INVESTIGATION OF THE INTERACTION OF ENVIRONMENTAL CHEMICALS WITH CANNABINOID-1 (CB1) RECEPTORS IN MAMMALIAN BRAIN USING THE [^{3}H]CP-55,940 BINDING ASSAY

by

Sudip Kumar Ghose
B.Sc., University of Calcutta 2002
M.Sc., Annamalai University 2004

PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ENVIRONMENTAL TOXICOLOGY

In the
Department of Biological Science

SIMON FRASER UNIVERSITY

Fall 2008

APPROVAL

Name: Sudip Kumar Ghose

Degree: Master of Environmental Toxicology

Title of Project:

Investigation of the interaction of environmental chemicals with cannabinoid-1 (CB1) receptors in mammalian brain using the [^{3}H]CP-55,940 binding assay

Examining Committee:

Chair: Dr. B. Crespi, Professor

Dr. R. Nicholson, Associate Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. C. Kennedy, Professor
Department of Biological Sciences, S.F.U.

Dr. F.C.P. Law, Professor
Department of Biological Sciences, S.F.U.
Public Examiner

5 November 2008
Date Approved

SFU SIMON FRASER UNIVERSITY
THINKING OF THE WORLD

STATEMENT OF ETHICS APPROVAL

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

(a) Human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

(b) Advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

(c) as a co-investigator, in a research project approved in advance,

or

(d) as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Bennett Library
Simon Fraser University
Burnaby, BC, Canada

ABSTRACT

Cannabinoid-1 (CB1) receptors are G protein-coupled receptors, which are widely distributed in mammalian brain. This investigation primarily examines several voltage-gated sodium channel targeting compounds for their ability to interfere with the binding of the CB1 receptor specific agonist [^{3}H]CP-55,940 to the CB1 receptors in a membrane fraction prepared from mouse brain. The study found that the pyrethroid insecticide [1*RS*]*trans*-permethrin is more potent at displacing radioligand (IC_{50} 7.1 μM) than its *cis* counterpart [1*RS*]*cis*-permethrin (IC_{50} 18.1 μM). Two other pyrethroids, deltamethrin and cypermethrin exhibited intermediate potency (IC_{50}s 9.3 and 11.2 μM, respectively). The dihydropyrazole insecticide RH3421 was the most potent within the group of insecticides tested with an IC_{50} value of 5.7 μM. Another dihydropyrazole, RH5529 was less potent (IC_{50} 16.5 μM). Other pesticides and drugs were inactive or barely active. The toxicological relevance of these findings remains to be clarified in future experiments.

Keywords: Cannabinoid-1 (CB1) receptors, mammalian brain, [^{3}H]CP-55,940 binding, voltage-gated sodium channels, pyrethroids, dihydropyrazoles

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation, gratitude and thanks to my senior supervisor Dr. Russell Nicholson who gave me the greatest inspiration and encouragement and patiently guided me through this project. His advice over the years has been instrumental throughout my time in the MET program. I am very grateful for his help.

I would also like to express my sincere gratitude to Dr. Chris Kennedy for agreeing to act as a member of my supervisory committee and providing his insightful perspectives and suggestions.

I also wish to thank Dr. Francis Law for his kindness in evaluating my thesis and serving as my Public Examiner.

I am also grateful to Dr. Chengyong Liao and Yin (Daniel) Duan for teaching me the binding assay technique.

Finally, heartiest thanks to my parents, sister and other family members for their endless support and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Δ^9-THC	Δ^9- tetrahydrocannabinol
CB1	Cannabinoid-1
CB2	Cannabinoid-2
TM	Transmembrane
GPCR	G protein-coupled receptor
SAR	Structure activity relationship
GTP	Guanosine-5'-triphosphate
GDP	Guanosine diphosphate
2-AG	2-arachidonoyl glycerol
2-AGE	2-arachidonoyl glycerol ether
DSI	Depolarization-induced suppression of inhibition
DSE	Depolarization-induced suppression of excitation
FAAH	Fatty acid amidohydrolase
mGluRs	Metabotropic glutamate receptors (mGluRs)
cAMP	Cyclic adenosine monophosphate
GIRK	G protein-coupled inwardly rectifying potassium channel
ATP	Adenosine triphosphate
B_{max}	Maximum binding
K_D	Equilibrium dissociation constant
[^{3}H]BTX-B	[^{3}H]batrachotoxin-A-20α-benzoate
VGSC	Voltage-gated sodium channel
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
IC_{50}	Concentration that causes 50% inhibition
S.E.M.	Standard error of mean

CHAPTER 1: BACKGROUND

1.1 Introduction

Cannabinoids are group of compounds, which are secondary metabolites and are present in the Cannabis plant (*Cannabis sativa*). The various members of this group of compounds are structurally related to tetrahydrocannabinol (THC), also known as Δ^9-THC, which is regarded as the main psychoactive substance present in the Cannabis plant. THC's most likely function in the Cannabis plant is to help provide protection from herbivores or pathogens (Abel, 1975). Cannabinoids, especially Δ^9-THC (Figure 1-1), represent the main exogenous ligands of cannabinoid receptors, which are a class of receptors within the G protein-coupled receptor super family (Matsuda et al., 1990). There are currently two subtypes of cannabinoid receptors, which have been identified in mammals, the CB1 and CB2 types. The CB1 receptor is expressed at high concentrations in certain areas of brain, especially in basal ganglia, cerebellum, hippocampus and cortex (Kreitzer, 2002). In addition, the CB1 receptor is expressed in different excretory glands, for example the pituitary gland and the thyroid gland. There is some evidence for its occurrence in the adrenal gland as well. The CB1 receptor is also present in different cell types in the body such as fat cells, liver cells (including Kupffer cells and stellate cells), muscle cells and digestive tract cells. The CB1 receptor has also been located on Leydig cells, sperm cells of the human reproductive system and in the ovary, oviduct and placenta (Begg et al., 2005). The other cannabinoid receptor, the CB2 receptor, is mainly

expressed on T cells of the immune system, specifically on macrophages and B cells (Miller, 2008).

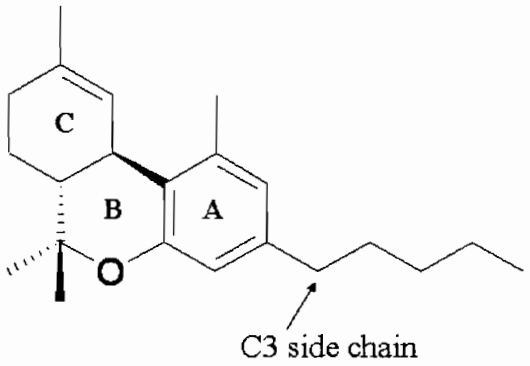


Figure 1-1 Structure of tetrahydrocannabinol (Δ^9-THC) showing ring systems A-C, the C3 side chain and the A ring hydroxyl group (Shim et al., 2002).

1.2 The structure of the CB1 receptor

The CB1 receptor is a G protein-coupled receptor (GPCR) which is composed of seven transmembrane helices (TM1-TM7) connected by three extracellular (E1, E2 and E3) and three cytoplasmic (C1, C2 and C3) loops (Figure 1-2) (Shim et al., 2003). The binding of an agonist to the receptor causes a conformational change in the TM and associated loop regions to transduce the agonist signal to the associated G protein. Highly conserved amino acid residues in the GPCR are responsible for maintaining its three-dimensional structure and function (Shim et al., 2003).

The homology model of the CB1 receptor was developed using the structure of bovine rhodopsin as a template. Rhodopsin was used as a template because it represents a classical example of a G protein-coupled macromolecule and 22 out of 34 amino acids present in the TM regions of rhodopsin are also found in the TM helical sequence of the human CB1 receptor (Figure 1-3). Monte Carlo (MC) and molecular dynamic (MD) simulations were used to identify potential binding conformations and binding sites of the CB1 receptor (Shim et al., 2003). Identifying the conformation of cannabinoid agonists required for binding to the CB1 receptor is extremely important as it not only indicates the binding interactions between the ligand and amino acid residues on the receptor, but also provides insight into the molecular mechanism of receptor activation (Shim et al., 2003). Site-specific mutation of the rhodopsin subfamily of receptors demonstrated that ligand binding sites are present within the TM core regions specifically in the crevices formed by TM3, TM5, TM6 and TM7 (Shim et al., 2002).

Mahmoudian (1997) modelled the docking of the naturally occurring cannabinoid agonist Δ^9-THC on the CB1 receptor by using bacteriorhodopsin as a structural template.

This research indicated the presence of calcium binding sites in the extracellular N terminus of this receptor. It was also proposed that the side chain of the ligand Δ^9-THC binds to the hydrophobic pocket of the human CB1 receptor, which consists of the aromatic rings of Trp-356 (TM-6) Trp-241 (TM-4) and the side chains of Met-240 (TM-4), Leu-359, Leu-360 (TM-6) and Ala-283 (TM-4). Moreover, the phenolic hydroxyl group of Δ^9-THC forms a hydrogen bond with the carboxyl group of Ala-198 (TM-3). Using an alternative approach Tao et al., (1999) demonstrated the binding of the synthetic CB1 receptor agonist CP-55,940 to the CB1 receptor using a projection map of rhodopsin as a template.

1.2.1 Homology model of the CB1 receptor

The homology model (Figure 1-4) was constructed by putting the amino acid sequences of TM helices of the CB1 receptor aligning with bovine rhodopsin including the DRY motif (D3.49, R3.50 and Y3.51) and three Pro residues (P4.60, P6.50 and P7.50) in the TM segments of GPCRs (Baldwin et al., 1997). The three extracellular loops of the CB1 receptor were constructed by using a random loop search method known as InsightII and the Brookhaven Protein Databank (PDB) (Berman et al., 2000) However, most of the GPCRs do not contain a disulfide bridge located in between the Cys residues at the extracellular end of TM3 and the E2 loop (Davidson et al., 1994). This specific disulfide bond is absent in the CB1 receptor since a Cys residue is not present in the TM3 region. However, a radioligand binding studies of CB1 receptors by Lu et al., (1993) and Hillard et al., (1995) suggested the importance of a disulfide bond for the binding of AC-bicyclic agonists. Moreover, Lu et al. (1993) also demonstrated that the disulfide reducing agent

dithiothreitol (DTT) could decrease the binding of the CB1 receptor agonist [^{3}H]CP-55,940 to the receptor again indicating the importance of a disulfide bridge.

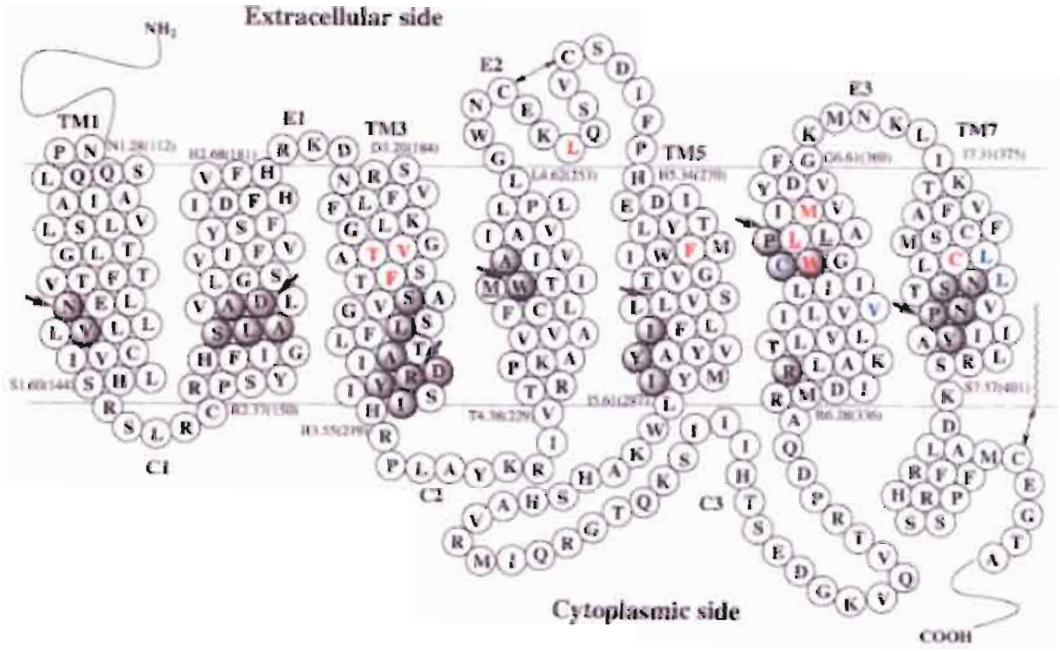


Figure 1-2 Structure of the CB1 receptor. Bold shaded circles represent highly conserved residues. The red letters indicate the hydrophobic residues, which interact with the C3 side chain of cannabinoid ligands (Tao et al. 1999).

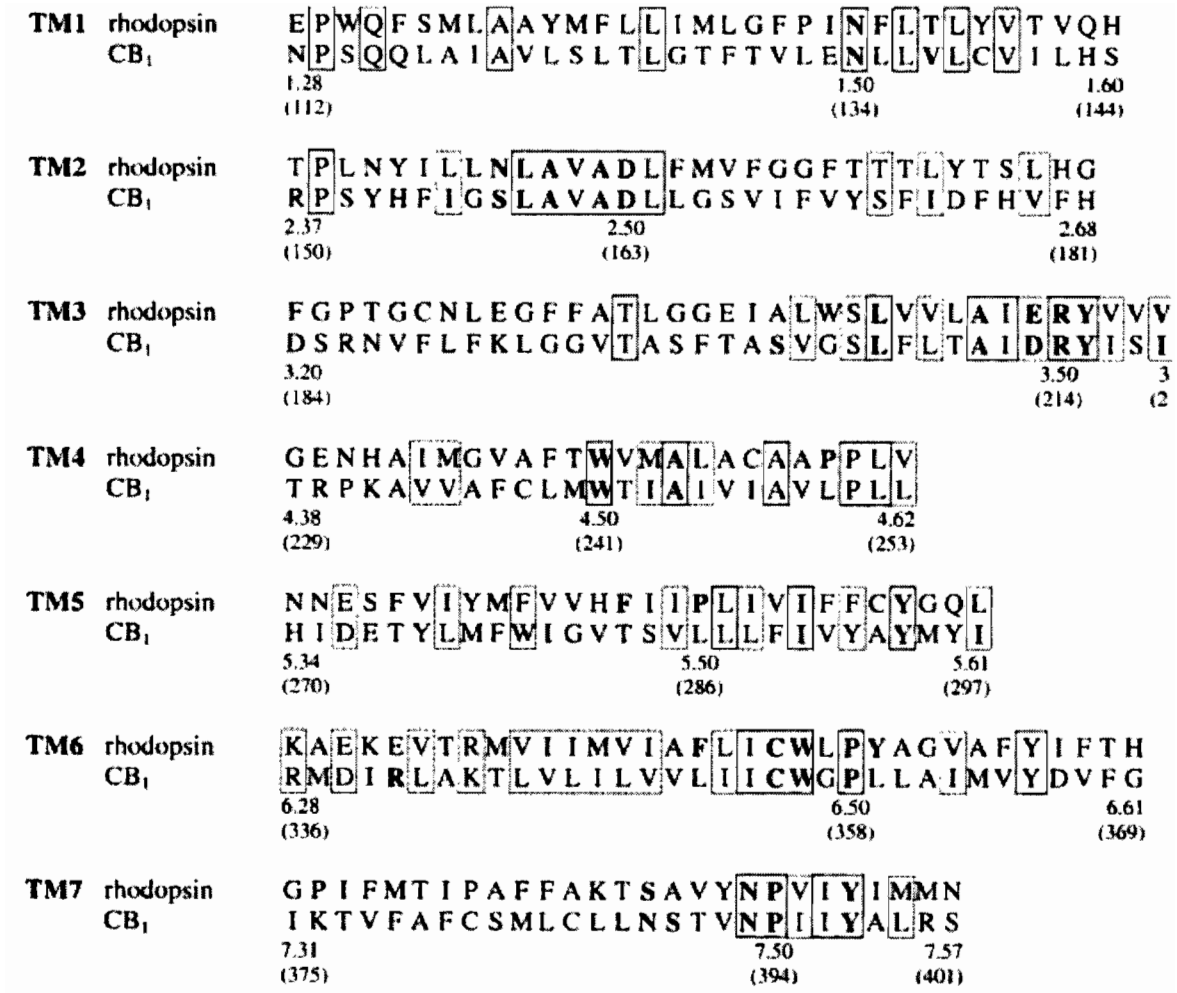


Figure 1-3 Arrangement of the TM helical sequences of the CB1 receptor compared with those of bovine rhodopsin. 22 out of 34 highly conserved residues in the rhodopsin subfamily (Baldwin et al., 1997) which are common to the human CB1 receptor are shown in bold letters contained within boxes (Shim et al., 2003).

Figure 1-4 The homology model of the CB1 receptor. Putative binding sites are shown as white dots as revealed by the binding site search (InsightII). Seven TM helical sequences, TM1 through TM 7 are depicted in different colours (red, orange, yellow, green, cyan, blue and purple respectively). Extracellular loops are colored in magneta (Shim et al., 2003).

1.2.2 Structural requirements for the binding of agonists to the CB1 receptor and the binding site requirements of the CB1 receptor

Shim et al., (2003) proposed two C3 side chain-binding conformations specifically C3-*out* and C3-*in* for the cannabinoid agonist CP55244. The C3 side chain (Figure 1-1) is positioned toward the extracellular region for the C3-*out* conformation but towards the interior helix for the C3-*in* conformation.

The docking study conducted by Shim et al., (2003) predicted that the C3-*in* binding sites for typical cannabinoid ligands such as CP55244 are localized approximately 2-6 turns away from the extracellular TM helical boundaries between TM 3-7 helices (Figure 1-6). Furthermore, in the C3-*in* binding mode, the C3 side chain of the cannabinoid agonist is positioned deep within the TM helical region and the E2 loop of the receptor extends into the binding crevice and engages in a binding interaction with the A and C/D ring moieties of the cannabinoid ligand. (Figure 1-6).

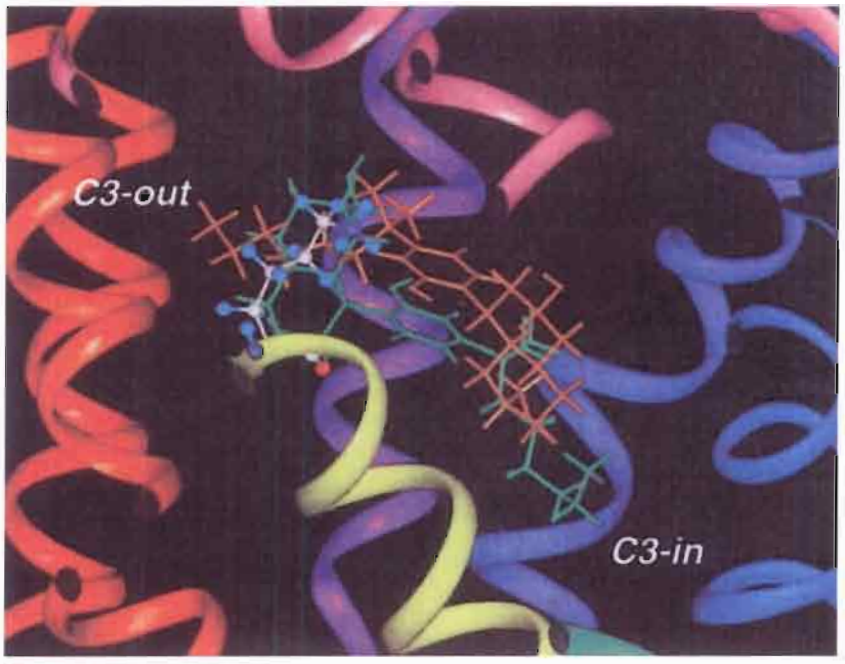


Figure 1-5 Two docking conformations of CB1 receptor agonist CP 55244, C3 *out* (orange) and C3-*in* (green) within CB1 receptor (Shim et al., 2003).

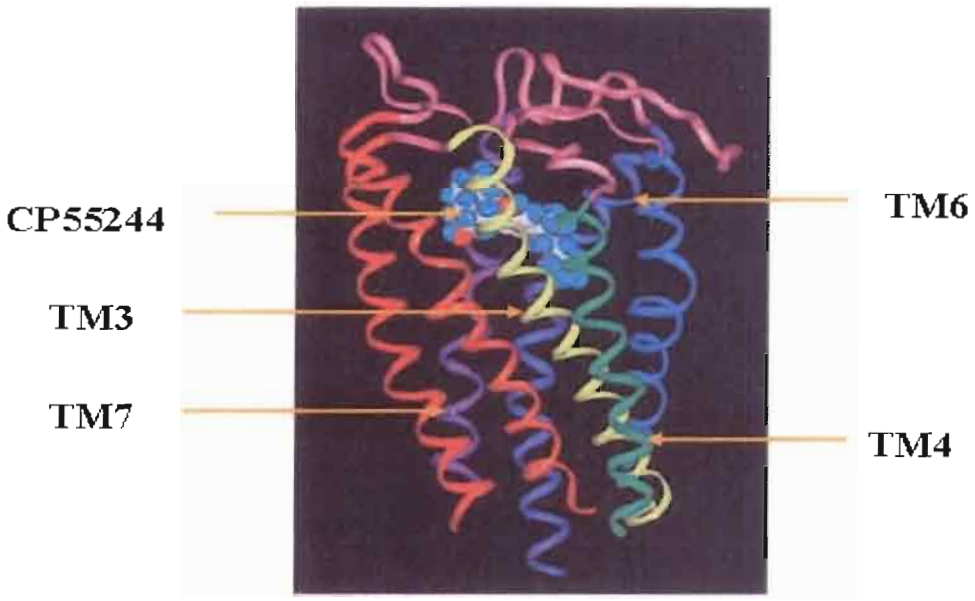


Figure 1-6 Docking of CB1 receptor agonist CP55244 within the binding crevices (TM segments) of the CB1 receptor (Shim et al., 2003).

Extensive structure activity relationship (SAR) studies for cannabinoid compounds identified the C3 side chain as a critical pharmacophoric element. It has been proposed that the existence of a hydrophobic pocket on the CB1 receptor facilitates a binding interaction with the hydrophobic C3 side chain of cannabinoid ligands (Melvin et al., 1993). Cannabinoid agonists also contain additional hydroxyl groups on the C-ring that have been considered as potential pharmacophoric elements (Shim et al., 2005). It was assumed that the hydrophobic C3 side chain of the cannabinoid ligand occupies the region in between TM6 and TM7 (Tao et al., 1999). However, Shim et al. (2003) demonstrated that binding region in between TM6 and TM7 appeared less able to accommodate to the bulky C3 side chain of cannabinoid agonists (Shim et al., 2003)

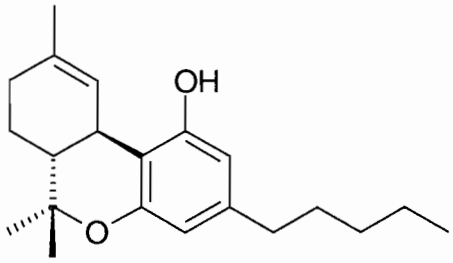
A knowledge of the three-dimensional (3D) molecular structure and the molecular topography of the ligand-binding regions of cannabinoid receptors is very important, as it will likely prove useful in the development of novel cannabimimetic drugs with potential therapeutic value (Xu et al., 2005).

1.3 Synthetic cannabinoids

The synthesis of cannabinoids in the laboratory was often based on the structure of plant cannabinoids and a large number of analogs have been produced and tested. However, newer compounds are no longer closely related to natural cannabinoids and neither are they based on the structure of the endogenous cannabinoids. Synthetic cannabinoids are useful in determining whether receptor active drugs are agonists or

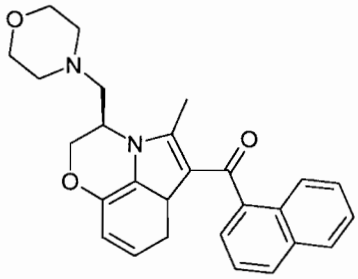
antagonists. Synthetic cannabinoids are also useful since they assist in the understanding of structure-activity relationships (SARs) of cannabinoid molecules (Lan et al., 1999).

Notable synthetic cannabinoids are WIN 55,212-2 (an agonist), CP-55,940 (an agonist) AM251 (an antagonist), SR 141716A (an antagonist). The structures of these compounds are provided in Figure 1-7.

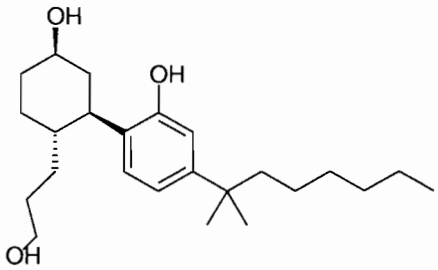


Natural cannabinoid tetrahydrocannabinol (Δ^9-THC)

SYNTHETIC AGONISTS

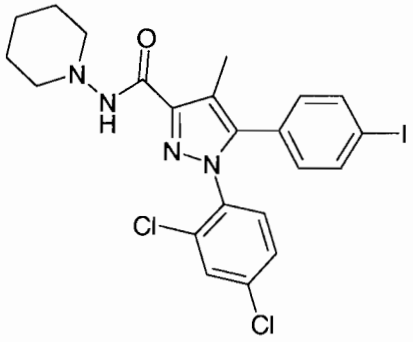


WIN 55,212-2

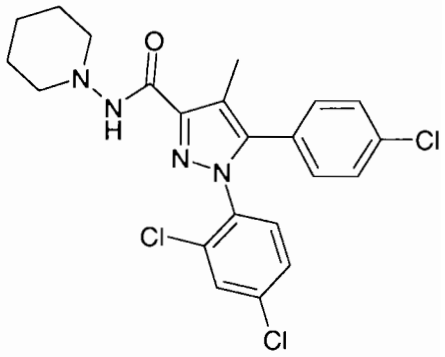


CP-55,940

SYNTHETIC ANTAGONISTS



AM251



SR 141716A

Figure 1-7 The structures of some important CB1 receptor agonists and antagonists

1.4 G proteins

Guanine nucleotide-binding proteins are commonly known as G proteins. These are important signal transducing molecules in cells, which function as "molecular switches". The G proteins are activated by G protein-coupled receptors (GPCRs) such as CB1 receptors when a ligand binds to the receptor. Structurally, G proteins are made up of three subunits, specifically the alpha (α), beta (β) and gamma (γ) moieties. G proteins are bound to the inside surface of the cell membrane and consist of G_α which is tightly associated with the $G_{\beta\gamma}$ dimer. When a ligand activates, the G protein-coupled receptor (GPCR), for example CB1 receptor, induces a conformational change in the G protein. This conformational change triggers the binding of guanisine-5'-triphospahte (GTP) to the G_α subunit and dissociation of the G_α subunit from the $G_{\beta\gamma}$ dimmer. This phenomenon activates a variety of signaling cascades and/or secondary messenger pathways in the cell. The G_α subunit eventually hydrolyzes attached GTP to guanosine diphosphate (GDP) by its inherent enzymatic activity (GTPase). As soon as GTP is hydrolysed this allows reassociation of G_α with $G_{\beta\gamma}$ subunit to complete the cycle (Figure 1-8) (Jahnichen, 2006).

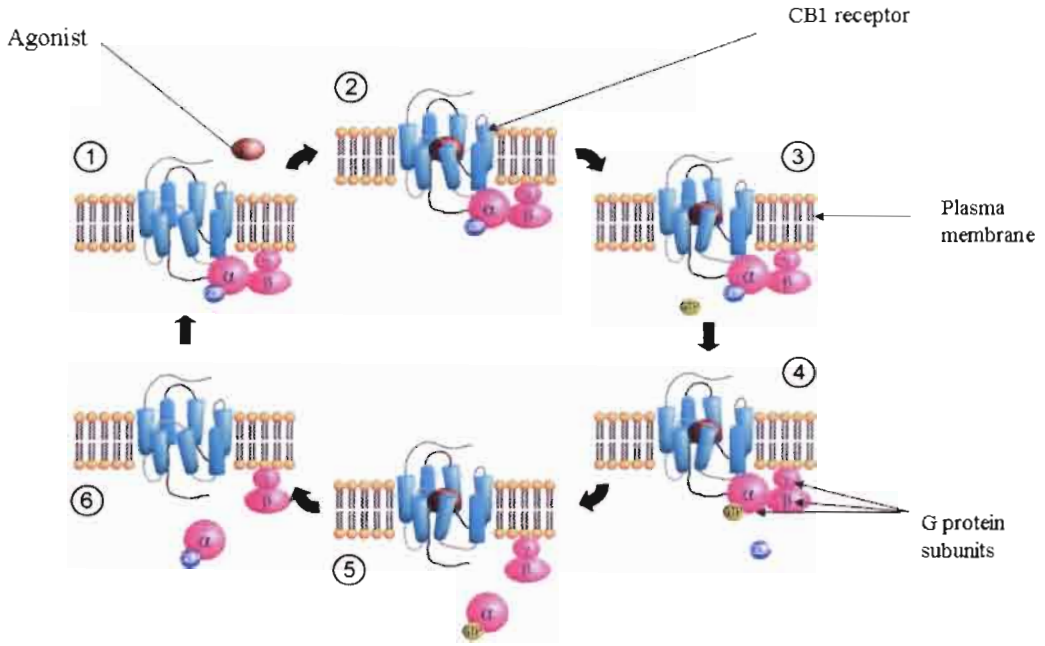


Figure 1-8 Activation cycle of G-proteins by Cannabinoid-1 (CB1) receptor agonists (Jahnichen, 2006).

1.5 Discovery of the endogenous cannabinoid system

The first endogenous cannabinoid discovered in human brain was arachidonoyl ethanolamide or anandamide, which consists of arachidonic acid with an amide linkage to ethanolamine (Devane et al., 1992). Anandamide exhibits similar behavioural (Crawley et al., 1993), pharmacological (Smith at el., 1994) and signal transduction effects (Felder et al., 1993) as the cannabinoid agonist Δ^9-THC. Anandamide is widely distributed in different parts of the body for example it is found in the central and peripheral nervous system, kidney, blood plasma, testis and different parts of the cardiovascular system (Martin et al., 1999). Another endocannabinoid, known as 2-arachidonoyl glycerol (2-AG), was isolated from mammalian brain and found to bind to the CB1 receptors (Sugiura et al., 1995). Subsequently, other endocannabinoids including N-arachidonoyl dopamine, 2-arachidonoyl glycerol ether (2-AGE, noladin ether) have been discovered (Piomelli, 2003; Di Marzo et al., 1998) as well as and other endocannabinoids such as N-arachidonyl GABA and N-arachidonyl glycine were isolated (Huang et al., 2001).

1.5.1 Depolarization-induced suppression of inhibition (DSI)

Depolarization-induced suppression of inhibition (DSI), as revealed by electrophysiological experimentation, represents the classical example of endocannabinoid function in the central nervous system. Although DSI was observed in the cerebellum and the hippocampus, it is suspected that this mechanism also operates in the other areas of brain such as in the basal ganglia, cortex, amygdala and the hypothalamus (Katona et al., 2001, Jo et al., 2005, Bodor et al., 2005, Matyas et al., 2006). Depolarization of a postsynaptic neuron results in increased permeability to

calcium ions. The influx of calcium ions into the post synaptic neuron elicits rapid biosynthesis of retrograde messengers (endocannabinoids) from lipid precursors in the membrane. The endocanabinoids travel in reverse fashion to the presynaptic cell and through a sequence of events inhibits voltage-gated calcium channels and open potassium channels. The inhibition of calcium channels prevents entry of calcium ions into the cell which results transient suppression of inhibitory neurotransmitter GABA (γ-aminobutyric acid) release. Thus, inhibition of inhibitory transmitter GABA by endocannabinoids is known as depolarization-induced suppression of inhibition or DSI (Liano et al., 1991; Pitler et al., 1992; Vincent et al., 1993; Pitler et al., 1994).

Other investigations support the central role of the CB1 receptor in DSI in the brain (Jo et al., 2005, Bodor et al., 2005). In addition, DSI was not observed in CB1 receptor knockout mice, suggesting the crucial role of CB1 receptor and also the role of CB1 receptor ligands, the endocannabinoids in DSI (Kreitzer & Regehr 2001, Ohno-Shosaku et al., 2002).

1.5.2 Depolarization-induced suppression of excitation (DSE)

Kreitzer et al. (2001) discovered that depolarization of Purkinje cells could also lead to a temporary reduction in the release of excitatory neurotransmitters, specifically glutamate and acetylcholine in climbing fibers and parallel fibers. This phenomenon is known as depolarization-induced suppression of excitation (DSE). The endocannabinoids also appear to mediate DSE (Cristino et al., 2006, Hajos et al., 2002). Depolarization of the post-synaptic neuron results in the biosynthesis of endocannabinoids from lipid precursor which eventually inhibit voltage-gated calcium channels through a series of events on presynaptic cell. The inhibition of calcium channel prevents the entry of

calcium ions into the postsynaptic cell which eventually inhibits release of excitatory neurotransmitters, for example glutamate (Kreitzer et al., 2001). Interestingly, Ohno-Shosaku et al. (2002) and Hajos et al. (2001) found DSE persists in CB1 receptor knock out mice. However, DSE is currently a largely unexplored phenomenon and more research is needed to draw any firm conclusions.

Conventional neurotransmitters are released from presynaptic nerve endings and activate receptors on the postsynaptic cell. In contrast, the endocannabinoids are known as retrograde messenger because they travel backwards as such they originate from the postsynaptic cell and act on the presynaptic nerve endings where CB1 receptors are present at high concentrations. Thus, endocannabinoids are known as retrograde messengers (Bisogno et al., 2005).

1.5.3 Biosynthesis of endocannabinoids

Kreitzer (2002) reviewed the biosynthesis of the endocannabinoid anandamide in the brain within the post synaptic cell. Depolarization of postsynaptic neurons results in the opening of voltage-dependent calcium channels and subsequently entry of calcium ion into the postsynaptic cell. The elevation of calcium ions triggers the biosynthesis of anandamide from the lipid precursor N-arachidonylphosphatidylethanolamine (NAPE) by phospholipase D (PLD). Anandamide leaves the postsynaptic cell and binds to CB1 receptor present on presynaptic cell which leads to activation of CB1 receptors. The activation of CB1 receptors also results in a conformational change in the associated G protein and when the $G_{\beta\gamma}$ dimmer dissociates from G_{α}, which inhibits presynaptic calcium influx (Kreitzer, 2002). Biosynthesis of 2-AG also occurs in brain but this

endocannabinoid is formed via a separate pathway which involves phospholipase C and diacylglycerol lipase (Mechoulam et al., 1995; Stella et al., 1997).

Following CB1 receptor activation, anandamide is readily taken up into the intracellular compartments where it is degraded by fatty acid amidohydrolase (FAAH) (Kreitzer, 2002). 2-AG is processed in a similar fashion. FAAH is distributed in the cell bodies and dendrites of many neurons (Egertova et al., 1998) and in fact many brain regions contain enzymes associated with both endocannabinoid synthesis and degradation (Kreitzer, 2002).

Moreover, Nicoll and Wilson (2002) discussed the biosynthesis of the endocannabinoid 2-AG in the brain. Depolarization at postsynaptic cell causes the opening of voltage-gated Ca^{2+} channels. The opening of calcium channels allows the entry of Ca^{2+} ions into the postsynaptic cell and synthesis of 2-arachidonoylglycerol from a lipid precursor. However, 2-AG biosynthesis is also activated by metabotropic glutamate receptors (mGluRs) (Nicoll and Wilson, 2002). Activation of metabotropic glutamate receptors (mGluRs) present on the postsynaptic cell results in the opening of calcium channels which in turn switches on the biosynthesis of the endocannabinoid, 2-AG through activation of phospholipase C. Specifically, phospholipase C generates diacylglycerol which is then cleaved by diacylglycerol lipase to yield 2-AG. The 2-AG is then free to exit the postsynaptic cell and diffuses retrogradely to activate the CB1 receptors present in the presynaptic nerve endings.

1.6 Activation of CB1 receptors

Activation of CB1 receptors by cannabinoid agonists results in a conformational change of the associated G protein structure. This in turn triggers the dissociation of G_{α} subunit from the $G_{\beta\gamma}$ dimer. The G_{α} subunit subsequently inhibits adenylyl cyclase in the cell. This results in a decrease of intracellular cAMP (cyclic adenosine monophosphate) as cAMP is synthesized from ATP by the help of adenylyl cyclase. Inhibition of adenylyl cyclase also decreases the protein kinase A activity which leads to reduced phosphorylation of A-type K^+ channels. A reduced phosphorylation status of A-type K^+ channels causes them to adopt an open configuration which increases efflux of potassium ions since the concentration of potassium ions is higher inside the cell (Lutz, 2002).

The other main pathway stimulated by CB1 receptor activation is cAMP-independent. Activation of CB1 receptors results in a conformational change to the G protein which leads to dissociation of G_{α} subunit from the $G_{\beta\gamma}$ dimer. The dissociated $G_{\beta\gamma}$ subunit then inhibits the N-type and P/Q-type voltage-dependent Ca^{2+} channels which blocks the influx of calcium ions into the cell. As Ca^{2+} plays an important role in neurotransmitter release from synaptic vesicle, blockade of calcium channels results in inhibition of neurotransmitter release. Moreover, the $G_{\beta\gamma}$ dimer interacts with G protein-coupled inwardly-rectifying potassium channels (GIRK) to open them which results in efflux of potassium ions from the post synaptic cell which leads to hyperpolarization of the cell. The activation of GIRK along with inhibition of N-type and P/Q-type voltage-

dependent Ca^{2+} channels which reduce the tendency of nerves to fire and represent important mechanisms underlying DSI and DSE as discussed earlier (Lutz, 2002).

1.7 Cannabinoids and *in vivo* behavioural effects

The Cannabis plant contains about 60 different cannabinoids including the psychoactive component Δ^9-tetrahydrocannabinol (Δ^9-THC) and other major non-psychoactive components for example cannabidiol and cannabinol. (Wiley and Martin, 2002). Δ^9-THC produces a variety of behavioural effects such as hypothermia, learning and memory impairment, analgesia, catalepsy-like immobilisation and hypoactivity (Wiley and Martin, 2002; Martin et al., 1991; Egashira et al., 2006). It is generally considered that these *in vivo* effects are due to the binding of Δ^9-THC to CB1 receptors (Hawakawa et al., 2007, Devane, 1988). Previous reports indicated that Δ^9-THC and other cannabinoids are as potent as morphine in the rat tail-flick assay, a test routinely used to quantify antinociception in laboratory animals (Dewey, 1986). Buxhaum (1972) also reported the potency of Δ^9-THC in rat tail-flick assay, and the hot-plate test. However, Bhargava and Matwyshyn (1980) observed insignificant analgesic activity for Δ^9-THC. Other laboratories found that the analgesic potency of Δ^9-THC is less than that of morphine. A previous study showed that Δ^9-THC has weak activity in the tail-flick test (Dewey, 1986). However, Martin (1985) clearly demonstrated that Δ^9-THC is a more potent analgesic than morphine when it is administered intravenously compared to subcutaneously. It is intriguing to consider the reason why potency of Δ^9-THC differs with its administration route. It may be because of differences in the access of Δ^9-THC to the blood stream and subsequent metabolism of this compound arises from different administration routes. Δ^9-THC has been shown to produce hypothermia in a dose-

dependent fashion in most laboratory animals (Dewey, 1986). Bhargava (1980) demonstrated that a single intraperitoneal injection of 10 mg/kg Δ^9-THC caused hypothermia in mice, which lasted for 5 or 6 hours. Hawakawa (2007) demonstrated that Δ^9-THC at different doses of 1, 3, 6 and 10 mg/kg decreased both locomotor activity and rectal temperature. Δ^9-THC also produced catalepsy-like immobilisation.

It has been shown by Herkenham et al. (1990) that the CB1 receptor is present at highest density within areas of brain such as hippocampus, basal ganglia and cerebellum. CB1 receptors have also been found in other regions of brain such as the nucleus accumbens, amygdala and hypothalamus (Fujiwara and Egashira, 2004; Egashira et al., 2006). The hippocampus modulates psychostimulant-induced behaviour via the mesolimbic dopamine system (Wise, 1998; Lisman and Grace, 2005). Stimulation of the hippocampus excites neurons in the nucleus accumbens and the ventral tegmental area. This could explain why activation of CB1 receptor in the hippocampus leads to hypoactivity and hypothermia (Hawakawa et al., 2007). However, catalepsy-like immobilisation is related to decreases in catecholaminergic and serotonergic neuron activity in the nucleus accumbens, amygdaloid nucleus and striatum, mediated through CB1 receptors (Fujiwara, 2001). These results demonstrated that the hypoactivity, hypothermia and catalepsy are likely mediated though CB1 receptor activation in specific regions of the brain (Hawakawa et al., 2007).

Varvel et al., (2005) demonstrated that marijuana smoke which has Δ^9-THC as the main psychoactive component also causes antinociception, hypothermia, catalepsy and hypomotility in rat. Marijuana produced significant ($p<0.001$) and dose-dependent antinociception at higher dose. Marijuana smoke equivalent to doses of 100-200 mg also

caused significant ($p<0.001$) decreases in rectal temperature. Locomotor activity was also depressed by marijuana ($p<0.001$) at all doses and catalepsy was also observed as well by following exposure to marijuana smoke ($p<0.001$) equivalent to a dose of 200 mg. Interestingly, pre-treatment with 10 mg of CB1 receptor antagonist SR 141716A along with marijuana smoke significantly blocked antinociceptive, hypothermic and cataleptic but not the locomotory depressant effects, all of which are symptoms of CB1 receptor activation (Varvel et al., 2004). It may be that locomotor depressant effects involve the inhibition of Na^+ channels.

1.8 Voltage-gated ion channels

Ion channels are integral membrane proteins, which play an important role in the normal activity of most living cells. The plasma membrane acts as a barrier separating cell components from the outside in order to maintain the proper ionic concentration of the cell. However, the ion channels allow particular ions to pass through the membrane down the concentration gradient or against it with the aid of an energy source (ATP). The difference in the concentration of particular ions results in an electrical potential difference between the cytoplasm and the external medium resulting in electrochemical gradients across the plasma membrane. Channels which are activated by a change in membrane potential are known as voltage-gated channels. The activation due to alternations in membrane potential cause the opening and closing of the particular channel (Denac et al., 2000).

1.8.1 Voltage-gated sodium channels

Voltage-gated sodium channels are integral membrane proteins, which allow the passage of sodium ions (Na^+) through the plasma membrane of various cells in particular neurons. The sodium channel has several binding sites, which are target of various neurotoxins and insecticides which are summarized in Table 1-1.

Table 1-1 Insecticide and neurotoxin binding sites associated with the Na^+ channel (Adapted from Catterall, 1980)

Binding site	Insecticides/drugs	Physiological effect
1	Tetrodotoxin (TTX) Saxitoxin (STX)	Inhibition of Na^+ transport
2	Veratridine (VTD) Batrachotoxin (BTX)	Persistent activation
3	North African scorpion α-toxin Sea anemone toxins (ATX)	Slowing of inactivation; enhancement of persistent activation
4	American scorpion β-toxins	Shift in voltage-dependent activation to more negative potential
5	Brevetoxins (PbTx) Ciguatoxins (CTX)	Repetitive activity; persistent activity
6	Pyrethroids DDT	Open up the sodium channels, repetitive firing
Unidentified	Dihydropyrazoles	Block

1.9 Depolarization of the plasma membrane

Voltage-gated sodium channels in the plasma membrane are generally closed in resting neurons. Depolarization of the plasma membrane due to an action potential causes a conformational change of the sodium channel. It results in the opening of the sodium channel on the cytosolic surface. The opening of the channel permits the entry Na^+ ions into the cell. Entry of sodium ions results in opening of more sodium channels and subsequently more Na^+ ions enter the cell, down the Na^+ concentration gradient. The influx of sodium ions and ensuing depolarization causes the opening of other important voltage-gated channels like calcium and potassium channels. The opening of potassium channels causes the outward movement of K^+ ions down the concentration gradient (Lodish et al., 2004). The number of potassium ions moving out correspond to the number of sodium ions moving in, to make the plasma membrane reach its original resting phase, which is negative inside, compared to outside of the cell. The return of the membrane to its original resting potential is known as repolarization. Moreover, after the repolarization, the nerve membrane cannot be restimulated immediately by another action potential. This brief period is known as refractory period. This is the time required to restore the gating mechanism of the sodium channels to a state which allows them to respond to another action potential (Denac et al., 2000) (Figure 1-9)

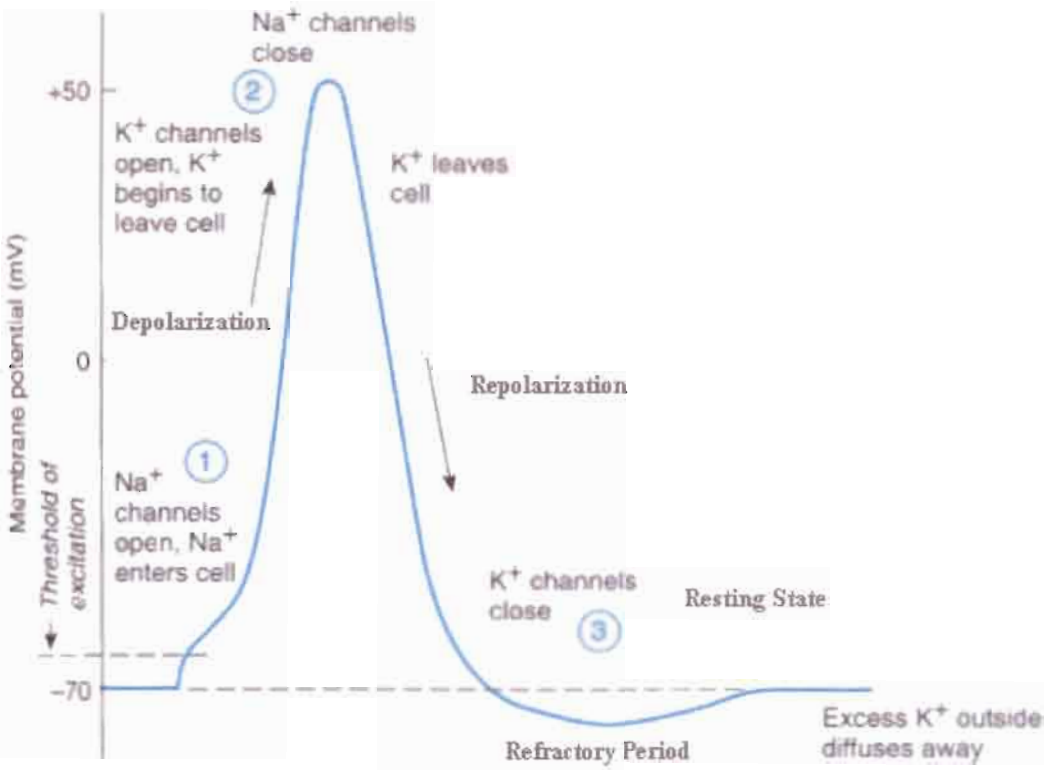


Figure 1-9 Neuronal action potential

Redrawn from www.mindcreators.com/NeuronBasics.htm

1.10 Binding theory

A radioligand is a radioactively labeled drug that can associate with a receptor, enzyme or any site of interest. Measuring the rate and extent of binding provides information on the number of binding sites, and as well as their affinity and accessibility for various drugs. Each molecule of radiolabeled ligand (L) binds to each molecule of receptor (R) to form ligand-receptor (LR) complex (Snyder, 1978).

$$L + R \rightleftharpoons LR$$

$$K_D [LR] = [L] [R]$$

The complex can break down to produce free ligand and unoccupied receptor. At equilibrium, the rate of the forward reaction is equal to the rate of backward reaction. The K_D is the dissociation constant, which depends on physical factors such as temperature and the chemical nature of the interacting substances (Obrenovitch, n.d). There are two general types of binding assay which are as follows:-

1.10.1 Competition experiments

Competition studies are based on the ability of unlabeled drugs to compete with the radiolabeled drug for the receptor. Competition experiments are used to determine the IC_{50} (concentration that causes 50% inhibition) of unlabeled drugs for a specific receptor. (Snyder, 1978)

1.10.2 Saturation experiments

Saturation radioligand binding studies determine specific binding of the radiloligand to the specific receptor at equilibrium and how this relates to the increasing concentrations of the radioligand. Saturation experiment allows calculation of the B_{max}, which is the maximum binding of a radioligand that is achieved when all of the receptor molecules are occupied by the radioactive drug. B_{max} is generally expressed in picogram of radioligand bound per milligram of protein. Saturation experiments are also useful in determining the affinity (K_D) of the radiolabeled drug for the receptor. K_D stands for equilibrium dissociation constant which is equal to the concentration of radioactive ligand required to occupy 50% of the receptors. As the Scatchard plots are utilized to analyze the data, so saturation experiments have come to be known as Scatchard experiments. The success of saturation experiments depend on the incubation time in order to reach the equilibrium state of the binding reaction. However, incubation time varies with the ligand, receptor, temperature and other experimental conditions.

The Scatchard equation is $B/F = B_{max} - B / K_D$ (Figure 1-10)

Where, B = Bound ligand

F = Free ligand

K_D = Equilibrium dissociation constant (Obrenovitch, n.d)

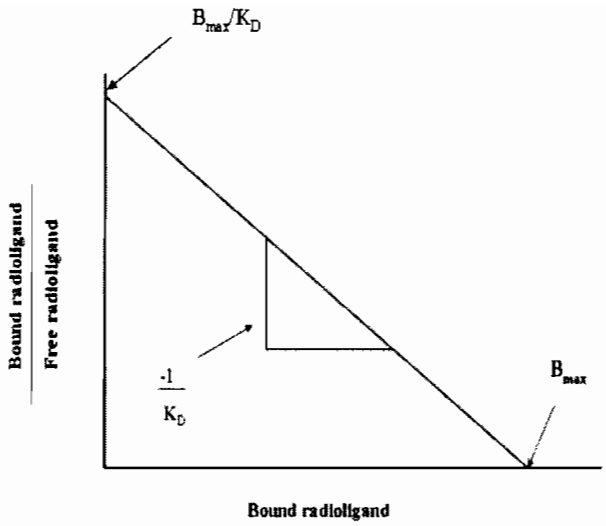


Figure 1-10 Example of a Scatchard plot

Redrawn from University of Barcelona website, 2002
www.pdg.cnb.uam.es/Guia_Glaxo/chap3b.html

1.11 Specific and non-specific binding

The radioligand often binds to more than one type of site in a membrane preparation isolated from the tissue in question. However, the radioligand will have a high affinity for some sites and often a much lower affinity for other sites. The site of primary interest in binding studies is the specific sites while other sites are called non-specific sites. The radioligand is designed to have high affinity towards a particular receptor in the membranes prepared from the target tissue. The radioligand might also binds with lower affinity to other sites available in the tissue for example, membrane, protein, lipid and to micelles that might form with the fat molecules, filter papers, glass tubes etc. Binding to all other sites except the site of interest is known as non-specific binding. Non-specific binding contributes to the total radioactivity bound as it remains with the tissue until the end of the experiment (Snyder, 1978).

1.12 Competitive and allosteric inhibition

A study compound can directly compete with the radioligand for the binding sites and subsequently reduces B_{max}, available for the radioligand, which is known as competitive inhibition. A competitive inhibitor can reduce the initial rate of association of the radioligand as well. In contrast, an allosteric mechanism is where a test compound doesn't bind to the site where the radioligand binds, but it binds to another site on the receptor. The binding causes a conformational change (allosteric) of the receptor where radioligand binds and subsequently decreases the affinity of the radioligand to the receptor i.e. increases K_D values. In allosteric inhibition, a test compound may also increases the rate of dissociation of radioligand from the receptor. Association (i.e.

attachment of radioligand with the receptors) and dissociation from the receptors occurs simultaneously (Snyder, 1978).

1.13 Agonists and antagonists

If the binding of a ligand to a receptor causes the conformational changes and activates a cellular response, then the ligand is known as agonist. On the other hand, when the binding of a ligand to the receptor does not cause any cellular responses but renders the agonist incapable of activating the receptor, is known as an antagonist (Obrenovitch, n.d).

1.14 Effects of cannabinoids on voltage-gated sodium channels

Investigations in our laboratory at SFU, demonstrated that the endocannabinoid arachidonyl ethanolamide (anandamide) and the synthetic CB1 receptor agonist WIN 55,212-2 inhibit the binding of [^{3}H]batrachotoxin-A-20α-benzoate to voltage-sensitive sodium channels. Moreover, the CB1 receptor antagonist AM251 at 1-2 μM was not able to attenuate the inhibitory capacity of anandamide and WIN 55,212-2, which shows clearly that action of these compounds is independent of CB1 receptor activation (Nicholson et al., 2003). Further studies in our lab demonstrated that several endocannabinoids for example, N-arachidonoyl glycerol dopamine (NADA), 2-AGE and 2-AG also inhibit the binding of [^{3}H]BTX-B to the sodium channels in the mouse brain (Duan et al., 2007). It was additionally demonstrated in saturation experiments (Scatchard analysis) that 2-AG, AGE or NADA reduce the number of binding sites (B_{max}) on sodium channel available to the radioligand [^{3}H]BTX-B and increases the K_D as well. It reveals that these endocannabinoids binds competitively to the sodium channel. Furthermore,

kinetic experiment demonstrated that 2-AG, NADA and AGE increase the rate of dissociation of [^{3}H]BTX-B from the sodium channel over time which strongly suggests that these endocannabinoids operate as allosteric inhibitors of all [^{3}H]BTX-B bindings. On the other hand inhibition of [^{3}H]BTX-B binding to sodium channel by endocannabinoids was unaffected by the presence CB1 receptor antagonist AM251 at 2 μM. It was therefore concluded that inhibitory effects of different endocannabinoids for example, 2-AG, NADA and 2-AGE on voltage-gated sodium channels also did not involve participation of the CB1 receptor (Duan et al., 2007).

The CB1 receptor antagonist AM251 at concentration above 2 μM was also shown to displace the binding of the radioligand [^{3}H]batrachotoxin-A-20α-benzoate to site 2 on sodium channel. Scatchard analysis of binding demonstrated that at these higher concentrations AM251 increases the K_D of the radioligand without changing the $B_{max.}$ Furthermore, kinetic experiments showed that AM251 accelerates the dissociation of radioligand [^{3}H]batrachotoxin-A-20α-benzoate from the sodium channel complex. This result suggests that the CB1 receptor antagonist AM251 allosterically binds to voltage-gated sodium channels. Consistent with the result it was found that AM251 inhibits sodium channel-dependent release of the excitatory (L-glutamic acid) and the inhibitory (GABA) neurotransmitter (Liao et al., 2003).

It can be concluded that different endocannabinoids and some synthetic CB1 receptor agonists and the antagonist AM251 at high concentrations, are able to inhibit voltage-gated sodium channels and ultimately the release of excitatory and inhibitory neurotransmitters from nerve endings. The mechanism is totally independent of any CB1

receptor activation. This could indicate an alternative pathway for DSI and DSE, which is separate from the mechanism discussed earlier.

1.15 Rationale of the project

The results discussed in the previous sections raised the possibility that CB1 receptors and VGSCs may possess binding regions with similar topographies. This formed the basis of a hypothesis that some of the pesticides, neurotoxins and pharmacological agents known to interfere with the sodium channel function in mammals (Deecher et al., 1991, Zhao et al., 2003; Ghiasuddin et al., 1985; Denac et al., 2003) may also have the ability to interact with CB1 receptors in mammalian brain.

However, there are several chemicals, pesticides, pharmacological agents etc is current use in various industries and agriculture whose action on the CB1 receptor of the brain is unknown. It has already been mentioned that activation of CB1 receptor causes hypothermia, antinociception, hypomotility, learning and memory impairment and catalepsy-like immobilisation in mammalian species (Wiley and Martin, 2002; Martin et al., 1991; Egashira et al., 2006). Hypomotility and catalepsy-like immobilisation due to CB1 receptor activation may make an animal susceptible to predator attack. In addition, impairment of memory or cognitive ability might cause changes in their migration pattern. Analgesic effects of environmental chemicals in mammals might make them unaware of the impending danger and reduce their ability to respond appropriately. Moreover, cumulative effects of these compounds might lead to change in their reproductive behaviour by affecting their ability to migrate normally. Therefore, I examined variety of environmental compounds of diverse structure in order to find out their capacity to interact with the CB1 receptor of mammalian brain. Many of the

compounds are initially considered to have a primary action on voltage-gated sodium channels.

One of the insecticides I used was the dihydropyrazole RH3421, which blocks voltage-gated sodium channels (VGSCs) (Salgado, 2003) and inhibits VGSC-dependent transmitter release from mouse brain synaptosomes (Nicholson et al., 1990). RH3421 shows certain structural similarities with the CB1 receptor antagonists AM251 and SR 141716A. In addition, subchronic doses of RH3421 cause hypomotility and pacificity in rats (Salgado, 2003) which are symptoms, quite like those produced by CB1 receptor activation. Therefore, there is a possibility that RH3421 might interact with CB1 receptor. We therefore examined 2 representative dihydropyrazole compounds in order to explore their ability to interfere with the CB1 receptor.

I also examined several pyrethroid insecticides. High doses of α-cyano pyrethroids such as cypermethrin and deltamethrin cause course tremors, choreoathetosis, clonic seizures and salivation (CS-syndrome; Verschoyle et al., 1980). On the other hand, enantiomers of compound such as permethrin (which lack an α-cyano group) produce fine and whole body tremors in the rat (T-syndrome; Verschoyle et al., 1980). The excitatory symptoms produced in rats by pyrethroids are due to Na^+ channel activation (Soderlund et al., 2002). However, other enantiomers of cyperpermethrin for example 1SαR (*cis & trans*) are not toxic to mammals. Moreover both α-cyano and non-α-cyano pyrethroids reduce motor activity (Crofton et al., 1984; Crofton et al., 1988) at low to moderate doses. These symptoms are also a sign of CB1 receptor activation. So there might be a possibility that some of the depressant *in vivo* actions of pyrethroid compounds could be due to CB1 receptor activation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and radioligand

Insecticides and chemicals: deltamethrin, cypermethrin, tetrodotoxin, veratridine, lamotrigine, carbamazepine, scorpion (*Leiurus quinquestriatus*) venom and unlabelled CP-55,940 were obtained from Sigma-Aldrich, Oakville, Ontario, Canada. Dihydropyrazole insecticides RH3421 and RH5529, isomers of permethrin, 1RS, *cis* and 1RS, *trans*, enantiomers of cypermethrin and the organochlorine DDT were obtained previously for other experiments in our laboratory from different agrochemical and pharmaceutical sources. Other chemicals were obtained from Sigma-Aldrich Canada. The radioligand [^{3}H]CP-55,940 (side chain-2, 3, 4(N)-^{3}H; 139.6, Ci/mmol) was obtained from Perkin Elmer Life and Analytical Sciences Canada Inc .

2.2 Preparation of the nerve membrane fraction

The nerve membrane fraction was isolated from the whole brain of a CD1 male mouse (20-25 g) as described by Quistad et al., (2002). Mice were killed by cervical dislocation and the whole brain was collected from a single mouse. The brain was cut into small fragments and homogenized in 10 ml buffer (100 mM Tris, 1 mM EDTA and pH adjusted to 9.0 with Tris base). The homogenized brain fragments were centrifuged for 10 minutes at 176 xg. The supernatant was collected and centrifuged once again for 20 minutes at 11290 xg. The pellet was then suspended in 2.5 ml of store solution (50 mM Tris, 1 mM EDTA, 3 mM $MgCl_2$, pH=7.4) and stored at -80^{o}C for future use.

Protein estimation was carried out as described by Peterson (1977). All the experimental procedures were performed by following Animal Care guidelines developed by Canadian Council on Animal Care guidelines and had the approval from the Simon Fraser University Animal Care Committee.

2.3 Estimation of protein concentration (Peterson, 1977)

This protein assay is the modified version from the original assay by Lowry et al., (1951). Peterson (1977) developed this modified version by including sodium dodecylsulfate (SDS) as one of the reagents of the assay. However, SDS does not interfere with the assay but in the presence of NaOH, it makes lipid transparent, and therefore prevents the scattering of light by lipid micro particles. Moreover, SDS is also helpful in dissolving the membrane and very importantly, releases all membrane proteins, which improves the quantification. Furthermore, the presence of alkali in the protein reagents provides an adequate solubilisation and denaturation of proteolipid proteins and membrane proteins. This protein assay is based on a linear standard curve. Bovine serum albumin (BSA) of 1 mg/ml concentration was used in order to construct a standard curve. In addition, reagent A, containing copper-tartrate carbonate, 0.8 M NaOH, 10% SDS, distilled water and reagent B, comprising of Folins B solution were also used indicated below.

Twelve tubes were prepared containing different amounts of BSA solution (1 mg/ml) for example 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 100 µl. Afterwards, distilled water was added into those tubes to make the total volume of each tube equal to 1 ml. Then 0.8 ml of reagent A was added into all tubes, vortexed and left at the room temperature for 10 minutes. Then, 0.5 ml of reagent B was added, vortexed and incubated

at room temperature for 60 minutes. The unknown protein samples were treated in a similar fashion. The absorbance of the samples was measured using a spectrophotometer set at 750 nm. The concentration of the protein samples was estimated by using the standard curve obtained from the diluted BSA solution.

2.4 The binding study using [^{3}H]CP-55,940

The method used in my project for the measurement of specific binding of the CB1 receptor agonist [^{3}H]CP-55,940 to the CB1 receptor was described by Quistad et al., (2002) which was highly reliable and adopted after some minor modifications. It is important to say that, several other published procedures were attempted with little success. In every experiment, binding in the absence and presence of unlabeled CP-55,940 was performed in triplicate and test compounds were assayed in duplicate. A minimum of three experiments were conducted on each compound. Binding reactions were initiated by adding 5 µl of solvent control (dimethylsulfoxide) to some specific tubes and 5 µl of unlabelled CP-55,940 to some specific tubes. Moreover, 5 µl of study compounds are added into respective test tubes in absence of unlabelled CP-55,940. This is followed by addition of 0.5 ml of ice-cold binding buffer (50 mM Tris, 1 mM EDTA, 1 mM $MgCl_2$ and adjusted to pH 7.4) containing bovine serum albumin 3 mg/ml and subsequently 25 µl of membrane fraction (150-180 µg membrane protein). The tubes were kept in room temperature for 15 minutes followed by the addition of [^{3}H]CP-55,940 (10 µl) in DMSO. The final concentration of radioligand [^{3}H]CP-55,940 inside the tube was 1.7 nM. The test tubes were incubated for 90 minutes at 30^{o}C. After the incubation the binding reaction was terminated by adding 1 ml of ice-cold wash buffer (0.9 % NaCl, 2 mg/ml BSA, pH adjusted to 7.4 with Tris base) to the incubation followed by vacuum

filtration using Whatman GF/C filters on a filtration unit (Hoefer FH 225V). The filter-trapped membrane fraction containing the CB1 receptors was washed 3 times with 3 ml of wash buffer (0.95 % NaCl; 2 mg/ml BSA). Filters were dried overnight and scintillant was added, and radioactivity associated with the membrane was measured by using liquid scintillation counting. Non-specific binding was measured in the presence of 10 μM of unlabelled CP-55,940. The specific binding signal calculated by subtracting nonspecific binding from the total binding and this averaged 80.9 ± 4.7 %.

2.5 Curve fitting, estimation of IC_{50}s and statistical analysis.

IC_{50} (concentration that causes 50% inhibition) values were calculated from concentration-inhibition curves by non-linear regression analysis using Prism 4 software (Graphpad Software Inc. San Diego, CA, USA). The results were expressed as means ± S.E.M. and significant differences between treatments were demonstrated by P values of less than 0.05 by using Student's t test.

CHAPTER 3: RESULTS

3.1 Effects of insecticides on the binding of [^{3}H]CP-55,940 to CB1 receptors

3.1.1 Effects of pyrethroids

Several pyrethroids including the non-α-cyano pyrethroid permethrin and the α-cyano pyrethroids cypermethrin and deltamethrin were investigated in order to determine their ability to interfere the binding of CB1 receptor agonist [^{3}H]CP-55,940 to the CB1 receptors. The two isomers of the non-α-cyano pyrethroid, permethrin specifically 1RS, *trans* and 1RS, *cis* were examined. Threshold inhibition values of inhibiting the radioligand binding to the CB1 receptor by these two isomers were estimated at about 1 μM and 2 μM for 1RS, *trans* and 1RS, *cis* isomers respectively. The potency of these compounds were evaluated by calculating IC_{50} (concentration causing 50% inhibition) which were 7.1 and 18.1 μM for 1RS, *trans* and 1RS, *cis* isomers respectively. The results indicate that *trans* isomer is more potent than its *cis* counterpart. Maximum inhibition of radioligand binding for 1RS, trans-permethrin was in between 80% and 90% and approx. 60% for 1RS, *cis*-permethrin (Figure 3-2). α-Cyano pyrethroids for example, deltamethrin and cypermethrin also showed inhibition of radioligand binding with threshold effects noticed at about 2 μM. The IC_{50} values of two α-cyano pyrethroids were 9.9 μM (deltamethrin) and 12.2 μM (cypermethrin). The maximum inhibitory responses were approximately 60% and 70-80 % for deltamethrin

and cypermethrin respectively (Figure 3-3). This result indicates deltamethrin is slightly more potent than cypermethrin.

The enantiomers of cypermethrin 1R,*trans*, αS and 1S,*trans*,αR were more potent than the 1R,*cis*, αS and 1S,*cis*, αR enantiomer. However, the inhibitory effects although significantly different were very small (Table 3-1).

Pyrethroid degradation products such as phenoxybenzyl alcohol, phenoxybenzoic acid and 2, 2, 3, 3-tetramethylcyclopropane carboxylic acid were also examined but did not show any inhibition on [^{3}H]CP-55,940 binding at 20 μM (Table 3-2).

3.1.2 Effects of dihydropyrazoles

The dihydropyrazole insecticide, RH3421 was the most potent within the group of insecticides tested giving a threshold inhibitory effect at about 1 μM. The maximum inhibitory response was 60% with IC_{50} value of 5.7 μM. The other dihydropyrazole insecticide RH5529 was less potent (IC_{50} = 16.5 μM) (Figure 3-4).

3.1.3 Effects of DDT and its breakdown products

The organochlorine insecticide DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane) which is able to activate the sodium channels and two of its breakdown products 4,4'-dichlorobenzhydrol and 4,4'-dichlorobenzophenone gave approximately 30% inhibition of [^{3}H]CP-55,940 binding at 20 μM (Table 3-3).

3.2 Effects of sodium channel-selective drugs on the binding of [^{3}H]CP-55,940 to CB1 receptors

Number of voltage-gated sodium channel modifying drugs for example, the anti-convulsants lamotrigine, carbamazepine and diphenylhydantoin were also examined and found to be inactive at 20 μM at inhibiting binding of [^{3}H]CP-55,940 (Table 3-4). Likewise, VGSC selective anaesthetics MS-222 and propofol were inactive (Table 3-4).

3.3 Effects of sodium channel-selective toxins on the binding of [^{3}H]CP-55,940 to CB1 receptors

At 20 μM the neurotoxins brevetoxin B, veratridine and tetrodotoxin which bind to sodium channel binding sites 1, 2 and 5 respectively were inactive at inhibiting the binding of [^{3}H]CP-55,940 to the CB1 receptors. Similarly, scorpion (*Leiurus quinquestriatus*) venom, which binds to sodium channel at site 3, showed very weak (approx. 19%) inhibition of [^{3}H]CP-55,940 binding at 60 μg/ml (Table 3-4).

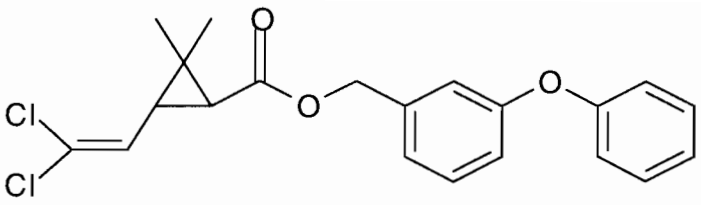
3.4 Effects of some other pesticides on the binding of [^{3}H]CP-55,940 to CB1 receptors

Numerous pesticides examined at my assay were inactive (at 20 μM) or barely active at inhibiting the binding of [^{3}H]CP-55,940 to the CB1 receptors (Table 3-5).

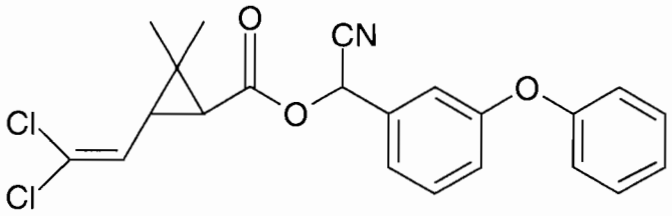
3.5 Binding comparisons with a classical synthetic cannabinoids and endocannabinoids

Our concentration-response data for the insecticides and drugs demonstrate that they are all substantially less potent than the CB1 receptor agonist WIN 55,212-2 and arachidonoylglycerol ether but more potent than 2-arachidonoyl glycerol, even in the

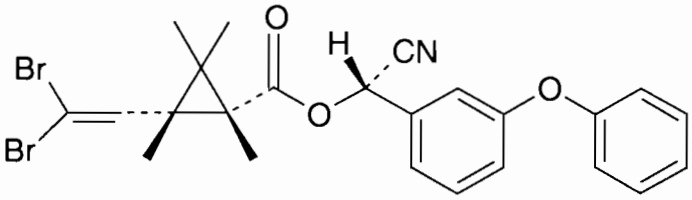
presence of 500 μM PMSF which prevents the degradation of this endocannabinoid (Table 3-6).



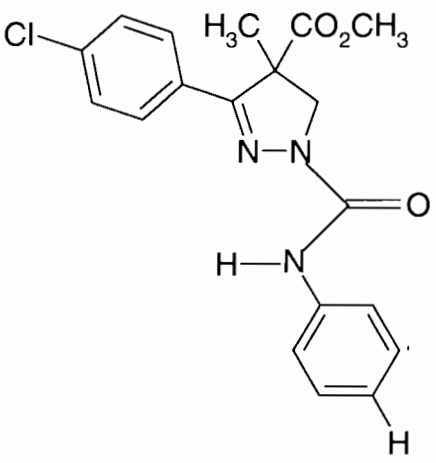
Permethrin



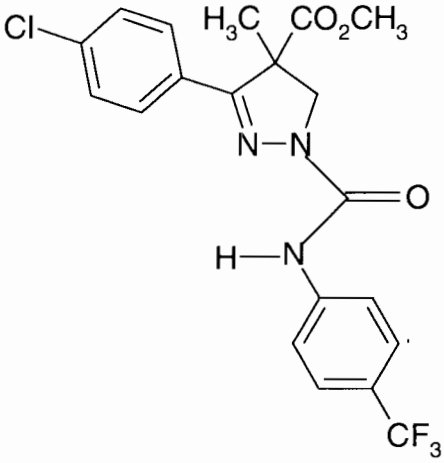
Cypermethrin



Deltamethrin



RH5529



RH3421

Figure 3-1 Structures of different test compounds

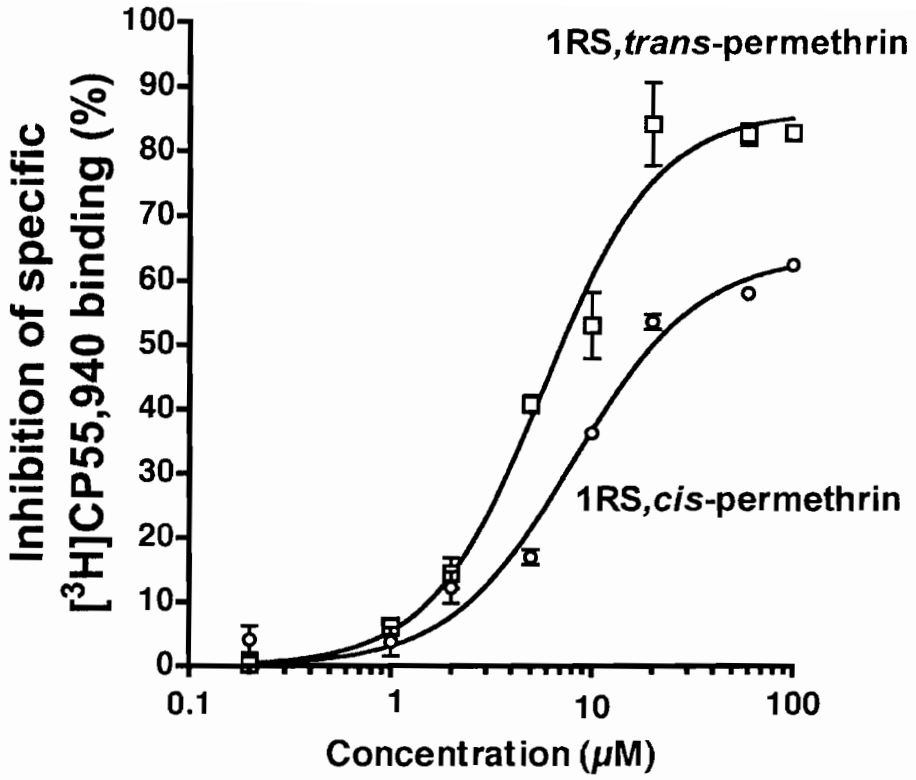


Figure 3-2 Competitive inhibition of specific [^{3}H]CP-55,940 binding to mouse whole brain membranes by the synthetic pyrethroids 1RS,*trans*-permethrin and 1RS,*cis*-permethrin. Each data point corresponds to the mean percentage inhibition of specific binding ± S.E.M. of 3 determinations, each performed in duplicate.

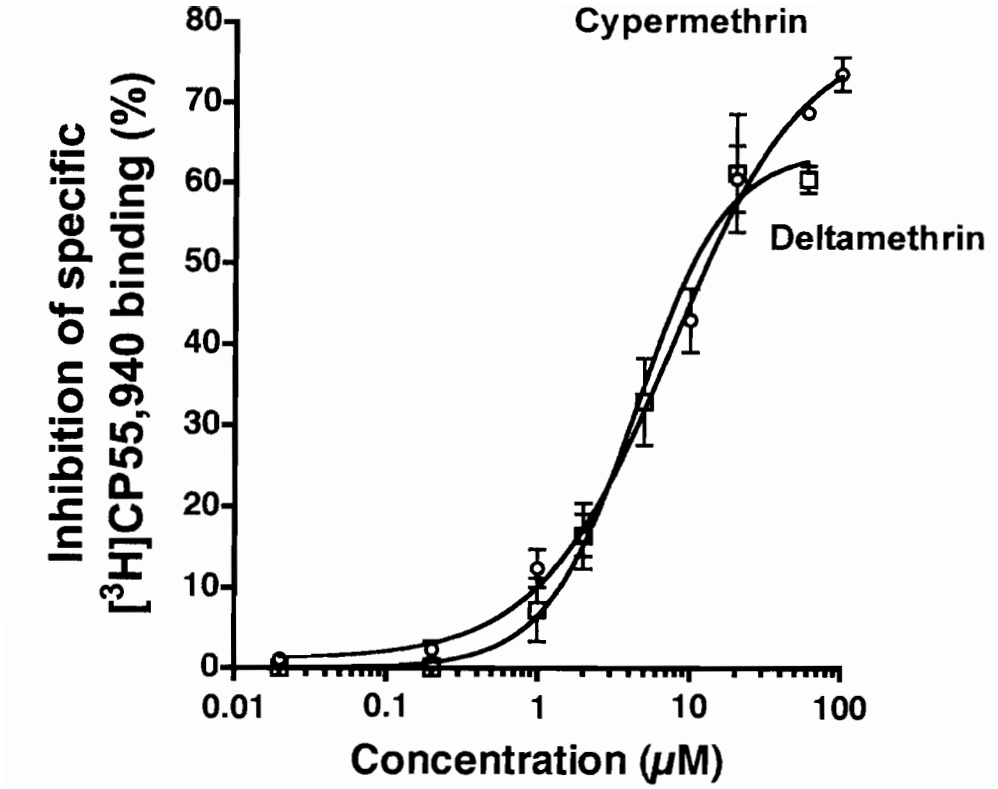


Figure 3-3 Inhibitory effects of the α-cyano-pyrethroids deltamethrin and cypermethrin on specific binding of [^{3}H]CP-55,940 to membranes prepared from mouse whole brain. Mean values ± S.E.M. of 3-4 determinations each performed in duplicate are displayed.

Table 3-1 Inhibitory effects of cypermethrin enantiomers on [^{3}H]CP-55,940 binding to CB1 receptors in mouse brain membranes. Values represent mean percentage inhibition of specific [^{3}H]CP-55,940 binding ± S.E.M. of 3 independent assays.

Enantiomers of cypermethrin (all at 10 μM)	Inhibition of [^{3}H]CP-55,940 binding (%)
1R, *cis*, αS	40.9 ± 0.9 *a, b, x*
1R, *trans*, αS	48.3 ± 1.1 *a, c, y*
1S, *cis*, αR	33.5 ± 0.9 *b, c, d,*
1S, *trans*, αR	42.3 ± 1.3 *y, d, x*

Significant differences were present in paired values designated as a ($P<0.05$), *b* ($P<0.05$), *c* ($P<0.001$) and *d* ($P<0.01$).

Paired values designated *x* and *y* are not significantly different ($P>0.05$).

Table 3-2 Inhibitory effects of pyrethroid breakdown products on [^{3}H]CP-55,940 binding to CB1 receptors in mouse brain membranes. Values represent mean percentage inhibition of specific [^{3}H]CP-55,940 binding ± S.E.M. of 3 independent assays.

Pyrethroid breakdown products (all at 20 μM)	Inhibition of [^{3}H]CP-55,940 binding (%)
Phenoxybenzyl alcohol	3.1 ± 1.9
Phenoxybenzoic acid	5.3 ± 3.4
2,2,3,3-tetramethylcyclopropane carboxylic acid	0.8 ± 0.9

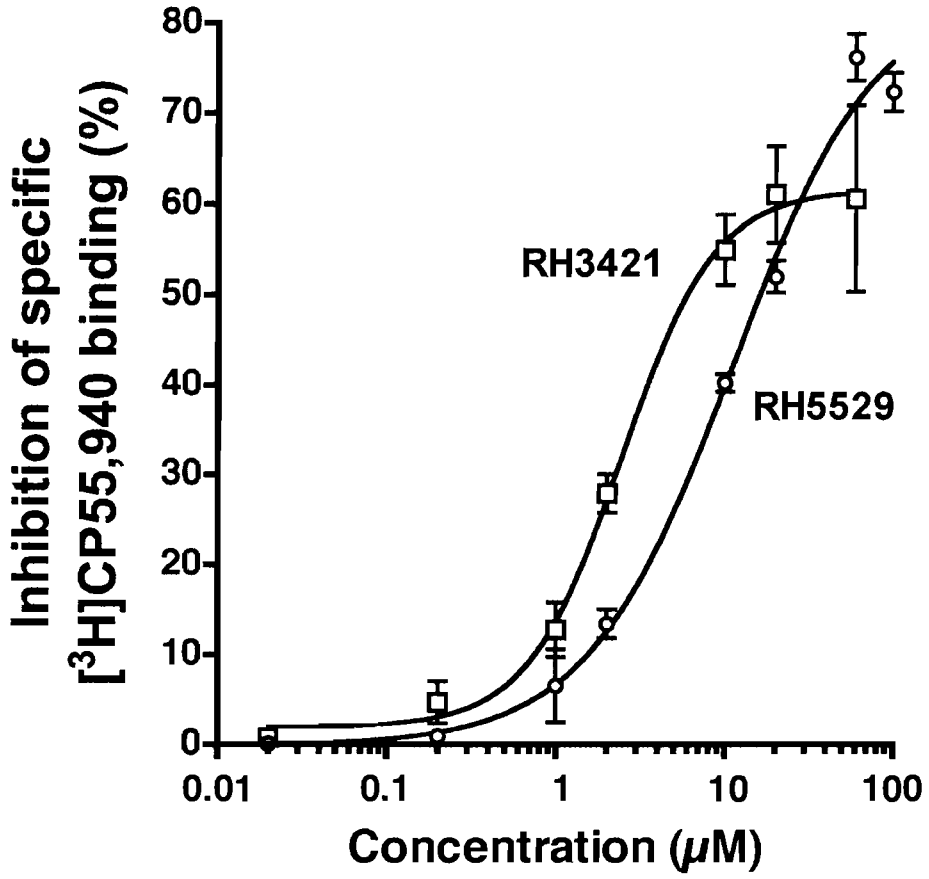


Figure 3-4 Concentration dependence for inhibition of the specific binding of [^{3}H]CP-55,940 to mouse brain membranes by the dihydropyrazole insecticides RH3421 and RH5529. The results signify means ± S.E.M. of 3 assays each conducted in duplicate.

Table 3-3. Inhibitory effects of DDT and two of its breakdown products (4,4'-dichlorobenzhydrol and 4,4'-dichlorobenzophenone) on [^{3}H]CP-55,940 binding to CB1 receptors in mouse brain membranes. Values represent mean percentage inhibition of specific [^{3}H]CP-55,940 binding ± S.E.M. of 3 independent assays.

DDT and degradation products (all at 20 μM)	Inhibition of [^{3}H]CP-55,940 binding (%)
DDT	32.9 ± 2.6
4,4'-dichlorobenzhydrol	27.0 ± 1.6
4,4'-dichlorobenzophenone	30.0 ± 6.8

Table 3-4 Voltage-gated Na^+ channel (VGSC) acting drugs and toxins found to be inactive or marginally active in the [^{3}H]CP-55,940 binding assay. Study compounds were present at 20 μM. Values in brackets represent inhibition of specific [^{3}H]CP-55,940 binding as the mean percentage ± S.E.M. of 3 or more assays conducted in duplicate. The negative value suggests a stimulation of binding which was not significant.

VGSC acting drugs and toxins (All at 20 μM)	Inhibition of [^{3}H]CP-55,940 binding (%)
Diphenylhydantoin	17 ± 3.3
Carbamazepine	7.6 ± 6.7
Lamotrigine	3.6 ± 5.3
Propofol	9.5 ± 2.3
MS-222	-1.4 ± 0.6
Tetrodotoxin	0.6 ± 1.6
Scorpion venom (*Leiurus quinquestriatus*)	18.7[a] ± 3.5
Veratridine	6.6 ± 2.7
Brevetoxin B	0.9[b]
20(*S*)-Protopanaxadiol	2.2 ± 3.5
Ginsenoside Rh_2	7.5 ± 4.5

[a] scorpion (*Leiurus quinquestriatus*) venom was present at 60 μg/ml.

[b] Our supply of brevetoxin-B permitted one experiment at 20 μM; the value provided is the mean of duplicate determinations.

Table 3-5 Inhibitory effects of various pesticides on [^{3}H]CP-55,940 binding to CB1 receptors in mouse brain membranes. Values represent mean percentage inhibition of specific [^{3}H]CP-55,940 binding.

Pesticides (All at 20 μM)	Inhibition of [^{3}H]CP-55, 940 binding (%)
N,N-diethyl-*m*-toluamide (DEET)	3.3
Ivermectin	-7.2
Fipronil	7.5
γ-hexachlorocyclohexane (lindane)	15.2
β-hexachlorocyclohexane	12.9
Dieldrin	26.9
Amitraz	7.5
Rotenone	37.8
3-iodo-propynyl butylcarbamate (IPBC)	11.4
Carboxin	4.7
Carbendazim	0.8
Amitrol	0.1
Atrazine	2.9
Triclopyr	-4.6
2-(thiocyanomethylthio)benzothiazole (TCMTB)	4.4
8-hydroxyquinoline	1.2
N-alkylamide	6.8

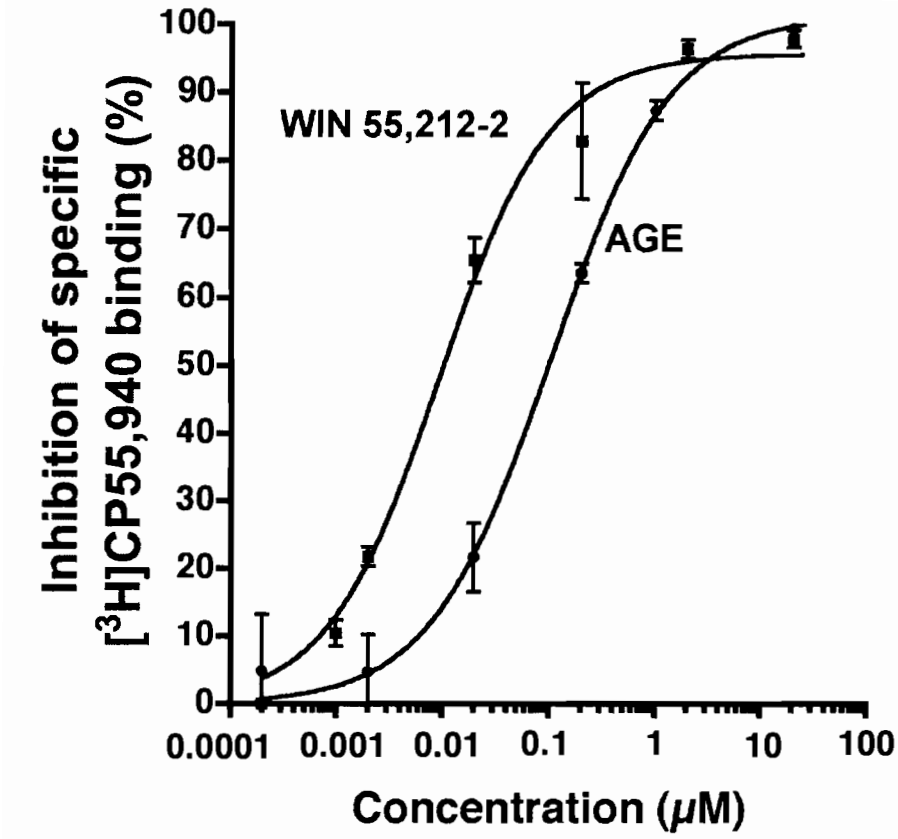


Figure 3-5 Inhibition curves for the endocannabinoid arachidonoylglycerol ether (AGE) and the synthetic CB1 receptor agonist WIN 55,212-2. Data points are the means ± S.E.M. of 3 experiments each carried out in duplicate.

Table 3-6 Inhibitory effects of 2-arachidonoyl glycerol on [^{3}H]CP-55,940 binding to CB1 receptors in mouse brain membranes. Values represent mean percentage inhibition of specific [^{3}H]CP-55,940 binding ± S.E.M. of 3 independent assays.

Endocannabinoids (all at 20 μM)	Inhibition of [^{3}H]CP-55,940 binding (%)
2-arachidonoyl glycerol	20.7 ± 3.4 *f*
2-arachidonoyl glycerol + PMSF (0.5 mM)	32.2 ± 1.7 *f*

Paired values designated as *f* are significantly different ($P<0.05$).

.

CHAPTER 4: DISCUSSION

The important finding from this investigation is that a number of insecticides including two dihydropyrazole and certain α-cyano and non-α-cyano pyrethroids, known primarily for their ability to interfere with Na^+ channel function, are able to inhibit the binding of CB1 receptor agonist [^{3}H]CP-55,940 to CB1 receptors in mammalian brain. However, it was also demonstrated in our binding assay that the CB1 receptor agonist WIN 55,212-2 and the endocannabinoid arachidonoyl glycerol ether (AGE) are significantly more potent as inhibitors of [^{3}H]CP-55,940 binding than these insecticides. On the other hand, the endocannabinoid 2-arachidonoyl glycerol (2-AG) is much weaker as an inhibitor, even in the presence of the phenylmethylsulfonyl fluoride (PMSF) which prevents the degradation of 2-AG by fatty acid amidohydrolase.

It was mentioned earlier that high doses of the 1R, *trans*, αS enantiomer of isomer cypermethrin is highly neurotoxic (excitatory) to mammals but on the other hand 1S, *trans*, αR enantiomer is not toxic at all to mammals. However, in our binding experiment the 1R, *trans*, αS and 1S, *trans*, αR forms were able to inhibit the binding of [^{3}H]CP-55,940 to the CB1 receptor with similar potency. This suggests that an action of pyrethroids on CB1 receptors could not possibly initiate excitatory signs. Moreover, in mammals cypermethrin (α-cyano) causes hypothermia (McDaniel and Moser, 1993; Verschoyle and Aldridge, 1980). Interestingly, it has also been observed that lower doses of α-cyano and non-α-cyano pyrethroids produce hypomotility (Crofton and Reiter, 1984; Crofton and Reiter, 1988; McDaniel and Moser, 1993). Both hypothermia and reduced

locomotor activity are cardinal signs of CB1 receptor activation. It is conceivable that depolarization following the opening of sodium channels during pyrethroid poisoning might open calcium channels as well which allows the entry of Ca^{++} ions. Entry of calcium ions releases endocannabinoids from the lipid precursor and eventually the CB1 receptor is activated by endocannabinoids produces the symptoms for example hypomotility, hypoactivity etc. However, I found that the 1S, *trans*, αR enantiomer of cypermethrin which is nontoxic to mammals binds to the CB1 receptors. In this regard, it would be very useful to ascertain whether pre-treatment with the highly potent and selective CB1 antagonist SR 141716A can increase or decrease the different excitatory and depressant signs of pyrethroid poisoning in mammals in order to gain more information on the role of pyrethroids as agonists or antagonists. If the pre-treatment with CB1 antagonist SR 141716A intensifies the excitatory effect then it could be concluded that the pyrethroid acts as an antagonist at the CB1 receptor. On the other hand, if pre-treatment of CB1 receptor agonist WIN 55,212-2, ameliorates the depressant signs then the pyrethroid may act as an agonist.

The dihydropyrazole insecticide RH3421 blocks voltage-gated sodium channels (VGSC) (Salgado, 1992) and inhibits the binding of [^{3}H]batrachotoxin 20-α-benzoate to rodent VGSCs with an IC_{50} of 0.3 μM (Deecher et al., 1991). This IC_{50} value of RH3421 is 20-fold lower than the IC_{50} value for the inhibition of [^{3}H]CP-55,940 binding to CB1 receptors (Salgado, 1992). It has been generally assumed that the depressant effects of RH3421 are due to the blockade of VGSCs. My investigation has clearly demonstrated that RH3421 has the capacity to interfere with CB1 receptors in the brain membrane fraction. Moreover, subchronic administration of RH3421 (>5000 mg/kg) by oral route to

rats showed symptoms including lethargy and pacificity (Salgado, 1992), which are both very similar to the symptoms of CB1 receptor activation. So, there is a possibility that centrally-mediated actions of RH3421 are due to cumulative effects of VGSC blockade and CB1 receptor activation. However, it is very important to establish whether RH3421 is acting as an agonist at the CB1 receptor.

The remaining sodium channel-acting compounds examined in this investigation for example neurotoxins (scorpion toxin), anticonvulsants (lamotrigine, diphenylhydantoin and carbamazepine), anesthetics (MS-222 and propofol), organochlorine DDT etc were not able to appreciably inhibit the binding of [^{3}H]CP-55,940 to the CB1 receptors. In addition, a variety of other structurally diverse pesticides and drugs were unable, or weakly able, to displace [^{3}H]CP-55,940 binding.

The results of this investigation have demonstrated that some neurotoxic insecticides for example pyrethroids and dihydropyrazoles could interfere with CB1 receptors in brain. It provided us a new idea about the mode of action of the compounds, which were initially considered to have primary effects on voltage-gated sodium channels. It has already been mentioned earlier that activation of CB1 receptor causes hypothermia, antinociception, hypomotility, learning and memory impairment and catalepsy-like immobilisation in mammalian species (Wiley and Martin, 2002; Martin et al., 1991; Egashira et al., 2006). Hypomotility, analgesic activity and catalepsy-like immobilisation due to CB1 receptor activation might make an animal unaware of the impending danger and reduce their ability to respond appropriately and the animal would be susceptible to predator attack. In addition, impairment of memory and cognitive ability might cause changes in animal behaviour and even migration patterns. Moreover,

cumulative effects of these compounds might lead to change in reproductive behaviour by affecting the ability to migrate normally. Although not described in my thesis, the antifouling agent tributyltin and related analogs also inhibited the binding of [^{3}H]CP-55,940 to CB1 receptors. Tributyltin is a chemical commonly found in the aquatic environment, which could affect CB1 receptor dependent physiological processes in aquatic organisms such as fish. It could be speculated that these insecticides along with other cannabimimetic compounds could intensify the effects of endocannabinoids in the brain.

It is possible that, structural components of these compounds could be exploited to design CB1 receptor-acting drugs with therapeutic potential for example in developing new analgesics and in treating sleeping and eating disorders as well as prevents emesis. At this point of the research this is not possible to state whether these study compounds directly inhibit the binding of [^{3}H]CP-55,940 by competing with the radiolligand for its binding site or if the study compounds might bind to an associated site on the CB1 receptor and allosterically change the configuration of the CB1 receptor leading to a decrease in the binding of [^{3}H]CP-55,940. Moreover, clarification is required as to whether the study compounds are able to increase the level of different endocannabinoids by inhibiting the degrading action of fatty acid amidohydrolase on endocannabinoids or facilitating the release of endocannabinoids from lipid precursors. Further, investigation like a functional assay for example using cAMP (cyclic adenosine monophosphate) is clearly needed to establish whether these compounds act as agonists or antagonists at the CB1 receptor. It is known that an agonist decreases the cAMP level whereas an antagonist increases the cAMP level. Steffens et al., (2004) demonstrated than CB1

receptor agonist CP-55,940 inhibited the forskolin-stimulated accumulation of cAMP in human synaptosomes. The maximum inhibitory response was 35% with IC_{50} value of 21 nM. On the other hand, CB1 receptor antagonist AM251 increased forskolin-stimulated accumulation of cAMP by 20%.

It has been discussed earlier that activation of CB1 receptor induces a conformational change in the CB1 receptor. This conformational change triggers the dissociation of the G_α subunit from the $G_{\beta\gamma}$ dimer and the G_α subunit eventually binds to GTP. Griffin et al., (1998) demonstrated that CB1 receptor agonists CP-55,244, WIN 55,212-2 and CP-55,940 increased the [^{35}S]GTPγS binding to G_α by 165 %, 156 % and 114 % respectively at rat cerebellar membranes. However, the CB1 receptor antagonist SR 141716A did not affect [^{35}S]GTPγS binding. It would be interesting to examine various compounds which demonstrated affinity to CB1 receptors in my project using these functional assays cAMP and [^{35}S]GTPγS in order to find out their role as agonists or antagonists. Moreover, Griffin et al., (1998) demonstrated that CB2 receptor selective compound deoxy-HU-210 has an affinity for the CB1 receptor as well and stimulated [^{35}S]GTPγS binding by 140 % which is more than the stimulation by CB1 receptor agonist CP-55,940. In addition, Gertsch et al., (2004) reported the role of alkylamides (found in *Echinacea*) as a potent immunomodulator and a ligand for CB2 receptors. However, in my binding assay an insecticidal *N*-alkylamide was shown to be very weak as an inhibitor (approx. 7%) at inhibition of [^{3}H]CP-55,940 binding at 20 μM (Table 3-5). It would be interesting to find out whether any of these compounds examined in our assay are capable of exerting immunomodulatory effects via peripheral CB2 receptors.

REFERENCES

Abel, E.L. (1975). Cannabis: effects on hunger and thirst. Behavioural Biology, 15,255-281

Baldwin, J. M., Schertler, G. F. X. & Unger, V. M. (1997). An alpha-carbon template for the transmembrane helices in the rhodopsin family of G protein-coupled receptors. *Journal of Molecular Biology, 272*(1), 144-164.

Begg, M., Pacher, P., Batkai, S., Osei-Hyiaman, D., Offertaler, L., Mo, F. M., et al. (2005). Evidence for novel cannabinoid receptors. *Pharmacology & Therapeutics, 106*(2), 133-145.

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., et al. (2000). The protein data bank. *Nucleic Acids Research, 28*(1), 235-242.

Bhargava, H. N. (1980). Effects of thyrotropin releasing hormone and histidyl-proline diketopiperazine on Δ^9-tetrahydrocannabinol-induced hypothermia. *Life Sciences, 26*(11), 845-850.

Bhargava, H. N., & Matwyshyn, G. A. (1980). Influence of thyrotropin releasing hormone and histidyl-proline diketopiperazine on spontaneous locomotor-activity and analgesia induced by Δ^9-tetrahydrocannabinol in the mouse. *European Journal of Pharmacology, 68*(2), 147-154.

Bisogno, T., Ligresti, A., & Di Marzo, V. (2005). The endocannabinoid signalling system: Biochemical aspects. *Pharmacology Biochemistry and Behavior, 81*(2), 224-238.

Bluher, M. (2007). The endocannabinoid system and new therapeutic options with rimonabant. *Diabetes Stoffwechsel Und Herz, 16*(1), 51-56.

Bodor, A. L., Katona, I., Nyiri, G., Mackie, K., Ledent, C., Hajos, N., et al. (2005). Endocannabinoid signaling in rat somatosensory cortex: Laminar differences and involvement of specific interneuron types. *Journal of Neuroscience, 25*(29), 6845-6856.

Bradshaw, H. B., & Walker, J. M. (2005). The expanding field of cannabimimetic and related lipid mediators. *British Journal of Pharmacology, 144*(4), 459-465.

Brizzi, A., Brizzi, V., Cascio, M. G., Bisogno, T., Sirianni, R., & Di Marzo, V. (2005). Design, synthesis, and binding studies of new potent ligands of cannabinoid receptors. *Journal of Medicinal Chemistry, 48*(23), 7343-7350.

Buxbaum, D. M. (1972). Analgesic activity of Δ^9-tetrahydrocannabinol in rat and mouse. *Psychopharmacologia, 25*(3), 275-&.

Casida, J. E., & Quistad, G. B. (2005). Serine hydrolase targets of organophosphorus toxicants. *Chemico-Biological Interactions,* 157, 277-283.

Catterall, W.A. (1980). Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annual Review of Pharmacology and Toxicology*, 20,15-43.

Compton, D. R., Johnson, M. R., Melvin, L. S., & Martin, B. R. (1992). Pharmacological profile of a series of bicyclic cannabinoid analogs - classification as cannabimimetic agents. *Journal of Pharmacology and Experimental Therapeutics, 260*(1), 201-209.

Crawley, J. N., Corwin, R. L., Robinson, J. K., Felder, C. C., Devane, W. A., & Axelrod, J. (1993). Anandamide, an endogenous ligand of the cannabinoid receptor, induces hypomotility and hypothermia *in-vivo* in rodents. *Pharmacology Biochemistry and Behavior, 46*(4), 967-972.

Cristino, L., De Petrocellis, L., Pryce, G., Baker, D., Guglielmotti, V., & Di Marzo, V. (2006). Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain. *Neuroscience, 139*(4), 1405-1415.

Crofton, K. M., & Reiter, L. W. (1984). Effects of 2 pyrethroid insecticides on motor-activity and the acoustic startle response in the rat. *Toxicology and Applied Pharmacology, 75*(2), 318-328.

Crofton, K. M., & Reiter, L. W. (1988). The effects of type-I and type-II pyrethroids on motor-activity and the acoustic startle response in the rat. *Fundamental and Applied Toxicology, 10*(4), 624-634.

Davidson, F. F., Loewen, P. C., & Khorana, H. G. (1994). Structure and function in rhodopsin replacement by alanine of cysteine residue-110 and residue-187, components of a conserved disulfide bond in rhodopsin, affects the light-activated metarhodopsin-ii state. *Proceedings of the National Academy of Sciences of the United States of America, 91*(9), 4029-4033.

Deecher, D. C., & Soderlund, D. M. (1991). RH-3421, an insecticidal dihydropyrazole, inhibits sodium channel-dependent sodium uptake in mouse-brain preparations. *Pesticide Biochemistry and Physiology, 39*(2), 130-137.

Denac, H., Mevissen, M., & Scholtysik, G. (2000). Structure, function and pharmacology of voltage-gated sodium channels. *Naunyn-Schmiedebergs Archives of Pharmacology, 362*(6), 453-479.

Devane, W. A., Breuer, A., Sheskin, T., Jarbe, T. U. C., Eisen, M. S., & Mechoulam, R. (1992). A novel probe for the cannabinoid receptor. *Journal of Medicinal Chemistry, 35*(11), 2065-2069.

Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S., & Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat-brain. *Molecular Pharmacology, 34*(5), 605-613.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., et al. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science, 258*(5090), 1946-1949.

Devane, W. A., Howlett, A. C., Johnson, M. R., Melvin, L. S., & Milne, G. M. (1987). Structural studies leading to the discovery of a physiologically relevant cannabinoid receptor-site in rat-brain. *Abstracts of Papers of the American Chemical Society, 194*, 17-MEDI.

Dewey, W. L. (1986). Cannabinoid pharmacology. *Pharmacological Reviews, 38*(2), 151-178.

Di Marzo, V., De Petrocellis, L., & Bisogno, T. (2005). The biosynthesis, fate and pharmacological properties of endocannabinoids. *Cannabinoids, 168*, 147-185.

Di Marzo, V., Melck, D., Bisogno, T., & De Petrocellis, L. (1998). Endocannabinoids: Endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends in Neurosciences, 21*(12), 521-528.

Duan, Y., Zheng, J., & Nicholson, R. A. (2008). Inhibition of [^{3}H]batrachotoxinin A-20 alpha-benzoate binding to sodium channels and sodium channel function by endocannabinoids. *Neurochemistry International, 52*(3), 438-446.

Egashira, N., Matsuda, T., Koushi, E., Mishima, K., Iwasaki, K., Shoyama, Y., et al. (2006). Involvement of 5-hydroxytryptamine (1A) receptors in Δ^9-tetrahydrocannabinol-induced catalepsy-like immobilization in mice. *European Journal of Pharmacology, 550*(1-3), 117-122.

Egertova, M., Giang, D. K., Cravatt, B. F., & Elphick, M. R. (1998). A new perspective on cannabinoid signalling: Complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. *Proceedings of the Royal Society of London Series B-Biological Sciences, 265*(1410), 2081-2085.

Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K., & Devane, W. A. (1993). Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal-transduction. *Proceedings of the National Academy of Sciences of the United States of America, 90*(16), 7656-7660.

Fujiwara, M., & Egashira, N. (2004). New perspectives in the studies on endocannabinoid and cannabis: Abnormal behaviors associate with CB1 cannabinoid receptor and development of therapeutic application. *Journal of Pharmacological Sciences, 96*(4), 362-366.

Ghiasuddin, S. M., & Soderlund, D. M. (1985). Pyrethroid insecticides-potent, stereospecific enhancers of mouse-brain sodium-channel activation. *Pesticide Biochemistry and Physiology, 24*(2), 200-206.

Griffin, G., Atkinson, P.J., Showalter, V.M., Martin, B.R., & Abood, M.E (1998). Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5-O-(3-[^{35}S]thio0-triphosdphte binding assay in rat cerebellar membranes. *The Journal of Pharmacology and Experimental Therapeutics,* 285(2), 553-560

Hajos, N., & Freund, T. F. (2002). Pharmacological separation of cannabinoid sensitive receptors on hippocampal excitatory and inhibitory fibers. *Neuropharmacology, 43*(4), 503-510.

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., Decosta, B. R., et al. (1990). Cannabinoid receptor localization in brain. *Proceedings of the National Academy of Sciences of the United States of America, 87*(5), 1932-1936.

Hillard, C. J., Edgemond, W. S., & Campbell, W. B. (1995). Characterization of ligand-binding to the cannabinoid receptor of rat-brain membranes using a novel method- application to anandamide. *Journal of Neurochemistry, 64*(2), 677-683.

Howlett, A. C. (1998). The CB1 cannabinoid receptor in the brain. *Neurobiology of Disease, 5*(6), 405-416.

Howlett, A. C., Bidautrussell, M., Devane, W. A., Melvin, L. S., Johnson, M. R., & Herkenham, M. (1990). The cannabinoid receptor-biochemical, anatomical and behavioral characterization. *Trends in Neurosciences, 13*(10), 420-423.

Jahnichen, S. (April, 2006). *Activation cycle of G-proteins by G-protein-coupled receptors.*Retrieved May 18,2008, from GNU Free Documentation License Website: http://www.gnu.org/copyleft/fdl.html

Jo, Y. H., Chen, Y. J. J., Chua, S. C., Talmage, D. A., & Role, L. W. (2005). Integration of endocannabinoid and leptin signaling in an appetite-related neural circuit. *Neuron, 48*(6), 1055-1066.

Katona, I., Rancz, E. A., Acsady, L., Ledent, C., Mackie, K., Hajos, N., et al. (2001). Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *Journal of Neuroscience, 21*(23), 9506-9518.

Kreitzer, A. C. (2005). Neurotransmission: Emerging roles of endocannabinoids. *Current Biology, 15*(14), R549-R551.

Kreitzer, A. C., & Regehr, W. G. (2001). Cerebellar depolarization-induced suppression of inhibition is mediated by endogenous cannabinoids. *Journal of Neuroscience, 21*(20), art. no.-RC174.

Kreitzer, A. C., & Regehr, W. G. (2001). Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron, 29*(3), 717-727.

Kreitzer, A. C., & Regehr, W. G. (2002). Retrograde signaling by endocannabinoids. *Current Opinion in Neurobiology, 12*(3), 324-330.

Liao, C. Y., Zheng, J., David, L. S., & Nicholson, R. A. (2004). Inhibition of voltage-sensitive sodium channels by the cannabinoid 1 receptor antagonist AM 251 in mammalian brain. *Pharmacology & Toxicology, 94*(2), 73-78.

Lisman, J. E., & Grace, A. A. (2005). The hippocampal-VTA loop: Controlling the entry of information into long-term memory. *Neuron, 46*(5), 703-713.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C.A., Krieger, M., & Scott, M.P et al. (2004). *Molecular Cell Biology*. W.H. Freeman and Company, NW.

Lutz, B. (2002). Molecular biology of cannabinoid receptors. *Prostaglandins Leukotrienes and Essential Fatty Acids, 66*(2-3), 123-142.

Mahmoudian, M. (1997). The cannabinoid receptor: Computer-aided molecular modeling and docking of ligand. *Journal of Molecular Graphics & Modelling, 15*(3), 149-+.

Martin, B. R. (1985). Structural requirements for cannabinoid-induced antinociceptive activity in mice. *Life Sciences, 36*(16), 1523-1530.

Martin, B. R., Compton, D. R., Thomas, B. F., Prescott, W. R., Little, P. J., Razdan, R. K., et al. (1991). Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. *Pharmacology Biochemistry and Behavior, 40*(3), 471-478.

Martin, B. R., Mechoulam, R., & Razdan, R. K. (1999). Discovery and characterization of endogenous cannabinoids. *Life Sciences, 65*(6-7), 573-595.

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., & Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cdna. *Nature, 346*(6284), 561-564.

Matyas, F., Yanovsky, Y., Mackie, K., Kelsch, W., Misgeld, U., & Freund, T. F. (2006). Subcellular localization of type 1 cannabinoid receptors in the rat basal ganglia. *Neuroscience, 137*(1), 337-361.

Mcdaniel, K. L., & Moser, V. C. (1993). Utility of a neurobehavioral screening battery for differentiating the effects of 2 pyrethroids, permethrin and cypermethrin. *Neurotoxicology and Teratology, 15*(2), 71-83.

Mechoulam, R., Benshabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., et al. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical Pharmacology, 50*(1), 83-90.

Miller, A.M, & Stella, N. (2008). CB2 receptor-mediated migration of immune cells: it can go either ways. *British Journal of Pharmacology,* 153(2), 299-308.

Moloney, G. P., Angus, J. A., Robertson, A. D., Stoermer, M. J., Robinson, M., Wright, C. E., et al. (2008). Synthesis and cannabinoid activity of 1-substituted-indole-3-oxadiazole derivatives: Novel agonists for the CB1 receptor. *European Journal of Medicinal Chemistry, 43*(3), 513-539.

Neuron basics, (n.d.) www.mindcreators.com.

Nicholson, R. A., Liao, C., Zheng, J., David, L. S., Coyne, L., Errington, A. C., et al. (2003). Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. *Brain Research, 978*(1-2), 194-204.

Nicholson, R. A., & Merletti, E. L. (1990). The effect of dihydropyrazoles on release of [^{3}H] GABA from nerve-terminals isolated from mammalian cerebral-cortex. *Pesticide Biochemistry and Physiology, 37*(1), 30-40.

Obrenovitch, T (n.d.). *Radioligand binding assays.*Retrieved July 15, 2008 from University of Bradford Website: http://www.brad.ac.uk/acad/pharmaco/Radioligand-Binding/sld001.htm

Ohno-Shosaku, T., Tsubokawa, H., Mizushima, I., Yoneda, N., Zimmer, A., & Kano, M. (2002). Presynaptic cannabinoid sensitivity is a major determinant of depolarization-induced retrograde suppression at hippocampal synapses. *Journal of Neuroscience, 22*(10), 3864-3872.

Peterson, G. L. (1977). Simplification of protein assay method of Lowry et al., which is more generally applicable. *Analytical Biochemistry, 83*(2), 346-356.

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *Nature Reviews Neuroscience, 4*(11), 873-884.

Pitler, T. A., & Alger, B. E. (1992). Postsynaptic spike firing reduces synaptic GABA(a) responses in hippocampal pyramidal cells. *Journal of Neuroscience, 12*(10), 4122-4132.

Pitler, T. A., & Alger, B. E. (1994). Depolarization-induced suppression of gabaergic inhibition in rat hippocampal pyramidal cells-G-protein involvement in a presynaptic mechanism. *Neuron, 13*(6), 1447-1455.

Pitler, T. A., & Alger, B. E. (1994). Suppression of GABAergic inhibition by postsynaptic depolarization involves a presynaptic, G-protein coupled mechanism. *Society for Neuroscience Abstracts, 20*(1-2), 890.

Quistad, G. B., Nomura, D. K., Sparks, S. E., Segall, Y., & Casida, J. E. (2002). Cannabinoid CB1 receptor as a target for chlorpyrifos oxon and other organophosphorus pesticides. *Toxicology Letters, 135*(1-2), 89-93.

Salgado, V. L. (1992). Slow voltage-dependent block of sodium-channels in crayfish nerve by dihydropyrazole insecticides. *Molecular Pharmacology, 41*(1), 120-126.

Scholtysik, G., & Williams, F. M. (1986). Antiarrhythmic effects of DPI 201-106. *British Journal of Pharmacology, 89*(2), 287-292.

Segall, Y., Quistad, G. B., Sparks, S. E., Nomura, D. K., & Casida, J. E. (2003). Toxicological and structural features of organophosphorus and organosulfur cannabinoid CB1 receptor ligands. *Toxicological Sciences, 76*(1), 131-137.

Shim, J. Y., Welsh, W. J., & Howlett, A. C. (2003). Homology model of the CB1 cannabinoid receptor: Sites critical for nonclassical cannabinoid agonist interaction. *Biopolymers, 71*(2), 169-189.

Smith, P. B., Compton, D. R., Welch, S. P., Razdan, R. K., Mechoulam, R., & Martin, B. R. (1994). The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *Journal of Pharmacology and Experimental Therapeutics, 270*(1), 219-227.

Soderlund, D. M., Clark, J. M., Sheets, L. P., Mullin, L. S., Piccirillo, V. J., Sargent, D., et al. (2002). Mechanisms of pyrethroid neurotoxicity: Implications for cumulative risk assessment. *Toxicology, 171*(1), 3-59.

Snyder, S.H. (1978). Overview of Neurotransmitter Receptor Binding. H.I. Yamamura, S.J.Enna & M.J.Kuhar (Eds). *Neurotransmitter Receptor Binding.* Raven Press, NJ.

Steffens, M., Engler, C., Zenther, J., & Feuerstein, T.J. (2004). Cannabinoid CB1 receptor-mediated modulation of evoked dopamine release and of adenylyl cyclase activity in the human neocortex. *British Journal of Pharmacology*, 141, 1193-1203

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., et al. (1995). 2-arachidonoylgylcerol- a possible endogenous cannabinoid receptor-ligand in brain. *Biochemical and Biophysical Research Communications, 215*(1), 89-97.

Tao, Q., Mcallister, S. D., Andreassi, J., Nowell, K. W., Cabral, G. A., Hurst, D. P., et al. (1999). Role of a conserved lysine residue in the peripheral cannabinoid receptor (CB2): Evidence for subtype specificity. *Molecular Pharmacology, 55*(3), 605-613.

University of Barcelona (2002). *Receptor Binding Techniques:Saturation Analysis.*Retrieved June 10, 2008, University of Barcelona Website: www.pdg.cnb.uam.es/Guia_Glaxo/chap3b.html

Varvel, S. A., Bridgen, D. T., Tao, Q., Thomas, B. F., Martin, B. R., & Lichtman, A. H. (2005). Δ^9-tetrahydrocannabinol accounts for the antinociceptive, hypothermic, and cataleptic effects of marijuana in mice. *Journal of Pharmacology and Experimental Therapeutics, 314*(1), 329-337.

Verschoyle, R. D., & Aldridge, W. N. (1980). Structure-activity relationships of some pyrethroids in rats. *Archives of Toxicology, 45*(4), 325-329.

Vincent, P., Armstrong, C. M., & Marty, A. (1992). Inhibitory synaptic currents in rat cerebellar purkinje-cell - modulation by postsynaptic depolarization. *Journal of Physiology London, 456*, 453-471.

Wiley, J. L., & Martin, B. R. (2002). Cannabinoid pharmacology: Implications for additional cannabinoid receptor subtypes. *Chemistry and Physics of Lipids, 121*(1-2), 57-63.

Willow, M., & Catterall, W. A. (1982). Inhibition of binding of [^{3}H]-labeled batrachotoxinin-A 20-alpha-benzoate to sodium-channels by the anticonvulsant drugs diphenylhydantoin and carbamazepine. *Molecular Pharmacology, 22*(3), 627-635.

Wilson, R. I., & Nicoll, R. A. (2002). Neuroscience - endocannabinoid signaling in the brain. *Science, 296*(5568), 678-682.

Xu, W., Filppula, S. A., Mercier, R., Yaddanapudi, S., Pavlopoulos, S., Cai, J., et al. (2005). Purification and mass spectroscopic analysis of human CB1 cannabinoid receptor functionally expressed using the baculovirus system. *Journal of Peptide Research, 66*(3), 138-150.

Zhao, X. L., Ikeda, T., Yeh, J. Z., & Narahashi, T. (2003). Voltage-dependent block of sodium channels in mammalian neurons by the oxadiazine insecticide indoxacarb and its metabolite DCJW. *Neurotoxicology, 24*(1), 83-96.