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Evaluation of Cannabinoid Receptor Interaction of Anandamide, the Endogenous Cannabinoid Receptor Ligand

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication

I dedicate this dissertation to Charles Ellis Adams, my husband. His devotion, encouragement and patience never wavered throughout the arduous period of my graduate education. I owe the completion of this doctorate solely to his steadfast support.

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I would like to express my sincere gratitude to the following people:

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List of Abbreviations

ACh	acetylcholine
ACTH	adrenocorticotropic hormone
AIDS	acquired immune deficiency syndrome
AAI	aminoalkylindole
anandamide, 22:4, n-6	docosatetraenylethanolamide
anandamide, 20:3, n-6	homo-γ-linolenylethanolamide
ATP	adenosine 5'-triphosphate
B_{max}	maximum binding
BSA	bovine serum albumin
℃	degrees centigrade
cAMP	cyclic adenosine 3',5'-monophosphate
CB_1	central cannabinoid receptor
CB_2	peripheral cannabinoid receptor
CBD	cannabidiol
CBF	cerebral blood flow
CBN	cannabinol
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
Ci	curie
CNS	central nervous system
CoA	coenzyme A

compound 1	*cis*-11-eicosanoyl-(2'-hydroxyethyl)amide
compound 2	all-*cis*-eicosapentenoyl-(2'-hydroxyethyl)amide
compound 3	eicosanoyl-(2'-hydroxyethyl)amide
compound 4	eicosanoyl-(2'-fluoroethyl)amide
compound 5	arachidonyl-(3'-hydroxypropyl)amide
compound 6	arachidonyl-(5'-hydroxypentyl)amide
compound 7	arachidonylsulfonamide
compound 8	arachidonyl-(4'-bromobenzenesulfon)amide
compound 9	arachidonylmorpholine
compound 10	arachidonyl-(2'-(4-benzenesulfonamide)ethyl)amide
compound 11	arachidonyl-(2'-phenoxyethyl)amide
compound 12	arachidonyl-(2'-fluoroethyl)amide
compound 13	arachidonyl-(2'-methoxyethyl)amide
compound 14	arachidonyl-(1'-methyl-2'-hydroxyethyl)amide
compound 15	2-methylarachidonyl-(2'-fluoroethyl)amide
compound 16	2,2-dimethylarachidonyl-(2'-hydroxyethyl)amide
compound 17	2-methylarachidonyl-(2'-hydroxyethyl)amide
compound 18	2-isopropylarachidonyl-(2'-hydroxyethyl)amide
compound 19	2-ethylarachidonyl-(2'-hydroxyethyl)amide
compound 20	arachidonyl-N-methyl-(2'-hydroxyethyl)amide
compound 21	2,N-dimethylarachidonyl-(2'-hydroxyethyl)amide
compound 22	R-arachidonyl-2'-(1'-hydroxy-4'-methyl)pentylamide
compound 23	S-arachidonyl-2'-(1'-hydroxy-4'-methyl)pentylamide
CRF	corticotropin-releasing factor

DA	dopamine
EDTA	ethylene diamine tetraacetic acid
ED_{50}	effective dose for 50% of the population
EEG	electroencephalogram
FDA	Food and Drug Administration
g	gram
$GABA_A$	γ-aminobutyric acid receptor (A subtype)
$GABA_B$	γ-aminobutyric acid receptor (B subtype)
G_i	inhibitory guanine nucleotide-binding protein
G protein	guanine nucleotide-binding protein
GTP	guanosine-5'-triphosphate
^3H	tritium
H_2O	water
hr	hour
IC_{50}	inhibitory concentration displacing 50% of the ligand
i.c.v.	intracerebroventricular
Ig	immunoglobulin
in	inch
IL	interleukin
IP_3	inositol triphosphate
i.p.	intraperitoneal
i.v.	intravenous
K_d	dissociation constant
K_i	inhibition constant
kg	kilogram
MAP	mitogen-activated protein

MeOH	methanol
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mmol	millimole
mM	millimolar
%MPE	percent maximal possible effect
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NE	norepinephrine
ng	nanogram
NIH	National Institute of Health
nM	nanomolar
11-OH-Δ^9-THC-DMH	11-hydroxy-Δ^9-tertrahydrocannabinol-dimethylheptyl
11-OH-THC	11-hydroxy-tetrahydrocannabinol
nor-BNI	*nor*-binaltorphimine
PCP	phencyclidine
PEI	polyethylenimine
PIP_2	phosphatidylinositol-bisphosphate
pM	picomolar
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
PG	prostaglandin

r	correlation coeficient
RNA	ribonucleic acid
SA	spontaneous activity
SAR	structure-activity relationship
S.E.	standard error
sec	second
TF	tail-flick
Δ^9-THC	Δ^9-tetrahydrocannabinol
Δ^8-THC	Δ^8-tetrahydrocannabinol
THC	tetrahydrocannabinol
THCCOOH	11-*nor*-carboxy-Δ^9-tetrahydrocannabinol
TNF	tumor necrosis factor
Tris HCl	2-amino-2-(hydroxymetyl)-1,3-propanediol hydrogen chloride
U.S.	United States of America
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
µmol	micromole

Abstract

EVALUATION OF CANNABINOID RECEPTOR INTERACTION OF ANANDAMIDE, AN ENDOGENOUS CANNABINOID RECEPTOR LIGAND

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 1996

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Recent evidence implicates anandamide as the endogenous ligand for the cannabinoid receptor. One purpose of this study was to determine the structural requirements for anandamide's receptor interaction and the influence of phenylmethylsulfonyl fluoride (PMSF), an enzyme inhibitor, on receptor affinity. A second objective was evaluation of the correlation between affinities of the analogs and *in vivo* pharmacological activities. The ability of anandamide and analogs to displace [^{3}H]-CP 55,940 was determined by a filtration assay. Displacement curves for anandamide in the presence of PMSF produced a K_i of 89 ± 10 nM; without PMSF the K_i increased to 5400 ± 1600 nM. Anandamide analogs were evaluated for their ability to produce antinociception and hypomotility. The levels of saturation and substituents for the ethanolamide and hydroxyl groups of the anandamide structure were critical to receptor

affinity and *in vivo* potency. Increasing the length of the N-substituent by one or two carbons decreased receptor binding affinity. Methylations at carbons 2 and 1' produced compounds stable in the absence of PMSF. Addition of larger alkyl groups at these positions or nitrogen methylation reduced receptor affinity and behavioral potency. These results indicate that methylations at specific carbons of anandamide confer stability *in vitro*. A final objective was to characterize anandamide's binding to the cannabinoid receptor in the CNS. Anandamide's receptor binding affinities and binding densities, as determined from autoradiographic experiments in rat brain, from selected brain areas were compared to the receptor binding densities and patterns of two other compounds, CP 55,940 and SR 147116A, that bind to the central cannabinoid receptor. The lack of difference between receptor affinity, receptor distribution and parallelism of the displacement curves indicate that anandamide, SR 141716A and CP 55,940 are binding to the same receptor in the same manner.

I. General Introduction

Although used for centuries for both medicinal and recreational purposes, no other drug of abuse, as defined by the United States Controlled Substances Act, arouses greater controversy than cannabis. Cannabis use is widespread throughout the world; in fact, it is the most prevalently used drug in many countries. Despite efforts to curtail its use in the United States, cannabis remains one of the most commonly abused drugs, ranking only behind the consumption of alcohol and cigarettes. According to the National Institute on Drug Abuse 1995 National Household Survey on Drug Abuse, approximately 59% of adults in the United States between the ages of 26 and 34 have used cannabis in their lifetime. Importantly, 2-3% of the population in the United States consume cannabis on a daily basis. Public debate centers upon the possible legalization of cannabis for certain therapeutic uses, such as glaucoma treatment, appetite stimulation in AIDS patients and suppressing nausea resulting from chemotherapy. By the early 1980s extensive research had provided information concerning the identification of cannabinoids in the plant and the physiochemical and biochemical properties of these compounds. Numerous breakthroughs in the past few years have greatly increased our understanding of cannabinoids. The history, chemistry, pharmacology and toxicology of cannabis in both animals and humans is extensive. Furthermore, the complex and often ambiguous health consequences and clinical utility of cannabinoids continue to be of vital concern to health care providers. Recent advances, such as the characterization and cloning of a specific cannabinoid receptor, identification of a second messenger system and isolation of an endogenous ligand provide insight for the direction of future research for this fascinating drug.

History and Prevalence of Use

References to the use of the plant *Cannabis sativa*, also known as Indian hemp, date back over twelve thousand years (Abel, 1979). Cannabis has long been used as a medicine in China, India, the Middle East, South Africa and South America. Egyptian, Chinese (2700 B.C.) and Assyrian (800 B.C.) sources indicate that it is one of the oldest drugs in history (Mechoulam and Feigenbaum, 1987). The earliest reference to the medicinal properties of cannabis dates back to 2700 B.C. (Grinspoon and Bakalar, 1993). The Chinese emperor Shen-Nung described cannabis in a book that later became the standard Chinese compendium of medicines. The Chinese used cannabis for treatment of constipation, malaria, rheumatic pains and female disorders. The euphoric properties of cannabis were discovered in India between 2000 and 1400 B.C., and cannabis was recommended medicinally for reducing fevers, producing sleep, stimulating the appetite, relieving headaches and curing venereal diseases (Mechoulam and Feigenbaum, 1987). In addition to its purported medicinal properties, the ancient Chinese and Greeks used cannabis to make ropes and clothes. Romans were also aware of the strength of cannabis rope and used it in naval construction. The plant was cultivated for its fiber early in American history at Jamestown, Virginia in 1611 (Grinspoon and Bakalar, 1993). Even today, interest continues in the hemp plant for the purpose of making clothes.

Cannabis was introduced into Western medicine several millennia later following the publication of a treatise in 1839 by W. B. O'Shaughnessy, a 30-year-old Irish physician serving in the British army in India (Lemberger, 1984; Snyder, 1971). He carefully reviewed literature on the uses of cannabis in Indian medicine that spanned nine hundred years and concluded that cannabis was a very safe drug. To further confirm the safety of cannabis, he conducted a series of experiments in animals to determine its effects and dosage limits (Snyder, 1971). He found that cannabis was not harmful in animals, and even high doses did not kill mice, rats or rabbits. He administered cannabis to patients

suffering from seizures, tetanus, rabies and rheumatism and recorded success, though side-effects, such as total catalepsy, sometimes occurred. He noted the anticonvulsive, analgesic, antianxiety and antiemetic properties of the drug. The reports of O'Shaughnessy made cannabis an acceptable form of medicine in England and other European countries (Mechoulam and Feigenbaum, 1987). At the turn of the twentieth century, the medicinal use of cannabis waned in the United States and Europe due to the development of synthetic medicines.

The rising fear of cannabis use in the United States began in the 1920s, and the use of cannabis was abolished in the United States in 1937 with the enaction of the Marijuana Tax Act (Musto, 1987). The Mexican term marijuana refers to cannabis leaves or other crude plant material. Despite legal measures in the United States, cannabis still became a major drug of abuse in the late 1960s, with peak usage occurring in the late 1970s and early 1980s. A United States Bureau of Census report in 1971 indicated that 40% of individuals between 18-25 years of age had experimented with cannabis, and 18% from the same age group currently used the drug. Drug usage data was also obtained in the United States with the National Household Survey on Drug Abuse and the Monitoring the Future Survey, which began collecting information in 1975 from young adults, college age students, twelfth grade students in public and private schools. Eighth and tenth graders were added in 1991. According to the Monitoring the Future Survey, 1979 was the peak year of use with 60.4% of twelfth graders having tried cannabis in their lifetime, and 50.8% of high school seniors in 1979 had used cannabis in the past year. In 1978, 37.1% of twelfth graders surveyed had used cannabis within 30 days, and 10.7% used cannabis on a daily basis. Following these peak years, cannabis use began a slow, but continuous decline, with the lowest levels of annual use occurring in 1992. In 1992, 21.9% of twelfth graders had used cannabis in the past twelve months, and 1.9% used the drug on a daily basis. The decline in use was linked to an increase in perceived risk and personal disapproval of

drugs (Hall, Johnston and Donnelly, in press). Surveys since 1992 indicated significant increases in all use categories (Johnston, O'Malley and Bachman, 1995). From 1992 to 1994, lifetime use in twelfth graders increased 5.6%; annual use increased 8.8%; 30-day use increased 7.1%, and daily use increased 1.7%. Although the current levels of cannabis use in the United States are still much lower than the peak periods, the recent increases represent an early warning that cannabis popularity could continue to increase, especially with the high school population. The recent upturn has been attributed to a decline in social disapproval of cannabis and in perceived risk, lower public attention to cannabis and an increase in prodrug messages in popular culture (Hall, Johnston and Donnelly, in press). The 1994 Monitoring the Future Survey reports that "perceived harmfulness" of cannabis experimentation for all age groups decreased. When asked in 1994 if "great risk" would result if cannabis was "smoked regularly", 65% of twelfth graders reported affirmatively. This response represents a 7.5% decrease from 1993. There was also an increase in participants who found that obtaining cannabis was "fairly or very easy." The increases in cannabis use, decline of perceived harmfulness and decline in social disapproval demonstrate an erosion of the anti-drug attitude in the United States (Hall, Johnston and Donnelly, in press).

Epidemiological evidence is also available from other countries, and these studies are reviewed by Hall *et al.* (in press). In Canada several school studies have shown similar trends to the United States, with a rise in use in the 1970s followed by a decline throughout the 1980s. However, the rates of illicit drug use were lower than in the United States. A national telephone survey reported that 23% of those sampled had ever tried cannabis (Hall, Johnston and Donnelly, in press). Cannabis is the most commonly used illicit drug in Australia. A 1993 national survey of adults demonstrated that one-third had tried cannabis. Large increases in use between 1988 and 1992, especially in males, were reported in the Netherlands from a national survey of students aged 10 to 18.

Although cannabis is used throughout the world, limited survey data are available in other parts of the world. Often these data provides only a crude indication of use. Survey methods are not reported, results are presented in a summary format and the levels of use of teenagers is often underreported. Nevertheless, these surveys do give an indication of overall levels of use. Limited data are available from Africa (Hall, Johnston and Donnelly, in press). A survey of 5000 workers reported a prevalence of 11.5% for having used cannabis. In a Moroccan survey from Tangier, two-thirds of 500 students reported trying cannabis. Reported rates of cannabis use in South American countries is lower than in Western countries, including the United States, Canada, Europe and Australia (Hall, Johnston and Donnelly, in press). In Brazil, two school-based surveys conducted in 1987 and 1989 demonstrated that 2.9% in 1987 and 3.4% in 1989 had used cannabis. Similar results were found in a 1992 National Household Survey on Drug Abuse in Colombia. Among 18- to 24-year-olds, 1.5% had used cannabis in the past year. In a survey conducted in Athens, Greece, which was based upon the substance use and attitudes sections of similar questionnaires of the World Health Organization and the United Nations, cannabis or hashish was the most frequently used illicit drug (Kokkevi, 1994). The highest lifetime rate of cannabis use was found in 25- to 35-year-old males (27.9%). A compilation of limited studies conducted in various hospitals in Lebanon indicated that hashish smoking is common, especially in rural areas where it often used daily (Hachem, 1994). One street study found 142 out of 198 participants were hashish users (Hachem, 1994). In Mexico, cannabis has been the most reported illicit drug of initiation in the past three years (Tapia-Conyer *et al.*, 1994). India has a long tradition of cannabis use associated with religious ceremonies. Yet, only very limited surveys are available. Surveys conducted in three Northern Indian states between 1989 and 1991 found a lifetime prevalence rate of 3% and current use rate of 1% (Hall, Johnston and Donnelly, in press). In Southern India a lifetime prevalence rate of 7% has been reported. Higher prevalence

rates of 10% to 27% exist among students. The limited data on cannabis use in African, Asian, Central and South American and Middle Eastern countries suggest that these countries have lower rates of lifetime cannabis exposure than many Western countries. Before definite conclusions are drawn, more complete and standardized surveys need to be conducted. However, it is clear that cannabis is used throughout the world, albeit at varying degrees.

The Cannabis Plant

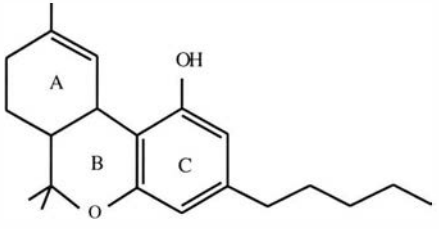
The flowering tops and leaves of the plant *Cannabis sativa,* subspecies indica, secrete a resin containing psychoactive compounds called cannabinoids. The highest concentration of cannabinoids in the plant is found in the flowering tops followed by the leaves. Small amounts of cannabinoids are found in the stem and roots, and none in the seeds. The cannabinoid content of the plant varies widely depending upon the climate, soil, cultivation and type of plant. The plant is cut, dried and incorporated into cigarettes with or without tobacco. Three types of plant preparations are used, as identified by the Indian names bhang, ganja and charas (Grinspoon and Bakalar, 1993). Bhang is made from dried leaves and tops of uncultivated plants and contains a low resin content. Ganja is obtained from the leaves and tops of cultivated plants and has a higher resin content. The first two preparations are referred to as marijuana. Charas, also known as hashish, is prepared from the resin itself and is 5 to 10 times more potent than marijuana. Plant products are also chewed, smoked in a waterpipe or eaten in baked goods.

A concern exists that the problem of elevated cannabis use may be compounded by recent increasing concentrations of Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary psychoactive constituent in the plant, found in confiscated cannabis. During the late 1960s, the average level of Δ^9-THC content was 1.5%. The levels steadily increased to the early 1980s when concentrations had doubled to 3.0-3.5% (ElSohly and Ross, 1994). Seized

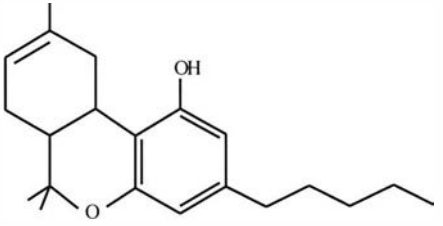
samples composed primarily of buds and sinsemilla (unfertilized flowering tops from the female *Cannabis* plant) contain much higher concentrations of Δ^9-THC. In fact, samples of cannabis sometimes contain concentrations of Δ^9-THC as high as 20%. Emphasis upon genetic experimentation and cross-breeding in recent years and developments in indoor hydroponic cultivation techniques have contributed to higher THC content in cannabis plants (Clarke, 1981). These efforts have enhanced THC levels in Dutch hemp ("Netherweed") to concentrations averaging 20% (Hall, Johnston and Donnelly, in press). One may argue that the elevation in levels of Δ^9-THC has not contributed to cannabis use since there was a decline in use during the time when levels had increased and then stabilized. On the other hand, if highly potent cannabis becomes readily available, the patterns in cannabis use could be affected.

Chemistry

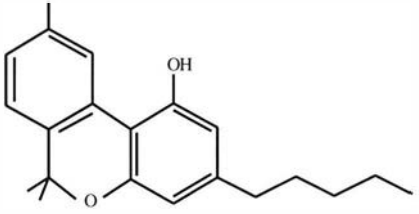
The cannabis plant contains over 400 chemical compounds. Approximately 60 of these compounds are cannabinoids. The term cannabinoid refers to the C21-compounds present in the plant and includes their transformation products and related analogs. The elucidation of the principal psychoactive constituents facilitated the ease of studying the pharmacological and behavioral effects of cannabis' specific constituents. Marijuana contains over 60 compounds which are structurally related cannabinoids. Four compounds important in terms of cannabinoid pharmacology are cannabinol (CBN), cannabidiol (CBD), (-)-Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC) and Δ^8-THC (Figure 1). Cannabinol was the first cannabinoid to be isolated and purified (Wood, Spivey and Easterfield, 1899), but its structure was not determined until later (Adams, Baker and Wearn, 1940). Also, cannabidiol was isolated and purified (Adams, Hunt and Clark, 1940), and its structure was later identified (Mechoulam and Shvo, 1963). The isolation of cannabinol and cannabidiol in the 1940s provided the general structure of the active principle of cannabis,



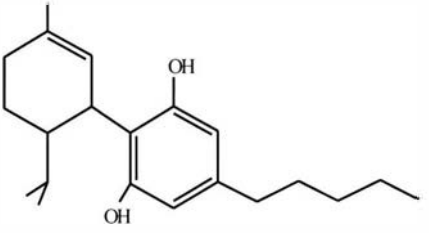
Δ^9-THC



Δ^8-THC



Cannabinol



Cannabidiol

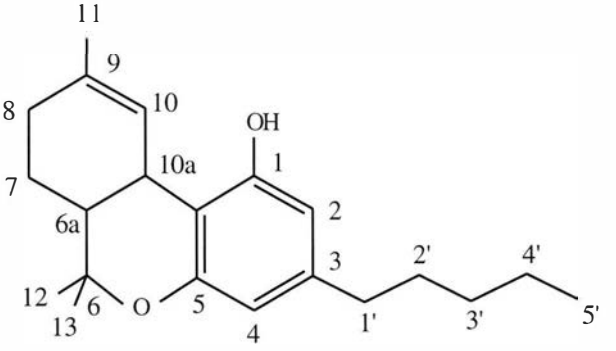
Figure 1. Structures of naturally occurring cannabinoids.

but CBN had little and CBD had no psychotomimetic activity (Adams, Baker and Wearn, 1940; Adams, Hunt and Clark, 1940). Mechoulam and his colleagues in the 1960s first isolated Δ^9-THC, later found primarily responsible for the psychoactive properties of the plant (Gaoni and Mechoulam, 1964). The synthesis of racemic (±)-Δ^9-THC was described in 1965 (Mechoulam and Gaoni, 1965). A second psychotomimetic compound was also identified as Δ^8-THC, a positional isomer of Δ^9-THC (Hively, Mosher and Hoffmann, 1966). The pharmacological profiles for the two components are similar, with Δ^9-THC possessing somewhat greater potency.

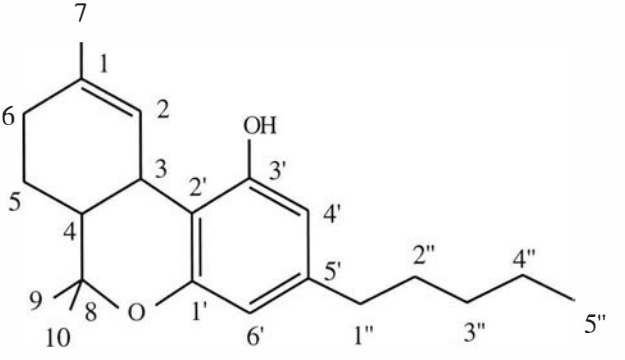
Two numbering systems exist for cannabinoids: the dibenzopyran and monoterpenoid numbering systems. The former is recognized in *Chemical Abstracts* and will be used when referring to both naturally occurring and synthetic cannabinoids (Figure 2). The formation of salts of naturally occurring cannabinoids is not possible since these compounds lack either a basic or acidic functional group. As a result, Δ^9-THC is a noncrystalline, waxy-liquid substance at room temperature. Δ^9-THC is unstable and loses potency upon storage. The main decomposition product of Δ^9-THC is CBN. The high degree of lipophilicity and instability of cannabinoids complicates the preparing and handling of cannabinoid solutions.

Structure-Activity Relationship Studies of Cannabinoids

Efforts were undertaken to synthesize and evaluate cannabinoid analogs for the purpose of separating desired pharmacological effects from adverse effects and for the elucidation of the biochemical and molecular mechanisms of cannabinoid action. Initially, due to the lipophilic nature of Δ^9-THC and the central depressant effects, cannabinoids were thought to mediate their actions through disruption of membrane ordering, similar to the mechanism of action of general anesthetics (Lawrence and Gill, 1975; Paton and Pertwee, 1972). Extensive structure-activity relationship (SAR) studies of cannabinoid

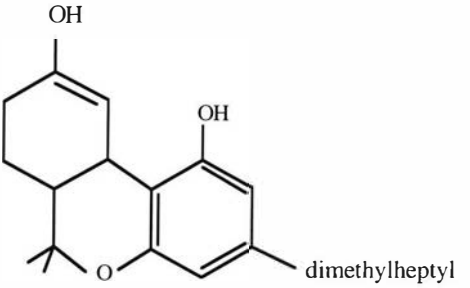


Dibenzopyran numbering

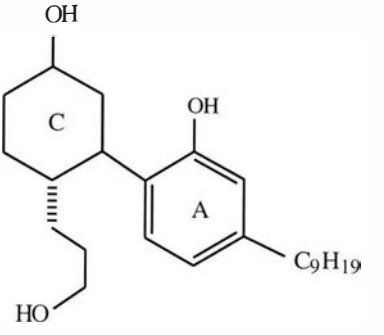


Monoterpene numbering

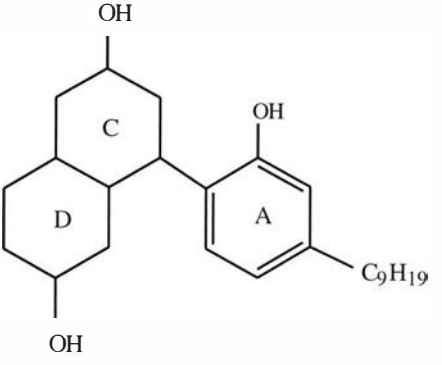
Figure 2. Cannabinoid numbering systems.



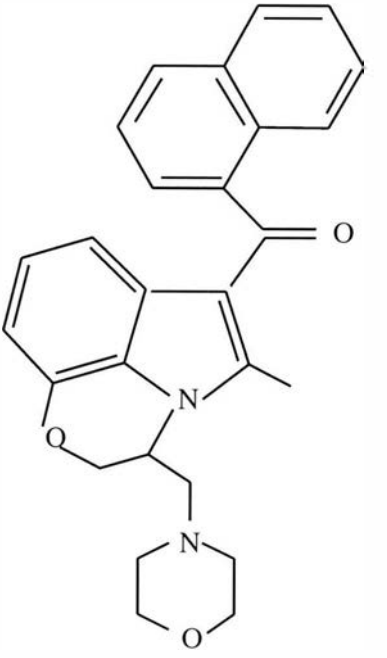
11-OH-Δ^9-THC-dimethylheptyl



CP 55,940



CP 55,244



WIN 55,212

Figure 3. Structures of 11-OH-Δ^9-THC-DMH, CP 55,940, CP 55,244 and WIN 55,212.

analogs revealed strict structural requirements for pharmacological activity and provided early evidence for a specific cannabinoid receptor. The first systematic SAR study in cannabinoids was conducted by Adams (1942) in the 1940s, and this work has been substantially supplemented with the pharmacological characterization of a wide variety of natural and synthetic cannabinoids, as reviewed by Razdan (1986). SAR studies are based primarily on animal behavioral paradigms such as dog static ataxia, drug discrimination and other specific mouse tests. While correlation does exist between potency in the dog static-ataxia test and the psychoactive component of cannabinoids in humans (Razdan, 1986), extrapolation from animal models to human marijuana intoxication is still problematic. However, Balster and Prescott (1992) did describe a good correlation between human data and drug discrimination in animals for approximately 20 cannabinoid compounds.

Chemically distinct subclasses of cannabinoid analogs exist with greater potency than Δ^9-THC. Potent compounds have resulted from numerous structural alterations made to the basic template of Δ^9-THC. Changes in side chain length or branching of the side chain at the C1' or C2' position (Figure 2) dramatically affects potency. Substitution of the pentyl group with a dimethylheptyl side chain and hydroxylation at carbon 11 of Δ^8-THC resulted in 11-OH-Δ^8-THC-DMH, a compound several hundred times more potent than Δ^8-THC in behavioral assays (Little *et al.*, 1989; Mechoulam *et al.*, 1988). A corresponding pharmacologically potent derivative of Δ^9-THC, 11-OH-Δ^9-THC-DMH (Figure 3), was also developed (Martin *et al.*, 1991; Razdan, 1987). Hydroxylation in the side chain of Δ^9-THC or Δ^8-THC at C3' increased behavioral activity 2-fold (Agurell *et al.*, 1976; Handrick *et al.*, 1982). Hydroxylation at the C5' position of Δ^8-THC had little effect upon behavioral activity; yet, hydroxylation at the C1' and C2' positions decreased activity (Agurell *et al.*, 1976). These studies suggest that the position of side chain substituents and the orientation of the side chain are important for cannabinoid activity. Unsaturation in

the side chain did not affect potency, even though unsaturation acts to restrict the orientation of the side chain.

Δ^9-THC possesses a phenolic hydroxyl, gem-dimethyl groups on the pyran ring and an oxygen in the pyran ring. Alterations have been made to these sites (Razdan, 1986). Substitution of the pyran oxygen with nitrogen or carbon atoms did not dramatically affect potency. Optimal activity resulted when the phenol group was either free or esterified. Etherification of the phenolic hydroxyl of Δ^8- and Δ^9-THC to O-methyl analogs eliminated activity. Removal of gem-dimethyl groups on the pyran ring decreased activity. Alterations in the location and number of unsaturations in the terpene ring of Δ^9-THC produced differing effects in potency. Optimal activity resulted with a double bond at the C9 position, as in Δ^9-THC; potency was somewhat reduced in Δ^8-THC, and potency was even further reduced with $\Delta^{6a\text{-}10a}$-THC and CBN, which has complete unsaturation.

Synthesis of the dimethylheptyl derivatives was based upon the three-point attachment theory and the necessity of an intact dibenzopyran ring system. A minimum of three points of attachment of Δ^9-THC were postulated to interact with a receptor: 1) a free phenolic hydroxyl group; 2) an appropriate substituent at the C9 position; and 3) a lipophilic side chain (Binder *et al.*, 1984; Howlett *et al.*, 1988). While attempting to develop a unique analgesic, a group at Pfizer prepared novel bicyclic cannabinoid analogs with pharmacological profiles similar to Δ^9-THC (Melvin *et al.*, 1984). They found that the benzopyran ring system can be extensively modified or removed without loss of activity (Johnson and Melvin, 1986). CP 55,940 (Figure 3), the most widely used compound in the series, possessed potency 4-25 times greater than Δ^9-THC, depending upon the pharmacological assay. Since the synthetic cannabinoids lacking a benzopyran ring represented the first significant deviation from the traditional THC structure, they were referred to as nonclassical cannabinoids. Due to the divergence in structure, great attention was placed upon proving that the bicyclic analogs were definitely THC-like. Evidence that

CP 55,940 is a cannabinoid emerged from studies showing that CP 55,940 and Δ^9-THC cross-generalized in rat and monkey drug discrimination, and cross tolerance developed between the two compounds (Gold *et al.*, 1992; Pertwee, Stevenson and Griffin, 1993).

The pharmacological activity of Δ^9-THC is stereoselective, with the (-)-trans isomer having 6-100 times more potency than the (+)-trans isomer, depending upon the pharmacological test (Dewey, Martin and May, 1984). However, the pharmacological profiles for the two enantiomers are similar. It has been argued that lack of complete enantioselectivity is due to contamination of the (+)-isomer with the (-)-isomer (Mechoulam, 1988). Indeed, synthesis of crystalline derivatives of THC resulted in much greater purity of enantiomers and enantioselectivity (Little *et al.*, 1987). Stereoselectivity for the psychoactive properties of cannabinoids was also demonstrated in human studies. The use of highly pure enantiomers of 11-OH-Δ^8-THC-DMH further established that cannabinoids exhibit enantioselectivity (Mechoulam *et al.*, 1988). The nonclassical tricyclic compounds CP 55,244 [(-)-ACD] (Figure 3) and CP 55,243 [(+)-ACD] showed enantioselectivity; CP 55,244 was significantly more potent than CP 55,243 in several of the behavioral tests (Little *et al.*, 1988). The existence of high enantioselectivity reinforced the notion that cannabinoids act through a specific receptor. Although progress in the development of potent cannabinoid agonists has been slower than for other centrally acting agents, potent agonists recently have emerged and contribute not only to SAR, but to the notion that a highly unique endogenous cannabinoid exists.

Although the bicyclic analogs contained unique characteristics, molecular modeling studies revealed that they still retained most of the structural characteristics of Δ^9-THC. However, a truly novel class of compounds has emerged that differs greatly from both classical and bicyclic cannabinoids. Pravadoline, a non-steroidal antiinflammatory agent, had analgesic properties, but interestingly did not interact with the opioid system or inhibit cyclooxygenase. The search for the compound's mechanism of action led to the

development of aminoalkylindoles, such as WIN 55,212 (Figure 3) (Ward *et al.*, 1990; Ward, Childers and Pacheco, 1989). These compounds, though structurally quite distinct from other cannabinoids, competed for binding to the cannabinoid receptor (Pacheco *et al.*, 1991) and possessed cannabimimetic properties, including antinociception, in mice and rats (Compton *et al.*, 1992a). The discovery of anandamide (arachidonylethanolamide) as a proposed endogenous cannabinoid ligand added yet another novel class to the cannabinoid pharmacophore of compounds that produce effects similar to Δ^9-THC (Figure 4) (Devane *et al.*, 1992). Confirming the similarity of anandamide with Δ^9-THC requires future testing, particularly in humans. The emergence of four chemically distinct classes of cannabinoids will provide probes for studying the diverse actions of cannabinoids, and these probes should facilitate the separation of the psychoactive properties from the pharmacological effects.

Animal Models and Pharmacology

The purpose of evaluating cannabinoids in animals is to establish a parallel relationship between animals and humans and to extrapolate the animal effects to humans. The development of a number of animal models in the mouse, rat, dog, rabbit and monkey have allowed researchers to predict the psychoactivity of novel compounds. Extensive reviews of these results are found elsewhere (Dewey, 1986; Razdan, 1986). Pharmacological effects have been measured with models such as dog ataxia (Loewe, 1947), the THC-seizure susceptible rabbit (Consroe, Martin and Fish, 1982), monkey overt behavior (Edery *et al.*, 1971; Edery *et al.*, 1972; Grunfeld and Edery, 1969), drug discrimination (Balster and Prescott, 1992) and a mouse behavioral battery consisting of spontaneous locomotor activity, hypothermia, immobility (catalepsy) and antinociception (Martin, 1985). Although cannabinoids have direct cellular actions on peripheral tissues, most of the behavioral and pharmacological effects studied by researchers appear to involve

the central nervous system (Dewey, 1986). The high lipophilicity of cannabinoids allows passage across the blood-brain-barrier.

Cannabinoids produce a unique syndrome of effects on the behavior of a wide variety of animal species. These behavioral effects are characterized at low doses as a mixture of depressant and stimulatory effects and at higher doses as predominantly CNS depression (Dewey, 1986). The depressant effects of psychotomimetic cannabinoids differ from other CNS depressants. Δ^9-THC and other psychoactive cannabinoids in mice produce a "popcorn" effect. Groups of mice in an apparently sedate state will jump (hyperreflexia) in response to auditory or tactile stimuli. As animals fall onto other animals, they resemble corn popping in a popcorn machine. This state of hyperreflexia is observed during the depressant stage at higher doses (Dewey, 1986). Cannabinoids generally cause a reduction in spontaneous locomotor activity (Little *et al.*, 1988) and a decrease in response rates with different reinforcement schedules (Carney *et al.*, 1979; Zuardi and Karniol, 1983).

With the drug discrimination model, animals use internal cues to discriminate between the subjective effects of different drug classes. In this paradigm, rats, pigeons or non-human primates are trained to make different responses for reinforcement contingent upon administration of either the training drug or vehicle (Gold *et al.*, 1992; Järbe and Hiltunen, 1987; Weissman, 1978). After successful discrimination training, other drugs may be administered to see if they produce similar stimulus characteristics as the training drug. A correlation exists between drugs that generalize to Δ^9-THC and bind to the central cannabinoid receptor. The bicyclic compounds (Gold *et al.*, 1992) and the aminoalkylindoles (Compton *et al.*, 1992b) substitute for Δ^9-THC, whereas drugs from other classes do not (Balster and Prescott, 1992). Ultimately, the best model for evaluating a drug's reinforcing effects and predicting abuse liability is a drug self-administration paradigm. Animals do not readily self-administer Δ^9-THC (Harris, Waters and McLendon,

1974), and CP 55,940 does not maintain intravenous self-administration with a fixed-interval schedule in rhesus monkeys (Mansbach *et al.*, 1994). The lack of reinforcing effects of cannabis is consistent with the mild dependence properties in humans. On the other hand, it is possible that animals will self-administer cannabinoids once appropriate models are discovered.

Cannabinoids also impair learning and memory in rodents (Carlini *et al.*, 1970) and non-human primates (Ferraro and Grilly, 1973). In rats, the delayed match to sample memory task (Heyser, Hampson and Deadwyler, 1993), Lashley III maze (Carlini *et al.*, 1970) and the eight arm radial-maze (Nakamura *et al.*, 1991) were used to measure memory disruption by cannabinoids. State-dependent learning studies have been used to examine the influences of drugs upon the process of conditioning, or memory formation and retrieval. State-dependent learning occurs when an association learned in one condition is more easily retrieved in that same condition. This paradigm has been useful for determining some of the disruptive effects of Δ^9-THC on memory and performance. The effects of Δ^9-THC upon state-dependent learning have been reported in tasks involving avoidance learning and conditioned suppression (Järbe and Mathis, 1992). Tolerance does develop to the disruptive and subjective drug-state effects of Δ^9-THC (Järbe, 1978; Järbe and Mathis, 1992).

The high numbers of cannabinoid receptors in the hippocampus may mediate the disruption in cognition (Herkenham *et al.*, 1991c; Jansen *et al.*, 1992; Thomas, Wei and Martin, 1992). Intrahippocampal administration of CP 55,940 produced a dose-dependent increase in the number of errors in the eight arm radial-maze test without elicitation of other pharmacological effects (Margulies and Hammer, 1991). Another study showed that the disruption in the delayed match to sample memory task induced by acute administration of Δ^9-THC was similar to that produced by damage to the hippocampus (Heyser, Hampson and Deadwyler, 1993). This disruption was associated with a specific decrease in

hippocampal cell discharge only during the encoding phase of the task; the effects were reversible within 24 hours of dosing. A number of other studies have examined the effects of cannabinoids on hippocampal ultrastructure, and are reviewed by Solowij (in press-a). While Δ^9-THC, CP 55,940 and WIN 55,212-2 all impaired working memory in rats, anandamide failed to do so in either the eight arm radial-maze or the delayed nonmatching to sample memory task (Crawley *et al.*, 1993). Lichtman *et al.* (1995) also found that CP 55,940, Δ^9-THC and WIN 55,212-2 administered systemically impaired spatial memory in rats, as assessed by the eight arm radial-maze, and retarded completion time; neither anandamide nor cannabidiol affected memory. Intrahippocampal administration of CP 55,940 impaired memory, but did not inhibit completion time. The intrahippocampal effects of CP 55,940 appeared specific to cognition since no other pharmacological effects were produced. The inability of anandamide to disrupt memory in rats illustrates a possible difference between the endogenous ligand and other cannabinoids and underscores the importance of further comparisons (Crawley *et al.*, 1993; Lichtman, Dimen and Martin, 1995). In a series of chronic studies, rhesus monkeys were trained for one year to perform operant tasks before one year of chronic cannabis administration (Slikker *et al.*, 1992). Task performance was impaired for over a week after cessation of use, but performance returned to baseline levels three weeks after cessation. The effects of chronic exposure were reversible with no apparent long term behavioral effects.

Mechanism of Action

Cannabinoids produce a myriad of pharmacological and behavioral effects which most likely involve numerous neural substrates that traverse the entire brain. The complexity of the pharmacological effects produced by cannabinoids is reflected in the above discussion on animal models. The most likely candidate for mediating the central effects of cannabinoids is a receptor mechanism. Discerning the mechanism of action for

cannabinoids transpired over several decades, and many difficulties encumbered the progress. Enantioselectivity provided initial evidence of receptor involvement, as discussed earlier. The high degrees of enantioselectivity in several of these analogs indicate a very specific mechanism of action, such as that involving a receptor. The lack of an appropriate radiolabeled ligand greatly hindered proving that cannabinoids exerted their interactions through a specific central receptor. Early attempts to identify a receptor in crude rat brain membranes by a ligand binding assay using [^{3}H]-Δ^8-THC failed (Harris, Carchman and Martin, 1978). Saturable binding did not result, and only 10% displaceable binding could be achieved. However, these investigators found displaceable binding in hepatocytes, which suggested that assessing receptor binding was feasible.

The synthesis and radiolabeling of the potent bicyclic cannabinoid CP 55,940 allowed identification of a receptor in rat brain membranes (Devane *et al.*, 1988). Analysis of the data revealed a single binding site that possessed saturable and reversible high affinity binding. Other labeled cannabinoids, such as the dimethylheptyl (DMH) derivative of [^{3}H]-11-OH-Δ^9-THC (Thomas, Wei and Martin, 1992) and [^{3}H]-WIN 55,212-2 (Compton *et al.*, 1992a; Haycock *et al.*, 1991), also bind to this receptor. This receptor displays selectivity for cannabinoids, as other classes of centrally acting compounds do not compete for cannabinoid binding (Howlett, Evans and Houston, 1992). Pharmacological potency of cannabinoids correlates well with their affinity for the cannabinoid binding site (Compton *et al.*, 1993). In addition, binding affinities correlated with *in vivo* potency in the rat drug discrimination model and psychotomimetic activity in humans (Balster and Prescott, 1992). These findings suggest that this receptor mediates most of the central cannabinoid effects across several different animal species.

According to autoradiographic studies, the distribution of the cannabinoid receptor is heterogeneous in several mammalian species, conserved and neuronally located (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991b; Herkenham *et al.*, 1991c). The

densest binding occurs in the basal ganglia (substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus and lateral caudate putamen), and the molecular layer of the cerebellum. Binding in these regions may explain cannabinoid interference with movement. Intermediate levels of binding were found in the CA pyramidal cell layers of the hippocampus, the dentate gyrus and layers I and VI of the cortex. Δ^9-THC disrupts short-term memory in humans (Chait and Pierri, 1992). Thus, cannabinoid effects on memory and cognition are consistent with receptor localization in the hippocampus and cortex. The hippocampus stores memory and codes sensory information. The presence of cannabinoid receptors in regions associated with mediating brain reward (ventromedial striatum and nucleus accumbens) suggests an association with dopamine neurons. Sparse levels were detected in the brainstem, hypothalamus, corpus callosum and the deep cerebellum nuclei. Low levels of receptors in brainstem areas controlling cardiovascular and respiratory functions is also consistent with the low of lethality of cannabis. Other ligands, such as [^{3}H]-WIN 55,212 (Jansen *et al.*, 1992) and [^{3}H]-11-OH-Δ^9-THC-DMH (Thomas, Wei and Martin, 1992), generated similar localization patterns. Binding has also been found in the peripheral B lymphocyte-enriched areas including the marginal zone of the spleen, nodular corona of Peyer's patches and the cortex of the lymph nodes (Lynn and Herkenham, 1994).

Prior to the characterization of a receptor, data demonstrated that cannabinoids inhibited adenylyl cyclase by probable interaction with an inhibitory G protein (G_i) (Howlett and Fleming, 1984). Researchers proposed that a cannabinoid receptor was linked to a G_i protein which, when activated, inhibited the activity of adenylyl cyclase. Adenylyl cyclase then cannot catalyze the conversion of ATP to the second messenger cyclic AMP (cAMP). The inhibition of adenylyl cyclase by cannabinoids took place in neuroblastoma cell membranes, rat brain slice membranes and cultured cerebellar neurons (Bidaut-Russell, Devane and Howlett, 1990; Howlett, Qualy and Khachatrian, 1986;

Pacheco, Ward and Childers, 1993). While extensive *in vitro* evidence exists for a cannabinoid receptor/adenylyl cyclase interaction, determining a pharmacological effect produced by adenylyl cyclase inhibition is difficult. Recently, Welch *et al.* (1995) demonstrated that pertussis toxin blocked the antinociceptive properties of cannabinoids in mice. Pertussis toxin prevents G_i proteins from interacting with receptors. This work suggests that the analgesic properties of cannabinoids might be due to cannabinoid receptor activation of a G_i protein. Furthermore, forskolin, which stimulates adenylyl cyclase, thereby producing increased levels of cAMP, and chloro-cAMP, a stable analog of cAMP, decreased cannabinoid-induced antinociception. Thus, both preventing G_i proteins from interacting with cannabinoid receptors and increasing the levels of cAMP interfered with the production of antinociception (Welch, Thomas and Patrick, 1995). These data suggest involvement of adenylyl cyclase in the antinociception of cannabinoids.

Definitive evidence for a specific cannabinoid receptor became apparent when it was cloned (Matsuda *et al.*, 1990). A clone isolated from a rat brain library had homology with other receptors that interacted with G proteins in the cell membrane. Yet, none of the traditional agonists of G proteins bound to this receptor clone. An identification breakthrough occurred with the discovery that the mRNA distribution of the receptor clone paralleled that of the cannabinoid receptor. Confirmation of the identity of the clone occurred when adenylyl cyclase was inhibited upon exposure to CP 55,940 and Δ^9-THC in cells transfected with this clone. Adenylyl cyclase in non-transfected cells did not respond to cannabinoids. The human cannabinoid receptor was subsequently cloned and found to have almost identical homology to the rat receptor (Gérard *et al.*, 1991). The cannabinoid receptor, abbreviated as CB_1, belongs to a G protein-coupled receptor sub-family which includes the adrenocorticotropin and melanotropin receptors (Mountjoy *et al.*, 1992). An amino-terminal variant, designated CB_{1A}, of CB_1 has been discovered in brain and several peripheral tissues (Shire *et al.*, 1995). CB_{1A} results from alternative splicing of CB_1

resulting in deletion of 60 amino acid residues in the amino terminus. Recently, a distinct peripheral cannabinoid receptor, designated CB_2, was identified in macrophages in the marginal zone of the spleen (Munro, Thomas and Abu-Shaar, 1993). Although CB_1 and CB_2 share only approximately 40% homology, Δ^9-THC and CP 55,940 demonstrate similar binding affinity for both receptor subtypes. The cloning of a peripheral receptor is consistent with previous data showing cannabinoid binding to mouse spleen cells (Kaminski *et al.*, 1992) and to the rat immune system (Lynn and Herkenham, 1994). CB_1 RNA transcripts have been identified in mouse spleen cells (Kaminski *et al.*, 1992) and human peripheral blood lymphocytes (Bouaboula *et al.*, 1993); CB_2 RNA transcripts are expressed in the rat spleen (Munro, Thomas and Abu-Shaar, 1993). The role of this receptor in the spleen remains unknown. Slipetz *et al.* (1995) demonstrated that the human peripheral receptor (CB_2) is functionally coupled to inhibition of adenylyl cyclase activity by a pertussis toxin-sensitive G-protein. A comparison of the pharmacology and signal transduction of the human CB_1 and CB_2 receptors indicate that both receptor subtypes display similar pharmacological and biochemical properties, except CB_2 did not couple to the modulation of Q-type calcium channels or inwardly rectifying potassium channels (Felder *et al.*, 1995). The discovery of a second receptor raises the possibility that other receptors with unique functional roles may exist.

Cannabinoids also produce effects through second messenger systems other than adenylyl cyclase. Initial evidence implicating calcium came from a study in which Δ^9-THC inhibited calcium uptake following depolarization in mouse brain synaptosomes (Harris and Stokes, 1982). Electrophysiological studies showed that cannabinoids inhibited an omega conotoxin-sensitive, high voltage-activated N-type calcium channel (Caulfield and Brown, 1992; Mackie and Hille, 1992). The inhibition of calcium channel activity was pertussis toxin-sensitive and stereospecific, suggesting a receptor-mediated process. In contrast, other data demonstrated that calcium influx in non-transfected cells occurred through a non-

receptor process (Felder *et al.*, 1992). However, high concentrations of cannabinoids were used, and calcium influx was also observed in non-transfected cells. Cannabinoids have also been reported to mediate an enhancement of A-type potassium channels in cultured hippocampal neurons through the cannabinoid receptor (Deadwyler *et al.*, 1993).

Several recent studies have explored the biological functions and gene regulation associated with the cannabinoid receptor. Bouaboula *et al.* (1995) discovered that CP 55,940, in addition to the inhibition of cAMP accumulation, induces the expression of the immediate-early growth-related gene *Krox-24* in astrocytoma cells. The transduction pathway between the cannabinoid receptor and *Krox-24* involves a pertussis toxin-sensitive GTP-binding protein and is independent of cAMP metabolism (Bouaboula *et al.*, 1995a). *Krox-24* induction was inhibited by the tyrosine kinase inhibitor herbimycin A, suggesting that a protein kinase may lie on the route between G_i and *Krox 24*. The authors concluded that one receptor subtype (CB_1) may be coupled to two separate effector systems. The precise role of the cannabinoid receptor in astrocyte cells remains under investigation. In another study, cannabinoids induced phosphorylation and activation of mitogen-activated kinases (MAP) in CHO cells transfected with the human CB_1 receptor (Bouaboula *et al.*, 1995b). The signal transduction between CB_1 and MAP kinases involves a pertussis-toxin-sensitive GTP-binding protein and is independent of cAMP metabolism. The coupling of CB_1 and mitogenic signal pathway may explain the mechanism of action underlying cannabinoid-induced *Krox-24* induction. Thus, activation of MAP kinase may be a candidate for regulation of *Krox-24* induction. Das *et al.* (1995) found that a cannabinoid receptor-mediated signaling pathway is present in the mouse uterus, and this organ has the capacity to synthesize anandamide. THC rapidly and transiently upregulated the gene encoding lactoferrin. Lactoferrin is an iron-binding glycoprotein proposed to be involved in immunomodulation and growth promotion. The authors propose that

pregnancy failures and fetal losses that have been reported with exposure to cannabinoids may be mediated via interactions with uterine cannabinoid receptors (Das *et al.*, 1995).

Other systems have been proposed for the signal transduction of cannabinoid receptor activation, though the evidence is not as compelling. Some data suggest that cannabinoids might activate the inositol phospholipid pathway. In this signaling pathway, a receptor activates a G protein (tentatively called G_p) that in turn activates phospholipase C. This enzyme cleaves PIP_2 (phosphatidylinositol-bisphosphate) into inositol triphosphate (IP_3) and diacylglycerol. Diacylglycerol activates protein kinase C, and IP_3 triggers calcium release from cellular compartments. One study presented evidence that Δ^9-THC decreased the formation of *myo*-inositol triphosphate in pancreatic islet cells (Chaudry *et al.*, 1988). This evidence implies that cannabinoids bind to a receptor that is linked to the inositol phospholipid pathway. Yet, another study demonstrated that protein kinase C distribution did not co-localize with cannabinoid binding (Herkenham *et al.*, 1991a). If cannabinoids did bind to receptors that activated this pathway, one would assume that cannabinoid binding would co-localize with components, such as protein kinase C, of the inositol phospholipid system. Other researchers showed that cannabinoids also stimulated the release of arachidonic acid and phospholipid turnover (Felder *et al.*, 1992). This effect lacked enantioselectivity, and high concentrations were necessary. Thus, these investigators ruled against receptor involvement (Felder *et al.*, 1992).

Based upon the discoveries over the past decade, one can postulate that a "cannabinoid" neurochemical system does exist. The function of this system and its interaction with other neurochemical systems remains unclear. It is well known that cannabinoids exert many of their actions by influencing several traditional neurotransmitter systems, as presented in other reviews (Dewey, 1986; Pertwee, 1988; Pertwee, 1992). The results from numerous studies suggest that several neurotransmitters and neuromodulators have a role in the neuropharmacology of cannabinoids. These substances

include acetylcholine (ACh), dopamine (DA), γ-aminobutyric acid (GABA), histamine, 5-hydroxytryptamine (5-HT), norepinephrine (NE), opioid peptides and prostaglandins (PGEs). The basis for some of the effects of cannabinoids are established through the interaction between cannabinoids and drugs that bind to other receptor types or drugs that alter the synthesis, storage, release or metabolism of transmitters and modulators (Pertwee, 1992). Cannabinoids have been shown to enhance the formation of NE, DA and 5-HT. Cannabinoids also stimulated the release of DA from rat corpus striatum, nucleus accumbens and medial prefrontal cortex. GABA turnover is enhanced by cannabinoids. The most commonly studied behavioral effects of cannabinoids include hypothermia, antinociception and changes in locomotor activity. Results from drug interaction studies for catalepsy and depression of spontaneous locomotor activity suggest that these effects are mediated by ACh acting at muscarinic and nicotinic receptors, GABA acting at $GABA_A$ and $GABA_B$ receptors and PGEs. The extrapyramidal system probably plays a role in catalepsy since intrapallidal administration of 11-OH-Δ^8-THC produced catalepsy (Pertwee and Wickens, 1991). Evidence exists that catalepsy results from interaction of Δ^9-THC with neurotransmitter systems in the basal ganglia (Gough and Olley, 1977; Gough and Olley, 1978). Hypothermia in rats and mice has been attributed to by DA, NE, 5-HT, GABA, histamine and opioid peptides. There is also evidence that alteration in thermoregulation occurs by the hypothalamus (Fitton and Pertwee, 1982) and brainstem activity (Hosko, Schmeling and Hardman, 1981). Possibly, enhanced serotonergic transmission (Davies and Graham, 1980) and modulated autonomic activity (Rosenkrantz, 1983) produce hypothermia. Results from hypothermia studies are often inconsistent, thus definite conclusions cannot be drawn about the neuronal pathway involved in cannabinoid-induced antinociception (Pertwee, 1992). Several endogenous compounds serve to inhibit nociception (NE, 5-HT, ACh, GABA, opioid peptides, PGE_1 and PGD_2), and some of these compounds interact with cannabinoids to produce antinociception.

An inwardly rectifying potassium channel co-expressed with the neuronal cannabinoid receptor in *Xenopus* oocytes was activated by WIN 55,212-2 (Henry and Chavkin, 1995). The precise role of calcium or potassium in the physiological actions of cannabinoids remains unknown. Cannabinoids also affect serotonin (5-HT) receptors. Anandamide, Win 55,212-2 and CP 55,940 inhibited the 5-HT_3-induced current in rat nodose ganglion neurons in a concentration dependent manner (Fan, 1995). 5-HT_3 receptors may be involved in cannabinoid-induced analgesia, antiemesis and possibly other behavioral effects.

Some experiments support the involvement of catecholamines, 5-HT, PGE_1 and opioid peptides. Interpretation of the actions of cannabinoids on neurotransmitter systems is often difficult since evidence exists that cannabinoids both inhibit and stimulate neuronal uptake. Relatively few studies have examined the long term-exposure of cannabinoids on brain neurotransmitter and neuromodulator levels. As reviewed by Solowij (in press-a), recent evidence suggests that few, if any, irreversible effects on brain chemistry exist due to Δ^9-THC administration. Although it appears that neurotransmitters are altered to produce some of the effects of cannabinoids, cause and effect have not been clearly established.

Anandamide as an Endogenous Ligand

The discovery of a receptor raised the question about the possible existence of an endogenous ligand and a separate cannabinoid neurochemical system. Due to the high lipophilicity of cannabinoids, Devane *et al.* (1992) searched for a compound in lipid extracts from porcine brain. They isolated anandamide, which competed for cannabinoid receptor binding and, like Δ^9-THC, inhibited electrically stimulated contractions in the murine vas deferens (Devane *et al.*, 1992). Anandamide, a fatty acid derivative, binds both to the cannabinoid receptor of the rat brain (Devane et al., 1992) and to murine Ltk- cells

transfected with the human cannabinoid receptor (Childers, Sexton and Roy, 1994; Felder *et al.*, 1993). Anandamide produced similar pharmacological effects to Δ^9-THC, such as antinociception, catalepsy, hypomotility and hypothermia (Fride and Mechoulam, 1993), and anandamide inhibited adenylyl cyclase (Felder *et al.*, 1993) and N-type calcium channels (Mackie, Devane and Hille, 1993). One study found that the effect of anandamide on adenylyl cyclase was region-specific, with maximal inhibition occurring in the cerebellum and striatum (Childers, Sexton and Roy, 1994). A comparison between anandamide and Δ^9-THC revealed that anandamide was 4 to 20 fold less potent and had a shorter duration of action than Δ^9-THC (Smith *et al.*, 1994). Both anandamide and Δ^9-THC affected the hypothalamo-pituitary-adrenal axis in a similar manner (Weidenfeld, Feldman and Mechoulam, 1994). Intracerebroventricular administration of anandamide decreased CRF levels in the median eminence and increased serum ACTH and corticosterone levels. Anandamide blocks adenylyl cyclase at the frog neuromuscular junction, providing evidence for the presence of a cannabinoid receptor at the motor nerve (Van der Kloot, 1994). Gap-junction conductance in striatal astrocytes is inhibited by anandamide, suggesting that anandamide may control intercellular communication in astrocytes and therefore neuron-glial interactions (Venance *et al.*, 1995).

Anandamide belongs to a widespread class of natural products, the *N*-acylethanolamines, whose precise biological role is not fully characterized. In pig and bovine brains, anandamide composes about 1% of the total *N*-acylethanolamines (Schmid *et al.*, 1995). Several months after its discovery, N-arachidonic acid-2-hydroxyethylamide (anandamide) was isolated from calf brain in an effort to find endogenous regulators of L-type calcium channels (Johnson *et al.*, 1993). Anandamide displaced the binding of a calcium channel antagonist, 1,4-dihydropyridine, in cardiac and cortical membranes. This independent finding serves to strengthen the hypothesis that anandamide is a chemical modulator.

One of the qualifications of a neurochemical system is the existence of a path for synthesis and degradation of a ligand. Deutsch and Chin (1993) showed that anandamide was rapidly taken into neuroblastoma and glioma cells. Synthesis was achieved in brain homogenates by incubating arachidonate with ethanolamine. Anandamide was hydrolyzed to arachidonic acid and ethanolamine by an amidase (anandamide amidohydrolase), which resides in the membrane fractions. Degradation also occurred in brain, kidney, liver and lung homogenates (Deutsch and Chin, 1993). The enzyme inhibitor phenylmethylsulfonyl fluoride (PMSF) prevented degradation, but not synthesis, of anandamide. Brain aminohydrolase activity is selective for anandamide and is enriched in areas of the brain with high density of cannabinoid receptors, such as the globus pallidus and hippocampus, which suggests that this enzyme activity may be involved in the inactivation of anandamide at its sites of action (Desarnaud, Cadas and Piomelli, 1995). Another group also found that the distribution of anandamide hydrolytic activity correlated with the distribution of cannabinoid-receptor binding sites; the cerebellum, cerebral cortex and hippocampus exhibited the highest metabolic activity, while the brainstem and white matter exhibited the lowest activity (Hillard *et al.*, 1995). The aminohydrolase was solubilized and purified from the microsomal fraction of porcine brain (Ueda *et al.*, 1995). Based upon their results, Ueda *et al.* (1995) suggested that the anandamide aminohydrolase and synthase activities were attributable to a single enzyme protein.

Evidence also exists that anandamide can be metabolized by cytochromes P450s. Mouse hepatic P450s, which are known to oxidize arachidonic acid, were found to metabolize anandamide into 10 different metabolites (Bornheim *et al.*, 1993). Pretreatment of mice with P450 inducers, such as dexamethasone, phenobarbitol and 3-methylcholanthrene, resulted in increased hepatic microsomal formation of several of the anandamide metabolites (Bornheim *et al.*, 1995). Antibodies against P450 3A prevented the formation of several of these metabolites. Metabolism of anandamide by brain

microsomes resulted in the formation of two metabolites, only one of which was partially inhibited by antibodies to P450 3A. The physiological consequences of P450-mediated anandamide metabolism remains unknown.

Two possible mechanisms for the formation of anandamide have been proposed. Devane and Axelrod (1994) and Kruszka and Gross (1994) propose that anandamide is formed by the enzymatically catalyzed condensation of arachidonic acid with ethanolamine. Both groups demonstrated the formation of anandamide by brain membrane preparations incubated with arachidonic acid and ethanolamine, and both laboratories found that arachidonic acid was the preferred substrate for the enzyme, and the reaction was specific for ethanolamine (Devane and Axelrod, 1994; Kruszka and Gross, 1994). Kruszka and Gross (1994) suggested that the condensation reaction is independent of coenzyme A and ATP. They also propose that arachidonic acid may react with a cysteine at the active center of the enzyme to form an arachidonyl-thiol ester enzyme intermediate. Interestingly, Devane and Axelrod (1994) found that anandamide synthase activity was highest in the hippocampus, followed by the thalamus, cortex and striatum, and lowest in the cerebellum, pons and medulla. The ability of brain tissue to enzymatically synthesize anandamide and the presence of receptors specific for anandamide suggest the existence of anandamide-containing neurons. Di Marzo and colleagues (1994) propose an alternative biosynthetic mechanism. They suggest that anandamide formation occurs through a phosphodiesterase-mediated cleavage of a novel phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine (Di Marzo *et al.*, 1994). In primary cultures of rat neurons, stimulation with the calcium ionophore ionomycin, which increases synaptic activity, led to the formation of anandamide. The newly synthesized anandamide is released into the external medium, where it can be inactivated by a rapid, saturable uptake mechanism. The findings of Di Marzo *et al.* (1994) suggest that anandamide biosynthesis is under physiological control. The condensation of arachidonic acid and ethanolamide has been

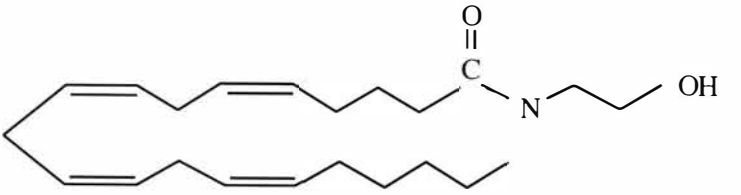
demonstrated to occur at conditions that are non-physiological, thus giving support to the pathway proposed by Di Marzo *et al.* (1994).

The establishment of a cannabinoid receptor and an endogenous ligand with biosynthetic and degradative pathways suggests the possible presence of a distinct neurochemical system. Anandamide may represent one member of a family of endogenous compounds (Figure 4). Two other compounds, homo-γ-linolenylethanolamide (20:3, n-6) and docosatetraenylethanolamide (22:4, n-6), isolated from bovine brain, also competed for cannabinoid receptor binding (Barg *et al.*, 1995; Hanus *et al.*, 1993) and inhibited the electrically evoked twitch response of the mouse isolated vas deferens (Pertwee *et al.*, 1994). Both compounds inhibited adenylyl cyclase, and this inhibition was blocked by pertussis toxin, indicating involvement of pertussis toxin-sensitive GTP-binding proteins (Barg *et al.*, 1995). The two new anandamides exerted similar behavioral effects to those observed with Δ^9-THC, including inhibition of motor activity, hypothermia, catalepsy and antinociception (Barg *et al.*, 1995). The maximal effect obtained with the two anandamides in the behavioral tests were smaller than the maximal effects of Δ^9-THC. Under certain conditions, the anandamides may function as partial agonists. In a separate study, vasa deferentia showed tolerance to the inhibitory effects of anandamide (20:3, n-6) and anandamide (22:4, n-6) when obtained from mice pretreated with Δ^9-THC (Pertwee *et al.*, 1994). Recently, another endogenous compound, 2-arachidonyl glycerol, was isolated from canine intestines (Figure 4) (Mechoulam *et al.*, 1995). This compound bound to CB_1 and CB_2, though with greater affinity to CB_1, and produced the typical cannabimimetic effects in mice. 2-Arachidonyl glycerol inhibited electrically evoked contractions of mouse isolated vasa deferentia, but was less potent and efficacious than Δ^9-THC. In addition, another group has reported that 2-arachidonyl glycerol bound to rat brain synaptosomal membranes, and its levels in the rat brain were about a thousand times higher than that of anandamide (Sugiura *et al.*, 1995). Mead ethanolamide has also been proposed as a novel

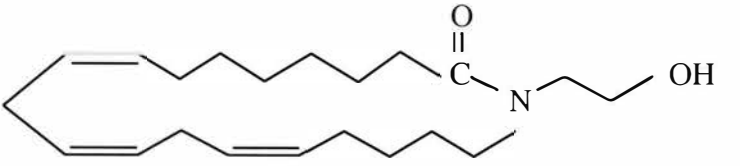
eicosanoid agonist for central and peripheral cannabinoid receptors (Priller *et al.*, 1995). Mead acid accumulates during periods of dietary fatty acid deprivation in rats. Priller *et al.* (1995) found that the chemically synthesized ethanolamide of mead acid bound to both CB_1 and CB_2 receptor subtypes, inhibited cAMP accumulation and N-type calcium currents in cells expressing the CB_1 receptor. Since anandamide is structurally related to the leukotrienes and prostaglandins, Hampson *et al.* (1995) assessed anandamide as a substrate for rat brain lipoxygenase. Lipoxygenase enzymes are important for the biosynthesis of eicosanoids, including a number of potent oxygenated metabolites such as leukotrienes, lipoxins and hepoxilins. Metabolites of this enzyme have been demonstrated to modulate neurotransmission. Anandamide did serve as a substrate for rat brain lipoxygenase. One of its metabolites, 12-hydroxyanandamide, had an affinity twice that of anandamide for the central cannabinoid receptor (Hampson *et al.*, 1995). 12-Hydroxyanandamide inhibited both forskolin-stimulated cAMP synthesis and the murine vas deferens twitch response, though it was less potent than anandamide. From these studies, a complicated picture is emerging of anandamide interacting with several different systems. Future research must answer numerous questions in order to advance our understanding of the physiology and neurochemistry of anandamide in the brain. Why does such a system exist? What is its physiological role? What would be the physical manifestations of an imbalance in this system?

A Novel Cannabinoid Receptor Antagonist

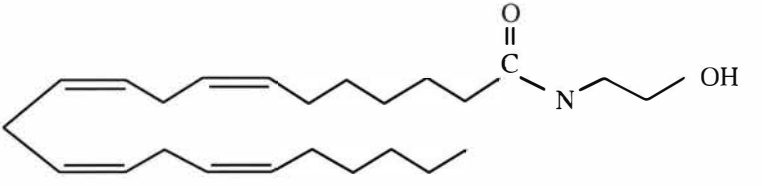
The recent discovery of a cannabinoid antagonist provides researchers with a valuable probe for elucidating the physiological and pathophysiological roles of the proposed cannabinoid system. The antagonist, SR 141716A, has high affinity for the CB_1 receptor, but not the CB_2 receptor (Figure 5) (Rinaldi-Carmona *et al.*, 1994). *In vitro*, it antagonized both cannabinoid-induced inhibition of adenylyl cyclase activity in rat brain



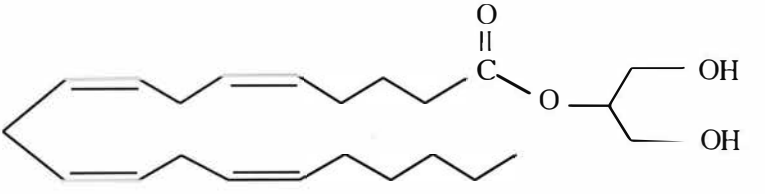
Anandamide (20:4, n-6)



Homo-γ-linolenylethanolamide (20:3, n-6)



Docosatetraenylethanolamide (22:4, n-6)



2-Arachidonyl glycerol

Figure 4. Structures of endogenous ligands of the cannabinoid receptor.

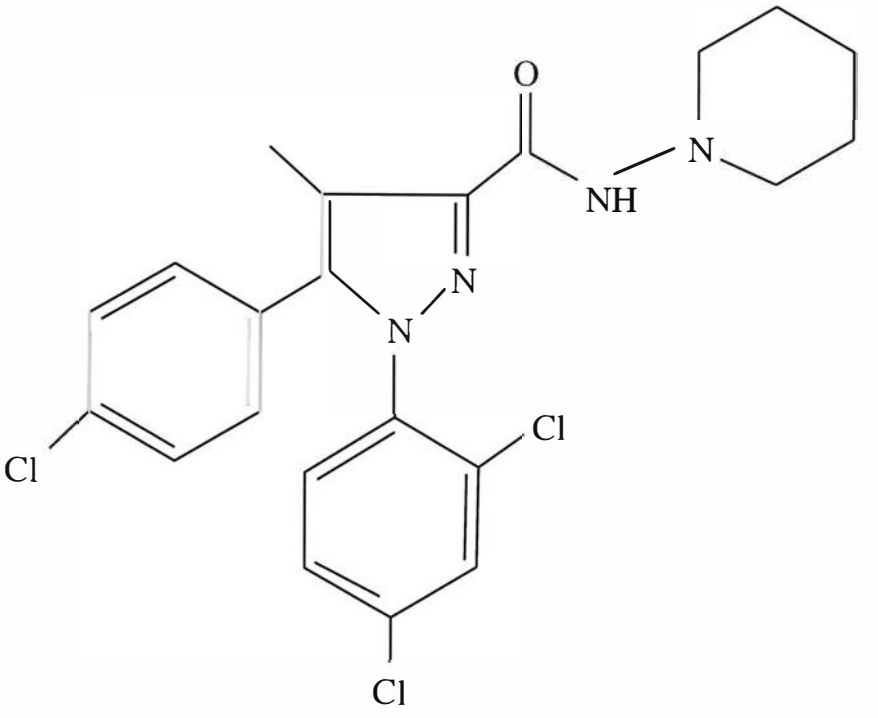


Figure 5. Structure of SR 141716A, the antagonist for the cannabinoid receptor.

membranes and mouse vas deferens contractions; *in vivo* it antagonized behavioral effects of cannabinoid agonists (Rinaldi-Carmona *et al.*, 1994). SR 141716A also antagonized the discriminative stimulus effects of both Δ^9-THC in rats and rhesus monkeys (Wiley *et al.*, 1995b) and CP 55,940 in rats (Wiley *et al.*, 1995a). This compound is the first reliable antagonist of cannabinoid discrimination and might be useful in blocking or reversing cannabis intoxication in humans. SR 141716A blocks activation of krox-24 gene expression in CHO cells transfected with the human CB_1 receptor (Bouaboula *et al.*, 1995a) and prevents cannabinoid-mediated blockade of long-term potentiation in the rat hippocampal slice (Collins, Pertwee and Davies, 1995).

Animal Tolerance and Dependence

Tolerance develops to the pharmacological effects of cannabinoids in a variety of animal species, including pigeons, rodents, dogs, monkeys and rabbits. Several review articles discuss the issues of tolerance and dependence (Compton, Dewey and Martin, 1990; Kaymakcalan, 1973; McMillan, Dewey and Harris, 1971; Wikler, 1976). Tolerance has occurred to antinociception (Martin, 1985), anticonvulsant activity (Colasanti, Lindamood and Craig, 1982), catalepsy (Pertwee, 1974), depression of locomotor activity (Karler, Calder and Turkanis, 1984), hypothermia (Thompson *et al.*, 1974), hypotension (Birmingham, 1973), corticosteroid release (Miczek and Dihit, 1980), ataxia in dogs (Martin *et al.*, 1976) and schedule-controlled behavior (McMillan *et al.*, 1970). Tolerance does not develop to all cannabinoid effects, such as ACTH secretion (Dewey, Peng and Harris, 1970). Often the levels of tolerance are markedly high with reported instances of 100-fold development. Other psychoactive cannabinoids, such as Δ^8-THC, the 11-hydroxy metabolites, nantradol and nabilone also produce tolerance (Kosersky, McMillan and Harris, 1974; Watanabe, Yamamoto and Yoshimura, 1983). Interestingly, tolerance also has been demonstrated in cultured cells. Tolerance developed to cannabinoid-induced

stimulation of prostaglandin E_2 production and arachidonate release (Burstein, Hunter and Renzulli, 1985) and to cannabinoid-inhibition of adenylyl cyclase activity (Dill and Howlett, 1988).

The precise mechanism for the development of tolerance remains unknown. Tolerance to drugs usually occurs by two main methods: changes in pharmacokinetics or pharmacodynamics. Several lines of evidence indicate that pharmacokinetics (absorption, distribution, metabolism and excretion) probably plays a minor role in tolerance production (Dewey *et al.*, 1973; Martin *et al.*, 1976; Siemens and Kalant, 1974). Thus, a pharmacodynamic event, such as receptor down-regulation, receptor conformational change and receptor internalization, more than likely attributes to tolerance development. These three events result in decreased receptor-ligand interaction. Changes at the cannabinoid receptor level following exposure to cannabinoids for a long period of time could result in conformational changes in the receptor which would produce an altered receptor structure, to which the ligand could not bind. Another possible pharmacodynamic event is receptor internalization. When receptor internalization occurs, receptors on the cell membrane are removed into the cytoplasm where they are either degraded or recycled. The number of receptors at the cell surface is decreased; therefore, binding to the receptor is decreased. Several groups have demonstrated cannabinoid receptor down-regulation in cannabinoid-tolerant animals (Oviedo, Glowa and Herkenham, 1993; Rodríguez de Fonseca *et al.*, 1994). Receptor down-regulation occurs when the number of receptors made by the cells is reduced. Oviedo *et al.* (1993) presented data suggesting that cannabinoid tolerance was due in part to agonist-induced receptor down-regulation. Altered binding in animals treated acutely with Δ^9-THC or CP 55,940 resulted from changes in affinity; in chronically treated animals, changes in binding were attributed to a lowering of binding capacity. Rodríguez de Fonseca *et al.* (1994) found that behavioral tolerance developed in rats chronically treated with Δ^9-THC. This tolerance was

accompanied with decreases in binding in the striatum and limbic forebrain. In a recent study, cannabinoid binding actually increased in brain areas, such as the cerebellum and hippocampus, after acute or chronic exposure to either anandamide or Δ^9-THC (Romero *et al.*, 1995). No changes were detected in the limbic forebrain or the medial basal hypothalamus, and after chronic exposure receptors were downregulated in the striatum. Interestingly, another study noted that cannabinoid receptor properties were not irreversibly altered by chronic exposure in either rat brain 60 days following 90 days of administration of Δ^9-THC or in monkey brain seven months after one year of exposure to cannabis smoke (Westlake *et al.*, 1991). Receptor down-regulation could either result from or cause alterations in gene transcription. Another study found that although a twenty-seven-fold behavioral tolerance to Δ^9-THC was observed, neither receptor binding nor mRNA levels in whole brain changed (Abood *et al.*, 1993). Fan *et al.* (1996) have demonstrated that an increase in cannabinoid receptor mRNA accompanies the down-regulation of the receptor in the cerebellum of tolerant mice. However, cause and effect has not been established (Fan *et al.*, 1996).

In light of the fact that most drugs which are used for recreational purposes produce some form of physiological dependence and that development of tolerance frequently occurs in conjunction with dependence, it would seem likely that physical dependence would also develop following chronic exposure to cannabinoids. One of the most common methods for demonstrating dependence, particularly for drugs which do not have a long duration of action, is to abruptly terminate chronic administration of the agent and observe the ensuing behavioral sequelae. Efforts to conduct abrupt withdrawal studies with cannabinoids have produced conflicting results. McMillan *et al.* (1971) failed to detect withdrawal symptoms upon termination of chronic administration of cannabinoids. A few reports have noted that abrupt cessation of cannabinoids produce certain behavioral changes. These alterations include increased grooming, motor activity (Kaymakcalan,

Ayhan and Tulunay, 1977), aggression (Beardsley, Balster and Harris, 1986) and susceptibility to electroshock-induced convulsions (Karler *et al.*, 1984). Yet, re-administration of a cannabinoid did not reverse these effects, and other laboratories could not duplicate withdrawal. Therefore, the capacity of cannabinoids to produce abrupt withdrawal remains ambiguous. A second approach for assessing dependence is to precipitate an abstinence syndrome in chronically treated animals by administering an antagonist. The lack of a cannabinoid antagonist prompted earlier investigators to attempt precipitated withdrawal with opioid antagonists. Naloxone was reported to precipitate withdrawal in rats treated chronically with Δ^9-THC, although the symptomatology differed somewhat from that described for opioid dependence (Hirschhorn and Rosecrans, 1974; Kaymakcalan, Ayhan and Tulunay, 1977). Fortunately, a selective and highly potent cannabinoid antagonist was developed recently (Rinaldi-Carmona *et al.*, 1994). This antagonist, SR 141716A, has proven to be effective in precipitating cannabinoid withdrawal. In one study rats were chronically infused with Δ^9-THC for four days and then administered the antagonist (Aceto *et al.*, 1995). A marked change in the Δ^9-THC-infused animals was evident approximately 10 minutes after the intraperitoneal injection of SR 141716A, and these effects subsided within an hour. The behavioral signs included head shakes, facial tremors, tongue rolling, biting, wet-dog shakes, eyelid ptosis, facial rubbing, paw treading, retropulsion, immobility, ear twitch, chewing, licking, stretching and arched back. The signs of facial rubbing and wet-dog shakes were quantified and found to be statistically greater than that observed in vehicle-infused rats. Similar results were observed by Tsou *et al.* (1995) who repeatedly injected rats with Δ^9-THC prior to an intraperitoneal challenge with SR 141716A. They observed that the most striking aspect of the withdrawal syndrome was the rapidly alternating sequences of aborted fragments of organized behavior (Tsou, Patrick and Walker, 1995). The syndrome may have resulted from alterations in the physiology of neural circuits in the basal ganglia. These studies

provide convincing evidence that cannabinoids can produce physical dependence. The challenge is to understand the relationship between these animal models and the use pattern of cannabinoids in humans. A high priority for future research is to identify the neuronal systems which subserve the cannabis withdrawal syndrome. Manipulation of these systems may provide a means for treating individuals who seek assistance in terminating their cannabis use.

Pharmacokinetics and Detection

Cannabis is usually smoked as a 0.5-1 g cigarette. The THC dose necessary to produce pharmacological effects in humans ranges from 2 to 22 mg for smoking (Martin, 1986). If only 10-25% of available THC enters the circulation when smoked, then the dose range is actually 0.2-4.4 mg. Animal studies have shown that the THC level in the brain is very small, with 1% of the administered dose available at peak concentration (Agurell *et al.*, 1986). If humans have a similar distribution, then only 2-44 μg THC would penetrate the brain. Following inhalation, Δ^9-THC is rapidly absorbed into the bloodstream and redistributed. Initial metabolism takes place in the lungs and liver to 11-hydroxy-THC (11-OH-THC). This metabolite is somewhat more potent than Δ^9-THC and more readily crosses the blood-brain barrier. More extensive metabolism in the liver converts 11-OH-THC to many inactive metabolites, including 11-nor-carboxy-Δ^9-THC (THCCOOH), the most abundant metabolite in plasma and urine. A study by Huestis *et al.* (1992) provides the first complete pharmacokinetic profile of THC and the appearance of metabolites during cannabis smoking. THC levels increase rapidly, peak prior to the end of smoking and quickly dissipate. Peak 11-OH-THC levels are lower than THC levels and occur immediately at the end of smoking. THCCOOH is detected minutes after smoking, and levels plateau for an extended period (Huestis, Henningfield and Cone, 1992). Δ^9-THC can be detected in blood at 7 and 18 ng/ml after a single inhalation of smoke from a

1.75 and a 3.55% THC marijuana cigarette, respectively (Huestis *et al.*, 1992). An entire cigarette will produce peak THC levels greater than 100 ng/ml (Cocchetto *et al.*, 1981; Huestis *et al.*, 1992; Lemberger *et al.*, 1972; Ohlsson *et al.*, 1980; Perez-Reyes, Owens and Di Guiseppi, 1981). Cannabis is also often consumed orally. Similar pharmacological effects to smoking result, but differences exist in the rate of onset and in the blood levels of cannabinoids. After oral ingestion, the levels of Δ^9-THC gradually increase over a period of 4 to 6 hours causing a delay in psychoactive effects (Wall *et al.*, 1983). 11-OH-THC is present in higher concentrations in blood after the oral route (Cone and Huestis, 1993).

Subsequent release of Δ^9-THC from lipid-rich tissues occurs slowly and produces a long elimination half-time. Estimates of elimination range from 18.7 hours to 4.1 days; the variability in half-life measures is due to the dependence of this measure upon assay sensitivity and timing of blood measurements (Cone and Huestis, 1993). Less variability is found in measurements of clearance. Recent data using sensitive detection techniques suggest that the elimination half-life in chronic users is actually three to five days (Johansson *et al.*, 1988). Conflicting reports exist for the clearance time of THC in light and chronic cannabis users. Lemberger *et al.* (1978) reported that the time to clear half of the dose from the body in a daily user (19 to 27 hours) is twice as fast than in an inexperienced user. Another study did not find significant differences in clearance rates between heavy and light users (Ohlsson *et al.*, 1982).

Since cannabinoids affect motor skills, having a reliable measurement of impairment similar to the breath test for alcohol intoxication is desirable. However, establishing a relationship between blood levels of THC or its metabolites and the degree of impairment has been difficult. This difficulty relates to the delay between peak blood concentrations and peak drug effects (Huestis *et al.*, 1992). Immediately after smoking, plasma levels are high while effects are low; whereas at later times, the situation reverses. Therefore, blood levels of THC could be useful for predicting impairment if the mode of

administration and time of use is known. In the absence of this critical information, attempts to develop 'cut-off' levels would have to be very conservative (i.e., the values would have to be rather high). Recently, models have been proposed to predict the time of cannabis exposure from plasma concentrations of THC and THCCOOH (Cone and Huestis, 1993). This model allows prediction of the elapsed time since cannabis use based on analysis from a single plasma sample. Additional research is needed to clarify the relationship between blood cannabinoid levels and behavioral effects.

Legal and moral concerns in the United States have led to increased efforts to detect cannabis use in the work place and in individuals whose performance is critical for general public safety. Initial screening tests are performed by immunoassay for the detection of cannabinoids in urine, and positive samples are verified by gas chromatography/mass spectrometry analysis. These assays were developed to detect the primary cannabinoid excreted in urine, which is THCCOOH. The development of "quick tests" for the detection of drugs of abuse results from the growing demand for simple, rapid and inexpensive on-site drug testing. The EZ-SCREEN® immunoassay test is highly sensitive for THCCOOH and has low cross-reactivity with other cannabinoids (Jenkins *et al.*, 1993). One of the most frequently asked questions is the length of time required for urinary levels to fall below detectable limits following smoking of a single cannabis 'joint'. Typically, THCCOOH can easily be detected two to three days following smoking of a single cannabis cigarette. Passive inhalation has become an attractive argument for explaining the presence of urinary cannabinoids. Yet Cone *et al.* (1990) demonstrated that Herculean efforts were required in order for passive inhalation to produce detectable urinary levels of THCCOOH. Measurement of urinary levels of cannabinoids should be conducted solely for the purpose of determining whether an individual has used cannabis. Attempts at assessing impairment would require considerable knowledge of the circumstances surrounding the last use.

Effects on Organ Systems

Central Nervous System

Since the brain is recognized as a principle target for cannabis, research has been conducted to study the effects of cannabinoids upon the central nervous system that extend beyond neurochemistry. The effects of cannabis on electroencephalographic (EEG) readings, cerebral blood flow (CBF) and brain morphology have been studied, as reviewed by Hall *et al.* (1994) and Solowij (in press-a). Long term alterations in EEG recordings have been observed in cats, rats and monkeys exposed to cannabinoids (Hall, Solowij and Lemon, 1994). In one chronic study, monkeys were exposed to cannabis smoke for six months (Heath *et al.*, 1980). Serious subcortical EEG alterations were noted, with the amygdala, hippocampus and septal region most profoundly affected. Quantitative EEG studies of cannabis in humans have been performed since the 1970s, and most reported an increase in alpha power (usually relative power or alpha abundance), decreased alpha frequency and a decrease in beta activity following acute exposure to THC (Fink *et al.*, 1976). These results are consistent with a state of drowsiness. Struve and Staumanis (1990) provide a review of the acute and chronic effects of cannabis use on the EEG recording and evoked potential studies in humans. Recently, Struve *et al.* (1994) reported that THC produced significant elevations in absolute alpha power, relative alpha power and interhemispheric alpha coherence over frontal and frontal-central areas in chronic users. They referred to this phenomenon as alpha hyperfrontality. In users with very long exposure (> 15 years) EEGs were characterized by increases in frontal-central theta activity in addition to hyperfrontality of alpha. These findings suggest that there may be a gradient of quantitative EEG change associated with long term cannabis exposure. Infrequent use did not produce persistent EEG change. With daily use, the topographic EEG became characterized with hyperfrontality of alpha. At some unknown point after cumulative exposure there was a downward shift in maximal EEG spectral power from the mid alpha

range to the upper theta/low alpha range. Exposure of 15-30 years resulted in increases of absolute power, relative power and coherence of theta activity over the frontal-central cortex. The relationship between EEG changes and performance on neuropsychological tests is not known.

Studies also have examined the effects of cannabis in humans upon two measures of brain activity, cerebral blood flow and cerebral metabolic rate. Drug-induced changes in these parameters are thought to represent a change in brain function (Mathew and Wilson, 1993). One study showed that acute cannabis exposure in inexperienced users produced a global CBF decrease, whereas in experienced users CBF increased in both hemispheres, but primarily in the frontal and left temporal regions. The authors attributed the decrease in CBF in inexperienced subjects to their increased anxiety following cannabis administration, and the increase in CBF in experienced users was attributed to pharmacological effects of cannabis (Mathew and Wilson, 1992). The increased blood flow correlated with the levels of intoxication (Mathew *et al.*, 1992). Acute Δ^9-THC increased cerebral metabolic rate in humans and animals, though in humans the effects on the metabolic rate are probably limited to specific brain areas such as the cerebellum or prefrontal cortex (Margulies and Hammer, 1991; Volkow and Fowler, 1993). One study compared the acute effects of cannabis on three control subjects (who had used cannabis no more than once or twice per year) and three chronic subjects (who had used cannabis at least twice per week for at least ten years) (Volkow and Fowler, 1993). Control subjects had an increase in metabolic activity in the cerebellum and prefrontal cortex, and the subjects' subjective sense of intoxication correlated with the degree of increase in metabolism in the cerebellar cortex. Chronic users showed less change in regional metabolism and reported fewer subjective effects, perhaps reflecting tolerance to the effects of cannabis.

Immune System

With efforts to use either cannabis or synthetic cannabinoids for therapeutic purposes, one should consider the potential effects on the immune system, especially in patients with a compromised immune system, as reviewed by Hall *et al.* (1994). Determining if cannabinoids impair the immune system is complicated by several factors. First, the majority of the studies have been conducted *in vitro* with animal and human cell cultures or *in vivo* in animals. Extrapolating these results to humans is further complicated by the very high doses of cannabinoids used in the studies. Second, the few *in vivo* human studies have produced conflicting results. Third, very few epidemiological studies assessing disease susceptibility in heavy chronic cannabis users have been conducted.

Cannabinoids probably exert their actions through both cannabinoid receptor and non-receptor, or nonspecific, mechanisms, since high concentrations are often needed to elicit an effect. A nonspecific indication of an effect on the immune system is a decrease in weight of lymphoid organs (Munson and Fehr, 1983). Cannabinoids reduced the weight of the thymus in monkeys, and in high doses cannabinoids affected the function of the stem cells and reduced the size of the spleen in rodents (Munson and Fehr, 1983).

The effects of cannabinoids on human, monkey and rodent macrophages have been studied both *in vivo* and *in vitro*. Cannabinoids can affect a macrophage's morphology, phagocytic and spreading ability, superoxide production and tumor necrosis factor and interleukin release. Rat alveolar macrophages were only moderately affected following 30 days exposure to cannabis smoke, with changes in morphology, superoxide production and oxygen consumption (Davies, Sornberger and Huber, 1979). Human pulmonary alveolar macrophages obtained from cannabis smokers displayed a suppression of superoxide production (Sherman *et al.*, 1991). Macrophages from monkeys exposed to cannabis smoke for up to one year had altered morphology, including an increase in the number of vacuoles, and protein expression (Cabral *et al.*, 1991). THC adversely affected

the phagocytic and spreading ability of macrophages from mouse peritoneal cultures (Lopez-Cepero *et al.*, 1986), and similar results occurred in human mononuclear phagocyte cultures (Spector and Lancz, 1991). Cytokine, or interleukin, production in macrophages was also altered by THC. Interleukin 1 (IL1) bioactivity and release were increased (Klein and Friedman, 1990; Shivers *et al.*, 1994), and antiviral factor production was suppressed (Cabral and Vasquez, 1992). Since tumor necrosis factor (TNF) levels were either increased (Shivers *et al.*, 1994) or decreased (Fischer-Stenger, Pettit and Cabral, 1993; Zheng, Specter and Friedman, 1992) depending upon the type of cell culture, the effect of cannabinoids on cytokine levels is probably modulatory.

The effects of cannabinoids on the humoral immunity (production of B lymphocytes) and cell-mediated immunity (T lymphocyte production) are inconsistent. Conflicting *in vivo* studies were generated in the 1970s, with cannabinoids either suppressing human and monkey leukocyte numbers and functions (Gupta, Grieco and Cushman, 1974; Nahas *et al.*, 1974) or not affecting lymphocytes (Lau *et al.*, 1976; Rachelefsky *et al.*, 1976; Silverstein and Lessin, 1974). These studies were often performed with human patients without controlling lifestyle factors. In monkey studies conducted during the same period, blood cell mitogen responses and serum antibody (IgG and IgM) levels were significantly reduced in monkeys chronically treated with THC for six months (Daul and Health, 1975). In another study, rhesus monkeys treated with THC for three weeks had elevated neutrophil levels; lymophocytes were not affected (Silverman *et al.*, 1982). A more recent study reported that in human outpatient cannabis abusers, the T cell CD4/CD8 ratio increased (Wallace *et al.*, 1988). CD4 and CD8 are cell-cell adhesion glycoproteins on the surface of T cells that act to stabilize the binding T cell receptors and antigen complexes on the target cell. However, Dax *et al.* (1989) demonstrated that in institutionalized patients receiving small amounts of cannabis for three weeks, white blood cell and subset lymphocyte counts and killer cell activity were unaffected. When the

amount of THC and length of exposure time increased, IgG antibody levels decreased; IgD antibody levels increased, and IgA and IgM levels were unaffected (Nahas and Ossweman, 1991). From these studies, one can conclude that cannabis smoking appears to produce moderate disturbances in lymphocyte activity in humans and monkeys *in vivo.* However, the clinical relevance of these findings are uncertain (Hollister, 1988).

Cannabinoids also affect the function of cultured human lymphocytes. THC suppresses leukocyte migration (Schwartzfarb, Needle and Chavez-Chase, 1974) and lymphoproliferation (Nahas, Morishima and Desoize, 1977). Again, these effects occurred upon exposure to high doses. Spector and Lancz (1991) showed that 11-OH-THC suppressed natural killer (NK) cell activity. The mechanism for some of the effects of THC might involve adenylyl cyclase activity since THC suppressed agonist-induced cAMP in lymphocyte cultures (Diaz, Spector and Coffey, 1993). Cytokine levels in human lymphoid cultures either increased or decreased (Watzl, Scuderi and Watson, 1991).

Many reports provide evidence that cannabinoids affect the immune system of rodents. *In vitro* studies performed with rodent lymphocytes indicate that cannabinoids suppressed antibody production (Bacztnsky and Zimmerman, 1983; Klein and Friedman, 1990), though the molecular mechanism for these effects remains unknown. B lymphocytes appear to be more sensitive to cannabinoid suppression than T lymphocytes (Klein *et al.*, 1985). Drug-induced suppression of antibody production is the most consistently reported observation in cannabinoid studies in the immune system. The effects of cannabinoids upon T lymphocyte proliferation do not always lead to suppression, suggesting that cannabinoids act as modulators (Luo *et al.*, 1992; Pross *et al.*, 1992).

Several studies have suggested that cannabinoids decrease host resistance to infection. Cannabinoids caused enhanced mortality in rodents to *Lysteria monocytogenes* and Herpes simplex type II virus (Morahan *et al.*, 1979). Extrapolating these results to humans is difficult since drug doses that had the greatest effect were in the 100 mg/kg

range. In more recent studies, bacterial infections in mice have been examined using THC in the range of 5 mg/kg (Klein *et al.*, 1993; Klein, Newton and Friedman, 1994). The effects of THC on resistance to infection depended on the dose and timing of injection. If mice were given two THC injections (8 mg/kg), one day before and one day after infection with *Legionella pneumophilia*, they displayed mortality within minutes of the second injection (Klein *et al.*, 1993; Klein, Newton and Friedman, 1994). Animal studies confirmed that cannabinoids decreased antibacterial (Ashfaq, Watson and ElSohly, 1987) and antiviral activity (Cabral, Lockmuller and Mishkin, 1986) of the host immune system.

Recent research has investigated the expression of central and peripheral cannabinoid receptors in immune tissues (Galiegue *et al.*, 1995). The CB_1 receptor is expressed in human immune-related tissues, including bone marrow, thymus and tonsils. Levels of CB_2 receptor expression were 10-100-fold higher in immune tissues than levels of CB_1 receptor expression. The rank order of CB_2 mRNA in human blood cell populations was B-cells > NK cells >> monocytes > polymorphonuclear neutrophil cells > T8 cells > T4 cells. An immunohistological analysis performed on tonsil sections showed that CB_2 expression was restricted to B-lymphocyte-enriched areas of the mantle of secondary lymphoid follicles. Expression of the receptors in components of the immune system provide further evidence for cannabinoid immunosuppressive events.

Several studies have examined the effects of endogenous ligands on the immune system. Although anandamide bound to the CB_2 receptor transfected in CHO cells, it did not inhibit CB_2 coupled adenylyl cyclase activity (Bayewitch *et al.*, 1995). Anandamide did inhibit T and B lymphocyte proliferation and at high doses induced apoptosis (Schwartz, Blanco and Lotz, 1994). The authors concluded that since anandamide is made in the brain and affects the immune system, it may participate in neuroimmune interactions (Schwartz, Blanco and Lotz, 1994). Lee *et al.* (1995) demonstrated that 2-arachidonyl glycerol, but not anandamide, exhibited biological activity on spleen cell function. These

findings suggest that centrally produced endogenous cannabinoids are most efficacious at mediating effects in neural tissue, and peripherally produced compounds exhibit greater efficacy on peripheral targets (Lee, Yang and Kaminski, 1995). Additional research is necessary to clarify the role of endogenous anandamides in the immune system.

Cardiovascular System

Cannabinoids also affect the cardiovascular system. THC can induce tachycardia, orthostatic hypotension and decreased platelet aggregation (Clark *et al.*, 1974; Merritt *et al.*, 1980; Schaefer *et al.*, 1979). In the rat, a transient pressor response is followed by hypotension and bradycardia (Dewey, 1986). Changes in the electrocardiogram include varied P and T waves and decreased ST segments (Johnson and Domino, 1971). Exposure to cannabinoids may aggravate pre-existing conditions such as angina and congestive heart failure. Hypotension and bradycardia result after prolonged exposure in humans (Benowitz and Jones, 1975). After high doses in humans, conjunctivae redden due to dilation of blood vessels and increased heart rate with a concomitant peripheral vasodilation (Dewey, 1986).

Recent work by Varga *et al.* (1995) implicates the involvement of the CB_1 receptor in the hypotensive action of anandamide. Anandamide produced a brief pressor response and a more prolonged depressor response. Only the depressor response was inhibited upon administration of the cannabinoid antagonist SR 141716A. In addition, either cervical spinal cord transection or blockade of α-adrenergic receptors attenuated the depressor response. These results suggest that the pressor component of anandamide's cardiovascular response results from a peripheral action not mediated by the CB_1 receptor or the sympathetic nervous system (Varga *et al.*, 1995). The depressor response is due to inhibition of sympathetic tone mediated by CB_1 receptors.

Human Psychopharmacology

Cannabinoids produce a variety of acute psychological effects in humans. THC is rapidly absorbed after smoking, and acute peak effects appear between 30 and 60 minutes. When cannabis is ingested, the onset of action is slower, and subjective effects last for 5-12 hours without a clear peak. Acute subjective effects are dose-dependent. It is still unknown whether cannabis hinders performance and produces a subtle hangover syndrome due to residual effects of cannabinoids during the day after smoking. The subjective acute effects of cannabis are very diverse. One characteristic of cannabis use is a state of intoxication or euphoria and relaxation, followed by drowsiness, sedation and sometimes depression (Hollister, 1986). Other symptoms accompanying euphoria include alterations of motor control, sensory functions and cognitive (decision-making) processes (Nahas, 1993). Users of cannabis also claim that the drug heightens sensitivity to external stimuli, brightens colors and enhances music appreciation. A recent extensive review concludes that at doses which produce a moderate level of intoxication, a wide range of learned and unlearned behaviors, including simple motor tasks and complex psychomotor and cognitive tasks were affected (Chait and Pierri, 1992). They concluded, after an evaluation of the literature, that cannabis adversely affected gross and simple motor tasks (body sway and hand tremor), psychomotor behavior (rotary pursuit, Digit Symbol Substitution, reaction time, accuracy in divided attention and sustained attention). Cannabis had weak effects on simple reaction time and inconsistent effects on hand-eye-coordination. Data from Heishman *et al.* (1990) indicate that cannabis can impair complex human performance in arithmetic and recall tests up to 24 hours after smoking.

Scientific evidence suggest that marijuana impairs memory and learning. Δ^9-THC causes its greatest and most consistent effects in short-term memory, as measured in free recall of previously learned items. The major impairment by cannabis in free recall studies produces substantial increases in memory intrusions (Chait and Pierri, 1992). Neither

immediate and sustained attention nor controlled retrieval from semantic memory were affected. Thus, THC probably impairs acquisition and working memory but not retrieval processes. The effects of cannabis upon recall in the digit span, recognition and paired-associate memory performance tasks have been inconsistent (Chait and Pierri, 1992; Schwartz, 1993). Generally, cannabis did not affect the retrieval of previously learned facts. Although the acute effects of THC on memory appear modest, there are concerns about the effects of chronic use upon adolescent development.

THC does alter time perception, producing an overestimation of elapsed time (Chait and Pierri, 1992). Associated with the altered time sense is temporal disintegration, which is defined as difficulty in retaining and coordinating memories and perceptions relevant to a goal the user is perusing (Melges *et al.*, 1970). The effect of changed time perception and short-term memory disruption might be reflected in decreased driving and occupational skills, but evaluation of work productivity in chronic users has not detected major decrements in work performance (Hollister, 1986).

Impairment of both cognition and motor control has been documented in a laboratory setting and proposed as a contributor to accident and traffic fatalities (Aussedat and Niziolek-Reinhardt, 1993) and non-vehicular accidents (Soderstrom *et al.*, 1993). However, based upon a review of the literature, no clear relationship has been shown between cannabis smoking and either seriously impaired driving performance or the risk of accident involvement. The extent that cannabis contributes to traffic accidents is not known with certainty. Results from laboratory studies and driving simulations are reviewed extensively by Chesher (1995) and Robbe (1994). Laboratory studies have shown performance impairment occurring after inhaled doses of cannabis as low as 40 µg/kg. Cannabis produces a dose-dependent impairment on specific skills, which become pronounced after 100-200 µg/kg doses. In particular, tracking, divided attention and

vigilance tests performance are affected by THC. In contrast, results from driving simulator and closed-course tests surprisingly indicate that THC in single-inhaled doses up to 250 µg/kg has relatively small effects on driving performance. Explaining the disparity in results obtained in laboratory studies and in driving simulations is difficult. Recently, Robbe (1994) performed a series of studies which evaluated the effects of cannabis smoking on actual driving performance and compared these results to the effects of alcohol on driving. Several driving tests were employed including maintenance of a constant speed and lateral position during uninterrupted highway travel, following a lead car with varying speed on a highway and driving in a city. Cannabis produced a moderate degree of impairment, which was related to the THC dose. At a dose of 300 µg/kg THC impaired road tracking ability and slightly impaired the ability to maintain a constant headway when following another car. A low THC dose (100 µg/kg) did not impair driving ability in the city to the same extent as a blood alcohol concentration of 0.04%. Drivers under the influence of marijuana tended to overestimate the level of impairment and compensate by concentrating on driving and/or slowing down. In contrast, drivers under the influence of alcohol tended to underestimate the effects of alcohol and not make allowances for impairment. Several studies have also attempted to determine the incidence of cannabis involved in road crashes in which the driver had consumed cannabis and was responsible for the collision. Three studies have reported that cannabis-bearing drivers were no more responsible than the non-drug-bearing drivers (Drummer, 1994; Terhune *et al.*, 1992; Williams *et al.*, 1985). This finding must await clarification until sample sizes are greatly increased. Robbe (1994) concluded that while campaigns to discourage the use of cannabis by drivers are warranted, concentrating upon cannabis alone may not be in proportion to the safety problem it causes.

Several factors complicate the interpretation of cannabis-induced impairment, such as co-use with other drugs, variability among individuals, development of tolerance and

intrinsic difficulties in conducting a systemic evaluation in the general population. Cannabis is often co-abused with other drugs, such as alcohol. Co-use of cannabis with alcohol (Wechsler *et al.*, 1984) or phencyclidine (PCP) (Poklis, Maginn and Barr, 1987) might augment cannabis' effects. Results indicate that performance disruption was greater for alcohol-induced impairment in combination with cannabis (Hollister, 1986). It has been reported that ethanol-induced dose-dependent decrements in performance skill required for automobile driving were further exacerbated by cannabis (Perez-Reyes *et al.*, 1988). Tolerance does develop during chronic exposure to high quantities of cannabis, but the degree of tolerance following intermittent exposure to cannabis is less definitive (Hollister, 1986). Detecting cannabis intoxication by motor performance in an experienced user may be difficult unless a complex performance task is assessed or if the user has had experience in the task (Chait and Pierri, 1992). Cannabis intoxication in an inexperienced user is readily detectable by many performance tests. Establishing a degree of correlation between the level of impairment and blood concentrations of cannabinoids would aid in determining causality in accidents. In chronic users, tolerance may develop to some of the acute effects. Thus, the level of intoxication is more difficult to detect in an experienced user except in novel tasks or tests requiring a great deal of skill or manual dexterity (Chait and Pierri, 1992). Given the confounding factors discussed above, it is unlikely that measures of Δ^9-THC and its metabolites will become standards for intoxication.

Human studies have been conducted to determine if a state-dependent learning effect exists for cannabis. The first evidence of a cannabis-induced state dependency was reported by Abel (1970). Subjects learned narrative material while exposed to cannabis and were tested in a sober or cannabis-intoxicated state (Abel, 1970). A greater deficit of recall was recorded for subjects tested in the sober state. Evidence also exists that the state-dependent learning effects of cannabis are most apparent in tasks using sequential memory (Hill *et al.*, 1973; Stillman *et al.*, 1974). The state-dependent learning effect for cannabis is

observed in memory tasks rather than psychomotor or adversely motivated tasks (Järbe *et al.*, 1993). Difficult tasks, such as active recall, are also affected by state-dependent learning (Järbe *et al.*, 1993). In order to determine the influence of the frequency of use upon cannabis' effects on memory, one study differentiated between heavy and social users (Cohen and Rickles, 1974). Subjects in the heavy-user group average smoking cannabis five to six times per week for a year. The social-user group smoked on weekends. The frequency of use had profound effects on the state-dependent learning effects of cannabis. In recall tests, social-users did exhibit state-dependent effects, whereas heavy users did not. The heavy-user group performed equally well whether intoxicated or not, and they performed better in recall than social-users.

Since one of the well known acute effects of cannabis is to impair cognitive functioning, it has long been suggested that chronic cannabis use may cause lasting cognitive impairments. Assessing the chronic effects of cannabis or any other psychoactive drug on cognitive functioning is often difficult since many factors other than drug use must be controlled. Difficulties encountered when attributing cognitive effects to psychoactive drugs include determining levels of cognitive impairment, which might have preceded drug use, determining the duration and frequency of drug use and taking into account effects of multiple drug use. It has been proposed that chronic use might result in long term memory impairment (Schwartz, 1993). Yet, previous reviews have generally concluded that evidence is insufficient to conclude that long term use of cannabis produces lasting gross cognitive impairment (Wert and Raulin, 1986). Solowij (in press-b) and Pope *et al.* (1995) have reviewed recent, more methodologically rigorous research which used improved test procedures and electrophysiological methods. These findings provide evidence that cannabis produces complex and subtle impairments, which are related to the duration of cannabis use. Impairments appear specific to higher cognitive functions, such as the organization and integration of complex information involving attention and memory

processes (Solowij, in press-b). It has been hypothesized that long term cannabis use impairs the frontal lobe, an area of the brain which functions in the temporal organization of behavior. This hypothesis is consistent with the altered perception of time and with cerebral blood flow studies which demonstrate greatest effects in the frontal lobe region. Recent studies also suggest that impairment assessed by sensitive measures of brain function can be detected after only five years of use. Not all individuals are affected equally by long term use, and often the effects are subtle. However, one should not underestimate the effects of even subtle impairment of cognitive functioning on daily life.

Great interest has been generated in the effects of cannabis upon adolescent development and educational performance and production of a cannabis-induced "amotivational syndrome." A modest statistical relationship may exist between cannabis and other illicit drug use and poor educational performance (Schwartz, 1993). Some individuals suffer no memory impairment at all, whereas those individuals who already have a learning disability are more susceptible to memory disruptions than a gifted student group (Schwartz, 1993). Attempts to verify the existence of a cannabis-induced "amotivational syndrome" have failed (Dewey, 1986; Foltin *et al.*, 1989; Foltin *et al.*, 1990; Hollister, 1986). The lack of motivation observed in some individuals more likely results from psychosocial problems and polydrug use rather than solely cannabis use (Taschner, 1983). Additional research should address the impact of long term cannabis use on cognitive development in adolescents.

Since THC produces diverse psychological effects in humans, it has been suggested that cannabinoids might induce psychopathological states (George, 1970; Talbott and Teague, 1969). However, identification of a specific "cannabis psychosis" even in chronic, heavy users has not occurred (Dewey, 1986; Hollister, 1986; Thornicroft, 1990). Cannabis does appear to worsen symptoms of some preexisting mental disorders, such as schizophrenia (Negrete, 1993). Even though paranoid schizophrenics recognize the

worsening of their disorder with cannabis use, many still continue to try to self-medicate themselves with the drug. Cannabis increases hallucinations and delusions and produces inconsistent results on the symptoms of social withdrawal and lethargy. While some investigators believe that cannabis use does lead to the development of schizophrenia, conclusive evidence does not exist that cannabis is a causative factor in the development of schizophrenia (Allebeck, 1993; Negrete, 1993). Individuals abusing cannabis who also develop psychiatric problems may suffer from rapid onset schizophrenia (Allebeck, 1993). Since most of these individuals are poly-drug users, it seems more likely that cannabis or any of the other abused drugs might act as a trigger for precipitating latent schizophrenia. The relative risk of developing psychiatric problems in the general population of cannabis users is apparently very small. Proper studies comparing the development of disorders in abusers and non-abusers have not been performed. However, given the world-wide and prevalent use of cannabis, one would expect to see more reported cases of cannabis-induced psychiatric disorders if cannabis readily caused them.

Human Tolerance and Dependence

In the late 60s and early 70s, there was considerable confusion regarding the development of tolerance to smoking cannabis. The well known phenomenon that many newcomers required several smoking episodes before experiencing the cannabis 'high' led to the hypothesis that "reverse tolerance" developed. The notion that tolerance could then develop to cannabis' psychotomimetic effects formed the basis of the proposed "reverse-reverse tolerance". There is no doubt that many factors, other than the inherent properties of Δ^9-THC, are contributors including potency of the cannabis, expectations, environmental influences, individual differences and frequency of use, to name just a few. Yet, convincing evidence exists for the development of tolerance to Δ^9-THC in humans (Jones, Benowitz and Bachman, 1976), as was described above for animals. Tolerance

developed to a variety of Δ^9-THC's effects, following oral administration, including cannabinoid-induced decreases in cardiovascular and autonomic functions, increases in intraoccular pressure, sleep disturbances and mood changes (Jones, Benowitz and Bachman, 1976). Results are less conclusive for behavioral tolerance. To achieve behavioral tolerance, high doses of Δ^9-THC were administered for a sustained period of time. In one study, tolerance to the subjective effects of Δ^9-THC developed after oral administration (10 mg) for several days; greater tolerance developed with increased amounts of the drug (Jones, 1983). Thus, if the doses of Δ^9-THC are small and infrequent, little behavioral tolerance develops. High doses must be given for long periods of time to produce tolerance.

Although it is established that chronic cannabis use does not result in severe withdrawal symptoms, numerous case reports attest to development of dependence (Jones, 1983). Several early reports came from countries where potent cannabis was used for long periods of time. Upon deprivation of cannabis, users experienced auditory and visual hallucinations and irritability (Fraser, 1949). Since that report, the development of tolerance and dependence have been studied under rigorous and controlled conditions (Jones, 1983; Jones and Benowitz, 1976; Jones, Benowitz and Bachman, 1976; Jones, Benowitz and Herning, 1981). In one study, a 30 mg dose of cannabis extract or Δ^9-THC was administered orally approximately six times per day for up to 21 days. The most prominent symptoms upon cessation of administration were increased irritability and restlessness. Other symptoms, though variable, included insomnia, anorexia, increased sweating and mild nausea. Objective symptoms were increased body temperature, weight loss and hand tremor. Re-administration of a cannabis cigarette or oral Δ^9-THC alleviated the objective and subjective effects, suggesting the establishment of a withdrawal symptom.

Potential Therapeutic Uses

The prevalence of cannabis use has resulted in intense efforts of cannabis research for the past several decades. Attempts have been made to discern the pharmacology of cannabis and the mechanism of action producing the psychoactive effects. In addition to exploring the euphoric effects of cannabis, emphasis has been placed upon the drug's therapeutic potential. Early crude preparations of cannabis were used to treat allergies and migraines and to facilitate childbirth (Mechoulam, 1986). The effective component, Δ^9-THC, was also used for alleviating pain, glaucoma, muscle spasticity, bronchial asthma and nausea (Hollister, 1986). However, the lack of evidence that cannabinoids are better than other drugs currently in use limits their clinical usefulness, and separating the undesired side effects of cannabis from the therapeutic effects has proven difficult. In addition, schedule II drugs require extensive record-keeping and cause other administrative problems. Pharmaceutical companies have marketed only Δ^9-THC which is used primarily as an antiemetic for cancer chemotherapy patients. The development of a cannabinoid analog possessing greater pharmacological selectivity is an important aim for future cannabinoid research.

Although many useful probes for determining the underlying mechanism of action for cannabinoids have been produced, no clinically relevant compound has emerged. The inability to separate the various pharmacological and psychoactive properties of the compounds remains the greatest impediment. Cannabinoids have generated interest over the centuries for their alleged ability to treat a wide range of disorders. Possible therapeutic uses include treatment of bronchial asthma, nausea, vomiting, pain, convulsions, glaucoma, muscle spasticity and loss of appetite (Hollister, 1986). Cannabinoids also represent a novel way to treat disorders not responding to traditional agents or therapies. Current debate in this country centers upon the possible legalization of cannabis for medicinal purposes. Proponents of legalization believe that the availability of THC for

medicinal purposes would eliminate the need for the crude plant product. While there may be some merit in legalization arguments, the development of a potent and selective cannabinoid possessing greater efficacy than current drugs would, of course, end the ongoing debate.

Cannabis has been used most frequently for treating refractory nausea and vomiting. In 1987, the United States Food and Drug Administration approved dronabinol, a Δ^9-THC formulation in sesame oil, for treatment of chemotherapy-induced nausea and vomiting not responding to other agents. Dronabinol has been useful, though some patients dislike the psychotropic effects and somnolence. Δ^9-THC has gained orphan status by the FDA to treat nausea from chemotherapy and to stimulate appetite in AIDS patients. Results from clinical trials have suggested that the drug improves appetite (Plasse *et al.*, 1991). However, one should remember that extensive animal studies indicate that cannabinoids adversely affect the immune system. Should a drug with possible immunosuppressive properties be given to patients who already have a compromised immune system? Only future research and more extensive clinical evaluation will determine if Δ^9-THC truly benefits these individuals.

Drug development also has focused upon the potent antinociceptive properties of cannabinoids. Great progress would be made in synthesizing an analgesic agent lacking the side effects and abuse liability of opioids. Unfortunately, cannabinoids produce antinociception at doses that also elicit other behavioral effects, such as sedation, hypothermia and catalepsy. Cannabinoids have a distinct pharmacological profile from the opioids and may act through a different mechanism for alleviating pain. Recent research demonstrated that a kappa receptor antagonist, *nor*-binaltorphimine (*nor*-BNI), blocked cannabinoid-induced antinociception, but did not affect the other behaviors (Smith, Welch and Martin, 1993). Perhaps this compound could be used to understand the mechanism of action for cannabinoid analgesia.

Dissertation Objectives

After years of research, anandamide has been proposed to be the elusive endogenous ligand for the cannabinoid receptor. Anandamide possesses many of the same pharmacological effects as Δ^9-THC and other exogenous cannabinoids, including production of antinociception, inhibition of locomotor activity, catalepsy and decrease in body temperature in mice. *In vitro*, anandamide mimics cannabinoids by inhibiting both forskolin-stimulated increases in adenylyl cyclase activity and the electrically-evoked murine vas deferens twitch response. Although anandamide does generate many of the same effects as other cannabinoids, differences do exist. The discovery of anandamide and other similar endogenous compounds provides the opportunity to investigate and understand the purpose of this novel neurochemical system. One of the problems encountered at the onset of anandamide research was the susceptibility of this compound to metabolism in both *in vitro* and *in vivo* systems. Developing a potent and stable anandamide analog would greatly facilitate determining the physiological and pharmacological role of anandamide. Examination of the structures of anandamide, CP 55,940, the potent bicyclic cannabinoid, and SR 141716A, the proposed antagonist of the central cannabinoid receptor, reveals great structural diversity. Anandamide is a fatty acid derivative. CP 55,940 is produced by the opening of the B ring in three ring system. Δ^9-THC and SR 141716A vastly differ in structure from either compound. The structural differences raise the question as to whether these three compounds are binding to the same receptor in the same manner.

While the discovery of anandamide provides an answer to the type of endogenous compound that binds to the cannabinoid receptor, many questions remain. The purpose of this dissertation is to investigate anandamide's interaction with the central cannabinoid receptor. The specific aims of this research are to:

1) Determine which structural features of anandamide are important for interaction with the central cannabinoid receptor, as assessed by *in vitro* binding studies and *in vivo* behavioral experiments

2) Structurally modify the template of anandamide in order to develop more potent and metabolically stable compounds

3) Characterize anandamide's binding to the cannabinoid receptor in the CNS by comparing anandamide's receptor binding affinities, as determined from autoradiographic experiments in rat brain from selected brain areas

4) Compare anandamide's receptor binding densities to the binding densities and patterns of two other compounds, CP 55,940 and SR 141716A, that bind to the central cannabinoid receptor

II. Evaluation of Cannabinoid Receptor Binding and *in Vivo* Activities for Anandamide Analogs

Introduction

Marijuana, or *Cannabis sativa*, is one of the oldest and most widespread drugs of abuse in use today. The psychoactive constituent of cannabis, Δ^9-THC, produces a unique pattern of behavioral effects, which include antinociception, catalepsy, anticonvulsive activity, hypothermia, hyperexcitability and depression of motor activity in a wide variety of animal species (Dewey, 1986). Structure-activity relationship studies suggested the existence of a specific cannabinoid receptor through which Δ^9-THC exerted behavioral and central effects (Razdan, 1986). The development of potent synthetic bicyclic cannabinoid agonists, such as CP 55,950, allowed for the characterization of a specific saturable high-affinity cannabinoid binding site in rat membranes (Devane *et al.*, 1988). The structure-activity profile indicated a correlation between cannabinoid receptor binding and pharmacological and behavioral effects (Compton *et al.*, 1993).

Our understanding of the cannabinoid system has progressed in a somewhat non-traditional manner in that characterization of the second messenger system proceeded the receptor binding studies. The expression of the cannabinoid receptor within the brain suggested the existence of an endogenous ligand. In 1992 a candidate ligand isolated from porcine brain displaced [^{3}H]-HU 243, a cannabinoid receptor ligand, from synaptosomal membranes in a dose-dependent manner (Devane *et al.*, 1992). The purified compound was determined to be arachidonylethanolamide, or anandamide, a derivative of arachidonic acid which bears no obvious structural similarity to either Δ^9-THC or CP 55,940. Like other cannabinoids, anandamide inhibited forskolin-stimulated cAMP production in CHO

cells expressing the human cannabinoid receptor and inhibited N-type calcium currents through a pertussis toxin-sensitive G protein (Felder *et al.*, 1993; Mackie *et al.*, 1993; Vogel *et al.*, 1993). Anandamide produced effects similar to Δ^9-THC, including antinociception, hypomotility, hypothermia and catalepsy in mice (Fride and Mechoulam, 1993; Smith *et al.*, 1994). However, anandamide differed pharmacologically from Δ^9-THC in that anandamide had a shorter duration of action in behavioral assays, was less potent and possessed different antinociceptive properties (Smith *et al.*, 1994).

It is highly likely that metabolism plays a role in the short duration of action of anandamide. The issue of metabolism was first raised with the report that PMSF (phenylmethylsulfonyl fluorine), an enzyme inhibitor of serine proteases, some thiol proteases and esterases such as erythrocyte acetylcholinesterase, interfered with the degradation of anandamide by inhibiting an amidase (Deutsch and Chin, 1993). Initial binding studies with anandamide competition for [^{3}H]-WIN 55,212-2 resulted in anandamide exhibiting low affinity for the receptor (Childers *et al.*, 1994). These researchers discovered that the addition of PMSF to the incubation condition allowed anandamide interaction with the receptor. Subsequent binding studies performed in our laboratory supported this finding in that the binding affinity of anandamide was enhanced several hundred fold with the addition of PMSF (Smith *et al.*, 1994). The fact that anandamide was found in brain and that both synthetic and degradative enzymes for anandamide are present in brain provide strong support for anandamide being an endogenous cannabinoid.

Anandamide had a shorter duration of action and more rapid onset than Δ^9-THC (Smith *et al.*, 1994). The shorter duration of action could be due to metabolic differences rather than separate mechanisms. Anandamide is metabolized to arachidonic acid by an amidase (Deutsch and Chin, 1993), whereas Δ^9-THC is not. Although anandamide produced antinociception, the mechanism by which it does so may be different than Δ^9-

THC since *nor*-BNI, a kappa opioid antagonist that blocks Δ^9-THC-induced antinociception, did not block anandamide-induced antinociception (Smith *et al.*, 1994). While much evidence supports anandamide being the endogenous ligand, this dissimilarity demonstrates that the actions of anandamide and Δ^9-THC are not identical.

At the present time, only limited structure-activity evaluations have been conducted with anandamide (Felder *et al.*, 1993; Mechoulam *et al.*, 1994). The first objective of this study was to determine the structural requirements important for anandamide's interaction with the cannabinoid receptor. To accomplish this objective, rapid filtration binding studies employing [^{3}H]-CP 55,940 as the radioligand were conducted in the presence and absence of PMSF. Behavioral activity was assessed by the ability of the analogs to produce hypomotility and antinociception. The affinities of anandamide analogs for the binding site were correlated with *in vivo* pharmacological potencies. These correlations were then compared to those obtained for other cannabinoid agonists. The second objective was to develop metabolically stable and potent analogs of anandamide. If compounds were stable in binding experiments both with and without addition of an enzyme inhibitor, then these compounds might also be stable *in vivo*. Analogs with increased potency and stability would aid in the determination of the physiological role of anandamide.

Materials and Methods

Male Sprague-Dawley rats (150-200 g) and male ICR mice (18-25 g), both from Harlan (Dublin, VA), received food and water *ad libitum* and were maintained on a 14:10 hr light/dark cycle. CP 55,940 was a gift from Dr. L. Melvin of Pfizer Inc., Central Research Division (Groton, CT). [^{3}H]-CP 55,940 and [^{3}H]-anandamide were purchased from DuPont-NEN (Wilmington, DE). [^{3}H]-Anandamide was labeled at carbons 5, 6, 8,

9, 11, 12, 14 and 15. All anandamide analogs were prepared by Dr. Raj Razdan of Organix Inc. (Woburn, MA).

Drug preparation and administration. For binding assays, compounds were prepared as 1 mg/ml stock solutions in absolute ethanol and were stored at -20 °C. If employed in behavioral assays, drugs were dissolved in a 1:1:18 mixture of ethanol, emulphor (International Specialty Products, Linden, NJ) and saline (0.9% NaCl) and were administered intravenously (i.v.) in the mouse tail vein in volumes of 0.1 ml/10 g of body weight.

Binding assays. Radioligand binding to P_2 membrane preparations was performed as described elsewhere (Compton *et al.*, 1993), with the exception that P_2 pellets were prepared from whole rat brains, not cortices. Ethanolic 1 mg/ml stock solutions of anandamide, anandamide analogs and CP 55,940 were diluted in buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM $MgCl_2$ and 5 mg/ml BSA) without evaporation of the ethanol (final concentration not exceeding 0.4%). Saturation studies were performed to determine the K_d of CP 55,940 in the presence and absence of PMSF (50 µM). [^{3}H]-CP 55,940 (50 pM - 10 nM), competing unlabeled CP 55,940 (1 µM) and sufficient buffer to bring the final reaction volume to 1 ml were added to siliconized glass tubes. Binding was initiated with the addition of 150 µg of membrane protein. In competition studies, analog concentrations ranging from 1 nM to 10 µM and a 1 nM concentration of [^{3}H]-CP 55,940 were used. Nonspecific binding was determined in the presence of 1 µM CP 55,940. Saturation and competition experiments were also performed with and without 50 µM PMSF. After a one-hour incubation at 30 °C, the reaction was terminated with the addition of 2 ml ice-cold buffer (50 mM Tris-HCl, 1 mg/ml BSA) followed by rapid filtration through PEI-treated filters. The assays were performed in triplicate, and the results represent the data from three to six independent experiments.

Behavioral evaluations. Mice were acclimated to the laboratory overnight. Depression of locomotor activity was measured by placing mice into individual photocell activity cages (11 x 6.5 in) 5 min following an i.v. injection. For the next 10 min the total number of beam interruptions in the 16 photocell beams per cage were recorded using a Digiscan Animal Activity Monitor (Omnitech Electronics Inc., Columbus, OH). Spontaneous activity (SA) was expressed as percent of control activity. Antinociception was assessed by measuring the tail-flick (TF) response to a heat stimulus (Dewey *et al.*, 1970). Control latencies of 2 to 4 sec were obtained for each animal with a standard tail-flick apparatus prior to drug or vehicle administration. A 10 sec maximum latency was set to avoid tissue damage. Mice then were re-tested 15 min after drug injection, and differences in latencies to the tail-flick response were recorded. Each dose tested in the antinociception and hypomotility assays represents a separate group of animals (six mice per group). Antinociception was expressed as the %MPE (maximum possible effect), which was calculated as:

$$\%\text{MPE} = \left[\frac{(\text{test latency} - \text{control latency})}{(10 \text{ sec} - \text{test latency})} \right] \times 100.$$

Time Course Study. Animals were injected i.v. with either anandamide, compound 16 or vehicle. At 5, 15, 30 and 60 min following injection, the animals were tested in the tail-flick assay. Separate groups of animals (six mice per group) were used for each time point and drug.

Anandamide Metabolism in the Receptor Binding Assay. [^{3}H]-Anandamide (0.1 μCi) (specific activity = 207 Ci/mmol), 1.5 μM anandamide, 150 μg membrane protein and sufficient buffer to bring the final reaction volume to 1 ml were

added to siliconized glass tubes. The experiment was performed either with or without PMSF (50 μM) in triplicate. Following a one-hour incubation at 30 °C, samples were extracted twice with 2 ml acidified ethyl acetate. The organic layer was filtered through glasswool, dried completely and re-suspended in 500 μl ethanol. Samples were analyzed by high performance liquid chromatography. The mobile phase was composed of MeOH:H_2O:acetic acid (85:15:0.05). A reverse phase C15 5 μm absorbance column was used with a 50 mm hand-packed guard column in line. Sample flow rate was 1 ml/min for 30 min through a radiomatic detector with a scintillant flow of 3 ml/min.

Data Analysis. The B_{max} and K_d values obtained from the Scatchard analysis (Rosenthal, 1967; Scatchard, 1951) of the saturation experiments were calculated from the KELL binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ). IC_{50} values for the analogs were determined and then converted to K_i values (Cheng and Prusoff, 1973). Statistical evaluation of parallelism between displacement curves generated in the presence and absence of PMSF were performed using ALLFIT (De Lean, Munson and Rodbard, 1978). Dose-response relationships were determined for each analog in the pharmacological assays. Antinociception and hypomotility data were converted to probit values, and ED_{50}'s were calculated by unweighted least-squares linear regression analysis of the log dose versus the probit values. Statistical analysis of the behavioral data was performed using ANOVA with Dunnett's t-test for comparison to the vehicle group. Comparisons between binding data (log K_i) and behavioral data (log ED_{50} values expressed in μmol/kg) were made with the Spearman rank order correlation test.

Results

Scatchard analysis. Saturation experiments were conducted in the absence and presence of 50 μM PMSF to determine if PMSF altered the affinity of [^{3}H]-CP 55,940 for

the binding site in whole brain P_2 membranes. Computer analysis of saturation data (means ± S.E., n = 5) without PMSF indicated a K_d of 580 ± 70 pM, a B_{max} of 2.09 ± 0.23 pmol/mg protein and a Hill coefficient of 0.96 ± 0.05. Incubation with PMSF produced a K_d of 1030 ± 130 pM, a B_{max} of 2.25 ± 0.19 pmol/mg protein and a Hill coefficient of 0.94 ± 0.02 (means ± S.E., n = 5). B_{max} and Hill coefficient values were the same in the presence and absence of PMSF. However, the addition of PMSF produced a statistical difference, as determined by Dunnett's t-test ($P < 0.05$), between the K_d values. The K_i's of the analogs were calculated using the appropriate K_d of [^{3}H]-CP 55,940.

Determination of Analog Affinity Constants. One of the main objectives of this investigation was the identification of structurally important features of anandamide required for cannabinoid receptor binding. Anandamide, an eicosanoid with cis double bonds at carbons five, eight, eleven and fourteen, is composed of arachidonyl and ethanolamide moieties joined through an amide linkage. The ability of anandamide to compete for [^{3}H]-CP 55,940 binding is depicted in Figure 6. The average K_i with PMSF was 89 ± 10 nM (n = 4). The average K_i increased to 5400 ± 1600 nM (n = 3), and the displacement curve shifted to the right when PMSF was not added. Since the affinity of anandamide was substantially decreased without the addition of an enzyme inhibitor, all displacement experiments were routinely performed in the presence of PMSF. Selected analogs were subsequently evaluated in the absence of PMSF to assess the influence of metabolism on binding. If an analog exhibited low affinity in the presence of the amidase inhibitor, additional binding studies were not repeated without PMSF.

Chemical modifications made to the backbone of anandamide created a diverse series of anandamide analogs. The first series of compunds consisted of changes in the double bond arrangement (Table 1). When a single double bond was placed at carbon 11, as in compound 1, a K_i of greater than 10,000 nM was obtained, as compared to anandamide's K_i of 89 nM. Any compound displaying a K_i of 10,000 nM or greater was

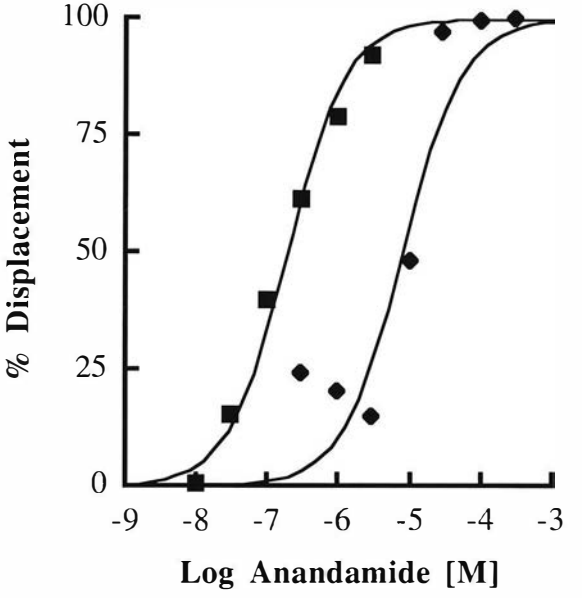


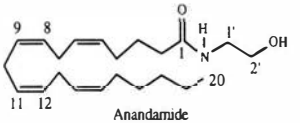
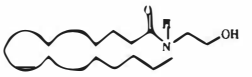
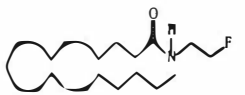
Figure 6. Anandamide displacement of [^{3}H]-CP 55,940 binding in the absence and presence of 50 μM PMSF. The data are presented as percent displacement of total binding. The K_i in the presence (■) of PMSF was 89 ± 10 nM; in the absence (◆) of PMSF the K_i equaled 5400 ± 1600 nM. K_i = mean ± S.E. of at least three experiments each performed in triplicate.

considered inactive in the binding assay. Adding a fifth double bond at carbon 17 resulted in an analog (compound 2) with a K_i of 1470 nM. Thus, these double bond changes in the anandamide backbone produced compounds with lower binding affinities to the receptor. K_i's were not determined without PMSF for compounds 1 and 2. Compounds 3 and 4 were completely saturated, and in compound 4 a 2'-fluoroethyl group replaced the 2'-hydroxylethyl group. However, these compounds were insoluble in ethanol which prevented the determination of their K_i's.

Analogs also were synthesized with changes in the ethanolamide constituent, as listed in Table 2. Replacement of the N-ethanolamide with either a hydroxypropyl or a hydroxypentyl group, as in compounds 5 and 6, respectively, resulted in analogs with lower affinity than that of anandamide. Therefore, progressively lengthening the N-substituent chain decreased the affinity of the analogs. In compound 7 substitution of the ethanolamide constituent with a sulfonamide functional group resulted in a compound with a K_i close to that of anandamide. The K_i increased significantly without PMSF, although to a somewhat lesser extent than with anandamide. Substitution with a bromobenzenesulfonamide (compound 8) created an inactive analog. The morpholine derivative of anandamide (compound 9) had an affinity that was approximately 10 times less than anandamide in the presence of PMSF.

A third series of analogs was synthesized in which several subsitutents were substituted for the hydroxyl in anandamide (Table 3). Substitution with bulky sulfonamide (compound 10) or phenoxyethyl (compound 11) groups produced analogs with affinities less than anandamide in the presence of PMSF, with the sulfonamide derivative having higher affinity than the phenoxyethyl derivative. Both compounds in the absence of PMSF had K_i's approximately two times higher than in the presence of PMSF. However, their affinities in the absence of PMSF were less than that of anandamide in the presence of PMSF. Substitution of the hydroxyl group with a fluorine moiety (compound 12), yielded

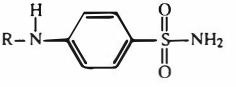
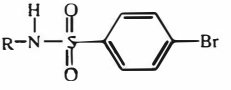
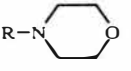
Table 1. Comparison of pharmacological potency and receptor affinities of anandamide analogs with varying degress of saturation.

Compound	Structure	K_i (PMSF) (nM)	K_i (nM)	S.A. ED_{50} (µmol/kg)	Tail-Flick ED_{50} (µmol/kg)
	 Anandamide	89 ± 10	5400 ±1600	50.3	17.4
1		> 10,000	nd[a]	77.2	110.3
2		1470 ± 500	nd	167.1	141.9
3		—[b]	—	50.3	261.5
4		—	—	5.3	10

[a]not determined

[b]unable to determine due to solubility problems

Table 2 Comparison of pharmacological potency and receptor affinities of anandamide analogs with substitutions for ethanolamide.

Compound	Structure	K_i (PMSF) (nM)	K_i (nM)	S.A. ED_{50} (μmol/kg)	Tail-Flick ED_{50} (μmol/kg)
5		189 ± 73	nd	43.7	61.4
6		1860 ± 300	nd	49.5	72.9
7		118 ± 29	1410 ± 470	37.5	148.2
8		> 10,000	nd	> 167	> 167
9		1150 ± 300	nd	20.7	25.4

[c]arachidonyl

Table 3. Comparison of pharmacological potency and receptor affinities of anandamide analogs with substitutions for hydroxyl.

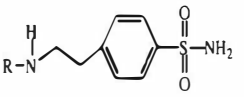
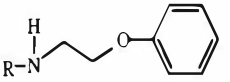
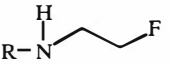
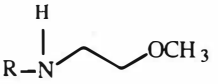
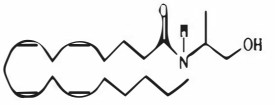
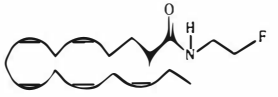
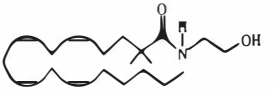
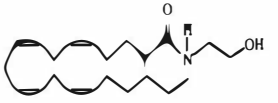
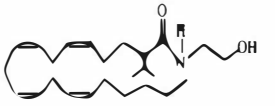
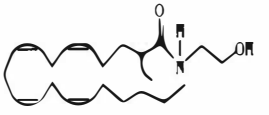
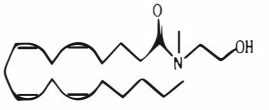
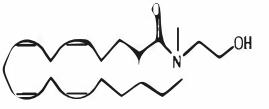
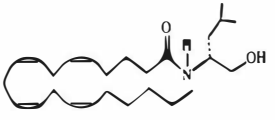
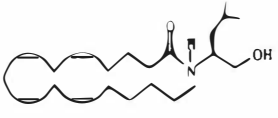
Compound	Structure	K_i (PMSF) (nM)	K_i (nM)	S.A. ED_{50} (μmol/kg)	Tail-Flick ED_{50} (μmol/kg)
10		163 ± 56	250 ± 70	44.6	142.4
11		400 ± 110	687 ± 107	23	74.1
12		8.6 ± 1.1	2130 ± 460	57.2	190
13		1820 ± 280	nd	186.1	> 276

Table 4. Comparison of pharmacological potency and receptor affinities of alkylated anandamide analogs.

Compound	Structure	K_i (PMSF) (nM)	K_i (nM)	S.A. ED_{50} (µmol/kg)	Tail-Flick ED_{50} (µmol/kg)
14		137 ± 13	87 ± 18	14.1	62.8
15		5.7 ± 12.1	15 ± 6	13.2	19.3
16		47 ± 2	41 ± 3	30.5	49.6
17		53 ± 11	137 ± 20	20.4	23.5
18		5030 ± 760	nd	> 152	> 152
19		461 ± 42	285 ± 58	18% @ 157	64.8
20		4980 ± 376	5240 ± 2025	98.3	32% @ 164
21		2330 ± 387	2250 ± 800	103	39% @ 160
22		5420 ± 1450	> 10,000	0% @ 149	32% @ 149
23		> 10,000	> 10,000	4% @ 242	30% @ 242

results resembling anandamide: a higher K_i in the absence of PMSF. Also, this compound had an affinity 10 times greater than that of anandamide in the presence of PMSF. An analog with a methoxy substitution (compound 13) had very low receptor affinity.

In the final series of analogs (Table 4), methyl derivatives were prepared with the intention of sterically interfering with binding to metabolic enzymes. These analogs, with the exception of compound 18 (isopropyl at carbon 2), produced K_i's similar to anandamide in the presence and absence of PMSF. The affinity of compound 17 (methylation at carbon 2) in the absence of PMSF was 2 times lower than anandamide in the presence of PMSF, yet compound 17 still had a higher affinity than many of the analogs. Compound 15, possessing methylation at carbon 2 and a fluorine substitution for the hydroxyl group, had high affinity with and importantly without PMSF. A previously discussed fluorine analog (compound 12) had a low K_i only with PMSF, supporting the idea that methylation at carbon 2 interferes with metabolism in this *in vitro* system. Insertion of an ethyl group at carbon 2 (compound 19) reduced the affinity of the analog in the presence of PMSF, compared to anandamide. Modest differences in binding were obtained in the absence of PMSF. However, unlike anandamide, the compound displayed slightly higher affinity without PMSF. Addition of an isopropyl group at carbon 2 (compound 18) greatly reduced affinity regardless of whether PMSF was present. Two analogs were made in which the nitrogen was methylated. Compound 20 (methylation at nitrogen) and compound 21 (methylations at nitrogen and carbon 2) had low affinities in the presence and absence of PMSF.

One of the criteria in establishing the existence of a receptor is proving enantioselectivity. A pair of enantiomers therefore were prepared with the intention of demonstrating enantioselectivity of an anandamide-like compound for the cannabinoid receptor. Recently, Abadji *et al.* (1994) demonstrated that (R)-(+)-arachidonyl-1'-hydroxy-2'-propylamide bound to the cannabinoid receptor with and without PMSF and

produced cannabimimetic activity *in vivo*. Compounds 22 and 23 (R- and S-forms, respectively) are 1'-isobutyl analogs of anandamide. Both compounds possessed a K_i of > 10,000 nM in the absence of PMSF. Any compound with a K_i of greater than 10,000 nM is generally found to be pharmacologically inactive, as is demonstrated here. With the inclusion of PMSF, the R-form bound with weak affinity, and the S-form was inactive.

Behavioral studies. The second objective of this study was to evaluate the analogs in two behavioral assays that assess cannabimimetic activity. Following an i.v. injection, the dose responsiveness of anandamide and anandamide analogs on spontaneous activity and production of antinociception was measured. From these data, ED_{50} values were calculated for each compound. The ED_{50} of anandamide was 50.3 µmol/kg for the hypomotility assay and 17.4 µmol/kg for the antinociception assay (Smith *et al.*, 1994).

Compounds 1 and 2 were not potent in producing either hypomotility or antinociception, which was consistent with their low affinity for the receptor. Saturation of anandamide resulted in an analog (compound 3) that had the same ED_{50} as anandamide for the spontaneous activity assay, yet it produced antinociception only at very high doses. Substitution of the hydroxyl group with a fluorine atom combined with complete saturation (compound 4) was more potent than anandamide for both tests.

Increasing the chain length for the N-substituent (compounds 5 and 6) and replacing the ethanolamide with a sulfonamide group (compound 7) reduced potencies for antinociception without changing ED_{50} values in the spontaneous activity assay. Compound 8 (bromobenzenesulfonamide) did not bind to the receptor, and it did not produce hypomotility and antinociception. An ED_{50} value was not calculated, since this compound did not produce a 50% effect. Therefore, the results for compound 8 and other very weak compounds are reported as greater than the highest dose tested (µmol/kg). Interestingly, an analog with morpholine substitution for ethanolamide (compound 9) was

more potent than anandamide in reducing spontaneous activity and just as potent in producing antinociception, yet this compound had rather low receptor affinity.

The hydroxyl constituent of the ethanolamide moiety was replaced with four different functional groups. Compounds 10 and 12 (sulfonamide and fluorine substitutions, respectively) were similar in potency to anandamide in the spontaneous activity test, but were 10 times less potent in the tail-flick assay ($ED_{50} > 140$). Phenoxy substitution produced compound 11 that was more potent than anandamide in depressing locomotor activity and approximately five times less potent in producing antinociception. Compound 13 (methoxy substitution) was inactive, a finding consistent with its very low binding affinity.

The 1' methylated analog (compound 14) was four times more potent in the spontaneous activity test, but the compound was not as potent as anandamide in the tail-flick assay. Methylation at carbon 2 (compound 17) yielded an analog that was more potent than anandamide in producing hypomotility and just as potent in producing antinociception. Similar results were obtained for compound 15 in which the hydroxyl group was replaced with a fluorine molecule. Compound 16 (dimethyl at carbon 2) was slightly more potent than anandamide for the spontaneous activity assay and less potent in the tail-flick test. An isopropyl derivative (compound 18) was inactive in both behavioral assays. Compound 19 was not as potent as anandamide in the tail-flick assay and produced little hypomotility. An ED_{50} value could not be calculated for compound 19 in the spontaneous activity assay since a statistically significant maximal possible effect was not obtained. Compounds 20 and 21 (methylation at nitrogen and methylations at nitrogen and carbon 2) produced little or no activity in the spontaneous activity and antinociception assays. The pair of enantiomers (compounds 22 and 23) neither produced a reduction in locomotor activity nor generated more than 32% effect in the tail-flick assay.

Linear correlations were evaluated between binding affinities (log K_i) and *in vivo* potencies (Figure 7). Spearman rank order statistics also were performed between the data. Compounds 3 and 4 were not included in the correlations since binding studies could not be performed with these insoluble analogs. Linear correlations between log SA and log K_i resulted in a correlation coefficient of 0.68 (Figure 7A), and they were statistically significant in the Spearman correlation test ($P < 0.05$, two-tailed). When log TF and log K_i were compared, a lower linear correlation resulted ($r = 0.51$), and correlations were not statistically significant (Figure 7B). All analogs were included when comparing log SA and log TF (Figure 7C). A correlation coefficient of 0.72 was obtained which was statistically significant at the $P < 0.01$ level (two-tailed).

Time course following i.v. administration for anandamide and compound 16 (2,2-dimethylarachidonyl-(2'-hydroxyethyl)amide) in the tail-flick assay. If a compound is stable without the addition of an enzyme inhibitor *in vitro*, then the same compound might have greater stability *in vivo*. Therefore, a time course study was performed to determine if one of the compounds stable in the absence of PMSF would have a longer duration of action than anandamide *in vivo*. Compound 16 (dimethyl at carbon 2) was selected since its pharmacological potency and receptor affinity, in the absence of PMSF, were similar to those of anandamide in the presence of PMSF. Tail-flick latencies were measured at 5, 15, 30 and 60 min following drug administration. As evident in Figure 8, the time course for compound 16 was parallel to that of anandamide (5 mg/kg). Therefore, the analog did not appear to be more stable than anandamide *in vivo*.

Anandamide metabolism in the receptor binding assay. In order to verify that PMSF prevented metabolism of anandamide rather than altering its inherent binding affinity, [^{3}H]-anandamide was incubated with rat brain membrane with the same conditions as in the receptor binding assay. A similar system has been used to detect a wide range of prostaglandin metabolites (Dr. Earl Ellis, MCV/VCU, personal communication). In the

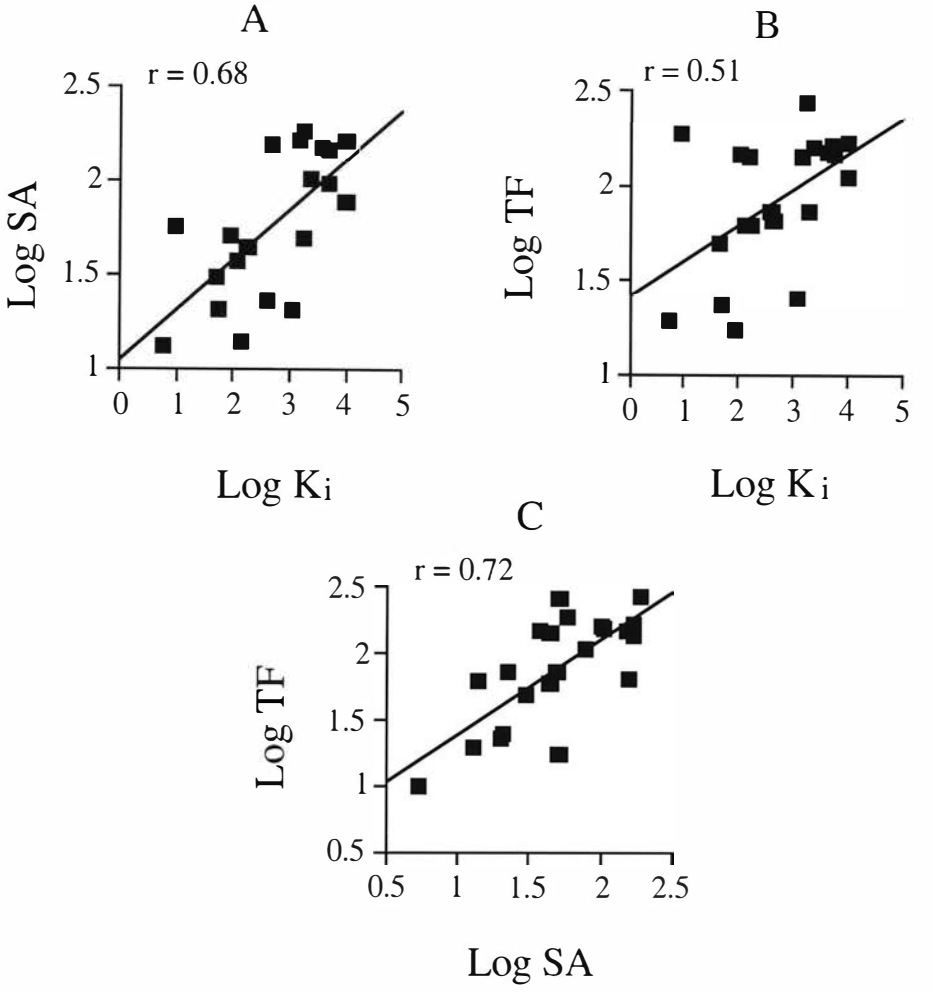


Figure 7. Relationships between pharmacological potency and receptor affinity for anandamide analogs. Correlations are based on log ED_{50} values (μmol/kg) and the log K_i values determined in the presence of PMSF. Graph A represents correlations between spontaneous activity (SA) and receptor affinity; graph B represents correlations between tail-flick (TF) and receptor affinity, and graph C represents correlations between the potencies in the SA and TF assays.

absence of PMSF [^{3}H]-anandamide was completely converted to [^{3}H]-arachidonic acid; no other peak was detected. Inclusion of PMSF protected [^{3}H]-anandamide from metabolism, as evidenced by the lack of [^{3}H]-arachidonic acid formation.

Discussion

In order to verify that Δ^9-THC and anandamide share a common receptor, SAR studies were conducted for correlative purposes. Extensive SAR studies have identified a number of structural features of Δ^9-THC important for receptor affinity and pharmacological potency (Razdan, 1986; Mechoulam *et al.*, 1987). These areas include the eleven position of the C ring, the phenolic A ring hydroxyl and the alkyl hydrophobic side chain attached to the A ring. Little structural similarity exists between anandamide, Δ^9-THC and CP 55,940, the potent synthetic cannabinoid agonist. Therefore, predicting the alignment of anandamide with the cannabinoid receptor is difficult. Also, due to structural and metabolic degradative pathway differences between anandamide and Δ^9-THC, identical results in all pharmacological assays should not be expected.

The observation that receptor binding for anandamide is greatly enhanced in the presence of PMSF, an amidase inhibitor, led to the reasonable assumption that PMSF inhibits enzymes that degrade anandamide (Childers, Sexton and Roy, 1994). The metabolic studies described herein confirmed that PMSF did prevent the conversion of anandamide to arachidonic acid when incubated with rat brain homogenate. Therefore, PMSF was included in [^{3}H]-CP 55,940 displacement studies for anandamide and its analogs. The finding that anandamide's receptor affinity was enhanced several hundred fold is consistent with these metabolic studies and the previous observations of Childers *et al.* (1994). PMSF also exerted a minor influence (a two-fold shift) on CP 55,940's receptor affinity which is not likely to be metabolic. While the PMSF action on CP 55,940

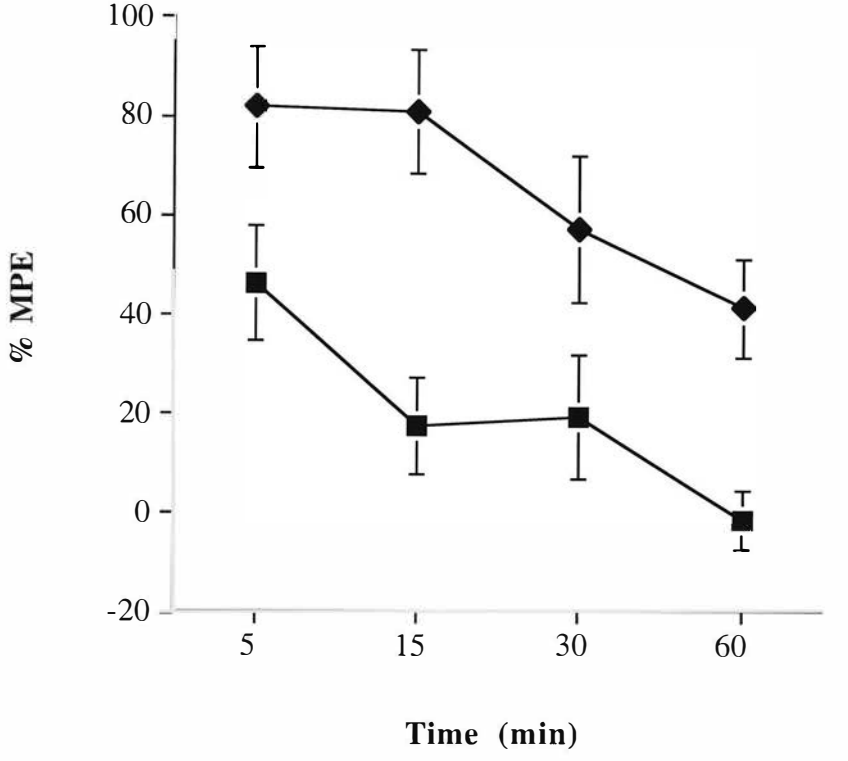


Figure 8. The time course effects of either 5 mg/kg anandamide (■) or 30 mg/kg of an alkylated anandamide analog (compound 16) (◆) on tail-flick latency following i.v. administration. A separate group of animals (six mice per group) was tested for each dose. The means ± S.E. are presented (n=6).

binding may lack biological significance, it underscores the importance of making appropriate corrections when conducting competition studies in the presence of PMSF. The identification of anandamide analogs with similar affinities in the presence and absence of PMSF indicates that the binding assays can be a useful tool for delineating between metabolically unstable and stable analogs.

Cannabinoids produce a wide variety of pharmacological properties in different animal species. A tetrad of behavioral tests in the mouse, including antinociception, depression of spontaneous activity, catalepsy and reduction in body temperature, have proven to be highly predictive of cannabimimetic activity (Martin *et al.*, 1981). In addition, the long duration of action of classical cannabinoids makes it possible to measure all four pharmacological effects in the same animal. For this study, anandamide analogs were evaluated for their ability to produce antinociception and depression of spontaneous activity. Since each test composing the cannabinoid tetrad has been shown to correlate with receptor binding, only two of the assays were selected to evaluate the anandamide analogs (Compton *et al.*, 1993). As determined by Compton *et al.* (1993) for 60 cannabinoids, the correlation coefficients between cannabinoid receptor binding and hypomotility production, antinociception, temperature reduction and catalepsy were, respectively, 0.91, 0.90, 0.89 and 0.85. In addition, the rapid onset of action of anandamide makes it impossible to measure all four pharmacological effects in the same animal.

This pharmacological evaluation permitted the identification of several sites in the anandamide structure important for anandamide's binding to the cannabinoid receptor and potency in the behavioral assays used to assess cannabimimetic activity. The degree of saturation is critical for receptor affinity and *in vivo* potency. Either removal of several of the *cis* double bonds or addition of a double bond at carbon 17 decreased affinity and potencies in both behavioral measures. These results are consistent with those of Felder *et al.* (1993) who noted that the addition of *cis* double bonds at carbons 3 and 17 drastically

increased the K_i. They also reported that removal of the double bond at carbon 3 did not alter receptor affinity. Complete saturation and hydroxyl substitution with a fluorine group increased potency in the spontaneous activity and tail-flick assays. Although these alterations are not comprehensive enough to precisely define an active conformation of the arachidonyl backbone, they demonstrated the importance of its conformation and the feasibility of restricting the backbone to an active conformation. Another structural feature of primary importance is the ethanolamide. Increasing the N-substituent chain length by one or two carbons decreased affinity and antinociceptive potency, but did not produce higher ED_{50} values for spontaneous activity. While an N-substituent appears to be critical for receptor affinity and behavioral potency, a thorough characterization has not been completed. For example, substitution with a bulky sulfonamide group produced a compound with equal affinity to the receptor, as compared to anandamide. However, substitution with an equally bulky bromobenzenesulfonamide inactivated anandamide. As for the ethanolamide, substitutions for the hydroxyl constituent affected both binding and behavioral paradigms. Surprisingly, substitution of the hydroxy group with a sulfonamide only decreased affinity two-fold when compared to anandamide. Yet, substitution with the much smaller methoxy group produced an analog with low affinity and very little potency in the behavioral tests.

Although the correlation was not high between receptor affinity and pharmacological potency, several explanations are possible to explain this result. Correlations made by Compton *et al.* (1993), which were higher than the anandamide correlations, were performed with a larger database of cannabinoids and with a greater range of structural diversity. With a larger number and wider range of compounds, a higher correlation might be obtained for anandamide compounds. On the other hand, anandamide may possess properties unique from other classical cannabinoids which need to be evaluated further. The possibility also exists that CP 55,940 and anandamide are

labeling different receptor subtypes; however, there is no direct evidence for receptor subtypes in brain at present.

One also should not ignore the role that differences in pharmacokinetics and metabolism may play between *in vitro* and *in vivo* assays. It is interesting that the correlation between the two pharmacological assays was higher than between either assay and receptor binding. One possible explanation for the discrepancy between *in vivo* and *in vitro* data is that the pharmacokinetics may vary for each analog. Obviously, it is not feasible to establish the pharmacokinetics of every analog in an SAR study. Also, pharmacological differences may exist between *in vitro* and *in vivo* experimental conditions. For *in vivo* experiments, a rapid time course is used and the behavioral effects produced by anandamide and the analogs are measured soon after drug administration. In the receptor binding assay, however, a longer time course, or incubation period, was selected. Additionally, in the *in vitro* assay, drugs are exposed to a small amount of brain tissue; whereas, *in vivo* experiments result in drug exposure to all tissues of distribution. As information on the synthesis and metabolism of anandamide emerges, it may be possible to determine the extent that pharmacokinetics is a contributing factor in SAR studies.

This SAR study has shown that it is possible to develop high affinity analogs. Two such analogs (compounds 12 and 15) produced higher binding affinities to the receptor than anandamide. Both of these analogs contained a fluorine substitution for the 2'-hydroxyl; additionally, compound 15 was methylated at carbon 2. Fluorine is an electron-dense atom, and substitution with fluorine might enhance receptor interaction. The compounds differed in respect to K_i's determined in the absence of PMSF. Compound 12 had a much higher K_i in the absence of PMSF; compound 15 had a K_i lower than anandamide's with and without PMSF. The increased affinity in the presence of PMSF, however, did not correlate with increased behavioral potency for both analogs. Compound

12 had the same potency as anandamide for the spontaneous activity test, yet this analog had low potency in producing antinociception. At this point, reconciling the difference between binding affinity and potency is not possible. Further synthesis of similar high-affinity analogs might lead to the development of compounds more stable and potent than anandamide. Potent and stable analogs would in turn facilitate studying the role of anandamide in *in vitro* and *in vivo* systems.

Methylations were made at several sites on the anandamide backbone in the hopes of blocking enzyme metabolism. The presence of bulky groups near the site of enzymatic cleavage produces steric hindrance. It is well known that hydrolysis of either peptides or esters is inhibited with the addition of increasingly larger alkyl groups. Therefore, a series of anandamide derivatives were prepared with alkyl groups at either the nitrogen, 1', 2 carbon or a combination of alkylations. By alkylating these sites, we postulated that the amidase activity would be inhibited, and the resulting compounds would have increased metabolic stability. PMSF was shown to have little influence on the binding affinities of several of the methylated compounds. Thus, one would expect these compounds to be active in the behavioral assays and have a longer duration of action. These compounds were more potent than anandamide in depressing spontaneous activity. Their ED_{50}'s for SA and TF, with the exception of one compound, were similar to that of anandamide. When the time courses for anandamide and one of the methylated derivatives were compared, parallel time courses resulted. However, a semilog plot suggested a longer half-life with the methylated compound. Therefore, predicting the stability of analogs *in vivo* based upon their sensitivity to PMSF should be done cautiously until there is a clearer understanding of the role that methylation is playing in receptor binding.

Receptor affinity and potency in the behavioral assays were reduced with the addition of an ethyl group at carbon 2, and substitution with an isopropyl group at the same position caused a 10-fold decrease in affinity and almost complete inactivation of

pharmacological activity. Thus, as alkyl groups of increasing bulkiness were added to carbon 2, interaction with the receptor was decreased. A size restriction therefore exists for this site with alkyl chains composed of two or more carbons presenting a steric hindrance and preventing optimal receptor interaction.

Additional evidence for steric hindrance due to larger alkyl chains was demonstrated for the carbon 1' site. A methylation at carbon 1' yielded an analog stable both in the presence and, importantly, absence of PMSF. This analog had the same affinity as anandamide for the cannabinoid receptor and also was pharmacologically active. When an isobutyl group was added at the 1' carbon both the R- and S- forms of the compound did not bind in the absence of PMSF, and only the R-form bound with low affinity in the presence of the enzyme inhibitor. Also, both analogs had very low pharmacological activity. For both the 1' and 2 carbons, receptor binding and behavioral activity depended upon the size of the alkyl group substituted at these sites. Addition of a single carbon produced analogs that were resistant to enzymatic degradation and, therefore, stable without PMSF. Substitution with larger groups also increased stability; however, these alkylations also reduced affinity and activity in the behavioral tests.

To further explore the role of methylation and its influence upon stability of the compounds, two compounds were synthesized with methylations at the nitrogen of anandamide. Nitrogen methylation dramatically reduced affinity and pharmacological activity in the tail-flick assay, and no difference existed in binding with and without PMSF. Similar results were obtained for a compound methylated at both the nitrogen and carbon 2. The decreased activity of these N-methylated compounds might be due to disruption of a hydrogen bonding process at the receptor active site, as has been suggested for the proton in the phenolic hydroxyl of Δ^9-THC (Semus and Martin, 1990). Thus, addition of a methyl group at the nitrogen prevents proper alignment and binding to the receptor.

In summary, these results indicated several areas of the structure which are important for receptor binding and behavioral potency. Alterations in the number of double bonds in the arachidonyl moiety, changes in the N-substituent, substitutions for the hydroxyl group and methylation at carbons adjacent to the nitrogen all influenced receptor affinity and pharmacological potency. Correlations between binding and pharmacological data were greatest when the analogs were either very potent or inactive. The greatest discrepancies existed between binding and behavioral data when the compounds possessed moderate binding affinity. This study clearly shows that stable and potent cannabinoid analogs can be developed from anandamide.

III. An Autoradiographic Comparison of Anandamide, CP 55,940 and SR 141716A Binding in Rat Brain

Introduction

Following the discovery of the cannabinoid receptor in brain tissue, autoradiographic experiments were performed to localize the receptor in brain. Autoradiography of cannabinoid receptors from several mammalian species, including human, reveals a conserved and unique pattern of distribution (Herkenham *et al.*, 1990). Binding was most dense in the outflow nuclei of the basal ganglia (the substantia nigra pars reticulata and globus pallidus), the hippocampus and the cerebellum. The high densities of receptors in the forebrain and the cerebellum explain the effects of cannabinoids on cognition and movement. High levels in the hippocampus provide a role for these receptors in cannabinoid impairment of memory. Sparse densities in the brainstem areas controlling cardiovascular and respiratory functions would explain why high doses of marijuana are not lethal. The distribution of cannabinoid receptors in rat brain also was determined with the aminoalkylindole [^{3}H]-WIN 55,212-2 and [^{3}H]-11-OH-Δ^9-THC-DMH (Jansen *et al.*, 1992; Thomas, Wei and Martin, 1992). Binding distribution was very similar between [^{3}H]-CP 55,940, [^{3}H]-WIN 55,212-2 and [^{3}H]-11-OH-Δ^9-THC-DMH confirming that these structurally diverse compounds bind to the same receptor.

The pattern of distribution of cannabinoid binding is consistent with data from in situ hybridization studies (Matsuda *et al.*, 1990). High levels of mRNA in the hippocampus were found in granule cells of the dentate gyrus and in cells in the pyramidal and molecular layers. Expression of message for the cannabinoid receptor was also high in

cells within the superficial and deep layers of the cerebral cortex and amygdala. The pattern of distribution is consistent with in situ hybridization data using oligonucleotide probes complementary to rat cannabinoid receptor cDNA (Mailleux and Vanderhaegen, 1992). In the hippocampus, high levels of mRNA for the cannabinoid receptor were found in granule cells of the dentate gyrus and in cells of the pyramidal and molecular layers of the hippocampus. Message for the receptor was also prevalent within the superficial and deep layers of the cerebral cortex and amygdala. In the human brain the distribution of the mRNA encoding for the cannabinoid receptor also has been studied using in situ histochemistry and oligonucleotide probes (Mailleux, Parmentier and Vanderhaegen, 1992). Microscopically, positive neurons were found in layers II-III and V-VI of the cerebral cortex, and in the hilus and the dendritic layers of the dentate gyrus and Ammon's horn of the hippocampus. Macroscopically, a small hybridization signal was found throughout all layers of the cortex, in the pyramidal cell layer of the hippocampus, in the caudate and putamen and in the granular and molecular layers of the cerebellum.

Binding experiments dealing with the fine neuronal localization of CB_1 receptors in the rat basal ganglia indicate that the receptors are not localized on dopamine cell bodies or terminals (Herkenham *et al.*, 1991b). Cannabinoid receptors in the basal ganglia are localized to striatal neurons, with very dense localization to the axons and terminals in the globus pallidus, entopeduncular nucleus and substantia nigra pars reticulata. This localization implies that the facilitation of cannabinoids upon nigrostriatal dopamine function might involve indirect, local and/or distal neuronal circuits. Within the basal ganglia, cannabinoid receptors occur both pre- and postsynaptically. In the cerebellum, cannabinoid receptors are neuronally localized to granule cell axons in the molecular layer (Herkenham *et al.*, 1991a).

In addition to Δ^9-THC, bicyclic compounds, dimethylheptyl analogs of THC and aminoalkylindoles, anandamide and SR 141716A have been added to the list of structurally

diverse compounds that bind to the cannabinoid receptor. Although anandamide produces many of the same effects as other psychoactive cannabinoids, differences do exist. Comparison between anandamide and Δ^9-THC revealed that anandamide was 4- to 20-fold less potent and had a shorter duration of action than Δ^9-THC (Smith *et al.*, 1994). Anandamide also acted as a partial agonist at the N-type calcium channels (Mackie, Devane and Hille, 1993). Anandamide produces antinociception like other cannabinoids, but anandamide is not active when administered i.c.v. (Smith *et al.*, 1994). Also, unlike other cannabinoids, anandamide's antinociception is not blocked by the kappa antagonist *nor*-BNI (Smith *et al.*, 1994).

The structure-activity relationship studies in the first part of this dissertation provided evidence that anandamide was binding to the cannabinoid receptor and producing pharmacological effects in mice, such as depression of spontaneous locomotor activity and generation of antinociception. The next objective in this dissertation was to determine whether anandamide might interact differently with receptors in some brain areas by using the technique of receptor autoradiography. Since anandamide does not have high affinity for the cannabinoid receptor, in comparison to other synthetic high-affinity THC analogs, [^{3}H]-anandamide was not used directly to llabel receptors. Performing autoradiography with compounds possessing weak affinity to a receptor introduces many technical problems. Weak ligands diffuse from the receptor, and the resulting autoradiogram is not clear. Fuzzy images cannot be accurately analyzed. To avoid this problem, [^{3}H]-CP 55,940 was selected. In these experiments anandamide (unlabeled) competed with [^{3}H]-CP 55,940 for binding to the cannabinoid receptor. The autoradiograms thus depict anandamide's displacement of labeled CP 55,940. The recent discovery of the cannabinoid antagonist SR 141716A also provided the opportunity to study the binding of this

compound. SR 141716A was not available in a tritiated form; therefore, autoradiograms of SR 141716A also show its displacement of labeled CP 55,940.

The purpose of the autoradiographic experiments was to determine if differences exist between the cannabinoid receptor population which binds CP 55,940, SR 141716A and anandamide. The technique of autoradiography was used to quantitatively compare cannabinoid receptor affinities, binding patterns and densities of anandamide, CP 55,940 and SR 141716A in different brain regions. If anandamide, CP 55,940 and SR 141716A are binding to the same cannabinoid receptor (CB_1) in the brain, then the binding localization should be the same in different brain regions for all three compounds. Differences in binding densities between regions for the three compounds might suggest that a receptor subtype for the central cannabinoid receptor exists. Furthermore, a second objective was to determine cannabinoid receptor affinities for anandamide, SR 141716A and CP 55,940 and compare the affinities for each compound between brain regions. Differences in affinities would indicate that a particular drug was binding to a brain region in a different manner than to cannabinoid receptors in other regions. Binding displacement curves from selected brain regions also were analyzed for parallelism for each compound.

Methods

Animals. Male Sprague-Dawley rats (150 - 200 g) from Harlan Laboratories were maintained on a 14:10 hr light/dark schedule and freely received food and water.

Chemicals. [^{3}H]-CP 55,940 was purchased from DuPont NEN (Wilmington, DE). CP 55,940 was a gift from Dr. L. Melvin of Pfizer Inc., Central Research Division (Groton, CT), and anandamide was kindly provided by Dr. Raj K. Razdan of Organix Inc. (Woburn, MA). Both compounds were prepared as 1 mg/ml stock solutions in absolute ethanol and stored at -20 °C. SR 141716A was obtained from Pfizer Inc. (Groton, CT),

and prepared as a 1 mg/ml solution in absolute ethanol. PMSF was dissolved in absolute ethanol as a 20 mg/ml stock solution.

Tissue Preparation. Following decapitation, rat brains were quickly removed and frozen in 2-methylbutane (-50 °C). The brains were embedded in M-1 embedding matrix and stored at -70 °C until sectioning. Brains were mounted onto cryostat chucks with TFM tissue freezing medium. Consecutive coronal brain sections (16 µm) were thaw-mounted onto slides coated with 0.5% gelatin and 0.05% chromium potassium sulfate. Sections were made at the stereotaxic coordinates 1.2 mm from bregma, 0.48 mm from bregma, -5.2 mm from bregma and -12.8 mm from bregma (Paxinos and Watson, 1986). Sections were stored desiccated at -70 °C prior to use in binding assays.

***In situ* Cannabinoid Binding Assays.** Assay conditions for cannabinoid binding have been described previously (Herkenham *et al.*, 1991c). Saturation experiments were first performed to determine the K_d of [^{3}H]-CP 55,940. This value then was compared to values reported in the literature. Coronal sections containing primarily frontal cortex and caudate-putamen (1.2 mm from bregma) were used for Scatchard analysis. Slides were allowed to return to room temperature and incubated for 2 hr in slide mailers at 37 °C in reaction buffer (50 mM Tris-HCl with 5% BSA, pH 7.4) Total binding was determined with seven concentrations of [^{3}H]-CP 55,940 (1.13, 2.3, 4.5, 7.5, 15, 22.5 and 30 nM); nonspecific binding mailers additionally contained 1 µM CP 55,940 (non-radiolabeled). Saturation experiments were also performed with 50 µM PMSF in the incubation buffer. Following incubation, slides were washed for 4 hr at 0 °C in 50 mM Tris-HCl with 1% BSA (pH 7.4). Sections were scraped from the slides with Whatman GF/C filters. The filters were placed in scintillation vials, and the tissue was solubilized overnight with 1.0 ml of TS-2. Samples were acidified with 10 µl of glacial acetic acid and counted by liquid scintillation spectrometry. Transformation of the data and calculation of

K_d values was accomplished using the LIGAND computer software developed by Munson and Rodbard (Munson and Rodbard, 1980) as supplied by Biosoft Inc. (Cambridge, U.K.).

Competition for [^{3}H]-CP 55,940 Binding. Due to the reported instability of anandamide (Childers, Sexton and Roy, 1994; Deutsch and Chin, 1993), optimal binding conditions were first determined in the presence and absence of the enzyme inhibitor PMSF. In the competition experiments, reaction buffers, incubation temperatures and times were identical to the *in situ* binding assay described above. Sections were made from 0.48 mm from bregma, -5.2 from bregma and -12.8 mm from bregma. For CP 55,940, SR 141716A and anandamide, nonspecific binding was determined using 10 μM CP 55,940, and total binding was determined for 10 nM [^{3}H]-CP 55,940 (approximately 40% receptor occupation). Eight concentrations of CP 55,940 ranging from 0.1 to 300 nM were assayed; concentrations of anandamide ranged from 0.01 to 10 μM, and concentrations of SR 141716A were 0.001 to 10 μM. Each experiment was conducted in at least triplicate. For anandamide displacement assays, additional experiments were performed either with 50 μM PMSF in the incubation buffer or with sections pretreated for 30 min in a buffer containing 50 μM PMSF prior to exposure of 50 μM PMSF in the incubation buffer. Anandamide sections were wiped from the slides, solubilized and counted. Optimal conditions for anandamide competition experiments occurred when sections were pretreated for 30 min with PMSF and exposed to PMSF in the incubation buffer.

[^{3}H]-CP 55,940 Autoradiography. Following the wash, slides were rapidly dried with a stream of cool air and stored in a desiccator overnight at 4 °C. Sections were apposed to tritium-sensitive film with [^{3}H]-microscales for three weeks before developing with a D-19 developer. Developed films were analyzed using the NIH Image 1.49 program. Levels of transmittance were converted to dpm/mg protein using a polynominal

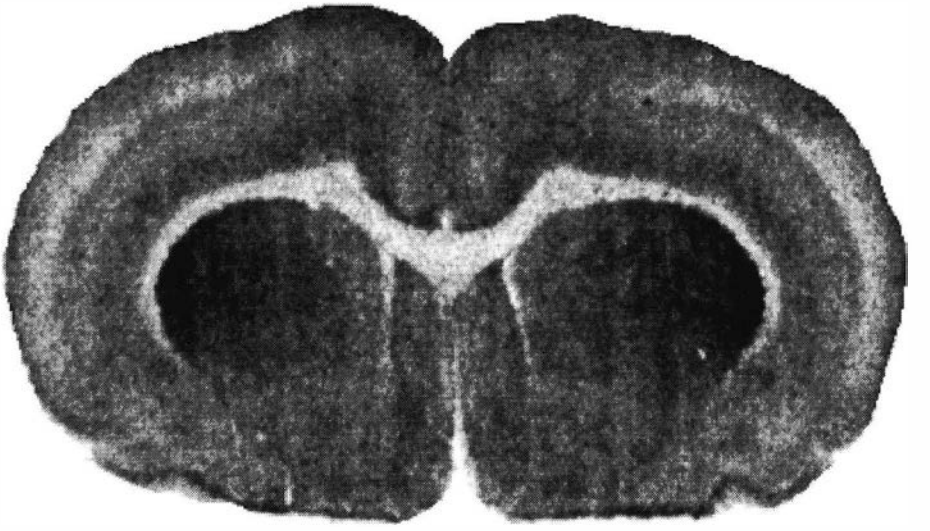
curve fit of the standards. Brain structures were outlined, and optical density in each area was measured. Curve-fitting of the displacement data and determination of K_i and B_{max} values for anandamide, CP 55,940 and SR 141716A were done using EBDA software. K_i and B_{max} values for anandamide in the substantia nigra and the molecular layer of the cerebellum were determined from autoradiograms apposed to film for one week.

Statistical Analysis. Significant differences between K_i values were determined using the ANOVA analysis (Scheffe post-hoc analysis). To determine if curves were parallel, data from a representative displacement curve from each brain area for anandamide, SR 141716A and CP 55,940 were analyzed using the ALLFIT curve-fitting program. B_{max} values were compared by linear correlations for Anandamide and CP 55,940 and SR 141716A and CP 55,940.

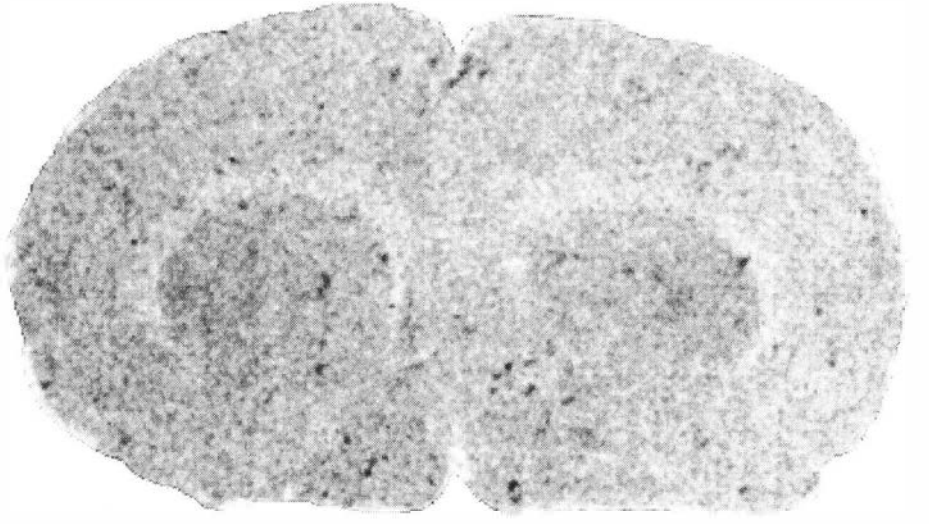
Results

Cannabinoid receptor affinity (K_d) was determined for CP 55,940 and expressed as a K_d value. Saturation experiments were performed both in the presence and absence of the enzyme inhibitor PMSF. Without PMSF a K_d value of 15.3 ± 1.2 nM (n = 5) was calculated, which correlated with value of 15 ± 3 nM obtained by Herkenham *et al.* (1990). In the presence of PMSF a K_d value of 12.3 ± 2.1 nM (n = 3) resulted, which is not statistically different from the K_i value obtained without PMSF. The presence of PMSF did not influence affinity to the cannabinoid receptor. Thus, all K_i's were calculated using the K_d value of 15.3 nM.

Since in the first part of this dissertation, it was determined that anandamide is susceptible to enzymatic cleavage, conditions were established for determining optimized receptor binding with anandamide in autoradiography experiments were established. Without inclusion of PMSF a K_i of 8030 ± 1110 nM (n = 3) resulted. PMSF (50 μM)



Total Binding



Nonspecific Binding

Figure 9. Autoradiogram of total and nonspecific binding of [^{3}H]-CP 55,940 to coronal sections of rat brain.

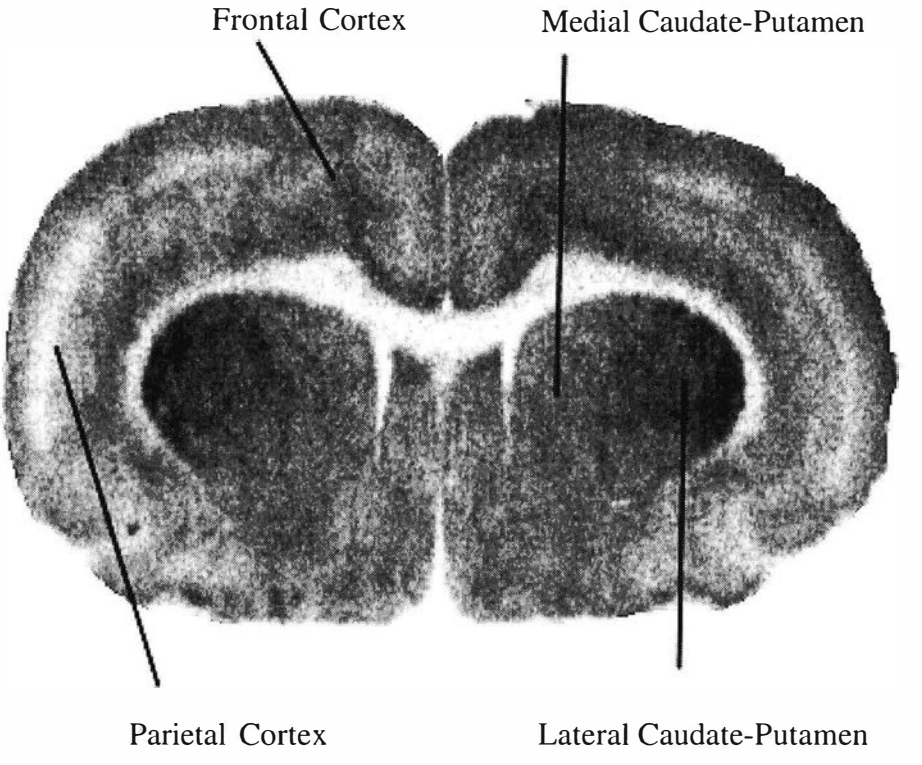


Figure 10. Autoradiogram of [^{3}H]-CP 55,940 binding to a coronal section of rat brain 0.48 mm from bregma.

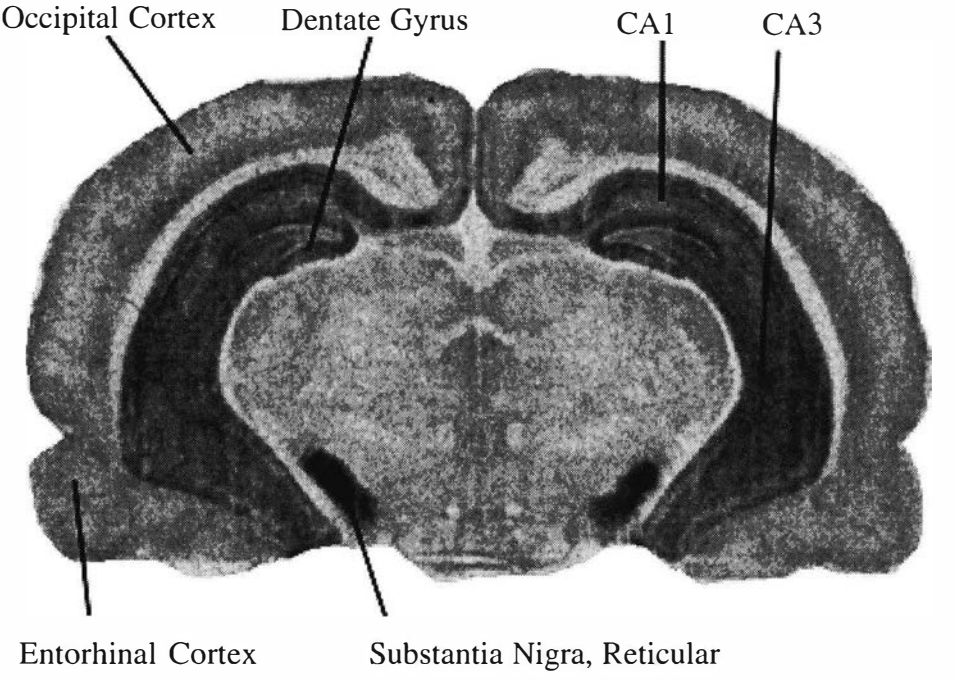


Figure 11. Autoradiogram of [^{3}H]-CP 55,940 binding to a coronal section of rat brain -5.2 mm from bregma.

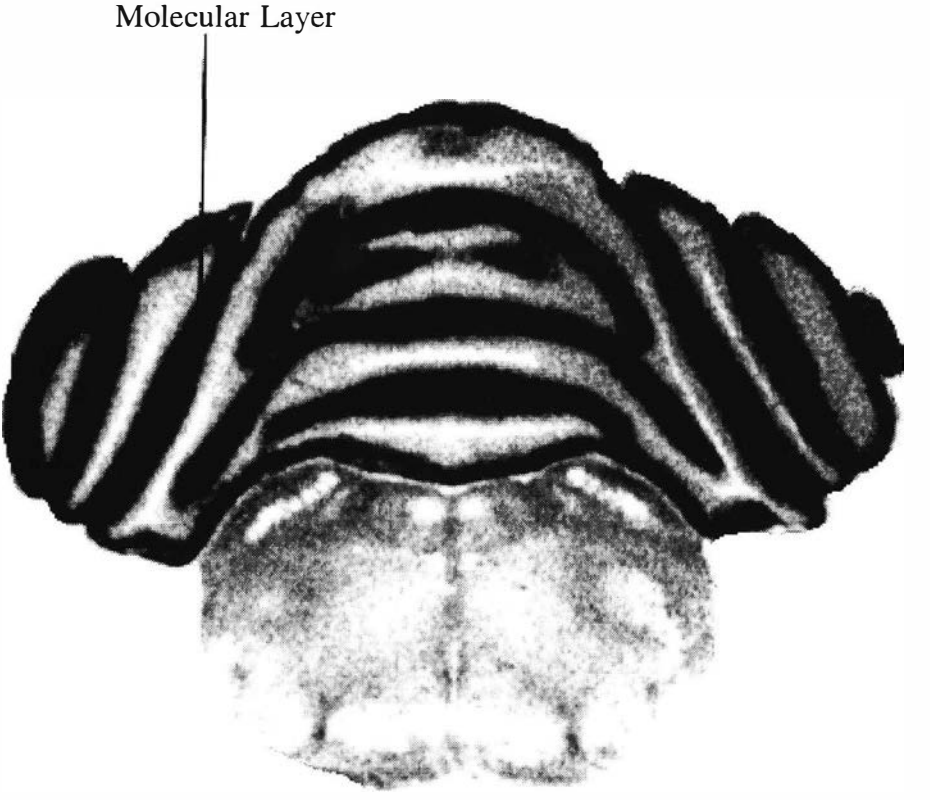
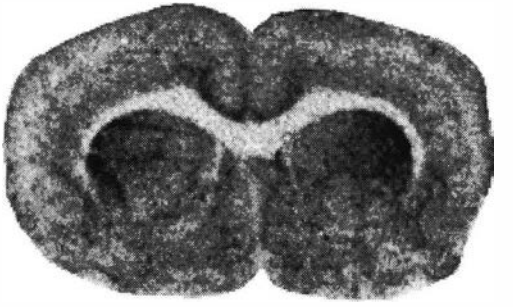
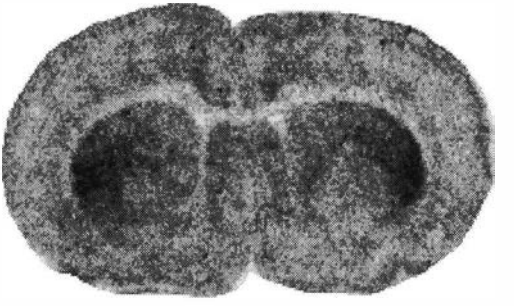


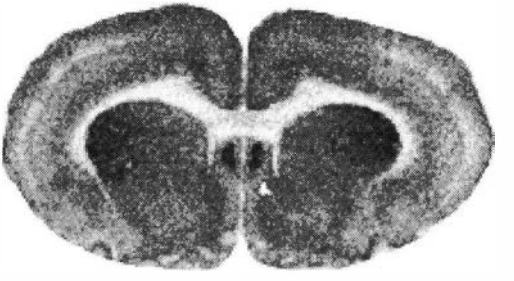
Figure 12. Autoradiogram of [^{3}H]-CP 55,940 binding to a coronal section of rat brain -12.8 mm from bregma.



SR 141716A (30 nM)



CP 55,940 (3 nM)



Anandamide (300 nM)

Figure 13. [^{3}H]-CP 55,940 displacement by SR 147161A (30 nM), CP 55,940 (3 nM) and anandamide (300 nM).

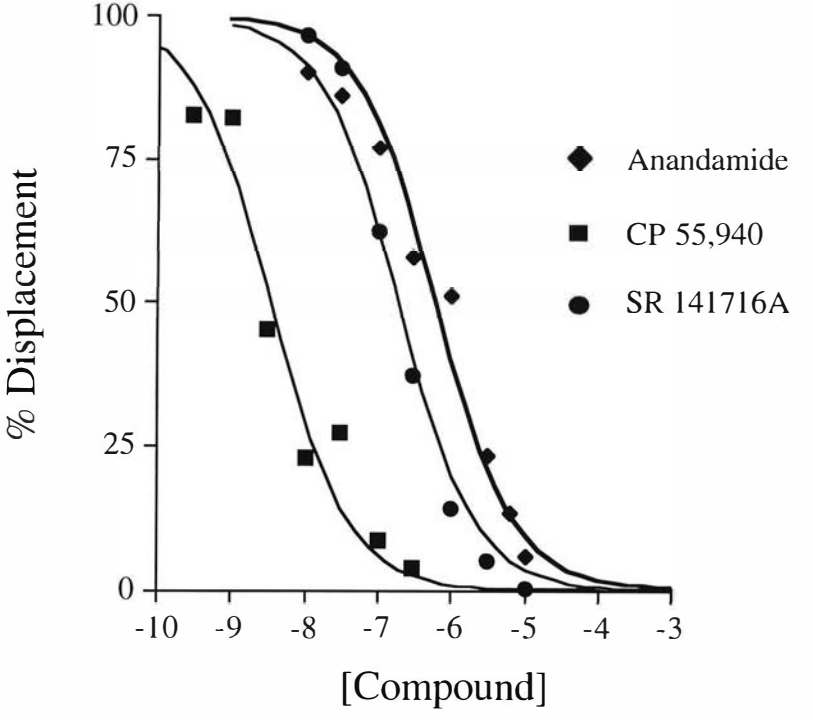


Figure 14. Anandamide, SR 141716A and CP 55,940 displacement of [^{3}H]-CP 55,940 in the lateral caudate-putamen. The curves are representative displacement curves.

then was added to the incubation buffer during the 2 hour reaction period. The K_i decreased to 2320 ± 540 nM (n = 6), but a K_i in this range demonstrates weak receptor affinity and suggests that anandamide is probably being degraded. Thus, slices were pretreated for 30 minutes with 50 µM PMSF and exposed to PMSF during the incubation time. A K_i of 608 ± 210 nM (n = 3) resulted from these assay conditions. These results indicate that anandamide is degraded without the addition of an enzyme inhibitor. Therefore, slices were exposed to PMSF before and during incubation for autoradiography experiments using anandamide.

Levels and patterns of cannabinoid receptor binding ([^{3}H]-CP 55,940) were in agreement with previously reported data (Herkenham *et al.*, 1991c; Jansen *et al.*, 1992; Thomas, Wei and Martin, 1992). High levels of binding were found in the substantia nigra pars reticulata, molecular layer of the cerebellum, dentate gyrus and CA1 and CA3 regions of Ammon's horn. Moderate levels of cannabinoid receptor binding was observed in all of the cortical areas and throughout most of the brain. Little binding was found in the brainstem or the corpus callosum indicating a lack of receptors in these areas.

A qualitative examination of the autoradiographic film demonstrates that only nonspecific binding results when an excess of unlabeled CP 55,940 (1µM) is added (Figure 9). Sections were made at three points in the rat brain. The stereotaxic coordinates were 0.48 mm, -5.2 mm and -12.8 mm from bregma. At 0.48 mm from bregma measurements were made in the lateral and medial caudate-putamen and the frontal and occipital cortices (Figure 10). At -5.2 mm from bregma measurements were made from the CA1 and CA3 regions from Ammon's horn, dentate gyrus, entorhinal and occipital cortices and substantia nigra (Figure 11). At -12.8 mm from bregma measurements were made from the molecular layer of the cerebellum (Figure 12).

Also apparent from a visual inspection of the developed brain images, is the similarity of the displacement patterns for SR 141716A, CP 55,940 and anandamide.

Table 5. K_i values for anandamide, CP 55,940 and SR 141716A.

Brain Region	Anandamide K_i (nM)	CP-55,940 K_i (nM)	SR 141716A K_i (nM)
Entorhinal Cortex	956**	10.2**	36 ± 4
Parietal Cortex	713 ± 7	6.7 ± 2.1	35 ± 14
Occipital Cortex	328 ± 86	17.5 ± 7.8	34 ± 7
Frontal Cortex	661 ± 145	8.5 ± 0.2	26 ± 10
Medial Caudate-Putamen	566 ± 93	9.0 ± 2.0	31 ± 8
Lateral Caudate-Putamen	515 ± 68	5.3 ± 1.3	52 ± 17
Dentate Gyrus	400 ± 72	8.5 ± 0.7	36 ± 5*
CA1	438 ± 87	6.0 ± 0.9	24 ± 3*
CA3	489 ± 176	7.4 ± 1.3	38 ± 5*
Substantia Nigra	512 ± 169	7.3 ± 2.9	95 ± 29*
Molecular Layer of the Cerebellum	861 ± 32	12.5 ± 4	114 ± 19*

*n = 6
**n = 2

Table 6. B_{max} values for anandamide, CP 55,940 and SR 141716A.

Brain Region	Anandamide B_{max} (pM)	CP-55,940 B_{max} (pM)	SR 141716A B_{max} (pM)
Entorhinal Cortex	44 ± 7	28 ± 7	48 ± 14
Parietal Cortex	50 ± 2	34 ± 4	66 ± 6
Occipital Cortex	59 ± 7	42 ± 7	43 ± 2
Frontal Cortex	76 ± 11	56 ± 7	94 ± 6
Medial Caudate-Putamen	108 ± 7	44 ± 2	107 ± 13
Lateral Caudate-Putamen	185 ± 33	109 ± 9	189 ± 19
Dentate Gyrus	211 ± 13	110 ± 22	149 ± 16*
CA1	258 ± 22	151 ± 29	147 ± 15*
CA3	771**	163 ± 33	150 ± 20*
Substantia Nigra	209 ± 4	212 ± 34	204 ± 35*
Molecular Layer of the Cerebellum	263 ± 44	250 ± 55	248 ± 22*

*n = 6
**n = 2

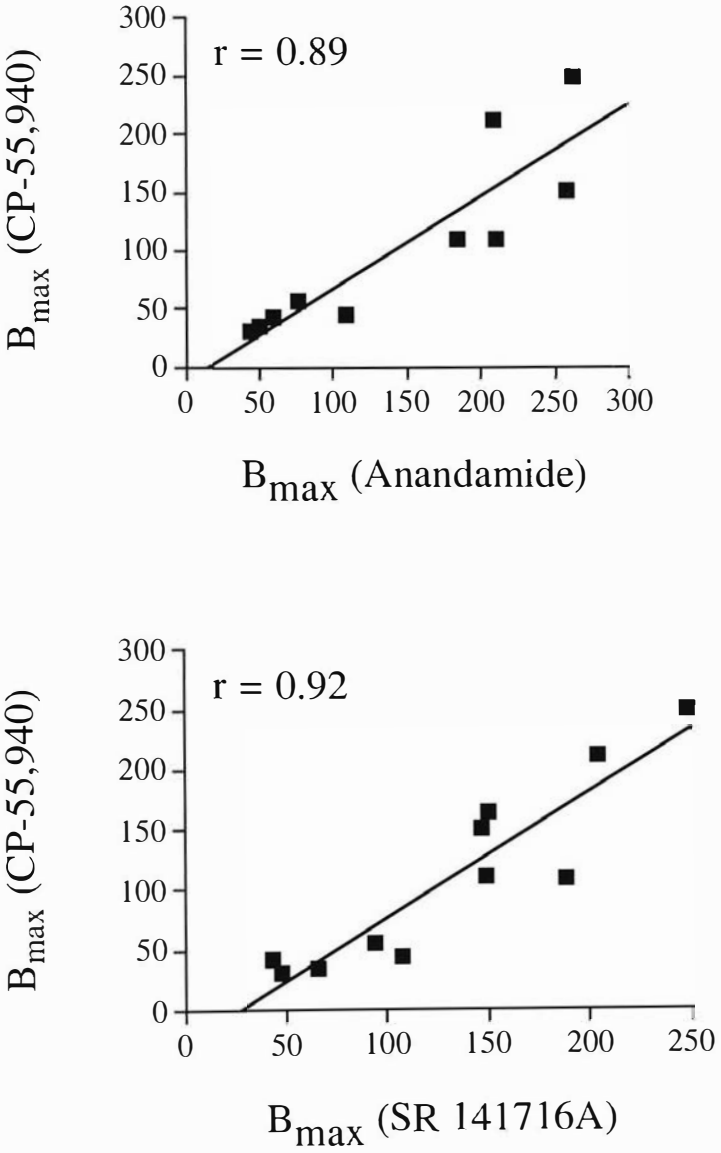


Figure 15. Relationship between B_{max} values for anandamide, SR 141716A and CP 55,940.

Figure 13 shows that binding density, qualitatively, appears not to differ between SR 141716A, CP 55,940 and anandamide at 30 nM, 3 nM and 3000 nM, respectively. These concentrations represent approximately 50% displacement of [^{3}H]-CP 55,940.

To quantitate results from autoradiography, measurements were made from the developed autoradiographic film of selected brain areas, and displacement curves were constructed. Figure 14 shows representative displacement curves for anandamide, SR 141716A and CP 55,940 in the lateral caudate-putamen. Since anandamide is a weaker ligand for the cannabinoid receptor, anandamide's displacement curve lies to the right of CP 55,940's curve. The curve for SR 141716A is to the right of CP 55,940's curve and to the left of anandamide's curve. SR 141716A has a higher affinity to the cannabinoid receptor than anandamide and has a lower affinity than CP 55,940. CP 55,940 is more potent than anandamide and SR 141716A. Similar curves were generated from the other regions.

Analysis of displacement data by EBDA generated K_i and B_{max} values. K_i values reflect the receptor affinity of the displacing compound, and B_{max} values are a measure of receptor number or density. K_i values for anandamide, SR 141716A and CP 55,940 from each brain area are found Table 5. The average K_i value for anandamide in the rat brain homogenate binding assay in the presence of PMSF was 90 nM, and the average K_i value for anandamide in the autoradiography experiments was 548 nM. Higher K_i and K_d values are obtained using in situ binding than in homogenate membrane binding. These differences are consistent for numerous ligands and probably reflect methodological differences between the two types of assays (Herkenham *et al.*, 1991c). Average K_i values were 9 nM for CP 55,940 and 47 nM for SR 141716A. Binding affinities were analyzed statistically to determine if differences existed for anandamide's affinity to the central cannabinoid receptor between different brain regions. Statistical analyses also were performed for SR 141716A and CP 55,940. Binding affinities for anandamide, SR

141716A and CP 55,940 were not statistically significant between brain areas. The K_i for the entorhinal cortex for anandamide and CP 55,940 were not included in the statistical analysis since the value was determined from an average of only two experiments. The cannabinoid receptor had the same affinity for anandamide in all regions analyzed. Binding affinities for CP 55,940 and SR 141716A also did not differ between brain regions.

A representative displacement curve was selected from each brain region for each compound. The curves for anandamide, CP 55,940 and SR 141716A were analyzed for parallelism by the program ALLFIT. All displacement curves for anandamide and CP 55,940 from each brain area were parallel. All displacement curves for SR 141716A, except for the entorhinal curve, were parallel.

B_{max} values for anandamide, CP 55,940 and SR 141716A are summarized in Table 6. B_{max}'s are listed from low to high based upon the values for CP 55,940. B_{max} values are listed in Table 6 according to areas with lower values (the cortices), areas with moderate values (the caudate-putamen) and areas with higher values. The molecular layer of the cerebellum had the highest B_{max} for anandamide (with the exception of CA3), CP 55,940 and SR 141716A. For an unknown reason, the B_{max} for anandamide in the CA3 region was extremely high and was calculated from only two assays; therefore, it was not used when determining correlations between anandamide and CP 55,940. The entorhinal cortex had the lowest B_{max}'s for anandamide and CP 55,940. The lowest value for SR 141716A was in the occipital cortex. To compare densities, linear correlations were made between CP 55,940 and SR 141716A. Correlations also were made between CP 55,940 and anandamide. Good correlation existed when comparing B_{max} values for anandamide to the B_{max} values of CP 55,940 ($r = 0.89$) (Figure 15). A correlation coefficient of 0.92 was obtained when B_{max} values for SR 147161A were compared to B_{max} values of CP 55,940.

Discussion

According to autoradiographic studies, the distribution of the cannabinoid receptor is heterogeneous in several mammalian species, conserved and neuronally located (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991b; Herkenham *et al.*, 1991c). The densest binding occurs in the basal ganglia (substantia nigra pars reticulata, globus pallidus, entropeduncular nucleus and lateral caudate putamen), and the molecular layer of the cerebellum. Binding in these regions may explain cannabinoid interference with movement. Intermediate levels of binding were found in the CA pyramidal cell layers of the hippocampus, the dentate gyrus and layers I and VI of the cortex. Δ^9-THC disrupts short-term memory in humans (Chait and Pierri, 1992). Cannabinoid effects on memory and cognition are consistent with receptor localization in the hippocampus and cortex. The hippocampus stores memory and codes sensory information. The presence of cannabinoid receptors in regions associated with mediating brain reward (ventromedial striatum and nucleus accumbens) suggests an association with dopamine neurons. Sparse levels were detected in the brainstem, hypothalamus, corpus callosum and the deep cerebellum nuclei. Low levels of receptors in brainstem areas controlling cardiovascular and respiratory functions is also consistent with the lack of lethality of marijuana.

The first step in autoradiographic binding, or any receptor binding technique, is to establish optimum binding conditions. The incubation times, temperature and BSA content were based upon those of Herkenham *et al.* (1991). A Scatchard analysis of saturation experiments using frontal cortex from the rat brain produced a K_d (15.3 nM) that corresponds to other K_d values in the literature (Herkenham *et al.*, 1991; Thomas *et al.*, 1992). Since anandamide is degraded in homogenate binding, it also was necessary to determine optimal binding conditions for anandamide. Consistent results were obtained when slices were pretreated with PMSF and exposed to PMSF during the reaction incubation. These results confirm previous reports of anandamide's instability in a

biological system. Since slices had to be both pretreated and exposed to PMSF during the entire two hour experiment, this finding suggests that levels of the enzyme that degrade anandamide are high in the brain. If anandamide is a neurotransmitter, then mechanisms must exist in the CNS to rapidly remove anandamide and prevent continuous stimulation. Therefore, one would not expect an endogenous compound to possess great stability. Development of more stable analogs would eliminate the need of exposing brain tissue to enzyme inhibitors. PMSF inhibits a wide variety of enzymes, not just amidases. Therefore, it was necessary to determine if PMSF produced an effect upon cannabinoid receptor affinity, as determined from saturation experiments. Unlike the homogenate receptor binding assay, PMSF did not influence cannabinoid receptor affinity for brain slice binding. The K_d determined in the presence of PMSF was not statistically different from the K_d obtained in the absence of PMSF. It is unknown why PMSF caused a two-fold shift in the receptor affinity in the homogenate receptor assay, but the shift is probably due to methodological differences between slice and homogenate binding.

Once conditions were established, a series of autoradiography experiments were performed using [^{3}H]-CP 55,940 to determine if anandamide, SR 141716A and CP 55,940 were binding to the same receptor in the brain. Following three weeks of apposure, the resulting autoradiograms showed the displacement of [^{3}H]-CP 55,940 by either anandamide, SR 141716A or CP 55,940 in the following brain areas: lateral and medial caudate-putamen, frontal, occipital, entorhinal and parietal cortices, dentate gyrus, substantia nigra and the molecular layer of the cerebellum. These areas were selected since cannabinoids affect the functioning of these regions. Also, they are all large enough so that a sufficient number of consecutive 16 µm slices can be made. Several of the areas, including the molecular layer of the cerebellum and the substantia nigra, have very dense levels of receptors. The dentate gyrus, CA1, CA3 and lateral caudate-putamen also have

dense receptor populations. The cortical regions have moderate levels of cannabinoid receptors.

The purpose of this section of the dissertation was to quantitate the binding of anandamide, SR 141716A and CP 55,940 in selected brain regions. Extensive autoradiographic experiments have been performed with CP 55,940 (Herkenham *et al.*, 1990). However, no such experiments have been performed with anandamide or SR 141716A. Results from these experiments would answer the question of whether CP 55,940, anandamide and SR 141716A were binding to the same receptor in the same manner. Analysis of measurements made from the developed film produced K_i and B_{max} values. K_i values are a measure of a compound's affinity for a receptor; B_{max} values represent the density of receptors. For anandamide, CP 55,940 and SR 141716A, no statistical difference existed between their K_i's in different brain regions. The K_i values from the entorhinal cortex for anandamide and CP 55,940 were not included in the statistical analysis since they were calculated from just two experiments. Also, K_i values for the substantia nigra and the molecular layer of the cerebellum were inconsistent when determined from film apposed for three weeks. Values for these two regions were therefore determined from film apposed for one week. Since anandamide is a weak ligand, it is possible that at the lower concentrations in anandamide's displacement curves the percentage of displacement is less accurate because the amount of displacement is overshadowed by the high numbers of receptors. If any of these compounds were binding to a receptor subtype possessing either a higher or lower K_i from the other regions, a statistical difference would result. No such differences were found for CP 55,940, anandamide or SR 141716A.

A second objective was to analyze representative curves for parallelism from each region for anandamide, SR 141716A and CP 55,940. Representative displacement curves from each brain region for anandamide were analyzed using the statistical program ALLFIT

to determine if they were parallel. Differences in parallelism would provide evidence that a compound was interacting with the cannabinoid receptor in a different manner from other regions. All 11 curves for anandamide were parallel, as were curves for CP 55,940. For an unknown reason, the entorhinal cortex curve for SR 141716A was not parallel to the other brain regions analyzed. Thus, these three compounds appear to bind to the CB1 receptor in a similar manner.

Cannabinoid receptor densities (B_{max}'s) were calculated for each brain region for anandamide, SR 141716A and CP 55,940. The purpose of calculating B_{max} values for each region was to determine if one compound might bind more or less in one brain area than in other regions. The relationship between B_{max} values for SR 141716A and CP 55,940 and anandamide and CP 55,940 were compared by linear plots of the respective values, and correlation coefficients were determined. A high correlation was obtained both when comparing the B_{max} values of SR 141716A and anandamide to those of CP 55,940. These correlations indicate that the three compounds are binding to the same population of receptors when comparing brain regions.

Several conclusions may be drawn from these results. The lack of difference between receptor affinity, receptor distribution and parallelism of the displacement curves indicates that anandamide, SR 141716A and CP 55,940 are binding to the same receptor in the same manner. No evidence of receptor subtypes in the brain was found.

IV. General Discussion

Marijuana is one of the most widely abused drugs throughout the world, yet it also has great therapeutic potential and has been used for centuries for medicinal purposes. The psychoactive constituent of marijuana, Δ^9-THC, produces a myriad of pharmacological effects in animals and humans. Great progress has been made in the past ten years regarding our understanding of the mechanism of action of cannabinoids. Cannabinoid receptors have been characterized both centrally and peripherally, and the distribution of the central cannabinoid receptor has been mapped throughout the central nervous system. The cellular mechanism of action of cannabinoids also has been more clearly defined. The discovery of anandamide as an endogenous ligand for the cannabinoid receptor creates the possibility of discovering a novel neurochemical system. The actions of cannabinoids and anandamide can better be elucidated with the recent discovery of an antagonist for the receptor. These advancements provide powerful tools for future research and should contribute to the expansion of our knowledge of the cannabinoid field.

These initial *in vitro* and *in vivo* experiments provided information that anandamide produced its effects by interacting with the cannabinoid receptor. Yet, differences do exist between anandamide and other cannabinoid agonists. Anandamide acted as a partial agonist at N-type calcium channels, whereas other cannabinoids inhibit N-type channels by acting as full agonists (Mackie, Devane and Hille 1993). Δ^9-THC decreased choice accuracy as the retention time increased in delayed nonmatching to sample memory task performance; anandamide did not affect memory in the same manner. Anandamide was less potent than Δ^9-THC in inhibiting adenylyl cyclase (Vogel *et al.*, 1993) and the

electrically evoked twitch response of the mouse isolated vas deferens (Devane *et al.*, 1992; Pertwee *et al.*, 1993). Anandamide was also less potent than Δ^9-THC in producing antinociception, hypothermia, depressed locomotor activity and catalepsy. After i.v. administration anandamide had a shorter duration of action for catalepsy and hypothermia (Smith *et al.*, 1994). Anandamide differed from Δ^9-THC in that anandamide produced all cannabinoid effects after i.t. administration except catalepsy, and that after i.p. administration the only effect anandamide produced to a significant degree was locomotor inhibition (Smith *et al.*, 1994). Furthermore, the *kappa* opioid antagonist, *nor*-BNI, which blocks Δ^9-THC-induced antinociception, did not alter antinociception after anandamide administration (Smith et al., 1994). This lack of antagonism for anandamide provided evidence for a distinct mechanism of action of anandamide-induced antinociception from that of Δ^9-THC. Thus, questions remain as to whether anandamide produces all of its central effects through the CB_1 receptor.

One of the purposes of this dissertation was to examine structure-activity relationships in anandamide analogs. In order to verify that Δ^9-THC and anandamide share a common receptor, SAR studies were conducted for correlative purposes. Extensive SAR studies with Δ^9-THC have resulted in a three-point receptor attachment theory. Δ^9-THC is believed to interact with cannabinoid receptors through a free phenolic hydroxyl group, an appropriate substituent at the C9 position, and a lipophilic side chain (Binder *et al.*, 1984; Howlett *et al.*, 1988). SAR have also helped to develop more potent analogs of the psychoactive constituents of marijuana, such as the bicyclic compounds and dimethylheptyl derivatives. Although SAR studies suggested that cannabinoids produced their effects through a specific receptor, initial binding studies conducted with [^{3}H]-Δ^9-THC were not successful because Δ^9-THC does not possess high affinity for the receptor and is highly lipophilic. The development of potent cannabinoids like CP 55,940 allowed for the

characterization of the cannabinoid receptor. [^{3}H]-CP 55,940 also was used to localize the cannabinoid receptor in rat brain slices (Herkenham *et al.*, 1991).

The shorter duration of action of anandamide could arise from dissimilarities in metabolism. The likelihood that metabolism plays a role in the actions of anandamide was raised with reports that PMSF, an enzyme inhibitor, interfered with the degradation of anandamide (Childers *et al.*, 1993; Deutsch and Chin, 1993). PMSF did not inhibit a synthase enzyme which catalyzes the formation of anandamide from arachidonic acid and ethanolamine. Initial competition binding studies with [^{3}H]-WIN 55,212-2 suggested that anandamide had low affinity for the receptor (Childers, Sexton and Roy, 1994). These researchers discovered that the addition of PMSF to the membrane preparation dramatically enhanced anandamide's affinity for the receptor. Subsequent binding studies performed in our laboratory supported this finding in that the binding affinity of anandamide was enhanced several hundred fold with the addition of PMSF (Smith *et al.*, 1994). Since the development of more potent analogs of Δ^9-THC greatly facilitated studying the pharmacological and physiological role of cannabinoids, a second objective was to develop metabolically stable analogs of anandamide. Fully investigating the mechanism of action of metabolically unstable and weak compounds is difficult.

To accomplish the two main objectives of understanding structural requirements important for anandamide's interaction with the central cannabinoid receptor and developing metabolically stable derivatives of anandamide, a series of analogs was synthesized with alterations to the main structural features of anandamide. Anandamide is composed of an arachidonyl group linked to ethanolamine through an amide bond. Double bonds are located at carbons 6, 8, 10 and 13, and a hydroxyl group is located at carbon 2'. Changes were made in the level of saturation of anandamide; substitutions were made for the ethanolamide and hydroxyl groups, and alkylations were made at certain sites of anandamide. The analogs were evaluated for their ability to displace [^{3}H]-CP 55,940 in a

filtration binding assay using rat brain membranes in the presence and absence of the PMSF. Behavioral activity was assessed by the ability of the analogs to produce hypomotility and antinociception in mice. The receptor affinities of anandamide analogs were correlated with *in vivo* pharmacological potencies in the hypomotility and antinociception assays and compared to those of anandamide. Thus, these results would provide information about the structural requirements important for anandamide's interaction with the cannabinoid receptor.

Results from these studies allowed for the identification of sites in anandamide's structure which were important for anandamide's interaction with the cannabinoid receptor and potency in the behavioral assays used to assess biological activity. The first series of analogs had alterations in the level of saturation. Removal of all of the cis double bonds with the exception of the double bond located at C10 or addition of another cis double bond at C17 produced compounds with lower affinity and weaker potency. Complete saturation obliterated antinociception. Although these changes are not comprehensive, conclusions may be drawn that the level of saturation is critical to anandamide's interaction with the cannabinoid receptor. The purpose of the double bonds is probably to introduce rigidity and restrict the movement of carbon tail. The double bonds force the carbon chain to assume a more restricted form. The arrangement of the double bonds more than likely restricts the backbone of anandamide to an active conformation and allows proper alignment of anandamide with the cannabinoid receptor.

One of the features of Δ^9-THC important to receptor affinity and pharmacological potency is the phenolic A ring hydroxyl. One may speculate that the hydroxyl group of anandamide corresponds to the hydroxyl group of Δ^9-THC. To test this hypothesis, several analogs were made with substitutions of the hydroxyl group. Substitutions with two bulky groups (sulfonamide and phenoxy) produced compounds that bound to the receptor with higher affinities than anandamide with and without PMSF; the difference in

affinities without PMSF was not as great as anandamide's affinity without PMSF. Yet, these substitutions interfered with the ability to produce antinociception. Although substitution with bulky groups produced weaker compounds, substitution with a much smaller methoxy group produced a basically inactive compound. The most interesting substitution involved a compound with a fluorine group; this analog had a 10-fold higher affinity to the receptor, but only in the presence of PMSF. Substitution of the ethanolamide group with bulkier substituents yielded analogs that did not bind as well to the receptor. The length of the chain attached to the nitrogen was important; increasing this chain decreased affinity and potency in producing antinociception. Based upon these results, one may conclude that the hydroxy group interacts with the receptor. Addition of bulky groups probably interfered with proper receptor alignment. Thus, the resulting analogs were not as effective agonists, and, consequently, they had reduced affinity and potency in the behavioral assays. The fluorine substitution actually produced a compound that bound better to the receptor, but it was not metabolically stable. Since fluorine is an electron-dense atom, it may allow better interaction with the active site of the cannabinoid receptor.

Since the enzyme that metabolizes anandamide acts by cleaving the amide bond, compounds were synthesized that might block the access of the enzyme to this site. Methylations at carbons 2 and 1' and a dimethylation at carbon 2 presumably enhanced the stability of the anandamide analogs as evidenced by an increase in their receptor affinity in the absence of PMSF. Thus, one may reason that the methylations in these compounds provided resistance to enzymatic degradation. Receptor affinity appears to be least affected by the substitution at carbon 2 versus carbon 1'. When larger and bulkier alkyl groups were added to these sites, receptor interaction decreased. Furthermore, replacement of the hydrogen attached to the nitrogen with a methyl group decreased both affinity and potency. The methyl group at the nitrogen prevented proper receptor alignment. Stable compounds

can be produced by methylating the anandamide structure, but a restriction on the size and placement of the attached alkyl groups exists.

The most promising analog in this study was a compound methylated at carbon 2 with a fluorine substitution for the hydroxy. The binding affinity of this analog was greater than anandamide in both the presence and, importantly, absence of PMSF. The combination of methylation and fluorine substitution produced a compound that appeared to be more stable. It also was more potent than anandamide in reducing spontaneous activity and had the same potency in producing antinociception. This compound provides an excellent model upon which to modify and synthesize compounds that have greater affinity and *in vivo* potency than anandamide.

It is not clear why the potency and receptor affinity of these methylated analogs were not enhanced in a comparable fashion. Anandamide is presumed to be rapidly degraded *in vivo* because of its relatively low potency. However, there are numerous other pharmacokinetic factors, coupled with its relatively low receptor affinity, which could account for its low *in vivo* activity. Only direct pharmacokinetic studies will answer these questions. It should be noted that Δ^9-THC and anandamide have time courses in tail-flick (only) which are not too dissimilar when equi-active doses are administered (Smith *et al.*, 1994). Anandamide may be vulnerable to enzymatic degradation in the receptor binding assay because of the long incubation period. Furthermore, anandamide may differ in regards to its time course.

One of the objectives of this study was to correlate receptor affinity and pharmacological potency for the analogs. A good correlation exists for cannabinoids regarding their receptor affinities and pharmacological potencies (Compton *et al.*, 1993). Several possible explanations exist to explain why correlation was not high for anandamide analogs. Previous correlation studies were performed with a much greater number of compounds possessing a wider range of potencies and affinities. On the other hand,

anandamide might be binding to different receptor subtypes, though there is no direct evidence for subtypes in the CNS. Although great care was taken to minimize differences in the fate of these compounds in the *in vivo* and *in vitro* conditions, it is likely that solubility factors, metabolism and tissue distribution were not the same for all of these compunds.

Several very recent papers have explored the structure-activity relationships of anandamide and provide support for the conclusions drawn in this dissertation. Edgemond *et al.* (1994) investigated the role of the hydroxyl group in the binding of anandamide using two analogs. For one analog the alkyl hydroxyl group was replaced with a hydroxyphenyl group, and the ethanolamide group of the second analog was replaced with a hydroxyphenyl group. Both compounds had weaker affinity for the CB_1 receptor than anandamide. Neither analog was a substrate for PMSF, which provides evidence that the amidase is selective for fatty acid ethanolamides. Recently, Pinto *et al.* (1994) studied the structure-activity requirements for a series of novel amides and rigid hairpin conformations typified by N-(2-hydroxyethyl)prostaglandin amides. Extending the hydroxyalkyl group by one carbon increased affinity to the CB_1 receptor by an order magnitude. Substituting the hydroxyalkyl moiety with a propyl group increased affinity by five-fold. They concluded that the bulk and length of the moiety attached to arachidonic acid are more important determinants of affinity for CB_1 than is hydrogen-bonding capability (Pinto *et al.*, 1994). Venance *et al.* (1995) performed a limited SAR study to determine if changes in the levels of saturation of anandamide would affect anandamide's ability to inhibit gap-junction conduction is striatal astrocyte cells. Analogs with 3, 2, 1 or 0 double bonds did not produce a significant effect on gap-junction inhibition. These results concur with the results presented in this dissertation that increasing the levels of saturation decreases activity. Finally, Welch *et al.* (1995) demonstrated that the fluorinated analog of anandamide (fluorine substitution of the hydroxyl) was more potent than either anandamide

or Δ^9-THC in producing antinociception following i.t. administration in the mouse. Results from this dissertation indicated that the fluorinated analog of anandamide had a higher K_i in the absence of PMSF, like anandamide, but had affinity greater in the presence of PMSF. It was similar in potency to anandamide in the spontaneous activity test, but was 10 times less potent than anandamide in producing antinociception following i.v. administration. The difference in results from those of Welch *et al.* (1995) in regards to antinociception might be due to the route of administration, i.v. versus i.t..

The behavioral and the binding data support the existence of a receptor whose binding region possesses site(s) that accommodates both cannabinoid and anandamide analogs. Molecular modeling approaches also support this hypothesis by demonstrating that there is the possibility of a common pharmacophore (Martin *et al.*, 1991). Specifically, an anandamide conformation has been obtained using Sybyl molecular modeling software. Thomas *et al.* (1996) performed a structure-activity analysis using several of the anandamide analogs described in this dissertation to determine a molecular conformation of anandamide that can be incorporated into a previously described model. A low energy conformation of anandamide and the classical cannabinoids can be obtained with the superposition of the oxygen of the carboxyamide with the pyran oxygen in Δ^9-THC, the hydroxyl group of the ethanol with the phenolic hydroxyl group of Δ^9-THC, the five terminal carbons and the pentyl side chain of Δ^9-THC and the polyolefin loop overlaying with the cannabinoid tricyclic ring (Thomas *et al.*, 1996).

Several recent experiments have explored the link between the presence of cannabinoid receptors in specific brain areas, including the striatum, globus pallidus, substantia nigra pars reticulata and hippocampus, to the production of cannabinoid-induced behaviors. Evaluating the effects of anandamide in the CNS is critical to understanding the role of the proposed cannabinoid, or anandaminergic, neurochemical system. Intrastriatal injection of WIN 55,212-2, CP 55,940 and anandamide induced turning behavior in mice

(Souilhac *et al.*, 1995). DA_1 and DA_2 antagonists or 6-hydroxydopamine lesions of the striatum blocked WIN 55,212-2 and CP 55,940-induced turning behavior, suggesting involvement of dopamine transmission in cannabinoid-induced turning. These results support the role of CB_1 receptor mediation upon nigrostriatal function in the basal ganglia

It has been proposed that Δ^9-THC produces catalepsy by enhancing GABAergic transmission mediated by $GABA_A$ receptors located in the globus pallidus, a brain area particularly rich in cannabinoid receptors. Wickens and Pertwee (1993) found that both anandamide and Δ^9-THC enhanced the ability of muscimol, a $GABA_A$ receptor agonist, to induce catalepsy when the drugs were administered into the globus pallidus. The relevance to humans that anandamide, when administered intrapallidally, also enhanced the ability of a $GABA_A$ agonist to enhance catalepsy in rats is unknown. It also has been proposed that cannabinoid receptors on striatonigral neuron terminals may regulate movement by disinhibiting the activity of the substantia nigra pars reticulata neurons; this disinhibition may occur by inhibiting the release of GABA into the substantia nigra pars reticulata (Miller and Walker, 1995). These results suggest that a possible way by which endogenous cannabinoids may regulate movement is by inhibiting striatonigral neurotransmission and thereby disinhibiting the activity of the substantia nigra pars reticulata neurons. Autoradiographic studies in rats have demonstrated that the cannabinoid receptor is located on the axon terminals projecting to the globus pallidus and substantia nigra (Herkenham *et al.*, 1991b). These neurons are selectively lost in Huntington's disease. Glass *et al.* (1993) used autoradiography to compare binding of [^{3}H]-CP 55,940 in the substantia nigra of Huntington's disease and normal human brains. In normal brains, cannabinoid receptors are discreetly located within the substantia nigra pars reticulata. In contrast, Huntington's disease brains have a massive loss (97%) of cannabinoid receptor binding in the substantia nigra pars reticulata (Glass, Faull and Dragunow, 1993). These results demonstrate that in the substantia nigra of the human brain, cannabinoid receptors are

located on striatonigral terminals which degenerate in Huntington's disease. Cannabinoid receptors are also present in the dentate gyrus and to a lesser extent in the entorhinal cortex. Cannabinoids have been found to decrease paired-pulse facilitation of synaptic transmission between perforant path axons and granule cells of the dentate gyrus (Kirby, Hampson and Deadwyler, 1995). These results provide evidence making it likely that cannabinoids, including endogenous compounds, play a role in controlling short-term plasticity of perforant path synapses.

Cannabinoid receptors in the hippocampus have been implicated to produce impairment of memory. Lichtman *et al.* (1995) studied the effects of cannabinoids on the spatial memory in rats. Systemic administration of CP 55,940 and WIN 55,212-2 disrupted performance in the eight arm radial-maze, but anandamide did not. These results suggest that systemic and intrahippocampal administration of cannabinoids acutely impair working memory. Interestingly, anandamide was also inactive in the delayed nonmatching to sample memory task in rats. The lack of effect of anandamide could be due to rapid degradation or species specificity. Another possibility is that anandamide, or other naturally occurring anandamides, might lack the memory-impairing effects of cannabinoids. These compounds would be useful therapeutic agents if they possess beneficial properties without impairing memory. Learning and memory processes result from hippocampal long-term potentiation formation. Cannabinoids prevent LTP in rat hippocampal slices. Anandamide also inhibits LTP (Collin *et al.*, 1995; Terranova *et al.*, 1995).

One of the hindrances of cannabinoid research for many years was the lack of an antagonist. The recent discovery of SR 141716A as the central cannabinoid antagonist should greatly facilitate studying the physiological and psychological purpose of an anandamide-based neurochemical system. If SR 141716A is the antagonist for the CB_1

receptor, then it should localize to the same population of cannabinoid receptors in the brain. Research has been conducted to assess the ability of SR 141716A to block the effects of cannabinoids and anandamide in both *in vitro* and *in vivo* systems. SR 141716A antagonized the effects of cannabinoids in the mouse vas deferens and in inhibiting adenylyl cyclase in cells transfected with the CB_1 receptor (Rinaldi-Carmona *et al.*, 1994). Inhibition of adenylyl cyclase in $N_{18}TG_2$ mouse neuroblastoma cells by the anandamides (22:4, n-6) and (20:3, n-6) was completely reversed by SR 141716A (Barg *et al.*, 1995). *In vivo*, SR 141716A antagonized cannabinoid-induced antinociception, hypothermia, catalepsy and spontaneous activity depression (Rinaldi-Carmona *et al.*, 1994). SR 141716A antagonized turning behavior in mice produced by intrastriatal administration of WIN 55,212-2, CP 55,940 and anandamide (Souilhac *et al.*, 1995). Anandamide's inhibition of LTP in rat hippocampal slices was reversed by the cannabinoid antagonist (Terranova *et al.*, 1995). SR 141716A has been used to reveal whether CB_1 receptor blockade alters electrophysiological functioning of DA neurons in the substantia nigra pars compacta, a target of the striato-nigral feedback system (Gueudet *et al.*, 1995). Acutely, SR 141716A increased SNC DA cell population response; after repeated administration, SR 141716A decreased SNC DA cell population response. These results suggest that CB_1 receptor blockade interrupts a cannabinoid endogenous tone controlling extrapyramidal function. Although SR 141716A blocks many of the effects of cannabinoids and anandamides, one study demonstrated that SR 141716A did not block anandamide's inhibition of gap-junction conductance in striatal astrocytes (Venance *et al.*, 1995). This result indicates that anandamide may modulate gap-junction permeability through a mechanism distinct from the CB_1 receptor. Thus, early research with SR 141716A indicates that it antagonizes the effects of cannabinoids and anandamide.

The purpose of the second part of this dissertation was to determine through autoradiographic experiments if CP 55,940, anandamide and SR 141716A, three

structurally diverse compounds, bound to the same populations of cannabinoid receptors. Since CP 55,940 is more potent than Δ^9-THC, it has been used extensively to investigate the effects of cannabinoids in both *in vitro* and *in vivo* experiments. It is important to compare the binding patterns and densities of well-studied cannabinoids, such as CP 55,940, with anandamide, the endogenous ligand to the cannabinoid receptor. Differences in receptor affinity and binding density between specific brain areas would provide evidence that an endogenous ligand does not mimic all of the effects of THC and other cannabinoids. If SR 141716A is truly an antagonist to the central cannabinoid receptor, then it should bind to the CB_1 receptor in a similar manner to the binding distribution of CP 55,940 and anandamide. The use of SR 141716A as a cannabinoid antagonist is critical to disseminating the function of an anandaminergic system in the central nervous system. Therefore, establishing that SR 141716A is binding to CB_1 receptor in the same brain areas as anandamide and CP 55,940 is of great importance in this field. No quantitative comparison has yet been made between CP 55,940, anandamide and SR 141716A.

Autoradiographic experiments were performed on rat brain slices to quantitate the receptor affinity and binding density for anandamide, CP 55,940 and SR 141716A to the central cannabinoid receptor. Measurements were made from 11 brain regions including the entorhinal, occipital, parietal and frontal cortex, the lateral and medial caudate-putamen, dentate gyrus, CA1 and CA3 regions of Ammon's horn, the substantia nigra pars reticulata and the molecular layer of the cerebellum. These areas were selected since cannabinoids have been shown to produce effects, such as memory disturbances, alterations in cognition and movement, through probable interaction with these regions.

Comparisons were made between receptor affinities (K_i's) for each drug for the selected brain regions. Statistical differences did not exist for anandamide's K_i's for the brain areas, nor were there any statistical differences between K_i's for CP 55,940 and SR 141716A. Thus, anandamide's affinity for the central cannabinoid receptor is the same

across all of the rat brain examined. This is also true for SR 141716A and CP 55,940. The next comparison was to determine if the three compounds were binding to the receptor in the same manner. This was established through determination of the parallelism for a representative curve from each region. All curves for anandamide and CP 55,940 were parallel. All curves, except for the entorhinal cortex, for SR 141716A were parallel. The physiological relevance of this difference remains to be established. The final comparison was made by correlating the binding densities for anandamide and SR 141716A with the binding densities of CP 55,940. High correlations existed, thus demonstrating that the density in the cannabinoid receptors binding anandamide, CP 55,940 and SR 141716A were very similar.

In summary, based upon an evaluation of receptor affinities for CP 55,940, anandamide and SR 141716A and a comparison of receptor densities, the three compounds appear to be binding to the same population of receptors in different brain areas. These results provide both visual and quantitative results that the central cannabinoid receptor binds all three compounds in a similar manner.

Even though the results in this dissertation provide no evidence for receptor subtypes, it does not rule out the possibility that subtypes do exist. The technique of autoradiography is not sensitive enough to detect small differences in receptor subpopulations. Pharmacological differences between anandamide and other cannabinoids could be due to interaction with a small number of as yet undiscovered subtypes. Differences could also be metabolic in nature or to as yet undiscovered mechanisms.

Speculating on the physiological role of the anandaminergic system is difficult. One cannot explain how a receptor can accommodate such a structurally diverse group of compounds. Although Δ^9-THC has been proposed to interact with the cannabinoid receptor through a three point attachment, it is unknown which areas of anandamide, the aminoalkylindoles and SR 141716A corresponds to these structural moieties in Δ^9-THC.

Future research will be essential in establishing the biological function of anandamide and the family of anandamides.

In summary, this dissertation investigated anandamide's interaction with the central cannabinoid receptor. Specific aims of this dissertation were met. Structural features of anandamide important for anandamide's interaction with the cannabinoid receptor were determined by *in vitro* and *in vivo* assays; anandamide was structurally modified to develop compounds with higher binding affinity to the cannabinoid receptor; anandamide's binding to the central cannabinoid receptor was characterized autoradiograpically in select brain regions; and anandamide, CP 55,940 and the cannabinoid antagonist, SR 141716A were shown to, respectively, bind to the same population of receptors in the same manner.

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Vita

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