Strathclyde Institute of Pharmacy and
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Electrophysiological and neurochemical investigation into the effects of cannabidiol in the CNS

by

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Copyright Statement

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Publications & Abstracts

Publications

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Abstract

Cannabis sativa has been used religiously and medicinally for centuries and the compounds it contains possess tremendous potential in the treatment of a plethora of disorders, both in the central nervous system (CNS) and in the periphery. The most widely studied of these compounds are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). However, cannabis use has been associated with psychological side effects, which have been attributed to THC acting via cannabinoid receptors. Despite this, the Canadian government recently approved a drug containing THC and CBD in equal parts for the treatment of painful spasms associated with Multiple sclerosis (MS). It is proposed that CBD inhibits the psychological effects of THC. However little is known about the effects of CBD on neuronal activity or about the mechanism through which it alters the effects of THC. It is the purpose of this study to evaluate the effects of CBD at the electrophysiological and neurochemical level as well as identifying potential mechanisms of action.

Whole cell patch-clamp experiments revealed that CBD inhibited spontaneous synaptic activity in rat primary hippocampal cultures via the activation of a $G_{i/o}$-protein coupled receptor (GPCR). Field recordings from rat hippocampal slices confirmed that CBD suppressed synaptic transmission, whilst enhancing paired-pulse facilitation (PPF) suggesting a presynaptic site of action. Both 5-HT_{1A} and CB_1 receptor antagonists inhibited the effect of CBD in hippocampal slices.

In situ hybridisation of immediate early genes (IEG) associated with neuronal activity revealed that CBD inhibited THC-induced increases in IEG expression in regions of the regions of the prefrontal cortex, agranular insular cortex and caudate putamen. Furthermore CBD administration was also shown to reduce expression of *Zif-268* and *Arc* in prefrontal cortical regions, the cingulate cortex and in the CA1 region of the hippocampus. Co-administration of CBD with a NMDA receptor antagonist (PCP) blocked the

prefrontal, but not the hippocampal, effects on *Arc* expression induced by CBD administration.

The findings presented in this thesis demonstrate that CBD modulates neuronal activity via direct or indirect activation of 5-HT_{1A} and CB_1 receptors and that CBD may modulate the effects of THC on regional brain activity through competing with THC at these sites of action. Therefore although these data support the evidence that CBD can inhibit the neuronal effects of THC, further investigation would be required to identify the potential consequences of long-term use of CBD on brain function.

Abbreviations

2-AG	2-arachidonylglycerol
5-HT	5-Hydroxytryptamine (Serotonin)
AC	Adenylyl Cyclase
aCSF	Artificial Cerebrospinal Fluid
AEA	Anandamide
AM-404	N-(4-hydroxyphenyl)arachidonoylethanolamide
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AP	Action Potential
ara-C	Cytosine-β-D-arabinofuranoside
Arc	Activity Related Cytoskeleton-associated Protein
ArPE	N-arachidonoyl phosphatidylethanolamine
ATP	Adenosine Triphosphate
BDNF	Brain-derived Neurotrophic Factor
BOLD	Blood-oxygen-level Dependence
BSA	Bovine Serum Albumin
Ca/CRE	Calcium/cAMP Response Element
CA1	Cornu Ammonis 1 (Region of Hippocampus)
CA3	Cornu Ammonis 3 (Region of Hippocampus)
cAMP	Cyclic Adenosine Monophosphate
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CBD	Cannabidiol
CBR	Cannabinoid Receptor
CCK	cholecystokinin
CNS	Central Nervous System
COMT	Catechol-O-methyl Transferase
DAG	1,2-diacylglycerol
DAGL	1,2-diacylglycerol Lipase
DEPC	Diethylpyrocarbonate
DG	Dendate gyrus
DLPFC	Dorsolateral Prefrontal Cortex
DMSO	Dimethyl Sulphoxide
DSE	Depolarisation-induced Suppression of Excitation
DSI	Depolarisation-induced Suppression of Inhibition
eCB	Endocannabinoid
EGTA	Ethylene glycol tetraacetic acid
EPSC	Excitatory Postsynaptic Current
EPSP	Excitatory Postsynaptic Potential
ERK	Extracellular signal-regulated kinases
FAAH	Fatty Acid Amide Hydrolase
fEPSP	Field Excitatory Postsynaptic Potentials
fMRI	Functional Magnetic Resonance Imaging
GABA	γ-aminobutyric acid

GPCR	G-Protein Coupled Receptor
GPR55	Orphan GPCR 55
GTP	Guanosine-5'-triphosphate
HDS	Hippocampal Dissection Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.p.	Intraperitoneal
i.v.	Intravascular
IEG	Immediate Early Gene
IPSC	Inhibitory Postsynaptic Current
LCGU	Local Cerebral Glucose Utilisation
LTP	Long-term Potentiation
MAO	Middle Artery Occlusion
MAP	Mitogen-activated protein
MAPK	MAP Kinase
MGL	Monoglyceride Lipase
mIPSC	Minature Inhibitory Postsynaptic Current
mPFC	Medial Prefrontal Cortex
mRNA	Messenger Ribose Nucleic Acid
NADA	N-arachidonoyl-dopamine
NAT	N-acyl transferase
NMDA	N-Methyl-D-aspartic acid
OEA	O-arachidonoyl-ethanolamine (Virodhamine)
PBS	Phosphate Buffered Saline
PCP	1-(1-phenylcyclohexyl)piperidine (Phencyclidine)
PE	Phosphatidylethanolamine
PET	Positron Emission Tomography
PI	Phosphatidylinositol
PLC	Phospholipase C
PLD	Phospholipase D
PN	Pyrimidial Neuron
PPF	Paired-pulse facilitation
PPI	Pre-pulse Inhibition
PTX	Pertussis Toxin
rCBF	Regional Cerebral Blood Flow
ROD	Relative Optical Density
SC	Schaffer collateral
SRE	Serum Response Element
SSC	Saline-sodium citrate
THC	Δ9-tetrahydrocannabinol
THCV	Tetracannabinivarin
TRPV1	Transient Receptor Potential Cation Channel, Subfamily V, Member 1
VGCC	Voltage-gated Calcium Channel
VOCC	Voltage-operated Calcium Channel

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Cannabis, in its many forms, is derived from a flowering plant which is indigenous to Central Asia where evidence of its cultivation dates as far back as 4000 B.C.; however it was not until 104 B.C. that use of cannabis as a therapeutic was documented for treatment of menstrual pain (Li, 1974). There is also historical evidence that suggests cannabis may also have been used in religious rituals in Central Asia and India: the sedation and euphoria experienced from the inhalation of the smoke produced by the burning of this plant was considered to be a religious experience and cannabis was therefore considered sacred by many religious cultures (Touw, 1981).

A physician who served in British-ruled India in the 19th Century introduced cannabis as medicinal drug to the western world. O'Shaughnessy performed the first recorded experiments in animals and humans using different preparations of the drug. In his work he described the therapeutic potential of cannabis for treatment of pain (rheumatism), muscular spasms associated with rabies and tetanus as well describing cannabis' anticonvulsant properties (O'Shaughnessy, 1843). The 20th century has seen an explosion in cannabis research including: the marketing by pharmaceutical companies of cannabis tinctures and synthetic analogues of cannabinoids for a host of medical conditions, the discovery and isolation of the psychoactive compound Δ^9-tetrahydrocannabinol (THC) and other cannabinoids and the identification of the receptor targets and endogenous ligands.

Despite the 20th century seeing an increase in the described applications of medicinal cannabis, it also seen cannabis being illegalised in many countries in the western world due to abuse of the drug and its use being associated with psychological conditions, such as schizophrenia. Cannabis is currently the most widely abused drug in the world, mainly due to the sedative and euphoric effects on the user, the lack of any significant negative effects following acute usage and the ease of availability and low cost.

Cannabinoids were first isolated and characterised in 1967 by Mechoulam and co-workers. It was later discovered that THC is the major psychoactive constituent of *Cannabis sativa*, and responsible for its psychotomimetic,

analgesic, antiemetic and anticonvulsive properties (Dewey, 1986; Hollister, 1986). *Cannabis sativa* contains over 60 constituents of which THC is the most widely studied psychoactive cannabinoid. Other cannabinoids isolated and characterised include: cannabidiol (CBD), cannabinol, cannabigerol and tetrahydrocannabivarin (THCV Mechoulam, 1970). CBD has been proposed to contribute to some of the desirable analgesic and antinociceptive effects of Δ^9-tetrahydrocannabinol (THC), while inhibiting the less desirable psychotic effects (Russo and Guy, 2006). Recently, a drug combining these naturally occurring components of *Cannabis sativa* (Sativex®) has been approved for clinical use in Canada to treat neuropathic pain in sufferers of multiple sclerosis (Wade et al., 2004; Wade et al., 2006){Wade, 2004 #54;Collin, 2007 #2;Wade, 2006 #52}. Furthermore, CBD has also been reported as a possible alternative treatment for the psychiatric disorder schizophrenia (Zuardi et al., 1995). However, the precise mechanism of action of CBD and its interaction with psychotomimetic agents, such as THC, has not been completely evaluated in human and animal studies. This thesis will critically assess the literature of studies using CBD and CBD in conjunction with THC and introduce preliminary data to evaluate the actions of CBD in neuronal tissue.

It was some years later that the receptor targets for THC were discovered; these are CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993). The identification of CB receptors as a target for THC has led to the identification of endogenous ligands (endocannabinoids). These include arachidonylethanolamide (anandamide or AEA) and 2-arachidonyl glycerol (2-AG) and it has been suggested that these can act as either neurotransmitters or neuromodulators (Pertwee, 2001). As well as naturally occurring cannabinoids there are several synthetic agonists and antagonists e.g. WIN 55,212-2, CP 55,940, HU-210, and SR 141716A. These synthetic compounds have proved to be useful tools in investigating CB receptor function as well as pharmacological treatments (Koe et al., 1985; Titishov et al., 1989; Rinaldi-Carmona et al., 1994; Showalter et al., 1996).

1.2 Cannabinoid Receptors

1.2.1 Identification of the molecular targets for THC

It was proposed that the effects of THC were a result of activation of specific receptors due to the finding that THC inhibited adenylyl cyclase (AC)-catalysed production of cyclic adenosine monophosphate (cAMP) (Howlett and Fleming, 1984). This theory was eventually confirmed when it was shown that this effect was sensitive to pre-treatment with pertussis toxin (PTX) (Howlett et al., 1986), which suggest that THC was acting through the activation of a $G_{i/o}$-protein coupled receptor (GPCR). Subsequently, this led to the identification and cloning of the CB receptor (Matsuda et al., 1990). This receptor was shown to localise in many brain regions leading to the suggestion that this receptor may mediate some of the behavioural effects of marijuana (Herkenham et al., 1990; Abood and Martin, 1992). In addition, the receptor was also shown to be located in the peripheral tissues, including the guinea-pig small intestine and the mouse bladder and vas deferens (Pertwee and Fernando, 1996; Pertwee et al., 1996a; Pertwee et al., 1996b). This receptor has now been widely accepted as the principal site of action of cannabinoids in the CNS and was termed cannabinoid receptor 1 (CB_1).

A second receptor was cloned from human promyelocytic leukemia cells (HL-60); these receptors have a 44% amino acid sequence homology to CB_1. This was termed CB_2 and further studies revealed it was localised to cells of the immune system, including B- and T-lymphocytes and microglia in the brain (Munro et al., 1993). CB_2 is also a 7 transmembrane receptor coupled to $G_{i/o}$-protein sharing similar properties to the CB_1 receptor in modulating cAMP synthesis.

1.2.2 G-protein coupling and signal transduction

1.2.2.1 $G_{i/o}$ coupling

Cannabinoid receptors (CBRs) are members of the G-protein coupled receptor superfamily. It is widely considered that CBRs preferably couple to $G_{i/o}$ proteins and that activation of the CB_1 receptor sub-type results in the cleavage of the heteromeric G-protein. The $G\alpha_{i/o}$ subunit stimulates inward

rectifying potassium channels (K^+_{ir}) and inhibits N- and P/Q-calcium channels (Mu et al., 1999) and directly inhibits adenylyl cyclase-catalysed production of cyclic adenosine monophosphate (cAMP) that inhibits A-type potassium channels (K^+_A) (Mu et al., 1999). Therefore CB_1 activation can inhibit neuronal excitability and neurotransmitter release. Several studies have demonstrated that both natural and synthetic CB_1 agonists inhibit both spontaneously occurring and electrically evoked glutamatergic synaptic transmission in a variety of systems, from primary cultures to acute slices. In cultured rat hippocampal neurons, THC has been shown to reduce postsynaptic Ca^{2+} spiking, a marker of synaptic transmission, by as much as 41% (EC_{50} = 20nM) (Shen and Thayer, 1999). WIN 55,212-2 application, however, resulted in a 100% reduction at a 10-fold lower concentration suggesting that THC may be only partially agonistic despite having a high affinity for CB_1 receptor. THC partially inhibited induced reduction of excitatory postsynaptic current (EPSC) amplitude (Shen and Thayer, 1999).

As well as modulation of ion channels, CB_1 receptor activation also results in the activation of mitogen-activating protein (MAP) kinase and extracellular signal-regulated kinase (ERK); this is involved in signal transduction leading to transcription and translation of modulatory proteins responsible for the long-term effects of receptor activation. For example, CB_1-dependent MAPK/ERK activation has been shown to result in the increase of the immediate early genes *Zif-268 (Krox-24, NGFI-A, Egr1 or ZENK)* and *c-fos* (Bouaboula et al., 1995; Derkinderen et al., 2003). These immediate early genes are transcription factors required for the induction of neurotrophic proteins. Specifically, expression of *Zif-268*, a zinc-finger protein, is responsible for the induction of proteins required for synaptic plasticity (Jones et al., 2001).

1.2.2.2 Alternative G-protein coupling

In studies examining the effects of the selective CB_1 agonist, WIN 55,212-2, on intracellular calcium ion concentration increases, it was found that the CB_1 (or a CB_1-like) receptor was coupled to a $G_{\alpha q/11}$ type G-protein in both hippocampal cultures and HEK-293 cells transfected with the CB_1 receptor

(Lauckner et al., 2005). Despite being blocked by the selective CB_1 antagonist, SR141716A, this effect was not reproduced by other synthetic CB_1 agonists and phytocannabinoids, including THC, CBD, 2-AG, HU-210 and CP55, 940 (Lauckner et al., 2005). A second study, evaluating intracellular calcium ion concentration in primary hippocampal cultures, both conflicted with and corroborated the previous study. This study showed that $G_{q/11}$-mediated phospholipase-C (PLC) activation was responsible for rises in intracellular calcium ion concentration. This effect was also seen following CBD and THC administration, which were ineffective in the previous experiment (Ryan et al., 2007). This suggests that the effects of $G_{q/11}$ mediated calcium increases were not due to activation of cannabinoid receptors. However, neither study identified a receptor responsible for this effect. However, these data may suggest an alternative receptor that can be activated or inhibited by cannabinoids. One such receptor may be the orphan GPCR known as GPR55 (Brown, 2007; Pertwee, 2007; Ryberg et al., 2007). GPR55 has been shown to couple to G_{13} and has also shown to mediate calcium influx (Henstridge et al., 2009; Henstridge et al., 2010); however, despite this receptor being activated by several cannabinoid receptor ligands, WIN 55,212-2 was shown to have no affinity for GPR55 (Ryberg et al., 2007). In addition, THC and CBD have opposing effects at GPR55, where THC is an agonist and CBD acts as an antagonist (Ryberg et al., 2007). Despite not providing clarity in the cannabinoid pharmacology, the potential involvement of a variety of receptors with which cannabinoid agonists have differential effects is worth taking into consideration before drawing any conclusions from cannabinoid pharmacological data.

1.2.2.3 Heterodimerisation with D_2 receptors

CB_1 receptors have also previously been shown to couple to G_s GPCR's in HEK-293 cells that have been transfected with both CB_1 and D_2 dopamine receptors (Koch et al., 2006). Activation of CB_1 receptors coupled to G_s protein resulted in an increase in cAMP production. These data suggest that CB_1 expressed in cells as heterodimers with D_2 receptors may function differently. These data may suggest the presence of other receptor targets for cannabinoids or alternative conformations of the CB_1 allowing differential G-

protein coupling and alter allosteric sites of action for CB_1-specific agonists and antagonists. The allosteric modulation of cannabinoid receptors due to dimerisation may provide an explanation of the phenomena discussed in the previous section (Section 1.2.2.2), altering the ability of ligands to bind and the G-proteins to which they couple to may create the illusion of multiple receptor targets.

1.2.3 Receptor localisation

CB_1 is expressed mainly in neuronal tissue and is localised to the rat cortex, globus pallidus, hippocampus, caudate putamen and cerebellum (Figure 1.1) and is responsible for most of the psychotropic side-effects associated with THC (Herkenham et al., 1990). The CB_2 receptor is primarily localised to cells and tissues of the immune system and is responsible for the immunosuppressive characteristics of THC, but identification of CB_2 in the brain stem and several brain regions, including the hippocampus, has linked cannabinoids with inflammatory control in the brain (Gong et al., 2006). More specifically, CB_1 receptors have been shown via electron microscopy and immunohistochemistry to localise to the presynaptic terminals of primary glutamatergic neurons and cholecystokinin (CCK)-positive γ-aminobutyric acid (GABA) ergic interneurons (Katona et al., 2000; Katona et al., 2006; Eggan and Lewis, 2007). The presynaptic location of these receptors coupled with the inhibitory effect of CB_1 receptors on synaptic transmission would suggest a form of retrograde signalling is at play (See section 1.3.3).

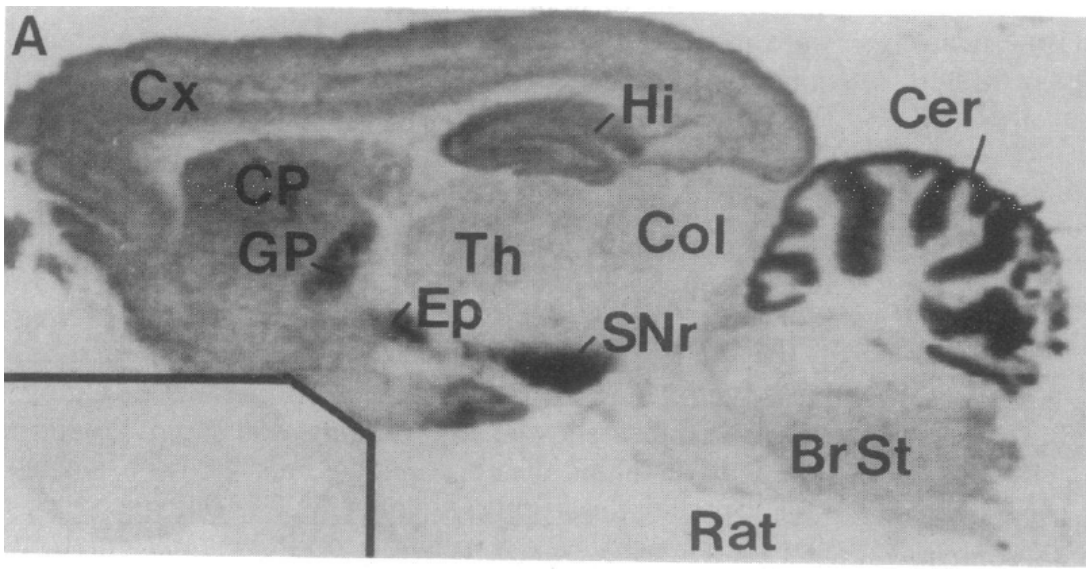


Figure 1.1 Autoradiograph of [^{3}H] CP-55, 940 binding, demonstrating the localisation of CB_1 receptor in the rat brain. Darker areas indicate a high level of expression of CB_1 in the cortex (CX), caudate putamen (CP), globus pallidus (GP), hippocampus (Hi), entopeduncular nucleus (Ep), substantia nigra (SNr) and cerebellum (Cer). There are, however, relatively low levels of CB_1 represented in the thalamus (Th) and the brain stem (BrSt). Taken from Herkenham et al. (1990).

1.3 Endocannabinoids and retrograde signaling

1.3.1 Endogenous ligands for CBRs

The identification of endogenously expressed receptors for THC led to the question: Are there endogenous ligands for these receptors? Investigating this question led to the identification of several endogenously produced molecules with a high affinity towards CB_1 and CB_2. These later were termed "endocannabinoids". The first of these to be identified was arachidonoylethanolamide (anandamide or AEA), and was shown to bind to CB_1 and CB_2 receptors at equal affinity. Anandamide has been shown to activate CB_1 receptors which results in a greater effect on contraction in the vas deferens compared with THC (Devane et al., 1992). Anandamide has also been shown to have a high affinity for transient receptor potential vanilloid 1 (TRPV1) receptors (Pertwee, 2005). Shortly after the discovery of anandamide, another endogenously released CB_1 receptor ligand, 2–arachidonoyl glycerol (2-AG), was identified which is derived from phosphotidylinositol (Mechoulam et al., 1994).

Although most studies on the endocannabinoid system have centered on the study of effects of AEA and 2-AG, there are other endogenous ligands for cannabinoid receptors, with differential affinities for either CB_1 or CB_2 receptors. These include: 2-arachidonyl glyceryl ether (noladin ether), *O*-arachidonoyl-ethanolamine (virodhamine or OEA) and N-arachidonoyl-dopamine (NADA). Noladin ether and NADA both bind to CB_1 receptors with the latter also activating TRPV1 receptors. NADA, however, displays a higher binding affinity for CB_2 receptors compared to CB_1 receptors, suggesting that it has a more significant role in the periphery.

1.3.2 Biosynthesis and degradation of eCBs

Anandamide is synthesised through a two-step process. Firstly phosphatidylethanolamine (PE) is converted to *N*-arachidonoyl PE (ArPE) by the enzymatic action of N-acyl transferase (NAT); ArPE is then broken down into anandamide by phospholipase D (PLD; Di Marzo et al., 1994). The activation of NAT is responsive to increases in intracellular calcium levels and activation of G_s-protein coupled receptors, which activate adenylyl

cyclase-induced production of cAMP; this suggests the release of anandamide may be activity-dependent. The synthesis of 2-AG in neurons occurs through the hydrolysis of phosphatidylinositol (PI) to 1,2-diacylglycerol (DAG) leading to the conversion to 2-AG through the activity of DAG lipase (DAGL) (Glass et al., 2000). Both anandamide and 2-AG undergo similar fates with regard to uptake and degradation, both being hydrolysed by fatty acid amide hydrolase (FAAH). 2-AG is also broken down by a monoglyceride lipase (MGL) to glycerol and fatty acids, which can be reused in phospholipid production. As well as providing useful tools for determining the functional role of the endogenous cannabinoid system, pharmacological modulators of the biosynthesis and degradation of eCBs have also been shown as a potential alternative to exogenous cannabinoids as therapeutics, especially in disorders where altered endocannabinoid levels may have a contributory role in the pathogenesis.

1.3.3 Neurophysiological function of eCBs

Localisation of the CB_1 receptor and the machinery, such as DAGL, required to synthesise endocannabinoids to pre- and postsynaptic terminals respectively (Silverdale et al., 2001), has led many to propose that endocannabinoids are involved in retrograde signalling. In 1992, two independent studies discovered a phenomenon in which repetitive depolarisation (also known as a "train") of Purkinje cells in the cerebellum or pyramidal cells in the hippocampus resulted in a reduction of inhibitory postsynaptic currents (IPSC) induced by GABA receptor activation on the depolarised cell (Kreitzer and Regehr, 2001a; Maejima et al., 2001; Straiker and Mackie, 2009). This phenomenon was referred to as depolarisation-induced suppression of inhibition (DSI). It was later shown that DSI was dependent on the activation of CB_1 receptors; this provided a potential physiological role for endocannabinoids (Maejima et al., 2001; Wilson and Nicoll, 2001).

It is proposed that depolarisation of the postsynaptic cell results in voltage-gated calcium channel (VGCC)-induced calcium entry, which activates DAGL in the postsynaptic cell membrane. DAGL hydrolyses PI to produce

2-AG which transverses across the synaptic space to activate the presynaptic CB_1 receptors, leading to a block of VGCC-mediated calcium influx in the presynaptic membrane, thus blocking calcium dependent exocytosis of vesicles containing inhibitory neurotransmitters (Fig 1.2). Although this describes DSI there has also been evidence to suggest that depolarisation-induced suppression of excitation (DSE) also occurs in Purkinje cells of the cerebellum (Kreitzer and Regehr, 2001b). However, it has been postulated that DSE may be mediated via another receptor, as CB1 has not been shown to localise to presynaptic glutamatergic terminals (Katona et al., 2000). The involvement of endocannabinoids in the modulation of neurotransmitter release could implicate them in diseases of the CNS and also as potential therapeutic target in disorders where synaptic transmission is elevated.

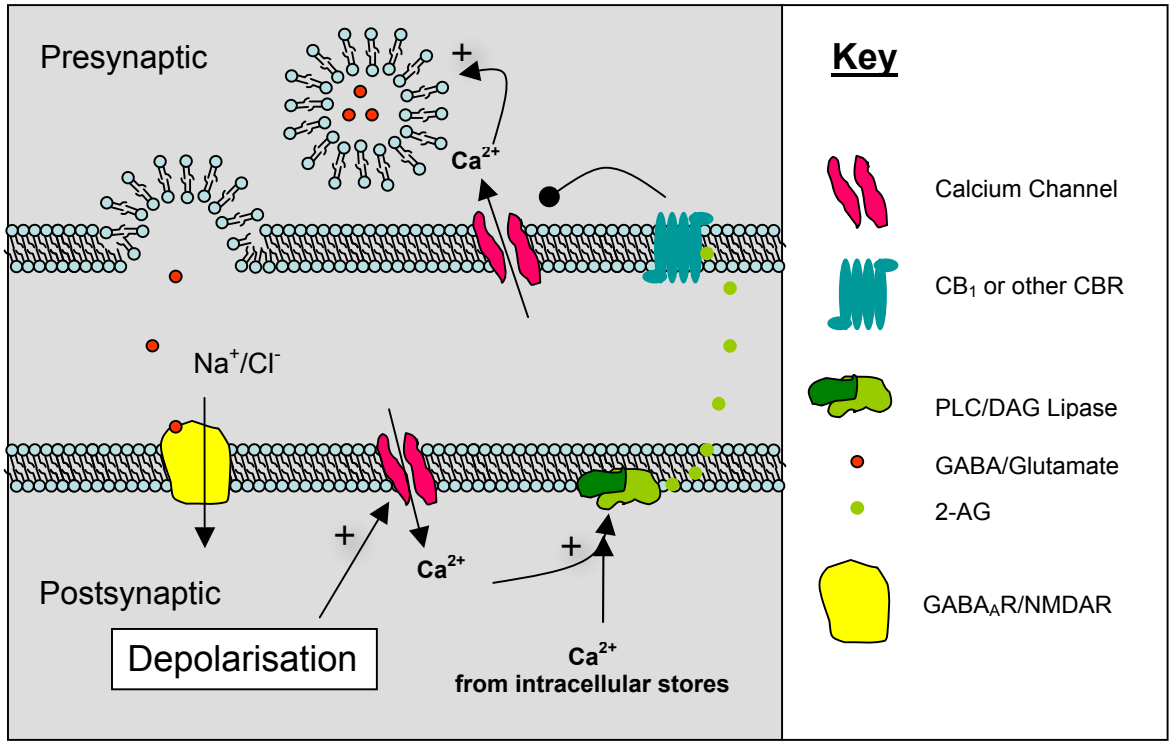


Figure 1.2 Illustration of endocannabinoid mediating DSI/DSE. Network-driven depolarisation of a postsynaptic cell activates VGCC at the postsynaptic membrane or release of calcium from intracellular stores may result in the synthesis of endocannabinoids (eCB), in this case 2-AG, through the activation of PLC/DAGL. 2-AG then translocates from the postsynaptic membrane to the presynaptic terminal membrane where it activates CB_1 receptors in the presynaptic neuron, which results in the inhibition of neurotransmitter release through blockade of calcium channels.

1.3.4 Modulation of eCB System in disease

There has been a recognized link between the levels of cannabinoid receptor expression and neurological disease states. Expression of the CB_1 receptor, or the mRNA encoding the receptor, has been shown to be decreased in animal models of neurodegenerative disorders such as Huntington's (Glass et al., 2000) and Parkinson's diseases (Beltramo et al., 1997; Silverdale et al., 2001). CB_1 expression was reduced in post mortem samples from patients suffering from depression (Koethe et al., 2007). It is speculated that effects in the CNS of synthetic pharmacological tools that modify biosynthesis or degradation of endocannabinoids, or direct activation of cannabinoid receptors, may be analogous to certain disorders which may be associated to a cannabinoid pathogenesis. The depressive side-effects caused by SR 141716 (rimonabant), the selective CB_1 antagonist marketed as an anti-obesity drug, has led to the hypothesis that the cannabinoid system may be involved in mental health homeostasis (Haney et al., 2005; Haney et al., 2007).

The association of cannabis use to psychosis has led many to suggest that the endocannabinoid system may be central to psychopathogenesis. It was found in patients suffering from schizophrenia that levels of AEA in the cerebrospinal fluid were 8 times that of control subjects (Giuffrida et al., 2004). Whether this is involved in pathogenesis or a consequence of the disorder is unclear, but it is known that CSF levels of AEA are increased in frequent cannabis users (Leweke et al., 2007). Potential links between cannabis use and this psychosis are covered in the next section of this chapter. Enhancing endocannabinoid signaling can reverse anxiety, a symptom found in chronic cannabis users. It has been shown that administration of AM404, an inhibitor of eCB degradation, is anxiolytic (Beltramo et al., 1997). This finding may suggest a potential role of the cannabinoid system in anxiety disorders.

1.4 Phytocannabinoids and CNS disorders

1.4.1 Therapeutic potential of cannabis

As discussed previously in this chapter, cannabis products, or cannabinoids, have been limited to use for treating nausea associated with chemotherapy in cancer patients, an appetite stimulant for patients suffering from AIDS (Haney et al., 2005; Haney et al., 2007) or to relieve neuropathic pain in patients suffering from multiple sclerosis and cancer (Mechoulam and Hanus, 2001; Russo and Guy, 2006). However, more and more potential uses have been investigated. Cannabis has been shown to inhibit the formation of beta amyloid plaques *in vitro*, which is the main pathogenesis in Alzheimer's disease (Eubanks et al., 2006). As well as this, THC and CBD have both been shown to suppress tumour growth in lung (Preet et al., 2008), breast (Caffarel et al., 2006; McAllister et al., 2007) and brain (Blazquez et al., 2008; Salazar et al., 2009) tissue both in animal and human studies. Considering cancer was responsible for 27% of all deaths in the UK in 2007 and killed around 6.7 billion people worldwide in 2004, cannabis derivatives could be of great interest in public health. However, it is worth noting that there has also some evidence that THC may enhance tumour growth (McKallip et al., 2005; Takeda et al., 2008), so further investigation into the mechanisms involved is required. Despite its therapeutic potential, the psychological side effects of cannabis have slowed the progress of research.

1.4.2 Cannabis: a risk factor in schizophrenia (-like) illness

The use of marijuana has been historically shown to result in psychological symptoms similar to that of schizophrenic patients. In fact, in a study of East Indian marijuana users, the author described the psychological state of these subjects as "schizoid" (Chopra and Smith, 1974). This was further corroborated by studies that involved acute administration of THC in healthy volunteers. In these studies it was reported that volunteers experienced psychotic and cognitive symptoms similar to those presented in patients suffering from schizophrenia (Morrison et al., 2009). Several studies have recognised that cannabis use is a major risk factor in developing psychological conditions in later life (Davison and Wilson, 1972; Hollister, 1986; Arseneault et al., 2002; Caspi et al., 2005; D'Souza et al., 2005; Grech et

al., 2005; 2005; Henquet et al., 2008a; Henquet et al., 2008b; Konings et al., 2008; Luzi et al., 2008; Morgan and Curran, 2008; Morrison et al., 2009). In one study it was shown that individuals whom have used cannabis on more than 50 occasions were 6 times more likely to be diagnosed with schizophrenia than non-users (Andreasson et al., 1987). Furthermore it has now become widely reported that cannabis use is a major environmental risk factor in developing some forms of schizophrenia when abuse occurs during adolescence (Henquet et al., 2005; Henquet et al., 2008a; Konings et al., 2008). This is of major significance since in the Drugs Misuse Statistics Scotland (2008) report it was revealed that 62% of individuals under the age of 20 who experiment with illicit drugs reported cannabis use. This proportion is increased to 85% of individuals under 15 years of age, suggesting cannabis is the drug of choice during adolescence. Cannabis (or the purified phytocannabinoid, THC) has also been shown in numerous studies to exacerbate the deficits in cognitive function in some individuals suffering from schizophrenia (Negrete, 1989; D'Souza et al., 2005; Grech et al., 2005; Grech, 2008). This may suggest that the endocannabinoid system may be involved in the pathogenesis of these symptoms.

As well as psychological evidence in humans, several preclinical studies in animals have shown THC to alter behaviour in models relevant to human psychosis and the cognitive deficits in schizophrenia. In mice, administration of THC (3 mg/kg) resulted in deficits in spatial working memory similar to those found in animals given phencyclidine (PCP), a drug used routinely as a positive control for psychosis (Varvel et al., 2001; Varvel et al., 2005). THC at doses of 1mg/kg also impaired affective flexibility in the rat attention set shift task (Egerton et al., 2005a). The impairment of performance in this task by THC was correlated to a reduction in the expression of the IEG *c-fos*, a marker of general neuronal activity, in the medial prefrontal cortex (mPFC) (Egerton et al., 2005a), an area that has a similar role in rat cognitive function to the dorsolateral prefrontal cortex (DLPFC) in humans (Levy and Goldman-Rakic, 2000).

1.4.3 Mechanism of THC-induced illness in the CNS

1.4.3.1 GABAergic hypothesis of schizophrenia-like illness

The cognitive impairments found in some schizophrenic patients have been proposed to be a result of reduced γ-aminobutyric acid (GABA) production in the DLPFC (Rao et al., 2000; Lewis et al., 2005). CB_1 receptors have been shown via electron microscopy and immunohistochemistry to localise to presynaptic terminals of cholecystokinin (CCK)-positive GABAergic interneurons (Katona et al., 2000; Eggan and Lewis, 2007). Since CB_1 receptor activation inhibits GABA release it is understandable that use of exogenous compounds that activate CB_1 receptors would therefore block presynaptic GABA release that may already be diminished in the schizophrenic condition. Administration of THC was shown by microdialysis to result in a reduction of GABA concentration in the prefrontal cortex (Pistis et al., 2002). It was also shown in this study that glutamate and dopamine concentrations were elevated (Pistis et al., 2002), which may have been due to THC administration resulting in a disinhibitory effect. This may go towards explaining the finding that cannabis consumption exacerbates symptoms in the schizophrenic condition. In mice, THC-induced deficits in cognitive function were reversed by a selective $GABA_A$ receptor antagonist, bicuculline, which would implicate the GABAergic system in the cognition-impairing effects of THC (Varvel et al., 2005).

1.4.3.2 Dopamine hypothesis of schizophrenia-like illness

Psychosis has also been linked with increased levels of the neurotransmitter dopamine, which may be crucial to the psychoactivity of cannabis (for review see Luzi et al., 2008). The dopamine theory of psychosis stems from several experiments where amphetamine and cocaine, which stimulate dopamine release, were shown to produce symptoms similar to those associated with schizophrenia (Harris and Batki, 2000). Furthermore, low doses of these drugs induced psychotic episodes in schizophrenic patients, an effect which was not demonstrated in healthy individuals. An interaction between the cannabinoid and dopaminergic system has been demonstrated at different levels. THC administration has been shown to cause an increase

in dopamine levels in the CNS, which may provide the simplest explanation (Diana et al., 1998; Pistis et al., 2002). THC administration sensitises rats to amphetamine-induced psycho-relevant behaviour (Gorriti et al., 1999). Both THC and WIN 55,212-2 have also been shown to increase firing frequency in dopaminergic neurons of the rat prefrontal cortex, an effect blocked by rimonabant (Diana et al., 1998). Rimonabant on its own has also been shown to inhibit dopamine production in the prefrontal cortex (Tzavara et al., 2003). A polymorphism that has been discovered in the gene encoding catechol-*O*-methyltransferase (COMT), a catabolic enzyme that deactivates dopamine, has been linked with the predisposition for cannabis-induced psychosis (Caspi et al., 2005).

1.4.3.3 Developmental hypothesis of psychosis

The role of the endocannabinoid system in neuronal development may provide evidence of another neurological consequence of cannabis abuse and the use of extracts as therapeutics. Recent developments have not only shown that the CB_1 receptors and endocannabinoids are involved in cortical development, but are essential in GABAergic neurite growth and axonal guidance to its target (Berghuis et al., 2007). Due to the implication of CB_1 specifically in the wiring of GABAergic neurons, the developmental hypothesis of cannabis-induced psychosis may be related to the GABAergic mechanism discussed above. This is also supported by the findings that CB_1 receptor specific knockout mice, $CB_1R^{Dlx5/6\text{-}Cre}$, showed a significant change in cortical morphology. This altered morphology has also been observed in the offspring of pregnant rats treated with the CB_1 receptor antagonist AM251 (Mereu et al., 2003; Antonelli et al., 2005). Although THC acts as a partial agonist at CB_1 receptors, it is possible that it may act as an inverse agonist if administered *in utero*, when tonic endocannabinoid is at its highest. This would result in a reduction in cortical development in the developing uterus. It is possible, although not investigated, that a similar situation occurs in adolescent CNS development. If true this may support the hypothesis that abuse of cannabis during adolescence increases the risk of psychotic illness in later life (Arseneault et al., 2002).

1.5 Cannabidiol

As previously discussed, the main psychoactive component of *cannabis sativa* is THC. However, there are also other components of interest, which although previously thought to have no intrinsic psychoactivity have been suggested to play a contributory role in the actions of THC (Moreira et al., 2006). Cannabidiol (CBD) has been described as a non-psychoactive component of *cannabis sativa.* Recent research has highlighted CBD to be of interest because of its potential as an alternative treatment for individuals suffering from schizophrenia (Zuardi et al., 1995; Long et al., 2006; Zuardi et al., 2006a; 2006b; Morgan and Curran, 2008). There is also interest in the ability of CBD to attenuate negative effects of THC while enhancing the more desired effects (Fadda et al., 2006).

1.5.1 CBD may protect against the adverse/unwanted effects of THC in the CNS

There have been a number of studies implicating an interaction between CBD and THC. However, the mechanisms of this interaction are not very well understood. An interaction between these two natural constituents of *cannabis sativa* has been under investigation since the early 1970s (Zuardi et al., 1982; Zuardi and Karniol, 1983; Leweke et al., 1999; Fadda et al., 2004; Russo and Guy, 2006). CBD was shown to attenuate the feeling of anxiety and psychological symptoms, such as depersonalisation and disconnected thought, induced by THC in healthy human volunteers (Zuardi et al., 1982). In a human model of illusionary perception, binocular depth inversion, CBD was shown to reduce the deficits induced by nabilone (a synthetic analogue of THC) in the ability to distinguish between convex or concave 3-dimensional shapes (Leweke et al., 1999). There is also a suggestion that a higher level of CBD in cannabis may reduce the risk of developing cannabis-related psychosis in the future. In a retrospective investigation hair samples were taken from volunteers and analysed for cannabinoid content. It was found that the hair samples from those volunteers suffering from mild psychological problems contained THC and no CBD, whereas volunteers whose hair samples contained similar levels of THC and CBD were not suffering from any psychological problems (Morgan and Curran, 2008). With the anecdotal suggestion that use of "skunk", an extremely potent

variety of cannabis which contains high levels of THC and little to no CBD, is becoming more popular, it may be of great interest in evaluating whether such users of this variety may be at greater risk in developing psychosis.

In preclinical studies, administration of CBD was shown to protect rats against THC-induced deficits in performance in the Morris water maze. A study showed that a CBD-rich extract, containing 50 mg/kg CBD and 4 mg/kg THC, had no significant effect on performance in this task. However, significant deficits were displayed in those rats treated with THC-rich extracts containing a concentration of THC above and below that present in the CBD-rich extract (Fadda et al., 2004). This lends some support to the protective effect of CBD against THC-induced cognitive deficits. However, as no comparisons were made to pure extracts an interaction with other cannabinoids in the extract cannot be ruled out.

Welburn et al (1976) and Karniol and Carlini (1973) showed conflicting results with regard to THC and CBD interactions, as the former showed attenuation of THC-induced antinociception in the abdominal stretch test while the latter showed CBD to increase THC-induced antinociception in the hot plate test (Karniol and Carlini, 1973; Welburn et al., 1976). A possible explanation stems from the work of Zuardi and Karniol (1983) who examined the effect of dose ratios on the ability of CBD to attenuate THC-induced behavioural alterations. They showed CBD inhibited the rate dependent effect in variable interval schedule behaviour induced by THC administration when 5 times the concentration of CBD compared to THC was given the variable interval schedule behaviour of rats were improved, this inhibition was not present when the ratio of CBD was increased to 20:1 (Zuardi and Karniol, 1983). These studies taken together would imply that in order for CBD to have any significant effect to protect against the effects of CBD, it is required at a much higher concentration to THC. However, GW Pharmaceuticals have produced a spray designed to administer 2.5 mg of CBD to every 2.7 mg of THC, in human subjects. Thus far, evidence suggests this to be beneficial to sufferers of multiple sclerosis, without any severe side effects (Wade et al., 2006; Collin et al., 2007). However, it will be some time

before it can be concluded that CBD at this ratio can protect against the chronic effects of THC.

1.5.2 Therapeutic potential of CBD

The use of CBD as therapeutic drug has mainly come to light of late due to its use in combination with THC in the treatment of the painful symptoms of the demyelinating disease multiple sclerosis (Ben Amar, 2006; Russo and Guy, 2006). Despite conflicting evidence, it is claimed that CBD has antinociceptive properties in its own right (Russo and Guy, 2006). CBD has been previously shown to lack antinociception in mice even at high doses (Sofia et al., 1975); this was later confirmed in a study in rats (Moreira et al., 2006).

The neuroprotective effects of CBD have been well established. CBD contains the phenolic ring structure that has been shown be responsible for the ability of some phytocannabinoids to protect cells against oxidative stress induced by reactive oxygen species (ROS; Hampson et al., 1998). It has been demonstrated that CBD protected streptozocin (STZ)-induced diabetic rats against the onset of retinopathy resulting from glucose-induced increase in oxidative stress (El-Remessy et al., 2003). Also, in a middle artery occlusion (MAO) model of stroke in mice (Mishima et al., 2005), CBD was shown to significantly reduce the infarct volume whether administered before or after occlusion. This is of great interest as there is a possibility of using CBD as a prophylactic treatment in those individuals at high risk of stroke. This ability to reduce infarct volume was blocked by WAY100635, a 5-HT_{1A} antagonist (Mishima et al., 2005). As well as this, CBD has also been shown to prevent pancreatic damage resulting in diabetes mellitus in mice, reduce swelling of cartilage tissue in arthritis and reduce blood pressure in patients with hypertension without lowering heart rate (Mechoulam and Hanus, 2001; Weiss et al., 2006).

1.5.3 Effects of CBD in behavioural studies

Several studies in rats and humans have shown CBD to have psychological effects on its own. These include anxiolytic-like and antipsychotic qualities.

In human studies, volunteers were given CBD and asked to fill out a self-evaluation. A significant number of these volunteers described themselves as feeling "Clear-minded" and "Quick-Witted" after taking CBD compared to when a placebo was given (Zuardi et al., 1982).

In preclinical studies, it was reported that CBD attenuates a conditioned contextual fear response in rats at 10 mg/kg (Resstel et al., 2006). In the elevated plus maze, a test of anxiety in rodents, rats given doses of 2.5, 5 and 10 mg/kg showed a higher tendency to enter the open arm of the maze (Guimaraes et al., 1990). In the Vogel conflict test, a test of anxiety behaviour where animals receive a punishment (electric shock) when it receives reinforcement (food or water). In this example, rats received an electrical shock when from a water bottle when drinking. CBD resulted in an increase water consumption at 10 mg/kg, but not at lower doses (Moreira et al., 2006) compared with control groups suggesting CBD administration resulted in an anxioltic effect. The anxiolytic effect of CBD in both the elevated plus maze and Vogel conflict test was shown to be blocked by the administration of a serotonin receptor antagonist, WAY 100635, which is a selective antagonist for 5-HT_{1A} receptor (Campos and Guimaraes, 2008).

CBD has also been proposed to act as an antipsychotic for the treatment of schizophrenia. Two very small studies showed that CBD was comparable to atypical antipsychotics in the treatment of schizophrenia (Zuardi et al., 1995; Zuardi et al., 2006b). However, given the small sample sizes (n=3 and n=1) these results should be viewed with extreme caution and a much larger study is required to investigate the potential of CBD as an anti-psychotic agent. There is some suggestion that CBD may be beneficial in treatment for some of the cognitive deficits found in some cases of schizophrenia. A recent study has shown that AM404, endocannabinoid uptake/degradation inhibitor can reverse cognitive deficits induced by phencyclidine (PCP), a NMDA antagonist used in some pharmacological models of schizophrenia (Vigano et al., 2009]. Although in this study the effect of CBD was not evaluated, CBD has been found to have a similar action to AM404 in

blocking the reuptake and degradation of endocannabinoids {Fernandez-Espejo, 2004 #125).

Sensory-motor gating deficits in schizophrenia can also be replicated in rodents treated with NMDA receptor antagonists. These deficits can be assessed using pre-pulse inhibition (PPI) of the acoustic startle response. It was shown that 5 mg/kg of CBD completely reversed disruption of PPI resulting from administration of 1 mg/kg MK-801 (Long et al., 2006). The ability of CBD to reverse these deficits was mediated via the TRPV1 receptor, a ligand-gated cation channel selective for calcium. This could be through either CBD acting at TRPV1 directly or through the ability of CBD to inhibit breakdown of anandamide, which has been shown to activate this receptor (Bisogno et al., 2001). For example, in normal conditions glutamate would be released and activate the NMDA receptor, resulting in an influx of Ca^{2+}, which would result in depolarisation of the cell membrane. However, when the NMDA receptor is blocked by MK-801 and CBD is administered, the Ca^{2+} influx generated through CBD activation of TRPV1 may be sufficient to depolarise the membrane. In this example the concentration of CBD and the availability of TRPV1 must be sufficient for this to occur. It is important for the advancement of knowledge in this area to elucidate the true mechanism of CBD.

1.5.4 Mechanisms of CBD action

Despite its many possible therapeutic applications, the mechanisms of CBD remain elusive. CBD was shown to have a low affinity to CB receptors in a binding assay (K_i>10μM; Bisogno et al., 2001) and more recently CBD was shown to act as a CB_1 receptor antagonist (K_B=79nM, K_i=4.9μM) and a CB_2 inverse agonist (K_B=65nM, K_i =4.2μM) (Thomas et al., 2007). However, CBD has also been shown to act as a full agonist at TRPV1 receptors (EC_{50}=3.5±0.3 μM) (Bisogno et al., 2001). In several studies of CBD effects on intracellular calcium ion concentration ($[Ca^{2+}]_i$), it was proposed that CBD causes an increase in $[Ca^{2+}]_i$ by increasing Ca^{2+} uptake via the voltage-gated calcium channels and release of Ca^{2+} from intracellular stores (Drysdale et al., 2006). Further experiments on the effect of CBD on $[Ca^{2+}]_i$ showed the presence of a

TRPV1 antagonist, capsazepine, resulted in a significant increase in CBD-induced $[Ca^{2+}]_i$ rises (Ryan et al., 2007). This may support the idea that the TRPV1 receptor is the main site of action of CBD.

Another potential mechanism that has recently become of interest is an action at the GPCR, 5-HT_{1A}. This is because a 5-HT_{1A} receptor antagonist, WAY100635, was shown to block the ability of CBD to protect against cerebral ischaemia as well as its ability to reduce anxiety in animal studies previously discussed (Campos and Guimaraes, 2008){Resstel, 2009 #179}. CBD at doses in the micromolar range was shown to displace a 5-HT_{1A} specific radioligand, [3H]8-OH-DPAT, as well as increasing [^{35}S]GTPγS binding in a transfection system (Russo et al., 2005).

There is also a possibility that CBD-induced effects may occur via a receptor that has not previously been characterised. A candidate receptor was found in the mesenteric artery activated by an abnormal CBD analogue (abnormal-CBD) and anandamide, resulting in vasorelaxation (Kunos et al., 2000), an effect attenuated by CBD. The presence of a "CB_3" receptor has also been suggested in pyramidal neurons in the CA1 region of the hippocampus (Hajos et al., 2001). This was due to WIN 55,212-2 showing an equal ability to suppress the activity of glutamatergic neurons in both CB_1 knock-out and wild type mice. However, a similar experiment showed that WIN 55,212-2 suppression of glutamatergic neurons was not present in $CB^{-/-}$ mice (Takahashi and Castillo, 2006). It was suggested by these authors that the differing results might be due to the methodology used. It has been suggested that the newly discovered cannabinoid receptor, GPR55, is the ever-elusive CB_3. However, the inability of anandamide to activate GPR55 as well as the currently uncharacterised functional consequences of GPR55 activation, have led most to rule out GPR55 as the proposed CB_3 receptor (Johns et al., 2007).

1.6 Summary

1.6.1 Summary

The principal aims of this thesis are to address the importance of the endocannabinoid system in neurophysiology and how modulation of this system with exogenous phytocannabinoids such as THC can contribute to the development of mental illnesses. Despite this, phytocannabinoids are of great interest as naturally occurring therapeutics in a host of debilitating and life-threatening conditions including, neuropathic pain and cancer.

Since the discovery of THC, the chemical identified to be responsible for most of the desirable and unwanted effects of cannabis, most pharmacological research has centred round its therapeutic potential. However, recently CBD has come to the forefront of phytocannabinoid research. Recent preliminary evidence suggests that it might be beneficial in treating the same conditions as THC without psychological side-effects as well as having a potential as an anti-psychotic agent in its own right. 3 specific aims shall therefore be examined in the work contained in this thesis:

1. To determine if CBD can modulate THC-induced alterations in synaptic activity in the hippocampus
2. To analyse the specific brain regions affected by THC administration and evaluate if these are modulated by CBD.
3. To evaluate whether CBD can reverse alterations in regional brain activity induced by a psychotomimetic agent, such as phencyclidine (PCP).

CHAPTER 2: MATERIALS & METHODS

2.1 Electrophysiology Part I: Dissociated hippocampal neurons

2.1.1 Primary hippocampal culture

Cultured hippocampal neurones have been used routinely in electrophysiological studies due to their ability to form synaptic connections and multi-cellular networks *in vitro* and due to their ability to maintain similar physiological state as *in vivo*. They are also a cost effective set-up to begin pharmacological investigations with, due to the number of experimental conditions that can be tested using cultures taken from a single animal. Most work optimising the conditions has been carried out by Brewer et al, who have shown that hippocampal cells cultured in a specialised serum-free media, Neurobasal-A (GIBCO) supplemented with B27, allowed for hippocampal neurons to be cultured for up to 4 weeks (Brewer et al., 1993) with a much higher viability than conventional media. They also showed that the use of the pineapple extract papain, resulted in a sufficient proteolysis of hippocampal tissue without compromising the viability of individual cells (Brewer, 1997).

Dissections were performed in a laminar flow hood, all surfaces and tools were sterilised using 70% ethanol. All animals were killed by cervical dislocation followed by decapitation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Brains were removed from Sprague-Dawley rats aged 1 to 2 days old and placed on filter paper. The cerebrum was split into two hemispheres down the midline and rolled exposing the cut area (Fig 2.1). The cerebellum was flipped at a 90° angle away from the cortex exposing the hippocampus. Cutting away the cortical tissue, the hippocampus was then removed using a spatula. Hippocampi were then placed in a sterile hippocampal dissection solution (HDS) consisting of:

- 116mM NaCl
- 5.4mM KCl
- 26mM $NaHCO_3$
- 1.3mM NaH_2PO_4
- 1mM $MgSO_4 \bullet 7H_20$

- 1mM $CaCl_2 \bullet 2H_20$
- 0.5mM EDTA
- 25mM Glucose

Hippocampi were finely chopped using a no. 11 scalpel blade before being incubated in 1.5 mg/ml papain (Sigma, UK), diluted in the above hippocampal dissection solution, for 20 mins at 37°C in a water bath to allow for the breakdown in connective tissue. Tissue was then transferred in to a BSA (Sigma, UK) solution, 10 mg/ml BSA in HDS. The tissue was then triturated using glass Pasteur pipettes that were fire polished, using an ethanol burner, to varying diameters large (fire-polished for < 5 seconds) to small (about the size of a ball-point pen tip). Tissue was taken up and expelled to and from the pipette 7 times per pipette. The cell suspension was then centrifuged for 3 minutes at 2000 rpm, the supernatant removed and the cells resuspended in 2ml per animal used of Neurobasal-A (GIBCO, UK) medium containing 2% B-27 (GIBCO, UK) and 1% L-glutamine (Invitrogen, UK) preheated to 37°C. 90 μl of cell suspension was applied to glass coverslips coated with 0.1mg/ml poly-L-lysine (Sigma, UK), 3 coverslips per 35mm tissue culture dish. The cells were incubated for 1 hour at 37°C and 5% CO_2. Excess media was removed and 2 ml of fresh medium added per dish and returned to the incubator at 37°C and 5% CO_2. After 5 days, 10μM of cytosine-β-D-arabinofuranoside (Ara-C) (Sigma, UK) was added and cells were returned to incubator until required.

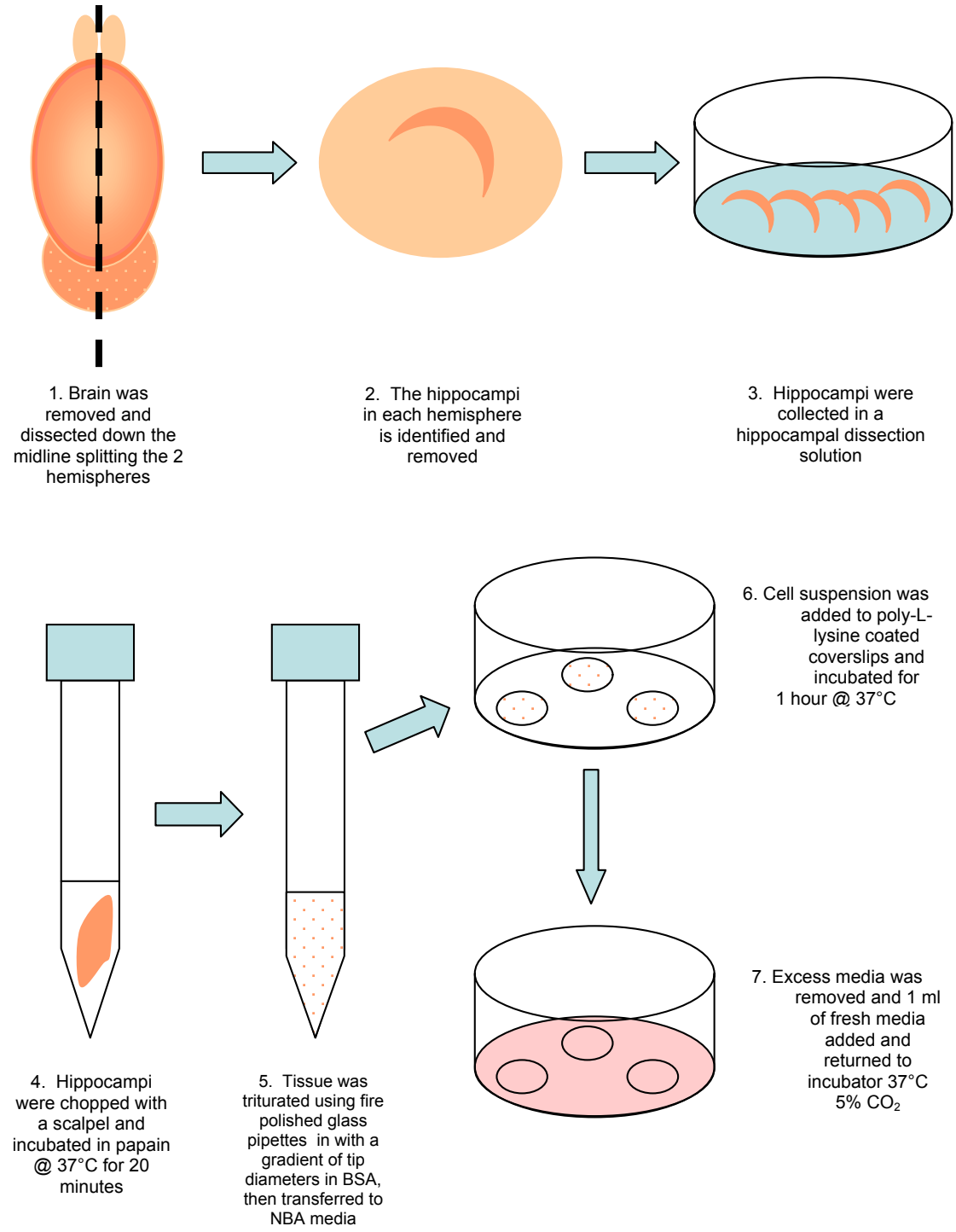


Figure 2.1 Preparation of primary hippocampal neurons. Diagram illustrating the steps in preparing primary dissociated hippocampal culture from dissection to plating dissociated cells.

2.2.2 Whole cell current clamp electrophysiology

Whole cell patch clamp technique has been used routinely to measure the effects of drugs on the function of neurons in the central and peripheral nervous system. This is largely the result of pioneering experiments by Hodgkin and Huxley in 1952, where they used glass pipettes to record the membrane potential in the squid giant axon. The refinement of this technique, using very fine glass pipettes coupled with the development of voltage-clamp devices allowed investigators to evaluate neuronal cell physiology. Fine fire-polished glass pipettes with a resistance (*R*) in the mega ohms (MΩ) range are able, with the aid of suction, to generate an extremely tight seal on a cell membrane, when the cell membrane within the area covered by the glass electrode is ruptured the space within the glass electrode, filled with an artificial intracellular solution, becomes part of the cell and therefore allows measurement in alteration of the intracellular ion concentration, this is known as a whole cell patch clamp. There are two main types of whole cell clamping, voltage clamp and current clamp. In voltage clamp recordings, the cells can be held at a specific voltage via injection of current (*I*) to generate a potential difference from the inside of the cell compared with the outside, known as membrane potential which is measured in volts (Vm). This allows the ability to activate voltage-dependent ion channels and measure the response of the cell to ion channel activation by measuring the changes in level of current required to maintain the clamped voltage. Current-clamp recordings allow the opposite, where alteration in the resting membrane potential can be measured in response to network driven synaptic activity. Due to the networked nature of the hippocampal cultures, utilization of current clamp will be the most relevant technique for this study. Therefore current clamp recordings were performed on hippocampal primary cell cultures between 12-16 days *in vitro*. Glass pipettes were pulled using DMZ universal puller (Zeitz) (4-6 MΩ) and filled with an internal solution containing:

- 150mM KCl
- 10mM HEPES

- 4mM Mg-ATP
- 0.3mM GTP
- 5mM NaPCr
- 0.5mM EGTA (all Sigma)
- pH was adjusted to 7.2 using 1M KOH and osmolarity was adjusted to 290 mOsm using sucrose if required

In order to transform the ionic activity of the cell into electrical activity that can be recorded using an Axopatch-200B (Axon Instruments, UK) patch-clamp amplifier, silver/silver chloride (Ag/AgCl) electrode is immersed in the intracellular solution. This allows transformation of ionic to electrical activity by exchanging Cl^- ions for electrons at the surface of the electrode. The analogue signal from the amplifier is then converted to a digital signal via a NIDAQ analogue to digital (A/D) convertor (National Instruments, UK; Fig 2.2). Current clamp recordings were then recorded to the hard drive using WinEDR v 2.7.9 software (J. Dempster, University of Strathclyde, UK).

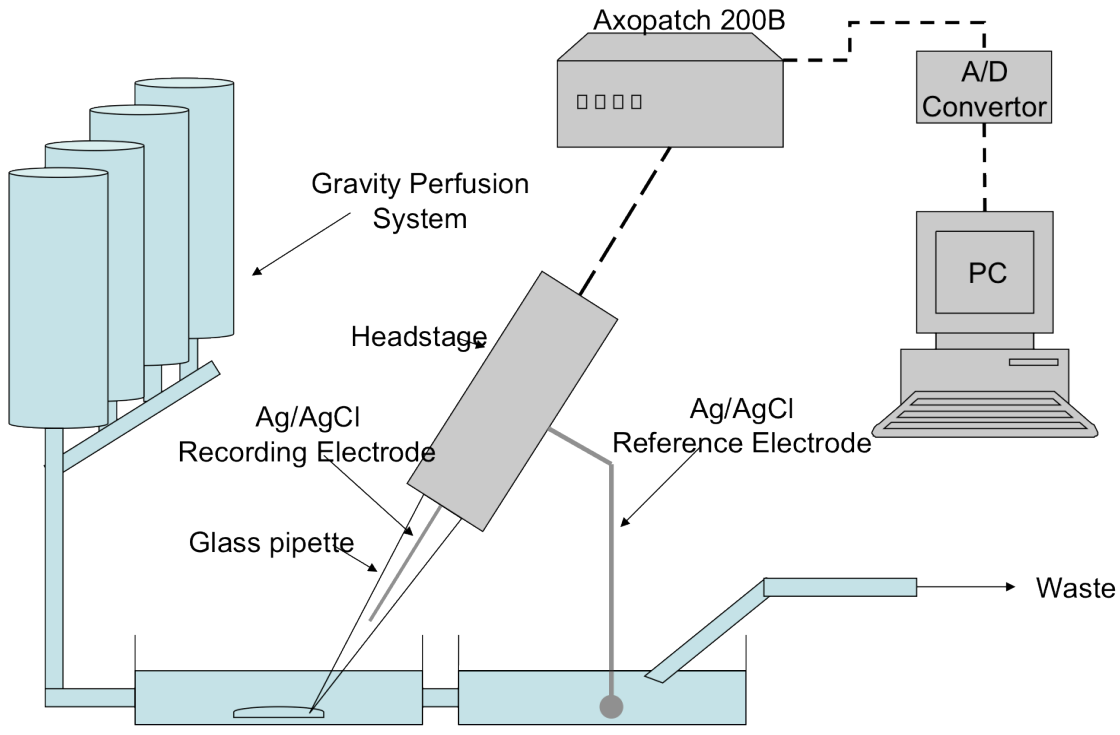


Figure 2.2 Schematic diagrams illustrating the set up of the patch-clamp recording apparatus.

Cells were identified for experiments using a TS100 light-microscope (Nikon, U.K.), where cells showing healthy cell body with neurites appearing to be well networked were selected. The visualisation and recording apparatus were mounted on an air-table contained in a Faraday cage to reduce potential for vibrations and electrical noise from outside devices interfering with the recording. All electrical equipment used in the visualisation and recordings were grounded through the amplifier.

Cells were perfused with an external solution containing the following:

- 140mM NaCl
- 5mM KCl
- 2mM $MgCl_2$
- 10mM HEPES
- 2mM $CaCl_2$
- 10mM D-glucose (all Sigma)
- pH was adjusted to 7.4 using 10M NaOH
- Osmolarity adjusted to 310 mOsm with sucrose if required

Data were analysed using WinEDR v 2.7.9 (J.Dempster, University of Strathclyde). Action potentials or events were detected by setting a detection threshold in the software of -30 to -45 mV, as the software did not distinguish whether the Vm was increasing or decreasing across the threshold an inter-event interval of 100ms was and frequency (Hz) of events was calculated. A Student's t-test was performed comparing Hz for 1 minute following drug application to that of the baseline frequency.

2.2 Electrophysiology Part II: Acute slice recording

2.2.1 Field recording in hippocampal slices

Although the hippocampal neurons in dissociated cultures do possess much of the same properties as *in vivo*, the fact that they are not developed in a physiological relevant environment means there may be differences between how neurons in the dissociated culture perform compared with neurons *in vivo*. Also, when recording from whole-cell patched cells the changing of the intracellular ionic concentration may affect the physiology of the cells an make them more susceptible to effects of drug administration. It is therefore worthwhile verifying that the effects of neuropharmacological drugs are present in a more physiological set up. The isolation and maintenance of neuronal slices for electrophysiological recording was developed by Yamamoto and McIlwain in 1966 and apart from some refinement, little has changed in the last 34 years (Yamamoto and McIlwain, 1966a; Yamamoto and McIlwain, 1966b). Briefly, saggital hippocampal slices (~400 μm thick) can be maintained *ex vivo* in oxygenated artificial cerebrospinal fluid (aCSF) and perfused with different drugs or neuromodulators as a neuropharmacological assay of synaptic function. Field recordings from the stimulated fibres of the Schaffer-collateral (SC) pathway in the CA1 (Figure 3.1) region of the hippocampus have been used for decades to gain a better understanding of synaptic function in the hippocampus. Simply, electrically evoked fibres release excitatory neurotransmitters, such as glutamate, which activate the ionotropic receptors on the postsynaptic membrane resulting in an influx of cations into the cell which alters the local potential of the aCSF, i.e. proximal to the membrane compared to that of the aCSF in another region of the bath. Although, this event is not enough to be detected with any great resolution, the activation of many fibres resulting in these events occurring at many sites results in a detectable transient change in extracellular cation concentration. The hippocampus contains both excitatory and inhibitory neurons which are both innervated by this stimulation, however, there are a significantly greater number of glutamatergic to GABAergic neurons meaning the net response of these

evoked fibres are excitatory in nature and are termed evoked field excitatory post-synaptic potentials (fEPSP) which are measured in millivolts (mV).

As well as being able to measure the neurophysiological effects of drugs, alterations to the stimulation protocol can help identify whether a drug is acting presynaptically or postsynaptically. Paired-pulse facilitation (PPF) is a form of short-term synaptic plasticity. This phenomenon was first discovered in 1938 in neuromuscular junctions, where it was discovered that when the investigators stimulated prejunctional fibres with multiple pulses, these multiple stimulations result in an increasingly larger muscular response (Boyd et al., 1938). This was later shown to be due to enhanced neurotransmitter release resulting from a build-up of calcium in the presynaptic junction that is not cleared by intracellular calcium chelators before the next pulse, therefore increasing the probability that the neurotransmitter will be released. Facilitation has be demonstrated in the CA1 region of the hippocampus, where a paired-pulse in the Schaefer-collateral fibres with an inter-pulse interval of less than 200ms has been shown enhance the fEPSP amplitudes of the second pulse (Creager et al., 1980). Activation of a presynaptic receptor which generally inhibts neurotransmitter release would reduce the first response, but the second response will be enhanced due to the slightly elevated calcium levels proximal to the synapse priming the response to the second response (for review see Thomson, 2000). Therefore, if a drug suppresses fEPSP response but enhances PPF then the drug is determined to be acting via a presynaptic mechanism.

2.2.2 Acute Slice Preparation

Whole brains were removed from 16 to 20 day old Sprague-Dawley rats and placed immediately in an ice-cold, oxygenated cutting solution containing:

- 100mM sucrose
- 26mM $NaHCO_3$
- 2mM NaH_2PO_4
- 2mM $MgSO_4$

- 3mM KCl
- 2mM $CaCl_2$
- 10mM D-glucose (All from Sigma, UK)

Using a vibratome, 400μm saggital slices were cut and place into oxygenated aCSF containing the same as above replacing the sucrose with 124mM NaCl. Areas from the frontal region, i.e. from the fornix forward, as well as the mid-brain were removed from the slice. These slices were then placed on a nylon mesh, submerged in oxygenated aCSF for 45 minutes to allow slices to equilibriate.

2.2.3 Evoked field EPSP recordings

Slices were placed in a submerged recording chamber. A perfusate of oxygenated aCSF was fed through by means of a gravity perfusion system. aCSF was maintained at a temperature of 30°C, by either using the coil-heater system encompassed by the recording chamber or by using an in-line heater. The in-line heater was used in later experiments due to problems with noise associated with the coil heater. A bipolar stimulating electrode was placed in the Schaffer-Collateral (SC) pathway in the CA1 region of the hippocampus (Fig 2.3; B). A paired-pulse stimulation protocol (Fig 2.3; A) was run using WinWCP (J.Dempster, University of Strathclyde, UK) that was delivered every 30 seconds via a DS3 isolated stimulator (Harvard Apparatus, UK). The postsynaptic response to this stimulation was recorded using a fire-polished pipette position in the stratum radiatium (aprox 0.5mm from stimulating electrode) made from borosilicate glass filled with 4M NaCl containing an Ag/AgCl electrode. This was attached via a headstage to an Axoptach 2D amplifier via a differential gain amplifier used to boost the signal. The analogue signal was converted to a digital signal using an A/D convertor board (Fig 2.4). The data were then aquired using on a PC running WinWCP software (J.Dempster, University of Strathclyde, UK). The stimulation current amplitude and polarity were altered until a maximal response was reached. The stimulation amplitude was adjusted to achieve 50% of the maximal response in the first pulse.

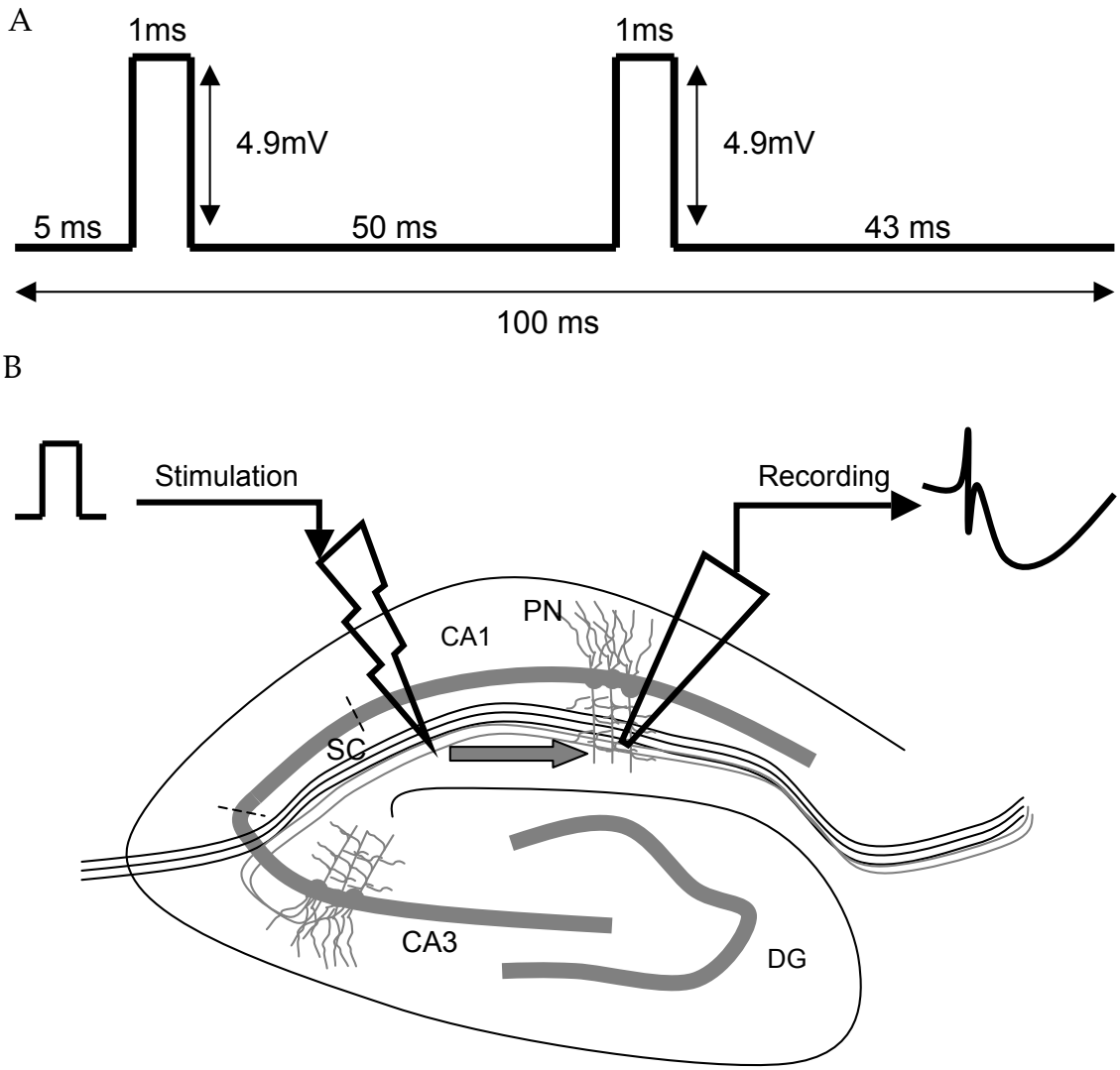


Figure 2.3 Illustratiion of the rat hippocampal slice recording. A. This illustrates the paired-pulse stimulation protocol used in experiments to determine whether drug effects are pre- or postsynaptic. B. This diagram shows the sagital hippocampus with the dark band representing the pyramidial neuron (PN) cell body layers in the CA1, CA3 and dendate gyrus (DG). The fibres of the Schaffer collateral pathway (SC) projecting from the CA3 to the CA1 are also show alongside an illustration of where the stimulation and recording electrodes are placed in measuring evoked field EPSP.

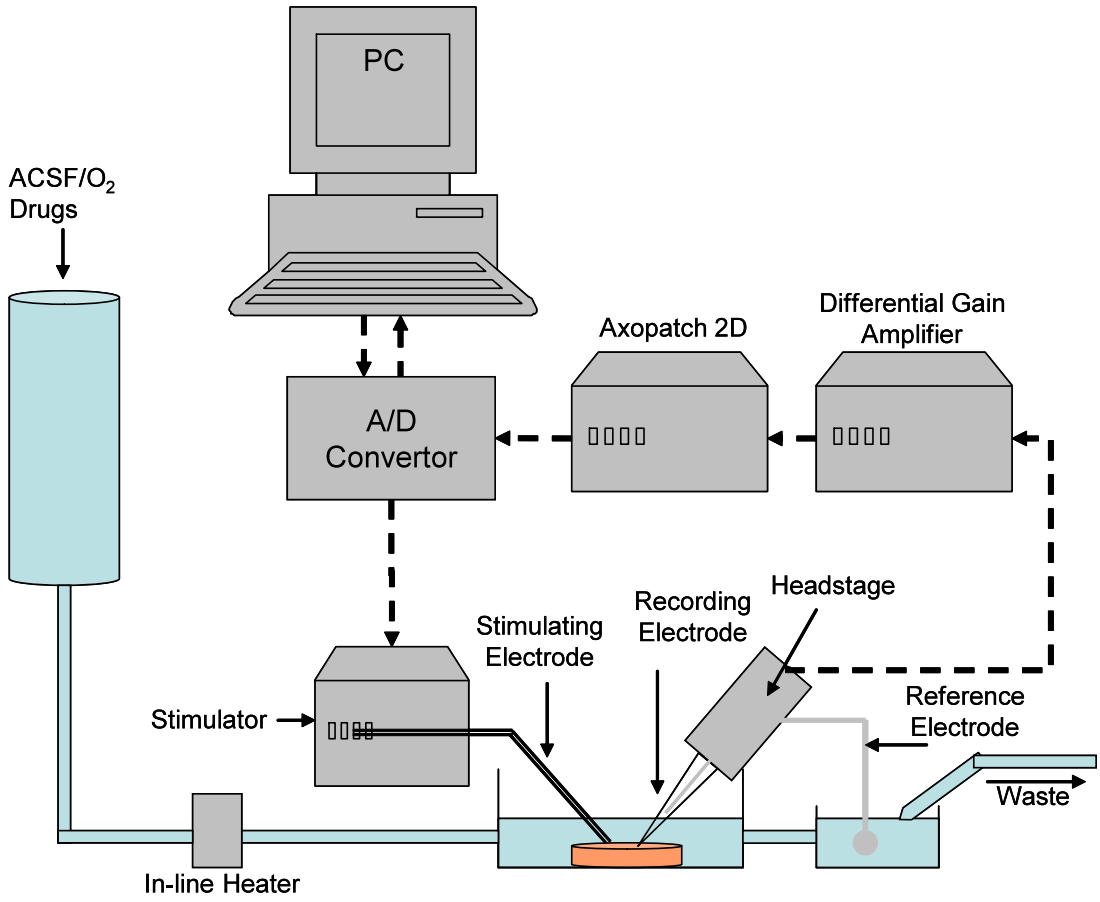


Figure 2.4 Schematic representation of the set-up of the electrophysiological slice recording apparatus

2.2.4 Waveform analysis

All data were acquired using an Axopatch-2B amplifier (Axon Instruments) and using WinWCP v 2.7.9 software (J.Dempster, University of Strathclyde). The rates of rise (or slope) were measured using WinWCP waveform analysis tool. The fEPSP slope was calculated by subtracting the value at 90% of the maximal response (vM_{90}) by the value at 10% of the maximal response (vM_{10}). This was then divided by the difference in time at these two points (t_{90}-t_{10}) to give a rate of rise in response. The paired pulse ratio (PP Ratio) was determined by dividing the peak amplitude of the second response by that of the first response.

2.3 In situ hybridisation of markers of neuronal activity

2.3.1 In situ hybridisation for imaging regional changes in IEG expression

IEGs have become a useful tool in neuroscience, where using techniques such as in situ hybridisation allows the investigator to identify regions of the brain in which the levels of IEG expression changes in response to exogenous stimuli, either directly or indirectly through altering the input from another region. Although the technique does not allow differentiation between these direct and indirect effects, the ability to visualise the regions affected by stimuli can give an indication of potential behavioural effects. The *in situ* hybridisation technique was based on the method described by Wisden and Morris (2002). Animals were randomly allocated into either a drug or vehicle group and received 1 mg/kg of drug solution by intraperitoneal (i.p.) injection. The control group received an equivalent volume of saline (1ml/kg). Both THC (Sigma) and CBD (Tocris) stock were stored in pure ethanol at -20°C. In order to re-suspend the THC/CBD in saline solution, it was first mixed with 2 parts Tween-80 (Sigma) by weight of pure drug and then the ethanol was evaporated off under nitrogen gas at 50°C. THC/CBD was resuspended in 0.9% saline up to a concentration of proportionate to the final dose i.e. for 5mg/kg dose stock solution was made to a concentration of 5mg/ml. PCP (Sigma) was dissolved in 0.9% saline to a concentration of 5mg/ml. After 75 mins animals were euthanized by cervical dislocation followed by decapitation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. The brain was carefully removed and frozen in isopentane (Sigma, UK), kept at -42°C on dry ice. The brain was then coated in embedding matrix (Thermo-Shandon, UK) and stored at -80°C until sectioning. All procedures were carried out in accordance with Home Office regulations.

This technique is extremely sensitive to RNAses, which can confound the experiment due to their differential effects. In order remove RNAses from equipment, all equipment was covered in aluminium foil and heated in a dry oven to 180°C for at least 4 hours, or overnight if possible. All solutions were also made using diethylpyrocarbonate (DEPC)-treated water. This was made adding 1 ml of DEPC (Sigma, UK) in 1000ml of distilled water in an autoclavable bottle and leaving overnight at room temperature. The water was then autoclaved to breakdown DEPC. 20μm thick coronal sections were cut using a cryostat (Leica Microsystem, Germany) maintained at -16°C, sections were taken at level of the prefrontal cortex, striatum and hippocampus at the stereotaxic co-ordinates; 3.72, 1.08 and -3.66 mm from bregma (Figs 2.6, 2.7, 2.8 respectively) respectively according to the stereotaxic atlas (Paxinos and Watson, 2007). These sections were thaw mounted onto poly-L-lysine coated microscope slides, two sections per animal, and allowed to dry at room temperature. Sections were then fixed in 4% depolymerised paraformaldehyde (Sigma, UK) prepared in phosphate buffered saline (PBS) for 5 mins, washed in PBS and dehydrated in a gradient of AR grade ethanol concentrations (70%, 95% and 100%), 5 mins in each concentration. Sections were then stored in 100% ethanol at 4°C.

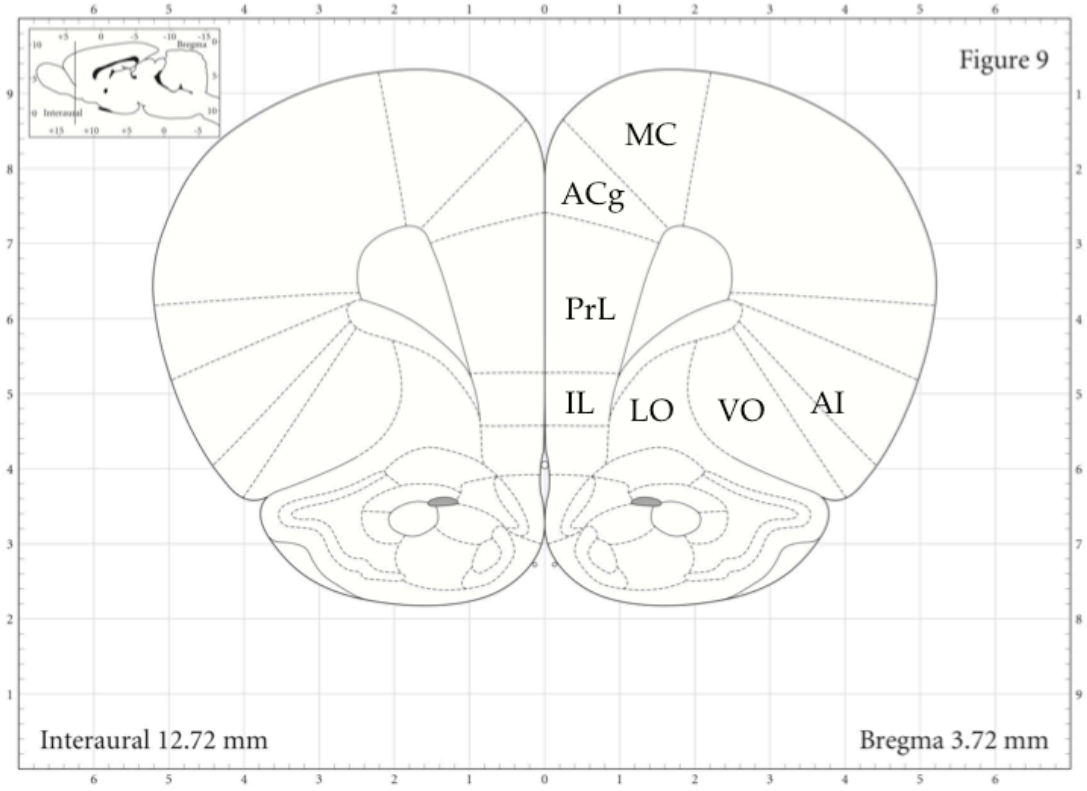


Figure 2.6 Schematic representation of a coronal section at the level of the prefrontal cortex in the rat brain PrL: prelimbic cortex; IL: infralimbic cortex; ACg: anterior cingulate cortex; VO: Ventral Orbital cortex; LO: lateral orbital cortex; MC: motor cortex; AI agranular insular cortex (Paxinos and Watson, 2007).

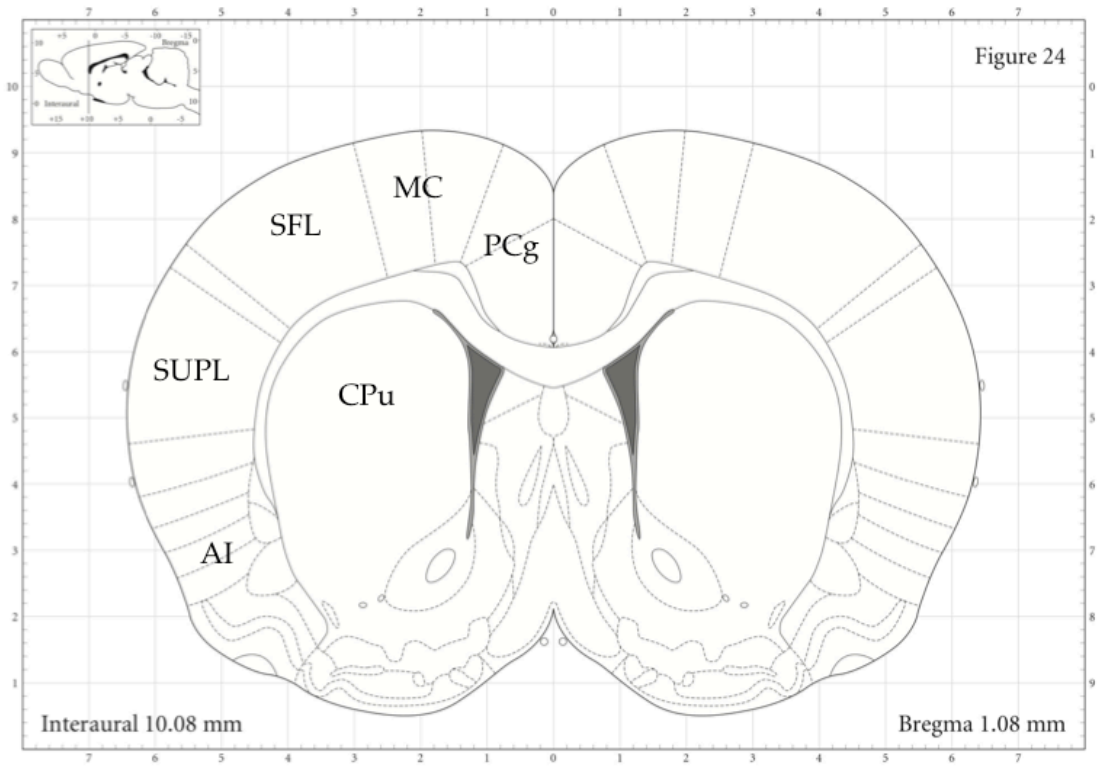


Figure 2.7 Schematic representation of a coronal section at the level of the striatum. CPu: caudate putamen; PCg: posterior cingulate cortex; MC: motor cortex; SFL: sensory cortex (forelimb); SUPL: sensory cortex (upper lip); AI: agranular insular cortex (Paxinos and Watson, 2007).

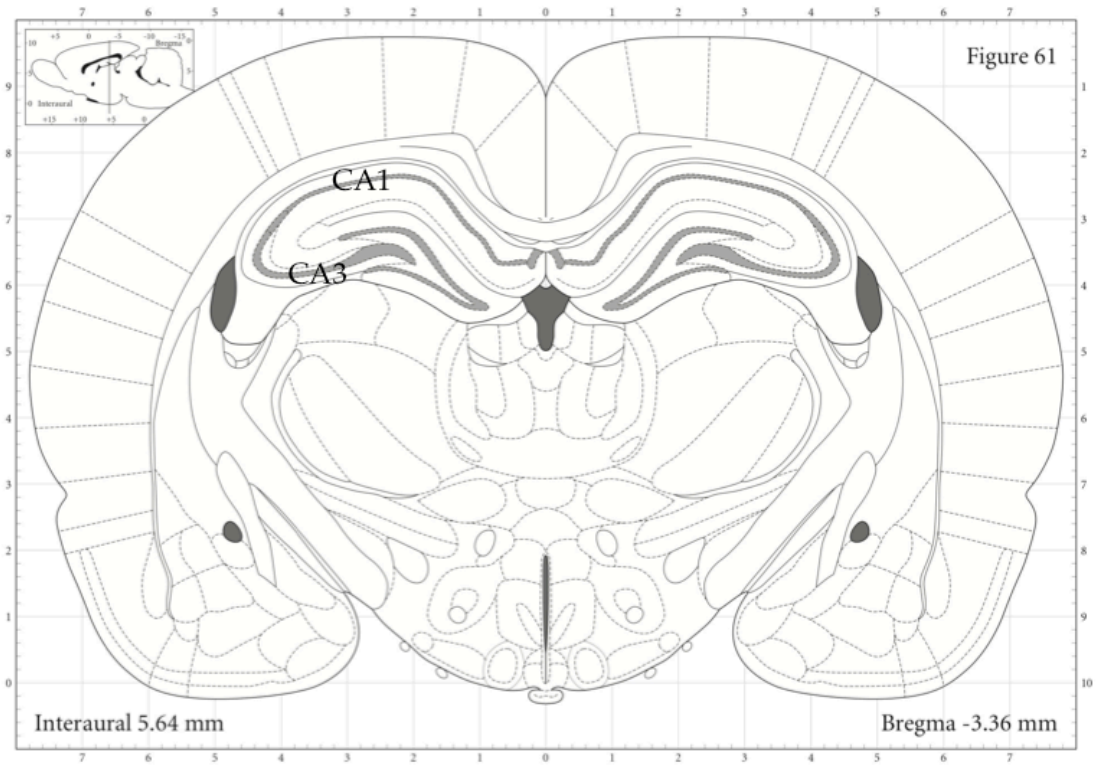


Figure 2.8 Schematic representation of a coronal section at the level of the hippocampus. Annotated to illustrate the CA1 and CA3 region of the hippocampus (Paxinos and Watson, 2007).

An oligonucleotide probe 45 nucleotides in length (45-mer) with a sequence complementary to mRNA encoding the immediate early genes:

- *Zif-268:* CCG TTG CTC AGC ATC ATC TCC TCC AGT TTG GGG TAG TTG TCC (Milbrandt, 1987; Cruachem, UK)
- *Arc:* CTT GGT GCC CAT CCT CAC CTG GCA CCC AAG ACT GGT ATT GCT GAA (Aston *et al*, 2001)
- *c-fos:* CAG GCG GGA GGA TGA CGC CTC GTA GTC CGC GTT GAA ACC CGA GAA (Curran *et al*; 1987; Cruachem, UK)

These were labelled with α[^{33}P]-dATP (PerkinElmer, UK) at the 3'-end of the 45-mer using a terminal deoxynucleotidyl transferase kit (Roche, UK) using a reaction mix containing:

- 1.2μl tailing buffer,
- 2.5μl oligonucleotide probe (5ng/μl)
- 1.5μl α[^{33}P]-dATP
- 0.6μl terminal deoxynucleotidyl transferase (TdT)
- Made up to a volume of 10μl using DEPC-treated water

This was incubated at 37°C for 1 hour to allow for efficient tailing of the 3' end. Adding 60μl of DEPC-treated water terminated the reaction. Probes were purified using QIAquick nucleotide removal kit (Qiagen, UK) as per the manufacturer's instructions. Radiolabeled probe was collected in 100μl of elution buffer and specific labelling was quantified by liquid-scintillation counting. Probes with a specific activity of 2.5-7.5 kBq/μl were used. These probes were stored at -20°C until required.

Sections were removed from storage and allowed to air dry. A hybridisation mix containing 4μl of radioisotope labelled probe and hybridisation buffer, made as follows:

- 10ml 20 x SSC (below)
- 25ml deionised formamide

- 25mM Na PO_4
- 1mM $Na_4P_2O_7$
- 5g dextran sulphate
- 5mg polyadenylic acid
- DEPC-water to 50ml

Approximately 200µl was applied to each section and this was covered with a piece of parafilm creating a uniform layer of hybridisation solution. Slides were placed in large petri dish with a piece of tissue soaked in 4 x SSC, made from a 20-fold stock containing 3M NaCl and 0.3M Sodium Citrate in DEPC-treated water, to prevent dehydration and sealed with parafilm. To test for non-specific hybridisation, test sections were coated with a hybridisation mix containing 200-fold excess of unlabelled oligonucleotide. The slides were incubated overnight at 42°C in an oven. Slides were placed in 1 x SSC where the parafilm was removed from the slides. Slides were transferred into fresh 1 x SSC at 60°C in a shaking water bath for 30 mins. The slides were then passed through a series of washing and dehydrating steps (1 x SSC, 0.1 x SSC, 70% ethanol and 95% ethanol). After air-drying for 30 mins, slides were exposed to Biomax MR-1 X-Ray film (Kodak, UK) for 5-10 days. Films were developed using an X-OMAT (Kodak, UK) automated film developer. Developed X-Ray films were analysed using the MCID (Imaging Research Inc, UK) densitometer software package. Anatomical regions of the brain were identified using a stereotaxic brain atlas (Paxinos and Watson, 2007). The relative optical density (ROD) was recorded for each selected region for both hemispheres by placing 4 – 8 selection squares (8 x 8 pixels) in the region of interest. The average ROD from sections used to test for non-specific hybridisation was subtracted from the average of these readings.

CHAPTER 3: CANNABIDIOL MODULATES SYNAPTIC ACTIVITY IN RAT HIPPOCAMPAL NEURONS

3.1 Introduction

3.1.1 Effects of cannabinoids on neuronal activity

In summary to that discussed previously in this thesis (Chapter 1), effects of cannabinoids in the CNS are mediated primarily via CB_1 receptors. These receptors are located preferentially on presynaptic sites of GABAergic neurons, although there is a suggestion that they localise at glutamatergic terminals, so that their activation by endogenous or exogenous ligands results in suppression of neurotransmitter release. Despite being discovered in 1969, and having known ability to alter levels of anxiety, the effects of CBD on neuronal activity have not been investigated. The effects of cannabinoids on neuronal activity in the nervous system have been well characterised and has been the topic of many reviews (Martin, 1986; Howlett et al., 1990; Ameri, 1999; Irving et al., 2002; Sim-Selley, 2003; Howlett, 2005). In humans and in animals, THC has been shown to alter expression of proteins associated with neuronal activity in several brain regions *in vivo*; this has been discussed in detail later in this thesis (Section 4.1.1)

3.1.2 Effects of cannabinoids on hippocampal dependent memory

THC has long been reported to impair all forms of memory in humans (for review see Ranganathan and D'Souza, 2006), including hippocampal-dependent spatial memory. This is not surprising considering the abundance of CB_1 receptors in the hippocampus (Herkenham et al., 1990; Katona et al., 1999; Katona et al., 2000). Plant-derived, endogenous and synthetic cannabinoids have all been shown to affect performance of rodents in tasks involving spatial working memory, whether the drugs were administered orally or via subcutaneous, intraperitoneal, intravenous or intrahippocampal injection. THC administration has been shown to reduce performance in the radial eight-arm maze in a CB_1 dependent manner (Lichtman et al., 1995; Lichtman and Martin, 1996; Mishima et al., 2001) an effect also imitated by synthetic CB_1 agonists. THC, and its analogues, have also been shown to inhibit performance in the Morris water maze (Mishima et al., 2001; Varvel et al., 2001; Diana et al., 2003; Fadda et al., 2004; Varvel et al., 2005; Tselnicker et al., 2007), and further studies investigating synthetic agonists at CB_1 receptors, including HU210 and WIN 55,212-2, would suggest

CB_1 activation impairs spatial memory (Ferrari et al., 1999; Robinson et al., 2007). Impairment of performance in the water maze task by HU210 was also correlated with a reduction in neuronal activity in the CA1 and CA3 region of the hippocampus (Robinson et al., 2007). However, one group showed that i.p. and intrahippocampal injection of WIN 55,212-2, AM251 (CB_1 inverse agonist) and AM404 (inhibitor of eCB uptake and metabolism) all impaired spatial working memory (Abush and Akirav, 2009), which complicates the issue as inactivation of CB_1 receptor would logically have an opposing effect to its activation.

3.1.3 Cannabinoid modulation of synaptic transmission in primary hippocampal cultures

Several groups of investigators have utilised primary hippocampal cultures to investigate the mechanisms of cannabinoid-induced effects on synaptic transmission. Proposed CB_1 agonists CP 54939 (EC_{50}=0.36nM; EC_{MAX}=100nM), CP 55940 (EC_{50}=1.2nM; EC_{MAX}=10μM), WIN 55,212-2 (EC_{50}=2.7nM; EC_{MAX}=0.1μM), THC (EC_{50}=20nM; EC_{MAX}=10μM) and anandamide (EC_{50}=70nM) were all shown to inhibit glutamatergic transmission in primary hippocampal cultures (Shen et al., 1996; Shen and Thayer, 1999; Sullivan, 1999). This was demonstrated in two studies that showed WIN 55,212-2-induced suppression of EPSC was inhibited by SR 141716 (Shen and Thayer, 1999; Sullivan, 1999). In these studies it was also shown that the maximal level of inhibition induced by THC and CP 55940 were approximately 40% of the maximal level of inhibition induced by the other drugs tested. These data suggested that THC and CP 55940 act as partial agonists at CB_1 receptors (Shen et al., 1996; Shen and Thayer, 1999). However, a recent study has suggested that THC may have a more significant effect on inhibitory currents in hippocampal slices (Laaris et al., 2010).

Irving et al (2000) demonstrated that WIN 55,212-2 (100 nM) also suppressed miniature inhibitory postsynaptic currents (mIPSC) in voltage clamp recordings in primary hippocampal cultures (Irving et al., 2000). This effect

was shown to be CB_1 receptor-mediated, as pre-application with SR 141716 inhibited this suppression (Irving et al., 2000). The use of specialised culture techniques in hippocampal neurons has also been used to demonstrate the electrophysiological phenomenon, DSI. Autaptic neuron culture is a technique in which neurons are cultured in a way in which they form synapses with themselves, allowing the investigator to measure retrograde signalling in neurons. Straiker and Mackie (2009) demonstrated that depolarisation of autaptic hippocampal neurons resulted in a reduction in relative IPSC charge in a CB_1 receptor dependent manner (Straiker and Mackie, 2009). Pharmacological and genetic manipulation of endocannabinoid synthesis and hydrolysis as well as application of exogenous endocannabinoids revealed that although anandamide, 2-AG and noladin ether were all involved in retrograde signalling in these neurons; 2-AG was found to be most fundamental in this form of short term plasticity (Straiker and Mackie, 2009). As well as affecting synaptic function it has also been demonstrated that THC can be toxic in hippocampal (Chan et al., 1998) and cortical cultures (Campbell, 2001), which may be a cause or a consequence of its effect on synaptic activity.

3.1.4 Effects of cannabinoids in intra- or extracellular recordings from acute hippocampal slices

Investigation into the effects of cannabinoids on transmission in acute hippocampal slices provides a more physiological insight into the effects of cannabinoids in the CNS. In acute hippocampal slices CB_1 receptor activation by WIN 55,212-2 and the endogenous cannabinoids AEA and 2-AG has been shown to inhibit both excitatory and inhibitory transmission in the CA1 region of the hippocampus (Ameri et al., 1999; Ameri and Simmet, 2000; Hajos et al., 2000; Hajos et al., 2001; Maejima et al., 2001; Hajos and Freund, 2002; Takahashi and Castillo, 2006; Bajo et al., 2009).

Several studies have shown that WIN 55,212-2 inhibits electrically evoked neurotransmission in the stratum radiatum of the CA1 region of hippocampal slices. Ameri et al (1999) demonstrated that WIN 55,212-2 (1 μM) significantly reduced evoked postsynaptic population spikes in

hippocampal slices an effect, which was blocked by pretreatment with CB_1 antagonist, SR 141716 (1 μM). Exposure of hippocampal slices to WIN 55,212-2 was also shown to reduce IPSCs, an effect which was not present in slices treated with CB_1 antagonists or slices from CB_1 knockout ($CB_1^{-/-}$) mice (Hajos et al., 2000; Hajos et al., 2001; Hajos and Freund, 2002). Both Hajos et al (2001) and Takahashi and Castillo (2006) also demonstrated that WIN 55,212-2 reduced excitatory synaptic transmission in intra- and extracellular recordings respectively. However, Hajos et al (2001) discovered that WIN 55,212-2-induced suppression of excitatory postsynaptic currents (EPSC) was maintained in slices taken from $CB_1^{-/-}$ mice, suggesting that suppression of excitatory transmission was not mediated via a CB_1-dependent mechanism (Hajos et al., 2001). This was also validated pharmacologically, where it was shown that the CB_1 antagonist, AM251, blocked IPSC, but not EPSC (Hajos and Freund, 2002). These findings are hardly surprising considering immunohistochemical evidence that would suggest that CB_1 receptors are selectively expressed on GABAergic presynaptic terminals (Katona et al., 1999). Interestingly, in recent studies WIN 55,212-2-induced suppression of EPSC and evoked excitatory postsynaptic potentials (EPSP) was not maintained in slices from $CB_1^{-/-}$ mice (Kawamura et al., 2006; Takahashi and Castillo, 2006). One possible explanation for the differences shown may be the age of animals used, but recent studies have shown that age had no effect on the ability of WIN 55,212-2 to inhibit EPSC in hippocampal slices (Kawamura et al., 2006; Kang-Park et al., 2007).

The effects of exogenous application of endocannabinoids have also been investigated in hippocampal slices. The arachidonic acid derivative, anandamide (1 and 10 μM), was shown to inhibit fEPSP in hippocampal slices (Ameri et al., 1999). As well as this effect being blocked by SR 141716, anandamide also enhanced paired-pulse facilitation (PPF) suggesting anandamide at these concentrations was acting via a presynaptic CB_1 receptor (Ameri et al., 1999). It was later shown by the same group that another endocannabinoid, 2-arachidonylglycerol (2-AG), also inhibited fEPSP while enhancing PPF in hippocampal slices, SR 141716 also blocked this effect. Depolarisation-induced suppression of inhibitory transmission, a

phenomenon known as DSI, has also been demonstrated in hippocampal slices. This form of retrograde signalling was first demonstrated in the cerebellum. However, Pitler and Alger (1992) confirmed that this was also present in pyramidal cells in the CA1 region of the hippocampus. They demonstrated that depolarisation of the postsynaptic cell reduced IPSC amplitude (Pitler and Alger, 1992). It was later discovered that endocannabinoids were key in this action, as described above.

A less clear picture exists for the effects of the phytocannabinoid, THC, on synaptic transmission in hippocampal slices, where it has been shown to have a biphasic effect on synaptic transmission in acute hippocampal slices (Foy et al., 1982; Chiaia et al., 1983; Kujtan et al., 1983). THC was found to increase field potential amplitude between 10pM and 0.1μM and reduced responses at a concentration of 1μM, which may suggest a dual site of action for THC.

3.1.5 Potential for CBD to alter neuronal activity

CBD has a low affinity for CB_1 receptors and has been shown to act as a CB_1 receptor antagonist and a CB_2 receptor inverse agonist (Thomas et al., 2007). Jones et al (2009) demonstrated that CBD was able to reduce epileptiform activity in hippocampal slices. In this study investigators induced epileptiform activity by either removing Mg^{2+} from the aCSF or by addition of 4-aminopyridine (4-AP). CBD was shown to both reduce burst amplitude and frequency (Jones et al., 2009). It was also shown in this study that CBD displayed low levels of affinity for CB_1 receptors, leading to the suggestion that CBD was acting through an alternative mechanism. However, taking into consideration the ability for CBD to inhibit the uptake and degradation of endogenous cannabinoids, such as anandamide, one may hypothesise an indirect route for CB_1-induced alteration of neuronal activity. A recent study has also suggested that CBD administration may have an effect on basal neuronal activity in the human hippocampus. Using functional magnetic resonance imaging (fMRI), Bhattacharyya et al (2009) demonstrated that administration of CBD in humans resulted in a reduction in regional brain

activity in several regions of the brain including the hippocampus (Bhattacharyya et al., 2009b). These data suggest that CBD may possess an ability to alter basal neuronal activity in this region.

CBD has been shown to bind and activate 5-HT_{1A} receptors (Russo et al., 2005) and this has been shown to mediate the anxiolytic and anti-ischaemic properties of CBD (Campos and Guimaraes, 2008; Resstel et al., 2009). Considering that activation of 5-HT_{1A} receptors by 8-OH-DPAT, which activates similar signal cascades to as CB_1 receptors, has been shown to reduce excitatory and inhibitory synaptic transmission in hippocampal slices (Schmitz et al., 1995a; Schmitz et al., 1995b; Schmitz et al., 1998), it is reasonable to conclude that should CBD act at 5-HT_{1A} receptors, CBD should induce reductions in synaptic transmission in hippocampal slices. CBD also acts either as an agonist or antagonist at TRPV1 channels (Bisogno et al., 2001), which were shown to be involved in CBD-induced reversal of MK-801-induced disruption of PPI (Long et al., 2006). However the extent to which TPRV1 receptor activation influences neuronal activity in the hippocampus has not been fully explored.

The effect of CBD on intracellular calcium may also be an indicator of potential to alter synaptic activity. Calcium is required for the release of neurotransmitters in the synapse (Hutter and Kostial, 1954), so any alteration in calcium may alter the synaptic activity of a cell. CBD has been shown to inhibit T-type voltage-gated calcium channels (Ross et al., 2008). This is suggestive that THC either directly or indirectly alters the excitability of neurons, so therefore could potentially inhibit neurotransmitter release. However, CBD has also been shown to increase intracellular Ca^{2+} levels in cultured hippocampal neurons, which was shown to be through initiating its release from intracellular stores (Drysdale et al., 2006; Ryan et al., 2009). The latter set of experiments may suggest that CBD may enhance neuronal activity in a hippocampal culture preparation.

In summary, despite the mounting evidence that suggests that CBD may possess the ability to alter synaptic transmission in hippocampal neurons, the

ability for CBD to alter basal transmission has not been investigated. It is therefore the aim of this study to:

1. Evaluate the potential for CBD to alter neuronal activity in primary hippocampal cultures and acute hippocampal slices.
2. Determine the potential mechanism underlying any effect.

3.2 Methods

3.2.1 Primary hippocampal culture electrophysiology

3.2.1.1 Culture preparation

As described in the previous chapter (Section 2.1), hippocampi were dissected from Sprague Dawley 1-2 day old neonates and cultured in Neurobasal-A medium (Invitrogen) containing 2% B-27 (Invitrogen) and 1% L-glutamine (Invitrogen). Dishes were incubated at 37°C and 5% CO_2 for 5 days. 10μM of cytosine-β-D-arabinofuranoside (Ara-C; Sigma) was added and cells were returned to incubator until required (12 – 16 days *in vitro)*.

3.2.1.2 Current-clamp electrophysiology

Fire-polished micropipettes made using borosilicate glass (4-6 MΩ) and filled with an internal solution containing as described in the methods section. Cells were perfused at a rate of 2ml/min with an external solution as described previously containing DMSO (1:10000); this was added to the external solution to counterbalance any potential effects of this solvent on neuronal activity. Current clamp recordings were acquired using an Axopatch-200B amplifier (Axon Instruments) and using WinEDR v 2.7.9 software (J.Dempster, University of Strathclyde). After whole–cell patch clamp was achieved, cells were allowed to equilibrate for at least 5 mins and only cells that maintained a resting membrane potential between -55 and -65 mV were used.

3.2.1.3 Drugs and experimental design

CBD, WIN 55,212-2, THC and AM251 were dissolved in DMSO so that the final bath concentration would contain 1 part DMSO in 10,000. WAY 100135, DL-AP5 and NBQX were dissolved in deionised water. CBD (0.1, 1 and 10 μM), WIN 55,212-2 (0.1 μM) or THC (10 μM) was added for 5 mins following at least a 5 min steady baseline. Cells were then perfused with fresh external solution to allow for wash out. In experiments using pertussis toxin (PTX), cells were incubated for 16-24 hours in a culture medium containing 200ng/ml of PTX. For experiments investigating potential receptor targets for CBD, AM251 (30nM) or WAY 100135 (30nM) was applied 1 minute prior and during CBD application. To affirm that spontaneous activity in these

cultures were synaptically driven, cells were treated with NMDA and AMPA receptor antagonists, DL-AP5 (100μM) and NBQX (20μM) respectively, for 5 minutes.

3.1.2.4 Data analysis and statistics

Data were analysed using WinEDR v 2.7.9 (J.Dempster, University of Strathclyde) and events were detected at a threshold of -30 to -45 mV with an inter-event interval of 100ms for every 60 seconds of recording. As the action potential (AP) frequency ranged between 13 and 398 events per minute these data were normalised to baseline recordings and % of inhibition was calculated. A Student's t-test was performed comparing action potential (AP) frequency, that has been normalised to the mean resting frequency, over 60 seconds following drug treatment with that of the frequency over 60 seconds immediately prior to drug application. A one-way ANOVA was performed to analyse the difference between the effects of CBD in the presence of AM251 or following pretreatment with PTX.

3.2.2 Acute slice electrophysiology

3.2.2.1 Slice preparation

Whole brains were removed from 16 to 20 day old Sprague-Dawley rats and placed immediately in an ice-cold, oxygenated cutting solution as described previously (Section 2.2). Using a vibratome, 400μm saggital slices were cut and place into oxygenated aCSF containing the same as above replacing the sucrose with 124mM NaCl. Areas from the frontal region, ie from the fornix forward, as well as the mid-brain were removed from the slice. These slices were then placed on a meshed grid, submerged in oxygenated aCSF for 45 minutes to allow slices to equilibrate to room temperature.

3.2.2.2 Acute slice recordings

Slices were perfused with oxygenated aCSF and bipolar stimulating electrode and a borosilicate glass recording electrode filled with 4M NaCl were placed in the Schaffer-Collateral pathway in the Striatum Radiatum

region of the CA1 (Fig 3.1). Slices were stimulated with a 0.05Hz pulse, the stimulation amplitude was adjusted to result in a 50% maximal response of the first pulse. After a 20 min stable baseline was achieved, drugs were added for 10 mins, this was immediately followed by a 20 minute wash out period. In control experiments DMSO (0.001%) was added to the perfusate and washed out prior to drug application, RS-baclofen (100μM; Sigma, UK), a $GABA_B$ receptor agonist was added for 10 minutes at the end of the experiment to ensure slice/system was responsive to drug treatment. WAY 100135 (300nM) and AM251 (2µM) were added to the aCSF 20 and 30 minutes prior to CBD application.

3.2.2.3 Data analysis and statistics

All data were acquired using an Axopatch-2B amplifier (Axon Instruments) and using WinWCP v 2.7.9 software (J.Dempster, University of Strathclyde). fEPSP slope and paired-pulse ration were calculated as described in section 2.2.3. Data were analysed using a Student's t-test comparing fEPSP slope and PP ratio prior to and following drug administration.

3.3 Results

3.3.1 Effect of cannabidiol and other cannabinoids on spontaneous neuronal activity in cultured hippocampal neurons.

Application of CBD (1 and 10μM) significantly reduced the frequency of spontaneously firing action potentials (AP) in dissociated hippocampal cultures after 5 minutes of drug treatment (10μM, 100%, n=4; 1μM, 87.7 ± 7.8%, n=5, P<0.001; Figure 3.1) compared to the control frequency measured over 5 minutes prior to drug treatment. Application with 0.1μM of CBD resulted in no significant change in AP frequency (18.54 ± 23.98%, n=4, n.s.; Fig 3.1). Application with CBD did not result in a significant change in resting membrane potential (1μM, 1.5 ± 1.6mV, n=5, P>0.05). Similar reductions in spontaneous AP frequency were observed following the application of the CB_1 agonist, WIN 55,212-2 (0.1μM, 53.3 ± 12.8%, n=5, P<0.01, Figure 3.2) and the CB_1 partial agonist, THC (10μM, 45.9 ± 10.9%, n=5, P<0.01, Figure 3.3) as has been shown previously (Shen and Thayer, 1998; Shen and Thayer, 1999; Roloff and Thayer, 2009). To confirm that the spontaneous activities in these cultures were synaptically driven, cells were perfused with the ionotropic glutamate receptor antagonists, DL-AP5 (100μM) and NBQX (20μM). A 5 minute application of DL-AP5 and NBQX inhibited AP firing suggesting that neuronal activity in these cultures were synaptically driven (Figure 3.8). In order to counterbalance any possible vehicle effects, DMSO (1:10000) was present in the extracellular solution throughout these experiments.

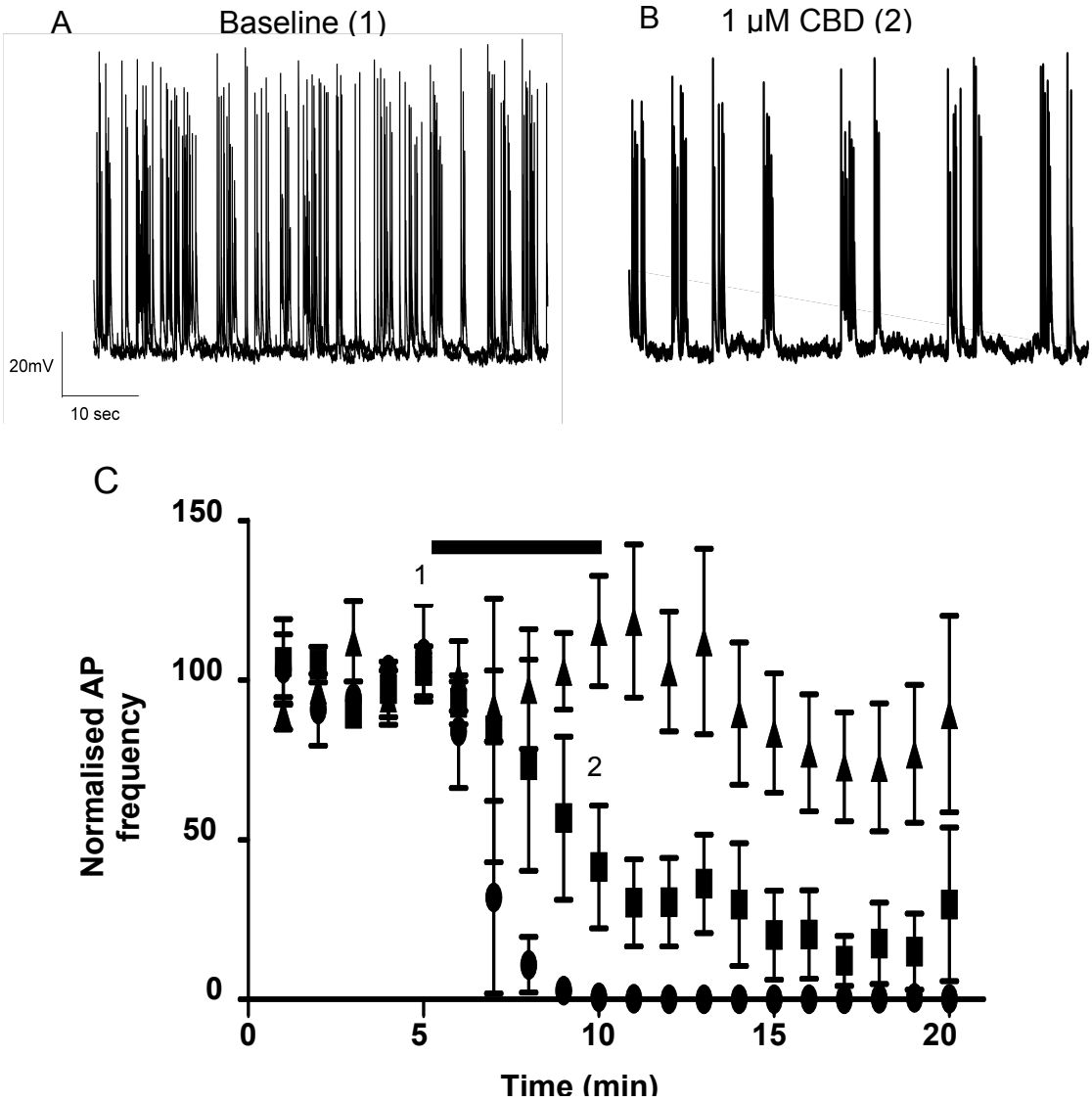


Figure 3.1 Application of CBD concentration-dependently inhibits AP firing rate in cultured hippocampal neurons. A and B are example traces of activity recorded from a hippocampal cell for 60 seconds during the baseline recording (A) and following a 5 minute application of CBD (1µM; B). C summarises the time course effect of CBD (0.1µM, triangles, n=5; 1µM, squares, n=5; 10µM, circles, n=4) on AP frequency. Data shown as normalised AP frequency per 60 seconds ± S.E.M.

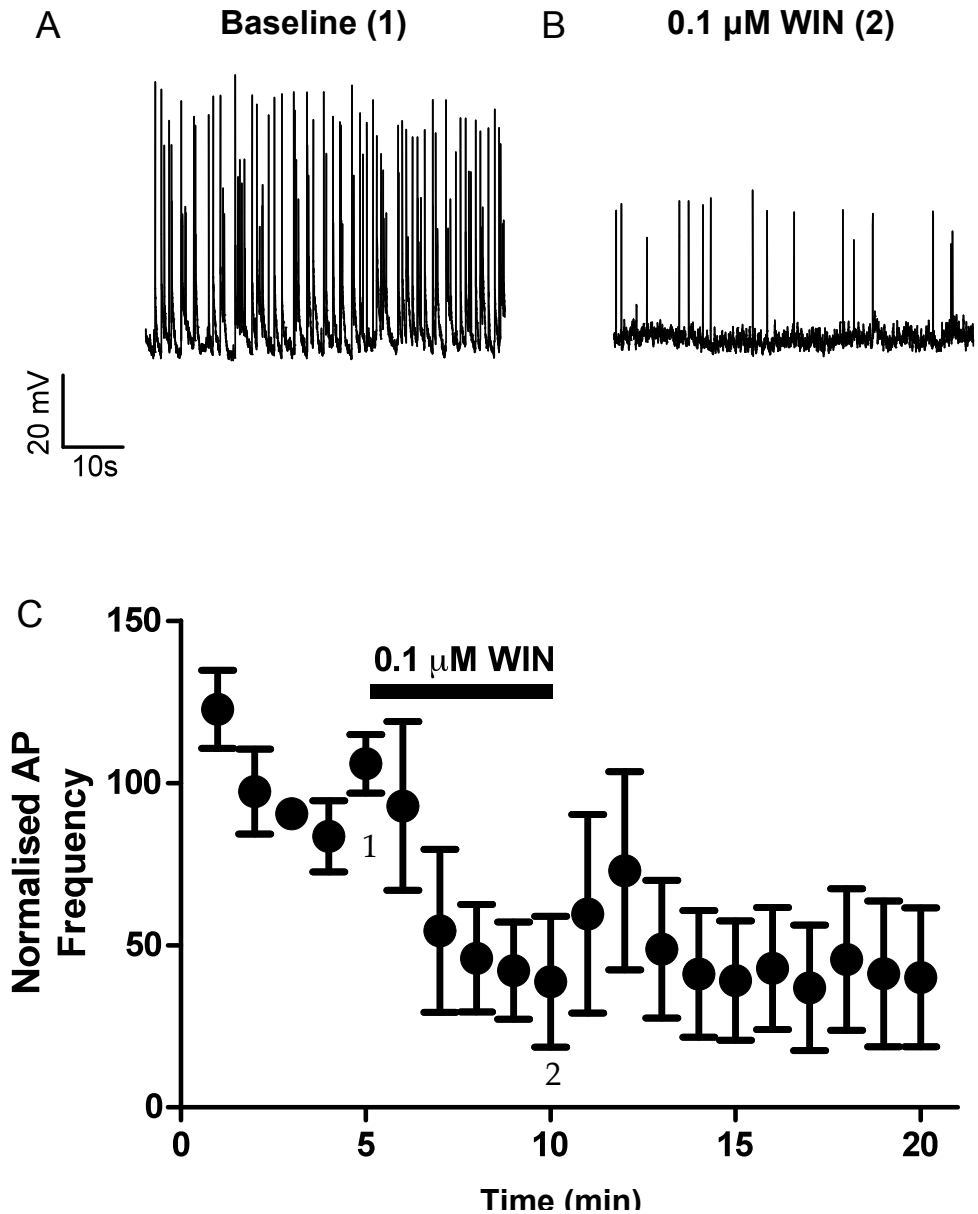


Figure 3.2 Application of a selective CB_1 agonist, WIN 55,212-2, reduces AP frequency in hippocampal cultures. A and B are example traces of activity recorded from a hippocampal cell for 60 seconds during the baseline recording (A) and following a 5 minute application of WIN 55,212-2 (0.1 µM; B). C summarises the time course effect of WIN 55,212-2 (0.1 µM, n=5)) on AP frequency. Data shown as normalised AP frequency per 60 seconds ± S.E.M.

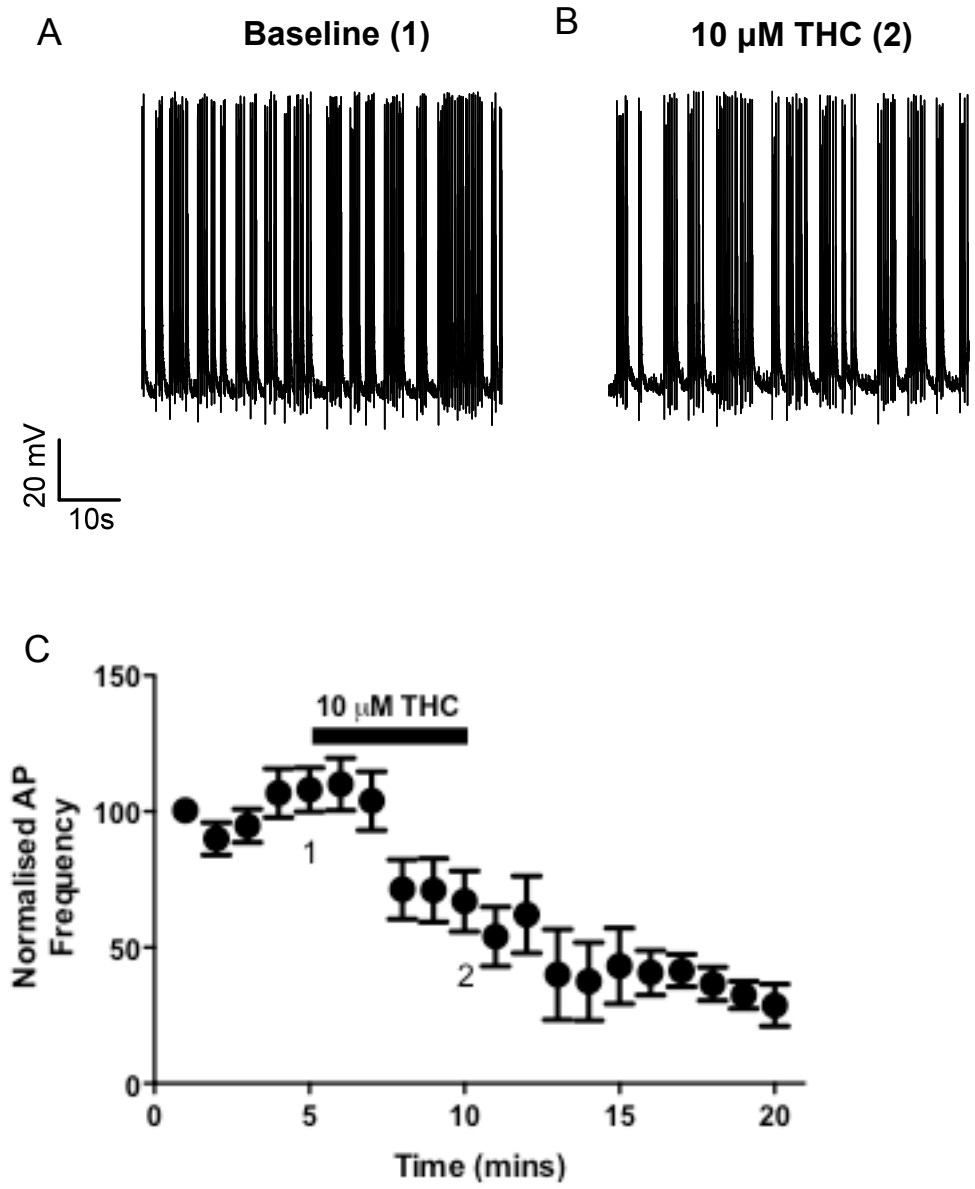


Figure 3.3 Application of THC reduces AP frequency in hippocampal cultures. A and B are example traces of activity recorded from a hippocampal cell for 60 seconds during the baseline recording (A) and following a 5 minute application of THC (10 µM; B). C summarises the time course effect of THC (10 µM, n=5) on AP frequency. Data shown as normalised AP frequency per 60 seconds ± S.E.M.

One-way ANOVA with a Dunnett's *post hoc* comparison revealed that pretreatment of cultures with PTX significant reduced the level of inhibition following CBD administration from 67% ± (CBD 1μM) to 19% ± (CBD 1μM + PTX), PTX treatment had no effect on basal AP firing frequency compared to that of naïve cells (PTX: 152.0 ± 28.9 vs. Naïve: 180.3 ± 42.6, $p>0.05$). PTX uncouples $G_{i/o}$ protein from the transmembrane receptor, thus deactivating this class of GPCR. In order to examine whether the CBD-induced effects involved CB_1 receptor activation, either directly or indirectly, a CB_1 receptor antagonist (AM251) was applied for 1-minute prior and during application with CBD. CBD (1μM) application resulted in a significant suppression of AP firing (55.5 ± 12.8%, n=4, $P<0.01$, Fig 3.5) in cells treated with AM251 (30nM) and there was no significant difference in the effect of CBD suggesting that the effects of CBD were not mediated via a CB_1 receptor dependent mechanism. A 1 minute application of AM251 had no significant effect on basal AP frequency compared to untreated cells (Untreated: 155.4 ± 46.30 vs. AM251: 149.2 ± 32.39, $p>0.05$). However, application of AM251 (30nM) on its own for longer than 1 minute resulted in a dramatic increase in AP firing rate that resulted in the depolarisation of the membrane potential to a level where events (AP) could not be detected (Figure 3.7). Therefore the ability of AM251 to block the effects of other cannabinoids could not be determined. This depolarisation was also present in cells treated with WAY 100135 (30nM), this meant that the possibility that the CBD-induced effects were mediated via 5-HT_{1A} receptors could not be explored in this preparation.

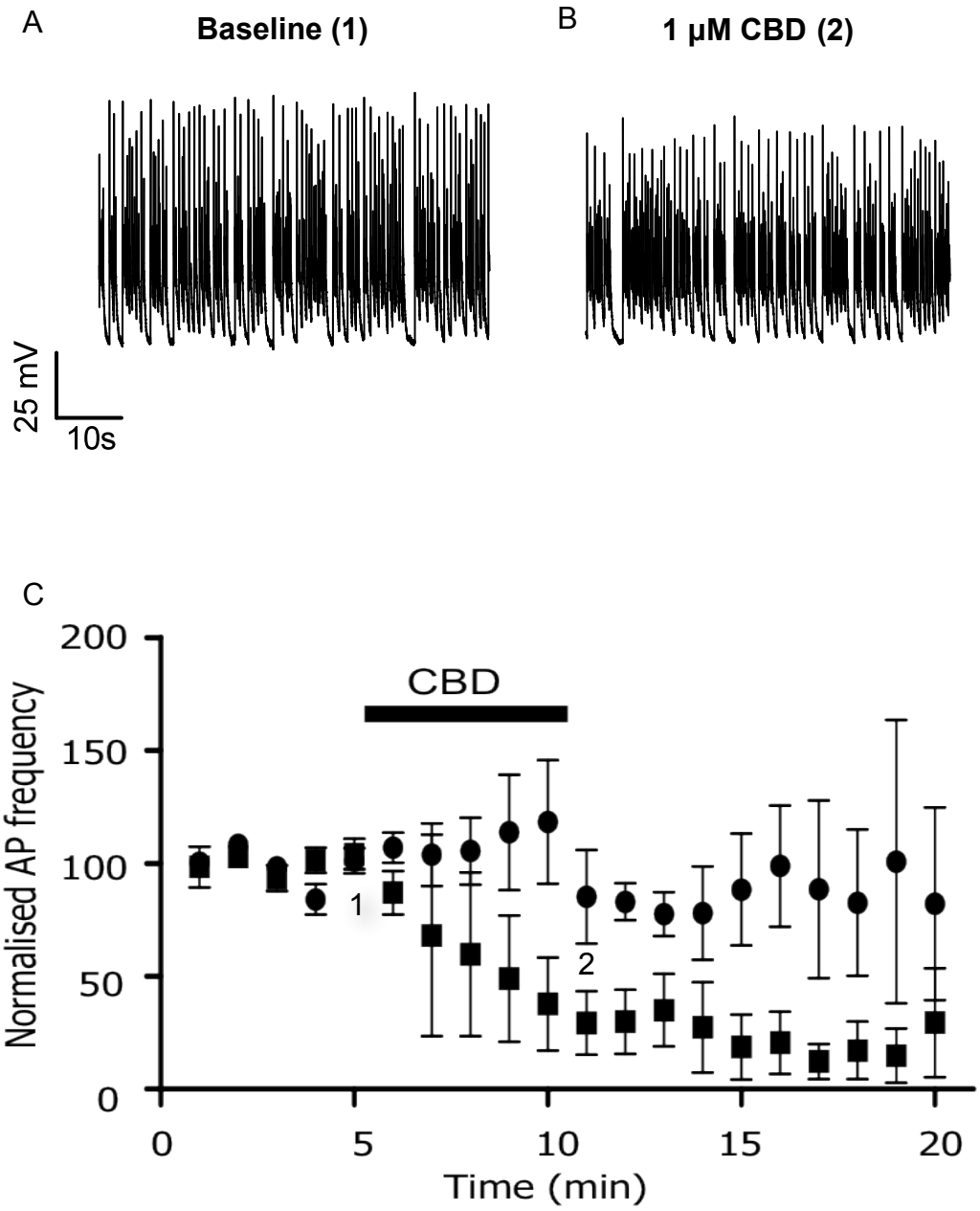


Figure 3.4 Incubation of hippocampal cultures with PTX blocks CBD-induced reductions in AP frequency. A, B and C are example traces of activity recorded from a hippocampal cell for 60 seconds during the baseline recording (A) and following a 5 minute application of CBD (1 µM; B). C summarises the time course effect of CBD (1 µM) on AP frequency in the cells treated with (circles, n=4) or without (squares, n=5) PTX overnight. Data shown as normalised AP frequency per 60 seconds ± S.E.M.

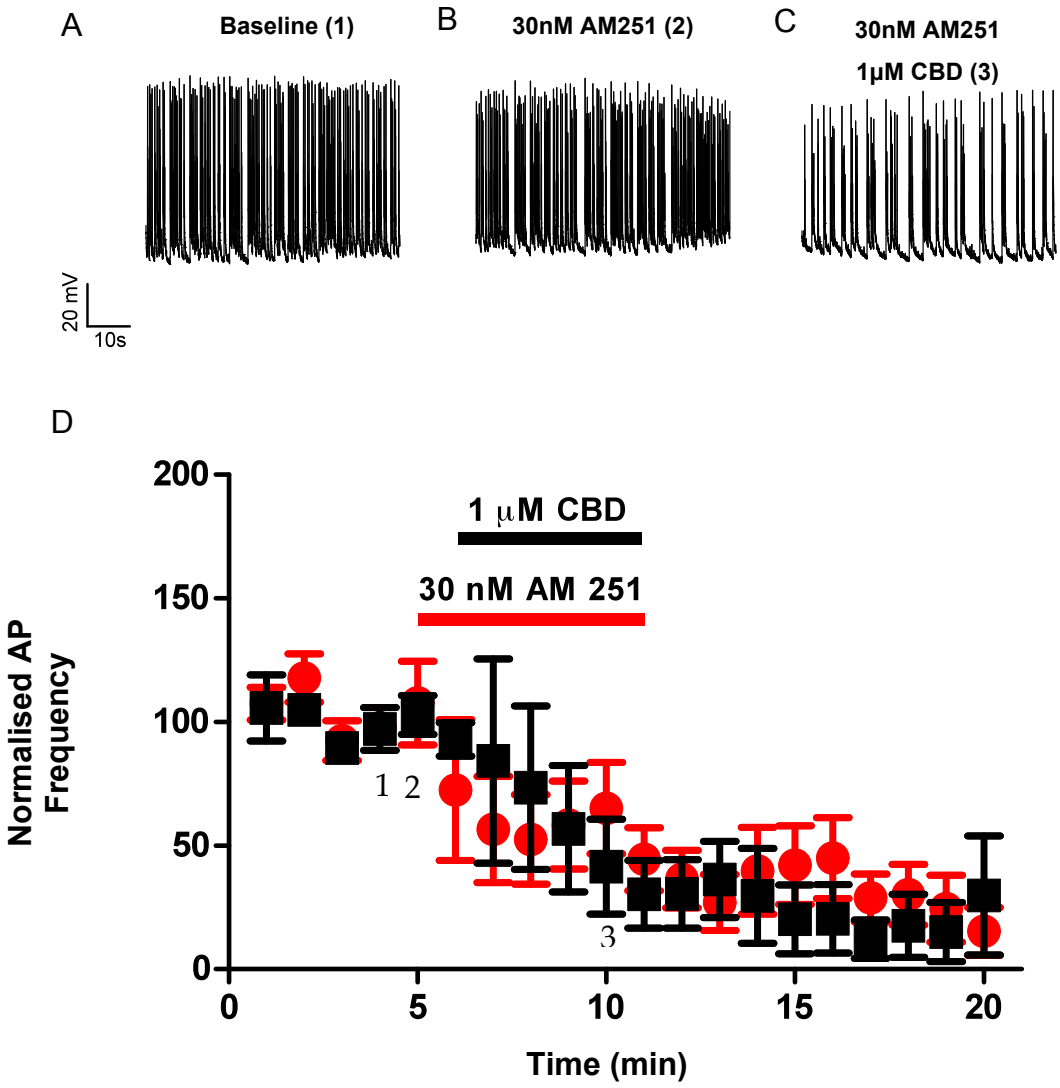


Figure 3.5 Pre-application of AM251 does not block CBD-induced reductions in AP frequency in hippocampal cultures. A, B and C are example traces of activity recorded from a hippocampal cell for 60 seconds during the baseline recording (A), 60 seconds of pretreatment with AM251 (30nM; B) and following a 5 minute application of CBD (1 μM) in the presence of AM251 (30nM; C). D summarises the time course effect of CBD (1 μM) on AP frequency in the presence (Red) or absence (Black) of AM251. Data shown as AP frequency per 60 seconds normalised to 5 mins prior to drug application ± S.E.M.

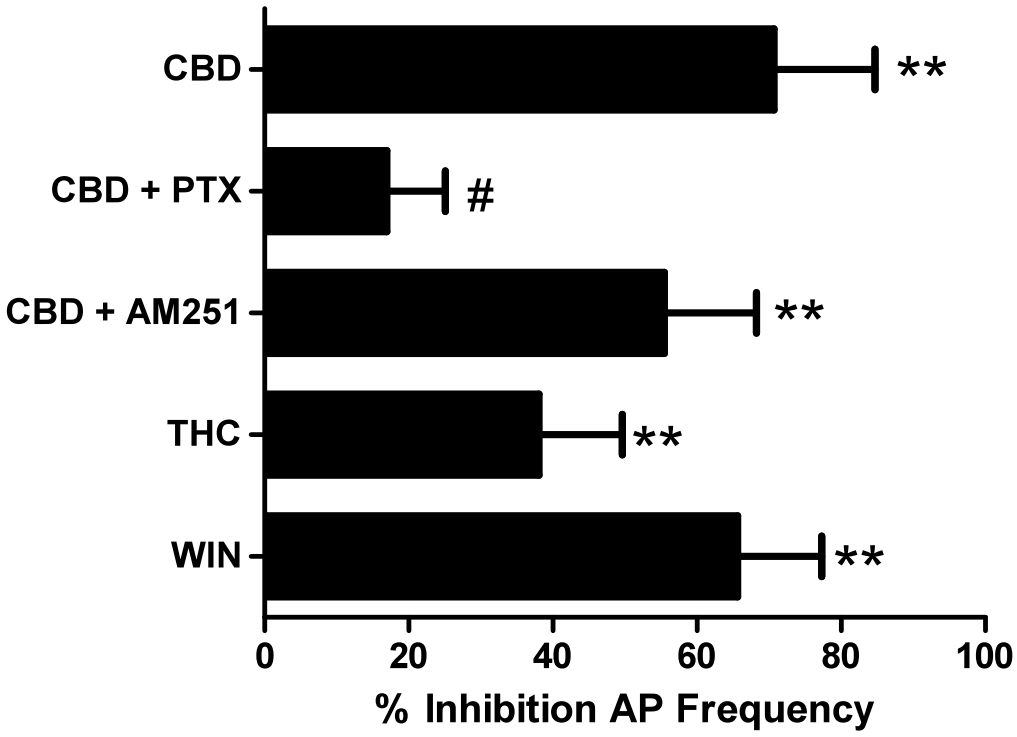


Figure 3.6 Summary of the data showing the levels of inhibition induced by drug treatments. Summary of the effects of the above treatments on AP frequency in hippocampal cultures. ** denotes $p<0.01$ vs respective baseline by t test; # denotes $p<0.05$ vs CBD (1 μM) by one-way ANOVA with a dunnett's *post hoc* analysis.

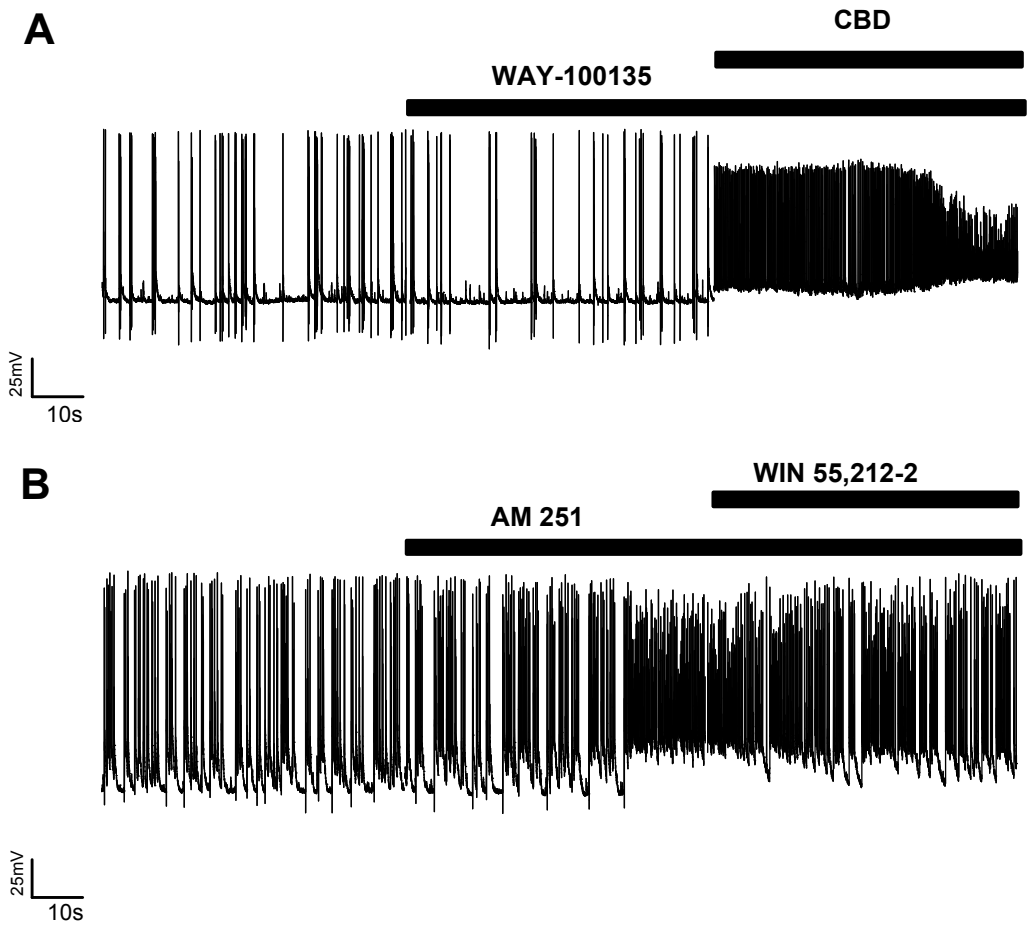


Figure 3.7 Application of both WAY 100135 and AM251 resulted in depolarisation of the resting potential. Sample traces over a 3-minute period illustrating the depolarisation effect of WAY 100135 (A; 30nM) and AM251 (B; 30nM)

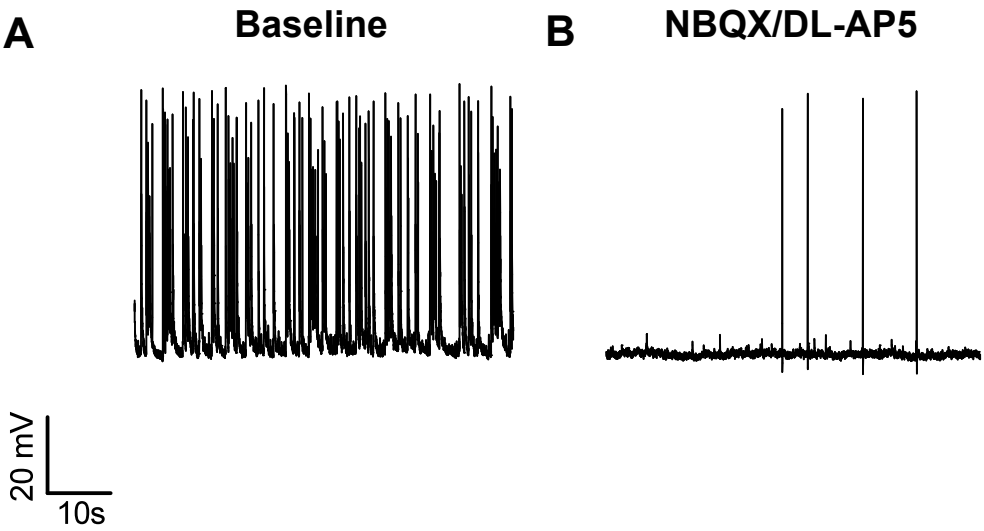


Figure 3.8 Spontaneous network activities in hippocampal cultures are synaptically driven. Representative sample traces from cells before (A) and after (B) a 5-minute co-application with NBQX and DL-AP5.

3.3.2 Effect of cannabidiol on evoked field excitatory postsynaptic potentials acute hippocampal slices

Having established the ability of CBD application to reduce synaptically driven network activity in hippocampal cultures, it was then investigated whether CBD modulates synaptic transmission in acute hippocampal slices. A 10-minute application of CBD (10μM) inhibited fEPSPs (37.0 ± 14.8%, n=6, $P<0.05$, Fig 3.9 B). Interestingly, this effect was reversed in these slices through washout with aCSF. The CBD-induced inhibitions were associated with a significant increase in paired-pulse facilitation (34.5 ± 11.5, n=6, $P<0.01$, Fig 3.9 C). This suggests that CBD is acting through a presynaptic receptor. Hence, we utilised antagonists against GPCRs that have been proposed to potentially underlie the actions of CBD. The selective 5-HT_{1A} receptor antagonist, WAY 100135 (300nM), abolished the CBD-induced inhibition of synaptic transmission (13.0 ± 8.1%, n=4, $P<0.01$ vs. CBD, Fig 3.4). Interestingly, the CB_1 receptor antagonist, AM251 (2μM), also blocked the CBD-induced inhibition of synaptic transmission (9.3 ± 7.0%, n=8, $P<0.01$ vs. CBD, fig 3.4). However, AM251 also result in a significant decrease in fEPSP amplitude on its own (18.1 ± 6.6%, n=5, $P<0.05$). In order to validate the response to drugs, slices were exposed to a negative and positive control, DMSO (0.0001% v/v) and RS-baclofen (100 μM) respectively. DMSO administration resulted in no observable change in EPSP rate of rise, where as RS-baclofen completely ameliorated any response (Fig 3.13). The ability of RS-baclofen to inhibit the evoked response validates that the slice and the recording/perfusion system was able to adequately detect a drug-induced effect.

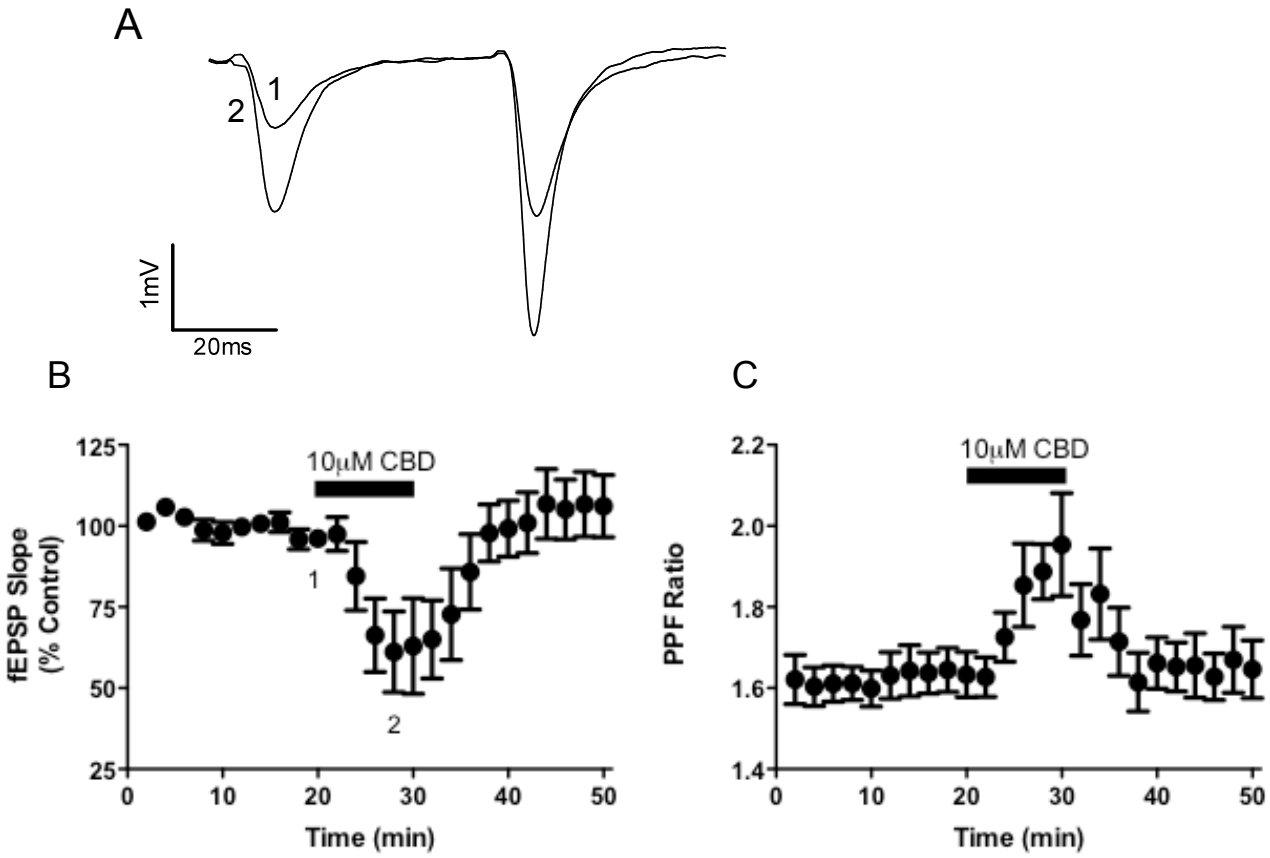


Figure 3.9 CBD inhibits evoked postsynaptic field potentials (EPSP) in acute hippocampal slices through a presynaptic mechanism. A is an example trace of a response to a paired-pulse stimulation prior to drug treatment (1) and following a 10 minute application of CBD (10 μM; 2). B represents the time course effect of the above CBD application on EPSP slope, shown as % of baseline (0-20 mins) ± S.E.M. (n=6), black bar indicates CBD application. C illustrates the time course effect of CBD on paired-pulse facilitation ratio.

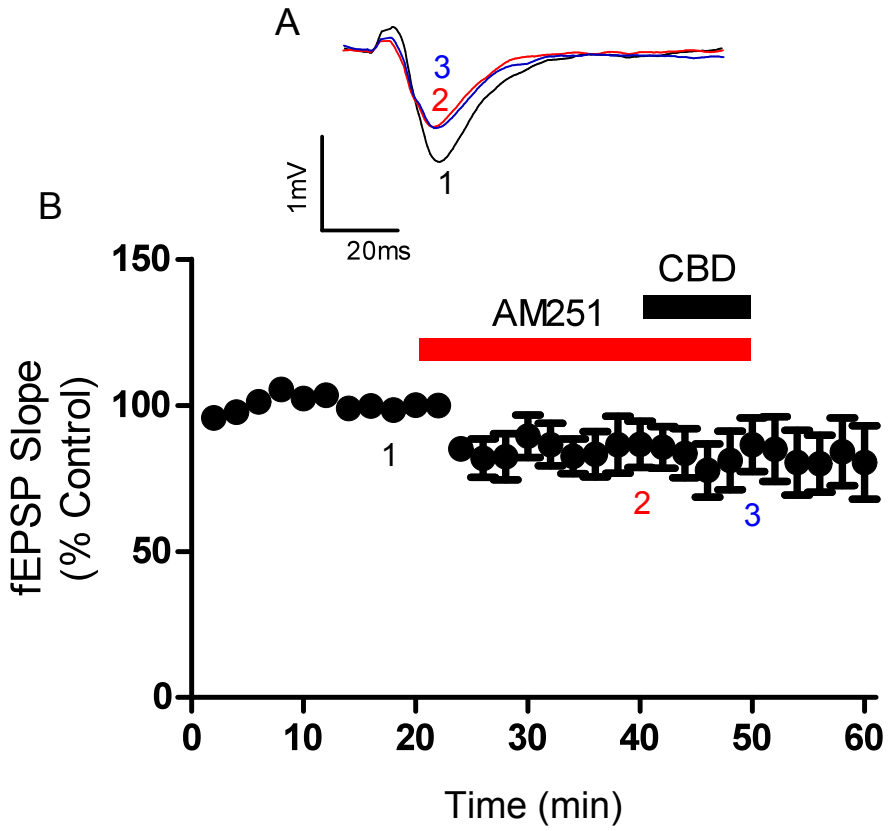
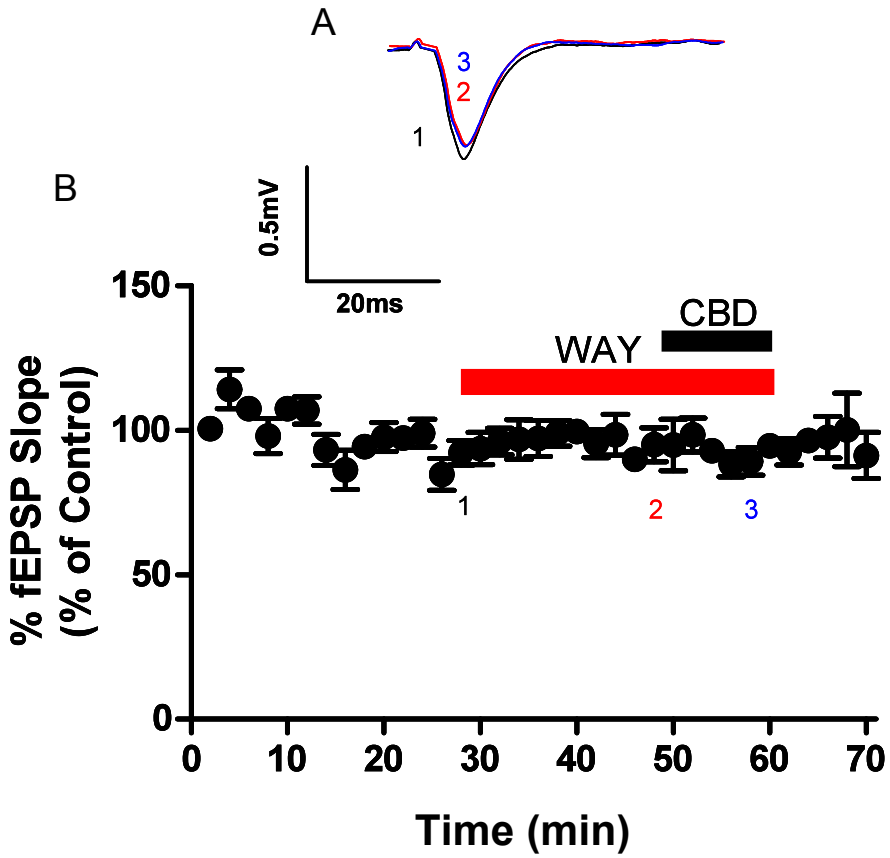


Figure 3.10 AM251 (2µM) blocks CBD-induced inhibition of evoked fEPSP. Representative time course (B) and corresponding evoked fEPSP traces from hippocampal slices (A; Baseline, Black; AM251, Red; CBD, Blue). Data presented as EPSP slope, shown as % of baseline (0-20 mins) ± S.E.M. (n=8).



3.11 CBD-induced inhibition of evoked fEPSP is blocked by 5-HT$_{1A}$ antagonist WAY 100135 (300nM). Representative time course (B) and corresponding evoked fEPSP traces from hippocampal slices (A; Baseline, Black; WAY 100135, Red; CBD, Blue). Data presented as EPSP slope, shown as % of baseline (0-20 mins) ± S.E.M. (n=4).

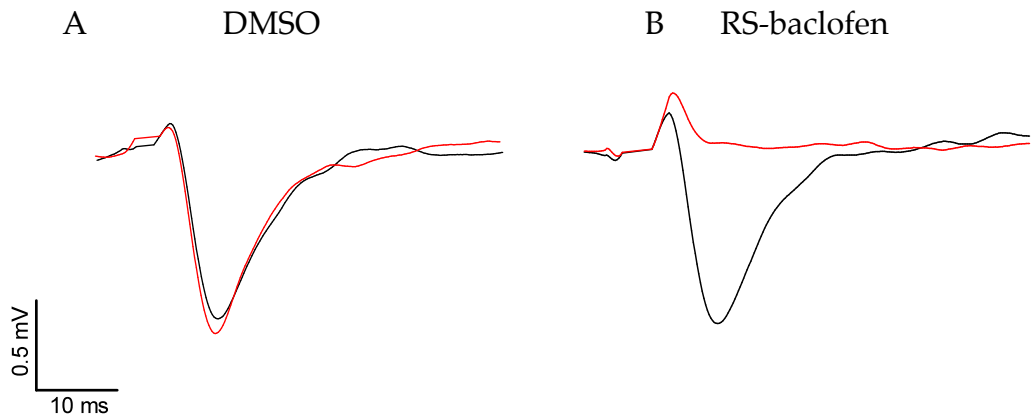


Figure 3.12 Electrically evoked fEPSP responses are not sensitive to the solvent (DMSO), but are abolished through application of a $GABA_B$ agonist (RS-baclofen). A and B are sample fEPSP responses at baseline (Black line) or in the presence of DMSO (1:10000; A; Red line) or RS-baclofen (100μM; B; Redline).

3.4 Discussion

These data show for the first time that CBD inhibits synaptic activity in both hippocampal cultures and acute slices. Although most cannabinoids (including synthetic, eCBs and phytocannabinoids) have been shown to alter synaptic activity, working via the CB_1 receptor, it has yet to be shown whether CBD shares this effect despite evidence to suggest CBD activates receptors known to modify a multitude of signalling mechanisms.

3.4.1 CBD modulates synaptic activity in cultured hippocampal neurons in a PTX sensitive manner

It has previous been shown that CBD application (1 and 10μM) resulted in an increase in intracellular Ca^{2+} in hippocampal neuronal cultures, through the activation of either a $G_{q/11}$ GPCR or through activating Ca^{2+} channels directly, an effect which one might expect would result in an increase in activity or an alteration in the membrane potential, due to Ca^{2+} driving neurotransmitter release (Drysdale et al., 2006; Ryan et al., 2006). However, these data show a clear concentration-dependent suppression in synaptic activity that, through the pre-treatment with PTX, can be concluded to be an effect dependent on the activation of a $G_{i/o}$ GPCR. However, with the spontaneous APs observed in our investigation being synaptically driven and with CBD decreasing intracellular Ca^{2+} levels in the neuronal soma when the excitability of the neurons were increased through increasing K^+ concentration or addition of the epileptiform inducing agent 4-AP (Ryan et al., 2009), a direct action of CBD on presynaptically located Ca^{2+} channels or their inhibition through the activation of presynaptic GPCRs cannot be discounted. With THC and WIN 55,212-2 both decreasing synaptic activity, in agreement with previous studies (Shen and Thayer, 1999; Sullivan, 1999; Ohno-Shosaku et al., 2001; Bajo et al., 2009; Straiker and Mackie, 2009), it may be concluded that the effects of CBD are not an artefact of our culture preparation.

3.4.2 Potential receptor targets for CBD in hippocampal cultures, a complicated issue

The finding that CBD inhibits synaptic activity via $G_{i/o}$ GPCR led immediately to investigating the two $G_{i/o}$ GPCRs discussed in previously in this chapter, CB_1 and 5-HT_{1A}, for which CBD has been shown to have an

affinity. It was found that the selective CB_1 inverse agonist, AM251 (30nM), did not prevent the reduction in synaptic activity induced by CBD (1μM). However, it may be questionable whether a pre-application of 1 minute would be sufficient enough time for AM251 to saturate the receptors prior to CBD application. This was unavoidable, as a longer pre-application with AM251 resulted in a dramatic increase in activity that eventually resulted in a depolarisation of the cell. Although this may invalidate this data, it in itself provides evidence that there is a strong endocannabinoid tone in this system that may allow for the proposition of indirect mechanism of CBD action. It was also attempted to investigate whether CBD elicits its actions via 5-HT_{1A}, however, pre-application of the selective 5-HT_{1A} antagonist, WAY 100135, also resulted in a hyperactive state also leading to cell depolarisation suggesting an endogenous serotoninergic tone.

3.4.3 Effect of CBD on synaptic activity in hippocampal slices

In order to investigate the effects of CBD on synaptic activity further, electrophysiological recordings were made in acute hippocampal slices, which provide a more physiological CNS system. Consistent with experiments in cultured hippocampal neurons, CBD reduced the amplitude of an evoked fEPSP, a measure of synaptic transmission, in the CA1 region of the hippocampus. The first observation from this study was that fEPSPs recovered to a baseline level following removal of CBD from the perfusate. This may be a consequence of a number of factors including, but not limited to, the support of microglia in the slice removing excess CBD or the fact that the responses are a result of stimulation and not a spontaneous occurrence. The second observation is that CBD enhances paired-pulse facilitation (PPF). An increase in paired-pulse facilitation ratio is indicative that CBD is acting via a presynaptic receptor (Baskys and Malenka, 1991). This coupled with the findings from cultured hippocampal neurons would suggest that CBD is acting via a presynaptic $G_{i/o}$ GPCR.

3.4.4 CBD, an agonist at presynaptic 5-HT_{1A} receptors

As discussed previously in this chapter, CBD has been shown to have little affinity for CB_1 receptors, which would be a logical presynaptic $G_{i/o}$ GPCR target for a cannabinoid. It was therefore hypothesised that CBD is inhibiting synaptic activity via a presynaptic 5-HT_{1A} receptor, which CBD has been shown to activate (Russo et al., 2005). To investigate the involvement of 5-HT_{1A} in the synaptic effects of CBD, WAY 100135, a selective 5-HT_{1A} antagonist which has been shown to block the anxiolytic effects of CBD observed in animal models of anxiety (Braida et al., 2007; Campos and Guimaraes, 2008; Resstel et al., 2009). In concurrence with this hypothesis, WAY 100135 blocked the effect of CBD on synaptic transmission. As activation of 5-HT_{1A} receptors has previously been shown to inhibit synaptic transmission in the hippocampus (Schmitz et al., 1995b; Pugliese et al., 1998; Schmitz et al., 1998), the observed effects with WAY 100135 are consistent with CBD mediating its effects through 5-HT_{1A} receptors. However, investigation into the effects of 8-OH-DPAT in acute hippocampal slices revealed that despite 8-OH-DPAT suppressing evoked fEPSP in above preparation (Bushell, personal communication), 8-OH-DPAT application resulted in no change in PPF ratio, suggesting the 5-HT_{1A} agonist is acting at a postsynaptic site. 5-HT_{1A} receptors have been shown to localise to pre- and postsynaptic membranes and it has been proposed that the anxiolytic effects of 8-OH-DPAT are mediated via receptors located presynaptically. However the role and localisation of 5-HT_{1A} receptors in the hippocampus is not fully understood.

In contradiction to the findings in hippocampal cultures it was found the CB_1 receptor inverse agonist, AM251, blocked that CBD mediated effects. Although CBD has not been shown to have any significant binding affinity for CB_1 receptors, the ability of AM251 to block CBD-induced inhibition of synaptic activity in slices may suggest indirect involvement of CB_1 in the actions of CBD. As CBD has been shown to inhibit the reuptake and metabolism of the eCB, AEA (Bisogno et al., 2001), there is the potential that CBD could lead to an increase in the eCB tone in the synapse leading to a decrease in synaptic activity through AEA activation of CB_1 receptor. It has

recently been shown that inhibition of AEA degradation results in a decrease in synaptic activity, which may add weight to this theory (Abush and Akirav, 2009). Further investigation by our group revealed that CBD-induced suppression of synaptic transmission was sensitive to another CB_1 receptor antagonist, LY 320135, which is structurally different to AM251.

In summary, application of CBD results in an inhibition of synaptic activity in primary hippocampal cultures and acute hippocampal slices. The inhibition of synaptic activity in hippocampal slices was shown to result from CBD acting at a presynaptic site that is sensitive to both CB_1 and 5-HT_{1A} receptor antagonists. These data show for the first time that CBD can alter basal neural activity, which may have implications in the proposed use of CBD for treatment of several diseases, including stroke, diabetes and cancer.

CHAPTER 4: CBD INHIBITS THC-INDUCED CHANGES IN NEUROCHEMICAL MARKERS OF CELLULAR ACTIVITY IN VIVO

4.1 Introduction

4.1.1 Immediate early genes as markers of neuronal activity

Immediate early genes (IEG) are genes that can be used as markers of cell activation to a variety of stimuli. They have been used extensively to map the regional effects of drugs of abuse, behaviour and electrical stimulation in the CNS and have been the topic of many reviews (Cochran, 1993; Hughes and Dragunow, 1995; Harlan and Garcia, 1998; Kovacs, 1998; Lanahan and Worley, 1998; Tischmeyer and Grimm, 1999; Clayton, 2000; Davis et al., 2003). Expression of an IEG is a genomic-reflex of a cell in response to receptor activation and the subsequent downstream signal transduction that results from this. The "genomic-reflex" analogy serves to explain that it is not perhaps the function of the protein that the IEG encodes that is of interest, but more that the IEG has been transcribed as a result of a stimulus (Clayton, 2000). Therefore examining the expression of IEGs is not descriptive of the function of the stimulus on neurons, but only serves as an indication that the neuron has been activated either endogenously or exogenously. That said the functions of some IEGs are specific enough to give an idea of the downstream consequences of the stimulus on the cell or system.

There have been over 30 IEGs identified to date (Lanahan and Worley, 1998) that can be divided into two main groups, based on the function of the protein that it expresses. The first are regulatory proteins, such as *c-fos*, *c-jun*, *JunB*, *Zif-268* or *Nur-77*. These are proteins which regulate the transcription of late response genes, which have a variety of functions from cell proliferation to cell death (Clayton, 2000). The second group encode effector proteins, such as brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc), which do not regulate transcription, but instead have a direct cellular function, such as cellular organisation.

Primarily the induction of IEGs may be induced by stimuli producing either neural excitation or differentiation (such as nerve growth factor). The intracellular signaling cascades resulting in *c-fos* induction may be stimulated

by compounds acting at G-protein coupled receptors (GPCRs) to increase cAMP, or via calcium influx through either NMDA receptor ion channels or voltage operated calcium channels (VOCC) (Herrera and Robertson, 1996). These cascades control the expression of the *c-fos* and *Zif-268* via interaction with two upstream regulatory elements termed the serum response element (SRE) and the calcium/cAMP responsive element (Ca/CRE) (Shen and Greenberg, 1990).

4.1.1.1 Zif-268

The regulatory IEG zif-268 (otherwise known as krox-24, NGFI-A, Egr-1 or ZENK) is a zinc-finger protein with high basal expression in pyramidal layers of the cerebral cortex, hippocampus and cerebellum (Davis et al., 2003). Expression of *Zif-268* mRNA has been linked to synaptic plasticity; the most notable finding was that LTP could not be induced in hippocampal neurons of *Zif-268* knockout mice (Jones et al., 2001). This coupled to the localised expression in the hippocampus has led to a proposed role for Zif-268 in learning and memory (Lanahan and Worley, 1998; Tischmeyer and Grimm, 1999; Jones et al., 2001; Bozon et al., 2003; Davis et al., 2003). *Zif-268* mRNA is transiently expressed following the activation of glutamatergic receptors (Wang, 1998).

4.1.1.2 c-fos

c-fos is also a regulatory gene, and is the IEG most widely used for mapping regional effects in response to stimuli due to its low levels of basal expression making it most sensitive to upregulation by stimuli. *c-fos* is a member of the leucine-zipper family of transcription factors. When translated, Fos forms a homodimer or heterodimer with c-jun, junB and binds to promoter sequences for genes encoding proteins with a variety of functions (Cochran, 1993; Yoneda et al., 2001). Analysis of the expression of *c-fos* mRNA or the protein it expresses has been used for decades as a marker of alteration of neuronal activity due external stimuli (Clayton, 2000). When activated, *c-fos* has a transient expression peaking at around 60 mins (Hughes and Dragunow, 1995; Kovacs, 1998).

4.1.1.3 Arc

Activity-regulated cytoskeleton-associated (Arc) protein, differs significantly to zif-268 and c-fos in its transcription signature and protein function. Arc is an effector member of the IEG family, where expression results in direct alteration in the trafficking of AMPA receptors to the synaptic membrane and therefore providing a direct link between Arc and synaptic plasticity (Chowdhury et al., 2006; Rial Verde et al., 2006). The temporal signature of *Arc* mRNA expression is unique. Upon activation of transcription of the Arc gene, Arc mRNA is immediately transported from the cell body to synapses in dendritic spines, where it is translated local to the synaptic membrane (Lyford et al., 1995).

4.1.2 Effects of cannabinoids on regional neuronal activity in the CNS in vivo

The effects of cannabinoids on regional brain activity have been well documented from human and rodents. Cannabis and its principal psychoactive component, THC, produce regionally specific alterations in the CNS, consistent with the profile of CB_1 receptor distribution. Alteration in activity in the regions targeted by THC may also be responsible for the cognitive deficits induced by cannabis consumption.

4.1.2.1 Effects of cannabinoids on neuronal activity in humans

In positron emission tomography (PET) studies, a method that can be used for imaging localisation of brain function by using radiolabelled substrates producing a measure of regional cerebral blood flow (rCBF), human subjects (with varying levels of cannabis abuse) were either given cannabis via inhalation of the vapours produced through burning in a cigarette or administered THC injection (i.v.). Acute administration of cannabis via cigarette, with THC concentrations ranging from 0 (placebo) - 3.55 %, resulted in a global increase in rCBF and regionally specific increases in blood flow in the frontal and temporal lobes 30-60 minutes after administration (Mathew et al., 1989; Mathew et al., 1992; Mathew and Wilson, 1993). It was also noted that global rCBF was reduced in chronic users compared to recreational users suggesting that long-term cannabis use

results in a reduction in basal cerebellar activity (Mathew et al., 1989). The acute administration of THC (i.v.) at concentrations ranging from 0 (placebo) – 0.25 mg/min or 2 mg (single dose), resulted in increases in blood flow and metabolic activity in the prefrontal, frontal and orbital cortices, anterior cingulate cortex and the cerebellum whilst resulting in a reduction of regional blood flow in the basal ganglia, hippocampus and amygdala. The above recordings were all taken at a resting state. Further studies have also measured regional functional alterations during the performance of cognitive tasks. Several studies in recreational cannabis users following a period of abstinence (around 2-4 days) or in subjects with no or little history of cannabis use found that administration of cannabis did not alter performance in tasks measuring working memory or attention despite altering activity in regions involved in these tasks. O'Leary et al (2002) found that cannabis intoxication in recreational cannabis users resulted in a significant increase in rCBF in the orbital frontal lobes, insula, anterior cingulated gyrus and cerebellum, but had no effect on an auditory attention task (O'Leary et al., 2002). Borgwardt et al (2008) found that THC did not alter performance in the Go/No-Go task, a task measuring response inhibition, in healthy (little or no cannabis use) volunteers, despite inhibiting regional activation (fMRI BOLD) in the right inferior frontal and anterior cingulate gyrus (Borgwardt et al., 2008). Bhattacharyya et al (2009) found that THC administration augmented parahippocamal gyral and ventrostriatal activation during performance in a verbal recall task without actually altering performance in the task itself (Bhattacharyya et al., 2009a). These data suggest that acute administration can alter neuronal activity without altering performance; therefore measuring alterations neuronal function may be a more sensitive mechanism in determining the neuronal effects of acute THC administration than behavioural studies.

4.1.2.2 Effects of cannabinoids on neuronal activity in rats

Functional imaging has also been used to localise the effects of cannabinoids on cerebral activity in animals. 2-deoxyglucose autoradiography has been widely used to map the regional effects of a wide variety of psychotropic drugs on local cerebral glucose utilisation (LCGU), a marker of metabolic

activity of cells. Several animal studies demonstrated that acute THC administration (i.p.) resulted in a general reduction in LCGU in regions analogous to human brain regions such as prefrontal, anterior and posterior cingulate cortices, nucleus accumbens, mediodorsal thalamus and both the CA1 and CA3 regions of the hippocampus (Brett et al., 2001; Freedland et al., 2002). This was shown in one study to be reversed by administration of a CB_1 receptor antagonist, SR141716A. In another study the authors demonstrated that despite THC resulting in decreased LCGU in several cortical and limbic regions when administered at doses of 2 and 10 mg/kg, when 0.2 mg/kg THC was administered it resulted in an increase in LCGU in the same regions. Therefore considering the range of doses of THC used in the human studies it could be concluded that these doses are more likely to be related to the lower dose of THC in rats (Margulies and Hammer, 1991).

4.1.3 Cannabinoid-induced changes in regional immediate early gene expression

As discussed previously (Section 2.3), IEGs have been used routinely to investigate the regional effects of drugs of abuse (for review see Harlan and Garcia, 1998). The most frequently used has been the regulatory IEG *c-fos*, which is a general marker of cellular activation. Several studies have shown THC (3.2, 5 or 10 mg/kg) increases in Fos protein or mRNA encoding *c-fos* in the cingulate cortex, prefrontal cortical regions, nucleus accumbens and caudate putamen (Mailleux et al., 1994; Miyamoto et al., 1996; McGregor et al., 1998; Porcella et al., 1998). Similarly, THC (5 mg/kg) administration was shown by our group to result in a significant induction of *c-fos* mRNA expression in the cingulate, ventral orbital and prelimbic cortices and the caudate putamen (Egerton, 2004). In another study by our group THC, when administered at a lower dose (1 mg/kg), produced a significant reduction in expression of *c-fos* mRNA (Egerton et al., 2005a). These data provide further support for the evidence of a biphasic effect of THC dependent on dose. The mechanism through which THC has an effect on *c-fos* expression has also been studied. In one study the ability of THC to induce an increase in expression of *c-fos* in the striatum and nucleus accumbens was blocked by treatment with a selective dopamine receptor

(D1) antagonist, SCH-23390 (Miyamoto et al., 1996). In a study by our group it was also shown that THC induced increases in *c-fos* expression were reversed through pre-treatment with MK-801 (also known as dizocilipine), a selective NMDA receptor antagonist (Egerton, 2004). The involvement of D1 and NMDA receptor activation in the induction of *c-fos* expression (see section 2.3) has been well documented. However, THC has not been shown to act directly at these receptors. These data would therefore suggest that induction of *c-fos* might be through an indirect action of THC. As discussed previously (section 1.4.3), administration of THC resulted in an increase in dopamine and glutamate concentration as demonstrated in micro-dialysis studies in the prefrontal cortex (Pistis et al., 2002). This is surprising since other approaches have shown that activation of CB_1 receptors inhibits neurotransmitter release (Kim and Thayer, 2000; Gerdeman and Lovinger, 2001; Huang et al., 2001; Kofalvi et al., 2005). However, considering that THC was also shown to reduce production of the inhibitory neurotransmitter, GABA, it could be concluded that the increase in the excitatory neurotransmitters may be due to THC blocking inhibitory tone within this region (Pistis et al., 2002).

Similar to its effects on *c-fos* expression, THC has also been shown to alter expression of *zif-268*, a marker of excitatory neuronal activity. THC (5 mg/kg i.p.) was shown to significantly increase mRNA expression of *zif-268* in the cingulate, frontal and parietal cortices as well as the caudate putamen 20 minutes after administration (Anis et al., 1983). Previously in our group THC (5 mg/kg) was shown to increase *zif-268* mRNA expression in caudate putamen, cingulate cortex and the CA1 region of the anterior and posterior hippocampus (Egerton, 2004). As with *c-fos* expression, THC-induced increases in *zif-268* expression were blocked by pre-treatment with MK-801. This also suggests an indirect effect of THC through its ability to increase regional glutamate production. However, it has also been shown that THC and selective CB_1 receptor agonists can induce *zif-268* expression through direct activation of the CB_1 receptor and subsequent MAP kinase activation (Derkinderen et al., 2003).

Despite the fact that the THC-induced effects on IEG expression have been well studied, there are no previous studies published that investigate the effect of THC on Arc expression. In the aforementioned study by our group, THC (5 mg/kg) induced widespread increases Arc expression, which was sensitive to MK-801 (Egerton, 2004). This is not that surprising considering Arc is associated with NMDAR-dependent plasticity and is involved in AMPA receptor trafficking (Chowdhury et al., 2006; Rial Verde et al., 2006). The investigation of the effects of a drug on expression of Arc, which is a member of the effector family of IEGs, is also useful in evaluating a possible functional consequence of a drug. Therefore the induction of Arc by THC may suggest that THC may influence synaptic plasticity, which has been shown in functional studies (Diana et al., 2003; Kim et al., 2008).

4.1.4 Effects of CBD on regional activity and potential interaction with THC-induced effects

Several studies, published after the experiments described in this thesis were carried out, have examined the regional effects of CBD on neuronal activity in humans. 600 mg of CBD was shown to reduce regional brain activity in the anterior and posterior cingulate cortex and the amygdala whilst volunteers were being shown fearful images (Fusar-Poli et al., 2009b). This was suggested to be due the ability of CBD to block connectivity from the amygdala to the cingulate cortex (Fusar-Poli et al., 2009a). CBD was also shown to produce regional effects opposite to those elicited by THC administration, by reducing neuronal activity in the striatum, hippocampus and amygdala whilst subjects performed memory recall, response inhibition or emotional tasks (Bhattacharyya et al., 2009b). Furthermore in the latter study pre-treatment with CBD blocked the neuronal effects elicited by THC in these tasks (Bhattacharyya et al., 2009b). This would indicate that in humans CBD reverses neuronal effects of THC; the fact that the two drugs have opposing effects on their own may suggest that they are working through different yet complementary mechanisms.

In preliminary unpublished studies within our group, CBD administration resulted in subtle changes in LCGU in rats. CBD administered at a concentration of 5 or 15 mg/kg resulted an increase in LCGU in the ventral pallidum and CA3 region of the hippocampus. A small number of animal studies, CBD has been shown to have some effect on expression the IEG protein, Fos. In one study investigators found an increase in Fos expression in the nucleus accumbens when rats were administered 120 mg/kg CBD (Guimaraes et al., 2004). However, this is a relatively high dose of CBD considering most experiments investigating the anxiolytic effect CBD used of doses less than 20 mg/kg. In a separate study CBD (10 mg/kg) was also shown to reduce expression of Fos in the prefrontal and infralimbic portions of the rat prefrontal cortex (Lemos et al., 2010). This could be suggestive that CBD alters activity in regions correlated to cognitive function in an opposing fashion to that observed by THC administration. As no studies have examined the ability of CBD to alter the THC-induced alterations in IEG expression the purpose of this experiment was two-fold.

1. To investigate the effect of CBD at various doses on basal IEG mRNA induction, in regions relevant to working memory and cognitive processing.
2. To investigate the ability of CBD to block THC-induced alterations in IEG mRNA expression in these regions.

4.2 Methods

4.2.1 Animals

Male hooded Lister rats (Harlan, UK) were purchased at a weight of 325-350 g and housed in a temperature-regulated room with a 12 hour light/dark cycle with *ad libitum* access to food and water. In all experiments handling was kept to a minimum, where animals were weighed and marked the day prior to experiments.

4.2.2 Drug administration

Animals were randomly allocated to 8 treatment groups (n=8). Animals each received two i.p injections concomitantly as per the table below.

	1ST Injection	**2nd Injection**
Group 1	Vehicle	Vehicle
Group 2	Vehicle	CBD (5 mg/kg)
Group 3	Vehicle	CBD (15 mg/kg)
Group 4	Vehicle	CBD (50 mg/kg)
Group 5	THC (5 mg/kg)	Vehicle
Group 6	THC (5 mg/kg)	CBD (5 mg/kg)
Group 7	THC (5 mg/kg)	CBD (15 mg/kg)
Group 8	THC (5 mg/kg)	CBD (50 mg/kg)

4.2.3 In situ hybridization

This was performed as described in section 2.3. Briefly, 75 minutes following drug administration animals were sacrificed by cervical dislocation and decapitation. Brains were removed and frozen before being sectioned using a cryostat. Sections were fixed in paraformaldehyde and dehydrated in ethanol. A hybridisation mix containing a radiolabelled 45-mer oligonucleotide probe with a complementary sequence to mRNA encoding

Arc, c-fos and *Zif-268* was applied to sections and incubated at 42°C overnight. Sections were washed, dehydrated and exposed to an X-Ray film for 5-10 days (depending on radioactivity). Films were than developed using a X-OMAT developer.

4.2.4 Densitometry and analysis

Developed X-Ray films were analysed using the MCID (Imaging Research Inc) densitometer software package. Anatomical regions of the brain were identified using a stereotaxic brain atlas (Paxinos and Watson, 2007). The relative optical density (ROD) was recorded for each selected region for both hemispheres. These readings were corrected for non-specific binding by subtracting the ROD for readings taken from the sections that were hybridised with an excess of unlabelled oligonucleotide. Data presented as mean ROD ± S.E.M. Two-way analysis of variance (ANOVA) was performed using Prism5 (GraphPad) to determine independent effects of CBD and THC administration as well as any interaction between the two treatment groups. Due to the number of groups, a two-way ANOVA may not be sensitive enough to detect minor changes between drug and control groups. Therefore, unpaired t-tests were performed using Prism5 (GraphPad) to analyse differences between groups treated THC (5 mg/kg) or CBD (5 mg/kg) alone and the control group (two injections of vehicle) so that the experiment can be compared to the effects of THC and CBD previously published. All analyses were performed on raw data, however data in the results description is presented as a percentage change. Percentage change was calculated by normalising all data to the control (vehicle treated animals) that was expressed as a percentage of the basal expression, to give a positive or negative percentage change then 100 is subtracted from this value. These values are represented in the text as mean ± S.E.M %.

4.3 Results

4.3.1 Effects of CBD and THC on IEG induction at the level of the prefrontal cortex

A two-way ANOVA revealed that there was no significant interactive or independent effect of the drug groups on expression of all IEGs. This is most likely due to there being only marginal changes observed in one out of the eight groups being analysed. Therefore, data were reanalysed by t-test to see if there was any significant difference between THC and CBD (5mg/kg) and vehicle treated animals.

4.3.1.1 Zif-268

In this study administration of THC (5 mg/kg) resulted in a significant increase in induction of mRNA encoding *Zif-268* in the anterior cingulate cortex (16.1 ± 6.8%; n=6; $p<0.05$; Fig 4.1, C), motor cortex (30.4 ± 8.4%; n=6; $p<0.01$; Fig 4.1, D), lateral orbital cortex (23.4 ± 6.8%; n=6; $p<0.05$; Fig 4.1, E), ventral orbital cortex (12.8 ± 5.2%; n=6; $p<0.05$; Fig 4.1, F) and agranular insular cortex (34.6 ± 10.9%; n=6; $p<0.05$; Fig 4.1, G) compared with vehicle treated animals. There was no significant increase detected in these regions in animals treated with both THC and CBD (5mg/kg) concomitantly compared with vehicle control suggesting that CBD (5mg/kg) was attenuating THC-induced changes in *Zif-268* expression. These findings corroborate with previous studies, where THC (5 mg/kg) resulted in a significant increase in *Zif-268* expression in the anterior cingulate cortex (Mailleux et al., 1994). THC was without effect on expression of *Zif-268* mRNA in the prelimbic and infralimbic cortices (Fig 4.1, A & B). Furthermore, THC administration was without effect when compared to control groups when co-administered with CBD (5, 15 or 50 mg/kg). This suggests that CBD attenuates the ability of THC administration to induce *Zif-268* expression in those regions described above. CBD (5 mg/kg) administration resulted a slight reduction in *Zif-268* expression in the prelimbic, infralimbic, motor, agranular insular and cingulate cortices, but these changes were not significant.

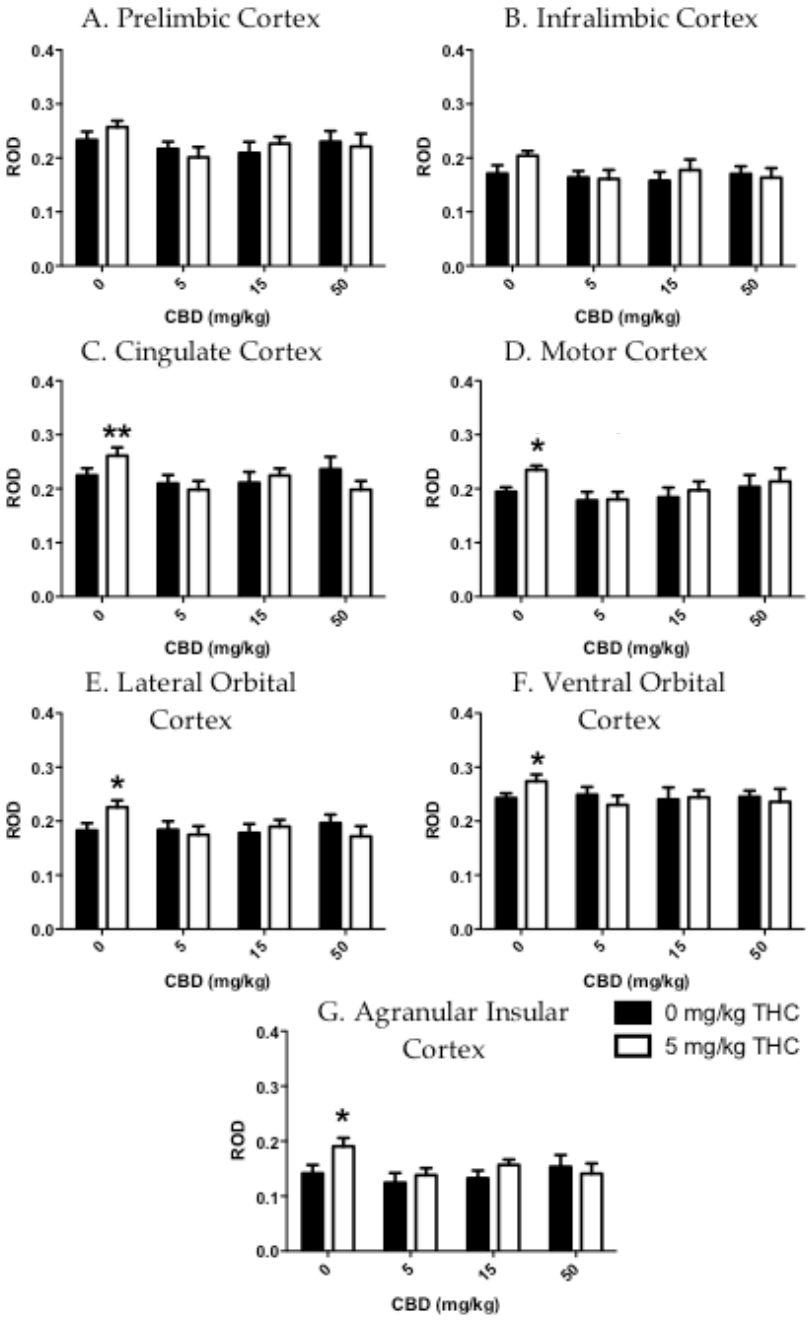


Figure 4.1 CBD blocks THC-induced increases of *Zif-268* mRNA expression in prefrontal cortical regions of the rat brain. These graphs illustrate the results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *Zif-268* mRNA in the prelimbic (A), infralimbic (B), cingulate (C), motor (D), lateral orbital (E) or ventral orbital (F) cortices or in the agranular insula (G). All data are expressed as mean ± S.E.M. relative optical density (ROD). * or ** denotes significant difference between treatment and control (two injections of saline; $p<0.05$ or $p<0.01$ respectively).

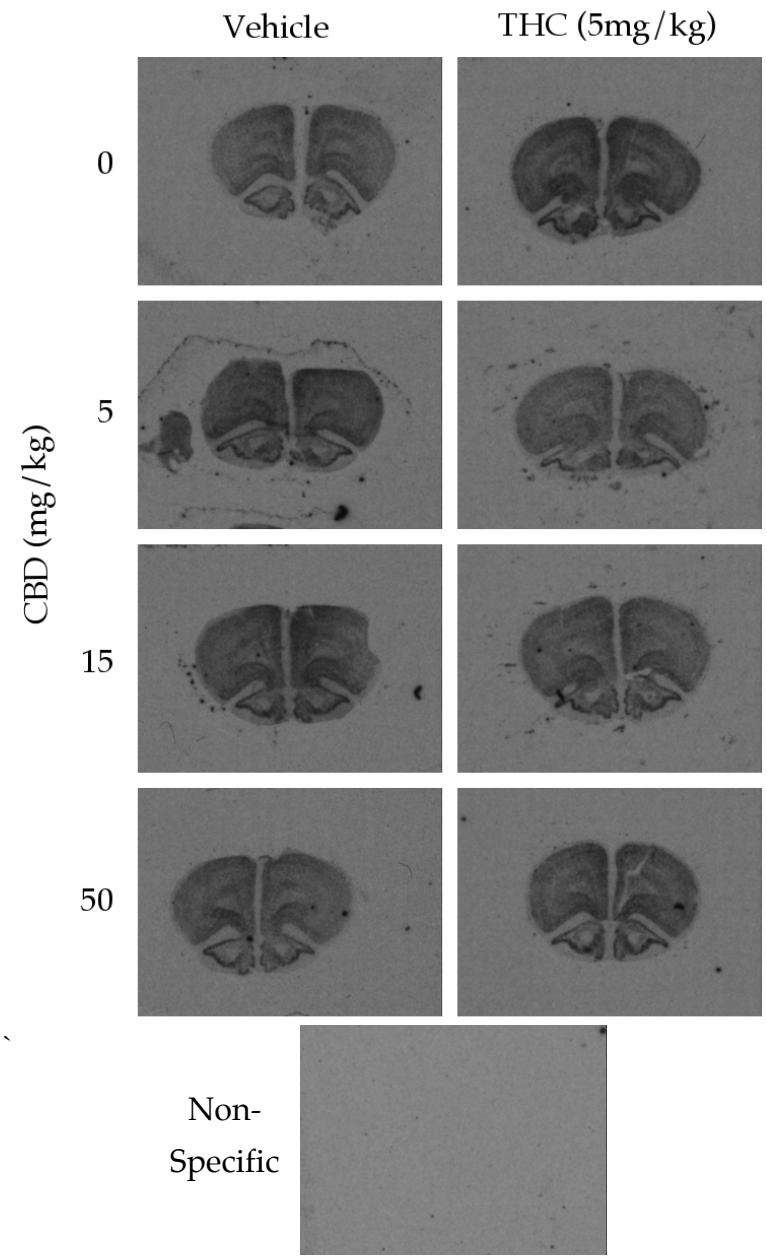


Figure 4.2 Autoradiogram illustrating the level of expression of *Zif-268* in response to CBD and THC treatment at the level of the prefrontal cortex. This autoradiogram are representative images illustrating the levels of *Zif-268* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.1.2 Arc

Despite its effects on the expression of *Zif-268*, a marker of excitatory activity in neurons associated with synaptic plasticity, THC administration had no significant effect on the expression *Arc* mRNA (Fig 4.3 A-G), another marker of excitatory activity in neurons associated with synaptic plasticity, in any regions measured in the prefrontal area (Fig 4.3). There was a trend for an increase in all regions examined in the group treated with THC (5 mg/kg) compared with vehicle control. Administration of CBD (5, 15 or 50 mg/kg) with vehicle or with THC (5 mg/kg) had no significant effect on *Arc* mRNA expression.

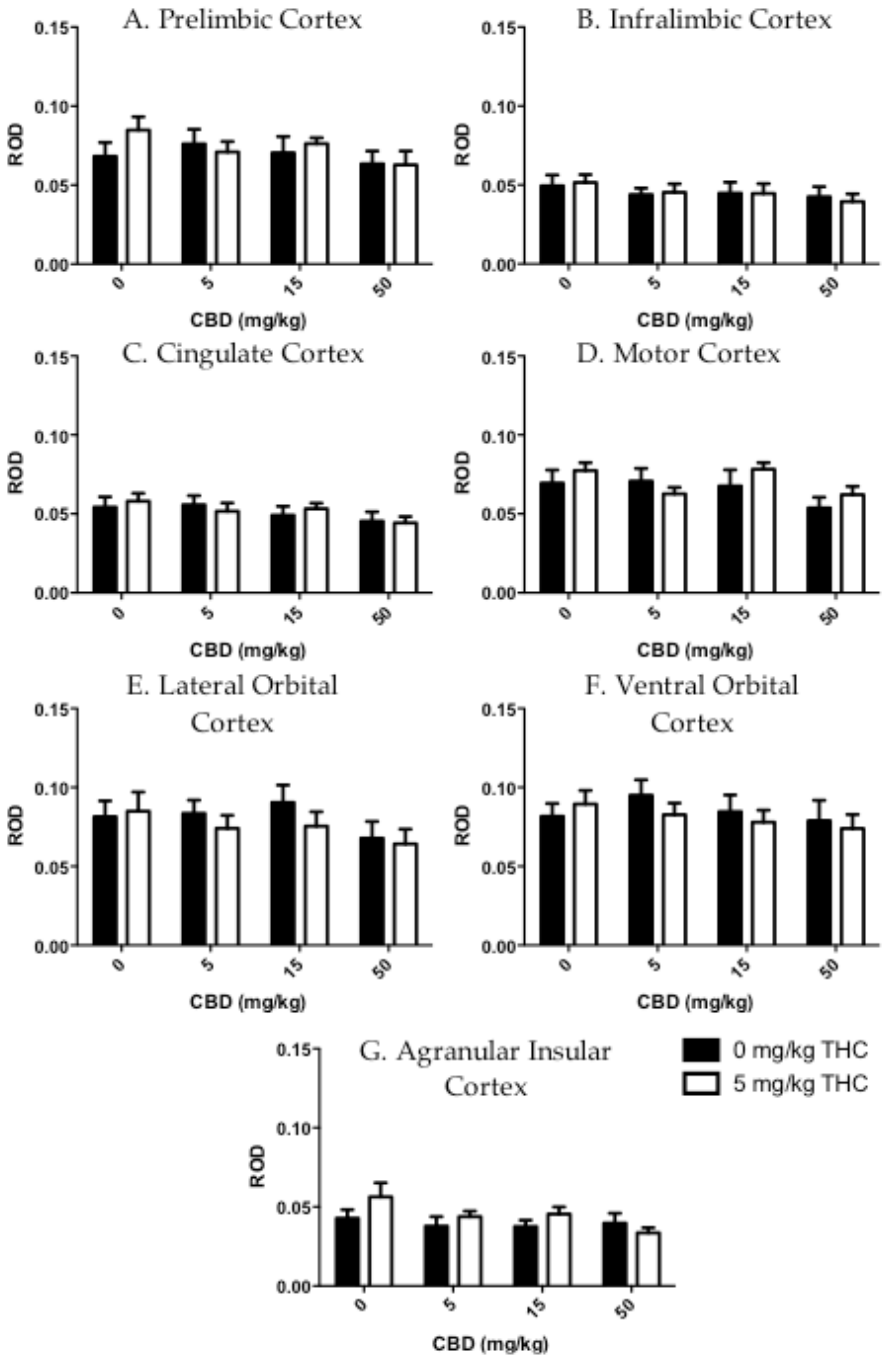


Figure 4.3 Neither THC nor CBD administration affected *Arc* mRNA expression in prefrontal cortical regions of the rat brain. These graphs represent the results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *Arc* mRNA in the prelimbic (A), infralimbic (B), cingulate (C), motor (D), lateral orbital (E) or ventral orbital (F) cortices or in the agranular insular cortex (G). All data are expressed as mean ± S.E.M. relative optical density (ROD). No significant changes were observed.

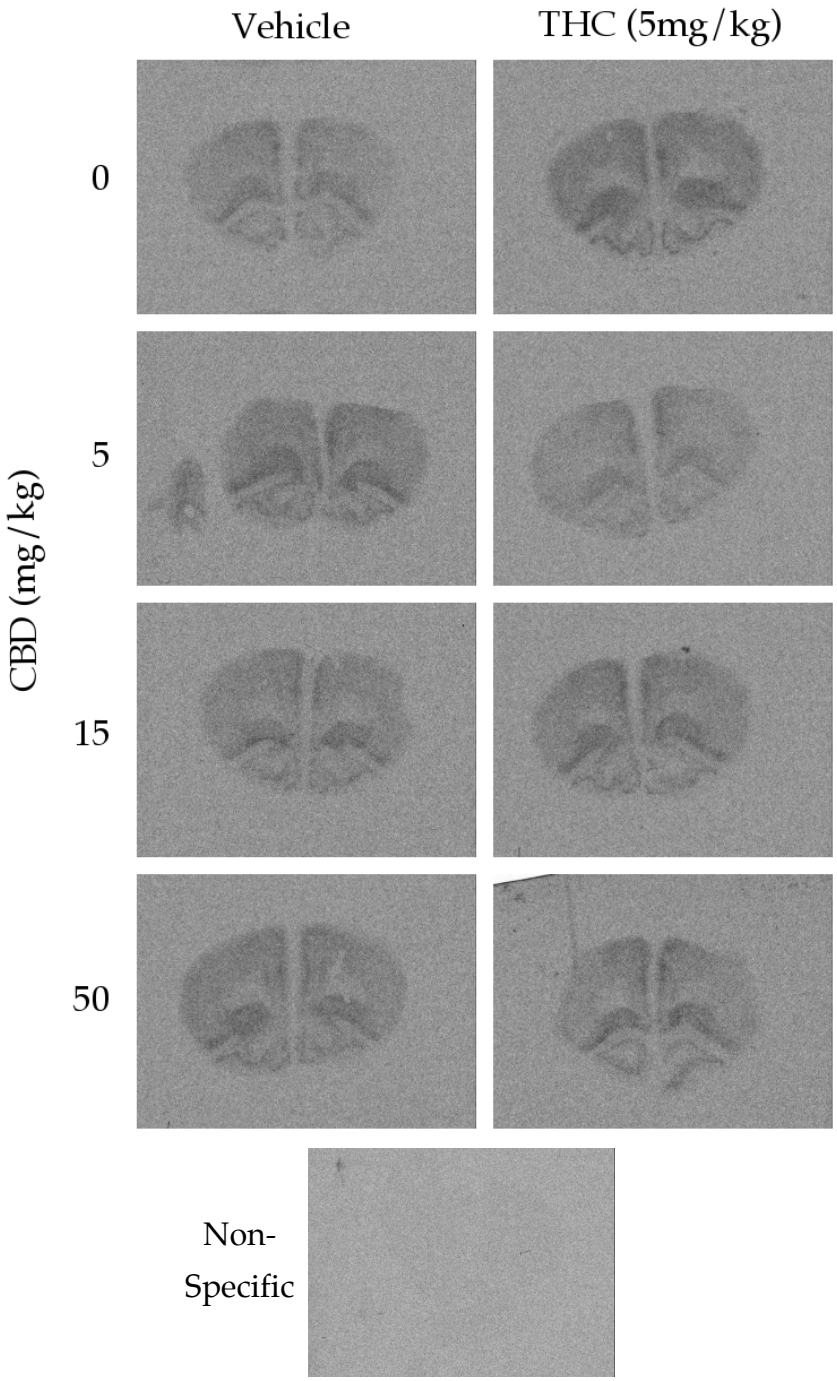


Figure 4.4 Autoradiogram illustrating the level of expression of *Arc* in response to CBD and THC treatment at the level of the prefrontal cortex. This autoradiogram is representative images illustrating the levels of *Arc* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.1.3 c-fos

Consistent with previous studies, administration of THC (5 mg/kg) resulted in a significant increase in *c-fos* mRNA expression in the cingulate cortex (36.6 ± 17.2%; n=5; $p<0.05$; Fig 4.5 C) and the agranular insular cortex (39.6 ± 20.5%; n=5; $p<0.05$; Fig 4.5 G) compared with vehicle control. Similar to the findings with *Zif-268*, THC administration had no effect on *c-fos* expression in animals when it was co-administered with CBD (5 or 50 mg/kg) compared with the control group. Interestingly the increase in *c-fos* expression compared to control in the cingulate cortex was conserved when THC was co-administered with 15 mg/kg CBD (40.6 ± 16.3%; n=8; $p<0.05$; Fig 4.5 C) but not when co-administered with 5 or 50 mg/kg of CBD. This effect of THC co-administered with CBD (15 mg/kg) was also present in the infralimbic cortex where this combination resulted in a significant increase in *c-fos* expression (25.2 ± 9.7%; n=8; $p<0.01$; Fig 4.5 B). In all other regions, with the exception of the lateral orbital cortex (Fig 4.5 E), THC administration produced a slight increase in *c-fos* compared to control, however this was not significant in any region. CBD (5 mg/kg) resulted in a significant increase in *c-fos* expression in the cingulate cortex (27.8 ± 10.9%; n=8; $p<0.05$; Fig 4.5 C). CBD (5, 15 or 50 mg/kg) had no effect on *c-fos* expression in any other region.

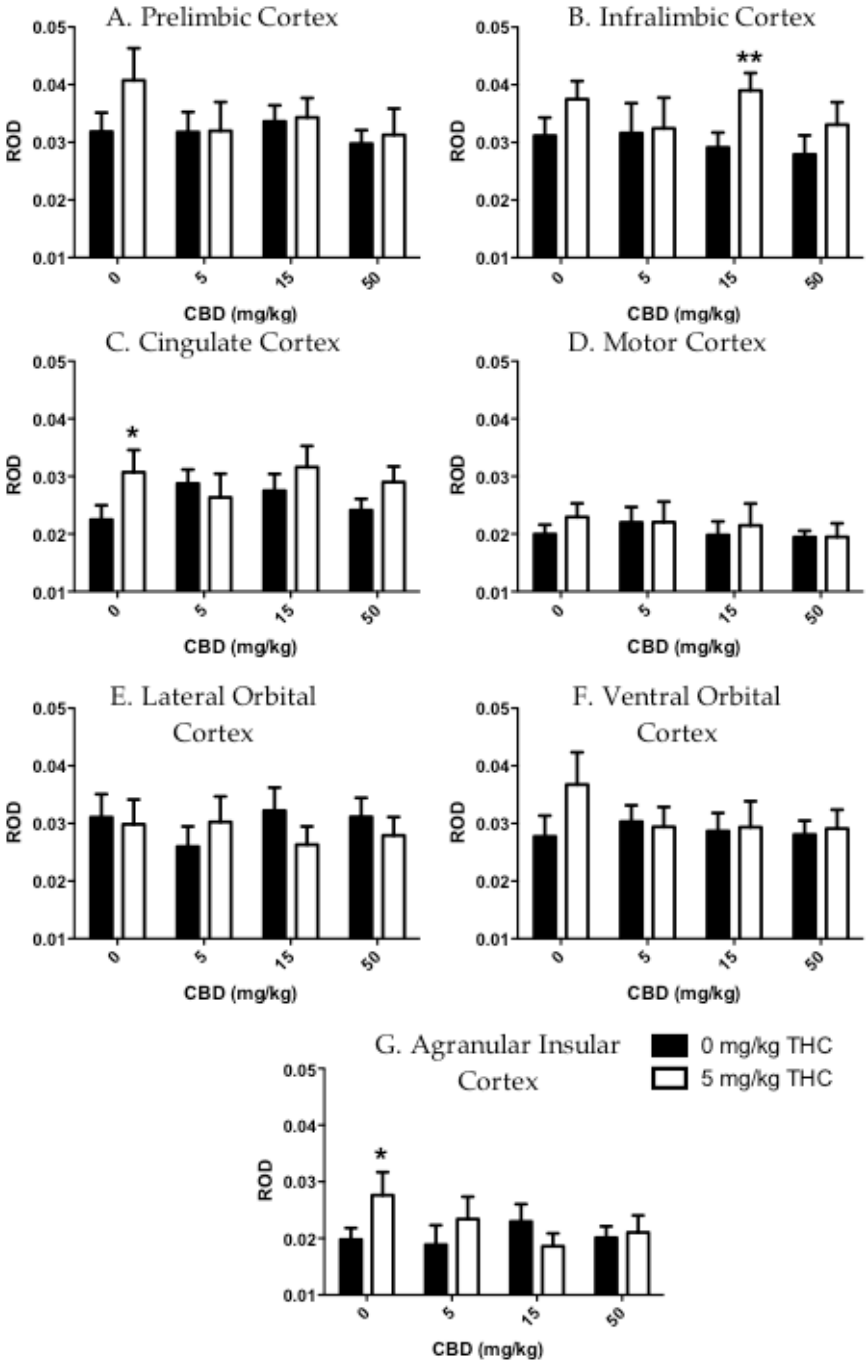


Figure 4.5 THC and CBD administration resulted in variable changes in *c-fos* mRNA expression in prefrontal cortical regions of the rat brain. These graphs illustrate results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *c-fos* mRNA in the prelimbic (A), infralimbic (B), cingulate (C), motor (D), lateral orbital (E) or ventral orbital (F) cortices or in the agranular insular cortex (G). All data are expressed as mean ± S.E.M. relative optical density (ROD). * or ** denotes significant difference between 0 and 5 mg/kg of THC ($p<0.05$ or $p<0.01$ respectively).

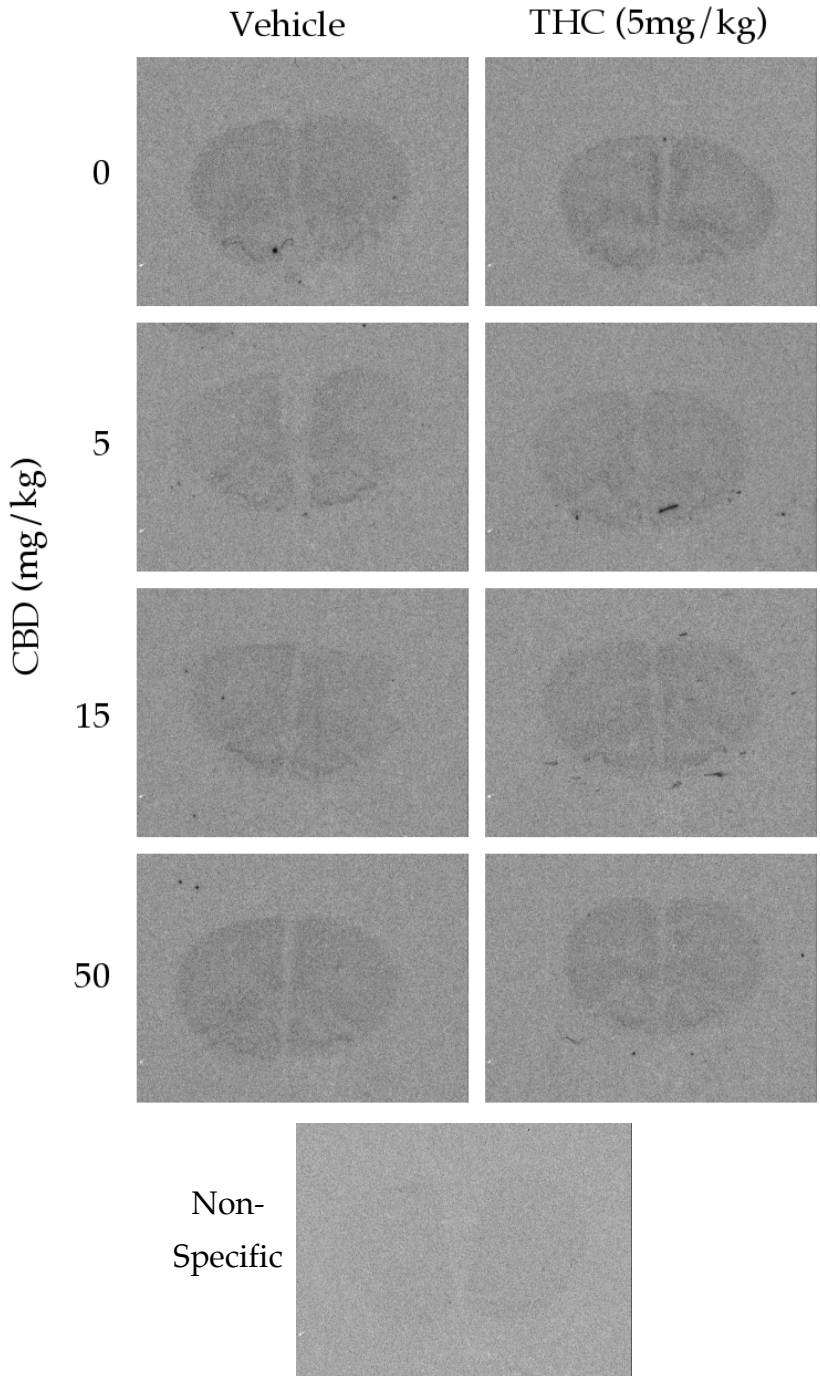


Figure 4.6 Autoradiogram illustrating the level of expression of *c-fos* in response to CBD and THC treatment at the level of the prefrontal cortex. This autoradiogram is representative images illustrating the levels of *c-fos* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.2 Effects of CBD and THC on IEG induction at the level of the striatum

4.3.2.1 Zif-268

Despite a trend suggesting that THC led to an increase in expression of *Zif-268* at the striatal level, there was only a significant increase expression of *Zif-268* in the caudate putamen (20.3 ± 9.7%; n=7; $p<0.05$; Fig 4.7, F) compared to control, this is in line with previous findings (Sharp, 1997). THC was without effect on *Zif-268* expression in the posterior cingulate cortex (Fig 4.7, A), motor cortex (Fig 4.7, B), sensory cortex (Fig 4.5, C, D) or the agranular insula (Fig 4.7, E). CBD (5, 15 or 50 mg/kg) was without a significant effect either on its own or in combination with THC in all regions of interest compared with vehicle control. Furthermore, THC administration was without effect compared to control when co-administered with CBD (5, 15 or 50 mg/kg). This suggests that CBD co-treatment attenuates the ability of THC administration to induce *Zif-268* expression in the caudate putamen.

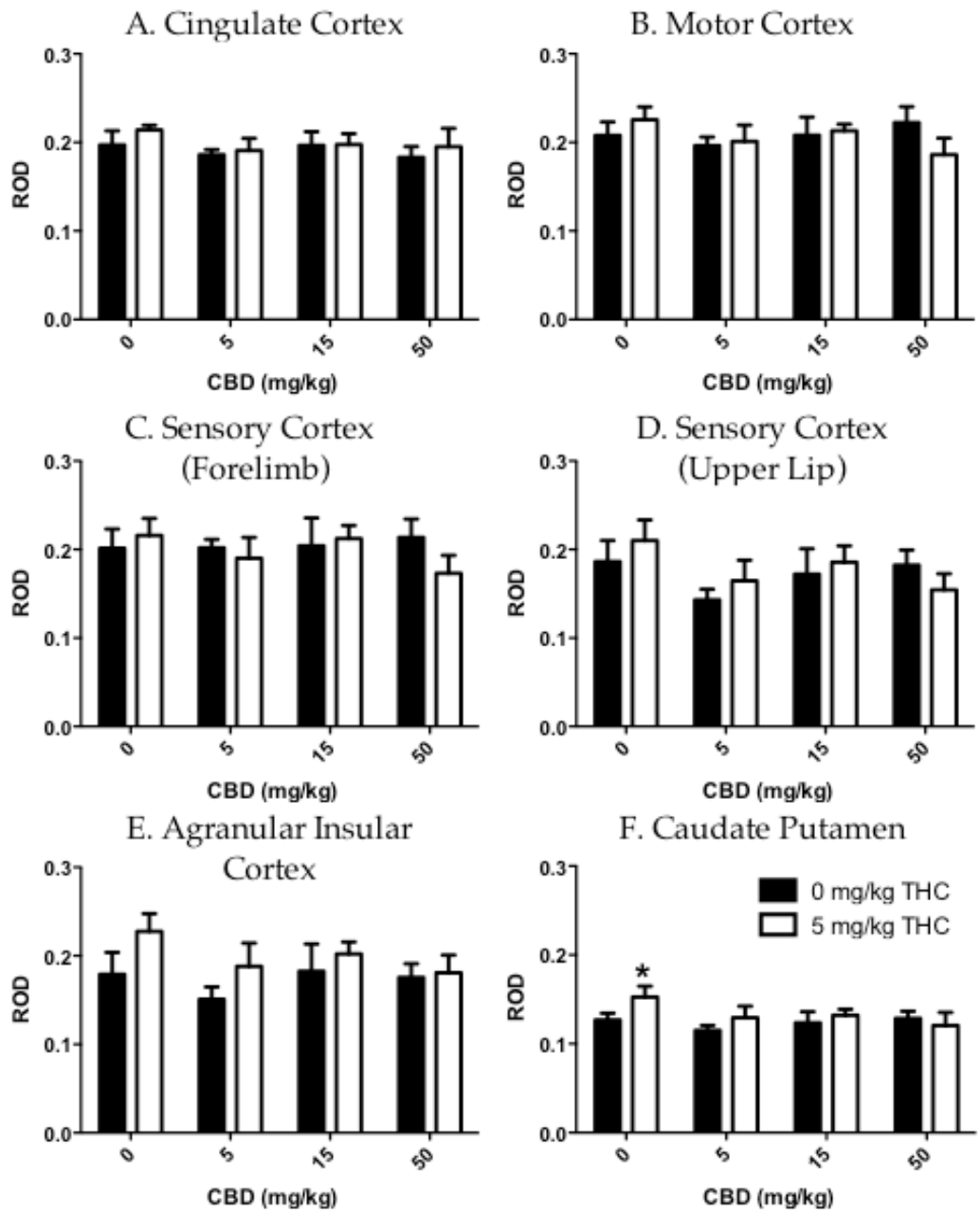


Figure 4.7 Effect of THC and CBD on *zif-268* mRNA expression at the level of the striatum of the rat brain. These graphs illustrate results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *zif-268* mRNA in the cingulate cortex (A), motor cortex (B), forelimb and upper lip region of the sensory cortex (C and D), agranular insular cortex (E) and caudate putamen (F). All data are expressed as mean ± S.E.M. relative optical density (ROD). * denotes significant difference between 0 and 5 mg/kg of THC ($p<0.05$).

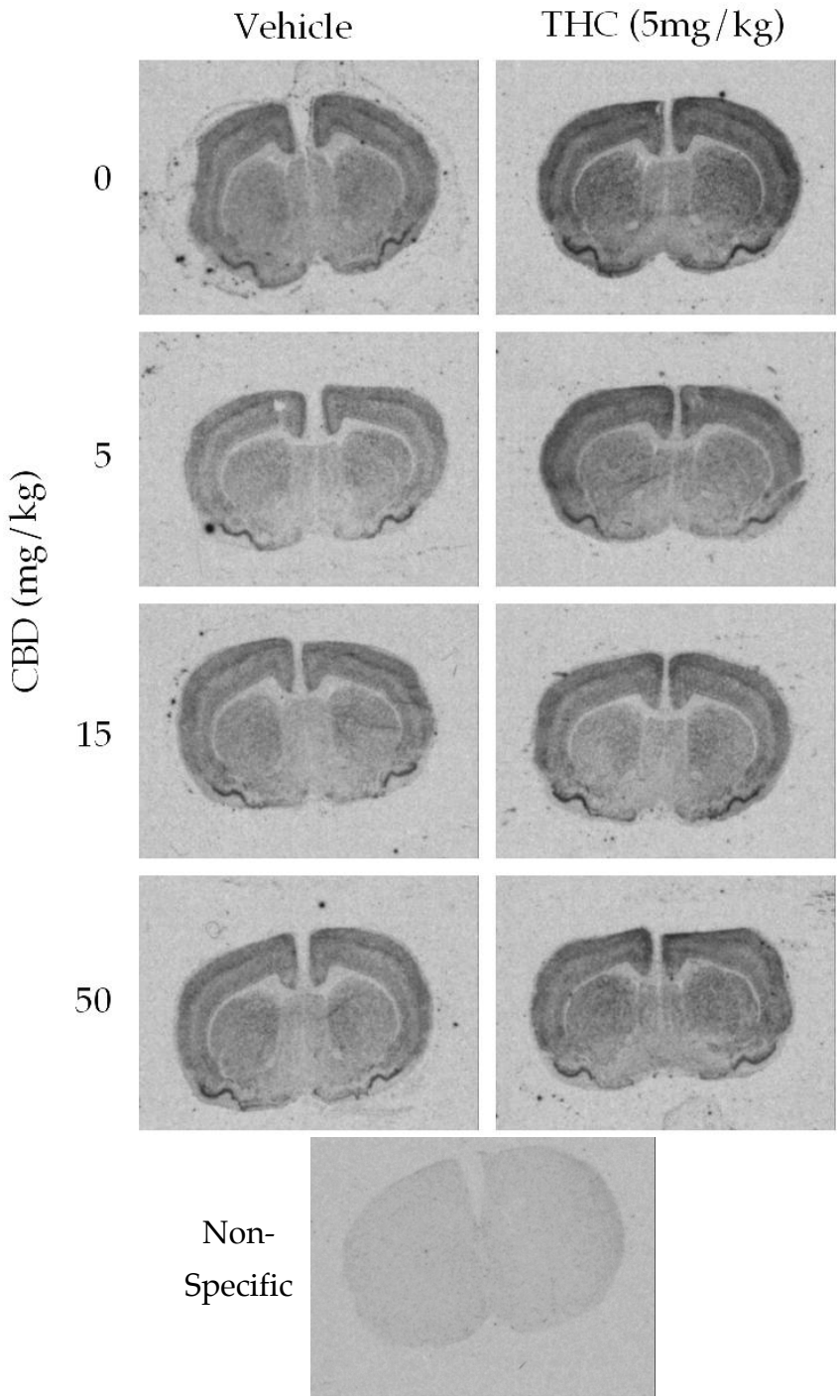


Figure 4.8 Autoradiogram illustrating the level of expression of *Zif-268* in response to CBD and THC treatment at the level of the striatum. These autoradiograms are representative images illustrating the levels of *Zif-268* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.2.2 Arc

Contrary to previous findings in our group, THC (5 mg/kg) did not significantly alter expression of *Arc* in either the caudate putamen or any of the selected cortical regions (Fig 4.9, A-F) when compared with control. There was a non-significant increase in expression of *Arc* in the caudate putamen (Fig 4.9, F) and agranular insular cortex (Fig 4.9, E). CBD (5, 15 or 50 mg/kg) was without effect either on its own or in combination with THC in all regions of interest compared with control.

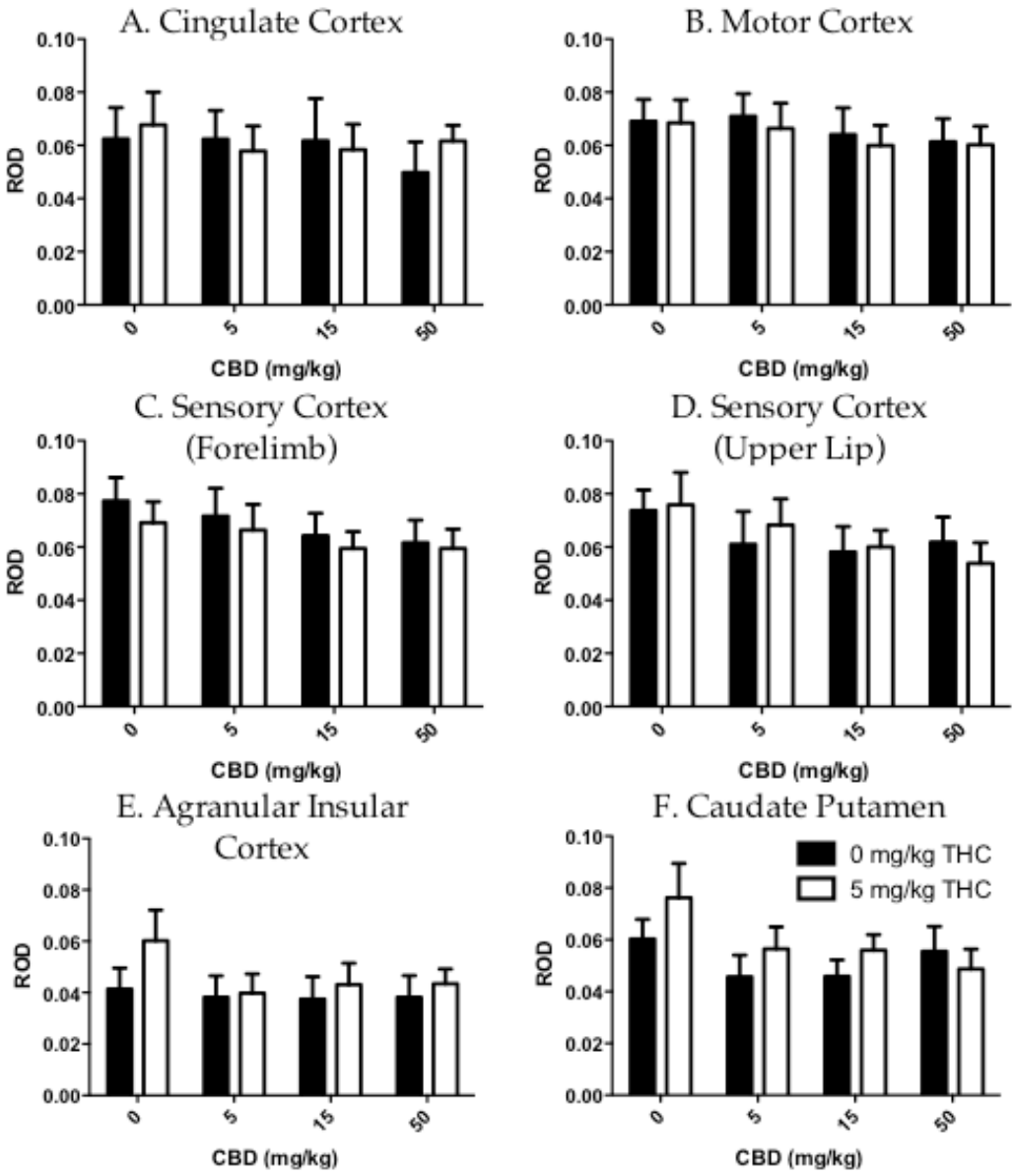


Figure 4.9 Effect of THC and CBD on *Arc* mRNA expression in striatal regions of the rat brain. These graphs illustrate results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *Arc* mRNA in the cingulate cortex (A), motor cortex (B), forelimb and upper lip region of the sensory cortex (C and D), agranular insular cortex (E) and caudate putamen (F). All data are expressed as mean ± S.E.M. relative optical density (ROD).

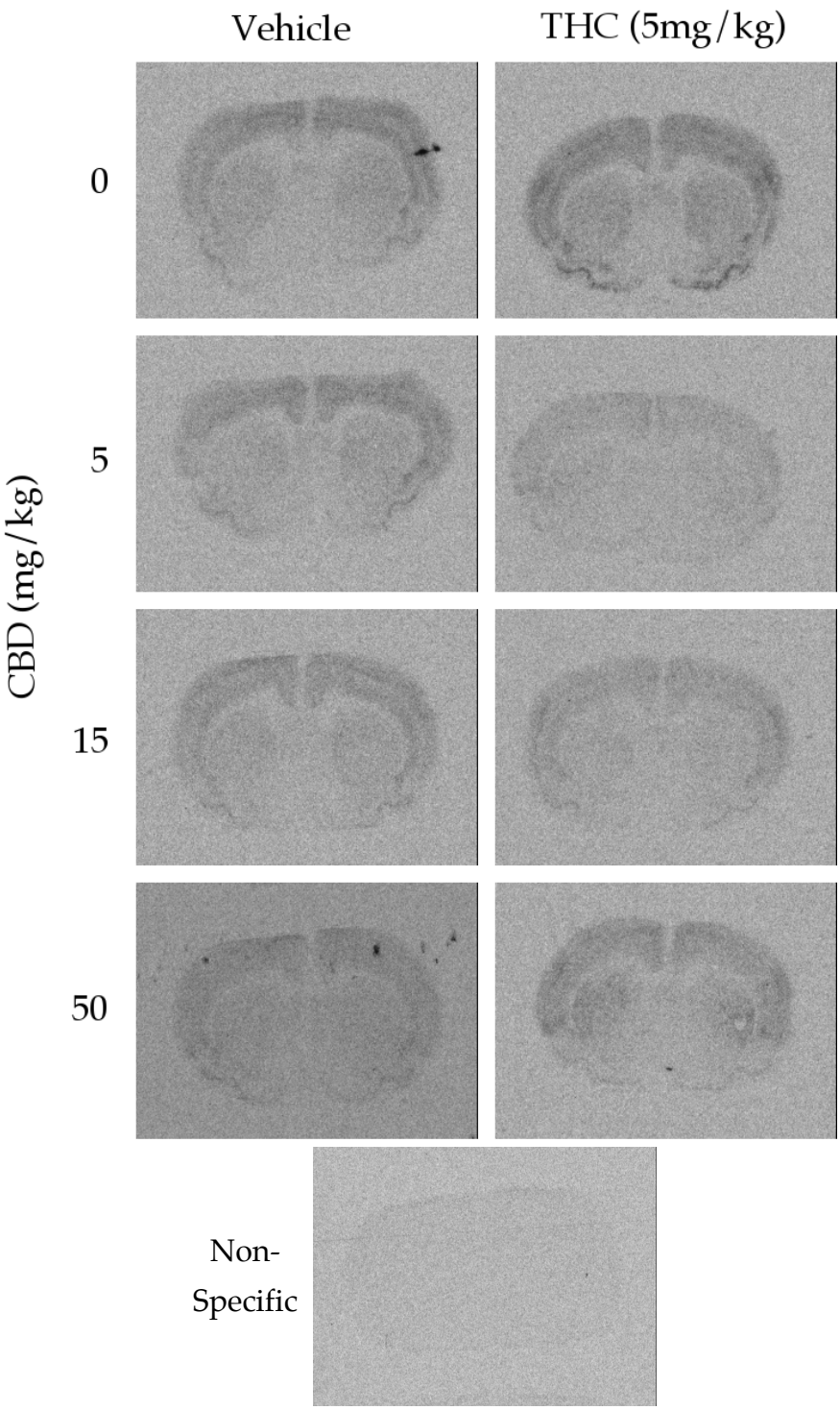


Figure 4.10 Autoradiogram illustrating the level of expression of *Arc* in response to CBD and THC treatment at the level of the striatum. These autoradiograms are representative images illustrating the levels of *Arc* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.2.3 c-fos

Similar to the effect THC administration had on *Zif-268* and *Arc* expression at this level, THC administration had no effect on *c-fos* expression in the posterior cingulate cortex (4.11, A), motor cortex (4.11, B), forelimb (4.11, C) and upperlip (4.11, D) regions of the sensory cortex and the caudate putamen (4.11, F) compared to control. There was a significant increase in *c-fos* expression in the agranular insular cortex of animals treated with THC compared to vehicle-treated animals (64.8 ± 30.75%; n=7; $p<0.05$; Fig 4.11, E). Animals treated with both THC and CBD (5, 15 or 50mg/kg) displayed no significant change in *c-fos* expression in the agranular insular cortex, suggesting CBD was blocking the ability of THC to induce expression in this region. CBD (5mg/kg) administration resulted in a trend in reduction in *c-fos* expression in all regions analysed in this region. Interestingly, CBD (15mg/kg) administration seemed to enhance *c-fos* expression in all these regions, which may suggest that CBD may have a biphasic effect on *c-fos* expression.

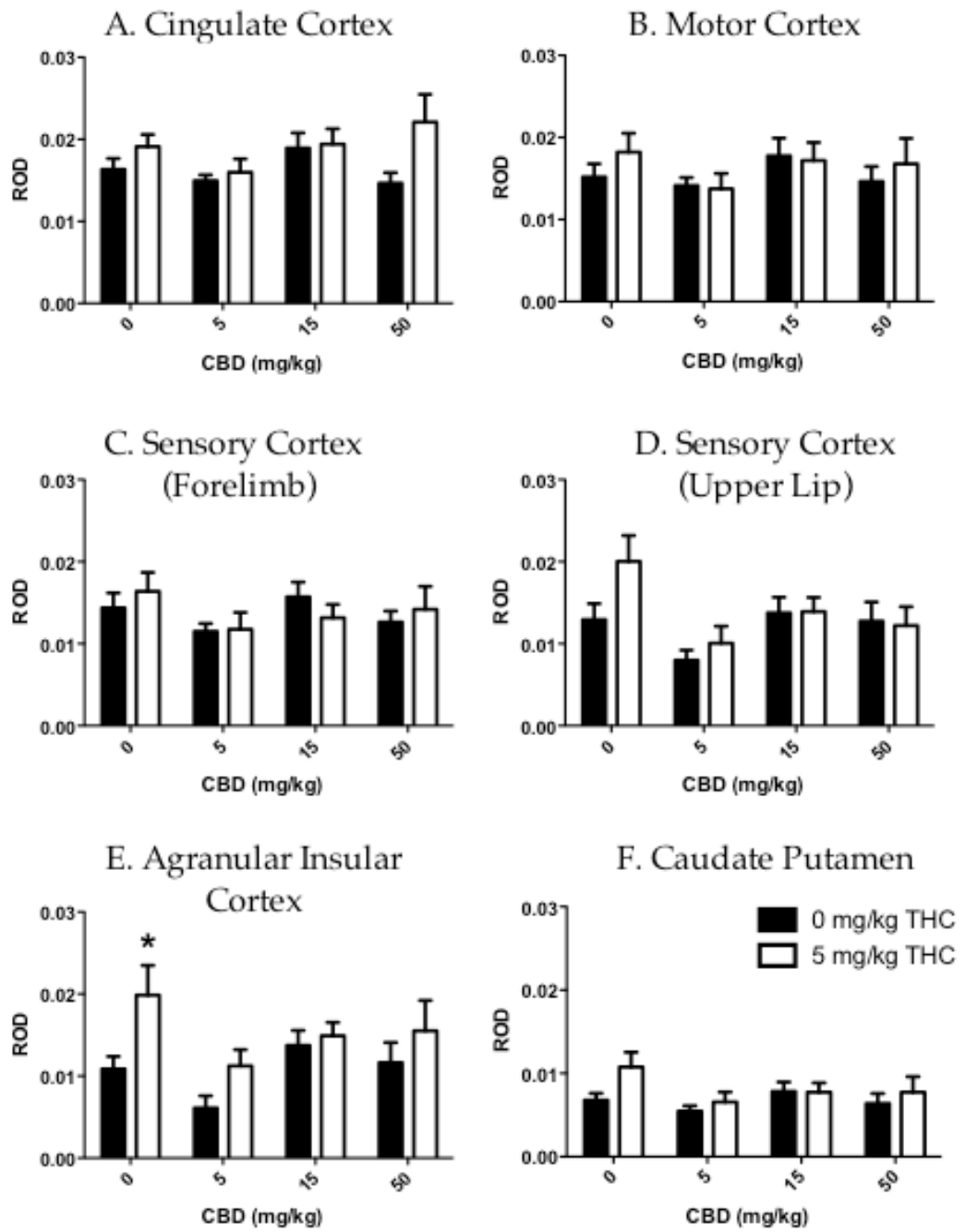


Figure 4.11 Effect of THC and CBD on *c-fos* mRNA expression in striatal regions of the rat brain. These graphs illustrate results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *c-fos* mRNA in the cingulate cortex (A), motor cortex (B), forelimb and upper lip region of the sensory cortex (C and D), agranular insular cortex (E) and caudate putamen (F). All data are expressed as mean ± S.E.M. relative optical density (ROD). * denotes significant difference between 0 and 5 mg/kg of THC ($p<0.05$).

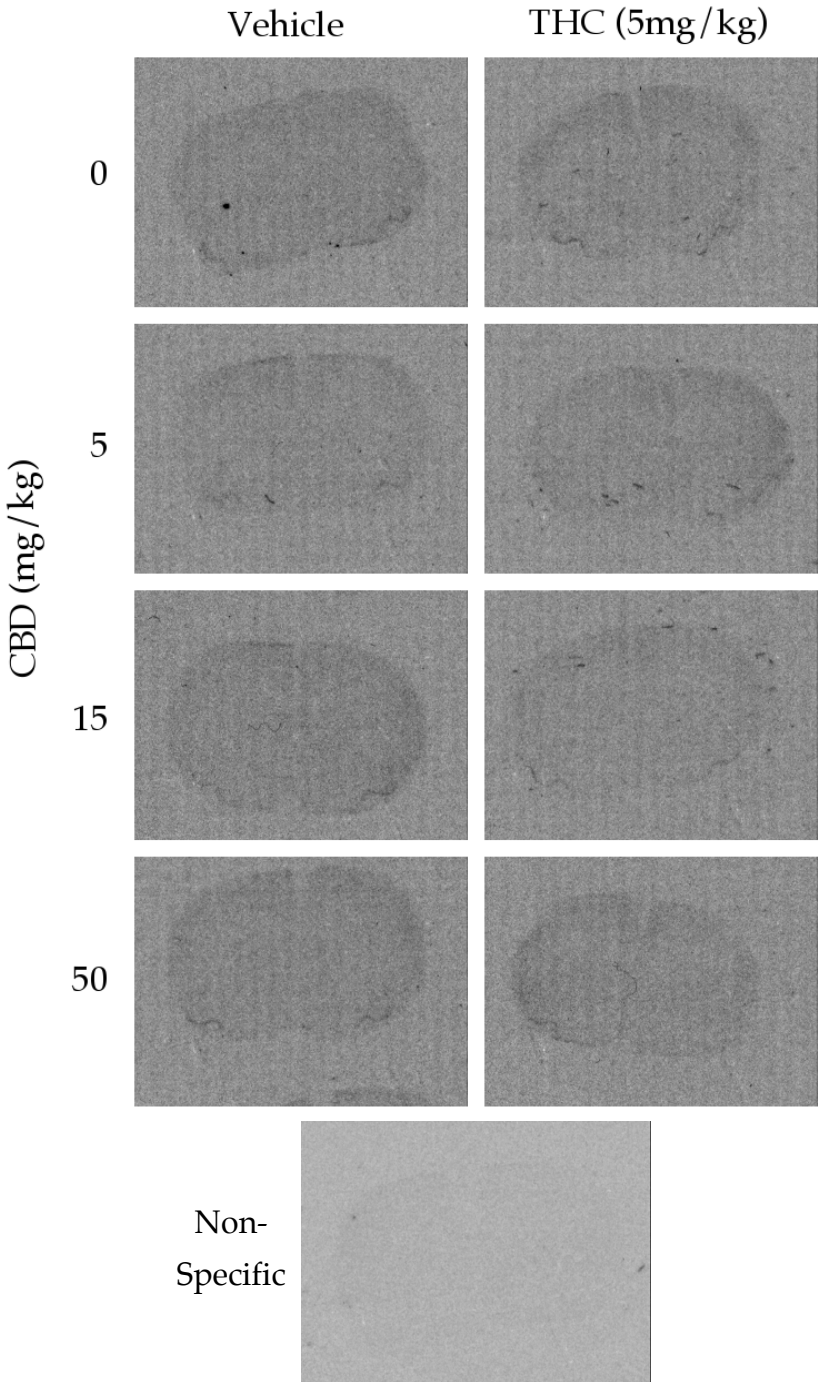


Figure 4.12 Autoradiogram illustrating the level of expression of *c-fos* in response to CBD and THC treatment at the level of the striatum. These autoradiograms are representative images illustrating the levels of *c-fos* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.3 Effects of CBD and THC on IEG induction in the Hippocampus

4.3.3.1 Zif-268

Neither THC (5 mg/kg) nor CBD (5, 15 or 50 mg/kg) administered independently or together, had any effect on expression of *Zif-268* expression in the CA1 or CA3 region of the hippocampus (Fig 4.13, A and B). This is surprising considering the ability of CBD and THC to alter hippocampal neuronal activity as discussed in the previous chapter.

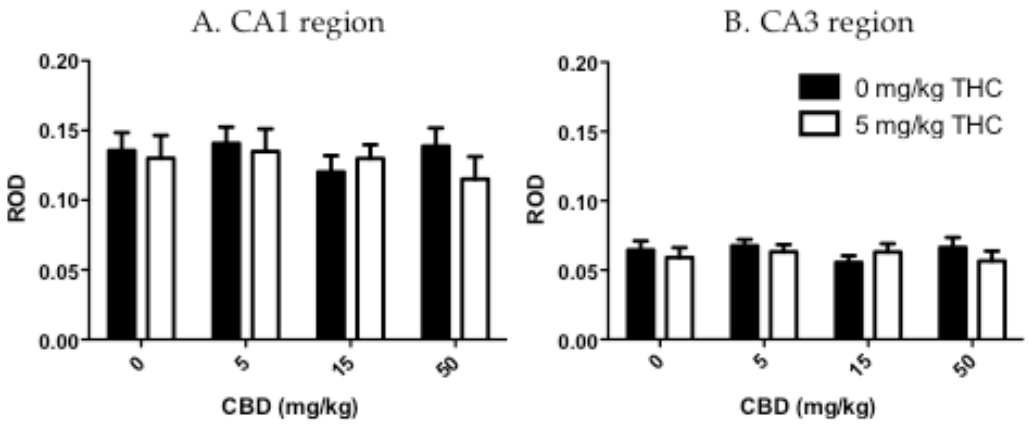


Figure 4.13 Effect of THC and CBD on *Zif-268* mRNA expression in the hippocampus of the rat brain. These graphs illustrate results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *Zif-268* mRNA in the CA1 (A) and CA3 (B). All data are expressed as mean ± S.E.M. relative optical density (ROD).

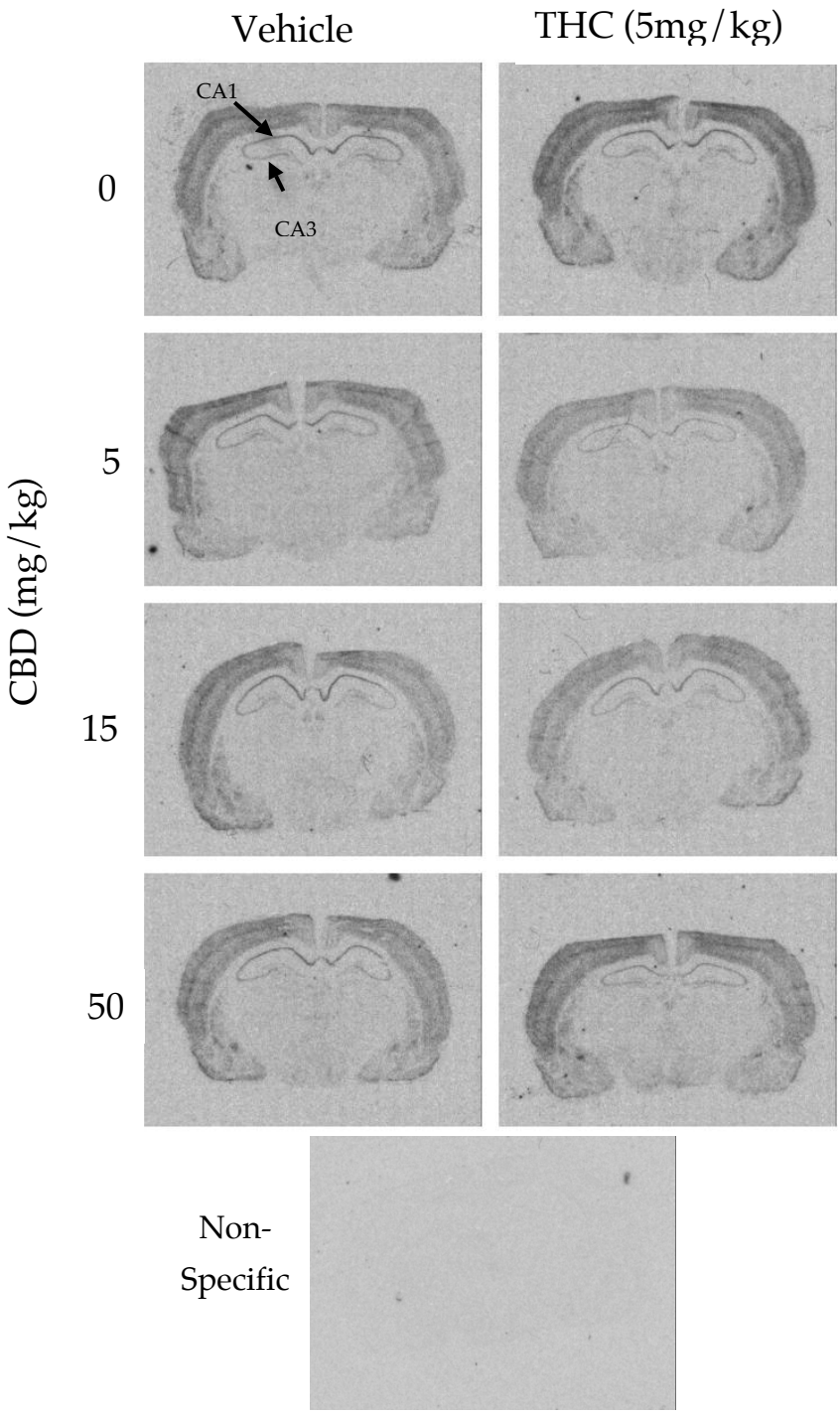


Figure 4.14 Autoradiogram illustrating the level of expression of *Zif-268* in response to CBD and THC treatment at the level of the hippocampus. These autoradiograms are representative images illustrating the levels of *Zif-268* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.3.2 Arc

THC (5 mg/kg) treatment also had little effect on *Arc* expression in either the CA1 or CA3 region of the hippocampus (Fig 4.15, A and B); this was also surprising considering previous data from our group. There was however a slight non-significant increase in expression *Arc* induced by CBD (5 mg/kg) in the CA1 region and by 50 mg/kg in the CA3 region.

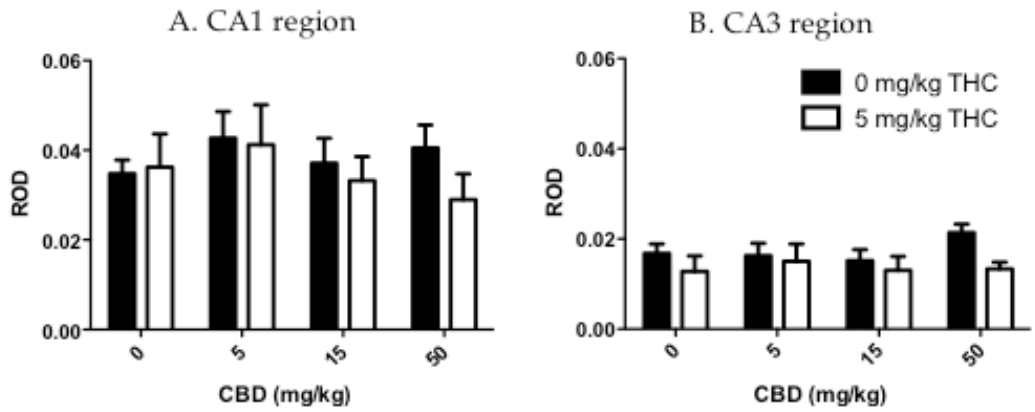


Figure 4.15 Effect of THC and CBD on *Arc* mRNA expression in the hippocampus of the rat brain. These graphs illustrate results of acute administration of CBD (0-50 mg/kg) on its own or co-administered with THC (0 or 5 mg/kg; black or white bars respectively) on expression of *Arc* mRNA in the CA1 (A) and CA3 (B). All data are expressed as mean ± S.E.M. relative optical density (ROD).

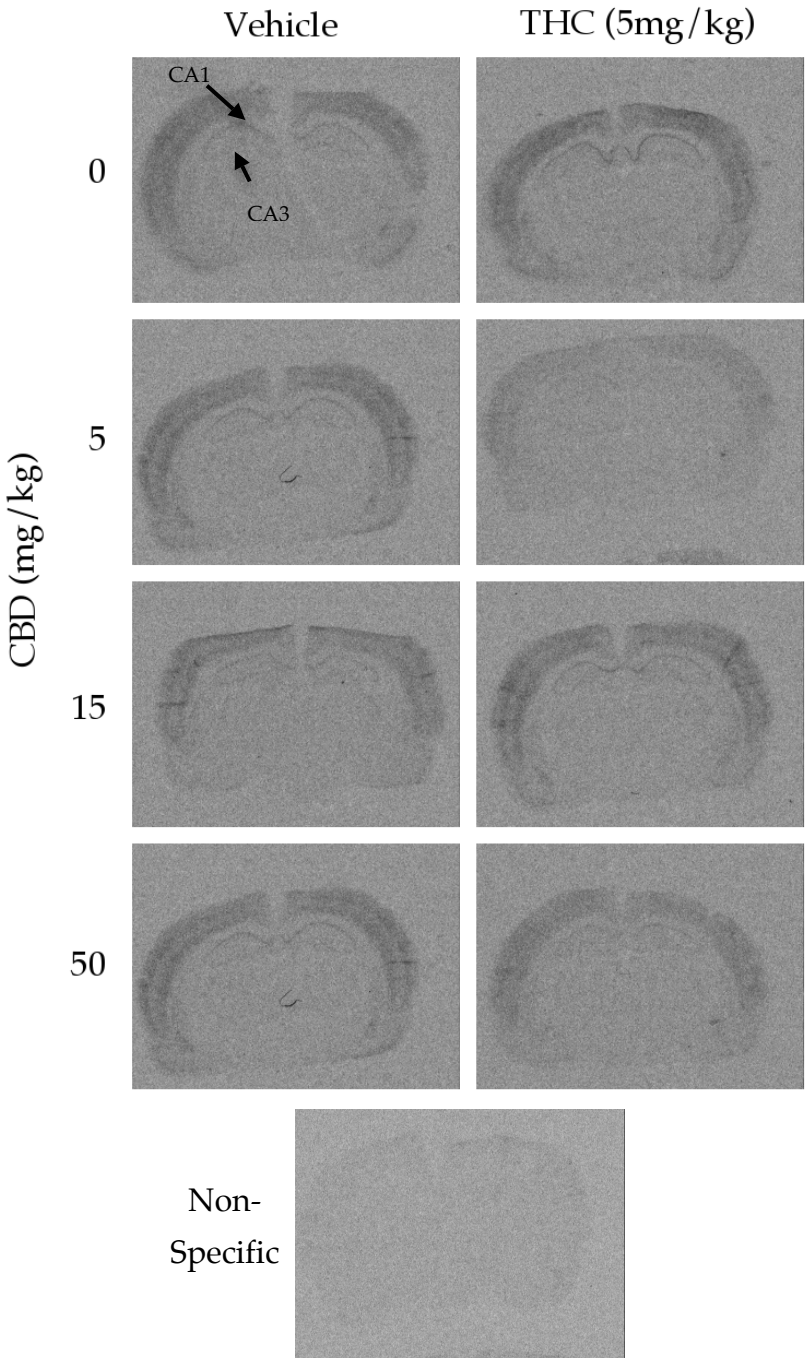


Figure 4.16 Autoradiogram illustrating the level of expression of *Arc* in response to CBD and THC treatment at the level of the hippocampus. These autoradiograms are representative images illustrating the levels of *Arc* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.3.3.3 c-fos

c-fos in general has a very low expression in the hippocampus (Fig 4.17) and although immunohistochemical studies have shown alterations of *c-fos* expression in this region, the resolution of in situ hybridisation technique along with the requirement for basal expression to identify regions of interest meant that it was impossible to accurately measure expression of *c-fos* in this region with any great level of accuracy.

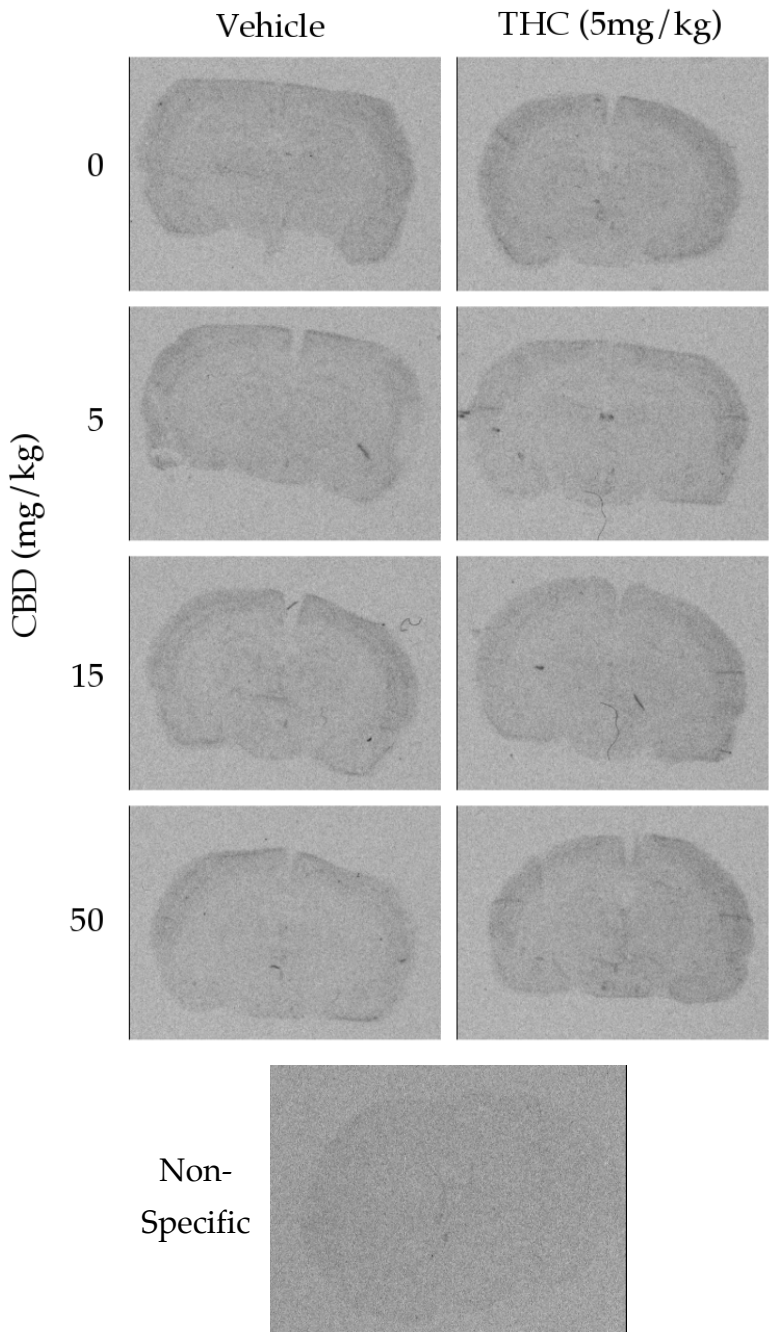


Figure 4.17 Autoradiogram illustrating the level of expression of *c-fos* in response to CBD and THC treatment at the level of the hippocampus. These autoradiograms are representative images illustrating the levels of *c-fos* expression following administration of CBD (0, 5, 15 or 50 mg/kg i.p.) with THC (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

4.4 Discussion

The main finding from the present study was that the significant increases in mRNA expression of *Zif-268* and *c-fos* in the cingulate cortex and caudate putamen and expression of *Zif-268* in the motor, orbital and agranular insular cortices following acute administration with 5 mg/kg of THC in were not present in animals receiving the same dose of THC co-treated along with CBD (5, 15 or 50 mg/kg). However despite trends suggesting effects of CBD on its own, it did not significantly alter any IEG expression in any region investigated. Although this study is not

4.4.1 THC and IEG expression: consistency with other studies

Some of the findings in the present study are consistent with findings previously published on THC-induced alterations in IEG expression (Shirayama et al., 1999). In the present study acute administration of THC (5 mg/kg) resulted in significant increases in *Zif-268* and *c-fos* mRNA expression in the cingulate cortex and caudate putamen 75 minutes after treatment. This is similar to the previous published findings of Mailleux *et al* (1994), who found significant increases in expression of *Zif-268* and *c-fos* in both the cingulate cortex and caudate putamen 20 minutes after administration of THC (5 mg/kg). Further to this, a study by Erdtmann-Vourliotis *et al* (1999) demonstrated a significant increase in *c-fos* in the caudate putamen 60 minutes after treatment with THC (25 mg/kg)(Erdtmann-Vourliotis et al., 1999). In addition to previously published findings, it is also noted here that acute administration of THC (5mg/kg) resulted in a significant increase in *Zif-268* expression in the agranular insular cortex, which is certainly pertinent due to its involvement in working memory, as well as the motor cortex and lateral and ventral orbital cortices.

Previously, it was shown by our group that THC (5 mg/kg) administration induced *c-fos*, *Zif-268* and *Arc* mRNA expression in the cingulate cortex, caudate putamen, which has been demonstrated in this thesis (Egerton, 2004). However, the previous study also noted several other changes in

mRNA expression that were not found in the data presented here. Notably, THC administration did not result in induction of Arc expression to the same degree as that shown by the previous study, which not surprising considering the variability in the Arc data (Section 4.4.3). THC administration did not alter expression of any IEG studied in the hippocampus, this is surprising considering the high levels of CB_1 receptor expression in this region and considering that both *Zif-268* and *Arc* expression was increased following THC in the study by Egerton (2004) in the CA1 region of the hippocampus. Also, considering the findings from the previous chapter (Chapter 3), where CBD inhibited synaptic transmission, CBD (5-15 mg/kg) administration *in vivo* did not alter expression the markers of excitatory activity, *Arc* and *Zif-268* in the hippocampus. Unfortunately, the effects of CBD on Zif-268 or Arc expression *in vitro* were not investigated therefore it could be reasoned that the effects of CBD may not be sufficient to alter the genomic response in hippocampal neurons.

4.4.2 Lack of THC and CBD induced changes in Arc expression

In this study neither THC nor CBD at any concentration affected expression of the effector IEG *Arc*. This was surprising considering it has been shown previously in our group that 5 mg/kg THC administration resulted in widespread increase in *Arc* expression in the regions measured in this study (Egerton, 2004). The first possible reason for this may be potential strain difference in the response to THC between the two studies. In the present study Lister hooded rats were used, where Long Evans rats were previously used in this group. However, the fact that the former did yield similar changes in other IEG's in the same regions to that seen in the latter which would suggest that strain effects of THC are not an issue. The second possible explanation is inconsistency at any stage of the experiment. All sections were hybridised at the same time, using the same probe and exposed to the same film which rules out variables in the latter stages of the experiment. However, this experiment was performed over two stages, with two batches of animals, the second batch being treated and the brains sectioned around 7 months after the first batch. Therefore batch differences of animals may be accountable for the lack of power in the data. It was also

noted that ROD of the first 10 animals treated was generally much higher than the rest of the animals regardless of treatment.

4.4.3 CBD attenuated THC-induced alteration IEG expression

Consistent with the hypothesis of this study CBD, especially at a concentration of 5 mg/kg, blocked the observed effects of THC (5 mg/kg) on expression of both *Zif-268* and *c-fos* in cingulate cortex and caudate putamen and the expression of *Zif-268* in the agranular insular cortex, motor cortex, lateral and ventral orbital cortices. It was recently demonstrated in humans that CBD and THC displayed opposing effects in the BOLD fMRI imaging in human subjects (Bhattacharyya et al., 2009b). These authors showed that acute THC or CBD administration resulted in a respective increase or decrease in regional brain activity in the striatum, amydala, hippocampus and occipital and superior temporal cortices. Furthermore when CBD was administered prior to THC in the same subject, there was no net effect. Similar to the authors in this study, the above data would lead one to conclude that this may underlie the nature of the interaction of THC and CBD and suggest that CBD may be protective of the acute effects of THC in a number of neuronal processes in the rat.

It was previously shown by Fadda et al (2004), that animals given extracts from cannabis which were rich in CBD (~50 mg/kg) performed normally in the Morris water maze, despite the extract containing around 4 mg/kg of THC, a concentration near that which induced deficits in performance in the same task (5 mg/kg). Despite the extracts containing other cannabinoids, which may have had an influence in these observed effects, the authors suggested that CBD was responsible for blocking the THC-induced disruption in performance in this spatial working memory task. The interaction of CBD and THC in the induction of *Zif-268* and *c-fos* expression in the anterior cingulate cortex, a region involved in adaptive responses and problem solving (Thomsen et al., 2010), may suggest functional interaction between THC and CBD in this region may be partly responsible for the effects of CBD on THC-induced deficits in tasks which this region plays a

major role. This is also true of the regional interaction between CBD and THC in the orbital cortices and agranular insular cortex which are also involved in cognitive processing and decision making in rats (Long et al., 2006).

These findings provide evidence for a regionally specific interaction between CBD and THC *in vivo* suggesting that CBD and THC have contradictory effects in the same neurons or neuronal networks. As previously discussed THC has been shown to increase release of the excitatory neurotransmitters glutamate and dopamine through activation of CB_1 receptors expressed preferentially on presynaptic terminals of GABAergic neurons, an effect that would result in *c-fos* and *Zif-268* induction via D1 and NMDA receptor activation as shown previously (Simpson and Morris, 1995). In the previous chapter, CBD was shown to inhibit evoked excitatory synaptic transmission. A possible explanation is that a downstream inhibitory effect of CBD on neurotransmission, through activation of presynaptic 5-HT_{1A} receptors, may cancel out any upregulation of excitatory transmission through a disinhibitory action of THC-induced CB_1 receptor activation on presynaptic GABAergic terminals. However, one must also consider other potential sites of action of THC and CBD. As previously discussed, THC acts as a partial agonist at a presynaptic receptor which suppresses excitatory transmission (Shen and Thayer, 1999) and a full agonist at receptors that suppress inhibitory transmission (Laaris et al., 2010). Partial agonism from exogenous application of THC may potentially alter endogenous activation of these receptors resulting in enhancement of glutamate release. Also CBD has been shown to act as an antagonist at CB_1 receptors (Thomas et al., 2007), which may support a more direct route of interaction between CBD and THC. However, in the current literature the effects of administration of CBD do not mimic that of CB_1 antagonists. Despite this, the potential of CBD to selectively modulate the ability of CB_1 receptors sensitivity to exogenous cannabinoids, by competing at a specific site on the receptor or by modulating the receptor allosterically, cannot be ruled out. There is also a suggestion that CBD administration may alter the pharmacokinetics of THC and other drugs of abuse. It was shown that pretreatment with CBD 30-60

minutes before THC administration led to a 3-fold increase in levels of THC in the brain. However, they also noted that when THC and CBD were administered at the same time, as they were in this study, there was little effect on brain THC levels (Reid and Bornheim, 2001). Further investigation would be required to investigate potential mechanisms of interaction *in vivo*. One possibility would be to investigate whether a selective 5-HT_{1A} receptor agonist or CB_1 receptor antagonist can replicate the action of CBD. An alternative would be to investigate whether CB_1 or 5-HT_{1A} receptor antagonists alter the ability of CBD to protect against THC administration-induced alterations in IEG expression

4.4.4 Functional implications

Alteration in function of the medial prefrontal cortex (mPFC), which is subdivided into the infralimbic, prelimbic, anterior cingulate, ventral orbital, lateral orbital and argranular insular cortices (Groenewegen and Uylings, 2000), has been shown to be involved in disruption of executive processing in tasks measuring working memory, behavioural flexibility and attention. As discussed in previous sections, acute administration of THC has been shown to alter the performance of animals and humans in tasks measuring executive processing. Disruptions of performance in these tasks have also been correlated with the ability of THC to alter neuronal activity in this region (Egerton et al., 2006).

In this study THC administration resulted in variable changes in expression of mRNA encoding *Zif-269*, *Arc* and *c-fos*. However, a general trend of an increase in IEG expression was noted in most divisions of the prefrontal cortex consistent with that shown previously (Mailleux et al., 1994; McGregor et al., 1998; Porcella et al., 1998; Egerton et al., 2006). It was also found in this study that co-administration of CBD with THC did not result in any change in expression of these markers of neuronal activity suggesting that CBD has the potential to cancel out THC-induced alteration in executive function. This coupled with the findings of human studies (Bhattacharyya et al., 2009b) provides evidence that CBD functionally interacts with THC in a regionally specific manner. These findings would strengthen the hypothesis that CBD protects against THC-induced cognitive deficits. Although this is an acute study it is reasonable to presume that if CBD prevents THC from having an acute effect through a complementary mechanism, it should prevent chronic administration of THC inducing long-term effects that resemble the cognitive deficits similar to that found in schizophrenia. Despite a trend for a reduction, CBD administration had no significant effect on the expression of any IEG in the PFC on its own. However, this was administered to naïve rats and it would be interesting to investigate the effect of CBD on alterations in

regional expression of IEGs that have been induced through a non-CB_1 mediated mechanism to rule out a direct interaction between CBD and THC.

Despite an isolated study by Fadda *et al* (2004), which illustrated a potential for CBD to inhibit THC-induced impairment in measure of working memory, the ability of CBD to inhibit disruption induced by THC in behaviours dependent on PFC function has not been studied. This may be a worthwhile investigation in the future and the data presented in this thesis would suggest that CBD would inhibit THC-induced disruptions in these tasks.

CHAPTER 5: EFFECT OF CBD ON REGIONAL CHANGES IN NEURAL MARKERS OF ACTIVITY INDUCED BY PCP

5.1 Introduction

5.1.1 Phencyclidine as a psychotomimetic agent

The NMDA receptor antagonist phencyclidine (PCP), and more recently ketamine are common drugs of abuse. PCP was widely used as an anaesthetic up until 1963, where patients reported experiencing hallucinations, paranoia and disorientation following treatment with the drug (for review see Steinpreis, 1996). Despite being an illegal substance, ketamine is still used in clinical research due to its ability to produce psychotomimetic symptoms similar to that found in patients suffering from schizophrenia. This has led to many groups utilising NMDA receptor antagonists in animal models of schizophrenia and the PCP models of schizophrenia are one of the most widely used pharmacological models of psychosis.

5.1.1.1 Pharmacology of PCP

PCP acts principally as a non-competitive NMDA receptor antagonist binding to the PCP binding site of the ionotropic glutamate receptor (Anis et al., 1983). The ability of PCP to block glutamatergic activity in synaptic transmission has led many to propose that glutamate hypofunction is a mechanism of pathogenesis in psychosis. This has been strengthened by findings suggesting mutations in genes encoding proteins involved in glutamate synthesis, release, deactivation and even the NMDA receptor itself as risk factors in developing psychoses (for review see Harrison and Owen, 2003). However, given that the specific NMDA antagonist MK-801 does not induce the same psychotomimetic effects as PCP in animal studies (Seillier and Giuffrida, 2009), it could be suggested that PCP induced psychosis may be mediated via an alternative site of action of the drug. PCP has also been shown to activate the σ_1 receptor, a receptor once thought to belong to the opioid receptor family (Sharp, 1997). Furthermore, σ_1 has been implicated in both humans and animals as a potential therapeutic target for treating the symptoms of psychological disease.

5.1.1.2 Effect of PCP in humans

Due to its wide use as both an illegal drug and as an anaesthetic the effects of PCP on human behaviour have been widely characterised. Administration of PCP in humans results in positive and negative symptoms and cognitive deficits similar to that found in schizophrenia patients. These included: positive symptoms such as auditory hallucinations, distortion of "self"-image, depersonalisation and paranoia, which have been well documented in individuals using the substance; negative symptoms, such as flattened emotional response, alogia, anhedonia and avolition; and disruption in cognitive behaviour, such as in attention and working memory (Smith et al., 1978; Smith and Wesson, 1980; Nicholi, 1984). At large doses, PCP administration results in catalepsy, sedation, convulsions and anaesthesia (for review see Domino, 1980). PCP also produces a disinhibitory and euphoric effect, which has made it popular as drug of abuse. As well as displaying in normal individuals symptoms similar to that experienced by schizophrenic patients, PCP has also been shown to exacerbate symptoms in schizophrenic patients (Itil et al., 1967). This has led many to propose that the mechanism of action of PCP may be related to the pathogenesis of schizophrenia. PCP is a NMDA receptor antagonist and this, and the action of similar drugs, such as ketamine, has formed the basis of the glutamate hypofunction hypothesis of the disease (for review see Javitt, 2007).

5.1.1.3 Effect of PCP in animals

As well as its well-characterised effects in humans, PCP produces a wide range of behavioural effects in animals ranging from altered locomotor activity to stereotypy such as ataxia, head bobbing, backward-walking, circling and head swaying (Sams-Dodd, 1996; Steinpreis, 1996). Although the positive psychological symptoms can be difficult to measure in animals, although it is argued that stereotyped behaviour is an indicator of the positive symptoms experienced by animals, the ability of psychotomimetic drugs to induce negative symptoms and disrupt performance in cognitive tasks has been studied extensively. PCP has been shown to dose-dependently induce social withdrawal, a behavioural correlate to the negative symptoms exhibited by schizophrenia patients (Sams-Dodd, 1996).

Acute and sub-chronic PCP administration has been shown to produce delayed effects on affective flexibility in an attention set-shifting task, in which animals treated with PCP (2.56 mg/kg) are unable to shift between perceptual dimensions (Egerton et al., 2005b; Egerton et al., 2008). This deficit in extra-dimensional set shifting in rats treated with PCP is comparable to that experienced in schizophrenia patients and was also correlated with altered neuronal activity in the prefrontal cortex. Furthermore, repeated (but not acute) treatment with PCP has been shown by several groups to induce deficits in performance in the Morris water maze, a test of spatial working memory (Kesner et al., 1993; Filliat and Blanchet, 1995; Kesner and Dakis, 1995; Beraki et al., 2008). However, it is proposed that acute administration of PCP does not induce impairment in spatial working memory because the dose required for altering behaviour in this task alters locomotor activity, which confounds performance in these tasks. PCP has also been shown to induce deficits in prepulse inhibition (PPI) in a variety of rodent studies (Egerton et al., 2008). PPI is a model of sensorimotor gating where an animals startle response following a stimulus (acoustic, light or tactile) is inhibited by administering a weaker prestimulus. This finding provides a direct link between the effects of PCP in animals and humans suggesting a common mechanism of action in both species. This PPI deficit is also present in animals treated with another NMDA receptor antagonist, MK-801, suggesting that PCP is functioning via NMDA receptor antagonism to disrupt sensorimotor gating. It is also worth noting that although chronic PCP treatment can result in long term deficits in performance in a number of cognitive tasks, PCP induced deficits in PPI are not sustained 72 hours after withdrawal of drug administration (Egerton et al., 2008), suggesting that deficits in PPI directly relate to NMDA receptor blockade and not through long term compensatory modulation of neuronal function.

5.1.2 Effect of PCP on regional neuronal activity

5.1.2.1 Effect of PCP on regional brain activity

Understanding the mechanism of action of PCP and brain regions affected by the drug can give a good indication of possible mechanism for psychoses. There has been very little investigation into the effect of PCP on regional brain activity in humans. However, functional brain imaging of patients administered with a similar drug, ketamine, suggest that NMDA receptor antagonism results in an increase in regional blood flow in the frontal cortex, anterior cingulate cortex and insula in humans (Langsjo et al., 2003). A further study demonstrated that ketamine administration resulted in decreases in the orbitofrontal cortex and subgenual cingulate and increases in the posterior cingulate cortex, thalamus and temporal cortical regions when measuring regional blood oxygen level-dependent (BOLD) signal by fMRI (Deakin et al., 2008). Ketamine administration was also shown to result in attenuation of increases in activity in the posterior cingulate cortex whilst patients performed a verbal memory task (Northoff et al., 2005). Interestingly, despite administration of ketamine increasing incidence of psychosis in these subjects, there was no significant alteration in performance in the cognitive task.

Most functional brain imaging studies involving PCP have therefore been conducted in animals. Acute PCP administration was shown to significant increase LCGU in the prelimbic and cingulate cortices, hippocampus and thalamus as well as sensory and motor cortices (Tamminga et al., 1987; Weissman et al., 1987). Interestingly, following subchronic or chronic PCP treatment (2.56 mg/kg; 5 days sequential or 25 days chronic intermittent respectively) a reduction in LCGU was shown in the prefrontal cortical regions of the rat brain 3 days after the last treatment (Cochran et al., 2003; Pratt et al., 2008), an effect that has been proposed to model "hypofrontality" found in schizophrenia patients (for reviews see Morris et al., 2005; Pratt et al., 2008)

5.1.2.2 Effect of PCP on IEG expression

As well as altering LGCU, the effects of PCP on regional induction of genes associated with neuronal activity have also been widely studied. Nakki et al (1996) were the first to analyse the effect of PCP (10 or 50 mg/kg; i.p.) on mRNA expression of several immediate early genes in rat. They found that 50 mg/kg PCP induced expression of most of the genes studied, including *c-fos* and *Zif-268,* in the prefrontal cortex, CA1 and CA3 regions and dentate gyrus of the hippocampus and the caudate putamen 30 minutes following treatment (Nakki et al., 1996). Furthermore, these authors observed a delayed onset of increased expression of these genes in the cingulate cortex 2 hours post PCP administration, suggesting that PCP activates the cingulate cortex indirectly. Those animals treated with a lower dose, 10 mg/kg, displayed lower levels of IEG induction; increases in mRNA expression were restricted to the prefrontal cortical regions. Gao et al (1998), using a similar approach, showed that 0.86 and 8.6 mg/kg PCP (i.p.) increased *c-fos* mRNA expression 1 hour after treatment in the prefrontal, dorsolateral, parietal, anterior and posterior cingulate cortices. PCP at 8.6 mg/kg also increased *c-fos* expression in the sensorimotor cortices. These effects reduced gradually over 6 hours post-treatment and were completely absent after 24 hours (Gao et al., 1998). The effects observed in this study were comparable to the selective NMDA antagonist, MK-801, which would suggest that these effects are mediated via the activity of PCP at this site. However, a previous study had indicated that PCP (40 mg/kg i.p.) induces Fos expression in the parietal and cingulate cortex by acting via σ_2 receptors, due to the induction of Fos by PCP being blocked with rimcazole, a selective σ_2 receptor antagonist (Sharp, 1997). PCP was also shown to give a dose-dependent increase in *Zif-268* expression in the retrosplenial cortex at doses ranging from 12.5 to 50 mg/kg (i.p.). There have also been several studies looking at the effects of PCP administration on *Arc* mRNA expression. Acute PCP (10 mg/kg; s.c.) administration resulted in increased expression of *Arc* in the prefrontal, posterior cingulate cortex and nucleus accumbens (Nakahara et al., 2000). This was later corroborated by a study in which a single dose of PCP (7.5 mg/kg; s.c.) also resulted in an increase in *Arc* mRNA in these regions

(Gotoh et al., 2002). Thomsen *et al* (2010) demonstrated that a sub-chronic treatment of PCP (5 mg/kg/day for 5 days) resulted in an increase in *Arc* expression in the medial prefrontal cortex, ventral orbital and lateral orbital cortices and the accumbens shell (Thomsen et al., 2010).

In summary, there are marked differences in IEG expression following administration of a range of different doses of PCP. Olney et al (1989) have shown NMDA receptor antagonism by PCP (ED_{50}= 2.83 mg/kg s.c.) or MK-801 (ED_{50}= 0.18 mg/kg s.c.) to be neurotoxic in the posterior cingulate cortex (Olney et al., 1989). This may be due to NMDA receptor blockade on GABAergic neurons thereby leading to an enhanced glutamate release from pyramidal cells. It has been suggested by the authors that lower doses of PCP that are not neurotoxic might be more relevant to schizophrenia.

5.1.3 CBD as a potential antipsychotic drug

As well as the potential for CBD to reverse the psychotomimetic effects of THC, several investigators have suggested that the anti-psychotic effect of CBD may not be limited to modifying THC-induced effects. As discussed in the introductory chapter of this thesis (Section 1.4.2), cannabis use or administration of THC results in psychotomimetic effects similar to that experienced in schizophrenia patients. As discussed in the previous sections of this chapter, PCP also shares the ability of THC to induce schizophrenia-like behaviour in humans. Considering that there is a potential ability of CBD to inhibit the psychotomimetic effects of THC, abuse of which is believed to be a risk factor for schizophrenia-like psychosis, CBD may also have the potential to inhibit the psychotic effects of other psychotomimetics, such as PCP.

Two small studies have suggested that CBD may be a novel treatment for patients suffering from schizophrenia, although these studies are very preliminary in nature as there were only 1-3 subjects studied (Zuardi et al., 1995; Zuardi et al., 2006a; Zuardi et al., 2006b). As well as this, CBD has also been shown to inhibit disruption of PPI in animals treated with MK-801 (Long et al., 2006). In this case, however, MK-801 was shown to be acting via

TRPV1 receptors. CBD has also been shown to block the reuptake and degradation of anandamide, which has also been shown to activate TRPV1 receptors (Bisogno et al., 2001). It could therefore be proposed that CBD is acting indirectly at TRPV1 receptors by blocking the removal of anandamide from the synaptic cleft and therefore allowing activation of TRPV1 by anandamide. This could be further enforced by looking at the study by Vigano et al (2009) who showed that blocking degradation of endocannabinoids using AM404, an anandamide reuptake and FAAH inhibitor, reversed PCP-induced disruptions in a novel object recognition task (Vigano et al., 2009). However there is evidence to suggest that AM404 also binds to TRPV1.

These data suggest that CBD may be able to reverse NMDA receptor-mediated sensorimotor gating and cognitive deficits, and supports that idea that CBD may have antipsychotic properties and potentially pro-cognitive effects. However the brain regions responsible for these effects are not clear. Therefore the aim of the present study are to identify brain regions affected by the psychotomimetic agent PCP and assess the ability of CBD to modify these changes or evoke changes on its own.

5.2 Methods

5.2.1 Animals

Male hooded Lister rats (Harlan) were purchased at a weight of 250-300g and housed in a temperature-regulated room with a 12 hour light/dark cycle with ad libtum access to food and water. Animals were kept for around 7 days in their home cage prior to experiments. In all experiments handling was kept to a minimum, animals were weighed and marked the day prior to experiments.

5.2.2 Drug administration

Drug groups were randomly allocated using a random number generator function in Excel (Microsoft). There were 4 treatment groups allocated (n=10) and each animal received 2 concomitant i.p. injections of CBD (5 :

	1st Injection	2nd Injection
Group 1	Vehicle	Vehicle
Group 2	Vehicle	CBD (5 mg/kg)
Group 3	PCP (5 mg/kg)	Vehicle
Group 4	PCP (5 mg/kg)	CBD (5 mg/kg

Dose selection for PCP (5 mg/kg) was a medial dose based on studies by Gao et al (1999) and Egerton et al (2005). The former demonstrated widespread changes in expression of *c-fos* and *Zif-268* in response to PCP administration at a dose of 8.6 mg/kg (i.p). The latter found that administration of 2.56 mg/kg PCP altered performance in an attention set-shift task.

5.2.3 In situ hybridization

This was performed as described in sections 2.3 and 4.2.3. Briefly, 75 minutes following drug administration animals were sacrificed by cervical dislocation and decapitation. Brains were removed and frozen before being sectioned using a cryostat. Sections were fixed in paraformaldehyde and dehydrated in ethanol. A hybridisation mix containing a radiolabelled 45-mer oligonucleotide probe with a complementary sequence to mRNA encoding *Arc, c-fos* and *Zif-268* was applied to sections and incubated at 42°C

overnight. Sections were washed, dehydrated and exposed to an X-Ray film for 5-10 days (depending on radioactivity). Films were than developed using a X-OMAT developer.

5.2.4 Densitometry and analysis

Developed X-Ray films were analysed using the MCID (Imaging Research Inc) densitometer software package. Anatomical regions of the brain were identified using a stereotaxic brain atlas (Paxinos and Watson, 2007). The relative optical density (ROD) was recorded for each selected region for both hemispheres. These readings were corrected for non-specific binding by subtracting the ROD for readings taken from the sections that were hybridised with an excess of unlabelled oligonucleotide.After receiving their injections animals were returned to their home cage for 75 minutes after which they were killed by cervical dislocation followed by decapitation. One–way analysis of variance (ANOVA) was applied to analyse the effects of administration of the different drug treatment groups on mRNA expression in each of the regions of interest. Where one-way ANOVA revealed significant effects of treatment group in mRNA expression levels, subsequent Students' Newman-Keuls post-hoc tests were employed to detect the source of significant effects. In all cases, statistical significance was defined as $p < 0.05$. This analysis was performed using GraphPad Prism 5 software.

5.3 Results

5.3.1 Effects of CBD and PCP on IEG induction at the level of the prefrontal cortex

At the level of the prelimbic cortex, as illustrated in Figure 5.1-5.6, CBD and PCP resulted in some regionally specific changes in expression of *Zif-268, Arc* and *c-fos. A* one-way ANOVA of the data obtained from regions of the prefrontal cortex revealed significant changes in all IEGs in some of the regions analysed (Table 1).

	Zif-268	***Arc***	***c-fos***
Prelimbic Cortex	F(3,34) = 1.858; p= 0.1553	F(3,34) = 2.634; p=0.0656	F(3,34) = 3.208; p=0.032*
Infralimbic Cortex	F(3,31) = 0.3872; p=0.7630	F(3,34) = 3.579; p=0.0237	F(3,34) = 4.021; p=0.0150*
Cingulate Cortex	F(3,34) = 2.809; p=0.0542	F(3,34) = 1.572; p=0.2141	F(3,34) = 0.5138; p=0.6755
Motor Cortex	F(3,34) = 5.030; p=0.0054*	F(3,34) = 1.975; p=0.1363	F(3,34) = 0.4834; p=0.6961
Lateral Orbital Cortex	F(3,34) = 0.8669; p=0.4677	F(3,34) = 2.158; p=0.1111	F(3,34) = 2.427; p=0.0824
Ventral Orbital Cortex	F(3,34) = 0.6727; p=0.5181	F(3,34) = 4.002; p=0.0153	F(3,34) = 2.474; p=0.0782
Agranular insular cortex	F(3,34) = 0.5815; p=0.6312	F(3,34) = 2.809; p=0.2821	F(3,34) = 1.067; p=0.3762

Table 1. The F and p values obtained from the one–way ANOVA for each IEG in respective subanatomical regions in the prefrontal cortex. * denotes a significant change detected by ANOVA ($p < 0.05$).

5.3.1.1 Zif-268

Contrary to previous findings by Gao et al (1998), PCP (5 mg/kg), either on its own or in combination with CBD (5 mg/kg), had no significant effect on expression of *Zif-268* mRNA 75 minutes after administration in any region analysed within the prefrontal region of the rat brain when compared to a

vehicle control (0.9% saline). However, the data does suggest a trend for a PCP-induced reduction in *Zif-268* expression in the prelimbic (Fig 5.1 A), motor (Fig 5.1 D), anterior cingulate (Fig 5.1 C), lateral orbital (Fig 5.1 F) and ventral orbital cortices (Fig 5.1 E). As in the previous chapter, a trend is apparent for CBD (5 mg/kg) to depress *Zif-268* expression in the prelimbic and cingulate cortices as well as the agranular insular cortex, with CBD administration resulting in a significant decrease in *Zif-268* expression in the motor cortex (31.0 ± 10.7%; n=9; $p<0.05$; Fig 5.1, D) compared with vehicle control. Interestingly, there was a significant increase in expression of *Zif-268* mRNA in the motor cortex of animals treated with both PCP and CBD compared to those animals treated with CBD on its own (49.5 ± 8.1 %; n=9; $p<0.001$; Fig 5.1, D). This would suggest that the NMDA receptor antagonist is blocking the CBD-induced reduction in the motor cortex.

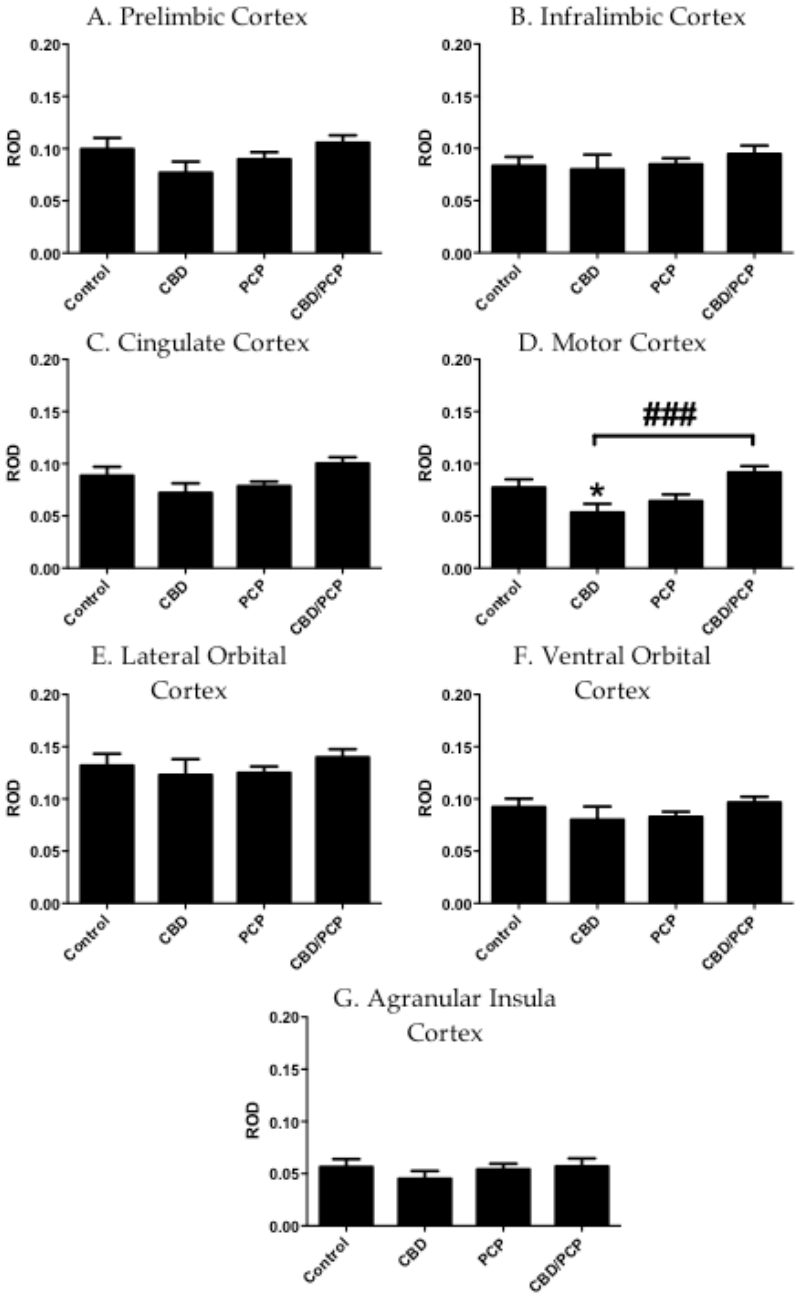


Figure 5.1 CBD administration results in limited reductions in *Zif-268* mRNA expression in prefrontal cortical regions of the rat brain. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *Zif-268* mRNA in the prelimbic (A), infralimbic (B), cingulate (C), motor (D), lateral orbital (E) or ventral orbital (F) cortices or in the agranular insular cortex (G). All data are expressed as mean ± S.E.M. relative optical density (ROD). * denotedenotes significant difference between drug treated and control animals s significant difference between 0 and 5 mg/kg of THC ((two injections of saline; $p<0.05$). ### denotes a significant difference between CBD and groups treated with both CBD and PCP ($p<0.001$).

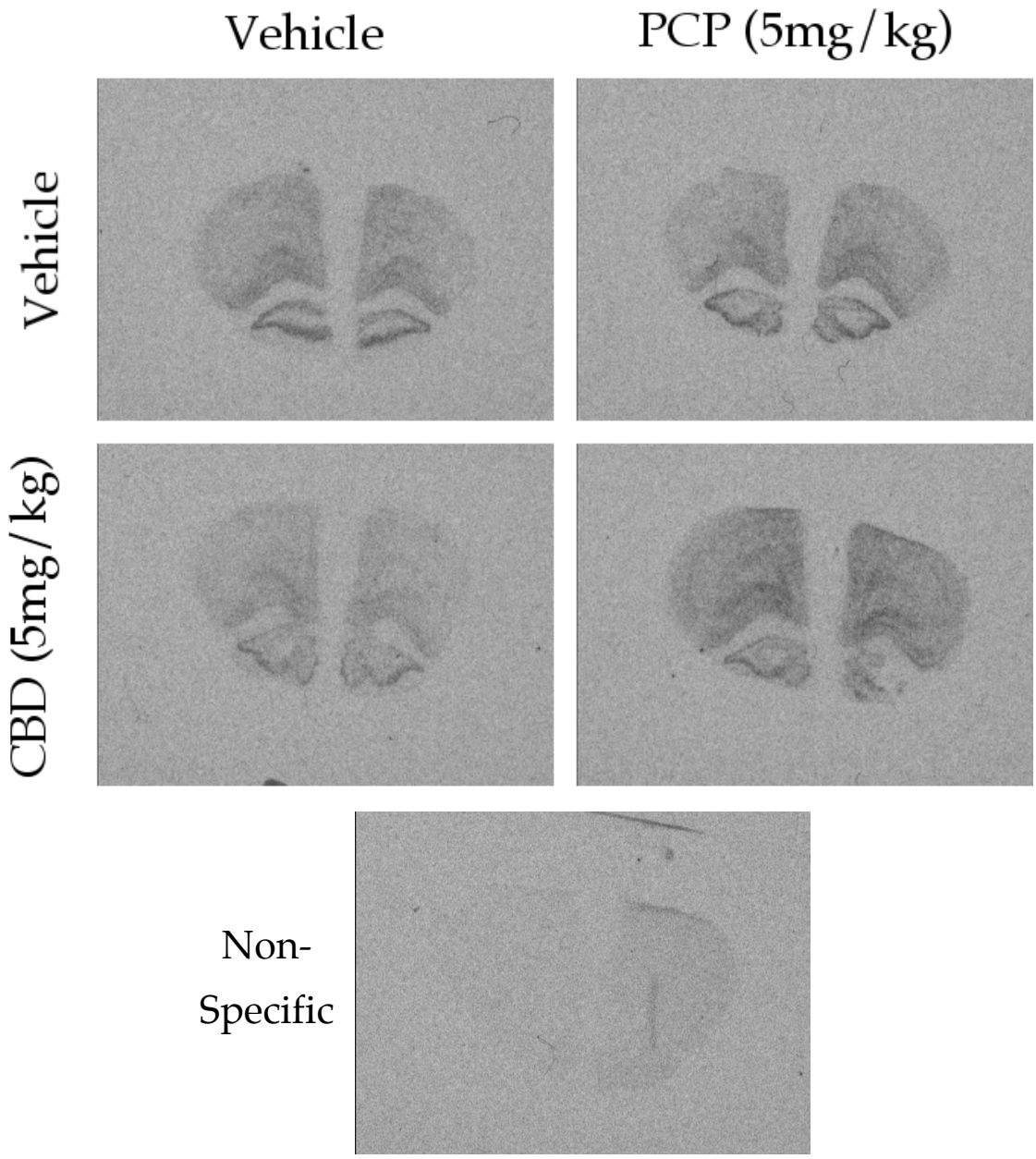


Figure 5.2 Autoradiograms illustrating the level of expression of *Zif-268* in response to CBD and PCP treatment at the level of the prefrontal cortex. These autoradiograms are representative images illustrating the levels of *Zif-268* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.1.2 Arc

As with *Zif-268*, PCP did not induce any significant changes in expression of *Arc*. However, CBD (5 mg/kg) administration resulted in a significant decrease in *Arc* expression in prelimbic (14.6 ± 8.3%; n=9; $p<0.05$; Fig 5.3, A), infralimbic (22.9 ± 6.6%; n=9; $p<0.05$; Fig 5.3, B), lateral orbital (26.9 ± 7.9%; n=9; $p<0.05$; Fig 5.3, E) and ventral orbital cortices (26.4 ± 6.7%; n=9; $p<0.01$; Fig 5.2, F) as well as non-significant decreases in the motor, cingulate and agranular insular cortices. Once again these decreases were not present in animals treated with both PCP and CBD, with these animals displaying a significant increase in *Arc* expression in prelimbic (28.8 ± 10.6%; n=9; $p<0.05$; Fig 5.3, A), infralimbic (23.7 ± 8.3%; n=9; $p<0.05$; Fig 5.3, B), lateral orbital (18.1 ± 5.9%; n=9; $p<0.05$; Fig 5.3, E) and ventral orbital cortices (19.1 ± 6.0%; n=9; $p<0.01$; Fig 5.3, F) compared with those treated with CBD on its own. This could also indicate that PCP is modulating the effects of CBD.

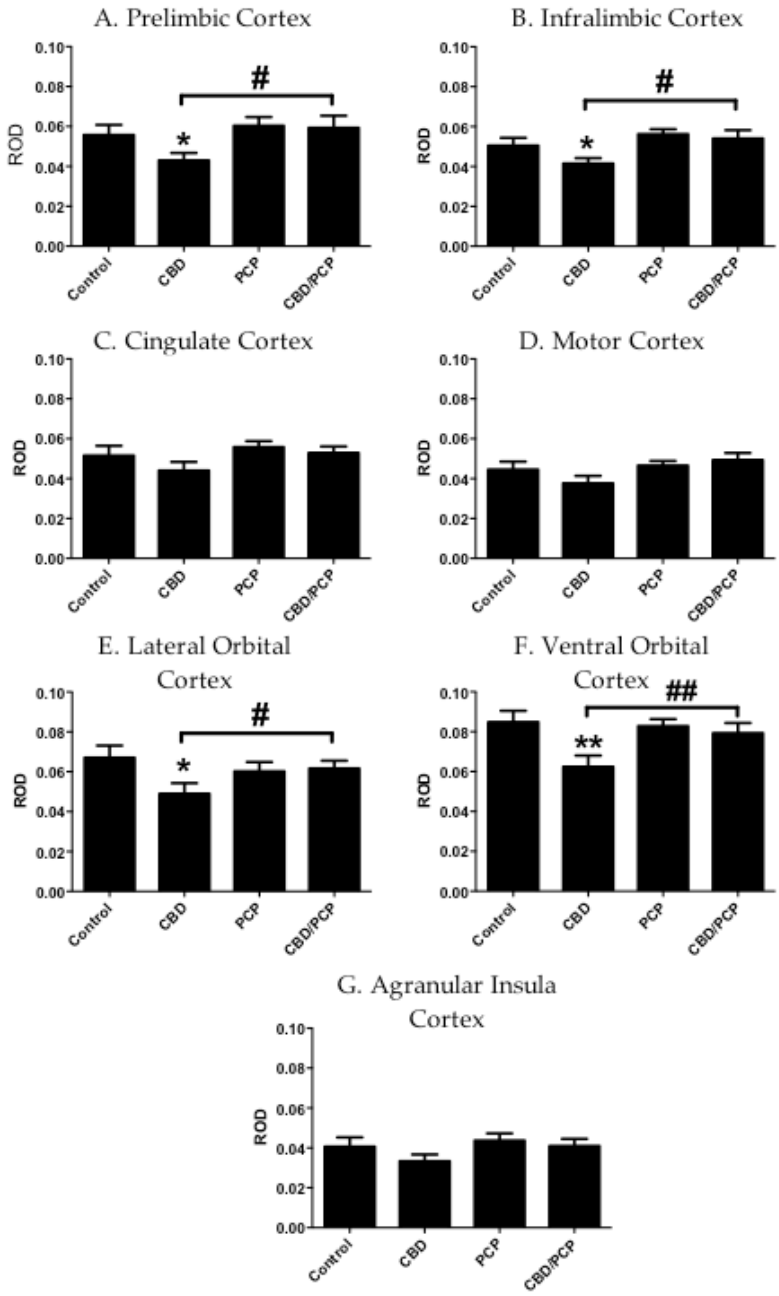


Figure 5.3 CBD administration results in reductions *Arc* mRNA expression in prefrontal cortical regions of the rat brain. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *Arc* mRNA in the prelimbic (A), infralimbic (B), cingulate (C), motor (D), lateral orbital (E) or ventral orbital (F) or agranular insula cortices (G). All data are expressed as mean ± S.E.M. relative optical density (ROD). * or ** denotedenotes significant difference between drug treated and control animals s significant difference between 0 and 5 mg/kg of THC ((two injections of saline; $p<0.05$ or $p<0.01$ respectively). # or ## deotes a significant difference between CBD and groups treated with both CBD and PCP ($p<0.05$ or $p<0.01$ respectively)

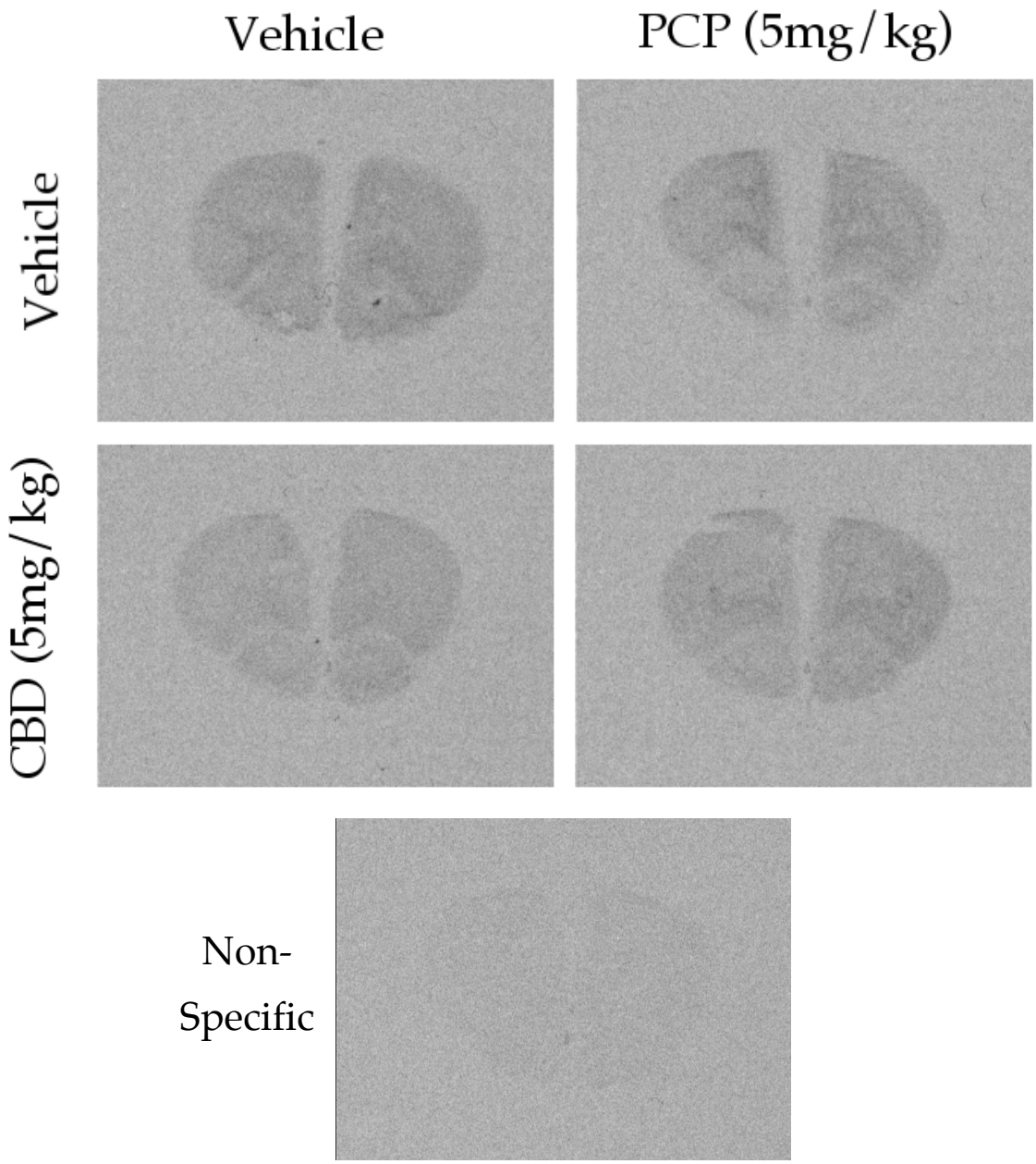


Figure 5.4 Autoradiograms illustrating the level of expression of *Arc* in response to CBD and PCP treatment at the level of the prefrontal cortex. These autoradiograms are representative images illustrating the levels of *Arc* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.1.3 c-fos

Consistent with previous studies administration of PCP resulted in a significant increase in expression of mRNA encoding *c-fos* infralimbic cortex (41.6 ± 14.8%; n=10; $p<0.05$; Fig 5.5, B). PCP also resulted in a trend for increased expression in the other regions studied at this level, most notably in the prelimbic (Fig 5.5, A) and lateral orbital cortex (Fig 5.5, E). These PCP-induced effects were not prevented by co-treatment with CBD; in fact CBD administration seemed to have an enhancing effect on *c-fos* expression in the ventral orbital cortex. There was no effect of CBD (5 mg/kg) on *c-fos* expression in prefrontal regions, which is consistent with findings in chapter 4 (Section 4.3.1.3).

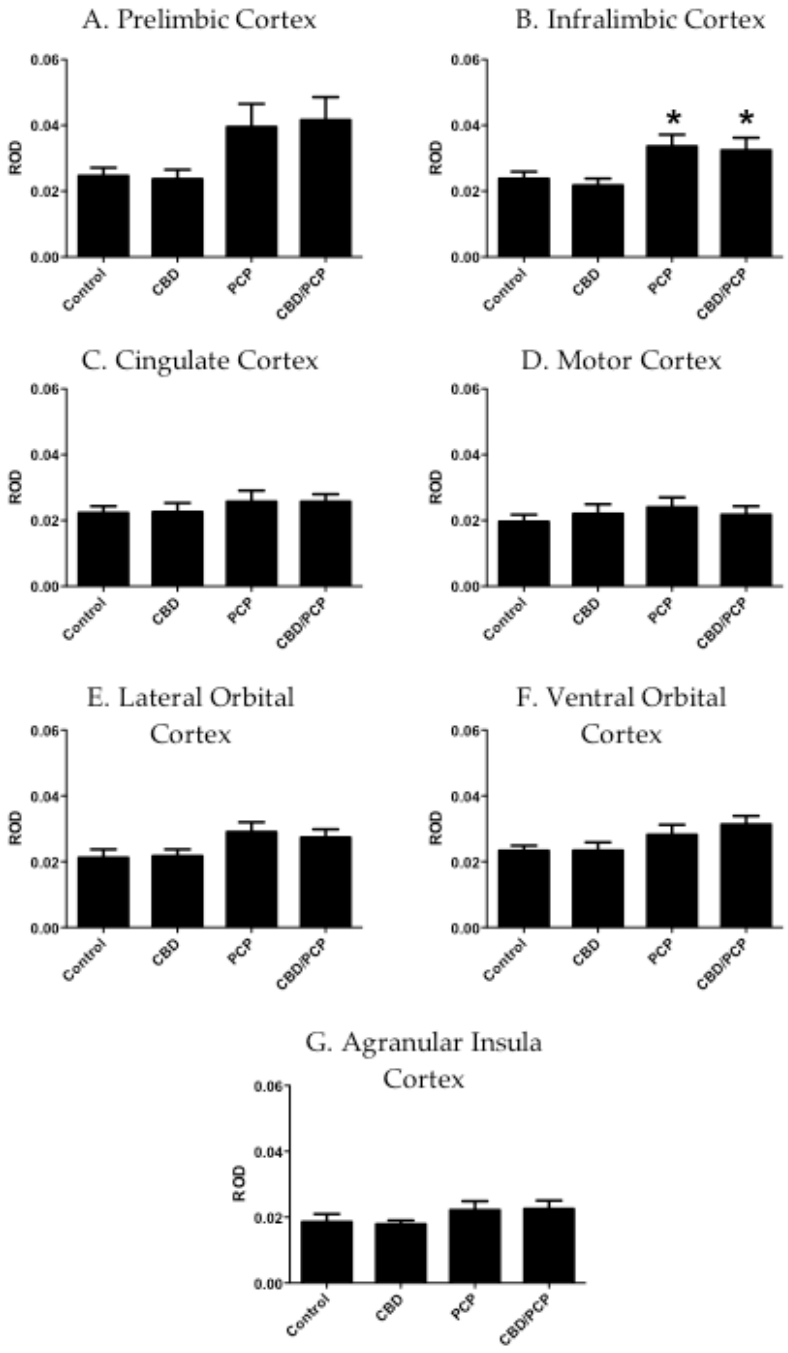


Figure 5.5 PCP administration increased *c-fos* mRNA expression some regions of the prefrontal cortex in the rat brain that was unaffected by co-treatment with CBD. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *c-fos* mRNA in the prelimbic (A), infralimbic (B), cingulate (C), motor (D), lateral orbital (E) or ventral orbital (F) cortices or in the agranular insular cortex (G). All data are expressed as mean ± S.E.M. relative optical density (ROD). * denotes significant difference between drug treated and control animals (two injections of saline; $p<0.05$ or $p<0.01$ respectively).

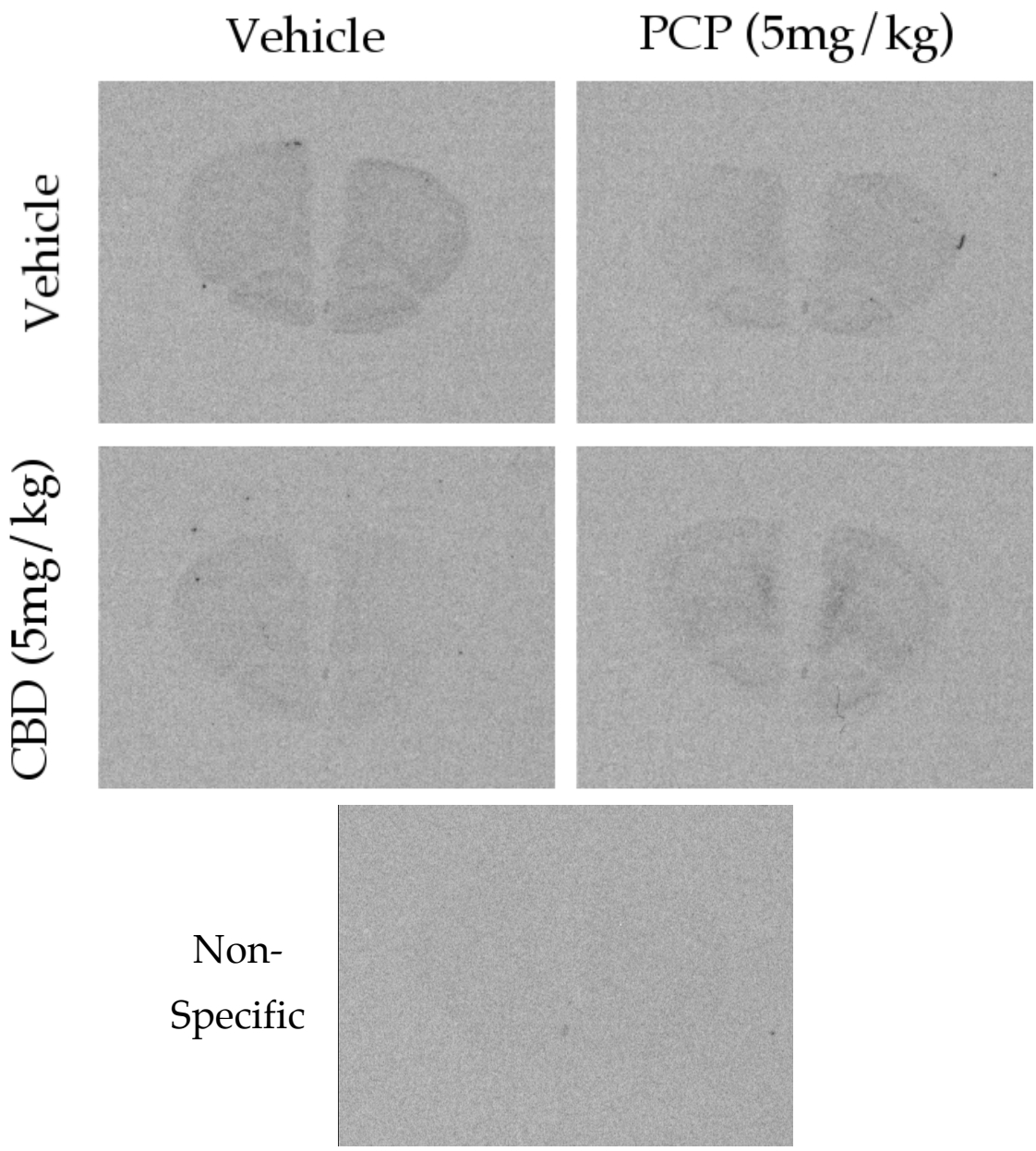


Figure 5.6 Autoradiograms illustrating the level of expression of *c-fos* in response to CBD and PCP treatment at the level of the prefrontal cortex. These autoradiograms are representative images illustrating the levels of *c-fos* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.2 Effects of CBD and PCP on IEG induction at the level of the striatum

At the level of the striatum, as illustrated in Figure 5.7-5.12, CBD and PCP resulted in regional specific changes in expression of *Zif-268, Arc* and *c-fos. A* one-way ANOVA of the data obtained for the various brain regions analysed at this level revealed significant changes in IEGs, most notably in *Arc* expression, in some of the regions analysed (Table 2).

	Zif-268	***Arc***	***c-fos***
Caudate putamen	F(3,34) = 5.621; $p = 0.0031$*	F(3,34) = 4.471; $p = 0.0095$*	F(3,34) = 1.067; $p = 0.3762$
Posterior cingulate cortex	F(3,31) = 2.769; $p = 0.0566$	F(3,34) = 4.852; $p = 0.0065$*	F(3,34) = 2.027; $p = 0.1258$
Motor cortex	F(3,34) = 2.768; $p = 0.0566$	F(3,34) = 3.028; $p = 0.0427$*	F(3,34) = 3.903; $p = 0.0169$*
Sensory cortex (forelimb)	F(3,34) = 4.018; $p = 0.0150$*	F(3,34) = 3.496; $p = 0.0259$*	F(3,34) = 2.782; $p = 0.0558$
Sensory cortex (upper lip)	F(3,34) = 1.792; $p = 0.1674$	F(3,34) = 5.601; $p = 0.0031$*	F(3,34) = 2.457; $p = 0.0797$
Agranular insular cortex	F(3,34) = 2.645; $p = 0.0648$	F(3,34) = 5.100; $p = 0.0031$*	F(3,34) = 5.022; $p = 0.0055$*

Table 2. The F and p values obtained from the one–way ANOVA for each IEG in respective subanatomical regions at the level of the striatum. * denotes a significant change detected by ANOVA ($p < 0.05$).

5.3.2.1 Zif-268

As was found in the prefrontal cortical region, PCP had no effect on *Zif-268* mRNA expression in cortical regions measured at the striatal level. However, PCP administration resulted in a significant reduction in mRNA expression in the caudate putamen (25.0 ± 5.3%; n=10; p<0.01; Fig 5.7, F) compared with control. Administration of CBD, on the other hand, resulted in a significant decrease in expression of mRNA encoding *Zif-268* in the

cingulate (26.1 ± 5.5%; n=9; p<0.01; Fig 5.7, A), motor (22.1 ± 7.8%; n=9; p<0.05; Fig 5.7, B) and sensory (26.2 ± 9.4%; n=9; p<0.05; Fig 5.7, B) cortices as well as in the caudate putamen (20.6 ± 6.1%; n=9; p<0.01; Fig 5.7, F) when compared with control. There was also a significant reduction in *Zif-268* expression compared with control in the caudate putamen of animals treated with both CBD and PCP (29.4 ± 5.7%; n=9; p<0.001; Fig 5.7, F). There were no differences between those animals treated with both CBD and PCP and control in any other regions measured, suggesting that PCP may be blocking the ability of CBD to reduce expression in the cingulate, motor and sensory cortices as discussed above.

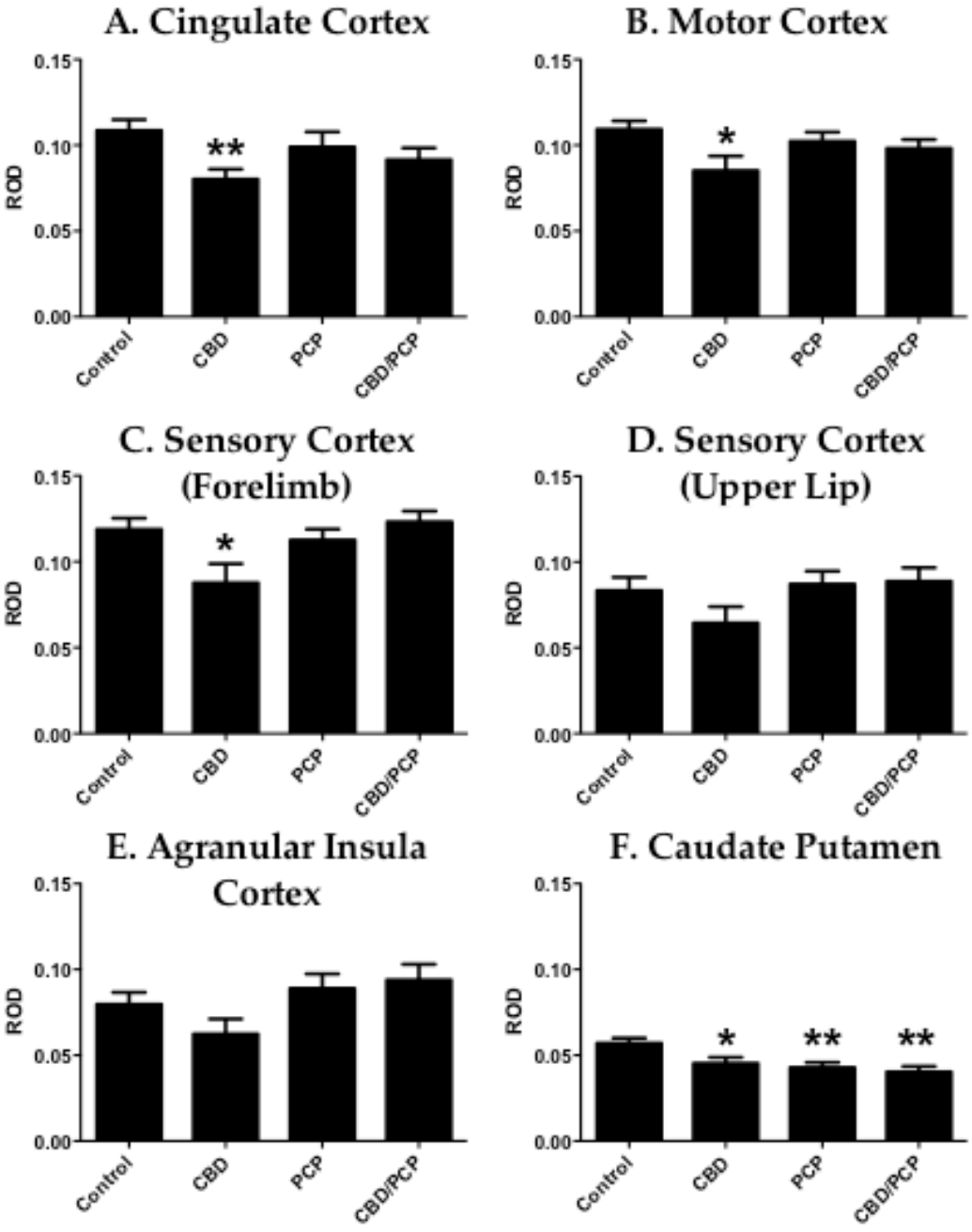


Figure 5.7 CBD administration results in a reduction of *Zif-268* mRNA expression in the caudate putamen and cortical regions at the level of the striatum. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *Zif-268* mRNA in the cingulate cortex (A), motor cortex (B), forelimb and upper lip region of the sensory cortex (C and D), agranular insular cortex (E) and caudate putamen (F). All data are expressed as mean ± S.E.M. relative optical density (ROD). *, ** or *** denotes significant difference between drug treated and control animals (two injections of saline; $p<0.05$, $p<0.01$ or $p<0.001$ respectively).

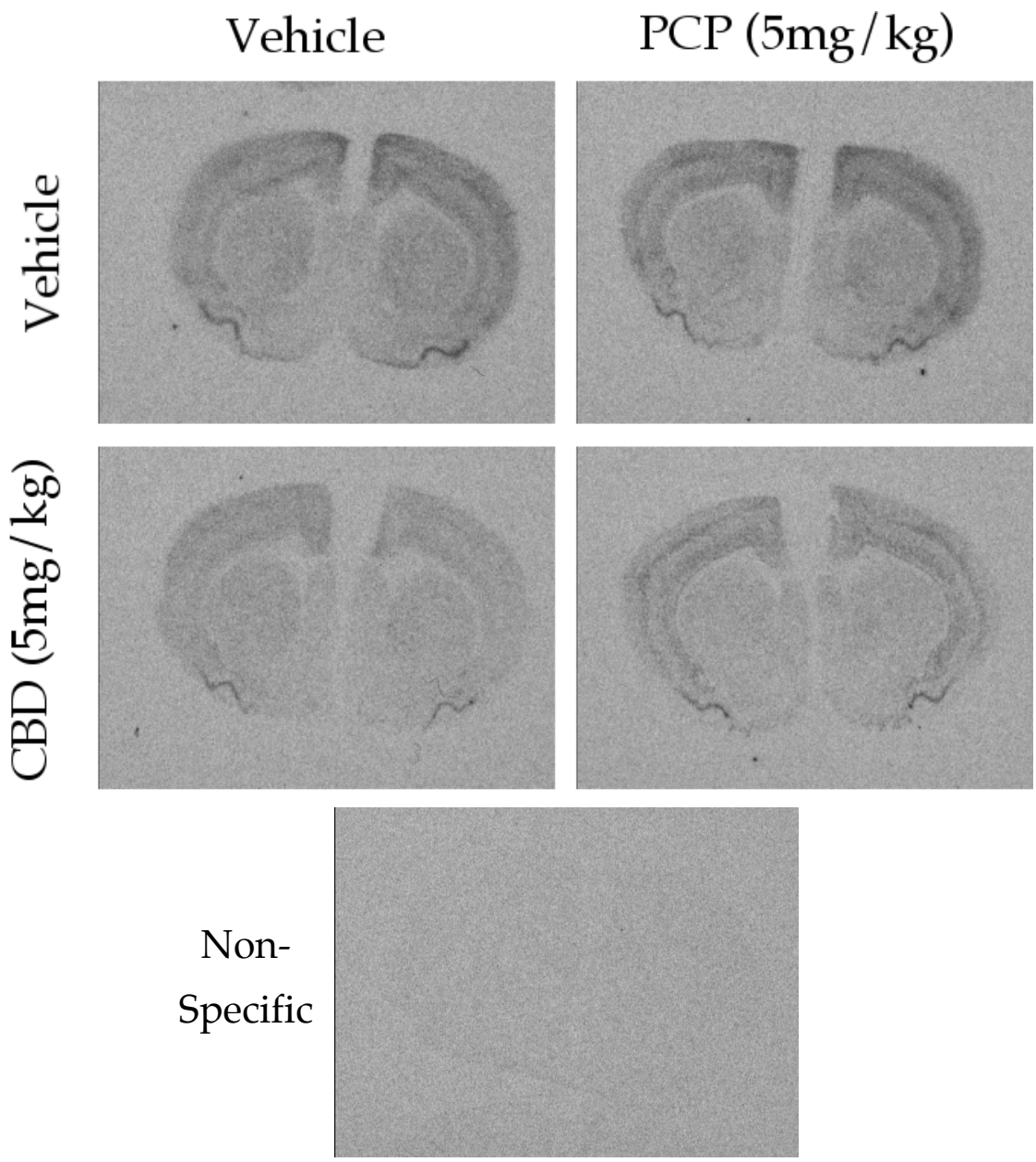


Figure 5.8 Autoradiograms illustrating the level of expression of *Zif-268* in response to CBD and PCP treatment at the level of the striatum. These autoradiograms are representative images illustrating the levels of *Zif-268* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.2.2 Arc

PCP administration produced regionally different effects on *Arc* expression at the level of the striatum. Administration of PCP resulted in a significant reduction in *Arc* expression in the posterior cingulate cortex (24.8 ± 8.2%; n=10; $p<0.05$; Fig 5.9, A) and caudate putamen (55.7 ± 6.2%; n=10; $p<0.05$; Fig 5.9, F) compared with control. PCP administration also resulted in a significant increase in expression in the agranular insular cortex (39.6 ± 12.9%; n=10; $p<0.05$; Fig 5.9, E) compared to control group. CBD administration resulted in a significant decrease in the posterior cingulate cortex (45.1 ± 8.0%; n=9; $p<0.01$; Fig 5.6, A) as well as a reduction in the motor (32.9 ± 11.6%; n=9; $p<0.05$; Fig 5.6, B) and sensory (40.4 ± 9.7%; n=9; $p<0.05$; Fig 5.6, D) cortex compared to controls. Animals co-administered with both CBD and PCP showed significantly higher levels of *Arc* expression than those administered with CBD alone in the motor, sensory and agranular insular cortex. These regions all showed the same pattern of *Arc* expression in response to CBD, PCP and CBD and PCP in combination, suggesting that PCP has the ability to modulate CBD-induced changes in *Arc* expression. The pattern of expression in response to drug treatments in the posterior cingulate cortex was different from that seen in the other regions. In the posterior cingulate cortex administration of both CBD and PCP alone and in combination significantly reduced expression of *Arc,* this suggests PCP and CBD have a similar effect in this region. Similarly, the expression profile of *Arc* following administration of these treatments in the caudate putamen, displayed a similar pattern to that seen with *Zif-268*.

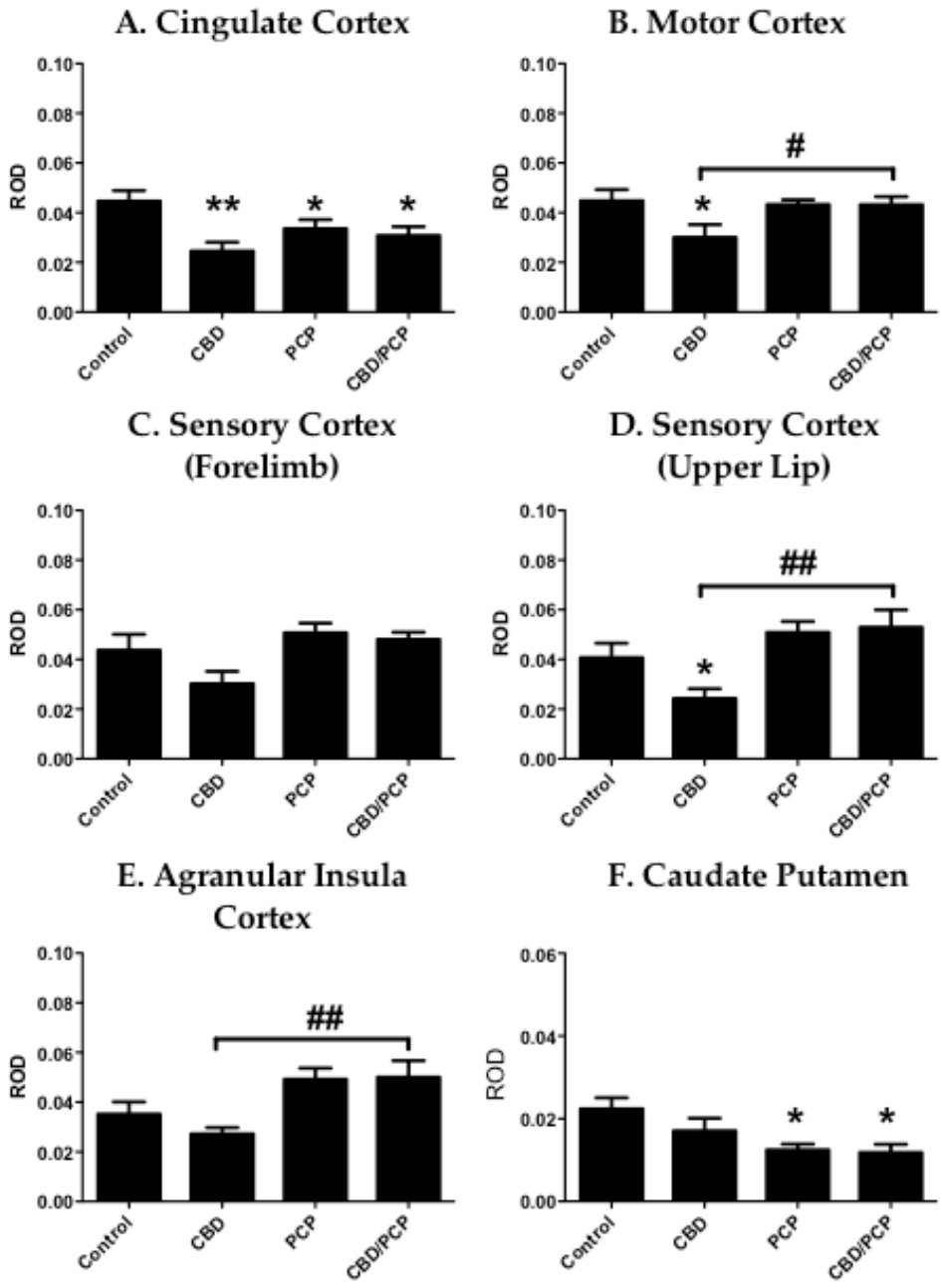


Figure 5.9 CBD and PCP administration resulted regional specific changes in *Arc* mRNA expression at the level of the striatum. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *Arc* mRNA in the cingulate cortex (A), motor cortex (B), forelimb and upper lip region of the sensory cortex (C and D), agranular insular cortex (E) and caudate putamen (F). All data are expressed as mean ± S.E.M. * or ** denotes significant difference between treatment and control animals (two injections of saline; $p<0.05$ or $p<0.01$ respectively). # or ## denotes significant difference between CBD/PCP and CBD treated animals ($p<0.05$ or $p<0.01$ respectively)

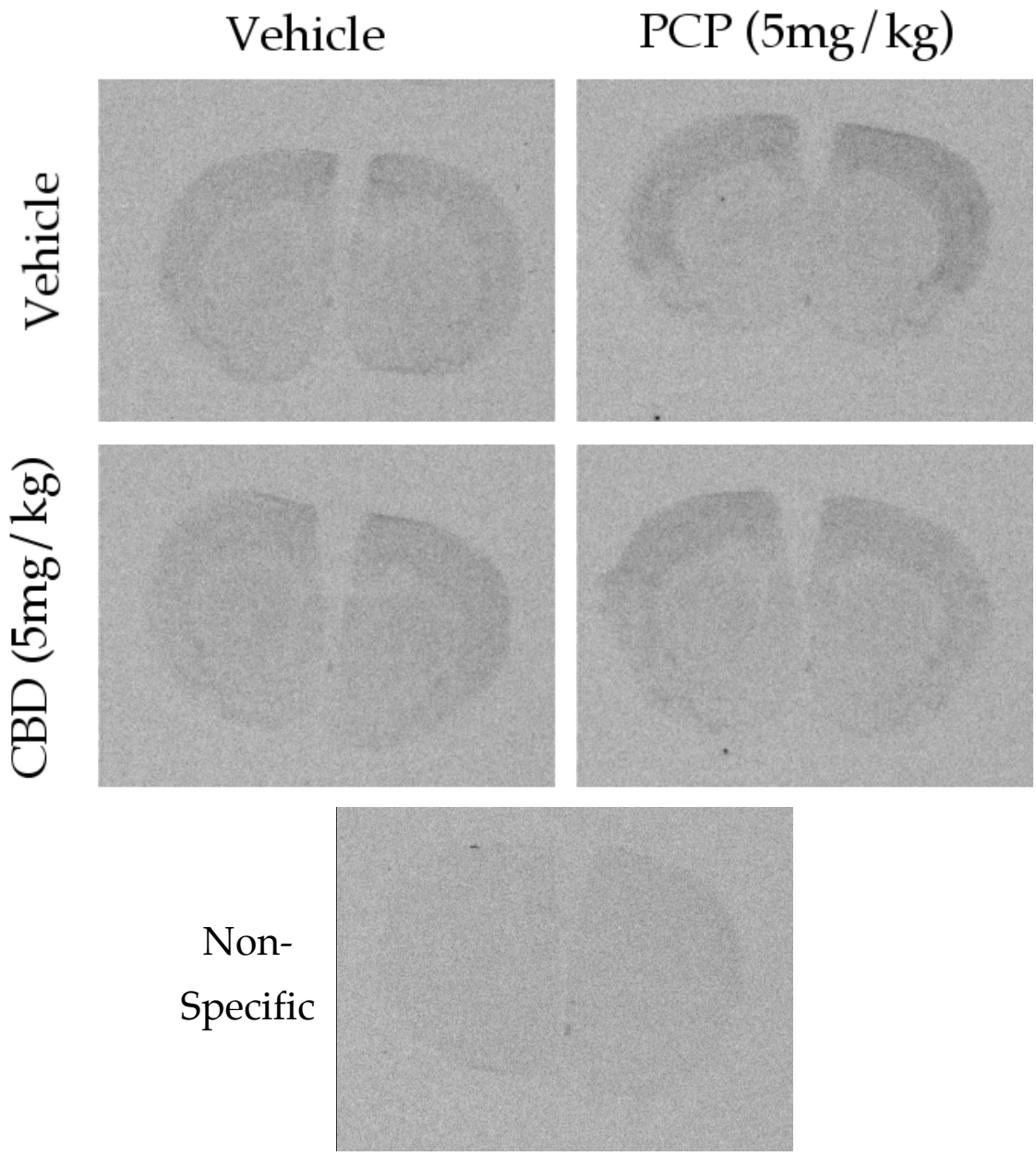


Figure 5.10 Autoradiograms illustrating the level of expression of *Arc* in response to CBD and PCP treatment at the level of the striatum. These autoradiograms are representative images illustrating the levels of *Arc* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.2.2 c-fos

Despite displaying a trend of increasing *c-fos* expression in the motor and sensory cortices, PCP administration only resulted in a significant increase in *c-fos* expression in the agranular insular cortex (70.8 ± 23.7%; n=9; $p<0.05$; Fig 5.11, E) compared with control. This effect was not reversed by co-application with CBD. A similar trend was found in the motor cortex (Fig 5.11, B), although PCP administration did not result in a significant change in *Arc* expression at this level. CBD application produced trends in reduction of *c-fos* expression in all regions measure at this level; however, CBD administration only resulted in a significant reduction in *c-fos* expression in the posterior cingulate cortex (36.3 ± 14.8%; n=9; $p<0.05$; Fig 5.11, A).

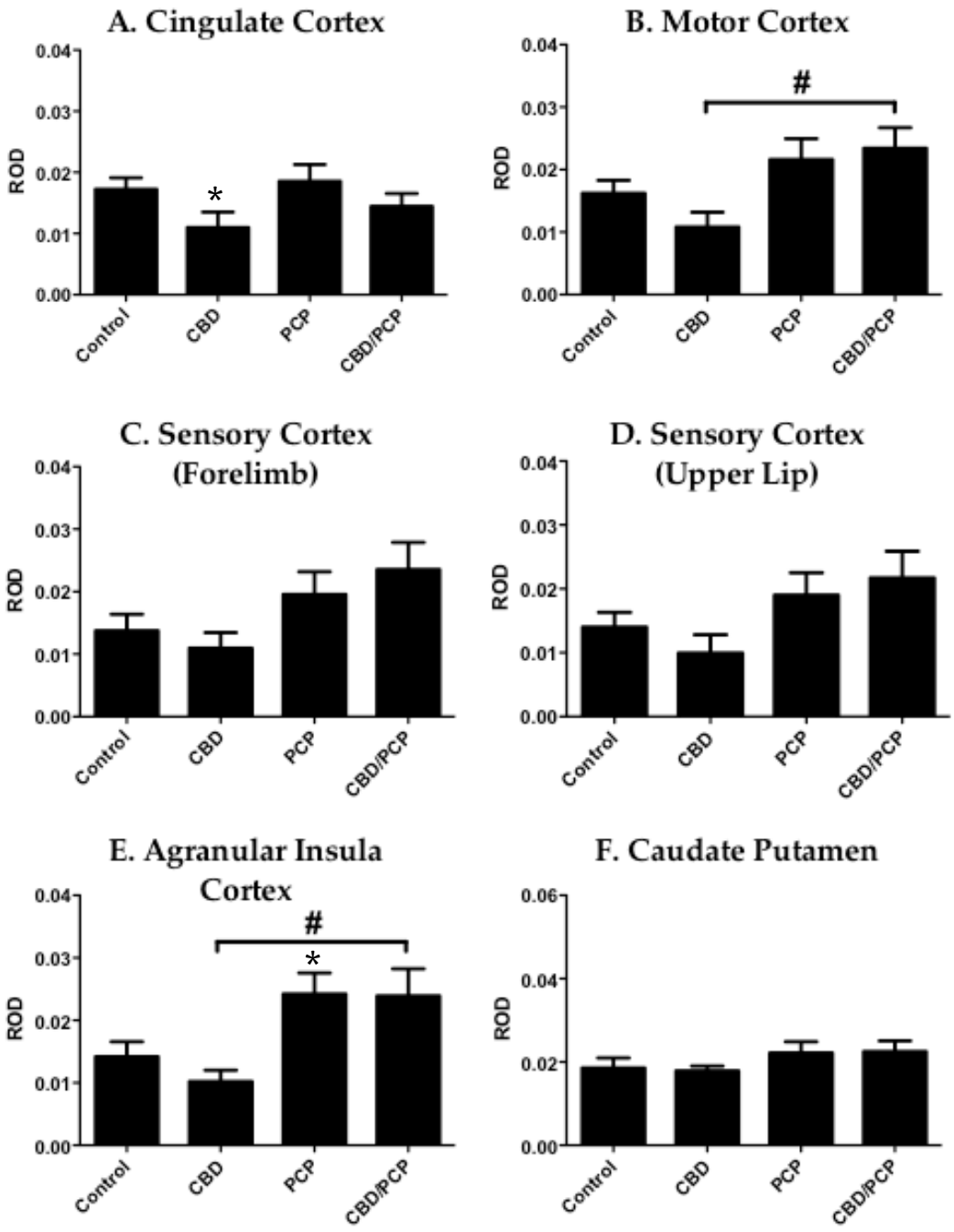


Figure 5.11 Effect of PCP and CBD on *c-fos* mRNA expression in striatal regions of the rat brain. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *c-fos* mRNA in the cingulate cortex (A), motor cortex (B), forelimb and upper lip region of the sensory cortex (C and D), agranular insular cortex (E) and caudate putamen (F). All data are expressed as mean ± S.E.M. # denotes significant difference between CBD/PCP and CBD treated animals ($p<0.05$).

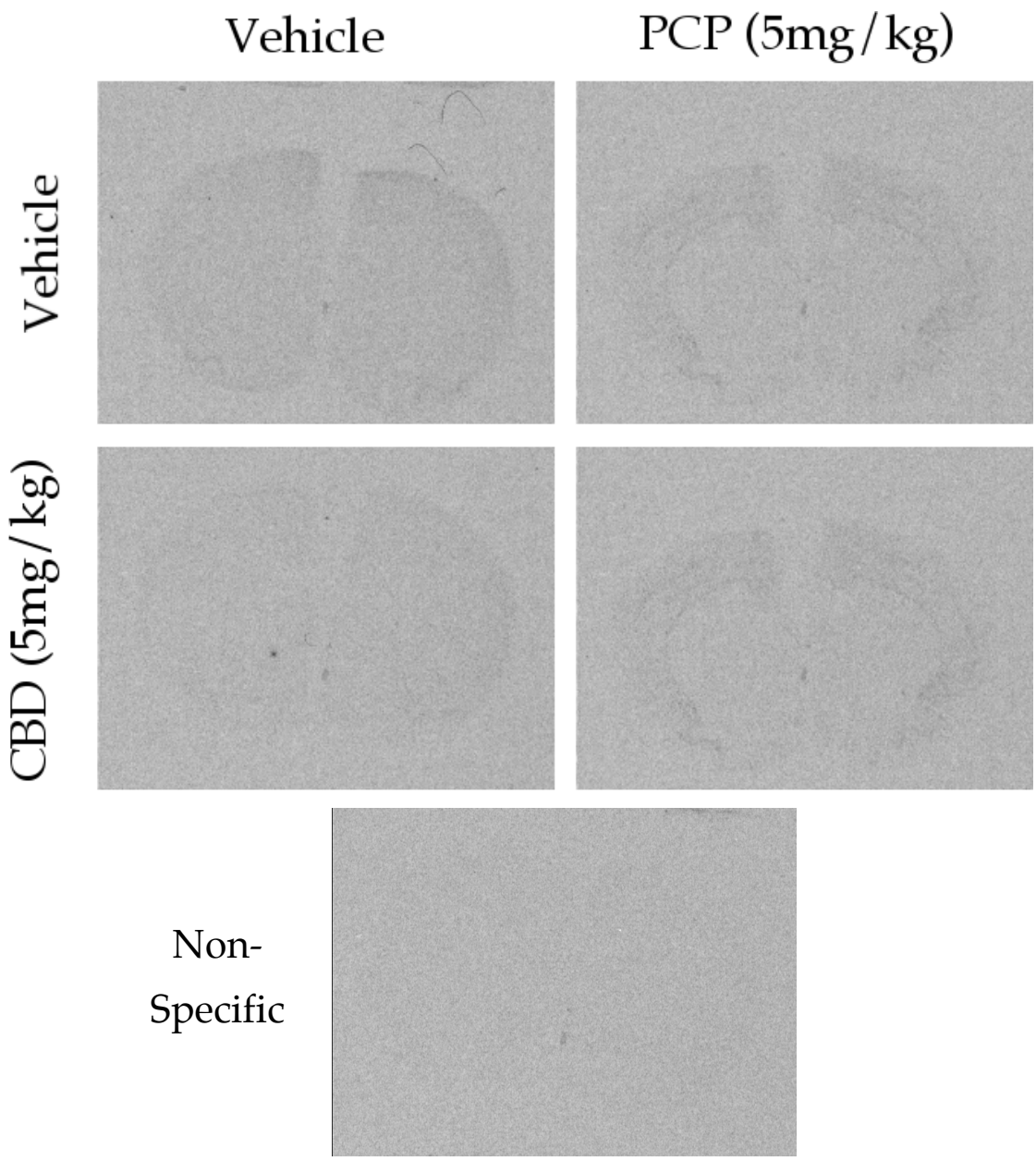


Figure 5.12 Autoradiograms illustrating the level of expression of *c-fos* in response to CBD and PCP treatment at the level of the striatum. These autoradiograms are representative images illustrating the levels of *c-fos* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.3 Effects of CBD and PCP on IEG induction in the hippocampus

At the level of the hippocampus, as illustrated in Figure 5.13-5.17, CBD and PCP both induced significant changes in *Zif-268* and *Arc* expression in the CA1 region, but not the CA3 region. *A* one-way ANOVA of the data obtained in this region revealed significant changes in all IEGs in some of the regions analysed (Table 3).

	Zif-268	***Arc***
CA1 region	F(3,34) = 4.664; $p = 0.0078$*	F(3,34) = 8.404; $p = 0.0003$*
CA3 region	F(3,34) = 0.09549; $p = 0.9620$	F(3,34) = 1.283; $p = 0.2963$

Table 3. The F and p values obtained from the one–way ANOVA for each IEG in respective subanatomical regions in the hippocampus. * denotes a significant change detected by ANOVA ($p < 0.05$).

5.3.3.1 Zif-268

Both PCP and CBD administration as well as combined treatment resulted in a significant reduction in *Zif-268* expression in the CA1 (CBD: 18.39 ± 4.1%; n=9; p<0.01; PCP: 14.4 ± 3.9%; n=10; p<0.01; CBD/PCP: 17.17 ± 4.1%; n=9; p<0.01; Fig 5.13, A). Interestingly, there was no change in expression of *Zif-268* induced by administration of any treatment in the CA3 region of the hippocampus (Fig 5.13, B)

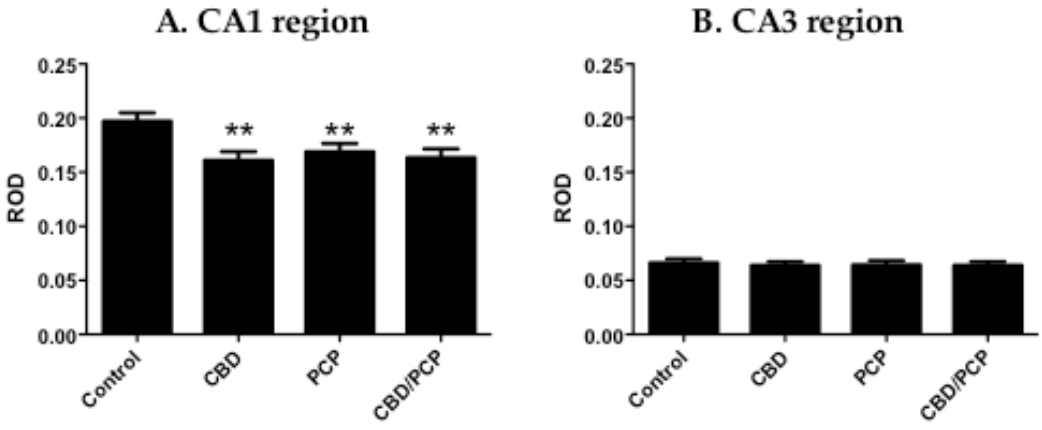


Figure 5.13 PCP and CBD administration induce reductions in *Zif-268* mRNA expression in the CA1 region of the hippocampus of the rat brain. These graphs illustrate results of acute administration of PCP or CBD (5 mg/kg) on their own or in combination on expression of *Zif-268* mRNA in the CA1 (A) and CA3 (B). All data are expressed as mean ± S.E.M. relative optical density (ROD). ** denotes significant difference between drug treated and control animals (two injections of saline; $p<0.01$).

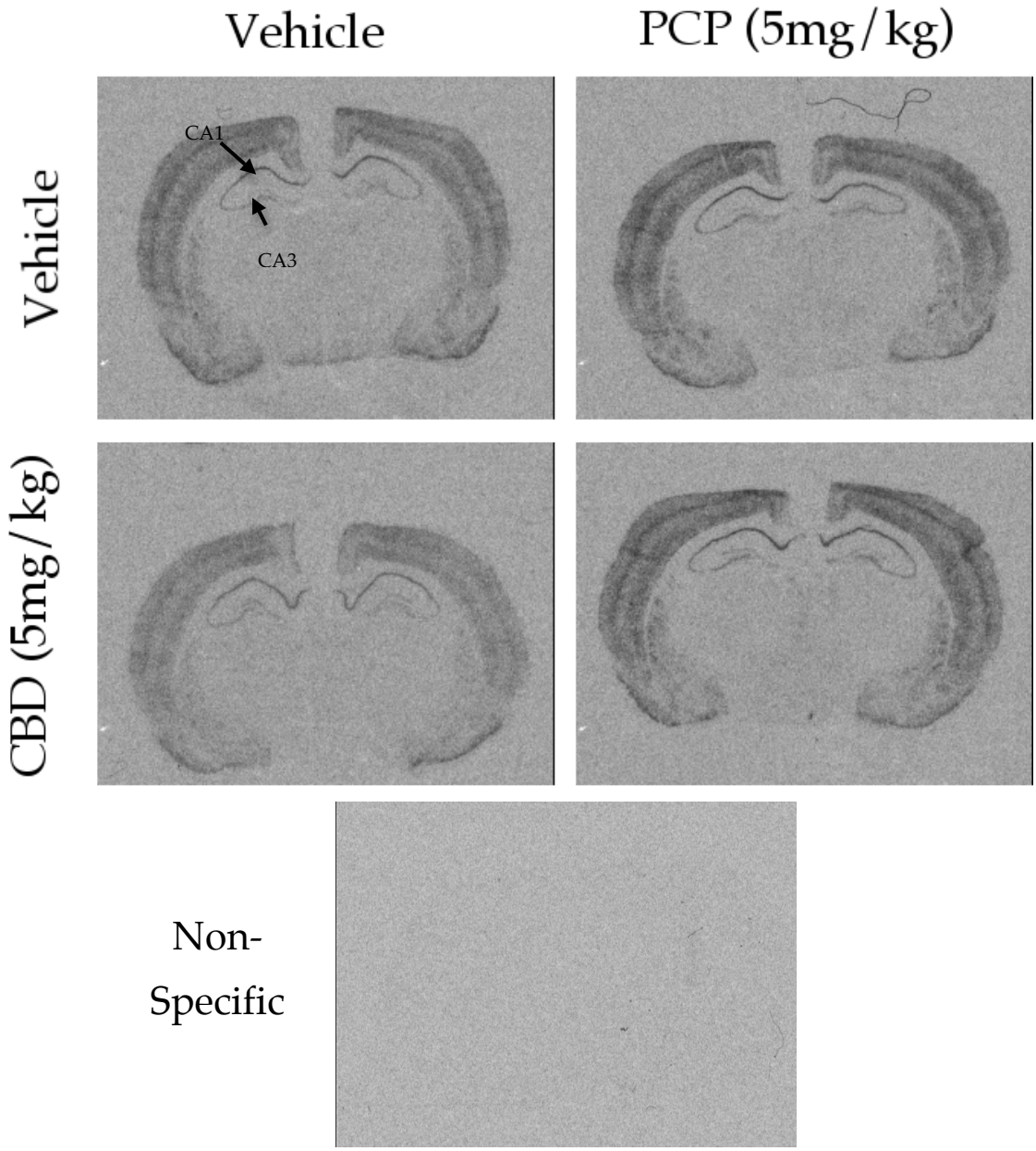


Figure 5.14 Autoradiograms illustrating the level of expression of *Zif-268* in response to CBD and PCP treatment at the level of the hippocampus. These autoradiograms are representative images illustrating the levels of *Zif-268* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.3.2 Arc

Both PCP and CBD administration on their own as well as combined treatment resulted in a significant reduction in *Arc* expression in the CA1 region (CBD: 34.6 ± 4.8%; n=9; $p<0.01$; PCP: 34.5 ± 6.8%; n=10; $p<0.01$; CBD/PCP: 41.3 ± 4.0%; n=9; $p<0.001$; Fig 5.15, A). However, only CBD resulted in a reduction in Arc expression in the CA3 region of the hippocampus (25.5 ± 4.5%; n=9; $p<0.05$; Fig 5.7, B)

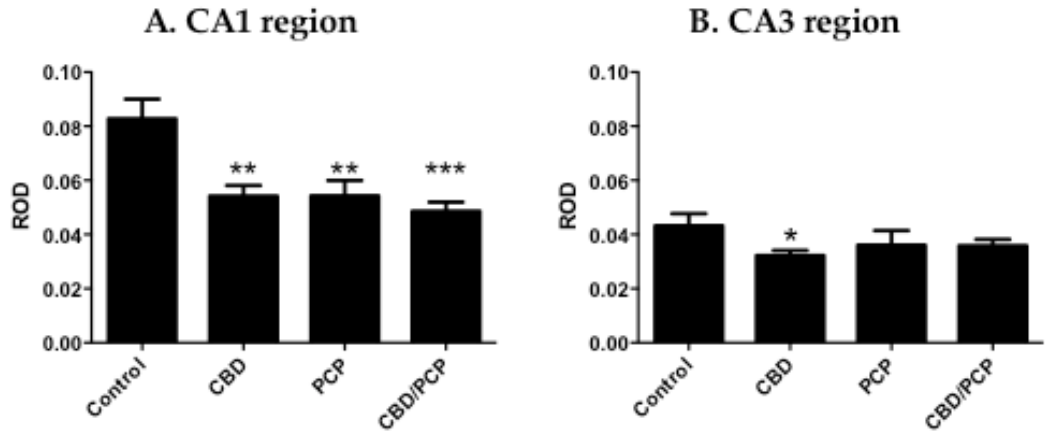


Figure 5.15 PCP and CBD administration causes reductions in *Arc* mRNA expression in the hippocampus of the rat brain. These graphs illustrate results of acute administration of PCP (5 mg/kg) or CBD (5 mg/kg) on their own or in combination on expression of *Arc* mRNA in the CA1 (A) and CA3 (B). All data are expressed as mean ± S.E.M. relative optical density (ROD). *, ** or *** denotes significant difference between drug treated and control animals (two injections of saline; $p<0.05$, $p<0.01$ or $p<0.001$ respectively).

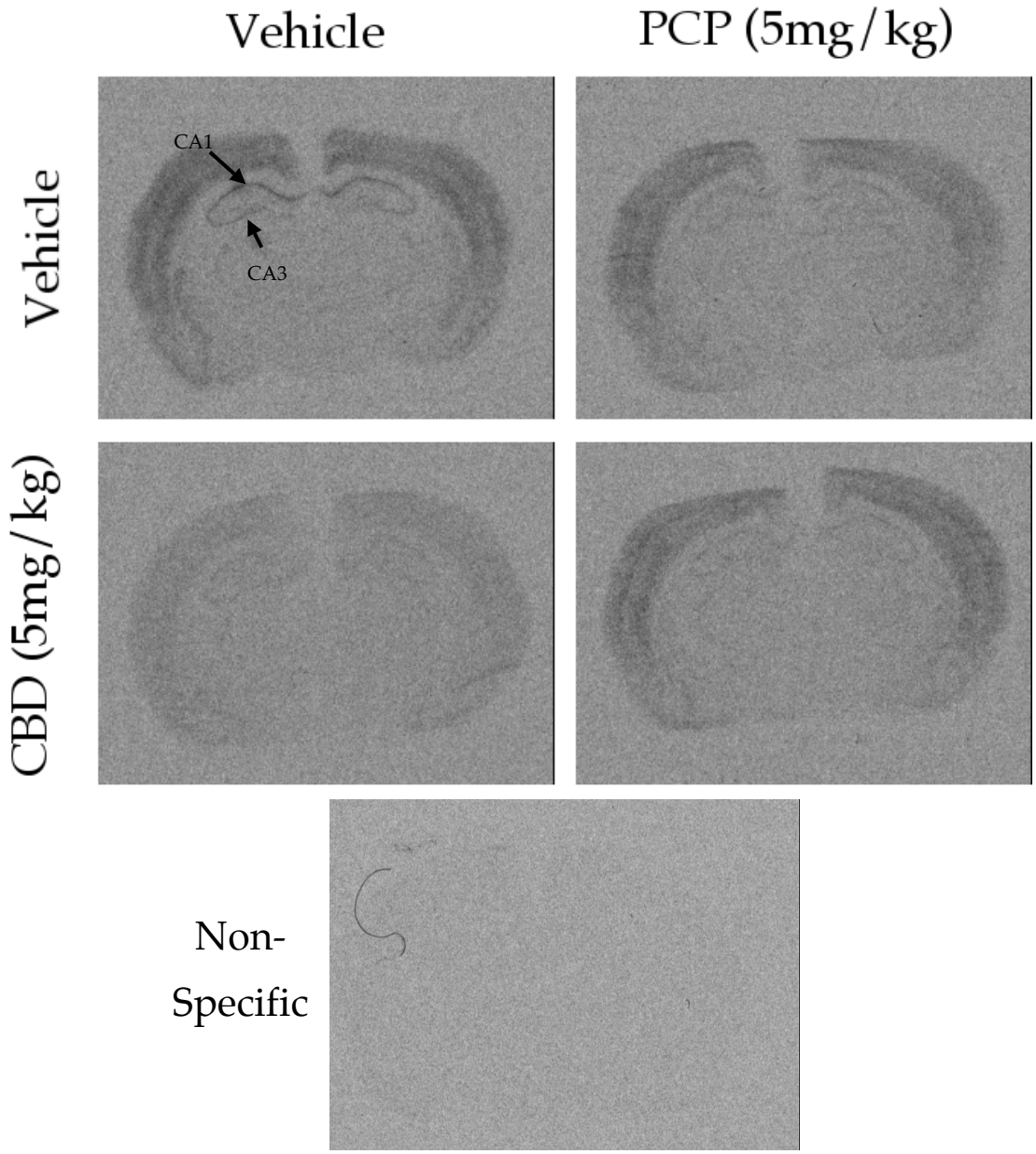


Figure 5.16 Autoradiograms illustrating the level of expression of *Arc* in response to CBD and PCP treatment at the level of the hippocampus. These autoradiograms are representative images illustrating the levels of *Arc* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

5.3.3.3 c-fos

As was discussed in the previous chapter, *c-fos* has a very low expression in the hippocampus (Figure 5.17) and although immunohistochemical studies have shown alterations of *c-fos* expression in this region, the resolution of *in situ* hybridisation technique along with the requirement for basal expression to identify regions of interest meant that it was impossible to accurately measure expression of *c-fos* in this region with any great level of accuracy.

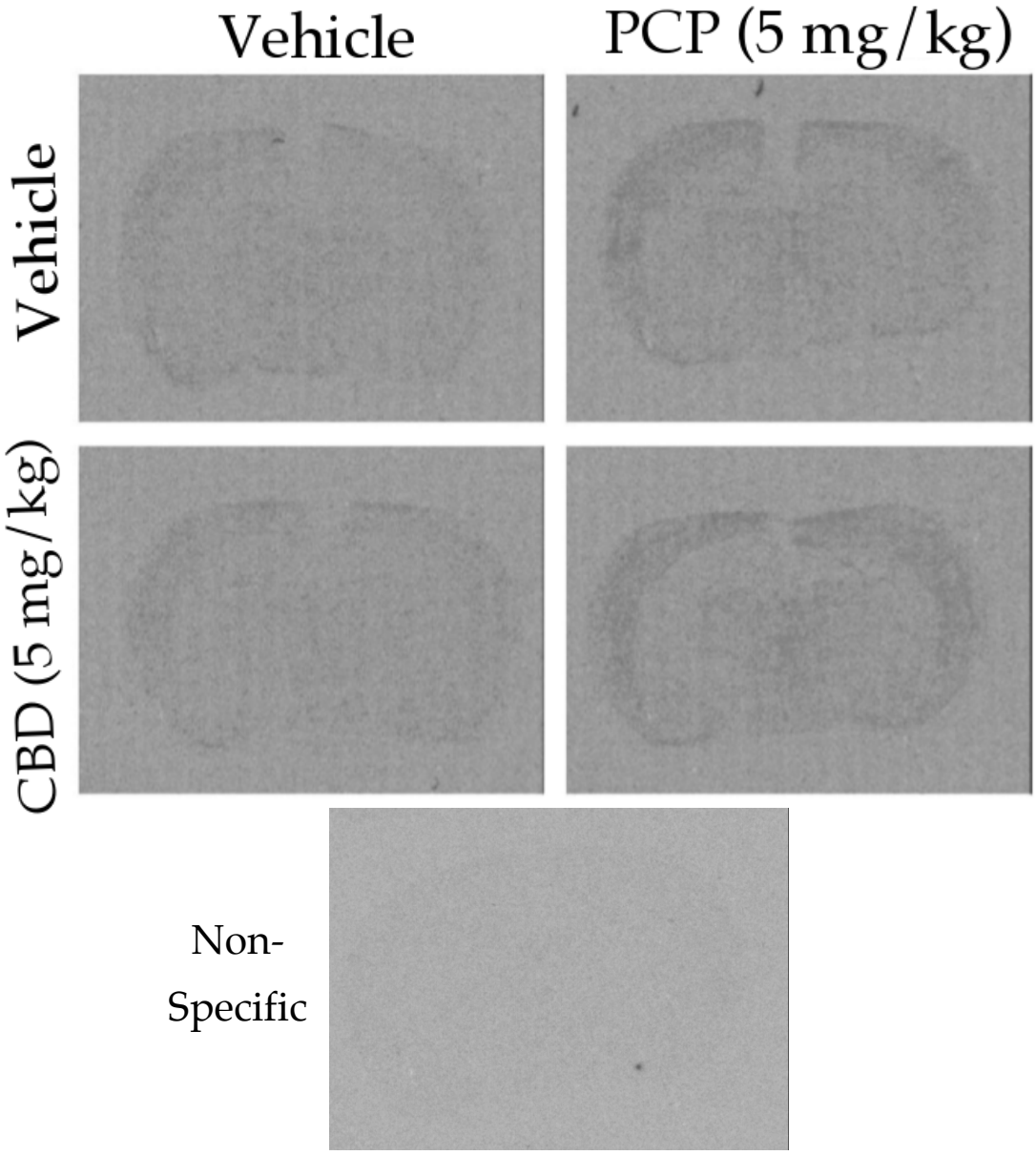


Figure 5.17 Autoradiograms illustrating the level of expression of *c-fos* in response to CBD and PCP treatment at the level of the hippocampus. These autoradiograms are representative images illustrating the levels of *c-fos* expression in controls (vehicle) and following administration of CBD (5 mg/kg) with PCP (5 mg/kg i.p.) or vehicle (saline with Tween-80) as well as autoradiogram demonstrating level of non-specific binding of radiolabeled oligonucleotide probe.

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5.4 Discussion

5.4.1 Effects of PCP on IEG mRNA expression: consistency with previous studies

The results in this study did demonstrate a degree in consistency with previous findings in the literature where the effects of PCP on regional IEG expression were examined. In concordance with previous studies by Gao et al (1998), PCP (5mg/kg; i.p.) administration resulted in a significant increase in *c-fos* expression in some prefrontal cortical regions. Despite the similarities between the effects of PCP on c-fos expression demonstrated here and in the aforementioned, the effects of PCP on *Zif-268* expression in cortical regions were not. This may lead one to propose that differences in dose may have been responsible for this fact. However, both doses used by Gao and co-workers that resulted in an effect (0.8 and 8.6 mg/kg; i.p.) lie either side of the dose used in the present study (5 mg/kg; i.p.). One variable that could possibly explain this lack of coherence between the two studies is the time at which measurements were taken. Gao et al (1998), measured expression levels at baseline then at 1, 3, 6, 24 and 48 hours after PCP administration, and noticed time dependent effects of PCP on *c-fos* and *Zif-268* mRNA expression. Notably, increases in expression in the prefrontal cortex 1 hour after PCP administration were not present at 3 hours after treatment. It could be that in the present study any increase in *Zif-268* expression may have subsided by the 75 minutes time point allowed for the drug to take effect. Further evidence to support this is the comparable effect of PCP on *Zif-268* mRNA expression in the CA1 region of the hippocampus of this study with the reduction in this region in the previous study at the 3 hour time point, while there was no effect at the 1 hour time point. The high doses used in the study by Nakki et al (1996), make it difficult to compare with this study. However, the increases in *c-fos* expression in the frontoparietal region, as shown by Nakki et al, are comparable to the increases in *c-fos* in the cortical regions measured in this study. Acute PCP administration did not result in the increases in *Arc* observed in previous acute studies. Thomsen et al (2010) demonstrated that a subchronic administration of PCP (5 mg/kg/day for 5 days) resulted in increases in expression of *Arc* in prefrontal cortical regions.

It should be noted that animals treated with PCP (5 mg/kg i.p.) in displayed signs of stereotyped behaviour, such as backward walking, head bobbing and circling in this study as previously described in the literature. This suggests that PCP administration at the dose used and in the hooded Lister strain was producing a psychotomimetic effect. It has been reported that several factors may influence the ability of drugs to induce IEG expression. These include, handling prior to experimentation, route of administration and time allowed for drug absorption. Stress and novelty have been shown to induce *c-fos, Zif-268* and *Arc* expression. Therefore there is a possibility that the process of administering the drugs may have increased basal levels of these IEGs therefore nullifying any change induced by PCP administration.

5.4.2 Cannabidiol produced regional selective alterations in IEG expression

In contrast to the results presented in chapter 4, CBD produced a significant reduction in mRNA expression of *Zif-268* and *Arc* in several prefrontal, striatal and hippocampal regions. More specifically CBD (5 mg/kg; i.p.) administration resulted in a reduction in expression of *Zif-268* in the cingulate, motor and sensory cortices, the caudate putamen and the CA1 region of the hippocampus. It was also found that CBD administration resulted in a significant decrease in *Arc* expression in the prelimic, infralimbic, lateral orbital ventral orbital, cingulate, motor and sensory cortices and also the caudate putamen and CA1 and CA3 regions of the hippocampus. This is comparable to that seen in human studies, where CBD administration resulted in reductions in regional neuronal activity in functionally similar regions in humans. The regional effects of CBD may also fit in with the hypothesis that CBD is acting through activation of presynaptic 5-HT_{1A} receptors. All regions where CBD resulted in a reduction of IEG expression, with the exception of the caudate putamen, have been shown previously to contain high levels of $5HT_{1A}$ receptor expression (Chalmers and Watson, 1991). These are presumably on presynaptic

terminals of afferents originating from the dorsal raphe nucleus. Unfortunately this region was not analysed for regional IEG expression so a potential effect of CBD in this region resulting in an effect on the regions it projects to cannot be ruled out. Inhibition of reuptake of 5-HT has previously been shown to result in an induction of *Zif-268* expression (Bhat and Baraban, 1993), therefore inhibition of basal 5-HT release through activation of a presynaptic G_i GPCR would therefore be hypothesised to result in a reduction in *Zif-268* expression. The ability of CBD to induce a reduction in *Zif-268* expression in the caudate putamen, in the absence of the proposed receptor target, may be due to the network effect of CBD administration. The caudate putamen has been shown to receive direct input from the dorsal raphe nuclei (Andersen et al., 1983). As well as this, the caudate also receives input from cortical regions (Ferry et al., 2000), which demonstrated a reduction in IEG expression, and therefore it could be concluded that the reduction of neuronal activity in this region may be due to the effects of CBD in cortical regions. The presence of significant changes in this experiment when no significant changes were caused by CBD in the previous experiment may be for a number of reasons. Firstly, this study was performed using one group of animals from the same batch, in contrast to the previous experiment, which was performed using two different batches of animals, which reduced the potential variability due to batch differences. Secondly, the data described in this chapter possessed greater statistical power due to higher and equal group sizes.

Administration of CBD resulted in general reductions predominantly in expression of *Arc* and *Zif-268*, which are widely regarded as markers of glutamatergic neurotransmission. More importantly, the proteins that these IEGs encode are fundamentally required for long-term potentiation (LTP), a form of synaptic plasticity where synaptic connections are strengthened. The reduction in markers of excitatory neuronal transmission in the CA1 region of the hippocampus adds further weight to that found in electrophysiological recordings in hippocampal cultures and acute slices (Chapter 3). In these *in vitro* findings CBD appeared to be acting via a presynaptic G_i-protein coupled receptor. Presumably, CBD is acting via a similar mechanism in

reducing *Zif-268* and Arc expression in the CA1 region of the hippocampus *in vivo* an effect that was mimicked by PCP administration and maintained by PCP/CBD co-administration. It could be postulated that, in the CA1 region of the hippocampus, CBD was acting in a mechanism as PCP resulting in reduced production of excitatory neurotransmitter, glutamate, and therefore reducing induction of *Zif-268* and *Arc*. However, with the exception of the cingulate cortex and caudate putamen where a similar trend was found, PCP blocked the effects of CBD administration on IEG expression in other regions suggests that the nature of interaction between CBD and PCP is regionally dependent. As this is an *in vivo* experiment there are many other possible interactions that cannot be ruled out. For example, PCP may affect the absorption, metabolism and distribution of CBD throughout the brain. There is also the possibility that PCP is acting at another in the respective neuronal circuit that disguises the CBD-induced effect. Interestingly, CBD and PCP administration had a similar ability in reducing *Zif-268* expression in the caudate putamen and *Arc* expression in the posterior cingulate cortex. This suggests that the effects of CBD and PCP are regionally selective.

5.4.3 CBD does not reverse PCP-induced changes in IEG expression

It was proposed in this study that because of the ability of CBD to reverse alterations in IEG expression induced by THC (Chapter 4) and because of the similarities between THC and PCP in inducing symptoms similar to that found in schizophrenia (Section 5.1), CBD may be able to alter the neuronal effects of PCP on IEG expression. In the present study PCP administration resulted in significant increase in *c-fos* in a variety of regions. These increases in *c-fos* expression were not blocked when PCP was co-administered with CBD suggesting that CBD may not block the behavioural effects of PCP. However, in contrast to the findings in previous publications (Gao et al., 1998), PCP had little effect on *Zif-268* or *Arc* mRNA expression in the regions measured, therefore the possibility that CBD may reverse PCP-induced alteration in *Zif-268* expression could not be investigated.

The ability of CBD to reverse MK-801-induced disruptions in PPI provides strong evidence that CBD can alter the behavioural effects of NMDA receptor antagonists. Gao et al (1998) has previously shown that both PCP and MK-801 administration resulted in marked increases in *c-fos* and *Zif-268* expression, which led to the proposal that this effect was mediated via inhibition of NDMA receptors in GABAergic neurons resulting in an increase in excitatory transmission and subsequent IEG induction (Gao et al., 1998). However, studies by Sharp et al (1997) may suggest an alternative mechanism for IEG induction. This study demonstrated that a sigma receptor antagonist inhibited PCP-induced expression of *c-fos*. They also went on to show that a selective sigma receptor ligand also induced *c-fos* expression, suggesting that sigma receptor activation may play some role in PCP-induced changes in expression of *c-fos*. The activation of the sigma receptor has been shown to enhance NMDA receptor activation, therefore the inability of CBD to reverse changes in *c-fos*, especially when CBD has had no effect on *c-fos* expression on its own, may not provide a clear picture of CBD/PCP interaction. Examination of the effect of CBD on PCP-induce disruption in PPI would be a useful experiment to determine whether the effects of CBD are limited to blocking MK-801 induced disruptions in this paradigm. However, given that there is a possibility that the psychotomimetic effects of PCP may be in part mediated via the sigma receptor, the ability of CBD to block one behavioural aspect, such as PPI, may not be evidence that CBD can block the psychological consequences of PCP.

5.4.4 Functional implications

The most interesting discovery in this study is the confirmation that CBD administration reduces neuronal activity in the hippocampus. However, in this study, CBD has been shown to alter neuronal activity *in vivo* using *in situ* hybridisation to visualise alterations in expression of genetic markers of neuronal activation. It is shown here that CBD administration significantly reduces expression of *Zif-268* and *Arc*, which are IEGs shown to be involved in forms of synaptic plasticity, such as LTP. This effect may be suggestive that CBD may modulate LTP and therefore have a potential role on learning

and memory. However, given that CBD administration has not been shown to alter performance in tasks that utilise the hippocampus, such as in the Morris water maze (Fadda et al., 2004; Fadda et al., 2006). This alteration in neuronal activity may not transcribe to behavioural activity. It would be useful to evaluate the effects of CBD administration on performance in other tasks measuring learning and memory, such as odour or spatial-span tasks.

CBD administration also displayed significant reductions in *Arc* expression in regions of the mPFC. This reduction could be a direct effect of CBD on the mPFC, but may also be related to alterations in activity of regions that interconnect with the PFC such as the hippocampus. There are well established glutamatergic connections between the hippocampus and the PFC (Jay et al., 1992). Interestingly, the ability of CBD to reduce *Arc* expression in the PFC, but not in the hippocampus, was blocked by co-administration with PCP. This finding may suggest a dependency of NMDA receptor activation in the effects of CBD in the hippocampal-PFC connection. In previous studies by our group (but not in this study), THC administration resulted in significant increases in *Arc* expression at doses shown to reduce performance in tasks measuring behavioural flexibility (Egerton, 2004; Egerton et al., 2005a). Given the opposing effects of CBD and THC in Arc expression, one may postulate that CBD can improve performance in these tasks. However, in these tasks animals are trained to perform at an optimal level, therefore improvements may be difficult to detect. Therefore more sensitive behavioural tasks should be employed that can detect positive as well as negative changes in performance.

In summary, it is demonstrated here for the first time that CBD administration can result in reductions in IEG expression in several areas including the posterior cingulate cortex (*Zif-268, Arc* and *c-fos*), regions of the prefrontal cortex (*Arc*), caudate putamen (*Zif-268* and *Arc*) and hippocampus (*Zif-268* and *Arc*). PCP administration only resulted in marginal changes in IEG expression that were not reversed by co-administration with CBD. These data may suggest that CBD does not possess the ability alter the

psychotomimetic effects of PCP, however further investigation would be required to conclude this.

Chapter 6: General Discussion

6.1 Summary and general discussion

Until recently, the ability of the non-psychoactive components of cannabis to alter neuronal activity had not been investigated. Cannabidiol has recently been shown to inhibit epileptiform activity in hippocampal slices *in vitro* as well as reducing basal neuronal activity in the human hippocampus *in vivo* (Bhattacharyya et al., 2009b; Jones et al., 2009). However, the work contained in this thesis, to the best of the author's knowledge, are the first conclusive demonstration that CBD can reduce basal neuronal activity *in vitro* using primary hippocampal cultures and acute hippocampal slices (Chapter 3) and using IEG expression as a marker of neuronal activity *in vivo* (Chapter 5). It also reflects the first attempts to investigating the potential mechanism through which CBD may mediate these neurosuppressive effects.

Pharmacological and physiological evidence presented in this thesis would suggest that CBD suppresses synaptic transmission in the CA1 region of hippocampal slices through activation of presynaptic receptor or receptors which are sensitive to CB_1 receptor blockade by AM251 or LY320135 (supplementary studies), and the $5\text{-}HT_{1A}$ receptor antagonist, WAY 100135. Interestingly, in supplementary experiments carried out by our group, 8-OH-DPAT also inhibited evoked fEPSP in hippocampal slices an effect, which was sensitive to AM251 but not LY320135. This suggests that CB_1 receptor activation may play a role in CBD-induced suppression of neural transmission. Considering CBD has been shown to have little affinity for CB_1 receptors it is postulated here that CBD may be acting in part indirectly via this receptor by enhancing endocannabinoid tone through inhibiting reuptake and degradation of endocannabinoids as shown previously (Bisogno et al., 2001). However, neither WAY 100135 (supplementary studies) nor AM251 blocked the effects of CBD in hippocampal cultures, which may suggest an alternative mechanism in cultures.

The sensitivity of the effects of CBD to WAY 100135 provides another potential mechanism of action of CBD. As mentioned above CBD enhanced

PPF in a WAY 100135 sensitive manner suggesting a presynaptic site of action. However, 8-OH-DPAT had no effect on paired-pulse facilitation, suggesting that the effects of this selective 5-HT_{1A} agonist are mediated via a postsynaptic site. Despite this there is evidence that 8-OH-DPAT acts at a presynaptic receptor and that activation of this receptor inhibits calcium entry (Schmitz et al., 1998), which is suggestive of the involvement of a $G_{i/o}$ GPCR. This has led the author to propose that CBD is mediating its effect on synaptic transmission through activation of the presynaptic site of action of 8-OH-DPAT. This proposed mechanism of action is strengthened by three key findings in the literature:

1. CBD has been shown to bind to 5-HT_{1A} receptors *in vitro* (Russo et al., 2005).
2. CBD-induced anxiolytic effects are sensitive to 5-HT_{1A} antagonists (Campos and Guimaraes, 2008; Resstel et al., 2009).
3. The anxiolytic effects of 8-OH-DPAT are mediated via presynaptic 5-HT_{1A} receptors *in vivo* (File et al., 1996).

The involvement of 5-HT_{1A} receptors in cannabinoid pharmacology is not exclusive to CBD. The anxiolytic effect of THC was inhibited by administration of WAY 100635 (Braida et al., 2007). In fact it is becoming increasingly evident that cannabinoids are not exclusive to cannabinoid receptors and may activate a wider range of receptors including 5-HT_{1A} (Marco et al., 2004; Braida et al., 2007; Mato et al., 2007; Campos and Guimaraes, 2008; Egashira et al., 2008; Cascio et al., 2010; Mato et al., 2010). This is not surprising if one were to consider the structural similarities between phytocannabinoids and serotoninergic agonists. CBD, THC, 8-OH-DPAT and 5-HT all possess a phenyl group (Fig 6.1), which may be an indication of a shared pharmacology. The phenolic hydroxyl group has been shown to be essential to the pharmacological effect of THC (Reggio et al., 1990; Semus and Martin, 1990). Removal of the phenyl group from spiperone, a non-selective 5-HT_{1A} receptor agonist, greatly decreased its affinity for this receptor (Metwally et al., 1998). This would suggest that further investigation is required to clear up cannabinoid pharmacology. One

may be able to gain a clearer picture of potential receptor targets for CBD and other cannabinoids by analysing the effect of the drugs in animals with these receptors knocked out.

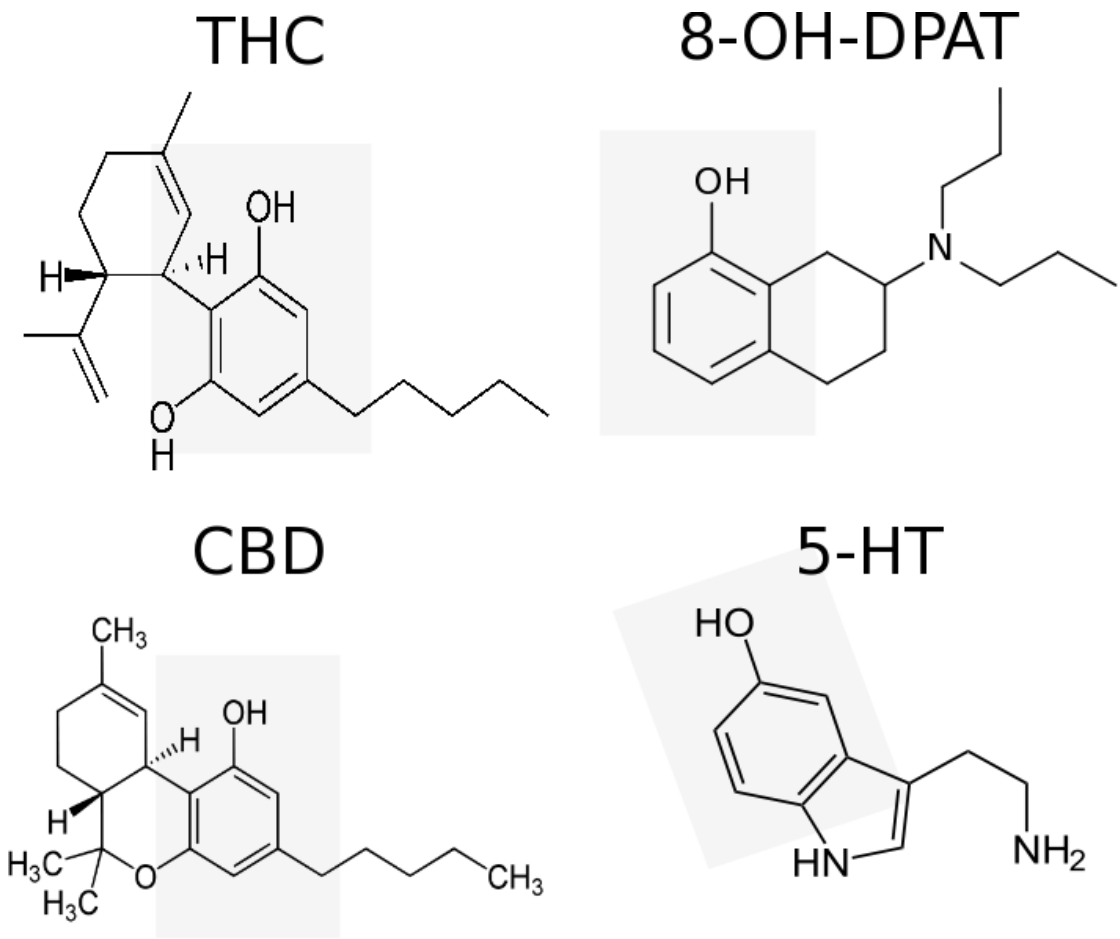

Figure 6.1 Illustration of the structural similarities between CB_1 and 5-HT_{1A} agonists. Chemical structures of CB_1 agonists THC and CBD and 5-HT_{1A} agonists 8-OH-DPAT and 5-HT with the common phenyl group (phenol) highlighted (grey box).

The experiments presented in this thesis also provide the first evidence that CBD can block neuronal effects induced by THC in animals, together with identification of potential regions of interaction. It has been previously shown that CBD administration can suppress some of the behavioural effects induced by THC including anxiogenesis in rodents and humans and impaired performance in tests of spatial working memory in rats. THC (5 mg/kg) administration was shown in this study to result in general regional increases in expression of the IEG *Zif-268* in prefrontal and parietal cortices. CBD administration on its own, particularly at a dose of 5 mg/kg, demonstrated a trend in the opposite direction on *Zif-268* mRNA expression in these regions. When administered together the opposing effects of THC and CBD seem to cancel each other out (Fig 6.2), resulting in a similar level of mRNA expression to that in animals treated with vehicle. This effect was most apparent in the PFC regions, which are involved in executive function. This may suggest that CBD may protect against THC-induced deficits in cognitive behaviour. As CBD has been reported in previous studies and in this thesis to mediate its effects via 5-HT_{1A} receptors, it is proposed here that CBD may inhibit THC-induced IEG induction through activation of this receptor. 5-HT_{1A} receptors have been identified as a potential target by pharmaceutical companies for the treatment of cognitive deficits in patients suffering from psychological illness (for review see Meltzer and Sumiyoshi, 2008).

In the second *in vivo* study, I investigated the ability of CBD to alter changes in regional IEG expression induced by administration of a psychotomimetic agent, phencyclidine (PCP). However, administration of PCP in this study did not replicate the effects of PCP on IEG that has been shown previously (Nabeshima et al., 1996; Nakki et al., 1996; Sugita et al., 1996; Sharp, 1997; Gao et al., 1998; Shirayama et al., 1999). This study was sensitive to CBD induced alterations in IEG expression in prefrontal cortical, striatal and hippocampal regions. Most notably, CBD administration produced significant reductions in *Arc* expression in the prelimbic, infralimbic and orbital regions of the PFC, caudate putamen, posterior cingulate, sensory and motor cortices, as well as in the CA1 region of the hippocampus. CBD

induced reductions in *Arc* expression in cortical regions appear to be dependent on NMDA receptor activation, since PCP administration blocked these reductions in expression of *Arc*. It has also been shown by our group that MK-801 pre-treatment blocks THC-induced alterations in *Arc* expression in similar regions discussed here (Egerton, 2004). Therefore, the role of NMDA receptors in cannabinoid pharmacology warrants further investigation.

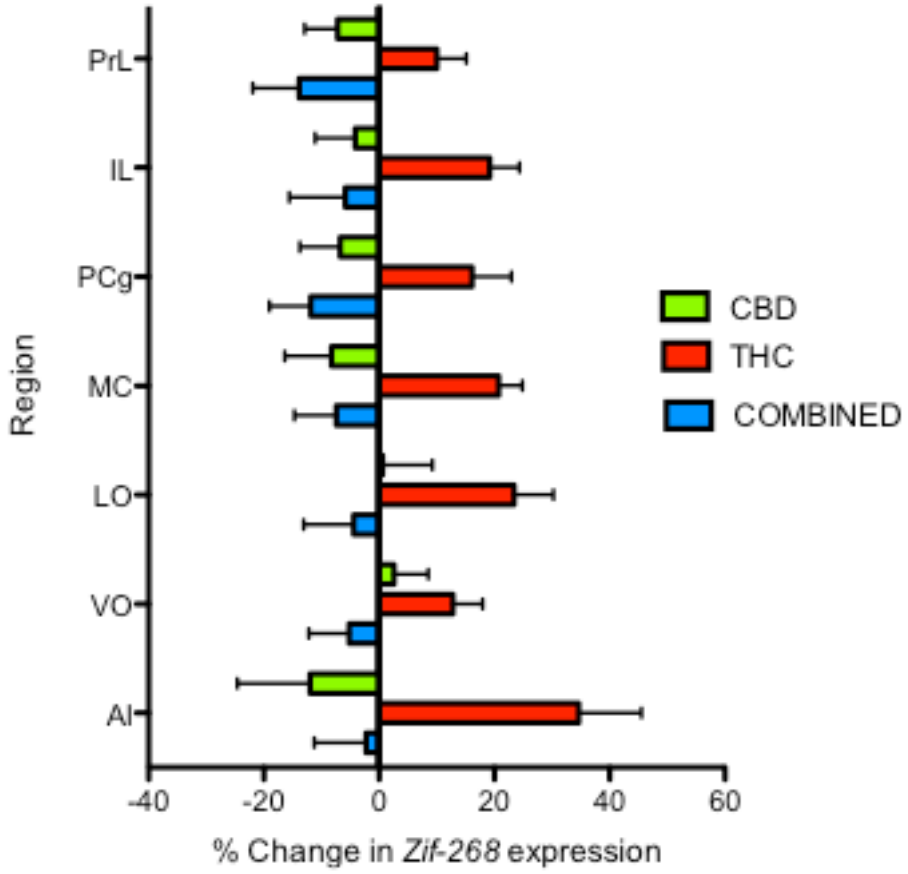

Figure 6.2 CBD and THC display opposing trends in *Zif-268* expression in prefrontal cortical regions of the rat brain. This graph illustrates the % change (± S.E.M.) in *Zif-268* expression following administration of CBD (5mg/kg; Green), THC (5mg/kg; Red) or THC and CBD combined (5mg/kg each; Blue) compared to the level of expression in vehicle treated animals.

6.2 Therapeutic implications

CBD has been proposed to be beneficial in a wide range of disorders. The data presented in this thesis show that CBD has an acute affect on neuronal activity both *in vitro* and *in vivo*. Previous studies using CBD in both animals and humans would also suggest CBD administered acutely does not produce psychological or physiological responses that would suggest that is detrimental to health. Further investigation into the long-term consequences of CBD would be required to corroborate these findings.

Data from the experiment evaluating the interaction of CBD and THC (Chapter 4) might suggest that CBD protects against the psychotropic effects of THC. These data when added to the fMRI data in human subjects (Bhattacharyya et al., 2009b) would suggest that the use of THC and CBD together in a medication, as described by Russo and Guy (2006), may prove to be a suitable method in utilising the potential analgesic and neuroprotective benefits of cannabinoids, without the psychological consequences. This idea is supported by data from trials using Sativex, where no adverse psychological side effects have been noted to date (Wade et al., 2004; Wade et al., 2006). It has been previously reported that around 30% of MS patients have self-medicated with cannabis in order to manage their symptoms. However, given that proportional levels of cannabinoids in strains of cannabis sold illegally are not known or controlled, the psychological effects of THC may outweigh the potential benefits. Therefore, the controlled nature of Sativex production provides an alternative solution for patients who have not responded to more conventional treatment.

Due to the inability of PCP administration to induce changes in regional IEG expression, used as a marker of neuronal function, the potential for CBD to act as an antipsychotic agent could not be evaluated. However, the ability of CBD to activate 5-HT_{1A} receptors would suggest that CBD does hold the potential to act as an antipsychotic agent. Activation of 5-HT_{1A} receptors may contribute to the alleviation of some of the symptoms of schizophrenia

(for reviews see Scatton and Sanger, 2000; Bantick et al., 2001; Meltzer and Sumiyoshi, 2008). CBD was shown to have some promise in treatment of schizophrenia in an anecdotal study (Zuardi et al., 1995; Zuardi et al., 2006b). However, more preclininical studies would be required to evaluate the potential, as there is some confusion over whether it is 5-HT_{1A} agonism or antagonism that improves cognition in schizophrenia (Meltzer and Sumiyoshi, 2008). For example, 8-OH-DPAT has been shown to disrupt cognitve perfomance in rats (Helsley et al., 1998; Ruotsalainen et al., 1998). As discussed in chapter 3, CBD and 8-OH-DPAT may act preferentially at 5-HT_{1A} receptors with a specific receptor localisation, with CBD acting preferentially at presynaptic receptors and 8-OH-DPAT shown to act both pre- and postsynaptically.

6.3 Future directions

Clearly the data contained in this thesis are preliminary in nature and further investigations into the mechanisms of action of CBD are required to generate a clearer picture. There are limitations to the pharmacological evaluation of the receptor-mediated mechanism of CBD-induced suppression of synaptic function because of lack of specificity of selective antagonists and agonsits. The effects of CBD on synaptic activity in CB_1 and 5-HT_{1A} knockout mice may provide a better picture of which of these receptors is required in its function. In the grander scheme, the affinity of cannabinoids for serotoninergic receptors and serotoninergic compounds for cannabinoid receptors should be evaluated more thoroughly. This may also provide an essential clue in the quest for the identity of the ever-elusive CB_3 receptor.

In order to clearly link the regional effects of CBD on IEG expression to the neurophysiological effects found in hippocampal slices and cultures, the mechanism of the CBD-induced reduction in IEG expression should be evaluated *in vivo*. Conversely, it would also be interesting to evaluate the effect of CBD on electrophysiological activity in the hippocampus *in vivo.* Considering the finding that PCP seemed to block the ability of CBD administration to depress *Arc* expression in the prefrontal cortex, it may be worthwhile repeating this experiment with another NMDA antagonist, such

as MK-801. The potential mechanisms of interaction between THC and CBD could also be investigated further. As discussed in section 4.4, investigation into the possibility of inhibiting the effects of CBD on IEG expression with antagonists for CB_1 and/or $5\text{-}HT_{1A}$ receptors may provide a better understanding of the mechanism of action of CBD. Evaluation on the effects other drugs with similar actions as CBD, such as AM404 or 8-OH-DPAT, on THC-induced changes in IEG expression could easily be incorporated into this experimental design.

The evaluation of neuronal function *in vitro* and *in vivo* is a useful tool in evaluating the effects and mechanisms of centrally acting drugs. However, one must also consider the functional consequences of these drugs on relevant behaviour. It is therefore proposed that further evaluation of the effects of both acute and chronic treatment with CBD in naïve and psychopathological models would be required to truly evaluate the potential of CBD to induce long-lasting functional alterations in the CNS and clarify potential therapeutic applications.

REFERENCES

Abood, M. E., and Martin, B. R. (1992). Neurobiology of marijuana abuse. Trends Pharmacol Sci *13*, 201-206.

Abush, H., and Akirav, I. (2009). Cannabinoids modulate hippocampal memory and plasticity. Hippocampus.

Ameri, A. (1999). The effects of cannabinoids on the brain. Prog Neurobiol *58*, 315-348.

Ameri, A., and Simmet, T. (2000). Effects of 2-arachidonylglycerol, an endogenous cannabinoid, on neuronal activity in rat hippocampal slices. Naunyn Schmiedebergs Arch Pharmacol *361*, 265-272.

Ameri, A., Wilhelm, A., and Simmet, T. (1999). Effects of the endogeneous cannabinoid, anandamide, on neuronal activity in rat hippocampal slices. Br J Pharmacol *126*, 1831-1839.

Andersen, E., Rigor, B., and Dafny, N. (1983). Electrophysiological evidence of concurrent dorsal raphe input to caudate, septum, habenula, thalamus hippocampus, cerebellum and olfactory bulb. Int J Neurosci *18*, 107-115.

Andreasson, S., Allebeck, P., Engstrom, A., and Rydberg, U. (1987). Cannabis and schizophrenia. A longitudinal study of Swedish conscripts. Lancet *2*, 1483-1486.

Anis, N. A., Berry, S. C., Burton, N. R., and Lodge, D. (1983). The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurones by N-methyl-aspartate. Br J Pharmacol *79*, 565-575.

Antonelli, T., Tomasini, M. C., Tattoli, M., Cassano, T., Tanganelli, S., Finetti, S., Mazzoni, E., Trabace, L., Steardo, L., Cuomo, V., and Ferraro, L. (2005). Prenatal exposure to the CB1 receptor agonist WIN 55,212-2 causes learning

disruption associated with impaired cortical NMDA receptor function and emotional reactivity changes in rat offspring. Cereb Cortex *15*, 2013-2020.

Arseneault, L., Cannon, M., Poulton, R., Murray, R., Caspi, A., and Moffitt, T. E. (2002). Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. Bmj *325*, 1212-1213.

Bajo, M., Roberto, M., and Schweitzer, P. (2009). Differential alteration of hippocampal excitatory synaptic transmission by cannabinoid ligands. J Neurosci Res *87*, 766-775.

Bantick, R. A., Deakin, J. F., and Grasby, P. M. (2001). The 5-HT1A receptor in schizophrenia: a promising target for novel atypical neuroleptics? J Psychopharmacol *15*, 37-46.

Baskys, A., and Malenka, R. C. (1991). Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. J Physiol *444*, 687-701.

Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A., and Piomelli, D. (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science *277*, 1094-1097.

Ben Amar, M. (2006). Cannabinoids in medicine: A review of their therapeutic potential. J Ethnopharmacol *105*, 1-25.

Beraki, S., Kuzmin, A., Tai, F., and Ogren, S. O. (2008). Repeated low dose of phencyclidine administration impairs spatial learning in mice: blockade by clozapine but not by haloperidol. Eur Neuropsychopharmacol *18*, 486-497.

Berghuis, P., Rajnicek, A. M., Morozov, Y. M., Ross, R. A., Mulder, J., Urban, G. M., Monory, K., Marsicano, G., Matteoli, M., Canty, A., *et al.* (2007). Hardwiring the brain: endocannabinoids shape neuronal connectivity. Science *316*, 1212-1216.

Bhat, R. V., and Baraban, J. M. (1993). Activation of transcription factor genes in striatum by cocaine: role of both serotonin and dopamine systems. J Pharmacol Exp Ther *267*, 496-505.

Bhattacharyya, S., Fusar-Poli, P., Borgwardt, S., Martin-Santos, R., Nosarti, C., O'Carroll, C., Allen, P., Seal, M. L., Fletcher, P. C., Crippa, J. A., *et al.* (2009a). Modulation of mediotemporal and ventrostriatal function in humans by Delta9-tetrahydrocannabinol: a neural basis for the effects of Cannabis sativa on learning and psychosis. Arch Gen Psychiatry *66*, 442-451.

Bhattacharyya, S., Morrison, P. D., Fusar-Poli, P., Martin-Santos, R., Borgwardt, S., Winton-Brown, T., Nosarti, C., CM, O. C., Seal, M., Allen, P., *et al.* (2009b). Opposite Effects of Delta-9-Tetrahydrocannabinol and Cannabidiol on Human Brain Function and Psychopathology. Neuropsychopharmacology.

Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., Moriello, A. S., Davis, J. B., Mechoulam, R., and Di Marzo, V. (2001). Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol *134*, 845-852.

Blazquez, C., Salazar, M., Carracedo, A., Lorente, M., Egia, A., Gonzalez-Feria, L., Haro, A., Velasco, G., and Guzman, M. (2008). Cannabinoids inhibit glioma cell invasion by down-regulating matrix metalloproteinase-2 expression. Cancer Res *68*, 1945-1952.

Borgwardt, S. J., Allen, P., Bhattacharyya, S., Fusar-Poli, P., Crippa, J. A., Seal, M. L., Fraccaro, V., Atakan, Z., Martin-Santos, R., O'Carroll, C., *et al.* (2008). Neural basis of Delta-9-tetrahydrocannabinol and cannabidiol: effects during response inhibition. Biol Psychiatry *64*, 966-973.

Bouaboula, M., Poinot-Chazel, C., Bourrie, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., and Casellas, P. (1995). Activation of

mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. Biochem J *312 (Pt 2)*, 637-641.

Boyd, T. E., Brosnan, J. J., and Maaske, C. A. (1938). THE SUMMATION OF FACILITATING AND INHIBITORY EFFECTS AT THE MAMMALIAN NEUROMUSCULAR JUNCTION. J Neurophysiol *1*, 497.

Bozon, B., Kelly, A., Josselyn, S. A., Silva, A. J., Davis, S., and Laroche, S. (2003). MAPK, CREB and zif268 are all required for the consolidation of recognition memory. Philos Trans R Soc Lond B Biol Sci *358*, 805-814.

Braida, D., Limonta, V., Malabarba, L., Zani, A., and Sala, M. (2007). 5-HT1A receptors are involved in the anxiolytic effect of Delta9-tetrahydrocannabinol and AM 404, the anandamide transport inhibitor, in Sprague-Dawley rats. Eur J Pharmacol *555*, 156-163.

Brett, R., MacKenzie, F., and Pratt, J. (2001). Delta 9-tetrahydrocannabinol-induced alterations in limbic system glucose use in the rat. Neuroreport *12*, 3573-3577.

Brewer, G. J. (1997). Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods *71*, 143-155.

Brewer, G. J., Torricelli, J. R., Evege, E. K., and Price, P. J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res *35*, 567-576.

Brown, A. J. (2007). Novel cannabinoid receptors. Br J Pharmacol *152*, 567-575.

Caffarel, M. M., Sarrio, D., Palacios, J., Guzman, M., and Sanchez, C. (2006). Delta9-tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. Cancer Res *66*, 6615-6621.

Campbell, V. A. (2001). Tetrahydrocannabinol-induced apoptosis of cultured cortical neurones is associated with cytochrome c release and caspase-3 activation. Neuropharmacology *40*, 702-709.

Campos, A. C., and Guimaraes, F. S. (2008). Involvement of 5HT1A receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats. Psychopharmacology (Berl) *199*, 223-230.

Cascio, M. G., Gauson, L. A., Stevenson, L. A., Ross, R. A., and Pertwee, R. G. (2010). Evidence that the plant cannabinoid cannabigerol is a highly potent alpha2-adrenoceptor agonist and moderately potent 5HT1A receptor antagonist. Br J Pharmacol *159*, 129-141.

Caspi, A., Moffitt, T. E., Cannon, M., McClay, J., Murray, R., Harrington, H., Taylor, A., Arseneault, L., Williams, B., Braithwaite, A., *et al.* (2005). Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. Biol Psychiatry *57*, 1117-1127.

Chalmers, D. T., and Watson, S. J. (1991). Comparative anatomical distribution of 5-HT1A receptor mRNA and 5-HT1A binding in rat brain--a combined in situ hybridisation/in vitro receptor autoradiographic study. Brain Res *561*, 51-60.

Chan, G. C., Hinds, T. R., Impey, S., and Storm, D. R. (1998). Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol. J Neurosci *18*, 5322-5332.

Chiaia, N., Foy, M., and Teyler, T. J. (1983). The hamster hippocampal slice: II. Neuroendocrine modulation. Behav Neurosci *97*, 839-843.

Chopra, G. S., and Smith, J. W. (1974). Psychotic reactions following cannabis use in East Indians. Arch Gen Psychiatry *30*, 24-27.

Chowdhury, S., Shepherd, J. D., Okuno, H., Lyford, G., Petralia, R. S., Plath, N., Kuhl, D., Huganir, R. L., and Worley, P. F. (2006). Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. Neuron *52*, 445-459.

Clayton, D. F. (2000). The genomic action potential. Neurobiol Learn Mem *74*, 185-216.

Cochran, B. H. (1993). Regulation of immediate early gene expression. NIDA Res Monogr *125*, 3-24.

Cochran, S. M., Kennedy, M., McKerchar, C. E., Steward, L. J., Pratt, J. A., and Morris, B. J. (2003). Induction of metabolic hypofunction and neurochemical deficits after chronic intermittent exposure to phencyclidine: differential modulation by antipsychotic drugs. Neuropsychopharmacology *28*, 265-275.

Collin, C., Davies, P., Mutiboko, I. K., and Ratcliffe, S. (2007). Randomized controlled trial of cannabis-based medicine in spasticity caused by multiple sclerosis. Eur J Neurol *14*, 290-296.

Creager, R., Dunwiddie, T., and Lynch, G. (1980). Paired-pulse and frequency facilitation in the CA1 region of the in vitro rat hippocampus. J Physiol *299*, 409-424.

D'Souza, D. C., Abi-Saab, W. M., Madonick, S., Forselius-Bielen, K., Doersch, A., Braley, G., Gueorguieva, R., Cooper, T. B., and Krystal, J. H. (2005). Delta-9-tetrahydrocannabinol effects in schizophrenia: implications for cognition, psychosis, and addiction. Biol Psychiatry *57*, 594-608.

Davis, S., Bozon, B., and Laroche, S. (2003). How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? Behav Brain Res *142*, 17-30.

Davison, K., and Wilson, C. H. (1972). Psychosis associated with cannabis smoking. Br J Addict Alcohol Other Drugs *67*, 225-228.

Deakin, J. F., Lees, J., McKie, S., Hallak, J. E., Williams, S. R., and Dursun, S. M. (2008). Glutamate and the neural basis of the subjective effects of ketamine: a pharmaco-magnetic resonance imaging study. Arch Gen Psychiatry *65*, 154-164.

Derkinderen, P., Valjent, E., Toutant, M., Corvol, J. C., Enslen, H., Ledent, C., Trzaskos, J., Caboche, J., and Girault, J. A. (2003). Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. J Neurosci *23*, 2371-2382.

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

Dewey, W. L. (1986). Cannabinoid pharmacology. Pharmacol Rev *38*, 151-178.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., and Piomelli, D. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature *372*, 686-691.

Diana, G., Malloni, M., and Pieri, M. (2003). Effects of the synthetic cannabinoid nabilone on spatial learning and hippocampal neurotransmission. Pharmacol Biochem Behav *75*, 585-591.

Diana, M., Melis, M., and Gessa, G. L. (1998). Increase in meso-prefrontal dopaminergic activity after stimulation of CB1 receptors by cannabinoids. Eur J Neurosci *10*, 2825-2830.

Domino, E. F. (1980). History and pharmacology of PCP and PCP-related analogs. J Psychedelic Drugs *12*, 223-227.

Drysdale, A. J., Ryan, D., Pertwee, R. G., and Platt, B. (2006). Cannabidiol-induced intracellular Ca2+ elevations in hippocampal cells. Neuropharmacology *50*, 621-631.

Egashira, N., Matsuda, T., Koushi, E., Higashihara, F., Mishima, K., Chidori, S., Hasebe, N., Iwasaki, K., Nishimura, R., Oishi, R., and Fujiwara, M. (2008). Delta(9)-tetrahydrocannabinol prolongs the immobility time in the mouse forced swim test: involvement of cannabinoid CB(1) receptor and serotonergic system. Eur J Pharmacol *589*, 117-121.

Egerton, A. (2004). Neurochemical and behavioural consequences of delta-9-tetrahydrocannabinol administration : a focus on the prefrontal cortex and striatum. University of Strathclyde, Doctoral Thesis.

Egerton, A., Allison, C., Brett, R. R., and Pratt, J. A. (2006). Cannabinoids and prefrontal cortical function: insights from preclinical studies. Neurosci Biobehav Rev *30*, 680-695.

Egerton, A., Brett, R. R., and Pratt, J. A. (2005a). Acute delta9-tetrahydrocannabinol-induced deficits in reversal learning: neural correlates of affective inflexibility. Neuropsychopharmacology *30*, 1895-1905.

Egerton, A., Reid, L., McGregor, S., Cochran, S. M., Morris, B. J., and Pratt, J. A. (2008). Subchronic and chronic PCP treatment produces temporally distinct deficits in attentional set shifting and prepulse inhibition in rats. Psychopharmacology (Berl) *198*, 37-49.

Egerton, A., Reid, L., McKerchar, C. E., Morris, B. J., and Pratt, J. A. (2005b). Impairment in perceptual attentional set-shifting following PCP administration: a rodent model of set-shifting deficits in schizophrenia. Psychopharmacology (Berl) *179*, 77-84.

Eggan, S. M., and Lewis, D. A. (2007). Immunocytochemical distribution of the cannabinoid CB1 receptor in the primate neocortex: a regional and laminar analysis. Cereb Cortex *17*, 175-191.

El-Remessy, A. B., Khalil, I. E., Matragoon, S., Abou-Mohamed, G., Tsai, N. J., Roon, P., Caldwell, R. B., Caldwell, R. W., Green, K., and Liou, G. I. (2003). Neuroprotective effect of (-)Delta9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. Am J Pathol *163*, 1997-2008.

Erdtmann-Vourliotis, M., Mayer, P., Riechert, U., and Hollt, V. (1999). Acute injection of drugs with low addictive potential (delta(9)-tetrahydrocannabinol, 3,4-methylenedioxymethamphetamine, lysergic acid diamide) causes a much higher c-fos expression in limbic brain areas than highly addicting drugs (cocaine and morphine). Brain Res Mol Brain Res *71*, 313-324.

Eubanks, L. M., Rogers, C. J., Beuscher, A. E. t., Koob, G. F., Olson, A. J., Dickerson, T. J., and Janda, K. D. (2006). A molecular link between the active component of marijuana and Alzheimer's disease pathology. Mol Pharm *3*, 773-777.

Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G., and Riedel, G. (2004). Differential effects of THC- or CBD-rich cannabis extracts on working memory in rats. Neuropharmacology *47*, 1170-1179.

Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G., and Riedel, G. (2006). Scopolamine and MK801-induced working memory deficits in rats are not reversed by CBD-rich cannabis extracts. Behav Brain Res *168*, 307-311.

Ferrari, F., Ottani, A., Vivoli, R., and Giuliani, D. (1999). Learning impairment produced in rats by the cannabinoid agonist HU 210 in a water-maze task. Pharmacol Biochem Behav *64*, 555-561.

Ferry, A. T., Ongur, D., An, X., and Price, J. L. (2000). Prefrontal cortical projections to the striatum in macaque monkeys: evidence for an organization related to prefrontal networks. J Comp Neurol *425*, 447-470.

File, S. E., Gonzalez, L. E., and Andrews, N. (1996). Comparative study of pre- and postsynaptic 5-HT1A receptor modulation of anxiety in two ethological animal tests. J Neurosci *16*, 4810-4815.

Filliat, P., and Blanchet, G. (1995). Effects of TCP on spatial memory: comparison with MK-801. Pharmacol Biochem Behav *51*, 429-434.

Foy, M. R., Teyler, T. J., and Vardaris, R. M. (1982). delta 9-THC and 17-beta-estradiol in hippocampus. Brain Res Bull *8*, 341-345.

Freedland, C. S., Whitlow, C. T., Miller, M. D., and Porrino, L. J. (2002). Dose-dependent effects of Delta9-tetrahydrocannabinol on rates of local cerebral glucose utilization in rat. Synapse *45*, 134-142.

Fusar-Poli, P., Allen, P., Bhattacharyya, S., Crippa, J. A., Mechelli, A., Borgwardt, S., Martin-Santos, R., Seal, M. L., O'Carrol, C., Atakan, Z., *et al.* (2009a). Modulation of effective connectivity during emotional processing by Delta9-tetrahydrocannabinol and cannabidiol. Int J Neuropsychopharmacol, 1-12.

Fusar-Poli, P., Crippa, J. A., Bhattacharyya, S., Borgwardt, S. J., Allen, P., Martin-Santos, R., Seal, M., Surguladze, S. A., O'Carrol, C., Atakan, Z., *et al.* (2009b). Distinct effects of {delta}9-tetrahydrocannabinol and cannabidiol on neural activation during emotional processing. Arch Gen Psychiatry *66*, 95-105.

Gao, X. M., Hashimoto, T., and Tamminga, C. A. (1998). Phencyclidine (PCP) and dizocilpine (MK801) exert time-dependent effects on the expression of immediate early genes in rat brain. Synapse *29*, 14-28.

Gerdeman, G., and Lovinger, D. M. (2001). CB1 cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum. J Neurophysiol *85*, 468-471.

Giuffrida, A., Leweke, F. M., Gerth, C. W., Schreiber, D., Koethe, D., Faulhaber, J., Klosterkotter, J., and Piomelli, D. (2004). Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. Neuropsychopharmacology *29*, 2108-2114.

Glass, M., Dragunow, M., and Faull, R. L. (2000). The pattern of neurodegeneration in Huntington's disease: a comparative study of cannabinoid, dopamine, adenosine and GABA(A) receptor alterations in the human basal ganglia in Huntington's disease. Neuroscience *97*, 505-519.

Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q. R., Tagliaferro, P. A., Brusco, A., and Uhl, G. R. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res *1071*, 10-23.

Gorriti, M. A., Rodriguez de Fonseca, F., Navarro, M., and Palomo, T. (1999). Chronic (-)-delta9-tetrahydrocannabinol treatment induces sensitization to the psychomotor effects of amphetamine in rats. Eur J Pharmacol *365*, 133-142.

Gotoh, L., Kawanami, N., Nakahara, T., Hondo, H., Motomura, K., Ohta, E., Kanchiku, I., Kuroki, T., Hirano, M., and Uchimura, H. (2002). Effects of the adenosine A(1) receptor agonist N(6)-cyclopentyladenosine on phencyclidine-induced behavior and expression of the immediate-early genes in the discrete brain regions of rats. Brain Res Mol Brain Res *100*, 1-12.

Grech, A. (2008). Should we advise patients with serious mental illness to stop using cannabis? Psychol Med *38*, 459; author reply 462-454.

Grech, A., Van Os, J., Jones, P. B., Lewis, S. W., and Murray, R. M. (2005). Cannabis use and outcome of recent onset psychosis. Eur Psychiatry *20*, 349-353.

Groenewegen, H. J., and Uylings, H. B. (2000). The prefrontal cortex and the integration of sensory, limbic and autonomic information. Prog Brain Res *126*, 3-28.

Guimaraes, F. S., Chiaretti, T. M., Graeff, F. G., and Zuardi, A. W. (1990). Antianxiety effect of cannabidiol in the elevated plus-maze. Psychopharmacology (Berl) *100*, 558-559.

Guimaraes, V. M., Zuardi, A. W., Del Bel, E. A., and Guimaraes, F. S. (2004). Cannabidiol increases Fos expression in the nucleus accumbens but not in the dorsal striatum. Life Sci *75*, 633-638.

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

Hajos, N., Katona, I., Naiem, S. S., MacKie, K., Ledent, C., Mody, I., and Freund, T. F. (2000). Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. Eur J Neurosci *12*, 3239-3249.

Hajos, N., Ledent, C., and Freund, T. F. (2001). Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience *106*, 1-4.

Hampson, A. J., Grimaldi, M., Axelrod, J., and Wink, D. (1998). Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. Proc Natl Acad Sci U S A *95*, 8268-8273.

Haney, M., Gunderson, E. W., Rabkin, J., Hart, C. L., Vosburg, S. K., Comer, S. D., and Foltin, R. W. (2007). Dronabinol and marijuana in HIV-positive

marijuana smokers. Caloric intake, mood, and sleep. J Acquir Immune Defic Syndr *45*, 545-554.

Haney, M., Rabkin, J., Gunderson, E., and Foltin, R. W. (2005). Dronabinol and marijuana in HIV(+) marijuana smokers: acute effects on caloric intake and mood. Psychopharmacology (Berl) *181*, 170-178.

Harlan, R. E., and Garcia, M. M. (1998). Drugs of abuse and immediate-early genes in the forebrain. Mol Neurobiol *16*, 221-267.

Harris, D., and Batki, S. L. (2000). Stimulant psychosis: symptom profile and acute clinical course. Am J Addict *9*, 28-37.

Harrison, P. J., and Owen, M. J. (2003). Genes for schizophrenia? Recent findings and their pathophysiological implications. Lancet *361*, 417-419.

Helsley, S., Siegel, T. L., Fiorella, D., Rabin, R. A., and Winter, J. C. (1998). WAY-100635 reverses 8-OH-DPAT-induced performance impairment in the radial maze. Prog Neuropsychopharmacol Biol Psychiatry *22*, 1179-1184.

Henquet, C., Di Forti, M., Morrison, P., Kuepper, R., and Murray, R. M. (2008a). Gene-Environment Interplay Between Cannabis and Psychosis. Schizophr Bull.

Henquet, C., Di Forti, M., Morrison, P., Kuepper, R., and Murray, R. M. (2008b). Gene-environment interplay between cannabis and psychosis. Schizophr Bull *34*, 1111-1121.

Henquet, C., Murray, R., Linszen, D., and van Os, J. (2005). The environment and schizophrenia: the role of cannabis use. Schizophr Bull *31*, 608-612.

Henstridge, C. M., Balenga, N. A., Ford, L. A., Ross, R. A., Waldhoer, M., and Irving, A. J. (2009). The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca2+ signaling and NFAT activation. FASEB J *23*, 183-193.

Henstridge, C. M., Balenga, N. A., Schroder, R., Kargl, J. K., Platzer, W., Martini, L., Arthur, S., Penman, J., Whistler, J. L., Kostenis, E., *et al.* (2010). GPR55 ligands promote receptor coupling to multiple signalling pathways. Br J Pharmacol.

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Rice, K. C. (1990). Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A *87*, 1932-1936.

Hollister, L. E. (1986). Health aspects of cannabis. Pharmacol Rev *38*, 1-20.

Howlett, A. C. (2005). Cannabinoid receptor signaling. Handb Exp Pharmacol, 53-79.

Howlett, A. C., and Fleming, R. M. (1984). Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. Mol Pharmacol *26*, 532-538.

Howlett, A. C., Johnson, M. R., and Melvin, L. S. (1990). Classical and nonclassical cannabinoids: mechanism of action--brain binding. NIDA Res Monogr *96*, 100-111.

Howlett, A. C., Qualy, J. M., and Khachatrian, L. L. (1986). Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol Pharmacol *29*, 307-313.

Huang, C. C., Lo, S. W., and Hsu, K. S. (2001). Presynaptic mechanisms underlying cannabinoid inhibition of excitatory synaptic transmission in rat striatal neurons. J Physiol *532*, 731-748.

Hughes, P., and Dragunow, M. (1995). Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. Pharmacol Rev *47*, 133-178.

Hutter, O. F., and Kostial, K. (1954). Effect of magnesium and calcium ions on the release of acetylcholine. J Physiol *124*, 234-241.

Irving, A. J., Coutts, A. A., Harvey, J., Rae, M. G., Mackie, K., Bewick, G. S., and Pertwee, R. G. (2000). Functional expression of cell surface cannabinoid CB(1) receptors on presynaptic inhibitory terminals in cultured rat hippocampal neurons. Neuroscience *98*, 253-262.

Irving, A. J., Rae, M. G., and Coutts, A. A. (2002). Cannabinoids on the brain. ScientificWorldJournal *2*, 632-648.

Itil, T., Keskiner, A., Kiremitci, N., and Holden, J. M. (1967). Effect of phencyclidine in chronic schizophrenics. Can Psychiatr Assoc J *12*, 209-212.

Javitt, D. C. (2007). Glutamate and schizophrenia: phencyclidine, N-methyl-D-aspartate receptors, and dopamine-glutamate interactions. Int Rev Neurobiol *78*, 69-108.

Jay, T. M., Thierry, A. M., Wiklund, L., and Glowinski, J. (1992). Excitatory Amino Acid Pathway from the Hippocampus to the Prefrontal Cortex. Contribution of AMPA Receptors in Hippocampo-prefrontal Cortex Transmission. Eur J Neurosci *4*, 1285-1295.

Johns, D. G., Behm, D. J., Walker, D. J., Ao, Z., Shapland, E. M., Daniels, D. A., Riddick, M., Dowell, S., Staton, P. C., Green, P., *et al.* (2007). The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. Br J Pharmacol *152*, 825-831.

Jones, M. W., Errington, M. L., French, P. J., Fine, A., Bliss, T. V., Garel, S., Charnay, P., Bozon, B., Laroche, S., and Davis, S. (2001). A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. Nat Neurosci *4*, 289-296.

Jones, N. A., Hill, A. J., Smith, I., Bevan, S. A., Williams, C. M., Whalley, B. J., and Stephens, G. J. (2009). Cannabidiol displays anti-epileptiform and anti-seizure properties in vitro and in vivo. J Pharmacol Exp Ther.

Kang-Park, M. H., Wilson, W. A., Kuhn, C. M., Moore, S. D., and Swartzwelder, H. S. (2007). Differential sensitivity of GABA A receptor-mediated IPSCs to cannabinoids in hippocampal slices from adolescent and adult rats. J Neurophysiol *98*, 1223-1230.

Karniol, I. G., and Carlini, E. A. (1973). Pharmacological interaction between cannabidiol and delta 9-tetrahydrocannabinol. Psychopharmacologia *33*, 53-70.

Katona, I., Sperlagh, B., Magloczky, Z., Santha, E., Kofalvi, A., Czirjak, S., Mackie, K., Vizi, E. S., and Freund, T. F. (2000). GABAergic interneurons are the targets of cannabinoid actions in the human hippocampus. Neuroscience *100*, 797-804.

Katona, I., Sperlagh, B., Sik, A., Kafalvi, A., Vizi, E. S., Mackie, K., and Freund, T. F. (1999). Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J Neurosci *19*, 4544-4558.

Katona, I., Urban, G. M., Wallace, M., Ledent, C., Jung, K. M., Piomelli, D., Mackie, K., and Freund, T. F. (2006). Molecular composition of the endocannabinoid system at glutamatergic synapses. J Neurosci *26*, 5628-5637.

Kawamura, Y., Fukaya, M., Maejima, T., Yoshida, T., Miura, E., Watanabe, M., Ohno-Shosaku, T., and Kano, M. (2006). The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. J Neurosci *26*, 2991-3001.

Kesner, R. P., and Dakis, M. (1995). Phencyclidine injections into the dorsal hippocampus disrupt long- but not short-term memory within a spatial learning task. Psychopharmacology (Berl) *120*, 203-208.

Kesner, R. P., Dakis, M., and Bolland, B. L. (1993). Phencyclidine disrupts long- but not short-term memory within a spatial learning task. Psychopharmacology (Berl) *111*, 85-90.

Kim, D. J., and Thayer, S. A. (2000). Activation of CB1 cannabinoid receptors inhibits neurotransmitter release from identified synaptic sites in rat hippocampal cultures. Brain Res *852*, 398-405.

Kim, H. J., Waataja, J. J., and Thayer, S. A. (2008). Cannabinoids inhibit network-driven synapse loss between hippocampal neurons in culture. J Pharmacol Exp Ther *325*, 850-858.

Koch, T., Wu, D. F., Yang, L. Q., Brandenburg, L. O., and Hollt, V. (2006). Role of phospholipase D2 in the agonist-induced and constitutive endocytosis of G-protein coupled receptors. J Neurochem *97*, 365-372.

Koe, B. K., Milne, G. M., Weissman, A., Johnson, M. R., and Melvin, L. S. (1985). Enhancement of brain [3H]flunitrazepam binding and analgesic activity of synthetic cannabimimetics. Eur J Pharmacol *109*, 201-212.

Koethe, D., Llenos, I. C., Dulay, J. R., Hoyer, C., Torrey, E. F., Leweke, F. M., and Weis, S. (2007). Expression of CB1 cannabinoid receptor in the anterior cingulate cortex in schizophrenia, bipolar disorder, and major depression. J Neural Transm *114*, 1055-1063.

Kofalvi, A., Rodrigues, R. J., Ledent, C., Mackie, K., Vizi, E. S., Cunha, R. A., and Sperlagh, B. (2005). Involvement of cannabinoid receptors in the regulation of neurotransmitter release in the rodent striatum: a combined immunochemical and pharmacological analysis. J Neurosci *25*, 2874-2884.

Konings, M., Henquet, C., Maharajh, H. D., Hutchinson, G., and Van Os, J. (2008). Early exposure to cannabis and risk for psychosis in young adolescents in Trinidad. Acta Psychiatr Scand *118*, 209-213.

Kovacs, K. J. (1998). c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Int *33*, 287-297.

Kreitzer, A. C., and Regehr, W. G. (2001a). Cerebellar depolarization-induced suppression of inhibition is mediated by endogenous cannabinoids. J Neurosci *21*, RC174.

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

Kujtan, P. W., Carlen, P. L., and Kapur, B. M. (1983). delta 9-Tetrahydrocannabinol and cannabidiol: dose-dependent effects on evoked potentials in the hippocampal slice. Can J Physiol Pharmacol *61*, 420-426.

Kunos, G., Jarai, Z., Batkai, S., Goparaju, S. K., Ishac, E. J., Liu, J., Wang, L., and Wagner, J. A. (2000). Endocannabinoids as cardiovascular modulators. Chem Phys Lipids *108*, 159-168.

Laaris, N., Good, C. H., and Lupica, C. R. (2010). Delta(9)-tetrahydrocannabinol is a full agonist at CB1 receptors on GABA neuron axon terminals in the hippocampus. Neuropharmacology.

Lanahan, A., and Worley, P. (1998). Immediate-early genes and synaptic function. Neurobiol Learn Mem *70*, 37-43.

Langsjo, J. W., Kaisti, K. K., Aalto, S., Hinkka, S., Aantaa, R., Oikonen, V., Sipila, H., Kurki, T., Silvanto, M., and Scheinin, H. (2003). Effects of subanesthetic doses of ketamine on regional cerebral blood flow, oxygen consumption, and blood volume in humans. Anesthesiology *99*, 614-623.

Lauckner, J. E., Hille, B., and Mackie, K. (2005). The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. Proc Natl Acad Sci U S A *102*, 19144-19149.

Lemos, J. I., Resstel, L. B., and Guimaraes, F. S. (2010). Involvement of the prelimbic prefrontal cortex on cannabidiol-induced attenuation of contextual conditioned fear in rats. Behav Brain Res *207*, 105-111.

Levy, R., and Goldman-Rakic, P. S. (2000). Segregation of working memory functions within the dorsolateral prefrontal cortex. Exp Brain Res *133*, 23-32.

Leweke, F. M., Giuffrida, A., Koethe, D., Schreiber, D., Nolden, B. M., Kranaster, L., Neatby, M. A., Schneider, M., Gerth, C. W., Hellmich, M., *et al.* (2007). Anandamide levels in cerebrospinal fluid of first-episode schizophrenic patients: impact of cannabis use. Schizophr Res *94*, 29-36.

Leweke, F. M., Schneider, U., Thies, M., Munte, T. F., and Emrich, H. M. (1999). Effects of synthetic delta9-tetrahydrocannabinol on binocular depth inversion of natural and artificial objects in man. Psychopharmacology (Berl) *142*, 230-235.

Lewis, D. A., Hashimoto, T., and Volk, D. W. (2005). Cortical inhibitory neurons and schizophrenia. Nat Rev Neurosci *6*, 312-324.

Li, H. L. (1974). An archaeological and historical account of cannabis in China. Economic Botany *28*, 437-448.

Lichtman, A. H., Dimen, K. R., and Martin, B. R. (1995). Systemic or intrahippocampal cannabinoid administration impairs spatial memory in rats. Psychopharmacology (Berl) *119*, 282-290.

Lichtman, A. H., and Martin, B. R. (1996). Delta 9-tetrahydrocannabinol impairs spatial memory through a cannabinoid receptor mechanism. Psychopharmacology (Berl) *126*, 125-131.

Long, L. E., Malone, D. T., and Taylor, D. A. (2006). Cannabidiol reverses MK-801-induced disruption of prepulse inhibition in mice. Neuropsychopharmacology *31*, 795-803.

Luzi, S., Morrison, P. D., Powell, J., di Forti, M., and Murray, R. M. (2008). What is the mechanism whereby cannabis use increases risk of psychosis? Neurotox Res *14*, 105-112.

Lyford, G. L., Yamagata, K., Kaufmann, W. E., Barnes, C. A., Sanders, L. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Lanahan, A. A., and Worley, P. F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron *14*, 433-445.

Maejima, T., Ohno-Shosaku, T., and Kano, M. (2001). Endogenous cannabinoid as a retrograde messenger from depolarized postsynaptic neurons to presynaptic terminals. Neurosci Res *40*, 205-210.

Mailleux, P., Verslype, M., Preud'homme, X., and Vanderhaeghen, J. J. (1994). Activation of multiple transcription factor genes by tetrahydrocannabinol in rat forebrain. Neuroreport *5*, 1265-1268.

Marco, E. M., Perez-Alvarez, L., Borcel, E., Rubio, M., Guaza, C., Ambrosio, E., File, S. E., and Viveros, M. P. (2004). Involvement of 5-HT1A receptors in behavioural effects of the cannabinoid receptor agonist CP 55,940 in male rats. Behav Pharmacol *15*, 21-27.

Margulies, J. E., and Hammer, R. P., Jr. (1991). Delta 9-tetrahydrocannabinol alters cerebral metabolism in a biphasic, dose-dependent manner in rat brain. Eur J Pharmacol *202*, 373-378.

Martin, B. R. (1986). Cellular effects of cannabinoids. Pharmacol Rev *38*, 45-74.

Mathew, R. J., and Wilson, W. H. (1993). Acute changes in cerebral blood flow after smoking marijuana. Life Sci *52*, 757-767.

Mathew, R. J., Wilson, W. H., Humphreys, D. F., Lowe, J. V., and Wiethe, K. E. (1992). Regional cerebral blood flow after marijuana smoking. J Cereb Blood Flow Metab *12*, 750-758.

Mathew, R. J., Wilson, W. H., and Tant, S. R. (1989). Acute changes in cerebral blood flow associated with marijuana smoking. Acta Psychiatr Scand *79*, 118-128.

Mato, S., Aso, E., Castro, E., Martin, M., Valverde, O., Maldonado, R., and Pazos, A. (2007). CB1 knockout mice display impaired functionality of 5-HT1A and 5-HT2A/C receptors. J Neurochem *103*, 2111-2120.

Mato, S., Vidal, R., Castro, E., Diaz, A., Pazos, A., and Valdizan, E. M. (2010). Long-term fluoxetine treatment modulates cannabinoid type 1 receptor-mediated inhibition of adenylyl cyclase in the rat prefrontal cortex through 5-hydroxytryptamine 1A receptor-dependent mechanisms. Mol Pharmacol *77*, 424-434.

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

McAllister, S. D., Christian, R. T., Horowitz, M. P., Garcia, A., and Desprez, P. Y. (2007). Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells. Mol Cancer Ther *6*, 2921-2927.

McGregor, I. S., Arnold, J. C., Weber, M. F., Topple, A. N., and Hunt, G. E. (1998). A comparison of delta 9-THC and anandamide induced c-fos expression in the rat forebrain. Brain Res *802*, 19-26.

McKallip, R. J., Nagarkatti, M., and Nagarkatti, P. S. (2005). Delta-9-tetrahydrocannabinol enhances breast cancer growth and metastasis by suppression of the antitumor immune response. J Immunol *174*, 3281-3289.

Mechoulam, R. (1970). Marihuana chemistry. Science *168*, 1159-1166.

Mechoulam, R., and Hanus, L. (2001). The cannabinoids: an overview. Therapeutic implications in vomiting and nausea after cancer chemotherapy, in appetite promotion, in multiple sclerosis and in neuroprotection. Pain Res Manag *6*, 67-73.

Mechoulam, R., Hanus, L., and Martin, B. R. (1994). Search for endogenous ligands of the cannabinoid receptor. Biochem Pharmacol *48*, 1537-1544.

Meltzer, H. Y., and Sumiyoshi, T. (2008). Does stimulation of 5-HT(1A) receptors improve cognition in schizophrenia? Behav Brain Res *195*, 98-102.

Mereu, G., Fa, M., Ferraro, L., Cagiano, R., Antonelli, T., Tattoli, M., Ghiglieri, V., Tanganelli, S., Gessa, G. L., and Cuomo, V. (2003). Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release. Proc Natl Acad Sci U S A *100*, 4915-4920.

Metwally, K. A., Dukat, M., Egan, C. T., Smith, C., DuPre, A., Gauthier, C. B., Herrick-Davis, K., Teitler, M., and Glennon, R. A. (1998). Spiperone: influence of spiro ring substituents on 5-HT2A serotonin receptor binding. J Med Chem *41*, 5084-5093.

Mishima, K., Egashira, N., Hirosawa, N., Fujii, M., Matsumoto, Y., Iwasaki, K., and Fujiwara, M. (2001). Characteristics of learning and memory impairment induced by delta9-tetrahydrocannabinol in rats. Jpn J Pharmacol *87*, 297-308.

Mishima, K., Hayakawa, K., Abe, K., Ikeda, T., Egashira, N., Iwasaki, K., and Fujiwara, M. (2005). Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. Stroke *36*, 1077-1082.

Miyamoto, A., Yamamoto, T., Ohno, M., Watanabe, S., Tanaka, H., Morimoto, S., and Shoyama, Y. (1996). Roles of dopamine D1 receptors in delta 9-tetrahydrocannabinol-induced expression of Fos protein in the rat brain. Brain Res *710*, 234-240.

Moreira, F. A., Aguiar, D. C., and Guimaraes, F. S. (2006). Anxiolytic-like effect of cannabidiol in the rat Vogel conflict test. Prog Neuropsychopharmacol Biol Psychiatry *30*, 1466-1471.

Morgan, C. J., and Curran, H. V. (2008). Effects of cannabidiol on schizophrenia-like symptoms in people who use cannabis. Br J Psychiatry *192*, 306-307.

Morris, B. J., Cochran, S. M., and Pratt, J. A. (2005). PCP: from pharmacology to modelling schizophrenia. Curr Opin Pharmacol *5*, 101-106.

Morrison, P. D., Zois, V., McKeown, D. A., Lee, T. D., Holt, D. W., Powell, J. F., Kapur, S., and Murray, R. M. (2009). The acute effects of synthetic intravenous Delta9-tetrahydrocannabinol on psychosis, mood and cognitive functioning. Psychol Med *39*, 1607-1616.

Mu, J., Zhuang, S. Y., Kirby, M. T., Hampson, R. E., and Deadwyler, S. A. (1999). Cannabinoid receptors differentially modulate potassium A and D currents in hippocampal neurons in culture. J Pharmacol Exp Ther *291*, 893-902.

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. Nature *365*, 61-65.

Nabeshima, T., Kitaichi, K., and Noda, Y. (1996). Functional changes in neuronal systems induced by phencyclidine administration. Ann N Y Acad Sci *801*, 29-38.

Nakahara, T., Kuroki, T., Hashimoto, K., Hondo, H., Tsutsumi, T., Motomura, K., Ueki, H., Hirano, M., and Uchimura, H. (2000). Effect of atypical antipsychotics on phencyclidine-induced expression of arc in rat brain. Neuroreport *11*, 551-555.

Nakki, R., Sharp, F. R., Sagar, S. M., and Honkaniemi, J. (1996). Effects of phencyclidine on immediate early gene expression in the brain. J Neurosci Res *45*, 13-27.

Negrete, J. C. (1989). Cannabis and schizophrenia. Br J Addict *84*, 349-351.

Nicholi, A. M., Jr. (1984). Phencyclidine hydrochloride (PCP) use among college students: subjective and clinical effects, toxicity, diagnosis, and treatment. J Am Coll Health *32*, 197-200.

Northoff, G., Richter, A., Bermpohl, F., Grimm, S., Martin, E., Marcar, V. L., Wahl, C., Hell, D., and Boeker, H. (2005). NMDA hypofunction in the posterior cingulate as a model for schizophrenia: an exploratory ketamine administration study in fMRI. Schizophr Res *72*, 235-248.

O'Leary, D. S., Block, R. I., Koeppel, J. A., Flaum, M., Schultz, S. K., Andreasen, N. C., Ponto, L. B., Watkins, G. L., Hurtig, R. R., and Hichwa, R. D. (2002). Effects of smoking marijuana on brain perfusion and cognition. Neuropsychopharmacology *26*, 802-816.

O'Shaughnessy, W. B. (1843). ON THE PREPARATIONS OF THE INDIAN HEMP, OR GUNJAH,*(Cannabis Indisa): Their effects on the Animal System in Health, and their utility in the treatment of Tetanus and other Convulsive Diseases. PROVINCIAL MEDICAL JOURNAL *123*, 363-369

.

Ohno-Shosaku, T., Maejima, T., and Kano, M. (2001). Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. Neuron *29*, 729-738.

Olney, J. W., Labruyere, J., and Price, M. T. (1989). Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. Science *244*, 1360-1362.

Paxinos, G., and Watson, C. (2007). The rat brain in stereotaxic coordinates, 6th ed. edn (Amsterdam: Elsevier).

Pertwee, R. G. (2001). Cannabinoid receptors and pain. Prog Neurobiol *63*, 569-611.

Pertwee, R. G. (2005). Pharmacological actions of cannabinoids. Handb Exp Pharmacol, 1-51.

Pertwee, R. G. (2007). GPR55: a new member of the cannabinoid receptor clan? Br J Pharmacol *152*, 984-986.

Pertwee, R. G., and Fernando, S. R. (1996). Evidence for the presence of cannabinoid CB1 receptors in mouse urinary bladder. Br J Pharmacol *118*, 2053-2058.

Pertwee, R. G., Fernando, S. R., Griffin, G., Ryan, W., Razdan, R. K., Compton, D. R., and Martin, B. R. (1996a). Agonist-antagonist characterization of 6'-cyanohex-2'-yne-delta 8-tetrahydrocannabinol in two isolated tissue preparations. Eur J Pharmacol *315*, 195-201.

Pertwee, R. G., Fernando, S. R., Nash, J. E., and Coutts, A. A. (1996b). Further evidence for the presence of cannabinoid CB1 receptors in guinea-pig small intestine. Br J Pharmacol *118*, 2199-2205.

Pistis, M., Ferraro, L., Pira, L., Flore, G., Tanganelli, S., Gessa, G. L., and Devoto, P. (2002). Delta(9)-tetrahydrocannabinol decreases extracellular

GABA and increases extracellular glutamate and dopamine levels in the rat prefrontal cortex: an in vivo microdialysis study. Brain Res *948*, 155-158.

Pitler, T. A., and Alger, B. E. (1992). Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. J Neurosci *12*, 4122-4132.

Porcella, A., Gessa, G. L., and Pani, L. (1998). Delta9-tetrahydrocannabinol increases sequence-specific AP-1 DNA-binding activity and Fos-related antigens in the rat brain. Eur J Neurosci *10*, 1743-1751.

Pratt, J. A., Winchester, C., Egerton, A., Cochran, S. M., and Morris, B. J. (2008). Modelling prefrontal cortex deficits in schizophrenia: implications for treatment. Br J Pharmacol *153 Suppl 1*, S465-470.

Preet, A., Ganju, R. K., and Groopman, J. E. (2008). Delta9-Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration in vitro as well as its growth and metastasis in vivo. Oncogene *27*, 339-346.

Pugliese, A. M., Passani, M. B., and Corradetti, R. (1998). Effect of the selective 5-HT1A receptor antagonist WAY 100635 on the inhibition of e.p.s.ps produced by 5-HT in the CA1 region of rat hippocampal slices. Br J Pharmacol *124*, 93-100.

Ranganathan, M., and D'Souza, D. C. (2006). The acute effects of cannabinoids on memory in humans: a review. Psychopharmacology (Berl) *188*, 425-444.

Rao, S. G., Williams, G. V., and Goldman-Rakic, P. S. (2000). Destruction and creation of spatial tuning by disinhibition: GABA(A) blockade of prefrontal cortical neurons engaged by working memory. J Neurosci *20*, 485-494.

Reggio, P. H., Seltzman, H. H., Compton, D. R., Prescott, W. R., Jr., and Martin, B. R. (1990). Investigation of the role of the phenolic hydroxyl in cannabinoid activity. Mol Pharmacol *38*, 854-862.

Reid, M. J., and Bornheim, L. M. (2001). Cannabinoid-induced alterations in brain disposition of drugs of abuse. Biochem Pharmacol *61*, 1357-1367.

Resstel, L. B., Joca, S. R., Moreira, F. A., Correa, F. M., and Guimaraes, F. S. (2006). Effects of cannabidiol and diazepam on behavioral and cardiovascular responses induced by contextual conditioned fear in rats. Behav Brain Res *172*, 294-298.

Resstel, L. B., Tavares, R. F., Lisboa, S. F., Joca, S. R., Correa, F. M., and Guimaraes, F. S. (2009). 5-HT1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. Br J Pharmacol *156*, 181-188.

Rial Verde, E. M., Lee-Osbourne, J., Worley, P. F., Malinow, R., and Cline, H. T. (2006). Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. Neuron *52*, 461-474.

Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Neliat, G., Caput, D., and et al. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett *350*, 240-244.

Robinson, L., Goonawardena, A. V., Pertwee, R. G., Hampson, R. E., and Riedel, G. (2007). The synthetic cannabinoid HU210 induces spatial memory deficits and suppresses hippocampal firing rate in rats. Br J Pharmacol *151*, 688-700.

Roloff, A. M., and Thayer, S. A. (2009). Modulation of excitatory synaptic transmission by Delta 9-tetrahydrocannabinol switches from agonist to antagonist depending on firing rate. Mol Pharmacol *75*, 892-900.

Ross, H. R., Napier, I., and Connor, M. (2008). Inhibition of Recombinant Human T-type Calcium Channels by {Delta}9-Tetrahydrocannabinol and Cannabidiol. J Biol Chem *283*, 16124-16134.

Ruotsalainen, S., MacDonald, E., Koivisto, E., Stefanski, R., Haapalinna, A., Riekkinen, P., Jr., and Sirvio, J. (1998). 5-HT1A receptor agonist (8-OH-DPAT) and 5-HT2 receptor agonist (DOI) disrupt the non-cognitive performance of rats in a working memory task. J Psychopharmacol *12*, 177-185.

Russo, E., and Guy, G. W. (2006). A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. Med Hypotheses *66*, 234-246.

Russo, E. B., Burnett, A., Hall, B., and Parker, K. K. (2005). Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochem Res *30*, 1037-1043.

Ryan, D., Drysdale, A. J., Lafourcade, C., Pertwee, R. G., and Platt, B. (2009). Cannabidiol targets mitochondria to regulate intracellular Ca2+ levels. J Neurosci *29*, 2053-2063.

Ryan, D., Drysdale, A. J., Pertwee, R. G., and Platt, B. (2006). Differential effects of cannabis extracts and pure plant cannabinoids on hippocampal neurones and glia. Neurosci Lett *408*, 236-241.

Ryan, D., Drysdale, A. J., Pertwee, R. G., and Platt, B. (2007). Interactions of cannabidiol with endocannabinoid signalling in hippocampal tissue. Eur J Neurosci *25*, 2093-2102.

Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N. O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., and Greasley, P. J. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol *152*, 1092-1101.

Salazar, M., Carracedo, A., Salanueva, I. J., Hernandez-Tiedra, S., Lorente, M., Egia, A., Vazquez, P., Blazquez, C., Torres, S., Garcia, S., *et al.* (2009). Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest *119*, 1359-1372.

Sams-Dodd, F. (1996). Phencyclidine-induced stereotyped behaviour and social isolation in rats: a possible animal model of schizophrenia. Behav Pharmacol *7*, 3-23.

Scatton, B., and Sanger, D. J. (2000). Pharmacological and molecular targets in the search for novel antipsychotics. Behav Pharmacol *11*, 243-256.

Schmitz, D., Empson, R. M., and Heinemann, U. (1995a). Serotonin and 8-OH-DPAT reduce excitatory transmission in rat hippocampal area CA1 via reduction in presumed presynaptic Ca2+ entry. Brain Res *701*, 249-254.

Schmitz, D., Empson, R. M., and Heinemann, U. (1995b). Serotonin reduces inhibition via 5-HT1A receptors in area CA1 of rat hippocampal slices in vitro. J Neurosci *15*, 7217-7225.

Schmitz, D., Gloveli, T., Empson, R. M., Draguhn, A., and Heinemann, U. (1998). Serotonin reduces synaptic excitation in the superficial medial entorhinal cortex of the rat via a presynaptic mechanism. J Physiol *508 (Pt 1)*, 119-129.

Seillier, A., and Giuffrida, A. (2009). Evaluation of NMDA receptor models of schizophrenia: divergences in the behavioral effects of sub-chronic PCP and MK-801. Behav Brain Res *204*, 410-415.

Semus, S. F., and Martin, B. R. (1990). A computergraphic investigation into the pharmacological role of the THC-cannabinoid phenolic moiety. Life Sci *46*, 1781-1785.

Sharp, J. W. (1997). Phencyclidine (PCP) acts at sigma sites to induce c-fos gene expression. Brain Res *758*, 51-58.

Shen, M., Piser, T. M., Seybold, V. S., and Thayer, S. A. (1996). Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. J Neurosci *16*, 4322-4334.

Shen, M., and Thayer, S. A. (1998). The cannabinoid agonist Win55,212-2 inhibits calcium channels by receptor-mediated and direct pathways in cultured rat hippocampal neurons. Brain Res *783*, 77-84.

Shen, M., and Thayer, S. A. (1999). Delta9-tetrahydrocannabinol acts as a partial agonist to modulate glutamatergic synaptic transmission between rat hippocampal neurons in culture. Mol Pharmacol *55*, 8-13.

Shirayama, Y., Hashimoto, K., Matsuki, H., Tsunashima, K., Iyo, M., Higuchi, T., and Minabe, Y. (1999). Increased expression of zif268 mRNA in rat retrosplenial cortex following administration of phencyclidine. Brain Res *839*, 180-185.

Showalter, V. M., Compton, D. R., Martin, B. R., and Abood, M. E. (1996). Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. J Pharmacol Exp Ther *278*, 989-999.

Silverdale, M. A., McGuire, S., McInnes, A., Crossman, A. R., and Brotchie, J. M. (2001). Striatal cannabinoid CB1 receptor mRNA expression is decreased in the reserpine-treated rat model of Parkinson's disease. Exp Neurol *169*, 400-406.

Sim-Selley, L. J. (2003). Regulation of cannabinoid CB1 receptors in the central nervous system by chronic cannabinoids. Crit Rev Neurobiol *15*, 91-119.

Simpson, C. S., and Morris, B. J. (1995). Induction of c-fos and zif/268 gene expression in rat striatal neurons, following stimulation of D1-like dopamine receptors, involves protein kinase A and protein kinase C. Neuroscience *68*, 97-106.

Smith, D. E., and Wesson, D. R. (1980). PCP abuse: diagnostic and psychopharmacological treatment approaches. J Psychedelic Drugs *12*, 293-299.

Smith, D. E., Wesson, D. R., Buxton, M. E., Seymour, R., and Kramer, H. M. (1978). The diagnosis and treatment of the PCP abuse syndrome. NIDA Res Monogr, 229-240.

Sofia, R. D., Vassar, H. B., and Knobloch, L. C. (1975). Comparative analgesic activity of various naturally occurring cannabinoids in mice and rats. Psychopharmacologia *40*, 285-295.

Steinpreis, R. E. (1996). The behavioral and neurochemical effects of phencyclidine in humans and animals: some implications for modeling psychosis. Behav Brain Res *74*, 45-55.

Straiker, A., and Mackie, K. (2009). Cannabinoid signaling in inhibitory autaptic hippocampal neurons. Neuroscience *163*, 190-201.

Sugita, S., Namima, M., Nabeshima, T., Okamoto, K., Furukawa, H., and Watanabe, Y. (1996). Phencyclidine-induced expression of c-Fos-like immunoreactivity in mouse brain regions. Neurochem Int *28*, 545-550.

Sullivan, J. M. (1999). Mechanisms of cannabinoid-receptor-mediated inhibition of synaptic transmission in cultured hippocampal pyramidal neurons. J Neurophysiol *82*, 1286-1294.

Takahashi, K. A., and Castillo, P. E. (2006). The CB1 cannabinoid receptor mediates glutamatergic synaptic suppression in the hippocampus. Neuroscience *139*, 795-802.

Takeda, S., Yamaori, S., Motoya, E., Matsunaga, T., Kimura, T., Yamamoto, I., and Watanabe, K. (2008). Delta(9)-Tetrahydrocannabinol enhances MCF-7 cell proliferation via cannabinoid receptor-independent signaling. Toxicology *245*, 141-146.

Tamminga, C. A., Tanimoto, K., Kuo, S., Chase, T. N., Contreras, P. C., Rice, K. C., Jackson, A. E., and O'Donohue, T. L. (1987). PCP-induced alterations in cerebral glucose utilization in rat brain: blockade by metaphit, a PCP-receptor-acylating agent. Synapse *1*, 497-504.

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., and Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. Br J Pharmacol *150*, 613-623.

Thomsen, M. S., Hansen, H. H., and Mikkelsen, J. D. (2010). Opposite effect of phencyclidine on activity-regulated cytoskeleton-associated protein (Arc) in juvenile and adult limbic rat brain regions. Neurochem Int *56*, 270-275.

Thomson, A. M. (2000). Facilitation, augmentation and potentiation at central synapses. Trends Neurosci *23*, 305-312.

Tischmeyer, W., and Grimm, R. (1999). Activation of immediate early genes and memory formation. Cell Mol Life Sci *55*, 564-574.

Titishov, N., Mechoulam, R., and Zimmerman, A. M. (1989). Stereospecific effects of (-)- and (+)-7-hydroxy-delta-6-tetrahydrocannabinol-dimethylheptyl on the immune system of mice. Pharmacology *39*, 337-349.

Touw, M. (1981). The religious and medicinal uses of Cannabis in China, India and Tibet. J Psychoactive Drugs *13*, 23-34.

Tselnicker, I., Keren, O., Hefetz, A., Pick, C. G., and Sarne, Y. (2007). A single low dose of tetrahydrocannabinol induces long-term cognitive deficits. Neurosci Lett *411*, 108-111.

Tzavara, E. T., Davis, R. J., Perry, K. W., Li, X., Salhoff, C., Bymaster, F. P., Witkin, J. M., and Nomikos, G. G. (2003). The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. Br J Pharmacol *138*, 544-553.

Varvel, S. A., Anum, E., Niyuhire, F., Wise, L. E., and Lichtman, A. H. (2005). Delta(9)-THC-induced cognitive deficits in mice are reversed by the GABA(A) antagonist bicuculline. Psychopharmacology (Berl) *178*, 317-327.

Varvel, S. A., Hamm, R. J., Martin, B. R., and Lichtman, A. H. (2001). Differential effects of delta 9-THC on spatial reference and working memory in mice. Psychopharmacology (Berl) *157*, 142-150.

Vigano, D., Guidali, C., Petrosino, S., Realini, N., Rubino, T., Di Marzo, V., and Parolaro, D. (2009). Involvement of the endocannabinoid system in phencyclidine-induced cognitive deficits modelling schizophrenia. Int J Neuropsychopharmacol *12*, 599-614.

Wade, D. T., Makela, P., Robson, P., House, H., and Bateman, C. (2004). Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. Mult Scler *10*, 434-441.

Wade, D. T., Makela, P. M., House, H., Bateman, C., and Robson, P. (2006). Long-term use of a cannabis-based medicine in the treatment of spasticity and other symptoms in multiple sclerosis. Mult Scler *12*, 639-645.

Wang, J. Q. (1998). Regulation of immediate early gene c-fos and zif/268 mRNA expression in rat striatum by metabotropic glutamate receptor. Brain Res Mol Brain Res *57*, 46-53.

Weiss, L., Zeira, M., Reich, S., Har-Noy, M., Mechoulam, R., Slavin, S., and Gallily, R. (2006). Cannabidiol lowers incidence of diabetes in non-obese diabetic mice. Autoimmunity *39*, 143-151.

Weissman, A. D., Dam, M., and London, E. D. (1987). Alterations in local cerebral glucose utilization induced by phencyclidine. Brain Res *435*, 29-40.

Welburn, P. J., Starmer, G. A., Chesher, G. B., and Jackson, D. M. (1976). Effect of cannabinoids on the abdominal constriction response in mice: within cannabinoid interactions. Psychopharmacologia *46*, 83-85.

Wilson, R. I., and Nicoll, R. A. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature *410*, 588-592.

Yamamoto, C., and McIlwain, H. (1966a). Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media in vitro. J Neurochem *13*, 1333-1343.

Yamamoto, C., and McIlwain, H. (1966b). Potentials evoked in vitro in preparations from the mammalian brain. Nature *210*, 1055-1056.

Yoneda, Y., Kuramoto, N., Kitayama, T., and Hinoi, E. (2001). Consolidation of transient ionotropic glutamate signals through nuclear transcription factors in the brain. Prog Neurobiol *63*, 697-719.

Zuardi, A. W., Crippa, J. A., Hallak, J. E., Moreira, F. A., and Guimaraes, F. S. (2006a). Cannabidiol, a Cannabis sativa constituent, as an antipsychotic drug. Braz J Med Biol Res *39*, 421-429.

Zuardi, A. W., Hallak, J. E., Dursun, S. M., Morais, S. L., Sanches, R. F., Musty, R. E., and Crippa, J. A. (2006b). Cannabidiol monotherapy for treatment-resistant schizophrenia. J Psychopharmacol *20*, 683-686.

Zuardi, A. W., and Karniol, I. G. (1983). Effects on variable-interval performance in rats of delta 9-tetrahydrocannabinol and cannabidiol, separately and in combination. Braz J Med Biol Res *16*, 141-146.

Zuardi, A. W., Morais, S. L., Guimaraes, F. S., and Mechoulam, R. (1995). Antipsychotic effect of cannabidiol. J Clin Psychiatry *56*, 485-486.

Zuardi, A. W., Shirakawa, I., Finkelfarb, E., and Karniol, I. G. (1982). Action of cannabidiol on the anxiety and other effects produced by delta 9-THC in normal subjects. Psychopharmacology (Berl) *76*, 245-250.