THE INHIBITION OF THE G-PROTEIN COUPLED CANNABINOID 1 (CB_1) RECEPTOR OF MAMMALIAN BRAIN BY PHTHALATE ESTERS *IN VITRO*

by

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ABSTRACT

This research investigates *in vitro* interactions of phthalates with G protein-coupled cannabinoid 1 (CB_1) receptors. Diesters, n-butylbenzylphthalate (nBBP), di-n-hexylphthalate (DnHP), di-n-butylphthalate (DnBP), di-2-ethylhexylphthalate (DEHP), di-isooctylphthalate (DiOP) and di-n-octylphthalate (DnOP) inhibited specific binding of CB_1 receptor agonist [^{3}H]CP-55940 to mouse brain membranes at micromolar concentrations. Monoesters, mono-2-ethylhexylphthalate (M2EHP) and mono-isohexylphthalate (MiHP) had low activity and mono-n-butylphthalate (MnBP) was inactive. Inhibition of CB_1 receptor agonist-stimulated binding of [^{35}S]GTPγS to the G-protein demonstrated that phthalates functionally impair this complex. DnBP, nBBP and DEHP also inhibited binding of [^{3}H]SR141716A, whereas inhibition by MiHP was comparatively weak and MnBP was ineffective. DnBP and nBBP reduced the association rate of [^{3}H]SR141716A with CB_1 receptors and rapidly enhanced the rate of [^{3}H]SR141716A dissociation, the latter being consistent with an allosteric mechanism for inhibition. Thus, phthalates act as relatively low affinity antagonists of CB_1 receptors and cause inhibition of cannabinoid agonist-dependent activation of the G-protein in vitro.

Keywords: Cannabinoid-1 (CB_1) receptors; mouse brain; G-coupled protein; [^{3}H]CP-55940 binding; [^{35}S]GTPγS binding; [^{3}H]SR141716A binding; phthalate di-esters; phthalate mono-esters.

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I would like to express my gratitude to my family, my mom, Art, Samantha and Ben, for their encouragement and for never doubting me. Also to my father, who gives me strength even is his absence.

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GLOSSARY

2-AG	2-Arachidonoylglycerol
ACEA	Arachidonoyl-2'-chloroethanolamide
ACPD	(1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid (mGluR agonist)
AM1241	1-(Methylpiperidin-2-ylmethyl)-3-(2-iodo-5-nitrobenzoyl)indole (selective CB_2 receptor agonist)
AM251	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (selective CB_1 receptor antagonist)
ATP	Adenosine triphosphate
B_{max}	Maximum binding
BSA	Bovine serum albumin
c-Myc	Cellular myelocytomatosis oncogene
cAMP	Cyclic adenosine monophosphate
CB_1-R	Cannabinoid-1 receptor
CB_2-R	Cannabinoid-2 receptor
CCNB1	Cyclin B1 gene
CCNB1IP1	Cyclin B1 interacting protein 1 gene
CNS	Central nervous system
CP-55940	(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethy lheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (non-selective CB receptor agonist)

DAG	1,2-diacylglycerol
DAGL	Diacylglycerol lipase
DBP	Dibutylphthalate
DEHP	Di-2-ethylhexylphthalate
DEP	Diethylphthalate
DiOP	Di-isooctylphthalate
DMP	Dimethylphthalate
DMSO	Dimethyl sulfoxide
DnBP	Di-*n*-butylphthalate
DnHP	Di-*n*-hexylphthalate
DnOP	Di-*n*-octylphthalate
DSI	Depolarization-induced suppression of inhibition
DUSP6	Dual specificity phosphatase 6 gene
ECS	Endocannabinoid system
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor gene
EGTA	Ethyleneglycoltetraacetic acid
eIPSC	Evoked inhibitory postsynaptic current
FAAH	Fatty acid amidohydrolase
FGFR2	Fibroblast growth factor receptor 2 gene
Fos	Finkel-Biskis-Jinkins murine osteosarcoma oncogene
GABA	γ-Aminobutyric acid

GDP	Guanosine diphosphate
$G_{i/o}$	Heterotrimeric G-protein α subunit (inhibits cAMP production)
GPCR	G protein-coupled receptor
GPR55	G protein-coupled receptor 5
$G_{q/11}$	Heterotrimeric G-protein α subunit (activates PLC)
GTPγS	Guanosine 5'-O-[gamma-thio]triphosphate)
HU-210	(6a*R*)-*trans*-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b*,*d*]pyran-9-methanol (synthetic THC-analogue)
IC_{50}	Concentration that results in 50% inhibition
K-Ras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
K_D	Equilibrium dissociation constant
Lyso-PI	Lysophosphatidylinostitol
Lyso-PLC	Lysophospholipase C
M1	Muscarinic acetylcholine receptor M1
M2EHP	Mono-2-ethylhexylphthalate
M3	Muscarinic acetylcholine receptor M3
MAFP	Methoxy arachidonyl fluorophosphonate (nucleophilic site displacer)
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
mGluRs	Metabotropic glutamate receptors
MiHP	Mono-isohexylphthalate
MnBP	Mono-*n*-butylphthalate

***N*-arachidonoyl PE**	*N*-arachidonoyl phosphatidylethanolamine
nAChR	Nicotinic acetylcholine receptor
nBBP	*n*-Butylbenzylphthalate
NTE-LysoPLA	Brain neuropathy target esterase–lysophospholipase
OP	Organophosphorus pesticide
PE	Phosphatidylethanolamine
PI	Phosphatidylinostitol
PKA	Protein kinase A
PLA1	Phospholipase A1
PLC	Phospholipase C
PNS	Peripheral nervous system
PPAR	Peroxisome proliferator-activated receptors
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SR141716A	Rimonabant (CB_1 receptor-selective antagonist)
SR144528	5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide (selective CB_2 receptor antagonist)
WIN55212-2	[(3*S*)-2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone monomethanesulfonate (non-selective CB receptor agonist)
Δ^9-THC	Δ^9-tetrahydrocannabinol

1: INTRODUCTION AND LITERATURE REVIEW

A modified version of this paper was accepted on June 23rd, 2011 by Neurochemistry International for publication as:

Bisset, K.M., Dhopeshwarkar, A.S., Liao, C., Nicholson, R.A. The G protein-coupled cannabinoid-1 (CB_1) receptor of mammalian brain: Inhibition by phthalate esters *in vitro*. (Note: the abstract found on page iii of this project write-up represents a truncated version of the abstract in the article noted above.)

1.1 Introduction

Cannabinoid receptors, along with endocannabinoids and the enzymes that are involved in their biosynthesis and degradation, are the main components of the endocannabinoid system (ECS) (Di Marzo, 2008). Interactions between cannabinoid receptors and their ligands play important roles in the processes of normal brain physiology, including pain and appetite, and in the normal functioning of the immune system (Piomelli, 2003). There are currently two known classes of cannabinoid receptors; the cannabinoid-1 receptor (CB_1-R) and the cannabinoid-2 receptor (CB_2-R) (Di Marzo, 2008), however, evidence of a novel non-CB_1/non-CB_2 cannabinoid receptor, GPR55, is mounting (Ryberg et al., 2007).

CB_1 receptors play an important part in the regulation of cardiovascular, gastrointestinal and respiratory function and in the perception of pain. CB_1

receptors also play roles in the release of hypothalamic hormones and peptides, which in turn influence food intake and the modulation of the pituitary-hypothalamus-adrenal axis, as well as in the regulation of male and female reproduction. CB_2 receptors on the other hand play an important role in cellular and humoral immune responses and in particular inflammation and chronic pain (Di Marzo et al., 2004). They have also been identified to play a role in cell metabolism, differentiation, proliferation and death (Guzman et al., 2002).

Other than endocannabinoids, natural and synthetic compounds are also capable of modulating the ECS. The most widely known natural agonist of cannabinoid receptors is Δ^9-THC, the main psychoactive ingredient found in the cannabis plant, *Cannabis sativa* (Figure 1-1) (Guzman, 2003). Δ^9-THC is functionally and structurally similar to endocannabinoids and has high binding affinity for cannabinoid receptors (Di Marzo et al., 2004). Plant cannabinoids, such as Δ^9-THC, act as receptor agonists to cause a multitude of pharmacological effects, some of which can be exploited for their therapeutic uses. Appropriate doses of cannabis are used therapeutically to stimulate appetite, suppress nausea and reduce pain (Adams & Martin, 1996). *Cannabis sativa* also has anti-inflammatory activity (Formukong et al., 1998), though it is difficult to separate unwanted psychoactive effects from those that are deemed beneficial. Unwanted effects of Δ^9-THC include impaired short-term memory, cognition and motor control, to name a few (Adams & Martin, 1996). Aside from natural cannabinoid ligands, some synthetic compounds are also capable of binding to cannabinoid receptors and modifying cannabinoid receptor function

(Figure 1-2). Some commonly known synthetic cannabinoids include non-selective agonists WIN55212-2 and CP55,940, and CB_1-selective antagonists AM251 and SR141716A (Piomelli, 2003; Di Marzo et al., 2004; Pertwee, 2006).

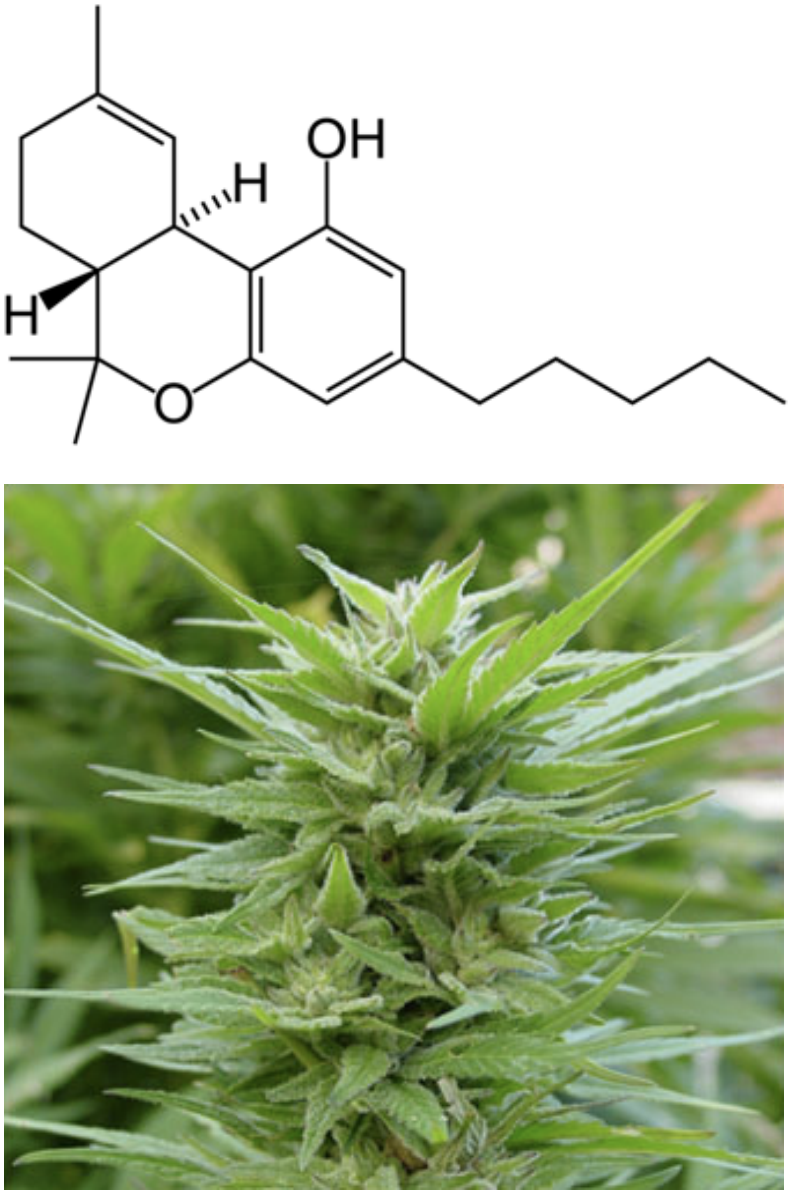

Figure 1-1 The chemical structure of Δ^9-THC (above), the major psychoactive ingredient found in the cannabis plant, *Cannabis sativa* (below).

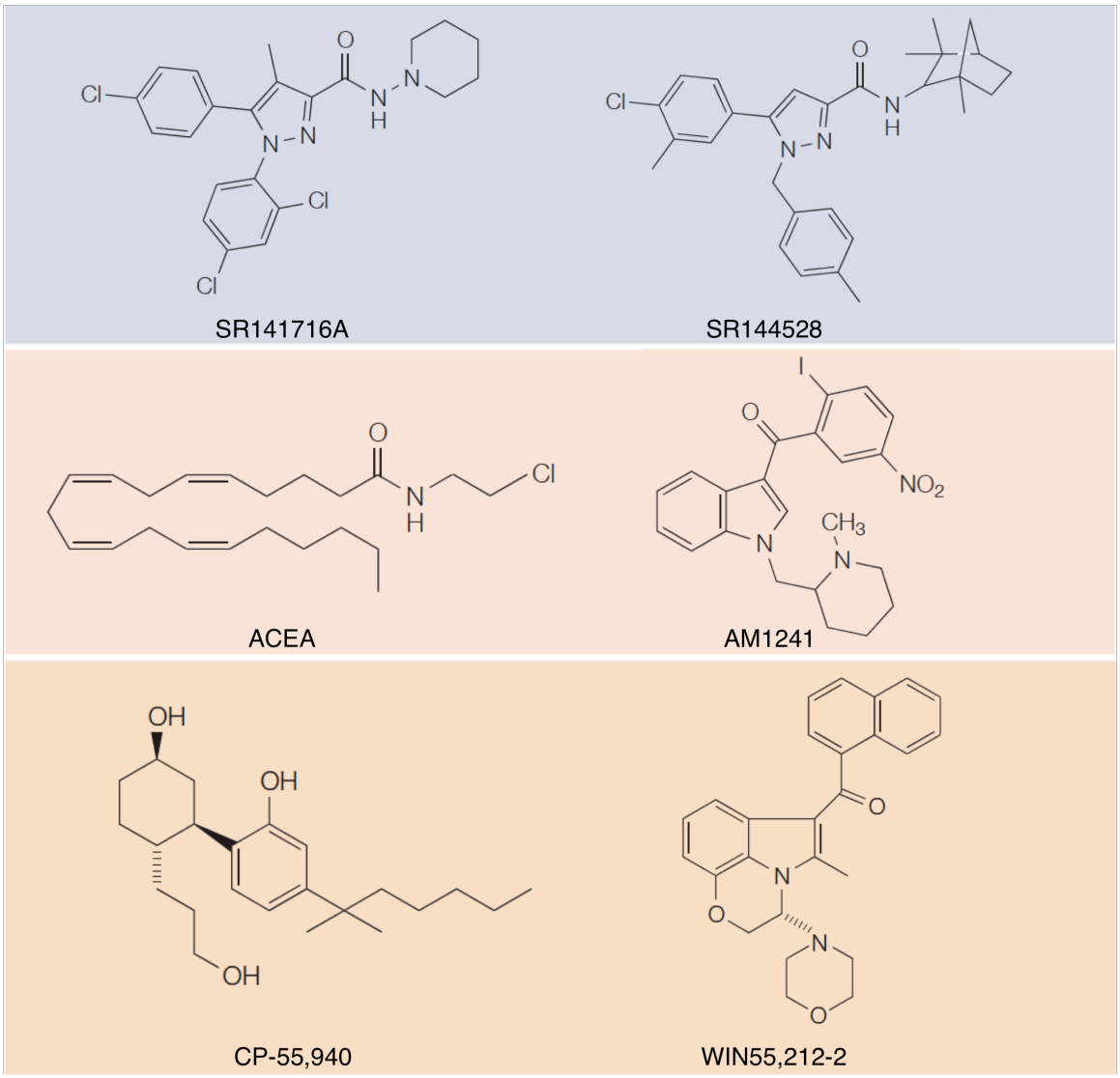

Figure 1-2 Chemical structure of several synthetic cannabinoids. Selective CB_1 (SR141716A) and CB_2 (SR144528) antagonists (Top). Selective CB_1 (ACEA) and CB_2 (AM1241) agonists (Middle). Nonselective CB agonists, CP55,940 and WIN55212-2 (Bottom). ACEA - arachidonoyl-2'-chloroethanolamide (modified from Piomelli, 2003; Di Marzo et al., 2004).

1.2 Endocannabinoids

The most commonly known and studied endocannabinoids include arachidonoyl ethanolamide, otherwise known as anandamide, and 2-arachidonoylglycerol (2-AG), while the lesser-studied endocannabinoids include virodhamine, noladin and *N*-arachidonoyldopamine (Figure 1-3) (Di Marzo et al.,

2004). Anandamide and 2-AG were first identified as endogenous ligands for cannabinoid receptors due to their ability to inhibit specific binding of a radiolabelled cannabinoid probe to synaptosomal membranes (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). All of the endocannabinoids that have been identified to date, including anandamide and 2-AG, are derivatives of a long-chain polyunsaturated fatty acid known as arachidonic acid.

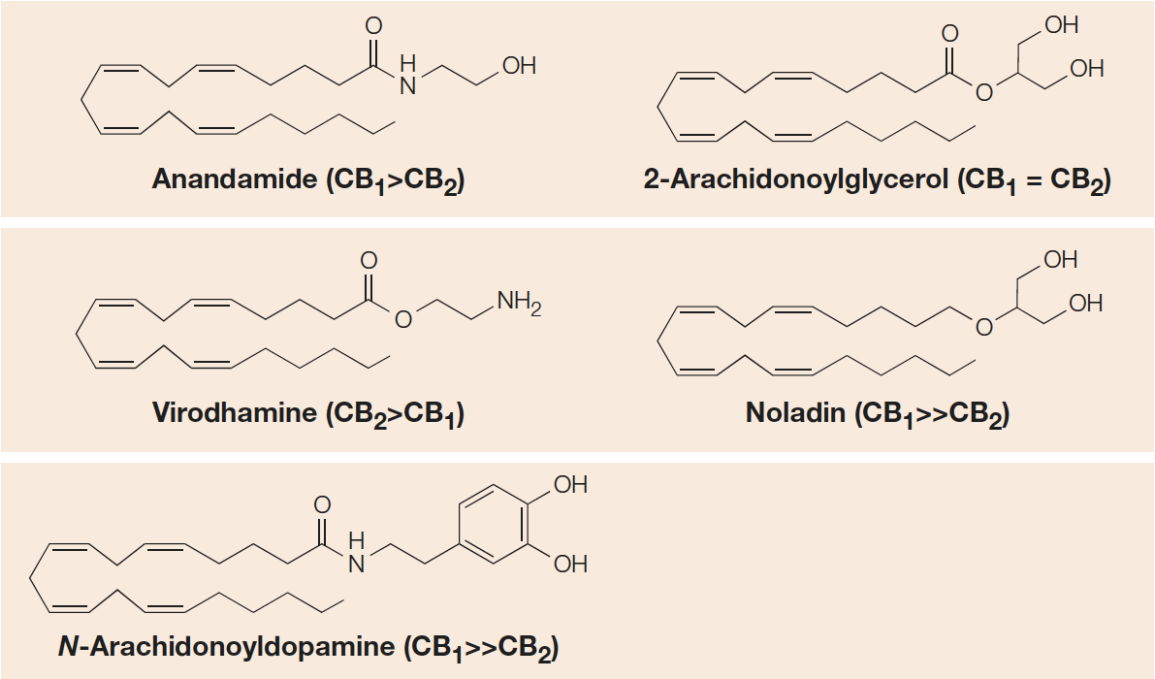

Figure 1-3 Chemical structures of endocannabinoids, including the two most well-studied endocannabinoids: anandamide and 2-arachidonoylglycerol. Receptor of preferential binding denoted in brackets (Di Marzo et al., 2004).

Each endocannabinoid has varying affinity for the two cannabinoid receptors: anandamide shows greater affinity for CB_1 receptors than CB_2 receptors, whereas 2-AG shows no preference over either, both noladin and *N*-arachidonoyldopamine have much greater affinity for CB_1 receptors than CB_2

receptors and virodhamine, unlike the others, has greater affinity for CB_2 receptors (Di Marzo et al., 2004).

Anandamide and 2-AG are widely distributed throughout the body, though 2-AG is present at much greater concentrations. These endocannabinoids have been found in the brain, retina, and various peripheral tissues that include the heart, spleen, liver, kidney, thymus, testis, uterus and skin (Sugiura et al., 2002).

1.2.1 Structures

Anandamide is a tetraenoic *N*-acylethanolamine whose chemical name is *cis*-5,8,11,14-eicosatetraenoylethanolamide (Sugiura et al., 2002; Thomas et al., 1996). 2-AG is a monoacylglycerol (Sugiura et al., 2002) whose chemical name is 1,3-dihydroxy-2-propanyl-*cis*-5,8,11,14-eicosatetraenoate. Their structures (Fig. 1-3) differ greatly from that of the classical tricyclic cannabinoid ligands like Δ^9–THC (Thomas et al., 1996), though they do have a structural resemblance to the eicosanoids (Piomelli, 2003).

1.2.2 Biosynthesis

1.2.2.1 Anandamide

Two synthetic pathways have been suggested for the biosynthesis of anandamide, one being its direct biosynthesis through the *N*-acylation of ethanolamine while the other is its transacylase-phosphodiesterase-mediated biosynthesis (Sugiura et al., 2002). Direct enzymatic biosynthesis of anandamide occurs when arachidonic acid and ethanolamine undergo *N*-acylation via fatty acid amide amidohydrolase (FAAH) (Sugiura et al., 2002). In 1994, investigations

conducted by Di Marzo et al. supported the role of anandamide as an endogenous neuronal messenger and demonstrated that it is produced and released by rat brain neurons in a calcium-dependent manner when neurons are stimulated by ionomycin (a Ca^{2+} ionophore), or by certain membrane-depolarizing agents including kainate, high K^{+}, and 4-aminopyridine. Their research suggests that anandamide may be the product of a single-step, phosphodiesterase-mediated cleavage of a novel phospholipid, *N*-arachidonoyl phosphatidylethanolamine (*N*-arachidonoyl-PE). A more recent study has indicated that the biosynthesis of anandamide in neurons is a two-step process (Figure 1-4) (Piomelli, 2003). The first step results in the formation of the anandamide precursor *N*-arachidonoyl-PE which is catalysed by *N*-acyltransferase, *N*-arachidonoyl-PE is then cleaved by phospholipase D to yield anandamide and phosphatidic acid as originally suggested by Di Marzo et al. (1994).

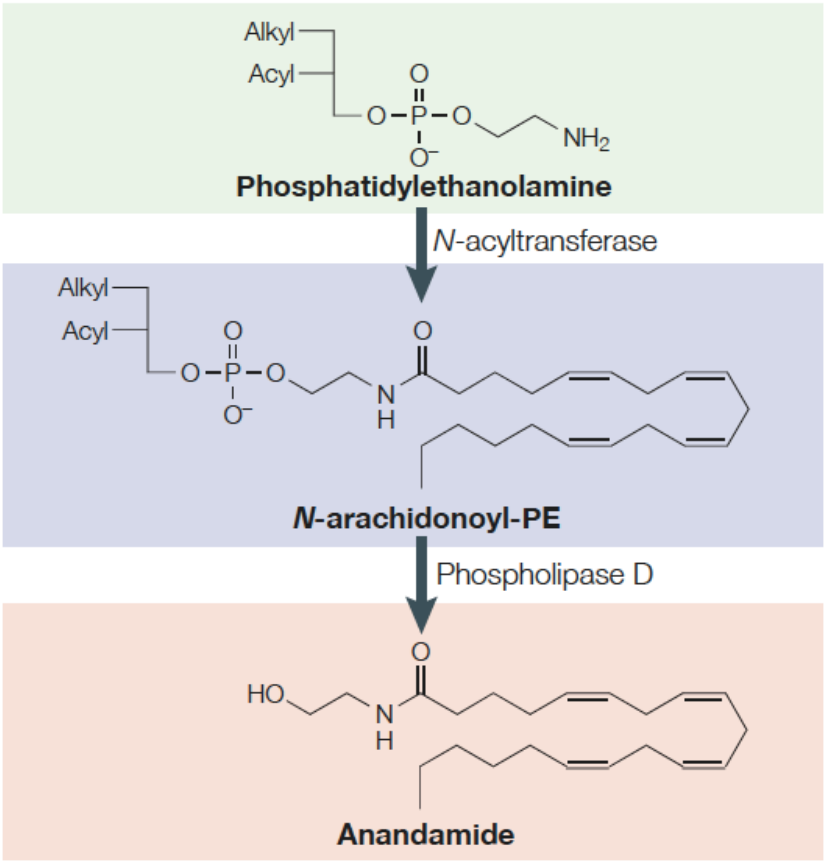

Figure 1-4 Anandamide biosynthesis in neurons. Phosphatidylethanolamine (PE) is biotransformed by *N*-acyltransferase into *N*-arachidonoyl-PE which is then cleaved by phospholipase D into anandamide and phosphatidic acid (Piomelli, 2003).

1.2.2.2 2-AG

2-arachidonoylglycerol plays a role in a diverse number of metabolic pathways, which may explain its high concentration and widespread distribution in brain tissue (Sugiura et al., 1995). 2-AG can be a precursor for one metabolic pathway (ie. the precursor of arachidonic acid) or the end-product of another (ie. the degradation product of inositol phospholipids) (Sugiura et al., 2002). Due to its central location in lipid metabolism it is complicated to define the biochemical pathways involved in its biosynthesis, however two possible routes have been

identified as depicted in Figure 1-5 (Piomelli, 2003). One possible pathway is initiated by the phospholipase-mediated formation of DAG (1,2-diacylglycerol). DAG is formed via the cleavage of phosphatidylinositol by phospholipase C (PLC). The hydrolysis of DAG to 2-AG is then catalyzed by calcium-stimulated diacylglycerol lipase (DAGL). Another route of 2-AG biosynthesis may be through the formation of a 2-arachidonoyl-lysophospholipid such as lyso-PI, which is catalyzed by phospholipase A1; the lysophospholipid is then hydrolyzed by lyso-PLC to form 2-AG (Piomelli, 2003).

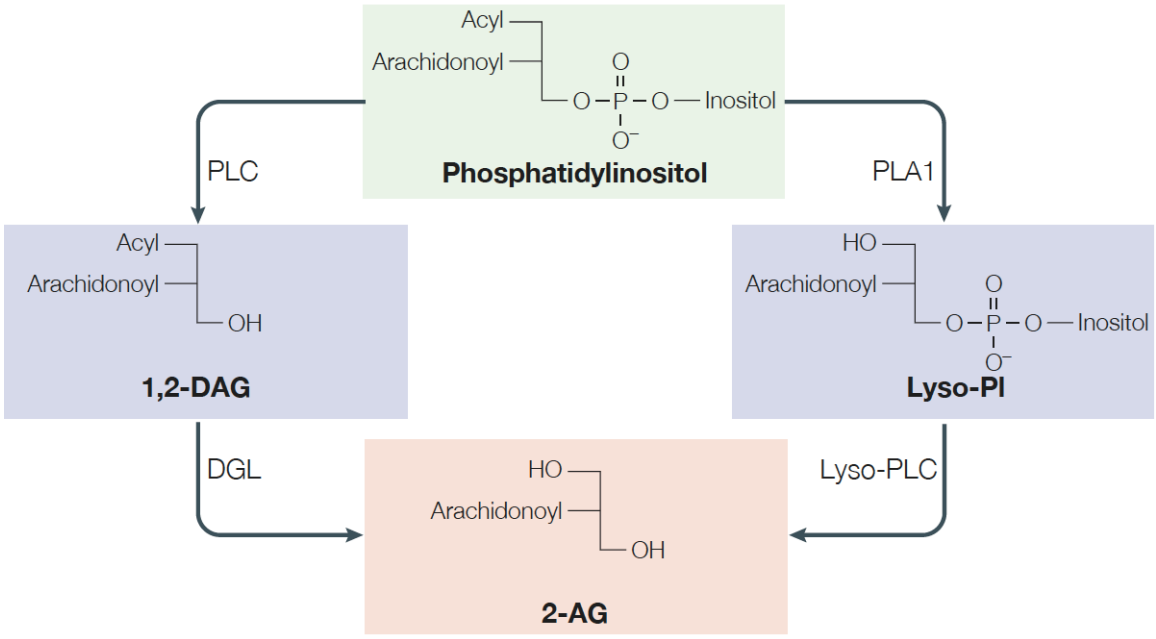

Figure 1-5 **Two possible pathways for the biosynthesis of 2-AG. Left, demonstrates one possible route via the cleavage of phosphatidylinositol (PI) by phospholipase C (PLC) to form 1,2-diacylglycerol (DAG). DAG is then catalyzed to 2-AG by diacylglycerol lipase (DAGL). The second route on the right shows another possibility where PI is catalyzed by phospholipase A1 (PLA1) to 2-arachidonoyl-lysophospholipid (Lyso-PI), which is then catalyzed by lyso-PLC to form 2-AG (Piomelli, 2003).**

1.2.3 Release

Due to their hydrophobic nature the release of endocannabinoids from neurons differs from classical transmitters and neuropeptides that can diffuse through the aqueous fluids surrounding neurons and within the synaptic clefts. Anandamide and 2-AG tend to maintain association with the lipid membranes of the neuron due to their lipophilic nature and thus they may not leave the cell in which they were produced, rather they may move laterally until they come into contact with membrane embedded CB receptors (Xie et al., 1996). However, anandamide has been found in the interstitial fluid of the brain indicating that it may also be able to move through membranes and access extracellular fluids (as reviewed by Piomelli, 2003).

Di Marzo et al. (1998) suggest that endocannabinoids are released in a method similar to the release of classical neurotransmitters, though postsynaptically as opposed to classical presynaptic release. They are released via selective, temperature-dependent and Na^+-independent transporters following postsynaptic membrane depolarization as a result of the influx of Ca^+ into the cell (Di Marzo et al., 1998) (Figure 1-6). Endocannabinoid release may also be induced via activation of a $G_{q/11}$-coupled receptor (as reviewed in Hashimotodani et al., 2007) (Figure 1-6). One study demonstrated that endocannabinoid release could occur independently of Ca^+ influx due to G-protein activation. In Purkinje cells they showed that activation of the metabotropic glutamate type 1 receptor (mGluR1), a G protein-coupled receptor family member, caused the release of endocannabinoids and subsequent inhibition of excitation from climbing fibres to

Purkinje cells in the cerebellum (Maejima et al., 2001). Though this study was the first to demonstrate the ability of endocannabinoids to be released without an influx of Ca^{2+}, many studies that followed demonstrated that the release of endocannabinoids could be the result of the activation of $G_{q/11}$-coupled receptors including mGluR1, M1 and M3 muscarinic receptors and orexin receptors (as reviewed by Hashimotodani et al., 2007). A study performed by Varma et al. (2001) found that activation of mGluR1 enhances depolarization-induced suppression of inhibition (DSI), whereas mGluR1 antagonists are able to block this effect (Figure 1-7). They also found that the administration of mGluR1 agonists to CB_1-R knockout mice did not have an effect on inhibitory postsynaptic currents. As a result of these findings they concluded that mGluR1-induced enhancement of DSI and suppression of IPSC is mediated by endocannabinoids (Varma et al., 2001). Therefore endocannabinoid release can be the result of a postsynaptic increase of Ca^{2+} or activation of a $G_{q/11}$-coupled protein receptor, and when both stimuli occur this can result in increased release (Hashimotodani et al. 2007).

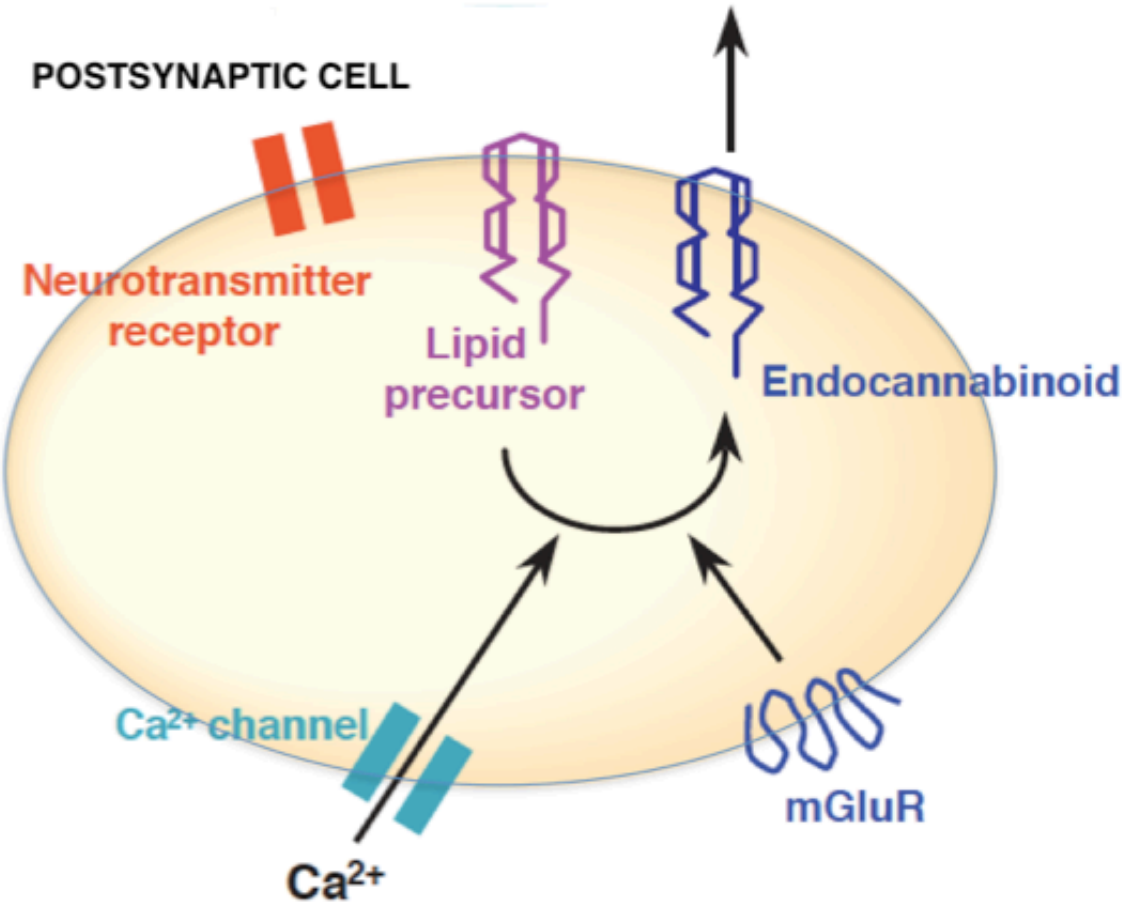

Figure 1-6 **Endocannabinoid release can be the result of either postsynaptic depolarization opening voltage-dependent Ca^{2+}-channels and the resultant influx of Ca^{2+} leading to the synthesis of endocannabinoids followed by their release or due to the activation of postsynaptic mGluRs. (Wilson & Nicoll, 2006).**

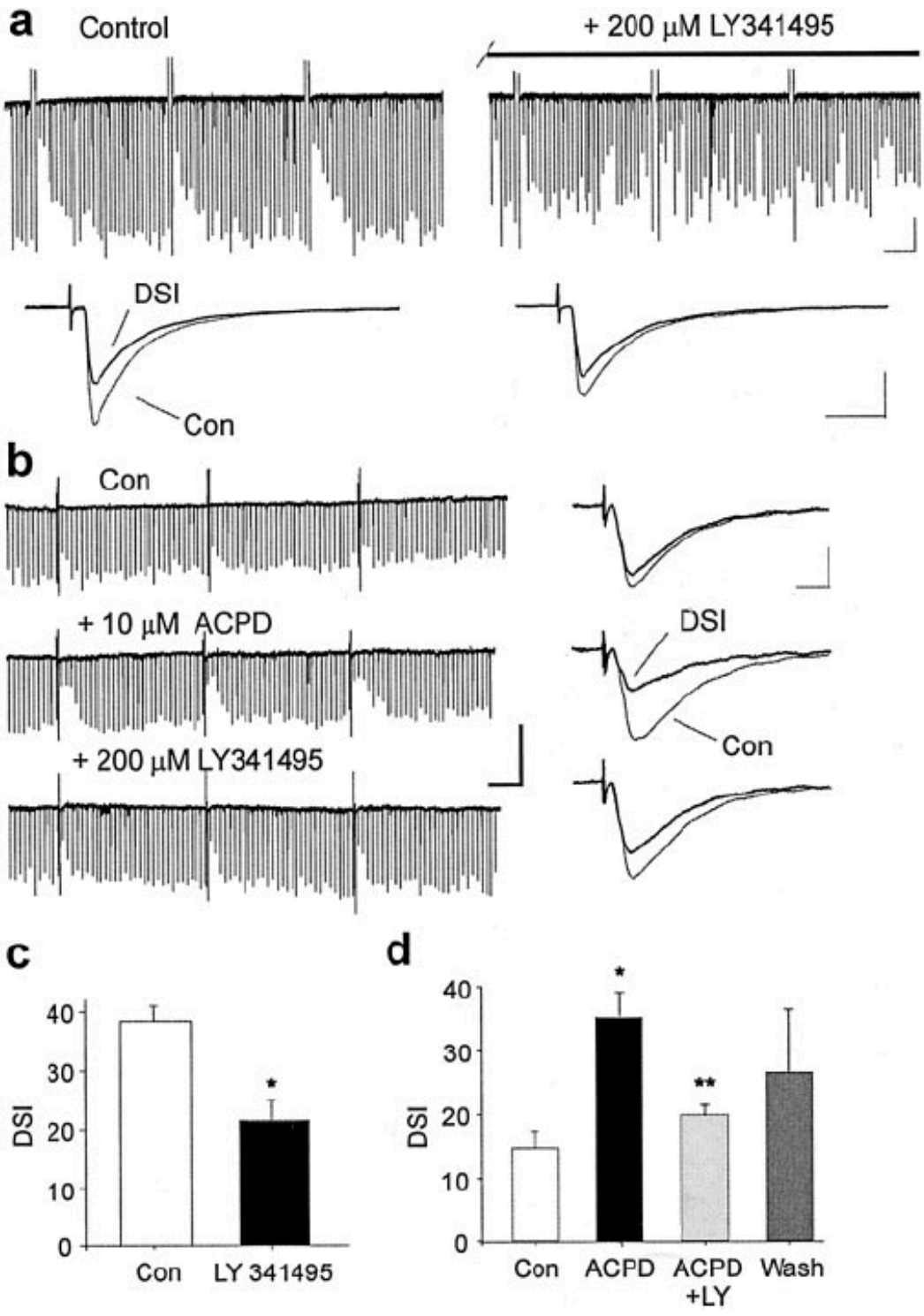

Figure 1-7 The mGluR1 antagonist LY341495 is able to decrease DSI and suppress ACPD-induced enhancement of DSI (ACPD is a mGluR agonist) (Varma et al., 2001).

1.2.4 Uptake and degradation

For degradation of these endocannabinoids to occur they must first be transported into the cell, once there hydrolysis can proceed. Anandamide and 2-AG are both capable of passively diffusing through the lipid membranes, though there may be an accelerated and selective carrier system that is responsible for moving these ligands into neurons and glial cells. This may be achieved by facilitated diffusion using energy-dependent carriers, similar to the internalization of fatty acids, eicosanoids and other biologically important lipids (Piomelli, 2003).

In one study, Piomelli et al. (1999) provided evidence that anandamide actions are terminated through a two-step process that includes carrier-mediated uptake into neurons and is followed by intracellular hydrolysis (Figure 1-8). They demonstrated that anandamide uptake occurs via a high-affinity Na^+-independent transporter that can be selectively inhibited by AM404, an anandamide analog. High substrate specificity was also shown since rat brain neurons were capable of internalizing anandamide but not its closely related analogues. They also suggest that 2-AG may function as a substrate due to recognition by the transporter of its non-ionizable head group and distal end hydroxyl moiety and because esters such as 2-AG can act as a hydrogen acceptor.

This area proves to be one of controversy, while Piomelli et al. (1999) believe a transporter is involved due to temperature dependency, selective inhibition and substrate specificity, others propose that uptake occurs by passive diffusion (Glaser et al., 2003) due to the findings that anandamide uptake does not require cellular energy (Beltramo et al., 1997; Hillard et al., 1997). Glaser et

al. (2003) refute the findings of Piomelli and colleagues (1999) by suggesting that the initial rates of accumulation need to be examined rather than long time points in order to eliminate the downstream effects including metabolism and intracellular sequestration of anandamide. They suggest that at short time points AM404 (which was determined to be a transport inhibitor at long time points by Piomelli et al. (1999)) does not inhibit anandamide uptake but rather it inhibits fatty acid amide hydrolase (FAAH). Therefore it is suggested that the increase in anandamide levels is in fact due to the inhibition of FAAH, the enzyme responsible for anandamide hydrolysis. Due to this finding and other evidence using immunofluorescence techniques that FAAH remains on intracellular membranes, they conclude that anandamide uptake is the result of simple and passive diffusion across the membrane (Glaser et al., 2003). This area of cannabinoid research remains hotly debated and definitive conclusions have yet to be reached.

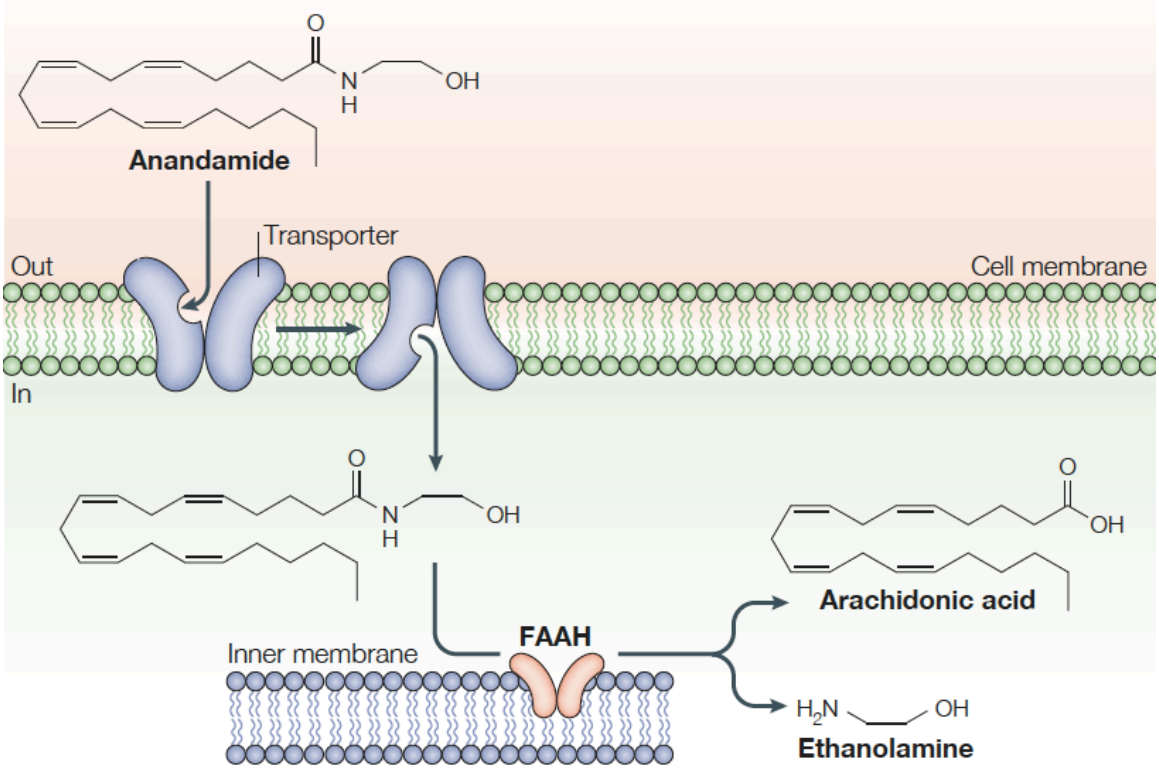

Figure 1-8 Mechanism of uptake and deactivation in neurons as proposed by Piomelli *et al*. (1999). Anandamide and 2-AG are internalized via a high-affinity transporter. Once internalized anandamide and 2-AG are hydrolyzed by two distinct serine hydrolases: fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively, into their inactive breakdown products (Piomelli, 2003).

1.2.4.1 Anandamide

The enzymatic degradation of anandamide occurs in many different tissues and cells of the body (Sugiura et al., 2002). It is rapidly hydrolysed following its re-uptake via anandamide amidohydrolase into free arachidonic acid and ethanolamine, which are then fed into other metabolic pathways (Di Marzo et al., 1994). The enzymatic activity of anandamide amidohydrolase was first detected in 1993 by Deutsch and Chin due to its ability to catalyze the hydrolysis of anandamide. This enzyme was later purified by Cravatt et al. (1996) and

named fatty acid amide hydrolase (FAAH). FAAH is an enzyme that is able to catalyze both the hydrolysis and biosynthesis of anandamide from free fatty acids and ethanolamine, as previously noted (Sugiura et al., 2002). In the 1996 study by Cravatt et al., FAAH was first identified as the enzyme responsible for the degradation of anandamide. In this study COS-7 cells transfected with FAAH cDNA expressed high levels of FAAH, whereas, the untransfected COS-7 cells contained negligible amounts of FAAH. The ability of these cells to convert ^{14}C-anandamide into its breakdown product arachidonic acid was then determined using thin-layer chromatography (Figure 1-9). It was found that due to the inability of untransfected cells to hydrolyse anandamide that FAAH is indeed the enzyme responsible for its degradation (Cravatt et al., 1996).

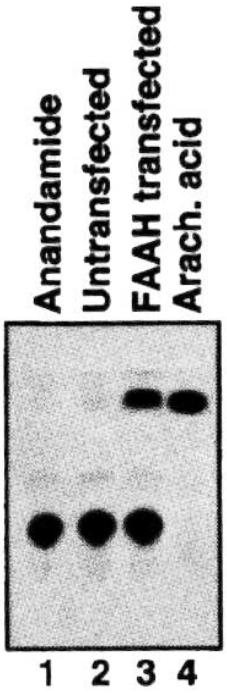

Figure 1-9 Thin-layer chromatography showing the conversion of ^{14}C-anandamide into arachidonic acid. Lane 1 and Lane 4 show anandamide and arachidonic acid standards, respectively. Lane 2 depicts the inability of untransfected cells to hydrolyse anandamide, whereas Lane 3 shows the ability of transfected cells to hydrolyse anandamide (Cravatt *et al*., 1996).

1.2.4.2 2-AG

2-AG is quickly metabolized into arachidonic acid and glycerol in a number of cells. Its rapid elimination is valuable due to its potency and its role in many biological activities that affect a diversity of tissues and cells of the body. In excess, 2-AG may cause deleterious and undesirable effects such as hypotension, relaxation of mesenteric arterial rings and a reduction of cellular responses induced by endothelin-1 in the brain capillary endothelium (Sugiura et al., 2002). When 2-AG passively diffuses into cells it is immediately degraded by enzymatic hydrolysis of the ester bond. Though many enzymes are capable of degrading 2-AG including artificially purified FAAH (Di Marzo et al., 1998)

presynaptic monoacylglycerol lipase (MAGL) is the primary enzyme (Di Marzo et al., 2004). In a study performed by Blankman et al. (2007) they characterized the ability of multiple enzymes to regulate 2-AG hydrolysis. Mouse brain cells were transfected with serine hydrolases (SH) and MAGL was demonstrated to be the most active SH (Figure 1-10).

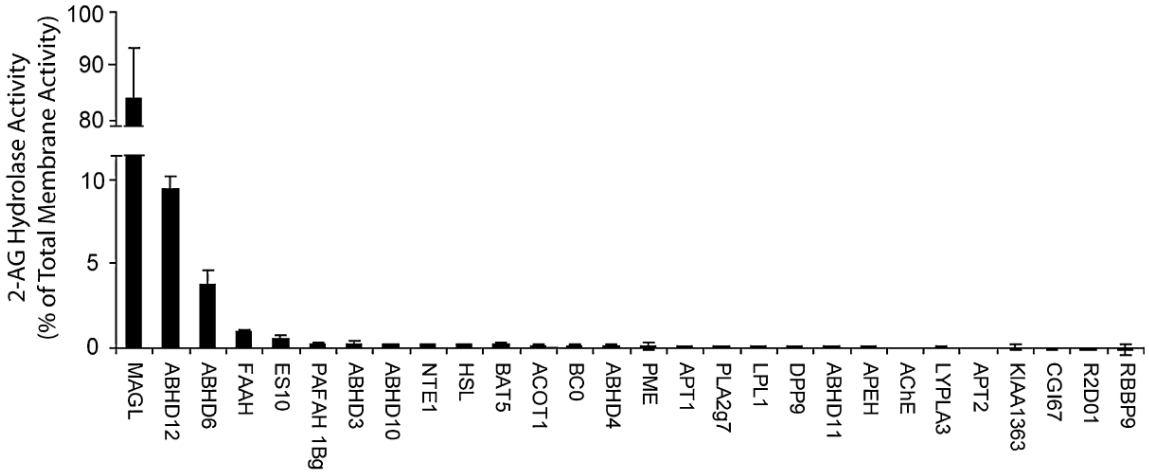

Figure 1-10 The relative abilities of mouse brain membrane serine hydrolases to degrade 2-AG. Using SH-transfected cell homogenates 2-AG hydrolase activity was assayed to determined the contribution of each enzyme to brain-membrane 2-AG hydrolase activity (50 mM Tris-HCl (pH 7.5), 50 mg protein/ml, 100 mM 2-AG, 10 min, room temperature). Results include average values ±SEM for two experiment with two separate transfections per enzyme where n=4 (Blankman *et al*., 2007).

1.3 Cannabinoid-1 receptors

The initial identification and characterization of a cannabinoid receptor in the brain was reported by Devane *et al*. (1988). This study provided supportive evidence for the existence of a cannabinoid receptor associated to a second messenger system through a G protein-coupled receptor (GPCR). Using a high specificity, radiolabeled cannabinoid agonist, [^{3}H]CP-55940, and carefully

optimized binding conditions, a K_d value of 139 pM and B_{max} value of 1.3 pmol/mg of protein were determined using Scatchard analysis (Figure 1-11). These K_d and B_{max} values are consistent with values found for other neuromodulatory receptors in the CNS. Due to their ability to inhibit adenylate cyclase in a way that is reversible, cell type-specific, potent and enantio-selective, it was hypothesized that cannabinoid compounds do indeed interact with a membrane-bound receptor. The work of this group produced results that were consistent with a GPCR that is associated with a second messenger system. Their findings of pH sensitivity and thermolability are consistent with a proteinaceous, neuromodulatory binding site (Devane *et al*., 1988).

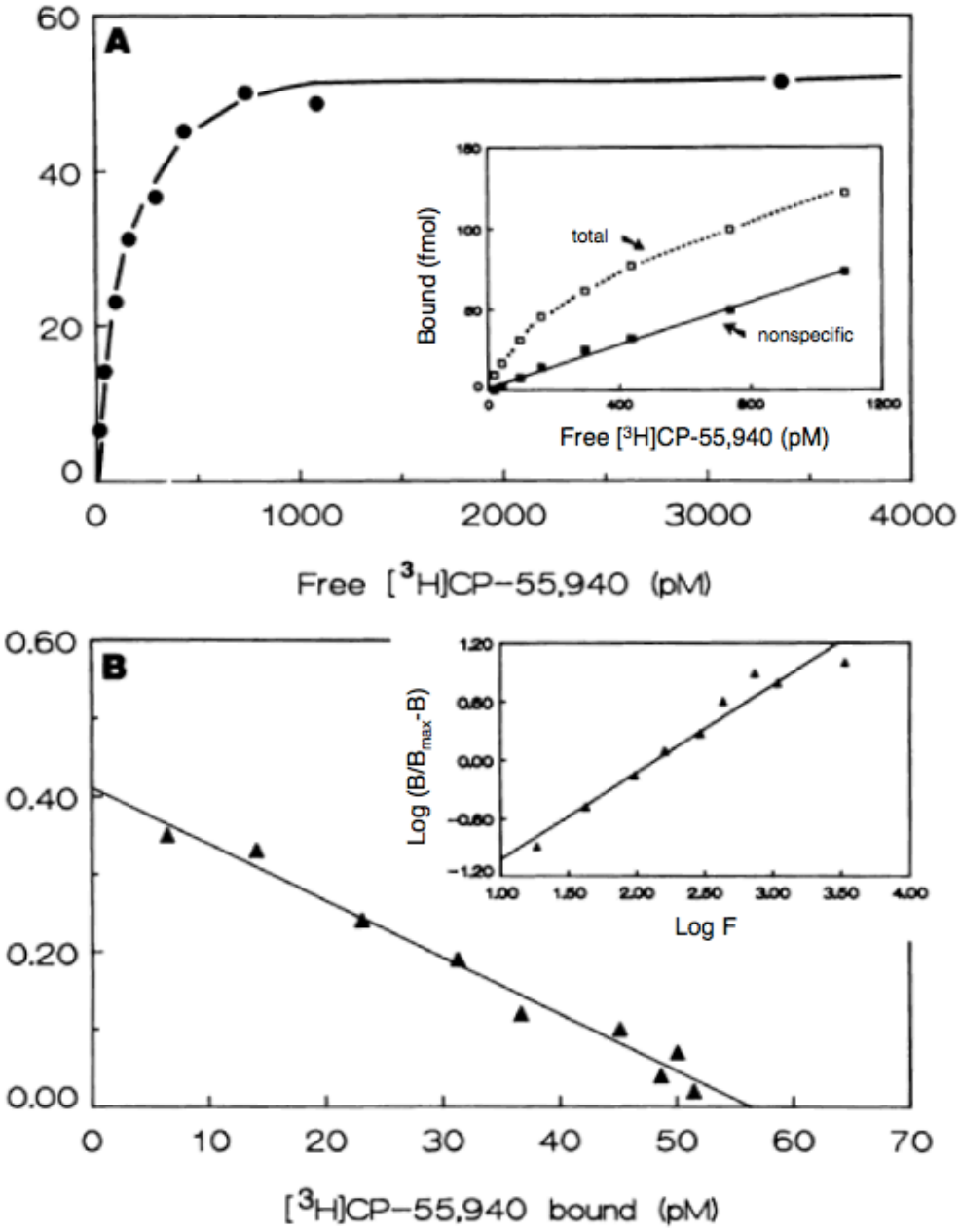

Figure 1-11 **Equilibrium binding for various concentrations of [^{3}H]CP-55940 incubated with rat brain membranes (43 µg of protein). (A) Depicts the saturation isotherm of specific binding. *Inset*: binding of [^{3}H]CP-55940 in the absence (□) or presence (■) of 1 µM DALN, the difference of which is used to determine specific binding. (B) Scatchard transformation of [^{3}H]CP-55940 binding data from (A) with the bound ligand being expressed in terms of concentration (pM). K_d and B_{max} values were 139 pM and 1.3 pmol/mg of protein respectively. *Inset*: The Hill transformation of data from (A)., where *F* is free drug concentration and *B* is specifically bound drug. The Hill coefficient (n_H) was calculated to be 0.90 for this experiment. The *lines* drawn represent the best fit as determined by least squares linear regression analysis. DALN is desacetyllevonantradol (Devane *et al.*, 1988).**

1.3.1 Structure

CB_1 receptors are part of the G protein-coupled receptor superfamily (Howlett *et al*., 2004) and are comprised of seven trans-membrane spanning domains with a C-terminal domain that (Howlett, 2005) couples to an intracellular $G_{i/o}$ heterotrimeric protein (Di Marzo *et al*., 2004). The $G_{i/o}$ heterotrimeric proteins are responsible for mediating the release of response hormones that lead to the inhibition of adenylate cyclase activity, which regulates the synthesis of cAMP from ATP (Devane et al., 1988). They can be activated by three major groups of lipophilic ligands, including the endocannabinoids, natural cannabinoids and synthetic cannabinoids (Di Marzo *et al*., 1998). The first cannabinoid receptor sub-type (CB_1) was cloned in 1990 by Matsuda et al., followed by the identification of a peripheral receptor, CB_2, in 1993 by Munro *et al*.. It was found that human CB_1 receptors are composed of 472 amino acids and those in rats are 473 amino acids long (Matsuda *et al*., 1990) (Figure 1-12). The CB_2 receptor was determined to be 360 amino acids long and shares 44% of its overall sequence with the CB_1 receptor, with 68% similarity through the trans-membrane domains (Munro *et al*., 1993).

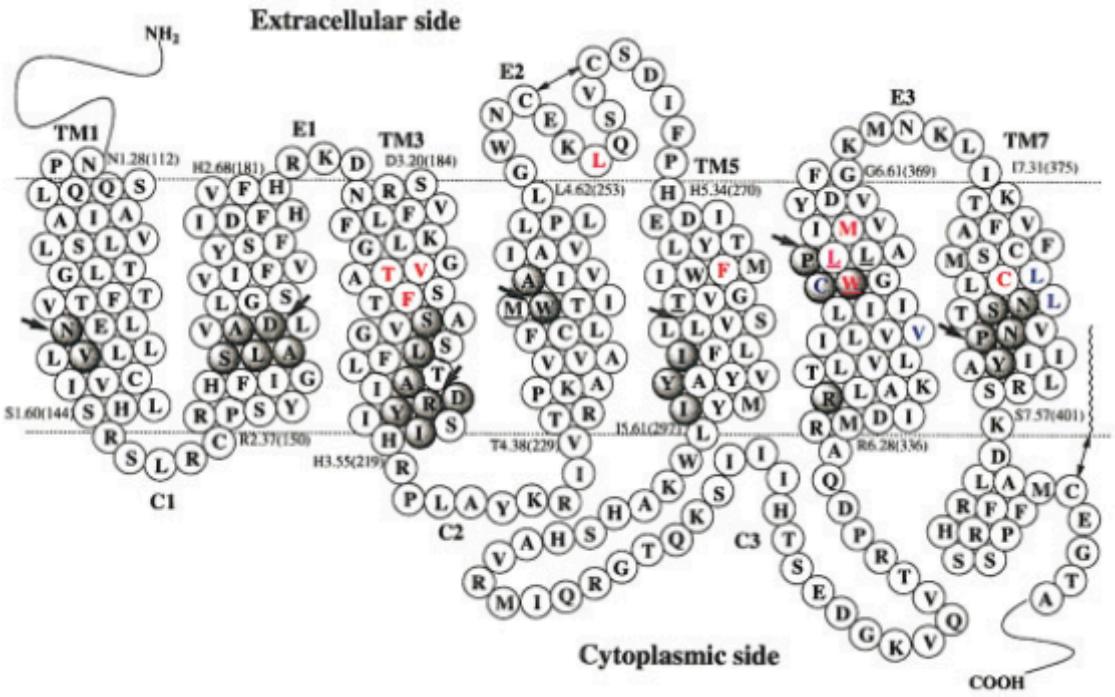

Figure 1-12 Two-dimensional representation of the 7-transmembrane, 473 amino acid CB_1 receptor found in rat brain. E1, E2 and E3 represent the three extracellular regions and C1, C2 and C3 represent the three intracellular regions. The shaded circles represent the highly conserved residues, and the arrows denote the most highly conserved residues of each helix (Shim et al., 2003).

1.3.2 Distribution

CB_1 receptors are one of the most abundant G protein-coupled receptors found in the brain (Howlett *et al*., 2004). In addition to their presence in the brain they have been found in various peripheral tissues exclusive of the immune system, whereas CB_2 receptor distribution has been historically associated with the immune system (Sugiura *et al*., 2002). However, in recent years evidence for low level presence of CB_2 receptors in the brain has begun to surface (Nunez *et al*., 2004; Van Sickle *et al*., 2005; Gong *et al*., 2006; Onaivi *et al*., 2006).

In rats the CB_1 receptors are primarily expressed in the central nervous system with distribution highest in the basal ganglia, cerebellar molecular layer, innermost layers of the olfactory bulb and parts of the hippocampal formation (Figure 1-13). Moderate densities are also found in the forebrain and sparse densities in the brain stem and spinal cord (Herkenham *et al*., 1991). Using *in vitro* labelling of brain sections with [^{3}H]CP55,940, a synthetic agonist, followed by quantitative receptor autoradiography, it was found that CB_1 receptors are similarly distributed in the human brain. Due to larger cortices in humans, densities can be more accurately defined: the highest being found in the limbic cortices and significantly lower densities in the primary sensory and motor areas (Glass *et al*., 1997). This localization suggests a role in motivational and cognitive information processing (Howlett *et al*., 2004). CB_1 receptors are also located, to a lesser extent, in the lungs, liver and kidneys. In both the CNS and PNS, CB_1 receptors are most commonly found associated with voltage-activated Ca^{2+} channels at the presynaptic neurons (Di Marzo *et al*., 1998).

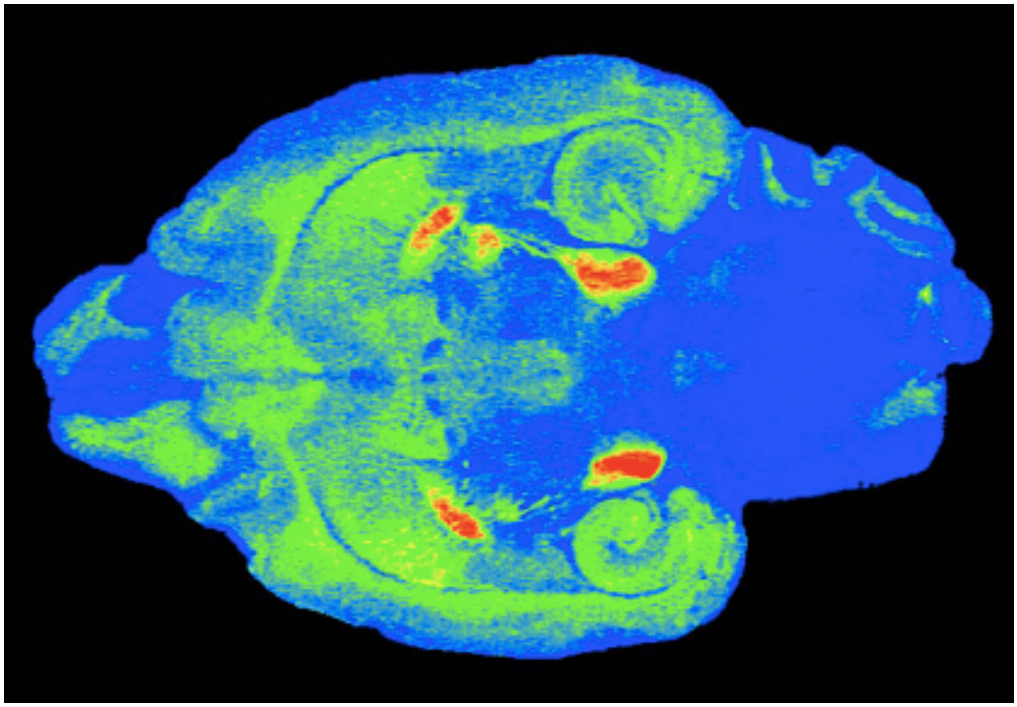

Figure 1-13 A horizontal section of rat brain showing autoradiography using [^{35}S]GTPγS to label cannabinoid-stimulated G-proteins, with WIN55212-2 as the agonist. High levels of activation can be seen in the globus pallidus and substantia nigra (sections of the basal ganglia), with moderately high activation in the hippocampus, cortex and cerebellum (Howlett *et al*., 2004).

1.4 The cannabinoid signal transduction pathway

In 2001, Wilson and Nicoll discovered the role of endocannabinoids in the mediation of retrograde signalling at the hippocampal synapses of the brain. They hypothesized that cannabinoids play a role in the depolarization-induced suppression of inhibition (DSI) due to three attributes. Those attributes being firstly that DSI requires Ca^{2+} influx into the postsynaptic neuron like endocannabinoid synthesis, secondly, DSI expression is presynaptical and does not affect the sensitivity of the postsynaptic membrane to GABA, this is consistent with the presence of CB_1-Rs on GABA-containing axons, and finally

DSI is pertussis toxin-sensitive which implies the involvement of G_i- or G_o-coupled receptors, like the CB_1 receptors. They determined that when hippocampal neurons are rapidly depolarized in a Ca^{2+}-dependent manner, both anandamide and 2-AG are released. Synthetic agonists acting at CB_1 receptors also cause a depression in the release of the neurotransmitter GABA, since they mimic the retrograde signalling caused by the release of endocannabinoids (Wilson & Nicoll, 2001). Retrograde signalling occurs, in this case, when a ligand travels from the postsynaptic neuron to the presynaptic neuron and inhibits the release of GABA. This process is also known as DSI (Nicoll & Alger, 2004). It was determined that this backward-acting ligand was 2-AG, and that CB-receptor blocking drugs were capable of preventing DSI, whereas those that act as agonists are capable of mimicking DSI (Figure 1-14). DSI is important in brain activity and works to temporarily reduce inhibition and improve the long-term potentiation of learning. Endocannabinoids are well-suited for this role due to their short-acting local effects (Nicoll & Alger, 2004).

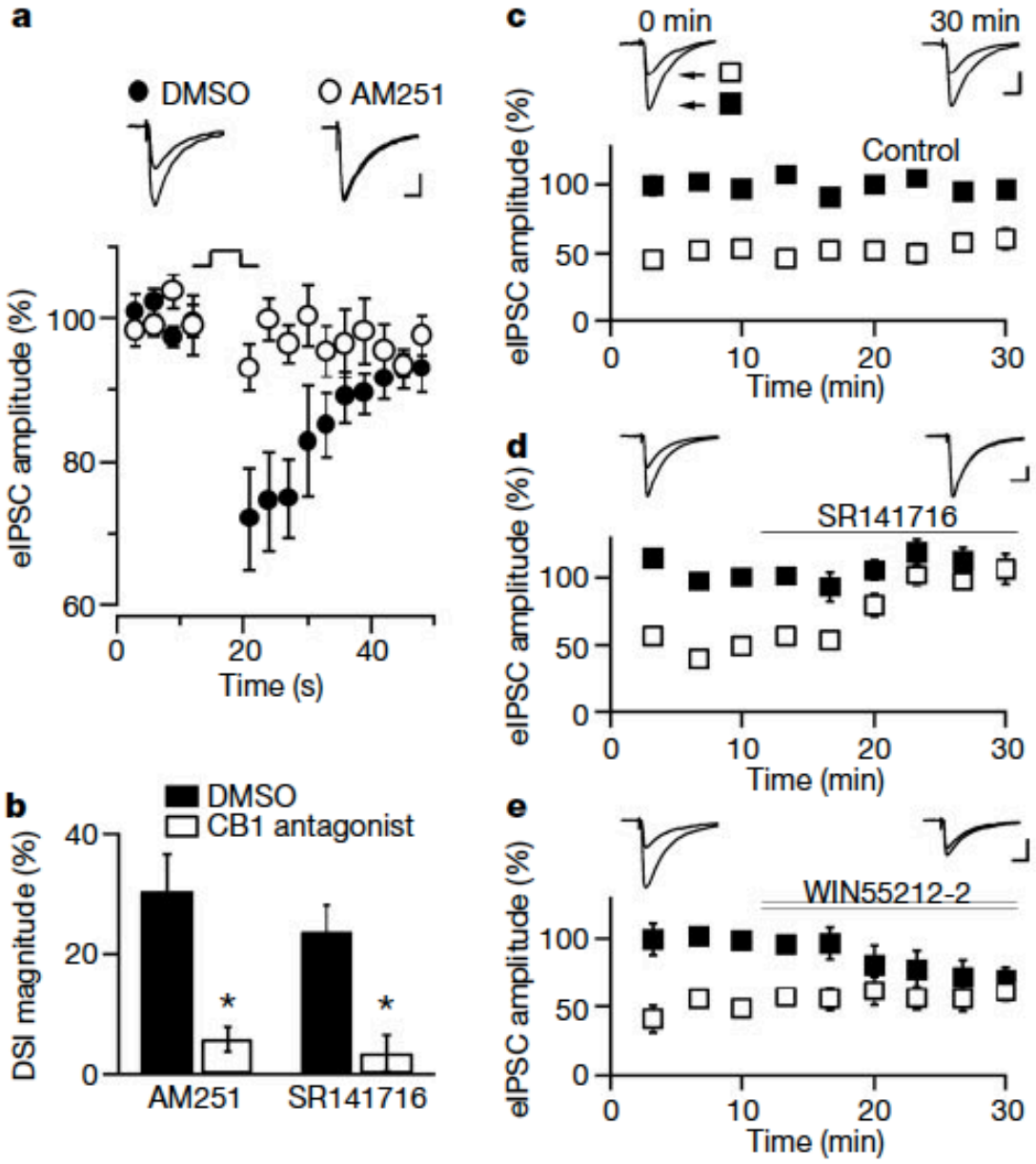

Figure 1-14 DSI requires the presence of endocannabinoids. (a) depicts the transient depression of evoked inhibitory postsynaptic currents (eIPSCs) caused by a brief depolarizing step in the holding potential of pyramidal neurons in the presence of AM251 (a CB_1 antagonist). (b) Summary of the effects of AM251 and SR141716A (CB_1 antagonist) on DSI magnitude. (c) control group shows steady DSI. (d) SR141716A blocked the ability of a depolarizing step to cause an eIPSC amplitude depression without affecting baseline eIPSC. (e) the effect of DSI on inhibitory transmission was mimicked by WIN55212-2 (a synthetic CB_1 agonist) and the DSI-resistant component of the eIPSC was not affected (Wilson & Nicoll, 2001).

Postsynaptic depolarization results in the opening of voltage-dependant Ca^{2+} channels, the subsequent influx of Ca^{2+} then activates the enzymes responsible for the synthesis of endocannabinoids from their lipid precursors (Wilson & Nicoll, 2006). Though, as previously discussed, the activation of postsynaptic mGluRs (metabotropic glutamate receptors) can also result in the generation of endocannabinoids through the activation of phospholipase C resulting in the production of diacylglycerol which is subsequently cleaved by diacylglycerol to yield 2-AG (Maejima et al., 2001). There is also some evidence of the peripheral involvement of ionotropic cannabinoid receptors (as reviewed by Akopian et al., 2008). The endocannabinoids then traverse the postsynaptic cell membrane in order to activate the presynaptic CB_1 receptors (Figure 1-15). This activation of the CB_1 receptors is followed by the stimulation of the $G_{i/o}$ heterotrimetic proteins that are coupled to the inhibition of adenylate cyclase, the enzyme responsible for synthesizing cAMP production from ATP. The inhibition of adenylate cyclase corresponds to the inactivation of protein kinase A (PKA) (Di Marzo et al., 2004). CB_1 activation may also stimulate mitogen-activated protein kinase (MAPK) which, along with PKA, is responsible for regulating the expression of some genes such as growth factor receptors (*FGFR2*, *EGFR*), oncogenes (*K-Ras*, *c-Myc*), tumour suppressor genes (*DUSP6*, *Fos*) and genes involved in the cell cycle (*CCNB1, CCNB1IP1*) (Di Marzo et al., 2004; González-Sarrías et al., 2009). The activation of the $G_{i/o}$-coupled protein receptors via CB_1 receptor stimulation is also directly coupled to voltage-activated Ca^{2+} channel inhibition, which then results in the stimulation of inwardly

rectifying K^+ (Di Marzo et al., 2004). Inactivation of the voltage-activated Ca^{2+} channel is a result of the direct inhibition by the G-protein βγ-subunits at synapses between GABA (γ-aminobutyric acid) interneurons and pyramidal cells of the hippocampus, whereas the G-protein α-subunit at synapses between parallel fibres and Purkinje cells in the cerebellum results in the activation of the K^+ channels (Piomelli, 2003). The resulting hyperpolarisation can result in a depression in response to depolarizing stimuli in the axon terminals and cause the inhibition of neurotransmitter release at the synapse (Di Marzo et al., 2004; Howlett, 2005).

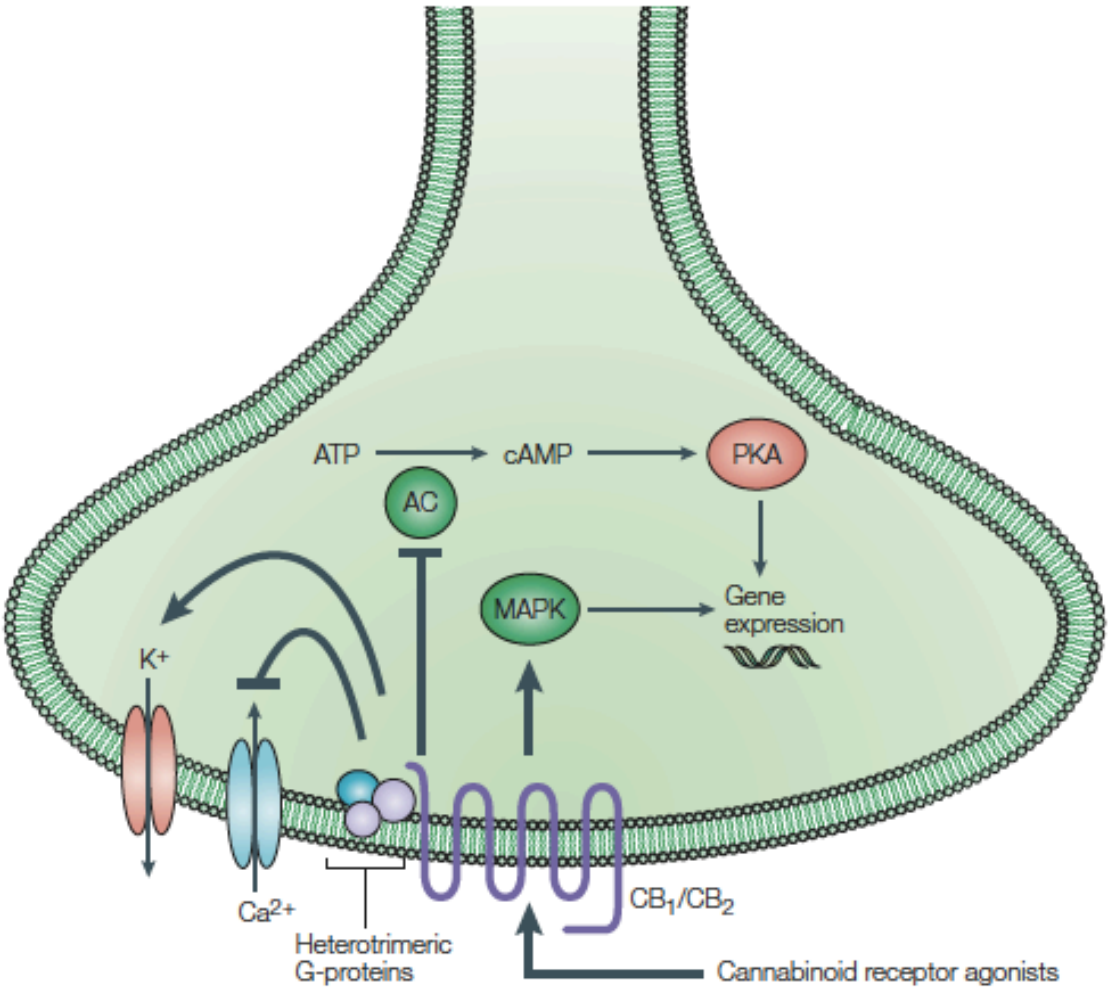

Figure 1-15 Cannabinoid receptor signalling pathway. The activation of CB receptors by agonist binding stimulates the $G_{i/o}$ heterotrimeric proteins, which are coupled to the inhibition of adenylate cyclase (AC), the enzyme responsible for synthesizing cAMP production from ATP. The inhibition of AC causes inactivation of protein kinase A (PKA) phosphorylation pathway. CB activation may also stimulate mitogen-activated protein kinase (MAPK) which, along with PKA, is responsible for regulating the expression of some genes. The activation of the $G_{i/o}$-coupled protein receptors via CB receptor stimulation is also directly coupled to voltage-activated Ca^{2+} channel inhibition, which then results in the stimulation of inwardly rectifying K^+ currents (Di Marzo et al., 2004).

1.5 Toxicology of natural CB-receptor modulators

The natural compounds that are capable of modulating normal endocannabinoid-receptor interactions come from the plant *Cannabis sativa. C.*

sativa produces approximately 60 unique compounds, termed cannabinoids, of which Δ^9-tetrahydrocannabinol (Δ^9-THC) is the main and most potent psychoactive ingredient (Guzman, 2003), and the only cannabinoid to bind to cannabinoid receptors with high affinity (Di Marzo et al., 2004). As an illicit drug *C. sativa* is commonly known as marijuana and has been popular in many developed countries since the 1960s, still today it represents one of the most widely abused drugs in the world (Adams & Martin, 1996). However, its use as a therapeutic drug dates back to 2600 B.C. when Chinese emperor, Huang Ti, suggested it be used to provide relief from cramps, rheumatic and menstrual pain (Di Marzo et al., 2004). In the 1940s the first cannabinoid to be identified was cannabidiol which proved to have very weak psychoactive properties (Mechoulam et al., 1998). It was not until 1964 that Δ^9-THC was isolated and its structure elucidated (Gaoni & Mechoulam, 1964). Finally in 1984, Howlett and Fleming made reliable indications to the existence of a cannabinoid receptor through which the cannabinoids exert their effects.

Plant cannabinoids mostly act as receptor agonists to cause a multitude of pharmacological effects, some of which are exploited for their therapeutic use as previously mentioned, however, one plant cannabinoid, Δ^9-tetrahydrocannabivarin, does act as an antagonist (Pertwee, 2006).

1.6 Toxicology of synthetic CB-receptor modulators

Some chemicals, other than those specifically designed as synthetic cannabinoids, may be able to alter endocannabinoid-receptor interactions through agonist or antagonist binding.

Quistad et al. (2002) found that some organophosphorus (OP) pesticides and their analogs are capable of inhibiting agonist binding to CB_1 receptors. Inhibition was assayed using [^{3}H]CP-55,940, and it was found that the binding of [^{3}H]CP-55,940 to CB_1 receptors in mouse brain was inhibited 50% by chlorpyrifos oxon at 14 nM and chlorpyrifos methyl oxon at 64 nM. Paraoxon, diazoxon, and dichlorvos were also found to result in 50% inhibition at higher concentrations between 1.2-4.2 μM, indicating that they are much less potent. Their subsequent findings suggest that these OP compounds may bind to a site other than the agonist site due to the their inability to completely displace the MAFP-sensitive component. MAFP is the unlabeled nucleophilic site displacer used to determine non-specific binding and is an OP inhibitor for agonist binding (Segall et al., 2003).

In another study, it was found that *O*-isopropyl dodecylfluorophosphonate, dodecanesulfonyl fluoride, and dodecylbenzodioxaphosphorin oxide are capable of inhibiting [^{3}H]CP-55,940 binding at an agonist site of the CB_1 receptor in mice brains that is proposed to be closely coupled to a nucleophilic binding site (Segall et al., 2003). It is suggested that these compounds inhibit binding via this coupled nucleophilic site or by direct inhibition at the agonist site and may also inhibit FAAH, the enzyme responsible for hydrolyzing anandamide, by phosphorylating it. With this study it was determined that these organophosphorus and organosulfur compounds with the highest potencies were capable not only of acting as CB_1 inhibitors but of also inhibiting esterase-

lysophospholipase (NTE-LysoPLA), a brain neuropathy target, which can lead to delayed toxicity (Segall et al., 2003).

1.7 Pharmacology

The cannabinoid system can be modulated and exploited by synthetic agonists, antagonists and inverse agonists. Here the roles of some of these compounds that have been synthesized and their utility in the field of cannabinoid pharmacology will be discussed.

1.7.1 Agonists

HU210, CP-55,940, and WIN55212-2, otherwise known as synthetic cannabinoids, can produce agonistic effects upon binding to cannabinoid receptors. These synthetic cannabinoids do not show a selective preference for either receptor but will bind with the same affinity to both CB_1 ad CB_2 receptors (Di Marzo et al., 2004). All three are useful agents in pharmacological studies concerning the function of cannabinoid receptors (Di Marzo, 2009). HU-210 was developed to work as a labelled probe to bind to cannabinoid receptors, it has a typical THC-like structure and was an important addition to the field of cannabinoid pharmacology (Mechoulam et al., 1998). HU-210 was the first of the potent and synthetic THC-analogues developed, followed by the development of 'non-classical' cannabinoids like the bicyclic CP-55,940 and the aminoalkylindole, WIN55212-2 (Pertwee, 2006).

1.7.2 Antagonists and inverse agonists

The development of selective cannabinoid receptor agonists soon followed with the development of the CB_1-selective antagonist SR141716A (Rinaldi-Carmona et al., 1994) and the CB_2-selective antagonist SR144528 (Pertwee, 2006). Again, the availability of selective CB_1 and CB_2 antagonists helped to greatly increase the tools available for research into cannabinoid pharmacology. There also exist some compounds that when administered alone are able to produce inverse agonist effects, where the resultant effect is in the opposite direction of those that are produced by CB_1 and CB_2 receptor agonists. These effects may be the result of antagonism of endocannabinoid release, though some effects have been noted in the absence of endocannabinoid release. This has lead to a hypothesis that some cannabinoid receptors exist in a constantly active state where inverse agonists work to shift these into a constitutively inactive state (see review by Pertwee, 2006).

1.8 Phthalate esters

1.8.1 History of use

Phthalate esters (PEs) are commonly used man-made chemicals that work as plasticizers (Figure 1-16). By weight they may contribute to up to 60% of plastic products (Rudel & Perovich, 2009). They have been in production for the past 50 years, with their yearly worldwide production reaching 4,300,000 tons/year for the last 25 years (Fromme et al., 2002; Peijnenburg & Struijs, 2006), though estimates as high as 6,000,000 tons/year exist (Rudel & Perovich, 2009). Due to their widespread use PEs have become ubiquitous environmental

contaminants (Heudorf et al., 2007). They are most commonly found in polyvinyl chloride (PVC) resins, however, they are also used in polyvinyl acetates, cellulosics and polyurethanes (Teil et al., 2006). The addition of PEs to these compounds works to enhance flexibility, elasticity, and self-lubricating properties of plastic products (Hauser & Calafat, 2005; Heudorf et al., 2007).

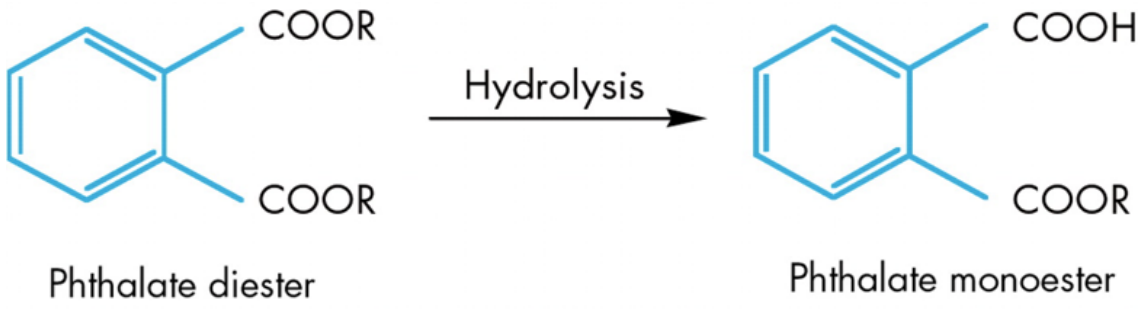

Figure 1-16 Generalized chemical structure of phthalate di- and mono- esters. Phthalate esters are comprised of paired ester groups on a benzene ring with *ortho* configuration (Hauser & Calafat, 2005).

Due to the presence of phthalates in plastics they are found in a wide range of consumer products from building materials and furniture to automobiles and clothing to food packaging and medical products. PE-containing products also include pharmaceuticals and pesticides (Heudorf et al., 2007). PEs are often found in scented products, including most personal care products such as cosmetics, lotions, perfumes and also in air fresheners and detergents (Wilson et al., 2004; Engel et al., 2009).

1.8.2 Human exposure

Phthalate esters can enter the environment due to losses during the manufacturing process or by leaching from the end products. PEs are

susceptible to leaching because they are not covalently bound to the polymeric matrix of the plastic (Fromme et al., 2002). Aside from leaching from the final product, PEs can also migrate or evaporate into both indoor air and atmospheric air, or food products and any other products that are in contact with the PE-containing plastic (Heudorf et al., 2007).

PEs are degraded in the environment due to photo degradation, biodegradation, and anaerobic degradation and therefore do not generally persist in the environment. However, due to their abundant sources they are constantly being released into the outdoor environment (Stales et al., 1997). Due to the quantity of phthalate sources found indoors, concentrations are generally higher indoors than outdoors. Also, the more volatile phthalate esters are found in higher concentration in the air, whereas, the heavier and less volatile PEs are more commonly concentrated into dust (Heudorf et al., 2007).

In many situations, humans are exposed to PEs for the duration of their lives, including the period of embryonic and fetal development. Exposure can result from ingestion, inhalation and dermal exposure. Due to the abundance of consumer products containing phthalate esters or food items being packaged in PE-containing materials, humans are exposed through direct contact and use, through PEs leaching into products or simply through widespread environmental contamination. Some phthalate ester-contain products are of particular concern for the significant quantities of PEs that are incorporated into them and their routes of human exposure, these include toys, food packaging, and medical devices (Heudorf et al., 2007). Exposures from these sources can be significant

due to the teething habits of children, the contact of PEs with food sources and the heavy reliance on plastic medical devices. Occupational exposure can also be significant due to the production of phthalate ester-containing consumer products (Hauser & Calafat, 2005).

Phthalate ester exposure from medical procedures can be considerable due to high levels of PEs leaching from plastic intravenous bags and tubing. In particular, those undergoing haemodialysis and premature babies may be exposed to much higher quantities of phthalate esters. One study examining the degree of exposure to di-2-ethylhexyl phthalate (DEHP)[1] in 11 haemodialysis patients estimated that, on average, a patient was being dosed with 105 mg DEHP during one 4-hr dialysis session, with an exposure range of 23.8 to 360 mg (Pollack et al., 1985). Similarly, premature babies that are intubated and placed in a plastic incubator receive high exposures to phthalates relative to body mass.

1.8.3 Pharmacokinetics

With high human exposure to phthalate esters it is necessary to understand the pharmacokinetics of such chemical substances.

Particular phthalate esters with short alkyl groups, for example di-*n*-butyl phthalate, are fairly water soluble (i.e. 0.5 g/100 ml), however most dialkyl phthalates, such as DEHP, are relatively insoluble due to their lipophilic structures. PEs generally have low volatility, particularly long-chain and branched

[1] the most commonly used and studied phthalate ester

phthalates, however, lower molecular weight phthalate esters have increased volatility, for example diethyl phthalate (DEP), dimethyl phthalate (DMP) and dibutyl phthalate (DBP) (Kluwe, 1982; Rudel & Perovich, 2009).

Phthalate esters are readily absorbed through the skin upon dermal contact and pulmonary tissue via inhalation due to their lipophilic nature. Some phthalate esters, particularly DEHP, can also be introduced directly into the blood stream through the use of plasticized medical equipment such as syringes, tubing, IV bags, etc. Other than occupational or medical exposure the most common route of phthalate ester absorption is through the ingestion of food or liquids that have contacted phthalate-containing products (Kluwe, 1982).

The dialkyl esters are mainly distributed to fat, absorptive and excretory organs including the gastrointestinal tract, liver, and kidneys though there is no evidence of accumulation due to their rapid metabolism and excretion. The monoesters on the other hand are not associated with adipose tissue but rather the intestines, heart, liver, kidney, lungs and muscle (Kluwe, 1982).

Dialkyl phthalates are generally and rapidly metabolized through hydrolysis to their monoester metabolites by enzymes found in many tissues of the body (Figure 1-15) (Kluwe, 1982; Silva et al., 2004). Some PEs with short chains can be excreted as their parent compounds, however, phthalate esters with longer chain lengths may require further modification after they are hydrolyzed to their monoester in order to achieve adequate polarity for excretion (Figure 1-17) (Kluwe, 1982). Monoesters may be further metabolized to oxidative metabolites, which can then be glucuronidated and excreted. The polar, lower

molecular weight phthalate esters are primarily excreted as their monoesters, whereas, the higher molecular weight phthalates undergo the multi-step oxidative pathway before excretion (Silva et al., 2004). Some research suggests that some toxic manifestations may be a result of these monoesters rather than diesters (Barr et al., 2003).

The major route of excretion for phthalate esters is through urinary excretion, with minimal amounts excreted through faeces and bile. The majority of administered phthalate esters, specifically DEHP and DBP, are cleared from the body with 24 hours of administration and virtually none remains 3-5 days after exposure (Kluwe, 1982).

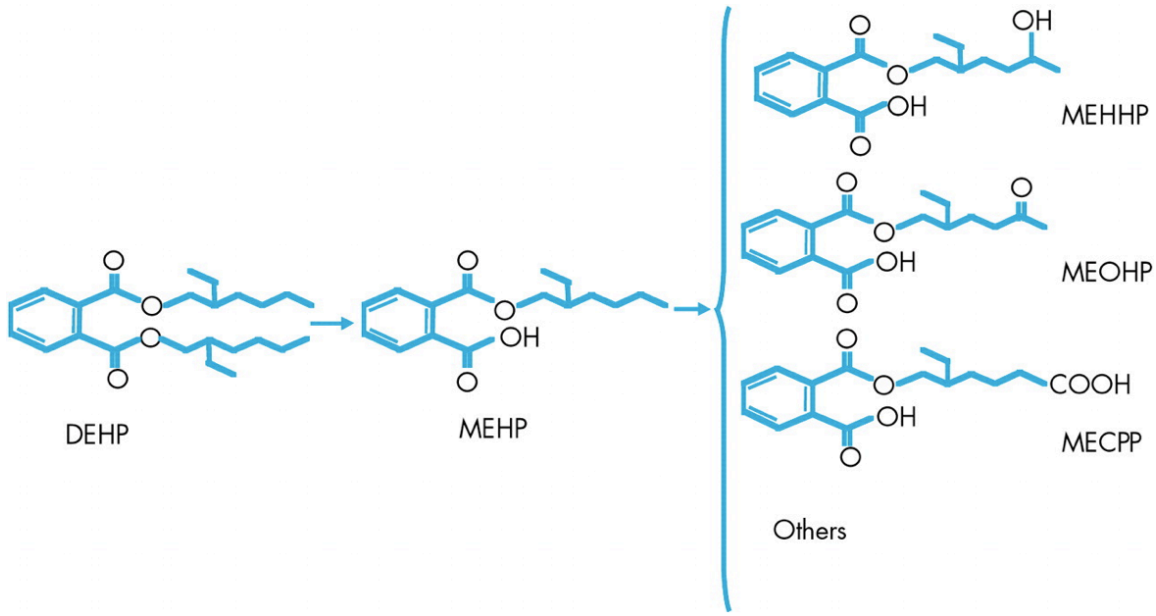

Figure 1-17 Metabolites of di-2-ethylhexyl phthalate (DEHP) found in humans, including mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP) and mono-2-ethyl-5-carboxypentyl phthalate (MECPP) (Hauser & Calafat, 2005).

Regardless of the efficient and quick metabolism and elimination of most phthalate esters the constant exposure to which humans are subjected to likely results in a steady-state concentration due to chronic low-level exposure from their presence in many commonly used products (Duty et al., 2003)

1.8.4 Toxicology

Available mammalian data suggests a range of possible toxic effects due to phthalate ester exposure including reproductive and developmental toxicity and carcinogenesis (Hoppin et al., 2002). While phthalate esters possess low acute toxicity with LD_{50} values ranging from 1-30 g/kg, chronic exposure from several PEs produces these notable toxicities (Heudorf et al., 2007). There also exists evidence of hepatic and renal impairment following administration of phthalates to rodents, and for selected phthalate esters adverse effects were noted in thyroid gland tissue and testes (Kavlock et al., 2002; Heudorf et al., 2007). Differences detected between species and between male and females have been found to be significant (Heudorf et al., 2007).

Phthalate esters also likely influence brain function. Kim et al. (2009) reported a positive association between phthalate ester exposure (measured as urinary phthalate metabolites) and symptoms of attention deficit hyperactivity disorder in school-age children. A number of phthalate esters modify brain function following intraperitoneal injection as evidenced by their ability to modify hexobarbital sleep time in rodents (Calley et al., 1966). Additionally, the cholinergic system of vertebrate brain is sensitive to phthalate esters. Various diesters (and to a lesser extent monoesters) inhibit nicotinic acetylcholine

receptor-mediated calcium signalling in human neuroblastoma cells (Liu et al., 2009; Lu et al., 2004) and fish exposed to dietary dibutyl- and diethylhexyl-phthalates show reduced brain acetylcholinesterase activity (Jee et al., 2009).

Exposure to phthalate esters is also known to be correlated to adverse reproductive and developmental effects. Several PEs have been shown to alter androgen production, rendering male foetuses the most vulnerable (Rudel & Perovich, 2009). Effects noted in animal studies include disrupted epididymal development, hypospadias, crytorchidism, retained nipples and reduced fertility though these effects occur at concentrations that are greater than levels to which the general population is exposed (as reviewed by Rudel & Perovich, 2009). Male rats exposed to some phthalate esters experience reduced levels of testicular testosterone and insulin-like 3 hormone production (Howdeshell et al., 2008).

Studies observing the effects of exposure to humans are far less common than animal studies, however, one study has observed the effect of prenatal phthalate exposure on the reproductive tract development in male offspring (Swan et al., 2005). Swan and colleagues found that the metabolite concentrations of some phthalates in prenatal urine samples were inversely proportional to anogenital distance in male offspring. These findings are consistent with incomplete virilization, a phthalate-related syndrome seen in prenatally exposed rodents (Swan et al., 2005). A link has also been made between increased phthalate metabolite concentrations in urine and decreased sperm quality in adult men (Duty et al., 2005).

1.8.5 Regulation of phthalate esters in Canada

Effective June 10, 2010 Health Canada, under the *Phthalates Regulations*, will limit the allowable concentrations of six phthalate esters (DEHP, DBP, BBP, DiNP, DiDP and DnOP) in consumer products intended for use by children. DEHP, DBP and BBP concentrations in soft vinyl children's toys and child care products are not allowed to exceed 0.1%. The same restrictions are placed on DiNP, DiDP and DnOP where it is "reasonably foreseeable" that the product be placed in the mouth of a child under the age of 4 years (Health Canada, 2011).

1.9 Purpose of the study

Little emphasis has been placed on synthetic environmental chemicals and their potential to modify CB_1 receptor function in mammalian brain. However, it is known that binding of [^{3}H]CP-55940 to CB_1 receptors is strongly inhibited by certain organophosphorus and organosulfur compounds incorporating longer chain alkyl moieties (Segall et al., 2003), and also by various organophophorus pesticides (Quistad et al., 2002). We have previously demonstrated that at micromolar concentrations, methoprene and piperonyl butoxide (chemicals of low acute toxicity used in pest management) can inhibit CP-55940 action at CB_1 receptors *in vitro*, possibly by adopting endocannabinoid-like conformations or by methoprene acting alternatively as a flexible analog of Δ^9-tetrahydrocannabinol (Dhopeshwarkar et al., 2011). During exploratory investigations (conducted by Dr. Chengyong Liao) we also found that certain phthalate esters interfere with the

binding of [^{3}H]CP-55940, which may be of importance due to their abundance in our natural environment and the potential outcome of CB-receptor interactions.

In the present investigation we examined a group of phthalate diesters and monoesters for their ability to influence the binding of [^{3}H]CP-55940 and [^{3}H]SR141716A. These radioligands and also the endocannabinoid anandamide bind to closely associated loci within the CB_1 receptor binding pocket (McAllister et al., 2003). We then carried out experiments with [^{35}S]GTPγS to evaluate potential interference of phthalates with the functional coupling of the CB_1 receptor to its G-protein (Selley et al., 1996; Petitet et al.,1997).

1.10 References

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2: METHODS & MATERIALS

A modified version of this chapter represents the methodology section of the paper entitled "The G protein-coupled cannabinoid-1 (CB_1) receptor of mammalian brain: Inhibition by phthalate esters *in vitro*" by Bisset, K.M., Dhopeshwarkar, A.S., Liao, C. and Nicholson, R.A., which was accepted for publication in Neurochemistry International on June 23rd, 2011.

2.1 Radioligands, drugs and study compounds

Radioligands, [^{3}H]CP-55940 (side chain-2,3,4-[^{3}H]; specific activity 174.6 Ci/mmol), [^{3}H]SR141716A (specific activity 56 Ci/mmol) and 5'-*O*-(γ-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS; specific activity 1250 Ci/mmol), were obtained from Perkin Elmer Life and Analytical Sciences, Canada. Phthalate esters were obtained from Sigma-Aldrich, Canada (see Figure 2.1 for structures). CP-55940, AM251, WIN55212-2, SR1417161A, GDP, DMSO, EDTA, HCl, Trisma base, $MgCl_2.6H_2O$, BSA, NaCl, EGTA and phenylmethanesulfonylfluoride (PMSF) were also purchased from Sigma-Aldrich, Canada.

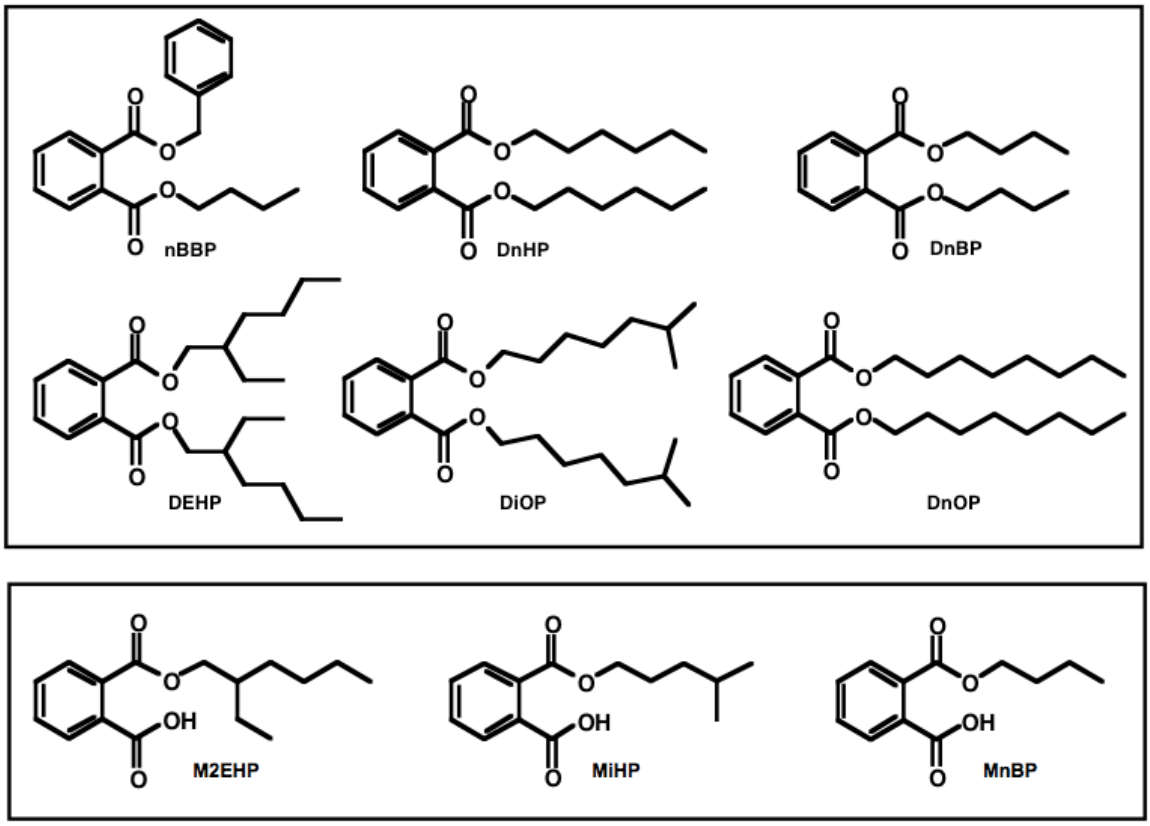

Figure 2-1 ***Upper panel.*** **The structures of phthalate diesters: *n*-butylbenzylphthalate (nBBP); di-*n*-hexylphthalate (DnHP); di-*n*-butylphthalate (DnBP); diethylhexylphthalate (DEHP); di-isooctylphthalate (DiOP) and di-*n*-octylphthalate (DnOP).** ***Lower panel.*** **The structures of phthalate monoesters: mono-2-ethylhexylphthalate (M2EHP), mono-isohexyl-phthalate (MiHP) and mono-*n*-butylphthalate (MnBP).**

2.2 Animals

Male CD1 mice (20-25g) obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) were used for all experiments. Animals were provided with food (rodent Lab Diet 5001) and water *ad libitum*. Mice were subjected to a 12 hr light/12 hr dark cycle and were maintained at 21 ± 2°C and 50 ± 10% relative humidity. All procedures were carried out in accordance with the Canadian Council on Animal Care standards regarding the use of animals in

research. Prior approval for procedures was obtained from the Animal Care Committee at Simon Fraser University, Burnaby, British Columbia.

2.3 Determination of the effects of study compounds on the binding of [^{3}H]CP-55940 and [^{3}H]SR141716A to CB_1 receptors in mouse brain

Through the evaluation of several published procedures for the measurement of specific binding of [^{3}H]CP-55940 to CB_1 receptors, the method as described by Quistad et al. (2002) was adopted with minor modification for the current investigation.

2.3.1 Membrane preparation

Mice were rapidly euthanized using cervical dislocation and all membrane preparation procedures were carried out at 0-4°C. After cervical dislocation whole mouse brains were briefly rinsed in ice-cold buffer (Trisma base (100 mM), EDTA (1 mM) adjusted to pH 9 with HCl) to remove excess blood. Brains were then homogenized (10 up/down strokes) in fresh ice-cold buffer (1 brain/10 ml buffer) using a motor driven homogenizer (pestle rotation approx. 1500 rpm). Homogenates were then divided into two centrifuge tubes and subsequently centrifuged in a Beckman J2HS centrifuge at 900 x g for 10 minutes in a JA20 rotor. The supernatant containing the neuronal membranes was then collected into a clean centrifuge tube and centrifuged at 11,500 x g for 25 minutes. The resulting pellets were thoroughly resuspended to a protein concentration of approximately 6.5 mg/ml in storage buffer (Trisma base (50 mM), EDTA (1 mM) and $MgCl_2.6H_2O$ (3 mM), adjusted to pH 7.4 with HCl) by moving the suspension

in and out (6 times) of a 5 ml syringe through an 18 gauge needle with its tip cut at an angle. Aliquots were then stored at -80°C for no longer than 1 month.

2.3.2 Determination of protein concentration in membrane preparations

Following membrane preparation, protein concentration was estimated using the procedure as described by Peterson (1977). Peterson (1977) simplified the assay for the determination of protein concentration as originally outlined by Lowry et al. in 1951. The improved method allows for the analysis of dilute protein solutions particularly of membrane-associated proteins and removes unwanted substances to avoid interference (Peterson, 1977).

The protein assay uses bovine serum albumin (BSA) at known concentrations to create a linear standard curve from which the unknown concentration of the membrane preparation can be determined. An initial solution with 1 mg/ml fatty acid free BSA was prepared, along with reagents A and B. Reagent A contains copper tartrate carbonate (0.1% copper sulfate, 0.2% potassium tartrate, 10% sodium carbonate), 0.8 M NaOH, 10% sodium dodecyl sulfate (SDS) and distilled water in equal parts, whereas, reagent B contains 1 part Folins B reagent to 4 parts distilled water.

Using nine 1.5 ml Eppendorf tubes, various concentrations of BSA were made. In tubes labelled one through nine, 1 mg/ml of BSA was added in 0, 5, 10, 20, 40, 50, 60, 80, and 100 µl aliquots. Distilled water was then used to bring the volume of each tube up to 100 µl. Reagent A (0.8 ml) was added to each tube, they were then vortexed and left to incubate at room temperature for 10 minutes.

Following the initial incubation, 0.5 ml of reagent B was added to each tube, which were then vortexed and left to incubate at room temperature for another 60 minutes. The absorbance of each tube was measured in a spectrophotometer (at 750 nm). Measured absorbances were plotted against the protein concentrations of diluted BSA solutions to produce a standard curve.

The same procedure was carried out using aliquots (2, 5 and 10 µl in duplicate) of membrane preparation for which the protein concentrations were unknown. Their measured absorbances were plotted onto the standard curve in order to determine protein concentration.

2.3.3 [^{3}H]CP-55940 and [^{3}H]SR141716A binding assay

When needed for experiments, aliquots of frozen membrane preparation were thawed on ice and thoroughly resuspended by moving the membranes through the 18g needle of a 5 ml syringe 5-6 times and then thoroughly vortexed, a procedure that helped reduce variability between replicates. For assays, compounds (in DMSO; 5µl) were added to borosilicate glass culture tubes (13 x 100 mm; Kimble-Chase; without siliconization) using 10µl glass Hamilton syringes, followed by binding buffer (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 (170.67 ± 0.84 µg protein, as measured using the method described by Peterson (1977)) were then added to each tube and the mixture was vortexed and incubated for 15 minutes at room temperature. Following the incubation period, [^{3}H]CP-55940 was added to each tube (in 10 µl DMSO; final radioligand concentration 1.0 nM; final DMSO concentration 2.8%), the contents of the tubes

were thoroughly vortexed and incubated for 90 minutes at 30°C with gentle shaking. Binding reactions were terminated by the addition of ice-cold wash buffer (0.9% NaCl containing 2 mg/ml BSA; 1 ml) followed by the collection of membranes by rapid vacuum filtration on pre-soaked (60 minutes; 4°C) Whatman GF/C filters. The filter-trapped membranes were immediately washed with ice-cold wash buffer (3 x 4 ml). Filters were completely air dried prior to adding scintillant (4 ml: BCS, Amersham Bioscience UK) and radioactivity was quantified with liquid scintillation counting. Specific binding was determined by subtracting non-specific binding, measured in the presence of unlabelled WIN55212-2 (10 μM), from total binding. A schematic representation of the binding methodology is provided in Figure 2-2. The specific binding signal averaged 76.8 ± 1.1 %. This is very similar to the 80% obtained by Quistad et al. (2002) using 1 μM WIN55212-2. Under the present assay conditions the IC_{50} for WIN55212-2 was 6 nM and maximum displacement of [^{3}H]CP-55940 by WIN55212-2 was achieved at both 1 and 10 μM. In each experiment, binding in the absence and presence of WIN55212-2 was assayed in triplicate and test compounds were performed in duplicate. At minimum, three experiments were conducted for each treatment.

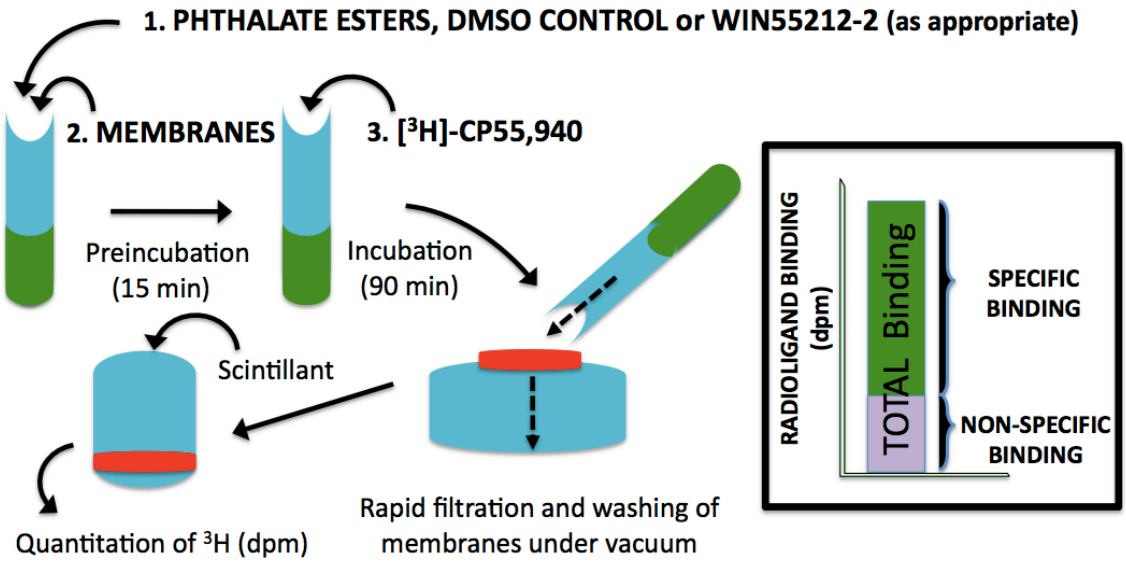

Figure 2-2 **[^{3}H]CP-55,940 binding assay schematic: Whole brains were removed from CD1 mice following rapid cervical dislocation and the 11,500 x *g* neuronal membrane pellet prepared for binding studies as described in Materials and Methods.**

Selected phthalate esters were also evaluated in the same binding assay using the CB_1 receptor antagonist [^{3}H]SR141716A (sp. act. 56 Ci/ mmol; Perkin Elmer Life and Analytical Sciences, Canada) in place of [^{3}H]CP-55940.

2.3.4 Competitive displacement assays

For competitive displacement assays an identical experimental procedure to that described above was used. [^{3}H]SR141716A was present at 1.2 nM and AM251 (at 2 μM) was introduced to estimate the specific binding signal, which averaged (71.0 ± 0.7%). For association experiments, membranes were either preincubated with the phthalate ester for 15 min. before [^{3}H]SR141716A addition or received simultaneous application of phthalate and radioligand. Dissociations were initiated on membranes equilibrated with [^{3}H]SR141716A using either a

saturating concentration of AM251 or this concentration of AM251 plus the phthalate ester.

In each experiment with [^{3}H]CP-55940 or [^{3}H]SR141716A, binding in the absence and presence of unlabeled WIN55212-2 or AM251 was performed in triplicate and test compounds were assayed in duplicate. A minimum of three independent experiments were performed for every treatment.

2.4 Determination of the effects of study compounds on CP-55940-stimulated [^{35}S]GTPγS binding to the G_{α}-protein

2.4.1 Membrane preparation

The method used to isolate the mouse whole brain membrane fraction and determine the effects of phthalates on agonist-stimulated [^{35}S]GTPγS binding generally followed the procedure published by Breivogel & Childers, (2000). Whole brains were quickly removed from two mice and homogenized for 15 seconds in 10 ml of ice-cold isolation buffer (Trisma base (50 mM), MgCl2.6H20 (3 mM), EGTA (0.2 mM), NaCl (100 mM) with pH adjusted to 7.4) using a tissue fragmenter (Polytron Kinematica GmBH; setting 6). The suspension was centrifuged in a Beckman J2HS centrifuge (24,000 x g for 25 min at 2 °C) and the pellet was then resuspended in fresh ice-cold isolation buffer and re-centrifuged. The washed membrane pellet was thoroughly dispersed in isolation buffer, then protein concentration was adjusted to approx. 7 mg/ml before aliquots were transferred to a -80 °C freezer.

2.4.2 CP-55940-stimulated [^{35}S]GTPγS binding assay

Prior to experimentation, the membrane fraction was thawed on ice and carefully dispersed as described in the previous section. This procedure helped improve the reproducibility between replicates without obvious loss in agonist-stimulated [^{35}S]radioligand binding. Binding experiments were performed using guanosine 5'-*O*-(γ-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) of sp. act. 1250 Ci/ mmol.

The phthalate esters (dissolved in DMSO; 5 µl) or DMSO control (as required) were placed in borosilicate glass tubes (13 x 100 mm; siliconized 24 h prior to assay with Sigmacote [Sigma-Aldrich Canada]) and then 500 µl of isolation buffer (pH 7.4) was added which contained fatty-acid free bovine serum albumin (1 mg/ml), guanosine diphosphate (GDP; 100 µM), dithiothreitol (20 µM), [^{35}S]GTPγS (0.14 nM final concentration) and adenosine deaminase (0.004 units/ml). The membrane fraction (70.1 ± 4.2 µg protein/assay) was then added and, after thorough vortexing, an initial 15 min. incubation was carried out at room temperature. The CB_1 receptor agonist CP-55940 (100 nM final concentration; in 5 µl DMSO) or DMSO solvent control were then added and, after thorough mixing, incubations were continued for 1.5 h at 30 °C with gentle shaking. The final concentration of DMSO was 1.9%. Incubations were terminated by introduction of 2 ml of ice-cold wash buffer (50 mM Trisma base:HCl; pH 7.4) which was immediately followed by rapid vacuum filtration through pre-soaked (90 minutes; room temperature) Whatman GF/B filters. Membranes trapped on filters were subjected to three 4 ml washes with the same buffer. Filters were then left to air dry over night. Finally, membrane-bound ^{35}S

was measured using liquid scintillation counting. All assays were conducted in triplicate. Specific binding of [^{35}S]GTPγS (90.7 ± 1.4%) was calculated by subtracting [^{35}S]GTPγS bound in the presence of 100 μM unlabelled GTPγS from total binding. 100 nM CP-55940 stimulated the basal specific [^{35}S]GTPγS binding signal by 57.7 ± 0.6% and the effect of phthalates on this signal was investigated.

2.5 Data analysis

Values are given as mean ± S.E.M. All values of IC_{50} (concentration of phthalate ester producing 50% inhibition) were estimated from the concentration:response relationships defined by non-linear regression analysis using Prism 4 (GraphPad Software Inc., San Diego, CA, USA). Linear regression analysis was also carried out with Prism 4.

2.6 References

Breivogel, C.S., Childers, S.R. 2000. Cannabinoid agonist signal transduction in rat brain: Comparison of cannabinoid agonists in receptor binding, G-protein activation, and adenylyl cyclase inhibition. *Journal of Pharmacology and Experimental Therapeutics*. 295:328-336.

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3: RESULTS

This chapter represents the results section of the paper entitled "The G protein-coupled cannabinoid-1 (CB_1) receptor of mammalian brain: Inhibition by phthalate esters *in vitro*" by Bisset, K.M., Dhopeshwarkar, A.S., Liao, C. and Nicholson, R.A., which was accepted for publication in Neurochemistry International on June 23rd, 2011.

3.1 Effects of phthalate esters on binding of [^{3}H]CP-55940 to CB_1 receptors.

The effects of the di- and mono-esters on the binding of [^{3}H]CP-55940 to CB_1 receptors in mouse whole brain membranes are shown in Figures 3-1 and 3-2. Apart from MnBP, all compounds produced concentration-dependent inhibition of [^{3}H]CP-55940 binding. Within the diester series, nBBP and DnHP were the most potent as indicated by IC_{50}s of 27.4 μM (95% CI = 20.7-36.5 μM) and 33.9 μM (95% CI = 26.5-38.5 μM) respectively. DnBP, DEHP and DiOP were of intermediate potency (IC_{50}s of 45.9 μM (95% CI = 35.9-58.6 μM), 47.4 μM (95% CI = 41.5-54.1 μM) and 55.4 μM (95% CI = 45.8-67.0 μM) respectively), while DnOP was the weakest (IC_{50}: 75.2 μM (95% CI = 65.9-87.2 μM). Based on the level of inhibition at 150 μM (the maximum concentration employed), BBP, DnHP and DnBP were the most efficacious (85-100% inhibition), followed by DEHP and DiOP (60-70% inhibition), while DnOP displayed lower efficacy (50-60% inhibition). At 150 μM MiHP and M2EHP achieved less than 50% inhibition of

[^{3}H]CP-55940 binding and MnBP was inactive. In a separate series of experiments, PMSF failed to modify the inhibitory effects of nBBP and DnBP on [^{3}H]CP-55940 binding (Table 3-1).

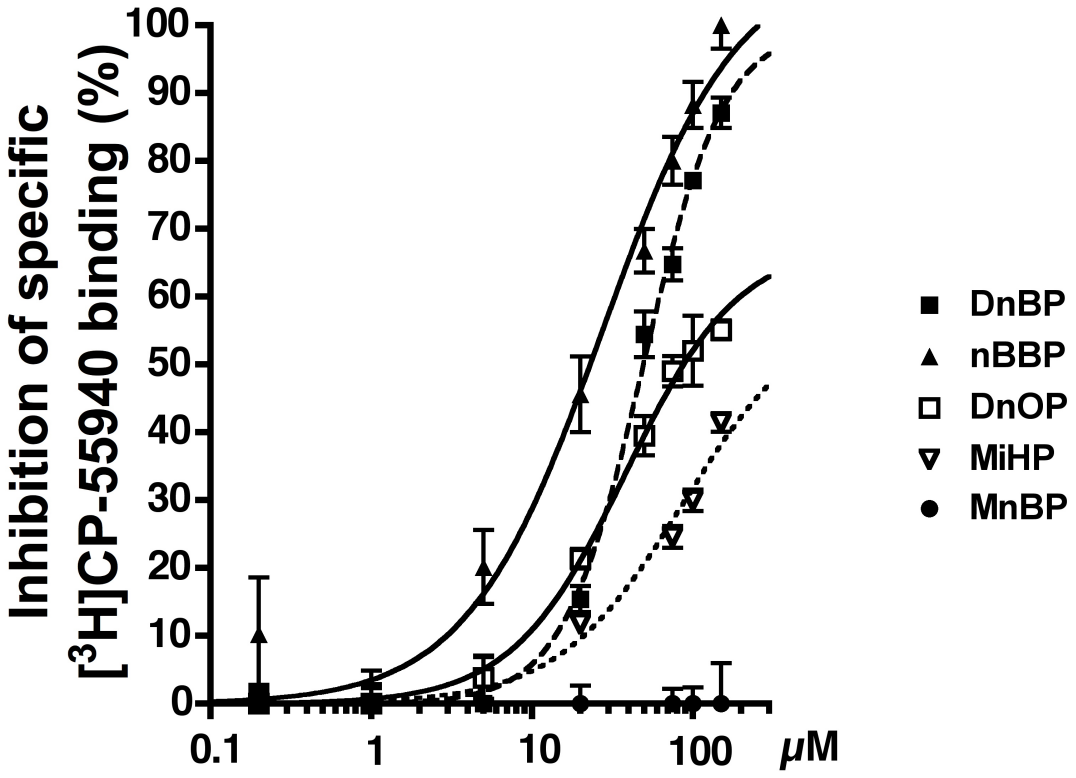


Figure 3-1 Inhibitory effects of phthalate esters (DnBP, nBBP, DnOP, MiHP and MnBP) on the binding of [^{3}H]CP-55940 to mouse brain CB_1 receptors *in vitro*. Each point represents the mean ± SEM of 3 independent experiments.

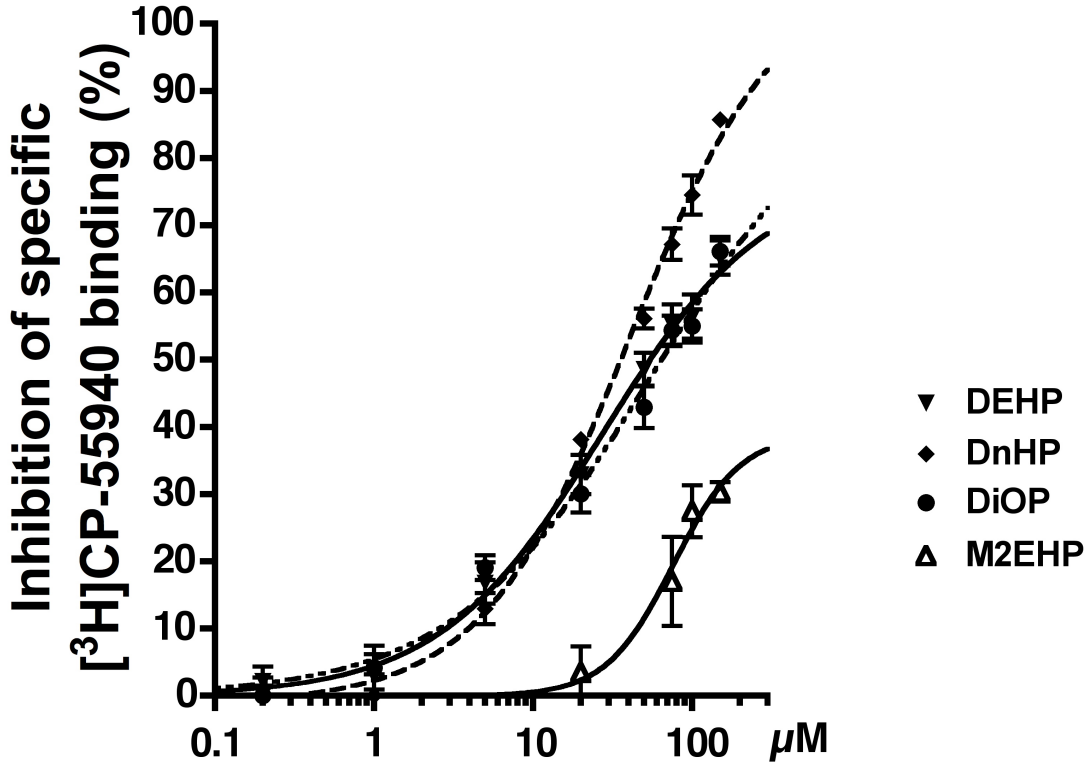


Figure 3-2 **Inhibitory effects of phthalate esters (DEHP, DnHP, DiOP and M2EHP) on the binding of [^{3}H]CP-55940 to mouse brain CB_1 receptors *in vitro*. Each point represents the mean ± SEM of 3 independent experiments.**

Table 3-1 **Inability of PMSF to influence the inhibitory effects of n-butylbenzylphthalate (nBBP) and di-n-butylphthalate (DnBP) on [^{3}H]CP-55940 binding to mouse brain membranes. Phthalate esters were present in the assay at 20 μM and PMSF was used at 50 μM. Each value represents the mean ± S.E.M. of 3-6 independent experiments.**

Treatment	*Inhibition of specific binding (%)*
PMSF	-2.82 ± 3.18
nBBP	28.25 ± 2.11
nBBP + PMSF	27.01 ± 4.55
DnBP	20.50 ± 1.40
DnBP + PMSF	22.61 ± 3.31

3.2 Effects of selected phthalate esters on binding of [^{3}H]SR141716A to CB_1 receptors.

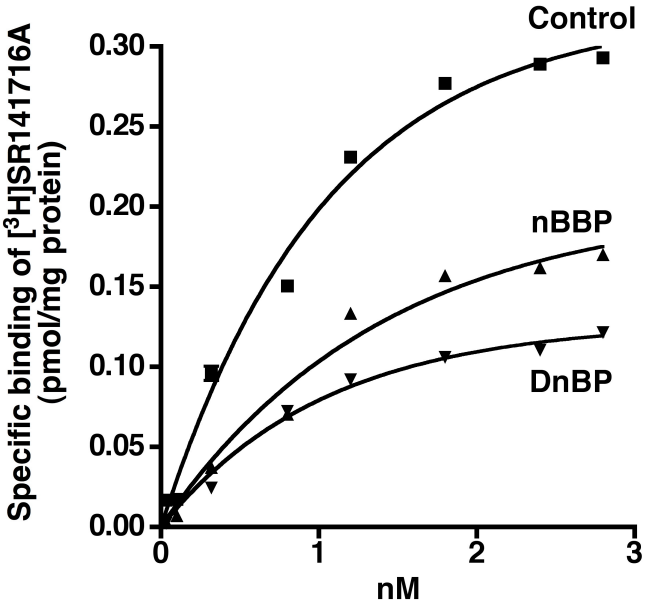
Table 3-2 shows the inhibitory effects of selected di- and mono-esters on the specific binding of [^{3}H]SR141716A to CB_1 receptors of mouse brain. In the case of the diesters nBBP, DBP and DEHP, the extent of inhibition of [^{3}H]SR141716A binding at concentrations that achieve 50% inhibition of [^{3}H]CP-55940 binding resulted in inhibitory effects that were approximately 35-45% higher (nBBP, DnBP) and 25% lower (DEHP). The monoester MnBP (100 μM) had no effect on specific binding of [^{3}H]SR141716A while MiHP produced approximately 33% inhibition. Results provided by Amey S. Dhopeshwarkar.

Table 3-2 Inhibitory effects of *n*-butylbenzylphthalate (nBBP), di-*n*-butylphthalate (DnBP), diethylhexylphthalate (DEHP), mono-isohexylphthalate (MiHP) and mono-*n*-butyl phthalate (MnBP) on the specific binding of [^{3}H]SR141716A to mouse brain membranes. Diesters were present at concentrations producing 50% inhibition of [^{3}H]CP-55940 binding. Each value represents the mean ± S.E.M. of 3 independent experiments (Results provided by Amey S. Dhopeshwarkar).

Treatment	*Inhibition of specific binding (%)*
nBBP (27 μM)	67.82 ± 1.71
DnBP (46 μM)	72.30 ± 3.23
DEHP (47 μM)	37.42 ± 3.48
MiHP (100 μM)	33.23 ± 4.15
MnBP (100 μM)	0

3.3 Influence of selected phthalates on the saturation binding of [^{3}H]SR141716A to CB_1 receptors

The control saturation binding curve was constructed by measuring the specific binding of [^{3}H]SR141716A to CB_1 receptors at equilibrium over a range of radioligand concentrations (0.032 to 2.8 nM). Experiments were concurrently performed in the presence of nBBP or DnBP (Figure 3-3). Analyses revealed that phthalates have negligible effect on the K_d of radioligand binding but reduce the B_{max} by 37% (nBBP) and 60% (DnBP). Results provided by Amey S. Dhopeshwarkar.



	K_d (nM)	B_{max} (pmol/mg)
Control	0.726	0.323
nBBP	0.969	0.203
DnBP	0.718	0.128

Figure 3-3 The effect of nBBP and DnBP (both at 35 µM) on the equilibrium binding of [^{3}H]SR141716A to CB_1 receptors of mouse whole brain. K_d and B_{max} values are displayed for each treatment and 95% confidence intervals were as follows: control (K_d 0.628 to 0.859. B_{max} 0.303 to 0.343), nBBP (K_d 0.761 to 1.333. B_{max} 0.176 to 0.229) and DnBP (K_d 0.624 to 0.846. B_{max} 0.120 to 0.136). R^2 values were 0.9877 (control), 0.9756 (nBBP) and 0.9887 (DnBP). Data points represent the means ± SEMs of 3 independent experiments (most SEM bars are obscured by data symbols) (Results provided by Amey S. Dhopeshwarkar).

3.4 Effects of selected phthalates on the kinetics of [^{3}H]SR141716A binding.

The association time course of [^{3}H]SR141716A between 0 and 3 minutes in the absence of test compounds (Figures 3-4a and 3-4b) aligns with data published by Rinaldi-Carmona et al., (1996) using synaptosomes. nBBP (35 μM) and DnBP (50 μM) reduce the ability of [^{3}H]SR141716A to equilibrate with CB_1 receptors both when applied in advance of the [^{3}H]SR141716A (Figure 3-4a) and to a lesser extent when introduced simultaneously with radioligand (Figure 3-4b). When combined with a saturating concentration of AM251, nBBP (35 μM) and DnBP (50 μM) increased the dissociation of [^{3}H]SR141716A:CB_1 receptor complex to levels much greater than that produced by a saturating concentration of AM251 alone (Figure 3-5). Results provided by Amey S. Dhopeshwarkar.

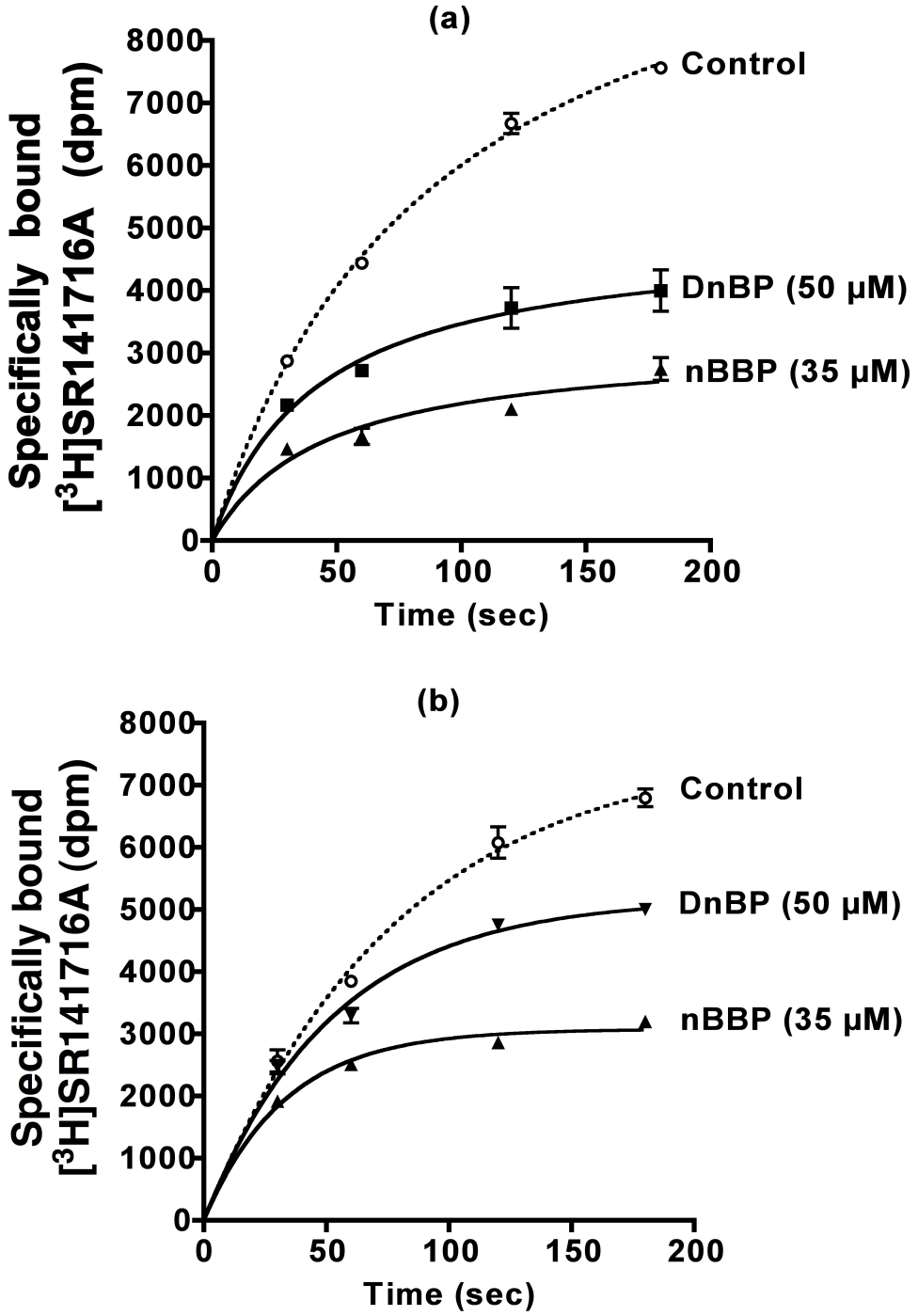


Figure 3-4 **Influence of nBBP (35 µM) and DnBP (50 µM) on the time course of association of [^{3}H]SR141716A with CB_1 receptors of mouse brain. In a) membranes received the standard 15 min preincubation with phthalate esters prior to [^{3}H]SR141716A addition. In b) the phthalate ester and [^{3}H]SR141716A were applied simultaneously (Results provided by Amey S. Dhopeshwarkar).**

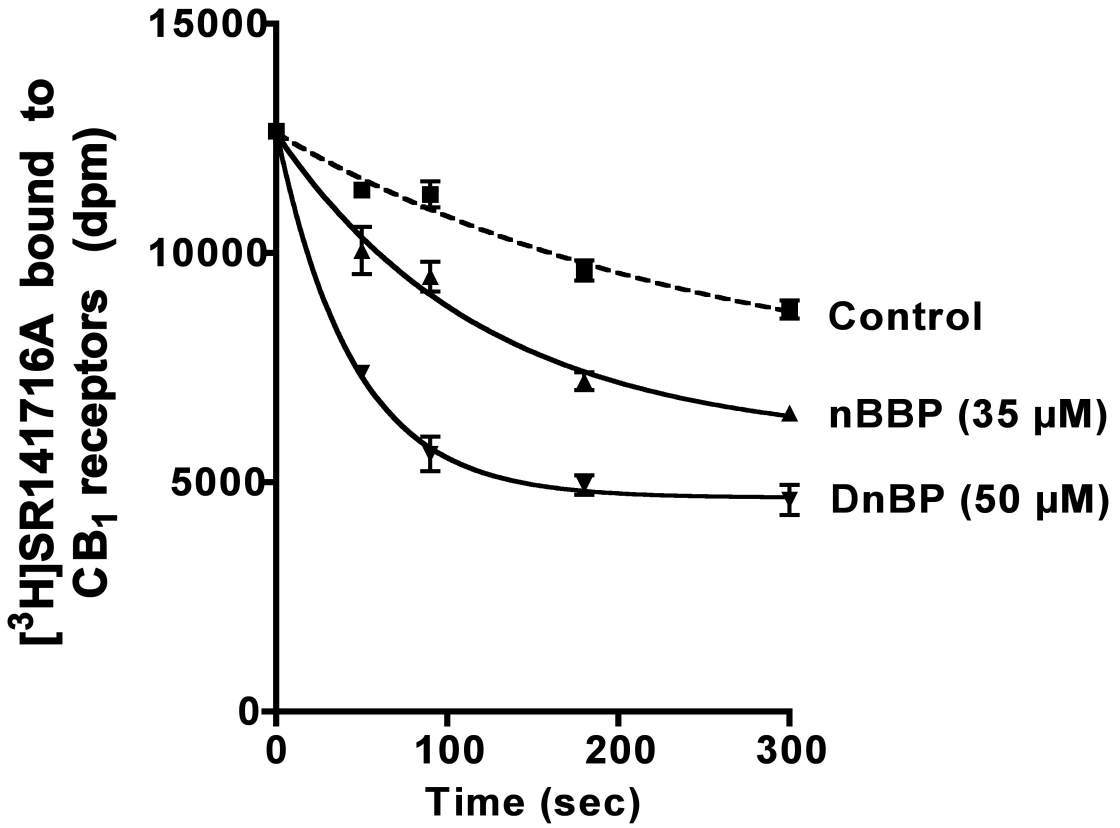


Figure 3-5 **Dissociation of the [^{3}H]SR141716A:CB_1 receptor complex (initiated by challenge with 5 µM AM251) in the absence (control) or in the presence of 35 µM nBBP or 50 µM DnBP. All curves incorporated a dissociation start point (y = 8392 dpm) estimated by extrapolating control curve to x = 0). Data represent mean ± SEM of at least 3 independent experiments, each performed in triplicate (Results provided by Amey S. Dhopeshwarkar).**

3.5 Effects of phthalates on CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding to the G_α-protein

Both diesters and monoesters also had the capacity to inhibit CB_1 receptor agonist-activated binding of [^{35}S]GTPγS to the G_α-protein and in agreement with [^{3}H]CP-55940 binding data, the diesters were consistently more active (Figure 3-6). Inhibitory effects of the study compounds on [^{3}H]CP-55940 binding and CP-55940-stimulated binding of [^{35}S]GTPγS to the G-protein were closely associated (r^2 = 0.7844; Figure 3-7).

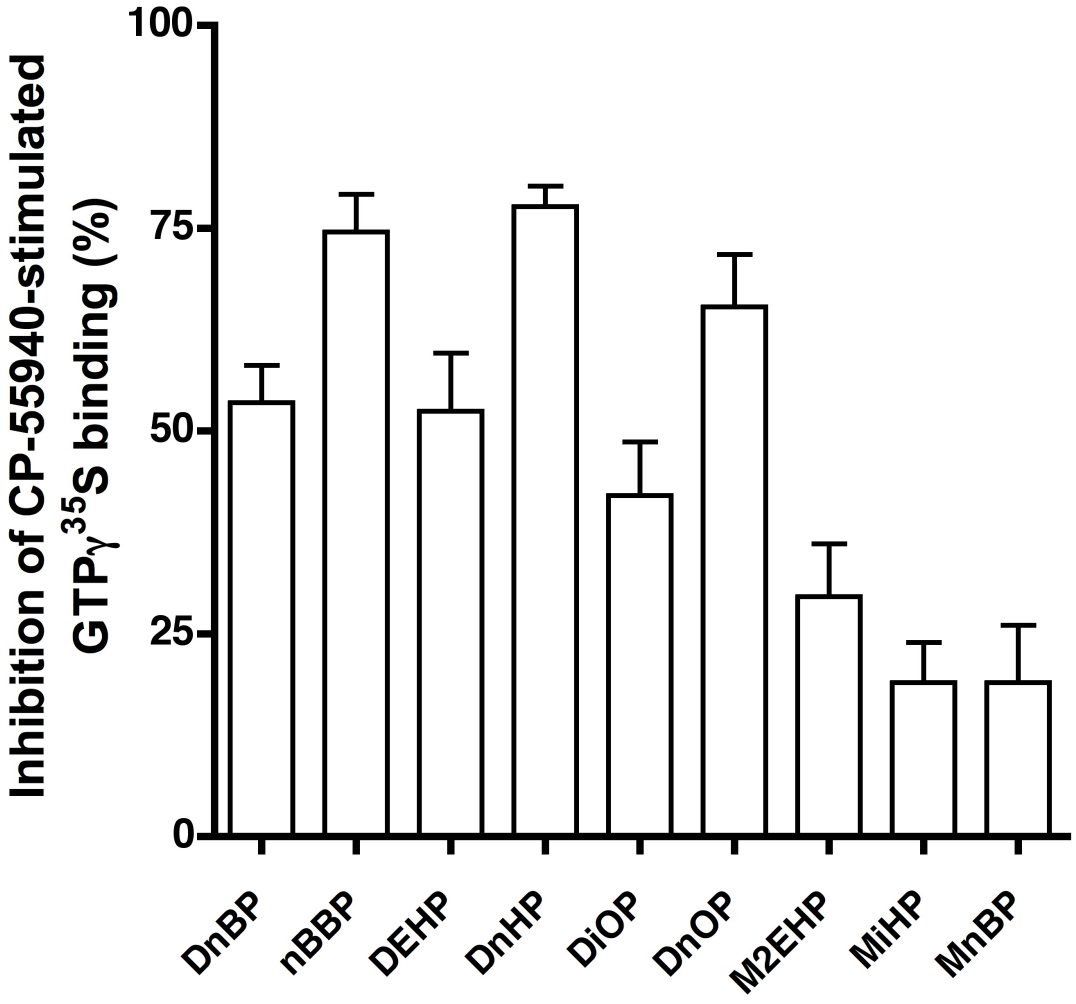


Figure 3-6 **Inhibition of CP-55940-stimulated binding of [^{35}S]GTPγS to the G_{α} protein by phthalate esters. Phthalate esters were assayed at 75 µM throughout. Each column represents the mean and error bar the SEM of 7 independent experiments.**

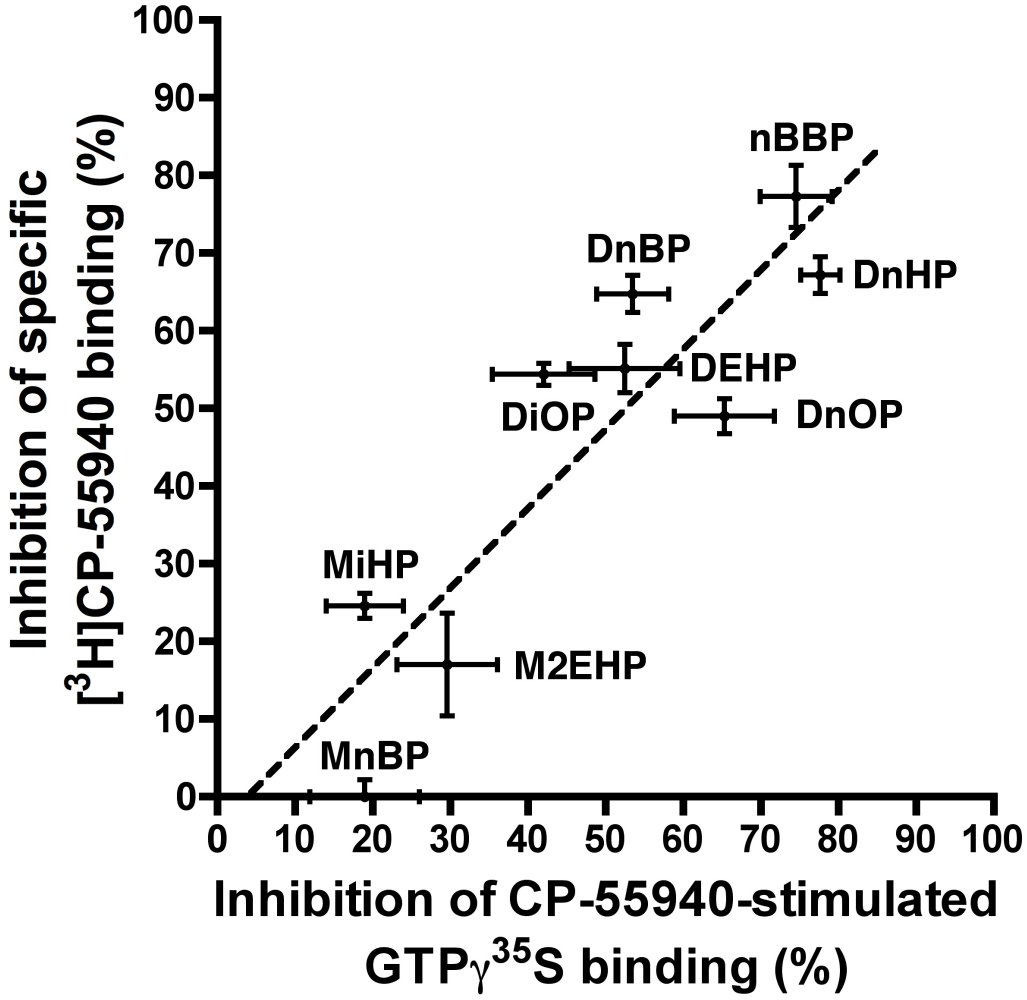


Figure 3-7 Relationship between the ability of study compounds to inhibit the binding of [^{3}H]CP-55940 and CP-55940-stimulated binding of [^{35}S]GTPγS in mouse whole brain membrane fractions. All assays were performed using phthalate esters at 75 µM; R^2 = 0.7844.

3.6 Reference

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4: DISCUSSION

This chapter represents the discussion section of the paper entitled "The G protein-coupled cannabinoid-1 (CB_1) receptor of mammalian brain: Inhibition by phthalate esters *in vitro*" by Bisset, K.M., Dhopeshwarkar, A.S., Liao, C. and Nicholson, R.A., which was accepted for publication in Neurochemistry International on June 23rd, 2011.

The present investigation demonstrates that certain phthalate esters interfere with the binding of [^{3}H]CP-55940 and [^{3}H]SR141716A to CB_1 receptors of mouse brain at micromolar concentrations *in vitro*. Since we found that CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding is also decreased by phthalate esters, these compounds appear to inhibit activation of the associated G-protein receptors by operating as low affinity CB_1 receptor antagonists.

In the [^{3}H]CP-55940 binding assay, the IC_{50} values of the phthalate esters nBBP, DnHP, DnBP and DEHP lie in the 27-47 μM range, which put them at similar potency to *cis*-9,10-octadecenyl-α-methylethanolamide an analog of the sleep-inducing lipid *cis*-oleamide (Boring et al., 1996), of higher potency than thujone, *cis*-oleamide, *cis*-9,10-octadecenylethanolamide (Boring et al, 1996; Meschler and Howlett, 1999), but of lower potency compared to the antagonists sanguinarine, chelerythrine, piperonyl butoxide and (S)-methoprene (Dhopeshwarkar et al., 2011). In distinct contrast to thujone and *cis*-oleamide and its analogs (Boring et al., 1996; Meschler and Howlett, 1999), the phthalate

esters were able to transduce CB_1 receptor modulatory effects to the G-protein. It must be emphasized that nBBP, DnHP, DnBP and DEHP are obviously much weaker as inhibitors of [^{3}H]CP-55940 binding, [^{3}H]SR141716A binding and CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding when compared to the antagonists SR141716A and AM251 which exert their effects in the low nanomolar range (Rinaldi-Carmona et al., 1995; Gatley et al., 1997).

Within the group of phthalate esters examined in the present investigation, a broad range of CB_1 receptor inhibitory effects were demonstrated. Our data indicate that the diesters are more potent inhibitors of [^{3}H]CP-55940 binding than the monoesters. A similar differential was also noted by Liu et al., (2009) in studies on the inhibitory effects of phthalates on Ca^{2+} transients triggered by nAChR activation in human neuroblastoma cells. In contrast, the potency of the monoester MEHP as an inhibitor of follicle stimulating hormone binding to Sertoli cells was reported to be at least three orders of magnitude higher than for the diester DEHP; the latter phthalate showing no activity at 100 μM (Grasso et al., 1993).

Our results suggest that the overall relationship between phthalate diester structure and inhibitory effects on [^{3}H]CP-55940 binding to CB_1 receptors is complex. nBBP and DnHP showed the highest inhibitory potencies (IC_{50}s = 27.4 and 33.9 μM respectively) combined with robust efficacy (85% or greater inhibition at maximum concentration). Reducing the length of each *n*-hexyl group of DnHP by 2 carbons (i.e. giving DnBP), reduces inhibitory potency but efficacy is retained at approximately 85%. By contrast, DEHP, which can be considered

as a bis 2-ethyl analog of DnHP or a bis 2-propyl analog of DnBP, demonstrates reduced potency and efficacy against DnHP, and similar potency with reduced efficacy compared to DnBP. The phthalate diesters with the longer alkyl substituents (DiOP and DnOP) exhibited lower inhibitory potencies (IC_{50}s 55.4 and 75.2) and efficacies were also comparatively low (55-65%). The critical nature of the diester configuration for inhibition of [^{3}H]CP-55940 binding is emphasized by comparison of nBBP and DnBP (which are amongst the most effective compounds studied) with MnBP (a phthalate devoid of activity).

For the experiments with PMSF, we reasoned that using phthalates of intermediate (DnBP) and higher (nBBP) potency at < IC_{50} would offer a sensitive basis for assessment. Moreover, DnBP and nBBP (study compounds with alkyl and aryl substituents respectively) might be expected to show different susceptibilities to breakdown by serine hydrolases. Nonetheless, based on our experiments, there was no evidence that serine hydrolases limit the inhibitory effect of either of these analogs in the [^{3}H]CP-55940 binding assay.

The equilibrium binding and dissociation data using [^{3}H]SR141716A provide a useful insight into the mechanism by which nBBP and DnBP inhibit radioligand binding. The saturation isotherms demonstrate that these phthalates act by eliminating binding sites for radioligand (i.e. B_{max} is reduced), without affecting the affinity of radioligand for the remaining sites (i.e. K_d is unchanged). Moreover, the dissociation experiments strongly suggest that these compounds act allosterically with respect to the [^{3}H]SR141716A binding site, since under our assay conditions any access by phthalate diesters to the radioligand binding site

is completely prevented by the saturating levels of AM251. The dissociation data also argue against an irreversible or tight binding of phthalate esters to the [^{3}H]SR141716A recognition site, another potential explanation of the reduced B_{max} and unchanged K_d. The time courses for dissociation of [^{3}H]SR141716A in the presence of nBBP and DnBP indicate that the binding of phthalates to this allosteric binding site and subsequent negative modulation of radioligand binding occurs very rapidly. Rapid engagement of phthalates with a site coupled allosterically to the [^{3}H]SR141716A binding site is also consistent with the reduced levels of nBBP and DnBP binding in the association experiments. However, the association profiles in the presence of nBBP and DnBP are likely markedly influenced by the effect of these compounds on availability of receptors (B_{max}) that can bind [^{3}H]SR141716A. Overall, our results indicate that a critical mechanism underlying inhibition of [^{3}H]SR141716A binding to CB_1 receptors involves phthalates engaging with a site that is distinct from but negatively coupled to the radioligand recognition site. The proposed binding region for phthalate esters on the CB_1 receptor may represent a novel target that could be exploited therapeutically by phthalate ester analogs or other drugs to produce downregulation of endocannabinoid action in the brain.

Unlike phthalate diesters, MEHP and other monoesters inhibit the binding of follicle stimulating hormone (FSH) to G-protein coupled FSH receptors, an action that may involve direct engagement of MEHP with the G-protein (Grasso et al., 1993). The allosteric inhibition of [^{3}H]SR141716A binding to the CB_1 receptor by phthalate diesters could also arise from a direct interaction with its G-

protein as we have postulated for chelerythrine and sanguinarine (Dhopeshwarkar et al. 2011). However, in contrast to the findings of Grasso et al., (1993), we found that monoesters are, at best, exceptionally weak inhibitors of CB_1 receptor radioligand binding. Therefore, negative allosteric coupling between a phthalate diester recognition site on the CB_1 receptor and the radioligand binding site is likely a more fruitful area for future exploration.

Phthalate diesters have potential to access the brain, since a number of these compounds interfere with barbiturate-induced sleep duration following systemic administration (Calley et al., 1966) and phthalate ester exposure in school children has been associated with a behavioural (attention-deficit/hyperactivity) disorder (Kim et al. 2009). The presynaptic CB_1 receptor plays a fundamental role at many synapses in mammalian brain and activation of this complex by endocannabinoids promotes a variety of physiological and behavioural responses. Moreover, downregulation of CB_1 receptors and other components of the endocannabinoid system in human epilepsy is associated with increased excitability in neuronal networks and has been linked to reduced seizure thresholds (Ludanyi et al., 2008). A critical question is whether brain CB_1 receptors are exposed to phthalate diesters *in vivo* at concentrations that are sufficient to interfere with the activation of this signalling pathway by endocannabinoids. Phthalate esters undergo extensive ester cleavage in the gastrointestinal tract and hydrolysis would be expected to limit the ability of diesters to reach the brain particularly after acute oral exposure. However, individuals receiving higher exposure to phthalate esters on a continuous basis

(perhaps as a result of occupational exposure) or hospital patients exposed to phthalates released from medical devices may be more likely to accumulate these chemicals in the brain. In the present investigation, threshold inhibitory effects of DEHP, DnOP, DiOP and nBBP on [^{3}H]CP-55940 binding are evident between 1 and 10 μM. Even concentrations within this range in brain may be sufficient to antagonize endocannabinoid-mediated signalling at CB_1 receptors to an extent that causes low level synaptic perturbations and subtle pathophysiological and affective responses.

Finally, it must be stressed that further studies aimed at determining 1) phthalate ester levels in brain following short term systemic and chronic exposures and 2) the ability of these compounds to modify critical effects of cannabinoid agonists in intact animals are essential to improve our understanding of the potential phthalate diesters might have in modulating the endocannabinoid system *in vivo*.

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