CHARACTERIZATION OF ENVIRONMENTAL CHEMICAL ACTION AT THE CB_1 RECEPTOR OF MAMMALIAN BRAIN AND INVESTIGATION OF SYNAPTOSOMAL EXOCYTOSIS AS A MEANS TO ASSESS FUNCTIONAL EFFECTS OF CB_1 RECEPTOR MODULATORS

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Abstract

This investigation first examined various environmental chemicals and natural products (including pesticides, mitochondrial function-disrupting chemicals, benzophenanthridines alkaloids and tributyltins) for their capacity to bind to mouse brain cannabinoid-1 (CB_1) receptors and modify the functional coupling of the CB_1 receptor to its G protein. Many of these compounds inhibited binding of [^{3}H]CP-55,940 and CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding to the G protein at micromolar concentrations. While benzophenanthridines exhibited inverse agonist-like profiles, the majority of the other compounds acted as antagonists. Some environmental chemicals may have potential to interfere with endocannabinoid-mediated synaptic signaling *in vivo* and behavioral/physiological outcomes.

This research also explored the effects of CB_1 receptor agonists and an antagonist/inverse agonist (AM251) on exocytosis (as measured with acridine orange) in a purified synaptosomal fraction from mouse brain. Cannabinoid agonists inhibited KCl-induced exocytosis. Paradoxically, AM251 was also inhibitory and failed to relieve CB_1 receptor agonist-dependent inhibition of exocytosis.

Keywords: G protein; endocannabinoid; Cannabinoid-1 (CB_1) receptor; [^{3}H]CP-55,940; [^{35}S]GTPγS; Acridine orange (AO)

Dedication

To My Beloved Mom and Dad

Acknowledgements

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Table of Contents

List of Figures

List of Tables

Glossary

Δ^9-THC	Δ^9-Tetrahydrocannabinol
CBN	Cannabinol
CBD	Cannabidiol
Δ^8-THC	Δ^8-Tetrahydrocannabinol
CBG	Cannabigerol
CBC	Cannabichromene
CBL	Cannabicyclol
CBNA	Cannabinolic acid
CBGA	Cannabigerolic acid
CBEA	Cannabielsoic acid
CB_1	Cannabinoid-1 receptor
CB_2	Cannabinoid -2 receptor
$CB_1^{-/-}$	CB_1 receptor knock out
GPCR	G protein-coupled receptor
Cys	Cysteine
Lys	Lysine
GTP	Guanosine-5'- triphosphate
GDP	Guanosine-5'-diphosphate
2-AG	2-Arachidonyl glycerol

2-AGE	2-Arachidonyl glyceryl ether
NADA	N–Arachidonyl-dopamine
NAEs	*N*-Arachidonyl ethanolamides
FAAH	Fatty acid amide hydrolase
NAPEs	*N*-Arachidonoyl-phosphatidylethanolamine
PLD	Phospholipase D
NAT	*N*-Acyl transferase
cAMP	Cyclic adenosine monophosphate
PLC	Phospholipase C
PI	Phosphatidylinositol
DAG	1,2-diacylglycerol
DGL	Diacylglycerol lipase
PLA1	Phospholipase A1
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
eCB-STD	Endocannabinoid-mediated short-term depression
eCB-LTD	Endocannabinoid-mediated long-term depression
GABA	γ-Aminobutyric acid
IPSCs	Inhibitory post synaptic currents
EPSCs	Excitatory postsynaptic currents
MAGL	Monoacylglycerol lipase
VGSCs	Voltage-gated sodium channels
VGCCs	Voltage-gated calcium channels

TTX	Tetrodotoxin
STX	Saxitoxin
K-ir	Inwardly rectifying potassium current
TBT	Tributyl tin
PMSF	Phenylmethane sulfonyl chloride
AO	Acridine orange
IC_{50}	Concentration that causes 50% inhibition
S.E.M.	Standard error of mean
4-AP	4-aminopyridine
VTN	Veratrine

1: Literature review and rationale

1.1 Historical Significance of Cannabinoids

Cannabis sativa is a dioecious flowering plant belonging to the family *Cannabaceae* that has one genus *Cannabis* (Childers & Breivogel, 1998; Hanus et al., 2001; Turner et al., 1980). Preparations of *Cannabis sativa* have been in use for thousands of years for their recreational and therapeutic properties but a scientific understanding of *Cannabis sativa* did not begin to develop until the mid-19th century (Childers & Breivogel, 1998; ElSohly & Slade, 2005; Hanus, 2009; Svizenska et al., 2008) (Figure 1-1). The historical record indicates use of cannabis by the ancient Chinese, Egyptian, Greek, Indian, Persian and Arabic civilizations for its fibre, oil, therapeutic effects, psychoactive manifestations and for religious purposes (Childers & Breivogel, 1998; Hanus, 2009; Pertwee, 2006). The ancient Chinese pharmacopoeia Pen Ts'ao recommended use of Ma-fen (flowers of the female marijuana plant) as a remedy for menstrual fatigue, rheumatism, malaria, beri-beri, constipation and lack of concentration, but also warned that overuse can lead to patients "seeing demons and communicating with spirits". Today, this latter effect can be linked to the psychomanipulatory properties of marijuana (Hanus, 2009). The Assyrians who ruled over a large part of Middle East (3,000 - 2,000 BC) also had cannabis as a major medicine in their pharmacopoeia (Hanus, 2009). The ancient Egyptian medical textbook Ebers Papyrus, which is the oldest complete medical compilation in existence, mentions the use of cannabis to treat rotting toe-nails and to induce uterine contractions (Hanus,

2009). The ancient Indian religious scripture Atharva Veda, regards the cannabis plant as one of the five most sacred plants in India, being prescribed mainly to protect from disease and to promote longevity (Hanus, 2009). The famous Greek physician Pedanius Discorides mentioned cannabis in Materia Medica as a good remedy to treat ear pain, inflammation and oedema (Hanus, 2009). It was ultimately these various ancient reports that prompted the Western world to conduct scientific investigations into the various therapeutic claims (Childers & Breivogel, 1998). Sir William B. O'Shaughnessy, an Irish physician working in Calcutta, India was the first person to describe the therapeutic value of cannabis in scientific terms (Di Marzo, 2006). O'Shaughnessy's work on cannabis is generally considered to be the start of cannabis research, which gained momentum particularly in the latter part of the 20th century (Di Marzo, 2006).

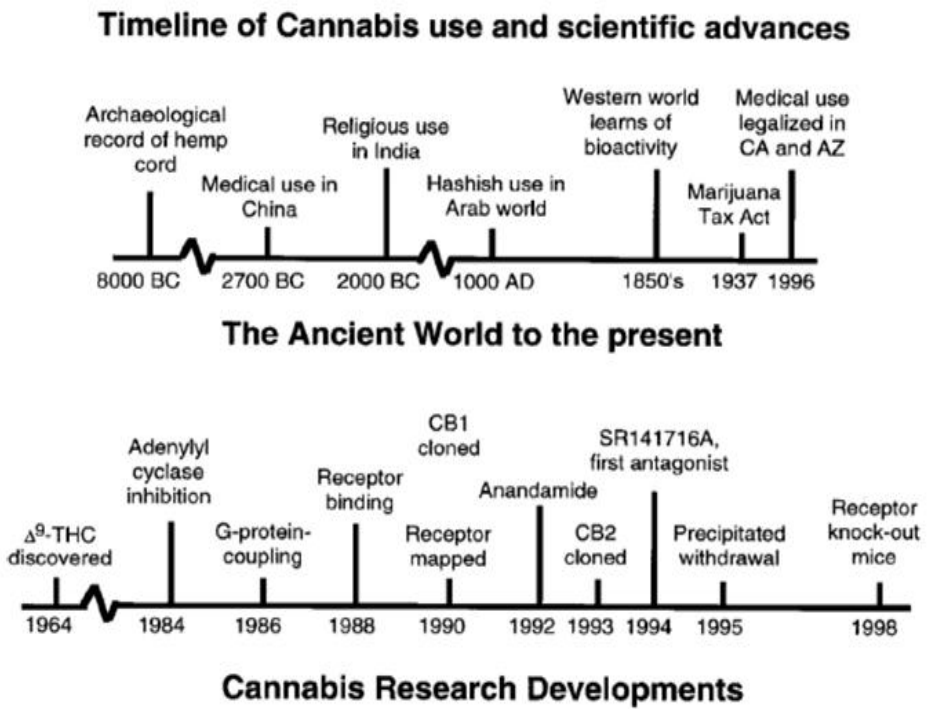


Figure 1-1 Landmarks in the history of cannabis use and advances in research on the pharmacological actions of cannabinoids (Childers & Breivogel, 1998).

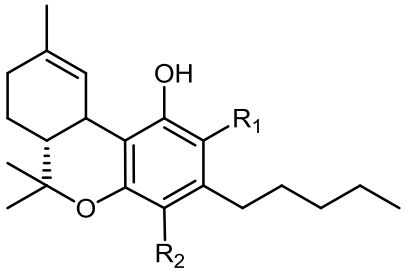
1.2 Chemical Constituents of *Cannabis sativa*

The two main preparations of *Cannabis sativa*, widely used illegally for recreational purposes, are marijuana and hashish (Hanus, 2009). Marijuana consists of the dried leaves and female flowering tops whereas hashish is the resinous extract obtained from the female flowering tops of *Cannabis sativa* (Hanus, 2009). A huge variety of natural products are present in *Cannabis sativa* and those compounds which have C_{21} terpenophenolic structures are referred to as cannabinoids (ElSohly and Slade, 2005; Turner et al., 1980). To date 538 compounds have been identified from *Cannabis sativa* plant and out of those 108 are referred to as cannabinoids (ElSohly & Slade, 2005; Hanus, 2009). There are 10 main types of cannabinoids and fourteen different cannabinoid subtypes (Hanus, 2009). The term phytocannabinoid is used for the cannabinoids obtained from plant origin. Cannabinoids are secondary metabolites and Δ^9-tetrahydrocannabinol (Δ^9-THC) (Figure 1-2 and Figure 1-5) is involved in providing protection to plants from pathogens and herbivores (Abel, 1975). Cannabinoid alkaloids have also been identified and some possess some cannabimimetic activity, but considering their low concentrations in cannabis, it is unlikely that they contribute significantly to the pharmacological actions of cannabis (Mechoulam & Hanus, 2000).

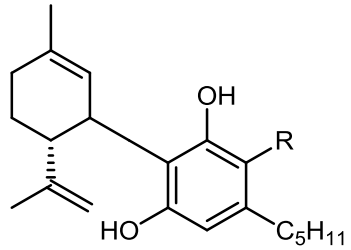
The compound present in cannabis extracts that was mainly responsible for its pharmacological effects remained elusive for some time because of difficulties with its chemical characterization (Mechoulam, 2000). In the 1930s, constituents like cannabinol (CBN) (Adams et al., 1940) and cannabidiol (CBD) (Adams et al., 1940) were isolated and structurally defined (Figure 1-2). CBN was the first natural cannabinoid to be obtained in pure form but was wrongly assumed to be the main psychoactive component

of cannabis (Mechoulam & Hanus, 2000). Subsequent experiments showed that CBN and CBD differed significantly in their UV spectra and biological activity from the active constituent in cannabis (Mechoulam & Hanus, 2000; Mechoulam, 2000). Although CBD was isolated earlier, its correct structure and stereochemistry was not reported until 1963 by Mechoulam & Shvo using NMR. With more sophisticated isolation and anyalytical techniques becoming available the primary psychoactive component present in *Cannabis sativa* was identified by Gaoni & Mechoulam (1964a) as Δ^1- tetrahydrocannabinol (Δ^1-THC) which was later renamed Δ^9-tetrahydrocannabinol (Δ^9-THC) (Mechoulam, 2000). The absolute configuration of Δ^9-THC was assigned in 1967 (Mechoulam & Gaoni, 1967). Δ^9-THC was shown to cause sedation in the rhesus monkeys at low doses. Numerous studies have subsequently shown that administration of Δ^9-THC to mice to causes antinociception, reduction in locomotor activity and hypothermia (Smith et al., 1994; Martin et al., 1991). After the identification of Δ^9-THC, numerous other constituents including neutral cannabinoids and cannabinoid acids were identified (Mechoulam & Hanus, 2000; Mechoulam, 2000). Another weakly psychoactive component of cannabis, Δ^8-THC (Figure 1-2) was isolated from marijuana (Hively et al., 1966). However, it is not certain whether Δ^8-THC is a natural component of cannabis or whether it is produced as an artifact during drying of the plant (Hanus, 2009). Other non-psychotropic cannabinoids like cannabigerol (CBG) (Gaoni & Mechoulam, 1964b), cannabichromene (CBC) (Gaoni & Mechoulam, 1966) and cannabicyclol (CBL) (Crombie & Ponsford, 1968) have been reported (Figure 1-2). Various cannabinoid acids such as cannabinolic acid (CBNA), cannabigerolic acid (CBGA), cannabielsoic acid (CBEA), cannabidiolic acid (CBDA) (Figure 1-2), Δ^9-THC acids (A & B) and Δ^8-THC

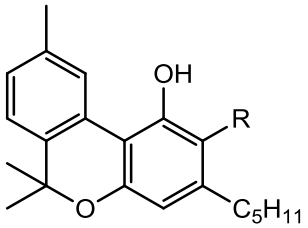
acid were described (Hanus, 2009; Mechoulam & Hanus, 2000; Mechoulam, 2000; Yamauchi et al., 1967) (Figure 1-2). Cannabinoid acids are important plant constituents from the biochemical perspective because they feature early in the biosynthesis and can be easily decarboxylated to produce neutral cannabinoids (Mechoulam & Hanus, 2000; Mechoulam, 2000; Yamauchi et al., 1967). In this way, Δ^9-THC acid A and B can serve as precursors for Δ^9-THC but interestingly, cannabinoid acids are devoid of psychoactive properties (Mechoulam & Hanus, 2000; Mechoulam, 2000; Yamauchi et al., 1967).



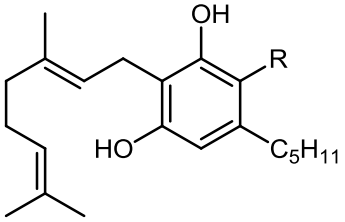
$R_1 = R_2 = H$, Δ^9-Tetrahydrocannabinol (Δ^9-THC)
$R_1 = COOH$, $R_2 = H$, Δ^9-THC acid A (Δ^9-THCA)
$R_1 = H$, $R_2 = COOH$, Δ^9-THC acid B (Δ^9-THCB)



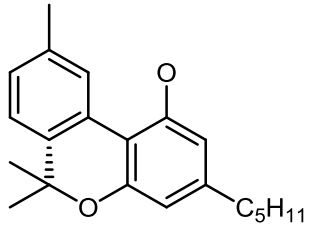
R = H, Cannabidiol
R = COOH, Cannabidiolic acid



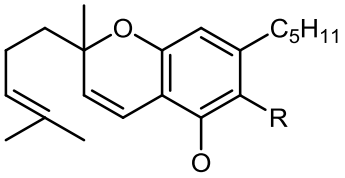
R = H, Cannabinol
R = COOH, Cannabinolic acid



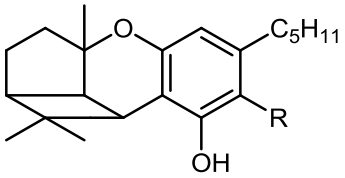
R = H, Cannabigerol
R = H, Cannabigerolic acid



Δ^8-tetrahydrocannabinol (Δ^8-THC)



R = H, Cannabichromene
R = COOH, Cannabichromenic acid



R = H, Cannabicyclol
R = COOH, Cannabicyclolic acid

Figure 1-2 The structures of important phytocannabinoids. All structures were redrawn using ChemBioDraw Ultra from the structures reported in Hanus (2009).

1.3 Cannabinoid Receptors

1.3.1 Cannabinoid-1 (CB_1) Receptor

Two views regarding the mode of action of cannabinoids initially emerged. One supported the presence of a specific cannabinoid receptor through which cannabinoids mediate their pharmacological actions (Howlett, 1984; Howlett et al., 1986). This superceded an earlier view that due to their highly lipophilic nature cannabinoids produce their effects through perturbations to the neuronal membrane as was originally proposed for anaesthetics and solvents (Begg et al., 2005; Mechoulam & Hanus, 2000; Mechoulam, 2000). Eventually a high affinity stereoselective cannabinoid receptor was identified in rat brain using a tritium labeled, synthetic cannabinoid (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP-55,940) (Figure 1-5; Devane et al., (1988)). This receptor is now known to be heterogeneously distributed in various areas of mammalian brain with regions of high density within the cerebellum, hippocampus, basal ganglia and cortex (Herkenham et al., 1990; Tsou et al., 1988). Structure activity studies confirm that the binding site of [^{3}H]CP-55,940 is the same as the one through which cannabinoids exert their pharmacological effects *in vivo* (Herkenham et al., 1990). This centrally located cannabinoid-1 (CB_1) receptor is a 473 amino acid G protein-coupled receptor (Matsuda et al., 1990). The human CB_1 receptor consists of 472 amino acids and the gene coding for the CB_1 receptor is located on chromosome 6 (Gerard et al., 1990). The CB_1 receptor of rat, human and mouse have 97-99% amino acid sequence similarity. An amino terminal splice variant for the human CB_1 receptor (CB_{1A}) has been reported (Shire et al., 1995). This species is sixty-one amino acids shorter than the CB_1 receptor and CB_{1A} has been detected at low levels in brain and

many peripheral tissues (Shire et al., 1995). A similar splice variant of the mouse CB_1 receptor was also reported (Shire et al., 1995). Yet another splice variant of the CB_1 receptor (hCB1b) was identified while cloning the amino terminal splice variant identified earlier by Shire et al. (1995) at AstraZeneca (Ryberg et al., 2005). These splice variants (hCB1a & hCB1b) of the full length CB_1 (hCB1) receptors were stably transfected into HEK293 cells but interestingly showed very low binding affinity for anandamide (an endogenous cannabinoid receptor ligand) (Ryberg et al., 2005). On the other hand 2-arachidonyl glycerol (2-AG; another endogenous ligand for the cannabinoid receptor) had significant binding affinity, for the splice variant CB_1 receptors but it was lower than the affinity of 2-AG for the hCB1 (Ryberg et al., 2005).

Herkenham et al. (1990) also showed that the CB_1 receptor targeted by [^{3}H]CP-55,940 was functionally coupled to a guanine nucleotide binding protein, a modulatory complex closely linked with second messenger systems. The low density of the CB_1 receptors in brain stem areas likely explains why high doses of Δ^9-THC are not fatal since the brain stem is central to the regulation of respiratory and cardiovascular function (Herkenham et al., 1990). The distribution pattern of the CB_1 receptor in the central nervous system (CNS) largely accounts effects of cannabinoids on motor and cognitive functions (Herkenham et al., 1990).

Autoradiographic evidence suggests that in some areas of brain CB_1 receptor numbers exceed 1 pmol/mg of protein, a value considerably higher than that of neuropeptide receptors and commensurate with that of glutamate and GABA receptors (Herkenham et al., 1990). The CB_1 receptor is also expressed albeit at very levels in the human testis, human heart muscle, the spleen, tonsils, small intestine, urinary bladder,

liver, lung, kidney, bile duct, colon, stomach, vas deferens, adrenal gland, uterus, prostrate and ovary (Bonz et al., 2003; Gerard et al., 1991; Shire et al., 1995; Adami et al., 2002; Banerjee et al., 2011).

1.3.2 Cannabinoid-2 (CB_2) Receptor

The other main subtype of the cannabinoid receptor is the cannabinoid-2 receptor (CB_2) which is primarily located in the cells of the immune system, the spleen and tonsils (Kaminski et al., 1992). Like the CB_1 receptor, the CB_2 receptor is a G protein-coupled receptor consisting of 360 amino acids (Munro et al., 1993). Interestingly, very low levels of CB_2 receptors have been detected in some areas of the brain and these are up-regulated in response to chronic pain (Benito et al., 2008). The CB_2 receptor also represents an attractive target for novel drugs that can be used in the treatment of neurodegenerative disorders like Alzheimers, HIV-induced encephalitis and multiple sclerosis (Benito et al., 2008). The CB_1 and CB_2 receptor have 68% homology in the transmembrane region and an overall 44% similarity (Munro et al., 1993). The generally accepted notion is that there are two types of cannabinoid receptors (CB_1 and CB_2). However, with the development of CB_1 receptor knock-out mice ($CB_1^{-/-}$), the presence of an unknown cannabinoid receptor in the brain has been hypothesized (Breivogel et al., 2001). More recently, an orphan G protein-coupled receptor GPR55 has been hypothesized to be a cannabinoid receptor but its role has to be evaluated more before it can be classified as a part of endogenous cannabinoid system (Hanus, 2009).

1.4 Structure of the CB_1 Receptor

CB_1 receptors are cell membrane receptors belonging to the large family of G protein-coupled receptors (GPCRs) (Matsuda et al., 1990). GPCRs are aptly named because these receptors couple to hetrotrimeric G proteins and act as relay molecules in transmitting signals from humoral molecules to the intracellular pathways, thereby defining the cell signalling profile of the respective receptor (Patny et al., 2006). GPCRs represent an extremely attractive target for the pharmaceutical industry. This is evident from the fact that 52% of the drugs on the market today act through GPCRs and 60-70% of drugs in the developmental stage are being developed to target GPCRs directly or indirectly (Patny et al., 2006).

The CB_1 receptor is one of the most abundant and heterogeneously distributed GPCRs in the mammalian brain, which attests to the functional importance of these receptors in regulating CNS function (Herkenham et al., 1990). Like typical GPCRs, the CB_1 receptor has seven transmembrane helices (TM1-TM7) connected by three extracellular loops (E1-E3) and three cytoplasmic loops (C1-C3) (Shim et al., 2003) (Figure 1-3). The CB_1 receptor belongs to the Class A rhodopsin-like family of GPCRs with an extracellular glycosylated N terminus and an intracellular carboxyl terminus (Shim et al., 2011; Stadel et al, 2011). Docking of Δ^9-THC on to the CB_1 receptor, using bacteriorhodopsin as a structural template revealed calcium-binding sites in the extracellular N terminus region (Mahmoudian, 1997). The side chain of Δ^9-THC was predicted to bind to the hydrophobic pocket of the CB_1 receptor consisting of residues Met-240, Trp-241 (TM-4), Trp-356, Leu-359, Leu-360 (TM-6), and Ala-283 (TM-5) (Mahmoudian, 1997). Mahmoudian (1997) also predicted the formation of hydrogen

bond between the phenolic hydroxyl group of Δ^9-THC and the carboxyl group of Ala-198 (TM3).

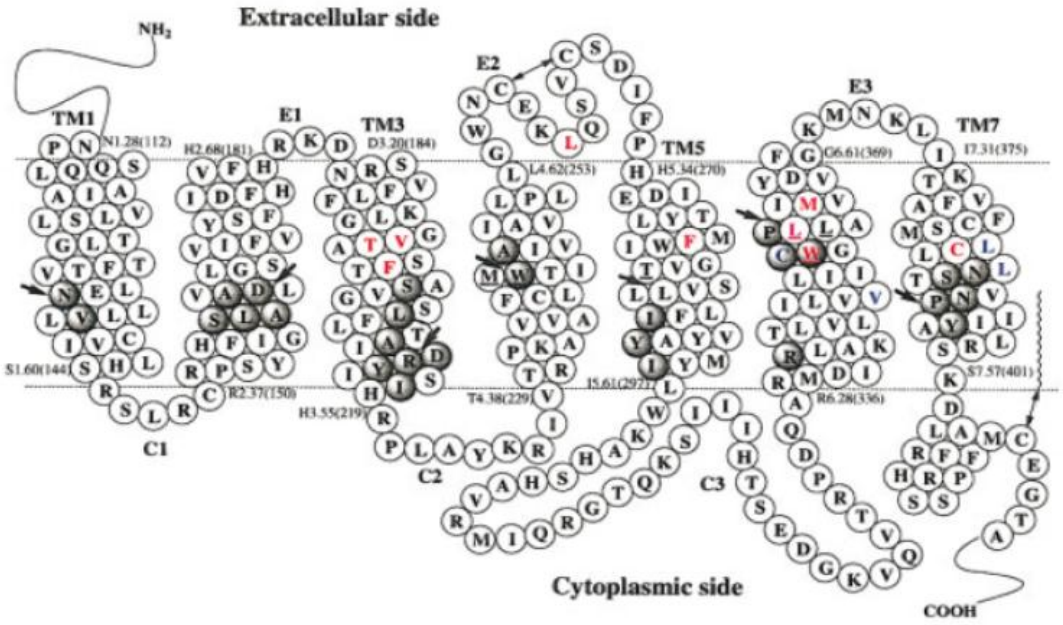


Figure 1-3 Two-dimensional structural representation of the CB_1 receptor. The CB_1 receptor, like a typical GPCR, shows seven transmembrane (TM) helices that are connected by three extracellular loops (E1-E3) and three intracellular loops (C1-C3). Bold shaded circles represent highly conserved residues. The residues deemed to be important for interacting with the C3 hydrophobic chain of cannabinoid ligands shown in red were proposed by Shim et al. (2003), residues in blue were proposed by Tao et al. (1999), and underlined residues were proposed by Mahmoudian (1997).

1.4.1 Homology model of the CB_1 receptor and importance of the E2 loop in CB_1 receptor structure and function

A homology model was constructed for the CB_1 receptor using the X-ray crystal structure of bovine rhodopsin (Palczewski et al., 2000) as a template (Shim et al., 2003). The inactive conformation of bovine rhodopsin indicates that several regions within the TM area (TM2, TM4, TM5 and TM7) do not have an α-helical structure and rhodopsin's ligand (retinal) binds covalently to the lysine (Lys) residue in TM7 in the binding pocket that forms from the TM helices and the E2 loop (Shim et al., 2003). Bovine rhodopsin was chosen as a template because 22 out of 34 highly conserved residues in TM region of rhodopsin are also present in the TM region of the CB_1 receptor (Shim et al., 2003) (Figure 1-4). The X-ray crystallographic structure of rhodopsin at high resolution showed that the centre residues of the E2 loop are inserted in the binding pocket formed by TM2, TM3 and TM7 and this represents an important region of the ligand binding site (Palczewski et al., 2000; Shim et al., 2003). Replacement of the E2 loop of the CB_1 receptor by the E2 loop of the CB_2 receptor resulted in the inability of the bicyclic cannabinoid agonist [^{3}H]CP-55,940 to bind. However, this modification did not affect the binding of N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) (a high affinity CB_1 receptor antagonist/inverse agonist) (Shire et al., 1996) (Figure 1-7). This result clearly indicates the importance of the E2 loop of the CB_1 receptor for the bicyclic cannabinoid agonist to bind whereas it had no importance for pyrazole antagonist binding (Shire et al., 1996). Intriguingly, replacement of the TM4 to TM5 region of the CB_1 receptor by the equivalent moiety of the CB_2 receptor resulted in complete loss of affinity for SR141716A but had no effect on [^{3}H]CP-55,940 binding (Shire et al., 1996). This emphasizes the importance of the TM4

to TM5 region of the CB_1 receptor for SR141716A to bind (Shire et al., 1996). Most GPCRs have a disulphide bond between the cysteine (Cys) residue located on the extracellular side of TM3 and the Cys residue located at the E2 loop. This disulphide linkage is considered important in GPCRs for maintaining structure and function (Shim et al., 2003; Shim et al., 2011). However, such a disulphide linkage is not possible in the CB_1 receptor because TM3 does not possess the critical Cys residue (Fay et al., 2005). The lack of a disulphide linkage renders the E2 loop unable to insert deep into the core region and this allows the TM helices to pack much closer together thereby preventing water from entering the binding region (Nebane et al., 2008). However, mutational studies have predicted an alternative disulphide linkage between two Cys residues on E2 (Fay et al., 2005). This disulphide bridge is predicted to form between Cys (257) and Cys (264) on the E2 loop (Fay et al., 2005; Shire et al., 1996). Radioligand binding studies in the presence of dithiothreitol (DTT) and other sulfhydryl blocking agents reported a decrease in the binding of [^{3}H]CP-55940 and this indicates the importance of at least one free sulfhydryl group (Fay et al., 2005; Lu et al., 1993). Out of a total of 13 Cys residues present in the CB_1 receptor only 5 highly conserved Cys residues are required for ligand binding and coupling to G proteins (Fay et al., 2005). Further experiments by Fay et al. (2005), showed that out of the 5 highly conserved Cys residues, mutants having only Cys (257) and Cys (264) which form a disulphide linkage, can also bind ligands and couple to G proteins. Recently the structure of the CB_1 E2 loop was determined using a stimulated annealing (SA) and molecular dynamics (MD) approach (Shim et al., 2011). These researchers compared E2 in the reduced state (i.e., with the two Cys residues in dithiol form) with E2 in oxidized state (i.e., with the two Cys residues covalently linked by the

disulphide bridge) in a fully hydrated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer. The reduced state of E2 interacts less with the TM helical bundle promoting attachment of TM5 to TM3 whereas E2 in oxidized state interacts more with the TM helices and promotes detachment of TM5 from TM3 (Shim et al., 2011). This result further highlights the importance of the E2 loop in maintaining the structure of the CB_1 receptor and ultimately regulating receptor function (Shim et al., 2011).

1.4.2 Importance of CB_1 receptor C-terminal domain in modulating CB_1 receptor structure and function

Recently, the three-dimensional structure of the full length CB_1 receptor carboxyl-terminal tail region has been reported (Ahn et al., 2009). The human CB_1 receptor carboxyl terminal has 73 residues, but does not have significant homology with the carboxyl terminal of CB_2 receptors (Stadel et al., 2011). In the C-terminal region the TM-7 is continuous with a small cytoplasmic helical extension, helix 8 (H8), and contains a highly conserved NPXXY motif (Tiburu et al., 2011). The NPXXY motif is important for GPCR activation and conformational switching between different GPCR activity states. Moreover, H8 is a constituent of the receptor site involved in coupling to G proteins (Tiburu et al., 2011). In the CB_1 receptor, TM-7 and H8 are also very important for ligand binding and receptor function (Barnett-Norris et al., 2002; Kapur et al., 2007; Shim et al., 2003). Mutation of a serine residue in TM7, results in a decrease in the binding of the agonists CP-55,940, (-)-11-hydroxydimethylheptyl-Δ^8-tetrahydrocannabinol (HU-210; Figure 1-6) and (-)-11β-hydroxy-3-(1',1'-dimethylheptyl)hexahydrocannabinol (AM-4056) but not that of the agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-

naphthalenyl)methanone (WIN-55,212; Figure 1-6) and the antagonist/inverse agonist SR-141716A (Kapur et al., 2007). Kapur et al. (2007) have predicted that a serine residue likely induces a helix bend in TM7 which provides binding space for CP-55,940. A peptide corresponding to the carboxyl terminus of the CB_1 receptor was shown to be critical for G protein activation (Howlett et al., 1998). Truncation of the C-terminal region of the CB_1 receptor inhibited CB_1 receptor-mediated inhibition of voltage-gated calcium channels, suggesting the importance of this region in G protein activation and signal transduction events (Nie & Lewis, 2001a). Truncation of the CB_1 receptor C-terminal region leads to increase in constitutive activity of the CB_1 receptor (Nie & Lewis, 2001b). Mutational studies also support the idea that the C-terminal region is important for CB_1 receptor internalization (Hsieh et al., 1999).

1.4.3 Binding interactions between the CB_1 receptor and cannabinoid agonists

Numerous structure activity studies have highlighted the C3 side chain, the A ring OH and C/D ring OH as the main pharmacophores within phytochemical cannabinoid ligands that are important for binding at the CB_1 receptor (Howlett et al., 1988; Melvin et al., 1984; Melvin et al., 1993; Milne & Johnson, 1981) (Figure 1-5). The hydrophobic C3 side chain apparently interacts with the putative hydrophobic pocket of the CB_1 receptor (Melvin et al., 1993). A C3 side chain of 7-8 carbon length is considered optimum for CB_1 receptor binding affinity (Melvin et al., 1993). Shim et al. (2003), proposed two conformations *C3-out* and *C3-in* of the C3 side chain of the cannabinoid agonist CP-55,244 (Figure 1-6) that interact with the binding pocket of the CB_1 receptor (Figure 1-8). In this model CP-55,244 docks in such a way that its A ring hydroxyl forms a hydrogen

bond with the side chain of Lys (192) located in third TM helical domain of the CB_1 receptor (Shim et al., 2003). Thus Lys (192) is considered a critical residue for CP-55,940 binding and mutation of this residue results in inhibition of bicyclic agonist binding although it has no effect on the binding of the aminoalkylindole cannabinoid agonist WIN55,212-2 (Chin et al., 1998). In another study, mutation of Lys (192) to alanine was shown to reduce CP-55,940, HU-210 and anandamide binding but had no effect on WIN-55,212-2 binding (Song & Bonner, 1996), thus confirming the earlier report by (Chin et al., 1998). The *C3-out* conformation represents the C3 side chain of CP-55,244 positioned towards the extracellular region whereas the *C3-in* conformation represents the C3 side chain of CP-55,244 positioned towards the helix interior (Shim et al., 2003). In both C3 conformations of CP-55,244, CP-47,497 (Figure 1-5) and CP-55,940 there is an opportunity for formation of a hydrogen bond between the A-ring OH and the side chain NH of Lys (192). In the *C3-in* conformation, there is an additional hydrogen bond formation between the side chain O of glutamic acid (Glu) (258) and the A-ring OH (Shim et al., 2003). In the *C3-in* conformation, the C-ring C12 hydroxyl propyl group of CP-55,940 or the D-ring C14 hydroxy-methyl of CP-55,244 forms an additional hydrogen bond with the side chain O of glutamine (Gln) (261) (Shim et al., 2003). It was also reported that introduction of an hydroxyl group at the C-ring at carbon 12 or the D-ring at carbon 14 results in increased binding affinity and this supports the idea that this interaction with the CB_1 receptor is a crucial ligand-receptor interaction (Shim et al., 2003). This additional hydrogen bond formation involving the C-ring carbon 12 or the D-ring carbon 14 hydroxyl group is only possible with the *C3-in* conformation and so the binding energy contribution by *C3-in* conformation is greater as compared with *C3-out*

conformation (Shim et al., 2003). The C-ring carbon 9 hydroxyl group is absent in classical cannabinoids like Δ^9-THC, and thus its contribution to ligand binding through a hydrogen bond formation is not relevant for in cannabinoid agonists like CP-55,940, CP-47,497 and CP-55,244 which do have a hydroxyl group at carbon 9 of the C-ring. In accordance with the *C3-in* conformation, the C-ring carbon 9 hydroxyl group was indicated as unlikely involved in any interaction, whereas in the *C3-out* conformation it was contributing to ligand binding (Shim et al., 2003). Shim et al. (2003) has predicted the *C3-in* conformation to occupy a region 2-6 turns away from the extracellular TM helical boundaries between TM helices 3-7. Earlier Tao et al. (1999) suggested that the C3 side chain occupies a region between TM6 and TM7, but according to the model of Shim et al. (2003) this region may be less capable of docking the relatively bulky hydrophobic side chain. According to Shim et al. (2003), in the *C3-in* conformation, the more lipophilic C3 side chain can insert deep into the binding pocket when the less polar A- and C/D-rings are present where hydroxyl groups are aligned towards the more polar region near the extracellular surface. In the model of Shim et al. (2003), the E2 loop of the CB_1 receptor extends deep into the binding pocket and interacts with the different structural constituents of the A and C/D rings. It is noteworthy that the earlier models of Tao et al. (1999) and Mahmoudian (1997) predict the residues associated with the binding pocket to be from TM4-TM7 and TM3-TM7, but its only the Shim et al. (2003) model that involves a residue from the E2 loop as part of the hydrophobic binding pocket. Taken together these findings suggest that the *C3-in* conformation is the most likely conformation promoting the binding of non-classical bicyclic and tricyclic cannabinoids to the CB_1 receptor (Shim et al., 2003).

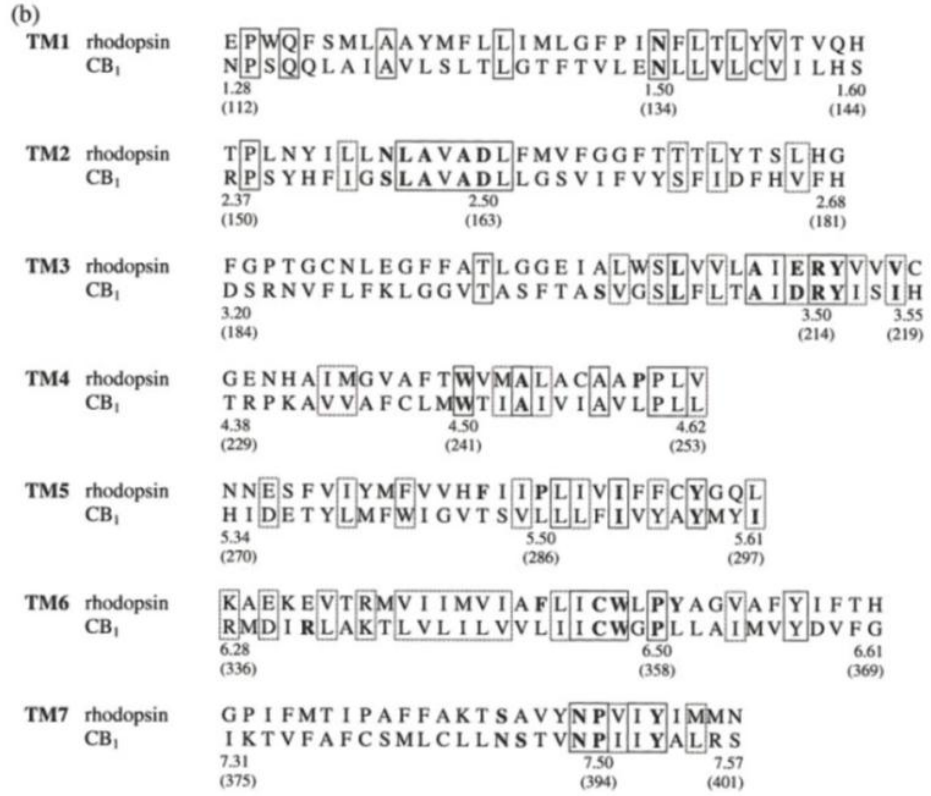
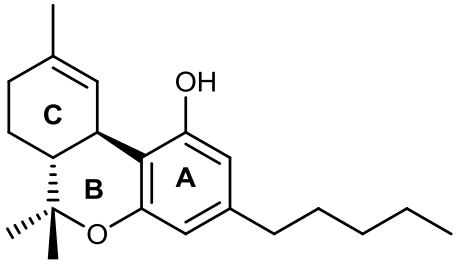
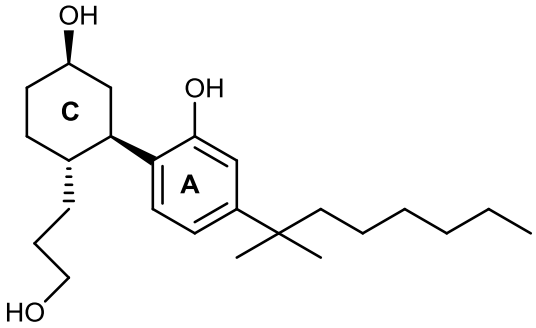


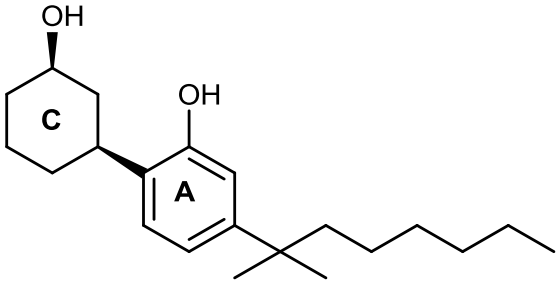
Figure 1-4 Alignment of the TM region of rhodopsin and the CB_1 receptor reveal a number of highly conserved residues common to both. 22 out of the 34 highly conserved residues present in the TM regions of rhodopsin (Baldwin et al., 2007) are also present in the TM region of the CB_1 receptor and are represented in bold letters placed inside boxes (Shim et al., 2003).



Classical cannabinoid agonist Δ^9-THC (Gaoni and Mechoulam, 1964a; Little et al., 1988)

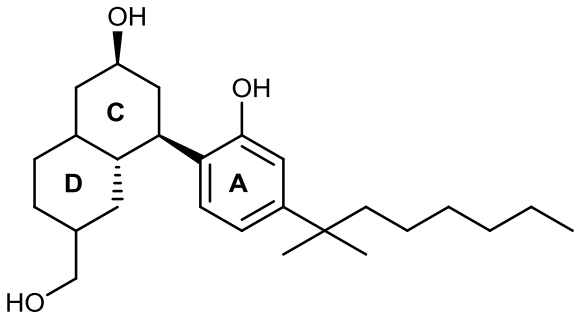


CP-55,940 (Non-classical AC bicyclic cannabinoid agonist (Little et al., 1988))

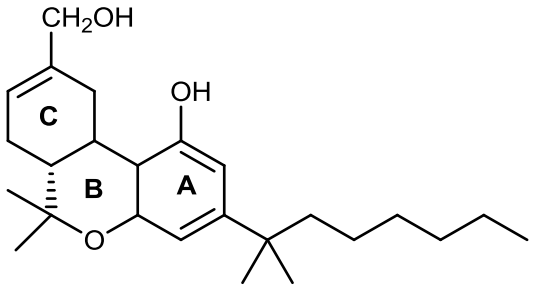


CP-47,497 (Non-classical AC bicyclic cannabinoid agonist (Compton et al., 1992))

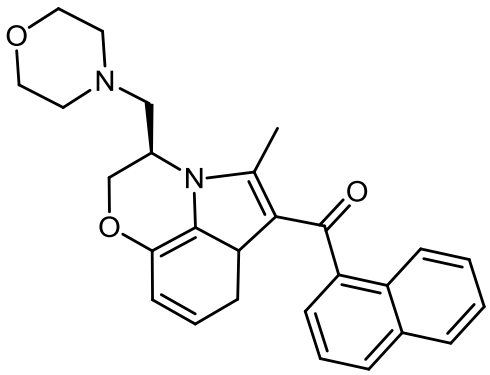
Figure 1-5 The structures of Δ^9-THC and bicyclic CB_1 receptor agonists. All structures were redrawn using ChemBioDraw Ultra by referring to structures reported in Hanus (2009), Piomelli (2003) and Shim et al. (2003).



CP-55,244 (Non-classical ACD tricyclic cannabinoid agonist (Little et al., 1988))

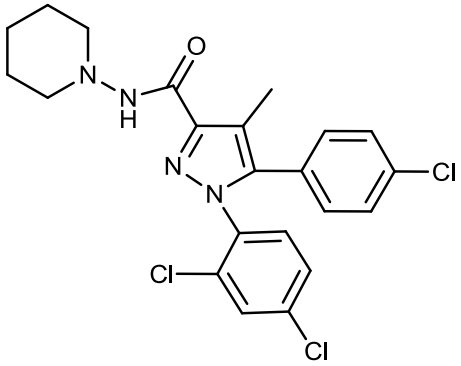


HU-210 (High affinity cannabinoid agonist) (Mechoulam, 2000)

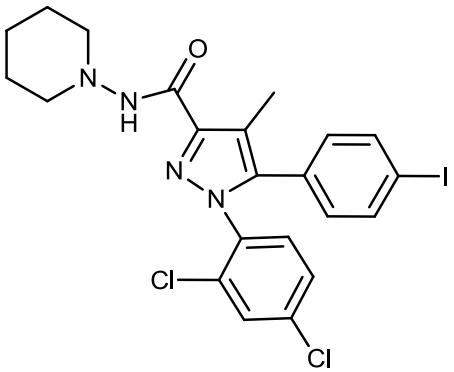


WIN-55,212-2 (Aminoalkylindole cannabinoid agonist (Compton et al., 1992))

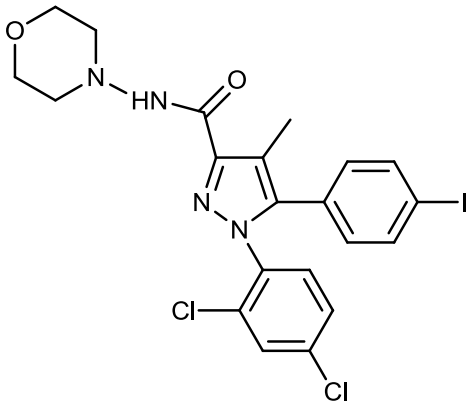
Figure 1-6 Structures of tricyclic and aminoalkylindole CB_1 receptor agonists. All structures were redrawn using ChemBioDraw Ultra by referring to structures reported in Hanus (2009), Piomelli (2003) and Shim et al. (2003).



SR141716A (CB_1 receptor antagonist/inverse agonist (Rinaldi Carmona et al., 1994))



AM251 (CB_1 receptor antagonist/inverse agonist (Dennis et al., 2008))



AM281 (CB_1 receptor antagonist/inverse agonist (Cosenza et al., 2000))

Figure 1-7 Structures of some important CB_1 receptor antagonists/inverseagonists. All structures were redrawn using ChemBioDraw Ultra by referring to structures reported in Hanus (2009), Piomelli (2003) and Shim et al. (2003).

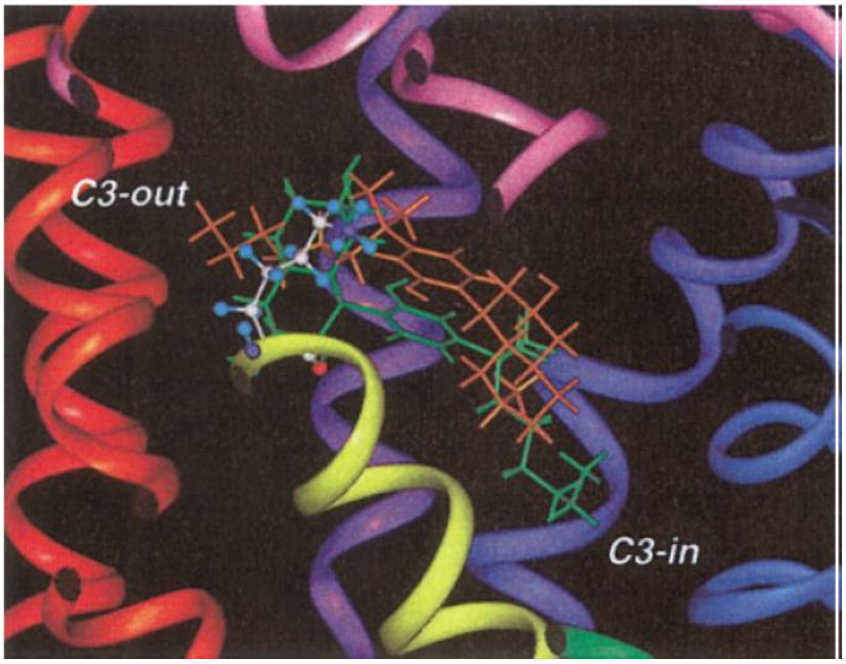


Figure 1-8 Two possible conformations (*C3-out* shown in orange and *C3-in* shown in green) that can be adopted by CP-55,244 when docking with the CB_1 receptor (Shim et al., 2003).

1.5 Agonist, Antagonist, Inverse agonist, Intrinsic Activity and Reverse Intrinsic Activity at GPCRs

An agonist is a ligand that when it occupies a receptor, triggers the stimulus that is needed for the biological response associated with that receptor to occur (Schutz & Freissmuth, 1992). Thus, agonists have intrinsic activity. The efficacy of a ligand also depends upon the receptor density and the coupling efficiency of the cell or tissue and thus a ligand might behave as partial agonist in one system and display the full profile of an agonist in the other system (Milligan et al., 1995). A competitive antagonist on the other hand binds to the receptor at the same site as the agonist and blocks the occupancy of the agonist and thus prevents the agonist from triggering the biological response (i.e. antagonists normally do not have intrinsic activity) (Schutz & Freissmuth, 1992). Drugs acting at GPCRs have varied intrinsic efficacies ranging from 0 for an antagonist to 1 for a full agonist (Milligan et al., 1995). Not all antagonists have zero intrinsic efficacy since some display biological activity which is opposite to that of the agonist (Milligan et al., 1995). These antagonists which have reverse biological activity are said to exhibit negative efficacy or negative intrinsic activity and are termed as inverse agonists (Milligan et al., 1995; Schutz & Freissmuth, 1992).

According to basic receptor theory, receptors exist in equilibrium between two conformations, an inactive state (R) and an active state (R*) (Figure 1-9). These two states can be readily interchanged. Agonists tend to have a higher affinity for the active state (R*) and thus shift the equilibrium towards the active conformation and preferentially stabilize the active state. It is the active state that can couple to the respective G proteins more effectively and thus enabling the sequence of events to be initiated. An inverse agonist tends to stabilize the inactive state and thus promotes the

uncoupling of the G proteins from their respective receptor and thus reduction in basal activity can be explained by this logic. A neutral antagonist does not have preferential affinity for any state and therefore in its presence the dynamic equilibrium between the two states remains unchanged. In this state the intrinsic activity seen is called basal activity (without the influence of an agonist). Receptor density also affects basal activity and with the increase in density there is a higher number of receptors that would be in their active conformation. It has been shown using cell lines that with an increase in expression of the receptor there is a parallel increase in basal activity (Milligan et al., 1995; Milligan & Bond, 1997; Schutz & Freissmuth, 1992).

GPCR agonists have been shown to cause receptor internalization, recycling and down regulation depending upon the period of exposure (Barker et al.,1994; Barak et al., 1997; Milligan & Bond, 1997; Molino et al., 1997; Vonzastrow & Kobilka, 1992). Interestingly, an inverse agonist was also shown to down-regulate receptor number whereas an antagonist failed to show this effect (Barker et al., 1994). According to the receptor model the active state of the GPCR promoted by the agonists which activate coupling to G proteins is mostly responsible for desensitization and down regulation and thus an inverse agonist which promotes the inactive state of a receptor should up-regulate the receptor number (Milligan & Bond, 1997). Inverse agonists like cimetidine and rantidine at the histamine H_2 receptor were shown to up-regulate the H_2 receptors whereas the neutral antagonist burimamide, as expected, did not induce upregulation (Smit et al., 1996).

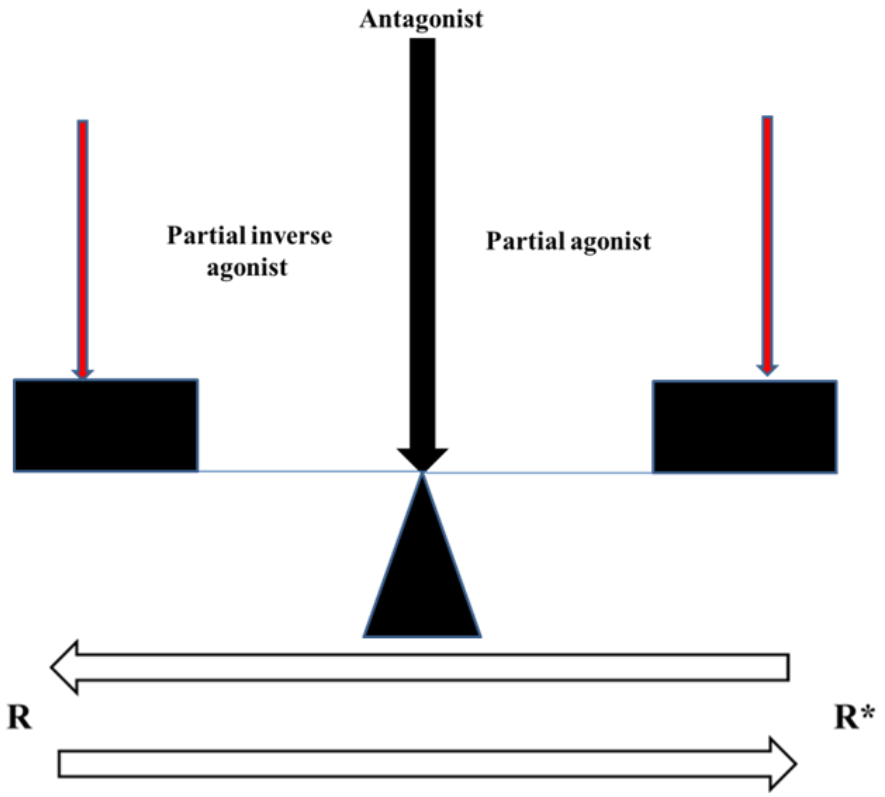


Figure 1-9 Different ligands preferentially shift the equilibrium to different extents between the active state (R*) and the inactive state (R) of a receptor. An agonist tends to favour R*, whereas an inverse agonist favours R. Neutral antagonists do not have any preference for either state and thus do not alter the position of the equilibrium. The red arrow on the extreme left represents the full shift of equilibrium towards R by an inverse agonist whereas the red arrow on the extreme right represents the shift of equilibrium towards R* by an agonist. This figure has been redrawn from Milligan et al. (1995).

1.5.1 Activation cycle of GPCRs

GPCRs as the name suggests, are receptors that couple to heterotrimeric G-proteins. GPCRs are important in regulating various physiological processes and thus an important target for pharmaceutical agents such as analgesics, antihistamines, neuroleptics, antidepressants and drugs that treat cardiovascular disorders. G proteins consist of a Gα subunit and a dimer of Gβ and Gγ subunits. G proteins in their inactive state have the Gα subunit bound with guanosine diphosphate (GDP) and they exist as Gα(GDP)βγ (Harrison & Traynor, 2003). When a ligand activates the G-protein-coupled receptor, the affinity of Gα subunit increases for guanosine-5'-triphosphate (GTP) in comparison to guanosine diphosphate (GDP) (Griffin et al., 1998; Harrison & Traynor, 2003). GTP binds to the Gα subunit, and the binary complex (Gα-GTP) separates from the Gβγ subunit (Griffin et al., 1998). After separating out, both the subunits (Gα-GTP and Gβγ) interact with various effectors systems. Eventually, hydrolysis of GTP to GDP occurs via GTPase activity of the Gα subunit and it reverts to its original form (Gα-GDP). Finally, Gα-GDP and Gβγ subunits combine returning to the inactive state composition. Interestingly, GPCRs may activate G proteins in the absence of agonist by adopting an active receptor conformation (Harrison & Traynor, 2003).

1.5.2 [^{35}S]GTPγS binding assay

The [^{35}S]GTPγS binding assay can be used to measure the G-protein activation by a particular ligand that interacts with a GPCR (Breivogel et al., 1998; Breivogel & Childers, 2000; Breivogel et al., 2001; Harrison & Traynor, 2003). A major advantage with this assay is that it is a functional assay and measures one of the first steps when a ligand occupies a GPCR (Harrison & Traynor, 2003). Any event measured downstream

of the G protein may be subjected to modulation, but the [^{35}S]GTPγS binding assay measures the first step after receptor occupancy and is free from such interference (Harrison & Traynor, 2003).

In this assay, endogenous GTP is replaced by [^{35}S]GTPγS which binds to the Gα subunit when the receptor is activated to form Gα[^{35}S]GTPγS . The γ-thiophosphate bond is resistant to hydrolysis by the GTPase of Gα, and thus [^{35}S]GTPγS-labeled Gα subunits accumulate and these can be quantified by measuring the [^{35}S]-label bound (Griffin et al., 1998; Harrison & Traynor, 2003).

1.6 Endocannabinoids

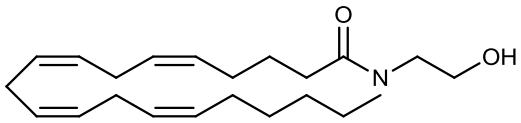
It was logical to think that the brain might not have a high affinity cannabinoid binding site for a plant constituent only, and investigations designed to look for an endogenous cannabinoid soon got underway. The calcium ionophore A23187 in the presence of Ca^{2+} was observed to release a substance that interfered with [^{3}H]CP-55940 binding (Evans et al., 1992). This was the first indication that a substance released in a Ca^{2+}-dependent manner interfered with the dynamics of [^{3}H]CP-55940 binding in a dose dependent manner. Subsequently, Devane et al. (1992) reported the isolation and structural characterization of an endogenous cannabinoid which they named anandamide (Figure 1-10). Anandamide binds to the CB_1 receptor (Devane et al., 1992), and inhibits the twitch response of isolated murine vas deferens (Devane et al., 1992). Moreover when administered intraperitoneally (i.p.) anandamide produces various dose-dependent *in vivo* symptoms such as analgesia, hypothermia, catalepsy and hypomotility (Fride & Mechoulam, 1993). Soon after discovery of anandamide in porcine brain (Devane et al., 1992), another endocannabinoid 2-arachidonyl glycerol (2-AG) (Figure 1-10) was

isolated from rat brain (Sugiura et al., 1995). Mechoulam et al. (1995) also reported the isolation of 2-AG from canine gut. 2-AG has been reported in both vertebrate and invertebrate tissues and interestingly 2-AG binds to both CB_1 and CB_2 receptors (Stefano et al., 2000; Gonsiorek et al., 2000). Another endocannabinoid, 2-arachidonyl glyceryl ether (noladin ether) (Figure 1-10) was then isolated from porcine brain (Hanus et al., 2001). Hanus et al. (2001) described the binding of 2-arachidonyl glyceryl ether (2-AGE) to CB_1 receptors in mouse brain and linked this with symptoms of sedation, hypothermia, intestinal immobility and mild antinociception. Interestingly, 2-AGE also has the ability to bind weakly at the CB_2 receptor (Hanus et al., 2001). Porter et al. (2002) reported another endocannabinoid virodhamine (Figure 1-10) (which consists of arachidonic acid and ethanolamine joined by an ester linkage). Interestingly, virodhamine behaved as an antagonist at the CB_1 receptor and was a full agonist at the CB_2 receptor (Porter et al., 2002). Levels of virodhamine were found to be 2-9 times more than anandamide in peripheral tissues which express CB_2 receptors, which points towards affinity of virodhamine for CB_2 receptor (Porter et al., 2002). N–arachidonyl-dopamine (NADA) (Figure 1-10) acts as an agonist at vanilloid receptors and has also been shown to have some activity at CB_1 receptors (Huang et al., 2002).

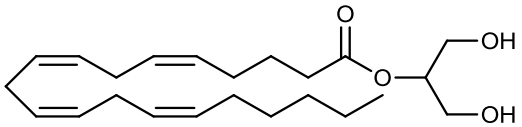
1.6.1 Levels of anandamide and 2-AG in the CNS

Different pharmacological effects of endocannabinoids are reliant upon the physiological presence of endocannabinoids in the CNS and therefore it is important to understand the processes responsible for their production and metabolism. In the previous section, I have mentioned several endocannabinoids that have been discovered, but there are strong lines of evidences to suggest that anandamide and 2-AG are the two main

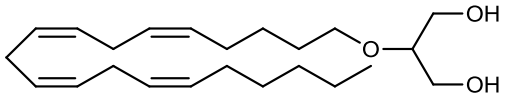
endocannabinoids. Understandably, most reports in the literature focus on their levels in the brain, their biosynthesis and inactivation processes (Gomez-Ruiz et al., 2007). Endocannabinoids differ a lot from other signalling molecules in that they are not stored inside synaptic vesicles but are synthesized on demand from their precursors. However similar to other neurotransmitters their action is terminated by deactivation or by metabolic breakdown (DiMarzo et al., 1994; Giuffrida et al., 2001; Gomez-Ruiz et al., 2007). The concentration of anandamide in sheep, pig and cow brain and post-mortem generation of anandamide has been measured by gas chromatography/mass spectrometry (Schmid et al., 1995). Anandamide was not detected in freshly extracted sheep brain, whereas in cow brain, the level was 4 ng/g and in pig brain it was 6 ng/g (Schmid et al., 1996). There was a significant increase in the level total *N*-arachidonyl ethanolamides (NAEs) but anandamide levels lagged in comparision to other NAEs when measured at an ambient temperature (Schmid et al., 1996). Anandamide levels measured by Devane et al. (1992) while trying to identify an endogenous cannabinoid were 0.13 mg/kg of tissue.



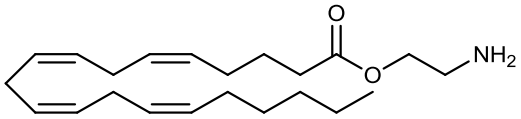
Anandamide



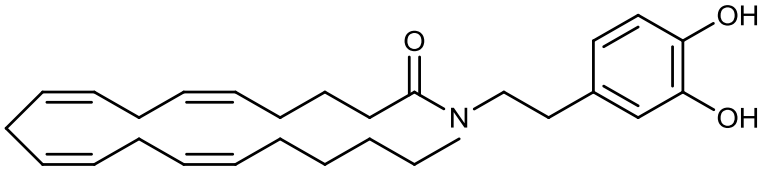
2-Arachidonylglycerol (2-AG)



2-Arachidonyl-glyceryl-ether (Noladin ether)



Virodhamine



Arachidonyl dopamine (NADA)

Figure1-10 Structures of important endocannabinoids. All structures were redrawn using ChemBioDraw Ultra and standard structures were previously described by Gomez-Ruiz et al. (2007), Hanus (2009), Mechoulam & Hanus (2000) and Piomelli (2003).

1.6.2 Biosynthesis of anandamide

After the discovery of anandamide, the putative endogenous ligand for central cannabinoid receptors by Devane et al. (1992), studies were directed towards exploring the biochemical processes responsible for its biosynthesis. In the 1960s, formation of *N*-acylethanolamines from free fatty acids and ethanolamine was reported, although there was no mention of arachidonic acid (Bachur and Udenfrie., 1966; Colodzin et al., 1963). Some time after this, the enzymatic formation of anandamide from arachidonic acid and ethanolamine via anandamide synthase was described (Deutsch and Chin, 1993). The role of phenylmethylsulfonylchloride (PMSF) as a potent inhibitor of amidases (anandamide amidase) involved in the degradation of anandamide, was also reported in this early work (Deutsch & Chin, 1993). Ueda et al. (1995), partially purified the amido-hydrolase involved in the breakdown of anandamide and demonstrated its ability to catalyze the biosynthesis of anandamide from arachidonic acid and a high concentration of ethanolamine. Based on a number of parameters investigated by Ueda et al. (1995), it is the same type of enzyme that participates in the synthesis and as well as the degradation of anandamide. It is now established that this condensation reaction is catalysed predominantly by fatty acid amide hydrolase (FAAH) operating in reverse fashion (Ueda et al., 1995). Anandamide was also reported to be synthesized in rabbit brain from free arachidonic acid and ethanolamine (Kruszka & Grossi, 1994). This synthesis of anandamide in rabbit brain was heat labile and independent of calcium, ATP and coenzyme A (Kruszka & Grossi, 1994). Formation of anandamide from ethanolamine and free arachidonic acid was also demonstrated in bovine brain (Devane & Axelrod, 1994). Devane & Axelrod (1994) incubated brain membranes with arachidonic acid and [^{14}C]ethanolamine and the main radioactive product had the same R_f value as pure

anandamide when assessed by thin layer chromatography. Considering the high concentration of ethanolamine and the reverse activity of FAAH required for this condensation reaction, this pathway may not be physiologically relevant for the biosynthesis of anandamide (Devane & Axelrod, 1994; Kruszka & Grossi, 1994; Sugiura et al., 1996).

In rat brain, neurons treated with the Ca^{2+} ionophore ionomycin or with various membrane depolarizing agents like high K^+, kainate and 4-aminopyridine (4-AP) biosynthesize anandamide (DiMarzo et al., 1994). Chelation of extracellular calcium with EGTA prevented the effect of high K^+, indicating that the biosynthesis of anandamide is Ca^{2+}-dependent (Dimarzo et al., 1994). Another biosynthetic route involving *N*-arachidonoyl-phosphatidylethanolamine (NAPEs) cleavage by phosphodiesterase was also proposed for the biosynthesis of anandamide and its precursors (DiMarzo et al., 1994). The mechanism proposed by DiMarzo et al. (1994), was explored by Sugiura et al. (1996) and they presented evidence that suggesting that this mechanism might be more relevant physiologically than the earlier possibility involving *N*-acylation of ethanolamine catalyzed by FAAH. According to the mechanism proposed by Sugiura et al. (1996) anandamide is released from a membrane lipid precursor *N*-arachidonyl-phosphatidylethanolamine (NAPE) by the action of a phospholipase D (PLD) (Figure 1-11). PLD has been cloned and has no homology previously characterized PLD enzymes (Okamoto et al., 2004). When overexpressed in COS-7, cells this novel phospholipase caused anandamide production (Okamoto et al., 2004). This enzyme has been confirmed in brain, heart and testis (Okamoto et al., 2004). Biosynthesis of NAPE is triggered by a Ca^{2+}-dependent *N*-acyl transferase (NAT), which integral to the membrane and transfers

the arachidonic acid group from the *sn*-1 position of phosphatidylcholine to the head group of phosphatidylethanolamine (DiMarzo et al., 1994; Sugiura et al., 1996). The activity of NAT is increased in the presence of the calcium ionophore ionomycin or depolarizing agents like veratradine. This enzyme is also regulated by cAMP, which activates by phosphorylation through cAMP-dependent protein kinase A (Cadas et al., 1996; de Fonseca et al., 2005).

1.6.3 Biosynthesis of 2-AG

The pathway for biosynthesis of 2-AG is different from that of anandamide since 2-AG is a monoglyceride. The concentration of 2-AG is approximately 200-fold greater than that of anandamide in the brain (Stella et al., 1997; Sugiura et al., 1995). 2-AG is also considered the main ligand for the peripheral CB_2 receptors (Sugiura et al., 2000). 2-AG can be generated by the action of phospholipase C (PLC) on phosphatidylinositol (PI) to generate 1,2 diacylglycerol (DAG) (Stella et al., 1997). 1,2 Diacylglycerol is then converted to 2-AG by diacylglycerol lipase (DGL). In cortical neurons, inhibition of PLC and DGL reduces Ca^{2+}-dependent accumulation of 2-AG, indicating involvement of this pathway in 2-AG biosynthesis (Stella et al., 1997).

An alternative mechanism proposed for 2-AG biosynthesis is the production of 2-arachidonyl-lysophospholipid by the action of phospholipase A1 (PLA1) on phosphatidylinositol. The 2-arachidonyl-lysophospholipid is then hydrolyzed by lyso-PLC to yield 2-AG. There is no evidence to support this mechanism, but considering there is a high level of PLA1 in brain, it might represent a possible alternative (Piomelli, 2003).

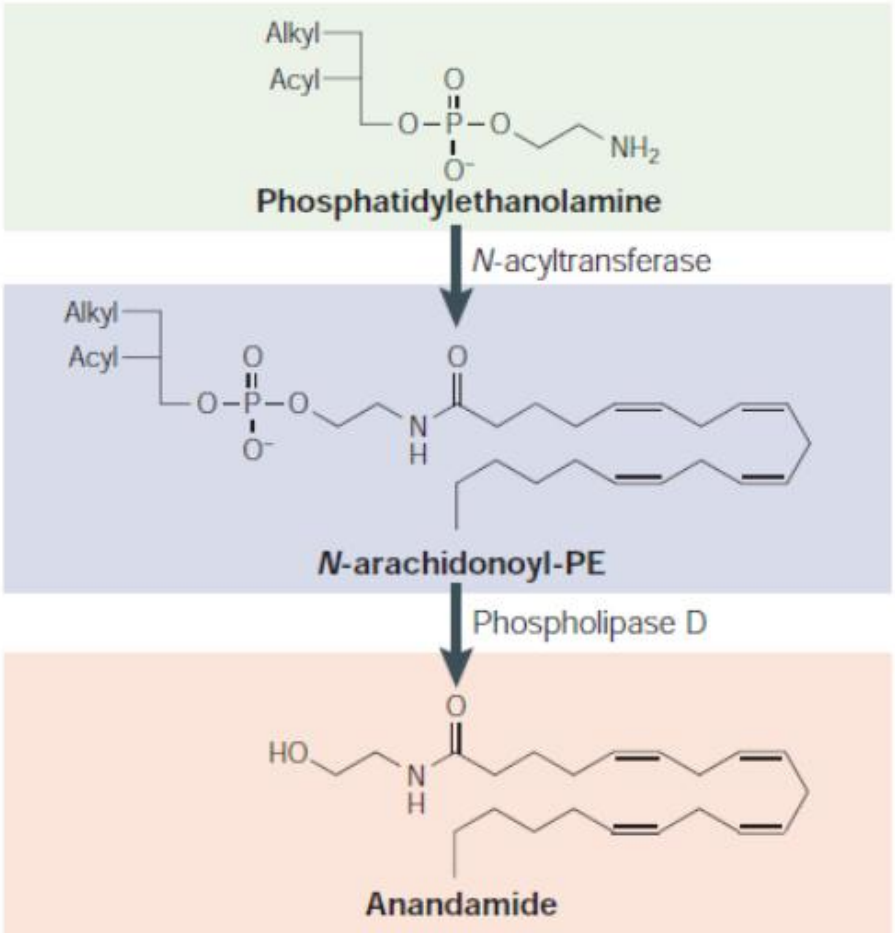


Figure 1-11 Biochemical pathway for the formation of anandamide in neurons. *N*-acyl transferase generates *N*-arachidonylphosphatidylethanolamine which is then cleaved by phospholipase D to generate anandamide (Piomelli, 2003).

1.6.4 Translocation of endocannabinoids to the CB_1 receptor

Classical transmitters and peptide neurotransmitters reach their target receptors by diffusion across the aqeous fluid of the synaptic cleft space. However, this mechanism cannot hold for endocannabinoids since they are hydrophobic molecules (Piomelli, 2003). One of the mechanisms suggested for the transport of endocannabinoids involves fast lateral movement through the membrane leaflet (Makriyannis et al., 2005; Xie et al., 1996). However, it has been shown that free anandamide can accumulate in brain interstitial fluids and incubation medium, which strongly indicates that anandamide, can resist being partitioned into the lipid environment (Piomelli, 2003). It is well-documented that endocannabinoids act as retrograde signalling molecules in the CNS and travel from the post-synaptic cell to the pre-synaptic cell and inhibit neurotransmitter release by activating CB_1 receptors (Kreitzer & Regehr, 2001b; Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001). Sideways movement of endocannabinoids through the plasmalemma is unlikely to fully explain this phenomenon (Piomelli, 2003). It has been suggested that endocannabinoids might reach their target receptors by binding to lipocallins (lipid binding proteins) which are expressed in high levels in the brain. It is also possible that as yet unidentified carriers might be involved in transporting endocannabinoids to their cellular targets (Piomelli, 2003).

1.6.5 Role of endocannabinoids in the CNS

CB_1 receptors are one of the most widely and highly expressed GPCRs in mammalian brain (Herkenham et al., 1990). Their presence in the brain cannot be justified solely on the basis of targets of the plant constituent Δ^9-THC since CB_1 receptors have been shown to mediate important regulatory functions in the CNS.

Endocannabinoids are released from the post-synaptic neurons and their release appears to be either activity-dependent or as a constant (basal) release (Kano et al., 2009). Two important physiological roles attributed to endocannabinoids in the CNS are depolarization-induced suppression of excitation (DSE) and depolarization-induced suppression of inhibition (DSI) whereby endocannabinoids suppress synaptic transmitter release. This suppression of neurotransmitter release can be temporary which is referred to as endocannabinoid-mediated short-term depression (eCB-STD), or persistent which is referred to as endocannabinoid-mediated long-term depression (eCB-LTD) (Kano et al., 2009). In the coming section I will discuss the retrograde signalling by endocannabinoids in the CNS and how this concept was established.

1.6.6 Retrograde signaling by the endocannabinoids in the CNS as a basis for DSI

In mammalian CNS, γ-aminobutyric acid (GABA) is an important inhibitory neurotransmitter (Pitler & Alger, 1992). An increase in intracellular calcium concentration $[Ca^{2+}]_i$ can reduce GABA-activated Cl^- conductance (Inoue et al., 1986). An increase in $[Ca^{2+}]_i$ up to 0.5 μM increased the run-down of the $GABA_A$ response, which was only suppressed and reversed when $[Ca^{2+}]_i$ was reduced to 0.01 μM (Chen et al., 1990). N-(6-Aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), a calmodulin inhibitor, reduced the effect of increased $[Ca^{2+}]_i$ on the $GABA_A$ response. Chen et al. (1990) have proposed that the increase in $[Ca^{2+}]_i$ concentration decreases the $GABA_A$ response most likely by activation of Ca^{2+}/calmodulin-dependent phosphatase. The spontaneous $GABA_A$ inhibitory post synaptic currents (IPSCs) were suppressed transiently in rat hippocampal CA1 pyramidal cells subjected to a series of action potentials, and this suppression was generally enhanced by the calcium channel agonist

(methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methylphenyl)-pyridine-5-carboxylate) (BAY K8644), and blocked by buffering with EGTA or 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) (Pitler & Alger, 1992). Depolarization of cerebellar Purkinje cells causes transient inhibition of inhibitory GABAergic synaptic currents and this inhibition was blocked after removal of extracellular Ca^{2+} from the bath solution (Llano et al., 1991). Llano et al. (1991), proposed a calcium-dependent diffusible messenger, which travels retogradely and mediates this decrease in synaptic current. This transient suppression in inhibitory GABAergic currents is termed as depolarization-induced suppression of inhibition (DSI) (Pitler & Alger, 1992). The experimental evidence provided by Pitler & Alger (1992) and Llano et al. (1991) suggests a calcium-dependent process mediating suppression of IPSCs. Glutamate was first implicated as a possible mediator of DSI but endocannabinoids were eventually demonstrated to be effective in mediating DSI (Kano et al., 2009). Important evidence in this regard comes from experiments with cultured hippocampal cells where WIN-55,212-2 (0.1 μM) suppressed IPSCs mediated by $GABA_A$ receptors a response blocked by AM251 and AM281 (Cosenza et al., 2000; Ohno-Shosaku et al., 2001). Moreover experiments using CA1 pyramidal neurons confirmed that WIN-55,212-2 suppressed evoked inhibitory post synaptic currents (eIPSCs) and this suppression was also blocked by CB_1 receptor antagonists (Wilson & Nicoll, 2001). AM404 (Figure 1-12) (an inhibitor of the anandamide/2-AG transport) decreased baseline amplitude of eIPSCs and this effect was also antagonized by CB_1 receptor antagonists AM251 or SR141716A (Wilson & Nicoll, 2001). AM404 does not affect the kinetics of DSI as indicated by Wilson & Nicoll (2001). These authors propose

that this might be due to signal transduction events inside the presynaptic terminal or due to diffusion of cannabinoids from their site of release (Wilson & Nicoll, 2001). Botulinum toxin E light chain (BTE) and botulinum toxin B (BTB) had no effect on DSI which further suggests that the retrograde signal does not require vesicular fusion process for endocannabinoids to be released. This evidence further strengthens the view that endocannabinoids are synthesized on demand and then travel retrogradely to the presynaptic site unlike other neurotransmitters, which are released in the synaptic cleft through a vesicular fusion process (Kano et al., 2009; Wilson & Nicoll, 2001; Wilson et al., 2001).

1.6.7 Involvement of the metabotropic glutamate receptors in mediating DSI

Activation of group 1 metabotropic glutamate receptors (mGluRs) also triggers the biosynthesis of endocannabinoids (Maejima et al., 2001; Wilson & Nicoll, 2002). In cerebellar Purkinje cells, activation of metabotropic glutamate receptor subtype 1 suppressed neurotransmitter release. This effect was blocked by the CB_1 receptor antagonist and mimicked by CB_1 receptor agonists (Maejima et al., 2001). However, experiments using the broad-spectrum metabotropic receptor antagonist LY341495 and $GABA_B$ receptor antagonist CGP55845 failed to block DSI in cerebellar Purkinje neurons, clearly suggesting non-involvement of these receptors in mediating DSI (Kreitzer & Regehr, 2001b). WIN-55,212-2 suppressed IPSCs and AM251 blocked DSI, indicating that CB_1 receptors may mediate DSI (Kreitzer & Regehr, 2001b). A time lag before maximal DSI is observed and a complete block of DSI by pertussis toxin in rat hippocampal pyramidal cells supports the idea that DSI is mediated by a $G_{i/o}$ (Pitler & Alger, 1994). Significantly, DSI is not observed in CB_1 receptor-knockout mice (Varma

et al., 2001; Wilson et al., 2001) and agonists of metabotropic glutamate receptors did not have any effect on IPSCs in CB_1 receptor knockout mice (Varma et al., 2001).

1.6.8 Retrograde signalling by the endocannabinoids in the CNS and DSE

Interestingly, in the cerebellum, depolarization of cerebellar Purkinje cells also inhibits parallel fiber and climbing fiber excitatory postsynaptic currents (EPSCs) for tens of seconds (Kreitzer & Regehr, 2001a). This phenomenon where the release of excitatory neurotransmitter glutamate is suppressed is termed depolarization-induced suppression of excitation (DSE) (Kreitzer & Regehr, 2001a). DSE was shown to have a presynaptic locus and like DSI, DSE can be blocked by buffering postsynaptic calcium with BAPTA (Kreitzer & Regehr, 2001a). DSE was blocked by AM251, mimicked by WIN-55,212-2 and was unaffected by antagonists of the metabotropic glutamate receptors, $GABA_B$, and adenosine A1 receptors. These data further suggested that DSE, like DSI, is mediated by endocannabinoids through a Ca^{2+}-dependent mechanism and this argues strongly against the earlier view that glutamate might be the retrograde messenger (Kreitzer & Regehr, 2001a).

1.6.9 Reuptake of endocannabinoids after interacting with the CB_1 receptor

Endocannabinoids like anandamide and 2-AG have are known to be degraded by intracellular enzymes but precisely how endocannabinoids are transported from the extracellular side to the intracellular compartment is not fully understood (Piomelli, 2003). Accumulation of [^{3}H]anandamide inside neurons and astrocytes was reported to be inhibited by AM404 and this suggested a carrier-mediated transport mechanism (Beltramo et al., 1997). Accumulation of [^{3}H]anandamide inside astrocytes was Na^+

gradient- and energy-independent, inhibited by unlabelled anandamide and not affected by inhibiting FAAH (Beltramo et al., 1997). This latter result indicates that hydrolysis of anandamide is not the driving force behind anandamide transport (Beltramo et al., 1997). Accumulation of [^{3}H]anandamide inside astrocytes was also unaffected by the presence of cannabinoid agonists and antagonists suggesting that receptor internalization cannot be the mechanism for endocannabinoid transport (Beltramo et al., 1997). Other reports reinforce the idea that FAAH activity is not involved in anandamide transport, although Piomelli (2003) suggested that it would be prudent to examine this mechanism using FAAH knock-out mice. Glaser et al. (2003) reported that AM404 failed to block anandamide transport when observed at 40 sec and also that AM404 inhibited FAAH *in vitro*. Glaser et al. (2003) concluded that anandamide transport is a simple diffusion process governed by the downstream metabolic processes and ruled out a specific anandamide transport carrier.

1.6.10 Metabolic processes and synthetic compounds degrading endocannabinoids

The fatty acid primary amide, *cis*-9-octadecenoamide (oleamide) was isolated from the cerebrospinal fluid of sleep-deprived cats and administration of synthetic oleamide induced a sleep-like state in rats (Cravatt et al., 1995). Oleamide hydrolase (Patterson et al., 1996) which catalyzes the conversion of oleamide to the inactive acid oleic acid (Cravatt et al., 1995), also biotransforms anandamide to arachidonic acid (Cravatt et al., 1996). Oleamide hydrolase was renamed fatty acid amide hydrolase (FAAH) on the basis of the variety of structurally different fatty acid amides it can hydrolyze (Cravatt et al., 1996). FAAH is an intracellular membrane bound serine hydrolase that is intimately involved in the breakdown of anandamide (Cravatt et al.,

1996; Ueda et al., 1995). COS-7 cells expressing anandamide amidohydrolase (the enzyme responsible for the hydrolysis of anandamide and oleamide) were four times faster in hydrolyzing 2-AG compared to anandamide. Anandamide inhibited [^{14}C]2-AG hydrolysis in a concentration-dependent manner (Goparaju et al., 1998). The results of Goparaju et al. (1998) indicate that FAAH can hydrolyze both fatty acid amides as well as monoacylglycerols (MAGs). It is important to note that FAAH does have the ability to hydrolyse monoacyl glycerol substrates such as 2-AG but FAAH is no longer considered as the main metabolizing enzyme for 2-AG (Dinh et al., 2002; Lichtman et al., 2002; Saario et al., 2004). Considering reports that demonstrate the non-selective nature of FAAH, it could be hypothesized that several fatty acid amides and MAGs might act as competitors for the active site of FAAH and thereby serve as regulators of endogenous levels of endocannabinoids. Oleamide does not bind strongly to CB_1 receptor (Boring et al., 1996) but it does display cannabinoid type behavioural effects in mice (Mechoulam et al., 1997). When anandamide and oleamide were administered together, the combination significantly decreased the ED_{50} in various cannabinoid behavioural tests as compared to when anandamide was administered alone. Also, in mouse neuroblastoma N18TG2 cells, oleamide was shown to inhibit FAAH-mediated [^{14}C]anandamide hydrolysis (Mechoulam et al., 1997). Display of cannabinoid type behavioral effects by oleamide might be due to its competition with anandamide for FAAH, thereby it increasing the level of anandamide, which could explain the lower ED_{50} observed for the oleamide and anandamide combination (Mechoulam et al., 1997). This phenomemon has been named as “the entourage effect” (Mechoulam et al., 1998). Inhibition of [^{3}H]CP-55,940 binding by various cannabinoids was investigated in $FAAH^{-/-}$ and $FAAH^{+/+}$ mice and these results

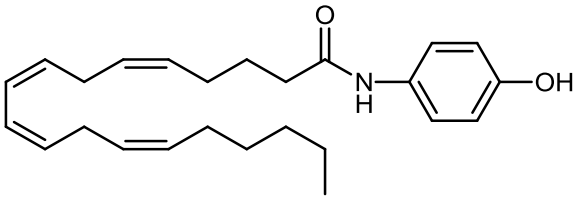
further empasize the important role of FAAH in regulating the levels of anandamide in CNS (Lichtman et al., 2002).

Deletion of the FAAH gene has no effect on levels of 2-AG hydrolysis pointing towards lack of involvement of FAAH in breakdown of 2-AG (Lichtman et al., 2002). 2-AG is believed to be hydrolysed predominantly by a serine hydrolase called monoacylglycerol lipase (MGL) and this activity is distinct from FAAH (Goparaju et al., 1999; Saario et al., 2004). Recently MGL has been cloned from rat brain and its mRNA found to be widely distributed in the very regions where CB_1 receptors are also present in high density (Dinh et al., 2002). Immunohistochemical experiments have suggested a presynaptic location for MGL compared as to FAAH which has postsynaptic location (Dinh et al., 2002). The presynaptic location of MGL also suggests that MGL is very important in terminating retrograde signalling events by 2-AG (Piomelli, 2003) and MGL is now widely accepted as the main enzyme responsible for inactivating 2-AG actions (Dinh et al., 2002).

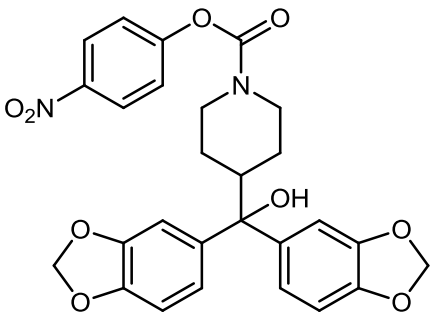
DSE (Kreitzer & Regehr, 2001a) and DSI (Pitler & Alger, 1992; Pitler & Alger, 1994) mediated by endocannabinoids are heavily reliant upon the enzymatic machinery regulating metabolism of endocannabinoids. The effect on magnitude and duration of DSI and DSE by various enzyme inhibitors has been studied by several researchers (Kim & Alger, 2004; Makara et al., 2005). COX-2 enzymes have been shown to oxidize 2-AG with high selectivity, which points to the role of 2-AG as a natural substrate for COX-2 (Kozak et al., 2000). Recombinant human cyclooxygenase-2 (hCOX-2) was shown to metabolize anandamide whereas recombinant human cyclooxygenase-1(hCOX-1) was inactive (Yu et al., 1997). As predicted, the selective COX-2 inhibitors nimesulide and

meloxicam increased the timecourse and magnitude of DSI in rat hippocampal slices (Kim & Alger, 2004). Moreover, AM251 treatment abolished DSI before and after application of nimesulide indicating involvement of the CB_1 receptor in COX-2-mediated modulation of DSI (Kim & Alger, 2004).

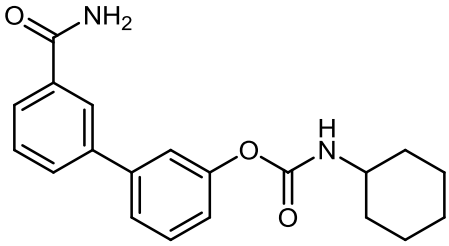
When anandamide is applied alone to hippocampal slices, it has no substantial depressant effect on the amplitude of IPSCs but when combined with cyclohexyl carbamic acid 3'-carbomoylbiphenyl-3-yl ester (URB597; Figure 1-12), a selective and highly potent FAAH inhibitor (Kathuria et al., 2003) it markedly depressed IPSCs effectively (Kim & Alger, 2004). The selective MAGL inhibitor 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184) (Long et al., 2009) and the non-selective MAGL inhibitor methyl arachidonyl fluorophosphonate (MAFP) also enhanced DSI/DSE (Pan et al., 2009) (Figure 1-12). The depressant effect of 2-AG on IPSCs was not altered by URB-597, indicating high specificity of URB-597 for FAAH (Kim & Alger, 2004). These results suggest that synthetic inhibitors of FAAH and MGL activity can promote the levels of anandamide/2-AG in the CNS and in doing so they can alter the intensity of DSI/DSE. The possibility of regulating the levels of the endocannabinoids without directly targeting CB_1 receptor may offer a novel therapeutic strategy for upregulating the endocannabinoid system.



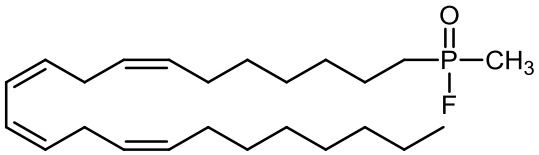
AM404



URB-597



JZL184



MAFP

Figure 1-12 Chemical structures of endocannabinoid deactivation inhibitors. AM404 (anandamide/2-AG transport inhibitor) (Beltramo et al., 1997), URB-597 (FAAH inhibitor) (Kathuria et al., 2003), JZL184 (highly selective and potent MAGL inhibitor) (Long et al., 2009) and MAFP (non-selective MAGL inhibitor) (Deutsch et al., 1997; Pan et al., 2009). All structures were redrawn using ChemBioDraw Ultra by referring to those reported in Giuffrida et al. (2001), Long et al. (2009), Piomelli (2003) and Deutsch et al. (1997).

1.7 Regulation of Adenylate Cyclase Activity and cAMP Levels by the CB_1 Receptor

Adenylyl cyclases are almost exclusively membrane bound integral proteins although non-membrane bound adenylyl cyclase has been reported in mammalian sperm regulated by bicarbonate ions (Chen et al., 2000). Adenylyl cyclase catalyzes the production of cAMP from adenosine triphosphate (ATP). cAMP is an important secondary messenger which affects a variety of biological processes. Cloning of known adenylyl cyclases has revealed that they consist of a large polypeptide (1080-1248 amino acids) of complex 3D structure (Cooper et al., 1995). According to the predicted model, segments of this enzyme cross the plasma membrane twelve times in two separate bundles (six transmembrane segments in each bundle) (Cooper et al., 1995). In addition, there is a discrete cytosolic component associated with each bundle which is where ATP binds (Cooper et al., 1995). Howlett & Fleming (1984) showed inhibition of forskolin-enhanced adenylate cyclase activity in plasma membranes from N18TG2 clone of neuroblastoma cells by Δ^8-THC, Δ^9-THC, levonantradol and desacetyllevonantradol. The biologically inactive stereoisomer of levonantradol was much less potent, which points towards this pharmacological effect being mediated through the CB_1 receptor (Howlett & Fleming, 1984). Howlett et al. (1986) showed that inhibition of adenylate cyclase in the presence or absence of forskolin by cannabinoids did not occur in the soluble form of adenylate cyclase from rat sperm, nor did it occur in C6 glioma or S49 lymphoma cell membrane-localized adenylate cyclase. The specific inhibition of membrane bound adenylate cyclase by cannabinoids further pointed towards inhibition of adenylate cyclase through a specific cannabinoid receptor (Howlett et al., 1986). Howlett et al. (1986) also

showed that pertussis toxin attenuates the inhibition of secretin-stimulated cAMP levels by cannabinoids, indicating involvement of G_i in cannabinoid-induced inhibition of adenylate cyclase. In rat brain slices, desacetyllevonantradol reduced forskolin-stimulated cAMP levels in the hippocampus, the frontal cortex and the striatum but a biphasic effect was seen in the cerebellum (Bidautrussell et al., 1990). Anandamide inhibited forskolin-stimulated cAMP levels in Chinese hamster ovary (CHO) cells transfected with CB_1 receptor and the decrease in cAMP levels was inhibited by pertussis toxin (Felder et al., 1993). Moreover, 2-AG reduced forskolin-stimulated cAMP levels and this was attenuated by both SR141716A and pertussis toxin (Stella et al., 1997). Based on the available evidence, it can be concluded that upon CB_1 receptor stimulation, adenylate cyclase activity is inhibited in a pertussis toxin-sensitive manner and this inhibition of adenylate cyclase activity can be blocked by CB_1 receptor antagonists.

1.8 Regulation of Ion Channels by the CB_1 Receptor

1.8.1 Voltage-gated sodium channels (VGSCs)

Different cannabinoids (endocannabinoids and synthetic cannabinoids) have been reported from our laboratory to block VGSCs and in doing so they function in a CB_1 receptor independent fashion. The effect of cannabinoids on VGSCs is reviewed in detail in the sections which explain the rationale behind my project, and also in Chapter 2. In this section a brief overview of VGSCs and the toxins that target different regions on VGSCs is provided. VGSCs are integral membrane proteins, which are responsible for the rapid rising phase of action potential (Catterall et al., 2007). During this phase, rapid influx of Na^+ takes place through VGSCs which forces the membrane potential from negative to positive (Catterall et al., 2007). VGSCs are composed of a major subunit (α)

and other auxillary subunits (β) (Catterall et al., 2007; Catterall, 2010). The α-subunit is sufficient to form a pore by itself (Catterall et al., 2007). The α-subunit is made of four homologous domains (I-IV) and each domain is composed of six transmembrane (S1-S6) segments connected together (Catterall et al., 2007). The extracellular loop connecting the fifth and sixth transmembrane segment in each domain enters the plasma membrane and this portion forms the narrow extracellular side of the pore whereas the sixth segment forms the broader intracellular side of the pore (Catterall et al., 2007; Catterall, 2010). Site 1 on VGSCs is targeted by tetrodotoxin (TTX), saxitoxin (STX) and μ-conotoxins (Narahashi et al., 1964; Hille, 1968; Hille, 1975; Ritchie and Rogart, 1977; Noda et al., 1989; Catterall et al, 2007). Site 1 lies in the pore loop region which is thought to provide ion selectivity and this logically explains why site 1 toxins block Na^+ conductance (Noda et al., 1989; Terlau et al., 1991; Heinemann et al., 1992). Site 2 toxins including batarachotoxin, veratridine, grayanotoxin and aconitine act allosterically on a site distinct from pore or voltage sensing regions and cause continuous activation, block inactivation of the channel(Catterall, 1977; Catterall et al., 2007). Batrachotoxin has been reported to target the sixth transmembrane segment (S6) in domain I of the rat sodium channel α-subunit whereas grayanotoxin activity was completely blocked by a mutation in the sixth transmembrane segment (S6) in domain IV of the α-subunit (Trainer et al., 1996; Kimura et al., 2000). Receptor site five on VGSCs is targeted by toxins like brevotoxin and ciguatoxin (Catterall et al., 2007). Receptor site five toxins, like receptor site 2 toxins, allosterically target VGSCs and mediate their inhibitory effects by causing increased activation and blocking inactivation of VGSCs (Catterall and Risk, 1981; Huang et al., 1984; Lombet et al., 1987; Catterall et al., 2007). Receptor site 3 on VGSCs is targeted

by polypeptide toxins like α-scorpion toxins and sea anemone toxins (Catterall et al., 2007). These toxins slow down or inhibit the inactivation of VGSCs (Catterall, 1979; Catterall and Beress, 1978; Catterall et al., 2007). Receptor site 6 on VGSCs is targeted by δ-conotoxins and these toxins also slow down the inactivation of sodium channels like receptor site 3 toxins (Fainzilber et al., 1994; Leipold et al., 2005; Catterall et al., 2007). Receptor site four on VGSCs is targeted by β-scorpion toxins and more recently a spider toxin has been reported to interact with this site (Corzo et al., 2003; Catterall et al., 2007). β-scorpion toxins have been reported to induce increased activation of sodium channels (Cestele et al., 1998; Catterall et al., 2007).

1.8.2 Voltage-gated calcium channels (VGCCs)

Activation of the CB_1 receptor by cannabinoids has important implications for the gating of different types of VGCCs in the CNS (Caufield & Brown, 1992; Mackie and Hill, 1992; Mackie et al., 1993; Mackie et al., 1995; Cain and Snutch, 2011). This effect on VGCCs has been shown to play an important role in CB_1 receptor-mediated modulation of neurotransmitters in the CNS. In this section a brief description of different types of VGCCs affected by the activation of CB_1 receptors and the toxins which target them is presented. VGCCs are a family of integral membrane proteins that regulate Ca^{2+} ion influx and are expressed in almost all excitable cells, and in large number of non-excitable cells (Cain and Snutch, 2011; Catterall, 2000). Genetically-based pathophysiological conditions that affect the expression of the VGCCs can lead to chronic diseases (Cain and Snutch, 2011; Catterall et al., 2007). In addition to the role of VGCCs in modulating neuronal excitability, Ca^{2+} ion's play a vital role in various other physiological processes like muscle contraction and regulation of a pleothora of

intracellular events (Ertel et al., 2000; Catterall, 2000; Cain and Snutch, 2011). VGCCs are comprised of different subunits of which α1 subunit represents the major subunit (Ertel et al., 2000; Catterall et al., 2007; Cain and Snutch, 2011). The α1 subunit is composed for four domains (I-IV) and each domain comprises of six transmembrane α helices (S1-S6) and a intermembrane loop between S5 and S6 (Catterall, 2000; Catterall et al., 2007; Catterall and Few, 2008). The α1 subunit has regions that are directly involved in Ca^{2+} influx (the pore region comprises of S5, S6 with the intermembrane interposed) and the voltage-sensing region (S1 to S4). Many regions of VGCCs are targeted by secondary messengers, drugs and toxins (Ertel et al., 2000; Catterall, 2000; Catterall and Few, 2008; Catterall et al., 2007). Ten types of α1 subunits have been reported in vertebrates and they are placed in three families which distinguish themselves on the basis of their function and how they are regulated (Ertel et al., 2000; Catterall et al., 2007; Cain and Snutch, 2011). Ca^{2+} currents recorded in different cells have different physiological and pharmacological signatures and through various electrophysiological studies, calcium channels have been classified as L-type, N-type and P/Q type etc. (Catterall, 2000; Ertel et al., 2000). However, Ertel et al. (2000) have proposed a more systematic way of classifying VGCCs. For example, Ca_v (1.2) refers to Ca^{2+} being the principal ion, v in subscript refers to voltage being the main regulator, 1 refers to the subfamily of α subunit (presently there are 3 subfamilies) and 2 refers to the order of discovery of that α subunit in that subfamily (Ertel et al., 2000) .

The first family (Ca_v1.1 -1.4) of VGCCs mediates L-type Ca^{2+} currents and these are expressed in brain, heart, pituitatry glands, adrenal glands, kidney, retina, pancreas, cochlea and skeletal muscles (Ertel et al., 2000; Catterall et al., 2007). L-type Ca^{2+}

channels require a relatively strong depolarizing stimulus for activation and are blocked by dihydropyridines, phenylalkylamines, and benzothiazepines (Reutr et al., 1983; Ertel et al., 2000; Catterall et al., 2007). L-type calcium channels support currents of a relatively long lasting nature and are very important in muscle and endocrine cells where they are responsible for causing muscle contraction and hormone secretion (Nowycky et al., 1985; Ertel et al., 2000; Catterall, 2000; Catterall et al., 2007).

The second subfamily ($Ca_v2.1$-2.3) of VGCCs mediate N-type, P/Q-type and R-type Ca^{2+} currents and these are expressed in brain, peripheral nervous systems (PNS), cochlea, pituitary, heart and retina (Ertel et al., 2000; Catterall, 2000; Catterall et al., 2007). These channel types are largely responsible for regulating synaptic transmission in the CNS and the PNS and can be blocked by different polypeptide toxins (Catterall, 2000; Catterall et al., 2007). For example, N-type Ca^{2+} channels are sensitive to inhibition by cone snail peptide ω-conotoxin GVIA and P-type Ca^{2+} channels are blocked by spider toxin ω-agatoxin IVA. Q-type Ca^{2+} channels are less sensitive to ω-agatoxin IVA but both P- and Q- type VGCCs are potently inhibited by ω-conotoxin MVIIC. In contrast R-type Ca^{2+} channels are both insensitive to peptide toxins and dihydropyridines (McCleskey et al., 1987; Mintz et al., 1992; Randall and Tsien, 1995; Catterall, 2000; Catterall et al., 2007).

The third subfamily ($Ca_v3.1$-3.3) mediates T-type Ca^{2+} currents and these are expressed in brain, heart, kidney, liver, cochlea and retina (Ertel et al., 2000; Catterall, 2000; Catterall et al., 2007). T-type VGCCs are typically activated at low voltages (low depolarizing stimuli) for very short duration compared to L-type VGCCs and no specific blocker has been reported for T-type VGCCs (Carbone and Lux, 1984; Nowycky et al.,

1985; Catarall, 2000; Catarall et al., 2007). These channels are important for repetitive firing of neurons and impulse generation in the heart (Perez-Reyes, 2003).

1.8.3 Inhibition of voltage-gated calcium channels by cannabinoids

WIN55,212-2 inhibits ω-conotoxin-sensitive, high-voltage-gated calcium currents (I_{ca}) in the NG108-15 cell line (Mackie & Hille, 1992). Pertussis toxin treatment blocked the action of WIN55,212-2 but application of a non-hydrolysable cAMP analog and a phosphodiesterase inhibitor had no effect (Mackie & Hille, 1992). These results therefore point to the possible role of a GTP binding protein in mediating inhibition of N-type calcium channels through the cannabinoid receptor without involvement of cAMP (Mackie & Hille, 1992). Anandamide inhibits N-type I_{ca} in the N18 neuroblastoma cell line in a pertussis toxin-sensitive manner (Mackie et al., 1993). The Q-type I_{ca} was found to be inhibited by cannabinoid agonists in CB_1 transfected murine tumour line (AtT-20) and this inhibition like that of the N-type I_{ca} was voltage-dependent and pertussis-toxin sensitive (Mackie et al., 1995). L-type Ca^{2+} channels have also been reported to be inhibited by WIN-55,212-2 and anandamide in a pertussis toxin-sensitive manner in cat cerebellar vascular smooth muscles (Gebremedhin et al., 1999). In this preparation, inhibition of L-type Ca^{2+} channels by cannabinoids led to dilation of the preconstricted cerebral smooth muscles and this effect was antagonized by SR141716A. Based on their results, Gebremedhin et al. (1999) proposed that the CB_1 receptor along with endocannabinoids regulate cerebral arterial tone by interefering with the Ca^{2+} influx through L-type Ca^{2+} channels.

1.8.4 Regulation of potassium ion currents by cannabinoids

Voltage-gated potassium channels are important ion channels since they are involved in the outward movement of potassium ions, which helps the plasma membrane potential to return to a resting state (Catterall et al., 2007). Anandamide and WIN-55,212-2 act as full agonists in activating the inwardly rectifying potassium current (K-ir) through a pertussis toxin-sensitive G protein and inhibiting a high voltage activated calcium current (I-Ca^{2+}) in the CB_1 transfected murine tumour line AtT-20 (Mackie et al., 1995). Depolarization of cerebellar Purkinje cells reduced the firing rate of nearby interneurons and this reduction in firing was blocked by the CB_1 receptor antagonist AM251 (Kreitzer et al., 2002). This reduction in firing of interneurons was shown to be due to an increase in a K^+ current (Kreitzer et al., 2002). This finding is of particular significance as it shows that an endocannabinoid-mediated inhibitory effect is not always restricted to the presynaptic neuron but can also impact distant regions by inhibiting the firing of interneurons (Kreitzer et al., 2002). Inhibition of cAMP accumulation by cannabinoid agonists was also found to indirectly increase A-type potassium currents in hippocampal cells (Deadwyler et al., 1995). The majority of studies suggest a presynaptic location for the regulation of ion channels by the CB_1 receptor. However, WIN-55,212-2 and methandamide were shown to reduce a postsynaptic K^+ M-current (IM) in rat hippocampal slices and this reduction was reversed by SR141716A (Schweitzer, 2000). Thus, it is possible that both an indirect mechanism operating through the presynaptic cell and a direct mechanism through the postsynaptic cell can contribute to regulation of activity in certain regions of the brain.

1.9 Vesicular Exocytosis and Vesicular Recycling

Chemical synapses represent specialized sites for the interaction of pre-synaptic and post synaptic cells. Synaptic activity can vary considerably from slow neuropeptide containing synapse to fast synapse of the hippocampus. Electric stimuli triggers the release of neurotransmitters packed inside the synaptic vesicles from specialized sites in the presynaptic cell. The neurotransmitters released in the synaptic cleft then interact with different ion channels on the postsynaptic locus and this result in either propagation or dampening of the action potential. The delicate balance between exocytosis of neurotransmitters and vesicular recycling helps in maintaining synaptic activity at the synapse (Haucke et al., 2011). In the coming section, a brief description of vesicular exocytosis and vesicular recycling is provided.

Synaptic vesicles contain 1500–2000 neurotransmitter molecules (Haucke et al., 2011). The synaptic vesicular pool is thought to be of three functional types. First, one is the readily-releasable pool (RRP) which is released rapidly on stimulation but gets exhausted readily during extended synaptic activity. Second is the recycling pool of vesicles, which release their contents during moderate stimulation. Third is the reserve pool, which represents the major pool of synaptic vesicles. Depolarization of a neuron opens VGCCs and this triggers rapid influx of Ca^{2+} ions. Vesicular exocytosis depends upon formation of a complex between SNARE (N-ethylmaleimide-sensitive factor attachment protein receptor) proteins on vesicular and plasma membrane (Haucke et al., 2011). Two proteins, NEM sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPS) (Malhotra et al., 1988; Clary et al., 1990) are involved in synaptic vesicle fusion. These proteins interact with vesicle-attached SNARE (v-SNARE), such as

synaptobrevin, and target membrane SNAREs, syntaxin and SNAP-25. This leads to a formation of a macromolecular complex, which spans both membranes and provides energy for the fusion of vesicular membrane with plasma membrane. Synaptotagmin, another protein located on vesicular membrane acts as a Ca2+ sensor and causes vesicular exocytosis when Ca^{2+} binds to it (Haucke et al., 2011).

Two theories have been described for synaptic vesicular recycling (Heuser & Reese, 1973; Ceccarelli et al., 1972; Ceccarelli et al., 1973; Morgan et al., 2002). Heuser & Reese (1973) propose a coating mechanism whereby synaptic vesicles are first coated, passed through endosomes before they can be filled with neurotransmitters again. This process is slow (30 to 60 sec) and occurs at a distinct site away from the active zone where exocytosis burst took place (Heuser & Reese, 1973; Morgan et al., 2002).

Ceccarelli et al. (1972 & 1973) proposed another mechanism for vesicular recycling. This theory is more popularly called "Kiss and run-endocytosis" (Morgan et al., 2002). According to this mechanism synaptic vesicles release their contents and then pinch back without collapsing with the membrane and does not involve a coating mechanism (Ceccarelli et al., 1972; Ceccarelli et al., 1973; Morgan et al., 2002). Several reports have indicated that both of these recycling mechanisms might be operational in the nerve terminal (Morgan et al., 2002).

1.10 Rationale Behind My Research

In our laboratory anandamide was reported to 1) inhibit veratridine-dependent depolarization of synaptneurosomes 2) inhibit veratridine-dependent release of L-glutamate and GABA from synaptosomes 3) inhibit [^{3}H]batrachotoxinin A 20-alpha-

benzoate binding to the voltage-sensitive Na^+ channels and 4) inhibit TTX-sensitive repeated firing in cortical neurons (Nicholson et al., 2003). None of the effects of anandamide on VGSCs was reversed by AM251, suggesting a CB_1 receptor independent action of cannabinoids on VGSCs (Nicholson et al., 2003). Interestingly, AM251 on its own was found to have similar effects (Liao et al., 2004). In continuation of this line of research, synthetic compounds like CP-55,9440 and ethyl arachidonate were tested for their effect on [^{3}H]batrachotoxinin A 20-alpha-benzoate binding to site 2 on sodium channels and voltage-gated sodium channel- (VGSC-) dependent depolarization of nerve membranes (Duan et al., 2008b). CP-55,9440 was more potent then ethylarachidonate in both inhibiting binding of [^{3}H]batrachotoxinin A 20-alpha-benzoate and inhibition of veratridine-dependent depolarization of nerve membranes (Duan et al., 2008b). Interestingly AM251 failed to prevent effects of both CP-55,9440 and ethyl arachidonate again supporting the idea of a CB_1 receptor-independent effect (Duan et al., 2008b).

A number of endocannabinoids such as 2-AG, 2-AGE and NADA were then examined using the [^{3}H]batrachotoxinin A 20-alpha-benzoate binding assay (Duan et al., 2008a). 2-AG, 2-AGE and NADA significantly inhibited [^{3}H]batrachotoxinin A 20-alpha-benzoate binding to the sodium channel (Duan et al., 2008a). As before, AM251 was not able to attenuate effects of these endocannabinoids again pointing towards CB_1 receptor-independent effects (Duan et al., 2008a). Kinetic experiments, suggested an allosteric mechanism behind the inhibition of [^{3}H]batrachotoxinin A 20-alpha-benzoate binding by anandamide, 2-AG, 2-AGE and NADA (Duan et al., 2008a; Nicholson et al., 2003).

1.10.1 Hypothesis 1

Based on the results above we concluded that voltage-gated sodium channels have a binding region through which endocannabinoids may exert their modulatory effects. It is noteworthy that CB_1 receptor-independent inhibitory effects of anandamide and synthetic cannabinoids at voltage-gated sodium channels and transmitter release have since been confirmed by other groups (Kelley & Thayer, 2004; Kim et al., 2005).

In essence, if there are binding topographies for cannabinoid drugs on both CB_1 receptors and VGSCs then we hypothesized that there may exist VGSC-acting ligands that can engage with the CB_1 receptors.

In addition to VGSC-acting compounds that might also engage CB_1 receptor, we became interested in the possibility that other environmental chemicals that can access the CNS may have the ability to interact with CB_1 receptors. We addressed this hypothesis by testing a variety of other pesticides and related chemicals. Some were selected based on their structural resemblance with the known endocannabinoids particularly in view of a several prior reports which have implicated organophosphorus pesticides and related chemicals as inhibitors of [^{3}H]CP-55,940 binding (Deutsch et al., 1997; Martin et al., 2000; Quistad et al., 2002; Quistad et al., 2006). Many of the compounds that interfere with mitochondrial function have alkyl chain systems that could potentially mimic the alkyl chains of CP-55,940 or Δ^9-THC in their binding to a sub region of the CB_1 receptor. Also, tributyl tin (TBT) compounds have some similarity with organophosphorus compounds i.e. phosphorous center versus Sn center but most

importantly unlike organophosphorus compounds, would not be expected to inhibit (i.e."stanyllate") FAAH or MAGL.

1.10.2 First objective

In order to test whether various VGSC-acting compounds and other environmental chemicals can interfere with the dynamics of [^{3}H]CP-55,940 binding we employed the [^{3}H]CP-55,940 binding assay. It is a well established assay used to investigate potential ligands for their interaction with the CB_1 receptor (Deutsch et al., 1997; Dhopeshwarkar et al., 2011; Martin et al., 2000; Quistad et al., 2002). Interestingly, it was tritium labelled CP-55,940 that was developed originally by Devane et al. (1988) to confirm the existence of the CB_1 receptor in the brain and this ended the long existing argument about whether such a receptor existed. CP-55,940 is a high affinity cannabinoid agonist with a similar biological activity to Δ^9-THC and by labelling it with tritium to high specific activity, the pharmacology of the CB_1 receptor could be characterized and the density and distribution of receptors in the brain can be quantified (Devane et al., 1988; Herkenham et al., 1990; Howlett et al., 1988; Little et al., 1988). Using this assay I explored various VGSC-acting insecticides e.g. oxadiazine insecticides (MP062 and JT333), compounds that interfere with mitochondrial function e.g. (famoxadone, surangin B and coenzyme Q_2) and various tributyltin (TBT) compounds e.g. TBT-benzoate, TBT-acetate and phenylethynyl TBT.

1.10.3 Second objective

Our [^{3}H]CP-55,940 binding assay results supported our first hypothesis that there is likely a binding topography common to both the VGSCs and the CB_1 receptor.

However, whether these test chemicals acted as agonists or antagonist/inverse agonists at the CB_1 receptor was unknown. My second objective was therefore to classify all test compounds which were shown to be active in the [^{3}H]CP-55,940 binding assay as agonist or antagonist/inverse agoinists at the CB_1 receptor. This objective was achieved by employing the [^{35}S]GTPγS binding assay. All the test compounds were investigated for their ability to affect [^{35}S]GTPγS binding (basal binding) on their own and their ability to inhibit 100 nM CP-55,940-stimulated [^{35}S]GTPγS binding.

1.10.4 Third objective

After pharmacologically characterizing test chemicals as agonists or antagonist/inverse agonists at the CB_1 receptor, I was interested in investigating the effect of cannabinoids in the exocytosis assay using synaptosomes as determined by acridine orange (AO) release. One would predict that depolarization-induced release of AO should be inhibited by cannabinoid (CB_1) agonists and that this inhibition may be counteracted by CB_1 antagonists or inverse agonists. Such an assay should have value as a non-radioactive functional assay in the characterization of synaptic actions of CB_1 receptor agonists and antagonists including environmental chemicals. This objective and the experimental approach employed are explained in more detail in Chapter 3.

1.11 Pharmacological Profile of Test Chemicals used in my Research

The pharmacological profile of the main study compounds investigated in my research is provided below. The primary modes of action test chemicals such as dihydropyrazole insecticides (Figure 1-15), pyrethroid insecticides (Figure 1-14), TBT

compounds (Figure 1-15) and benzophenanthridine alkaloids (Figure 1-16) are described in detail in Chapter 2.

1.11.1 Oxadiazine insecticides

In accordance with our line of thought I tested the oxadiazine insecticide indoxacarb (MP062) and its decarbomethoxylated derivative (JT333) known to inhibit VGSC function in insects (Wing et al., 1998; Lapied et al., 2001; McCann et al., 2001) for their potential to bind to CB_1 receptors using the [^{3}H]CP-55,940 binding assay and further functionally characterized them using the [^{35}S]GTPγS binding assay (for oxadiazine structures see Figure 1-15). The decarbomethoxylated metabolite of indoxacarb (JT333) has been shown to be the main metabolite responsible for the insecticidal action (Wing et al., 1998; Lapied et al., 2001). Indoxacarb is the first commercialized pyrazoline type insecticide developed to counter resistance to organophosphorus, carbamate and pyrethroid insecticides (McCann et al., 2001; Zhao et al., 2003). MP062 and JT333 also act on sodium channels in mammalian nervous system (Tsurubuchi et al., 2001; Zhao et al., 2003). However, in mammalian neurons JT333 was more effective in blocking sodium currents than its parent compound indoxacarb (Tsurubuchi et al., 2001). The effect of both JT333 and MP062 on tetrodotoxin- (TTX-) sensitive and TTX-resistant VGSCs was compared in rat dorsal root ganglion neurons (Zhao et al., 2003). JT333 was again more effective than its parent compound indoxacarb in inhibiting the TTX-sensitive and TTX-resistant component with both compounds having higher inhibitory action on TTX-sensitive VGSCs as compared to TTX-resistant VGSCs (Zhao et al., 2003). The affinity of MP062 and JT333 for the inactivated state of sodium channels and their ability to cause voltage-dependent block was similar to the

mechanism described for dihydropyrazole insecticides (Salgado, 1992) and this seems logical because the oxadiazine class of insecticides was derived from the structurally similar dihydropyrazole class of insecticides (McCann et al., 2001; Zhao et al., 2003)

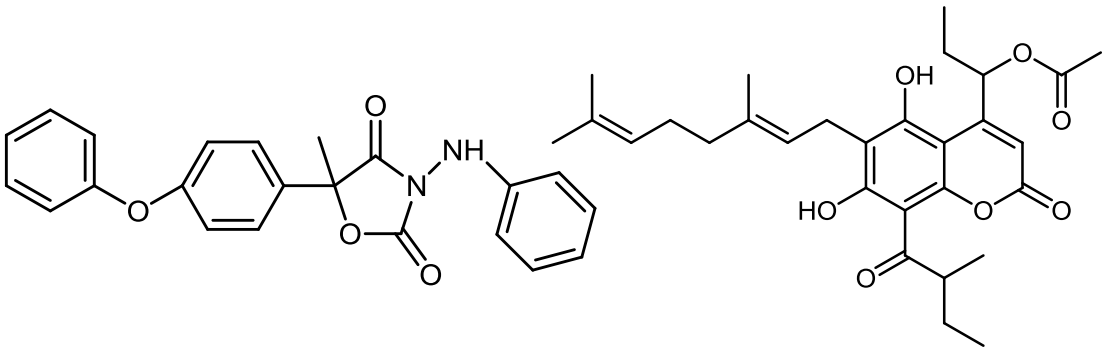
1.11.2 Componds interfering with mitochondrial function

Famoxadone is an oxazolidinone fungicide, which achieves its fungicidal action by targeting the Q_O binding domain of the mitochondrial bc_1 complex. This mechanism is similar to that through which myxothiazol acts (Jordan et al., 1999; Pember et al., 2005). Famoxadone has a broad activity against fungal pathogens of plants with low mammalian toxicity and minimal environmental impact (Sternberg et al., 2001). Surangin B is a 4-(1-acetoxypropyl) coumarin obtained from *Mammea longifolia* and it also possesses bactericidal properties (Joshi et al., 1969). In our laboratory, surangin B was found to have insecticidal properties, antifungal properties and also neurotoxic potential since it stimulated the release of endogenous amino acids from synaptosomes by interfering with mitochondrial function. This latter effect was found to be mainly due to blockade of electron transport chain at Q_i site of bc_1 complex in bovine heart mitochondria (Deng & Nicholson, 2003; Deng & Nicholson, 2005; Nicholson & Zhang, 1995). Antimycin A is an antibiotic secreted by several species of *Streptomyces* that interferes with electron transfer in complex III and is well established as a selective Q_i site inhibitor (Kido and Spyhalski, 1950; Miyoshi et al., 1995). Antimycin A has insecticidal and antifungal activity (Kido and Spyhalski, 1950). Myxothiazol is another natural product antibiotic obtained from various species of *Streptomyces* (Gerth et al., 1980). Like famoxadone, myxothiazol achieves its antifungal properties by targeting the Q_o site of mitochondria1 complex III (Thierbach and Reichenbach, 1981). Oligomycin is also an antibiotic but this

substance targets the ATP synthase and through this action directly blocks production of ATP (Tzagoloff et al., 1968; Glaser and Norling, 1983). See Figure 1-13 for structures of mitochondrial function disrupting chemicals.

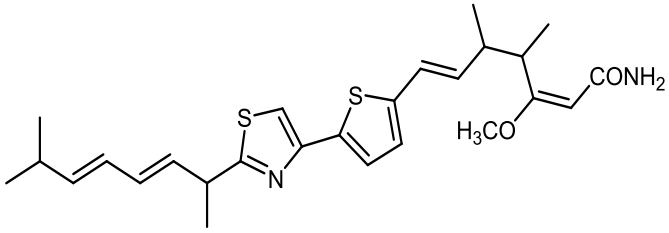
1.11.3 TBT compounds

TBT compounds are organic derivatives of tin (Okoro et al., 2011). TBT compounds are highly toxic to both target and non-target organism and their use as biocides in antifouling paints has been banned in several countries since 2008 (Qin et al., 2010; Oliveria and Stanelli, 2010; Okoro et al., 2011). TBT compounds are also used to protect wood as insecticidal and antifungal agents (Okoro et al., 2011). TBT compounds have been reported to induce female masculinization (Titley-O'Neal et al., 2011). In many aquatic species, exposure to TBT compounds has resulted in decreased cell viability and significant DNA damage (Hagger et al., 2005). Humans are also at risk of being exposed to TBT compounds either directly by contact or through the food chain. A study based in Canada showed presence of organotin compounds in drinking water samples collected from residential areas which had new polyvinylchloride (PVC) piping (Sadiki and Williams, 2009). Human liver samples have been reported to contain butyl tin derivatives (Takahashi et al., 1999). Detectable levels of organotin compounds have been found in several commonly used commodities like diapers, sanitary napkins, polyurethane gloves and baking parchments (Takahashi et al., 1999). The transfer of organotin tin compunds from baking parchment to the food revealed a significant route through which humans can be exposed (Takahashi et al., 1999).

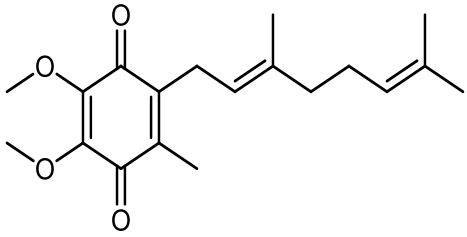


Famoxadone

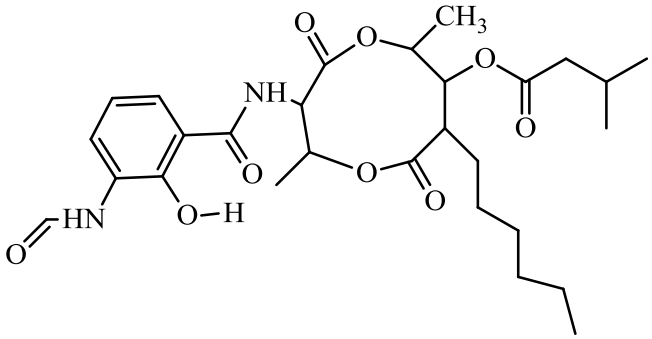
Surangin B



Myxothiazol A

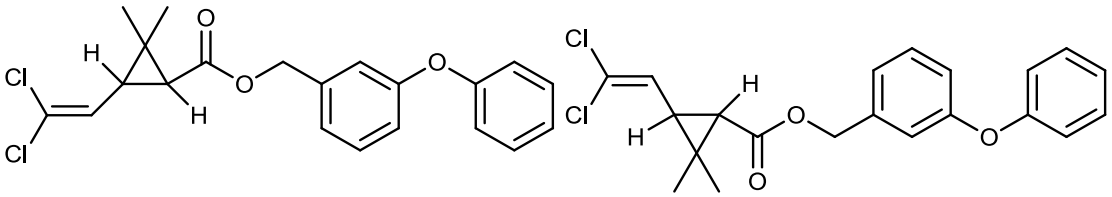


Coenzyme Q_2



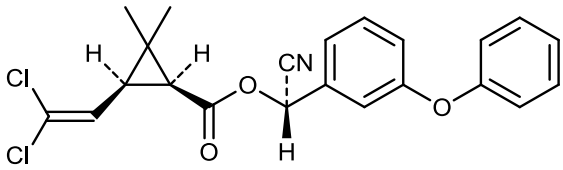
Antimycin A

Figure 1-13 Structures of mitochondrial function disrupting chemicals. All structures were redrawn using ChemBioDraw Ultra. Structures previously reported in Zhang et al. (2010).

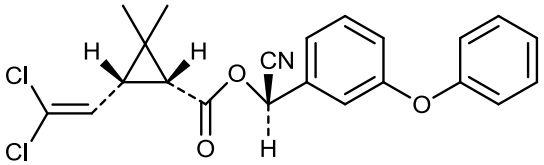


1 RS, *trans*-permethrin

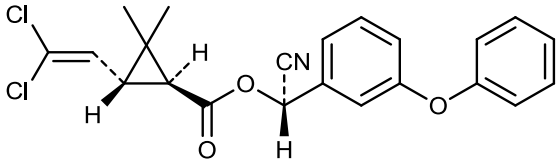
1RS, *cis*-permethrin



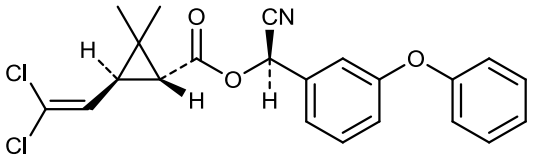
[1S, *cis*, αR] cypermethrin



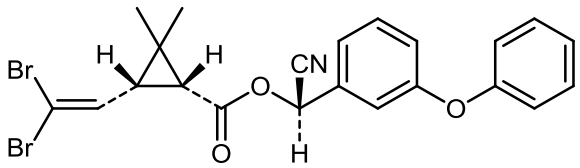
[1R, *cis*, αS] cypermethrin



[1S, *trans*, αR] cypermethrin

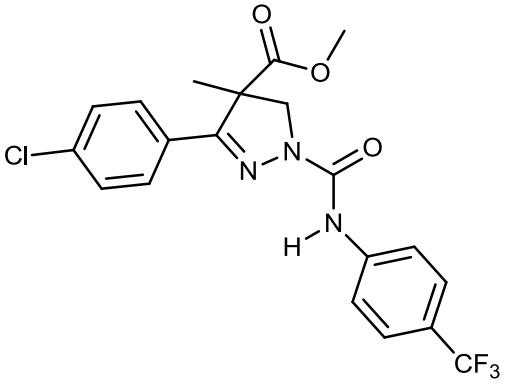


[1R, *trans*, αS] cypermethrin

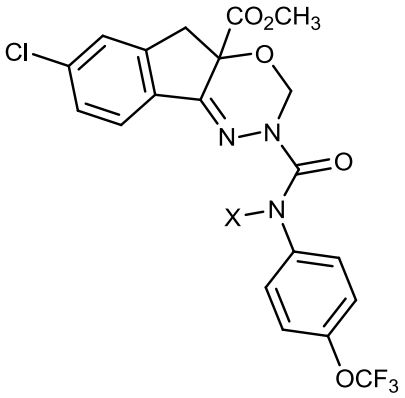


[1R, *cis*, αS] deltamethrin

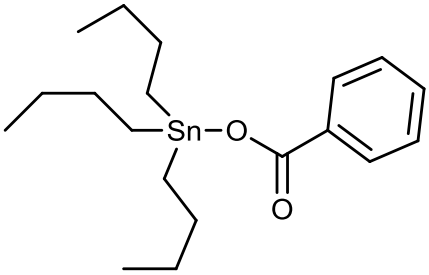
Figure 1-14 Structures of pyrethroid insecticides (α-cyano and non α-cyano) tested in the present investigation. All structures were redrawn using ChemBioDraw Ultra. Structures previously reported in Verschoyle & Aldridge (1980) and Vijverberg & Berken (1990).



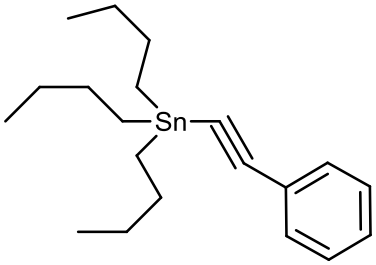
RH3421; RH5529 (CF_3 = H)



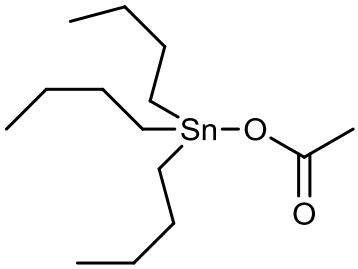
X = CO_2CH_3 (MP062); X = H (JT333)



TBT Benzoate



TBT Phenylethynyl tin



TBT Acetate

Figure 1-15 Structures of dihydropyrazole insecticides, oxadiazine insecticides and TBT compounds used in the present investigation. All structures were redrawn using ChemBioDraw Ultra. Structures previously reported in Zhao et al. (2003) and McCann et al. (2001).

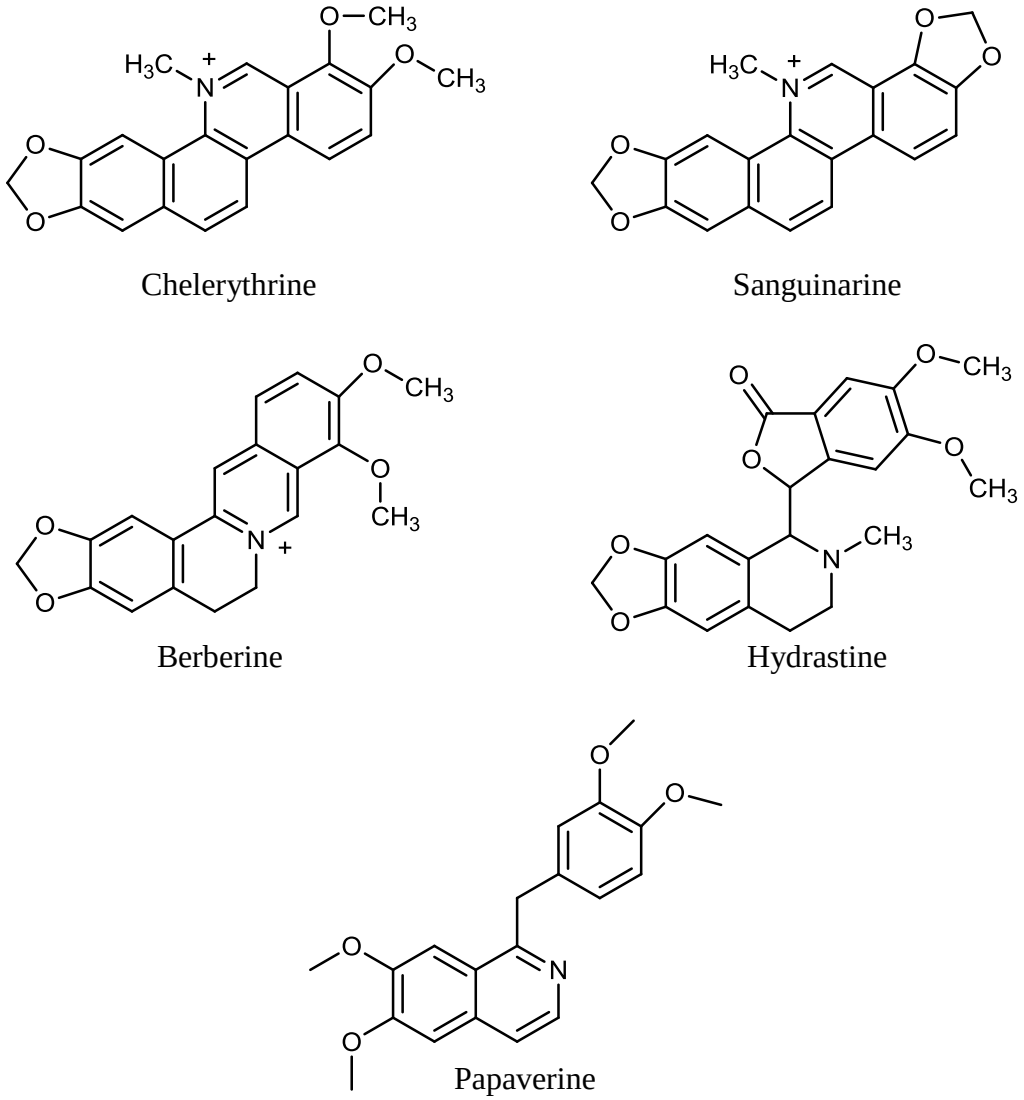


Figure 1-16 Structures of benzophenathridine, isoquinoline and phthalide isoquinoline alkaloids tested in the present investigation. All structures were redrawn using ChemBioDraw Ultra. Structures previously described by Dhopeshwarkar et al. (2011).

2: Characterization of environmental chemicals action at the mammalian cannabinoid-1 (CB_1) receptor using radioligand binding

2.1 Abstract

Cannabinoid-1 (CB_1) receptor coupled G-protein complexes are hetrogeneosly expressed in high densities in the mammalian central nervous system (CNS). The activation of this receptor complex by CB_1 receptor agonists modulates cAMP levels, gating of voltage-gated Ca^{2+} channels (VGCCs) and activation of inwardly rectifying K^+ channels in the cells that express them. The endocannabinoid system is also a target for environmental chemicals like organophosphorus pesticides, alkylfluorophosphonates, benzodioxaphosphorin oxides and alkanesulfonyl fluorides etc. The present investigation indicates the presence of binding topographies common to both voltage-sensitive sodium channels (VGSCs) and the CB_1 receptor, since several members of the VGSC-targeting insecticidal classes including pyrethroids, oxadiazines and dihydropyrazoles interfered with [^{3}H]CP-55,940 binding to the CB_1 receptor. Three other classes of environmental chemicals including mitochondrial function disrupting chemicals, tributyl tin (TBT) compounds and benzophenathridine alkaloids were found to bind to CB_1 receptors. The functional significance of the interaction of study compounds with the CB_1 receptor was also investigated using the [^{35}S]GTPγS binding assay. The majority of the study compounds were found to have antagonistic activity at the CB_1 receptor barring a few

like chelerythrine and sanguinarine which displayed inverse agonist-like activity. Our results suggest that the study compounds are able to engage with the CB_1 receptor *in vitro* and this action does not involve FAAH/MAGL. This investigation offers a new perspective with which to consider how the study compounds might act in the brain and the possibility that certain of these compounds may be suitable as templates for development of novel CB_1 receptor antagonists which have therapeutic potential.

2.2 Introduction

The cannabinoid-1 (CB_1) receptor, the main target of the pyschoactive phytocannabinoid Δ^9-THC (Gaoni & Mechoulam, 1964) is a G protein-coupled receptor (Matsuda et al., 1990) that is heterogeneously distributed in different regions of mammalian brain with high densities occurring in the cerebellum, the hippocampus, the basal ganglia and the cortex (Herkenham et al., 1990; Tsou et al., 1998). The other main subtype of cannabinoid receptors is the cannabinoid-2 receptor (CB_2), another G protein-coupled receptor located primarily in immune tissues although a few reports indicate its presence at low levels in the brain (Kaminski et al., 1992; Munro et al., 1993; Benito et al., 2008). Various endogenous ligands like anadamide (Devane et al., 1992), 2-arachidonyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), 2-arachidonyl glyceryl ether (2-AGE) (Hanus et al., 2001), virodhamine (Porter et al., 2002) and N–arachidonyl-dopamine (NADA) (Huang et al., 2002) have been reported to act at the CB_1 receptor although the weight of evidence suggests that anandamide and 2-AG are the main endogenous ligands regulating the cannabinoid system. Anandamide, 2-AG and 2-AGE have been characterized as agonists at the CB_1 receptor and *in vivo* administration of these endocannabinoids results in *in vivo* symptomology similar to that

seen with Δ^9-THC, such as analgesia, hypothermia, hypomotility and catalepsy (Fride & Mechoulam, 1993; Hanus et al., 2001; Martin et al., 1991; Mechoulam et al., 1995; Smith et al., 1994). In addition to endogenous agonists like anandamide and 2-AG acting at the the CB_1 receptor, synthetic compounds elicit agonist effects at the CB_1 receptor. These include non-classical cannabinoids such as the synthetic bicyclic compounds CP-55940 and CP-47,947 etc. (Compton et al., 1992a; Devane et al., 1988; Johnson et al., 1982; Little et al., 1988; Weissman et al., 1982) and aminoalkylindole derivatives like WIN-55,212-2 (Compton et al., 1992b; Dambra et al., 1992). Administration of these synthetic CB_1 receptor agonists generally produces *in vivo* symptomology similar to that of Δ^9-THC, anandamide, 2-AG and 2-AGE (Compton et al., 1992a; Compton et al., 1992b; Fride & Mechoulam, 1993; Hanus et al., 2001; Little et al., 1988; Martin et al., 1991; Mechoulam et al., 1995; Smith et al., 1994).

The diarylpyrazole SR141716A is a high affinity competitive antagonist at the CB_1 receptor with very low affinity for peripheral CB_2 receptors. This compound antagonizes the *in vitro* effects of cannabinoid agonists (RinaldiCarmona et al., 1994) as well as the *in vivo* effects of Δ^9-THC (Compton et al., 1996) and WIN55212-2 (RinaldiCarmona et al., 1994). In addition to these antagonist effects, SR141716A can produce inverse agonist like activity at the CB_1 receptor. Thus, SR14176A stimulates locomotor activity in mice at doses greater than 5 mg/kg (Compton et al., 1996), potentiates the release of [^{14}C]acetylcholine from hippocampal brain slices (Gifford & Ashby, 1996), increases cannabinoid agonist-suppressible cAMP production (Meschler et al., 2000), and reduces basal [^{35}S]GTPγS binding to human recombinant CB_1 receptors by 22% (Landsman et al., 1997). When administered by intrathecal route, SR141716A

also causes thermal hyperalgesia (Richardson et al., 1997). These observations point towards an inverse agonist effect of SR141716A at the CB_1 receptor. Interestingly, rimonabant which has as its active ingredient SR141716A, was approved in the European Union (EU) to treat obesity since by antagonizing the agonist action of endocannabinoids at the CB_1 receptor, it reduces appetite (Ward & Raffa, 2011). However, unwanted psychiatric side effects were found with rimonabant and this led to its withdrawal from the EU market and denial of US FDA approval (Ward & Raffa, 2011). Other CB_1 receptor antagonist/inverse agonists withdrawn from various stages of clinical trials included MK-0364 by Merck, CP-945,598 by Pfizer, SR147778 by Sanofi and SLV-319 by Solvay (Ward & Raffa, 2011). Recently the peptide hemopressin, has been identified as an antagonist/ inverse agonist at the CB1 receptor and *in vivo* administration by different routes have shown similar analgesic effects to other CB_1 receptor agonists (Heimann et al., 2007).

In Chapter 1 we hypothesized a binding topography common to both CB_1 receptors and VGSCs and considered whether certain VGSC-targeting ligands can interact with the CB_1 receptor. Further we were interested that if there is such an interaction with the CB_1 receptor whether these ligands can interfere with the functional coupling of G proteins to the CB_1 receptor and in doing so whether they act as agonists or antagonist/inverse agonist at the CB_1 receptor.

In order to address this objective we first employed the [^{3}H]CP-55,940 binding assay in which the CB_1 receptor agonist [^{3}H]CP-55,940 binds to the CB_1 receptor with high affinity at a binding pocket also shared by endocannabinoids, aminoalkylindoles, diarylpyrazoles (Devane et al., 1988; Song & Bonner, 1996; McAllister et al., 2003). The

binding of this radioligand can be inhibited by classical CB_1 receptor agonists and antagonists (Gatley et al., 1997). Secondly we used the [^{35}S]GTPγS binding assay which is used to measure G protein activation by a ligand occupying the GPCR (Lorenzen et al., 1993; Traynor, 1995). On the basis of G protein activation, a ligand that interferes with [^{3}H]CP-55,940 binding can be characterized as an agonist, partial agonist, neutral antagonist and inverse agonist at the CB_1 receptor (Landsman et al., 1997; Petitet, 1997; Griffin, 1998; Breivogel & Childers, 2000; Breivogel et al., 2001). A clear advantage with the [^{35}S]GTPγS binding assay is that it is a functional assay that measures the first transduction step occurring after the ligand occupies the receptor (Harrison & Traynor, 2003). Any step measured downstream from the G protein may be subjected to additional modulatory inputs, but since the [^{35}S]GTPγS binding assay measures the first step after receptor occupancy it is free from these potentially confounding effects (Harrison & Traynor, 2003).

Our interest in VGSC-targeting ligands for their possible interaction with the CB_1 receptor was increased for other reasons. For example, the dihydropyrazole insecticide RH3421 which blocks VGSCs (Salgado, 1992) and inhibits VGSCs-dependent release of [^{3}H]GABA from synaptosomes (Nicholson & Merletti, 1990), bears some structural resemblance to potent CB_1 receptor antagonists like SR141716A and AM251. Interestingly, several pyrazole analogs of SR141716A have also been reported to have agonist-like activity at the CB_1 receptor (Wiley et al., 2001) and the hypomotility, lethargy and pacificity observed in rats after sub-chronic administration of RH3421 (Salgado, 1992) is similar in some respects to the behavioural effects of cannabinoid agonists (Martin et al., 1991). We considered it a possibility that the action of RH3421

might involve a cannabinoid component. Both dihydropyrazole insecticides RH3421 and RH5529 were shown to decrease K^+-induced increases in intrasynaptosomal [Ca^{2+}] by blocking voltage-gated calcium channels (Zhang & Nicholson, 1993; Zhang & Nicholson, 1994). Cannabinoid agonists have also been shown to inhibit K^+-induced increases in intracellular Ca^{2+} by blocking VGCCs (Mato et al., 2009). In addition to dihydropyrazole insecticides, we also tested the oxadiazine class of insecticides as the chemistry of this class evolved directly from dihydropyrazoles (McCann et al., 2001). The oxadiazine class of insecticides, like dihydropyrazoles also block VGSCs in mammalian neurons (Tsurubuchi et al., 2001; Zhao et al., 2003). At high doses the α-cyanopyrethroids cypermethrin and deltamethrin cause seizures, tremors, choreoathetosis, clonic seizures and salivation (CS-syndrome; Verschoyle & Aldridge, 1980)), whereas 1RS, *trans* and *cis* isomers of permethrin which lack the α-cyano group produce fine and whole body tremor (T-syndrome; Verschoyle & Aldridge, 1980). In mammals, the toxicity to pyrethroids arises primarily from sodium channel activation (Soderlund et al., 2002). In some situations both α-cyano and non-α-cyano pyrethroids reduce motor activity in rats (Crofton & Reiter, 1984; Crofton & Reiter, 1988; McDaniel & Moser, 1993). Reduction in motor activity is a prominent *in vivo* behavioural response to cannabinoids (Little et al., 1988; Martin et al., 1991), and it is possible that motor activity reductions caused by pyrethroids may be partially mediated through CB_1 receptors.

In addition to testing VGSC-specific insecticides for their interaction with CB_1 receptor we were also interested in probing the CB_1 receptor with other environmental chemicals. There are few reports describing the ability of pesticides and other toxic substances to inhibit the binding of [^{3}H]CP-55,940 to mammalian CB_1 receptors, thereby

suggesting a possible interaction with the CB_1 receptor *in vivo*. Methylfluorophosphonate (MAFP) and some of its analogs are potent inhibitors of [^{3}H]CP-55,940 binding and FAAH activity (Deutsch et al., 1997; Martin et al., 2000). Various fatty acid sulfonyl fluorides inhibit [^{3}H]CP-55,940 binding (Deutsch et al., 1997). Organophosphorus pesticides, for example chlorpyrifos oxon and chlorpyrifos methyl oxon, are extremely potent inhibitors of [^{3}H]CP-55,940 binding to CB_1 receptors with their IC_{50} values being in the low nanomolar range, whereas thion insecticides like fenthion, parathion and fenitrothion exhibit IC_{50} values in the high micromolar range (Quistad et al., 2006). Organophosphorus compounds like isopropyl dodecylfluorophosphonate (IDFP), ethyl octylphosphonofluoridate (EOFP), methyl octylphosphonofluoridate (MOFP) and methyl arachidonylflurophosphonate (MAFP) also inhibit MAG lipase and FAAH with very high potency as indicated by IC_{50} values in the low nanomolar range (Quistad et al., 2006). Administrations of IDFP, EOPF and MOPF at 10 mg/kg by the intraperitoneal route to mice caused robust inhibition of brain MAGL and FAAH. This inhibition of MAGL by *in vivo* administration of alkylphosphonofluoridates leads to increased levels of 2-AG and hypomotility (Quistad et al., 2006). Hypomotility seen in mice is exclusively due to increase in 2-AG levels and was shown to not to be influenced by simultaneous inhibition of FAAH by alkylphosphonofluoridates (Quistad et al., 2006).

In accordance with our objectives, we tested a group of mitochondrial function-interfering chemicals for their ability to interact with the CB_1 receptor and modify G protein activity. These compounds included fungicides like famoxadone and myxothiazol which primarily target the Q_0 binding domain of the mitochondrial bc_1 complex (Jordan et al., 1999; Pember et al., 2005) and compounds like surangin B which blocks electron

transport at the Q_i site of the bc_1 complex in bovine heart mitochondria and also exhibits insecticidal and antifungal properties (Deng & Nicholson, 2005; Nicholson & Zhang, 1995).

We also developed strong interest in quaternary benzophenanthridine alkaloids like chelerythrine and sanguinarine for their interaction with the CB_1 receptor. Also in our laboratory, we have shown that chelerythrine chloride blocks the *cis*-oleamide-mediated inhibition of veratridine-dependent depolarization of mouse brain synaptosomes (Laurence S. David: unpublished data). This result indicates a sodium channel action of chelerythrine. On the basis of structural considerations, we hypothesized that the pseudo-base form of the benzophenanthridine alkaloids might engage the CB_1 receptor in a similar fashion as Δ^9-tetrahydrocannabinol (Δ^9-THC) or Δ^9-tetrahydrocannabivarin (Dhopeshwarkar et al., 2011). Other structurally related compounds including isoquinoline alkaloids like berberine and papaverine as well as the phthalide isoquinoline ((-)-β-hydrastine) were also tested using [^{3}H]CP-55,940 and [^{35}S]GTPγS binding assays.

Lastly, I also looked at several tributyltin (TBT) compounds. Preliminary experiments suggested that TBT benzoate and phenylethynyl TBT were quite potent inhibitors of [^{3}H]CP-55,940 binding and thus TBT compounds were more fully characterized in terms of inhibitory effects on [^{3}H]CP-55,940 binding and then evaluated for their influence on [^{35}S]GTPγS binding.

2.3 Materials and Methods

2.3.1 Radioligands, drugs and study compounds

[^{3}H]CP-55940 [(1R,3R,4R)-3[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxy-propyl) cyclohexan-1-ol; side chain-2,3,4-[^{3}H]; specific activity 139.6 and 174.6 Ci/mmol) and guanosine 5'-O-(γ-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS; specific activity 1250 Ci/mmol) were procured from Perkin Elmer Life and Analytical Sciences, Canada. Chelerythrine, berberine, sanguinarine, papaverine, (-)-β-hydrastine, cypermethrin, deltamethrin, phenylmethane sulfonyl chloride (PMSF), TBT benzoate, phenylethynyl TBT, antimycin, CP-55,940, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-H-pyrazole-3-carboxamide (AM251), TBT acetate, DMSO, 2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)-methanone (WIN55,212-2), sigmacote, antimycin and coenzyme Q_2 were purchased from Sigma-Aldrich, Canada. The oxadiazines JT333 and MP062 were kindly donated by Dr. Keith Wing E. I. Dupont de Neumors and Company, Wilmington, Delware, USA. 1RS, *cis* and 1RS, *trans* isomers of permethrin, enantiomers of cypermethrin, RH3421, RH5529 and +/- famoxadone, were from previous laboratory stocks as supplied by agrochemical or pharmaceutical companies. Surangin B was also available from previous investigation in our laboratory. All chemicals used for making buffers and other reagents used were obtained from Sigma Aldrich-Canada. All chemicals were of the highest purity available.

2.3.2 Animals

Male CD1 mice (20-25 g) were obtained from Charles River Laboratories, (Saint-Constant, Quebec, Canada). All mice orders were placed through animal care facility

(ACF) at Simon Fraser University, Burnaby, Canada. Mice were maintained on a 12-hour light and dark regime with food and water provided *ad libitum*. All procedures using mice were in accordance with Canadian Council on Animal Care guidelines as developed for research using animals and had prior approval of the Simon Fraser University Animal Care Committee.

2.3.3 Preparation of the membrane fraction from mouse brain for the [^{3}H]CP-55,940 binding assay

The membrane fraction isolation procedure was adopted from Quistad et al. (2002) with some minor modifications. Mice were euthanized by cervical dislocation and then whole mouse brains were removed within 20 seconds and cut in small fragments using a razor blade. All isolation and membrane preparation procedures were done at 0-4 °C. Fragments of mouse brain were transferred to ice-cold buffer (100 mM Trisma base, 1 mM EDTA with pH adjusted to 9.0 with HCl) and homogenized (10 up and down strokes) using a motor driven homogenizer (pestle rotation 1500 rpm). The amount of ice-cold buffer added was 10 ml per brain. Homogenates were centrifuged in a Beckman J2HS centrifuge at 900 x g for 10 minutes in a JA20 rotor and the resulting supernatant was further centrifuged at 11,500 x g for 20 minutes. The membrane pellet was resuspended in storage buffer (50 mM Trisma base, 1 mM EDTA, 3 mM $MgCl_2$, with pH adjusted to 7.4 with HCl) and the protein concentration adjusted to 6.5 mg/ml. Protein quantification was performed according to the method of Peterson (1977). Aliquots of 100 µl were apportioned in snap top plastic vials and stored in the -80 °C freezer.

2.3.4 [^{3}H]CP-55,940 binding study

[^{3}H]CP-55,940 binding assay procedure as described by Quistad et al. (2002) with minor modifications was used to evaluate the ability of environmental chemicals to interact with the mammalian CB_1 receptor. Stock solutions of test compounds were made in DMSO and the required concentration (in 5 µl DMSO) was added to non-siliconized borosilicate glass culture tubes (13x100 mm; Kimble-Chase). Binding buffer was added next (500 µl; Trisma base (50 mM), EDTA (1 mM), $MgCl_2.6H_2O$ (3 mM), BSA (fatty acid free; 3 mg/ml) adjusted to pH 7.4 with HCl). Membranes were transferred to the laboratory from the -80 °C freezer, thawed on ice for 20 minutes, and then thoroughly resuspended. Membranes (154.3 ± 3.5 µg protein) were then added to each tube, vortexed and left at room temperature for 15 minutes. [^{3}H]CP-55940 (in 10 µl DMSO) was added next and the final concentration of the radioligand in each tube was 1.7 nM. Tubes were then incubated at 30 °C for 90 minutes with gentle shaking. Incubation was terminated by adding 1 ml ice-cold wash buffer (0.9% NaCl containing 2 mg/ml BSA) and membranes were collected on GF/C filters by rapid filtration under vacuum (Hoefer FH 225 V filtration unit). Membranes trapped on GF/C filters were washed with 4 ml of ice-cold wash buffer three times. Filters were thoroughly air dried and 4 ml of scintillant (Amersham Bioscience UK) was added. Membrane bound [^{3}H] was quantified by liquid scintillation counting (Beckman LS 6500). In each assay non-specific binding was also calculated in the presence of 10 µM CP-55,940 and this value was deducted from total binding signal to obtain the specific binding which averaged 82.76 ± 4.5%. In each experiment every test compound was tested in duplicate and the binding in presence and absence of unlabelled CP-55,940 was performed in triplicate. A minimum of three experiments were conducted for each treatment.

2.3.5 Isolation of mouse brain membranes for [^{35}S]GTPγS binding assay

The procedure used for isolating mouse brain membranes to investigate the effects of test compounds on basal and CP-55,940-stimulated [^{35}S]GTPγS binding were adopted from Breivogel & Childers (2000) with slight modifications. Two mice were euthanized by cervical dislocation and whole brains removed within 20 seconds. All membrane preparation procedures were done at 0-4 °C. After removal, the brains were immediately transferred to the isolation buffer (Trisma base (50 mM), $MgCl_2.6H_2O$ (3 mM), EGTA (0.2 mM), NaCl (100 mM) with pH adjusted to 7.4 with HCl). Mouse brains were homogenized (Polytron Kinematica GmBH; speed setting 6 for 15 seconds). The homogenate was then passed through nylon mesh and the filtrate was further homogenized (5-6 up and down gentle strokes) using a glass homogenizer (5 ml; Wheaton, USA). The homogenate was centrifuged in a Beckman J2HS centrifuge (JA20 rotor) at 24,000 x g for 25 minutes. The resulting membrane pellet was washed by resuspension in 15 ml isolation buffer and centrifugation in a Beckman J2HS centrifuge (JA20 rotor) 24,000 x g for 25 minutes. The final pellet was homogenized (Teflon homogenizer with 10-15 strokes) in isolation buffer and the protein concentration was adjusted to 7 mg/ml. Protein quantification was performed as per the method of Peterson (1977). Aliquots (100 µl) were then placed in vials and stored at -80 °C until the assay was performed.

2.3.6 [^{35}S]GTPγS binding study

The [^{35}S]GTPγS binding procedure was adopted from Breivogel & Childers (2000) and Moore et al. (2000) with minor modifications. Test compounds were added (in 5 µl DMSO) to the respective tubes and control tubes were set up with DMSO

vehicle. The total volume of DMSO was kept constant at 15 µl per tube. Assay buffer (500 µl; isolation buffer (pH 7.4) containing fatty acid free bovine serum albumin (1 mg/ml), guanosine diphosphate (GDP; 100 µM), dithiothreiotol (20 µM), [^{35}S]GTPγS (0.14 nM) was then added. Brain membranes were transferred to the laboratory from the -80 °C freezer and left to thaw out on ice for 30 minutes. The brain membranes were then thoroughly dispersed with a 1000 µl pipette and diluted with an equal volume of isolation buffer. Adenosine deaminase (0.004 units/ml) was then added to the membrane suspension and an incubation (30 °C for 30 minutes with gentle shaking) was carried out. After this, brain membranes (70.1 ± 4.2 µg protein) were added to each tube. All tubes were then vortexed and left to preincubate at room temperature for 15 minutes. Finally CP-55,940 (100 nM final concentration; in 5 µl DMSO) was added to all tubes except the control tubes which represented the basal [^{35}S]GTPγS binding signal. In experiments where effects of test compounds were evaluated on basal binding, there was no addition of CP-55,940. Non-specific binding was measured in the presence of unlabelled GTPγS (100 µM) and subtracted from the total binding signal to get specific binding. All the tubes were thoroughly vortexed and incubated at 30 °C for 90 min with gentle shaking. The incubation was terminated by adding 2 ml of ice-cold wash buffer (Trisma base: HCl; pH 7.4) and rapid filtration under vacuum (Hoefer FH 225 V) through GF/B filters (pre-soaked in wash buffer for 30 minutes). Filtration was followed by washing membranes trapped on GF/B filters with 4 ml of ice-cold wash buffer three times. Filters were left to air dry for 12 hours and then 4 ml of scintillant (Amersham Bioscience UK) was added. Membrane-bound [^{35}S] was quantified by liquid scintillation counting (Beckman LS 6500). Assay tubes (borosilicate glass culture tubes; 13 x 100 mm) were

siliconized with Sigmacote (Sigma-Aldrich Canada) followed by drying in an oven (45 minutes at 65 °C). All assays were performed in triplicate. Basal binding measured by subtracting non-specific binding averaged 96.1 ± 1.9% and increase in [^{35}S]GTPγS basal binding by 100 nM CP-55,940 averaged 69.8 ± 2.3%.

2.3.7 Data analysis and IC_{50} estimation

Concentration-inhibition curves were drawn and IC_{50}s with 95% confidence interval values were based on non-linear regression analysis of data using Prism 4 software (Graphpad Software Inc. San Diego, CA, USA). Results are represented as mean ± S.E.M.

2.4 Results

2.4.1 Effect of mitochondrial function-disrupting chemicals on [^{3}H]CP-55,940 binding to mammalian CB_1 receptors

The present work explored the possibility that chemicals like famoxoadone, surangin B and coenzyme Q_2 known to interfere with different aspects of mitochondrial function interfere with the binding of the CB_1 receptor selective ligand [^{3}H]CP-55,940. Famoxadone was a full inhibitor of [^{3}H]CP-55,940 binding, achieving threshold inhibitory effects at 2 µM and an IC_{50} of 11.26 µM (95% CI = 8.61 - 14.73 µM) (Figure 2-1). Coenzyme Q_2 which is a synthetic alkyl-1,4-benzoquinone inhibited [^{3}H]CP-55,940 binding with an IC_{50} of 8.16 µM (95% CI = 6.25 - 10.51 µM) (Figure 2-1). Other synthetic alkyl-1,4-benzoquinones like decylubiquinone (IC_{50} 21.7 µM; 95% CI 18.9 – 24.9 µM) and decylplastoquinone (IC_{50} 20.1 µM; 95% CI 18.0 – 30.1 µM) were approximately 50% less potent than famoxadone and coenzyme Q_2. Surangin B, which blocks electron transport exhibited intermediate inhibitory potency as indicated by an

IC_{50} of 16.34 µM (95% CI = 12.59 - 21.19 µM) (Figure 2-1). Other mitochondrial function modifying chemicals like myxothiazole and stigmatellin at 20 µM were shown to more weakly inhibit [^{3}H]CP-55,940 binding by 39.2 ± 3.1% and 27.3 ± 0.8% respectively and antimycin A, oligomycin, 2-heptyl-4-hydroxyquinoline N-oxide and carbonylcyanide-3-chlorophenylhydrazone produced minimal (< 15%) inhibition of [^{3}H]CP-55,940 binding at 20 µM .

2.4.2 Effect of mitochondrial function disrupting chemicals on basal [^{35}S]GTPγS binding

Various chemicals that interfere with mitochondrial function were tested for their ability to affect basal [^{35}S]GTPγS binding. Table 2-1 shows the effect of different concentrations of various mitochondrial function disrupting chemicals on basal [^{35}S]GTPγS binding. None of these chemicals were able to stimulate [^{35}S]GTPγS binding and this ruled out any possible agonist nature at the CB_1 receptor. Surangin B when tested at higher concentrations (100 µM) and myxothiazole (60 µM) encroached 41.50 ± 3.50% and 33.51 ± 0.98% respectively into the basal component of [^{35}S]GTPγS binding. Other modulators of mitochondrial function were less effective in this regard (< 22% encroachment into the basal component of [^{35}S]GTPγS binding) (Table 2-1).

2.4.3 Effect of mitochondrial function disrupting chemicals on CP-55,940 stimulated [^{35}S]GTPγS binding

Famoxadone, completely inhibited CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding with a IC_{50} value of 4 µM (95% CI = 3.1 - 5.3 µM) (Figure 2-2). Threshold inhibitory effects for famoxadone were evident around 1 µM. Famoxadone tested at 30 µM completely inhibited agonist stimulated [^{35}S]GTPγS binding and also encroached

into basal binding (by approximately 25%). The naturally occurring coumarin insecticide surangin B, was a full inhibitor of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding and gave an IC_{50} value of 3.96 µM (95% CI = 3.43 - 4.57 µM) (Figure 2-3). A threshold inhibitory effect of surangin B was evident around 2.5 µM and at 20 µM agonist-stimulated [^{35}S]GTPγS binding was completely abolished. Encroachment into the basal binding component was also observed with cold CP-55,940 present. Myxothiazole (IC_{50} 19.76 µM; 95% CI = 17.61 - 22.18 µM) and coenzyme Q_2 (IC_{50} 34.48 µM; 95% CI = 27.02 - 44.00 µM), both were less potent than than famoxadone and surangin B, but at higher concentrations also completely inhibited CP-55,940-stimulated [^{35}S]GTPγS binding (Figure 2-3 and Figure 2-4). Other mitochondrial poisons like antimycin A, oligomycin and decylubiquinol (all tested at 40 µM), all achieved lower (respectively 9.30 ± 5.95%, 12.04 ± 7.23% and 49.24 ± 9.24%) inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding.

2.4.4 Effect of sodium channel-selective insecticides on [^{3}H]CP-55,940 binding to the mammalian CB_1 receptors

The oxadiazine insecticides JT333 and MP062, known to block voltage-gated sodium channels were tested for their ability to interfere with the dynamics of [^{3}H]CP-55,940 binding to the mammalian CB_1 receptor. MP062 and its decarbomethylated form JT333 achieved only partial inhibition (50-60% of maximum) of radioligand binding. JT333 (IC_{50} 10.02 µM; 95% CI = 7.02 - 13.81 µM) was very close in inhibitory potency to MP062 (IC_{50} 11.1 µM; 95% CI = 6.99 - 17.62 µM) with threshold inhibitory effects evident around 2 µM for both the compounds (Figure 2-5). Other sodium channel-selective insecticides such as pyrethroids (1RS, *trans* permethrin, 1RS, *cis* permethrin,

and cypermethrin) and dihydropyrazoles (RH3421 and RH5529) were examined in our laboratory by Mr. Sudip Ghose for their ability to interfere with [^{3}H]CP-55,940 binding to the mammalian CB_1 receptor (Table 2-2).

2.4.5 Effect of sodium channel-selective insecticides on basal [^{35}S]GTPγS binding

VGSC-targeting insecticides like JT333, MP062, RH3421, RH5529, 1RS, *trans* permethrin, 1RS, *cis* permethrin, cypermethrin and enantiomers of cypermethrin were tested at appropriate concentrations for their ability to stimulate [^{35}S]GTPγS binding on their own (Table 2-3), and all failed to stimulate [^{35}S]GTPγS binding on their own.

2.4.6 Effect of sodium channel-selective insecticides on CP-55,940-stimulated [^{35}S]GTPγS binding

Several insecticides known to target sodium channels were clearly shown to interfere with the binding of [^{3}H]CP-55,940, but it was to be determined if such an interaction of these insecticides with the CB_1 receptor can affect the G protein coupling machinery associated with the CB_1 receptor. MP062 and JT333 inhibited CP-55,940-(100 nM-) stimulated [^{35}S]GTPγS binding (maximum inhibition 55-60% for both) with an IC_{50} value of 11.2 μM (95% CI = 2.5 - 47 μM) and 30.0 μM (95% CI = 20.5 – 35.0 μM) (Figure 2-6 and Figure 2-7). The dihydropyrazole insecticide RH3421 (IC_{50} 6.5 μM; 95% CI = 4.6 - 9.2 μM) was approximately three times more potent than RH5529 (IC_{50} 19.7 μM; 95% CI = 10.2 - 38.3 μM) in inhibiting CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding (Figure 2-8 and Figure 2-9). Threshold inhibitory effects on agonist stimulated [^{35}S]GTPγS binding were evident around 0.5 μM for RH3421 and around 2 μM for RH5529. RH3421 achieved approximately 100% inhibition at 50 μM whereas RH5529 was only able to achieve approximately 80% inhibition at the highest

concentration tested (75 µM). The pyrethroid insecticide 1RS, *trans*-permethrin (IC_{50} 10 µM; 95% CI = 7.8 – 25.6 µM) was more potent than 1RS, *cis*-permethrin (IC_{50} 35.6 µM ; 95% CI = 15 - 79 µM) (Figure 2-10 and Figure 2-11). Both pyrethroid insecticides achieved complete inhibition of agonist stimulated [^{35}S]GTPγS binding. The α-cyano pyrethroid cypermethrin also partially (approximately 55%) inhibited CP-55,940- (100 nM-) stimulated [^{35}S]GTPγS binding with an IC_{50} value of 27 µM (95% CI = 20.4 – 37 µM) (Figure 2-12). Different enantiomers of cypermethrin were also tested at 40 µM and they varied in their ability to inhibit agonist stimulated [^{35}S]GTPγS binding (Table 2-4). The α-cyano pyrethroid deltamethrin was not able to inhibit agonist stimulated [^{35}S]GTPγS binding despite using new batches and repeated testing (data not shown).

2.4.7 Effect of benzophenanthridine alkaloids on CP-55,940-stimulated [^{35}S]GTPγS binding and basal binding

Benzophenanthridine alkaloids like chelerythrine, sanguinarine, berberine, papaverine and 1R, 9S-(-)-β-hydrastine were tested previously in our laboratory using the [^{3}H]CP55,940 binding assay. Based on these results I used the [^{35}S]GTPγS binding assay to characterize these five alkaloids for influencing the G protein coupling cycle associated with the CB_1 receptor. Sanguinarine and chelerythrine both potently inhibited CP-55,940 stimulated [^{35}S]GTPγS binding with sanguinarine being slightly more potent than chelerythrine as estimated from IC_{50}s of 1.22 µM (95% CI = 1.05 - 1.50 µM) and 2.09 µM (95% CI = 1.05 - 1.50 µM) (Figure 2-13 and Figure 2-14). Sanguinarine and chelerythrine both completely inhibited agonist stimulated [^{35}S]GTPγS binding. In the CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding assay chelerythrine caused an additional 54.92 ± 2.54% and 67.52 ± 3.40% encroachment into basal signal at 20 µM

and 50 µM respectively. Sanguinarine in a similar fashion produced an additional 50.12 ± 2.84%, 68.28 ± 0.56% and 74.76 ± 1.05% encroachment into the basal signal at 4 µM, 10 µM and 50 µM respectively. The effect of chelerythrine and sanguinarine on basal [^{35}S]GTPγS binding was also evaluated in our laboratory by Mr. Amey Dhopeshwarkar and both benzophenanthridine alkaloids produced a marked reduction in the basal binding signal. AM251 also inhibited the CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding but AM251 did not encroach into basal binding at high micromolar concentrations in marked contrast to chelerythrine and sanguinarine (Table 2-5). The isoquinoline alkoloids berberine and papaverine and phthalide isoquinoline (-)-β-hydrastine were unable to inhibit agonist-stimulated or basal [^{35}S]GTPγS binding at 2 µM, 20 µM and 40 µM (Table 2-6; data for 40 µM only shown).

2.4.8 Effect of tributyltin (TBT) compounds on [^{3}H]CP-55,940 binding to mammalian CB_1 receptors

TBT benzoate, TBT acetate and phenylethynyl TBT were tested for their ability to interfere with [^{3}H]CP-55,940 binding to mammalian CB_1 receptors. TBT benzoate (IC_{50} 2.56 µM; 95% CI = 1.68 - 3.91 µM) and TBT acetate (IC_{50} 2.72 µM; 2.28 - 3.27 µM) were quite potent inhibitors of [^{3}H]CP-55,940 binding with their individual IC_{50} values differing only slightly (Figure 2-15). Phenylethynyl TBT (IC_{50} 14.76 µM; 9.83 - 22.16 µM) was less potent and at its maximum effect concentration also displaced [^{3}H]CP-55,940 to a lesser extent (80%) compared to the other TBT compounds which were full inhibitors of [^{3}H]CP-55,940 binding (Figure 2-15).

2.4.9 Effect of tributyltin (TBT) compounds on basal and CP-55,940-stimulated [^{35}S]GTPγS binding

TBT benzoate and phenylethynyl TBT inhibited agonist-stimulated [^{35}S]GTPγS binding with similar potency as estimated from IC_{50s} of 1.43 µM (95% CI = 1.35 - 1.53 µM) and 1.87 µM (95% CI = 1.71 - 2.02 µM) (Figure 2-16). TBT benzoate achieved complete inhibition of agonist-stimulated [^{35}S]GTPγS binding around 2.5 µM and in addition encroached 51.03 ± 3.24% into the basal binding at 5 µM. Phenylethynyl TBT produced complete inhibition of agonist-stimulated [^{35}S]GTPγS binding around 4 µM and, in addition, encroached 48.98 ± 14.64% and 68.53 ± 8.81% into basal binding signal at 10 µM and 20 µM respectively. TBT benzoate on its own reduced basal binding by 22.12 ± 3.60% and 50.87 ± 1.87% at 2.5 µM and 5 µM respectively. Phenylethynyl TBT on its own also reduced basal binding signal by 63.06 ± 3.62% at 20 µM.

2.4.10 Effect of membrane disrupting detergent dodecyl maltoside on binding of [^{3}H]CP-55,940 to the CB_1 receptor

The detergent and membrane protein-releasing agent *n*-dodecyl β-D maltoside was tested at 2 µM, 60 µM and 100 µM and inhibited [^{3}H]CP-55,940 binding to the mouse CB_1 receptor by -2.2 ± 4.7%, 2.4 ± 5.0% and 14.4 ± 2.0% respectively. It is evident from these results that inhibition of [^{3}H]CP-55,940 binding observed for other test chemicals reported in this investigation is unlikely to be due to non-specific release of the CB_1 receptor complex from the neuronal membrane during interaction but involves a CB_1 receptor-mediated and pharmacologically specific effect.

2.4.11 Figures and tables

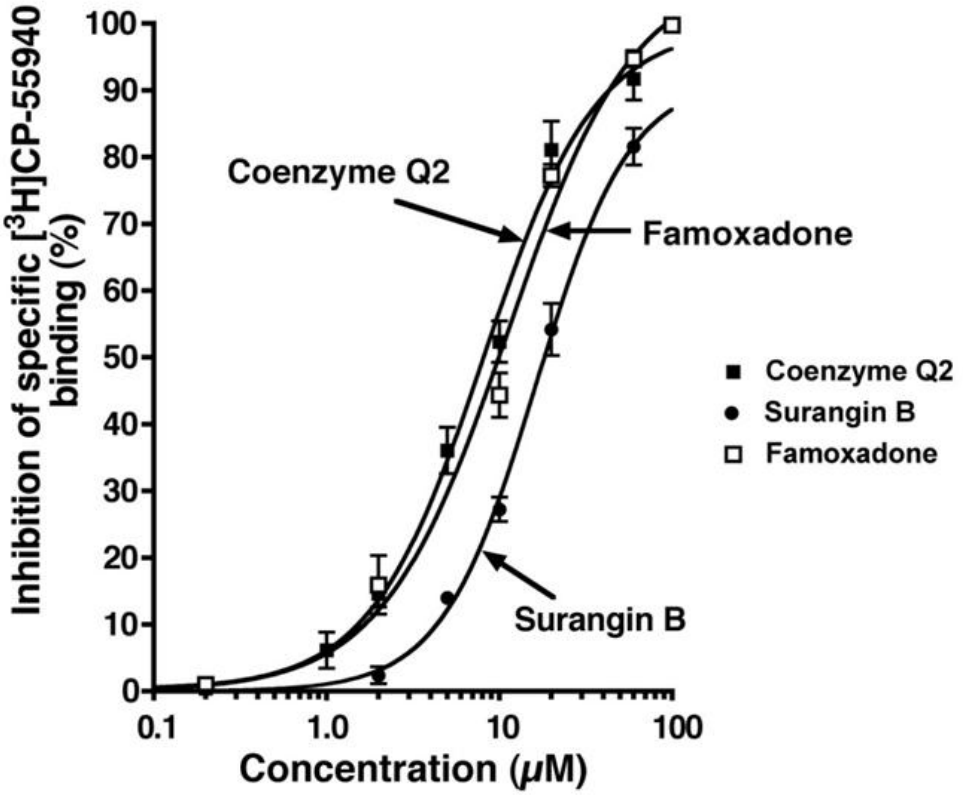


Figure 2-1 Concentration-dependent inhibition of specific [^{3}H]CP-55,940 binding to mouse brain CB_1 receptors by coenzyme Q_2, famoxadone and surangin B. Each data point represents the mean ± S.E.M. percentage inhibition of specific [^{3}H]CP-55,940 binding determined by three independent assays each performed in duplicate.

Table 2-1 Inhibition of the basal component of [^{35}S]GTPγS binding by various mitochondrial function disrupting chemicals. Each value represents mean ± S.E.M. percent decrease in [^{35}S]GTPγS basal binding determined by three independent assays each performed in triplicate.

Compound	**Concentration (μM)**	**Percent inhibition of basal [^{35}S]GTPγS binding**
Famoxadone	20	17.03 ± 3.01
Surangin B	50	12.94 ± 0.86
Surangin B	100	41.50 ± 3.50
Coenzyme Q_2	100	21.05 ± 2.73
Myxothiazole	60	33.51 ± 0.98
Antimycin	40	9.81 ± 4.24
Oligomycin	40	5.32 ± 1.21

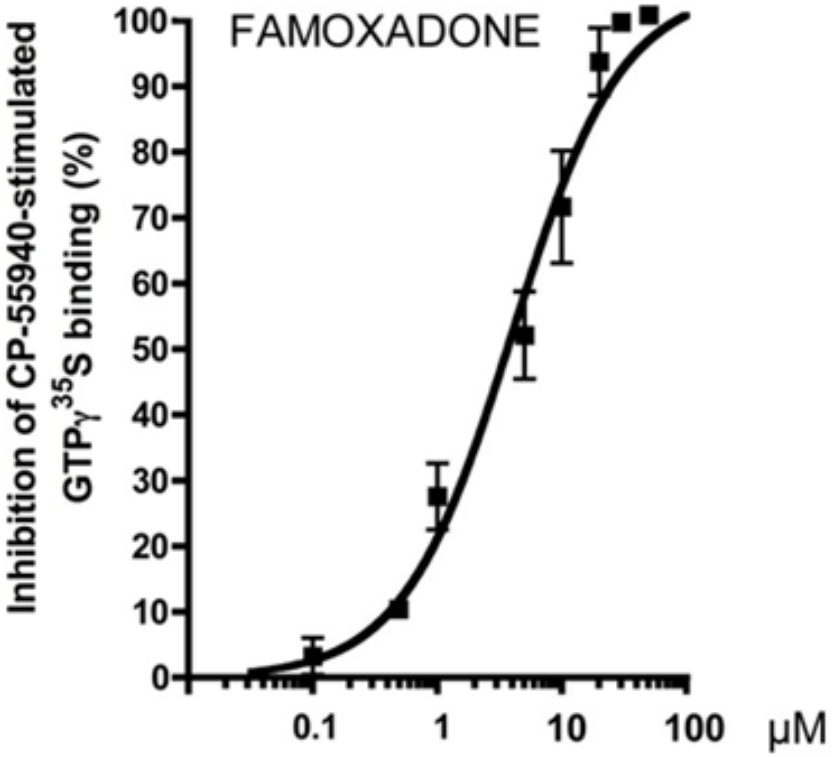


Figure 2-2 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by famoxadone. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

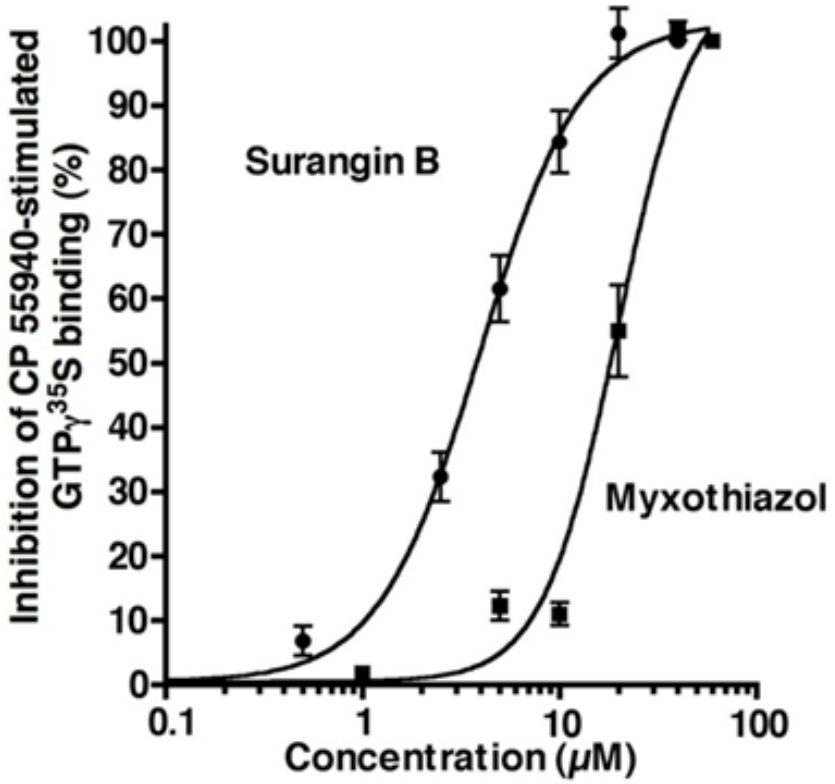


Figure 2-3 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by surangin B and myxothiazol. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

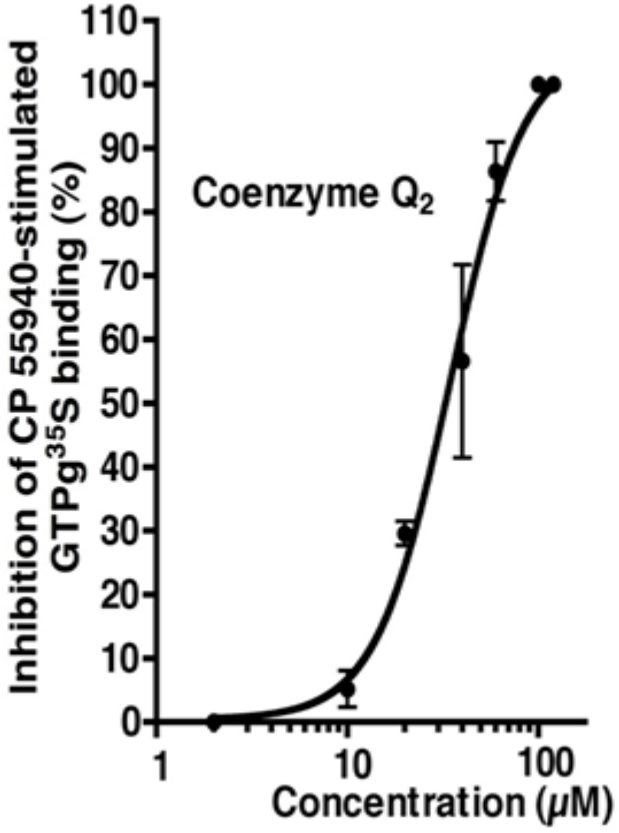


Figure 2-4 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by coenzyme Q_2. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

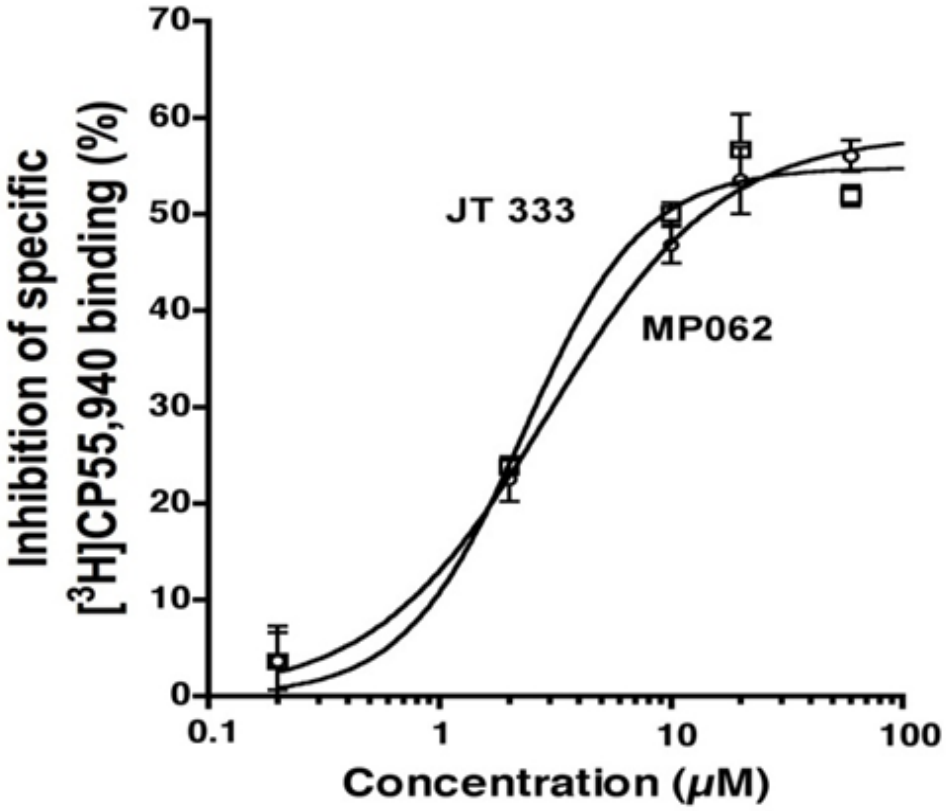


Figure 2-5 Concentration dependent inhibition of specific [^{3}H]CP-55,940 binding to the mouse brain CB_1 receptors by JT333 and MP062. Each data point represents the mean ± S.E.M. percentage inhibition of specific [^{3}H]CP-55,940 binding determined by three independent assays each performed in duplicate.

Table 2-2 IC_{50} values for various VGSC targeting insecticides for inhibition of specific [^{3}H]CP-55,940 binding to mouse brain CB_1 receptors. All compounds included in this table were tested by Mr. Sudip Ghose as part of his M.E.T project thesis.

Insecticide	**Inhibition of [^{3}H]CP-55,940 binding (IC_{50})**
1RS, *trans*-permethrin	7.1 µM
1RS, *cis*-permethrin	18.1 µM
Cypermethrin	12.2 µM
RH3421	5.7 µM
RH5529	16.1 µM

Table 2-3 Percent inhibition of basal [^{35}S]GTPγS binding by various VGSC-targeting insecticides. Each value represents mean ± S.E.M. percent decrease in [^{35}S]GTPγS basal binding determined by three independent assays each performed in triplicate.

VGSC targeting insecticide	**Concentration (μM)**	**Percent inhibition of basal [^{35}S]GTPγS binding**
JT333	20	8.19 ± 1.08
MP062	20	7.35 ± 1.91
RH3421	20	12.34 ± 2.42
RH3421	50	14.04 ± 4.30
RH5529	20	7.48 ± 1.62
RH5529	75	17.89 ± 1.88
1RS, *trans*-permethrin	75	12.26 ± 0.90
1RS, *cis*-permethrin	75	10.46 ± 0.75
Cypermethrin	80	7.20 ± 1.71
1S-*cis*-alpha R-cyano	40	0.76 ± 0.56
1R-*cis*-alpha S-cyano	40	7.59 ± 0.67
1S-*trans*-alpha R-cyano	40	15.30 ± 1.21
1R-*trans*-alpha S-cyano	40	10.39 ± 1.50

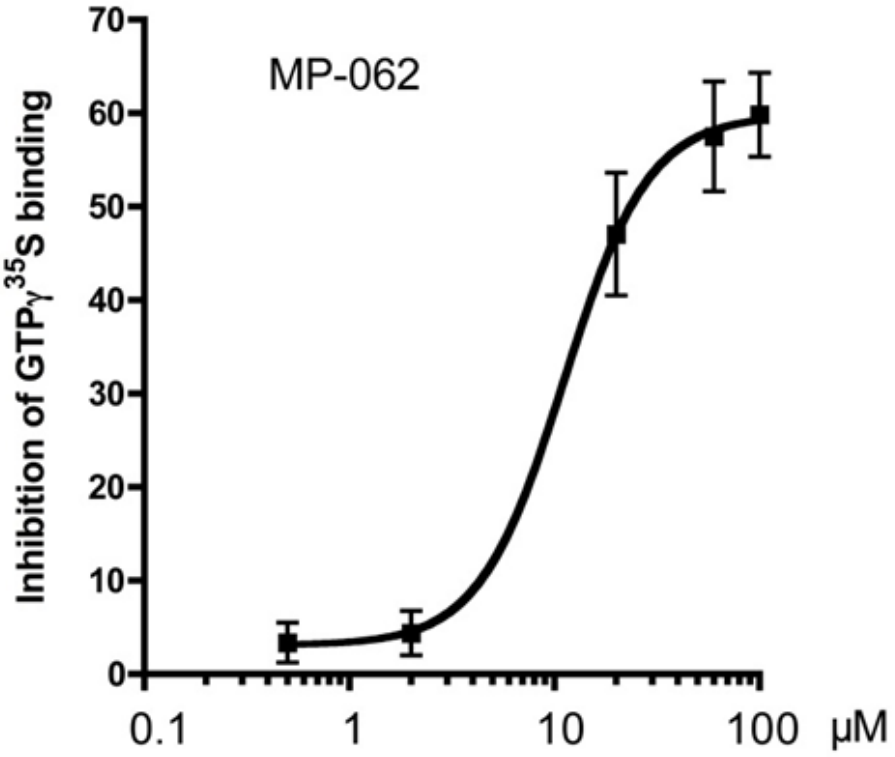


Figure 2-6 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by MP062. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

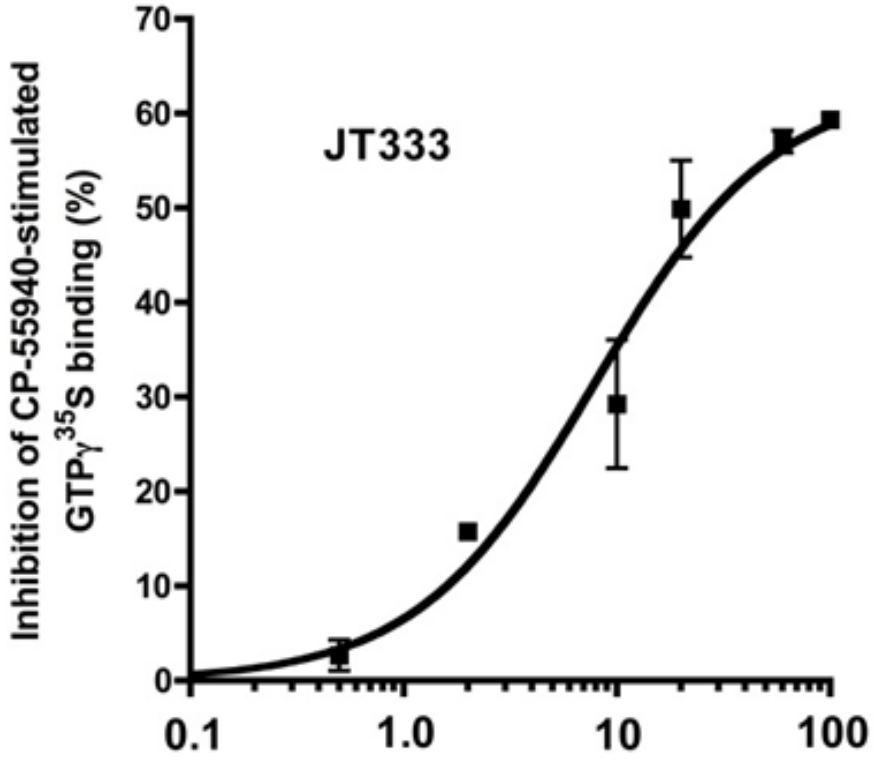


Figure 2-7 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by JT333. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

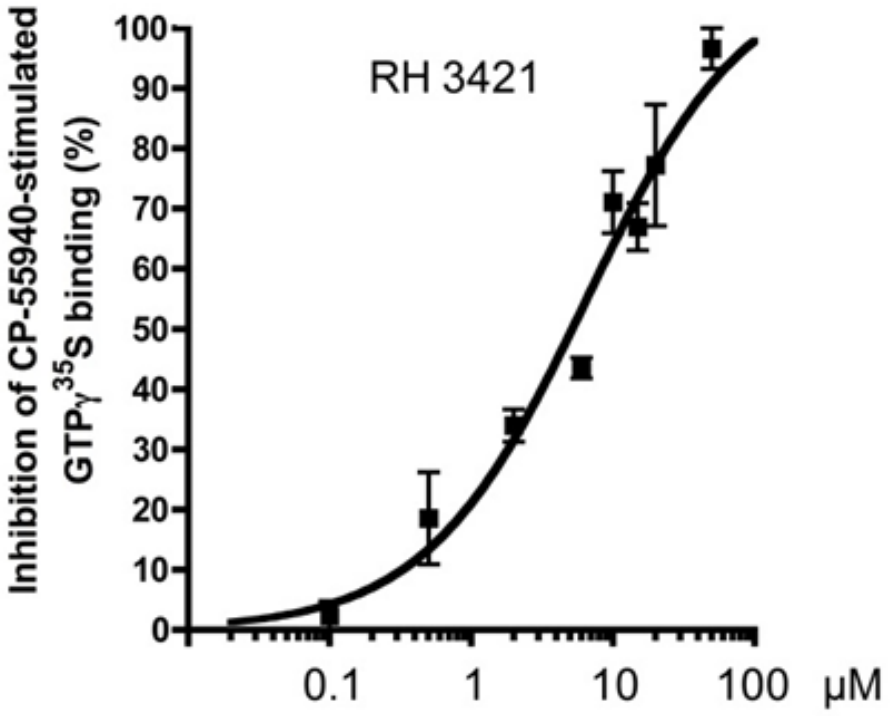


Figure 2-8 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by RH3421. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

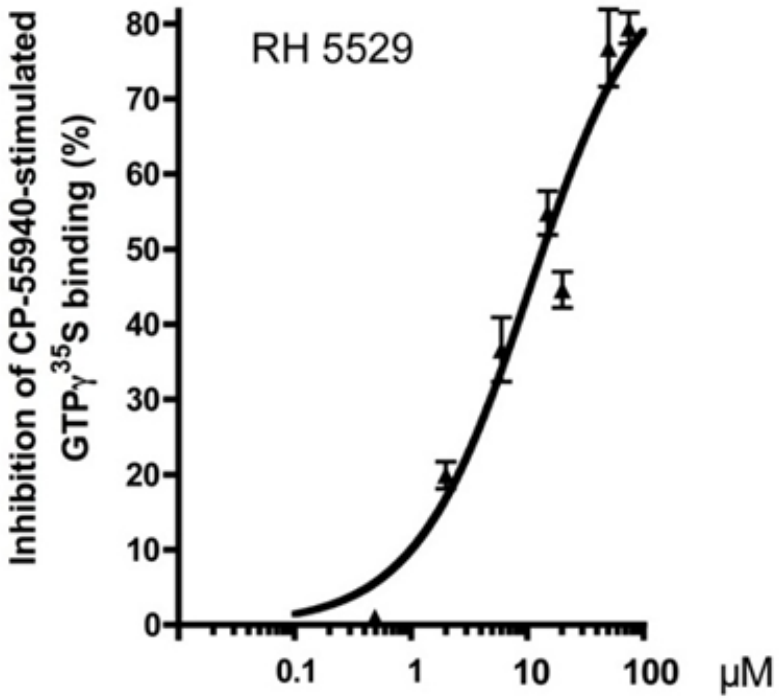


Figure 2-9 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by RH5529. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

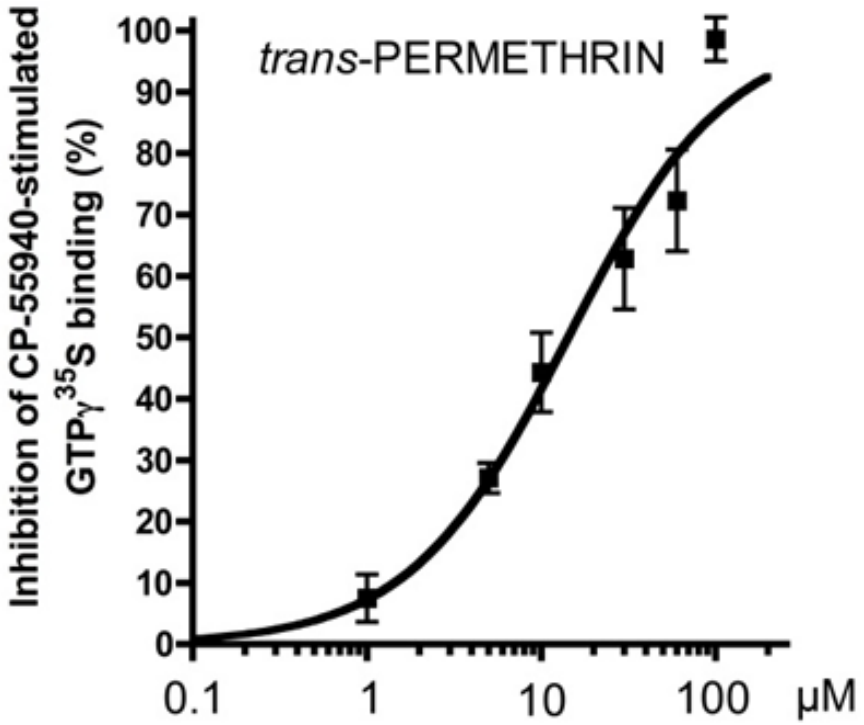


Figure 2-10 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by1 RS, *trans*-permethrin. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

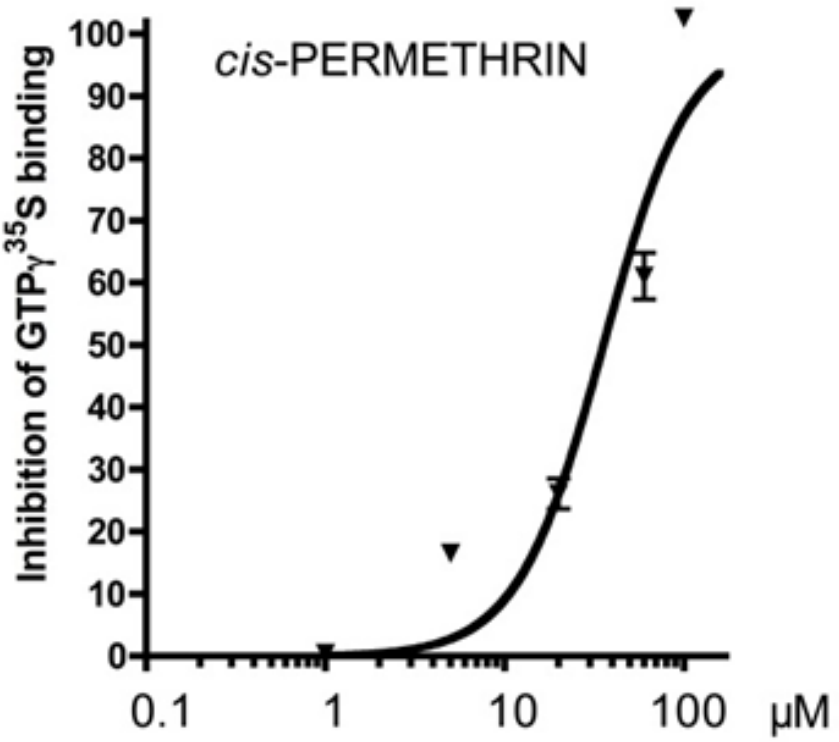


Figure 2-11 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by1 RS, *cis*-permethrin. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

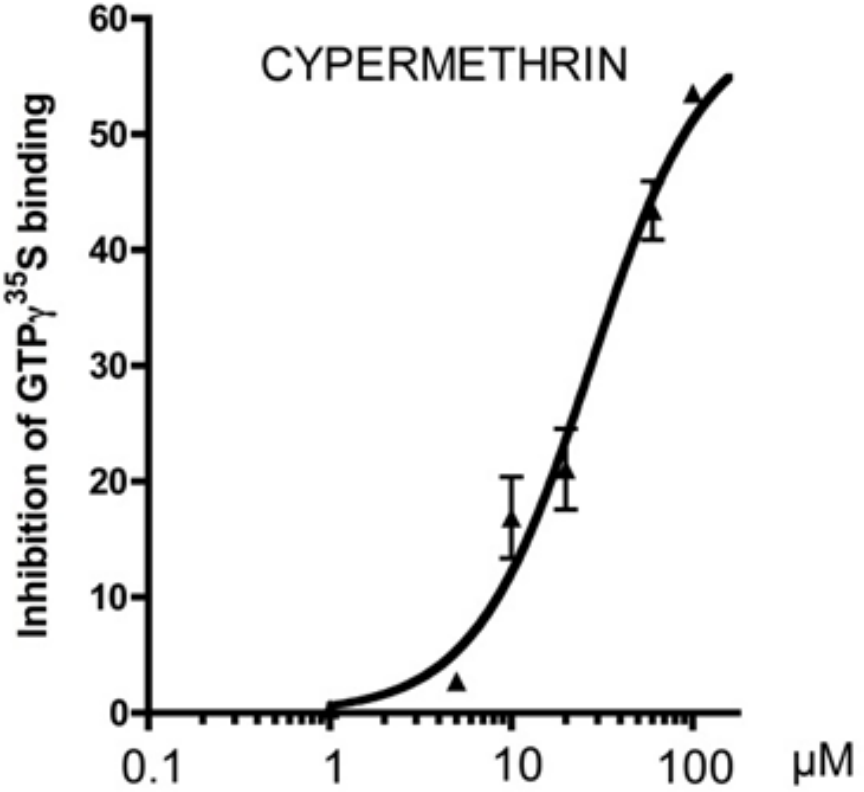


Figure 2-12 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by cypermethrin. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

Table 2-4 Percentage inhibition of 100 nM-CP-55,940 stimulated [^{35}S]GTPγS binding by cypermethrin enantiomers. Each data point represents the mean percentage inhibition (± S.E.M.) of CP-55,940-stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

Cypermethrin enantiomers (40 µM)	**Inhibition of CP-55,940 (100 nM) stimulated [^{35}S]GTPγS binding (%)**
1S-*cis*-alpha R-cyano cypermethrin	13.52 ± 7.80
1R-*cis*-alpha S-cyano cypermethrin	29.75 ± 6.43
1S-*trans*-alpha R-cyano cypermethrin	42.57 ± 6.30
1R-*trans*-alpha S-cyano cypermethrin	62.40 ± 2.31

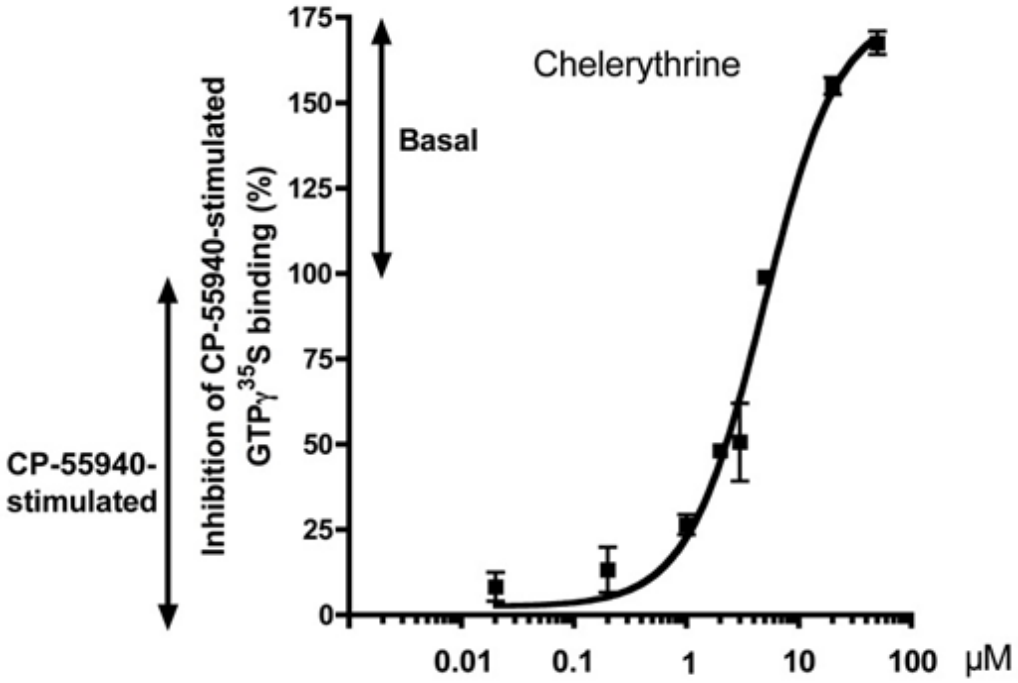


Figure 2-13 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by chelerythrine. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

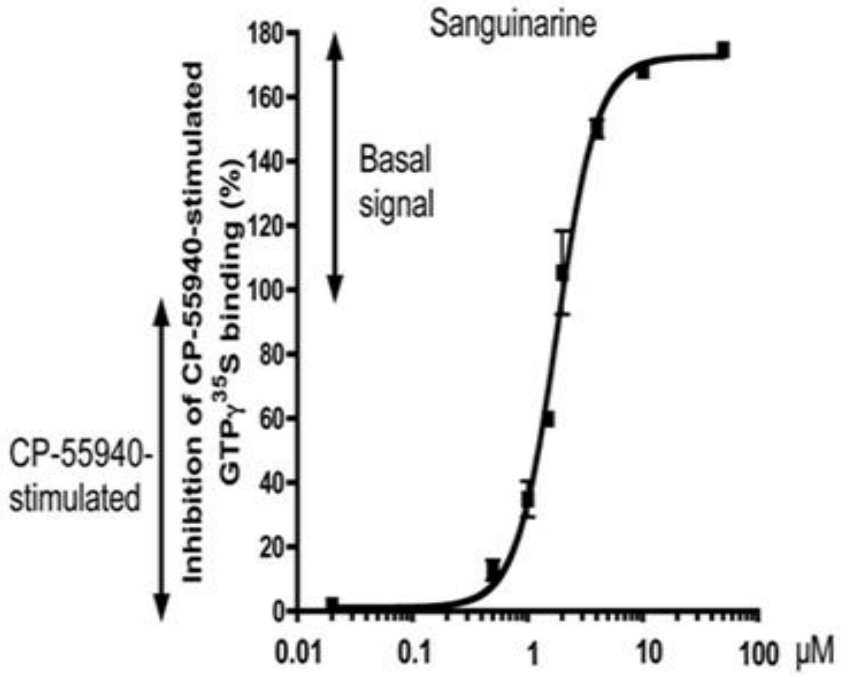


Figure 2-14 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by sanguinarine. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

Table 2-5 Inhibition of 100 nM CP-55,940-stimulated and basal [^{35}S]GTPγS binding to mouse brain membranes by AM251. Data represents mean ± S.E.M. of three independent experiments performed in triplicate. ND = not determined

AM251 (μM)	**Inhibition of CP-55,940-stimulated [^{35}S]GTPγS binding (%)**	**Inhibition of basal [^{35}S]GTPγS binding (%)**
0.01	59.59 ± 2.66	ND
1.0	98.93 ± 1.62	ND
10.0	100	7.61 ± 6.32
20.0	ND	10.01 ± 1.37

Table 2-6 Lack of effect of isoquinoline type compounds on CP-55940-stimulated and basal [^{35}S]GTPγS binding to mouse brain membranes. Compounds were tested at 40 µM. Each value represents mean ± S.E.M. of three independent experiments performed in triplicate.

Compound (40 µM)	**Inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding (%)**	**Effect on basal [^{35}S]GTPγS binding (+ = % increase; - = % decrease)**
Berberine	2.82 ± 0.89*	1.98 ± 0.35
1R, 9S-(-)β-Hydrastine	2.23 ± 2.23	- 0.50 ± 5.77
Papaverine	5.79 ± 1.77	3.90 ± 2.32

* represents a % increase in CP-55940-induced stimulation

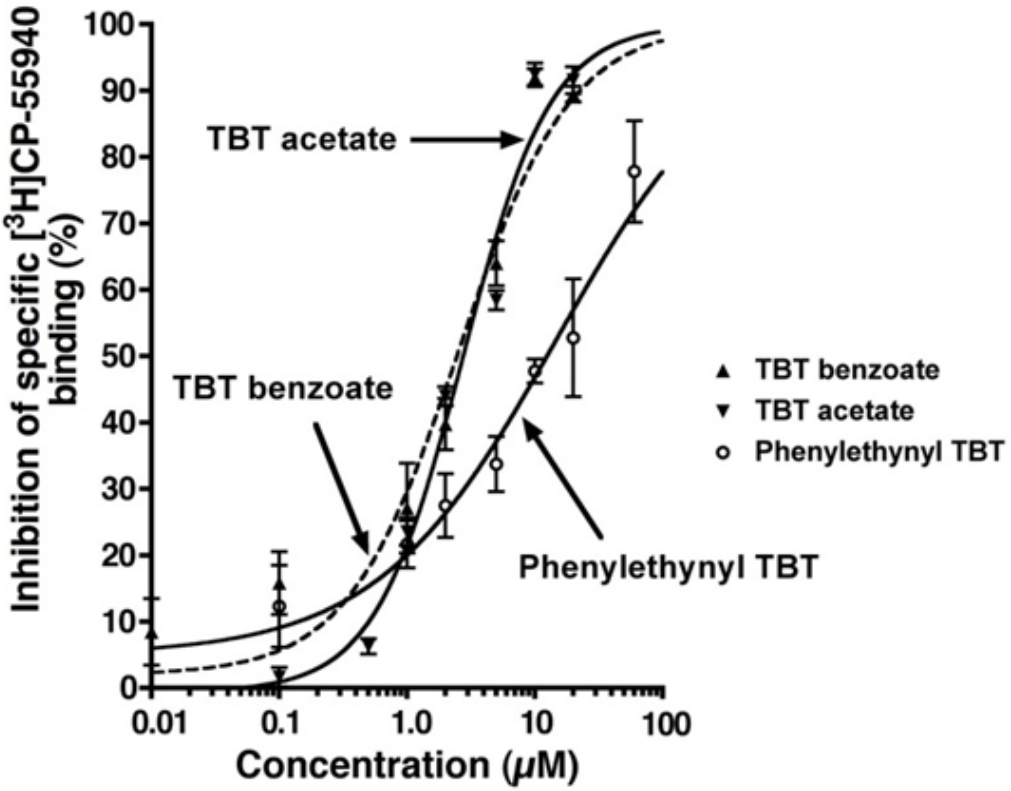


Figure 2-15 Concentration-dependent inhibition of specific [^{3}H]CP-55,940 binding to mouse brain CB_1 receptors by TBT benzoate, TBT acetate and phenylethynyl TBT. Each data point represents the mean ± S.E.M. inhibition of specific [^{3}H]CP-55,940 binding for three independent assays, each performed in triplicate.

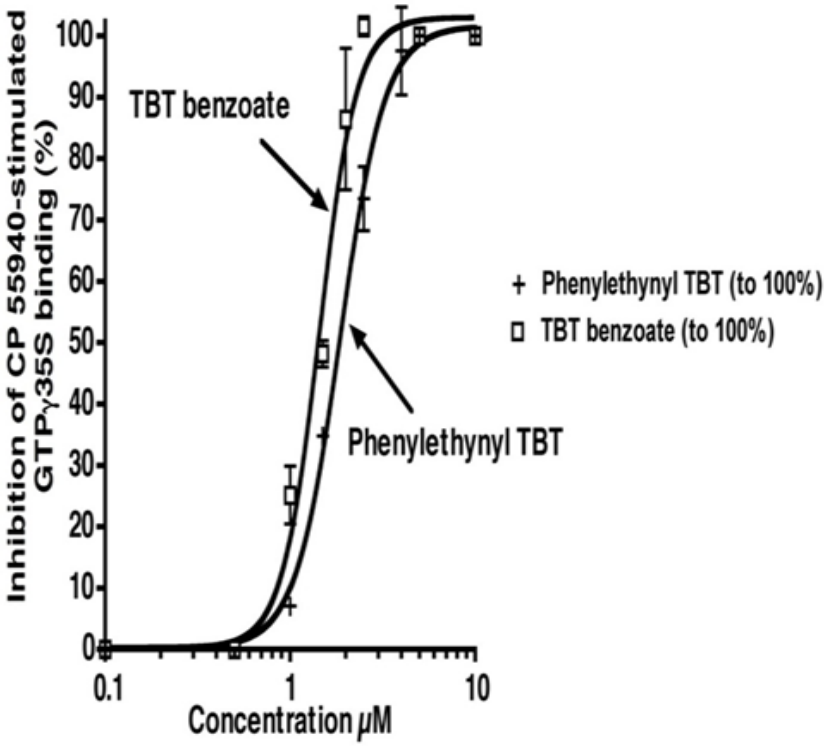


Figure 2-16 Concentration-dependent inhibition of CP-55,940 (100 nM)-stimulated [^{35}S]GTPγS binding by TBT benzoate and phenylethynyl TBT. Each data point represents the mean ± S.E.M. percentage inhibition of CP-55,940 stimulated [^{35}S]GTPγS binding determined by three independent assays each performed in triplicate.

2.5 Discussion

Earlier reports (Nicholson et al., 2003; Liao et al., 2004; Duan et al., 2008a; Duan et al., 2008b) from our laboratory suggested a possible binding topography common to both CB_1 receptors and VGSCs. In our laboratory we initially tested this hypothesis by examining in the [^{3}H]CP-55940 binding assay various candidate molecules belonging to the pyrethroid, oxadiazine and dihydropyrazole classes of insecticides which are well-known to mediate their insecticidal effects by primarily targeting VGSCs.

If indeed there was an interaction between different VGSC targeting ligands and the CB_1 receptor, we were interested in establishing what the functional consequence of such an interaction was, and importantly whether these test compounds act as agonists, antagonists or inverse agonists at the CB_1 receptor. CB_1 receptors are G protein-coupled receptors and CB_1 receptor agonists and antagonists can be differentiated by [^{35}S]GTPγS binding assay (Petitet, 1997; Landsman et al., 1997; Breivogel & Childers, 2000; Breivogel et al., 2001).

We were aware as we embarked on this study of other reports which also suggested the interaction of some pesticides and toxic substances (e.g., organophosphorus pesticides, alkylflurophosphonates, benzodioxaphosphorin and alkanesulfonyl fluorides) with the CB_1 receptor based on their ability to inhibit [^{3}H]CP-55,940 binding to mouse brain CB_1 receptors (Deutsch et al., 1997; Martin et al., 2000; Quistad et al., 2002; Segall et al., 2003; Quistad et al, 2006). Some of these toxic substances are extremely potent inhibitors of radioligand binding with their IC_{50s} in very low nanomolar range (IC_{50s} 1 – 3 nM) (Martin et al., 2000; Segall et al., 2003). These reports further prompted us to look at other classes of toxic substances like 1) mitochondrial function disrupting chemicals 2)

benzophenanthridine alkaloids, isoquinoline alkaloids and phthalide isoquinoline alkaloid and 3) TBT compounds for their influence on the radioligand binding.

The results clearly show that VGSC-targeting non α-cyano pyrethroid insecticides, certain α-cyano pyrethroid insecticides dihydropyrazole insecticides and oxadiazine insecticides inhibit binding of [^{3}H]CP-55,940 binding to CB_1 receptors. This decrease in radioligand binding is unlikely to be due to the release of CB_1 receptors from the neuronal membranes, since the membrane detergent dodecylmaltoside tested at 100 μM produced minimal inhibition of [^{3}H]CP-55,940 binding. Moreover, other VGSC-targeting drugs & toxins tested by Mr. Sudip Ghose were inactive or minimally active in the [^{3}H]CP-55,940 binding assay indicating that we are indeed looking at a specific effect. In addition to this range of activities, the dose-response relationships observed for inhibition of [^{3}H]CP-55,940 binding with pyrethroids, dihydropyrazoles and oxadiazines indicate a specific CB_1 receptor-mediated pharmacological effect.

Interaction of pyrethroid insecticides with VGSCs has been studied using [^{3}H]batrachotoxinin 20α-benzoate and the pyrethroid radioligand [^{3}H]RU5847 (Brown et al., 1988; Rubin et al., 1993; Trainer et al., 1997), while the association of α-cyano pyrethroids with the convulsant binding site on $GABA_A$ receptor have utilized [^{35}S]TBPS (Casida & Lawrence, 1985; Lawrence & Casida, 1983). Non α-cyano pyrethroids like permethrin inhibited [^{3}H]batrachotoxinin 20α-benzoate binding to the mouse brain VGSCs but at higher concentrations inhibitory effects were weak whereas the α-cyano pyrethroid deltamethrin allosterically stimulated the binding of [^{3}H]batrachotoxinin 20α-benzoate to mouse brain VGSCs with 50% of its maximal effect achieved around 7 μM (Brown et al., 1988; Rubin et al., 1993). The concentrations of pyrethroids required to

inhibit the binding of [^{3}H]CP-55,940 reported in this investigation are very similar to the concentrations required for the inhibition of [^{3}H]batrachotoxinin 20α-benzoate binding to VGSCs and [^{35}S]TBPS to $GABA_A$ receptors (Casida & Lawrence, 1985).

The mammalian neurotoxicity profile is highly excitatory when animals are exposed to high doses of 1R, *trans*, αS enantiomers of cypermethrin but 1S, *trans*, αR enantiomers are without effect (Verschoyle & Aldridge, 1980). However in the present investigation both the enantiomeric forms were of similar activity in inhibiting [^{3}H]CP-55,940 binding and therefore even if both enantiomers were acting as inverse agonist at the CB_1 receptor, the lack of sterochemical specificity rules out the CB_1 receptor as being involved in producing *in vivo* excitatory symptoms. A number of non α-cyano pyrethroids and α-cyano pyrethroids have also been reported to reduce motor activity at low doses (Crofton & Reiter, 1984; Crofton & Reiter, 1988; Mcdaniel & Moser, 1993; Wolansky et al., 2006) and cypermethrin has also been reported to cause hypothermia (Mcdaniel & Moser, 1993; Verschoyle & Aldridge, 1980). Hypomotility and hypothermia are two important signs of CB_1 receptor activation, and so it was important to determine if pyrethroid insecticides act as agonists at the CB_1 receptor. My [^{35}S]GTPγS binding data reported in this chapter clearly argues against the possible agonist nature of pyrethroids at the CB_1 receptor since all the pyrethroid insecticides tested (cypermethrin and its enantiomers, 1RS, *trans*-permethrin, 1RS, *cis*-permethrin) were not able to stimulate [^{35}S]GTPγS binding and neither did they reduce basal [^{35}S]GTPγS binding significantly to be considered as inverse agonist at the CB_1 receptor. However, all the pyrethroid insecticides (except deltamethrin) inhibited CP-55,940-stimulated [^{35}S]GTPγS binding, thus supporting an antagonist behaviour at the CB_1

receptor. Pyrethroids cause depolarization and enhanced release of neurotransmitters (Brooks & Clark, 1987; Eells & Dubocovich, 1988; Symington et al., 2007), and such synaptic excitation should trigger widespread release of endocannabinoids. By antagonizing endocannabinoid action at CB_1 receptors it is possible that some pyrethroids may also prevent any suppression of the excitation resulting from Na^+- or Ca^{2+}- channel activation i.e. self-reinforce the excitation process. It would be interesting to investigate whether pretreatment with highly potent CB_1 receptor antagonists can enhance the excitatory signs of pyrethroid poisoning in mammals and whether excitatory effects of pyrethroids can be reduced by CB_1 receptor agonists. In this latter regard CB_1 receptor agonists may have antidotal properties.

Oxadiazine insecticides (JT333 and MP062), like pyrethroid insecticides, inhibited [^{3}H]CP-55,940 binding. The concentration needed for the parent oxadiazine indoxacarb (MP062) to inhibit VGSCs (Zhao et al., 2003) is very similar to that required for inhibition of [^{3}H]CP-55,940 binding however the decarbomethoxylated metabolite (JT333) is more potent in blocking VGSCs than inhibiting [^{3}H]CP-55,940 binding. Both oxadiazine insecticides were unable to stimulate [^{35}S]GTPγS binding but did inhibit CP-55,940-stimulated [^{35}S]GTPγS binding and therefore have antagonist activity at the CB_1 receptor.

The dihydropyrazole insecticide RH3421 is a potent blocker of VGSCs (Salgado, 1992) and inhibits the binding of [^{3}H]batrachotoxinin 20 α-benzoate to mouse VGSCs with an IC_{50} of 0.3 μM (Deecher et al., 1991). The IC_{50} for inhibition of VGSCs is therefore *circa.* 20-fold lower than the IC_{50} (5.7 μM) calculated from our data for inhibition of [^{3}H]CP-55,940 binding by RH3421 and clearly indicates that VGSCs are the

main target for the dihydropyrazole insecticide. When administered subchronically to rats, RH3421 produces cumulative neurotoxicity characterized by periods of lethargy, pacificity and convulsions (Salgado, 1992). These poisoning symptoms are generally believed to be due to VGSC blockade but it is possible that RH3421 might achieve sufficient concentrations in the brain considering it is lipophilic and metabolically stable insecticide to bind to the CB_1 receptor. However apart from the convulsive effects, the poisoning symptomology following sub-chronic administration of RH3421 is more closely aligned with a CB1 receptor agonist.

In synaptosomes prepared from mouse brain both RH5529 and RH3421 (in a similar fashion to TTX) inhibited veratridine-induced increase in intraterminal [Ca^{2+}] by blocking VGSCs (Zhang & Nicholson, 1993; Zhang & Nicholson, 1994). Interestingly both RH3421 and RH5529 in a TTX-insensitive manner also blocked K^+-induced increase in intraterminal [Ca^{2+}] highlighting that both dihydropyrazoles block voltage-gated calcium channels (VGCCs) directly (Zhang & Nicholson, 1993; Zhang & Nicholson, 1994). We originally considered that the inhibition of VGCCs might be due to dihydropyrazoles activating CB_1 receptors in the mouse brain synaptosomes.There is plethora of reports published where cannabinoid agonist have been reported to block N- and/or P/Q type Ca^{2+} channels suggesting possible parallels between cannabinoid agonists and dihydropyrazole insecticides. Cannabinoid agonists also have also been reported to decrease K^+-induced increase in Ca^{2+} influx in oligodendrocytes, 4-aminopyridine induced Ca^{2+} influx and K^+-induced increase in Ca^{2+} influx in cerebellar granule neurons (Wang, 2003; Mato et al., 2009; Nogueron et al., 2001). This decrease in Ca^{2+} influx by cannabinoid agonists has CB_1 receptor-dependent and CB_1 receptor-

independent components (Mato et al., 2009). In order for this line of reasoning to be correct dihydropyrazoles must act as agonists at the CB_1 receptor. However, they were not able to stimulate [^{35}S]GTPγS binding in the present investigation and this clearly rules out the agonist hypothesis. Both dihydropyrazoles inhibited CP-55,940-stimulated [^{35}S]GTPγS binding, demonstrating an antagonist effect at the CB_1 receptor. Another possible explanation is based on the finding that the CB_1 receptor antagonist AM251 also reduced K^+-induced increase in Ca^{2+} influx in oligodendrocytes in both wild type and CB_1 knock-out mice. This prompted the authors to suggest that AM251 was acting through a CB_1 receptor-independent route (Mato et al., 2009). Another highly potent CB_1 receptor antagonist SR14176A also reduced Ca^{2+} influx in cerebellar granule neurons in a pertussis toxin-insensitive manner and therefore most likely through a CB_1 receptor-independent pathway. These results made us think that since dihydropyrazole insecticide RH3421 has some structural similarity with the highly potent CB_1 receptor antagonists SR14176A and AM251, it might be behaving in a similar way to produce the CB_1 receptor agonist-like action of reducing Ca^{2+} influx. It is also likely that the dihydropyrazoles on their own are directly inhibiting VGCCs but it would be interesting to find what type of Ca^{2+} channels these insecticides block. Based on [^{35}S]GTPγS binding data it is evident that dihydropyrazole insecticides are not agonists at the CB_1 receptor. However, dihydropyrazoles may be capable of promoting low level CB_1 receptor antagonist response after exposure but these symptoms would be likely be overwhelmed by sodium channel blocking effects.

Mitochondrial function disrupting chemicals like famoxadone, surangin B, myxothizol and coenzyme Q_2 also inhibited [^{3}H]CP-55,940 binding and as far as I am

aware this represents the first report describing the interaction of these chemicals with the CB_1 receptor. These chemicals bind to the CB_1 receptor at micromolar concentrations and are not able to stimulate [^{35}S]GTPγS binding on their own. However, in a concentration-dependent manner, they inhibit CP-55,940-stimulated [^{35}S]GTPγS binding and thus clearly point towards antagonist effects at the CB_1 receptor. Surangin B and myxothiazol were also able to encroach into the basal component of [^{35}S]GTPγS binding but considering the high concentrations required to produce this effect, a characterization as inverse agonist at the CB_1 receptor needs caution. The ability of surangin B, myxothiazol and coenzyme Q_2 to interact with the CB_1 receptor is likely due to in part due to the nature of their alkyl chains which may represent much less potent mimics of the alkyl pharmacophore of Δ^9-THC.

Benzophenathridine alkaloids like chelerythrine and sanguinarine were earlier shown in our laboratory to inhibit [^{3}H]CP-55,940 binding. The IC_{50s} of chelerythrine and sanguinarine in the [^{3}H]CP-55,940 binding assay is in the range of 1 - 2 μM, which places these two benzophenathridine alkaloids close in potency to cannabidiol (Compton et al., 1993) and virodhamine (Steffens et al., 2005). In contrast, highly potent CB_1 receptor ligands like Δ^9-tetrahydrocannabinol and Δ^9-tetrahydrocannabivarin (a competitive CB_1 receptor antagonist) inhibit [^{3}H]CP-55,940 binding with IC_{50s} in the low nanomolar ranges (Devane et al., 1988; Thomas et al., 2005). Chelerythrine chloride has also been reported to inhibit protein kinase C (Herbert et al., 1990). It is highly unlikely that inhibition of [^{3}H]CP-55,940 binding is due to kinase-mediated phosphorylation since the membrane fraction employed in our binding buffer is unlikely to support this mechanism. Both chelerythrine and sanguinarine like AM251 inhibited agonist-

stimulated [^{35}S]GTPγS binding. When these alkaloids were tested at greater than maximum effect concentration, both strongly encroached into the basal component of [^{35}S]GTPγS binding whereas AM251 when tested at higher concentrations was not able to produce this effect. In our laboratory both chelerythrine and sanguinarine were tested for their effect on basal [^{35}S]GTPγS binding by Amey Dhopeshwarkar and here, also unlike AM251, both benzophenathridine alkaloids strongly inhibited the basal binding. Based on the ability of chelerythrine and sanguinarine to modulate the G protein-coupling cycle associated with the CB_1 receptor, it would be reasonable to classify both chelerythrine and sanguinarine as inverse agonists at the CB_1 receptor (Dhopeshwarkar et al., 2011).

We hypothesize that chelerythrine and sanguinarine might not be binding to the CB_1 receptor like Δ^9-THC and other phytocannabinoids but might exert their inhibitory effect by targeting the guanine nucleotide recognition site on the associated G protein (Dhopeshwarkar et al., 2011). We believe this because both sanguinarine and chelerythrine have been reported to inhibit the binding of the fluorescent GTP probe to the GTP binding site on the protein Rac1b in a competitive way (Beausoleil et al., 2009). Both benzophenathridine alkaloids may be therefore inhibiting agonist binding by targeting the guanine nucleotide binding site on the associated G protein and thus this mechanism could represent a retrograde allosteric inhibition whereby a ligand is acting at a different site other than the receptor itself and inhibiting binding of the agonist in a reverse fashion, a mechanism entirely different from phytocannabinoids and other CB_1 receptor ligands which compete with [^{3}H]CP-55,940 binding (Devane et al., 1988; Compton et al., 1993; Dhopeshwarkar et al., 2011). Such an alternative mechanism of

targeting CB_1 receptor might offer a novel therapeutic strategy to help reduce endocannabinoid tone in the CNS. This strategy might also be extended to other GPCRs. Rimonabant which was marketed to treat weight disorders was withdrawn because of unwanted psychiatric side effects and therefore the benzophenathridines may offer prototypic molecules which target the G protein-complex coupled to the CB_1 receptor that may eventually lead to new chemistries beneficial in treating weight disorders (Dhopeshwarkar et al., 2011). The basal binding signal for [^{35}S]GTPγS binding reflects the sum of all GTP binding sites. The [^{35}S]GTPγS binding response to CP-55,940 is 4 to 5 times more sensitive to inhibition by chelerythrine and sanguinarine than the basal [^{35}S]GTPγS binding response. It would be interesting to test these compounds in a cell line transfected with the CB_1 receptor where only the receptor of interest plus its G-protein is expressed and that would further improve our understanding of the mechanism by which benzophenathridines act as potential inverse agonists at the CB_1 receptor.

We also considered that our study compounds might not directly interact with the CB_1 receptor at all, but might display their effects by inhibiting endocannabinoid degrading enzymes (FAAH or MAGL) that might be present in the membranes used in the binding assay, thus increasing the level of anandamide and 2-AG in the system (Dhopeshwarkar et al., 2011). Such a mechanism was shown to be operational for organophosphorus pesticides (Noumara, 2008). Such a mechanism cannot be operating in our system because 1) data from our laboratory has shown that 0.5 mM PMSF (a concentration which fully inhibits FAAH and about 50% MAGL) has no effect on [^{3}H]CP-55,940 binding and 2) if such an mechanism was operational in our system then increased levels of anandamide and 2-AG resulting from enzyme inhibition would have

increased [^{35}S]GTPγS binding, an effect we have never observed with benzophenanthridines.

As stated earlier, chelerythrine has been shown to inhibit protein kinase C but such a mechanism is unlikely to contribute to the effect it produces in our system. Stimulation of protein kinase C leads to phosphorylation of CB_1 receptors and this inhibits WIN-55,212-2-mediated activation of inwardly rectifying potassium current (K-ir current) and agonist-mediated inhibition of P/Q-type calcium channels (Garcia et al., 1998). If our results were as a result of protein kinase C inhibition then we would have seen no effect or possibly a stimulatory effect in [^{35}S]GTPγS binding, and since we did not see this profile with chelerythrine this strengthens our conclusion that this effect is protein kinase C-insensitive (Dhopeshwarkar et al., 2011).

TBT compounds are used as antifouling agents but there has been a concerted effort 2008 onwards in many countries to ban their environmental use as they are very toxic to both target and non-target organisms (Qian et al., 2010; Oliveira & Santelli, 2010). Triphenyltin is also used in pesticide formulations (Oliveira & Santelli, 2010). Unfortunately, TBT compounds can readily enter the food chain and TBT compounds can readily access mussels and fish (Oliveira & Santelli, 2010). We found TBT benzoate and phenylethynyl TBT inhibited basal [^{35}S]GTPγS considerably at low concentrations. Again, it is not possible that they are inhibiting FAAH and MAGL like organophosphorus pesticides (Nomura et al., 2008) since if they were, they would have increased the [^{35}S]GTPγS binding signal.

In the present investigation, our results have shown that our hypothesis concerning binding topographies common to both VGSCs and CB_1 receptors is supported

since a number of VGSC-targeting insecticides can interact with the CB_1 receptor and behave as antagonists *in vitro*. This research considered alternative mechanisms that neurotoxic insecticides might interact with the nervous system in addition to the well-established VGSC-directed actions. The symptoms of poisoning of some insecticides were more consistent with a CB_1 receptor agonist component but my [^{35}S]GTPγS binding data clearly supports antagonist actions. The antagonist effect at the CB_1 receptor suggest that these insecticides could still interfere with normal "endocannabinoid tone" in the CNS if they build to sufficient concentrations. This research also highlights three novel classes of chemical which include 1) mitochondrial function-disrupting chemicals, 2) benzophenanthridine alkaloids and 3) TBT compounds that interact at micromolar concentrations with the CB_1 receptor of mouse brain. Also a possible retrograde allosteric inhibition involving interaction of benzophenathridine alkaloids with the G protein component of the CB_1 receptor is proposed. We have put forward strong evidence that both chelerythrine and sanguinarine act as inverse agonists at the CB_1 receptor, however *in vivo* studies would be needed to support this claim fully. This report also supports the widely held view that a highly specific structural configuration is needed for a ligand to behave as agonist at the binding pocket on the CB_1 receptor, since none of our study compounds acted as agonist (Howlett et al., 1988; Little et al., 1988; Compton et al., 1992; Shim et al., 2003). CB_1 receptors are widely expressed in mammalian brain and modulate important functions in the CNS (Herkenham et al., 1990). There are few therapeutic products based on cannabinboids availaboratoryle in the market like Sativex® (Δ^9-THC and cannabidiol in 1:1 proportion used to treat neuropathic pain in multiple sclerosis patients), Marinol® and Cesamet® (synthetic Δ^9-THC to treat nausea and

vomiting associated with cancer chemotherapy and anorexia in AIDS patients), Cannabinor (binds to the CB_2 receptor and is currently under investigation as an analgesic) and Health Canada sells cannabis herb for medicinal purposes (Hanus, 2009; Grotenhermen., 2006). In spite of these products being marketed, the therapeutic potential of cannabinoid drugs has likely not yet been realized because of difficulties associated with separating therapeutic effects from the undesirable psychiatric side effects. However, non-psychotropic cannabinoids like cannabidiol may have significant therapeutic potential in the future (Ward & Raffa, 2011; Russo et al., 2007). Cannabidiol, has been reported to have antitumour activity (Ligresti et al., 2006), to reduced gastrointestinal hypermotility in inflammatory bowel disease (Capasso et al., 2008), and to reduce seizure activity in various animal models (Mechoulam et al., 2007; Zuardi, 2008). It also has good antioxidant properties which might be useful in neurodegenerative diseases like Parkinson's, Alzheimer's and Huntington's disease (Izzo et al., 2009).

In summary this part of the investigation has demonstrated a range of environmental chemicals with diverse structures that interact with CB_1 receptors at low to moderate micromolar concentrations. Some may serve as templates for designing more potent CB_1 receptor-selective antagonists, which in turn might have therapeutic potential. There is a distinct possibility that some of the chemicals examined in this study might also interact with the CB_2 receptor and therefore interfere with immunomodulatory outcomes. Studies are underway in our laboratory to investigate possible interactions with the CB_2 receptor. There is also a possibility that in addition to the centrally located CB_1 receptor, some of the study compounds may also modulate CB_1 receptor function in other

organ systems, for example, the gastrointestinal system (Adami et al., 2002; Coutts & Izzo, 2004) and the reproductive system (Banerjee et al., 2011).

3: Effect of cannabinoids on exocytosis in synaptosomes as determined by acridine orange (AO) release

3.1 Abstract

This investigation examined the actions of CP-55,940 and WIN 55212-2 [both agonists of the cannabinoid-1 (CB_1) receptor] and AM251 (a CB_1 receptor antagonist) on synaptic vesicle exocytosis (as determined by release of the fluoroprobe acridine orange) in synaptosomes prepared from mouse brain. Veratrine, 4-aminopyridine and KCl produced robust exocytotic signals when synaptosomes were carefully washed in physiological saline immediately following their loading with acridine orange. Exocytotic responses to 4-aminopyridine and veratrine were strongly inhibited both by tetrodotoxin (indicating participation of VGSCs only) and also when assays were conducted using Ca^{++}-free saline. Exocytosis stimulated by KCl was totally suppressed in the absence of Ca^{++} and was unaffected by tetrodotoxin. The CB_1 receptor agonists CP-55,940 and WIN 55,212-2 produced a concentration-dependent inhibition of KCl-dependent synaptic vesicle exocytosis to a maximum of 38.3 ± 4.6% (CP-55940) and 47.8 ± 0.4% (WIN 55212-2) at 2 µM. Paradoxically, the CB_1 receptor antagonist AM251 caused a 57.3 ± 6.5% reduction in KCl-induced exocytosis at 2 µM and was not able to antagonize the inhibitory effects of either agonist. Selected environmental chemicals found to display antagonist effects at the CB_1 receptor (see Chapter 2), also failed to antagonize the inhibitory effects of these CB1 receptor agonists.

The results using veratrine, 4-aminopyridine and KCl as depolarizing agents corroborate several known functional responses of synaptosomes and, in particular, verify that voltage-sensitive Ca^{++} channels (but not voltage-sensitive Na^{+} channels) are functionally responsive to KCl challenge. The effects of CP-55,940 and WIN 55,212-2 on KCl-induced synaptic vesicle exocytosis are consistent with the well known effects of CB_1 receptor agonists on the transmitter release process. In contrast, AM251 appears to be blocking either Ca^{++} channels directly (as has been proposed in a few other reports) or some other downstream process activated by KCl-dependent depolarization. Further studies are required to fully evaluate the potential of the acridine orange exocytosis assay in profiling drugs that act on presynaptic CB_1 receptors.

3.2 Introduction

CB_1 receptors are located predominantly on the presynaptic neuronal membrane where they interface directly with G proteins and together they form the initial complex of a presynaptic negative feed-back system regulating the transient and long term components of neurotransmitter release at the synapse (Howlett et al., 1986; Katona et al., 1999; Kawamura et al., 2006; Chevaleyre et al., 2006). Depolarization of postsynaptic neurons triggers Ca^{2+}-dependent synthesis of endocannabinoids, from where they migrate towards presynaptic CB_1 receptors and activate them (Wilson & Nicoll, 2001). Stimulation of the CB_1 receptor-dependent G protein machinery activates inwardly rectifying potassium channels (Deadwyler et al., 1995; Mackie et al., 1995), inhibits adenylate cyclase activity (Howlett & Fleming, 1984; Howlett, 1984; Howlett et al., 1988) and inhibits voltage-sensitive N- and/or P/Q-type Ca^{2+} channels (Mackie & Hille, 1992; Mackie et al.,1993; Mackie et al., 1995; Twitchell et al., 1997; Wang, 2003;

Kushmerick et al., 2004). The net effect is the downward modulation of neurotransmitter release at the synapse (Kreitzer & Regehr, 2001a & b; Wilson & Nicoll, 2001; Wilson et al., 2001; Wilson & Nicoll, 2002; Chevaleyre et al., 2006). This downward modulation will then reduce the stimulation of the post-synaptic neurons thus completing the negative feed back loop operated by endocannabinoids. This mechanism is of great physiological relevance in regulating synaptic plasticity in the CNS (Chevaleyre et al., 2006). Various neurotransmitters have been reported to have their release regulated by cannabinoids including glutamate (Shen et al., 1996; Levens et al, 1998;Wang, 2003), acetylcholine (Gifford & Ashby, 1996; Gessa et al, 1997; Gifford et al., 1997a & b; Gifford et al., 2000), noradrenaline (Schlicker et al, 1998) and GABA (Miller & Walker, 1995).

WIN-55,212-2 inhibited Ca^{2+}-dependent 4-aminopyridine- (4-AP)-induced release of L-glutamate from rat hippocampal synaptosomes in a pertussis toxin sensitive manner identifying a G protein-mediated inhibition of N- and P/Q-type Ca^{2+} channels (Wang, 2003). In Purkinje cells bath application of WIN-55,212-2 decreased parallel fibre excitatory post-synaptic currents (Levenes et al., 1998). In both brain slices and synaptosomes isolated from the hippocampal and cortical regions of rat brain, WIN-55,212-2 reduced [^{3}H]acetylcholine release (Gifford & Ashby, 1996). CP-55,940 reduced [^{3}H]acetylcholine release, whereas it had no effect on [^{3}H]norepinephrine release from hippocampal brain slices (Gifford et al., 1997). AM281 antagonized the WIN-55,212-2-mediated decrease in [^{14}C]acetylcholine release from superfused hippocampal slices (Gifford et al., 1997).

Synaptosomes are isolated pinched-off nerve terminals, which functionally mimic *in vivo* nerve terminals very closely. Synaptosomes represent an excellent tool to study

neurotransmitter release since they retain all the metabolic machinery for the uptake and storage of neurotransmitter and they release neurotransmitters when appropriate stimuli are applied (Whittaker et al, 1964; Whittaker, 1993; Dunkley et al, 2008). A large number of studies have made use of synaptosomes to study neurotransmitter release and even four decades after the initial report of their isolation, they remain a very popular *in vitro* model to study neurotransmitter release (Whittaker, 1993; Gifford et al., 2000; Deng & Nicholson, 2003; Nicholson et al., 2003; Wang, 2003). Depolarization of the synaptosomal plasma membrane by elevated concentrations of external K^+, the calcium ionophore ionomycin, Na^+ channel activators like veratridine and veratrine, and the K^+ channel blocker and indirect sodium channel activator 4-AP, have been shown to induce neurotransmitter release (SanchezPrieto et al., 1987; Nicholson et al., 2003; Wang, 2003).

Acridine (AO) is a weakly basic pH-sensitive fluorescent dye that has been used to study exocytosis and endocytosis in synaptosomes (Zoccarato et al., 1999; Melnik et al., 2001). AO in its un-ionized (amine) form can freely cross biological membranes but when protonated inside the acidic interior of synaptic vesicles it becomes trapped inside (Melnik et al., 2001). AO accumulation is mostly restricted to those organelles expressing V-type ATPase, an ATP-energized H^+ pump which is abundant in synaptic vesicles and sensitive to inhibition by bafilomycin (Floor et al., 1990; Moriyama & Futai, 1990; Zoccarato et al., 1999; Melnik et al., 2001). Addition of AO to a synaptosomal preparation results in immediate quenching of the fluorescence signal as AO is taken up into acidic compartments. Upon depolarization with high external K^+, exocytosis results in a fraction of the dye being externalized causing a fluorescence increase. This is followed by vesicle endocytosis which can be tracked as a decrease in fluorescence signal

often going below the pre-depolarization baseline as some of the externalized dye is taken up (Zoccarato et al., 1999; Melnik et al., 2001). The reuptake of AO is abolished in the presence of bafilomycin when it is added just before depolarizing with KCl (Zoccarato et al., 1999). This result supports the idea that the reuptake of AO is entirely dependent upon the V-type ATPase (Zoccarato et al., 1999). The slower reuptake of AO into synaptic vesicles is consistent with the time required for vesicular cycling and this explains why the reuptake phase is slower compared to vesicular release of AO (Zoccarato et al., 1999; Melnik et al., 2001).

In the present investigation, I studied the effect of cannabinoids on AO exocytosis from synaptosomes isolated from mice brain. This assay could represent a novel and rapid method for assessment of cannabinoid receptor agonists, antagonists and inverse agonists and to best of our knowledge no report has specifically explored the effect of cannabinoids on AO exocytosis. I was interested in this possibility because, as previously discussed, AO exocytosis is a Ca^{2+}-dependent effect. Moreover, activation of the CB_1 receptor inhibits different types of voltage-gated Ca^{2+} channels (VGCCs) and therefore if the CB_1 receptor is activated by an agonist it would inhibit VGCCs and this would in turn inhibit Ca^{2+} influx into synaptosomes. The ultimate effect should be a reduction in exocytosis (AO release) and this process should be antagonized by a cannabinoid receptor antagonist. If successful, this method could be used directly to investigate the CB_1 receptor antagonist effects of the chemicals described in Chapter 2.

In the present investigation, synaptosomes were chosen as an *in vitro* model as it has been shown previously by Wang (2003) to be a suitable model to study effects of cannabinoid agonists and antagonist on release of glutamic acid. Wang (2003)

demonstrated that cannabinoid agonists partially inhibit glutamate release through a CB_1 receptor dependent inhibition of VGCCs.

3.3 Material and Methods

3.3.1 Reagents and chemicals

AO hydrochloride, DMSO, CP-55,940, AM251, WIN-55,212-2, EGTA, acetone and all reagents used to make buffers were purchased from Sigma Aldrich-Canada. Micro syringes (Hamilton, USA) were purchased from Science Stores at SFU, Burnaby, Canada. The magnetic stirrer used for stirring the contents of the quartz cuvette and Percoll was purchased from Perkin Elmer. A metal needle fabricated by Dr. Russell A. Nicholson was used for layering different Percoll solutions. 10 ml polycarbonate centrifuge tubes with inserts for the JA-20 rotor of the Beckman J2HS was used for isolating synaptosomes. 4-AP, veratrine and TTX were present in the laboratory from previous investigations.

3.3.2 Animals

Male CD1 mice (20-25 g) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada). All orders for mice were placed through Animal Care Facility (ACF) at Simon Fraser University, Burnaby, Canada. Mice were maintained on a 12 hour light and dark cycle and supplied with food and water *ad libitum*. All procedures using mice complied with Canadian Council on Animal Care standards for the use of animals in research and had approval of the Simon Fraser University Animal Care Committee.

3.3.3 Preparation of synaptosomes

Synaptosomes were prepared by employing the Percoll gradient procedure developed by Dunkley et al. (2008) with few minor modifications adopted from Tarasenko et al. (2006). Percoll gradient solutions (3%, 10%, 15% and 23%) were prepared on the day of the experiment and stored on ice. Two 12 ml polycarbonate centrifuge tubes were mounted on a metal stand inside the cold room (0-4 °C) and 2 ml of the 23% percoll gradient solution was transferred to the bottom of both polycarbonate tubes using a 3 ml syringe with a 9-10 cm long satinless steel needle. Then, carefully and slowly (over a period of 2 minutes with uniform release) 2 ml of 15% Percoll gradient solution was layered on the top of 23 % layer. Similarly 10% and then 3% Percoll gradient solutions were layered on top of 15% layer. A clear schlieren line could be observed at the interface of any two Percoll layers. Polycarbonate tubes with the Percoll gradients were carefully transferred to an ice-bucket inside the cold room and stored until needed. Mice were euthanized first by cervical dislocation and whole mouse brains were removed within 15 seconds. All isolation and subsequent membrane preparation procedures were done at 0-4 °C. All the glassware, buffers and centrifuge tubes used in the synaptosomal isolation procedure were prechilled before the start of experiment by placing them in an ice-filled bucket and storing the ice-bucket inside a cold room (0-4 °C). Two mouse brains were transferred to a 15 ml glass beaker and rinsed 3 times with 5 ml of ice-cold homogenizing buffer [sucrose (0.32 mM), EDTA (1 mM) and Trisma base (5 mM) with pH adjusted to 7.4 with HCl] to remove excess blood. After rinsing, the brains they were transferred to 15 ml of homogenization buffer in a glass Teflon-glass tissue grinder. Mouse brain tissue was homogenized with 10 even strokes (one up and one down) using a motor driven homogenizer (pestle rotation 500-800 rpm.). The

homogenate was transferred to a 50 ml centrifuge tube and centrifuged in a Beckman J2HS centrifuge using JA-20 rotor at for 15 minutes (1,000 x g based on the average radius of the centrifuge tube). The supernatant ($S1_A$) obtained from the first centrifugation was stored on ice and the pellet (P1) was resuspended gently (5 up and down strokes) using a glass homogenizer (7 ml; Uniform, England). The homogenate was further diluted to 30 ml with the homogenizing buffer and centrifuged again for 15 minutes at 1000 x g. The supernatant $S1_B$ was pooled with the $S1_A$ supernatant and centrifuged at 15000 x g for 30 minutes. The resulting supernatant (S2) was discarded and the pellet (P2) was gently homogenized using a glass homogenizer (1 ml; Uniform, England). The homogenate was further diluted to 2 ml with homogenization buffer and kept on ice. 1 ml of the homogenate was carefully and very slowly layered on the top of the 3 % Percoll layer in each polycarbonate tube. Each polycarbonate tube was inserted into the rubber inserts to position them in the rotor. Both polycarbonate tubes were then centrifuged at 31,000 x g at 4 °C for precisely 6 minute 28 seconds. This time takes into account the time our J2HS centrifuge takes to reach 20,000 rpm. plus 5 min centrifugation at 20,000 rpm. Both polycarbonate tubes were then placed on ice and fractions at the 10% - 15% interface and 15% - 23% were collected. Both fractions were pooled in a 100 ml glass beaker (held in ice) and diluted with ice-cold homogenization buffer up to 80 ml over a period of 30 minutes with gentle mixing. After dilution, the suspension was divided equally between two 50 ml centrifuge tubes and centrifuged at 20,000 x g at 4 °C for 30 minutes. Supernatants in both tubes were discarded and the two synaptosomal pellets were gently resuspended in 1 ml assay buffer [(NaCl (140 mM), KCl (4 mM), $KHCO_3$ (5 mM), $MgCl_2.6H_2O$ (1 mM), Na_2HPO_4 (1.2 mM), HEPES (10

mM), glucose (10 mM), BSA (1 mg/ml) and $C_3H_3NaO_3$ (4 mM))] using a glass homogenizer (1 ml; Uniform, England). The final synaptosomal homogenate was equally distributed in each of 8 snap top vials and all vials were placed in ice. The final protein concentration in each aliquot is approximately 650 µg per vial as determined using the method of Peterson (1977).

3.3.4 Acridine orange exocytosis assay

Experiments using the pH-sensitive fluorescent indicator acridine orange (AO) were conducted according to the methods published by Zoccarto et al. (1999) and Melnik et al. (2001) with modifications. 875 µl of assay buffer and 5 µl of acridine orange (5 µM final concentration) were added to synaptosomes contained in the snap top vial. Synaptosomes were then incubated at 35 °C with gentle shaking for 10 minutes. After 10 minutes the synaptosomes were centrifuged for 30 seconds using a Beckman Microfuge E. The synaptosomal pellet was gently resuspended using a 1 ml air displacement pipette in 1 ml of fresh assay buffer after removing the supernatant. Resuspension was done slowly (5-7 times). The resulting homogenate was further centrifuged for 30 seconds and the supernatant removed. The final synaptosomal pellet was gently resuspended using the 1 ml pipette (5-7 times). The washed synaptosomal suspension was further diluted to 2.5 ml using assay buffer maintained at 35 °C. The resulting synaptosomal suspension loaded with acridine orange was then transferred to a stirred quartz fluorescence cuvette thermostated at 35 °C. The system was equilibrated for 5 minutes and then 2 µl DMSO, Ca^{2+} (2 mM) or TTX (7 µM) or EGTA (2 mM) according to the needs of the particular experiment was added within the first 1 minute. The total volume of DMSO in each assay was kept constant at 2 µl. If the effect of an antagonist was to be investigated on acridine

orange exocytosis (whether AM251 or study compounds), they were introduced in the next minute in 1 µl DMSO by microsyringe (Hamilton, USA). The cannabinoid agonists CP-55,940 or WIN-55,212-2 were added in 1 µl DMSO using a microsyringe in the last 30 seconds before starting the fluorescence recording. An excitation wavelength of 490 nm was used and the emission intensity was continously sampled at 530 nm using a Perkin Elmer LS-50 fluorescence spectrophotometer. The magnetic stirrer was set at high speed and slit widths were set at 5 for both emission and excitation beams. Synaptosomes were depolarized at 600 seconds using KCl (30 mM final concentration), veratrine (50 µM final concentration based on the molecular weight of veratridine the major component) or 4-aminopyridine (40 µM or 100 µM final concentration), and the experiment was terminated at 1000 seconds. At the end of each fluorescence run, the quartz cuvette was washed with running hot water for 30 seconds and then dipped in fresh acetone kept in two 50 ml beakers (three times in each beaker) and left to dry. The acetone in each beaker was discarded and filled with new acetone for the next run. Addition of control vehicle (2 µl DMSO) did not affect acridine orange exocytosis.

3.3.5 Data analysis

Peak heights of AO exocytosis were measured using the vertical analysis tool in Perkin Elmer LS-50 fluorescence spectrophotometer and percentage inhibition was calculated in comparison to the control. Time resolved fluorescence data points (0 to 1000 sec) were transferred from the Perkin Elmer LS-50 fluorescence spectrophotometer to Graphpad prism (5.0) and fluorescence traces were then constructed. Percentage inhibition of AO exocytosis was calculated as mean ± S.E.M. based on a minimum of 3 – 4 independent experiments conducted for each concentration of study compound. Where

appropriate, two-tailed Student's t test with 95% confidence interval was performed using Graphpad prism (5.0).

3.4 Results

3.4.1 Effect of KCl on synaptosomes loaded with AO

Depolarization of synaptosomes by KCl (30 mM) in the presence of Ca^{2+} (2 mM) caused robust exocytosis of AO (Figure 3-1). Rapid exocytosis of AO was followed by endocytosis and the fluorescence signal went below the starting basal fluorescence level (i.e. the pre-KCl baseline) (Figure 3-1). In the absence of Ca^{2+} and in presence of EGTA (2 mM) there was not a single fluorescence unit increase, clearly showing this release of AO to be fully Ca^{2+}-dependent (Figure 3-1). In presence of TTX (7.2 μM) there was no significant effect (NS; $P > 0.05$) on Ca^{2+}-dependent release of AO when synaptosomes were depolarized by KCl (Figure 3-1). The lack of effect of TTX on KCl-induced AO exocytosis shows that VGSCs are not involved in KCl-stimulated release of AO from synaptosomes (Figure 3-1 and Figure 3-2). In cultured optical nerve oligodendrocytes, 50 mM KCl-induced Ca^{2+} influx was unaffected by 1 μM TTX (Mato et al., 2009) in agreement with my result that VGSCs do not participate in the Ca^{2+} increase following depolarization with KCl. Another report has also confirmed the lack of Na^{+} channel involvement in KCl-induced elevation of Ca^{2+} (Galvan & Sitges, 2005). My fluorescence traces of exocytosis and endocytosis of AO from synaptosomes, when depolarized by KCl are qualitatively very similar to those reported previously by Zoccarato et al. (1999) and Tarasenko et al. (2006). The peak heights observed for KCl-, veratrine- and 4-AP-induced AO exocytosis were more intense than the earlier reports.

3.4.2 Effect of veratrine (VTN) on AO exocytosis

Veratrine (50 µM), like KCl also caused robust exocytosis of AO from synaptosomes but strong endocytosis (quenching of fluorescence signal) was not observed after depolarization with VTN (Figure 3-2). This behavior is consistent with the earlier report by Liao & Nicholson (2007). The effect of VTN (50 µM) was blocked significantly ($P < 0.05$) by TTX (7.2 µM), clearly indicating involvement of VGSCs in exocytosis induced by VTN (Figure 3-2). The effect of EGTA (2 mM) was quantitatively similar to the effect seen in presence of TTX ($P > 0.05$) and again emphasizing the role of Ca^{2+} in AO exocytosis (Figure 3-2).

3.4.3 Effect of 4-AP on AO exocytosis

4-AP at 40 µM and 100 µM depolarized synaptosomes and activated AO exocytosis. The release pattern was similar to VTN but again unlike stimulation by KCl, a massive endocytosis response was not seen. The increase in fluorescence signal by 4-AP (100 µM) was blocked significantly ($P < 0.05$) in presence of 7.2 µM TTX indicating involvement of VGSCs in depolarization by 4-AP (Figure 3-3). This result accords with another report demonstrating involvement of tetrodotoxin-sensitive Na^{+} channels in the activation of presynaptic Ca^{2+} channels by 4-AP (Galvan & Sitges, 2005). Removal of Ca^{2+} with EGTA addded decreased 4-AP-induced AO exocytosis, which again aligns with the results of Galvan & Stiges. (2005). The effect of TTX and removal of Ca^{2+} with EGTA present greatly reduced 4-AP induced AO release and therefore emphasizes the importance of Ca^{2+} influx and activation of TTX-sensitive VGSCs in this response (Figure 3-3).

3.4.4 Effect of CP-55,940 on K^+-induced AO exocytosis from mouse brain synaptosomes

CP-55,940 inhibited 30 mM K^+-induced AO exocytosis from mouse brain synaptosomes in a concentration-dependent manner (Figure 3-4). There was no obvious effect of CP-55,940 on the shape of the exocytosis and endocytosis profile of AO release when CP-55,940 decreased the height of the AO fluorescence signal after K^+ challenge. This change in peak height is represented as percent inhibition compared with the control (Figure 3-4). The percent inhibition (mean ± S.E.M.) of K^+-induced AO exocytosis by 50 nM and 2 µM of CP-55,940 was 5.50 ± 0.74 and 38.34 ± 4.60 respectively. This result is in agreement with other reports discussed in the Introduction section where cannabinoid agonists have been shown to decrease neurotransmitter release.

3.4.5 Effect of WIN-55,212-2 on K^+-induced AO exocytosis from mouse brain synaptosomes

WIN-55,212-2 like CP-55,940 (in a concentration-dependent manner and without altering the peak shape of AO release) reduced the peak height of AO exocytosis when stimulated by KCl (Figure 3-5). The percent inhibition (mean ± S.E.M.) of the K^+-induced AO exocytosis peak height by 50 nM and 2 µM WIN-55,212-2 was 9.29 ± 1.07% and 47.83 ± 0.38% respectively. Both the minimum and maximum inhibitory effects observed for WIN-55,212-2 at 50 nM and 2 µM were greater than those observed for CP-55,940 suggesting WIN-55,212-2 to be more effacacious and possibly of higher potency in our system than CP-55,940.

3.4.6 Effect of AM251 on K^+-induced AO exocytosis from mouse brain synaptosomes

AM251 also paradoxically reduced K^+-induced AO exocytosis from mice brain synaptosomes (Figure 3-6). We originally predicted AM251 should have no effect on K^+-induced AO exocytosis or may even enhance AO exocytosis considering it is an antagonist/inverse agonist at the CB_1 receptor. The percent inhibition (mean ± S.E.M.) of the K^+-induced AO exocystosis peak height by 50 nM and 2 µM of AM251 was 6.86 ± 2.34 and 57.28 ± 6.52 respectively (Figure 3-6). The maximum inhibition produced by AM251 was greater than that of CP-55,940 and WIN-55,212-2 based on effects at the highest concentration of 2 µM (Figure 3-6).

3.4.7 Effect of AM251 on WIN-55,212-2- and CP-55,940-mediated inhibition of K^+-induced AO exocytosis in synaptosomes isolated from mouse brain

I next explored the effect of AM251 on WIN-55,212-2-mediated inhibition of K^+-induced AO exocytosis. AM251, as suspected from my previous results, was not able to block WIN-55,212-2-mediated inhibition of AO exocytosis. In my experiments I had four controls which included 400 nM WIN-55,212-2, 1 µM WIN-55,212-2, 400 nM AM251 and 1 µM AM251 so as to test whether a low or high concentration of AM251 can antagonize WIN-55,212-2's effect. The combinations tested were 400 nM WIN-55,212-2 versus 400 nM AM251, 400 nM WIN-55,212-2 versus 1 µM AM251, and 1 µM WIN-55,212-2 versus 1 µM AM251. None of these combinations supported the idea that AM251 blocks the inhibitory effects of WIN-55,212-2. Representative data for WIN-55212 (1 µM) versus AM251 (1 µM) has been shown (Figure 3-7). In an identical experimental design, I tested the same AM251 combinations with CP-55,940 as the agonist. Like WIN-55,212-2, the inhibitory effects of CP-55,940 on K^+-induced AO

exocytosis were not antagonized by AM251, giving further support to a paradoxical (CB_1 receptor-independent) mechanism of action of AM251in this system (Figure 3-8).

3.4.8 Effect of CP-55,940 and AM251 on VTN- and 4-AP-induced AO exocytosis from synaptosomes isolated from mouse brain

I also felt it is important to investigate the effect of cannabinoids on VTN-induced and 4-AP-induced AO exocytosis since a previous published report from our laboratory has shown that at low micromolar concentrations, cannabinoids inhibit veratridine-(VTD-) dependent depolarization of synaptosomes and also VTD-induced release of L-glutamate and GABA from synaptosomes (Nicholson et al., 2003). Inhibition of VTN-induced AO exocytosis based on peak height reduction by 50 nM CP-55,940 was not significant ($P > 0.05$) whereas inhibition caused by 2 µM CP-55,940 was significant ($P < 0.05$) supporting our earlier findings (Nicholson et al., 2003) that the inhibitory effects on VGSCs become evident at low micromolar concentrations (Figure 3-9). Inhibition produced by AM251 at both 500 nM and 2 µM was not significant ($P > 0.05$) (Figure 3-9). CP-55,940 and AM251 both tested at 2 µM significantly ($P < 0.05$) inhibited 4-AP induced peak height of AO exocytosis (Figure 3-9). CP-55,940 and AM251 both tested at 1 µM had no additional effect on KCl-induced AO exocytosis in presence of TTX (7 µM) and thus ruling out involvement of VGSCs in the depolarization produced by KCl (data not shown). The stability testing of synaptosomes was also monitored and there was no decrease in the AO response to KCl until at least 4.5 hours. On any day all experiments were completed in less than 4 hours.

3.4.9 Effect of selected environmental chemicals on AO exocytosis

Despite the clearly anomalous behaviour of AM251 in the exocytosis assay several environmental chemicals displaying more potent antagonist effects in the [^{3}H]CP-55,940 and [^{35}S]GTPγS binding assays were investigated for their effect on K^{+}-induced AO exocytosis from synaptosomes. The environmental chemicals examined in the exocytosis assay included TBT benzoate (1.5 and 5 µM), phenylethynyl TBT (10 µM), RH3421 (20 µM and 50 µM) and RH5529 (50 µM). TBT benzoate tested at 1.5 µM did not increase inhibition ($P > 0.05$) whereas at 5 µM it added significantly ($P < 0.05$) to the inhibition caused by 1 µM CP-55,940. Phenylethynyl TBT tested at 10 µM also significantly ($P < 0.05$) added to the inhibition caused by 1 µM CP-55,940 (Figure 3-10 a). RH3421 tested at 20 µM, 50 µM and RH5529 tested at 50 µM significantly increased the inhibition caused by 1 µM CP-55,940 (Figure 3-10 b). The increase in inhibition in presence of RH3421 and RH5529 can be explained based on the earlier reports (Zhang and Nicholson, 1994; Zhang and Nicholson, 1995) where these dihydropyrazole insecticides were shown to decrease K^{+}-induced Ca^{2+} influx in synaptosomes. In summary, none of the test compounds tested at the mentioned concentrations were able to antagonize CP-55,940-mediated inhibition of K^{+}-induced AO exocytosis from the synaptosomes (Figure 3-10 a & b).

3.4.10 Figures

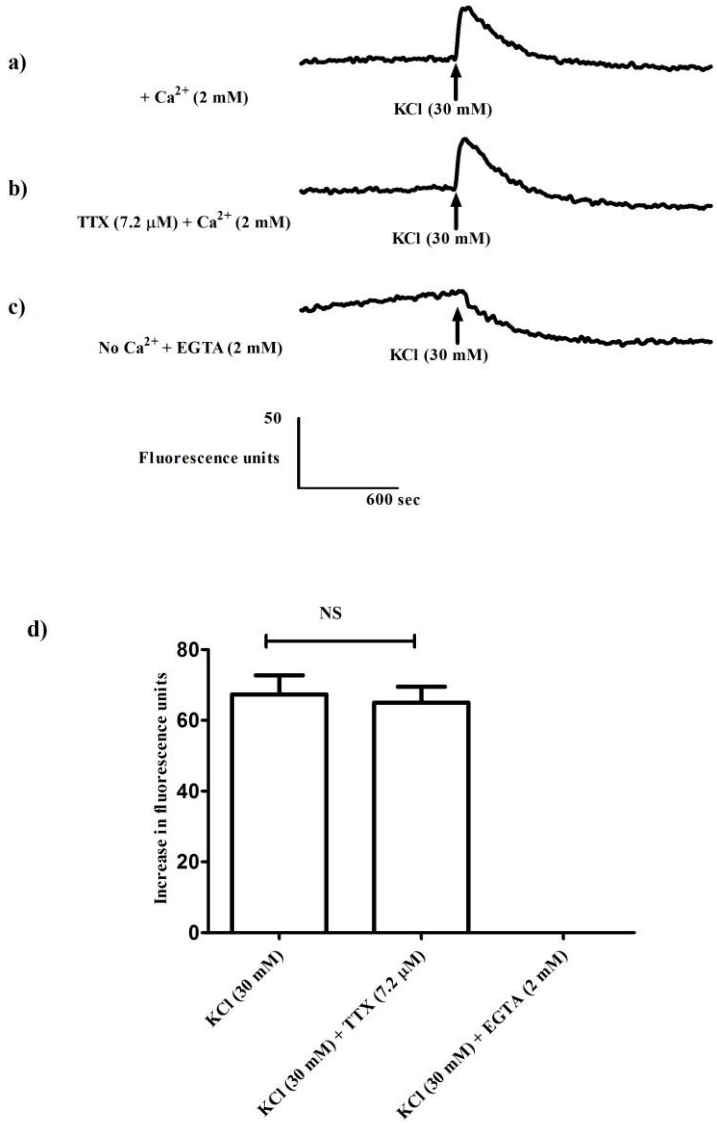


Figure 3-1 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 30 mM KCl with Ca^{2+} present. (b) AO fluorescence recorded in the presence of TTX (7.2 µM) when synaptosomes were depolarized by 30 mM KCl. (c) KCl cannot induce AO exocytosis in the absence of Ca^{2+}. Traces a - c are representative of results from 3 – 4 independent experiments. (d) Change in fluorescence units (mean ± S.E.M.) when synaptosomes were depolarized with KCl (30 mM) in presence of, Ca^{2+} (2 mM), TTX (7.2 µM) + Ca^{2+} (2 mM) and in the absence of Ca^{2+} with EGTA (2 mM) added. (NS = $P > 0.05$).

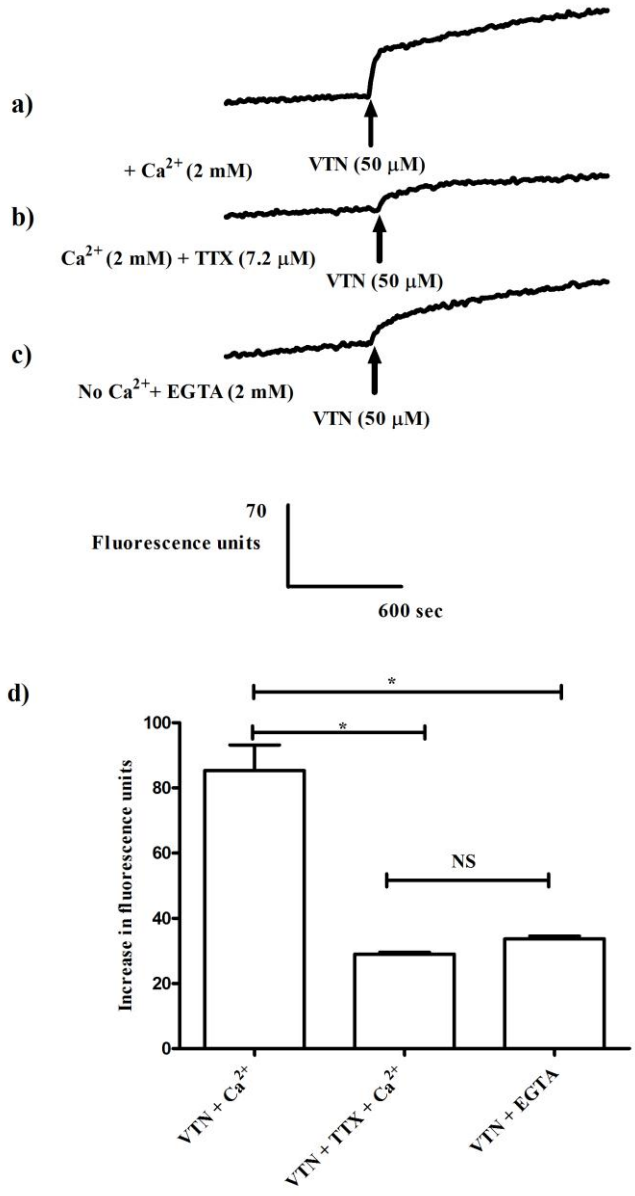


Figure 3-2 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 50 µM VTN with Ca^{2+} present. (b) TTX (7.2 µM) blocks VTN-induced AO exocytosis by 65 % approximately. (c) EGTA (2 mM) produces an effect similar to TTX. Traces a - c are representative of results from 3–4 independent experiments. (d) Changes in fluroescence units (mean ± S.E.M.) when synaptosomes were depolarized with VTN (50 µM) in the presence of, Ca^{2+} (2 mM), TTX (7.2 µM) + Ca^{2+} (2 mM) and in the absence of Ca^{2+} with EGTA (2 mM) added. (* = $P < 0.05$ and NS = $P > 0.05$).

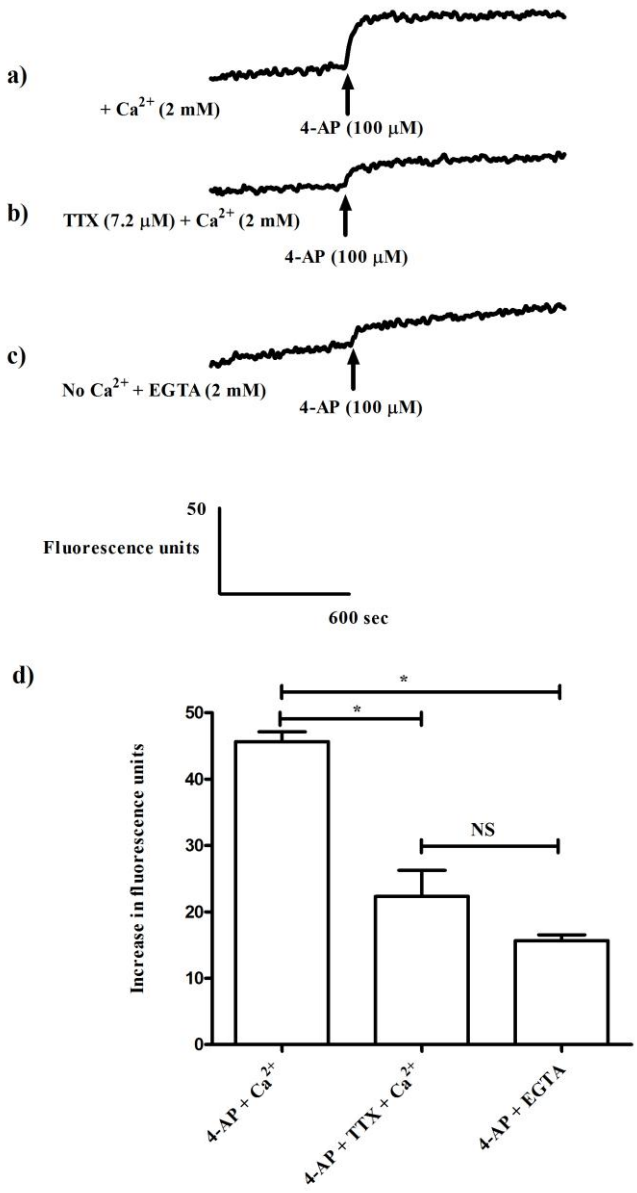


Figure 3-3 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 100 µM 4-AP with Ca^{2+} present. (b) TTX (7.2 µM) blocks 4-AP-induced AO exocytosis by 50% approximately. (c) The Effect of EGTA (2 mM) is similar to the effect seen with TTX. Traces a - c are representative of results from 3–4 independent experiments. (d) Changes in fluorescence units (mean ± S.E.M.) when synaptosomes were depolarized with 4-AP (100 µM) in the presence of, Ca^{2+} (2 mM), TTX (7.2 µM) + Ca^{2+} (2 mM) and in the absence of Ca^{2+} with EGTA (2 mM) added. (NS = $P > 0.05$ and * is significant $P < 0.05$).

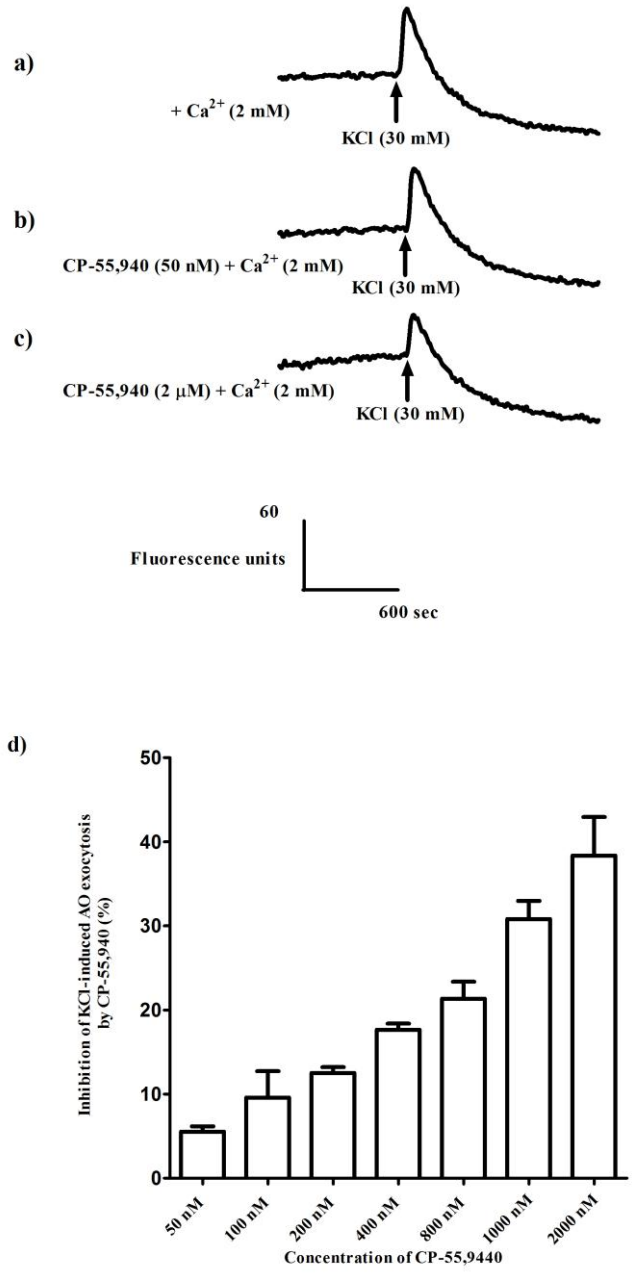


Figure 3-4 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 30 mM KCl with Ca^{2+} present. (b) 50 nM CP-55,940 reduces KCl-induced AO peak height increase by 5.50 ± 0.74% (c) 2 µM CP-55,940 reduces KCl-induced AO peak height increase by 38.34 ± 4.60%. Traces a - c are representative of results from 3–4 independent experiments. (d) Percent inhibition (mean ± S.E.M.) of KCl-induced AO peak height increase by CP-55,940 (50 nM to 2 µM).

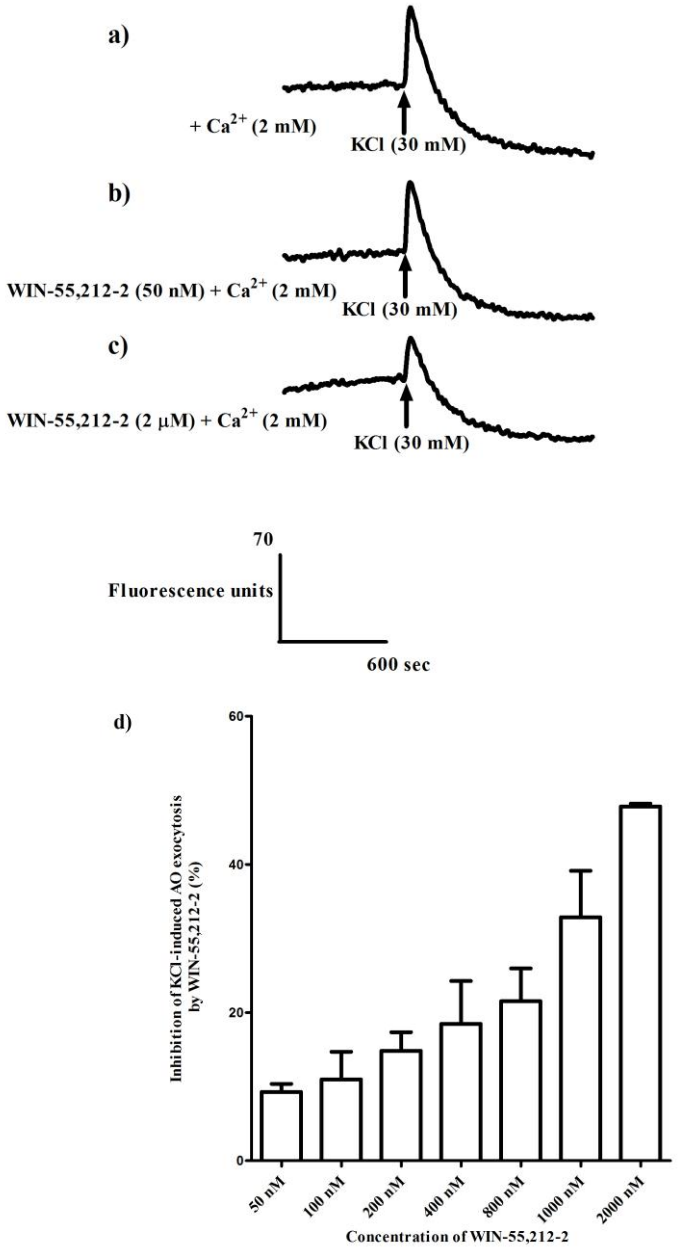


Figure 3-5 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 30 mM KCl with Ca^{2+} present. (b) 50 nM WIN-55,212-2 reduces KCl-induced AO peak height increase by 9.29 ± 1.07%. (c) 2 µM WIN-55,212-2 reduces KCl-induced AO peak height by 47.83 ± 0.38%. Traces a - c are representative of results from 3–4 independent experiments. (d) Percent inhibition (mean ± S.E.M.) of KCl- induced AO peak height increase by WIN-55,212-2 (50 nM to 2 µM).

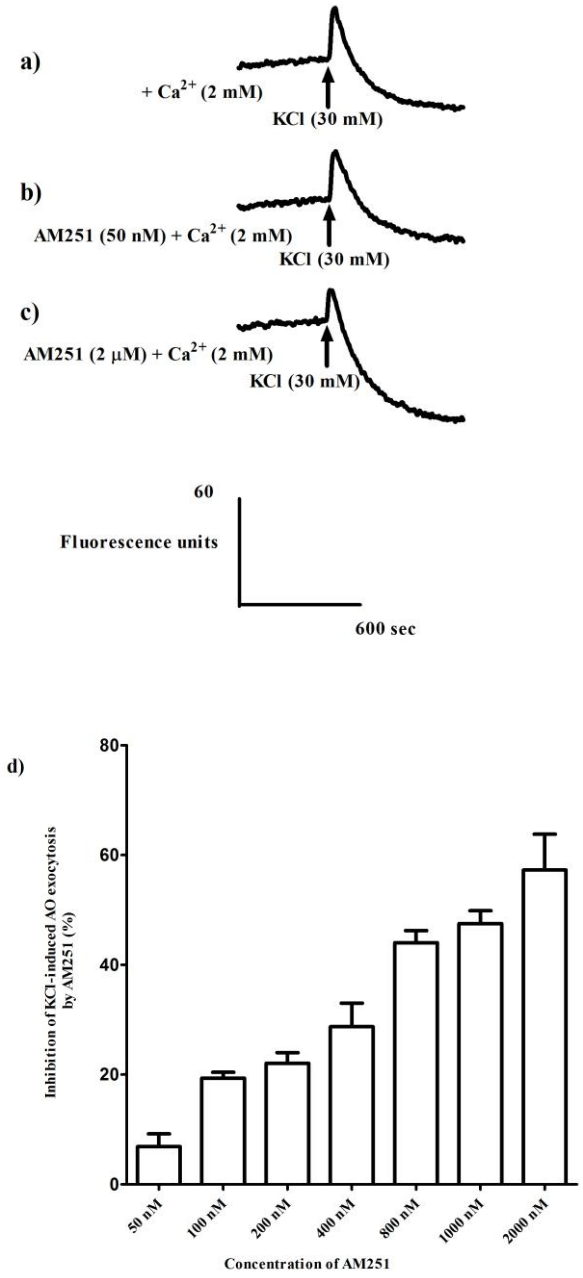


Figure 3-6 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 30 mM KCl with Ca^{2+} present. (b) 50 nM AM251 reduced KCl-induced AO peak height increase by 6.86 ± 2.34%. (c) 2 µM AM251 reduced KCl-induced AO peak height increase by 47.83 ± 0.38%. Traces a - c are representative of results from 3–4 independent experiments. (d) Percent inhibition (mean ± S.E.M.) of KCl-induced AO peak height increase by AM251 (50 nM to 2 µM).

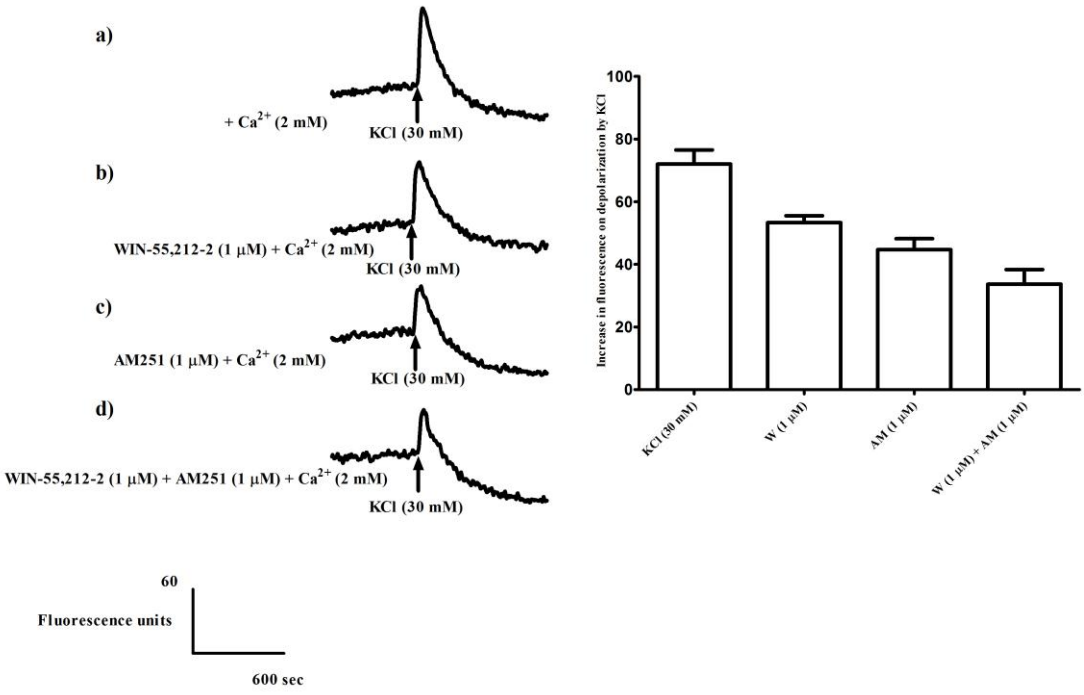


Figure 3-7 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 30 mM KCl with Ca^{2+} present. (b) 1 μM WIN-55,212-2 reduced KCl-induced AO peak height increase by 25.70 ± 1.85%. (c) 1 μM AM251 reduced KCl-induced AO peak height increase by 38.05 ± 1.92% (d) 1 μM AM251 is not able to antagonize the inhibitory effect of 1 μM WIN-55,212-2. Traces a - d are representative of results from 3–4 independent experiments. (e) Increase in AO exocytosis when synaptosomes are depolarized by 30 mM KCl (control) and in presence of 1 μM WIN-55,212-2 (W), 1 μM AM251 (AM) and 1 μM WIN-55,212-2 + 1 μM AM251.

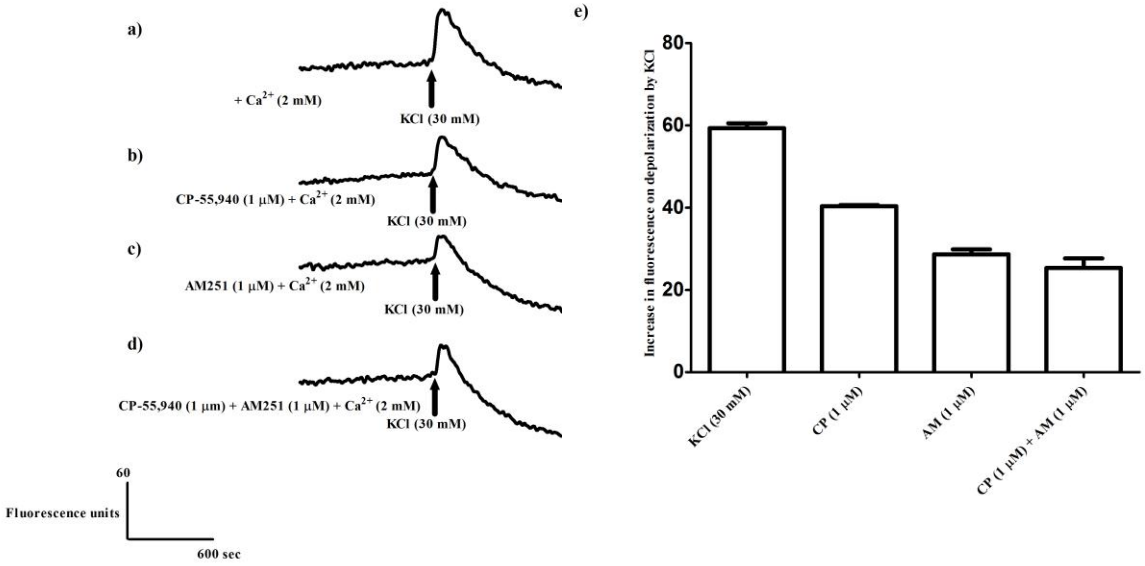


Figure 3-8 (a) Depolarization-induced exocytosis observed as an increase in AO fluorescence when synaptosomes were challenged by 30 mM KCl with Ca^{2+} present. (b) 1 µM CP-55,940 reduced KCl-induced AO peak height increase by 31.98 ± 1.09%. (c) 1 µM AM251 reduced KCl-induced AO peak height increase by 51.57 ± 2.98%. (d) 1 µM AM251 is not able to antagonize the inhibitory effect of 1 µM CP-55,940. Traces a - d are representative of results from 3–4 independent experiments. (e) Increase in AO exocytosis when synaptosomes are depolarized by 30 mM KCl (control) and in presence of 1 µM CP-55,940 (CP), 1 µM AM251 (AM) and 1 µM CP-55,940 + 1 µM AM251.

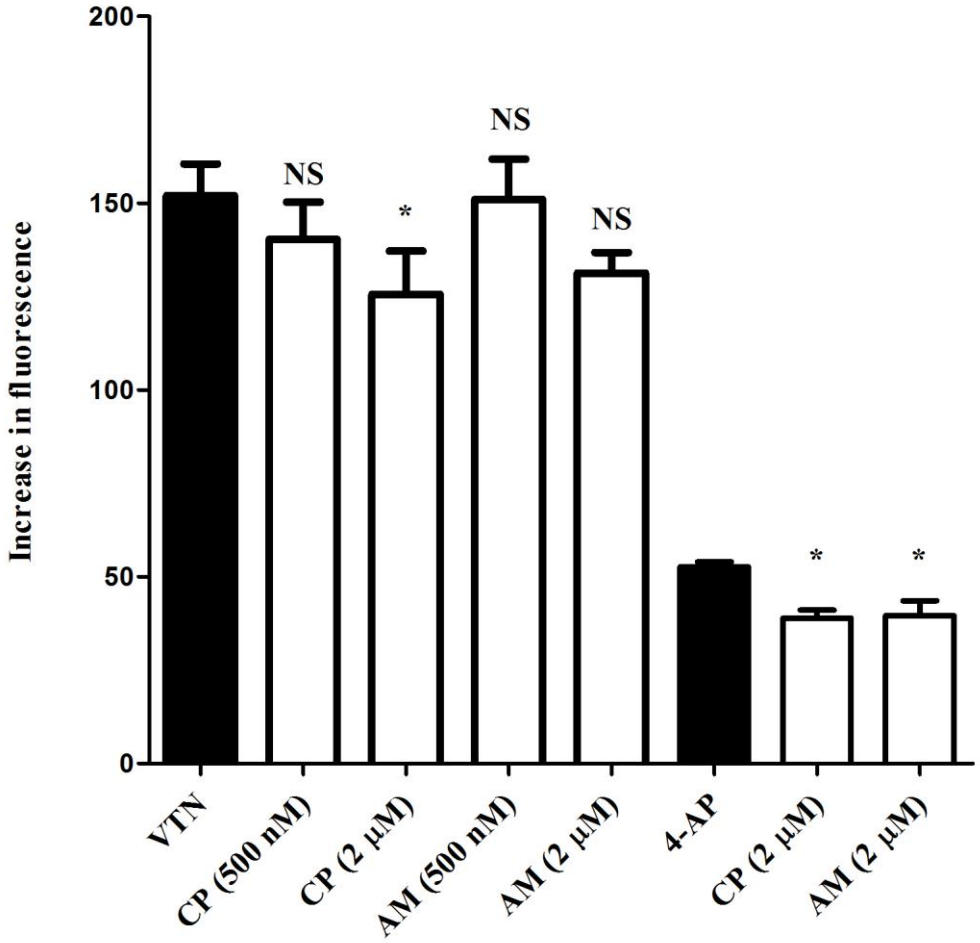


Figure 3-9 Inhibition of VTN- and 4-AP-induced AO exocytosis by CP-55,940 (CP) and AM251 (AM). VTN and 4-AP were used to depolarize synaptosomes at 50 µM and 100 µM respectively. NS is non-significant ($P > 0.05$) and * represents a significant difference ($P < 0.05$).

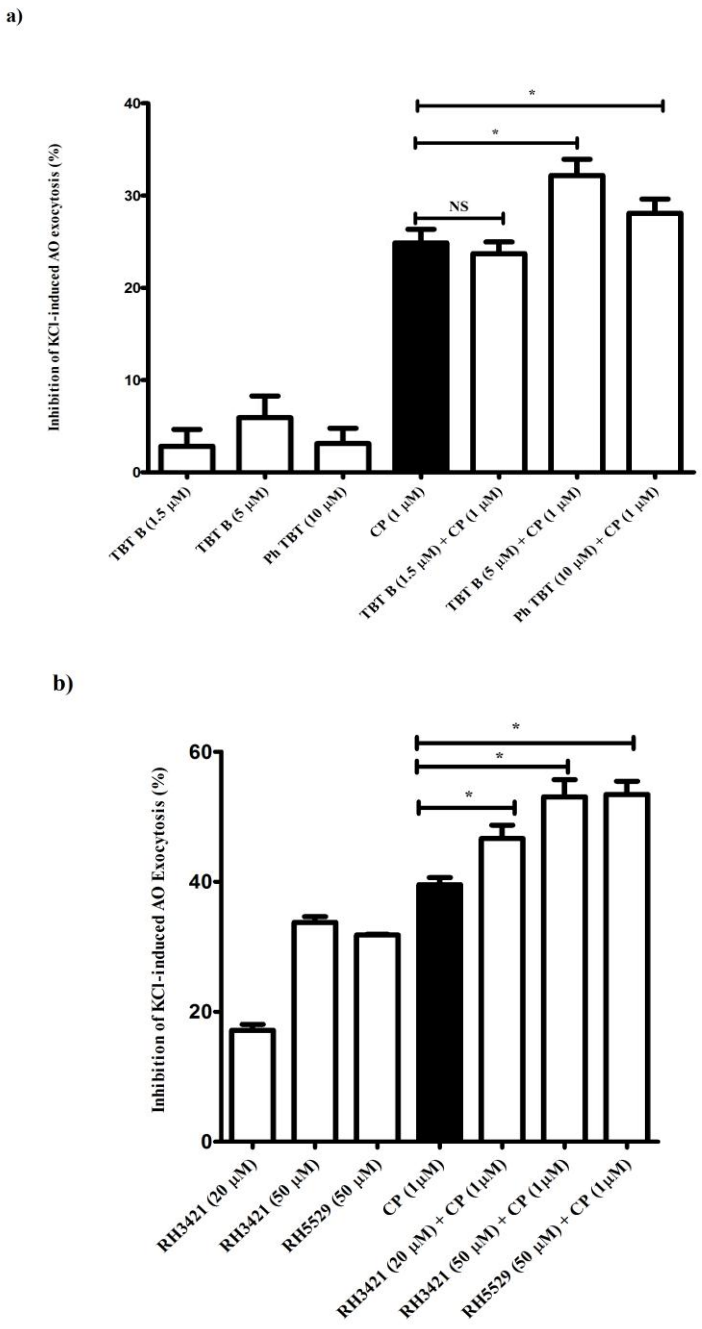


Figure 3-10 Inhibition of K^+-induced AO exocytosis from synaptosomes by selected environmental chemicals. a) TBT benzoate (TBT B) and phenylethynyl TBT (Ph TBT) and b) RH3421 and RH5529 tested at several concentrations were not able to antagonize the inhibitory effect of 1 µM CP-55,940 on K^+-induced AO exocytosis. NS represents non-significant ($P > 0.05$) and * represents a significant effect ($P < 0.05$).

3.5 Discussion

The present investigation was initiated to investigate the effect of cannabinoids on AO exocytosis and to investigate whether a depolarization- (KCl-) activated AO exocytosis assay might serve as a functional assay to delineate cannabinoid agonists and antagonists. Development of this assay may offer several advantages over conventional cannabinoid agonist/antagonist delineating assays such as it is a non-radioactive, safe, cost and time effective assay and it potentially measures an important functional consequence of CB_1 receptor activation on vesicular exocytosis which represent an index of release of physiological neurotransmitters. It also has potential to be a rapid throughput fluorescence micotitre plate assay. The AO release profile is very similar in nature to that of glutamate and GABA and therefore it makes a useful tool for predicting the release pattern of these important neurotransmitters in nerve endings (Zoccarato et al., 1999; Raiteri et al., 2002). Since the [^{35}S]GTPγS binding assay used to delineate CB_1 receptor agonist/antagonist effects measures the first step after receptor activation (Harrison & Traynor, 2003), it can only be used to approximate the effect on neurotransmitter release dynamics by novel CB_1 receptor modulators. Modulation of adenylate cyclase activity by CB_1 receptor agonists and antagonist can also serve as another alternative assay to classify CB_1 receptor agonists and antagonists. However, this assay also cannot convincingly predict the effect on neurotransmitter release as this ultimate downstream event following receptor activation is subjected to many other modulatory influences (Harrison & Traynor, 2003). Wang (2003) has reported the partial inhibition of 4-AP-induced glutamate release from synaptosomes by WIN-55,212-2 and this inhibition was shown to be blocked by the CB_1 receptor antagonist AM281. Thus, the enzyme-linked

fluorometric glutamate assay can serve as an excellent assay to delineate CB_1 receptor-selective agonists and antagonists but its utility as a tool to delineate novel CB_1 receptor agonist and antagonist warrants further investigation. In the present investigation depolarization by various depolarizing agents such as 1) high external KCl (30 mM), veratrine (50 µM) and 3) 4-AP (40 µM and 100 µM) caused AO exocytosis from synaptosomes isolated from the mouse brain. The AO exocytosis signal to noise ratio was enhanced considerably by our modification of the already established methods of Melnik et al. (2001) and Zoccarato et al. (1999). On Dr. Nicholson's suggestion, I introduced a two-step centrifugation and resuspension after loading the synaptosomes with AO. This procedure effectively removes excess *extracellular* AO and therefore the background fluorescence is lowered and this vastly improves the AO signal to noise ratio.

High K^+ in the range of 30 mM to 50 mM has been used by various researchers to depolarize neuronal membranes for studying neurotransmitter release (SanchezPrieto et al., 1987; Raiteri et al., 2002; del Carmen Godino et al., 2007). Depolarization of the neuronal membrane by K^+ triggers the opening of VGCCs which leads to vesicular exocytosis (Raiteri et al., 2002; del Carmen Godino et al., 2007). However high external K^+ in 35 mM to 50 mM range has in some instances been reported to induce a neurotransmitter efflux profile driven by mechanisms other than the classical external Ca^{2+} entry (Raiteri et al., 2002). Therefore the concentration of K^+ in the present investigation was kept at 30 mM to mimic physiological release conditions rather than any potentially pathological changes accompanying higher external K^+ challenges (Raiteri et al., 2002). Interestingly in the present investigation the 30 mM KCl-induced AO exocytosis signal was entirely dependent on external Ca^{2+}. Hence it can be said that

AO exocytosis is occurring through the classical exocytosis mechanism. However, Raiteri et al. (2002) have reported some residual AO fluorescence even after the removal of external Ca^{2+} and they believe that this residual fluorescence is due to Na^{+} ions being mobilized from the extracellular side to the intracellular component with Na^{+} then activating the mitochondrial Na^{+}/Ca^{2+} exchanger (Raiteri et al., 2002). This release of Ca^{2+} from the mitochondria then further triggers release of Ca^{2+} from the adjacent endoplasmic reticulum and thus contributing to non-classical exocytosis (Raiteri et al., 2002). The concentration of KCl used by Raiteri et al. (2002) was 50 mM and this concentration was used to stimulate pathological conditions. This may explain why the concentration of KCl (30 mM) used in the present investigation produced no residual fluorescence in absence of external Ca^{2+}. Also in the present investigation EGTA (200 µM) was also supplemented in the Ca^{2+} free external medium so as to make sure there is no contaminating Ca^{2+}. These results closely parallel ,the results of Zoccarato et al. (1999) and Melnik et al. (2001) where no increase in fluorescence is seen when Ca^{2+} is chelated by EGTA.

In the present investigation cannabinoid agonists like CP-55,940 and WIN-55,212-2 inhibited KCl-induced AO exocytosis in a concentration-dependent manner. The inhibition of AO exocytosis aligns with the earlier reports, which described the inhibition by cannabinoid agonists on release of various neurotransmitters from brain slices and synaptosomes (Gifford & Ashby, 1996; Gifford et al., 1997a; Gifford et al., 1997b; Gifford et al., 2000; Wang, 2003). Now the question arises about what is the mechanism involved in cannabinoid agonist-mediated inhibition of AO exocytosis. Cannabinoid agonists interfere with the excitatory synaptic transmission in cerebellar

slices by reducing Ca^{2+} influx through N-type, P/Q-type, and R-type calcium channels (Brown et al., 2004). Other work has shown WIN-55,212 to partially inhibit 4-AP-induced glutamate release from synaptosomes by inhibiting N- and P/Q- type Ca^{2+} channels (Wang, 2003). In another report WIN-55,212-2 reduced KCl-induced glutamate release from cerebrocortical synaptosomes by reducing Ca^{2+} influx through N- and P/Q-type Ca^{2+} channels (Godino et al., 2005a). It has also been shown that activation of the CB_1 receptor activates presynaptic K^+ channels and this can contribute indirectly to the reduction of Ca^{2+} influx by shortening the time course of the action potential (Alger, 2002; Godino et al., 2007). Godino et al. (2005b) have shown using a membrane potential-sensitive cationic carbocyanine dye that WIN-55,212-2-mediated reduction of 30 mM KCl-induced glutamate release from synaptosomes is not due to activation of presynaptic K^+ channels but exclusively due to the modulation of VGCCs. WIN-55,212-2 reduced high external 30 mM K^+ triggered glutamate release from synaptosomes and this reduction was not reversed by K^+ channel blockers whereas the low (5 and 10 mM) external K^+-triggered glutamate release reduced by WIN-55,212-2 was totally reverted back by K^+ channel blockers (Godino et al., 2007). Also the low external K^+ (5 and 10 mM) triggered glutamate release was sensitive to TTX and the high (30 mM) external K^+ triggered glutamate release was insensitive to TTX showing non-involvement of voltage-gated Na^+ channels on depolarization with high external K^+ (Godino et al., 2007). My results correspond well with these findings since the AO exocytosis signal induced by 30 mM KCl was insensitive to TTX and also based on results of Godino et al. (2005 a & b) and Godino et al. (2007), therefore the AO exocytosis signal induced by 30 mM KCl in the present investigation is unlikely to be due to CB_1 receptor activation induced

presynaptic K^+ channel activity but more likely brought about by CB_1 receptor-activated inhibitory G protein influence on VGCCs. In our laboratory, endocannabinoids and synthetic cannabinoid ligands like AM251 have been found to inhibit VGSCs but such inhibition cannot contribute to the inhibition of KCl-induced AO exocytosis because as already stated this effect is TTX-insensitive (Nicholson et al., 2003; Duan et al., 2008a; Duan et al., 2008b). Also, the maximum concentration of cannabinoid ligands used in the present investigation is 2 µM which is just below the concentration required to see most threshold effects on VGSCs. In reports published by Wang (2003) and Godino et al. (2007) the concentration of WIN-55,212-2 used is 5 µM which clearly has potential to block VGSCs and this effect might be partially contributing to the inhibition they found. However, Godino et al. (2007) provide a counter argument that since cannabinoid-induced inhibition of VGSCs reported by Nicholson et al. (2003) was CB_1 receptor-independent and also because the inhibition caused by WIN-55,212-2 (at 5 µM and 10 µM) was completely reversed by AM281, it is less likely that the mechanism involving inhibition of VGSCs by cannabinoids is a contributing factor.

Veratrine and 4-AP were also used as depolarizing agents to investigate their effects on synaptosomes loaded with AO. When high external K^+ is used to depolarize, the VGSCs likely open very transiently but close immediately as the voltage swings rapidly to positive making participation of voltage-sensitive Na^+ channels almost negligible. However, depolarizing agents like veratrine and 4-AP cause neurotransmitter release by activating VGSCs (Godino et al., 2007). Like KCl, there are different concentration-dependent mechanisms involved in 4-AP-induced release of neurotransmitters (Galvan & Sitges, 2004). A low concentration of 4-AP (50 µM) has

been shown to induce a mainly external Ca^{2+}-dependent release of [^{3}C]glutamate and [^{3}H]GABA from synaptosomes whereas high concentrations of 4-AP (2 mM) cause release that is only partially dependent upon external Ca^{2+}, thus indicating other mechanism in addition to classical exocytosis coming into play at higher concentrations (Tapia & Sitges, 1982). In an another study, the release of [^{3}H]dopamine was totally independent of external Ca^{2+} when 1 mM 4-AP was used to depolarize rat striatal synaptosomes. In contrast, when 4-AP was used at 100 µM, release was mostly dependent upon external Ca^{2+} (Carvalho et al., 1995). Ca^{2+} influx through N-type, P-type and possibly Q-type has been implicated in 4-AP-induced [^{3}H]dopamine release from synaptosomes (Carvalho et al., 1995). The increase in Na^+ influx triggered by 100 µM 4-AP was almost completely blocked by TTX, whereas Na^+ influx caused by 200 µM and 1 mM 4-AP was only inhibited about 35% blocked by TTX (Galvan & Sitges, 2004). Activation of TTX-sensitive VGSCs by 4-AP leads to Na^+ influx which then triggers Ca^{2+} influx through VGCCs and this eventually results in neurotransmitter release (Galvan & Sitges, 2004). Based on the above data the concentration of 4-AP selected for the present investigation was 100 µM and also few experiments were also conducted at 40 µM. Robust AO exocytosis signals were observed with 100 µM 4-AP of which a large proportion was blocked by TTX showing a significant involvement of TTX-sensitive VGSCs.

As stated earlier in the present investigation, CP-55,940 and WIN-55,212-2 activate the presynaptic CB_1 receptors which then triggers the inhibition of the VGCCs. Inhibition of VGCCs should decrease the Ca^{2+} flux into the presynaptic nerve terminals when depolarized by high external K^+. A decrease in Ca^{2+} influx would logically result in

decrease in AO exocystosis. It is important to restate that my results clearly show that KCl-induced AO exocytosis is an entirely Ca^{2+}-dependent effect and no exocytosis is observed if Ca^{2+} is removed. Thus it gives another line of evidence that cannabinoid agonists can only interfere with the dynamics of AO exocytosis if they are interfering in some way with the Ca^{2+} entry mechanism of synaptosomes. Based on the findings of others (above) and my own results it can be concluded that both WIN-55,212-2 and CP-55,940 inhibit AO exocytosis through an inhibitory action of the CB_1 receptor-coupled G protein subunit on VGCCs. In my experiments it was important to investigate whether the cannabinoid antagonist AM251 can block the inhibitory effects of cannabinoid agonists on KCl-induced AO exocytosis. If AM251 was acting in a highly specific fashion (i.e. only targeting CB1 receptors) it would be logically predicted that this compound when tested on its own should not interfere with the dynamics of depolarization-induced AO exocytosis or possibly slightly enhance it considering AM251 is an antagonist/inverse agonist at the CB_1 receptor. Paradoxically, when tested on its own AM251 strongly inhibited KCl-induced AO exocytosis in a concentration-dependent manner. AM251 was also unable to reverse the inhibitory effects of cannabinoid agonists on AO exocytosis. This type of anomalous behaviour of AM251 has also been noted in an another study where AM251 inhibited KCl-induced Ca^{2+} influx in cultured optical nerve oligodendrocytes (OLs) isolated from both wild type and CB_1 knockout mice to the same extent (approximately 70%) (Mato et al., 2009). Also, AM251 was not able to reverse the inhibitory effect of a cannabinoid agonist (Mato et al., 2009). This made the authors claim that AM251 is acting through a non-specific mechanism independent of CB_1 receptor activation (Mato et al., 2009). In the experiments of Mato et al. (2009), even the

cannabinoid receptor agonist was able to inhibit Ca^{2+} influx to a certain extent in OLs from CB_1 knockout mice although to a much lower extent than in oligodendrocytes from wild type mice, indicating that the depolarization-induced Ca^{2+} can be modulated by both CB_1 receptor-dependent and CB_1 receptor-independent mechanisms. Interestingly, cannabidiol (CBD) which is a non-psychoactive constituent present in marijuana, was shown to increase intracellular Ca^{2+} in cultured optic nerve OLs and decreased oligodendrocyte viability (Mato et al., 2010). This increase in cytosolic Ca^{2+} was suggested to originate from mitochondria (Mato et al., 2010).

In another report, CB_1 receptor agonists like CP-55940, WIN-55,212-2 and anandamide decreased Ca^{2+} influx in cerebellar granule neurons through a pertussis toxin-sensitive mechanism whereas highly potent CB_1 receptor antagonist SR-141716A also exhibited the type of decrease shown by cannabinoid agonists but through a pertussis toxin-insensitive pathway indicating a CB_1 receptor-independent effect (Nogueron et al., 2001). With AM251 being an extremely close structural analog of SR141716A, a similar mechanism may explain why AM251 is reducing AO exocytosis.

Although I did not see any reversal of the inhibitory effects of cannabinoid agonist in the KCl-induced AO exocytosis with AM251, I went ahead to investigate some environmental chemicals that were shown using [^{35}S]GTPγS in Chapter 2 to behave as antagonists at the CB_1 receptor. I tested one low and one high concentration of each environmental chemicals and investigated whether they could reverse the inhibitory effect of a cannabinoid agonist. None of the test chemicals was able to reverse cannabinoid agonist-mediated inhibition of exocytosis. The cannabinoid agonist and antagonist controls in these experiments were at 400 nM and 1 μM. I chose 400 nM as

the lowest concentration because at this concentration, a well-resolved inhibition of AO was produced by cannabinoid agonists. However, in the [^{35}S]GTPγS binding assay the CP-55,940 concentration was 100 nM and it is possible that the concentrations of cannabinoid agonist used in the AO exocytosis were too high for these lower potency environmental chemicals to antagonize. However, if these environmental chemicals reach the CNS, they will not be competing with synthetic cannabinoid agonists like CP-55,940 and WIN-55,212-2. In contrast, they would be competing with less stable and more transiently acting endocannabinoids and it may be more likely that under these conditions antagonistic effects at the CB_1 receptor could readily occur.

This investigation answered some important questions about the effects of cannabinoids on depolarization-induced AO exocytosis from synaptosomes. Based on my results and other published reports we can conclude that both CP-55,940 and WIN-55,212-2 are inhibiting AO exocytosis most likely through an inhibitory action of CB_1 receptor on VGCCs. 4-AP-induced AO exocytosis is largely dependent upon TTX-sensitive VGSCs. Based on the results of Wang (2003) and Godino et al. (2005a) N-type and P/Q type Ca^{2+} channels are likely to be targets for CP-55,940 and WIN-55,212-2 in the present investigation. This investigation also supported the anomalous behaviour of AM251 which is well-supported by other published reports.

Future work should focus on intrasynaptosomal Ca^{2+} determinations assays (e.g. using the fluoroprobe, fura-2) to investigate the effect of AM251 on depolarization-induced increase in free cytoplasmic Ca^{2+} and as this approach could shed some light on to why AM251 is inhibiting AO exocytosis. Wang (2003) and Godino et al. (2007) antagonized the inhibitory effect of WIN-55,212-2 on glutamate release using AM281

and thus it would be interesting to use this pharmacological agent in the AO exocytosis assay to see if it antagonizes the inhibitory effect of cannabinoid agonist on AO exocytosis. However, it is apparent (see Figure 1-7) that AM281 like SR141716A shows only very minor structural difference when compared to AM251. In the present experiments we also considered the possibility that AM251 is having some non-specfic interaction with the fluorescent dye (acridine orange) which is preventing the reversal of cannabinoid agonist-mediated effect. However, this is unlikely because AM251 fails to affect AO fluorescence in the absence of synaptosomes. It would be important to conduct AO exocytosis assays in the presence of pertussis toxin and to further investigate the role of the CB_1 receptor in interfering with the dynamics of depolarization-induced AO exocytosis from synaptosomes. On the basis of the data generated from the present investigation depolarization-induced AO exocytosis may not be a valid functional assay to delineate cannabinoid antagonist effects. However as mentioned earlier when experimental results with fura-2, AM281 and pertussis toxin become available, one would be in a stronger position to decide on the utility of this assay as a functional method to profile cannabinoid agonists and antagonist.

4: Conclusions and future prospects

My master's research supports our hypothesis that there are binding topographies common to VGSCs and the CB_1 receptor. VGSC-targeting insecticides like JT333, MP062, RH3421, RH5529, 1R, *trans*-permethrin, 1R, *cis*-permethrin and cypermethrin were characterized as antagonists at the CB_1 receptor. Some of the neurotoxic poisoning symptoms like reduced motor activity, hypothermia, lethargy and pacificity observed with α-cyano pyrethroids, non α-cyano pyrethroids and RH3421 (Verschoyle and Aldridge, 1980; Crofton and Reiter, 1984; Crofton and Reiter, 1988; Mcdaniel and Moser, 1993; Wolansky et al., 2006; Salgado, 1992) are attributed mainly to VGSC blockade. Considering these signs are also important symptoms of CB_1 receptor agonist intoxication profile, we origionally considered that CB_1 receptor activation (i.e. agonist action) might partially contribute to the neurotoxicity profile. My results show that this is unlikely since the pyrethroids did not display agonist actions at the CB_1 receptor. The antagonistic nature of these insecticides at the CB1 receptors offers new perspective with which to view the poisoning symptomology. For example, inhibition of endocannabinoid effects at the CB_1 receptors may intensify the overall excitability in the animal triggered by sodium channel activation. If this is true, potent cannabinoid (CB_1) agonists may have value in the treatment of pyrethroid poisoning (i.e. they may have antidote properties).

This research also highlights three new chemical classes like 1) mitochondrial function-disrupting chemicals 2) benzophenathridine alkaloids and 3) TBT compounds that interact with the CB_1 receptor at micromolar concentrations. My data support the

idea also presents evidence that the effects are being mediated through the CB_1 receptor rather than being of a non-specific nature.

Mitochondrial function disrupting chemicals like famoxadone, surangin B, myxothiazol and coenzyme Q_2 were shown to interact with the CB_1 receptor *in vitro* and interfere with agonist-stimulated G protein activation cycle. Based on my results these mitochondrial function disrupting chemicals may have neurotoxic potential through this mechanism if they are able to build up to sufficient concentrations inside the brain. It is likely that the alkyl chain system in these mitochondrial function-disrupting chemicals is facilitating engagement with the binding to the binding pocket of the CB_1 receptor. It would be interesting to synthesize these compounds without the alkyl chain system or replace the alkyl chain systems with those of Δ^9-THC or CP-55,940 and then investigate their binding affinity and functional consequence of such a binding at the CB_1 receptor.

Benzophenathridine alkaloids like chelerythrine and sanguinarine displayed inverse agonist behaviour at the CB_1 receptor and may target guanine nucleotides rather than act through the receptor level like classical inverse agonists. This retrograde allosteric mechanism might offer an alternative therapeutic strategy to target GPCRs (Dhopeshwarkar et al., 2011). Interestingly both chelerythrine and cannabinoids like Δ^9-THC and CP-55,940 have been reported to decrease thermal hyperalgesia (De Vry et al., 2004; Chen et al., 2006) and interrupt short-term memory (Essman, 1984; Saachetti and Bielavaska, 1998). However, chelerythrine was found to be an antagonist/inverse agonist at the CB_1 receptor and it is possible that chelerythrine might undergo some biotransformation *in vivo* and that a biotransformation product might be responsible for cannabinoid agonist-like properties. Chelerythrine is also protein kinase C inhibitor

(Herbert et al., 1990), but our results together with other reports suggests that it is not the mechanism operating in our investigation (Dhopeshwarkar et al., 2011). It is also possible that the pseudobase forms of chelerythrine and sanguinarine could engage with the CB_1 receptor in a similar fashion to Δ^9-THC or Δ^9-THCV (Dhopeshwarkar et al., 2011).

In the various assays conducted in the present investigation, all test compounds were competing against relatively high affinity CB_1 receptor ligands like CP-55,940. In the CNS the environmental chemicals would be competing with compounds like anandamide and 2-AG that engage with the CB_1 receptor but undergo rapid breakdown. It is possible that these chemicals may be considerably more efficacious at CB_1 receptors than the *in vitro* binding and functional assays show. In this context, experiments to examine the ability of study compounds to inhibit endocannabinoid [^{3}H]anandamide binding to the CB_1 receptor may be helpful. Recently our laboratory has reported several phthalate esters to allosterically interfere with the CB_1 receptor signalling (Bisset et al., 2011). In the present investigation, I demonstrate the interaction of the environmentally toxic TBT compounds with the CB_1 receptors. The TBT compounds show antagonist and possible inverse agonist behaviour at the CB_1 receptor. TBT compounds are unlikely to inhibit FAAH/MGL as has been shown for organophosphorus compounds (Nomura et al., 2008) and it is more likely that they are binding to the CB_1 receptor. However, TBT compounds could act directly or via an allosteric mechanism and whether this interaction is direct or allosteric has to be investigated in the future.

The endocannabinoid machinery is very important for regulating short and long-term synaptic transmission and any blockade in this system at any level could in theory promote adverse psychiatric or physiological manifestations. For example, rimonabant

(CB_1 receptor antagonist/inverse agonist) which was approved in the EU union to treat obesity was discontinued because patients developed significant psychiatric effects (Ward and Raffa, 2011). If same line of reasoning is followed, these insecticides and other test chemicals could also work in a similar fashion by disrupting endocannabinoid tone in the CNS. Subtle psychiatric manifestations are likely to be missed in standard toxicological testing of environmental chemicals.

CB_1 receptors are also found (albeit at low levels) in human testis, human heart muscles, the spleen, tonsils, small intestine, urinary bladder, liver, lung, kidney, bile duct, colon, stomach, adrenal gland, prostrate and ovary (Bonz et al., 2003; Gerard et al., 1991; Shire et al., 1995). There is also a possibility that these environmental chemicals could also interact with the CB_1 receptor in these organ and tissues and affect various tissue-specific physiological processes. For example, they could antagonize inhibition of gastric fluid secretion or reduced gut motility mediated by cannabinoid agonists (Adami et al., 2002; Coutts & Izzo, 2004). In the reproductive system (testes) they might interfere with cannabinoid agonist effects on male fertility (Banerjee et al., 2011). Future studies should also investigate potential effects of study compounds at CB_2 receptors as already discussed.

Different preparation of *Cannabis sativa* have been in use for thousands of years but still the therapeutic potential of this ancient plant only been partially realized (Hanus, 2009; Svizenska et al., 2008). Ancient civilizations treated cannabis as a panacea but modern science has so far not been able to transform these ancient claims into therapeutic reality (Hanus, 2009; Childers and Breivogel, 1998). A lot of effort and money has been devoted to cannabis research but the majority of compounds failed at the clinical trial

stage (Ward and Raffa, 2011). *Cannabis sativa* is a complex plant and it is very likely that the euphoric and therapeutic effects are due the cocktail of different chemical constituents present in it and using it as a holistic medicine could be more beneficial rather than trying to find the active chemical constituent (Hanus, 2009). It might also be this reason that isolated cannabis constituents have not been therapeutic blockbusters. However, non-psychoactive component of marijuana (cannabidiol) is reported to have therapeutic benefits without the psychoactive manifestations exhibited by Δ^9-THC (Russo et al., 2007). Cannabidiol has a significant potential in the treatment of inflammation, diabetes, cancer and neurodegenerative disorders (Pertwee, 2008; Zuardi, 2008; Mechoulam et al., 2007). In clinical trials and subsequent usage by many people, Sativex ® (Δ^9-THC and cannabidiol in 1:1 proportion) has helped patients drastically improve their sleep and helped with a wide variety of pain conditions including, peripheral neuropathic pain, intractable cancer pain, and pain associated with rheumatoid arthritis and multiple sclerosis, with an acceptable adverse event profile (Russo et al., 2007). The other beneficial effect with Sativex ® is that no tolerance has been reported in the last 4 years of its usage (Russo et al., 2007). Δ^9-THCV is an antagonist at CB_1 receptor and holds promise as an anti-obesity drug (Izzo et al., 2009). In this investigation we have highlighted a lot of different chemical structures which interact with the CB_1 receptor. Some of the chemicals can be considered as templates for the synthesis of novel and more potent CB_1 receptor antagonists and can be explored for their therapeutic potential. Future investigation in our laboratory would be directed towards studying the interaction of these environmental chemicals with the CB_2 receptor using spleen isolated from mouse. As already stated many environmental chemicals highlighted in this study

could also interact with the peripheral CB_2 receptor and affect immunomodulatory effects mediated through the CB_2 receptor.

In the second part of my research, I explored the effect of cannabinoids on exocytosis from synaptosomes using AO. This assay offers the potential as a non-radioactive functional assay to delineate CB_1 receptor agonists and antagonists. I further confirmed lack of involvement of VGSCs in high external K^+-induced exocytosis. Participation of TTX-sensitive VGSCs was shown in 4-AP- and veratrine-induced AO exocytosis. My results together with other reports (Wang, 2003; Godino et al., 2005a) suggests that cannabinoid agonists (CP-55,940 and WIN-55,212-2) are inhibiting KCl-induced AO exocytosis by the inhibitory action of the CB_1 receptor coupled G protein machinery on N-type and P/Q-type VGCCs. Most importantly, this investigation showed the paradoxical behaviour of AM251 a finding supported by other published reports (Mato et al., 2009; Nogueron et al., 2001). Based on my results and other published reports it seems like AM251 is acting in a non-specfic manner (CB_1 receptor independent) and reducing Ca^{2+} influx in to the synaptosomes. Future experiments using the fluoroprobe fura-2, pertussis toxin and AM281 are recommended to shed further light on the paradoxical nature of AM251's action. Until this data becomes available AO exocytosis assay cannot be used to characterize antagonists at the CB_1 receptor.

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