity in the striatum of three-month-old rats.

Figures 17a, 17b, 17c, and 17d depict striatal basal adenylate cyclase activity in the absence of stimulatory chemicals (a), or in the presence of 100 µM GTP (b), 100 µM forskolin (c), or 10 nM sodium fluoride (NaF) (d). Figure 17b also depicts D1-stimulated and D2-inhibited adenylate cyclase activity in the presence of GTP. The activity is defined as nmoles cAMP/mg protein.

Each value represents the mean ± the standard deviation of three individual experiments. The symbols * and ** indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.
Figure 18. The effect of chronic ethanol treatment on adenylate cyclase activity in the nucleus accumbens of three-month-old rats.

Figures 18a, 18b, 18c, and 18d depict basal adenylate cyclase activity of the nucleus accumbens in the absence of stimulatory chemicals (a), or in the presence of 100 µM GTP (b), 100 µM forskolin (c), or 10 nM sodium fluoride (NaF) (d). Figure 18b also depicts D1-stimulated adenylate cyclase activity in the presence of GTP. The activity is defined as nmoles cAMP/mg protein.

Each value represents the mean ± the standard deviation of three individual experiments. The symbols * and ** indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.
Figure 19. The effect of chronic ethanol treatment on adenylate cyclase activity in the frontal cortex of three-month-old rats.

Figures 19a, 19b, 19c, and 19d depict basal adenylate cyclase activity of the frontal cortex in the absence of stimulatory chemicals (a), or in the presence of 100 µM GTP (b), 100 µM forskolin (c), or 10 nM sodium fluoride (NaF) (d). Figure 19b also depicts D-1 stimulated adenylate cyclase activity in the presence of GTP. The activity is defined as nmoles cAMP/mg protein.

Each value represents the mean ± the standard deviation of three individual experiments. The symbols ' and ′ indicate that values from control and ethanol-treated animals differ significantly at p<0.05 and p<0.01, respectively.
one month results in significant alteration in adenylate cyclase activity in the nucleus accumbens of three-month-old Fisher 344 rats, although the adenylate cyclase activity of the striatum and the frontal cortex was not affected by ethanol treatment.

Basal, D1-stimulated, and D2-inhibited adenylate cyclase activities in the striatum were not significantly altered by chronic ethanol treatment (p>0.05). Striatal adenylate cyclase activity in the presence of forskolin, GTP, and sodium fluoride was not effected by ethanol treatment (p>0.05).

In the nucleus accumbens, although basal adenylate cyclase activity was not significantly different in ethanol-treated animals (p>0.05), adenylate cyclase activity in the presence of 100 μM dopamine was significantly increased (70%) in ethanol-treated animals (p<0.05). D1-stimulated adenylate cyclase activity was not significantly altered in the nucleus accumbens of ethanol-treated animals (p>0.05). Adenylate cyclase activity in the presence of forskolin, GTP, and sodium fluoride was not effected by ethanol treatment (p>0.05). Dopamine D2-inhibited adenylate cyclase activity was not measurable in the nucleus accumbens. This finding concurs with the observations of Stoof and Verheijden (1986) and Kelly and Naharski (1987).

Basal and D1-stimulated adenylate cyclase activity was
not significantly altered in the frontal cortex of ethanol-treated animals (p>0.05). Since D2 sites were not measurable in the frontal cortex, D2-inhibited adenylate cyclase activity was not analyzed. Adenylate cyclase activity of the frontal cortex in the presence of forskolin, GTP, and sodium fluoride was not significantly altered in ethanol-treated animals (p>0.05).

Analysis of G Proteins

This study analyzed the effects of chronic ethanol treatment on the content of the G proteins in several brain regions. The α subunits of Gs and Go were measured using monoclonal antibodies directed against these subunits, Gia was not analyzed due to the unavailability of specific antibodies to this protein. The brain regions studied include the striatum, globus pallidus, and substantia nigra of the nigrostriatal pathway and the frontal cortex, nucleus accumbens, ventral pallidum, and the ventral tegmentum of the mesolimbic dopamine pathway. β-Actin was analyzed as a control to monitor for equivalency for protein loading.

The samples were separated on a 10% (v/v) SDS-polyacrylamide gel and the separated proteins were transferred to nitrocellulose filters. The filters were incubated with the antibody for the G proteins or actin (primary antibody), followed by incubation with [125I]-IgG (secondary antibody). The filters were then exposed on film.
The autoradiograms for Gs, Go, and actin were scanned by a densitometer. Optical density (O.D.) values were assigned to each sample and these values were used to calculate ratios for Gs and Go relative to actin. Actin served as a control for differences in the loading of gel lanes. This allowed for a correction factor due to unequal protein loading onto the gel. The ratios were defined as arbitrary densitometer units (ADU). Three individual samples (control and ethanol) from each brain region were separated on a gel. Three additional individual samples were similarly analyzed. Thus, a total of six samples were analyzed for each brain region.

The results of this study are shown in Figures 20 and 21. Figure 20 depicts the ratio of ADU for Gs/actin in each brain region, while Figure 21 depicts the ratio of ADU for Go/actin. The values represent the mean ± the standard deviation of three individual samples separated on the same gel. Similar results were obtained from gels containing additional samples. In all brain regions analyzed, neither the Gs protein nor the Go protein were significantly altered (p>0.05). The results indicate that chronic ethanol exposure does not alter the protein content of the Gs or Go proteins in the striatum, globus pallidus, substantia nigra, nucleus accumbens, frontal cortex, ventral pallidum, and ventral tegmental area from three-month-old rats which consumed ethanol for one month prior to sacrifice.
Figure 20. The effects of chronic ethanol exposure on the protein content of Gs in three-month-old rats.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>0.84 ± 0.14</td>
<td>0.80 ± 0.06</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>3.10 ± 0.49</td>
<td>3.13 ± 0.32</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>4.55 ± 1.82</td>
<td>4.29 ± 1.80</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>2.79 ± 0.66</td>
<td>2.91 ± 0.08</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.30 ± 0.51</td>
<td>1.28 ± 0.54</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>2.42 ± 0.99</td>
<td>2.66 ± 1.38</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>1.01 ± 0.05</td>
<td>1.07 ± 0.14</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of the Gs/actin ratio in arbitrary densitometer units (ADU) obtained from three individual samples separated on the same gel. These results are similar to the results obtained from other gels. There was no significant difference in the protein content in similar samples of Gs from control and ethanol-treated rats in all brain regions analyzed (p>0.05).
**FIGURE 20**

**Gs**

- **Frontal Cortex**
- **Nucleus Accumbens**
- **Ventral Pallidum**
- **Ventral Tegmentum**

- **Control**
- **Ethanol**

**G Protein/Actin (ADU)**

- **Striatum**
- **Globus Pallidus**
- **Substantia Nigra**

- **Control**
- **Ethanol**
Figure 21. The effects of chronic ethanol exposure on the protein content of Go in three-month-old rats.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>3.33 ± 1.27</td>
<td>3.49 ± 1.16</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>8.44 ± 1.17</td>
<td>8.89 ± 1.59</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>4.22 ± 0.45</td>
<td>4.11 ± 1.02</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>4.85 ± 2.18</td>
<td>5.27 ± 2.61</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.82 ± 0.17</td>
<td>0.88 ± 0.23</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>0.98 ± 0.03</td>
<td>0.94 ± 0.06</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>0.53 ± 0.23</td>
<td>0.51 ± 0.11</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of the Go/actin ratio in arbitrary densitometer units (ADU) obtained from three individual samples separated on the same gel. These results are similar to the results obtained from other gels. There was no significant difference in the protein content in similar samples of Go from control and ethanol-treated rats in all brain regions analyzed (p>0.05).
FIGURE 21

Go

G PROTEIN/ACTIN (ADU)

FRONTAL CORTEX NUCLEUS ACCUMBENS VENTRAL PALLIDUM VENTRAL TEgmentUM

G PROTEIN/ACTIN (ADU)

STRIATUM GLOBUS PALLIDUS SUBSTANTIA NIGRA

CONTROL ETHANOL

CONTROL ETHANOL
Assessment of mRNA Levels of G Protein

The mRNA levels of several G proteins were measured in brain regions of control and ethanol-treated animals. The cDNA probes for the α subunits of Gs, Go, Gi1, Gi2, and Gi3 (obtained from Dr. Randall Reed; Jones and Reed 1987) were used to analyze the mRNA levels of brain areas of the nigrostriatal and mesolimbic dopamine pathways.

Total RNA was loaded onto Nytran filters utilizing a slot blot apparatus. The cDNA probes isolated from plasmids were subjected to a DNA digest and separated on a 0.7% (w/v) agarose gel to screen for purity (Figure 22). The cDNA probes were labeled with $^{32}$P-CTP by random priming and hybridized to the filters. The filters were exposed to film to generate autoradiograms.

The autoradiograms for the G proteins and actin were scanned by densitometer and ratios were calculated for each G protein (G protein/actin). These values were defined as arbitrary densitometer units (ADU). Slot blot analyses were repeated 3-5 times and G protein/actin ratios, obtained from scanning individual autoradiograms, were analyzed by the t-test for paired comparison to account for day to day differences in control ADU values.

Typical slot blots for each G protein and actin are shown in Figures 23 to 27. The values listed in the legend represent the ratio of G protein to actin for each blot. There was a 20% decrease in mRNA levels for Go in the
The DNA probes for the G proteins were extracted from the plasmid, pGEM-2, and digested with the restriction enzyme, Eco RI. The DNA fragments were separated on a 0.7% (v/v) agarose gel to estimate the size of the cleavage products in order to determine the purity of preparation.

Lane 1: Gi₃ (3.1 kb)
Lane 2: Gi₂ (1.8 kb)
Lane 3: Gi₁ (2.0 kb)
Lane 4: Go (2.1 kb)
Lane 5: Gs (0.7 and 1.1 kb)
Lane 6: Size markers from HindIII and BamHI digest of phage τ.
Figure 23. Ratio of mRNA for Gs/actin in 3-month-old control and ethanol-treated rats.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>1.84</td>
<td>1.63</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.89</td>
<td>0.94</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>1.00</td>
<td>0.85</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>1.32</td>
<td>1.19</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>1.24</td>
<td>1.33</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>0.60</td>
<td>0.57</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>1.17</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Each value represents the ratio of G protein/actin in arbitrary densitometer units (ADU) from a typical slot blot analysis. The differences in ratio values obtained from 3-5 paired experiments were statistically analyzed using the t-test for paired comparisons. There were no significant differences in Gs content in all brain regions analyzed ($p>0.05$). This figure represents a typical slot blot for Gs. Equal amounts of RNA were diluted to 1:10 and 1:1000 and all three dilutions were loaded onto the Nytran filter. The first sample of each set represents an ethanol-treated animal.
FIGURE 23

- FRONTAL CORTEX
- STRIATUM
- NUCLEUS ACCUMBENS
- GLOBUS PALLIDUS
- SUBSTANTIA NIGRA
- VENTRAL PALLIDUM
- VENTRAL TEGMENTUM

ETHANOL CONTROL

Gs
Figure 24. Ratio of mRNA for Go/actin in 3-month-old control and ethanol-treated animals.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.21</td>
<td>1.19</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>1.07</td>
<td>1.11</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>1.22</td>
<td>1.44</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>0.92**</td>
<td>1.14</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>1.00</td>
<td>0.96</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>1.05</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Each value represents the ratio of G protein/actin in arbitrary densitometer units (ADU) from a typical slot blot analysis. The differences in ratio values obtained from 3-5 paired experiments were statistically analyzed using the t-test for paired comparisons. There was a significant decrease in the Go content of the substantia nigra (p<0.01) (**). There were no significant differences in the other brain regions analyzed (p>0.05). This figure represents a typical slot blot for Go. Equal amounts of RNA were diluted to 1:10 and 1:1000 and all three dilutions were loaded onto the Nytran filter. The first sample of each set represents an ethanol-treated animal.
<table>
<thead>
<tr>
<th>Structure</th>
<th>Ethanol Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td></td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td></td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td></td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td></td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td></td>
</tr>
</tbody>
</table>

Go
Figure 25. Ratio of mRNA for Gi₁/actin in 3-month-old control and ethanol-treated animals.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>0.87</td>
<td>0.81</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.59</td>
<td>0.67</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>0.84</td>
<td>0.89</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>0.38</td>
<td>0.47</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>0.90</td>
<td>0.97</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>0.50</td>
<td>0.54</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>0.70</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Each value represents the ratio of G protein/actin in arbitrary densitometer units (ADU) from a typical slot blot analysis. The differences in ratio values obtained from 3-5 paired experiments were statistically analyzed using the t-test for paired comparisons. There were no significant differences in Gi₁ content in all brain regions analyzed (p>0.05). This figure represents a typical slot blot for Gi₁. Equal amounts of RNA were diluted to 1:10 and 1:1000 and all three dilutions were loaded onto the Nytran filter. The first sample of each set represents an ethanol-treated animal.
<table>
<thead>
<tr>
<th>FRONTAL CORTEX</th>
<th>ETHANOL CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRIATUM</td>
<td></td>
</tr>
<tr>
<td>NUCLEUS ACCUMBENS</td>
<td></td>
</tr>
<tr>
<td>GLOBUS PALLIDUS</td>
<td></td>
</tr>
<tr>
<td>SUBSTANTIA NIGRA</td>
<td></td>
</tr>
<tr>
<td>VENTRAL PALLIDUM</td>
<td></td>
</tr>
<tr>
<td>VENTRAL TEGMENTUM</td>
<td></td>
</tr>
</tbody>
</table>
Figure 26. Ratio of mRNA for Gi\textsubscript{2}/actin in 3-month-old control and ethanol-treated animals.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>0.83</td>
<td>0.87</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.88</td>
<td>0.78</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>0.63</td>
<td>0.60</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>0.84</td>
<td>0.95</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>1.36</td>
<td>1.10</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>1.40</td>
<td>1.25</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>1.20</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Each value represents the ratio of G protein/actin in arbitrary densitometer units (ADU) from a typical slot blot analysis. The differences in ratio values obtained from 3-5 paired experiments were statistically analyzed using the t-test for paired comparisons. There were no significant differences in Gi\textsubscript{2} content in all brain regions analyzed (p>0.05). This figure represents a typical slot blot for Gi\textsubscript{2}. Equal amounts of RNA were diluted to 1:10 and 1:1000 and all three dilutions were loaded onto the Nytran filter. The first sample of each set represents an ethanol-treated animal.
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Control</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig 26

Gi2
Figure 27. Ratio of mRNA for Gi₃/actin in 3-month-old control and ethanol-treated animals.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Ethanol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>0.71*</td>
<td>0.53</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.83</td>
<td>0.67</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>0.62</td>
<td>0.48</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>0.73</td>
<td>0.56</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>0.52</td>
<td>0.63</td>
</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>1.13</td>
<td>0.86</td>
</tr>
<tr>
<td>Ventral Pallidum</td>
<td>0.31*</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Each value represents the ratio of G protein/actin in arbitrary densitometer units (ADU) from slot blot analysis. The differences in ratio values obtained from 3-5 paired experiments were statistically analyzed using the t-test for paired comparisons. There was a significant increase in Gi₃ content in the frontal cortex and ventral pallidum (p<0.05) (*). There was no significant differences in Gi₃ content in the other brain regions analyzed (p>0.05). This figure represents a typical slot blot for Gi₃. Equal amounts of RNA were diluted to 1:10 and 1:1000 and loaded onto the Nytran filter. The first sample of each set represents an ethanol-treated animal.
substantia nigra \( (p<0.01) \). In all brain regions except the substantia nigra, there was a 30-40% increase in \( \text{Gi}_3 \) but this difference was only significant in the frontal cortex and ventral pallidum \( (p<0.05) \). There were no significant differences in mRNA levels for \( \text{Gs} \), \( \text{Gi}_1 \), and \( \text{Gi}_2 \) in all brain regions analyzed. The results of this experiment indicate that three-month-old rats exposed to ethanol for one month displayed region specific alterations in the mRNA levels of \( \text{Go} \) and \( \text{Gi}_3 \), while the mRNA levels of \( \text{Gs} \), \( \text{Gi}_1 \), and \( \text{Gi}_2 \) were not significantly changed.

**Cholesterol and Phospholipid Levels**

This study examined the cholesterol and phospholipid levels in synaptic plasma membranes prepared from whole brains of control and ethanol-treated rats. The results presented as the mean ± the standard deviation were compared for statistical significance using Student's 't' test (Table 2).

In comparison to control animals, there was a 42% increase in cholesterol levels \( (p<0.01) \), while the levels of phospholipid were not significantly different \( (p>0.05) \). There was also a significant increase \( (31\%) \) in the ratio of cholesterol to phospholipid \( (p<0.05) \). This study demonstrates that consumption of an ethanol liquid diet for one month resulted in a significant increase in cholesterol levels and in the cholesterol/phospholipid ratio of synaptic
plasma membranes.
Table 2

Effects of Chronic Ethanol Consumption on Cholesterol and Phospholipids in Synaptic Plasma Membranes from 3-Month-Old Fisher 344 Rats

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (µmol/mg protein)</th>
<th>Phospholipid (µmol/mg protein)</th>
<th>Cholesterol/Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.532 ± .068**</td>
<td>1.017 ± .150</td>
<td>0.529 ± .078*</td>
</tr>
<tr>
<td>Control</td>
<td>0.375 ± .102</td>
<td>0.948 ± .129</td>
<td>0.403 ± .128</td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard deviation of values obtained from individual synaptic membrane preparations from seven rats. The symbols * and ** indicate that values from control and ethanol-fed rats are significantly different at p<0.05 or p<0.01, respectively.
Summary

Tables 3 and 4 represent a summary of the results of this research.
# Table 3

## Components of the Mesolimbic Dopamine Pathway

<table>
<thead>
<tr>
<th></th>
<th>Frontal Cortex</th>
<th>Nucleus Accumbens</th>
<th>Ventral Pallidum</th>
<th>Ventral Tegmentum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dopamine</strong></td>
<td>†</td>
<td>NC</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td><strong>DOPEG</strong></td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td><strong>D1 Site</strong></td>
<td>†</td>
<td>†</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>G Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Gs) mRNA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>protein</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td><strong>AC Activity</strong></td>
<td>NC</td>
<td>†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>D2 Site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiperone</td>
<td>ND</td>
<td>NC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NPA</td>
<td>–</td>
<td>NC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Conversion</td>
<td>–</td>
<td>NC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>G Protein</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(Gi2) mRNA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td><strong>AC Activity</strong></td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>G Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Go) mRNA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>(Gi1) mRNA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>(Gi3) mRNA</td>
<td>†</td>
<td>NC</td>
<td>†</td>
<td>NC</td>
</tr>
<tr>
<td>protein</td>
<td>(Go)</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC: no change    ND: not detected    (†): not determined
<table>
<thead>
<tr>
<th></th>
<th>Striatum</th>
<th>Globus Pallidus</th>
<th>Substantia Nigra</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dopamine</strong></td>
<td>NC</td>
<td>NC</td>
<td>↓</td>
</tr>
<tr>
<td><strong>DOPAC</strong></td>
<td>NC</td>
<td>NC</td>
<td>↑</td>
</tr>
<tr>
<td><strong>D1 Site</strong></td>
<td>NC</td>
<td>NC</td>
<td>ND</td>
</tr>
<tr>
<td><strong>G Protein</strong></td>
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<td></td>
<td></td>
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<tr>
<td>(Gs)</td>
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<td></td>
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</tr>
<tr>
<td>mRNA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>protein</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td><strong>AC Activity</strong></td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D2 Site</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiperone</td>
<td>NC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NPA</td>
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<td></td>
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</tr>
<tr>
<td>Conversion</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G Protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Gi2)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td><strong>AC Activity</strong></td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G Protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Go)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Gi1)</td>
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<tr>
<td>(Gi3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Go)</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC: no change       ND: not detected   (·): not determined
The objective of this research was to examine the effect of chronic ethanol exposure on several components of the dopaminergic system. It was hypothesized that chronic ethanol consumption either alters the ability of the dopamine signal to effect cellular changes through signal transduction or alters the concentration of dopamine itself. These studies represented a comprehensive examination of the membrane components of the dopaminergic system. Specifically, the components analyzed included the dopamine D1 and D2 receptors, the guanine nucleotide binding proteins (G proteins), and adenylate cyclase activity. In addition, steady-state levels of dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) were analyzed. These components were measured in brain regions associated with the nigrostriatal or mesolimbic dopamine pathways. Alterations in any of these components could contribute to ethanol-induced changes in motor activity or to the reinforcing effects of ethanol.

These studies demonstrated that consumption of a 6.6% (v/v) ethanol-containing liquid diet for one month affected several components of the dopamine system in three-month-old
Fisher 344 rats. Specifically, there was a loss of D1 receptors in the frontal cortex and nucleus accumbens. In contrast, there was no change in D1 receptor affinity or density in the striatum and globus pallidus. In addition, the number and affinity of spiperone binding sites and the number of propylnorapomorphine high affinity sites, as well as their conversion from the high affinity state to the low affinity state in the striatum and nucleus accumbens were unchanged by chronic ethanol consumption. The concentrations of dopamine and DOPAC were increased at least two-fold in the frontal cortex, ventral pallidum, and ventral tegmental area, while the levels were unchanged in the striatum and globus pallidus. In the substantia nigra, dopamine concentration was decreased and that of DOPAC was increased. The dopamine concentration in the nucleus accumbens was normal, but that of DOPAC was decreased. An analysis of Gs and Go proteins demonstrated minimal, if any changes, in either protein content or mRNA levels in all brain regions analyzed. Concerning the inhibitory G proteins, the mRNA levels of Gi1 and Gi2 were unchanged in all brain regions, however, there was a trend towards an increase in the mRNA levels of Gi3 in all regions except the substantia nigra. This increase was only significant in the frontal cortex and ventral pallidum. In the frontal cortex and striatum, no change was seen in dopamine-sensitive adenylate cyclase activity, although D1-stimulated adenylate cyclase activity
and D1-stimulated adenylate cyclase activity in the presence of forskolin were increased in the nucleus accumbens. These results suggest a selective ethanol effect on several components of the dopamine system.

**Dopamine and DOPAC Concentrations**

This study examined the effects of chronic ethanol exposure on dopamine and DOPAC content in the striatum, globus pallidus, and substantia nigra which are components of the nigrostriatal pathway (Fibiger et al. 1974; Lindvall and Bjorkland 1979), and in the frontal cortex, nucleus accumbens, ventral pallidum, and ventral tegmental area which are components of the mesolimbic dopamine pathway (Lindvall et al. 1978; Nauta et al. 1978). Although, other laboratories have assessed dopamine and DOPAC content and turnover following acute ethanol exposure, this is the first extensive assessment of the effects of chronic ethanol consumption on the concentrations of dopamine and DOPAC in several brain regions. In the present study, the concentrations of dopamine ranged from 2.2 ng/mg protein in the frontal cortex to approximately 250 ng/mg protein in the striatum and globus pallidus in control rats. The concentrations of DOPAC ranged from 2.6 ng/mg protein in the frontal cortex to 234 ng/mg protein in the nucleus accumbens of control rats. Other investigators have reported dopamine and DOPAC concentrations are highest in the striatum and
lowest in frontal cortex (Fadda et al. 1980; Westerink 1983; Ehrenstrom and Johansson 1985; Haikala 1987; Wetherell et al. 1989). The dopamine concentrations in the nucleus accumbens and substantia nigra in the present study are comparable to values reported by Bacopoulous et al. (1978).

Chronic ethanol consumption had a region-specific effect on dopamine and DOPAC levels in three-month-old Fisher 344 rats. In the frontal cortex, ventral pallidum, and ventral tegmental area, there was a two-fold increase in dopamine and DOPAC levels in the ethanol-treated animals. The dopamine content in the substantia nigra was slightly decreased but the level of DOPAC was increased two-fold. In the nucleus accumbens, dopamine concentration was not altered by chronic ethanol treatment, while that of DOPAC was decreased. Neither the dopamine nor DOPAC levels in the striatum and globus pallidus were affected by ethanol consumption. Some of the brain regions associated with the mesolimbic dopamine pathway are those affected by chronic ethanol treatment. In contrast, the content of dopamine and DOPAC in the striatum and globus pallidus which are components of the nigrostriatal pathway were not affected by chronic ethanol treatment.

An increase in dopamine content as observed in the frontal cortex, ventral pallidum, and ventral tegmental area may reflect an increase in dopamine synthesis, a decrease in dopamine release, or a decrease in dopamine turnover.
Increased dopamine synthesis would occur if there were increased activity of tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of the catecholamine neurotransmitters (Cooper et al. 1986). This enzyme can be activated by phosphorylation by any of at least three protein kinases: cAMP-dependent protein kinase, Ca\(^{+2}\)/calmodulin-dependent protein kinase, and Ca\(^{+2}\)/diacylglycerol-dependent protein kinase (Nestler and Greengard 1989). Phosphorylation of tyrosine hydroxylase increases its catalytic activity by increasing the affinity of the enzyme for its tetrahydropteridine cofactor and by decreasing its affinity for its end-product inhibitor (Cooper et al. 1986). Ethanol may activate tyrosine hydroxylase by activating one or more protein kinase through an increased activation of second messenger systems (cAMP, phosphoinositide, Ca\(^{+2}\)) (Rabin and Molinoff 1981; Lynch and Littleton 1983; Hudspith et al. 1985; Saito et al. 1985; Gonzales et al. 1986; Messing et al. 1986; Dolin et al. 1987). The present studies suggest the cAMP-dependent phosphorylation and activation of tyrosine hydroxylase may not occur in the frontal cortex since normal adenylate cyclase activity was found in this area. However, the present studies cannot exclude the possibility that ethanol may have increased tyrosine hydroxylase phosphorylation via altered phosphoinositide (IP\(_3\)) formation or calcium (Ca\(^{+2}\)) influx.
Within the ventral tegmental area, D2 receptors are reportedly autoreceptors on dendrites of dopaminergic neurons which project to the nucleus accumbens (Wang 1981; White and Wang 1984). In contrast, D2 receptors are lacking on ventral tegmental area dopaminergic cell bodies which project to the frontal cortex (Chiodo et al. 1984). In both projection areas of the ventral tegmental area (the frontal cortex and nucleus accumbens), D1 and D2 receptors are found postsynaptically (White and Wang 1984, 1986; Galloway et al. 1986). In addition, D2 receptors are also found postsynaptically in the nucleus accumbens (White and Wang 1986).

With the above receptor localization in mind, an elevation in ventral tegmental area dopamine and DOPAC may be indicative of an increase in the dendrodendritic release of dopamine. The increased release of dopamine, acting on the D2 autoreceptors, could inhibit firing of the projection from the ventral tegmental area to the nucleus accumbens. As a consequence of decreased firing, one would expect decreased release of dopamine, as suggested by the lower DOPAC levels in this region. In light of the apparent lack of a D2 autoreceptor in the ventral tegmental area on cells which project to the prefrontal cortex (Chiodo et al. 1984) and the finding of elevated cortical dopamine and DOPAC, a similar analogy cannot be made with the mesocortical system.

On the other hand, dopamine was decreased in the
substantia nigra. Dopamine may be decreased if dopamine synthesis is decreased as a result of decreased activation (decreased phosphorylation) of tyrosine hydroxylase by the second messenger systems mentioned above. Alternatively, dopamine levels may be decreased if dopamine release is increased. Dopamine release could be increased by an ethanol-induced activation of second messenger systems which phosphorylate synapsin I. Synapsin I, a component of the regulation of neurotransmitter release, is associated with the synaptic vesicles located in the nerve terminal (Browning et al. 1985; De Camilli et al. 1986). Synapsin I is phosphorylated by cAMP-dependent protein kinase and Ca\(^{+2}\)/calmodulin-dependent protein kinase. The dephosphorylated protein inhibits neurotransmitter release, whereas the phosphorylated protein facilitates release (Llinas et al. 1985). Consequently, phosphorylation of synapsin I could facilitate dopamine release and result in decreased dopamine levels as seen in the substantia nigra. If this explanation for a decrease in dopamine levels in the substantia nigra is valid, it would suggest that phosphorylation of synapsin I is not similarly affected in other brain regions which did not demonstrate a decrease in dopamine levels.

It is also possible that ethanol may inhibit the enzymes responsible for the metabolic degradation of catecholamines: monoamine oxidase (MAO) and
catechol-O-methyltransferase (COMT). Differences in the concentration of DOPAC in the brain regions of ethanol-treated animals may reflect an ethanol-induced alteration in MAO activity. Presently, additional information is needed to substantiate this interpretation.

In summary, three-month-old Fisher 344 rats fed an ethanol-containing diet for one month display unique regional responses. These differential effects for each brain region may reflect complex regulatory mechanisms specific for each region.

Dopamine Receptors

The present study provides information concerning the effects of chronic ethanol exposure on the dopamine D1 and D2 receptors in rat brain. This is the first report that examines the D1 and D2 receptors in brain regions other than the striatum in chronically ethanol-treated rats. This is also the first report of the effects of ethanol on the conversion of the D2 receptor from the high affinity state to the low affinity state.

Although the present study was unable to detect D2 receptors in the frontal cortex, ventral pallidum, ventral tegmental area, globus pallidus and the substantia nigra, a more sensitive method, using quantitative autoradiography, detected D2 sites in these regions (Boyson et al. 1986; Charuchinda et al. 1987; Richfield et al. 1989). Similarly,
quantitative autoradiography has demonstrated D1 receptors in several brain regions in which these receptors were below the level of detectability for homogenate binding studies. These areas include the ventral pallidum, ventral tegmental area, and the substantia nigra (Richfield et al. 1987; 1989).

Total dopamine D1 sites were measured using the selective D1 antagonist, SCH 23390 (Billard et al. 1984). Binding studies indicate that [3H]-SCH 23390 binds stereospecifically with high affinity to a single site, the D1 receptor site (Billard et al. 1984; Schulz et al. 1984). In the present study, the Kd values of control rats ranged from 0.3 nM in the globus pallidus to 2.0 nM in the frontal cortex. The Bmax values ranged from 0.2 pmol/mg protein in the frontal cortex to 0.8 pmol/mg protein in the nucleus accumbens. The Kd and Bmax values of this study are consistent with those in previous reports of D1 binding in the rat striatum (Billard et al. 1984; Schulz et al. 1985; Hess et al. 1986; Manik et al. 1988).

The present study demonstrated regional alterations in dopamine D1 sites in ethanol-treated rats. A decrease in D1 sites was observed in the nucleus accumbens and frontal cortex, while the D1 sites in the striatum and globus pallidus were not changed by ethanol treatment. The affinity of the SCH 23390 for D1 site was not significantly different in these four regions. Other studies have reported that
ethanol increases, decreases, or has no effect on striatal SCH 23390 D1 sites. Hietala et al. (1990) observed the number and affinity of D1 receptor sites in the frontal cortex and striatum were not significantly altered by 5 weeks of ethanol treatment. However, Hruska (1988) reported a 40% increase in striatal D1 sites after 3 weeks of ethanol administration, while Lucchi et al. (1988) reported a 25% decrease in striatal D1 sites after 25 days of ethanol treatment. Both investigators observed no significant differences in the affinity of striatal D1 sites. The effects of ethanol exposure on D1 sites in other brain regions have not been reported.

The discrepancies among the present report of striatal binding sites and previous findings are possibly explained by technical differences, specifically species, dose and mode of ethanol administration, or assay conditions. For example, Lucchi et al. (1988) administered 6% (v/v) ethanol in the drinking water to Sprague-Dawley rats. The mean daily intake of ethanol was 2-3 g/kg body weight. Hruska (1988) used a liquid diet with an ethanol concentration of 6.2% (v/v). Adult male Sprague-Dawley rats were placed on the diet for 14 or 21 days and the amount of ethanol consumed daily ranged from 12-17 g/kg body weight. Hietala et al. (1990) also used a liquid diet containing 6% (v/v) ethanol. Wister rats were maintained on the diet for 5 weeks. In the present study, male Fisher 344 rats were pair-fed a 6.6%
(v/v) ethanol-containing diet for 4 weeks. The average daily intake was 20-25 g/kg body weight. It is possible the different feeding models, length of treatment, daily intake, or species differences may account for the discrepancies in these findings. Nevertheless, the present findings corroborate some findings observed in other binding assays. Namely, ethanol treatment does not alter the affinity of the dopamine D1 receptor site and an ethanol effect in one region does not necessarily define the effect in all regions.

It is interesting to note the selective decrease in D1 sites in the nucleus accumbens and frontal cortex, brain areas which are components of the mesolimbic dopamine pathway (Lindvall et al. 1978; Nauta et al. 1978). The mesolimbic dopamine pathway is recognized as an important component of reward circuitry and has been implicated in the reinforcement of several drugs of abuse (Phillips and Fibiger 1978; Lyness et al. 1981; Bozarth and Wise 1981; Mathews and German 1984). Thus, changes in the dopamine D1 receptors may in some way be associated with rewarding effects of ethanol and thus may potentially be involved with the development of ethanol dependence.

Total dopamine D2 sites were measured using the D2 antagonist, spiperone (Leyson et al. 1978). Spiperone binds specifically and with high affinity to a single class of dopamine D2 sites (Leslie and Bennett 1987). The Kd values
in this study ranged from 0.08 nM in the nucleus accumbens to 0.15 nM in the striatum of control animals. The Bmax values in the striatum were 0.1 pmol/mg protein. The Kd and Bmax values in this study are in agreement with reported Kd values and Bmax values of other binding assays in the rat striatum (Briley and Langer 1978; Leyson et al. 1978; Owen et al. 1979; List & Seeman 1981). Little information is available about the kinetics of spiperone binding, as measured by homogenate binding assays in the nucleus accumbens and frontal cortex.

The density and affinity of dopamine D2 sites in the nucleus accumbens and striatum were not altered by ethanol exposure in this study. Similar to the reports of others regarding ethanol's effect on the D1 sites, various laboratories have observed an increase, decrease, or no change in D2 sites after ethanol treatment. Hietala et al. (1990) demonstrated that the affinity and number of striatal D2 sites, measured by [3H]-spiperone, were not significantly altered by 3 or 5 weeks of ethanol treatment. In addition, the specific binding of [3H]-haloperidol to striatal D2 sites was unaffected in rats treated with ethanol for 11-15 days (Muller et al. 1980). In contrast, Hruska (1988) reported an increase (15-20%) in striatal D2 sites measured by spiperone after 3 weeks of ethanol treatment, while Lucchi et al. (1988) observed a decrease (27%) in these sites after 25 days of ethanol exposure. Furthermore,
Syvalahti et al. (1988) found a decrease (38%) in the number of striatal D2 sites in rats treated with ethanol for 32 weeks. As stated previously, the difference in findings may be attributed to technical differences including species, dose and duration of ethanol treatment, and assay conditions.

The effect of chronic ethanol exposure on the high affinity state of the dopamine D2 site and its ability to convert from the high affinity state to the low affinity state were analyzed with the D2 agonist, propylnorapomorphine (Creese et al. 1979; Titeler and Seeman 1979). This D2 agonist binds with high affinity to an apparently homogeneous receptor population, representing the high affinity agonist binding site (Sibley et al. 1982). Guanine nucleotides decrease the binding capacity for \(^{[3\text{H}]}\)-propylnorapomorphine by inducing a conformational change in the high affinity state of the receptor (Sibley et al. 1982), a mechanism involving the guanine nucleotide regulatory proteins (G proteins) (Boeynaems and Dumont 1975).

This is the first report that examines agonist binding in brain regions of control and ethanol-treated rats. The Kd values of control animals ranged from 0.1 nM in the striatum to 0.4 nM in the nucleus accumbens. In the presence of GTP, the Kd values ranged from 0.2 nM in the striatum to 0.7 nM in the nucleus accumbens. Reported Kd values range from 0.2
nM in bovine anterior pituitary to 1.5 nM bovine striatum (Sibley et al. 1982; Creese et al. 1979). In the presence of GTP, the K\textsubscript{d} value reported in the anterior pituitary was 0.3 nM (Sibley et al. 1982). The B\textsubscript{max} values of control animals in the present study ranged from 0.1 pmol/mg protein in the nucleus accumbens to 0.2 pmol/mg protein in the striatum and in the presence of GTP, the B\textsubscript{max} values ranged from 0.07 pmol/mg protein in the nucleus accumbens to 0.1 pmol/mg protein in the striatum.

In the present study, approximately 50% of the striatal D\textsubscript{2} high affinity sites were converted to the low affinity state in the presence of GTP; in the nucleus accumbens approximately 35% of the sites were converted in the presence of GTP. Sibley et al. (1982) reported a 50% conversion rate in the anterior pituitary. Chronic ethanol treatment did not alter the binding of \[^3\text{H}\]-propylnorapomorphine to the high affinity site or the conversion of the D\textsubscript{2} high affinity site to the low affinity site in the striatum and nucleus accumbens.

In summary, this study demonstrated that three-month-old Fisher 344 rats exposed to ethanol for the month displayed a selective ethanol effect on the dopamine receptors. There was a significant decrease in the number of D\textsubscript{1} sites in the nucleus accumbens and frontal cortex while the D\textsubscript{1} sites in the striatum and globus pallidus were not altered. In these four regions, the affinity of SCH 23390
was not affected by ethanol treatment. The density and affinity of the dopamine D2 sites in the striatum and nucleus accumbens were not significantly different in ethanol-treated rats. Also ethanol exposure did not change the affinity, number, or conversion of the D2 high affinity site.

The number of D1 receptors was decreased in both the nucleus accumbens and frontal cortex of ethanol-fed rats. Although a decrease in cortical D1 receptor number could be potentially explained as a compensatory down regulation in response to the increased dopamine release (increased DOPAC) in the cortex, this explanation cannot explain the decrease in D1 receptors in the nucleus accumbens since DOPAC is decreased in this area. Alternative explanations for the decreased number of dopamine receptors are: 1) receptor desensitization, 2) agonist-induced endocytosis, 3) decreased gene transcription. For example, ethanol may induce receptor desensitization of the D1 receptor or may inhibit transcription of the gene for the D1 receptor, resulting in the loss of D1 receptor sites. It is also possible that the selective effect of ethanol on D1 receptors in distinct brain areas may reflect a different cellular localization of the D1 and D2 receptors.

Molecular mechanisms of receptor desensitization have been described using the β-adrenergic receptor as a model (Sibley et al. 1987). A similar mechanism may exist for
other receptor types including the dopamine D1 receptor. Receptors assume an altered conformation during desensitization. This conformational change is promoted by covalent receptor modification. Phosphorylation plays a role in desensitizing receptors linked to the G proteins (Klein et al. 1989). Receptors may be phosphorylated by a variety of protein kinases but a novel cAMP-independent protein kinase (βARK) appears to be involved in the homologous desensitization of the β-adrenergic receptor (Benovic et al. 1986). This enzyme only phosphorylates the agonist-occupied form of the receptor. When a cell is stimulated by a β-adrenergic agonist, βARK is translocated from the cytosol to the plasma membrane where it phosphorylates the agonist-occupied receptor (Strasser et al. 1986). The phosphorylated receptor is uncoupled from adenylate cyclase and sequestered within the cell into low density membrane vesicles. Within the vesicle, the receptor is dephosphorylated by a phosphoprotein phosphatase and then is functionally regenerated. When the agonist is removed, the receptor is redistributed to the plasma membrane. βARK may serve as a nonspecific adenylate cyclase-coupled receptor kinase and may play a role in a generalized mechanism for homologous desensitization for several receptors including the dopamine receptors.

The loss of dopamine D1 receptors in the nucleus accumbens and frontal cortex may be explained by an ethanol
effect on the mechanism described above. Ethanol may promote receptor phosphorylation via an increased activation of one or more second messenger pathway, which in turn may activate one or more protein kinases. An activated protein kinases would increase the phosphorylation state of the receptor, maintaining the receptor in an inactive form. On the other hand, ethanol may inhibit phosphatase activity, thus maintaining the phosphorylated state of the receptor and in turn, inhibit the recycling of the receptors into the plasma membrane resulting in a loss of receptors. Presently, additional information regarding ethanol's effects on this mechanism is needed to substantiate this interpretation.

Another mechanism which may account for the down regulation of receptors is receptor-mediated endocytosis. Ligand-induced receptor degradation occurs when the endosomes containing the receptors fuse with lysosomes (Chuang et al. 1982). It is possible that ethanol mediated changes in dopamine concentrations or other neurotransmitters levels may promote agonist-induced receptor degradation resulting in receptor down-regulation.

Adaptive changes in receptor density may also be regulated at the level of gene transcription. Ethanol may alter the activation or inhibition of transcriptional factors that control the rate of transcription. A decrease in transcription may result in a decreased receptor number. The recent availability of cDNA probes for the dopamine
receptors will provide a means to examine this mechanism of receptor regulation.

It is interesting to note the selective loss of the D1 receptors in the nucleus accumbens and frontal cortex. It is possible the D1 and D2 receptors are differentially regulated within each brain region or cell type at the level of gene transcription. It is possible that the genes for the D1 and D2 receptors require at least one tissue specific transcriptional factor in order for transcription to occur. Although, the precise pharmacological actions of ethanol are unknown, however, the differential responses to ethanol reveal the complex neuronal interactions involved in normal physiological activity.

Regardless of the cause of the decreased number of D1 receptors in the frontal cortex and nucleus accumbens, it is doubtful whether this decrease is responsible for altered cellular function because in the presence of a saturating concentration of GTP dopamine-stimulated adenylate cyclase activity was not altered in either region. Additional components of this signal transduction pathway, namely the synthesis and concentration of Gsα as well as the basal, NaF, and forskolin-stimulated adenylate cyclase activity were unaltered in these regions from ethanol-fed rats. Consequently, the loss of D1 receptors from the frontal cortex and nucleus accumbens may represent a loss of spare receptors.
Adenylate Cyclase Activity

In the present study, cAMP levels were assessed as an indicator of adenylate cyclase activity. The response of adenylate cyclase to stimulation by forskolin, GTP, and sodium fluoride was also analyzed as a means to define the site of ethanol's action. Basal, D1-stimulated, and D2-inhibited adenylate cyclase activity was not altered in the striatum of ethanol-treated animals. There was no change in the stimulation of striatal adenylate cyclase activity by forskolin, GTP, and sodium fluoride. Similarly, adenylate cyclase activity in the frontal cortex was not altered by ethanol treatment. In contrast, basal adenylate cyclase activity in the presence of dopamine was significantly increased in the nucleus accumbens of ethanol-treated animals, D1-stimulated activity and basal adenylate cyclase activity in the presence of forskolin or sodium fluoride were unchanged in this region. It is possible that there was a sufficient amount of GTP in the tissue resulting in the stimulation of adenylate cyclase by dopamine; however, further studies will be necessary to determine the site of this ethanol effect.

In studies by other laboratories, chronic ethanol treatment generally produced a decrease in adenylate cyclase activity. Dopamine-stimulated and norepinephrine-stimulated adenylate cyclase activity was reduced in the striatum and cerebral cortex, respectively (Tabakoff and Hoffman 1979;
Lucchi et al. 1983; Saito et al. 1987). The stimulation of adenylate cyclase by various agonists and agents acting through Gs or adenylate cyclase also was decreased after chronic ethanol exposure (Saito et al. 1987). Studies in cultured cells demonstrated a similar response, a decrease in agonist-stimulated adenylate cyclase after chronic ethanol treatment (Richelson et al. 1986; Rabin 1990). In contrast, Rabin et al. (1987) reported an increased response to dopamine-stimulated, GTP-stimulated, and sodium fluoride-stimulated adenylate cyclase activity in the striatum of chronic ethanol-treated rats. The discrepancies among the present study and other studies may be attributed to different laboratory protocols. For example, some studies examined adenylate cyclase activity in ethanol-withdrawn animals which may not represent the effects of chronic ethanol exposure. Mode of ethanol administration, dose of ethanol, or animal species also may contribute to these different findings.

An increase in cAMP levels may be the result of increased levels of adenylate cyclase. Ethanol may alter the activation of one or more transcription factors which regulate the gene for adenylate cyclase. Alternatively, ethanol may inhibit the degradation of either the mRNA or the protein for adenylate cyclase. Such alterations would result in an increased synthesis of cAMP.

Another possible explanation for increased cAMP levels
may be decreased phosphodiesterase activity. Phosphodiesterase hydrolyzes the phosphodiester linkage of cAMP terminating the biological activity of adenylate cyclase. As stated previously, ethanol may alter the activation of the transcription factors regulating the expression of the gene for phosphodiesterase. Decreased phosphodiesterase levels may result in increased levels of cAMP. Phosphodiesterase is regulated by cAMP-dependent protein kinase (Northup et al. 1989). Phosphorylation of this enzyme decreases its affinity for calmodulin, thereby decreasing its activation. Alternately the phosphorylation state of phosphodiesterase may be modulated via the dopamine-stimulated phosphorylation and activation of DARPP-32, potent inhibitor of protein phosphatase-1 (Hemmings et al. 1989). Inhibition of protein phosphatase-1 would maintain phosphodiesterase in its phosphorylated inactive state.

G Proteins

In the present study, mRNA and protein levels of the $\alpha$ subunit of several G proteins were assessed by slot blot analysis and Western analysis. In all brain regions surveyed, the mRNA levels of the $\alpha$ subunits of $G_s$, $G_i_1$, and $G_i_2$ were not altered by ethanol exposure. There were significant differences in the mRNA levels of $G_o$ in the substantia nigra and $G_i_3$ in the frontal cortex and ventral
pallidum. The protein levels of the α subunits of Gs and Go were not affected by ethanol treatment. The D1 stimulation of adenylate cyclase appears to involve Gs, whereas D2 inhibition of adenylate cyclase may be coupled to Gi₂ (Senogles et al. 1990). Ethanol-induced alterations of dopamine-sensitive adenylate cyclase activity may not be mediated through the G proteins since ethanol does not affect G protein synthesis. Although there was a decrease (20%) in the Go mRNA level in the substantia nigra, the protein level of Go in the substantia nigra was not altered. A change in mRNA levels of this magnitude may not result in differences in protein levels.

The results of this study concur with findings reported concerning G protein content in the cerebral cortices of chronically treated mice (Hoffman and Tabakoff 1990). The investigators observed no significant alterations in the levels of the α subunits of the Gs and Go proteins. However, studies of ethanol-treated culture cell lines differ. Mochley-Rosen and colleagues (1988) demonstrated decreases in mRNA and protein levels of the α₁ subunit in chronic ethanol-treated NG 108-15 cells. Charness and colleagues (1988) reported a decrease in the content of the α₁ subunit in NG 108-15 and N1E-115 cell lines, but not in the N18TG2 cell line. They also found a dose-dependent increase in the α₁ subunit in the N1E-115 cell line with no change in the NG 108-15 and N18TG2 cell lines. In the present study, there
was a significant increase in the mRNA levels of Gi₃ in the frontal cortex and ventral pallidum which may result in an increase in the protein content as observed by Charness et al. (1988).

In theory the presently observed ethanol-induced changes in adenylate cyclase activity may involve alterations of the synthesis of the α subunits of the G proteins. Mochley-Rosen and colleagues (1988) suggest the quantity of α₁ is the rate-limiting step in the activation of adenylate cyclase. However, this hypothesis is not supported by the present study because the mRNA and protein levels of α₁ were not altered by ethanol exposure. Hoffman and Tabakoff (1990) suggest the possibility that chronic ethanol exposure causes posttranslational modification of the α subunit that may alter its interaction with receptors and effectors. In the present study, this possibility could not be tested utilizing the selected experimental procedure.

In summary, three-month-old rats exposed to ethanol for one month display regional specific changes in G protein mRNA levels, but protein content of Gₛ and Gₒ were not altered. Although there were small changes in the mRNA levels of Gₒ and Gi₃, these differences may not affect the overall function of the G proteins. The results of this study suggest ethanol's action may be at the level of adenylate cyclase rather than at the level of the G proteins.
Cholesterol/Phospholipid Ratio of Synaptic Plasma Membranes

Although a significant increase in the cholesterol/phospholipid ratio of synaptic plasma membranes was observed, it is unlikely that this membrane change was responsible for the observed changes in components of the dopaminergic system. Whereas the present studies report a decrease in dopamine D1 receptor number in certain brain areas, earlier studies from this laboratory have shown that D1 receptor number is not significantly affected by changing the cholesterol/phospholipid ratio (Maguire and Druse 1989). In contrast, artificial elevation of the cholesterol/phospholipid ratio does inhibit dopamine-stimulated adenylate cyclase (Maguire and Druse 1989), while the ethanol-induced increase in membrane cholesterol did not inhibit this enzyme in any brain region examined. Thus, the observed changes in membrane-associated components of the dopaminergic systems appear to be caused by factors other than changes in the membrane cholesterol content.

Concluding Remarks

The results of the present studies have demonstrated that several components of the dopaminergic system in three-old-month Fisher 344 rats are altered after one month of chronic ethanol exposure. Those brain areas which are affected are associated with the mesolimbic dopamine pathway. There was a loss of the D1 dopamine receptor in the
nucleus accumbens and frontal cortex of chronic ethanol-treated rats. Dopamine and DOPAC levels were increased two-fold in the frontal cortex, ventral pallidum, and ventral tegmental area, while DOPAC levels were decreased in the nucleus accumbens of chronic ethanol-treated rats. In the nucleus accumbens, there was an increase in basal adenylate cyclase activity in the presence of dopamine.

These changes in the brain regions of the mesolimbic dopamine pathway are notable because this pathway is a component of the reward circuitry (Phillips and Fibiger 1978). Several drug self-stimulation studies suggest that dopamine neurons in the accumbens and prefrontal cortex play a critical role in the reinforcement of cocaine (Roberts and Koob 1980; Goeders and Smith 1986). It is also possible the mesolimbic dopamine neurons may be involved in the rewarding effects of ethanol (Koob and Weiss 1990; Lewis and June 1990; McBride et al. 1990; Samson et al. 1990).

In three-month-old Fisher 344 rats fed an ethanol-containing diet for one month prior to sacrifice, specific components of the mesolimbic and mesocortical dopamine systems were significantly altered. Whether these changes are translated into behavioral abnormalities is not known. Although, it does not appear that the observed changes in receptor number contribute to signal transduction in regards to the adenylate cyclase pathway, we cannot rule
out the possibility that other dopamine-sensitive signal transduction pathways may be altered. Furthermore, it is not known whether the observed changes are readily reversed upon withdrawal of the ethanol diet or whether they represent the first stages of a potential neurotoxicity.

The differential responses of each brain region to chronic ethanol may reflect different mechanisms of regulation as discussed previously. In addition, regulation through other neurotransmitter systems should be considered as a possible explanation for ethanol's selective effects. For example, GABA, serotonin, and neurotensin may have a role in the regulation of the dopamine system (Mereu and Gessa 1985; Nemeroff and Cain 1985; Erwin and Korte 1988; Galloway 1990). Thus, alterations in components of the dopamine system may be secondary to the effects of ethanol on other neurotransmitter systems. Consequently, ethanol's unique effect on a given neurotransmitter may reflect the interaction of effects produced on a number of interrelated pathways.
CHAPTER VI

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VITA

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ABSTRACTS


PUBLICATIONS

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

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