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Investigation Into Select IsoquinolInes as a Putative Causal Agent of Ethanol Addiction in Mammals

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AS A PUTATIVE CAUSAL AGENT OF ETHANOL ADDICTION IN MAMMALS

by

Jacob Christopher Strawbridge

Abstract of a Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

May 2009

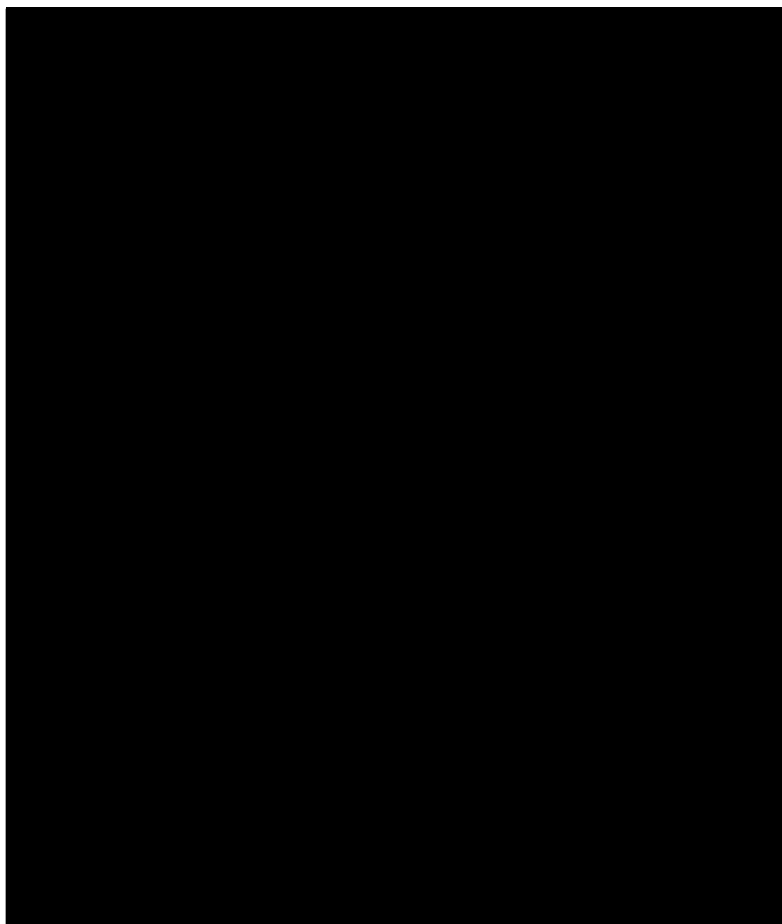The University of Southern Mississippi

INVESTIGATION INTO SELECT ISOQUINOLINES

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ABSTRACT

INVESTIGATION INTO SELECT ISOQUINOLINES

AS A PUTATIVE CAUSAL AGENT OF ETHANOL ADDICTION IN MAMMALS

by Jacob Christopher Strawbridge

May 2009

Alcohol is the most abused drug in America today. The neurological root of the problem is still a much debated subject and many differing views exist on the nature of alcoholism, be it a social dysfunction or a neurochemical imbalance. Some researchers have proposed that a skewed metabolism of dopamine that results in the formation of tetrahydroisoquinoline (THIQ) alkaloids plays a major role in the neurochemical acquisition and maintenance of alcohol addiction. In an effort to better understand the relationship of THIQ's to alcohol addiction, a series of experiments have been conducted. The chiral separation via high pressure liquid chromatography (HPLC) of various salsolinol (SAL) and salsolinol-derived compounds using a macrocyclic substituted sugar, sulfated β-cyclodextrin, as a chiral mobile phase additive was investigated. These HPLC separations yield data on a cheap and effective method for resolving enantiomers of simple catecholamines. The second set of experiments involved measuring the ethanol intake of rats given intracerebroventricular (ICV) injections of racemic tetrahydropapaveroline (THP) and R-(+)-THP. A similar experiment was conducted with tetrahydroberbine (THB). Finally, rats were administered ethanol solutions via gavage

and the local cerebrospinal fluid (CSF) in the lateral ventricle of the rats' brains was sampled via microdialysis. These dialysate solutions were assayed for THP concentration using high pressure liquid chromatography with electrochemical detection (HPLC-ECD). The results of our experiments indicate that THP is formed *in vivo* in the rat brain and THP levels increase in response to ethanol ingestion. This finding appears to support the hypothesis that THP plays an important role in the etiology or symptoms of alcoholism in humans.

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care and statistics.

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LIST OF ABBREVIATIONS

Abbreviation

1. NIAAA .... National Institute of Alcohol Abuse and Alcoholism

2. NAD^+ .... nicotinamide adenine dinucleotide

3. CNS .... central nervous system

4. K^+ .... potassium ion

5. Cl^- .... chloride ion

6. Na^+ .... sodium ion

7. Ca^{+2} .... calcium ion

8. DA .... dopamine

9. DOPA .... dihydroxyphenylalanine

10. MAO .... monoamine oxidase

11. DOPAC .... 3,4-dihydroxyphenylacetic acid

12. THIQ .... tetrahydroisoquinolines

13. SAL .... salsolinol

14. 3-CSAL 3-carboxysalsolinol

15. 1-CSAL 1-carboxysalsolinol

16. ICV .... intracerebroventricular

17. THP .... tetrahydropapaveroline

18. NADH .... protonated nicotinamide adenine dinucleotide

19. MAO-A .... monoamine oxidase-A

20. pmol .... picomoles

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CHAPTER I

INTRODUCTION AND BACKGROUND

Ethanol and Alcoholism

Throughout history, ethanol has played an important part in human society. It has inspired uncounted artists, musicians, and poets. However, one cannot speak of ethanol without coming to grips with alcoholism. Alcoholism has been defined by Koob, a prominent addiction researcher, as a complex behavioral disorder characterized by excessive consumption of ethanol, the development of tolerance and dependence, and impairment in social and occupational functioning [1]. Alcoholism is a serious health concern in America, as alcohol is the most widely used psychoactive drug [2] and is the third leading cause of preventable death in America [3]. A relative comparison of the alcohol-related healthcare costs versus the corresponding health care costs for all other drugs of abuse can be seen in Figure 1 [4].
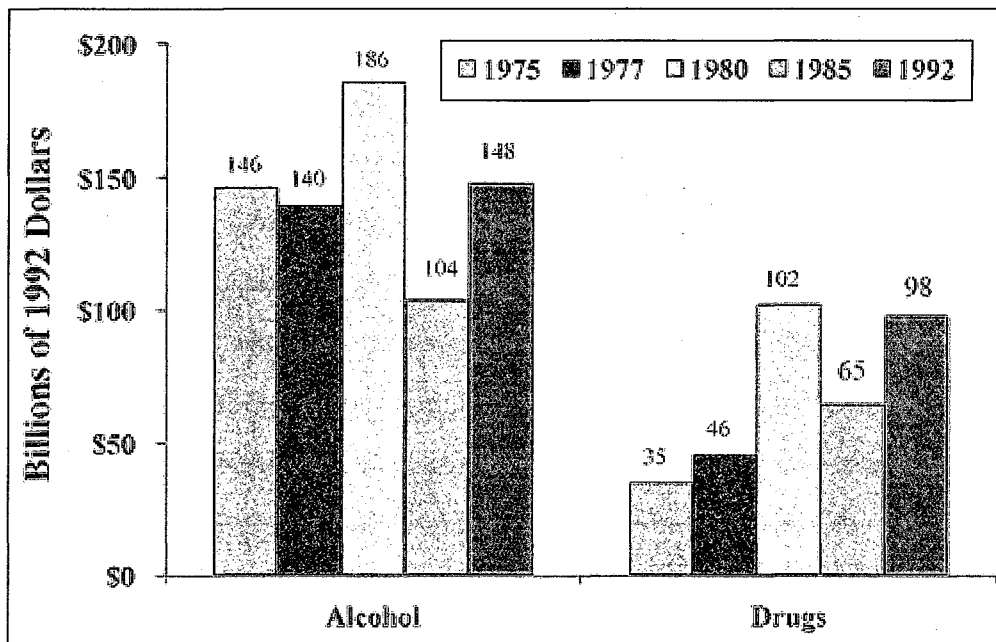
Figure 1: Comparison of Estimates From the Major Cost-of-Illness Studies for Alcohol and Drug Abuse, Adjusted for Inflation and Population Growth [4]

In January 1987, the National Institute of Alcohol Abuse and Alcoholism (NIAAA) stated that two-thirds of the American population drank ethanol. However, ten percent of those who drank accounted for one half of all the alcohol consumed [5]. In 1994, it was estimated that nearly eight million Americans were alcoholics [6]. Clearly, the etiology of alcoholism merits further study.

A better understanding of the phenomena associated with craving of ethanol and the reward mechanisms involved could lead to more efficient treatments for alcoholics. Due to the complexity of alcohol addiction in humans, a problem that has psychological, genetic, sociological, and ethical components, researchers rely on the use of animal

models, genetic profiling of family histories, and anecdotal accounts to assess the

neurochemistry of ethanol addiction.

The metabolism of ethanol in the liver and brain of mammals is well established

[7, 8]. As shown in Figure 2, ethanol is converted to acetaldehyde by alcohol

dehydrogenase found in cytosol, the liquid component of the cytoplasm, and the cofactor

nicotinamide adenine dinucleotide (NAD^+). Acetaldehyde is then oxidized by an NAD^+-

dependent aldehyde dehydrogenase found in mitochondria and NAD^+ to form the acetate

ion. This acetate product is then eliminated from the body.
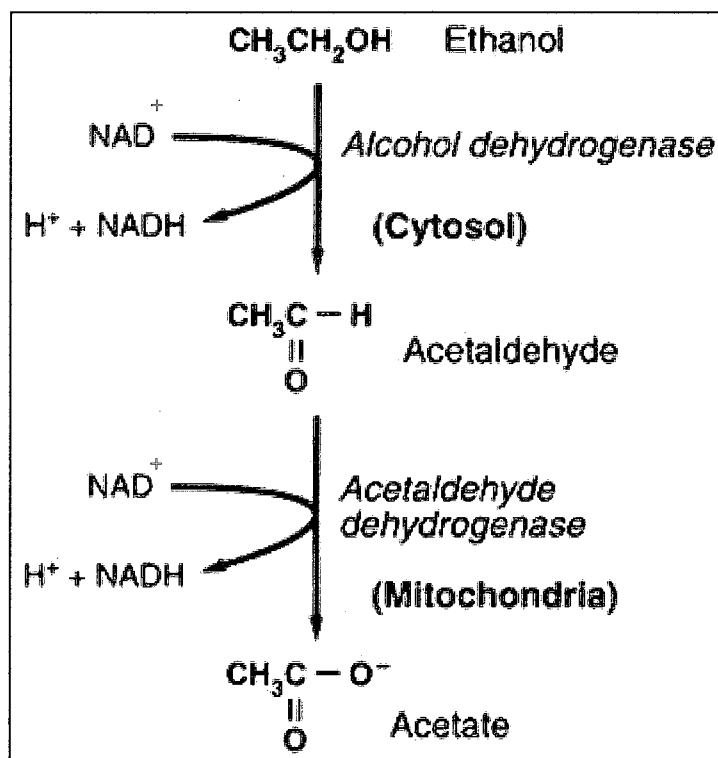
Figure 2: The Metabolism of Ethanol

Ethanol exerts its intoxicating effects on the central nervous system (CNS) by shifting the normal processes of information conduction and transmission in an indirect manner. A nerve cell must maintain a certain concentration of K^+, Cl^-, and Na^+ in the extracellular fluid in order to generate action potentials and allow Ca^{+2} to release neurotransmitters. When an action potential reaches the terminus of the axon, Ca^{+2} ions enter the presynaptic terminal through voltage-gated ion channels specific for Ca^{+2} and signal the release of the stored dopamine into the synaptic cleft.

Insights into the mechanisms by which ethanol affects CNS activity have been derived primarily from neurochemical assessments of ethanol's actions. Ethanol is known to inhibit sodium and potassium ion channels in the brain [9-12]. Ethanol has also been shown to increase calcium ion binding in the brains of rats and mice [13], resulting in a decrease in free calcium ions in select brain areas [14].

While these discoveries may help explain the effects of ethanol on nerve signal conductance, the complexity of the neural environment of even experimental animals has produced a controversy in the neuroscience community over the exact mechanism by which ethanol dependence operates. The current controversy revolves around the causal agents of dependence and whether changes in the dopaminergic, the endogenous opioid systems, or other circuits play the most important role in the etiology of alcoholism.

Dopamine as a Neurotransmitter

Dopamine (DA) is one of the major neurotransmitters in the CNS. Figure 3 shows

five of the prominent neurotransmitters. Dopamine, also called hydroxytyramine, is a

monoamine intermediate formed from dihydroxyphenylalanine (DOPA) during the

metabolism of the amino acid tyrosine [15]. Figure 4 demonstrates the synthesis of

dopamine in the CNS.
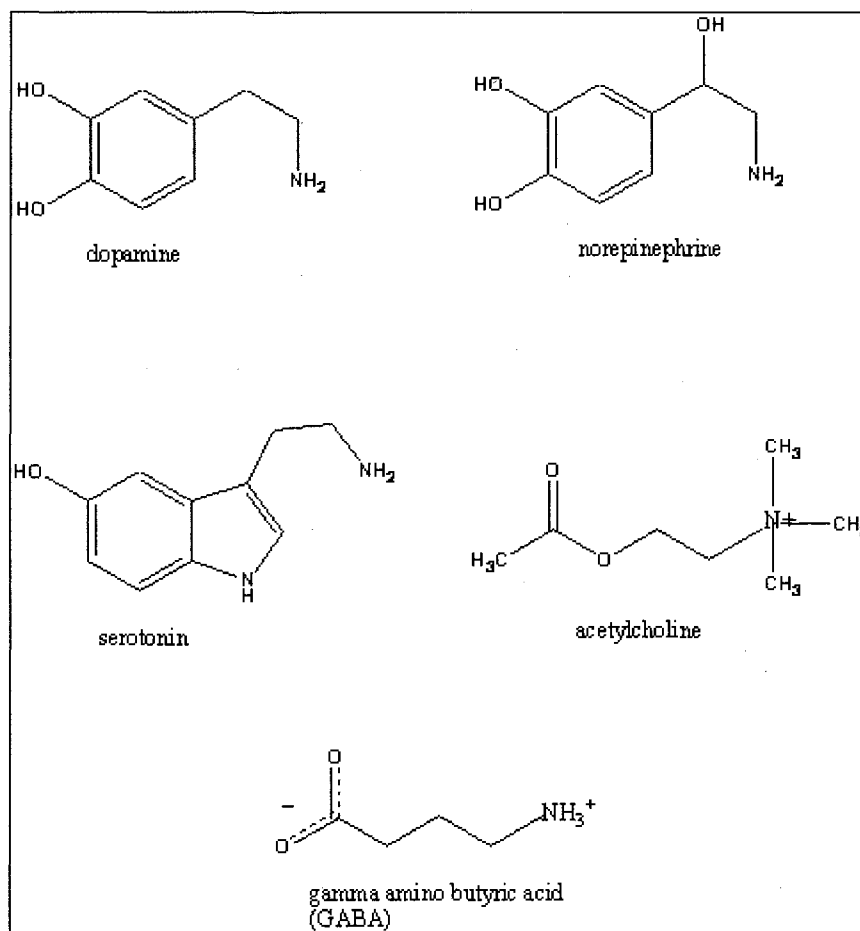
Figure 3: Five Major Simple Neurotransmitters
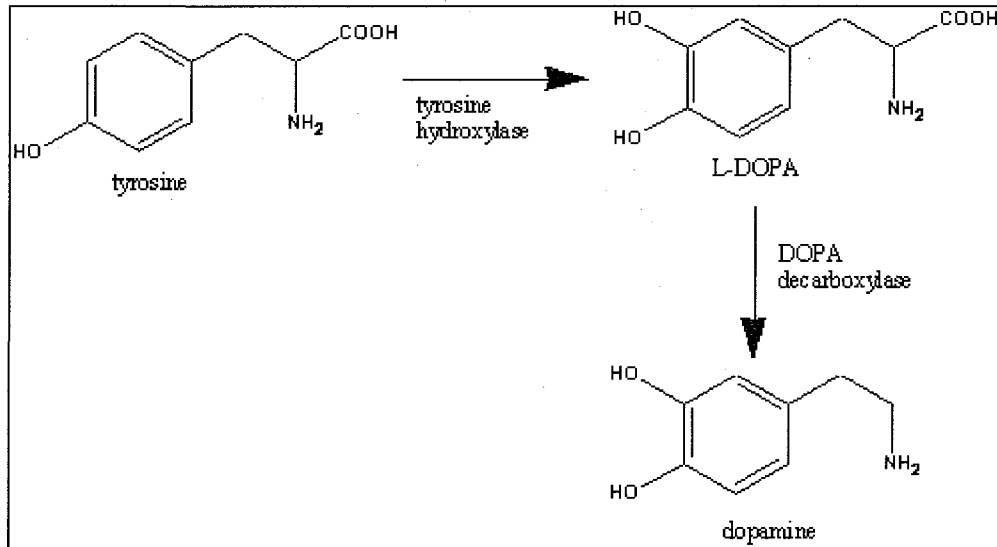
Figure 4: Dopamine Synthesis *In Vivo* From Tyrosine

Dopamine is commonplace in the medial forebrain bundle and important in the substantia nigra, basal ganglia, and corpus striatum of the brain [16-19]. Figure 5 shows a variety of dopamine pathways located in the human brain. Dopamine can be both excitatory and inhibitory, depending on the postsynaptic receptor. It has been implicated in several functions, including locomotion, learning, attention, temperature regulation [15], and as a reinforcing agent for drugs of abuse via the nucleus accumbens and the mesolimbic reward pathway [1, 20-27].

frontal lobe

basal ganglia
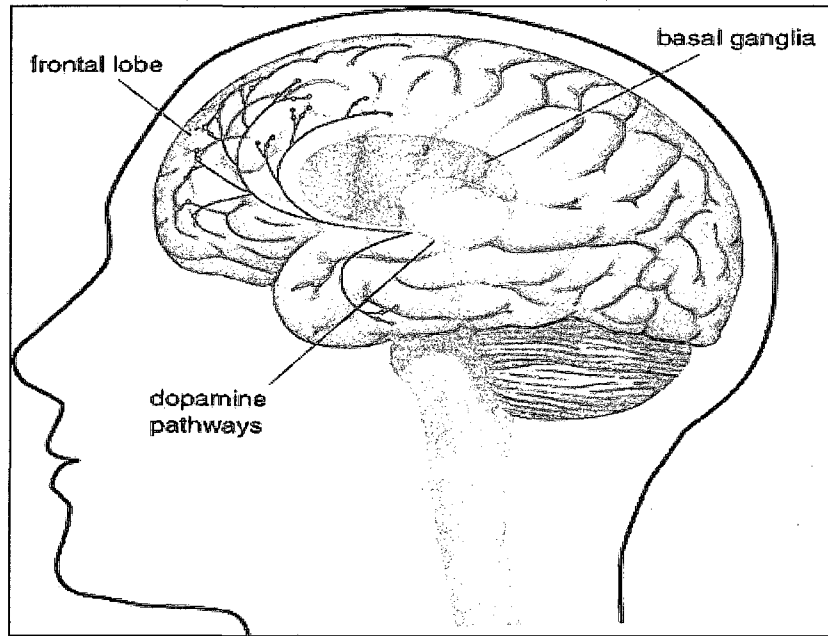
dopamine
pathways

Figure 5: An Overview of Dopamine Pathways in the Human Brain

Dopamine, once synthesized, is stored in vesicles in the presynaptic neuron's terminal button. Ca^{+2} ions enter the presynaptic axon and signal the release of dopamine into the synaptic cleft. This released dopamine then binds with postsynaptic receptors and generates a signal, either excitatory or inhibitory, in the postsynaptic neuron. This process is then repeated in the next series of dopaminergic neurons. A schematic of a normal dopaminergic synapse appears as Figure 6.
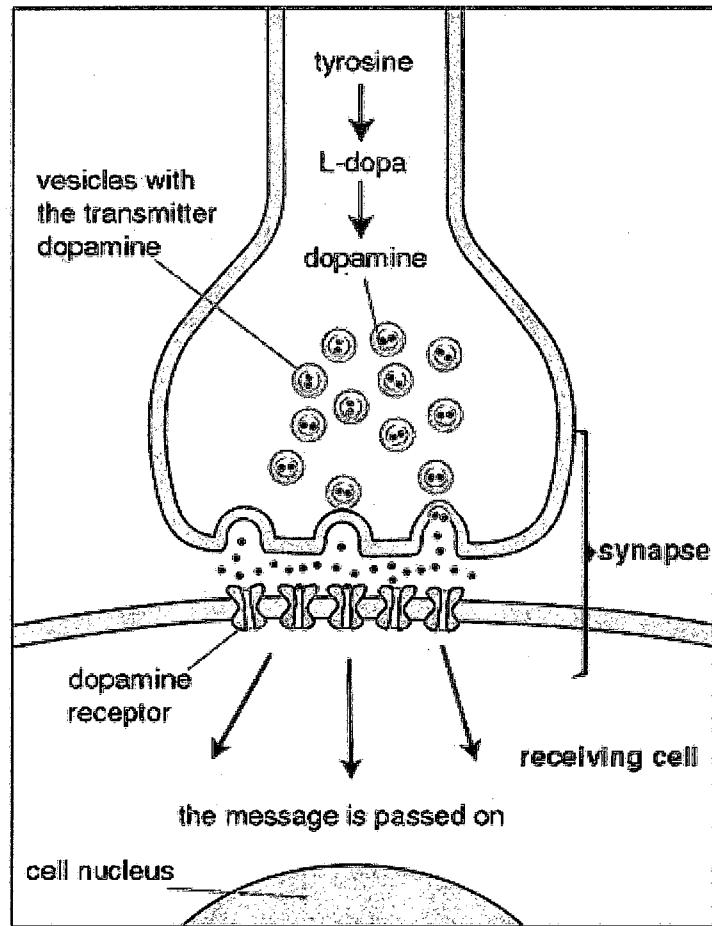
Figure 6: A Diagram of a Dopaminergic Synapse

The metabolism of dopamine is, in part, a simple oxidation. Dopamine is oxidized

by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetaldehyde (dopaldehyde).

Dopaldehyde is oxidized by aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic

acid (DOPAC). Figure 7 shows the known dopamine metabolism in mammals [28-31].
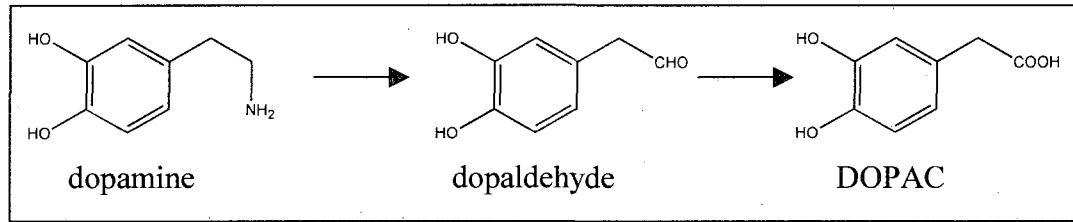
Figure 7: Dopamine Metabolism

Catecholamine Theory of Alcohol Addiction

Two slightly different mechanisms for the development of alcoholism were

suggested independently by separate groups some thirty years ago. The first involved the

formation of tetrahydroisoquinoline (THIQ) alkaloids from the condensation of dopamine

or DOPA with acetaldehyde, the first metabolite of ethanol [32]. This condensation

reaction produced a 1-methyl-THIQ, salsolinol (SAL), and its carboxylated derivatives,

the D and L isomers of 3-carboxysalsolinol (D-3-CSAL and L-3-CSAL). Another

condensation of dopamine with pyruvic acid produces the 1-carboxysalsolinol (1-CSAL)

derivative of SAL. Figure 8 shows the relevant reactions involved in these condensations.

This theory was supported by the discovery that SAL could be formed *in vitro* by

perfusing bovine adrenal gland homogenates with acetaldehyde solutions [32]. Further

studies confirmed that SAL was formed *in vivo* in rat [33, 34] and in man [35, 36]. More

recently, it was shown that the condensation of dopamine and acetaldehyde to form SAL

is enzymatically driven, hence forming a majority of the R-(+)-isomer of SAL [37, 38].

Other studies have shown that intracerebroventricular (ICV) perfusion of SAL can elicit a

drinking response from alcohol avoiding animals [39, 40]. Recently, SAL has been the

object of study because of the neurotoxicity of one of its metabolites that may be a

contributing factor in the development of Parkinson's disease [41-43].
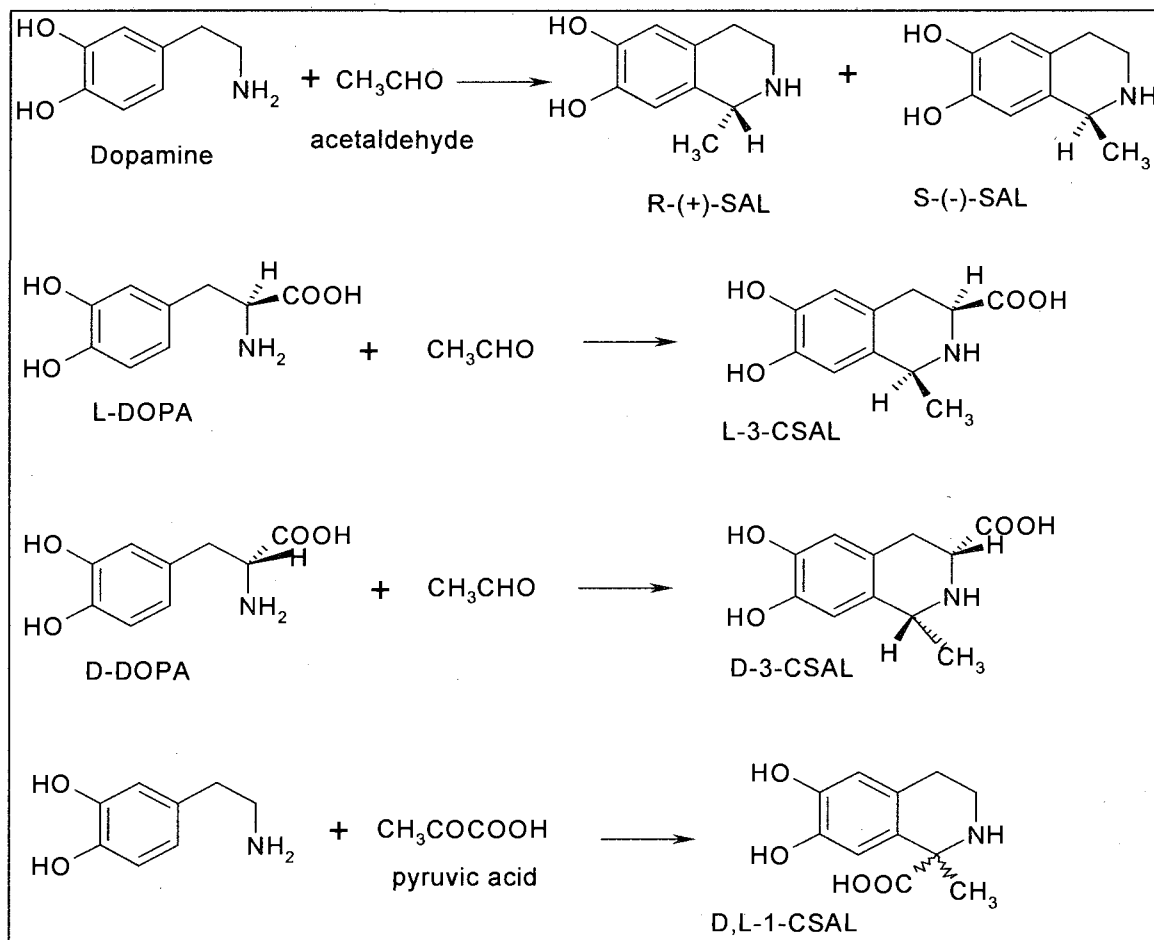
Figure 8: The Formation of SAL and CSAL

Another group of researchers [31] proposed the second major catecholamine

theory of alcoholism at virtually the same time. This theory held that the presence of

acetaldehyde could inhibit the conversion of dopaldehyde to DOPAC, as acetaldehyde

has a high affinity for aldehyde dehydrogenase also [31]. Alcohol, by way of its primary

metabolite acetaldehyde, facilitates the formation of tetrahydropapaveroline (THP) by

competitively inhibiting aldehyde dehydrogenase *in vitro*. Under circumstances of substantial alcohol ingestion *in vivo*, the limited ability of the brain to oxidize aldehydes could contribute to the formation of THP [28-31]. This inhibition would lead to high levels of dopaldehyde accumulating in the brain and could lead to the condensation of dopamine with dopaldehyde to form 1-benzyl-THIQ, tetrahydropapaveroline [31].

THIQ's were originally isolated in plants under strongly acidic conditions by the Pictet-Spengler condensation reaction [44]. It was subsequently demonstrated that THIQ alkaloids can be formed under physiologic conditions of pH and temperature from the condensation of aldehydes, such as acetaldehyde, with catecholamines, such as dopamine and its N-methyl congener, epinephrine [45].

Davis and Walsh found that THP could be formed *in vitro* by incubating rat brain stem homogenates rich in dopamine, with acetaldehyde and NADH, which is used as a cofactor to retard the further metabolism of acetaldehyde [31]. The presence of THP in rat brain was verified by infrared spectroscopy, gas chromatography, and thin-layer chromatography [29-31]. Other researchers used thin-layer chromatography to demonstrate the formation of THIQ alkaloids *in vitro* by perfusing bovine adrenal gland with dilute acetaldehyde solutions [32]. This hypothesis was also supported by reports that THP occurred *in vivo* in the brains of rats [33, 34, 46] and humans [35, 36, 47, 48]. Thus, buildup of THP following alcohol intake, might have pharmacological effects relevant to the etiology of alcoholism.

Although the majority of ethanol is metabolized by alcohol dehydrogenase, catalase, a ubiquitous enzyme found in large amounts in peroxisomes, can oxidize ethanol as a co-substrate with hydrogen peroxide to yield water and acetaldehyde [49].

Hydrogen peroxide is produced in brain tissue when monoamine oxidase (MAO) oxidizes biogenic amines. Thus, catalase can use hydrogen peroxide to metabolize ethanol to acetaldehyde in the brain, resulting in a large concentration of acetaldehyde localized at dopaminergic neurons [8].

Alternatively, acetaldehyde inhibits the degradation of dopamine to dihydroxyphenylacetic acid. This increases the availability of aldehydes for participation in a Pictet-Spengler condensation with intact dopamine. If aldehyde dehydrogenase is occupied with the metabolism of acetaldehyde, the biogenic aldehyde could condense with its parent amine, resulting in THP formation. In fact, a recent report has shown that pharmacological inhibition of MAO-A reduced the volitional consumption of ethanol, perhaps by preventing the formation of both biogenic aldehydes and acetaldehyde and preventing the formation of the alkaloidal products [8].

Since THP is a precursor in the synthesis of morphine and other alkaloids in *Papaver somniferum*, the opium poppy, it was suggested that the formation of THP might contribute to the development of addiction by acting as a false transmitter or opiate agonist [29, 31, 50]. Additionally, THP might be further metabolized to form the opiates themselves. Figure 9 shows this proposed "skewed" metabolism of dopamine resulting in the formation of THP and hypothesized pathways (appearing as a dashed reaction arrow) by which THP could be converted to opiate alkaloids and tetrahydroxyberberine compounds [31]. Recently, S-(-)-THP was documented as being present in human brain tissue at concentrations of 0.12 to 0.22 pmol/g [51]. Morphine has also been reported to be endogenous to the CNS [52].
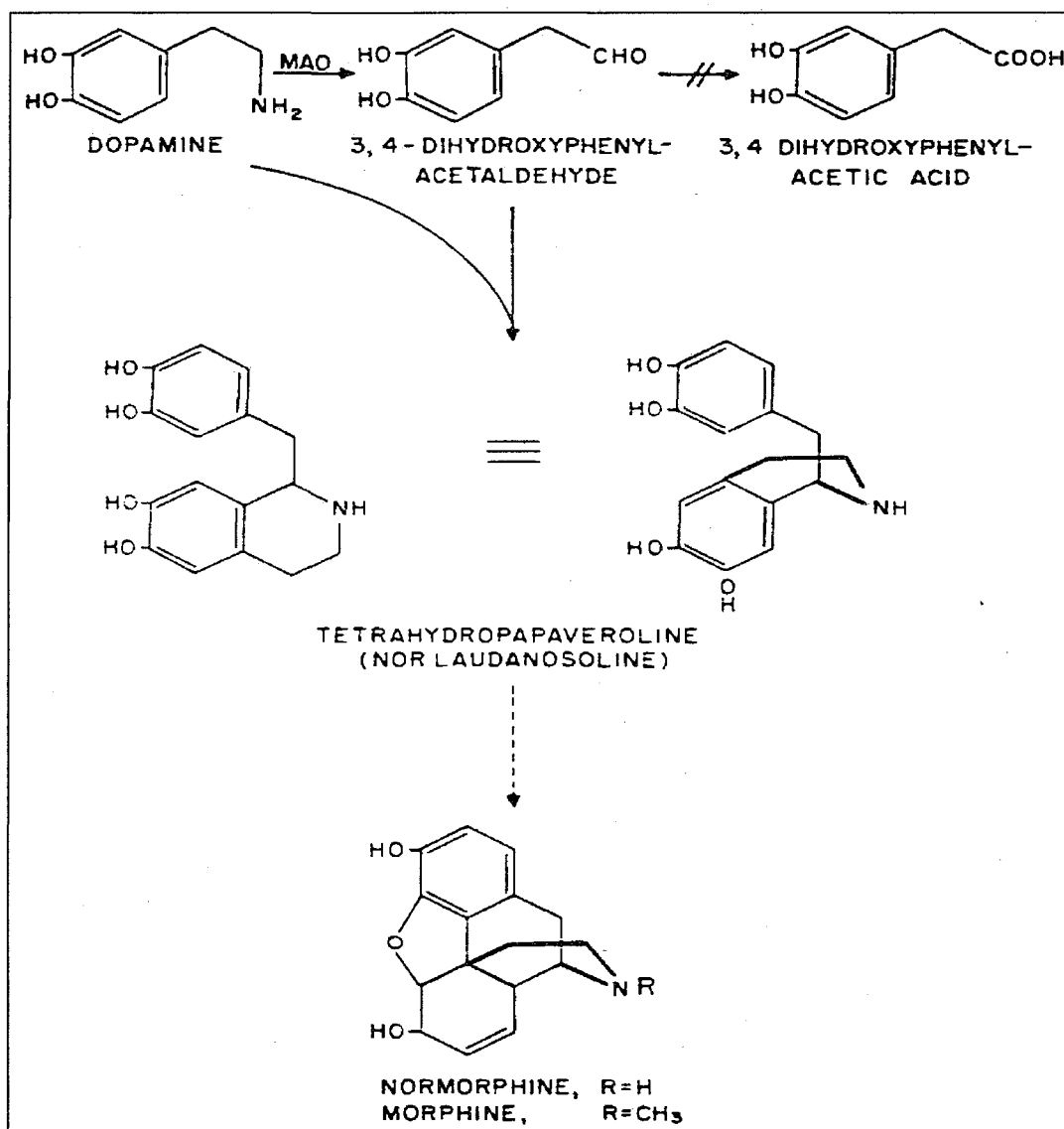
Figure 9: Proposed Dopamine Metabolism Resulting in the Formation of

THP and Possibly Opiate Alkaloids [31]

While no conclusive evidence exists as yet for the formation of these opiate

alkaloids directly from THP *in vivo*, the efficacy of THP in eliciting a volitional drinking

response in experimental animals is well documented. Myers and Melchior reported

initially that ICV infusions of THP would induce spontaneous volitional consumption of relatively high concentrations of ethanol by Sprague-Dawley rats, an inbred strain that normally avoids even low levels of ethanol [53-55]. Perfusion cannulae were implanted in the lateral ventricle of rats by stereotaxic surgery. Figure 10 shows a cutaway diagram of the lateral ventricles (marked as LV) of a rat. It should be noted that the lateral ventricles serve as a reservoir of cerebrospinal fluid (CSF) for most of the medial forebrain bundle.
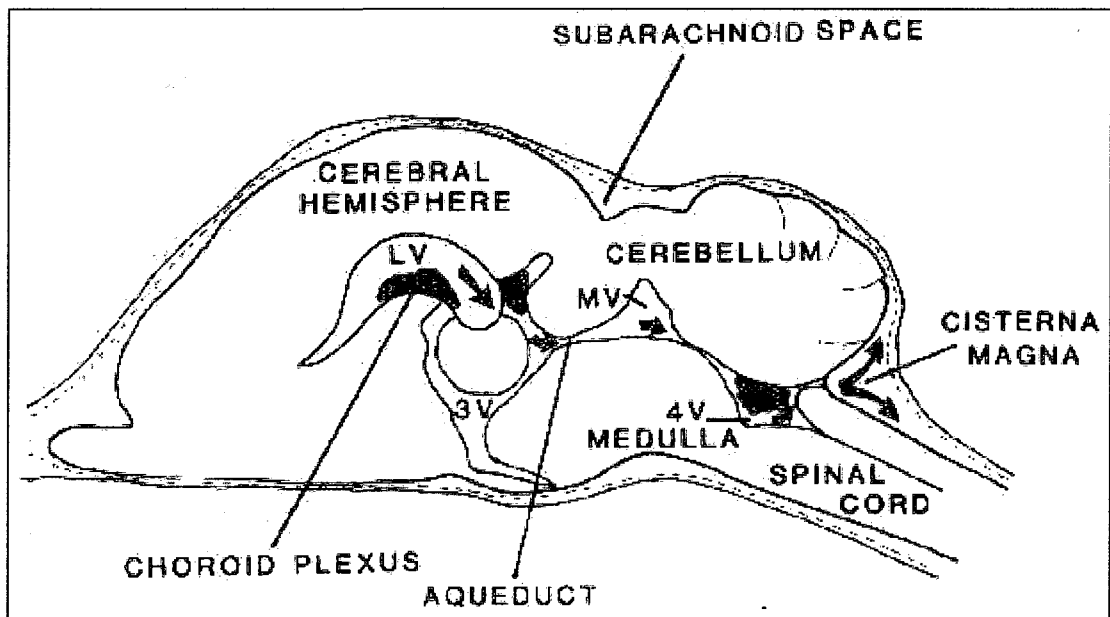
Figure 10: A Sagittal Cutaway Diagram of a Rat Brain Showing the Lateral Ventricle (LV) and Other Prominent Brain Features

The animals were then screened for alcohol preference using a three-bottle, twelve-day test. The bottle positions were randomized every day. One bottle was empty, the second contained water, and the third contained an ethanol solution in water. The

alcohol concentration was gradually increased from 3% by volume to 30% by volume over the twelve-day period [53]. Once baseline alcohol consumption for each animal was established, the pool of experimental rats was divided into two groups. One group was given ICV injections of a relatively high concentration of a racemic mixture of THP, and the other given a low dosage of racemic THP. The S-(-)-isomer was also tested. The ICV injections were made every fifteen minutes around the clock for fourteen days.

The rats were then screened for alcohol preference using the same initial method. Drastic changes were noted in all of the rats' drinking preferences. Halfway through the twelve-day protocol, rats that avoided even low concentrations of alcohol drank increasing amounts [53]. Rats receiving the concentrated injections of THP increased their alcohol consumption to half of their daily fluid intake [53]. Rats receiving the low concentration doses of THP drank alcohol in concentrations of up to 25% by volume [53]. These changes persisted until the end of the twelve-day protocol, with some animals' blood ethanol levels reaching 0.2% [53]. Symptoms of intoxication such as ataxia were noted in these animals that drank heavily, as well as symptoms of withdrawal when these rats were not drinking ethanol, such as tremors, hyperactivity, rearing, chewing, and jerking motions [53].

Another team of researchers [56] replicated this experiment. They confirmed that ICV injections of 5.2 to 41.6 nmoles/day is sufficient to elevate a Sprague-Dawley rat's alcohol consumption but that doses of 104 nmoles/day produced an aversion to ethanol [56]. It was also reported that the highest volumes of alcohol consumption occurred when the alcohol solution was 11% to 15% by volume [56]. This group of rats refused higher concentrations of ethanol and their blood alcohol levels did not reach intoxication,

although some animals did develop seizures [56]. No other symptoms of intoxication or withdrawal were reported. THP-induced changes in alcohol preference in Sprague-Dawley rats have been found to be very long lasting [53, 56, 57]. They may even be permanent, as animals injected with THP tested ten months later still exhibited a strong alcohol preference [56].

These studies [53, 56-59] suggest a possible role for THP in the etiology of ethanol craving. However, it remains to be seen whether THP acts as a false transmitter, or as a morphine precursor. All available evidence revolves around the treatment of experimental animals with morphine [60-72] or opiate antagonists such as naltrexone [73-83] or naltrindole [84, 85]. Morphine has been shown to potentiate alcohol drinking in rats with long term effects similar to those for THP-induced alcoholism [74]. Naloxone and naltrindole have been reported to reduce the alcohol intake of rats that prefer alcohol [63, 70, 74, 86]. So while no clear conclusions about the relationship between THP and the opioidergic neural circuits in the brains of mammals can be drawn, the circumstantial evidence points to some form of linkage between the two.

A number of abused drugs, which have independent pharmacological effects and are otherwise classified as having separable sites and mechanism of action, all activate the mesolimbic dopamine path. Drugs as diverse as cocaine, alcohol, nicotine, and heroin have been shown to release dopamine from nerve terminals at the nucleus accumbens (NAcc) of the basal forebrain [24, 26, 27, 87]. Pretreatment with naloxone, an opioid antagonist which shows higher affinity for the μ opioid receptor [88, 89] or with naltrindole, a δ opioid receptor antagonist [90], attenuated the release of dopamine at the NAcc evoked by administration of alcohol. Opiate antagonists, naltrexone and naloxone

have also been found to suppress volitional alcohol consumption initiated by THP infusion [74, 91, 92].

Mapping studies [93-95] have found that the ventral tegmental area (VTA) and the nucleus accumbens are among the most behaviorally responsive sites to THP microinfusion. Of the THIQ alkaloids, THP appears to be the most potent releaser of dopamine at forebrain sites, including the NAcc [96]. More recently, microinjections of THP into the VTA were found to augment extracellular efflux of dopamine from the core of the NAcc [97] of high ethanol preferring (HEP) rats. Conversely, THP suppressed dopamine efflux from the shell of the NAcc. Collectively, this evidence suggests that THP might exert behavioral actions via modulation of the mesolimbic dopamine circuit.

While this hypothesis is supported by some investigators, others view THP-induced alcohol consumption in experimental animals as a pharmacological effect [98, 99]. These researchers call into question whether THIQ alkaloids are ever formed endogenously in quantities sufficient to exert a significant effect on the behavior of the animal. Still other researchers have called into question techniques used by investigators that have been able to simulate alcoholism using THIQ's such as THIQ purity, surgical techniques, dosing regimens, and animal care protocols [60, 100-102].

Nonetheless, there are few known compounds that exert such a profound effect on volitional consumption of alcohol. At the least, these compounds may have utility in probing sites and mechanisms in the central nervous system (CNS) involved in the mediation or control of alcohol consumption.

Increases in alcohol consumption have been reported previously following central injection of racemic THP as well as the (S)-enantiomer of THP [53]. Heretofore there

have not been any published reports of alterations in alcohol preference induced by the (R)-enantiomer of THP. The (S)-enantiomer has been proposed to be a major precursor of morphine in the opium poppy [31]. It has been proposed that R-(+)-THP could be converted to morphine via a racemization reaction [103-107] and low doses of morphine have been found to promote alcohol consumption [64, 67, 70, 86, 108-110].

The (S)-enantiomer has been detected in the striatum of rats fed alcohol *ad libitum* for 18 months, suggesting that a THP biosynthetic pathway exists in the mammalian brain [111]. Moreover, a stereochemical analysis reported the presence of S-(-)-THP, but not R-(+)-THP, in each of four human brains [51]. Thus, further investigation of the (R)-enantiomer may yield additional pertinent information. If R-(+)-THP has no effect on alcohol consumption, then one might expect S-(-)-THP to be twice as effective as the racemic mixture.

On the contrary, prior reports have not found substantial differences in efficacy between S-(-)-THP and racemic THP [53]. This suggests that the (R)-enantiomer should be capable of stimulating alcohol consumption. Alternative to the hypothesis that morphine is synthesized from the alkaloid, THP may act as an opiate agonist. Because of the relative symmetry of the molecule, receptors may be unable to distinguish between the two alkaloid isomers. In either scenario, one might expect each enantiomer to be equally effective.

Experiments were conducted to re-evaluate whether, and to what degree, centrally administered (±)-THP or (+)-THP might induce alcohol consumption. Additional experiments evaluated whether simple repeated exposure to increasing concentrations of alcohol might result in volitional drinking. Control groups were used to evaluate whether

the stereotaxic procedure itself could impair the ability of the animal to discriminate between alcohol and water.

CHAPTER II

EXPERIMENTAL

HPLC Resolution of the Enantiomers of Dihydroxyphenylalanine and Selected Salsolinol

Derivatives Using Sulfated β-Cyclodextrin as a Chiral Mobile Phase Additive

Reagents

Racemic and optically active salsolinol was prepared in our laboratory using

routes described in the literature [112]. Briefly, condensation of 3,4-

dimethoxyphenethylamine, the dimethoxy analog of dopamine, with acetic anhydride,

subsequent cyclization to 1-methyl-6,7-dimethoxy-3,4-dihydroisoquinoline, followed by

reduction with sodium borohydride in ethanol gave salsolidine (Figure 11), the

dimethoxy analog of salsolinol.
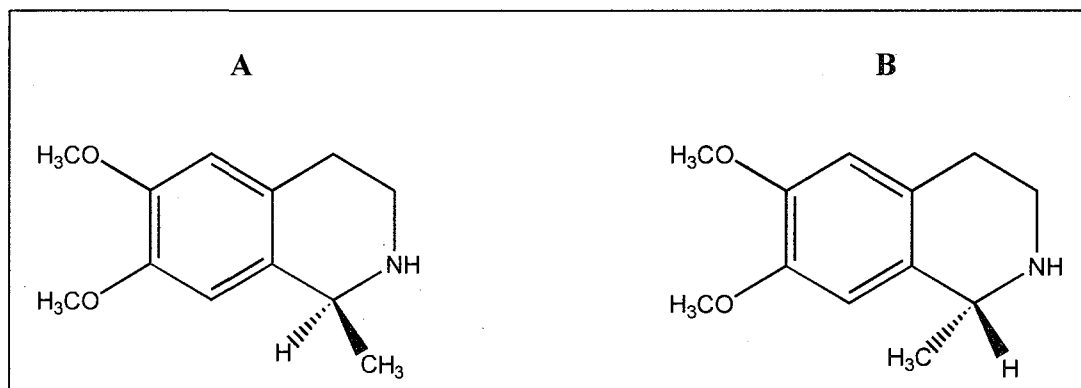
Figure 11: Structure of (A) S-(-)-salsolidine; (B) R-(+)-salsolidine

Demethylation of salsolidine in boiling concentrated hydrobromic acid gave

racemic salsolinol in analytical purity. Resolution of salsolidine followed by hydrobromic

acid catalyzed demethylation provided the optical isomers of salsolinol (Figure 12).
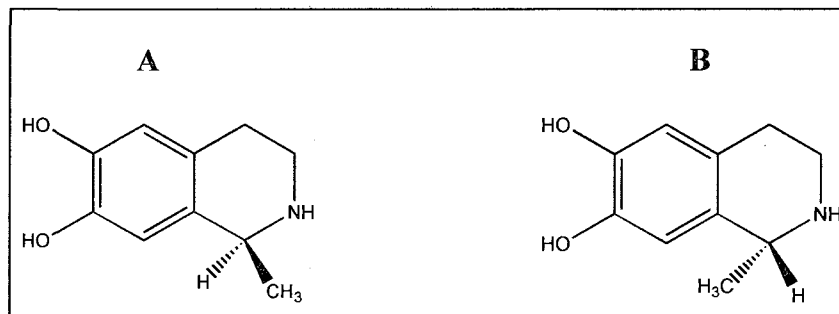
Figure 12: Structure of (A) S-(-)-salsolinol; (B) R-(+)-salsolinol

L-DOPA and racemic DL-DOPA (Figure 13) were obtained from commercial

sources (Sigma-Aldrich, St. Louis, MO).
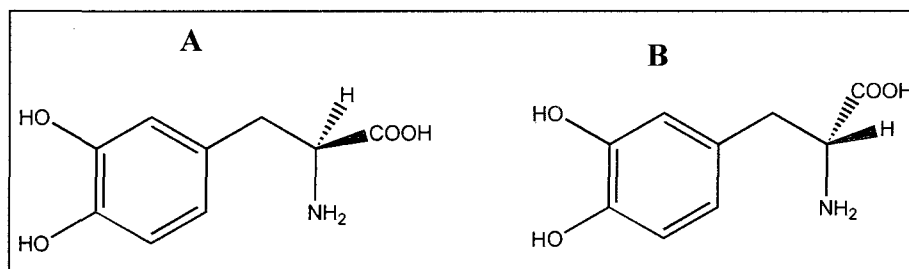
Figure 13: Structure of (A) L-(-)-DOPA; (B) D-(+)-DOPA

The 3-carboxy-tetrahydroisoquinoline derivatives were prepared by reaction of L-

DOPA or racemic DL-DOPA with acetaldehyde to give, respectively, L-3-

carboxysalsolinol and DL-3-carboxysalsolinol (Figure 14). Crystallization during work-up provided the *cis* isomers as the major product [113].
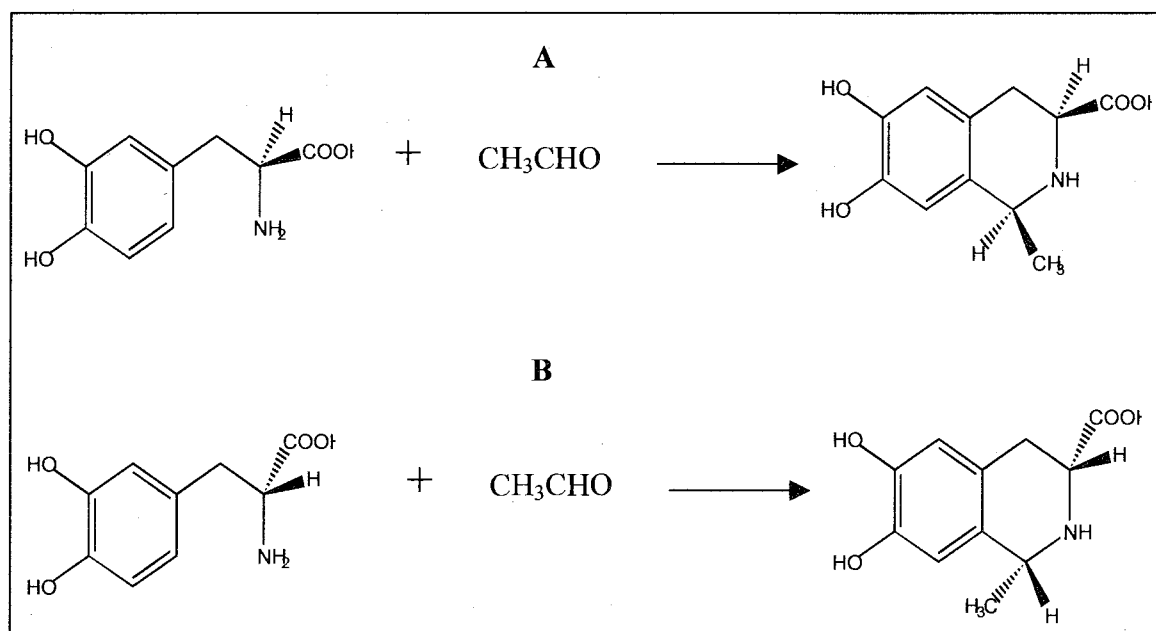
Figure 14: Condensations of (A) L-DOPA with Acetaldehyde to Form L-3-Carboxysalsolinol; (B) D-DOPA with Acetaldehyde to Form D-3-Carboxysalsolinol
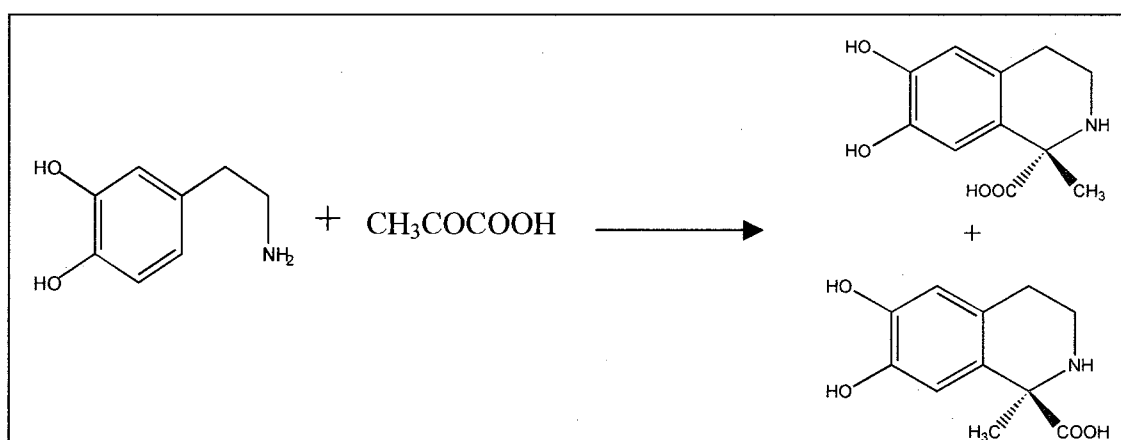
Figure 15: Condensation of Dopamine with Pyruvic Acid to Form a Racemic Mix of 1-Carboxysalsolinol

Racemic 1-carboxysalsolinol was synthesized in our laboratory using the reaction of dopamine with pyruvic acid (Figure 15) as described in the literature [114].

Sulfated β-cyclodextrin (lot number 09718HR) was purchased from Aldrich Chemical Co. Sulfated β-cyclodextrin is a cyclic sugar made up of seven glucose units joined "head-to-tail" by alpha-1,4 linkages and has several (13-19) of the 21 inner and outer rim hydroxyl groups converted to sulfate functionalities (Figure 16) [115-117].

A   B
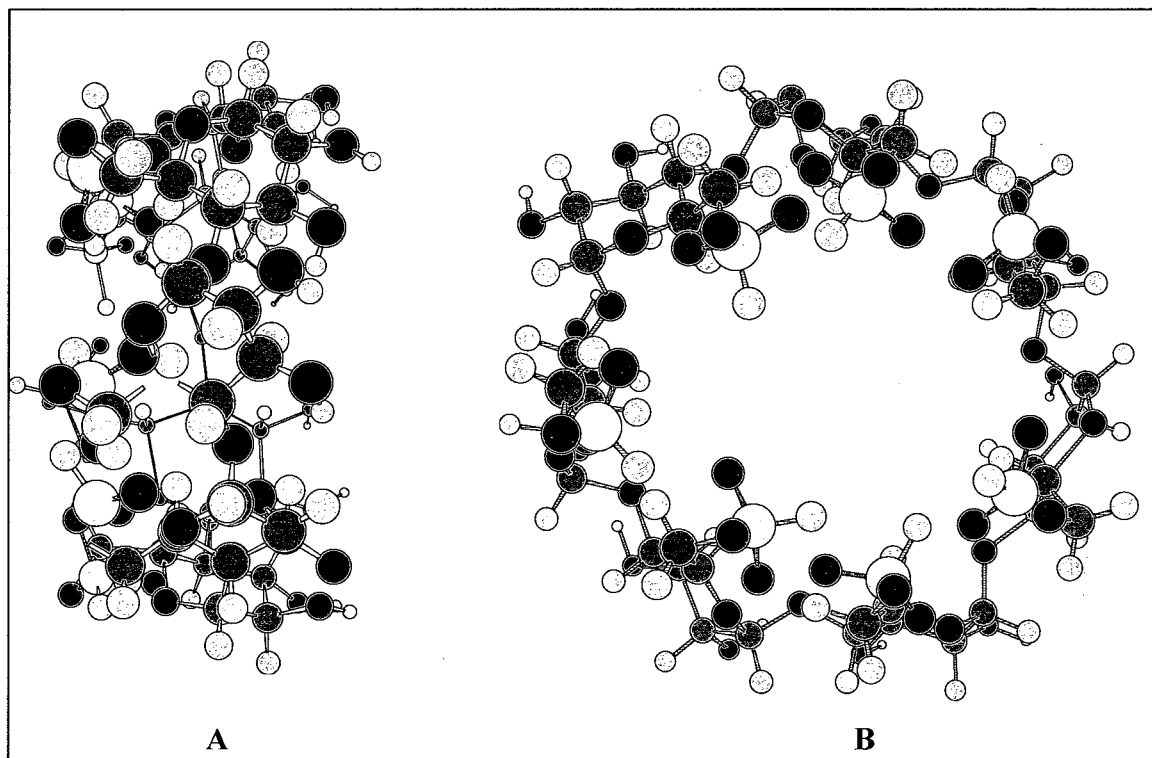
Figure 16: Two Views of Sulfated β-Cyclodextrin (A) Side View; (B) Front as Seen From the Smaller Diameter "Inner" Rim

While direct molecular weight measurements were not available, carbon-hydrogen analysis values provided by the vendor indicated an average molecular weight of 3,000 Daltons.

Chromatography

An isocratic HPLC consisting of a micrometering pump (Rainin XLS), a valve-loop injector (Rheodyne 7125), a variable UV detector (PE SpectroMonitor III) set to measure absorbance at 280 nm, and a column oven (PE) were used in these studies. Subambient column temperatures were maintained using stirred water baths. Econosphere (4.6 mm ID x 250 mm, 5 μm ODS) and Adsorbosphere HS (4.6 mm ID x 150 mm, 3 μm ODS) reversed-phase columns, purchased from Alltech Associates (Grace Davison, Deerfield, IL) were employed. Econosphere columns were used primarily to chromatograph salsolinol while Adsorbosphere columns, which have greater carbon loading were used for the carboxylated substances.

Mobile phases consisted of 0.05 M phosphoric acid with the pH adjusted to a value of 2.5 with sodium hydroxide. No adverse effects were noted from the use of acidic mobile phases. Sulfated β-cyclodextrin was added as indicated in the text, to give solutions of 0 to 2 mM. Concentrations greater than 1 mM for Econosphere columns and 2 mM for Adsorbosphere columns gave retention times very near the void retention time, t_0. Columns were equilibrated by passing 15 to 20 column volumes of mobile phase through them before measurements were made.

Capacity factor, k', values were determined using the formula,

$$k' = (t_r - t_o)/t_o, \hspace{3cm} \text{(Eqn. 1)}$$

where t_r is the retention time of an analyte peak and t_o the retention time of an unretained

solute, obtained using injections of solutions of $NaNO_3$ or $NaNO_2$.

Resolution, R_s, was determined using the equation,

$$R_s = 2\Delta t_r/(w_1 + w_2),\qquad\text{(Eqn. 2)}$$

where Δt_r is the difference in retention times between peaks and w_1 and w_2 are the widths

of the peaks of the two enantiomers.

Selectivity, α, was calculated using the equation,

$$\alpha = k'_2/k'_1,\qquad\text{(Eqn. 3)}$$

where the k' values are for two peaks of interest.

Thermodynamics

Thermodynamics operating during the chromatographic process were assessed

using the van't Hoff expression [118, 119],

$$lnk' = (-\Delta H/RT) + (\Delta S/R) + ln\phi.\qquad\text{(Eqn. 4)}$$

In this expression k' is the capacity factor, R the gas constant, ΔH and ΔS the enthalpy

and entropy of transfer of the solute from the mobile to the stationary phase, and ϕ the

volume phase ratio of the stationary and mobile phases. The value of 0.385 was used to

approximate ϕ as suggested by Sander and Field for octadecasilyl (ODS) packings [120].

Approximately the same value for ϕ was obtained when the method described by Cole

and Dorsey [118, 119, 121] was used together with values of percentage carbon loading

and mass of packing in a typical column obtained from the manufacturer.

Plots of lnk' vs. 1/T were obtained. Slopes of these lines were taken to be equal to $-\Delta H/R$ and the intercept to be equal to $(\Delta S/R) + \ln\phi$. Uncertainties in ϕ should affect all ΔS measurements equally.

A Re-evaluation of the Role of Tetrahydropapaveroline in

Ethanol Consumption in Adult Male Sprague-Dawley Rats

Subjects

Thirty-one (31) male Sprague-Dawley rats (Harlan, Indianapolis, IN) obtained from the Frederick, MD plant, weighing between 280 and 340 g at the time of surgery, were used for this experiment. Animals were housed individually in hanging 11 inch by 15 inch wire cages on a reversed 12:12 light/dark cycle with light onset at 20:00. Food was available *ad libitum*. Room temperature was maintained at 23 ± 1 °C. All procedures involving animals were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern Mississippi (APPENDIX).

Drug Preparation

Artificial cerebrospinal fluid was used as a vehicle solution for all experimental injections. A liter of artificial cerebrospinal fluid (aCSF) was made containing 7.46 g NaCl, 0.19 g KCl, 0.14 g CaCl$_2$, and 0.19 g MgCl$_2$ dissolved in deionized water to which was added 0.1 g ascorbic acid to retard the oxidation of THP. The aCSF was filtered through a 0.2 μm nylon filter. The THP used in these experiments was synthesized in our laboratory (Figure 17) [122-124]. Optically pure R-(+)-THP was synthesized in our laboratory by Kenneth D. McMurtrey using established methods [125].
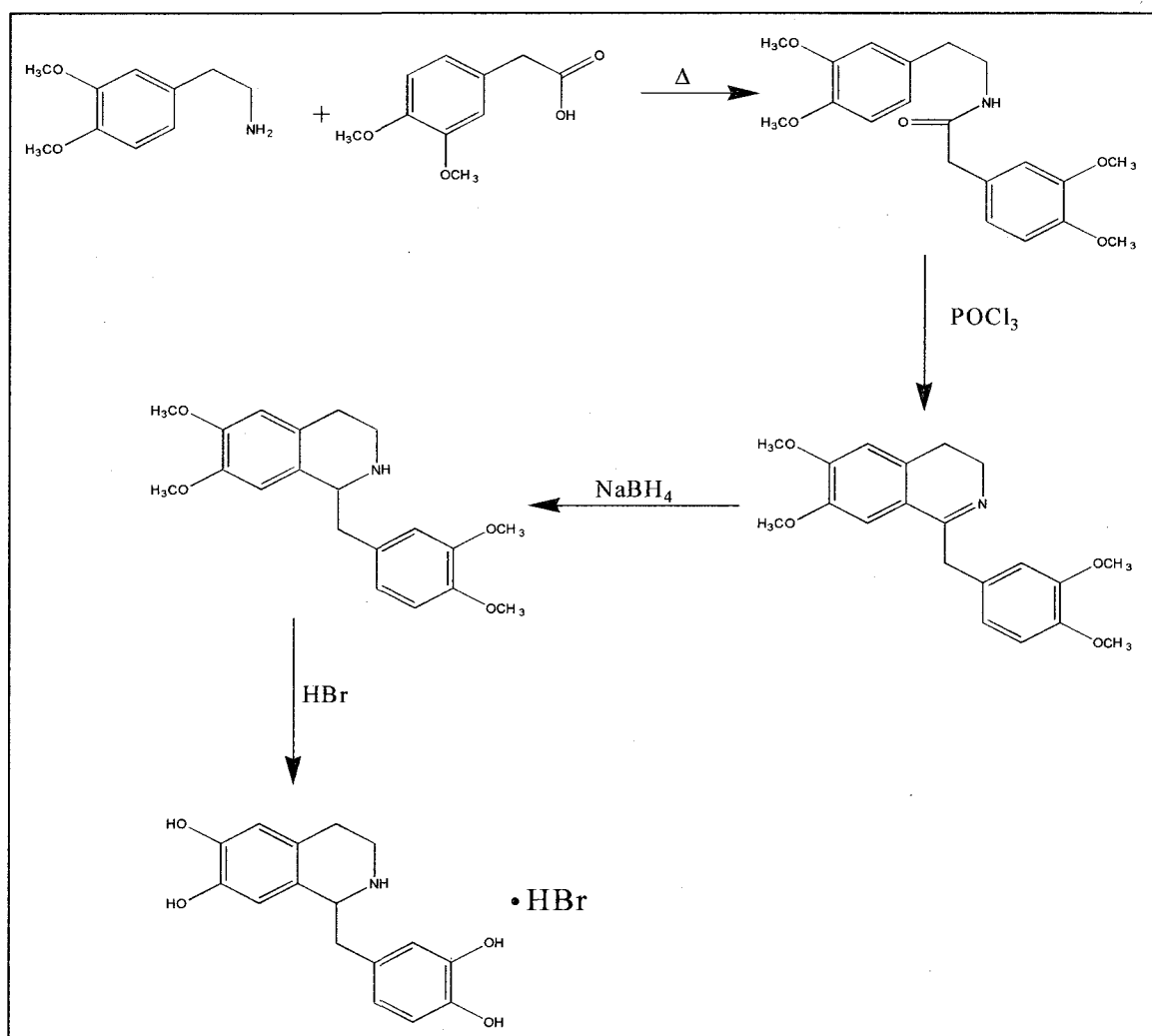
Figure 17: Synthesis of Racemic Tetrahydropapaveroline Hydrobromide

NMR Analysis

A 350 MHz Bruker NMR was used to obtain proton nuclear magnetic resonance spectra for R-(+)-THP in D_2O to obtain proof positive that our sample is in fact THP.

Chiral and Achiral HPLC Analysis

Samples of synthesized R-(+)-THP were chromatographed using both achiral and chiral reversed phase HPLC with a UV detector. An isocratic HPLC consisting of a micrometering pump (Rainin XLS), a valve-loop injector (Rheodyne 7125), and a variable UV detector (PE SpectroMonitor III) set to measure absorbance at 280 nm. The columns used were common octadecasilyl Luna (4.6 mm ID x 150 mm, 5μm ODS-2), and Prodigy (4.6 mm ID x 150 mm, 5μm ODS-2) reversed-phase columns, purchased from Phenomenex via Alltech Associates.

The mobile phase used for achiral analysis was 75 % 0.05 M phosphoric acid: 25% acetonitrile with the pH adjusted to a value of 2.5 with sodium hydroxide. The mobile phase used for chiral analysis was 90 % 0.05 M phosphoric acid: 10% methanol with the pH adjusted to a value of 3.0 with sodium hydroxide. S-β-CD was then added to this mobile phase in the amount of 2.0 g/L. These achiral and chiral mobile phases were used in concert to assay both the identity and the enantiomeric excess of our R-(+)-THP sample. In each case, the column was equilibrated by passing 15 to 20 column volumes of mobile phase through it before measurements were made.

Grouping of Experimental Animals

The Sprague-Dawley rats were split into experimental groups. Animals in one group received one of two doses of racemic THP in the amount of 0.65 μg/μL (n=6) or 1.3 μg/μL (n=4) in aCSF. All other reagents were purchased from Sigma (St. Louis,

MO). A counterpart solution of (+)-THP was prepared in a similar fashion. A second group of animals received one of two doses of the (+)-THP, either 0.66 µg/µL (n=8) or 1.4 µg/µL (n=4) in aCSF. Controls received either aCSF containing no racemic THP or (+)-THP, or no injections at all (n=9).

Alcohol Preference Testing

This experiment utilized the two-choice, three-bottle free access paradigm [126] of exposure to incremental concentrations of alcohol (3 to 30% over twelve days). Three calibrated 100 mL drinking tubes were positioned equidistantly at the front of each animal's cage. One tube contained a solution of ethanol in which the concentration was increased daily in the following manner: 3, 4, 5, 6, 7, 9, 11, 13, 15, 20, 25, and 30 percent alcohol by volume. Each solution was prepared with 95% ethanol (Everclear drinking spirits) and distilled water. A second tube contained only water and the third tube was empty.

These tubes were rotated each day according to a predetermined random sequence to prevent the development of a position habit. By raising the concentration of the ethanol offered, a baseline preference or aversion was established for each animal. Daily measures of alcohol and water consumption were taken at 16:00. To evaluate the possibility of fluid loss due to evaporation, an empty cage was fitted daily with the appropriate fluids. Metal trays lined with newsprint were carefully inspected (and replaced) daily for evidence of fluid spillage. Loss of fluid due to evaporation and spillage was negligible.

Intraventricular Administration of THP

Each rat was anesthetized with ketamine: xylazine anesthesia (25:5 mg/kg or 1 mL/kg body weight; IP Figure 18) prior to stereotaxic surgery.

Figure 18: Intraperitoneal (IP) Injection of Anesthetic

Once anesthesia was confirmed via tail and foot pinch, the animal's head was shaved and placed into a Kopf stereotaxic instrument (Figure 19) with the head centered and the incisor bar set 3.0 mm above the interaural line.
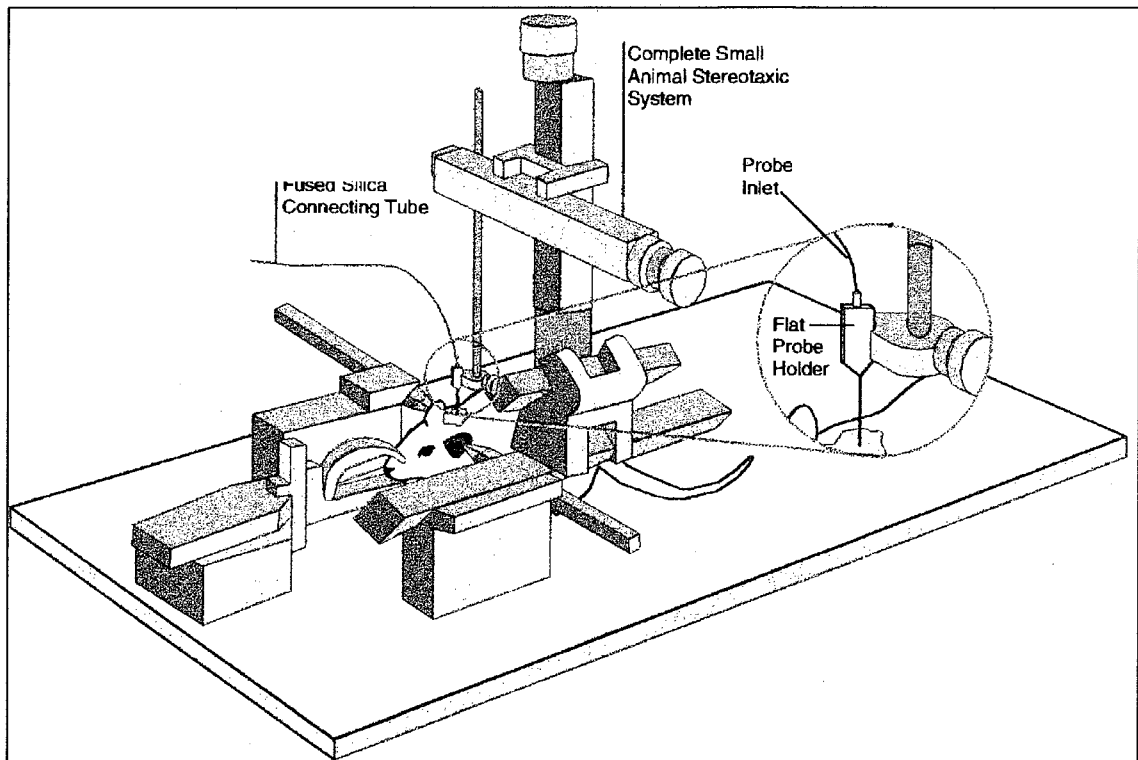
Figure 19: Schematic of a Kopf Stereotaxic Surgical Instrument

A stainless steel (22 ga., 13 mm in length) guide cannulae (Small Parts Inc., Miami Lakes, FL) was affixed to the probe holder of the Kopf apparatus. A small (ca. 1.5 inches) longitudinal incision was made in the scalp with a scalpel. The incision was held open by hemostats. The fascia was then scraped away to reveal the skull. The skull is wiped and dried with surgical gauze and any bleeding is stopped with a surgical cautery (Aaron, Inc.).

The tip of the guide cannula was placed at bregma, a landmark feature of the skull where the sagittal and coronal sutures meet (Figure 20).

Figure 20: Schematic of Landmark Features Found on the Rat Skull

Once bregma was located, the guide cannula tip was positioned above the lateral

ventricles according to Paxinos and Watson [127]. The coordinates from bregma were:

AP -0.5 mm; ML 1.5 mm; flat skull [127]. A mark was made at this point with a No. 2

pencil. A burr hole was drilled through the skull at this mark. The guide cannula was

swung aside on the boom arm and small surgical anchor screws (available from Small

Parts, Inc.) were inserted into the calvarium (Figure 21).

Figure 21: Photograph of Placement of Stereotaxic Anchor Screws

The guide cannula was inserted into the skull through the burr hole and lowered

3.0 mm to the lateral ventricle of the animal [127]. Cranioplastic cement (Lang Dental)

was packed around the screws and guide cannulae in a pedestal shape to fix them all in

place. The incision was then closed around the pedestal with standard silk surgical

sutures.

ICV Injections

Dummy cannulae (28 ga. stainless steel tubing from Small Parts, Inc.) were prefitted to extend 0.5 mm beyond the tip of the guide cannula so that the tip of the injection cannula rested in the lumen of the lateral ventricle. This prefitted 13.5 mm length of 28 ga. stainless steel stylet was then inserted into the guide cannulae to prevent obstructions. Injection needles of the same length and material were attached by polyethylene tubing to a 50 μL tuberculin HPLC syringe. An automated Harvard Apparatus Model PHD 2000 (South Natick, MA) syringe pump was used to deliver intracerebroventricular (ICV) infusions of drug (or vehicle) in a volume of 5 μL per infusion over two minutes. Each animal received two injections (09:00 and 16:00) per day for three consecutive days. This dosing regimen has been shown previously with THP to induce significant increases in alcohol consumption [113]. The first injection commenced after a seven day post-surgery recovery period.

Alcohol Preference Test Scheduling

A twelve-day alcohol preference test was administered prior to the surgery. A second twelve-day alcohol preference post-test was given following the three injection days. In a subset of animals, three twelve-day tests were conducted: one sequence prior to surgery, a second sequence after surgery but prior to central injections, and the third sequence following three days of injections. In a subset of animals (n=15) that had been treated with either (±)-THP or (+)-THP, volumetric water and alcohol consumption was recorded every four hours around the clock to evaluate the distribution of alcohol and water consumption over the course of a 24 hour period.

Standard histological techniques were used to verify placement of guide cannulae. All animals were sacrificed with a lethal overdose of sodium pentobarbital (Nembutal, 100 mg/kg BW), and perfused intracardially with 0.9% saline and 10% phosphate-buffered formalin. The brains were removed and stored in formalin for at least 24 hours. Serial coronal sections (40 μm) were made in a cryostat (Microm HM 505 N) at -30 °C through the site of implant onto gelatinized glass slides which were stained with cresyl violet dye, then coverslipped. Cannula tip placements were visually compared to line drawings from a stereotaxic atlas [127].

Statistical Analyses

All analyses were conducted using SPSS software. All values are expressed as mean ± SEM. The α level of significance used for all statistical tests was $p \leq 0.05$. A one-way ANOVA for independent groups was used to compare the three control conditions: vehicle-treated animals, sham-operated animals, and naïve animals. A mixed-model ANOVA (drug treatment over days) was used to analyze the effect of drug treatment. The Huyn-Feldt correction was used since there was a violation of the assumption of sphericity. Tukey's HSD post-hoc tests were used to compare treatment conditions. To evaluate whether differences in alcohol consumption were significant on each day, 95% confidence intervals were determined around the mean of the control condition for each day. To evaluate whether repeated exposure to alcohol caused any alteration in alcohol consumption, a one-way ANOVA for repeated measures was used on animals that had completed three consecutive twelve-day preference tests without receiving a drug treatment.

A Re-evaluation of the Role of Tetrahydropapaveroline in

Ethanol Consumption in Another Population of Adult Sprague-Dawley Rats

Subjects

Subsequent to the first round of treatments performed on rats obtained from the

Frederick, MD plant, ten (10) male Sprague-Dawley rats (Harlan, Indianapolis, IN) from

the Prattville, AL plant weighing between 280 and 340 g at the time of surgery, were

used for this experiment. All conditions with regards to housing, light cycle, temperature,

and animal care were identical to the previous ICV experiment involving rats obtained

from the Harlan site in Frederick, MD. All procedures involving animals were conducted

in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and

the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of

the University of Southern Mississippi (APPENDIX).

Drug Preparation

Artificial cerebrospinal fluid was used as a vehicle solution for all experimental

injections. A liter of artificial cerebrospinal fluid (aCSF) was prepared in the same

manner as the previous ICV experiment involving rats obtained from the Harlan site in

Frederick, MD. The aCSF was filtered through a 0.2 µm nylon filter. The THP used in

these experiments was synthesized in our laboratory (Figure 17) [122-124]. Optically

pure R-(+)-THP was synthesized in our laboratory by Kenneth D. McMurtrey using

established methods [125]. All other reagents were purchased from Sigma (St. Louis,

MO).

Grouping of Experimental Animals

One group of animals received a dose of racemic THP in the amount of 0.65

µg/µL in aCSF. A counterpart solution of R-(+)-THP was prepared in a similar fashion.

A second group of animals received a dose of aCSF containing (+)-THP in the amount of

0.67 µg/µL. Control animals received aCSF containing no THP.

Animals in the first group received racemic THP in the amount of 0.65 µg/µL in

aCSF (n=3). A counterpart solution of R-(+)-THP was prepared in a similar fashion. A

second group of animals received R-(+)-THP in the amount 0.67 µg/µL in aCSF (n=3).

Controls received either aCSF containing no THP (n=2), or no injections at all (n=2).

Alcohol Preference Testing

This experiment utilized the two-choice, three-bottle free access paradigm [126]

of exposure to incremental concentrations of alcohol (3 to 30% over twelve days). Three

calibrated 100 mL drinking tubes were positioned equidistantly at the front of each

animal's cage. One tube contained a solution of ethanol in which the concentration was

increased daily in the following manner: 3, 4, 5, 6, 7, 9, 11, 13, 15, 20, 25, and 30

percent alcohol by volume. Each solution was prepared with 95% alcohol (Everclear

drinking spirits) and distilled water. A second tube contained only water and the third

tube was empty. These tubes were rotated each day according to a predetermined

random sequence to prevent the development of a position habit. By raising the

concentration of the ethanol offered, a baseline preference or aversion was established for

each animal. Daily measures of alcohol and water consumption were taken at 16:00. To

evaluate the possibility of fluid loss due to evaporation, an empty cage was fitted daily

with the appropriate fluids. Metal trays lined with newsprint were carefully inspected

(and replaced) daily for evidence of fluid spillage. Loss of fluid due to evaporation and spillage was negligible.

Intracerebroventricular Administration of THP

Each rat was anesthetized with ketamine: xylazine anesthesia (25:5 mg/kg or 1 mL/kg body weight; IP) prior to stereotaxic surgery. Once anesthesia was confirmed via tail and foot pinch, the animal was shaved and placed into a Kopf stereotaxic instrument with the head centered and the incisor bar set 3.0 mm above the interaural line.

A stainless steel (22 ga., 13mm in length) guide cannulae (Small Parts Inc., Miami Lakes, FL) was affixed to the probe holder of the Kopf apparatus. A small (ca. 1.5 inches) longitudinal incision was made in the scalp with a standard surgical scalpel. The incision was held open by hemostats. The fascia was then scraped away to reveal the skull. The skull is wiped and dried with surgical gauze and any bleeding is stopped with a surgical cautery.

The tip of the guide cannula was placed at bregma, a landmark feature of the skull where the sagittal and coronal sutures meet. Once bregma was loc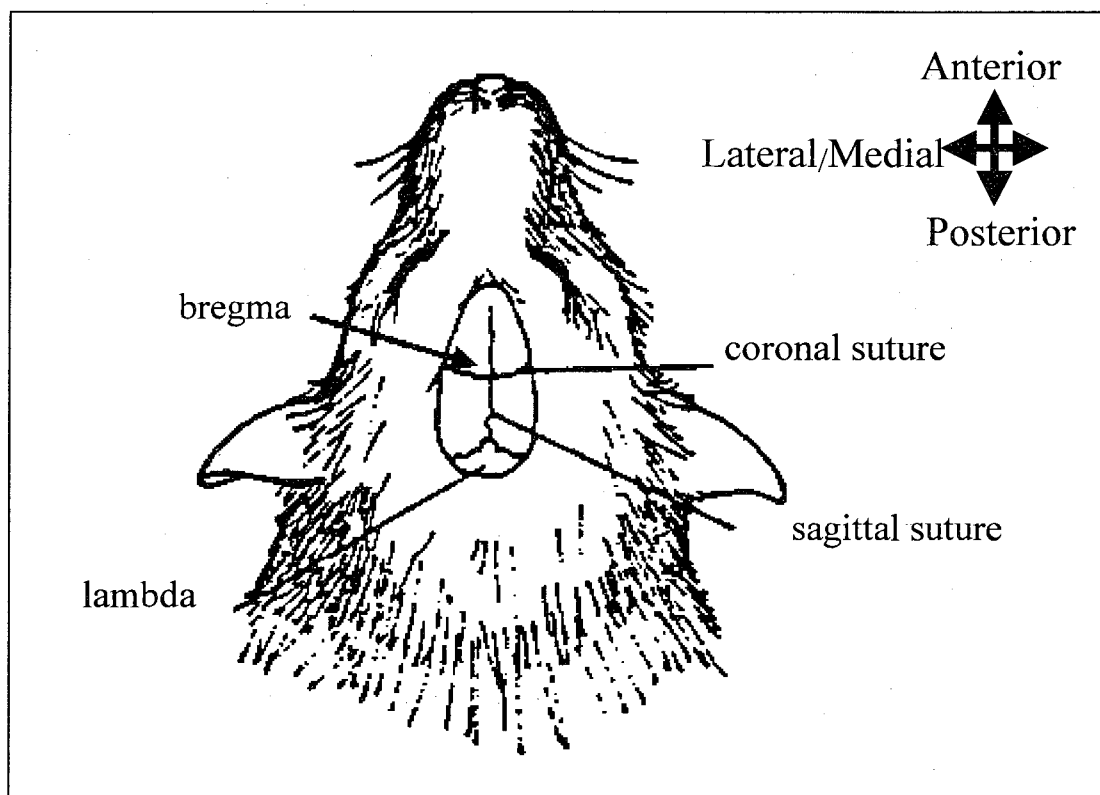ated, the guide cannula tip was positioned above the lateral ventricles according to Paxinos and Watson [127]. The coordinates from bregma were: AP -0.5 mm; ML 1.5 mm; flat skull [127]. A mark was made at this point with a No. 2 pencil. A burr hole was drilled through the skull at this mark. The guide cannula was swung aside on the boom arm and small surgical anchor screws (available from Small Parts, Inc.) were inserted into the calvarium.
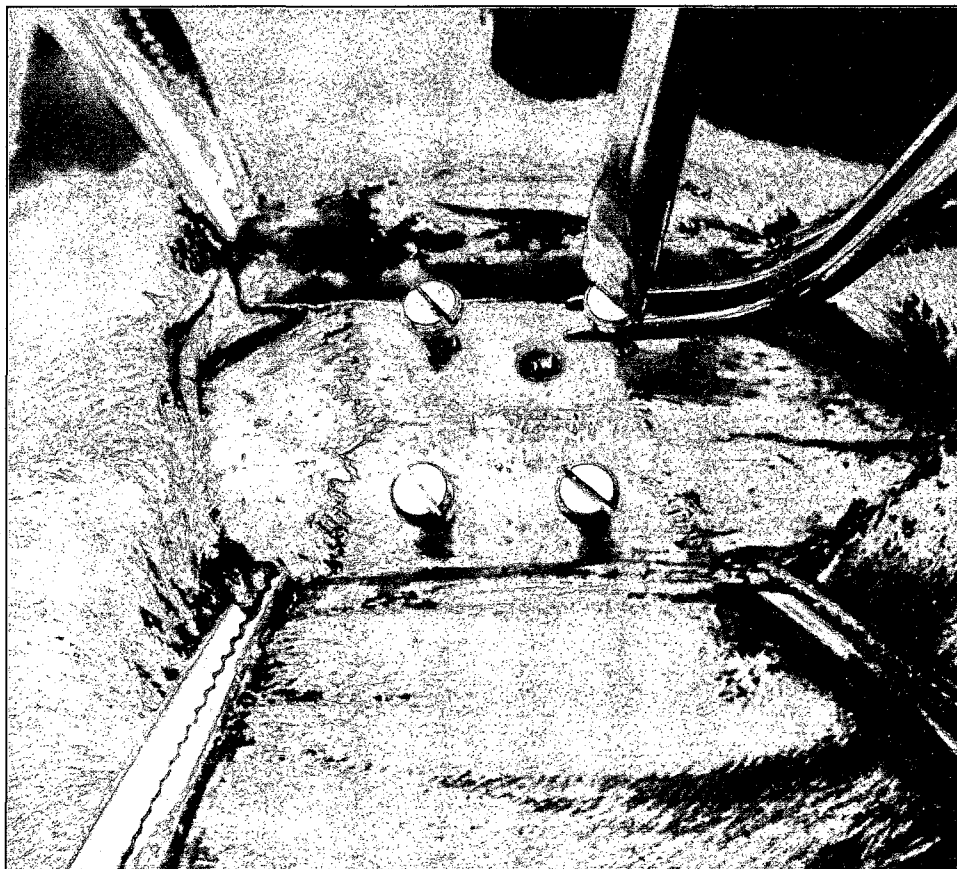
The guide cannula was inserted into the skull through the burr hole and lowered 3.0 mm to the lateral ventricle of the animal. Cranioplastic cement (Lang Dental) was packed around the screws and guide cannulae in a pedestal shape to fix them all in place.

ICV Injections

Dummy cannulae (28 ga. stainless steel tubing from Small Parts, Inc.) were prefitted to extend 0.5 mm beyond the tip of the guide cannula so that the tip of the injection cannula rested in the lumen of the lateral ventricle. This prefitted 13.5 mm length of 28 ga. stainless steel stylet was then inserted into the guide cannulae to prevent obstructions. Injection needles of the same length and material were attached by polyethylene tubing to a 50 μL tuberculin HPLC syringe. An automated Harvard Apparatus Model PHD 2000 (South Natick, MA) syringe pump was used to deliver intracerebroventricular (ICV) infusions of drug (or vehicle) in a volume of 5 μL per infusion over 2 minutes. Each animal received two injections (09:00 and 16:00) per day for three consecutive days. This dosing regimen has been shown previously with THP to induce significant increases in alcohol consumption [113]. The first injection commenced after a seven day post-surgery recovery period.

Alcohol Preference Test Scheduling

A twelve-day alcohol preference test was administered prior to the surgery. A second twelve-day alcohol preference post-test was given following the three injection days. In a subset of animals, three twelve-day tests were conducted (n=10): one sequence prior to surgery, a second sequence after surgery but prior to central injections, and the third sequence following three days of injections. In a subset of animals (n=15) that had been treated with either THP racemate or R-(+)-THP, volumetric water and alcohol consumption was recorded every four hours around the clock to evaluate the distribution of alcohol and water consumption over the course of a 24 hour period.

Standard histological techniques were used to verify placement of guide cannulae. All animals were sacrificed with a lethal overdose of sodium pentobarbital (Nembutal, 100 mg/kg BW), and perfused intracardially with 0.9% saline and 10% phosphate-buffered formalin. The brains were removed and stored in formalin for at least 24 hours. Serial coronal sections (40 μm) were made in a cryostat (Microm HM 505 N) at -30 °C through the site of implant onto gelatinized glass slides which were stained with cresyl violet dye, then coverslipped. Cannula tip placements were visually compared to line drawings from a stereotaxic atlas [127].

Statistical Analyses

All analyses were conducted using SPSS software. All values are expressed as mean ± SEM. The α level of significance used for all statistical tests was $p \leq 0.05$. A one-way ANOVA for independent groups was used to compare the control conditions. A mixed-model ANOVA (drug treatment over days) was used to analyze the effect of drug treatment. The Huyn-Feldt correction was used since there was a violation of the assumption of sphericity. Tukey's HSD post-hoc tests were used to compare treatment conditions. To evaluate whether differences in alcohol consumption were significant on each day, 95% confidence intervals were determined around the mean of the control condition for each day. To evaluate whether repeated exposure to alcohol caused any alteration in alcohol consumption, a one-way ANOVA for repeated measures was used on animals that had completed three consecutive twelve-day preference tests without receiving a drug treatment.

An Evaluation of the Role of 2,3,10,11-Tetrahydroxyberbine in

Ethanol Consumption in Adult Male Sprague-Dawley Rats

Subjects

Eleven (11) male Sprague-Dawley rats (Harlan, Indianapolis, IN) obtained from

the Frederick, MD plant, weighing between 280 and 340 g at the time of surgery, were

used for this experiment. All conditions with regards to housing, light cycle, temperature,

and animal care were identical to the previous experiments involving ICV injections of

THP. All procedures involving animals were conducted in accordance with the NIH

Guidelines for the Care and Use of Laboratory Animals and the guidelines set forth by

the Institutional Animal Care and Use Committee (IACUC) of the University of Southern

Mississippi (APPENDIX).

Drug Preparation

Artificial cerebrospinal fluid was used as a vehicle solution for all experimental

injections. A liter of artificial cerebrospinal fluid (aCSF) was prepared in the same

manner as the previous experiments involving ICV injections of THP. Ascorbic acid was

added in the amount of 0.1 g to retard the oxidation of THB (structure depicted in Figure

22). The aCSF was filtered through a 0.2 μm nylon filter. The THB used in these

experiments was synthesized in our laboratory according to methods found in the

literature [123, 124]. All other reagents were purchased from Sigma (St. Louis, MO).
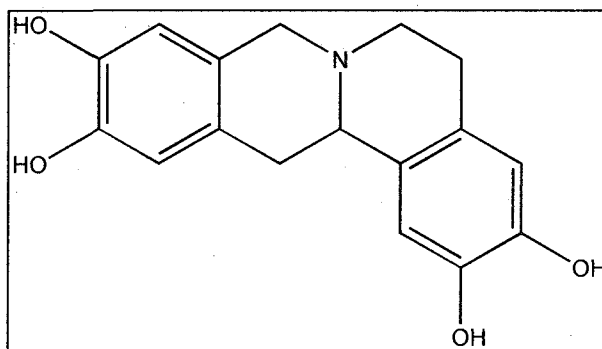
Figure 22: Structure of THB

Animals received a dose of THB in the amount of 0.65 μg/μL in aCSF (n=6) in the form of 5 μL over a period of two minutes. Control animals received aCSF containing no THB in the same volume and time period (n=4).

Alcohol Preference Testing

This experiment utilized the two-choice, three-bottle free access paradigm [126] of exposure to incremental concentrations of alcohol (3 to 30% over twelve days). Three calibrated 100 mL drinking tubes were positioned equidistantly at the front of each animal's cage. One tube contained a solution of ethanol in which the concentration was increased daily in the following manner: 3, 4, 5, 6, 7, 9, 11, 13, 15, 20, 25, and 30 percent alcohol by volume. Each ethanol solution was prepared with 95% alcohol (Everclear drinking spirits) and distilled water. A second tube contained only water and the third tube was empty. These tubes were rotated each day according to a predetermined random sequence to prevent the development of a position habit.

By raising the concentration of the ethanol offered, a baseline preference or aversion was established for each animal. Daily measures of alcohol and water

consumption were taken at 16:00. To evaluate the possibility of fluid loss due to evaporation, an empty cage was fitted daily with the appropriate fluids. Metal trays lined with newsprint were carefully inspected (and replaced) daily for evidence of fluid spillage. Loss of fluid due to evaporation and spillage was negligible.

Intracerebroventricular Administration of THB

Stainless steel (22 ga., 13mm in length) guide cannulae (Small Parts, Miami Lakes, FL) were stereotaxically implanted into the right lateral ventricle of each rat under ketamine: xylazine anesthesia (25:5 mg/kg body weight; IP) seven days prior to the beginning of central injections. The coordinates from bregma were AP -0.5 mm; ML 1.5 mm; flat skull [127]. After anchor screws (available from Small Parts, Inc.) were inserted into the calvarium, cranioplastic cement (Lang Dental) was packed around the screws and guide cannulae to fix them in place.

Dummy cannulae (28 ga. stainless steel tubing, Small Parts, Inc.) were prefitted to extend 0.5 mm beyond the tip of the guide cannula so that the tip of the injection cannula rested in the lumen of the lateral ventricle. These prefitted 13.5 mm lengths of 28 ga. stainless steel stylets were then inserted into the guide cannulae to prevent obstructions.

Injection needles of the same length were attached by polyethylene tubing to a 50 µL tuberculin HPLC syringe. An automated Harvard Apparatus Model PHD 2000 (South Natick, MA) syringe pump was used to deliver intracerebroventricular (ICV) infusions of drug (or vehicle) in a volume of 5 µL per infusion over 2 minutes. Each animal received two injections (09:00 and 16:00) per day for three consecutive days. This dosing regimen has been shown previously with THP to induce significant increases

in alcohol consumption [113]. The first injection day commenced after a seven day post-surgery recovery period.

A twelve-day alcohol preference test was administered prior to the surgery. A second twelve-day alcohol preference post-test was given following the three injection days. Standard histological techniques were used to verify placement of guide cannulae. All animals were sacrificed with a lethal overdose of sodium pentobarbital (Nembutal, 100 mg/kg BW), and perfused intracardially with 0.9% saline and 10% phosphate-buffered formalin. The brains were removed and stored in formalin for at least 24 hours. Serial coronal sections (40 µm) were made in a cryostat (Microm HM 505 N) at -30 ° C through the site of implant onto gelatinized glass slides which were stained with cresyl violet dye, then coverslipped. Cannula tip placements were visually compared to line drawings from a stereotaxic atlas [127].

Statistical Analyses

All analyses were conducted using SPSS software. All values are expressed as mean ± SEM. The α level of significance used for all statistical tests was $p \leq 0.05$. A one-way ANOVA for independent groups was used to compare the control conditions. A mixed-model ANOVA (drug treatment over days) was used to analyze the effect of drug treatment. The Huyn-Feldt correction was used since there was a violation of the assumption of sphericity. Tukey's HSD post-hoc tests were used to compare treatment conditions. To evaluate whether differences in alcohol consumption were significant on each day, 95% confidence intervals were determined around the mean of the control condition for each day. To evaluate whether repeated exposure to alcohol caused any alteration in alcohol consumption, a one-way ANOVA for repeated measures was used

on animals that had completed three consecutive twelve-day preference tests without receiving a drug treatment.

Investigation of the Effects of Ethanol Consumption on Tetrahydropapaveroline Levels in the Cerebrospinal Fluid of Adult Male Sprague-Dawley Rats Using Microdialysis and High Pressure Liquid Chromatography with Electrochemical Detection

Subjects

Three (3) male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing between 280 and 340 g at the time of surgery, were used for this experiment. Animals were housed individually in hanging 11 inch X 15 inch wire cages on a reverse 12:12 light/dark cycle with light onset at 20:00. Food was available ad libitum. Room temperature was maintained at $23 \pm 1 \,^{\circ}C$. All procedures involving animals were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern Mississippi (APPENDIX).

Implantation of Microdialysis Probe Guide Cannulae

Stainless steel (21.5 ga., 13 mm in length) guide cannulae (Small Parts, Miami Lakes, FL) were stereotaxically implanted into the right lateral ventricle of each rat under ketamine: xylazine anesthesia (25:5 mg/kg body weight; IP) seven days prior to the beginning of microdialysis sampling. The coordinates from bregma were AP -0.5 mm; ML 1.5 mm; flat skull [127]. After anchor screws (available from Small Parts, Inc.) were inserted into the calvarium, cranioplastic cement (Lang Dental) was packed around the screws and guide cannulae to fix them in place.

Dummy cannulae (28 ga. stainless steel tubing, Small Parts, Inc.) were prefitted to extend 0.5 mm beyond the tip of the guide cannula so that the tip of the dummy cannula would rest in the lumen of the lateral ventricle. These prefitted 13.5 mm lengths of 28 ga. stainless steel stylets were inserted into the guide cannulae to prevent obstructions.

Microdialysis Recovery Determination

In order to determine the optimal flow rate for microdialysis experiments, a simulation of microdialysis conditions was conducted. A 2mL aliquot of a 0.69 µM solution of THP in aCSF was placed in a 3 mL vial for each flow rate trial. A CMA/11 microdialysis probe with a 2.0 mm cuprophane dialysis membrane having a 6,000 Dalton cutoff range was placed in the 0.69 µM "parent" solution and perfused with aCSF using a Harvard Apparatus syringe pump at 0.5, 1.0, and 1.5 µL/minute. Six 20 µL samples of dialysate were collected at each flow rate and THP concentration of each dialysate sample was determined using high pressure liquid chromatography with an electrochemical detector.

The HPLC apparatus used consisted of a Rheodyne 7125 injector valve, a Rainin XLS delivery pump, a Econosphere (4.6 mm ID x 250 mm 5 µm ODS) HPLC column, and a BAS LC-3 amperometric detector using a glassy carbon electrode cell. The potential of the working electrode was set to 700 mV. The mobile phase consisted of 0.05M $NH_4H_2PO_4$ with a 5% acetonitrile component with a pH of 4.00.

Microdialysis Sampling of Rat CSF

An animal holding pen was constructed from a large (ca. 3.5 gallon) plastic planting pot available at any garden or home improvement store. The drain holes were blocked with a large paper plate and appropriate bedding and food pellets were placed in

the pen. Two iron ring stands were placed on either side of the holding pen and a crossbar transecting the diameter of the pen was suspended approximately six inches above the rim of the pen to act as a tether point for polyethylene tubing and dialysate collection vials (Figure 23). A drinking tube filled with water was clamped to one iron ring stand and lowered into the pen to provide animals with fluid *ad libitum*.
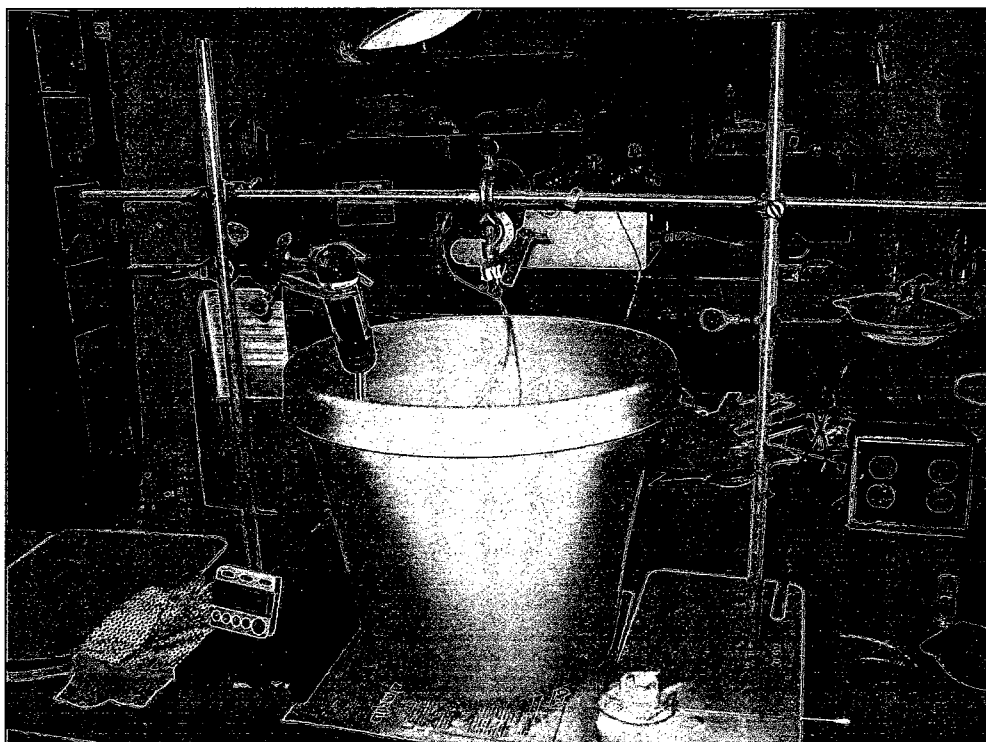
Figure 23: Microdialysis Holding Pen and Support Setup

A CMA/11 microdialysis probe was connected to a Harvard Apparatus syringe pump using approximately one meter of polyethylene tubing and CMA tubing connectors. The CMA/11 microdialysis probe is tipped with a 2.0 mm cuprophane membrane that allows the diffusion of neurotransmitters into the perfusate solution, aCSF prepared in the same fashion as the aCSF used for ICV injections.

The syringe pump was set to deliver aCSF to the probe at 1.0 µL/min. After a visual inspection to insure that the dialysate fluid ran clear and the membrane was not perforated, approximately 200 cm of polyethylene collection tubing was connected using CMA tubing connectors. After a 30 minute equilibration period, the collection of dialysis samples began. Dialysate was collected from each animal in five 20 minute intervals. After collection of baseline THP samples for assessment of basal THP levels, rats were force fed via a gavage tube 5 mL of 20% ethanol in distilled water ten minutes prior to their introduction into the microdialysis chamber and subsequent insertion of the CMA/11 probe.

HPLC Analysis of Ventricular CSF Using Amperometric Electrochemical Detection

The HPLC apparatus used consisted of a Valco rotary injector valve, a LDC delivery pump, an Econosphere (4.6 mm ID x 250 mm, 5 µm ODS) HPLC column, and an amperometric detector using a glassy carbon electrode cell. Dr. Newton Fawcett of the University of Southern Mississippi chemistry and biochemistry department constructed this detector. This amperometric detector was used because of its excellent signal to noise ratio. Dr. Fawcett used high-grade gold connector pins, which last much longer and yield a better signal than conventional manufacturer components, in the circuit design. The potential of the working electrode was set to 700 mV. The mobile phase consisted of 0.05M $NH_4H_2PO_4$ buffered to a pH of 3.00 with NaOH. The column was flushed after each chromatographic run until THP was no longer detectable to avoid carryover into the next sample.

CHAPTER III

EXPERIMENTAL RESULTS, DISCUSSION, AND CONCLUSIONS

Experimental Results on HPLC Resolution of the Enantiomers of

Dihydroxyphenylalanine and Selected Salsolinol Derivatives Using Sulfated β-

Cyclodextrin as a Chiral Mobile Phase Additive

Deng and co-workers used β-cyclodextrin (β-CD) in combination with ion-

pairing reagents such as sodium heptyl sulfate to separate the stereoisomers of salsolinol

[128-131]. They reported modest maximum selectivity of approximately 1.08. This

experiment examined the use of commercially available sulfated β-cyclodextrin (S-β-

CD) and demonstrates that it efficiently separates the stereoisomers of salsolinol when

used alone without heptane sulfate or other ion-pairing reagents.

Indeed, S-β-CD yields a selectivity of approximately 1.08 at even the very low

concentration of 0.1 mM, whereas concentrations of about 15 mM are required for β-CD

and these chiral separations are successful only in the presence of an ion-pairing

reagent[128-131]. S-β-CD is capable of much greater degrees of separation of salsolinol

enantiomers than β-CD. This experiment yielded an α value of 1.59 and a resolution of

3.5 for R-(+)- and S-(-)-salsolinol using 1 mM S-β-CD concentration without an ion-

pairing reagent. Greater concentrations of S-β-CD should provide even greater α and R_s

values. The limiting factor for salsolinol appears to be the very low retention times

accompanying the use of higher concentrations of the chiral selector. With a 1 mM

concentration of S-β-CD the value of k' for (+)-SAL is less than 1.

Stoichiometry of the complex formed between SAL stereoisomers and sulfated β-

cyclodextrin appears to be 1:1. A study of the effects of increasing S-β-CD concentration

on retention of the stereoisomers of SAL indicates that variation of the reciprocal of the

capacity factors of R-(+)-, and S-(-)-SAL are linear (Figure 24). This is the relationship

expected if SAL and S-β-CD form a 1:1 complex [132].
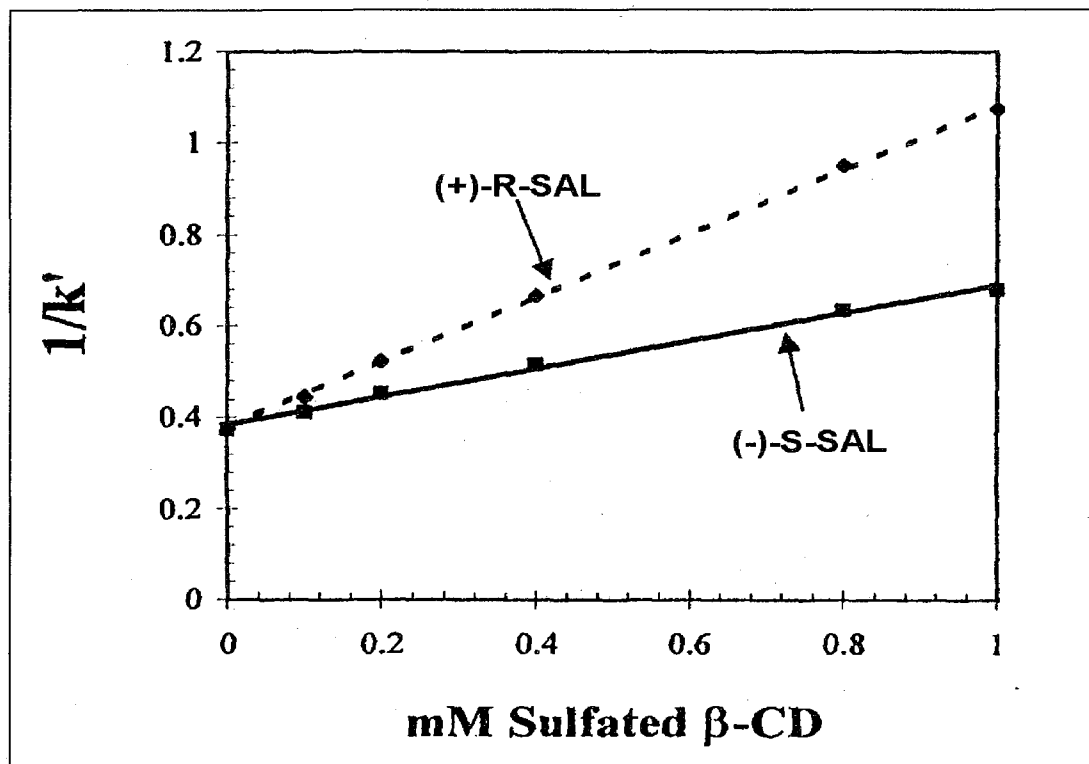
Figure 24: Plot of the Reciprocal of the Capacity Factor Versus Varying

Concentrations of Chiral Mobile Phase Additive

L-DOPA, D-DOPA and related carboxylated derivatives of salsolinol were

chromatographed on a C_{18} reversed phase column with an aqueous mobile phase

containing varying amounts of the chiral selector, sulfated β-cyclodextrin (S-β-CD). In

contrast to the linear relationships observed in the case of SAL stereoisomers, the

carboxyl-substituted compounds give nonlinear curves when 1/k' for these materials is

plotted against S-β-CD concentration (Figure 25). This nonlinearity is taken as evidence

that the equilibrium system between S-β-CD and the carboxylated compounds are more complex than that operating for salsolinol [132].
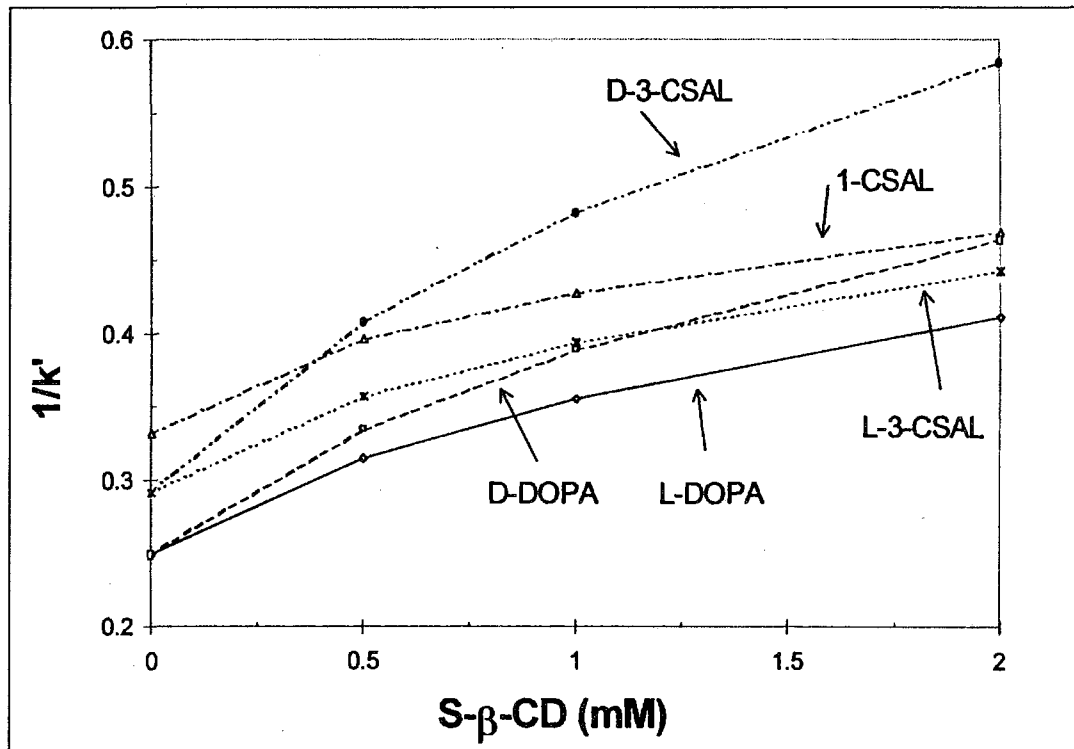
Figure 25: Plot of the Reciprocal of Capacity Factor of Chiral Separations of DOPA, 1-CSAL, and 3-CSAL Versus Chiral Mobile Phase Additive

In an attempt to gain some insight into the mechanisms that may be operating, the effects of temperature on retention of the various carboxylated compounds were investigated with a mobile phase containing 2 mM S-β-CD. Representative chromatograms of resolution of the compounds studied are given in Figure 26.
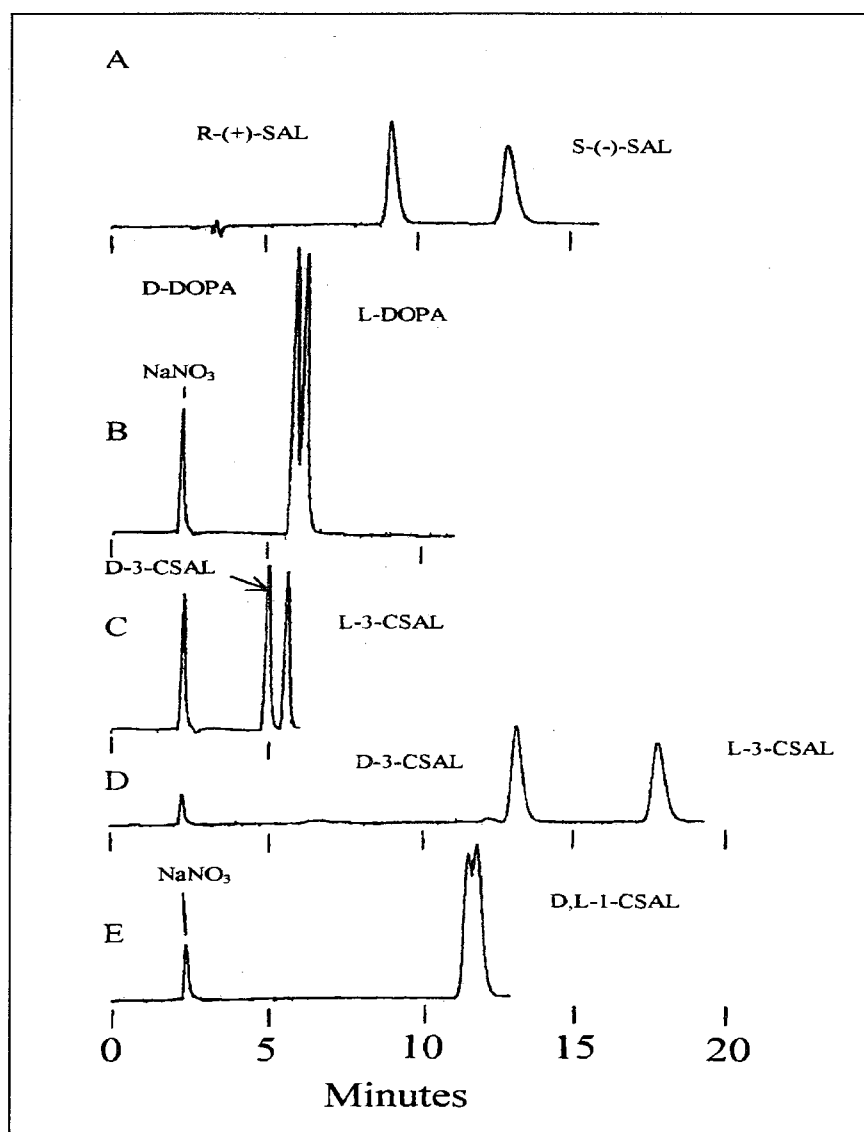
Figure 26: Representative Chromatograms of Chiral Separations of (A) SAL at 20 °C; (B) DOPA at 35 °C; (C) 3-CSAL at 35 °C; (D) 3-CSAL at 0 °C; (E) 1-CSAL at 0 °C. All Chromatograms on Same Time Scale With Tick Marks at Five Minute Intervals

Salsolinol separates well at room temperature (trace A) and 3-CSAL gives good

resolution at 35 °C (trace C). Resolution of D- and L-DOPA is not complete at 35 °C

(trace B), although resolution improves when the temperature of the column is decreased

(not shown). Lower temperatures generally improve resolution. This improvement is represented by chromatogram D which is the separation of D- and L-3-CSAL at 0 °C with a resolution slightly greater than 5. In contrast 1-CSAL isomers are separated only slightly (trace E). Even this degree of separation is achieved only at 0 °C.

The data obtained were used to prepare van't Hoff plots (Figure 27). The most immediately obvious characteristic of these plots are the breaks in the curves immediately below room temperature, approximately 15 °C to 20 °C. Curves between 20 °C and 35 °C have different slopes and intercepts than those obtained from measurements between 0 °C and 13 °C. Different slopes and intercepts indicate differences in ΔH and ΔS values. When plotted seperately these two sections of the curve have differing slopes, indicating different enthalpy and entropy values. The "low temperature" van't Hoff plot appears as Figure 28. The "high temperature" van't Hoff plot appears as Figure 29.
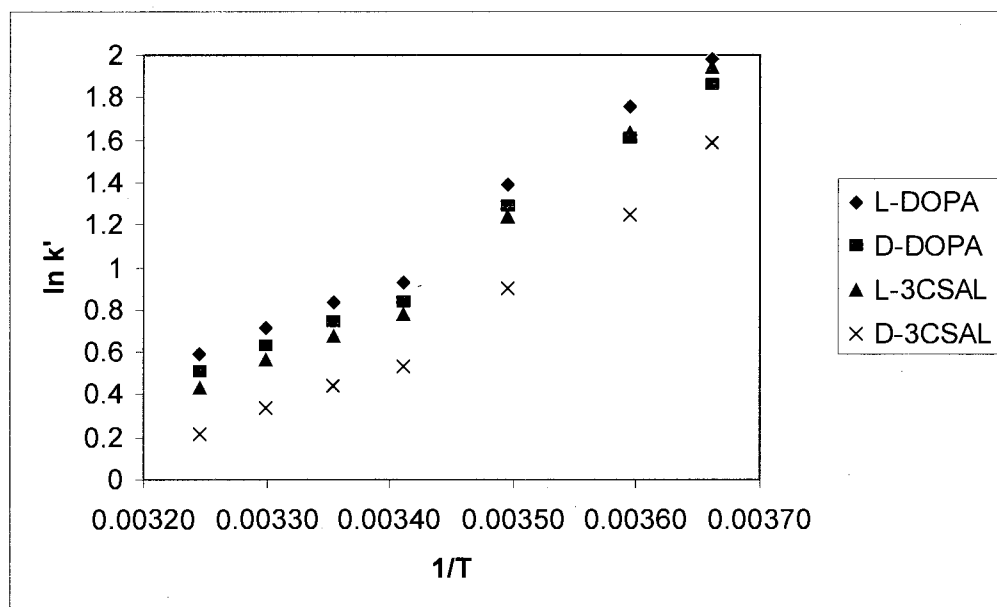
Figure 27: Van't Hoff Plot for Chiral Separations of Selected Catecholamines Showing a Change in Slope Between "High Temperature" and "Low Temperature"
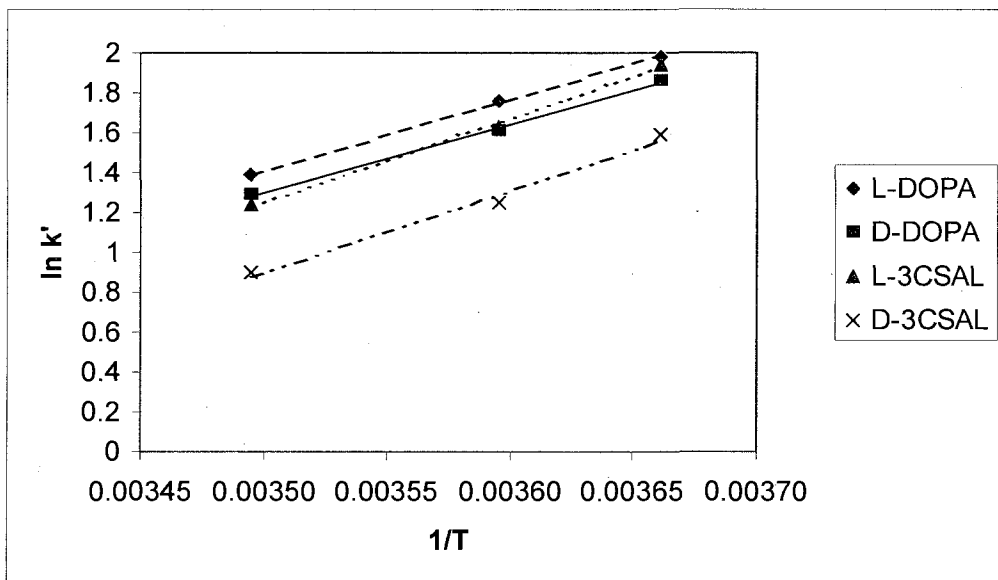
Figure 28: Van't Hoff Plot of Low Temperature Seperations of DOPA and 3-CSAL with 2 mM Chiral Moile Phase Additive S-β-CD
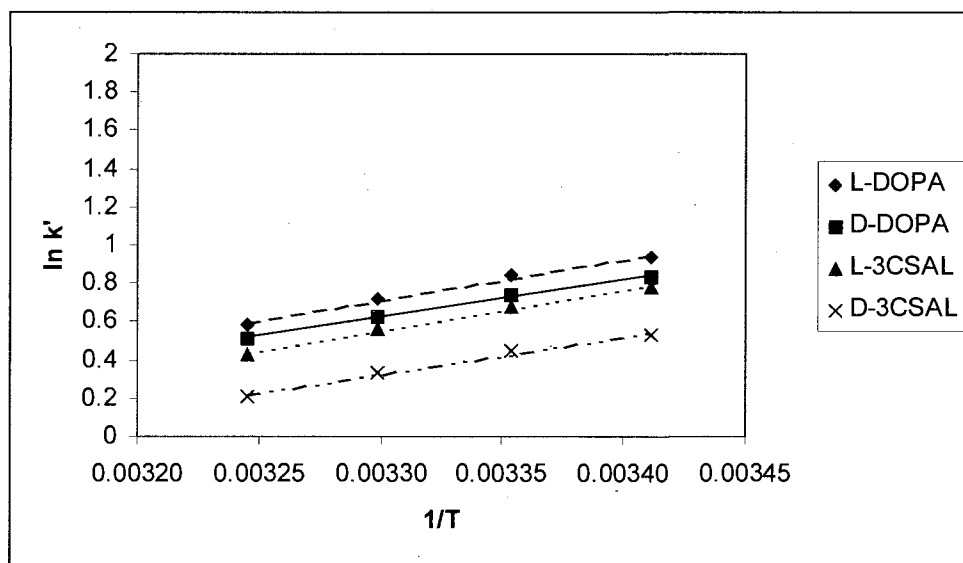
Figure 29: Van't Hoff Plot of High Temperature Seperations of DOPA and 3-CSAL with 2 mM Chiral Moile Phase Additive S-β-CD

Van't Hoff curves for the compounds using a mobile phase that did not contain the chiral selector were obtained and similar breaks were found. Thermodynamic data obtained from the slopes and intercepts of the curves are given in Tables 1-4. As may be seen, all thermodynamic variables are negative. Furthermore, absolute values for both ΔH^o and ΔS^o increase when the temperatures used in their determinations decrease. In all cases ΔH^o values are negative and thus favorable for retention, but in all cases ΔS^o values are also negative and therefore unfavorable. Contributions by unfavorable entropy values are more than offset by favorable enthalpy values.

Table 1: Thermodynamic Variables Associated With Chromatography of the Test Substances with Mobile Phase Containing 2 mM S-β-CD Over Relatively High Temperatures (20 °C–35 °C)

Substance	ΔH^o (kJ/mol)	ΔS^o (J/Kmol)
L-DOPA	- 17.7	- 45.0
D-DOPA	- 16.5	- 41.3
L-3-CSAL	- 18.0	- 47.0
D-3-CSAL	- 16.3	- 43.3

Table 2: Thermodynamic Variables Associated With Chromatography of the Test Substances with Mobile Phase Containing 2 mM S-β-CD Over Relatively Low Temperatures (0 °C–13 °C)

Substance	ΔH^o (kJ/mol)	ΔS^o (J/Kmol)
L-DOPA	- 30.7	- 87.8
D-DOPA	- 29.3	- 84.0
L-3-CSAL	- 36.0	- 107.8
D-3-CSAL	- 35.2	- 108.0

Table 3: Thermodynamic Variables Associated With Chromatography of the Test Substances with Mobile Phase Containing no Chiral Selector S-β-CD Over Relatively High Temperatures (20 °C–35 °C)

Substance	ΔH^o (kJ/mol)	ΔS^o (J/Kmol)
DOPA	- 18.4	- 49.1
3-CSAL	- 18.7	- 50.0
1-CSAL	- 15.6	- 39.2
SAL	- 15.2	- 33.2

Table 4: Thermodynamic Variables Associated With Chromatography of the Test Substances with Mobile Phase Containing no Chiral Selector S-β-CD Over Relatively Low Temperatures (0 °C–13 °C)

Substance	ΔH° (kJ/mol)	ΔS° (J/Kmol)
DOPA	- 33.7	- 100.8
3-CSAL	- 34.9	- 104.8
1-CSAL	- 34.0	- 103.1
SAL	- 22.7	- 57.8

The breaks in the van't Hoff curves are interpreted in terms of the retention model developed by Dorsey and Dill [133]. These data indicate a phase transition in the stationary phase hydrocarbon chains occurring over approximately the 15 °C to 20 °C range. Such phase transitions in reversed-phase stationary phases are relatively well known. They have been described as a "reversible melting-like transition of long-chain hydrocarbon ligands" which occur in high bonding density monomeric silica based C_{18} packings in the vicinity of room temperature [119, 134, 135].

At higher temperatures the extended chains allow relatively easy penetration of solute molecules into the stationary phase while the more compacted chains at lower temperature provide less opportunity of solute penetration. The effect is most clearly seen in the entropy values. Entropies of retention are double in the negative unfavorable direction at lower temperatures than they are at higher ones. Absorption of solute molecules by the stationary phase at the lower temperature conformation is more costly in terms of the entropy of chain ordering if the chains are more compact at these

temperatures.

The chiral compound S-β-CD appears to be an efficient and cost effective chiral selector for resolution of salsolinol and 3-carboxysalsolinol optical isomers. However, it is less effective for D- and L-DOPA, giving complete resolution only at subambient temperatures. It is least effective of all for 1-carboxysalsolinol. Retention of these materials is promoted by favorable enthalpies which (except for the case of 1-CSAL) more than make up for unfavorable changes in entropy.

Experimental Results from ICV Injections of Relevant Compounds on Volitional Ethanol

Consumption in Adult Male Sprague-Dawley Rats

THP Characterization by Proton NMR

A one dimensional proton NMR scan of the sample of R-(+)-THP in D_2O yielded the following spectrum (Figure 30).
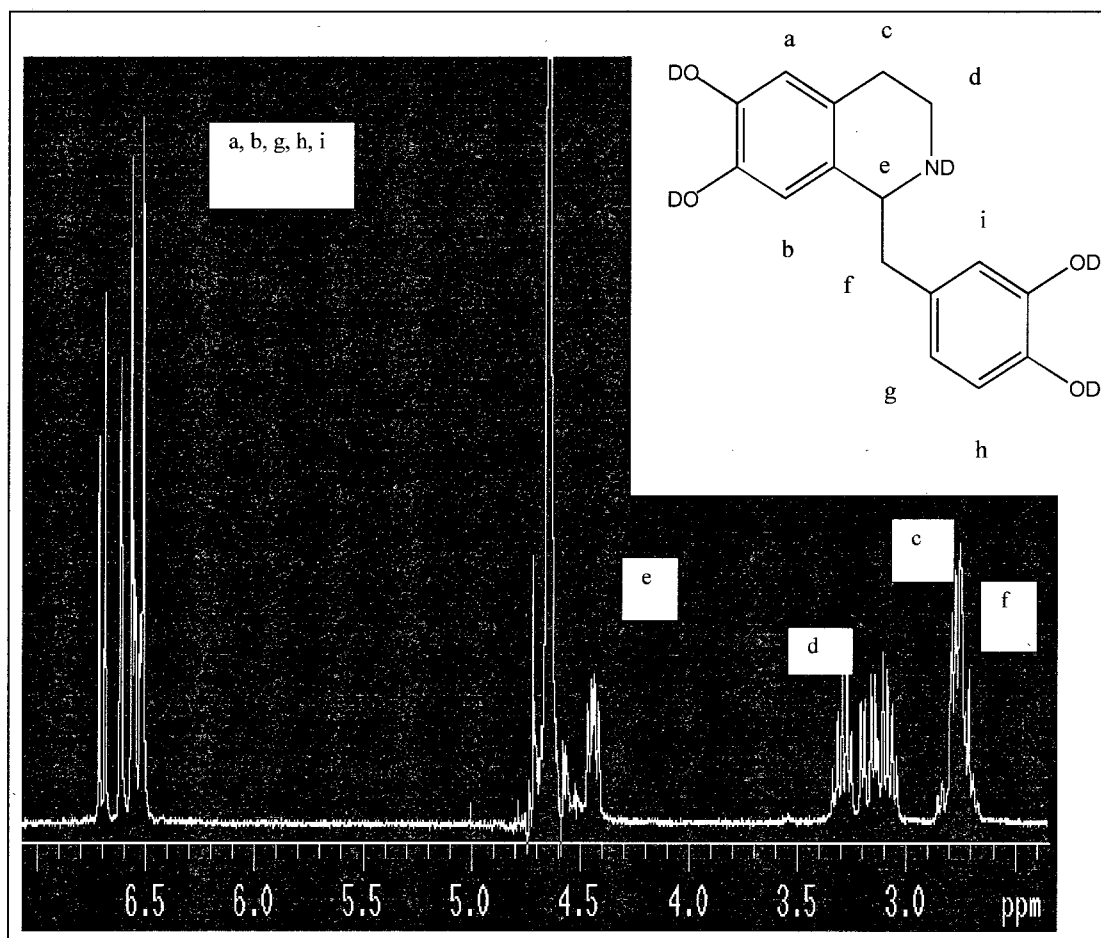
Figure 30: Proton NMR Spectrum and Peak Assignments for R-(+)-THP

Protons attached to aryl moieties (positions "a", "b", "g", "h", and "i") exhibit the greatest chemical shift and show as peaks from 6.7-6.5 ppm. The HDO peak from the exchange of labile hydrogen between the analyte and D_2O solvent appears, as expected, as a large peak at approximately 4.7ppm. A chemical shift of approximately 4.45 ppm is observed for the proton in the position "e". Proton "d" is heavily split and shows as a multiplet from 3.3-3.1 ppm. Proton "c", attached to the nitrogen-containing portion of the molecule, overlaps slightly with proton "f" at approximately 2.8-2.7 ppm. These interpretations match those obtainable from the Sadtler Index [136] and those obtained by previous researchers [137, 138]. Hence, the identity of this sample of synthesized stereoisomer of THP is confirmed.

HPLC Analysis in Achiral and Chiral Environments

Injection of samples of R-(+)-THP and racemic THP onto a commercially available C_{18} ODS HPLC column with an achiral mobile phase yielded the following chromatograms (Figure 31). Trace A is a sample of R-(+)-THP dissolved in a solution of identical composition as the mobile phase with sodium nitrate added as a t_0 marker. Trace B is an identical solution that has been spiked with an additional aliquot of racemic THP. The coelution of these compounds from this column indicates that the samples behave identically in an achiral environment. This is indicative of extreme similarity in the hydrophilic nature of these compounds.
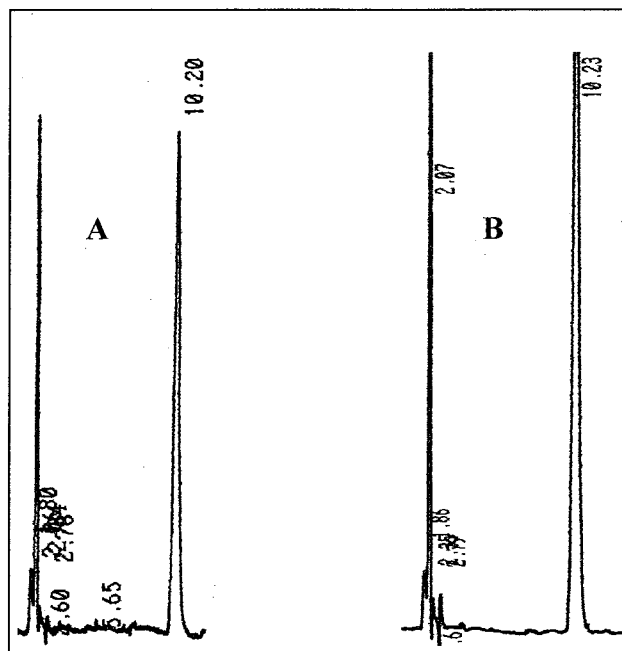
Figure 31: Achiral HPLC Chromatographs Showing Retention Times of (A)

NaNO₃ and R-(+)-THP; (B) NaNO₃, R-(+)-THP, and Racemic THP

Additionally, samples of R-(+)-THP and racemic THP were injected onto a

commercially available C_{18} ODS HPLC column with an chiral mobile phase (2.0 g/L S-β-

CD) yielded the following chromatograms (Figure 32).
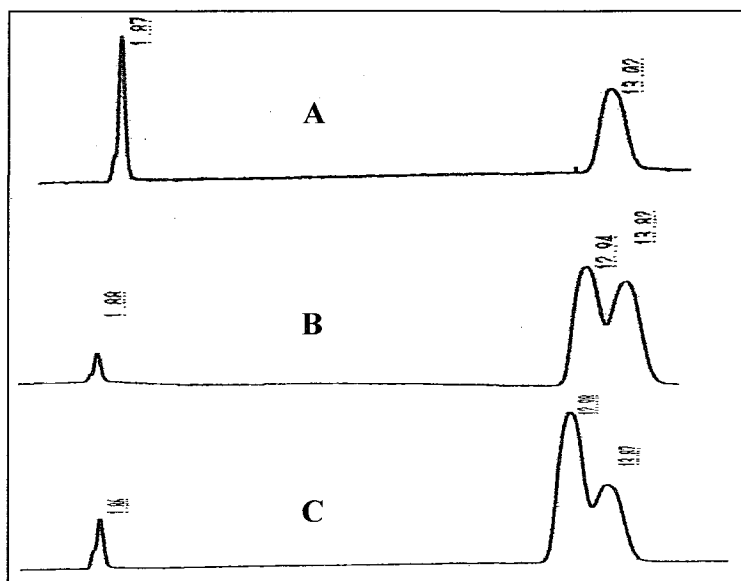
Figure 32: Representative Chiral HPLC Chromatograms of (A) R-(+)-THP and NaNO₃; (B) NaNO₃ and THP Racemate; (C) NaNO₃, THP Racemate, and R-(+)-THP

Chromatogram A is a sample of R-(+)-THP dissolved in an aliquot of mobile phase with sodium nitrate added as a t_0 marker. Trace B represents a sample of THP racemate chromatographed under the same conditions. The two peaks in chromatogram B proved to be of equal area after integration, verifying that the two THP stereoisomers are present in equal proportions in the racemate. Chromatogram C depicts an equimolar sample of R-(+)-THP and THP racemate.

Upon integration, the leftmost peak of R-(+)-THP has doubled in area. This indicates that the sample of R-(+)-THP and the R-stereoisomer of the racemate coelute in a chiral environment. These data, when coupled with the proton NMR spectra, confirm the identification of both samples as authentic THP.

The Effect of ICV Injections of THP on Ethanol Consumption in Adult Male Sprague-

Dawley Rats Obtained from the Frederick, MD Harlan Site

Only data from animals having cannulae tips within the right lateral ventricle or at

the dorsal rim of the ventricle were included in the statistical analysis. Histological 40 μm

sections were made on a cryostat (Microm HM 505 N) at −30 °C and stained with cresyl

violet. These sections were visually inspected on a lightfield microscope. A

representative histological section is shown in Figure 33.

0.7 mm
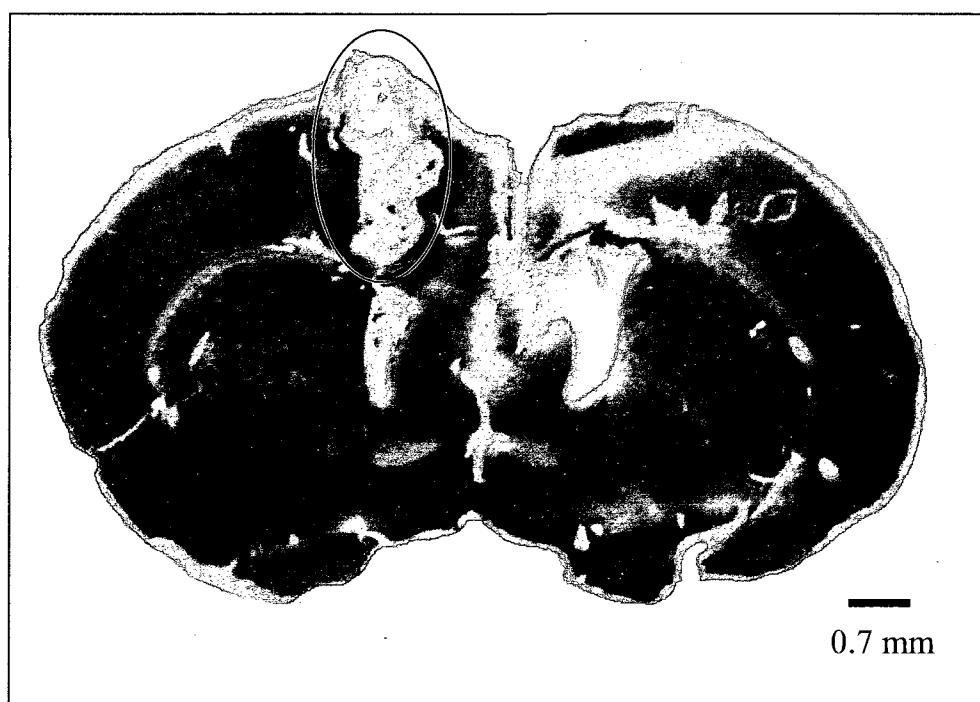
Figure 33: Representative Coronal Brain Section Showing Typical Cannula

Placement (Outlined Area Indicates Cannula Tract)

The control condition in this experiment consisted of CSF-treated animals, sham-operated animals, and naïve animals. A one-way ANOVA for independent groups revealed no significant differences in alcohol intake between these three control conditions, $F(2,6) = 2.274, p = 0.184$. Hence, these data were collapsed into one control condition. From this analysis, one can rule out the possibility that the surgical procedure itself might affect an animal's preference for (or ability to discriminate between) the tastes of alcohol and water.
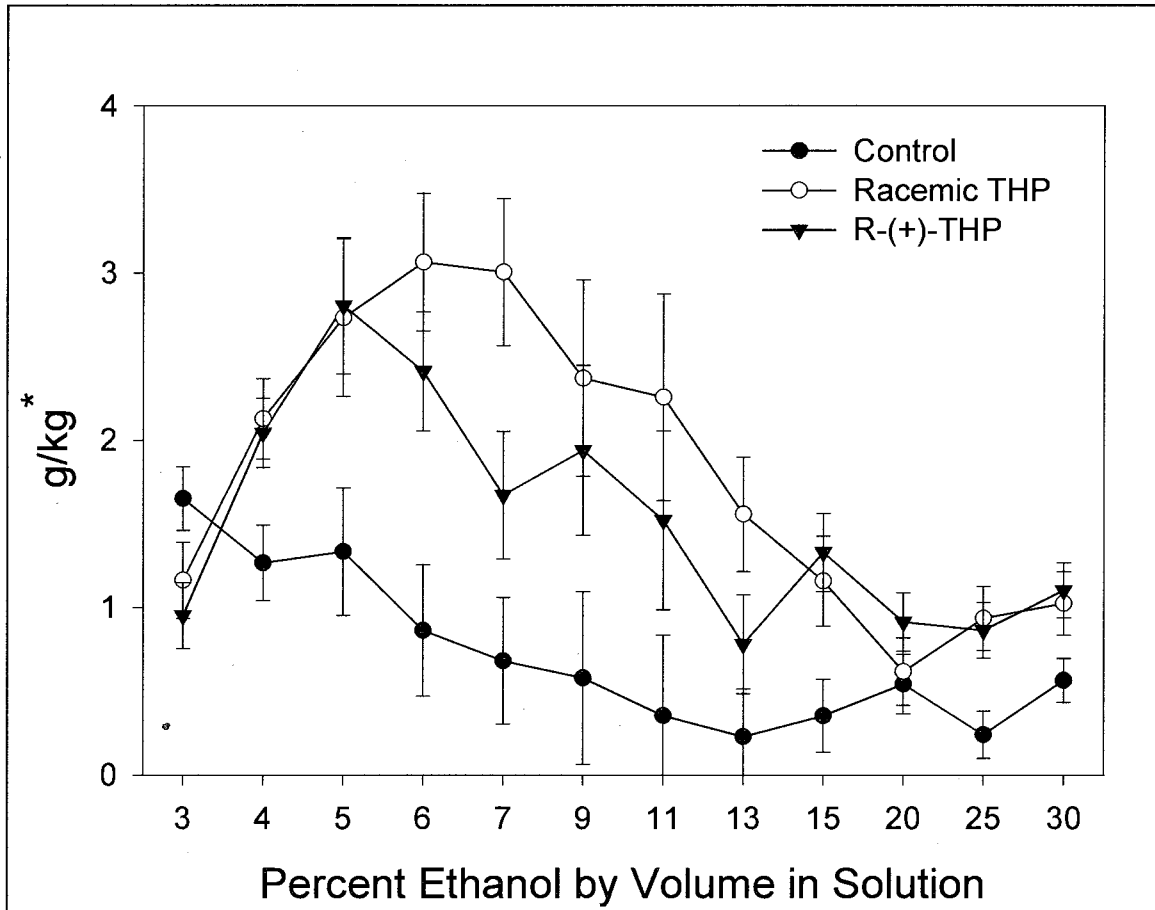
Figure 34: Graph Showing Ethanol Consumption in Response to 20 nmoles/day ICV Injections of R-(+)-THP and Racemic THP
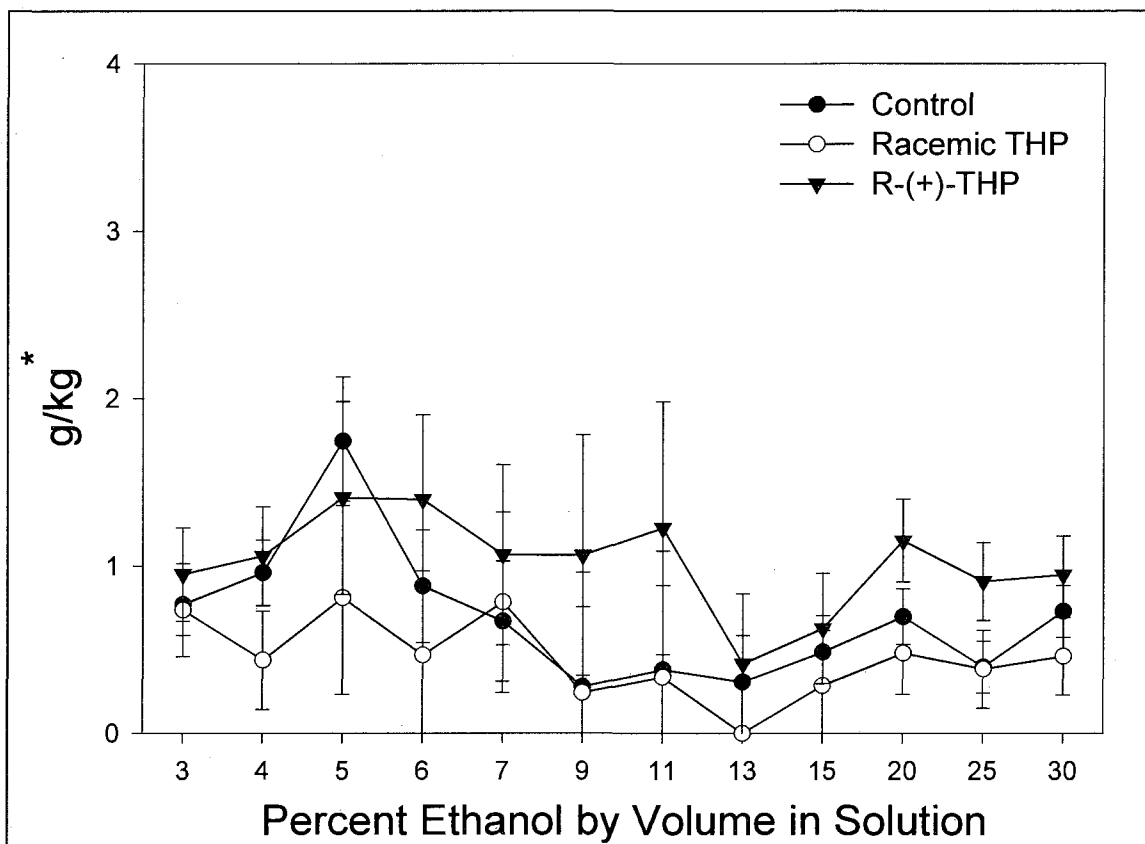
Figure 35: Graph Showing Ethanol Consumption in Response to 40

nmoles/day ICV Injections of R-(+)-THP and Racemic THP

The effect of acute ICV injections of ($\pm$)-THP or (+)-THP on the preference test is

shown in Figures 34 and 35. The effects of the lower doses (0.65 and 0.66 μg/μL) of

either ($\pm$)-THP or (+)-THP on alcohol intake were strikingly different than the effects of

the higher doses (1.3 and 1.4 μg/μL) of either compound. As a result, separate mixed-

model ANOVA's were conducted for the lower and higher doses of the alkaloids. For

the lower doses, the main effect of animals across days was significant, $F(11,231)=5.648$,

$p<0.001$. The main effect of drug treatment was also significant, $F(2,21)=9.139$,

$p \leq 0.001$. In addition, the interaction effect (drug treatment over days) was significant, $F(22, 231)=3.847$, $p<0.001$.

The effects of lower doses of (±)-THP or (+)-THP are illustrated in Figure 34. Post-hoc comparisons revealed a significant increase in consumption of absolute alcohol following ICV administration of the lower dose (0.65 µg/µL) of (±)-THP, $p=0.002$. Compared to controls, the lower dose (0.66 µg/µL) of (+)-THP also significantly enhanced alcohol intake, $p=0.017$. Differences in alcohol consumption between (±)-THP-treated animals and (+)-THP-treated animals were not significant, $p=0.493$.

The 95% confidence interval test determined that low doses of (±)-THP resulted in a significant increase in intake of alcohol from 7%-13% concentrations of ethanol. Low doses of (+)-THP significantly enhanced consumption of alcohol from 4%-11% concentrations and again at 15%-20% concentrations. At no time did drug treatment result in a decrease in alcohol consumption relative to control.

While lower doses of racemic THP induced a preference for alcohol across a mid-range of ethanol concentrations, the magnitude of the increase was not as pronounced as those observed in the initial reports of THP-induced drinking [53, 139]. This may be due to differences in dosing regimen. For example, more dramatic increases have been observed across a similar range of doses (i.e., 0.1-10.0 µg/µL), perhaps because the dosing regimen was extended throughout the entire 12-day preference test [53, 54, 74, 91, 95, 101, 140-143]. Although less dramatic, the data reported here indicates that repeated central infusion of THP during the test period is not necessary to maintain a change in alcohol preference.

In contrast to the prominent effects of low doses, ICV administration of the higher doses of (±)-THP (1.3 μg/μL) and (+)-THP (1.4 μg/μL) did not significantly alter alcohol consumption (Figure 35). A mixed-model ANOVA revealed that neither the main effect of animals across days nor the main effects of drug treatment were significant. Additionally, the interaction effect (drug treatment over days) was not significant.

A summary of the average g/kg ethanol dosage consumed by each experimental group appears as Table 5. While these data are not as dramatic as those reported by other researchers [53-55, 58, 74, 91, 93, 95, 97, 141, 144-148], they are compelling nonetheless.

Table 5: Average Grams of Ethanol per Kilogram Body Weight Consumed by Each Experimental Group Over the Course of the Entire ICV Experiment

Experimental Group	Number of Subjects	Mean g/kg
±THP (20 nmoles/day)	6	1.83 ± 0.25
+THP (20 nmoles/day)	8	1.53 ± 0.22
Control	9	0.69 ± 0.22
±THP (40 nmoles/day)	4	0.45 ± 0.18
+THP (40 nmoles/day)	4	1.02 ± 0.18

A subset of animals from the control condition was subjected to three consecutive twelve-day alcohol preference tests due to the fact that previous researchers' report of habituation [149]. A one-way ANOVA for repeated measures revealed no significant differences in alcohol consumption among these animals, $F(2,26)=0.452$, $p=0.642$.

Repeated exposure to escalating concentrations of alcohol solutions alone did not alter

alcohol intake. One can, therefore, rule out habituation or sensitization as possible

explanations for changes in patterns of alcohol consumption in this experiment.

In a subset of animals (n=17) treated with either (±)-THP or (+)-THP, alcohol and

water measurements were taken every four hours around the clock during the twelve-day

preference test (Figure 36). Most of the intake of both alcohol solutions and water

occurred during the nocturnal period, with more than a two-fold decline in consumption

of all fluid occurring immediately thereafter, in the first full four hours of light. Thus, it

may be assumed that rats do, in fact drink at a specific point in their circadian cycle.
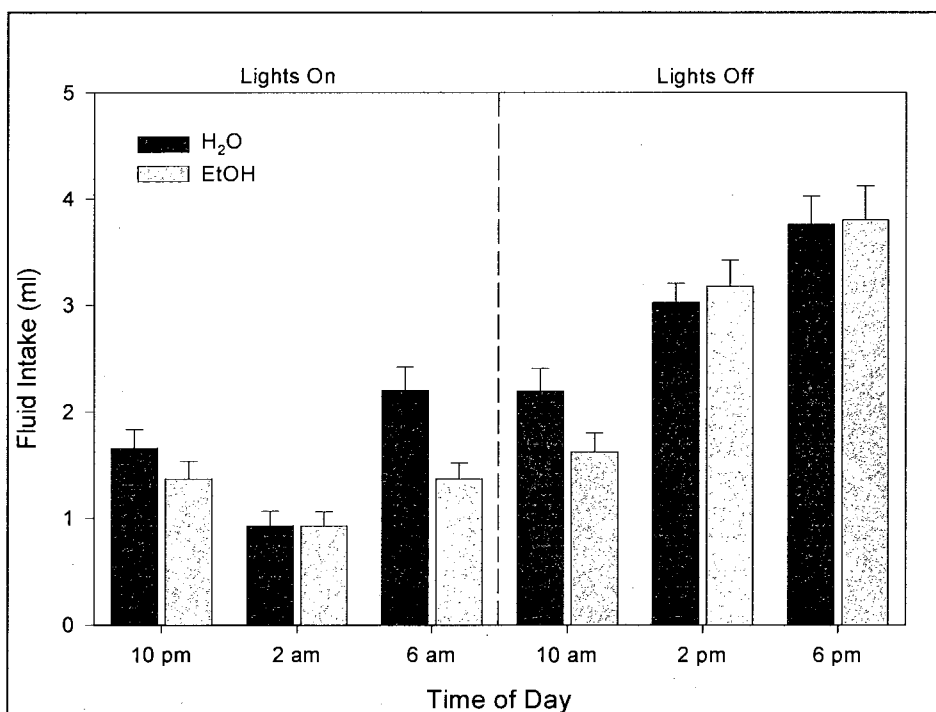
Figure 36: Bar Graph Showing Fluid Consumption Distribution For Four Hour

Periods Across Twelve-Day Ethanol Preference Tests

Myers and associates have conducted numerous experiments demonstrating volitional alcohol consumption following ICV or site-specific injections of THP, as well as other THIQ alkaloids, in rats [150]. However, some investigators have not observed any drinking following ICV injections of THP [102]. Even if THP-induced drinking does occur, some investigators have expressed doubts concerning the potential relevance of these data [99, 151, 152]. Moreover, there are few laboratories that have actually confirmed the finding of an increase in alcohol consequent to THP administration [56, 95, 142]. In the present study, it was found that both (±)-THP and (+)-THP (0.65-0.66 μg/μL; ICV) induced significant increases in alcohol intake (Figure 34). Most of the alcohol was consumed within an eight hour period.

The lack of effect of the higher doses (1.3-1.4 μg/μL; ICV) of either compound was unexpected (Figure 35 or Table 5). Duncan and Deitrich reported enhanced consumption of alcohol after ICV administration of THP at doses of 10.4 and 41.6 nmoles/day [56]. The doses used in the present study are 20 and 40 nmoles of THP per day. The principle methodological difference between the two studies concerns the dosing regimen. In contrast to the shortened dosing regimen used here, Duncan and Deitrich gave injections of 0.02 μg/μL every 15 minutes around the clock, starting two days prior to and continuing throughout the preference test [56]. Given the minimal dosing regimen used in this study (i.e., two injections/day for three days), one might expect a response to the higher of the two doses tested. Alcohol consumption, instead, increased in response to the lower dose.

Other investigators have reported similar findings. Clow et al. [142] found that the most marked behavioral response occurred in response to the lower of two doses (0.1

µg compared to 1.0 µg) of THP. Even lower doses (50 ng) of THP, delivered intraventricularly, have been shown to increase absolute intake of alcohol from 1.0 g/kg to 3.5 g/kg/day using the same dosing regimen as the one utilized presently [141]. Site-specific injections of picomolar concentrations of THP into limbic structures have been found to enhance alcohol consumption [93, 94]. Thus, the efficacy of the lower dose of either (±)-THP or (+)-THP in the present study was not surprising. Additionally, our findings are consistent with prior reports [53, 56, 94, 139] which demonstrate that the augmented drinking response does not depend on continuous THP infusion. Enhanced drinking continues long after THP clearance.

Diminishing alcohol consumption at higher doses of THP has been reported previously. Indeed, higher doses of THP are capable of attenuating alcohol consumption [94]. An inverted U-distribution best describes the dose-response function [150], with THP serving as an opiate agonist at lower doses and as an opiate antagonist at higher doses [94]. This may explain the lack of response to higher doses of THP tested in the present experiment. If relatively few injections of 1.3-1.4 µg/µL of THP are sufficient to induce such a ceiling effect [56], then why would a more chronic treatment regimen actually enhance alcohol intake? Perhaps a long-term alteration in receptor sensitivity occurs. Alternatively, perhaps molecular alterations in signal transduction processes may occur in response to chronic treatment with THP.

While increases in alcohol consumption have been reported previously following ICV injection of (±)-THP and (-)-THP [53], this is the first known report of an increase in alcohol consumption caused by (+)-THP. The magnitude of the response did not differ significantly from the response to racemic THP. In fact, the response was extended to

higher concentrations (15% and 20%). These findings are consistent with previous reports that (-)-THP induces volitional alcohol intake comparable to (±)-THP [53].

Alternative to the hypothesis that morphine is synthesized from the alkaloid, THP may act as an opiate agonist. Because of the relative symmetry of the molecule, receptors may be unable to distinguish between the two isomers. In either case, one might expect both enantiomers would exhibit efficacy equal to the racemic mixture [53]. Unfortunately, this data cannot discriminate between these two hypotheses.

Aldehydes form as a result of the enzymatic degradation of alcohol by alcohol dehydrogenase in the liver [31]. These peripherally formed aldehydes may react with catecholamines such as dopamine to form THP, as well as other THIQ alkaloids, shown to induce ethanol intake. The presumption that THIQ alkaloids are relevant to the etiology of alcoholism appears to rest on the assumption that peripherally formed THIQ alkaloids can cross the blood-brain barrier, and exert their behavioral effects by binding to centrally located receptors. Picomole quantities of THP have been detected in brain tissue using high performance liquid chromatography (HPLC) coupled with electrochemical detection following peripheral administration of either THP [153] or alcohol [154].

The highest concentrations of THP were found at axons associated with the mesolimbic reward system (e.g., olfactory bulb, frontal cortex, hypothalamus). Substantial basic and clinical research findings implicate opioid modulation of this dopamine circuit in alcohol addiction [155-157]. Given the structural similarity of THP to morphine, the presence of THP in the mesolimbic dopamine system is noteworthy. The discriminative stimulus effects of THP and ethanol are apparently dissimilar to those

of opiates [158]. Therefore, the biochemical connection is not likely to be direct. Nonetheless, once it has crossed over the blood brain barrier, THP was mostly highly concentrated in structures [154] that contain both dopamine and opioid receptors.

The notion that ingested alcohol could conceivably lead to a buildup of THIQ levels in the brain has been experimentally tested [159]. Peripherally administered ethanol (2 g/kg; IP) resulted in an increase in THP and dopamine levels measured in the extracellular space of the nucleus accumbens in alcohol-preferring (AA) and alcohol-avoiding (ANA) rats. AA rats were generally less sensitive to changes in THP, salsolinol, or dopamine than ANA rats. The increases in THP following ethanol administration were more pronounced in the ANA rats [159].

The fact that peripheral infusion of THP did not influence alcohol consumption in this experiment presents a dilemma for the multiple-metabolite theory. One plausible hypothesis is that endogenously formed THP does not contribute to any significant degree to the etiology of alcohol addiction. In this event, THP and other THIQ alkaloids are still pharmacological tools useful for investigating neural mechanisms that regulate alcohol ingestion. Since the necessary compounds for endogenous THP production are also present in the mammalian brain, it is also possible that centrally formed THP plays a role in alcohol consumption. However, the reason for the lack of response to chronic, peripheral infusion of THP remains unknown.

The present experiments confirm previous reports of volitional alcohol consumption following central administration of THP. Increases in alcohol intake were observed following administration of the racemic compound, as well as the R(+) – isomer of THP, but only at the lower doses tested (0.65-0.66 µg/µL). To our knowledge, the

finding that the R(+) – isomer of THP can potentiate alcohol consumption has not been reported previously. Increased alcohol consumption in response to low doses of racemic THP was observed mostly in the mid-range of concentrations tested (7-13%). The R(+) – isomer compound induced significant alcohol intake across a mid-range of concentrations (4-11%), but also at much higher concentrations (15 and 20%).

Furthermore, most of the ethanol was consumed within a four hour period each day, maximizing the pharmacological effects of ingested ethanol. At present, it is still unclear whether or not endogenous THP plays a significant role in the etiology and maintenance of alcohol addiction. Nonetheless, the present data verify that central administration of THP and its R(+) – isomer can significantly alter drinking behavior in rats such that unsweetened alcohol solutions become preferred over water.

The Effect of ICV Injections of THP on Ethanol Consumption in Adult Male Sprague-Dawley Rats Obtained from the Prattville, AL Harlan Site

A population of adult male Sprague-Dawley rats was obtained from the Harlan site located in Prattville, AL. These animals were treated in exactly the same manner as the experimental animals obtained from the Frederick, MD site in regards to all experimental procedures and animal care. This population of rats already, on average, drank large amounts of ethanol prior to any ICV injections.

This was odd, because the conventional animal research wisdom is that Sprague-Dawley rats are alcohol-aversive. This occurrence of untreated animals drinking relatively large amounts of alcohol was heretofore unreported. When contacted about this anomalous behavior, representatives from Harlan, Inc. expressed little surprise. They

claimed that the outbred strains of laboratory rats that Harlan vends are isolated at each individual site.

Therefore, rats from different plants could be considered, for all intents and purposes, to be separate strains with respect to many factors. These factors include heart disease, debilitating aging effects, growth curves, respiratory defects, and death rate. The representative knew nothing of the potential for alcohol drinking differences, but noted that very few labs specify which site they prefer their rats to be shipped from. Of these few labs, even fewer conduct addiction-related research, so it was not unusual that Harlan had no records of the propensity for these rats from the Prattville site to consume ethanol.

Only data from animals having cannulae tips within the right lateral ventricle or at the dorsal rim of the ventricle were included in the statistical analysis. Histological 40 μm sections were made on a Microtome cryostat and stained with cresyl violet. These sections were visually inspected on a lightfield microscope. Data from this population of rats is shown in Figure 37.
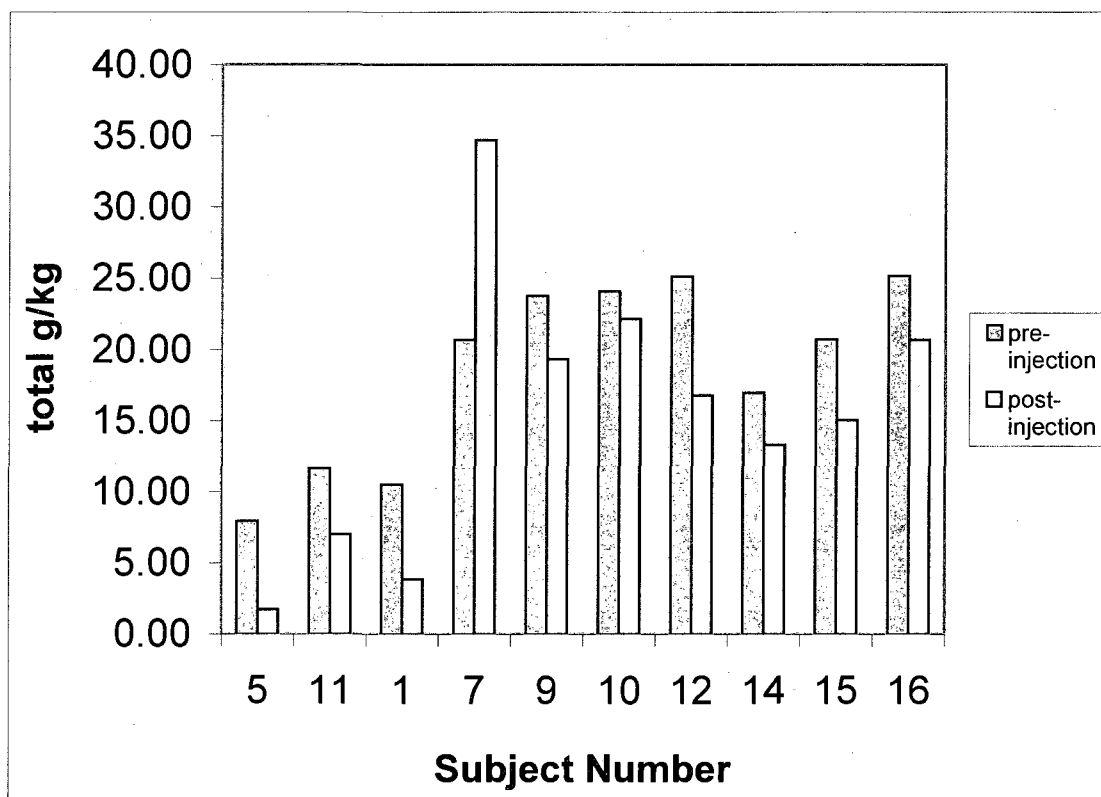
Figure 37: Bar Graph Depicting Total g/kg Consumption of Prattville, AL Rats Across Entire Twelve-Day Ethanol Screening (Rats 5,11, and 1 treated with (+)-THP; Rats 7, 9, 10, and 12 treated with aCSF; Rats 14, 15, and 16 treated with THP racemate)

As can be seen from the Figure 37, no THP treatments resulted in elevated ethanol consumption. In fact, the only animal to dramatically increase its ethanol consumption was Rat 7, a control animal. Control animals, rats 7, 9, 10, and 12, drank significantly more ethanol over the course of the experiment than treated animals. While rats treated with (+)-THP, rats 1, 5, and 11, drank approximately the same amount of ethanol as other tested Sprague-Dawley rats from Frederick, MD, THP treatments

resulted in a decrease in alcohol preference. Rats 14-16 drank elevated levels of ethanol before treatment and decreased their drinking with subsequent treatment of THP racemate.

Presently, there is no satisfactory explanation for the behavior observed from these Prattville, AL rats. These rats may already have elevated levels of THP present in their brain. Raising the endogenous levels of THP through ICV injections may result in an aversive dose, as other researchers have observed in previous dose-response curve studies of THP [56, 142]. Alternately, the Prattville, AL Harlan plant may have unknowingly bred their rats selectively for a high ethanol preference, as Myers and co-workers have with their HEP rats [97, 160, 161].

The Effects of ICV Injections of THB on the Ethanol Preference of Adult Male Sprague-Dawley Rats Obtained from Harlan's Frederick, MD Site

Only data from animals having cannulae tips within the right lateral ventricle or at the dorsal rim of the ventricle were included in the statistical analysis. Histological 40 μm sections were made on a Microtome cryostat and stained with cresyl violet. These sections were visually inspected on a lightfield microscope.

These experimental subjects were treated in the exact same manner as previous animals with regard to animal care, surgical, injection and cannulae placement verification procedures. The only notable difference in the treatment of these animals was in the identity of the tested compound. These animals were given ICV injections in the amount of 20 nmoles/day of THB. Data from this experimental group appears as Figure 38.
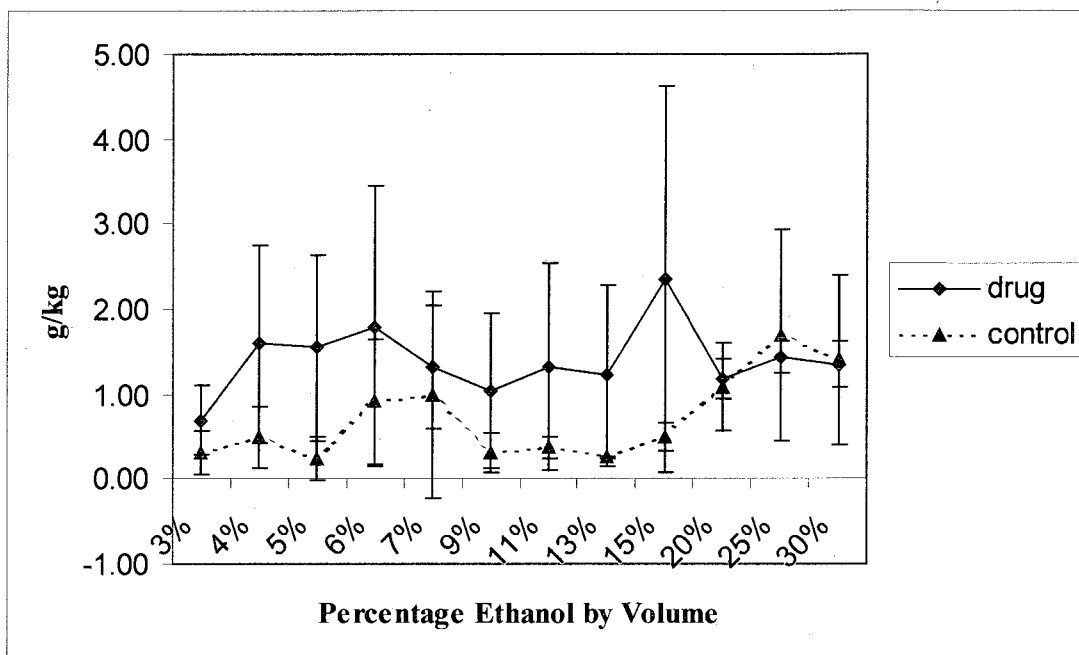
Figure 38: Graph of Control and Experimental Groups of ICV THB-Treated Rats

As can be seen in the graph, no significant increases are observed when the whole population of rats is examined. However, the large standard deviations are the result of three subjects' dramatic increases in ethanol consumption.

As THB is not a precursor to biological opiate alkaloids [162], it is unlikely in the extreme that opiate biosynthesis plays any part in these observed results. Also, since THB, compared to THP, has a relatively low affinity for opioid receptors [163, 164], it is unlikely that this very limited success is due to THB acting as an opioid agonist. Rather, it is probably due to the action of THB on dopaminergic receptors, for which it has a relatively high affinity [164].

Effects of Ethanol Consumption on Tetrahydropapaveroline Levels in the Cerebrospinal

Fluid of Adult Male Sprague-Dawley Rats In Vivo

Microdialysis Recovery Determination

The amperometric electrochemical detector used in this series of experiments was

calibrated by injecting various concentrations of THP Hydrobromide in artificial CSF

(aCSF) onto the C_{18} ODS column used for future dialysate analyses and plotting the

detector response as a function of peak area counts. This detector response is given in

Figure 39.
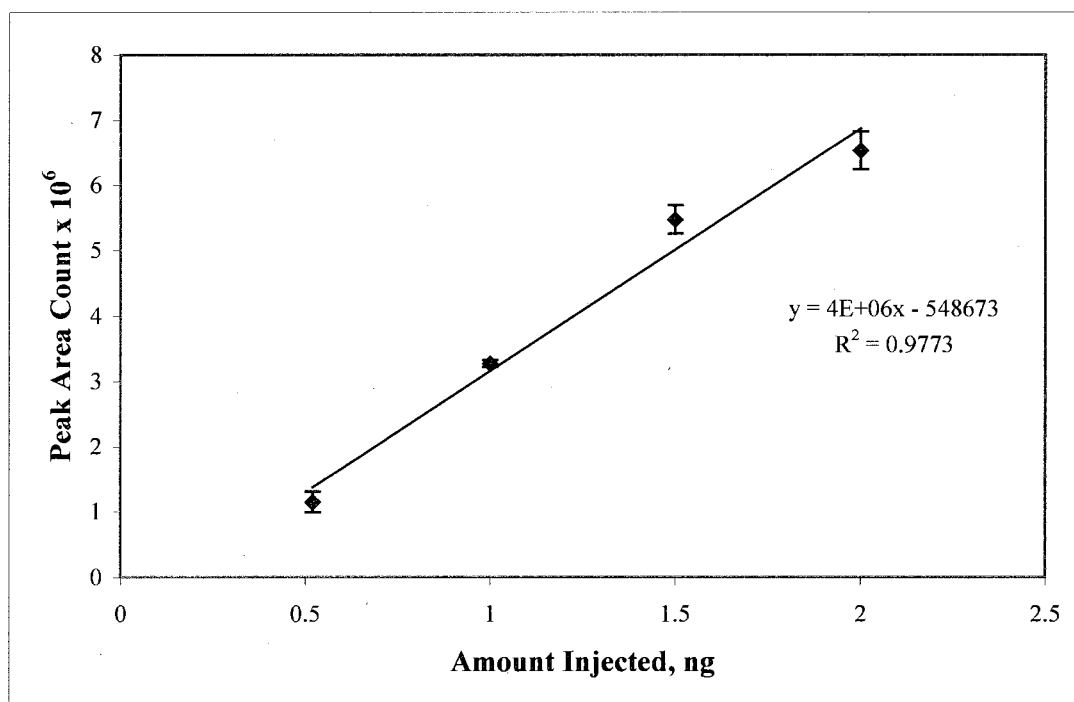
Figure 39: Detector Response of Amperometric Detector to THP

Hydrobromide

Flow rates of 0.5, 1.0, and 1.5 μL/minute were examined to determine the optimal

flow rate for an *in vivo* microdialysis experiment. Since a calibration curve had been

established for the detector, determining the percentage of recovered from the dialysis of

the 0.69 µM "parent" solution was a simple matter of measuring the concentration of the

dialysate and expressing its concentration as a percentage of the "parent". Results from

the flow rate experiment are seen as Figure 40.
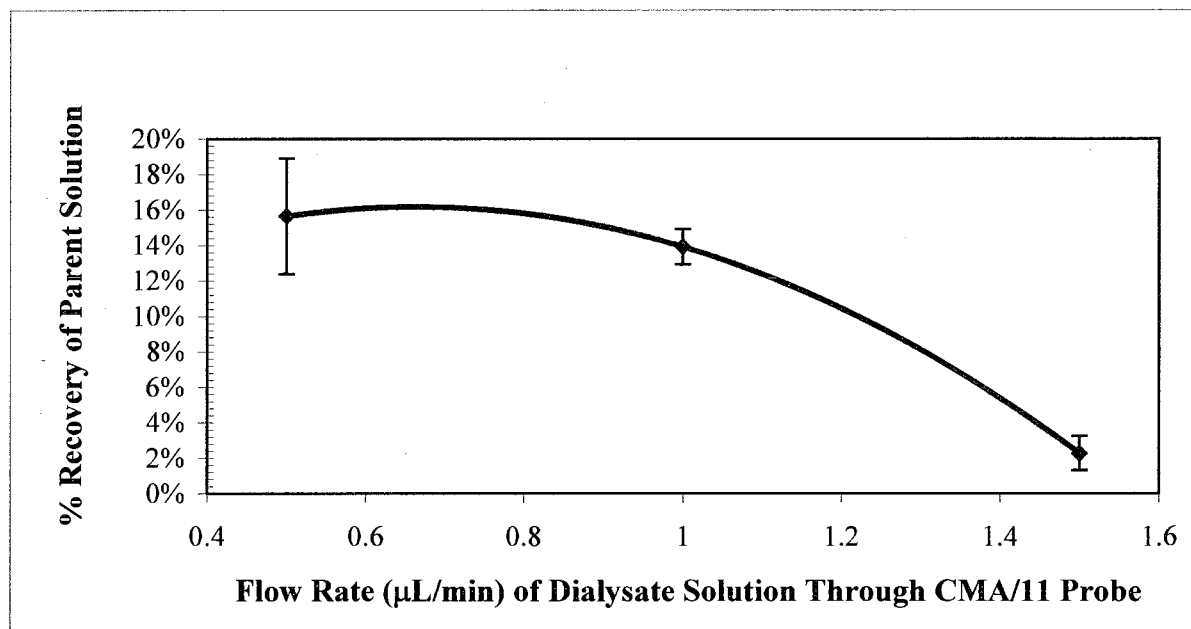
Figure 40: Percent Recovery of THP Hydrobromide in Dialysate Solution at Three

Flow Rates as Measured by HPLC-ECD

From this flow rate calibration, it can be seen that the optimal flow rate at which

to operate a CMA/11 microdialysis probe for maximum recovery of THP Hydrobromide

and repeatability is 1.0 µL/minute. This flow rate was used throughout the *in vivo*

microdialysis experiment.

In Vivo Microdialysis Results

Exposure to ethanol prior to collection of dialysate definitely increased

endogenous THP levels when compared to basal levels. Representative chromatograms

of analyzed dialysates appear as Figures 41 and 42. The circled peaks in Figures 41 and

42 have retention times identical to those of prepared THP standards.
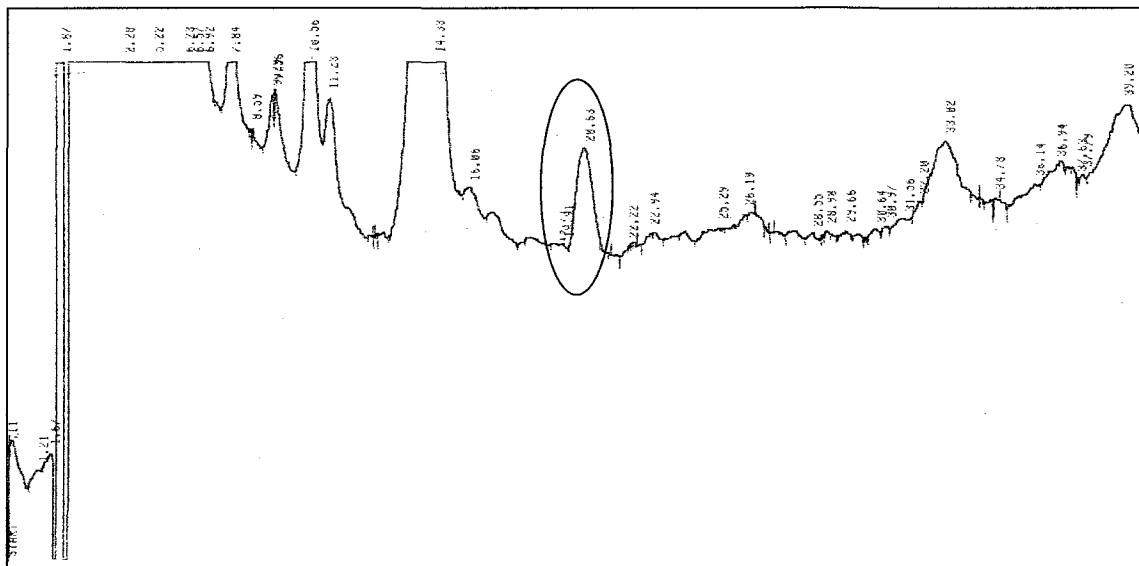
Figure 41: Representative Chromatogram of Dialysate from Rat M2 Before Ethanol

Exposure
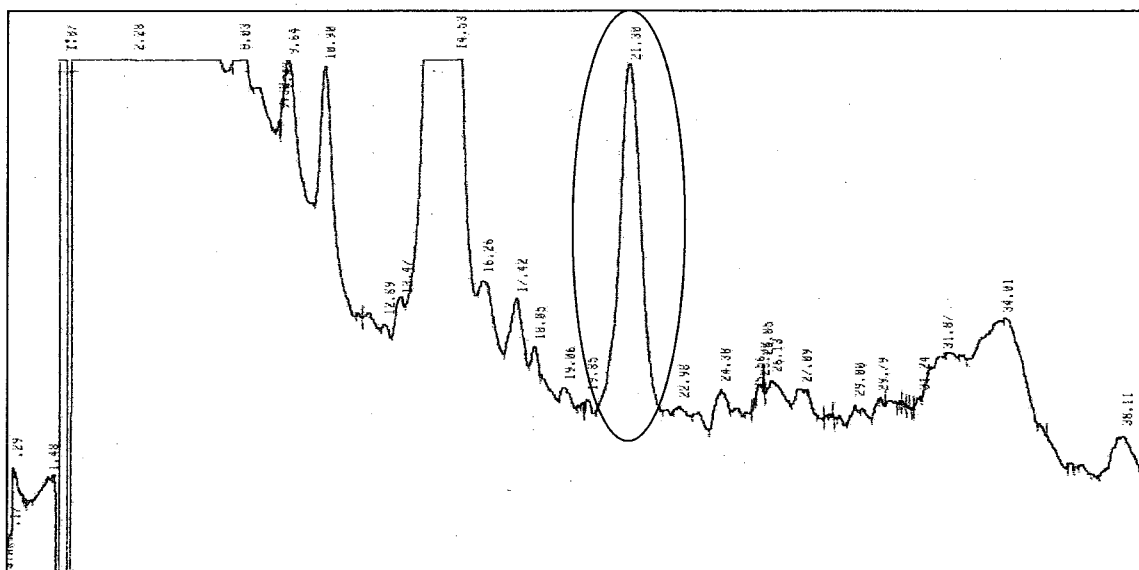
Figure 42: Representative Chromatogram of Dialysate from Rat M2 After Ethanol

Exposure

The data from the *in vivo* microdialysis on the three experimental subjects appears as Figures 43-46. Amounts of THP in the dialysis solution were calculated using the detector response curve displayed as Figure 39. Application of the CMA/11 probe recoveries displayed in Figure 40 allows for the estimation of the concentration of THP *in vivo* within the lateral ventricles of the experimental subjects shown in Figures 47-49. This experiment was originally intended to be a larger study with seven experimental subjects, but three rats damaged their implants prior to final THP level assessment. One set of data was lost due to a loss of communication between the electrochemical detector and the HP 3396A integrator.
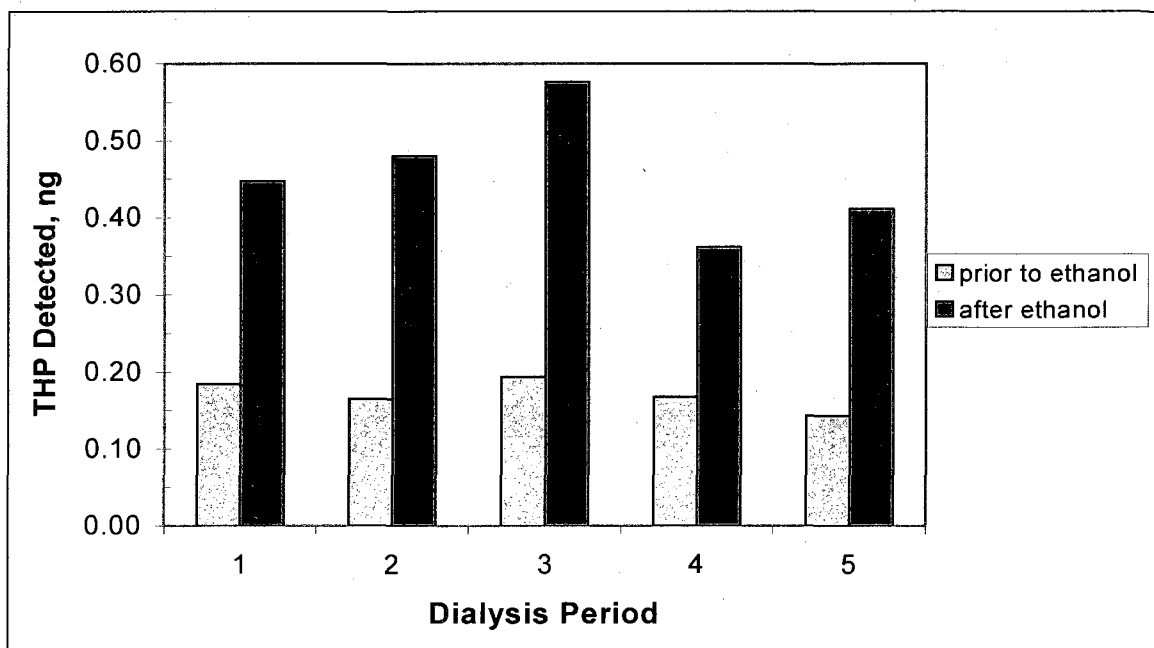
Figure 43: Bar Graph showing Elevated Levels of Dialysis THP (Rat M1) After

Exposure to Ethanol
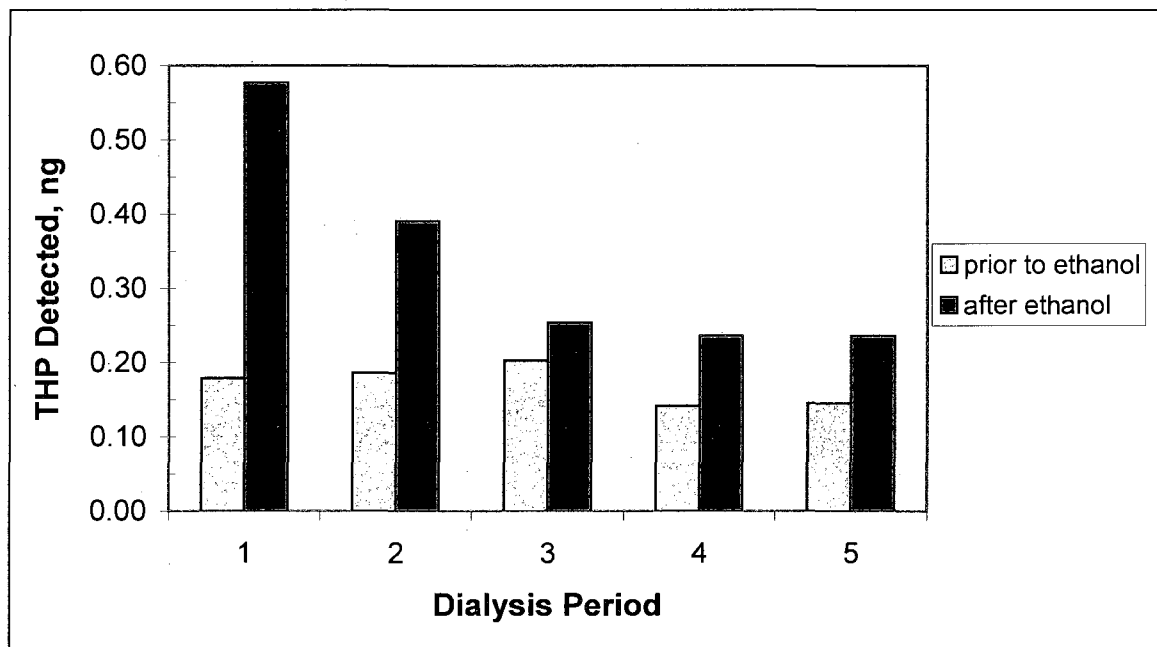
Figure 44: Bar Graph showing Elevated Levels of Dialysis THP (Rat M2) After

Exposure to Ethanol
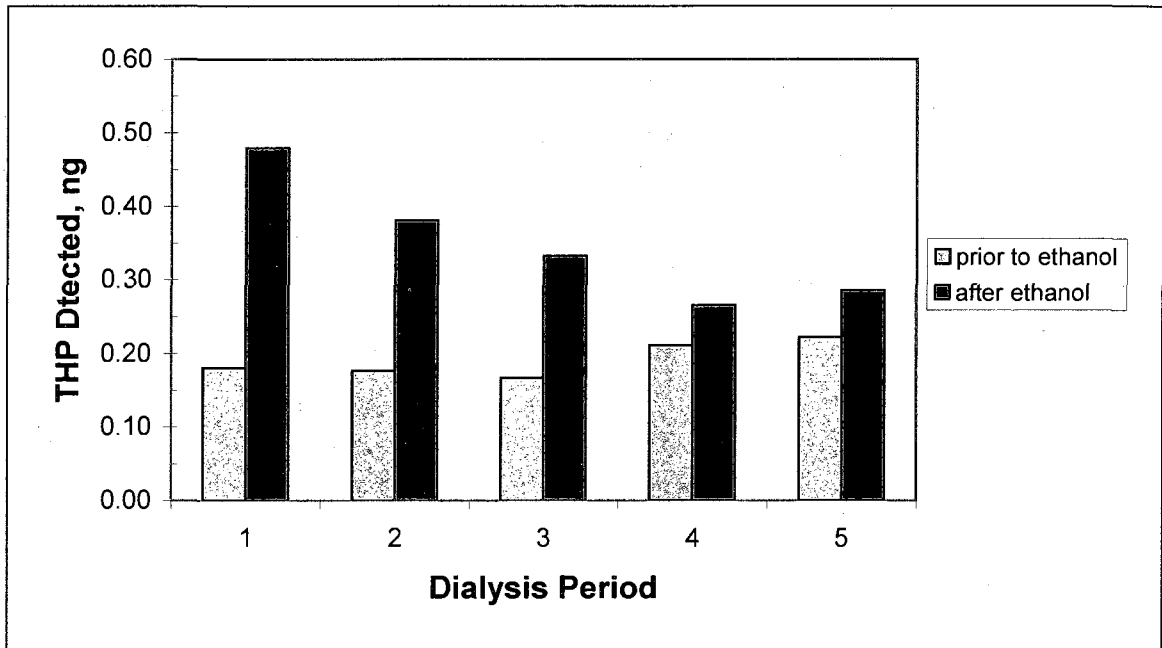
Figure 45: Bar Graph showing Elevated Levels of Dialysis THP (Rat M7) After

Exposure to Ethanol
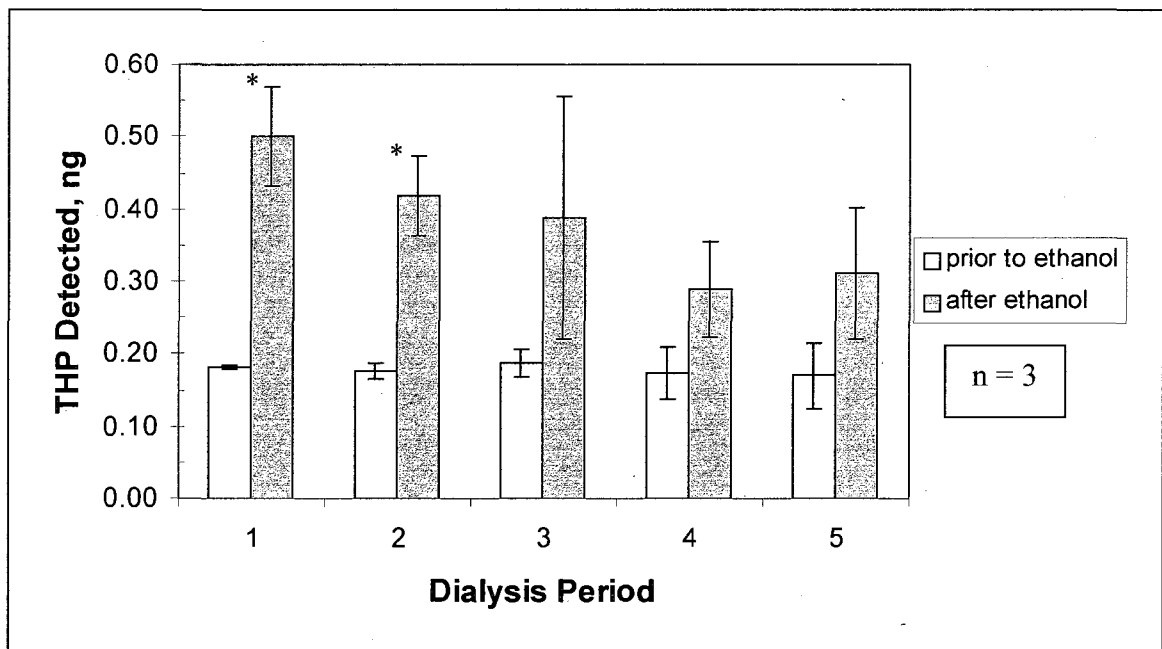
Figure 46: Composite Bar Graph showing Elevated Levels of Dialysis THP of All

Rats After Exposure to Ethanol
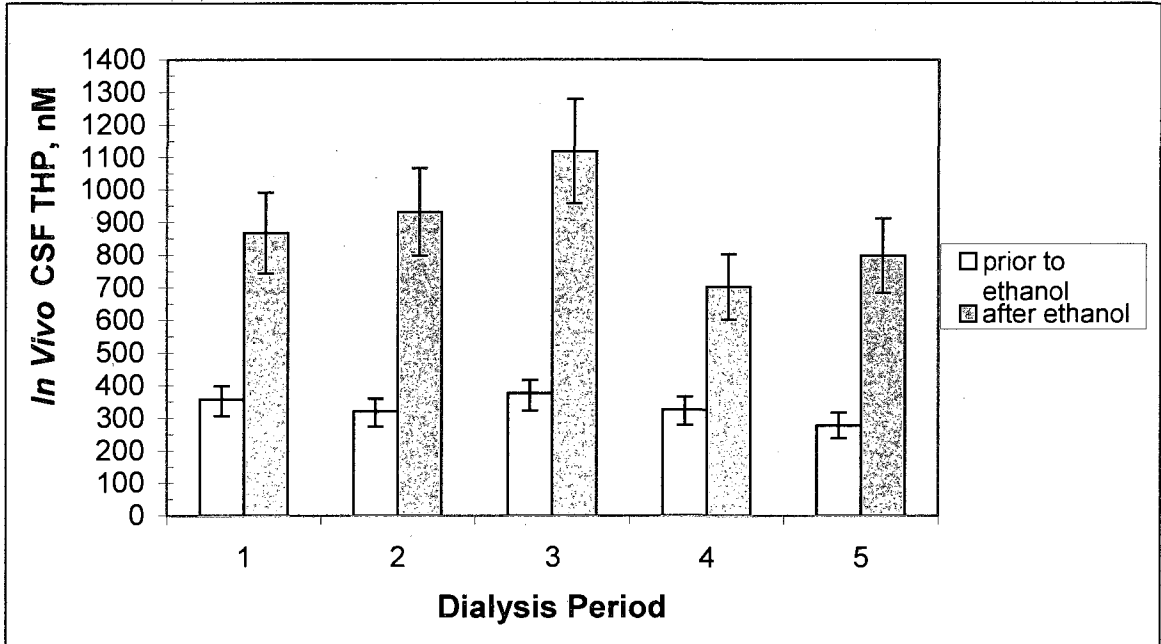
Figure 47: Bar Graph showing Calculated *In Vivo* THP Concentration of the

Lateral Ventricle (Rat M1)
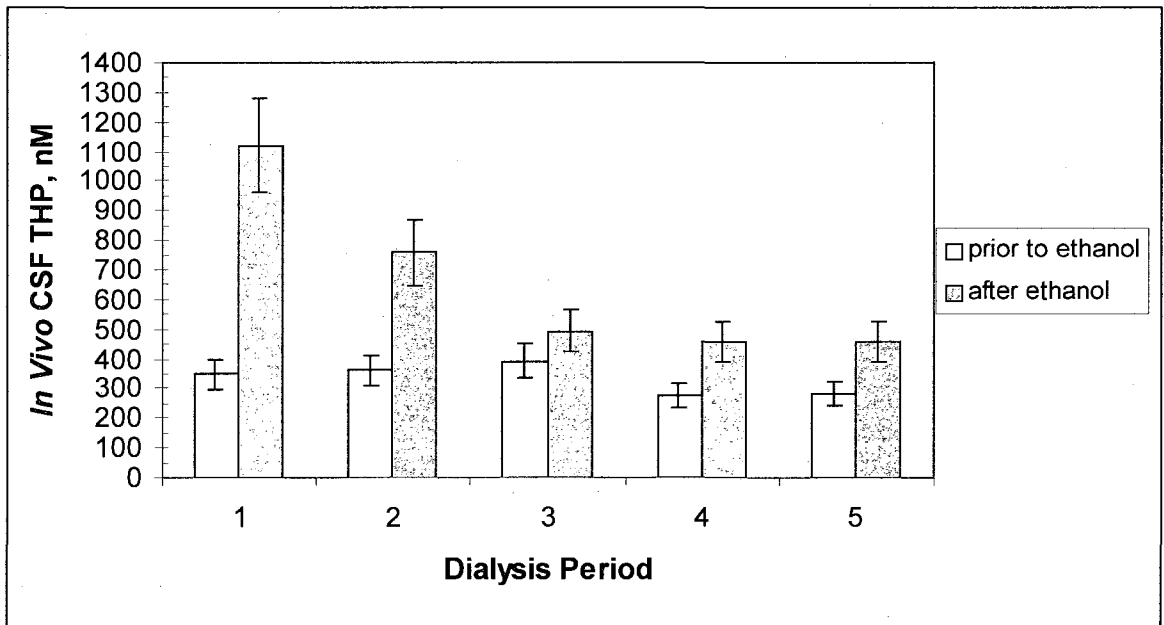
Figure 48: Bar Graph showing Calculated *In Vivo* THP Concentration of the

Lateral Ventricle (Rat M2)
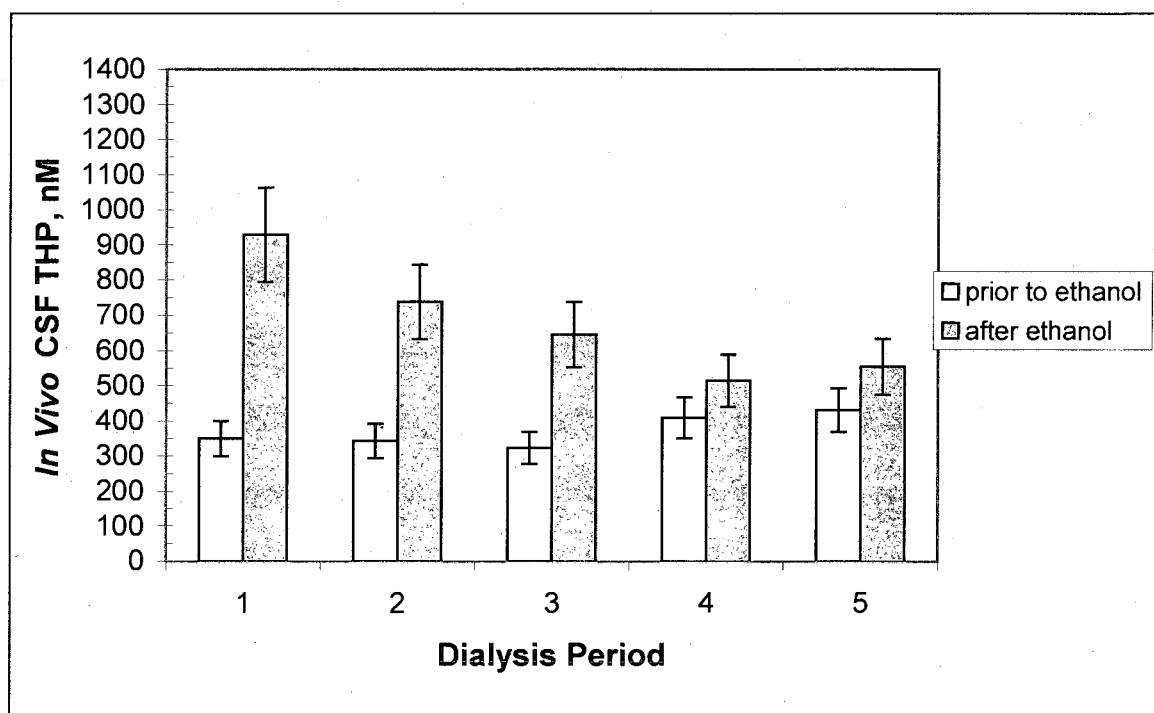
Figure 49: Bar Graph showing Calculated *In Vivo* THP Concentration of the Lateral Ventricle (Rat M7)

General Discussion of the Relevance of Results

Chiral HPLC columns for the separation of enantiomeric mixtures employing chiral stationary phases are quite expensive, have delicate bonded phases, and are well documented [116, 128, 165-168]. A desirable alternative to these columns would be a cheap and effective method of creating a chiral environment that aids in the separation of these stereoisomers. S-β-CD is just such a compound. It vastly improves resolution of catecholamines compared to unsubstituted cyclodextrins [129, 130].

While several researchers have conducted studies of cyclized macromolecules or ion-pairing agents in HPLC mobile phase additives [115, 117, 129-131, 169, 170], no published studies have used subambient temperatures to enhance the thermodynamics of

separations. The separation of carboxylated salsolinol derivatives using chiral mobile phase additives is difficult, at best. However, subambient temperatures coupled with the chiral mobile phase additive S-β-CD allow meager resolution of even these traditionally inseparable enantiomers.

Chiral recognition is an important function for biological systems. Just as only one stereoisomer of glucose is important for terran biosystems, only one isomer of morphine, the S-isomer, has analgesic effects. Likewise, it was previously thought that only one isomer of THP was effective at eliciting an alcohol-drinking response in aversive animals. Only the S-isomer was singled out for further study by Myers and co-workers [58] or Duncan and Deitrich [56]. Sango et al. found only the S-isomer of THP to be present in the brains of rats exposed to ethanol [51]. Tabakoff et al. found S-(-)-THP to be present biological fluids of human alcoholics [171]. The experiment conducted with ICV THP injections in Sprague-Dawley rats indicate that R-(+)-THP is just as effective at evoking alcohol-addictive responses from rats.

Whether this result is due to the conversion of R-(+)-THP to the S-isomer or the inability of THP-reactive receptors to distinguish between the two is unclear. Also unclear is the precise manner in which THP evokes alcohol drinking in aversive rats. THP may be converted to morphine *in vivo* [52, 103, 104, 106, 107, 111, 112, 172-175], or THP may act as an opiate agonist and bind with opioid receptors found in the brain [146, 163, 164, 176, 177]. It was suggested by one group of researchers that THP may have a two-pronged effect, binding with dopaminergic and opioid receptors alike [164], depending on the conformational rotamers of THP.

THB has very little affinity for opiate receptors, but a much larger affinity for dopaminergic neuroreceptors [164]. Hence, it is reasonable to surmise that if THB is successful at evoking volitional alcohol consumption in ethanol-aversive rats, dopaminergic neurons may be more involved than receptors that are selective for opiates in the etiology of alcoholism. However, if THB is unsuccessful at evoking volitional alcohol drinking, the converse is most likely true. Since ICV injections of THB were successful in inducing alcohol consumption in few experimental animals, it is likely that the truth is more complicated than this hypothesis.

Nonetheless, one fact is undeniable. The gastric gavage of solutions relatively high in ethanol concentration did result in a marked increase in endogenously formed THP in experimental animals. This fact alone implicates tetrahydroisoquinolines in the etiology of alcohol addiction and vindicates the theories of early alcohol addiction researchers such as Davis and Walsh [28-31] and Myers [53-55, 58, 96, 139, 178, 179].

It is noteworthy that, during the microdialysis experiment, the maximum levels of THP were detected within the first three periods of the experiment. THP levels then appeared to drop towards basal concentrations. This increase in initial endogenous levels of THP following alcohol exposure is startling. While one researcher has assayed THP concentration in brain tissues following ethanol exposure [154], no other researcher to date has published such a change in THP levels in CSF in "real time" from a freely behaving subject [33, 159, 180-189]. While not conclusive by any means, these experiments, both the ICV administration of THP and the dialysis of THP from CSF following the administration of an ethanol solution, definitely lend credence to the theory

that THP and related alkaloids play an important role in the acquisition and maintenance of alcohol addiction.

Observation of the amounts of THP detected in the CSF through microdialysis indicate that endogenous THP levels, both basal and elevated through ethanol administration, in the lateral ventricle CSF are approximately four orders of magnitude less than the demonstrated effective ICV dose of 20 nanomoles of THP per day. This finding would appear to support earlier reports that ICV doses as low as 50 ng are sufficient to induce elevated ethanol consumption in rats [141].

Future experiments expanding on this line of research should replicate these experiments with much larger experimental groups from varied rat colonies and facilities. Additionally, future experiments should explore the relationship of opiate receptor density versus ethanol consumption elicited via ICV injection of THP. Also of interest is the relative persistence of endogenously formed THP within the CSF of subject M1. Whether this is a function of relatively low metabolic rate of the disposition of THP within this particular subject is, as yet, unclear. Future researchers should also focus on the determination of individual neurochemical and neuroanatomical variations as contributing factors to the etiology of alcohol addiction.
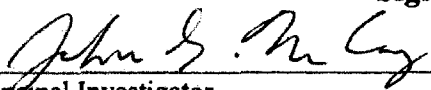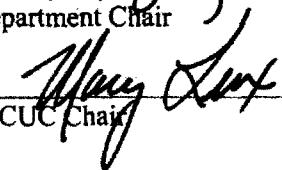
APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) PROPOSAL

University of Southern Mississippi
Application for Use of Animal Subjects

Principal Investigator's Name (Please Print): John G. McCoy	Department: Psychology
Office Phone Number: 601-266-4617	Mail Box: 5025

Signature Approvals

Principal Investigator   4-9-03

Department Chair   Date   4-7-03

IACUC Chair   Date   5/5/03

IACUC Official Approval

216-003
Protocol #

9/30/05
Expiration Date

Renewal #   Expiration Date

Renewal #   Expiration Date

I. FUNDING INFORMATION

Is this application associated with a grant? ☑Yes ☐No
>> If yes, list the title used on the grant application to assure proper notification of the
>> approval to the funding agency.

>> If no and you do not require verification of approval for an outside funding source, you
>> may list the fund source as "departmental." The duration dates for departmentally funded
>> projects must not exceed three years.

Funding Agency or Fund Source: NIH: Academic Research and Enhancement Award (AREA); and NIH (Center for Psychiatric Neuroscience Small Grant)
Grant or Project Title: Modeling Alcohol Addiction: Role of Mammalian Alkaloids
Grant or Project Duration (beginning and ending dates): 6/1/03 - 6/1/06
This application is (check one) ☑New ☐Addendum/Modification ☐Renewal (required every three years and must be reviewed by IACUC to ensure that all current federal guidelines are being met)

II. ASSURANCE STATEMENTS

• • • • • •

PRINCIPAL INVESTIGATOR

I certify that I have truthfully and completely described the use of animals for this project/grant and that I will notify the Institutional Animal Care and Research Advisory Committee in writing of any changes in this information prior to proceeding with the animal use. Furthermore, the activities I plan do not unnecessarily duplicate previous experiments.

As a Principal Investigator, I accept and will conform to all federal, state, and institutional laws or guidelines concerning: care and use of animals in research, teaching, or testing; efforts to minimize animal pain and distress; training of any research personnel or students handling animals as described herein; and consideration of alternatives to animal use in research.

_____   4-9-03
Principal Investigator's Signature   Date

• • • • • •

CO-INVESTIGATOR
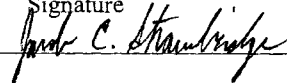
I understand that my name is listed on this project as a co-investigator. I have read this application and understand that only the described procedures are to be conducted.

            Name (**typed**)   Signature
1 Ken D. McMurtrey, Ph.D. _____ _____ 4/10/03

2 _____

• • • • • •

RESEARCH ASSOCIATE

I understand that my name is listed on this project as a research associate (this would include all graduate and/or undergraduate students involved in this project). I have read this application and understand that only the described procedures are to be conducted.

            Name (**typed**)   Signature
1 J. Chris Strawbridge _____ _____ C. Strawbridge 4/10/03

2 _____

3 _____

• • • • • •

DEPARTMENT CHAIR APPROVAL

I understand that responsibility for assessing the quality of animal research must be shared by both the department and the IACUC. My signature as Department Chair certifies that the proposed research has been reviewed and approved as having scientific merit.

_____   4-9-03
Signature of Department Chair   Date

III. NONTECHNICAL (LAY) SUMMARY OF PROJECT

The lay summary should be written in **non-scientific terms that can easily be understood by a layperson** and should include the following:

*   a description of the potential benefits of the project
*   an overview of the goals of the project
*   a statement that mentions all of the species to be used and their importance to the project

Please limit your description to the space provided in the box below.

Ingested ethanol is metabolized, to a large extent, by liver alcohol dehydrogenase to acetaldehyde, which can condense with dopamine to form salsolinol (SAL). Alterhnatively, dopaldehyde can condense with dopamine to form tetrahydropapaveroline (THP). another isoquinoline alkaloids. Patients with alcoholic parents had lowered (R)-SAL and (S)-SAL levels compared with family history negative alcoholics, suggesting genetic association of disturbance of SAL biosynthesis and alcoholism. In rats, intraventrentricular injections of SAL or THP have been shown to induce long-lasting preferences for alcohol solutions over water. However, the critical issue is not whether TIQ's can have behavioral effects, but whether endogenously formed TIQ's are produced in sufficient amounts to exert a significant influence on volitional alcohol consumption. Only one published report has attempted to address this issue. These results were consistent with the hypothesis that alcohol infusion can influence central formation of TIQ's.

The proposed experiments will evaluate levels of SAL or THP from the shell and core of the nucleus accumbens using in vivo microdialysis following oral infusion of ethanol (1.0, 1.5, 2.0 or 2.5 g ethanol/kg), delivered by gavage in a 5 mL volume. Sprague-Dawley rats will be used. To evaluate whether voluntary versus involuntary oral administration of ethanol influences extracellular SAL levels differentially, separate experiments will employ the 12-day "step-up" procedure in which animals are free to select an escalating concentration of ethanol or water. Briefly, three graduated 100 mL drinking tubes were placed in front of each animal's cage. One tube contained deionized water. A second tube was empty, and a third contained an alcohol solution in water that increased in ethanol concentration over the course of the 12 days. The concentration of ethanol (Everclear) was increased each day in the following manner: 2, 3, 4, 5, 7, 9, 11, 13, 15, 20, 25, and 30% ethanol by volume. Prior experiments in our laboratory and others have confirmed that most Sprague-Dawley rats avoid even low concentrations of ethanol in this paradigm. However, a smaller percentage of animals will voluntarily select even high concentrations of unsweetened ethanol (15-20%) over water. These alcohol-preferring animals can be used to evaluate the effects of voluntary ethanol consumption on SAL levels in the nucleus accumbens. The proposed experiments will shed light on the unresolved and controversial issue of whether endogenously formed TIQ alkaloids may be involved in the etiology and/or maintenance of an addiction to alcohol.

IV. JUSTIFICATION FOR USE OF ANIMALS

Type of animal (one per form)

	Amphibians		Birds		Fish		Mice
	Rabbits		Reptiles	✔	Rats		
	Other (please specify)						

Sex ___✔___ Male   _____ Female   _____ Either or mixed

Strain/species Sprague-Dawley rats (Harlan, Indainapolis, IN)

Size or Age 60 days of age

1. Why must you use animals in the proposed studies?

   The proposed set of experiments examine the hypothesis that endogenous THP formation in the brain may be a critical factor in the etiology of alcohol addiction. To answer this question directly, a microdiaylsis tube is inserted through a surgically implanted chronic, indwelling metal cannula. For obvious reasons, it is impossible to conduct this experimental procedure in humans. An in vivo model is necessary to ascertain whether or not THP formation occurs in the brain in sufficient quantities to influence further drinking behavior of the animal. The findings will be important from both a theoretical and clinical point of view. From the a clinical viewpoint, it would provide a potential target for development of new pharmacological approaches to treat alcoholism.

2. Was a literature search performed to ensure that there is no duplication with the work that is being proposed? ___✔___ Yes   _____ No
   If yes, then please provide details about the method used for the literature search (i.e. database used) including when the search was performed and what keywords were used.

   An Internet search using Pubmed was conducted in March 2003. The following keywords were used: tetrydropapaveroline, salsolinol, microdialsysis, isoquinoline alkaloids.

V. NUMBER OF ANIMALS TO BE USED PER YEAR

1. Please explain how you determined the number of animals needed to accomplish the proposed research. For experiments in which there will be statistical analysis of data collected from the animals, please briefly describe the type of analysis that will be performed and how that effected the number of animals to be used per group (or per time point) for each experiment or each series of similar experiments.

    Levels of THP will be assessed via microdialysis following either involuntary oral administration of ethanol or voluntary consumption of ethanol. The experimental procedure for involuntary oral administration of ethanol is as follows. Levels of THP will be assessed following oral administration of 1.0, 1.5, 2.0 or 2.5 g ethanol/kg, delivered by gavage in a 5 mL volume. Control animals will receive an equal volume of water by gavage. Power analyses were conducted utilizing GPOWER (Faul & Erdfelder, 1992) to determine the sample size for this experiment. Based on the power observed in previous experiment (Sallstrom et al., 1999), a strong effect size is expected. Current anlysis revealed that a total sample size of N = 55 (n = 11 per group) should be sufficient to observe significant differences between groups and achieve an anticipated power greater than 0.80.

    Another N = 55 (n = 11 per group) animals will be used to evaluate Salsolinol following oral administration of 1.0, 1.5, 2.0 or 2.5 g ethanol/kg, delivered by gavage in a 5 mL volume or an equal volume of the vehicle. The rationale for the number of animals is the same as for the THP experiments (above).

2. Write the number of animals to be used per procedure category for **each year** of the project in the table below. (If you write in the number of animals for one year only, the Committee will assume that number is adequate for the full duration of the project.) If an animal will be used for procedures of varying categories, write the number only in the category for the maximum level of pain/distress that the animal may experience. Please see the instruction sheet for examples of experimental use of animals and the category applicable for that research.

Category	Description of Procedure Category	Numbers of Animals per Year 1	2	3
1	Procedure will produce minimal, transient, or no pain/distress (e.g. minor injections or collections)			
2a	**Nonsurgical** procedures **will be** performed using anesthetics, analgesics or tranqulizers to alleviate possible pain/distress.			
2b	**Nonsurvival surgical** procedure **will be** performed using anesthetics, analgesics or tranquilizers to alleviate possible pain/distress.			
2c	**Survival surgical** procedures **will be** performed using anesthetics, analgesics or tranquilizers to alleviate possible pain/distress.	37	37	36
3	Procedures may produce pain/distress which **will not** be relived by anesthetics, analgesics, or tranquilizers,			

VI. EXPERIMENTAL PROCEDURES

In this section a technical description of the use of the animals in the proposed research to be conducted should be given. This section can be either written in a narrative description or drawn as a flow diagram (examples are given in the instructions that accompany this form). Either choice should include all technical details necessary for the committee to fully understand the use of the animals in the proposed research. The type of information that should be included is the number of animals to be used per procedure, the treatment and control groups, duration of the proposed research, care of the animals during the proposed research, and what will be done with the animals at the end of the research. Conciseness will be appreciated, but you may use additional pages if necessary.

Although rats are known to be relatively resistant to infections, subacute infections resulting in behavioral and physiological changes have been demonstrated (Bradfield et al., 1992). The risk of postoperative infection will be minimized by adhering to aseptic surgical practices (Cunliff-Beamer, 1993/4). Our facility and procedures have been reviewed by Tom Ricks, D.V.M. (see below). We have a lab area dedicated to stereotaxic surgical procedures which is equipped with mounted surgical lighting. An industrial steam pressure cleaner is used to wash animal cages / racks. Glassware is cleaned with Alconox / Liquiox. Quatricide PV brand germicide is also used for cage cleaning. Instruments, gauze pads, and cotton tip swabs will be initially sterilized in an autoclave (121 °C for 15 min) in Johnson Science Tower. When multiple surgeries are performed per day, instruments are washed with disinfectant and re-sterilized with a portable Hot Bead Sterilizer. Sterile surgical drapes may be used to cover the animal and also help to prevent hypothermia. A 60-W desk lamp will also be used to warm the animal while it is anesthetized. Lab coats and sterile gloves are worn by the surgeon. The immediate surgical area is sterilized using 70% ethyl alcohol. During surgery, instruments will be maintained in a solution of Zephiran chloride.

Surgical Procedures: For microdialysis experiments, animals will be anesthetized with ketamine/xylazine anesthesia (25:5 mg/kg body weight; IM). Atropine (0.3 ml of a 0.54 mg/ml) solution may also be administered if excessive secretions of the mouth and lung are present. Also, a 2-ounce rubber squeeze ball and tube will be used to aspirate mucous from the back of the tongue if breathing becomes labored. The animals head from behind the tips of the ears to the eyes and across the skull will be shaved with an electric razor prior to the incision. Chlorhexiderm Surgical Scrub will be used to clean the incision site. The incision will be about 2.5 cm in length and will be performed using sterile #10 scalpel. Following the initial incision, the periosteum is scraped to either side of the skull and the membrane is then clipped with bulldog clamps. After cleaning and drying of the skull with cotton swabs and saline, a pencil point is made at the designated coordinates. Both bregma and lambda reference points are used to verify the appropriate site for drilling. Following the implant of the cannula for microdialysis probe, the entire area is cleaned with Betadine or Chlorhexiderm. Dissolved silk suture (3.0) is used to sew the fascia and skin together around the area of the pedestal holding the cannula or probe.

The implementation of the guide cannula and the microdialysis probe into the nucleus accumbens will follow procedures for which there is an established precedent (Sallstrom et al., 1999). Under ketamine / xylazine anesthesia, a guide cannula will be implanted. Using the bregma reference point, stereotaxic coordinates will be anterior 1.7 mm, lateral 1.3 and ventral -6.5 mm (Paxinos and Watson, 1986). The rats will be placed individually in a macrolon cage (size 34 X 30 X 25 cm) and tethered to the swivel in a noise-insulated room with two cages present. After three days, a CMA 10 probe (2 mm dialysis membrane) will be inserted and perfused with artificial cerebrospinal fluid (aCSF: 137 mM Na+, 1.2 mM Ca2+, 2,4 mM K+, 144.2 mM Cl -, 1.2 mM Mg 2+, 0.9 mM NaH2PO4.H2O and 1.4 mM Na2HPO4.2H2O; pH 7.0) at a flow rate 1.0 •l/min, with sampling at intervals of 20 minutes for up to 200 minutes following oral gavage administration. There will be a 24 hour delay before collection of dialysate begins in order to minimize stress associated with implantation of the intracerebral dialysis probe. The first 5 samples determine basal levels of THP or salsolinol from CSF prior to ethanol administration.

Experimental Procedure: Levels of THP or salsolinol will be assessed following oral administration of ethanol. Levels of THP will be assessed following oral administration of 1.0, 1.5, 2.0 or 2.5 g ethanol/kg, delivered by gavage in a 5 mL volume. A 5 mL oral infusion of 20% ethanol by gavage would equate to a 2.63 g/kg dose for a typical 300 g rat. This amount has been shown to induce clear signs of intoxication (Cowen, Chen, Jarrott & Lawrence, 1998). Animals in the control group will receive oral infusion of an equal volume of water. Our dose range of ethanol is comparable to those evaluated by other investigators (Lucas & McMillan, 2002; Fadda, Mosca, Columba & Gessa, 1989).

Perfusion for Histology: After completion of the experiment, the position of the probe will be evaluated by fixing the brain in a formalin solution by cardiac perfusion. Animals will be deeply anesthetized with an IP injection of 60 mg/kg sodium pentobarbital. A lack of response to a paw pinch is necessary before the perfusion procedure begins. With the rat laying on its back, the paws are pinned to a dissecting tray at an angle so that the runoff of blood, saline, or fixative can be collected in the basin. Large scissors are used to cut through the abdominal wall (with the scissor tips pointed upward to avoid cutting organs. The incision is made about halfway between the animal's pelvic region and ribcage. A cut is made anteriorly until the sternum is reached and then cut laterally along either border of the ribs. The body wall is then pinned back so that the diaphragm is exposed. A cut is made along the border between the diaphragm and the ribs, exposing the heart and lungs. A cut is made on either side of the ribs as laterally as possible, forming a flap that can be lifted up. A hemostat is attached to the sternum, the ribs are lifted anteriorly, and the heart is exposed. The pericardium around the heart may be removed with a pair of blunt forceps. A cannula is inserted into a small incision in the left ventricle and another incision is made in the right atrium with fine-tip forcep to drain blood (exsanguination). When the fluid leaving the heart is clear (using about 150 ml of saline), the valve to the saline is shut off and the valve from the formalin container is opened up and 300-400 ml of formaline is slowly infused into the animal.

Bleeding Procedure: Blood alcohol level will be tested in a subset of animals treated with ethanol. Blood will be drawn by tail snip procedure in which an incision is made on the last 1 mm of tissue. Blood is drawn into heparinized capillary tubes and transferred into aliquots for storage at -80 degree F freezer.

VII. ANIMAL DISPOSITION

Based on the "Report of the AVMA Panel on Euthanasia 2000" the following methods of euthanasia are considered either acceptable or conditionally acceptable. Place a checkmark next to the procedure(s) that will be used to euthanize any animals for this project. The animals will be euthanized by the attending veterinarian, the animal facility supervisor, a laboratory animal technician, or a researcher who has received special permission from the attending veterinarian and the IACUC. If the researcher is desiring special permission to perform the euthanasia please indicate in the appropriate space below. You must provide scientific justification (including a detailed literature search) in order to obtain approval for a method not listed below. If the species you are working with is not listed below, please consult with the attending veterinarian for an acceptable method prior to submission of this form.

Species	Acceptable Method	Conditionally Acceptable Method
Amphibians	_____ Barbiturates _____ Inhalant anesthetics _____ CO_2 _____ Double pithing _____ TMS _____ MS 222 _____ Benzocaine hydrochloride	_____ Decapitation and pithing _____ Stunning and decapitation
Birds	_____ Barbiturates _____ Inhalant anesthetics _____ CO_2	_____ Cervical dislocation _____ Decapitation
Fish	_____ Barbiturates _____ 2-phenoxyethanol _____ CO_2 _____ TMS _____ MS 222 _____ Benzocaine hydrochloride	_____ Decapitation and pithing _____ Stunning and decapitation/pithing
Rabbits	_____ Barbiturates _____ Inhalant anesthetics _____ CO_2	
Reptiles	_____ Barbiturates _____ Inhalant anesthetics _____ CO_2	_____ Decapitation and pithing _____ Stunning and decapitation
Rodents	✔ Barbiturates _____ Inhalant anesthetics ✔ CO_2	_____ Decapitation _____ Cervical dislocation (in mice and rats (< 200 g))

VII. ANIMAL DISPOSITION (CONT.)

1. Species not listed above will need to be specified here along with the method of euthanasia to be employed and the signature of the attending veterinarian acknowledging that this method is acceptable.

    _____

    Signature of attending veterinarian   Date

2. Does the researcher desire permission to perform the euthanasia? ___✔___ Yes _____ No
    If yes, then please specify why you wish to perform the euthanasia yourself.

    To ensure that the microdialysis probes are localized within the nucleus accumbens, histological sectioning and staining of brain tissue is necessary. Cryosectioning, in turn, requires that the brain tissue be fixed using formalin solution. Thus, intracardial perfusions are necessary for these experiments.

3. Will you have tissues that may be made available to other investigators with approved, active protocols? Please note that the other investigator can have an approved, active protocol with either our institution or another institution. If the other investigator is at another institution a copy of their approved, active protocol will be necessary to have on file before the tissues can be released. _____ Yes __✔__ No

VIII. FACILITIES FOR ANIMAL USE

1. Where will the animals be housed?

    _____ Johnson Science Tower animal facility
    (rabbits must be maintained in this facility, as well as all animals involved in biohazardous research that aren't being conducted at the Toxicology laboratory at GCRL or animals involved in surgical procedures)

    __✔__ Owings-McQuagge animal facility
    (only rodents may be maintained in this facility)

    _____ Wet lab in Walker Science Building
    (only fish, reptiles, and amphibians may be maintained in this facility)

    _____ Toxicology laboratory at GCRL

    _____ Wet lab facilities at GCRL

    _____ Field (requires information to be provided in Appendix H)

    _____ other (specify)

2. Describe any special housing requirements that may be needed, especially if the animal will be exposed to hazardous materials.

3. Will animals be maintained outside the above stated facility overnight for periods longer than twelve hours? _____ Yes __✔__ No
    If yes, provide the following information:

    a.   Proposed location(s) where animals will be housed?_____

    b.   Estimated number of animals or cages to be housed at any given times?
    _____

    c.   Length of time animals will be housed? _____

    d.   Reason(s) why animals must be housed outside designated animal facilities? (Note: convenience is not adequate justification for housing outside of approved animal facilities)

IX. ANIMAL MEDICAL EMERGENCIES

In the table below, list the names of individuals who have authority to approve animal treatment or euthanasia. If the designated individual(s) cannot be reached, then the Animal Research Facility veterinarian will provide supportive care or euthanatize animals suffering unrelenting pain.

Name	Work Phone Number	Emergency Phone Number
John G. McCoy	266-4617	266-5197
Julie Rich	266-5617	297-8936

X. REQUIRED APPENDICES
(This page **MUST** be submitted with the application)

	Yes? ✔	No? ✔
A. ANTIBODY PRODUCTION Will animals be used for antibody production? If yes, then complete and attach Appendix A.		✔
B. NONSURGICAL PROCEDURES NOT INCLUDING ANTIBODY PRODUCTION Will animals be subjected to nonsurgical procedures (i.e. injections (not including those routinely used to administer euthanasia agents), radiographs, oral treatments, etc.? If yes, then complete and attach Appendix B.	✔	
C. BREEDING COLONIES Will pregnant animals be required or will a breeding colony be established specifically for this project? If yes, then complete and attach Appendix C.		✔
D. SURGICAL PROCEDURES Will animals be subjected to nonsurvival and/or survival surgical procedures? If yes, then complete and attach Appendix D.	✔	
E. PROLONGED PHYSICAL RESTRAINT OF UNANESTHETIZED ANIMALS Will unanesthetized animals be subjected to physical restraint for periods of longer than one hour? If yes, then complete and attach Appendix E.		✔
F. PROCEDURES INVOLVING PAIN/DISTRESS WITHOUT PAIN/DISTRESS MEDICATION Will animals be subjected to procedures that may produce pain/distress which will not be relieved by anaesthetics, analgesics, or tranquilizers? If yes, then complete and attach Appendix F.		✔
G. USE OF GENETICALLY MANIPULATED ANIMALS Will genetically manipulated animals be used? If yes, then complete and attach Appendix G.		✔
H. WILD ANIMAL AND/OR FIELD RESEARCH Will wild animals be used in this research and/or will wild animals be held in the field while research is being conducted on them? If yes, then complete and attach Appendix H.		✔
I. SAFETY Will living animals be exposed to any of the following: recombinant DNA, infectious agents, toxic chemicals, flammable or explosive materials, and/or carcinogens? If yes, then complete and attach Appendix I.		✔

NONSURGICAL PROCEDURES NOT INCLUDING ANTIBODY PRODUCTION

1. Place a check by the procedures that will be used in this application.

☑Oral treatments   ☑Injections (IV, SQ, IM, etc.)   ☑Blood collections
☐Topical applications   ☐Non-surgical catheter use   ☐Fluid collections
☐Radiographs   ☐Intranasal administrations   ☑Behavioral studies
☐Exercise studies   ☐Abnormal noise levels   ☐E.K.G.
☐Environmental O_2 variations   ☐Abnormal light levels or cycles   ☐Ocular exams
☐Blood pressure monitoring   ☐Food or fluid restrictions   ☐Irradiation
☐Exposure to abnormal temperature ☐Other (describe)

2. Will the use of paralytics be required for these procedures?
☐Yes   ☑No
(Paralyzing drugs must be used only while the animals are fully anesthetized/sedated. If paralytics will be used, blood pressure and heart rate must be monitored. Describe the methods that will be used to monitor for possible elevations in blood pressure and heart rate to ensure that adequate levels of anesthesia/sedation are maintained while the animals are paralyzed).

Will the use of anesthetics be required for these procedures? ☑Yes   ☐No
List the anesthetic agent(s) that will be used.

Agent	Dose (mg/kg body wt)	Route	Frequency
ketamine/xylazine anesthesia	25:5 mg/kg body weight	IM	Once prior to surgery

Signature of the appropriate animal facility supervisor and/or attending veterinarian

3. Briefly describe the technique to be used for the procedure(s). Include in your description the frequency and the maximum number of procedures to be conducted per animal.

An intramuscular injection of ketamine / xylazine anesthesia (25:5 mg/kg BW) will be given prior stereotaxic surgery (one cannula implant per animal). This anesthetic takes full effect within about 20 minutes. The surgical stage of anesthesia is characterized by reduced muscle tone, no spontaneous movement, no reaction to painful stimuli (paw or tail pinch) and lack of eyeblink when eyelid is touched. Respiration should be regular. Because ketamine prevents the eyelids from closing, an eye lubricant (Paralube Vet ointment) is applied.

SURGICAL PROCEDURES

An investigator planning on performing surgical procedures must submit documented proof of formal training with this application. The training must have occurred within the last five years. Even with documentation it will still be necessary for all initial surgical procedures to be performed under the supervision of the attending veterinarian or an approved substitute. The following questions should be filled out with the assistance of the animal facility supervisor and/or the attending veterinarian. Questions 4 through 6 and 11 require the signature of the animal facility supervisor and/or the attending veterinarian prior to submission of this application.

1. Will nonsurvival surgical procedures be conducted for this application?
    ☐Yes   ☑No
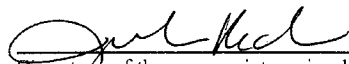
2. Will survival surgical procedures be conducted for this application? If animals will recover from anesthesia for any time following surgery, it must be considered as a survival procedure.
    ☑Yes   ☐No

3. Which of the following parameters will be used to determine the pre-operative health status?
    ☑Activity level   ☐Heart rate
    ☑General physical condition   ☑Respiratory rate
    ☐Body temperature   ☑Body weight
    ☐Blood chemistries   ☐Other (specify)

4. Please specify when, prior to surgery, food and fluids will be withheld.
    Food will be withheld the evening prior to surgery. Water will be available ad lib.

5. Will pre-operative, medications (i.e. antibiotics, anticholinergics, tranquilizers, etc.) be used?
    ☐Yes (list them below)   ☑No

Medication	Dose (mg/kg body wt)	Route	Frequency

_____
Signature of the appropriate animal facility supervisor and/or attending veterinarian

6. List the anesthetic agent(s) that will be used.

Agent	Dose (mg/kg body wt)	Route	Frequency
ketamine/xylazine anesthesia	25:5 mg/kg body weight	IM	one or two (40% supplemental)

Signature of the appropriate animal facility supervisor and/or attending veterinarian
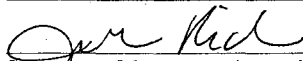
7. Will paralytic agents be used? ☐ Yes (list them below)   ☑ No
(Paralyzing drugs must be used only while the animals are fully anesthetized/sedated. If paralytics will be used, blood pressure and heart rate must be monitored. Describe the methods that will be used to monitor for possible elevations in blood pressure and heart rate to ensure that adequate levels of anesthesia/sedation are maintained while the animals are paralyzed).

Agent	Dose (mg/kg body wt)	Route	Frequency

Signature of the appropriate animal facility supervisor and/or attending veterinarian

8. For each surgical procedure that is to be conducted, address the following:
   a. List the site(s) that will be used for the incision(s).

   The incision will be about 2.5 cm in length along the midline of the scalp from a point between the ears and proceeding anteriorly.

b.   Describe how the surgical site(s) will be prepared.

The animals head from behind the tips of the ears to the eyes and across the skull will be shaved with an electric razor prior to the incision. Chlorhexiderm Surgical Scrub will be used to clean the incision site. The incision will be performed using a sterile #10 scalpel. Following the initial incision, the periosteum is scraped to either side of the skull and the membrane is then clipped with bulldog clamps.

c.   For all survival procedures, describe the type(s) of closure materials that will be used (i.e. clips, types of suture materials, etc.).

3.0 Surgical silk suture material is used.

d.   For survival procedures conducted on non-rodent mammals, list the basic suture patterns to be used (i.e. continuous, simple interrupted, mattress, etc.) for underlying tissues and skin.

A simple interrupted suture pattern will be used. Usually one or two sutures anterior to the cement pedestal and one or two sutures posterior to the pedestal are sufficient.

e.   Provide a brief description of the nonsurvival and/or survival surgical procedures.

After cleaning and drying of the skull with cotton swabs and saline, a small hole is drilled in the calvarium where the guide cannula is to be lowered. Three additional half-holes are drilled in a triangular region around the cannula hole. Anchor screws are inserted to a depth of 1 mm to provide support for the cannula pedestal. The surface of the skull is cleaned with saline and cotten swabs. A think paste of dental acrylic (methacrylate and powder) is prepared in a watchglass. A think metal spatula is used to apply the dental acrylic to the skull and anchor screws. Successive layers of acrylic are applied until the anchor screw heads are completely covered. Following the implant of the guide cannula, the entire area is cleaned with Betadine or Chlorhexiderm. Silk suture (3.0) is used to sew the fascia and skin together around the area of the pedestal holding the cannula or probe. Following surgery, animals are placed on a warming pad.

f.   If nonsurvival procedures (animals will not be allowed to regain consciousness) will be conducted, explain how long the animals will be maintained under anesthesia prior to euthanasia.

9.   Will any animal be subjected to multiple, major survival surgical procedures? ☐Yes   ☑No
     If yes, answer questions a-c below.
     a.   Are the surgeries related components of the project? Explain how they are related and why they
          are a scientific necessity.

     b.   How many surgeries in addition to the primary surgery will be conducted per animal?

     c.   How long will animals be allowed to recover between surgeries?

10. During anesthesia what methods or parameters will be used to monitor the animal?

     To prevent hypothermia, an insulated pad will be placed beneath the animal and a heating pad will be used post-surgery. Normal rectal
     temperature should be 37.5 degrees C. Labored breathing in an anesthetized animal be produced by excess mucous that needs to be
     cleared from the mouth. Placing the animal on a 35 degree incline (nose down) will allow fluids to drain from the respiratory tract. A 2
     ounce rubber squeeze ball and tube may be used to clar mucous from the mouth. Gasping and wheezing indicates the need for aspiration.
     Aspiration is accomplished by inserting a rubber tube connected to a 5 cc syringe about 3 cm into the throat. Repeated aspiration,
     however, should be avoided since it only produces more mucous. A rat that feels no pain breaths deeply and regularly throughout the
     surgery. Shallow or irregular breathing may indicate either that the animal is too lightly anesthetized or conversely, it may indicate an
     anesthetic overdose. Pedal and corneal reflexes will be tested throughout surgery to assess level of anesthesia. Twitching and leg
     movement also identify an animal that is too lightly anesthetized. A supplemental (40% of initial injection volume) dose may be
     administered. If an animal is overanesthetized, the surgery should be stopped and breathing may be assisted rubber tube.

11. Animals that will be allowed to regain consciousness following surgery must be closely monitored until
     they regain the ability to control their head movement and maintain sternal recumbency. From the list
     below, check the parameters that you will use to monitor the animals' recovery from anesthesia.

     ☑Body Temperature   ☐Heart Rate   ☑Respiratory Rate
     ☐Palpebral Reflex   ☐Swallow Reflex   ☑Response to External Stimuli
     ☑Muscle Control   ☐Mucous Membrane Re-fill Times ☐Blood Pressure
     ☐Other (specify)

12. The postoperative period is considered at an end when the skin sutures are removed or the wound is healed.

    a.   How frequently will animals be monitored during this period?

        Body weight, water consumption and appearance will be monitored daily. Fluids (1-cc of saline) may be given SC.

    b.   Describe any anticipated, clinically significant, adverse effects that may result from the surgical manipulation and the care that will be provided should they occur.

        Signs of postsurgical distress include a hunched back, failure to groom, reduced body weight and food intake, and extreme vocalization in response to handling. If any of these signs are observed, Dr. Ricks will be consulted. The analgesic listed below has been recommended due to its relatively long duration of action (8-12 hours).

    c.   From the list below, check the parameters that will be used to determine the presence of postoperative pain/distress.

☑Body Weight   ☑Appearance   ☑Response to External Stimuli
☑Respiratory Rate   ☐Heart Rate   ☑Unprovoked behavior
☐Body Temperature   ☑Body Posture   ☐Other Clinical Signs (explain)

13. Will postoperative antibiotics and/or analgesics be given?
    ☑Yes (list them below)   ☐No

Medication	Dose (mg/kg body wt)	Route	Frequency
Buprenorphine	0.02-0.05 mg/kg	SC	As prescribed by veterinarian

Signature of the appropriate animal facility supervisor and/or attending veterinarian

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