Cannabinoids for the control of experimental multiple sclerosis

Pryce, Gareth

Cannabinoids for the control of experimental multiple sclerosis

GARETH PRYCE

Neuroimmunology Unit,
Neuroscience & Trauma
Barts and the London School of Medicine and Dentistry
Queen Mary University of London
UK

This thesis is dedicated in memory of my Dad, Glyn Pryce (1927-2010), with love and thanks.

ACKNOWLEDGEMENTS

I would like to thank David Baker and Gavin Giovannoni for their supervision and providing me with the opportunity to undertake this thesis.

I would like to thank J.Ludovic Croxford, Sam Jackson, Sarah Al-Izki from the lab past and present; Ana Cabranes (RIP) and the Javier Fernadez-Ruiz laboratory in Madrid, Spain; the Vincenzo Di Marzio Laboratory Naples, Italy; the Andy Irving Laboratory, Dundee; the laboratories of Elga De Vries and Sandra Amor at the Free University Amsterdam, The Netherlands and the laboratories of Ruth Ross and Roger Pertwee in Aberdeen and Alison Hardcastle University College London for providing me with supportive data and particularly Cristina Visintin and David Selwood of University College London and Canbex for providing data from contract Research organizations. I thank Dr. D. Argentieri and Dr. D Ritchie for providing access to RWJ compounds and Dr. M. Ferrari for providing CT3. David Selwood and Cristina Visintin are thanked for supply of VSN16. Jonathan Long and Ben Cravatt are thanked for providing JZL184. Catherine Ledent, Sarah A. Thomas, Beat Lutz, Giovanni Marsicano and Benjamin Cravatt are thanked for supply of mice and materials.

I would also like to thank The Multiple Sclerosis Society of Great Britain and Northern Ireland, the National Multiple Sclerosis Society, USA, Aims2Cure; the Brain Research Trust; the Bloomsbury Bioseed Fund; The Wellcome Trust, Canbex and the Pharmaceutical Industry and Barts and the London School of Medicine and Dentistry for support of various aspects of research. I acknowledge RW Johnson, Raritan, New Jersey, USA in part funded experiments involving RWJ compounds. Altantic Ventures part funded some of the early CT3 studies. I am also indebted to the National Institute for Drug Abuse chemical supply programme for donating chemicals for this study.

Lastly, but certainly not least I would like to thank my Mum, Dad, my sister Jan and my partner Karen for their support and encouragement. I would particularly like to thank David Baker for his unswerving friendship, support, help, encouragement and cajoling when needed. It has been incredibly rewarding from an intellectual and personal level to work with him for the last thirteen years and hopefully for many more to come.

My very great thanks to all!

ABSTRACT

There have been numerous studies reporting that cannabinoids, both exogenous and endogenous, have a potential beneficial function during incidences of neurological damage. Using gene knockout mice and cannabinoid-selective agents, this study demonstrates the diverse actions of cannabinoids with a particular focus on experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. The results presented here report on the action of stimulators of cannabinoid receptors in the nervous system (CNS) on; immune function, as a mechanism of suppressing autoimmune attack of the central nervous system, as agents to suppress neurodegenerative events leading to disease progression and as agents that can control signs of disease that occur as the consequences of autoimmune neurodegeneration such as spasticity. Tetrahydrocannabinol the psychoactive component in cannabis and the CB_1 cannabinoid receptor appears to be central to many of the therapeutic actions of cannabis but also to the side-effect potential of cannabinoid drugs. This study reports on methods to avoid psychoactive side-effects of conventional brain-penetrant CB_1 receptor agonists whilst exploiting the therapeutic potential of the cannabinoid system in order to control spasticity. This was achieved by targeting mechanisms of endocannabinoid degradation, particularly using fatty acid amide hydrolase inhibitors. Furthermore, this study also reports the development of novel cannabinoid compounds that are excluded from the brain and inhibit spasticity and also demonstrates the mechanism of exclusion of CNS-excluded cannabinoid CB_1 receptor agonists. This study provides further evidence for the efficacy of cannabinoid compounds during an ongoing CNS disease and also their efficacy for treating the consequences of CNS autoimmune disease, which hopefully, will give additional impetus for further clinical investigations of cannabinoid agents in not only multiple sclerosis but also other neurodegenerative diseases of the CNS.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABH	Biozzi Antibody High Mouse
AEA	Anandamide (N-arachidonoylethanolamine)
Ag	Antigen
2-AG	2-Arachidonoyl Glycerol
BBB	Blood:Brain:Barrier
BDNF	Brain-derived Neurotrophic Factor
cAMP	cyclic Adenosine Monophosphate
CB	Cannabinoid
CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CNS	Central Nervous System
CO_2	Carbon dioxide
Cre	Cre (**c**auses **re**combination) Recombinase
Cre-Lox	Cre-Lox P-mediated recombination
CREAE	Chronic Relapsing experimental Allergic Encephalomyelitis
CsA	Cyclosporin A
CSF	Cerebrospinal Fluid
DAB	Diaminobenzidine chromogen
DAG Lipase	DiAcylGlycerol Lipase
DCP	DMSO:Cremophor:PBS
DMSO	DiMethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EAE	Experimental Allergic Encephalomyelitis
ECB	Endocannabinoid
ECP	Ethanol:Cremophor:PBS
EDSS	(Kurtzke) Expanded Disability Status Scale
ELISA	Enzyme Linked Immunosorbant Assay
FAAH	Fatty Acid Amide Hydrolase
GABA	Gamma Amino Butyric Acid
GPCR	G Protein-Coupled Receptor
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous

kDa	KiloDalton
Lck	Leukocyte-specific protein tyrosine kinase
MAG	Myelin-associated Glycoprotein
MAG lipase	Monoacylglycerol lipase
MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin basic protein
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger Ribonucleic Acid
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
Nes	Nestin
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-apartic Acid
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Per	Peripherin
p.i.	Post-inoculation
PLP	Proteolipid Protein 1
PPAR	Peroxisome Proliferator-Activated Receptor
PPMS	Primary Progressive multiple Sclerosis
RNA	Ribonucleic Acid
RRMS	Relapsing-Remitting Multiple Sclerosis
s.c.	Sub-cutaneous
SCH	Spinal Cord Homogenate
SD	Standard Deviation of the Mean
SEM	Standard Error of the Mean
SPMS	Secondary Progressive Multiple Sclerosis
Taq	Taq polymerase
Th	T helper lymphocyte
THC	Δ^9-Tetrahydrocannabinol
TMB	Tetramethylbenzidine chromogen
TNFα	Tumour Necrosis Factor Alpha
TRPV1	Transient Receptor Potential cation channel, subfamily V, member 1
VR1	Vanilloid receptor (See TRPV1)

CHAPTER ONE

INTRODUCTION

Over recent years, experimental data on the role of cannabinoids in physiological processes has revealed that there are few areas of physiology where the actions of cannabinoids do not exert some influence, from the control of neuronal signaling to the regulation of bone formation and homeostasis. It is also becoming increasingly clear that many neurological diseases share common mechanisms of neuronal damage and one of primary importance appears to be perturbation of excitatory neuronal signalling resulting in excitotoxic neuronal death. The location of these events and the type of neuronal damage leads to the clinical manifestation of each disease, and to the development of symptoms such as spasticity seen in multiple sclerosis. Cannabinoids can regulate neurotransmitter release and signalling plus elements of oxidative stress that may be neurotoxic in excess. There may a number of neurological diseases such as multiple sclerosis, which should be amenable to cannabinoid therapy, not only for symptom relief but also as neuroprotective strategies to modulate disease progression. Cannabinoids may be particularly attractive as they display low toxicity and with correct dose titration should be well tolerated. In addition, agents that enhance endocannabinoid levels, by inhibition of uptake/degradation, which are already elevated at sites of injury may also be an attractive approach, as it will bring a more targeted strategy of action whilst potentially limiting unwanted psychoactive side effects.

1.1. *The Cannabinoid system*

The cannabinoid system is a relatively novel regulatory pathway that was revealed, like the opioid system, following the study of plant-derived narcotics and it is now clear that it is a fundamental element of the biology of the nervous system and many other areas of the body. Since the identification and cloning of the predominantly neuronally expressed cannabinoid receptor CB_1 (Devane et al., 1988; Matsuda et al., 1990), there has been a huge increase in research into this new field. The cloning of a second, peripheral cannabinoid receptor, CB_2 (Munro et al., 1993), which is expressed primarily on cells of the immune system, revealed the ubiquity of cannabinoid receptor signalling in many physiological processes. These are the classical cannabinoid receptors which are characterised by their agonism with the prototypic cannabinoid of *Cannabis sativa* that is Δ-9 tetrahydrocannabinol (THC). Both of these receptors show constitutive activity, as

evidenced by the inverse agonism of many CB receptor antagonists. The identification of endogenous ligands such as anandamide (Devane et al,, 1992) and 2-arachidonoyl glycerol (2-AG), (Mechoulam et al., 1995; Stella et al., 1997) termed endocannabinoids, for these receptors has identified a functional endogenous cannabinoid system, raising the possibility of utilizing aspects of the endocannabinoid system for potential therapeutic benefit, particularly in neurological disease. A putative third CB receptor, GPR55 has also been described (Baker et al., 2006; Ryberg et al., 2007) and recently a fourth putative receptor GPR18 (McHugh et al., 2010). The transmitter-gated channel transient receptor potential vanilloid type-1 receptor (TRPV1), associated with detection of noxious stimuli, is agonized by the endocannabinoid anandamide (Zygmunt et al., 1999) and recent evidence also indicates that cannabinoids have activity at the nuclear receptor family, peroxisome proliferator- activated receptors (PPARs, O'Sullivan, 2007; Stahel et al., 2008).

1.1.1. *Cannabinoid receptors - CB_1*

Cannabinoid receptors are members of the 7-transmembrane-spanning receptor rhodopsin-like superfamily which appear to bind their ligands in the central core of the membrane-spanning helices (McAllister et al.,2002). The CB_1 receptor sequence shows a high degree of conservation across mammalian species, whereas the CB_2 receptor shows a greater degree of interspecies difference (Howlett et al., 2002). CB_1 is among the most highly expressed and ubiquitous G-protein coupled receptors in the brain (Moldrich and Wenger, 2000), with densities similar to gamma aminobutyric acid (GABA) and glutamate receptors (Herkenham et al., 1991). The cannabinoid receptors are coupled to the intracellular signaling G protein $G_{i/o}$ in a *Bordatella pertussis* toxin-sensitive manner (Howlett, 1984). CB_1 can also couple to the G-protein G_s under conditions where $G_{i/o}$ signalling is blocked by *B. pertussis* toxin and under these conditions activate adenylyl cyclase activity (Bonhaus et al., 1998). The CB_1 receptor coupling to G-proteins is relatively inefficient, with each cannabinoid receptor coupling to three G-proteins, compared to twenty for mu and delta opioid receptors (Breivogel et al., 1997). This low level of coupling may reflect that the high level of receptor expression renders a high level of amplification unnecessary (Howlett et al., 2004). CB_1 receptor agonism inhibits adenylyl cyclase activity with a concomitant decrease in cytosolic cAMP levels, leading to inhibition of neurotransmitters from synaptic vesicles and stimulation of p42/p44 and p38 mitogen-activated protein kinase (MAPK) activity (Bouaboula et al.,1995a; Bouaboula et al., 1995b). CB_1 agonism liberates $G_{i/oa}$ proteins coupling to the inhibition of adenylyl cyclase, and the associated depletion of intracellular cAMP leads to the inactivation of the protein kinase A pathway (PKA)

and reduction in ion-channel phosphorylation, leading to reductions in neuronal activity due to hyperpolarisation of axon terminals (Childers and Deadwyler, 1996; Demuth and Molleman, 2006). PKA and cyclic AMP levels can also regulate gene expression and may influence long-term levels of gene expression (Demuth and Molleman, 2006). CB_1 mediated inhibition of ion channels is associated with the reduction of the presynaptic release of glutamatergic (excitatory) and GABAergic (inhibitory) neurotransmitters from synaptic vesicles (Wilson and Nicoll, 2002). CB_1 activation results in the triggering of intracellular protein kinases critical to the mediation of synaptic strength, in particular the stimulation of extracellular signal-related kinase (ERK) and focal adhesion kinase (FAK) in response to a decrease in intracellular cAMP.

CB_1 receptor stimulation inhibits N and P/Q-type calcium channels by the interaction of $G_{i/o}$ proteins with these channels in a pertussis toxin sensitive manner. CB_1 agonists may inhibit L-type calcium currents and inhibition of this effect is seen with CB_1 agonists in a PTX and SR141716A sensitive manner (Gebremedhin et al., 1999), in contrast stimulation has been seen by cannabinoids in neuroblastoma cells (Rubovitch et al., 2002). The increase of intracellular calcium in response to CB_1 stimulation has been reported in several cell types and evidence suggests that CB_1 receptor stimulation is coupled to phospholipase C activation via $G_{i/o}$ proteins which increase intracellular inositol triphosphate triggering the release of Ca^{2+} from intracellular stores (Sugiura et al., 1996). The apparently paradoxical increase in intracellular Ca^{2+}, when cannabinoids decrease neuronal excitability by inhibiting voltage-dependent calcium channels may itself be inhibitory by activating Ca^{2+} -dependent K^+ channels, leading to hyperpolarisation and preventing Ca^{2+} influx (Demuth, 2006 #66). Though in the main CB_1-mediated, there is also evidence for the direct modulation of ion channels by cannabinoids, inhibition of the T-type calcium channel by the endocannabinoid anandamide, appears to be a direct effect of ligand binding to this channel (Chemin et al., 2001). CB_1 receptors couple positively to G-protein coupled inwardly rectifying potassium channels (GIRK), this stimulation is reported to be a mechanism for the inhibition of neurotransmitter release independent from the cAMP/PKA pathway (Mackie et al., 1995). This activation of GIRK channels can be inhibited by stimulation of protein kinase C which may act by phosphorylation of the CB_1 receptor, restoring neural excitability and synaptic strength (Garcia et al., 1998). This may also be a mechanism to restore a normal level of neuronal excitability when there are high levels of endocannabinoids present. The endogenous cannabinoid anandamide in cerebellar granule cells can directly inhibit the acid-sensitive background K^+ channels TASK-1 and TASK-3, which can be reproduced with the cannabinoid agonist WIN55,212-2. Inhibition of these channels leads to depolarisation and enhances excitability (Maingret et al., 2001). In contrast, cannabinoids inhibit I_M

and I_K K^+ currents in hippocampal neurons (Hampson et al., 2000; Schweitzer, 2000). Both anandamide and WIN55-212 demonstrate the ability to directly inhibit voltage-dependent sodium channels, depressing synaptic transmission and reducing neurotransmitter release (Nicholson et al., 2003).

CB_1 receptors although primarily expressed in the CNS are also expressed in; reproductive tissues, gut, vascular endothelia, muscle, sympathetic ganglia, bladder, lung and cells of the immune system (Galiegue et al., 1995). Expression of CB_1 by cells of the immune system is dependent on activation state, with T cells showing a decrease in CB_1 expression post-activation whereas B cells displayed increased CB_1 expression after mitogen stimulation (Klein et al., 2003). The order of CB_1 receptor expression in human peripheral leukocytes is B cells> NK cells> Polymorphonuclear cells > CD8 T cells> monocytes > CD4 T cells (Bouaboula et al., 1993). T cell CB_1 receptor expression is influenced by agonism of the CB_2 receptor (Borner et al., 2007). The expression of CB_1 receptors in the CNS is heterogeneous and found in; cerebral cortex, hippocampus, caudate-putamen, substantia nigra pars reticulata, globus pallidus, cerebellum, periaqueductal grey, rostral ventromedial medulla, superior colliculus, thalamus and amygdala (Herkenham et al., 1991). In contrast, there is a low level of CB_1 receptor expression in the brain stem, which may explain the low level of toxicity associated with cannabinoids (Howlett et al., 2004). The expression of CB_1 in particular areas of the CNS accounts for the specific pharmacological effects of CB_1 agonists, namely; control of motor function, effects on cognition and memory, control of the hypothalamus/pituitary axis and effects on the detection and transmission of painful stimuli.

CB_1 receptors are primarily located at the axonal presynaptic terminals of central and peripheral nerves, which have the function of reducing the release of a number of neurotransmitters from synaptic vesicles into the synaptic cleft (Wilson and Nicoll, 2002; Freund, 2003). The release of excitatory or inhibitory neurotransmitters is inhibited depending on the type of neuron terminal where the CB_1 receptors are expressed. An example of this is in the hippocampus, where the predominant expression of CB_1 is at the inhibitory neurotransmitter GABAergic neural terminals, with the result that inhibitory signals are reduced leading to a net increased excitation in this brain area. This phenomenon has been observed via endocannabinoid release in electrically stimulated hippocampal slices termed depolarization suppression of inhibition (DSI), (Wilson and Nicoll, 2002). In contrast, in the cerebellum CB_1 receptor expression is predominantly on glutamatergic (excitatory) terminals so here, receptor agonism leads to a net reduction in excitation (Kreitzer et al., 2002). This has also been modelled *in vitro* in tissue slices and is described as depolarization suppression of excitation (DSE). This indicates the central role of the cannabinoids in synaptic strengthening and

plasticity. This retrograde endocannabinoid signalling to inhibit neurotransmitter release appears to be primarily mediated by 2-AG, as gene deleted mice for the enzyme DAG Lipase α, responsible for the production of 2-AG show the abolition of retrograde signaling in the brains of animals (Gao et al., 2010; Tanimura et al., 2010).

Nitric oxide production has been reported in several cell types in response to CB_1 receptor stimulation via the stimulation of nitric oxide synthase (Fimiani et al., 1999). Nitric oxide is a prominent second messenger with a role in the CNS in thermoregulation and also a supportive role in retrograde synaptic inhibition by cannabinoids (Makara et al., 2007). The reduction of the inflammatory-mediated release of nitric oxide via glial cells by CB_1 receptor agonists is a potentially neuroprotective mechanism in neuroinflammatory events, protecting nerve cells from the toxic effects of Nitric oxide (Cabral et al., 2001).

1.1.2. *CB_2 receptor*

The human CB_2 receptor shows only a 45% homology with the CB_1 receptor and is expressed primarily but not exclusively by cells of the immune system (Munro et al., 1993). The CB_2 receptor has only been cloned in mammals. The cannabinoid system is implicated in the modulation of aspects of immune cell function via action on the CB_2 receptor. In the immune system, the highest levels of CB_2 expression, as assessed by mRNA levels, are seen in B-cells > natural killer cells > monocytes > polymorphonuclear neutrophils > CD8 T cells > CD4 T cells (Galiegue et al., 1995). The level of expression is higher than is seen for the CB_1 receptor in these cells. CB_2 receptor expression is also dependent on the differentiation and activation state of cells (Klein et al., 2003). Microglial cells in the CNS may express/upregulate CB_2 receptors in response to damaging events (Maresz et al., 2005; Ashton et al., 2007). Recently, CB_2 receptor expression has also been reported in other organ systems of the body, including; central nervous system, liver and bone, and is implicated in several models of organ-specific inflammatory conditions (Xu et al., 2007; Buckley et al., 2008). As with the CB_1 receptor, CB_2 inhibits adenylyl cyclase activity and activates p42/p44 ERK-MAP kinase (Bayewitch et al., 1995; Bouaboula et al., 1996). In contrast to CB_1, CB_2 receptors do not have a direct modulatory effect on ion channels and also in contrast to the CB_1 receptor, CB_2 does not couple to G_s in a pertussis toxin sensitive manner (Felder et al., 1995; Glass and Felder, 1997).

Endocannabinoid stimulation of CB_2 with 2-AG and activation of p42/p44 ERK-MAPK is associated with the migration of leucocytes (Walter et al., 2003), and appears to act in a ligand specific manner. There are numerous studies detailing the

modulatory roles of cannabinoids on cells of the immune system, such as the inhibition of T cell proliferation, the inhibition of cytokine production by T cells, shifting of Th1/Th2 ratios, reduction of immunoglobulin production, impairment of cytotoxic T cell activity and also the down-regulation of macrophage function such as migration in vitro (Klein et al., 2003). Definitive results as to the role of the CB_2 receptor in the process of inflammation remain elusive, with reports of both potentially stimulatory effects and suppressive effects of CB_2 agonism, depending on the models or cell types studied (Klein et al., 2003). In a mouse model of uveoretinitis, the CB_2 selective agonist JWH-133 has been shown to have significant disease-suppressing activity in a dose-dependent manner (Xu et al., 2007). These studies are complicated by the fact that experiments are performed in cells also expressing CB_1 or in the presence of selective agonists/antagonists of these receptors which may still show activity at other receptors. The fact that CB_2 deficient mice also fail to exhibit any obvious dysregulation of the immune system, such as a predisposition to autoimmunity, under normal conditions, would suggest that the involvement of the endogenous cannabinoid system on the immune system is complex, with the CB_2 receptor having a contributory role in immune modulation or immune cell development/maturation. This is reflected in CB_2 deficient mice having a reported deficiency in the splenic marginal zone, peritoneal B1a cells, splenic memory CD4+ T cells, and intestinal natural killer cells and natural killer T cells (Buckley, 2008). This deficiency is mimicked by gene-deficient mice lacking the signalling G-protein G alpha i2. This may further indicate that the CB_2 receptor may be involved in developmental aspects of cells of the immune system rather than the suppression of activation.

1.1.3. *Non $CB_{1/2}$ receptor mediated cannabinoid signalling*

1.1.3.1. *GPR55*

A third proposed cannabinoid-binding receptor has been recently reported by searching the patent database (Baker et al., 2006; Ryberg et al., 2007). This G protein-coupled orphan receptor has been identified as a novel metabotropic endocannabinoid binding receptor stimulated by the endocannabinoids anandamide and virodhamine plus the non cannabinoid receptor ligand pamitoyl ethanolamide. GPR55 is also stimulated by CP55940 and cannabidiol and abnormal cannabidiol, which have no activity at CB_1 or CB_2 (Ryberg et al., 2007). In contrast, it has also been reported that GPR55 does not show activity with conventional cannabinoid receptor ligands but may be a specific receptor for lysophoshatidylinositol (LPI), (Oka et al., 2007). Kapur et al., (2009), used an elegant alternative approach to determine GPR55 ligands by studying β-arrestin complex formation with activated

GPCRs. β-arrestins are intracellular proteins that bind and desensitize activated GPCRs forming stable GPCR/arrestin complexes.

Measurement of GPR55/arrestin complexes in GPR55 transfected ligand stimulated cell cultures revealed that LPI and also the CB_1 receptor antagonists/inverse agonists SR141716A and AM251 had GPR55 agonist activity in addition to the classical CB_1 receptor agonist CP55,940. Anandamide or 2-AG showed no activity at GPR55 in this assay (Kapur et al., 2009). The CB_1 antagonist SR141716A has also shown activity as a GPR55 antagonist (Lauckner et al., 2008).

LPI and the putative endocannabinoid N-Arachidonoyl-serine have been shown to stimulate endothelial cell signaling, vasodilation and angiogenesis in a partially GPR55-dependent manner (Bondarenko et al., 2010; Zhang et al., 2010).

In addition, GPR55 also appears to have a role in bone physiology by the regulation of osteoclast number and function. Osteoclast stimulation of function (polarization and resorption was effected by the endogenous GPR55 ligand LPI and the synthetic GPR55 agonist 0-1602 *in vitro* and was attenuated in osteoclasts from GPR55 knockout mice and by cannabidiol. Cannabidiol also reduced bone resorption *in vivo* and increased bone mass in treated animals (Whyte et al., 2009)

GPR55 is highly expressed the adrenal glands, gut and the CNS, particularly in large dorsal root ganglion neurons of the spinal cord. Activation of this receptor produces a slow rise in intracellular calcium, probably independent of G_i and G_s proteins and inhibition of the M type K^+ current in response to THC and anandamide stimulation (Lauckner et al., 2008). This suggests that the GPR55 receptor may be pronociceptive and further evidence for this is provided by the observation that GPR55 gene knockout mice show resistance to inflammatory or neuropathic pain, suggesting that antagonists of this receptor may reduce painful stimuli (Staton et al., 2008).

In summary, current pharmacological data concerning the role of GPR55 as a putative cannabinoid receptor and its role in physiological processes is conflicting and much more research is warranted before a coherent role for this receptor can be proposed.

1.1.3.2. *Vanilloid receptor TRPV-1*

The TRPV1 receptor is a protein that has been primarily associated with activation by noxious stimuli such as; heat and hydrogen ions. TRPV1 is also activated by capsaicin, which is the pungent ingredient of chilli peppers. TRPV1 was initially reported as being expressed by sensory neurons where opening of the channel triggers calcium influx, neurotransmitter release and transmission of painful or noxious stimuli. Numerous studies have demonstrated that the endocannabinoid

anandamide can also activate the TRPV1 receptor, although the binding site may be at cytosolic sites of the receptor (De Petrocellis et al., 2001). Anandamide can also mimic the vasodilatory properties of capsaicin at the TRPV1 receptor, which is blocked by the antagonist capsazepine. This vasodilatory property of anandamide is not cannabinoid receptor-dependent, as it is not blocked by CB_1 receptor antagonists (Zygmunt et al., 1999). TRPV1 expression has been reported in the CNS where TRPV1 activation has also been observed in slice cultures of rat hippocampus. Thus anandamide can have inhibitory effects via CB_1 receptors and stimulatory effects via TRPV1. The true potency of anandamide at the TRPV1 receptor remains subject to conjecture as physiologically the response to capsaicin or anandamide intravenous injection are quite different with regard to an irritant effect being observed with the classical agonist capsaicin (Pryce, unpublished observation). Indeed, it is postulated the high levels of anandamide necessary to observe TRPV1 stimulation as opposed to inhibition at lower concentrations, may not be relevant under physiological conditions (Nemeth et al., 2003). Lower levels of anandamide (10μM) stimulated neuropeptide release from peripheral sensory nerve terminals where the inhibitory CB_1 receptor was antagonised by SR141716A, again a condition not likely to have physiological relevance (Nemeth et al., 2003). Also, a study investigating the actions of anandamide in mice deficient in both CB_1 and the enzyme fatty acide amide hydrolase, responsible for anandamide inactivation, indicated that the CB_1 receptor is the predominant target for the behavioural effects of anandamide (Wise et al., 2007). Anandamide does not induce desensitisation of the TRPV1 receptor as do conventional exogenous ligands (Dinis et al., 2004) and its limited ability to activate this receptor may serve to limit the inappropriate stimulation of TRPV1, resulting in a pain signal, in the absence of a relevant pain-inducing stimulus. It has also been suggested that anandamide acts as a conditional activator of TRPV1 with a potent activation potential in the presence of activating compounds such as inflammatory mediators (Singh Tahim et al., 2005).

1.1.3.3. *Peroxisome proliferator-activated receptors (PPARs)*

Recent observations point to the potential activity of cannabinoids on the peroxisome proliferator-activated receptors (PPAR) α and γ. These are a family of nuclear receptors consisting of 3 isoforms, α, δ and γ. PPARs heterodimerise with the retinoid X receptor and bind to PPAR response elements of DNA sequences which trigger the transcription of target genes upon ligand activation of these receptors (Burstein, 2005). Ligand binding elicits the recruitment of regulator proteins binding to a third site on PPARs which are proposed to regulate

transactivation. PPARs primarily influence the transcription of genes involved in the regulation of metabolism, cell differentiation and inflammation. PPAR receptor activation has been shown to inhibit pro-inflammatory gene transcription via the repression of the transcription factor nuclear factor κB (NFκB), (reviewed in Stahel et al., 2008).

The ligand spectrum of PPARs is large with fatty acids and eicosanoids having reported activity. Cannabinoids and their metabolites can activate PPARα, with compounds such as the 2-AG metabolite oleolylethanolamide (OEA) and palmitoylethanolamide (PEA) showing ligand activities that are not as a result of classical cannabinoid receptor binding capacity (O'Sullivan, 2007). PEA is a weak agonist at the CB_1 receptor but modulates anti-inflammatory activity by the activation of PPARα (Lo Verme et al., 2005). Endocannabinoids, such as anandamide, or the putative endocannabinoids noladin ether and virhodamine, all show binding to and activation of PPARα (O'Sullivan, 2007). The metabolite of Δ-^{9} THC, ajulemic acid or CT-3 (Burstein, 2000), a therapeutically promising cannabinoid, has been shown to activate the PPARγ receptor, inducing an anti-inflammatory effect (Liu et al., 2003). The endocannabinoids anandamide and 2-AG have also been reported to bind to this receptor, suppressing interleukin 2 release from T cells (Bouaboula et al., 2005; Rockwell et al., 2006), in a PPARγ antagonist sensitive manner and PPARγ agonists inhibit the activation of microglia and astrocytes, inhibiting the release of proinflammatory cytokines and the neurotoxic agent nitric oxide (Storer et al., 2005). In addition, PPARγ agonists have been reported to ameliorate disease in experimental models of multiple sclerosis and also in a patient with secondary progressive MS, supporting the potential beneficial role of cannabinoids as PPAR agonists in neurological inflammatory disease (Niino et al., 2001; Natarajan and Bright; 2002; Pershadsingh et al., 2004; Loria et al, 2010).

1.1.3.4 *GPR18*

The G-protein-coupled receptor GPR18 gene was cloned and found to be expressed at a high level in testis and spleen and a lower level of expression in tissues associated with the endocrine and immune systems (Samuelson et al., 1996; Gantz et al., 1997). GPR18 is expressed significantly in lymphoid cell lines, but not in non-lymphoid hematopoietic cell lines. The expression of GPR18 was higher in peripheral T lymphocyte subsets (CD4(+), CD4(+)CD45RA(+), CD4(+)CD45RO(+), CD8(+), and CD19(+))B cells than in monocytes and lymphoid cell lines, and the level of expression increased after stimulation with the T cell mitogen phytohaemagglutinin (Kohno et al., 2006). Lipid library screening revealed the

putative endocannabinoid N-arachidonoylglycine (NAGly) to be a ligand for GPR18, producing an increase in intracellular calcium in GPR18 transfected cells together with an inhibition of forskolin-stimulated cAMP production which was *B. pertussis* toxin sensitive (Kohno et al., 2006).

GPR18 stimulation by NAGly has recently been reported to be a potent stimulator of proliferation, migration and MAP Kinases in the immortalized mouse microglial cell line BV-2 and GPR18 transfected HEK293 cells at sub nanomolar concentrations, which is *B. pertussis* toxin sensitive (McHugh et al., 2010). Abnormal cannabidiol (Abn-CBD) and 0-1602, synthetic isomers and agonists of the previously proposed but unidentified "Abn- CBD receptor" modulating vasodilation (Járai et al., 1999), also stimulate migration in these cell lines in a *B. pertussis* toxin sensitive manner. These effects on migration are blocked by the "Abn-receptor" antagonist 0-1918 and suggest that GPR18 is the purported Abn-CBD receptor (McHugh et al., 2010).

As activation and recruitment of microglia to inflammatory lesions is seen in both EAE and MS, the observation that GPR18 may be involved in this process and contribute to lesion development suggests that the modulation of GPR18 may have a novel therapeutic role in MS.

1.2. $CB_{1/2}$ *receptor agonists/antagonists*

The prototypic cannabinoid Δ-^{9} THC, the main psychoactive component of *Cannabis sativa* was identified in 1964 (Gaoni and Mechoulam, 1964). Before the identification of specific receptors, it was postulated that cannabinoids exerted their effects by interaction with and perturbation of membrane lipids and associated proteins. The identification of the cannabinoid receptors revealed that cannabinoids exert their effects by directly binding to these receptors. THC is a partial agonist at both CB_1 and CB_2, exhibiting less efficacy at CB_2 than CB_1 (Pertwee, 2008). THC and its derivatives are termed classical cannabinoids, other cannabinoid receptor agonists are the non-classical cannabinoid derivatives as represented by CP55,940 (Johnson et al., 1981), aminoalkylindoles such as WIN 55-212-2 (Pacheco et al., 1991; Compton et al., 1992), and the endogenous cannabinoid receptor ligands anandamide and 2-arachidonylglycerol. Both CP55,940 and WIN 55-212-2 show equal affinity for both CB_1 and CB_2 receptors. Anandamide shows a modest selectivity for CB_1 receptors which can be enhanced by structural modification to give the derivatives R-methanandamide, ACEA, ACPA and O-1812 (Pertwee, 2008). Examples of CB_2 receptor selective agonists are JWH133 (Huffman et al., 1999), a classical cannabinoid and the less selective JWH 015, an aminoalkylindole (Griffin et al., 1997). The first cannabinoid receptor antagonists to be developed were the CB_1 receptor selective SR141716A (Rimonabant, Accomplia™) and the CB_2 selective antagonist SR144528 (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998).

It is important to stress that both cannabinoid receptor selective agonists and antagonists lose their selectivity at higher concentrations whereupon both receptors will be blocked/stimulated to some extent.

1.2.1. *Inverse agonism*

CB_1 antagonists can produce experimental effects that are opposite to the responses seen with direct CB_1 receptor agonism. This may be a direct result of the antagonism of effects produced by endogenous endocannabinoids but also reflects the ability of these antagonists to induce "inverse agonism" by inhibition of the spontaneous coupling of CB_1 receptors to their effector signalling pathways, indicating the constitutive activity of these receptors (Pertwee, 2005a). The same situation is also seen with antagonism of the CB_2 receptor, indicating that this receptor is also constitutively active (Rhee and Kim, 2002). This has prompted the search for neutral antagonists which could discriminate between tonic activity derived from endocannabinoid release, to CB receptors compared to tonic activity via the constitutive activity of these receptors.

1.3. *Endocannabinoids*

Endocannabinoids are defined as endogenous compounds capable of binding to and stimulating endogenous cannabinoid receptors. The first endogenous ligand for cannabinoid receptors or endocannabinoid was isolated from porcine brain by (Devane et al., 1992). This fatty acid arachidonic acid derivative was identified as arachidonoyl ethanolamide and named anandamide, derived from the Sanskrit for "inner bliss". Anandamide displayed agonist activity in assays identifying CB_1 agonist effects. Subsequently, a second major endocannabinoid, 2-arachidonyl glycerol (2-AG), was identified as having CB_1 ligand activity, isolated from canine gut (Mechoulam et al., 1995), Anandamide shows slightly more binding activity to CB_1 receptors than CB_2 and shows partial agonist activity at CB_1 and CB_2 receptors with higher efficacy at CB_1 than CB_2. 2-AG has been reported to have a higher efficacy than anandamide at both CB_1 and CB_2, with a similar affinity to anandamide for both receptor types (Pertwee, 2005b). As 2-AG is detected at greater levels than anandamide in nervous tissue, with levels in the nanomole range compared to picomoles for anandamide, it is considered to be the primary retrograde signalling endocannabinid at both CB_1 and CB_2 receptors (Sugiura and Waku, 2000; Sugiura et al., 2002), which is further confirmed by the observation that retrograde signaling is abolished in DAG Lipase α (chiefly responsible for 2-AG synthesis) knockout mouse brains (Gao et al., 2010; Tanimura et al., 2010). These may be considered to be the 2 main endocannabinoids but other novel

endocannabioids have been reported to have activity at cannabinoid receptors, isolated from brain homogenates. These include; a 2-AG analogue, 2-arachidonoyl glyceryl ether (noladin ether) a selective CB_1 agonist which is refractory to enzymatic hydrolysis (Hanus et al., 2001), the anandamide analogue, O-arachidonyl-ethanolamine (virhodamine), a CB_1 antagonist and partial CB_2 agonist (Porter et al., 2002), and the arachidonyl amino acids *N*-arachidonyl-Dopamine (NADA), a selective CB_1 agonist versus CB_2 also showing activity at vanilloid receptors (Bisogno et al., 2000), *N*-aracidonyl Glycine (NAGly), an inhibitor of inflammatory pain via a non-CB_1 dependent mechanism (Burstein et al., 2000) and a ligand for the putative cannabinoid receptor GPR18 (Kohno et al., 2006), *N*-arachidonyl γ-aminobutyric acid (NAGABA), *N*-arachidonylalanine (NAAla), (Huang et al., 2001) and N-arachidonoyl serine (ARA-S) which has vasodilatory and pro-angiogenic properties (Milman et al., 2006; Zhang et al., 2010). The characterization of these additional "endocannabinoids" is limited and at present, their physiological relevance remains unclear as to their status as true endocannabinoids (Alexander and Kendall, 2007).

Endocannabinoids, in contrast to conventional neurotransmitters appear to be released 'on demand' from membrane precursors via multi-step enzymatic pathways, rather than being released from intracellular stores, in response to increased intracellular calcium after neuronal activation or via the stimulation of metabotropic receptors coupled to $G_{q/11}$ proteins. Anandamide is synthesised via a number of pathways where the enzyme N-acyl-phosphatidyethanolamine (NAPE)-selective phospholipase D plays a vital role (Okamoto et al., 2004). However NAPE-PLD deficient mice did not have altered anandamide levels suggesting other pathways can generate anandamide synthesis (Leung et al., 2006). Another pathway for the generation of anandamide is proposed via phospholipase C-mediated hydrolysis of NAPE to yield phosphoanandamide, which is then dephosphorylated by a number of phosphatases (Leung et al., 2006; Basavarajappa, 2007; Di Marzo, 2008).

Phospholipase C (PLC) catalyzes the formation of the 2-AG precursor, 1,2-diacylglycerol (DAG) from membrane phosphoinositides (Wang and Ueda, 2009). The crucial enzyme in the biosynthesis of 2-AG is diacylglycerol lipase (DAG lipase) where the α isoform is localised to postsynaptic dendritic spines (Bisogno et al., 2003; Yoshida et al., 2006). Studies in mice genetically engineered to lack DAG lipase α or β, revealed that the the major biosynthetic enzyme for 2-AG production in the CNS is DAG lipase α. DAG lipase α deficient mice showed; a greatly reduced level of 2-AG in the brain and spinal cord, an absence of endocannabinoid mediated retrograde suppression of neurotransmitter release and also a compromised level of

adult neurogenesis, which is also observed in DAG lipase β knockout mice (Gao et al., 2010, Tanimura et al., 2010).
Alternatively a phospholipase A_1 (PLA_1) hydrolyses phosphoinositol precursors to produce a lyso 2-arachidonoyl phosphoinositol (LAPL) and hydrolysis of this via lyso phopolipase C (LPLC) can also produce 2-AG (Leung et al. 2006; Basavarajappa, 2007, Jung et al., 2007; Di Marzo, 2008).

It has been postulated that endocannabinoids are first removed from the extracellular space by a diffusion-facilitated transporter, re-uptake mechanism present in cell membranes (Beltramo et al., 1997; Dainese et al., 2007), prior to enzymatic degradation. Indeed, compounds, which inhibit this anandamide re-uptake activity such as AM404, VDM11, UCM707, OMDM1, OMDM2, 0-2093 can all be shown to have anti-spastic activity, without cannabimimetic adverse effects (Baker et al., 2001; de Lago et al., 2004; de Lago et al., 2006; Ligresti et al., 2006). However, the re-uptake transporter has yet to be cloned and there has been evidence questioning the existence of a specific–re uptake transporter (Day et al., 2001; Deutsch et al., 2001; Glaser et al., 2003). In particular, the prototypic transport inhibitor, AM404, has been reported by some to have to have CB_1 binding affinity, transient receptor potential vanilloid receptor ($TRPV_1$) agonist activity and be a FAAH inhibitor (Beltramo et al., 1997; Jarrahian et al., 2000; Ralevic et al., 2001). Each of these could contribute to the therapeutic activities of putative transport inhibitors (Baker rt al., 2001; Brooks et al., 2002). However, not all transport inhibitors appear to have TRPV1 or FAAH activity and can increase endocannabinoid levels *in vivo* (De Petrocellis et al., 2000; Lopez-Rodriguez et al., 2001; Ortar et al., 2003). Therefore, if a specific transport molecule does not exist, these agents may act competitively to allosterically inhibit biochemically-compatible sites of diffusion within the plasma membrane, as has been reported for interference in some receptor systems (Barann et al., 2002). More recently, it has been reported that intracellular fatty acid binding proteins (FABPs) function as carriers for anandamide, facilitating its degradation by FAAH (Kaczocha et al., 2009).

Once endocannabinoids enter the cell they are enzymatically degraded (Deutsch et al., 2002; Dinh et al., 2002; Fezza et al., 2002; Blankman et al., 2007). Allthough both anandamide and 2-AG are substrates for fatty acid amide hydrolase (Cravatt et al., 2001; Deutsch et al., 2002), *in vivo* FAAH is the major degradative enzyme of anandamide, but not 2-AG, which is degraded by Monoglycerol lipase (MAG lipase) and two novel serine hydrolases alpha-beta-hydrolase domain 6 and 12 (ABHD6 and ABHD12), (Dinh et al., 2002; Dinh et al., 2004; Blankman et al., 2007).

MAG lipase knockout mice display highly elevated levels of 2-AG in the CNS with a concomitant desensitization of CB_1 receptors and a significant reduction in the cannabimimetic activity of CB_1 receptor agonists (Chanda et al., 2010). This CB_1 receptor desensitization is also observed in mice which were repeatedly administered a MAG lipase inhibitor with accompanying loss of analgesic activity, impaired endocannabinoid-dependent synaptic plasticity and physical dependence (Schlosburg et al., 2010).

Whilst MAG lipase has been considered to be the major enzyme involved in 2-AG hydrolysis, recent observations indicate that ABHD6 also controls the accumulation and efficacy of 2-AG at cannabinoid receptors (Marrs et al., 2010), further adding to the complexity of the endocannabinoid signaling network. A recent study of patients with polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC) has revealed that these patients have mutations in the ABHD12 gene, leading to a presumed loss of function which indicates a putative essential function of ABHD12 in 2-AG hydrolysis in the central, peripheral nervous systems and the eye and in addition suggests any proposed pharmacological modulation of 2-AG degradation should be proceeded with with caution (Fiskerstrand et al., 2010).

When pharmacologically specific inhibitory agents have been generated and assessed in models of MS, it may be possible to determine the role of 2-AG and other putative endocannabinoids in control of signs/symptoms, however the analgesic and anti-spastic activity of compounds that inhibit FAAH indicate that anandamide at least controls signs, which can also be shown by exogenous endocannabinoid administration and knockout mouse studies (Baker et al., 2000; Walker et al., 2002; Lichtman et al., 2004). Potent FAAH inhibitors have been generated (Boger et al., 2000; Kathuria et al, 2003) which have symptom modifying potential, but it remains to be seen whether inhibitors of endocannabinoid degradation have a sufficiently strong therapeutic clinical benefit in spasticity in MS, although this approach may offer promise for the future.

1.4. *Multiple Sclerosis and experimental models*

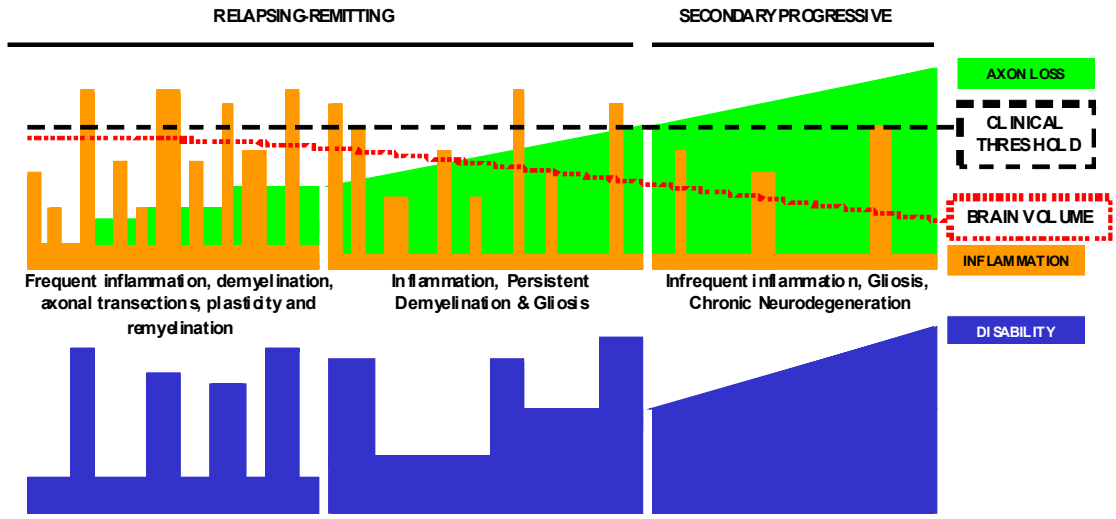
1.4.1. *Multiple Sclerosis*

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) and is the most common cause of non-traumatic neurological disability in young adults of northern European descent (Compston and Coles, 2002). This disease affects about 100,000 people within the UK. The

absolute number of cases of MS around the world has steadily increased, possibly as a result of improved diagnosis amongst other factors and affects 2-3 million people worldwide (Kurtzke, 1993). The incidence of MS is geographically restricted and occurs with high incidence in Northern Europe and in regions colonized by white Northern Europeans such as Canada and Northern USA, Australia and New Zealand with a gradient of higher incidence further from the equator (Compston and Coles 2002). MS is more common in females compared to males with an increasing ratio of 3:1, with a more pronounced incidence in females in younger MS patients with relapsing-remitting disease (RRMS), (Runmarker and Andersen, 1993). Disease is influenced by genetics, as evidenced by an increased concordance of MS in monozygotic twins (~30%) compared to dizygotic twins (~5% concordance rate) and is polygenically controlled (Compston and Coles 2002, Compston and Coles 2008). Disease is associated with the expression of certain MHC haplotypes such as HLADRb*1501 (Prat et al., 2005) Other susceptibility loci include the cytokine IL-7 and IL-2 receptors, the adhesion molecule LFA-3 (CD58) and the c-type lectin domain family 16 member A (Hafler et al., 2007, De Jager et al., 2009, Hoppenbrowers et al., 2009). However the observation that the concordance of disease in identical twins demonstrates that other, environmental, factors may influence susceptibility. Migration studies from low to high incidence areas suggest that the environmental trigger is acquired before the age of fifteen [Compston and Coles, 2002]. Some have suggested that it may relate to age of infection and there are thoughts that this could relate to Epstein Barr Virus (EBV) infection (Sumaya et al., 1980; Ascherio and Munger, 2010). The vast majority of people with MS are infected with EBV compared to 90% of the general population and there is increased frequency of MS in people who developed glandular fever (Handel et al., 2010). Another hypothesis is that this environmental influence may relate to sunlight exposure and vitamin D production (Hayes et al., 1997; Freedman et al., 2000). This is indirectly supported by the geographic distribution of people with MS (Sadovnick and Ebers, 1993). Vitamin D levels can influence the immune response and may even be important *in utero* (Willer et al., 2005). Importantly a number of genes associated with MS, such as certain HLA haplotypes, contain vitamin D responsive elements in their promoter regions that can influence expression and may link environment and genetic susceptibility elements (Ramagopalan et al., 2009; Ramagopalan et al., 2010). MS most commonly (about 80%) presents as a series of relapsing-remitting episodes of loss of neurological function that eventually develops into a chronic, secondary progressive (SPMS) phase with no remission and increasing disability over time, which correlates with CNS atrophy and axonal loss, particularly in the spinal cord (Bjartmar et al., 2000), Figure 1.1. In about 10-15% of people, particulary in those with an onset later in life, disease becomes progressive (Primary progressive MS) from onset (Compston

and Coles 2002; Compston and Coles 2008). As such about 80% of people with MS will be severely disabled within 25 years from disease onset.

Figure 1.1. *Disease course in multiple sclerosis.*



An initial period of repeated inflammatory episodes results in blood:brain barrier dysfunction and in some occasions relapsing neurological deficit induced by persistent demyelination. This creates a chronic neurodegenerative microenvironment, seen by brain atrophy, which reaches a threshold beyond which clinical disease progresses unabated (adapted from Compston & Coles 2002).

Disease is associated with blood:brain barrier dysfunction and mononuclear cell infiltration that arises around post-capillary venules and leucocytes then invade the brain parenchyma leading to an expanding ring of macrophage-mediated myelin-destruction. This leads to the pathological hallmark of MS, which is demyelination of the white and grey matter, due to loss of oligodendrocytes and myelin. Although initially there is remyelination (shadow plaques), the capacity to repair eventually becomes exhausted and astrogliotic scars are formed within demyelinated plaques. Whilst lesion load is decreased following successful immunosuppressive treatment (Polman et al., 2006; Jones and Coles; 2010), suggesting that leucocyte inflammation is the damaging force in MS, it has also been suggested that damage to the astrocyte or oligodendrocyte may be the primary event followed by infiltration of mononuclear cells (Barnett and Prineas, 2004; Parratt and Prineas, 2010).

As the disease evolves, inflammatory attacks increase the burden of demyelination and a dystrophic environment leads to eventual axonal loss, which impairs normal neurotransmission. This leads to the development of additional distressing symptoms such as incontinence, limb tremor, pain, spasms, fatigue and spasticity, which have a major negative impact on quality of life indices (Compston and Coles, 2002; Confavreux and Vukusic, 2006). RRMS is the most common clinically

presenting form of MS, with an incidence of approximately 85%, with the typical age of onset being the early third decade of life. RRMS is characterised by acute or sub-acute onset of neurological dysfunction lasting for more than 24 hours, usually resolving within weeks to complete or partial recovery. The frequency of relapses varies over time but there appears to be a clear trend for relapses to be more common in the initial years of the disease and recovery from these early relapses to be more complete (Weinshenker et al., 1989). The time taken to convert to a secondary progressive neurodegenerative phenotype can vary widely between individuals and may reflect differences in an individual's ability to cope with episodes of neuronal insult, perhaps consistent with genetic control and heterogeneity of disease (Compston and Coles, 2002). In approximately a quarter of cases, neurological disability does not reach a level where it impinges on daily living but conversely in around 15% of cases the progression to disability is rapid. The prognosis for patients is better in cases where sensory symptoms dominate the course of disease and there is a complete recovery from these symptoms at remission whereas the prognosis is poorer when there is motor involvement such as deficits of pyramidal, visual, sphyncteric and cerebellar systems (Amato and Ponziani, 2000). Frequent relapses and incomplete recovery plus a short time period between the initial neurological event and the subsequent relapse also have a poorer prognosis. There is also a poorer prognosis for the disease in older men who develop MS (Weinshenker et al., 1989; Compston and Coles, 2002). However, once a threshold of disability has been reached, disability progression is remarkably uniform (Confavreux et al., 2000), and approximately 90% of RRMS patients will develop progressive disease after 25 years of clinical follow up (Weinshenker et al., 1989). It may be that given enough time, all RRMS patients will eventually convert to the progressive phase of the disease. A recent study demonstrated that disablility progression seems to follow a two stage course, the first stage, corresponding to clinical disease onset to irreversible Kurztke expanded disability status scale (EDSS) 3 (Moderate disability in one of eight functional systems (FS), or mild disability in three or four FS. Fully ambulatory) being dependent on ongoing focal neuroinflammation and a second stage, from irreversible disability scale 3 to irreversible disability scale 6 (Intermittent or unilateral constant assistance (cane, crutch, brace) required to walk about 100 meters with or without resting), which is independent of ongoing focal neuroinflammation and where neuroprotective strategies are indicated, rather than immunomodulatory therapies which are indicated for the phase one stage of MS (Leray et al., 2010).

Whilst immune-mediated conduction block and destruction of CNS myelin, followed by lesion resolution and limited myelin repair, may account for the relapsing-remitting nature of the disease, what is less clear are the mechanisms that account

for the conversion to the chronic neurodegenerative secondary phase, which appears to be independent of, though worsened by, the accumulated neuronal dysfunction accompanying relapses (Bjartmar et al. 2003). A gradual degeneration of predominantly the pyramidal and cerebellar systems evolves which is often accompanied by sphincter and sexual dysfunction (Amato and Ponziani, 2000). In addition, a subtype of MS, primary progressive MS (PPMS) presents as a progressive degenerative phenotype in 10-15% of patients after an initial bout of CNS inflammation, which along with secondary progressive MS is largely refractory to currently available MS therapies such as immunomodulation (Miller and Leary, 2007), and where neuroprotective strategies are urgently indicated. Clinically, PPMS develops at a later age than RRMS, with onset in the fourth decade rather than the third decade as seen in RRMS (Andersson et al., 1999), and with a lower female preponderance. The presence of inflammatory cells of the immune system in active lesions, particularly in the white matter has lead to the hypothesis that MS is primarily an autoimmune T- cell mediated demyelinating disease. The presence in active inflammatory perivascular lesions of CD4 and CD8 positive T cells, monocytes and B cells has provided evidence for this hypothesis (Traugott et al., 1983a; Traugott et al., 1983b; Hauser et al., 1986).

Further evidence from experimental animal models of MS showing the central importance of T cell-mediated demyelination in the pathogenic process has lead to the dominant paradigm of MS as an autoimmune disease where self-tolerance to CNS antigens is lost, leading to autoimmune-mediated destruction of myelin in the CNS. T cells in the periphery become activated and migrate to the CNS, initiating disease episodes. However, the cause of the initiating factors leading to the activation of T cells recognising CNS antigens, the identity of these antigens and the mechanism underlying the episodic nature of relapses have remained elusive. This has lead to a recent hypothesis that proposes that MS is primarily a neurodegenerative disease accompanied by secondary inflammatory demyelination (Trapp and Nave, 2008). As to whether inflammation is a primary or secondary event in the disease process, results from clinical trials thus far have revealed that patients with established secondary progressive MS continue to accumulate disability, despite the cessation of relapses following treatment with the anti-CD52 antibody, alemtuzumab/Campath-1H, which produces a profound long-lasting lymphocyte depletion. In contrast, patients in the earlier stages of relapsing–remitting disease showed an improvement in disability scores which was maintained at a lower level at 36 months (Coles et al., 1999; Coles et al, 2006). When this therapy is administered early in the disease course there was a marked inhibition of relapse and apparent recovery of motor deficits (Coles et al., 2008; Jones et al., 2010) in an ongoing trial which should reveal whether early intervention in

suppressing inflammatory lesions in the CNS can halt disease progression due to neurodegeneration. This indicates the importance of the primary inflammatory response in the development of progressive MS due to neuronal loss. It will be of interest to follow up these patients over a long period of time to establish whether the cessation of neuroinflammation completely halts disease progression and associated neurodegeneration.

Over recent years, axonal pathology during MS has been re-examined and it has been established that CNS atrophy and axonal loss occurs, coincident with inflammatory lesion formation, early in the relapsing-remitting phase. This may be accommodated initially by remodelling of neuronal circuits (neural plasticity) or an increase in the number of neural precursors in some lesional areas contiguous with subventricular zones (Chang et al., 2008). However, as the disease continues, a threshold is reached and beyond which, permanent impairment and increasing disability is established (Bjartmar et al., 2000; Confavreux et al., 2000; Bjartmar et al., 2003; Confavreux and Vukusic, 2006). This suggests that axonal loss rather than myelin damage is the key determinant of progressive disability in MS. In addition, a doubling in the levels of glutamate, an excitatory amino acid that has been shown to be neurotoxic in excess is seen in the CSF of MS patients undergoing an inflammatory episode (Stover et al., 1997).

In experimental allergic encephalomyelitis (EAE), an animal model of MS induced by the development of autoimmunity against myelin antigens, 15-30% of spinal cord axons can be lost before permanent locomotor impairment is noted (Bjartmar et al., 2000; Wujek et al., 2002). After a number of relapse events, permanent disability develops with significant axonal loss (40-80%, as also occurs in MS), in the spinal cord (Wujek et al., 2002) and the development of hind limb spasticity and tremor (Baker et al., 2000), which may reflect as the preferential loss of inhibitory circuits in certain locations of the spinal cord and their influence on signalling to skeletal muscles. Whilst inflammatory events are associated with axonal transections, chronic demyelination may contribute to a slow degenerative process. Demyelinated axons must redistribute ion channels, particularly sodium channels, to maintain neurotransmission along the length of the axon (Waxman, 2001), placing an increased metabolic burden on the demyelinated neuron. Demyelination makes the axon particularly vulnerable to damage in the presence of toxic mediators such as nitric oxide (Smith et al., 2001), released by activated macrophages or resident activated microglial cells. This has lead to the examination of the potential of partially reducing the activity of these sodium channels using the sodium channel blocker lamotrigene to reduce the level of axonal degeneration (Bechtold et al., 2006). The use of the sodium channel blocker phenytoin, whilst

having a beneficial effect in experimental MS, on withdrawal, a rapid exacerbation of the disease was observed accompanied by significant mortality. This may indicate that in these experiments sodium channel blockade is immunosuppressive rather than being definitively neuroprotective *per se*, where neuroprotection is observed via reduction in CNS inflammation and therefore reducing the inflammatory insult to the CNS (Black and Waxman, 2008). Recently trials of lamotrogine in secondary progresssive MS, indicated some slowing of the loss of motor function, although the anti-inflammatory effects and inhibition of swelling of nerve tissue masked influences on nerve loss being detected by reduction of loss of brain volume (Kapoor et al. 2010).

As increasing numbers of axons are lost, this creates an extra burden on the remaining neurons and potential excitotoxicity due to increased activity on these neurons within the neural circuitry. Thus, a slow amplifying cascade of neuronal death may be triggered, which could occur independently of significant inflammation. This would be compatible with the slow progression in secondary progressive MS and the inability of potent immunosuppressive agents to inhibit this aspect of disease despite their efficacy in reducing blood:brain barrier dysfunction and the reduction of relapse rate (Coles et al., 1999; Confavreux and Vukusic, 2006). During all neurodegenerative diseases, symptoms occur because homeostatic control of neurotransmission is lost, and may result from increased neurotransmission by excessive signalling of excitatory circuits or loss of inhibitory circuits or *vice versa*. As it appears that an important function of the cannabinoid system is the modulation of neurotransmitter release via CB_1 receptor expression at pre-synaptic nerve terminals (Wilson and Nicoll, 2002), this raises the possibility of therapeutic intervention in CNS events for symptom control by the manipulation of this system.

1.4.2. *Experimental allergic encephalomyelitis (EAE)*

The most widely utilised animal model of multiple sclerosis is EAE, which can be induced in susceptible animal strains by immunisation with; CNS-derived antigens such as spinal cord homogenate, Myelin Associated glycoprotein (MAG), Myelin oligodendrocyte glycoprotein (MOG), Proteolipid protein 1 (PLP1) infection with neurotropic viruses or the adoptive transfer of encephalitogenic T cell lines (Denic et al., 2010). Spontaneous transgenic mouse EAE models have also recently been reported which have a preponderance of myelin-specific T cell receptors (Ellmerich et al., 2005; Bettelli et al 2006; Friese et al., 2006). EAE is most commonly induced in mouse strains but there are also rat models and these have tended to have

supplanted animal models such as the guinea pig due to the large number of reagents available for the study of immunological parameters in particular. In addition there are some primate models of EAE, particularly the common marmoset which has tended to replace the use of rhesus macaque monkeys ('t Hart et al., 2005).

Disease type and the associated CNS pathology can vary widely depending on the animal model used ranging from an acute monophasic disease in the Lewis rat strain, an acute progressive form with little remission seen in the C57BL/6 mouse strain, to a relapsing-remitting phenotype leading to secondary progression due to neurological loss seen in the Biozzi antibody high (ABH) mouse (Baker et al., 1990). EAE in the ABH mouse is typically induced by immunisation with whole spinal cord homogenate in complete Freund's adjuvant leading to a chronic relapsing-remitting phenotype with secondary progression. In contrast, immunisation with a peptide of myelin oligodendrocyte glycoprotein (MOG) 35-55, which is commonly used for disease induction in the C57BL/6 mouse strain, results in a progressive chronic disease with minimal remission as seen in that strain (Amor et al., 2005). The relapsing-remitting phenotype of disease in the ABH mouse and the development of a chronic secondary progressive phase of disease, mirroring the type of disease most commonly seen in patients with MS, indicates this may be a particularly relevant model to investigate the pathology of the disease and potential therapeutic strategies both immunological and neuroprotective.

ABH mice express a unique major histocompatibility (MHC) haplotype K^d D^q L^q which has been designated H-2 ^{dql} (Baker et al., 1990). In addition, ABH mice share the MHC class II molecule A^{g7} with the non-obese diabetic mouse strain (NOD) but lack a functional E region due to a defect in the E^a molecule (Liu et al., 1993). In EAE studies, ABH mice express significant levels of IgG1 but no detectable levels of IgG2a are observed (Amor et al., 2005). During clinical episodes of neuroinflammation, ABH mice show perivascular infiltration of mononuclear immune cells comprising of CD4 positive T lymphocytes and macrophages (Butter, 1991a), predominantly in the spinal cord. This cellular accumulation in the CNS correlates with disease severity. Inflammatory cell infiltration is accompanied by increased expression of MHC class II molecule expression by microglia and perivascular macrophages and expression of adhesion molecules on endothelial cells of the CNS vasculature (Butter et al., 1991b; O'Neill et al., 1991). In the acute phase of the disease, there is little evidence of demyelination, but demyelination is observed during the relapse phase of the disease (Amor et al., 2005). Demyelinating inflammatory lesions are also seen in the optic nerve, (also a hallmark of early MS), accompanied by impaired visual responses and impaired axonal protein transport

(O'Neill et al., 1998). EAE in the ABH mouse is accompanied by increasing levels of axonal degeneration, particularly in the spinal cord during the course of disease and this degeneration leads to the development of signs of neurological impairment, such as decreased locomotor performance and clinical signs such as spasticity and tremor (Baker et al., 2000; Petzold et al., 2003). There are also increased levels of expression of neuron-specific sodium channels in demyelinated axons to maintain saltatory conduction in chronic progressive EAE ABH mice, which is mirrored in MS brain tissue (Black et al., 2000; Craner et al., 2003).

In an important recent study, it has been reported that using *in vivo* imaging techniques that a subset of T cells (Th17) identified by the production of the cytokine IL-17 are capable of forming direct contact with neurons/axons in a manner analogous to synapse formation in an antigen independent manner, which is of interest as MS and EAE have been considered to be triggered by antigen-specific immunity to myelin antigens on oligodendrocytes. Formation of these Th17/neural synapses is accompanied by significant increases in intracellular neuronal Ca^{2+} levels leading to axonal transaction and neuronal death via Wallerian degeneration or apoptosis. A possible mechanism for this neuronal Ca^{2+} increase appeared to be via direct glutamate release from Th17 cells and the triggering of excitotoxicity via NMDA receptors, which can be modulated by treatment with the NMDA antagonist MK-801 and to a lesser extent blocking of Na^{+} channels with phenyoin (Siffrin et al., 2010).

It is important in the use of EAE models for neuroprotective studies, that therapeutic strategies for neuroprotection are not confused with anti-inflammatory activity on disease. Many potential neuroprotective agents can also be immunosuppressive due to the co-expression of receptors on cells of the immune system as well as the CNS and a reduction in inflammatory infiltrates can be interpreted as being neuroprotective as a consequence of a reduction in the level of inflammatory attack. Potential neuroprotective agents should be titrated so that there is an equivalent level of disease in treatment and control groups before true neuroprotection can be demonstrated. It is surprising that in many if not the majority of "neuroprotective" studies in EAE, suppression of disease activity is routinely confused with neuroprotective properties of the therapeutic agent being studied and highlights the importance of interpreting results correctly.

1.5. *Cannabinoids and symptom management in MS*

To date, the primary area of investigation of the cannabinoids in MS has been that of symptom relief, in particular bladder incontinence, tremor and particularly limb spasticity, as patients claim that these particular symptoms are alleviated by cannabis and this has been supported by some early reports (Consroe et al., 1997; Pertwee, 2002). Current therapies for spasticity include the GABA receptor agonist Baclofen, Tinazidine and Benzodiazepines (Paisley et al., 2002). Intrathecal Baclofen is commonly used for the treatment of severe refractory spasticity (Kita and Goodkin, 2000). The anti-convulsant, Gabapentin and local administration of botulinum toxin have also showen efficacy in clinical trials (Kita and Goodkin, 2000; Paisley et al., 2002) The pathophysiology of spasticity remains poorly understood but it may reflect a preferential loss of inhibitory circuitry in the spinal cord resulting in excessive levels of stimulatory signals. Under normal circumstances, inhibitory signals are sent via the corticospinal tract to the spinal cord, but following injury, damage to the corticospinal tract, a hallmark of MS, causes disinhibition of the stretch reflex leading to a reduction in the triggering threshold. This results in excessive contraction of the muscles, sometimes even at rest (Brown, 1994; Adams and Hicks, 2005; Nielsen et al., 2007) The hypertonic mouse mutant *hyrt* (Gilbert et al., 2006), show spastic signs in the hind limbs associated with a reduction in the level of inhibitory GABA A receptors in lower motor neurons. Loss of GABAergic inputs or GABA receptor expressing neurons may produce the spasticity seen in multiple sclerosis as neurodegeneration progresses and explain the efficacy of GABA agonists such as Baclofen. Improved treatment regimes for spasticity are urgently needed, as agents that directly interfere with neurotransmitter activity are often associated with undesirable side-effects (Paisley et al., 2002). Experimental data in MS models in mice have indicated the anti-spastic and anti-tremor effects of cannabinoids and CB_1 agonists (Baker et al., 2000; Baker et al., 2001) and any CB_1 agonist that reaches the CNS has the potential to inhibit spasticity. Furthermore and importantly, antagonism of the cannabinoid system produces a worsening of these signs, indicating the presence of an endogenous cannabinoid tone, which is modulating these signs to some degree via the release of endocannabinoids in response to elevated neuronal excitation (Baker et al., 2000, Baker et al., 2001). Surprisingly, although there is limited data to suggest that CB_2 is expressed by nerves (Howlett et al., 2002), CB_2 receptor agonists or their metabolites could inhibit spasticity (Baker, 2000). In addition, endocannabinoid (particularly anandamide) levels are raised in the spinal cords and brains of mice, which show hind limb spasticity, but not in animals, which have equivalent levels of neurodegeneration but without associated limb spasticity (Baker et al., 2001). This further indicates the presence of an endocannabinoid tone, which is elevated as a

result of spasticity and tremor in these animals. Indeed administration of compounds, which elevate endogenous anandamide levels, either via inhibition of re-uptake or enzymatic degradation by fatty acid amide hydrolase (FAAH), also reduces the level of spasticity in mice (Baker et al, 2001). These observations provide objective evidence, to underpin patient perceptions of the efficacy of cannabis on MS symptoms.

In MS patients, it has been reported that a cannabis extract administered as a sublingual spray, shows efficacy in the treatment of bladder incontinence, showing both a decrease in emptying episodes and an increase in bladder retention volume (Brady et al., 2004). A second study as part of the Cannabis in Multiple Sclerosis (CAMS) study of patients, treated with a cannabis extract or Δ-^{9} THC, reported a significant reduction in episodes of urge incontinence compared to placebo (Freeman et al., 2006). This suggests that cannabinoids can compensate for the dysregulation of bladder neural circuitry that frequently accompanies disease progression in multiple sclerosis.

A number of controlled and blinded trials have been undertaken on spasticity in MS (Killestein et al., 2002; Zajicek et al., 2003; Wade et al., 2004; Vaney et al., 2004; Zajicek et al., 2005; Wade et al., 2006; Collin et al., 2007). Although oral cannabis at doses that lack overt psychoactivity, has so far shown no or a marginal effect in treating spasticity as assessed by the Ashworth Scale (Killestein et al., 2002), there was an improvement in the time taken to complete a ten metre walk (Zajicek et al., 2003). Oral administration of cannabinoids is hampered compared to other routes due to variable absorption and metabolism including a significant first pass effect through the liver which complicates dose-titration (Agurell et al., 1986; Mattes et al., 1993; Grotenhermen, 2003). However, similar studies with a sublingual cannabis extract spray (Sativex®), has likewise had a minimal impact on objective outcomes such as the Ashworth Scale (Wade et al., 2003; Wade et al., 2004; Collin et al., 2007), but have had consistent, subjective patient assessed, perceived improvements in spasms and spasticity. These apparently negative results may be largely due to the insensitivity of the Ashworth Scale in detecting positive effects of anti-spastic therapies where many of the currently prescribed drugs fail to show efficacy using this measure (Shakespeare et al., 2003).

As cannabis affects cognitive processes (Curran et al., 2002), it can be argued that whilst patients feel subjectively improved due to mood modulation, these may not be objectively demonstrable at cannabinoid doses that do not induce significant cannabimimetic psychoactive effects (Killestein et al., 2002; Zajicek et al., 2003; Wade et al., 2004; Zajicek et al., 2005; Wade et al., 2006; Pertwee, 2007).

However, positive effects, with few exceptions (Killestein et al., 2002), have been reported following treatment with THC or medical cannabis extracts (Zajicek et al., 2003; Wade et al., 2004; Vaney et al., 2004; Wade et al., 2006; Collin et al., 2007). Importantly, patients on clinical trials suggest that only certain signs such as spasticity, pain and sleep disturbances are notably being affected suggesting that these positive effects are unlikely to be simply due to a generalized perception of improvement following drug administration (Zajicek et al, 2003; Wade et al., 2004; Vaney et al., 2004; Wade et al., 2006; Collin et al., 2007).. This suggests some positive benefit of cannabinoids and further evidence of the efficacy on MS related spasticity following long-term administration of THC was reported showing a positive improvement on the Ashworth Scale in patients treated with THC for one year (Zajicek et al., 2005).

The apparently limited evidence thus far of the efficacy of cannabis in symptom management in MS; may reflect the poorly designed nature of many of the early trials, with subjective rather than quantitative outcome measures and insufficient appreciation of the pharmacokinetic problems such as first pass effects via the liver with the oral route of administration (a clinically preferred route compared to smoking Agurell et al., 1986; Pertwee, 2002). It would appear that routes of delivery, which facilitate rapid entry to the bloodstream and then to the CNS, are preferable to the orally administered route. Such routes are; aerosol inhalation, rectal suppository or sublingual spray (Grotenhermen, 2003). This may account for the anecdotal claims that smoked cannabis, which is rapidly absorbed and has no first pass effects, allowing self-titration of therapeutic effect, is preferable to orally administered Δ^9-THC, which is slowly absorbed and subjected to first pass metabolism in the liver plus there is little chance of self titration (Agurell et al., 1986; Consroe et al., 1997; Pertwee, 2002). The biology of the cannabinoid system indicates that CB_1 mediates both the psychoactive and the majority of the potentially therapeutic effects of cannabis, and therefore its use will invariably be associated with side effects, which some people may find intolerable (Killestein et al., 2002; Baker et al., 2003). However, through individual dose-titration, these may be limited to achieve a therapeutic window where a benefit is achieved whilst unwanted side-effects are limited. A study on cannabinoid-mediated control of tics associated with Tourette's syndrome suggests it is indeed possible to have a positive therapeutic outcome without significant cognitive impairment (Muller-Vahl et al., 2003). Furthermore, oral Δ^9-THC and Nabilone (a synthetic analogue of THC), are licensed anti-emetics, producing a therapeutic benefit within tolerable side-effect limits. Therefore, usage in any clinical indication will be a balance between treatment of a particular condition and the acceptability of side effects.

The mounting evidence for the potential benefit of cannabis on MS –associated spasticity has recently lead (June, 2010) to the approval for prescription in the UK of the cannabis extract Sativex® for the treatment of spasticity in MS.

1.6. *Cannabinoids in Autoimmunity*

There is a perception that cannabis may affect disease course in MS (Consroe et al., 1997), but quantifiable data are however lacking, and the clinical course of disease is notoriously variable (Compston and Coles, 2002; Confavreux and Vukusic, 2006). Although CB_1 receptors are highly expressed in the neural compartments, they are also expressed on leucocytes, which may additionally express CB_2 receptors (Bouaboula et al., 1993; Galiegue et al., 1995; Howlett et al., 2002). Here, it is expressed particularly on B cells and macrophages, which can also produce endocannabinoids (Bisogno et al., 1997). The level of CB_1 and CB_2 receptor expression is affected by the degree of activation, with CB_2 levels being reduced on activation whereas CB_1 receptor levels are reported to be reduced or increased depending on the cell type studied and the activating agent used (Klein et al., 2003; Borner et al., 2007). The upregulation of CB_1 receptors on T cells in response to cannabinoids may enhance the immunomodulatory effects of cannabinoids (Borner et al., 2007) The function of the endocannabinoid system on leucocytes has yet to be fully elucidated, although it may function as a regulator of the development of haematopoietic cells, influence cellular activation, it may control the magnitude and migration of the immune response (Klein et al., 2005; Klein and Cabral, 2006; Baker et al., 2007; Correa et al., 2007) or shift the Th1/Th2 cytokine balance (Yuan et al., 2002) to a potentially disease suppressing Th2 phenotype.

There is abundant evidence that cannabinoids can influence the nature and level of cytokine production and leucocyte function (Klein et al., 2003) and have been shown to inhibit the development of disease in autoimmune (Lyman et al., 1989; Wirguin et al. 1994; Pryce et al., 2003; Ni et al., 2004; Fujiwara and Egashira, 2004; Cabranes et al., 2005; Sanchez et al., 2006; Palazuelos et al., 2008) and viral models of MS (Arevalo-Martin et al., 2003; Croxford and Miller, 2003) via CB_1 and CB_2-receptor dependent mechanisms. The CB_2 receptor regulates T cell apoptosis which is mediated by CNS-derived endocannabinoids and CB_2 selective agents (Sanchez et al., 2006; Lombard et al., 2007). In a mouse model of inflammatory retinal disease, experimental autoimmune uveoretinitis (EAU), the CB_2 selective agonist JWH-133 has been shown to have significant disease-suppressing activity in a dose-dependent manner (Xu et al., 2007). Stimulation of

the CB_2 receptor can reduce Th1/Th17 responses, particularly the inhibition of gamma interferon production reflected by the failure of upregulation of MHC class II on glial cells resulting in a reduction in antigen presenting capacity to T cells (Arevalo-Martin et al., 2003). In addition, the CB_1/CB_2 agonist WIN55,212-2 inhibits leucocyte migration into the CNS of EAE mice by a partially CB_2 receptor-dependent mechanism (Sanchez et al., 2006). However, it has also been reported that CB_2 receptor agonists and antagonists can inhibit leucocyte migration into tissues (Lunn et al., 2006, Oka et al., 2006).

In EAE, cannabinoid immunotherapy is typically associated with peripheral immunosuppression that prevents the events leading to leucocyte accumulation in the CNS, possibly at the level of initial sensitization (Lyman et al., 1989; Wirguin et al., 1994; Pryce et al., 2003; Ni et al., 2004; Fujiwara and Egashira, 2004; Cabranes et al., 2005; Sanchez et al., 2006; Palazuelos et al., 2008) and inhibition of T cell function. Importantly, disease inhibition in MS models using cannabinoids typically occurred at relatively high doses that induced cannabimimetic effects Lyman et al., 1989; Wirguin, 1994; Croxford and Miller, 2003). This form of immunosuppression does not appear to be associated with the direct stimulation of cannabinoid receptors on cells of the immune system but an indirect effect of the stimulation of neuronal CB_1 receptor stimulation. This results in the downstream production of immunosuppressive molecules such as glucocorticoids that have been shown to be potent modulators of the neuroinflammatory response in EAE (Pertwee, 1974; Wirguin, 1994; Bolton et al., 1997). However, in a clinical context, such dose levels necessary to achieve immunosuppression are unlikely to be achieved without severe psychoactive side effects (Zajicek et al., 2003). In addition chronic cannabis smokers are not overtly immunosuppressed although they may exhibit some immune perturbations (Rachelefsky et al, 1976; Bredt et al., 2002; Roth et al., 2002; Pacifici et al., 2003). Increases in proinflammatory cytokine levels such as TNFα in cannabinoid-treated patients have also been reported (Killestein et al., 2003) and there was no influence on cytokine levels compared to controls in a large study of MS patients treated with cannabinoids for spasticity alleviation (Katona et al., 2005).

Furthermore, people infected with human immunodeficiency virus smoke cannabis and oral THC is approved for appetite stimulation in the wasting syndrome associated with human immunodeficiency virus (HIV) infection where immunosuppression would be a major contraindication (Bredt et al., 2002). As HIV-induced disease is a problem of loss of immune control, this further suggests that

clinically useful doses of plant-based cannabinoids may not be overtly immunosuppressive and is further supported by observations of people with MS receiving cannabis, of a sufficient level to induce psychotropic effects (Killestein et al., 2003; Katona et al., 2005). Similarly, although patients in symptom control trials of cannabis were selected for stability of disease, the relapse rate of people taking oral THC and cannabis extract did not appear to be reduced (Zajicek et al., 2005).

Although the CB_1 receptor may offer more limited scope for control of immune responses, there is increasing evidence for influences of CB_2 receptors in limiting some of the detrimental elements of immune responses. Indeed, CB_2 receptors may control the activation and pattern of migration of leucocytes and microglial progenitors into the CNS (Ni et al., 2004; Palazuelos et al., 2008) and also upregulate the expression of the CB_1 receptor on T cells (Borner et al., 2007). Whilst there has been not reported differences in disease severity of EAE in CB_1 receptor and FAAH deficient mice (Pryce et al., 2003; Webb et al., 2008), CB_2-deficient mice exhibit elevated disease severity compared to wildtype animals (Palazuelos et al., 2008). This may suggest a degree of tonic immune system control by the endocannabinoid system. The high endogenous level of 2-AG within the CNS compared to the blood, may provide a sufficient level of stimulation of CB_2 receptors on T cells entering the CNS, which is not achieved in the circulation, to limit autoimmune responses and provide an additional mechanism for immune privilege for the CNS. Increases in anandamide levels have been found in the blood and cerebrospinal fluid of people with MS and these levels may increase in active disease and could serve to limit immune function (Centonze et al., 2007). In contrast, in another study in MS patients, reduced levels of endocannabinoids were seen in the cerebrospinal fluid of RRMS patients compared to controls, which was even lower in SPMS patients. A small increase was seen in patients undergoing a relapse but was still lower than controls (Di Filippo et al., 2008). FAAH-deficient mice which exhibit elevated levels of anandamide develop EAE of comparable onset and severity to wild type controls (Webb et al., 2008), indicating that any increases in endocannabinoid levels are not sufficient to impact on the level of inflammation. Few studies have attempted to pharmacologically manipulate the endocannabinoid tone to affect autoimmune function, but AM404 the anandamide re-uptake inhibitor inhibited the development of EAE not by effects on cannabinoid receptors but via virtue of the apparent capacity of AM404 to stimulate TRPV1 receptors (Cabranes et al., 2005). In contrast, other re-uptake inhibitors have been shown to exhibit cannabinoid mediated disease inhibitory effects in viral models of MS (Ortega-Gutierrez et al., 2005; Mestre et al., 2005).

The hybrid synthetic cannabinoid/vanilloid agonist arvanil has potent activity at the $TRPV_1$ vanilloid receptor and has been shown to produce a moderate effect on the immunosuppression of EAE via a $TRPV_1$-dependant mechanism (Malfitano et al., 2006; Marquez et al., 2006). Again this immunosuppression is observed at doses that are likely to induce immunosuppressive stress responses such as glucocorticoid release *in vivo* due to the highly noxious/cannabimimetic properties of this compound (Brooks et al., 2002).

In addition to CB_2-mediated T cell immune modulation, CB_2 receptors are expressed on microglial cells in MS lesions (Yiangou et al., 2006; Benito et al., 2007). CB_2 receptor stimulation can serve to directly or indirectly inhibit the development of a pro-inflammatory environment that leads to microglial activation, as shown by upregulation of major histocompatibility complex class II antigens and migration of microglial displaying an amoeboid phenotype that is central not only to the development of lesions but also repair of immune attack (Arevalo-Martin et al., 2003; Klegeris et al., 2003; Carrier et al., 2004; Maresz et al., 2005; Sheng et al., 2005; Witting et al., 2006).

Importantly, microglial activation and function appears to be central to the development of the processes associated with the "low-level" inflammatory neurodegenerative events that underpin clinical progression independent of autoimmune attack in MS and other neurodegenerative conditions via the release of toxic mediators such as nitric oxide, which is inhibited by cannabinoids via CB_1 (Waksman et al., 1999) and CB_2 (Eljaschewitsch et al, 2006). Although perhaps marginal, any immunosuppressive activity could be beneficial due to reductions in the level of the inflammatory insult, however, more importantly the biology of the cannabinoid system and experimental data may indicate that cannabinoids may be neuroprotective in an environment where neuronal damage is taking place (Pryce et al., 2003; Witting et al., 2006; Webb et al, 2008).

1.7 *Cannabinoids in neuroprotection and disease progression in MS and animal models*

There is abundant experimental evidence that cannabinoids can act as neuroprotective agents in both *in vitro* and *in vivo models* of neurodegeneration. Cannabinoids can protect cultured cortical neurons from oxygen and glucose deficiency in a CB_1 and CB_2 independent manner (Nagayama et al., 1999). Δ-^{9} THC

and cannabidiol have also been reported to reduce glutamate toxicity in cultured cortical neurons independent of CB_1 receptor activation (Hampson et al., 1998). CB_1 receptor dependent neuroprotection has been observed in kainate excitotoxicity toxicity in mouse spinal neurons with Δ-^{9} THC (Abood et al., 2001) and the synthetic cannabinoid WIN 55,212-2 has also shown to protect cultured hippocampal neurons from glutamate-mediated excitotoxicity in a CB_1 dependent manner (Shen and Thayer, 1998).

The major cause of permanent disability in MS is the underlying neurodegeneration that drives progressive MS and this has so far evaded any satisfactory treatment (Compston and Coles, 2002; Bjartmar et al., 2003; Dutta and Trapp, 2007). There is increasing evidence that cannabinoids, including the endogenous cannabinoid tone can limit acute neurodegeneration in; experimental cerebral ischaemia (Nagayama et al., 1999; Parmentier-Batteur et al., 2002), closed head trauma (Panikashvili et al., 2001; Hansen et al, 2001), and neurodegeneration induced by excitotoxic agents (Hansen et al., 2001; van der Stelt et al., 2001a; van der Stelt et al., 2001b; Hansen et al., 2002; Pryce et al., 2003). Cannabinoids have also been shown to have a protective effect in chronic models of neurodegeneration (Pryce et al., 2003; Bilsland et al., 2006; Docagne et al, 2007), however, recent data suggest that cannabinoids may not be a ubiquitous neuroprotective agent in all neurodegenerative diseases as in a transgenic animal model of Huntington's disease in R6/1 mice, neither THC, the synthetic CB_1 agonist HU210 or the FAAH inhibitor URB 597 affected the deterioration of motor performance over the disease course when administered prior to the development of motor impairment (Dowie et al., 2010). However, chronic treatment with URB 597 did preserve CB_1 receptor expression in the striatum, the early loss of which is a hallmark of this disease (Glass et al., 2000; Dowie et al., 2009), indicating that increased levels of anandamide may show some beneficial effects in this model.

The neurotoxic mechanisms during MS and experimental models are varied, with the potential agents of neuronal/axonal damage including; oxidative damage to mitochondria, release of inflammatory cytokines, nitric oxide release from activated macrophages/microglia and excitotoxicity due to excessive glutamate signalling leading to toxic levels of calcium ion influx. There is increasing evidence that elevated levels of glutamate are seen in both MS and EAE particularly during the active stages of disease (Stover et al., 1997; Sulkowski et al., 2009; Marte et al., 2010), accompanied by an increase in the level of expression of Group 1 metabotropic glutamate receptors and excitatory amino acid transporters (Sulkowski et al., 2009). Elevation of glutamate was also observed in the

progressive phase of EAE concomitant with increased levels of neourodegeneration, further implicating glutamate excitotoxicity as a mechanism for neuronal degeration in experimental MS (Marte et al., 2010). Modulation of the effects of elevated CNS glutamate levels has been reported to show disease amelioration in experimental studies (Pitt et al., 2000; Smith et al., 2000; Srinivasan et al., 2005; Bolton and Paul, 2006). Elevated levels of glutamate may result from; the down-regulation of enzymes responsible for the catabolism of glutamate (Hardin-Pouzet et al., 1997), the down-regulation or reversal of the actions of neuronal and astrocytic glutamate transporters (Ohgoh et al., 2002, Loria et al., 2010) or the direct release of glutamate from activated Th17 cells forming direct synapse-like contact with neurons (Siffrin et al., 2010) .

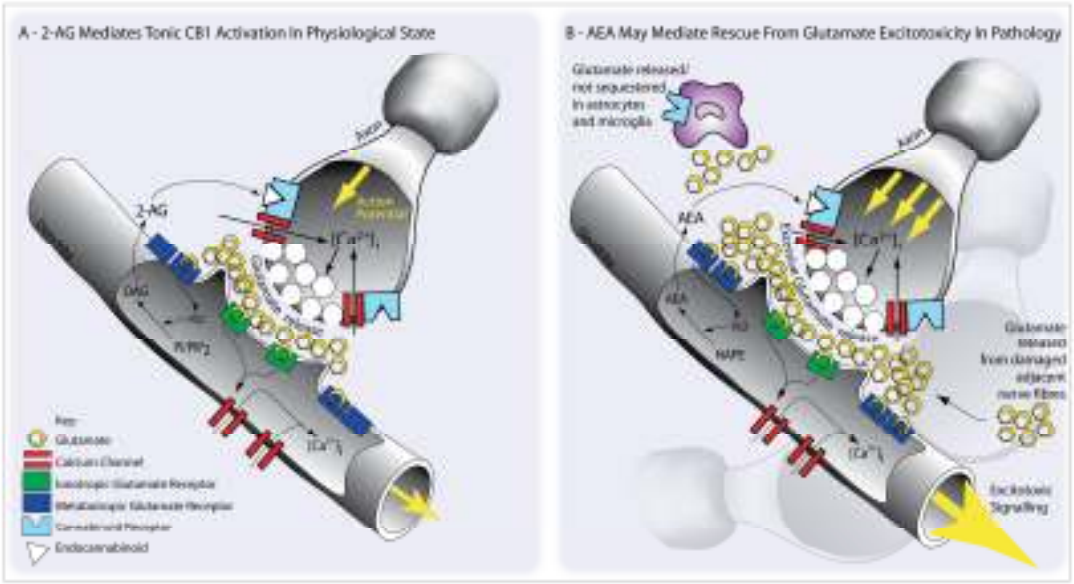
In addition, aberrant expression of sodium channels, which distort the firing patterns of neurons to prolong axonal conduction (Black et al., 2000; Craner et al., 2003), may render axons susceptible to damage from toxic mediators (Ferguson et al., 1997; Kornek et al., 2000; Lu et al. , 2000; Pitt et al., 2000; Smith et al., 2000; Smith et al., 2001; Waxman, 2001; Lo et al., 2002; Kapoor et al, 2003). A sustained increase in the levels of glutamate from glutamatergic nerve terminals produces an increased activation of post-synaptic glutamate receptors of the NMDA, AMPA/kainate subtypes that results in a sustained influx of Ca^{2+} ions into the neuron. Such a sustained calcium influx can activate calcineurin which further activates the apoptosis effector molecule caspase 3 (Polster and Fiskum, 2004) and death via the apoptotic pathway (Ahmed et al., 2002), in addition to Wallerian degeneration (degeneration of axons downstream of the primary insult) and necrosis (Perry and Anthony, 1999). In contrast to stroke and trauma, where these elements are rapid and often catastrophic, these elements accumulate slower and less aggressively in chronic neurodegeneration and thus there is a much greater treatment window for therapeutic modulation (Bjartmar et al., 2003; Dutta and Trapp, 2007). During MS and EAE inflammatory events may rapidly generate a damaging microenvironment (Compston and Coles, 2002; Bjartmar et al., 2003; Dutta and Trapp, 2007). The cannabinoid system can regulate potential degenerative events at multiple levels within the vasculature and CNS including; anti-oxidant activity, inhibition of glutamate release and signalling and in addition the cannabinoid response is negatively coupled with a number of calcium channels (Howlett et al, 2002). Initially, neurodegeneration occurs concomitantly with inflammation (Bjartmar et al., 2003; Dutta and Trapp, 2007) and cannabinoids can control the degree of neurodegeneration that develops as a consequence of immune attack of the CNS (Pryce et al., 2003; Eljaschewitsch et al., 2006; Centonze et al., 2007; Webb et al., 2008; Witting et al., 2006). In addition, in ABH mice that had established chronic EAE, where subsequent relapses were eliminated

by the reinduction of immune tolerance, degenerative signs continued to develop, indicating a continuing slow level of neurodegeneration in the absence of further inflammatory events. This may indicate the continuing presence of a neurodegenerative environment due to a lack of trophic support to neurons or the low-level release of neurotoxic mediators from activated microglial cells in the CNS (Pryce et al., 2005). Whilst not all neuroprotective elements of cannabinoids and the endocannabinoids may be mediated by CB_1 receptors, CB_1 receptors can act at many levels within the death cascade, which will ultimately lead to toxic ion influxes, cell metabolic failure and activation of death effector molecules, such as caspase 3 (Ahmed et al., 2002; Jackson et al., 2005) This would be consistent with the rapid neurodegeneration that accumulates in CB_1 receptor deficient mice after both excitotoxicity and importantly in an experimental model of multiple sclerosis, where CB_1 animals demonstrate a reduced ability to recover from the effects of inflammatory attack in the CNS (Pryce et al., 2003; Jackson et al., 2005). Furthermore, stimulation of cannabinoid receptors using WIN-55,212-2 or THC can induce neuroprotection from such an inflammatory attack, in the absence of overt immunosuppression that blocks relapsing disease (Pryce et al., 2003). Enhanced levels of endocannabinoids in quiescent chronic disease (Baker et al., 2001), not only provide a mechanism for control of excessive neurotransmission, they may also provide a neuroprotective mechanism in response to excessive excitotoxicity (Pryce et al., 2003; Jackson et al., 2005; Eljaschewitsch et al., 2006; Witting et al., 2006; Centonze et al., 2007). Further evidence for an intrinsic neuroprotective endocannabinoid tone, is provided by the observation that UCM707, an inhibitor of anandamide uptake can protect against AMPA-mediated excitotoxicity in neural cultures *in vitro,* which is reversed by blockade of the glial glutamate transporter GLT-1. UCM707 also ameliorated disease in a Theiler's virus model of MS in mice and reversed the downregulation of GLT-1 seen in this model (Loria et al., 2010).

The apparent reduction in endocannabinoid levels during active, immune attack has been attributed to neurodegeneration (Cabranes et al., 2005), although it may also reflect the reduced level of neuronal signalling to the brain from the spinal cord during the paralytic phase of the disease or the suppressive effects of inflammatory cytokine release on endocannabinoid production (Witting et al., 2006). A concomitant reduction in the level of CB_1 receptors and a reduction in the signalling ability of these receptors in motor related brain areas is observed in EAE mice in acute and also the chronic phase of disease where neurodegeneration is observed (Cabranes et al., 2006). Reduced levels of endocannabinoids have also been detected in the CSF of MS patients (Di Filippo et al., 2008), in contrast to the elevated levels of anandamide (but not 2-AG) reported in another study on CSF and plasma levels of endocannabinoids in MS patients (Centonze et al. 2007). The

reasons for this discrepancy is unclear but it may be noted that the study reporting an increased level of anadamide in MS patients also showed much greater levels of anandamide in control patients compared to previous studies in other CNS syndromes (Giuffrida et al., 2004; Sarchielli et al., 2007). Further studies are needed to establish which of these observations is correct. Elevated levels of anandamide in response to immune attack of the CNS may indicate that this is an endogenous neuroprotective response which may limit the damage associated with disease whereas decreased levels of anandamide may reflect a dysregulation in the endocannabinoid response to damaging events which may contribute to enhanced neurodegenration and disease progression particularly in SPMS in these patients. A schematic diagram of how 2-AG and anandamide may operate in both the normal physiological situation in the CNS and also during adverse events in the CNS is illustrated in Figure 1.2.

Figure1.2. *Mechanism of action of 2-Ag and anandamide in normal and pathological events in the CNS.*



This figure is reproduced with the kind permission of Sam Jackson.

The neuroprotective properties of cannabinoids may be operating via a number of mechanisms, such as; inherent anti-oxidant properties and scavenging of reactive oxygen species (Hampson et al., 1998; Marsicano et al., 2002), inhibition of caspase-3 processing (Iuvone et al., 2004), and inhibition of voltage-sensitive calcium channels reducing toxic levels of calcium influx (Shen and Thayer, 1998). CB_1 receptor stimulation with the synthetic cannabinoid WIN55,212-2, induces neuronal sprouting and increases in synaptic density which may be a significant neuroprotective stimulus (Tagliaferro et al., 2006). In EAE, an increased level of

activation of the transcription factor NFκB has been reported and inhibition of this increase attenuated clinical signs (Pahan and Schmid, 2000). The activation potential of the cytokine Interleukin-1, associated with neurodegeneration is blocked by the cannabinoid inhibition of the transactivation potential of NFκB by the synthetic cannabinoid WIN55,212-2 (Curran et al., 2005). Increases in the levels of the endocannabinoid 2-AG has also been reported to be associated with a reduction in NFκB transactivation and a reduction in inflammatory signalling pathways (Panikashvili et al., 2005; Panikashvili et al., 2006), which is abolished in CB_1 receptor knockout mice.

The p38 MAP kinase family of signalling molecules is also implicated in the neuroprotective actions of cannabinoids. The upregulation of extracellular-regulated protein kinases (ERK) by endocannabinoids enhances synaptic integrity and protects against excitotoxicity (Karanian et al., 2005a; Karanian et al., 2005b). Conversely, the downregulation of p38 MAPK by the non-psychoactive cannabinoid cannabidiol, and by Δ-^{9} THC via the CB_1 receptor has been reported to have a neuroprotective effect on retinal ganglion cells in an experimental diabetes model (El-Remessy et al., 2003; El-Remessy et al., 2006) via the inhibition of p38 MAP kinase signalling by proinflammatory cytokines. Δ-^{9} THC has also been reported to downregulate the increased levels of p38 MAPK due to NMDA-mediated apoptosis and reactive oxygen species release in neuronal AF5 cells *in vitro* (Chen et al., 2005). Many of the neuroprotective properties of cannabinoids are likely to include the modulation of the transcription factors involved in the release of proinflammatory mediators during both acute inflammatory episodes and also during states of chronic disease where these mediators may be released at a low level from resident cells in the CNS such as activated microglia. CB_1 and CB_2 receptor agonists block microglial activation and subsequent cognitive impairment due to neuronal loss associated with β-amyloid neurotoxicity in an animal model of Alzheimer's disease (Ramirez et al., 2005), which may have similarities to the low grade inflammation of progressive MS. This reduction in the toxicity of β-amyloid is also seen with cannabidiol, which is associated with a decrease in the activity of p28 MAP kinase and NFκB together with a reduction in nitrosative stress in neuronal PC12 cells *in vitro* (Esposito et al., 2006).

The identity of the endogenous neuroprotective cannabinoid has yet to be definitively resolved and may involve more than one CB_1/CB_2-mediated pathway, possibly dependent on the neural circuitry involved. The reports of the Ca^{2+}-dependent synthesis of anandamide and 2-AG (Di Marzo et al., 1994; Stella et al., 1997), indicates that endocannabinoids are produced in response to a potentially toxic Ca^{2+} influx to provide a feedback inhibition of excitotoxicity. There are

numerous studies showing upregulation of endocannabinoids in neurotoxic models. Some studies have reported beneficial effects of 2-AG in the neuroprotective action of endocannabinoids (Panikashvili et al., 2001; Witting et al, 2006), whereas others have reported beneficial effects of anandamide (Hansen et al., 2001; van der Stelt et al., 2001b), which is reported to be elevated in stroke and active MS (Schabitz et al., 2002; Eljaschewitsch et al., 2006; Centonze et al., 2007). Enhancement of anandamide levels via uptake inhibition or FAAH inhibition, protects against excitotoxic insult both *ex vivo* and *in vitro* (Karanian et al., 2005a,b). Furthermore, as FAAH-deficient mice exhibit an enhanced ability to recover from immune attack, it indicates that upregulation of anandamide levels exhibits a neuroprotective function during EAE (Webb et al., 2008). The function of 2-AG as a potentially neuroprotective agent will be clarified further once specific-inhibitory reagents and gene-deficient mice for the 2-AG synthesis and degradation pathways, such as the enzyme monoacylglycerol lipase, are studied.

Although CB_1 receptors can control some elements of the neurodegenerative process, CB_2 receptors, particularly on microglial cells, provides another target for control of neurodegeneration in an indirect manner via limiting the of release of toxic mediators such as proinflammatory cytokines and reduction in the release of Nitric oxide (Waksman et al., 1999; Klegeris et al., 2003; Carrier et al., 2004; Sheng et al., 2005; Maresz et al., 2005; Esposito et al., 2006; Kim et al., 2006a; Witting et al., 2006).

The capacity of cannabinoids to mediate beneficial effects in multiple sclerosis will be dependent on there being sufficient neural circuitry remaining intact. As cannabinoids can regulate both excitatory and inhibitory neural pathways, (Howlett et al., 2002) the outcome will be dependent also on the density and location of the cannabinoid receptor within the neural circuitry affected. Although many clinical endpoints were not met in symptom control trials (Zajicek et al., 2003), during clinical follow-up, after long-term administration of THC for one year, a number of positive effects were found (Zajicek et al., 2005), including the first report of a significant improvement in spasticity as assessed by the Ashworth scale. This either supports the concept that cannabinoids can slow neurodegenerative events during progressive MS, or that it can promote synaptogenesis, plasticity and repair, as cannabinoid receptor stimulation can promote neuronal sprouting and synapse formation that could promote compensation for the neurological deficit to occur (Galve-Roperh et al., 2006 Kim et al., 2006b; Tagliaferro et al., 2006; Berghuis et al., 2007; Hashimotodani et al., 2007). In addition, cannabinoids may promote repair, as neural progenitors can be stimulated to proliferate in response to

cannabinoid receptor stimulation (Aguado et al., 2007; Galve-Roperh et al., 2007; Molina-Holgado et al., 2007; Rubio-Araiz et al., 2008). The synthetic cannabinoid WIN55,212-2, stimulates adult neurogenesis by inhibiting the suppression of neurogenesis by nitric oxide (Kim et al., 2006b). This neurogenesis is greatly reduced in the brains of CB_1 receptor knockout mice (Jin et al., 2004). Further evidence for the involvement of the cannabinoid system in neurogenesis has been reported, where gene deleted mice for DAG Lipase α and/or β, the enzymes responsible for 2-AG production show a significantly compromised level of neurogenesis in the adult brain (Gao et al., 2010).

In addition, several studies have reported evidence for the influence of cannabinoids on neurotrophin signalling. Brain-derived neurotrophic factor (BDNF), is involved in interneuron migration and morphogenesis and endocannabinoids facilitate this via transactivation of the receptor tyrosine kinase TrkB (Berghuis et al., 2005). This cannabinoid regulation of neurotrophic factors and the importance of BDNF as a neuroprotective agent is evidenced by the absence of upregulation of BDNF due to excitotoxicity in CB_1 knockout animals. This is accompanied by enhanced neuronal loss which is reduced by the administration of exogenous BDNF (Khaspekov et al., 2004), and long-term administration of Δ-^{9}THC, upregulates BDNF production in several brain areas, which may indicate the adaptation of the CNS to cannabinoid exposure (Butovsky et al., 2005).

The neuroprotective effects of endocannabinoids or exogenous cannabinoid agonist administration may include short-term effects such as the inhibition of glutamate release and toxic mediators but also mediate longer term adaptations such as the generation of new neuronal formation and the differentiation of these neurons to compensate for neuronal loss during CNS insult. Currently, a large scale trial investigating the effect of long-term oral THC administration (http://www.pms.ac.uk/cnrg/cupid.php), where the potential neuroprotective benefits of cannabinoid therapy on symptom improvement or stabilisation will be investigated.

1.8 AIMS OF THIS STUDY

In summary, there is now persuasive evidence to suggest that cannabinoids could be useful therapeutic agents in the treatment of a variety of neurological diseases including MS. However the use of cannabis is not without the potential to induce adverse effects. The aim of this project was to use the EAE model to investigate further the potential of cannabinoids in:

1. Symptom (Spasticity) control.
2. Autoimmunity.
3. Progression of disease with respect to prevention of neurodegeneration.

The aim was to avoid CB_1 stimulation in brain centres controlling adverse physiological effects, using cannabinoid related agents and endocannabinoid modulators.

CHAPTER TWO

MATERIALS AND METHODS

2.1. *Animals*

Mice were from in-house bred stock that was maintained in a 12h light/dark cycle with controlled humidity and temperature. Animals were fed RM-1E diet and water *ad libitum*. All animal studies conformed to the United Kingdom Animals (Scientific Procedures) Act 1986.

2.1.1. *Laboratory Mice*

Biozzi ABH mice were from stock bred at the Institute of Neurology, University College London; Queen Mary University of London (QMUL), were purchased from Harlan UK Ltd, Bicester, Oxon UK or were donated by UCB, Cambridge, UK from stock held by Charles Rivers, Margate Kent. These mice were used for most studies.

Crl:CD-1®(ICR) mice originated at Charles Rivers (Crl), USA from caesarian-derived (CD) mice obtained from the Institute for Cancer Research (ICR. Chia et al. 2005). Crl:CD-1® and C57BL/6J were purchased from Charles Rivers, UK or were from stock bred at QMUL. SWR/JOlaHsd, SJL/JOlaHsd; NIH/OlaHsd, Hds:NIHS, Hds:ICR(CD-1®), Hds:NIMR and C57BL/6/OlaHsd were purchased from Harlan (Hds) UK Ltd.

2.1.2. *Transgenic Mice*

These mice were bred at the Institute of Neurology, UCL and QMUL.

2.1.2.1. *CB_1 Cannabinoid Receptor Knockout Mice*

CD-1.*Cnr1*^{tm1Map} mice, which are deficient in the CB_1 receptor (Ledent et al., 1999), were obtained from Catherine Ledent, Brussels, Belgium (Ledent et al., 1999). These were backcrossed onto the ABH mouse background for over 11 generations before intercross to produce congenic ABH.*Cnr1*^{tm1Map} mice [Pryce et al. 2003]. Functional knockout of the gene was demonstrated by the lack of hypothermia and sedation following administration of 20mg/kg WIN-55,212-2 i.p. in dimethylsulphoxide (DMSO), cremophor, and phosphate buffered saline (PBS) (1:1:18). These animals are termed **ABH.*Cnr1*$^{-/-}$** mice. Loss of receptor expression

was confirmed by CB_1-specific immunocytochemistry as performed by the Group of Dr. Vincenco Di Marzo, Naples, Italy (Cristino et al., 2006).

2.1.2.2. *CB_1 Cannabinoid Receptor Conditional Knockout Mice*

C57BL6.Cnr1^{tm1Ltz} transgenic mice were obtained from Dr Beat Lutz and Giovanni Marsicano, Munich, Germany. These express *Cnr1* genes that are flanked by Lox P sites (floxed) and can therefore be selectively excised following crossing with mice expressing Cre recombinase (Marsicano et al., 2002). C57BL6.Cnr1^{tm1Ltz} mice were backcrossed to ABH mice for at least 6 generations and typically 11 generations prior to intercross to produce congenic floxed CB_1 receptor ABH.Cnr1^{tm1Ltz} mice. These are termed **ABH.Cnr$^{f/f}$** mice

To selectively delete CB_1 receptors from nerves, B6.Cg-Tg(*Nes-cre*)1Kln/ mice expressing Cre-recombinase gene under control of the nestin (intermediate filament protein expressed in neural precursor cells, nerves and neuroglia) of the central and peripheral nervous systems) gene were purchased from the Jackson Laboratories. J (Tronche et al., 1999). These were backcrossed with ABH.*Cnr1*$^{-/-}$ for 7 generations. These were screened by Cre recombinase-specific PCR at each generation to produce **ABH.*Cnr1*$^{-/-}$.Tg (*Nes-cre*$^{-/+}$)** mice. These were crossed with ABH.Cnr1$^{f/f}$ to produce ABH.Cnr1^{tm1Map}/^{tm1Ltz} (ABH.*Cnr1*$^{-/f}$) CB_1 receptor heterozygote controls and ABH.Cnr1^{tm1Map}/^{tm1Ltz}.Tg$^{Nes\text{-}cre}$ neural CB_1 conditional deletor (**ABH.Cnr1$^{-/f}$. Tg$^{Nes\text{-}Cre\text{-}/+}$**) mice. These were screened using a Cre recombinase-specific PCR and, for functional loss of cannabinoid receptor from the brain, by the ability of mice to resist the hypothermic and sedative effects of 20mg/kg WIN55,212-2 i.p. It was also possible to assess deleter mice visually as they were notably smaller than non-deleter litter mates (Table 2.1).

Table 2.1 *Conditional Loss of CB_1 receptor from the nervous system.*

	Mean Weight ± SD	
	ABH.Cnr$^{-/f}$	**ABH.Cnr$^{-/f}$. Tg$^{Nes\text{-}Cre\text{-}/+}$**
Male	30.4 ± 2.6g (n=13)	24.4 ± 2.0g (n=8) P<0.001
Female	23.4 ± 1.7g (n=12)	21.3 ± 2.5g (n=9) P<0.05

ABH.Cnr1$^{f/f}$ mice were crossed with ABH.Cnr1$^{-/-}$.Tg$^{Nes\text{-}Cre\text{-}/+}$ mice and 7-8 old offspring were weighed. Mice with CB_1 deleted from the nervous system, exhibited a smaller size than their litter mates. No weight difference was evident between wildtype and global CB_1 receptor knockout mice.

To selectively delete CB_1 receptors from lymphocytes, B6.Cg-Tg(*Lck-cre*)548Jxm/J (Hennet et al., 1995) mice expressing Cre-recombinase gene under control of the thymidine kinase gene promoter were purchased from the Jackson Laboratories. J [Hennet et al. 1995]. These were backcrossed with ABH.*Cnr1*$^{-/-}$ for 7 generations. These were screened by Cre recombinase-specific PCR at each generation to produce **ABH.*Cnr1*$^{-/-}$.Tg (*Lck-cre*$^{-/+}$)** mice. These were crossed with ABH.Cnr1$^{f/f}$ to produce ABH.Cnr1^{tm1Map}/^{tm1Ltz} (ABH.*Cnr1*$^{-/f}$) controls and ABH.Cnr1^{tm1Map}/$^{tm1Ltz.}$Tg$^{Lck\text{-}cre}$ lymphocyte CB_1 conditional deletor (**ABH.Cnr1$^{-/f}$.Tg$^{Lck\text{-}Cre\text{-}/+}$**) mice. These were screened using Cre recombinase-specific PCR.

To selectively delete CB_1 receptor from peripheral nerves, transgenic C57BL/6-Tg *(Prph1-cre*)35Don/mmcd mice expressing Cre-recombinase gene under control of the peripherin 1 (intermediate filament protein of the peripheral nervous system) gene (*Prph1*) promoter (Zhou et al., 2002) were purchased from the mutant mouse regional resource center (UC Davies, CA, USA). These were backcrossed with ABH.*Cnr1*$^{-/-}$ for more than 15 generations. These were screened by Cre recombinase-specific PCR at each generation to produce **ABH.*Cnr1*$^{-/-}$.Tg (*Prph1-cre*$^{-/+}$)** mice. These were crossed with ABH.Cnr1$^{f/f}$ to produce ABH.Cnr1^{tm1Map}/^{tm1Ltz} (ABH.*Cnr1*$^{-/f}$) controls and ABH.Cnr1^{tm1Map}/$^{tm1Ltz.}$Tg$^{Prh1\text{-}cre}$ peripheral nerve CB_1 conditional deletor (**ABH.Cnr1$^{-/f}$. Tg$^{Lck\text{-}Cre\text{-}/+}$**) mice. These were screened using Cre recombinase-specific PCR.

2.1.2.3. *CB_2 Cannabinoid Receptor Knockout Mice*

B6.129P2-*Cnr2*^{tm1Dgen}*/J* homozygous CB_2 receptor knockout mice, which were produced by Deltagen Inc. (Wotherspoon et al., 2005) were purchased from Jackson Laboratories, USA. These mice had been backcrossed onto the C57BL/6 background for more than 5 generations at the time of arrival. These are termed **B6.*Cnr2*$^{-/-}$** mice. These mice were backcrossed, at least 4 generations prior to intercross, with the ABH mice to produce ABH.*Cnr2*^{tm1Dgen}, termed **ABH.*Cnr2*$^{-/-}$** mice. *Cnr2*^{tm1Dgen}*/J* mice lack CB_2 receptor expression. In contrast B6.*Cnr2*^{tm1Zim}, which were obtained from Dr. G. Kunos, NIH, Bethesda, MD, USA, only lack intracellular loop 3, transmembrane domains 6 and 7, and the carboxy terminus due to replacement of the 3', 341 nucleotide base pairs of *Cnr2* with a neomycin resistance cassette, that functionally inactivates CB_2 receptor (Buckley et al., 2000). These mice were bred at the University of Aberdeen by Prof. Ruth Ross.

2.1.2.4. *G-protein Coupled Receptor 55 Knockout Mice.*

B6.129-Gpr55^{tm1Lex}/J homozygous GPR55 receptor knockout mice, termed **B6.129-Gpr55$^{-/-}$** mice, which were produced by Lexicon Inc. were purchased from Jackson Laboratories, USA. (www.jax.org).

2.1.2.5. *Transient Receptor Potential Vanilloid Receptor 1 Knockout Mice.*

C57BLB6.*Trpv1^{tm1Jbd}* mice, which are deficient in transient receptor potential cation channel, subfamily V, member 1 (Vanilloid receptor-1), were obtained from Dr John B Davis, Glaxo Smith Kline, Stevenage, UK. These were backcrossed with ABH mice for 6 generations and screened for the expression of the neomycin resistance gene, prior to intercross to produce ABH.*Trpv1^{tm1Jbd}* mice. These were termed **ABH.*Trpv1$^{-/-}$*** Functional knockout of the gene was demonstrated following the lack of hypothermia and sedation following administration of 0.5mg/kg arvanil (Cayman Biochem, UK) i.v. in alchohol, cremophor, and phosphate buffered saline (1:1:18). Loss of receptor expression was confirmed by TRPV1-specific immunocytochemistry as performed by the Group of Dr. Vincenco Di Marzo, Naples, Italy (Cristino et al., 2006).

2.1.2.6. *Fatty Acid Amide Hydrolase Knockout Mice.*

C57BL/6/129.*Faah^{Tm1Crv}* mice, termed B6.*Faah$^{-/-}$* were obtained from Dr. Benjamin Cravatt, Scripps Institute, La Jolla, CA, USA (Cravatt et al., 2001). These had been backcrossed with C57BL/6 mice 4 generations at the time of arrival. Heterozygous mice were crossed to produce **B6.Faah$^{+/+}$** and **B6.Faah$^{-/-}$** litter mates. In addition, C57BL/6/129.Faah*Tm1Crv* were backcrossed for a least 11 generations prior to intercrossing to produce ABH.Faah^{Tm1Crv} mice termed here. **ABH.*Faah$^{-/-}$***. It has been reported that levels of FAAH can influence fertility and miscarriage (Maccarrone et al., 2002) and due to repeated episodes of poor breeding-performance, such as failure to produce or keep litters, the colony was maintained as male ABH.*Faah$^{-/-}$* x female ABH.*Faah$^{-/+}$* mice and offspring screened before use. Functional deletion of FAAH was demonstrated by hypothermia and sedating effect of 1mg/kg anandamide i.v.

Mass spectroscopic analysis of endocannabinoid: anandamide (AEA) and 2-AG levels using Liquid crystallography mass spectrometry techniques as used previously (Baker et al., 2001) was used to demonstrate an increase in the levels of anandamide In the spinal cord of ABH.*Faah$^{-/-}$* mice as previously reported to occur

(Cravatt et al., 2001). This was performed by Dr. Tiziana Bisogno in the group of Prof. Vincenco Di Marzo, Naples, Italy. The influence of *Faah* gene deletion on CB_1 receptor expression was assessed by ligand binding, *in situ* hybridization receptor signaling potential in the brains of FAAH-deficient mice as described previously (Cabranes et al., 2006). This was performed by Anna Cabranes in the group of Dr. Javier Fernades-Ruiz, Madrid, Spain.

2.1.2.7. *P-Glycoprotein Knockout Mice.*

Wildtype FVB and congenic FVB.*Abcb1a*^{Tm1Bor} /*Abcb1b*^{Tm1Bor} double transgenic (termed **FVB.*Abcb1a/Abcb1b*$^{-/-}$**) p-glycoprotein deficient mice originating from Taconic Farms Inc. Germantown, NY, USA were from stock bred at the Kings College London (Yau et al., 2007). These mice were provided by Dr Sarah A Thomas and Dr. Carmine M. Pariante, Kings College London and injections into these mice were performed at Kings College London by Brittany Mason.

2.2. *Genotyping of Animals*

2.2.1. *Production of Crude DNA*.

Initial tail tips and more latterly ear biopsies were removed from weaned mice, in some instances under local anaesthetic. DNA samples were prepared following digestion overnight at 60°C in 500μl 0.2μg/ml Proteinase K (Invitrogen, Paisley, UK) in Nucleon™ Reagent B lysis buffer pH8 (400mM Tris/HCl 400, 60mM EDTA 60, NaCl 150mM SDS 1%). 150μl 5M sodium perchlorate was added followed by a further 30minute incubation at 60°C . Equal volumes of chloroform were added, the sample vortexed, centrifuged for 4 minutes at 1400rpm in an Eppendorf microfuge. The aqueous phase was added to 2 volumes of cold ethanol to precipitate the DNA, which was then dissolved in water. In later experiments, DNA was isolated from ear biopsies using a Qiagen DNeasy extraction kit (Qiagen, Crawley, UK) using the protocol provided by the manufacturer.

2.2.2 *Polymerase Chain reaction (PCR).*

DNA from tail or ear biopsies were screened by PCR (Cycles 30-35; 94ºC 60 s, annealing temp 50, 55 0r 60º C, 72º C 60s) using Qiagen PCR core kit reagents (Qiagen, Crawley, UK).

Per sample reaction mixture;
Premix
Qiagen buffer (10x) 2.5 µl.
Qiagen dNTPs (10mM) 0.5µl.
Qiagen $MgCl_2$ (25 mM) 0.5µl.
H_2O 1.5µl.

For each PCR reaction;
Premix 5µl
Primer A (forward) 0.5µl.
Primer B (reverse) 0.5µl.
Q buffer(Qiagen) 5µl.
H_2O 8.7µl.
DNA sample from Dneasy extraction 5µl.
Qiagen Taq DNA polymerase 0.3µl.
Samples were run using a Hybaid Omigene thermal cycler (Hybaid, Cambridge, UK).
PCR products were analysed by 2% Agarose (Sigma, Poole, UK) in 1x Tris/Borate/EDTA (TBE) buffer (Sgma, Poole, UK) gel electrophoresis (120 volts) for 60-90 minutes.

Table 2.2 *Primer Sequences used for Screening Transgenic Mice.*

Target	Primer Sequence	Annealing Temp	Band size
Wildtype Cnr1	5'-CATCATCACAGATTTCTATGTAC-3' 5′-AGGTGCCAGGAGGGAACC-3';	55°C	366bp
Cnr1-deletion	5'-GATCCAGAACATCAGGTAGG-3' 5' AAGGAAGGGTGAGAACAGAG-3'	55°C	521bp
Wildtype& floxed-Cnr1	5'-GCTGTCTCTGGTCCTCTTAAA-3' 5'-GGTGTCACCTCTGAAAACAGA-3'	55°C	WT 210bp Transgene 255bp
CreRecombinase	5'-ACCAGCCAGCTATCAACTC-3' 5'-TATACGCGTGCTAGCGAAGATCTCCATCTTCCAGCAG-3'	55°C	300 bp
CB_2 wildtype	Forward 5′-GGGGATCGATCCGTCCTGTAAGTCT-3′	60ºC	350bp
	Reverse1 5′-GGAGTTCAACCCCATGAAGGAGTAC-3′		
CB_2 knockout allele	Reverse2 5′-GACTAGAGCTTTGTAAGGTAGGCGGG-3′		500bp
FAAH	Forward 5′-TAACTAGGCAGTCTGACTCTAG-3′		
	Reverse1 5′-ACTCAAGGTCAGCCTGAAACC-3′	50ºC	WT 200bp
	Reverse2 5′-TTTGTCACGTCCTGCACGACG-3′		Transgene 320bp
GPR55	Forward 5′-TCTGGATTCATCGACTGTGG-3′	55ºC	
	Reverse1 5′-CTCCACAATCAAGCTGGTCA-3′		WT 207bp
	Reverse2 5′-GTCACCCATCCAGGTGATGT-3′		Transgene 299bp
LacZ	5′-GAATCTCTATCGTGCGGTGGTTGA-3′	55°C	522 bp
	5′-GGATCGACAGATTTGATCCAGCGA-3′		

2.2.3. *P-glycoprotein and CNS exclusion pump activity.*

To determine whether mice exhibited wildtype brain-derived p-glycoprotein (*Abcb1a*^{mdr} multidrug resistance (mdr) pump) or harboured the 0.52kb viral insert at the intron22, exon23 boundary associated with the *Abcb1a*^{mds} multidrug susceptible (mds) variant (Jun et al., 2000; Pippert & Umbenhauer, 20010, DNA was subject to PCR. Negative control (C57BL/6) and positive control (p-glycoprotein-deficient mutant CF-1*Abcb1a*^{mds} *mice*) DNA was supplied from Charles Rivers, USA. Primers: 5'-ACAAGGTCAACCATGAGTCC-3' and 5'-AGTTGTTGTGTTCACCAAGTAG-3' in intron 22 and exon 23 of *Abcb1a*, respectively were designed to produce a PCR products of 883bp (wildtype allele) and about 1400bp for the mutant allele. Intron 22 and reverse primers 5'ctgttcatccgaatcgtgg3' within the long terminal repeat of the mouse leukaemia virus produced an 848bp product in mutant allele [Jun et al. 2000]. DNA was subject to polymerase chain reaction: 94°C 5min and 35 cycles 94°C 1min, 56° 1min, 72°C 5min and products detected by agarose electrophoresis. This was undertaken by Prof. Alison Hardcastle, Institute of Ophthalmology, UCL, London, UK.

2.2.4. *Ribonucleic Acid (RNA) extraction and Microarray.*

100 mg of cerebral cortex tissue from CT3 responder and non-responder CD1 mice was collected in 10 x volume RNAl*ater* (Qiagen, Crawley, UK) and stored at -70°C prior to homogenisation. This tissue was collected at least 2-3 weeks following phenotyping. Tissue was homogenised with a sterile autoclaved pestle and mortar in 1 ml of Trizol reagent (Invitrogen, Paisley, UK) under liquid nitrogen. The resulting Trizol plus tissue powder was transferred to a sterile 2 ml safelock microcentrifuge tube and stored at -70°C. For phase separation, homogenised samples were incubated at room temperature to allow the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml of Trizol sample, mixed by vortex mixer and incubated at room temperature for 3 mins. Samples were centrifuged at 12,000 x g for 15 mins in a bench-top centrifuge (Eppendorf, Cambridge, UK). The upper aqueous phase was collected and transferred to a new Eppendorf tube and an equal volume of 70% ethanol was added and mixed thoroughly with the sample. 700 µl of the sample was applied to the membrane of an RNeasy spin column (Qiagen, Crawley, UK), placed in a collection tube supplied by the manufacturer and centrifuged for 15s at 8000 x g. Flow-through was discarded and a second 700µl aliquot was added centrifugation repeated and flow-through discarded. After three washing steps, according to the manufacturer's protocol, the spin column was transferred to a new collection tube (supplied) and RNA was eluted from the spin column by the addition of 10µl of RNAse-free water

to the membrane and centrifugation at 8000 x g for 1 min. This elution step was repeated by the addition of a further 10µl of RNAse-free water and centrifugation. Samples were stored in safelock microcentrifuge tubes at -70°C prior to analysis by the Genome Centre, QMUL, London, UK. 1µg of RNA was subject to quality control and microarray analysis using Illumina® MouseRef-8 v2.0 Expression BeadChips (Illumina, Cambridge, UK), which detects approximately 25,600 well-annotated RefSeq transcripts and enables the interrogation of eight samples in parallel. GenomeStudio Data was analysed using GenomeStudio software (Illumina, Cambridge, UK). This was performed by Lia De Faveri and Charles Mein at the Genome Centre, QMUL, London, UK.

2.3 *Chemicals*

2.3.1. *Vehicles*

The cannabinoid compounds are very hydrophobic and they can create a problem with regard to the choice of vehicles. This has changed over the time of the project. Initially [Baker et al. 2000], this involved dissolving compounds in ethanol containing Tween 80 (Sigma Poole, UK) and using a rotary, vacuum evaporator over 2h the ethanol was removed and the compounds were then resuspended in PBS. Cannabinoid compounds tended to form cloudy solutions. Intralipid® 30% (Pharmacia, Milton Keynes, UK), used for i.v. formulation in humans was used as a vehicle for some experiments, but was subsequently found to slow movement of animals when assessed by open-field activity monitoring. The typical vehicle contains ethanol cremophor and PBS (ECP) in a 1:1:18 ration. The compound is dissolved in ethanol followed by the addition of cremophor (Sigma, Poole, UK) and PBS.. R(+)WIN55,212-2 is more soluble in DMSO than in ethanol and was used with cremophor and PBS (1:1:18) vehicle (DCP) mixture.

2.3.1. *CB_1-targeted Cannabinoid Receptor Reagents.*

The full CB_1/CB_2 receptor agonists *R*(+)WIN-55,212-2 (*R*(+)WIN-55) and its inactive enantiomer *S*(-)WIN-55,212-3 were purchased and CP55,940 were purchased from RBI/Sigma (Poole, UK) or Tocris Ltd (Bristol, UK). Δ^9Tetrahydrocannabinol (THC) was provided by National Institute for Drug Abuse (NIDA) Supply Program. In addition, the non-CB_1 receptor-binding compound cannabidiol (CBD) was generously provided by Dr. Malfait and Prof. Marc Feldmann, Imperial College London and Dr. Ruth Gallily, Hebrew University, Jersusalem, Israel or was purchased from Sigma (Poole, UK). The non CB_1/CB_2 selective agonist; RWJ352303 (Ki CB_1R =0.6nM Ki CB_2R=0.3nM. Forskolin-stimulated cAMP agonism in SKN cells

IC_{50} CB_1R =0.64nM, IC_{50} CB_2R = 0.14nM (Dr. D. Argentieri and Dr. D Ritchie, unpublished observations) was supplied by RW Johnson (Raritan, NJ, USA). These were dissolved in ECP or DCP prior to intraperitoneal (i.p.) or intravenous (i.v.) injection with typically 0.1ml. SR141617A (rimonabant), a CB_1 receptor antagonist was supplied by the NIDA drug-supply program.

2.3.2. *CB_2-selective Cannabinoid Receptor Reagents.*

The CB_2 selective agonist; JWH056 (Receptor Affinity. Ki CB_1=8770nM, K_i CB_2=32nM), was provided by Dr. J. Huffman, Clemson University, USA (Huffman et al.,1996). The CB_2 selective agonist; RWJ400065 (Binding Affinity. Ki CB_1R=600nM, K_i CB_2R =10nM. Forskolin-stimulated cAMP agonism IC_{50} CB_1R= 6600nM, IC_{50} CB_2R =6.6nM (Dr. D. Argentieri and Dr. D Ritchie, unpublished observations) compounds were provided R.W. Johnson (Raritan, NJ, USA). These were suspended in intralipid® 30% (Pharmacia, Milton Keynes, UK) prior to i.v. or i.p. injection in 0.1ml. In addition JWH133 (Ki CB_1 = 600nM, Ki CB_2 = 3.4nM) was purchased from Tocris, (Bristol,UK), and dissolved in ECP.

2.3.2. *CNS Excluded CB_1-Receptor Agonists.*

2-(2-hydroxy-ethylcarbamoyoxymethyl)-5,7-dimethyl-3-(2-methylsulphamoyl-phenyl)-4-oxo-3,4-dihydro-quinazoline-6-carboxylic acid ethyl (Adam-Worrall et al., 2007) termed here SAD488 was synthesized by Dr. Cristina Visintin, Wolfson Institute, UCL as described previously (Brain et al., 2003; Adam-Worrall et al., 2007) or was supplied by Novartis (Basel, Switzerland). 1',1'-dimethylheptyl-Δ^8-tetrahydrocannabinol-11-oic acid (CT3/ajulemic acid) (Rhee et al., 1997, Burstein et al., 2004) was supplied by Atlantic Ventures Inc, New York, USA. Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl) methanone (CRA13. Dziadulewicz et al., 2007; Gardin et al., 2009), termed here SAB378 and SAB722 a CNS-penetrant CB_1 agonist with IC_{50} CB_1R = 11nM and Cyclosporin A (CsA) were supplied by Novartis. The compounds were dissolved in ECP and were injected intravenously via a tail vein using a 30g needle, intraperioneally (i.p.), or were administered by oral gavage in a volume of 0.1ml.

2.3.3. *Endocannabinoid Degradation Inhibitors.*

1-(Oxazolo[4,5-b]pyridin-2-yl)-1-oxo-9(Z)-octadecene (Compound 29. Boger et al. 2003) termed here CAY10400 and 1-(Oxazolo[4,5-b]pyridin-2-yl)-1-oxo-6-phenylhexane (Compound 53 Boger et al. 2000) termed here CAY10402 were

supplied by Novartis Ag, Basel, Switzerland, or were purchased from Cayman Chemicals, UK. AM404 was purchased from Tocris Ltd (Bristol, UK). These were reconstituted (20mg/ml) in warmed ethanol prior to dilution in Intralipid™ vehicle (Intralipid 30%. Pharmacia, Milton Keynes, UK), prior to the injection intravenously of 0.1ml into the tail vein. The MAG Lipase inhibitor JZL184 was a generous gift of the Skaggs Institute, Scripps Research Institute, La Jolla, USA. This was reconstituted in Ethanol;Cremophor:PBS (1:1:18) and injected into the tail vein of spastic animals in a volume of 0.1 ml, 5 mg/kg.

2.3.4. *Inhibitors of CNS efflux Pumps.*

Cyclosporin A (CsA) was supplied by Novartis (Basel, Switzerland) this was injected i.v. at 50mg/kg in ECP (Hendrikse et al. 1998). Mitoxantrone (MX) was supplied by Lederle (Cyanamid, Gosport, UK) and 5mg/kg was injected i.p. (Baker et al., 1992). 3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)-(3-dimethyl-amino-3-oxopropyl)-thio)-methyl)thio)propanoicacid (MK571) was purchased from Merck chemicals Nottingham, UK. These compounds were dissolved in PBS before use. Compounds were administered 30 minutes prior to the subsequent administration of cannabinoids.

2.3.5. *Non-Cannabinoid Receptor Reagents.*

3-(5-Cyano-pent-1-enyl)-*N*-(2-hydroxy-1-methyl-ethyl)-benzamide(VSN15), 3-(5-dimethylcarbamoyl-pent-1-enyl)-N-(2-hydroxy-1-methyl-ethyl)benzamide (VSN16) and the VSN16S and VSN16R enantiomers were synthesised as described previously [Hoi et al., 2007]. VSN16 was sometimes dissolved in saline or water for intravenous or oral administration respectively. These were injected intravenously via a tail vein using a 30g needle or were administered by oral gavage in a volume of 0.1ml. Baclofen was purchased from RBI/Sigma (Poole, UK) and was dissolved in PBS prior to i.v. injection. Arvanil, a potent Trpv1 agonist, was purchased from Cayman Biochem, USA.

2.4 *Receptor Binding Assays.*

The compounds were tested in the the rat and mouse vas deferens in the Laboratories of Roger Pertwee and Ruth Ross, University of Aberdeen as described previously (Pertwee et al., 1992). Additional studies were performed by contract research organisations (CRO) on cell lines transfected with human receptors. These were performed by; CEREP, Poitiers, France; Euroscreen, Brussels, Belgium;

Multispan Inc, Hayward, California, USA; ChanTest, Rockville, Maryland, USA and also MDS Pharma services, Taipei, Taiwan, where it was also shown that VSN16R failed to reveal any evidence of cell cytotoxicity or mutagenicity at 30mg/ml (approximately 10mM) in the Ames Test (Maron and Ames, 1983), using the TA98, TA100, TA102 and TA1537 strains of *Salmonella typhimurium.*

2.5 *Pharmacokinetics.*

The stability to hepatic and plasma degradation of VSN16 was assessed *in vitro* by Inpharmatica, Cambridge, UK. Compounds (1μM) were incubated with either pooled mouse microsomes (0.1mg protein/ml) or pooled mouse plasma at 37^{o}C for 0, 5, 10, 20 and 40 minutes before termination with acetonitrile containing warfarin as analytical internal standard. Samples were centrifuged and the resultant supernatant analysed for parent compound. The mass responses at baseline were taken as the 100% reference values against which compound disappearance was measured. The natural log of the percentage remaining values was used to generate linear plots of disappearance of the compounds. Half-life values were calculated from the slope of these plots.

The stability of VSN16R was assessed *in vivo* by Inpharmatica. Blood (plasma) samples were obtained prior to and 5min-8hours after drug administration of 2-5mg/kg i.v. or 5mg/kg p.o. VSN16R into outbred mice and rats (n=3 per time-point). Immediately after blood collection the brain and spinal cord were removed and then stored at -20ºC prior to assay. Tissue samples were weighed, homogenized and centrifuged and the lysates generated. Brain lysates and plasma were assayed by liquid chromatography-mass spectrometric assay methods by Inpharmatica, Cambridge, UK. The detection limit was 2ng/ml in plasma and brain. Pharmacokinetic parameters were assessed using PK solutions 2.0 software (Summit Research Services, Montrose CO, USA) by Inpharmatica, Cambridge, UK

2.6. *Induction of Experimental Allergic Encephalomyelitis.*

2.6.1. *Preparation of Spinal Cord Homogenate.*

20-50 animals, typically ex-breeders were killed by CO_2 overdose. The head was removed using scissors and the spinal column was severed at the level of the pelvis, whilst holding the mouse. Once the cut is made the hindlimbs drop when the column is severed any blood will drain and the spinal cord becomes visible. A 20g needle attached to 20 ml syringe filled with distilled water was inserted into the spinal column. Tin foil was placed under the neck end of mouse to collect the cord.

The spinal cord was expelled from the cervical end by hydrostatic pressure. The pooled spinal cords were homogenised in a glass hand homogeniser. Parafilm M (Sigma, Poole, UK) was used to seal the aperture of the glass homogeniser and multiple holes were made in the parafilm with a 25g needle, to allow the water to escape when freeze dried. Homogenate was frozen in -70°C freezer overnight and placed in a Freeze dryer (Edwards, Crawley, UK and freeze dried for 24-48hour. The dried spinal cord homogenate from the homogenizer was removed, placed on foil and diced to a fine dust with a single edged razor blade to make a fine powder and stored in 7 ml Bijoux containers (Sterilin, Caerphilly, UK) at -20°C.

2.6.2 *Preparation of Inoculum for Spinal Cord-Induced Disease ABH Mice.*

20ml syringes (Becton Dickinson, Oxford, UK) were to make up the solution (i.e. multiples of 20ml). Firstly a stock solution was prepared (stock A), consisting of 4ml incomplete Freund Adjuvant (Difco, Becton Dickinson, Oxford, UK),16mg *Mycobacterium tuberculosis* H37Ra and 2mg *M butyicum* (Difco, Becton Dickinson, Oxford, UK), in a 5ml Bijou (Sterilin, Caerphilly, UK). This was kept for no longer than 1 month at 4°C. Stock Mycobacteria were stored at -70°C. Once a vial was opened it was stored in fridge/freezer. If the incidence of EAE dropped to about 50% it was usually that the *M tuberculosis* had lost its potency and needed replacing. Complete adjuvant: Freund's adjuvant was prepared by adding 11.5ml adjuvant incomplete Freund's adjuvant to 1ml stock A that was vortex-mixed before use.

The plunger from a 20ml syringe was removed and the barrel was plugged with a stopper cap (Scientific Laboratory Supplies, Nottingham, UK). 5ml sterile PBS was added and 33mg of freeze dried spinal cord homogenate (6.6mg/ml). This was mixed and then 5ml of Complete Freund's adjuvant was added (see above). The syringe was sealed with Parafilm and vortexed. A retort stand, boss and clamp was used to hold the 20ml syringe in place with the water level reaching the level of the adjuvant (containing a drop of detergent) in a waterbath sonicator (Bransonic Ultrasonicator, Sigma, UK) and sonicated for 10 min to thicken the mixture and dissociate the spinal cord homogenate. The adjuvant was vortexed and placed on ice to cool. A 1ml syringe (Becton Dickinson, Oxford, UK) was inserted into the 20ml syringe and the adjuvant was pumped using the 1ml syringe until it had thickened sufficiently that the solution did not disperse when a drop was added to water. The plunger was inserted into the 20ml syringe and the syringe was tapped on the bench such that the content moved towards the plunger and then the syringe cap was removed. A long (6cm) large bore needle was fixed to the syringe and insered into 1ml syringes with plungers pulled out to the 1ml mark. The

syringe was filled to 1ml and the barrel of the 1ml syringe was wiped with tissue paper to remove any excess adjuvant. A 16mm 25g needle (Becton Dickinson, Oxford, UK) was fixed to the 1 ml syringe. With the tip of the needle cover on the bench, the syringe was pushed very firmly onto the needle.

2.6.3 *Preparation of Inoculum for MOG-Induced Disease in C57BL/6 Mice.*

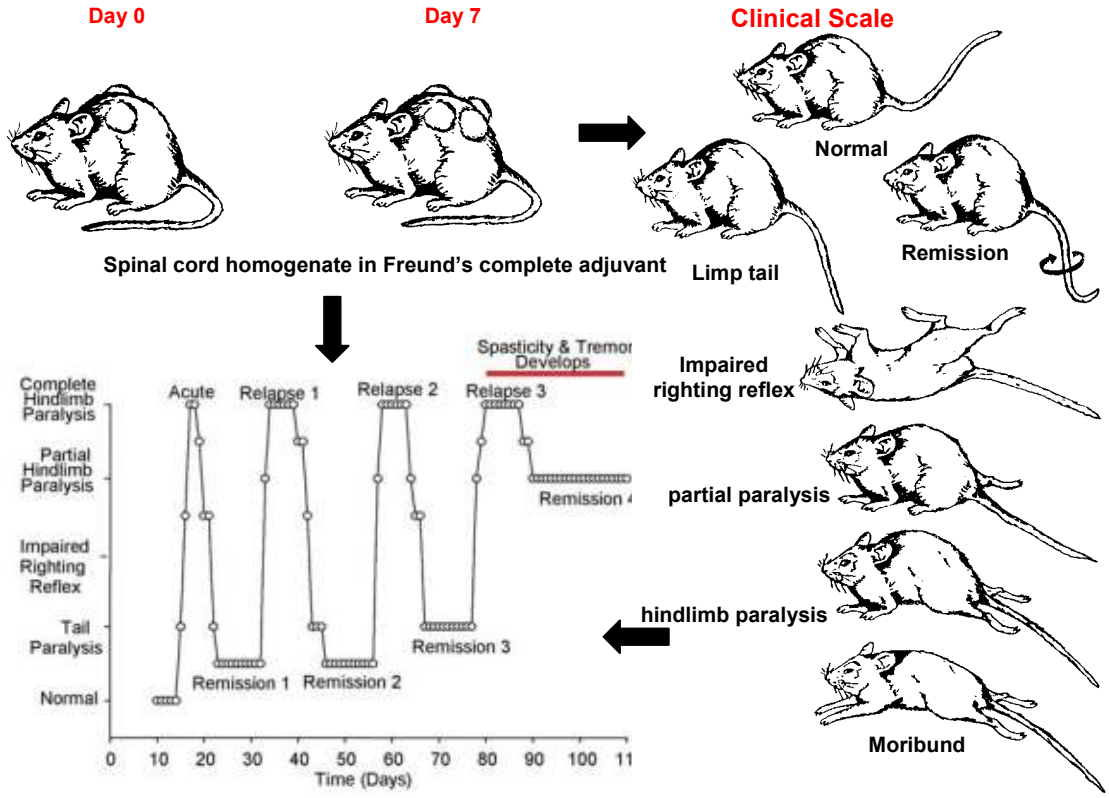
The procedure was followed as above in 2.5.2 except that the spinal cord homogenate is replaced with a synthetic peptide amide corresponding to the 35-55 amino acid residues of mouse myelin oligodendrocyte glycoprotein MOG^{35-55} made as a peptide amide.

2.6.4. *Injection of Animals.*

Disease was typically induced in 6-8 week male and or female mice. Mice were held at the nape of the neck between thumb and forefinger. The tail was held with the right hand with thumb and forefinger (tips facing the head) and the mouse was placed on the top of a wire mouse cage. The skin of the dorsal surface of the flank was lifted with thumb and forefinger (left hand) and the needle was inserted (facing towards the head) subcutaneously into the mouse. 0.15ml of adjuvant was injected into the right flank and another 0.15ml was injected into the left flank. This was day 0. The procedure was repeated one week later (day 7). Injections were below, more posterior to the original injections. EAE ABH disease developed at around day 14-15 (Baker et al. 1990, Amor et al. 1994). A relapse could be induced about 7-8 days after a further injection of neuroantigen in Freund's complete or incomplete adjuvant (O'Neill et al. 1991). ABH mice did not require the injection of *B pertussis* toxin (Sigma, Poole, UK), however, MOG-induced disease in C57BL/6 mice typically required the co-administration of 0.1ml of 200ng *B. pertussis* toxin in PBS on day 0 and day 7.

2.6.5 Clinical Disease Scoring.

Figure 2.1. *Induction and Assessment of Chronic Relapsing Experimental Allergic Encephalomyelitis.*



Animals were weighed and scored daily from day 10 onwards. At approximately day 13, mice lost more than 1.5g overnight. Weight loss continued for a few days. On about day 15, clinical signs start (Figure 2.3). This was ascending paralysis that started with the tail. This was scored as follows:

Normal = 0

Fully flaccid tail = 1. Tail is completely paralysed. Tail does not lift but has some tone. E.g. Tail can bend round finger. Lift the mouse by the scruff of the neck and the tail will helicopter = (1) = 0.5. This is the typical score of remission 1.

Impaired righting reflex. = 2. When turned on back, the animal does not right itself. If it rights itself slowly it received a score of (2) = 1.5.

Hindlimb paresis = 3. Significant loss of motor function of the hindlimbs. Hindlimb gait disturbance = (3) = 2.5. This is typical score of remission 2-3.

Complete hindlimb paralysis = 4. Both hind limbs drag. Limbs virtually paralysed but have some minor movement or one leg fully paralysed = 3.5= (4).

Moribund/Death = 5. If forelimbs became paralysed, the animal was euthanised. We have set a weight loss limit of about 35% from the day 10 weight. However, animals that will die lose the ability to thermoregulate and appear cold to the touch.

Relapse = Increase of Disease Score, usually accompanied with weight loss

The data are presented as the mean daily clinical score ± standard error of the mean (SEM) or the mean maximal clinical score of the group (Group Score) ± SEM; the mean maximal clinical score of the animals that developed clinical disease (EAE Score) ± SEM and the mean day of onset ± standard deviation (SD). Differences between groups were assessed using non-parametric, Mann Whitney U statistics using Minitab Software (O'Neill et al. 1992, Pryce et al. 2003a).

2.7. *Behavioral Testing.*

2.7.1. *Open-Field Activity Monitoring.*

Motor activity was assessed in a 27.9cm x 27.9cm open field activity monitor chambers and computer software (Med Associates Inc, St. Albans, VT, USA.) and typically performed in a darkened room. Recording were initiated once the mouse entered the chamber and continued for 5 minutes. These chambers, allowing 4 simultaneous recordings of individual mice, were fitted with infrared beams that could detect movement in the X, Y planes. The total distance travelled (cm) was recorded.

2.7.2. *Temperature Measurement.*

Temperature was monitored by using a K-type single input thermocouple thermometer (Portec, Wrestlingworth, UK), or (ATP Instrumentation, Ashby-de-la-Zouch, UK) , placed under the hindlimb in the inguinal region. The temperature was allowed to equilibrate for 30-60s and was recorded once the temperature failed to increase further(Brooks et al. 2002).

2.7.3. *Rotorod Activity Monitoring.*

Motor control and coordination was assessed on an accelerating (4 – 40 rpm. 12rpm/50s) RotaRod treadmill (ENV-575M. Med Associates Inc, St. Albans, VT, USA), during the remission phases of the disease, over a maximum 5 minute observation period. The trial was terminated when the mouse either fell from the

RotaRod spindle or if the mouse failed to tolerate the revolving drum shown by holding onto the RotaRod spindle rod for two consecutive turns.

2.7.4. *Gut Motility.*

Gut motility was assessed by counting and weighing the number of faecal pellets passed in 2 hours by mice into clean cages (Fride et al., 2004). Differences between groups were assessed using t tests using Sigmastat software (Aspire Software International, Ashburn, VA, USA).

2.7.5. *Bladder Volume.*

Bladder volumes were measured using a high resolution portable digital ultrasound system (Sonosite® MicoMaxx®, BCF Innovative Imaging, Livingston, Scotland, UK) with a 13-6 MHz 26 mm linear array transducer. The ultrasound system acquires a high resolution 2D image from which volumetric calculations were made. For bladder measurements, the abdomens were shaved using electric clippers (Andis D-4D Combo, Sandown Scientific Ltd, Hampton, Middlesex, UK) and an ultrasound gel (Alpha tube ultrasound scanning gel, BCF Innovative Imaging, UK) was applied. The animal was gently restrained, and the transducer was first placed longitudinally against the animal to capture the maximum bladder length and depth. Next; the transducer was rotated 90º in order to capture the maximum width of the bladder. The volume of bladder urine was automatically calculated by the ultrasound imaging software (Al-Izki et al., 2009).

2.7.6. *Spasticity Measurement.*

Following EAE induction and the development of chronic relapsing EAE, spasticity typically developed after 2-3 relapses, about 80-100 days post-induction (Baker et al., 2000). This was assessed during remission from active paralytic episodes by the force required to bend the hind limb to full flexion against a strain gauge (Baker et al., 2000). The limb was extended two-three times and then the limb was gently pressed against a strain gauge to full flexion. The measurement of left then right hindlimbs was repeated typically 5 times per time point. Analogue signals were amplified and then digitized and captured using either: a PCMDAS16S/12 PCMICA card (ComputerBoards Inc, Middleboro, MA, USA) and Dacquire V10 software (D. Buckwell, Institute of Neurology, UCL) on a Windows™ 98 platform or a DAQcard 1200 PCMICA card (National Instruments Austin, TX, USA) and Acquire V1 software (D. Buckwell, Insititute of Neurology, UCL) on the Windows™ XP platform. The data

were analyzed using Spike 2 software (Cambridge Electronic Design, UK) and a mean score for each limb at each time point was calculated and forces were converted to Newtons. Each group contained a minimum of 5 different animals and the results represent the mean ± SEM resistance to flexion force (N) or individual limbs, which were compared using repeated measures analysis of variance or paired t tests using SigmaStat software (Baker et al., 2001).

2.8. *Assessment of Immune Function in EAE.*

Lymph node cells from cannabinoid-treated C57BL/6 mice immunized with MOG^{35-55} in Freund's adjuvant without *B.pertussis* toxin as co-adjuvant were cultured with MOG^{35-55} peptide (Croxford & Miller, 2003; Fuller et al., 2004). Cytokine production was assessed using cytokine bead array analysis 48h after antigen pulsing and proliferation was assessed using ^{3}H-thymidine incorporation as described previously (Croxford & Miller, 2003). These studies were all performed by Dr J. Ludovic Croxford, Tokyo, Japan.

2.9. *Assessment of Neuroprotection in EAE.*

Axonal content was assessed using neurofilament-specific ELISA (Pryce et al., 2003a). Whole spinal cords were homogenized in 500 µl of barbitone buffer [11 mM barbital, 63 mM sodium barbital, 1.2 mM EDTA (Sigma)] containing a protease inhibitor cocktail and 4 mM EGTA in a glass homogeniser. Lipids were extracted from the sample by adding di-isopropyl-ether (Sigma) at 1: 5000 and centrifuging for 5 min at 20,000x*g*. The supernatant was frozen and stored in aliquots at –70°C, and the total protein was measured using the standard Lowry method. Ninety-six well microtiter plates (Maxisorp; Nunc, Rochester, NY, USA) were coated overnight at 4°C with the monoclonal antibody against neurofilament heavy chain (SMI35; Sternberger Monoclonals Inc., Lutherville, MD, USA) diluted in 0.05 M sodium carbonate (pH 9.6). This was washed (barbitone buffer containing 5 mM EDTA, 1% bovine serum albumin and 0.05% Tween-20 (Sigma, Poole, UK) and non-specific protein binding was blocked by incubation with 1% bovine serum albumin in barbitone buffer for 1 h at room temperature. Spinal cord homogenates were serially diluted to 1:10 000 in barbitone buffer containing 5 mM EDTA (Sigma, Poole, UK), and incubated at room temperature for 2 h. After washing, a rabbit polyclonal anti-neurofilament H antibody (N-4142; Sigma, Poole, UK), diluted 1:1000, was incubated at room temperature for 1 h. Following another wash, horseradish peroxidase-conjugated anti-rabbit immunoglobulin diluted 1:1000 was incubated for 1 h at room temperature. The tetramethylbenzidine (TMB) chromogenic reagent system (R & D Systems, Abingdon, UK) was used to detect

protein levels in the samples. Signal development was stopped using 1 M Phosphoric acid, and the plate was read at 450 nm, with a reference reading at 620 nm on a Synergy HT ELISA plate reader (BioTek, Vermont, USA). The antigen concentration for each sample was calculated from an internal standard curve ranging from 0 to 250 ng/ml (high-performance liquid chromatography-purified bovine neurofilament H; Affiniti Bioreagents, Golden, Colorado, USA). All samples were analysed in duplicate. This was performed by Samuel Jackson and Sarah Al-Izki .

2.10 *Immunopathology*

2.10.1. *Tissue Sections.*

Post-mortem central nervous system tissues from donors with multiple sclerosis were collected and classified by Prof. Paul Van der Valk and Dr Sandra Amor (Free University of Amsterdam, The Netherlands). The lesions were from patients with secondary progressive MS. Tissue was fixed in 10% formol saline and embedded in paraffin wax. All patients and controls, or their next of kin, had given informed consent for autopsy and use of their brain tissue for research purposes. This tissue was ethically obtained and used in accordance with law from The Netherlands and the UK. Spinal cord tissue was dissected from the spinal column of normal mice and mice with acute EAE phase paralysis (day 17 p.i.), first remission (day 27 p.i.), first relapse (day 37 p.i.), second remission (day 60 p.i.) and spastic chronic EAE (day 120 p.i.). This was fixed in 10% formol saline and embedded in paraffin wax.

2.10.2 *Immunocytochemistry*

For immunohistochemical stainings, 5 µm cryosections were cut, de-waxed and hydrated. Sections were incubated with (a) C219 (GeneTex, Irvine, CA, USA) mouse IgG2a (which is not made by mice such as ABH mice that express the *Igh1*^{b} immunoglobulin allotype) monoclonal antibody, which reacts with human, rat and mouse ABCB1 (b) 6D170 rat IgG monoclonal antibody (Europa Bioproducts, Cambridge, UK), which reacts with mouse and human ABCG2. Slides were incubated with EnVision Kit anti-rat/mouse-labeled horseradish peroxidase (DAKO, Glostrup, Denmark) for 30 minutes at room temperature. Peroxidase activity was visualised with 0.5 mg/ml 3,3'-diaminobenzidine tetrachloride (DAB; Sigma, St Louis, MO, USA) in PBS containing 0.02% H_2O_2. Between incubation steps, sections were thoroughly washed with phosphate-buffered saline (PBS). After a short rinse in tap water sections were incubated with haematoxylin for 1 minute and extensively washed with tap water for 10 minutes. Finally, sections were

dehydrated with ethanol followed by xylene and mounted with Entellan (Merck, Darmstadt, Germany). All antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA, Boehringer-Mannheim, Ingelheim, Germany), which also served as a negative control. This was performed by Dr. Sandra Amor and Wouter Gerritson, Free University Amsterdam, The Netherlands.

2.11. *Assay for CNS Drug exclusion pumps.*

P-glycoprotein function on human CMEC/D3 brain endothelial cells was measured as described previously (Kooij et al., 2009). Briefly, cells were cultured to confluent monolayers in 96-well plates. Subsequently, cells were stimulated with various reagents. Cells were then washed three times with PBS and incubated for 45 minutes at 37°C with fluorescent ABC transporter substrates. These were either: the p-glycoprotien substrate 2 μM rhodamine 123 (Sigma, Poole, UK) with or without a specific P-gp inhibitor 10 μM reversin 121 (Alexis, Exeter, UK) or the multi-drug resistance protein one (MRP-1/ABCC1) substrate, 2μM calciene AM (Sigma, Poole UK) with or without the MRP-1 inhibitor MK571 (Merck, Nottingham, UK) or CsA. After 45 minutes of incubation, cells were washed three times with PBS and fluorescence intensity was measured using a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany), excitation 485 nm, emission 520 nm or by a FACScan flow cytometer (Becton & Dickinson, San Jose, CA, USA). P-gp activity is expressed as ratios of fluorescence with modulator divided by fluorescence without modulator after subtraction of the fluorescence of the control. This was performed by Gijs Kooij and Elga De Vries, Free University Amsterdam, The Netherlands).

CHAPTER THREE

CONTROL OF AUTOIMMUNITY AND PROGRESSION BY CANNABINOIDS.

3.1 INTRODUCTION

Multiple sclerosis (MS) is considered to be an autoimmune, demyelinating disease of the central nervous system (CNS), which has a complex pathophysiology (Compston and Coles, 2002; Compston and Coles, 2008). There is now clear evidence that: (i) the immune response drives lesion formation and relapsing-remitting, clinical attacks and (ii) the progressive stages of MS result from neurodegenerative processes, which do not appear to respond to immunotherapy (Coles et al., 2006; Confavreux and Vukusic, 2006; Polman et al., 2006; Metz et al., 2007). These distinct but related, disease elements both produce nerve damage/loss that results in (iii) altered neurotransmission that lead to the development of a number of signs of disease, such as; spasticity, pain and bladder dysfunction (Compston and Coles, 2002). The inability of available medicines to control such symptoms has prompted people with MS to self-medicate and perceive benefit from taking cannabis (Consroe et al., 1997). MS patients also perceived an effect on relapsing disease, suggestive of immunosuppressive capabilities (Consroe et al., 1997). This latter aspect is notoriously difficult to predict and disease activity may naturally slow at a time when residual symptoms are becoming increasingly apparent and people may be taking cannabis for symptom control (Compston and Coles, 2002; Confavreux and Vukusic, 2006).

Although the experimental autoimmune encephalomyelitis (EAE) disease model of MS is most commonly used to study (auto)immune function, we have previously investigated distinct, non-immunological aspects of the disease and provided the first objective evidence for both control of signs of disease and neuroprotection by cannabinoids in EAE (Baker et al., 2000; Pryce et al., 2003a). This has gained some support from subsequent clinical studies (Wade et al., 2004; Rog et al., 2005; Zajicek et al., 2003, 2005) and the recent understanding of the biology of cannabis. This shows that cannabis signals to an endogenous cannabinoid system via cannabinoid receptors (CBR), which can regulate neurotransmission and cell death pathways (Howlett et al., 2002). However, plant cannabinoids have been shown to have the potential to inhibit the development of monophasic EAE (Lyman

et al., 1989; Wirguin et al., 1994) and suggest some potential to modulate immune function.

Although, leucocytes have low levels of the receptor, CB_1R is the most abundant G-protein coupled receptor expressed in the CNS (Howlett et al., 2002; Klein, 2005). The cannabimimetic effects of cannabis and THC, which in rodents are assessed using "tetrad" tests (catalepsy, hypomotility, analgesia and hypothermia), are due to central CB_1 receptor stimulation (Pertwee, 1972; Howlett et al., 2002; Varvel et al., 2005). In contrast, CB_2R is expressed chiefly by leucocytes, which suggest that cannabinoids may control immune function (Howlett et al., 2002; Klein, 2005; van Sickle et al., 2005). Therefore, unsurprisingly, CB_2R has been implicated in the control of inflammation in a number of studies (Noe et al., 2001; Walter et al., 2003; Ni et al., 2004; Steffens et al., 2005; Lunn et al., 2006). However, as leucocytes also express low levels of CB_1 receptors and all current CB_2 selective agents bind to CB_1 receptor and *vice versa* (Pertwee, 1999), the role of the CB_1 receptor in the control of immunity has not always been adequately addressed. Thus, whilst *selective* cannabinoid receptor agonists/antagonists have largely been used to elucidate cannabinoid function, the pharmacological approach is hampered by the lack of any totally *specific* pharmacological tools and the fact that elements of the cannabinoid system, to which these agents may bind, have yet to be identified (Pertwee, 1999; Howlett et al., 2002). Thus, although agents may be selective *in vitro*, at doses used *in vivo*, there is a particular potential for such cannabinoids to cross-react with the other receptors (Breivogel et al., 2001; Howlett et al., 2002; Begg et al., 2005; Baker et al., 2006). Whilst genetic depletion in animals is not without its limitations, cannabinoid gene knockout technologies have been important in identifying cannabinoid function and provide additional confidence in validating targets for therapy (Ledent et al., 1999; Zimmer et al., 1999; Buckley et al., 2000; Marsicano et al., 2002). Recent studies in cannabinoid gene-deficient animals, suggest that both CB_1R and CB_2R agonism may be of benefit in controlling autoimmunity in EAE (Maresz et al., 2007). We have investigated this further, using exogenous CB_1R and CB_2R-selective agents, and although CB_1R-mediated immunosuppression was detectable, it appears that cannabinoid-mediated neuroprotection may be more relevant to the clinical application of cannabis in MS.

3.2 *RESULTS*

3.2.1. *THC but not CBD, is immunosuppressive in EAE and inhibits T cell infiltration of the CNS.*

Previously, doses greater than 5mg/kg of THC have been shown to exhibit immunosuppressive effects in rat and guinea pig EAE (Lyman et al. 1989). We investigated this in mouse EAE and indeed THC greater than this dose, significantly delayed the onset and reduced the severity of spinal cord homogenate induced EAE in ABH mice (Fig. 3.1A, B). Low clinical scores in THC-treated animals were associated with the relative inhibition of mononuclear cell infiltration of the CNS (Fig. 3.1C). Infiltration was more readily detected in more severely affected animals (Fig. 3.1D). Lower doses of THC (<3mg/kg) failed to influence the development of EAE (Fig. 3.1A, B) and similarly CBD exhibited no apparent inhibitory effect on the development of EAE (Fig 3.1A). This contrasts with the anti-inflammatory effect of CBD in an autoimmune, arthritis model (Malfait et al. 2001). This lack of an immunosuppressive effect was evident in EAE, despite using similar dose ranges (Fig 3.1A), the same batches of CBD were used in previous arthritis studies and using a daily dosing protocol from the time of sensitization onwards.

3.2.2. *Cannabinoid-induced immunosuppression is associated with a reduction of Th1 cell differentiation.*

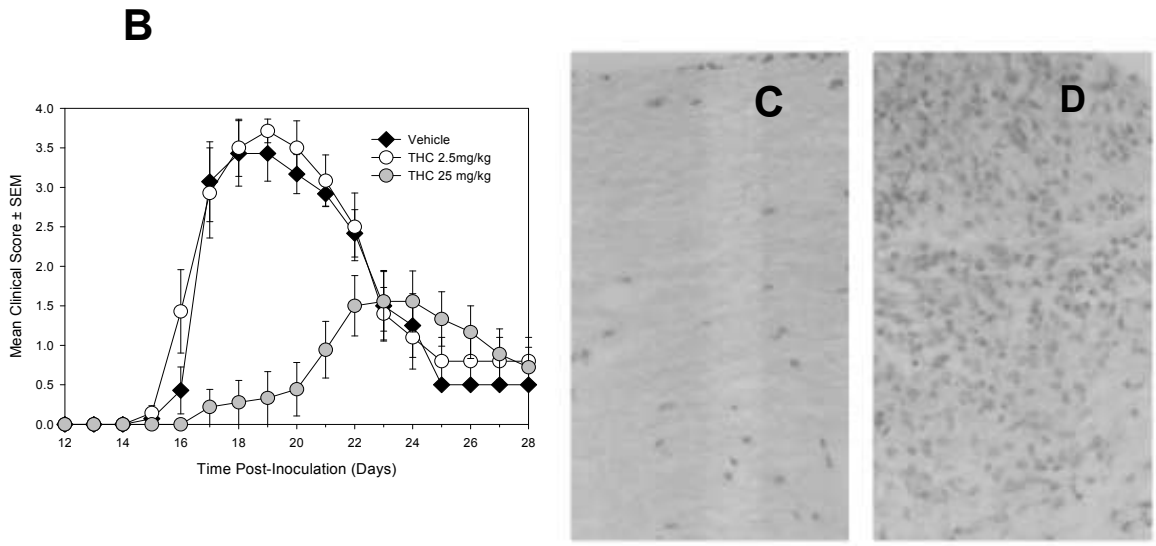
It has been shown that a short course of 20mg/kg *R*(+)WIN-55 exhibits immunomodulatory effects in the Theiler's virus model of MS in SJL mice (Croxford & Miller, 2003). To facilitate *in vitro* studies to examine cannabinoid induced immunosuppression in EAE, we investigated the effect of *R*(+)WIN55, a synthetic full CB_1/CB_2 agonist, on myelin peptide-induced EAE in C57BL/6 mice (Fig. 3.2). A single administration of 20mg/kg *R*(+)WIN-55 on either day 11 or day 15 post-inoculation did not induce a significant amelioration of the severity of EAE, (Fig. 3.2A, B), although the severity of animals treated on day 11 appeared to be reduced compared to vehicle or the CB-inactive enantiomer *S*(-)WIN-55 (Fig. 3.2A). However, by increasing the number of doses, a significant ($P<0.05$) immunosuppressive effect was seen on the clinical course that delayed onset and the severity of disease (Fig. 3.2C), which was not evident following similar injection of the potent CB_2 receptor agonist JWH133 (Fig 3.2D). This treatment was associated with an inhibition of mononuclear cell trafficking to CNS and therefore inflammation-induced demyelination (Fig. 3.2E, F). This treatment could inhibit *ex vivo* T cell recall responses to MOG^{35-55} (Fig. 3.3A) and antigen-induced interleukin-2; interferon-gamma and tumour necrosis factor alpha production (Fig. 3.3 B-D).

Treatment with *S*(-)WIN-55 exhibited no significant inhibitory effect (Fig. 3.3A-C). This suggested a cannabinoid receptor driven inhibition of Th1 responses by *R*(+)WIN-55 (Fig 3. 3B,C). There was no inhibition of Th2 cytokine production (Fig. 3. 3E, F) and interleukin-4 and interleukin-5 appeared to be moderately augmented, although this was probably not cannabinoid receptor dependent as *S*(-)WIN-55 induced a comparable response to *R*(+)WIN-55 (Fig. 3.3E,F).

Figure 3.1. *Immunosuppression of SCH-induced acute EAE in ABH mice by high dose Tetrahydrocannabinol.*

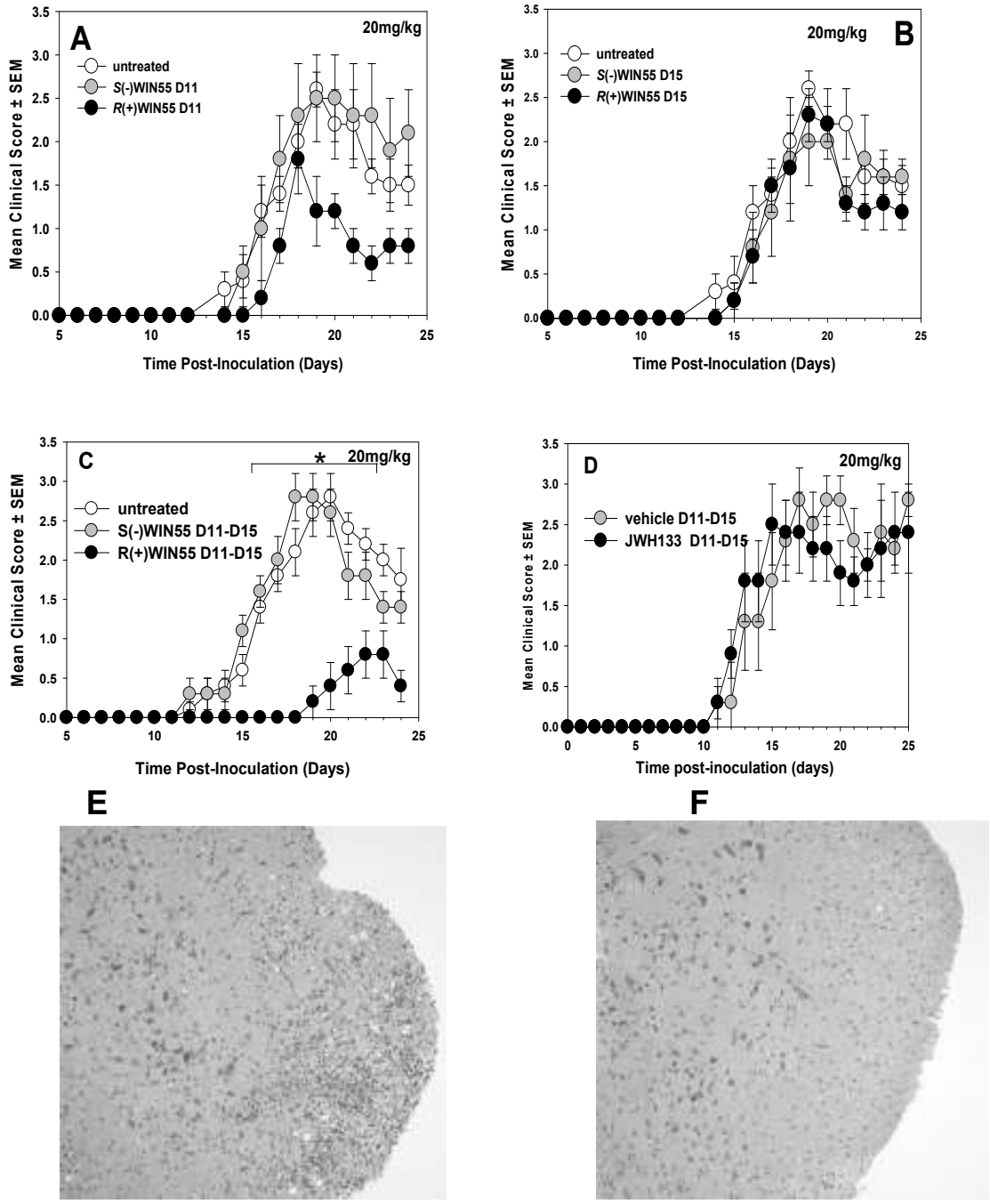
A

Treatment	Dose	No.EAE/Total	Group Score ± SEM	EAE Score ± SEM	Day of Onset ± SD
Untreated		26/26	3.9 ± 0.1	3.9 ± 0.1	17.1 ± 1.6
Vehicle	-	8/8	3.8 ± 0.1	3.8 ± 0.1	16.5 ± 0.9
THC	0.25mg/kg	9/9	3.8 ± 0.1	3.8 ± 0.1	16.2 ± 2.0
THC	2.5mg/kg	10/10	3.8 ± 0.1	3.8 ± 0.1	17.5 ± 1.9
THC	25 mg/kg	7/9	1.7 ± 0.4**	2.1 ± 0.3**	20.7 ± 1.8**
Vehicle	-	9/10	3.6 ± 0.4	3.9 ± 0.1	15.2 ± 0.8
THC	10mg/kg	6/7	2.3 ± 0.6*	3.1 ± 0.5**	17.2 ± 1.8**
THC	20mg/kg	2/8	0.8 ± 0.5***	3.0 ± 0.0	16.0 ± 1.8**
Untreated		11/12	3.3 ± 0.4	3.6 ± 0.2	16.2 ± 1.3
CBD	0.5 mg/kg	10/10	3.7 ± 0.2	3.7 ± 0.2	16.5 ± 1.2
CBD	5.0 mg/kg	8/10	3.0 ± 0.5	3.8 ± 0.1	16.9 ± 1.2
Untreated		12/12	3.8 ± 0.4	3.8 ± 0.2	14.9 ± 1.2
CBD	10mg/kg	7/7	4.0 ± 0.0	4.0 ± 0.0	14.8 ± 1.8
CBD	25 mg/kg	8/8	4.0 ± 0.0	4.0 ± 0.0	15.1± 0.4



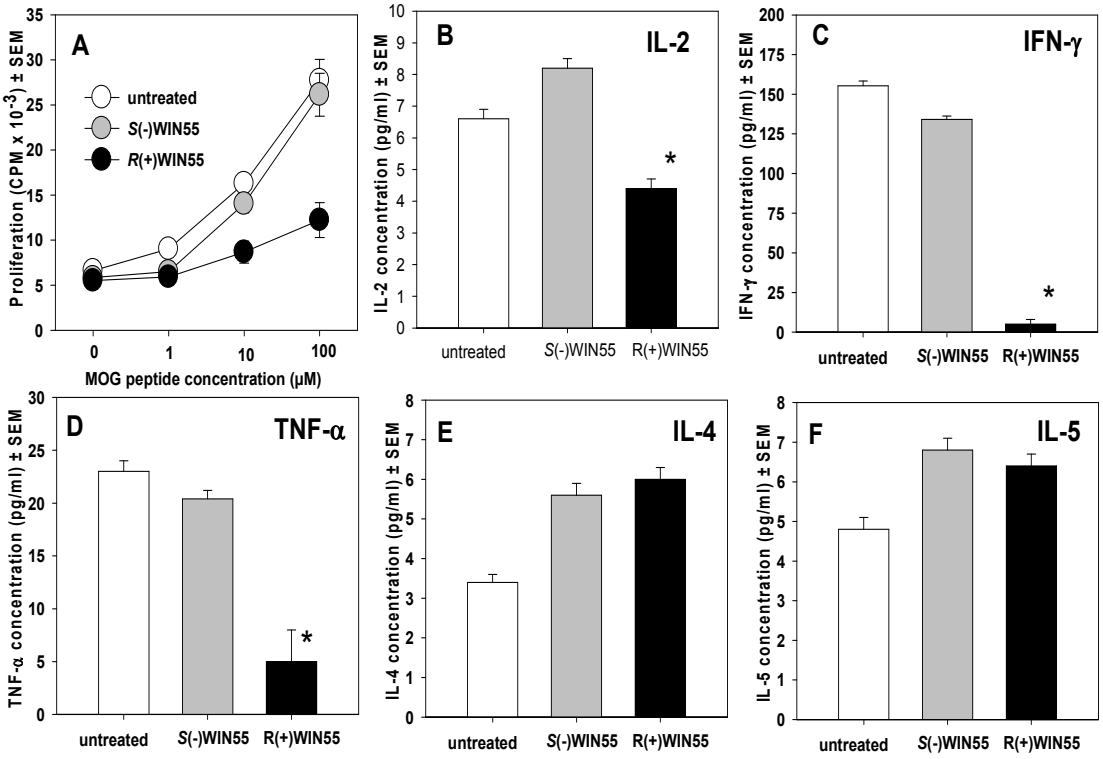
ABH mice were injected with mouse spinal cord homogenate in Freund's adjuvant on day 0 & 7. Animals were injected i.p. daily from day 10-22 with compounds either in Tween:PBS or DMSO:PBS. (A) The results indicate the mean maximal clinical score of the whole group, the mean maximal score of animals that developed EAE and the day of onset of signs. (B) The results indicate the mean daily clinical score ± SEM (C) Histological section of spinal cord tissue from an animal treated with 25mg/kg THC exhibiting mild disease (score 0.5). (D) Histological section of spinal cord tissue from an animal treated with 25mg/kg THC exhibiting paresis (score 3). **P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated controls.

Figure 3.2. *Immunosuppression of MOG-induced EAE by high dose R(+)WIN55 in C57BL/6 mice.*



C57BL/6 mice were injected with myelin oligodendrocyte glycoprotein peptide in Freund's adjuvant on day 0 and 7 using *B. pertussis* toxin as co-adjuvant. Animals were untreated or injected i.p. with either (**A-C**) 20mg/kg *R*(+)WIN55 or *S*(-) WIN55 on (**A**) day 11, (**B**) day 15 or (C) day 11-15 p.i. or (**D**) the CB_2R-selective agonist JWH-133 in Tween:PBS (n=6-9/group). The results indicate the mean daily clinical score ± SEM. Histology was performed on day 17 from cervical spinal cords from mice treated from day 11-15 with 20mg/kg of either (**E**) *S*(-) WIN55 or (**F)** *R*(+)WIN55. These were stained with haematoxylin and eosin to detect cellular infiltrates. *P<0.05 compared to untreated animals. **This was performed by J.Ludovic Croxford, Tokyo, Japan.**

Figure 3.3. *Inhibition of MOG-induced, Th1 T cell responses in EAE by high dose R(+)WIN55 in C57BL/6 mice.*



C57BL/6 mice were injected with myelin oligodendrocyte glycoprotein peptide in Freund's adjuvant on day 0 & 7, without the use of *B.pertussis* toxin as co-adjuvant. Animals were untreated or injected i.p. with 20mg/kg *R*(+)-WIN55 or *S*(-)-WIN55 in Tween:PBS on day 11-15 (**A**) Proliferative response from MOG peptide stimulated lymph node cells from animals injected with cannabinoids from day 11-15. (**B-F**) Cytokine ELISA of tissue culture supernatants from 48 h cultures of lymph node cells stimulated with 100μM MOG^{35-55} peptide. These detected either: (**B**) interleukin-2 (**C**) interferon gamma (**D**) tumour necrosis factor alpha (**E**) interleukin 4 or (**F**) interleukin 5. n=3/group * P<0.05 compared to untreated control animals. **This was performed by J.Ludovic Croxford, Tokyo, Japan.**

3.2.3.*Immunosuppression induced by cannabinoid receptor agonists is CB_1-mediated.*

To investigate the nature of the cannabinoid receptor(s) mediating immunosuppression, additional selective synthetic cannabinoid receptor agonists and antagonists were investigated (Fig. 3.2. Table 3.1). In addition to the peptide induced chronic EAE model in C57BL/6 mice, which is useful for performing *in vitro* experiments because T cell stimulation assays can be performed using a peptide, we also examined tissue homogenate induced disease in ABH mice that exhibit a distinct relapsing/remitting disease course, which is suited to monitoring drug effects on clinical course of disease (Pryce et al., 2003a). CB_1R deficient mice developed EAE of comparable severity to wildtype mice (Table 3.1., Table 3.2. Pryce et al. 2003a), although they exhibit poorer recovery due to accumulation of nerve damage (Pryce et al. 2003a). Following the twice daily injection of SR141617A (CB_1R-antagonist) in ABH mice there was a tendency for disease to develop with earlier onset but of comparable severity to that seen in wildtype mice (Table 3.1). Injection of SR144528 (CB_2R-antagonist) failed to affect the incidence, onset or maximal severity of disease (Table 3.1). In contrast, CB_1 receptor agonism inhibited EAE.

On a C57BL/6, (Table 3.2. Palazuelos et al. 2008), or C57BL/10 (Maresz et al. 2007), EAE-low susceptibility background mouse strain, there appeared to be greater susceptibility to EAE in CB_2 receptor deficient mice (Table 3.2). Although RWJ352303 in ABH mice (Table 3.1) and R(+)WIN55 in C57BL/6 (Fig 3.2C), both full CB_1R/CB_2R agonists, significantly ameliorated the development of EAE, the potent selective CB_2R agonists: RWJ400065 and JWH-133 (Xu et al. 2007) in ABH mice (Table 3.1) and JWH-133 in C57BL/6 mice (Fig. 3.2D), did not significantly inhibit disease. This suggested a CB_1R mediated immunosuppressive action and this was definitively shown using CB_1R-deficient mice where a high dose (10mg/kg i.p.) of RWJ352303 failed to influence the course of disease in *Cnr1*$^{-/-}$ mice (Table 3.1). This dose was used as it was known to be active for at least 24h and thus the lack of bioavailability of the compound at the cannabinoid receptors could be excluded, however this dose was too high to use in wildtype mice because of marked cannabimimetic effects following CNS penetration of the compound. Likewise, the immunosuppressive effect of THC was lost in *Cnr1*$^{-/-}$ mice (Table 3.2). Using conditional deletion to either remove CB_1R from T cells or nerves it was evident that immunosuppression remained when CB_1R was removed from T cells but the immunosuppressive actions of THC was lost when CB_1 R were conditionally deleted from the nerves in the brain (Table 3.2). This suggested that the immunosuppressive action of cannabinoids was probably an indirect effect following

stimulation of cannabinoid receptors in the brain. This was further supported by the lack of immunosuppression following administration of a CNS excluded CB_1R agonist.

It appears that CT3 is a CB_1R agonist that is excluded from the CNS (Ki CB_1R = 6-32nM, Ki CB_2R = 1nM) in rodents (Dyson et al., 2005, Hirgata et al.2007). At the doses used, up to 10mg/kg i.p., it did not induce cannabimimetic effects (Temperature change at 20-120min post-injection 10mg/kg CT3 both 0.0 ± 0.1°C). Likewise, although CT3 may have anti-inflammatory properties (Burstein et al., 2004), it failed to demonstrate evidence of immunosuppression and did not inhibit the development of EAE (Table 3.1).

3.2.4. *Immunosuppression is secondary to CNS cannabinoid receptor agonism and is associated with adverse physiological effects.*

To determine whether immunomodulation was due to direct effects of THC on lymphoid cells or due to immunomodulatory effects secondary to stimulation of CNS-expressed CB_1R, conditional knockout mice were generated (Table 3.2). Conditional exclusion of CB_1 from nerve cells in *Nes*-floxed CB_1-deficit mice prevented the capacity of THC to suppress EAE (Table 1B). However, loss of CB_1R in nerves not only stopped the immunosuppressive activity of the cannabinoids examined, but also inhibited the sedative potential of the cannabinoids. Although nestin is expressed in peripheral nerves (Hennet *et al.* 1995), the sedation was a central effect as cannabinoids induced sedation in ABH.*Peripherin*-Cre-floxed CB_1-deficit (Zhou et al. 2002), which delete CB_1R from the peripheral nervous system. Sedation was evident following administration of immunosuppressive doses of THC, RWJ352303 and notably *R*(+)WIN-55, which also induced a transient hypothermia that was absent in generalised and *Nes*-floxed; CB_1R-deficit mice (Fig. 3.4). This indicates that cannabinoid-induced immunosuppression occurs at doses that may cause adverse physiological responses such that they would be unlikely to be achieved in human use.

3.2.5. *Cannabinoid therapy at doses lacking overt immunosuppressive efficacy slow the accumulation of neurological deficit in relapsing EAE.*

In contrast to the immunomodulatory effect of 20mg/kg *R*(+)WIN-55 (Fig.3.1C), repeated administration of 5mg/kg failed to significantly inhibit the development of EAE in C57BL/6 mice (Fig 3.5A) and inhibition of *ex vivo* T cell proliferation and interferon gamma responses in MOG peptide induced disease in C57BL/6 mice, compared to untreated mice (J.L. Croxford. unpublished observations). Similarly

lower doses of *R*(+)WIN-55 (0.5mg/kg i.p. day 10-22. n=10/10 EAE Score 3.8 ± 0.1; Day of Onset 16.7 ± 1.6 compared to vehicle n=9/9 EAE Score 3.9 ± 0.1 Day of Onset 17.6 ± 1.5) and 5mg/kg R(+)WIN-55 did not prevent acute EAE in CB_1 receptor deficient (Not shown) or wildtype animals (Fig 3.5B) and did not prevent relapsing paralytic EAE in ABH mice (Fig. 3.5C,D). Whilst treatment started during the first remission (RM1), did not inhibit the development of relapse (RL) in mice, it was apparent that less residual deficit accumulated in *R*(+) WIN-55-treated animals and the clinical score significantly ($P<0.01$) diverged from vehicle-treated animals over time during the second (RM2) and third (RM3) remission periods (Fig. 3.5C,D). This was evident despite developing relapses of comparable maximal severity (Fig. 3. 5D). Similarly, *R* (+) WIN-55, treatment slowed the rate of loss of mobility and axons/nerves in the spinal cord (Fig. 3.5 E,F). Thus, whilst immunosuppression of relapsing disease was not induced, cannabinoid-treated animals could better withstand the damaging effects of relapsing EAE (Fig 3.5C, F). This was consistent with the reduced capacity of CB_1-deficient mice to tolerate inflammatory insults. Whereas ABH.*Cnr1* +/+ (n=6), ABH.*Cnr1* -/+ (n=7) and ABH.*Cnr1* -/- (n=10) mice developed paralytic acute EAE of comparable severity (4.0 ± 0.0), the residual deficit of CB_1 deficient mice (Minimum RM1 remission Score. 1.9 ± 0.3) was significantly ($P<0.05$) worse than either the wildtype ABH.*Cnr1* +/+ (RM1 Score. 0.5 ± 0.3) or ABH.*Cnr1* -/+ heterozygous (Minimal RM1 Score. 0.7 ± 0.3) mice. Therefore, this indicates that low dose cannabinoid treatment can induce neuroprotective effects, despite failing to affect the relapse rate that would be indicative of an immunosuppressive effect. This neuroprotective effect of WIN-55 was lost in ABH CB_1 deficient mice, where the observed rapid development of neurological impairment, assessed by clinical score after the acute phase of disease, was not ameliorated by 5mg/kg WIN-55 treatment that was neuroprotective in normal ABH mice (Figure 3.6). Due to the high level of residual neurological disability in these animals after the acute phase, it was not possible to assess motor impairment by rotarod analysis.

In addition to the neuroprotective effects seen with low dose administration of the synthetic cannabinoid receptor agonist *R*(+) WIN-55, neuroprotective effects in CREAE were observed with plant-based cannabinoids. An induced-relapse paradigmn was used as animals more rapidly accumulate damage, and disease is more synchronous, compared to spontaneous relapsing EAE. Therefore drug treatment effects can be observed more rapidly. Furthermore animals were subjected to rotarod analysis as a quantitative, objective outcome measure. Neuroprotection was detected following treatment with THC and also with the non-cannabinoid receptor binding non-psychoactive cannabis constituent cannabidiol (10 and 5 mg/kg), administered separately (Fig 3.7) or in combination (Figure 3.8),

when assessed by clinical score and rotarod performance (Fig 3.7, Fig 3.8). As found previously in acute disease (Fig 3.1A) 2.5mg/kg or less THC and CBD did not induce immunosuppression as animals developed a paralytic attack of comparable serverity to vehicle-treated animals (Fig 3.7). However, there was a better motor recovery from the effects of the relapse, as seen in remission compared to vehicle-treated animals.Although 2.5mg/kg THC was affective at controlling loss of motor function as assessed both clinically and via rotarod activity, CBD reached significance in only in the rotarod outcome measure. (Fig 3.8). This activity is associated with the relative sparing of spinal cord axons compared to vehicle treatment. Unfortunately a technical problem with the neurafilment assay prevented this being shown. The neuroprotective effect of THC was lost when the daily dose was lowered to 0.25mg/kg i.p., Likewise 1mg/kg CBD exhibited a minimal effect compared to 10mg/kg ip. CBD that significantly ($P<0.05$) limited the loss of motor function as a consequence of relapse (Fig 3.9). Therefore, CBD may possess neuroprotective effects in contrast to a lack of activity as an immunosuppressive (Fig 3.1) or symptom control agent (Baker et al., 2000).

Table 3.1 *Immunosuppression in EAE by synthetic cannabinoids is CB_1R-mediated.*

Treatment	Dose	No.EAE/Total	Daily group score ± SEM	Max EAE score ± SEM	Onset day ± SD
Vehicle	-	8/8	4.0 ± 0.2	4.0 ± 0.2	15.3 ± 0.7
RWJ352303 (CB_1R /CB_2R Agonist)	1mg/kg	6/7	2.6 ± 0.5***	3.0 ± 0.3**	19.0 ± 1.1***
RWJ400065 (CB_2R Agonist)	10mg/kg	8/8	3.7 ± 0.2	3.7 ± 0.2	16.3 ± 1.3
Vehicle	-	6/7	3.4 ± 0.6	3.9 ± 0.1	13.2 ± 1.7
JHH 133 (CB_2R Agonist)	0.1mg/kg	7/8	3.1 ± 0.6	3.6 ± 0.4	14.0 ± 1.8
Vehicle	-	9/9	4.0 ± 0.0	4.0 ± 0.0	14.8 ± 0.7
JWH133	1.5mg/kg	8/8	4.0 ± 0.0	4.0 ± 0.0	14.4 ± 1.5
Vehicle	-	7/7	3.8 ± 0.2	3.8 ± 0.2	16.1 ± 1.2
CT3 (CNS-excluded,CB_1R agonist)	0.01mg/kg	7/7	3.6 ± 0.6	3.6 ± 0.6	15.0 ± 0.9
CT3	0.1mg/kg	6/7	2.8 ± 0.8	3.3 ± 0.6	16.8 ± 1.8
CT3	10mg/kg	6/8	2.6 ± 0.7	3.5 ± 0.3	16.2 ± 1.7
Vehicle	-	25/26	3.8 ± 0.2	4.0 ± 0.2	15.2 ± 1.1
SR141617A (CB_1R Antagonist)	2 x 5mg/kg	6/6	4.2 ± 0.4	4.2 ± 0.4	13.8 ± 0.4**
SR144528 (CB_2R Antagonist)	2 x 5mg/kg	8/8	4.0 ± 0.0	4.0 ± 0.0	14.4 ± 1.4
Vehicle in ABH.*Cnr1*$^{-/-}$	-	7/7	4.2 ± 0.2	4.2 ± 0.2	16.4 ± 1.1
RWJ352303 in ABH.*Cnr1*$^{-/-}$	10mg/kg	8/8	3.8 ± 0.1	3.8 ± 0.1	16.0 ± 1.2

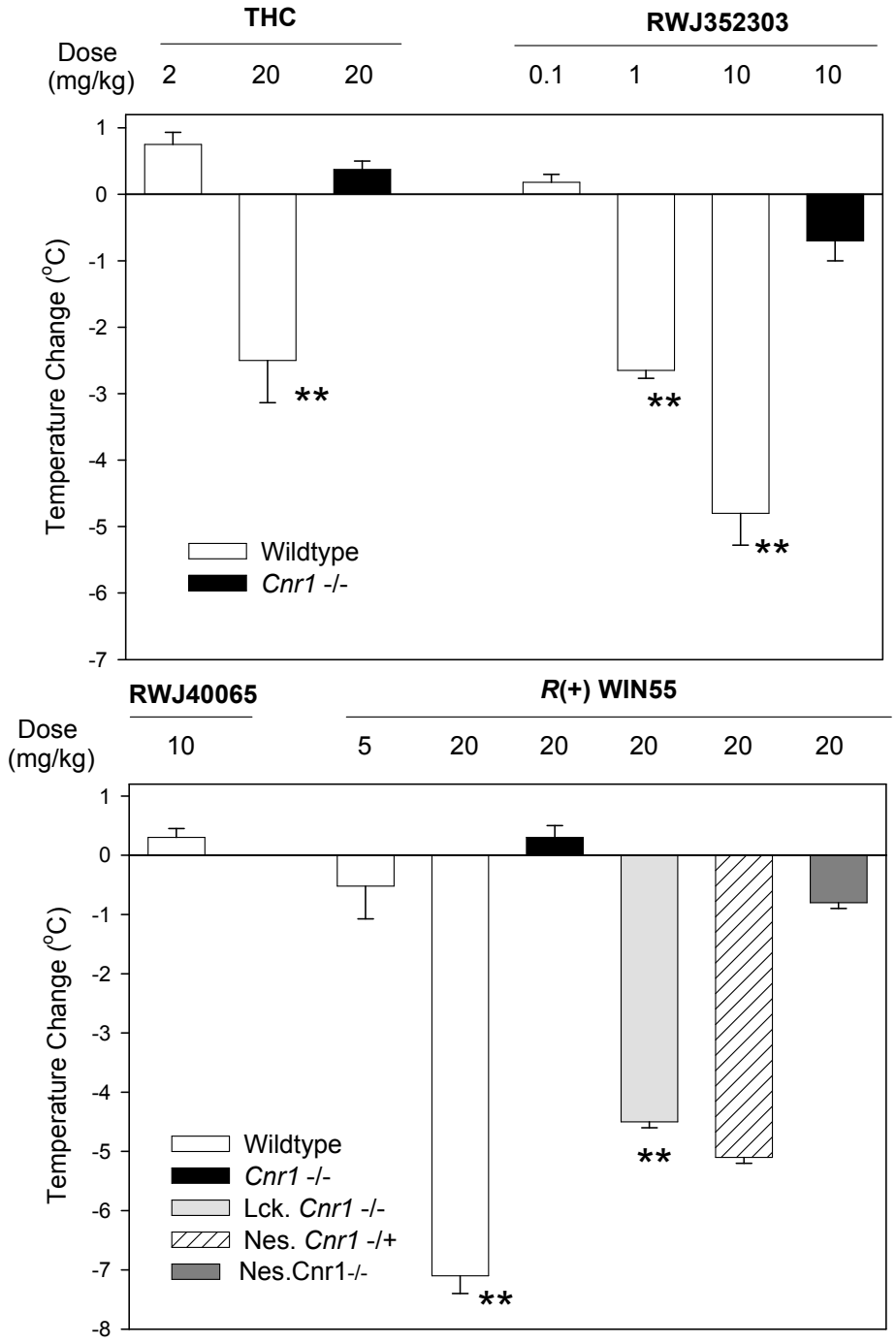
EAE was induced with SCH in ABH mice on day 0 & 7. These were injected daily i.p. from day 10-22 with either RWJ352303, CB_2 selective agonists JWH-133 or RWJ400065 or cannabinoid receptor selective antagonists SR141617A or SR144528. (n=6-8/group) in Tween PBS or DMSO:Cremophor:PBS. The results indicate the mean maximal clinical score of the whole group, the mean maximal score of animals that developed EAE and the day of onset of sign s or the daily clinical score ± SEM. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to vehicle-treated controls.

Table 3.2 *Immunosuppression in EAE by cannabinoids is mediated by CB_1R-expressed in the CNS.*

Strain	CB_1 Expression T cell	CNS	Treatment Dose	No. EAE	Group Score ± SEM	EAE Score ± SEM	Day of Onset ± SD
A. *Generalised CB_1 Knockout*							
ABH.*Cnr1*$^{+/+}$	+/+	+/+	untreated	6/6	4.0 ± 0.0	4.0 ± 0.2	16.3 ± 1.8
ABH.*Cnr1*$^{+/-}$	+/-	+/-	untreated	9/9	4.2 ± 0.2	4.2 ± 0.2	15.4 ± 1.0
ABH.*Cnr1*$^{-/-}$	**-/-**	**-/-**	untreated	15/15	4.1 ± 0.1	4.1 ± 0.1	16.3 ± 1.8
C57BL/6	+/+	+/+	untreated	1/10	0.1 ± 0.1	1.0 ± n/a	17.0 ± n/a
C57BL/6/*J.Cnr2-/-*	+/+	+/+	untreated	6/11	1.3 ± 0.4	2.4 ± 0.3	16.8 ± 1.7
ABH.*Cnr1*$^{+/-}$	+/-	+/-	vehicle	9/9	4.2 ± 0.2	4.2 ± 0.2	15.9 ± 1.2
ABH.*Cnr1*$^{+/-}$	+/-	+/-	THC 20mg/kg	4/8	1.3 ± 0.6**	2.6 ± 0.6**	17.0 ± 0.8
ABH.*Cnr1*$^{-/-}$	**-/-**	**-/-**	THC 20mg/kg	5/6	3.0 ± 0.8	3.6 ± 0.6	18.0 ± 1.8
B. *Conditional CB_1 Knockout*							
ABH wildtype	+/+	+/+	untreated	9/9	3.6 ± 0.1	3.6 ± 0.1	15.4 ± 1.1
ABH.*Cnr1*$^{f/f}$	+/+	+/+	untreated	6/6	3.3 ± 0.2	3.3 ± 0.2	15.0 ± 0.6
ABH.Trpv1$^{-/-}$	+/+	+/+	untreated	11/11	3.8 ± 0.1	3.8 ± 0.1	16.0 ± 1.0
ABH.*Cnr1*$^{-/f}$.Tg(*Nes-cre*)$^{-/-}$	+/-	+/-	vehicle	19/21	3.5 ± 0.3	3.8 ± 0.2	15.5 ± 1.5
ABH.*Cnr1*$^{-/f}$.Tg(*Nes-cre*)$^{-/+}$	+/-	**-/-**	vehicle	24/27	3.5 ± 0.2	3.9 ± 0.1	15.3 ± 1.3
ABH.*Cnr1*$^{-/f}$.Tg(*Lck-cre*)$^{-/+}$	**-/-**	+/-	vehicle	6/8	2.9 ± 0.6	3.9 ± 0.1	16.2 ± 1.3
ABH.*Cnr1*$^{-/f}$.Tg(*Lck-cre*)$^{-/-}$	+/-	+/-	vehicle	7/7	4.0 ± 0.0	4.0 ± 0.0	15.7 ± 1.3
ABH.*Cnr1*$^{-/f}$.Tg(*cre*)$^{-/-}$	+/-	+/-	THC 20mg/kg	6/11	0.8 ± 0.4***	1.5 ± 0.5**	16.5 ± 1.3
ABH.*Cnr1*$^{-/f}$.Tg(*Lck-cre*)$^{-/+}$	**-/-**	+/-	THC 20mg/kg	7/16	0.8 ± 0.3**	1.8 ± 0.4**	16.7 ± 1.4
ABH.*Cnr1*$^{-/f}$.Tg(*Nes-cre*)$^{-/+}$	+/-	**-/-**	THC 20mg/kg	7/9	2.7 ± 0.5	3.4 ± 0.2	15.0 ± 0.8

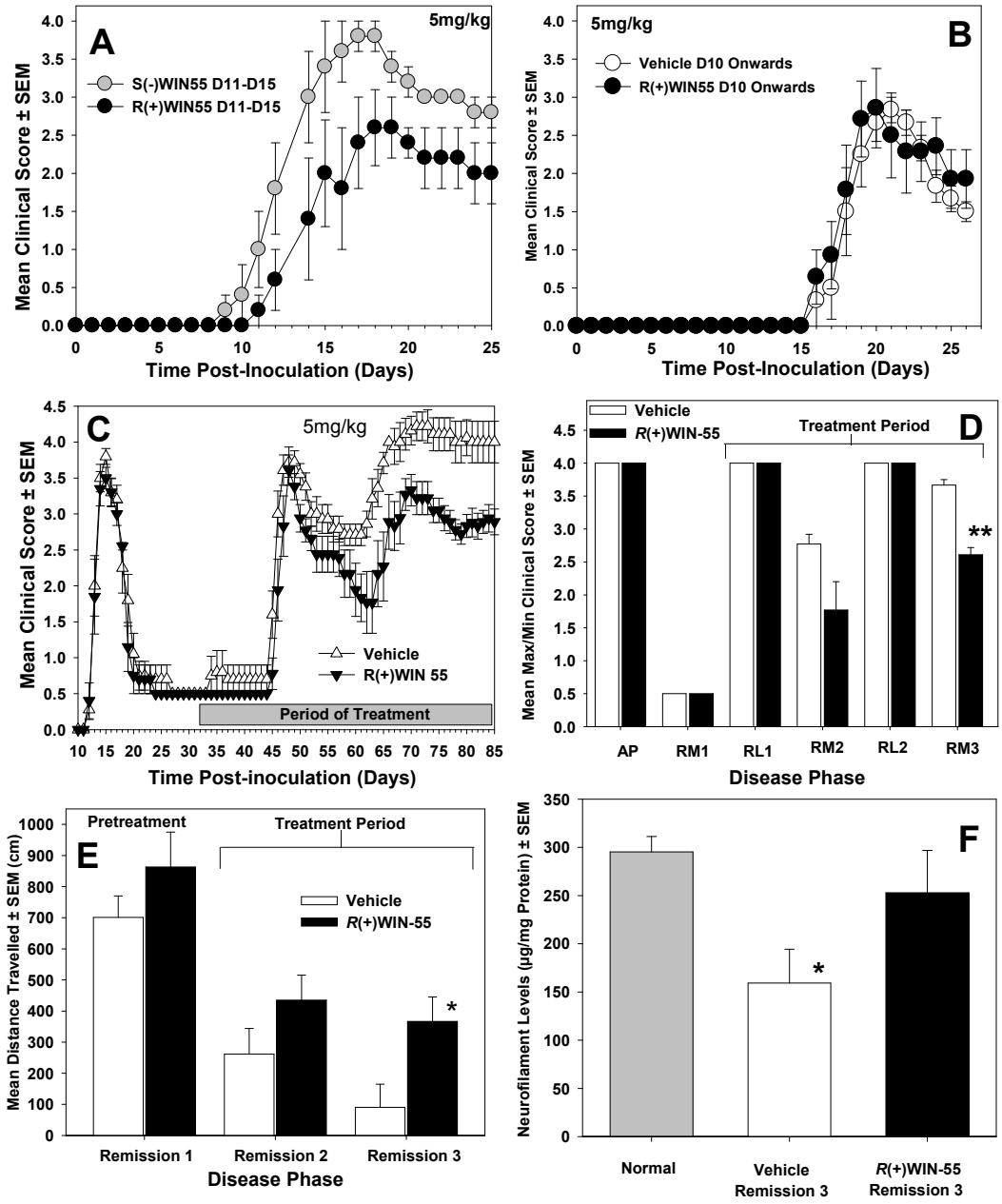
Mice that were either homozygous for the null expressing CB_1 construct (*Cnr1*$^{-/-}$) or litter-mates from crosses between *Cre* transgene heterozygotes, CB_1 null homozygous construct mice and mice homozygous for the "floxed" CB_1 construct (*Cnr1*$^{f/f}$) were injected with mouse spinal cord homogenate in Freund's adjuvant on day 0 & 7. The CB_1 genotype in T cells or the CNS is indicated for each strain. Animals were injected i.p. daily from day 10-22 with compounds dissolved in Ethanol:Cremophor:PBS. The results indicate the mean maximal clinical score of the whole group, the mean maximal score of animals that developed EAE and the day of onset of signs. *P<0.05, **P<0.01, ***P<0.001 compared to relevant vehicle-treated controls.

Figure 3.4. *Hypothermia induced by immunosuppressive doses of cannabinoids.*



Either: wildtype ABH (white box); ABH.Cnr1$^{-/-}$ (Black Box. Cnr1$^{-/-}$) CB_1 knockout; ABH.Cnr1$^{-/f}$.Tg(Lck-cre)$^{-/+}$ (Hatched Box. Lck$^{-/-}$) T cell CB_1 knockout; ABH.Cnr1$^{-/f}$.Tg(Nes-cre)$^{-/-}$ (Light Shaded Box. Nes$^{-/+}$), CNS CB_1 CB_1 heterozygote expressing mice or ABH.Cnr1$^{-/f}$. Tg(Nes-cre)$^{-/+}$ (Light Shaded Box. Nes$^{-/-}$) CNS CB_1 knockout or mice (n=4-5/group) were injected i.p. with either: THC, RWJ352303; RWJ40065 or R(+)-WIN55. Body temperature was measured at baseline and 20 minutes after injection. $P<0.01$ compared to baseline by paired *t* test analysis.

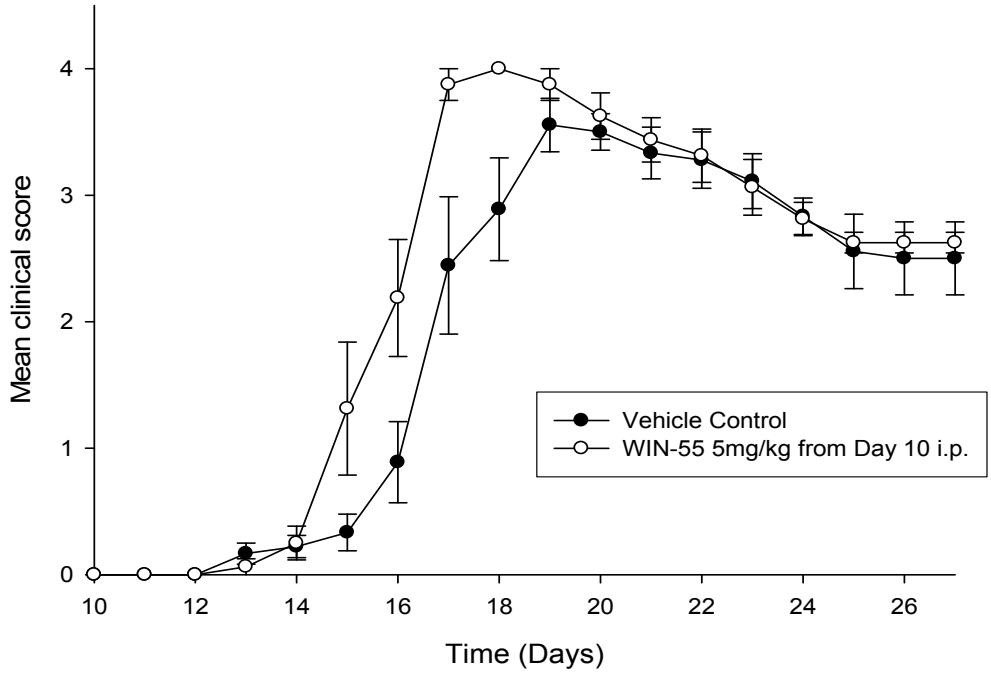
Figure 3.5. *Low dose R(+) Win-55 treatment fails to inhibit the development of acute or relapsing EAE, but slows the accumulation of neurological deficits due to inflammatory attack.*



EAE was induced with either: (**A**) MOG peptide in C57BL/6 mice, using *B. pertussis* toxin as co-adjuvant or (**B-F**) SCH in ABH mice in Freund's adjuvant on day 0 and 7. These were injected daily i.p. with 5mg/kg *R*(+)WIN55 or S(-)WIN55 in Tween PBS or DMSO:Cremophor:PBS on (**A**) day 11-15 (n=8/group) (**B**) day 10-25 (n=6-7/group) or (**C-F**) During the post-acute, remission period (RM1) from day 32 onwards (n=10-12/group). Mean ± SEM daily clinical scores, during (**A, B**) acute or (**C**) relapsing EAE. Four-vehicle treated animals were removed from the study due to the neurological deficit accumulated and were not included in further (**D-F**) analysis. (**D**) The maximum clinical score during acute phase paralysis (AP) or relapses (RL) and minimal clinical score during each remission (RM) of

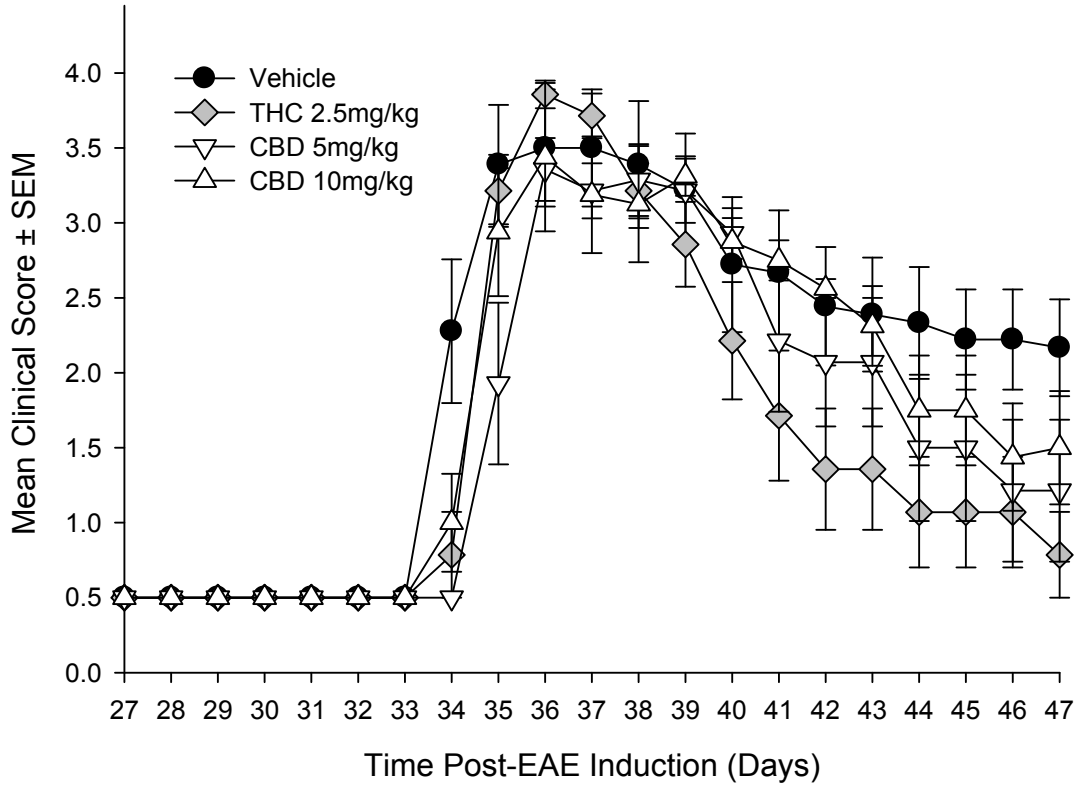
animals remaining at the termination of the experiment on day 85 p.i. (**E**) The movement activity over 5 minutes in an open field activity chamber before and after treatment on day 65 and 82 p.i. (n=8-10/group). This level of protection by WIN-55 was reproduced in one additional experiment. * P<0.05 compared to vehicle treated controls (**F**) The total neurofilament content of spinal cords in normal (n=4), vehicle or *R*(+)WIN55 (n=8-10/group). * P<0.05 compared to normal animals on day 85p.i.

Figure 3.6. *Low dose R(+)WIN-55 fails to slow the accumulation of neurological deficit in ABH/CB$_1$ knockout mice.*



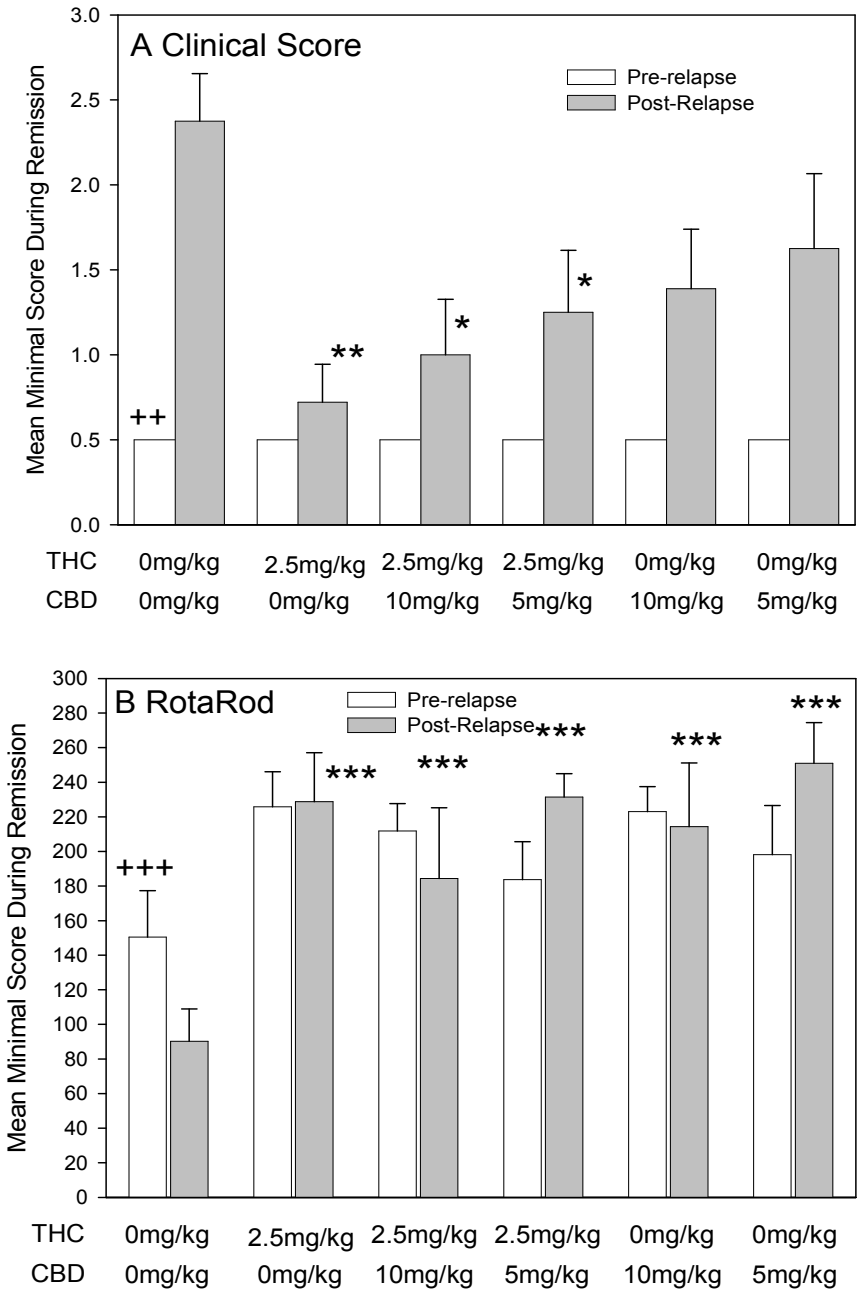
EAE was induced with SCH in ABH/CB$_1$ knockout mice in Freund's adjuvant on day 0 and 7. Animals were injected with 5 mg/kg WIN-55 in ECP i.p. or ECP alone. Neurological impairment was assessed by the mean clinical score ± SEM at the remission phase of disease at day 27 n= 8 animals per group. Due to the severity of the residual neurological deficits it was not possible to assess rotarod performance in these animals.

Figure 3.7. *Low-dose THC and cannabidiol therapy in relapsing EAE slows the development of neurological deficit during the relapse phase of disease in ABH mice.*



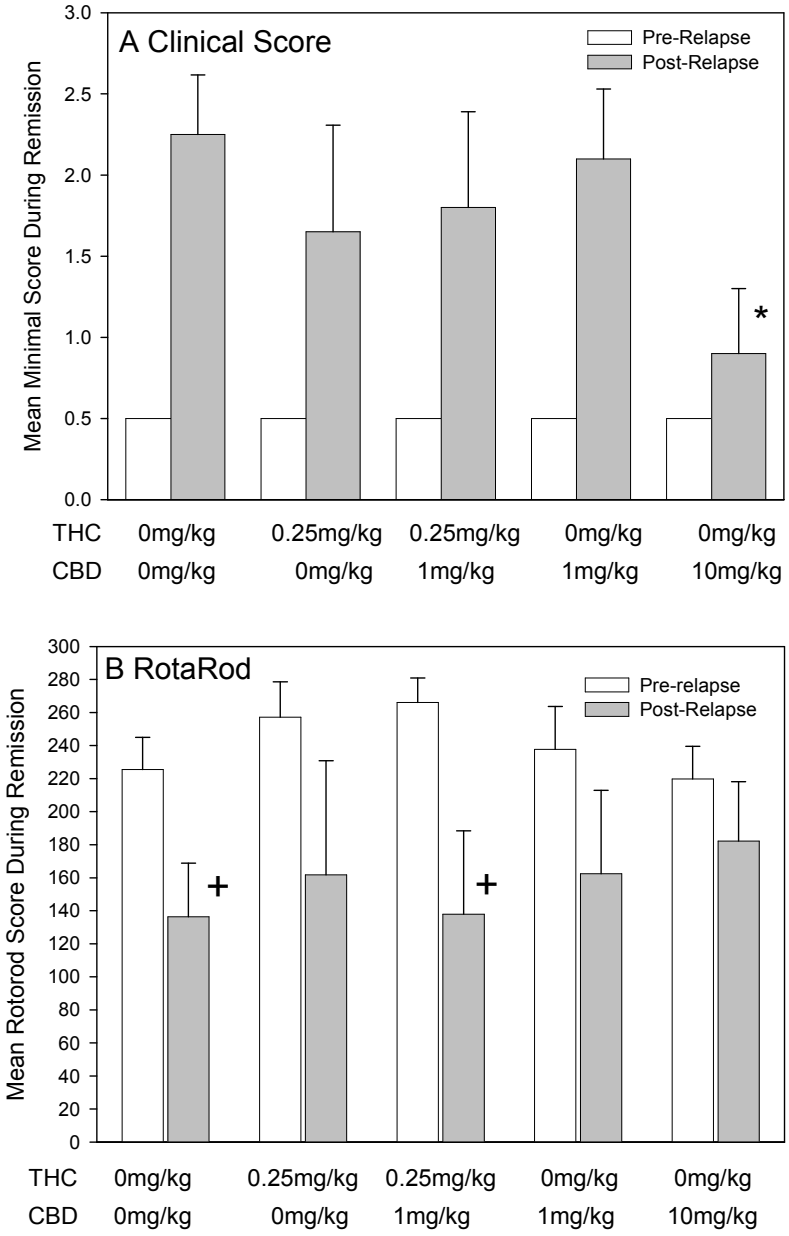
EAE was induced with SCH in ABH mice in Freund's adjuvant on day 0 and 7. Animals were allowed to undergo acute phase inflammatory attack and relapse was induced by re-immunisation with SCH in Freund's adjuvant at day 28. Animals were injected i.p. with 2.5 mg/kg THC, 10 or 5 mg/kg CBD in ECP, n= 7-9 animals per group. Results are mean ± SEM for the post-acute remission phase and relapse.

Figure 3.8. *THC and CBD treatment slows the development of neurological deficit due to relapsing EAE in ABH mice.*



EAE was induced with SCH in ABH mice in Freund's adjuvant on day 0 and 7. Animals were allowed to undergo an acute phase inflammatory attack and relapse was induced by re-immunisation with SCH in Freud's adjuvant at day 28. Animals were injected i.p. with 2.5 mg/kg THC, 10 or 5 mg/kg CBD or a combination of both, n= 7-9 animals per group. Neurological impairment was assessed by rotarod performance measurement post acute phase remission at day 27 and post-relapse remission phase at day 48. * P<0.05, ** P<0.01, ***P<0.001 compared to vehicle treated animals. +++ P<0.001 compared to pre-relapse levels.

Figure 3.9. *Cannabidiol treatment slows the development of neurological deficit due to relapsing EAE in ABH mice.*



EAE was induced with SCH in ABH mice in Freund's adjuvant on day 0 and 7. Animals were allowed to undergo an acute phase inflammatory attack and relapse was induced by re-immunisation with SCH in Freund's adjuvant at day 28. Animals were injected i.p. with 0.25 mg/kg THC, 1 or 10 mg/kg CBD or a combination of both. n= 5-9 animals per group. Neurological impairment was assessed by Rotarod performance measurement post acute phase remission at day 27 and post-relapse remission phase at day 48. * $P<0.05$ compared to vehicle treated animals. + $P<0.05$ compared to pre-relapse levels.

3.3. *DISCUSSION*

There is increasing evidence that MS is, at least in part, a neurodegenerative disease, which is associated with the development of neurological deficits in MS (Compston and Coles, 2002; 2008). There are a number of routes for neuroprotection and this can be achieved by preventing the immune response from either being generated or from entering the CNS. This will prevent direct CNS damage by the immune system. Another route is to slow nerve damage that occurs as a consequence of the immune attack. Cannabinoids have the potential to inhibit both of these pathways, suggesting that cannabinoids could influence the development of progressive MS, which has so far been refractory to treatment (Compston and Coles, 2002; 2008).

This study demonstrates that some cannabinoids have immunosuppressive potential as shown by recent studies in MS models using synthetic cannabinoids and THC (Lyman et al., 1989; Wirguin et al., 1994; Ni et al., 2004; Cabranes et al., 2005; Sanchez et al., 2006; Maresz et al., 2007; Palazuelos et al., 2008; Zhang et al., 2009). However, further evidence is provided here that this is mediated by the actions of CB_1R receptors. This results in downstream immunomodulatory actions that suppress T cell activity and prevent the accumulation of inflammatory cells within the CNS during EAE. Our studies have focused on the administration of agents once T cell priming had been initiated and thus therapy was targeted to effector T cell function. Encephalitogenic cells including the Th17 subset appear to, produce proinflammatory gamma interferon and tumour necrosis factor (Karin et al., 1994; Suryani et al., 2007), whose production was reduced in cannabinoid treated animals. However, as immunosuppression using exogenous agonists appears to be CB_1R-mediated and secondary to neuronal stimulation of release of immunosuppressive agents such as glucocorticoids, changes in cytokine production are downstream of the immunosuppressive mechanism.

Based on studies in CB_2R deficient animals that can show augmented EAE susceptibility, as shown here and in other studies (Maresz et al., 2007; Palazuelos et al.' 2008), the action of CB_2R-selective agonists in EAE have been investigated and currently, any immunosuppressive effect via CB_2R receptors in C57BL/6 or ABH mice has not been demonstrated. It has been reported previously that *R*(+)WIN-55 inhibits leukocyte migration into the CNS of C57BL/6 mice by a CB_2R-dependent mechanism (Ni et al., 2004; Xu et al., 2007). Recently, S(-) WIN55 has been reported to exhibit low potency pharmacological activity as a CB_2 antagonist/inverse agonist (Savinainen et al., 2005), but this failed to inhibit EAE. However, others have found that CB_2R inverse agonists inhibit leukocyte diapedesis

into tissues (Lunn et al., 2006; Oka et al., 2006). Similarly, it has also been reported recently that contact dermatitis is augmented in CB_1R and CB_2R deficient mice but similar to this study, exogenous CB_2R agonism failed to inhibit or even augment disease (Karsak et al., 2007). Thus, the role of CB_2R in the control of T cell autoimmunity is controversial. The myelin-specific T cell receptor transgenic mice used for CB_2R knockout studies in EAE, exhibited weak EAE-susceptibility compared to marked EAE susceptibility induced here in ABH mice. It is possible that the CB_2R-deficiency acted to produce greater numbers of encephalitogenic T cell precursors during lymphoid development, which could become of less significance if strong sensitizing signals are used for sensitization, rather than by influencing effector T cell function that would be therapeutically targeted here. Thus, the failure of exogenous agonists to inhibit disease may relate to timing of drug administration, such that drug-responsive elements during the sensitization process were not targeted. Furthermore, there could be problems in the pharmacokinetic profiles of the agents examined such that insufficient amounts were administered or that receptor tolerance following stimulation occurred that could account for the lack of efficacy. However, we have failed to find evidence for immunomodulatory effects of CB_2R agonists or antagonists at doses that have shown biological activity in other systems (Baker et al., 2000; Arevalo-Martin et al., 2003; Pryce and Baker, 2007; Xu et al., 2007). Importantly, the immunosuppressive activity of a CB_1R/CB_2R agonist (RWJ 352303) that could give long-term cannabimimetic effects, suggesting that it was bioavailable, was lost in CB_1R-deficient mice. This suggests that CB_2R may offer limited potential to induce immunosuppression. Furthermore, as THC and cannabidiol bind but exhibit limited or no agonism at CB_2R (Bayewitch et al., 1996; Thomas et al., 2007), this suggests that cannabis will be of limited use as an immunosuppressive agent via CB_2R. Furthermore, some cannabinoids have been reported to inhibit acute EAE via $TRPV_1$ vanilloid receptor activation (Cabranes et al., 2005). Thus, cannabinoids may have additional biological properties, possibly as yet unrecognised, which may account for their activity *in vivo*. Due to the lack of specificity of available pharmacological agents, we combined a pharmacological and gene knockout approach to investigate the nature of the cannabinoid receptor mediating immunosuppression in EAE.

The efficacy of THC was largely lost in CB_1R-deficient animals and was subsequent to stimulation of CB_1R expressed by nerves. Hypothalamic stimulation of CB_1R influences the regulation of neuropeptides that modulate hormonal systems such as; leptin, sex hormones and glucocorticosteroids that can influence susceptibility to EAE (Bolton et al., 1997; Murphy et al., 1998; Matarese et al., 2001; van den Broek et al., 2005). Glucocorticosteroid responses tonically control EAE susceptibility and are stimulated by doses of cannabinoids that cause suppression

of cytokine responses and immunosuppression (Pertwee, 1974; Wirguin et al., 1994; Bolton et al., 1997). However, demonstrating a causal link *in vivo* is technically challenging as genetic disruption of the glucocorticosteroid receptor is lethal and genetic inhibition of the lymphoid glucocorticosteroid receptor expression inhibits the development of T cells and prevents EAE from being induced (Tonche et al., 1999; Marchetti et al., 2002). Furthermore, we have shown that chemical adrenalectomy or the pharmacological blockage of glucocorticosteroid receptors markedly augments the sedating properties of cannabinoids, including marked and long-lasting CB_1R-dependent hypothermia, to such an extent that it prevents the appropriate experiments being undertaken in EAE (Pryce et al., 2003b).

The results concerning CB_1R mediated immunosuppression with synthetic cannabinoid receptor agonists and THC were totally consistent and show that immunosuppression was only evident at doses of THC and synthetic cannabinoids that induced significant cannabimimetic effects including sedation and hypothermia. Even allowing for the enhanced metabolic potential of rodents, the immunosuppressive doses of cannabinoids in animals are significantly greater than those achieved by recreational or medicinal use in humans (Lyman et al,, 1989; Grotenherman 2003; Zajicek et al., 2003). This suggests that irrespective of the mechanism(s) of immunosuppression, it is probably irrelevant to human medicinal use of cannabinoids where doses are titrated to avoid cannabimimetic effects. This provides another example where it is possible to demonstrate control of disease elements in animal models that are unlikely to be relevant to clinical use in human disease (Baker and Jackson, 2007). As such, although there have been instances of reported increased bronchial infections following smoking cannabis, there is essentially no good evidence that cannabinoids induce a serious, relevant immunosuppression in humans (Rachelefsky et al., 1976; Kraft and Kress 2004) and immunological studies of peripheral blood of patients in MS trials have so far failed to indicate any marked immune perturbations, including skewing of the T cell cytokine response (Killestein et al., 2003; Katona et al., 2005). Whilst the current cannabis trials in MS for symptom control have not been designed for the identification of immunosuppression, despite early indications, they have so far failed to demonstrate a reduction of relapses, indicative of an immunosuppressive effect (Zajicek et al., 2003; 2005). Similarly, a patient developed fulminant MS whilst taking SR141617A, during anti-obesity trials, suggesting that inverse agonism does not appear to inhibit disease (van Oosten et al., 2004). Importantly, THC is licensed for the treatment of wasting in acquired immune deficiency syndrome, where further immunosuppression is undesirable and would have hampered drug development if cannabinoids exhibited significant immunosuppressive potential.

However, it is increasingly being realised that neurodegeneration in progressive MS is the major cause of disability (Compston and Coles, 2002; Coles et al., 2006; Confavreux and Vukusic, 2006). Studies in CB_1R deficient mice indicate that such mice accumulate nerve loss as a result of inflammatory insults (Pryce et al., 2003a; Jackson et al., 2005). This has been attributed in part due to the development of a deficiency of endocannabinoids, with neuroprotective potential, during immune attack in some EAE studies (Cabranes et al., 2005; Witting et al., 2006; Centonze et al., 2007) and in support of this, Faah-/- mice, which have elevated levels of anandamide, accumulate less nerve damage as a consequence of EAE (Webb et al. 2008). Although a lower dose of *R*(+)WIN-55 failed to inhibit relapsing EAE, consistent with the lack of immunosuppression, it slowed the accumulation of neurological deficits and nerve loss resulting from inflammatory attack. This suggests that cannabinoid receptor stimulation may be more important in mediating neuroprotection rather than immunosuppression.

This has also been reported in EAE studies in DA rats where high dose (10 and 20 mg/kg) *R*(+)WIN 55 treatment had immunomodulatory effects on relapse, whereas low dose (5 mg/kg) treatment had no influence on relapse severity but did produce a significant reduction in axonal degeneration (Hasseldam and Johansen, 2010). It has been shown previously that *R*(+)WIN-55 can inhibit the development of autoimmune-independent neurodegeneration in models of motor neuron disease as shown by a number of outcomes such as histology and neurophysiology (Bilsland et al., 2006). Furthermore, we have demonstrated that *R*(+)WIN-55 can induce neuroprotection in acute neuroinflammation of the eye (Pryce et al., 2003a) and show here that chronically administered cannabinoids can inhibit chronic, autoimmune-dependent neurodegeneration, which is lost in CB_1 receptor deficient animals. In addition, low dose (2.5 mg/kg) administration of the phytocannabinoid THC, a dose which does not produce immunosuppression of disease, is also effective in reducing neurological impairment during the relapse phase of EAE, both alone and in combination with the non-psychoactive non-cannabinoid receptor binding cannabinoid constituent CBD. The observation that CBD also has neuroprotective properties is intriguing as it has no overt activity at either CB_1 or CB_2 receptors and does not have the psychoactive properties of THC. The neuroprotective action of CBD may result from firstly; anti-oxidant properties protecting neurons from the toxic effect of reactive oxygen species release by inflammatory cells (Hampson et al., 1998), secondly, restoration of neuronal mitochondrial Ca^{2+} homeostasis and inhibition of apoptosis (Ryan et al., 2009), or as a Na^{+} channel antagonist (D. Selwood, unpublished observations), which may be reflected in the ability of CBD to reduce epileptiform activity and seizures *in vitro* and *in vivo* (Jones et al., 2010). In addition, it has been recently reported that

cannabidiol can inhibit synaptic transmission in both hippocampal slices and cultures *in vitro* in a CB_1 indirect manner by the presumed augmentation of endocannabinoid levels and also in a direct $5HT_{1A}$ receptor dependent manner which can be abolished by *B. pertussis* toxin, which may provide futher evidence for the neuroprotective properties of this compound (Ledgerwood et al., 2010).
These results provide support for the notion that cannabinoids may offer a neuroprotective potential in MS (Zajicek et al., 2005). This is currently being investigated in trials of long-term administration of THC in progressive MS (http://www.pms.ac.uk/cnrg/cupid.php).
Currently Sativex® contains a 1:1 mixture of cannabidiol, but it is feasible that the concentration of CBD could be increased to improve the neuroprotective capacity.

These data are consistent with the ability of cannabinoids to inhibit a number of cell death pathways (Howlett et al., 2003). While CB_1R-deficient animals may more rapidly develop neurodegeneration during EAE (Pryce et al., 2003a), which is resistant to cannabinoid therapy as shown here, suggesting a CB_1R-dependent mechanism that may include: control of excitotoxic glutamate activity; metabolic failure and toxic ion influxes, CB_2R-mediated control of microglial function in neurodegeneration *in vivo*, as well as $CB_{1/2}$-independent effects requires further elucidation (Howlett et al., 2002; Pryce et al., 2003a; Walter et al., 2003; Kim et al., 2006). Current cannabinoid receptor antagonists are cross-reactive with other receptors making interpretation of pharmacological blockade of therapeutic compounds more difficult (Baker et al., 2000; Howlett et al., 2002) and further studies combining pharmacological agents with CB receptor deficient mice are warranted to more precisely determine the neuroprotective role of the cannabinoid system. However, although cannabinoids have the potential for modulating immune responses, results here and elsewhere indicate that their effects on aspects of neuroscience relating to neuroprotection and symptom control are of more relevance to the control of MS.

CHAPTER FOUR

CONTROL OF SPASTICITY IS CB_1, NOT CB_2 RECEPTOR MEDIATED.

4.1. *INTRODUCTION*

There has been recent interest in the therapeutic potential of cannabis for the control of a number of symptoms, notably spasticity that often develops as a consequence of multiple sclerosis (MS. Consroe et al., 1997; Pertwee, 2003). Using cannabinoid agonists and antagonists, we were the first group to provide objective, experimental evidence for the tonic control of spasticity by the cannabinoid system in the EAE model of MS (Baker et al., 2000; Baker et al., 2001). This supported patient claims for the use of medicinal cannabis (Consroe et al., 1997) and has been validated by the modest improvements of symptoms in more recent clinical trials of cannabinoids in MS (Zajicek et al. 2003; Wade et al., 2004; Brady et al., 2004; Vaney et al., 2004; Zajicek et al., 2005; Freeman et al., 2006; Collin et al., 2007). Although the exact cause of spasticity is not definitively known, it is clear that this results from alterations in the balance, possibly secondary to selective neuronal loss, between excitatory and inhibitory neural circuits (Brown, 1994; Dutta et al., 2006). This results in the loss of control of neurotransmission between the muscles and the central nervous system resulting in uncontrolled spastic movements, which in some instances can be treated using GABA receptor agonists (Brown, 1994; Ivanhoe and Reistetter, 2004). Since the initial observations in EAE (Baker et al., 2000), the CB_1 receptor and endocannabinoid system has been shown to regulate synaptic neurotransmission (Wilson and Nicoll, 2001; Howlett et al., 2002) and this action would be consistent with cannabinoid control of spasticity.

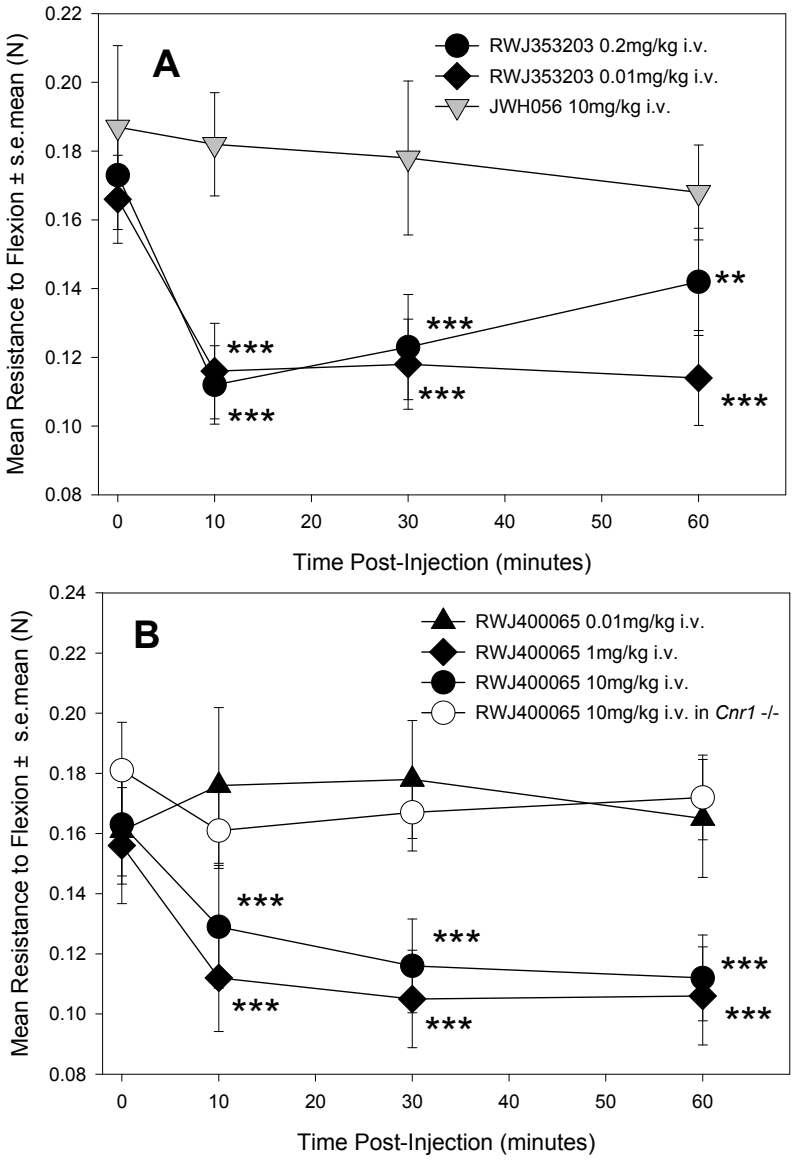
In contrast to CB_1, there is limited evidence to indicate that normal nerve tissues express CB_2 receptors and they appear to be restricted to leucocytes (Howlett et al. 2002, Van Sickle et al. 2005), although they are expressed by glial cells and may be unregulated in inflamed brain tissue (Wotherspoon et al. 2005, Maresz et al. 2005) and therefore may not be anticipated to control problems of neurotransmission. Surprisingly however, a CB_2 agonist ameliorated and an antagonist transiently worsened spasticity in EAE (Baker et al., 2000), suggesting that CB_2 agonists could provide therapies that avoid the psychoactive effects associated with CB_1 agonism (Baker et al., 2000; Howlett et al., 2002; Varvel et al., 2005). In animals, cannabimimetic potential is determined by activity in "tetrad" (hypomotility, hypothermia, ring catalepsy and analgesia) tests, which show no response due to CB_2 stimulation (Howlett et al., 2002). However, currently there are no absolutely specific cannabinoid reagents (agonists or antagonists) available, which solely act on either of the CB_1 or CB_2 receptors and although they may be

selective to one or other of the cannabinoid receptors *in vitro*, at the doses used *in vivo*, there is the potential for cannabinoids to cross-react with the other CB receptor(s) (Pertwee, 1999; Howlett et al., 2002). Furthermore, there is increasing evidence for additional receptors that mediate cannabimemetic effects (Hajos et al., 2001; Howlett et al., 2002; Friede et al., 2002; Begg et al., 2005; Baker et al., 2006), which further complicates the interpretation of pharmacological data. Therefore, receptor-deletion using transgenic technology (Zimmer et al., 1999; Brooks et al., 2002) provides a level of certainty of the role of the CB receptor subtype that is not provided by CB receptor antagonism alone. This was used to re-evaluate CB_2-mediated control of spasticity during EAE.

4.2. *RESULTS*

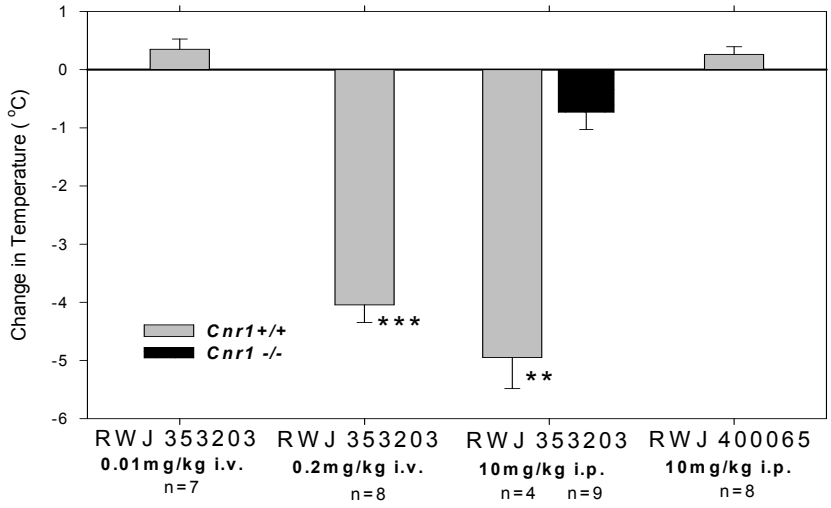
In an attempt to validate our previous studies, which showed an anti-spastic activity of CB_2 agonists (JWH133 [Receptor Affinity. Ki CB_1=680nM CB_2 =3nM.), (Baker et al., 2000), additional compounds were investigated. Surprisingly, 10mg/kg i.v. JWH056, which is less potent at CB_2, but with a lower affinity for CB_1 (Ki > 8μM) than JWH133, failed to inhibit spasticity at 10-60 minutes after injection i.v. (Figure 4.1A), whereas RWJ352303, a potent non-selective CB_1 agonist inhibited spasticity (Figure 4.1 A). However, a dose-dependent anti-spastic activity was detectable following injection i.v. of a potent CB_2 agonist RWJ400065 (Figure 4.1B). This compound has similar binding affinities to JWH133 and failed to induce observable catalepsy, ptosis and hypothermia (Figure 4.2), indicative of CB_1 receptor-mediated effects (Figure 4.2). In contrast RWJ352303 had the potential to induce “tetrad-like” effects (Figure 4.2), but was still active as an anti-spastic agent (Figure 4.1A), at doses that did not induce "tetrad-type" effects, shown here by hypothermic responses (Figure 4.2). However, when 10mg/kg i.v. RWJ400065 was injected into ABH.*Cnr1*-/- mice; there was no apparent anti-spastic activity (Figure 4.1B). To clarify this further, commonly used high affinity CB_1/CB_2 non-selective agonists were examined. However, there was no evidence of inhibition of spasticity in CB_1-deficient mice with either CP55,940 or *R*(+)WIN-55, 212-2 compared to significant ($P<0.001$) inhibitory activity in wildtype mice (Figure 4.3). This suggested that CB_1 and not the CB_2 receptors were actually mediating the inhibitory effects of some CB_2 agonists.

Figure 4.1. *Inhibition of spasticity with $CB_{1/2}$ agonists is CB_1-mediated.*



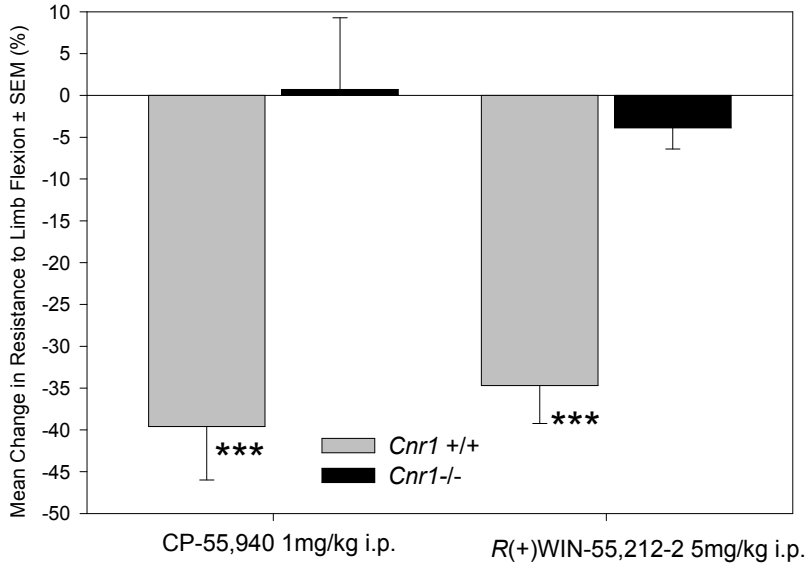
Following the development of spasticity ABH mice were injected i.v. with either: (A) the non-selective agonist RWJ353203 or the CB_2-selective agonist JWH056 or (B) the CB_2-selective RWJ400065 agonist. These received 0.2mg/kg (n=17 limb), 0.01 mg/kg (n=13 limbs) RWJ353203 or 10 mg/kg JWH056 (n=7 limbs) or 0.01 mg/kg (n=12 limbs), 1 mg/kg (n=16 limbs) or 10 mg/kg (n=16limbs) RWJ400065 in wildtype or CB_1-deficient mice (n=12 limbs) in intralipid. The resistance to flexion was measured against a strain gauge. **P<0.01, ***P<0.001 compared to baseline.

Figure 4.2. *Hypothermia induced by cannabinoids.*



Wildtype or CB_1-deficient mice were injected either i.v. or i.p. with the non-selective agonist RWJ353203 or CB_2-selective agonist RWJ400065 in intralipid. The change in body temperature (mean ± SEM) 20 minutes following injection compared to baseline was assessed. **P<0.01, ***P<0.001 compared to baseline by paired *t* tests.

Figure 4.3. *Spasticity is controlled by the CB_1 receptor.*



Wildtype or CB_1-deficient mice (*Cnr1* -/-) were injected intraperitoneally with the full CB_1/CB_2 agonists CP-55,940 (n=8/group) or *R*(+)WIN-55,212-2 (n=14/group). To facilitate visualisation of differences between groups, results are expressed as the mean ± SEM percentage change in the resistance to hindlimb flexion compare to baseline, 10 minutes after the injection of compound. ***P<0.001 compared to baseline by paired *t* tests.

4.3. *DISCUSSION*

Whilst, this study confirms our previous observation (Baker et al., 2000) that "tetrad inactive", apparent CB_2 agonists can show anti-spastic activity, this does not appear to be due to the direct activity of CB_2 receptors. This most likely occurs because CB_2 agonists/antagonists (Baker et al., 2000), or possibly their *in vivo* metabolites, have some affinity for CB_1 receptors that may actually mediate the inhibitory effects. The biology of cannabis and the cannabinoid system now indicates that both tetrahydrocannabinol and CB_1 receptors are the major mediators for both therapy in spasticity and also the adverse side-effects (Howlett et al., 2002; Wilkinson et al., 2003; Varvel et al., 2005). It will be virtually impossible to truly dissociate these two effects, using cannabis. Clinical studies indicate that there is a substantial variability of individuals to tolerate cannabis and tetrahydrocannabinol (Zajicek et al., 2003; Wade et al., 2004; Brady et al., 2004). The apparent therapeutic window, prior to psychoactive effects, appears to be very small and is consistent with the modest effects in symptom control observed so far (Zajicek et al., 2003; Wade et al., 2004; Brady et al., 2004; Zajicek et al., 2005; Freeman et al., 2006), which nevertheless validate our original observations in animal models (Baker et al., 2000; 2001; Wilkinson et al., 2003). This variability of individuals to tolerate cannabinoids means that it will be difficult to adequately dose-titrate with potent CB_1 agonists and that weak CB_1 agonists, such as at the level found with some CB_2 agonists may be preferable for clinical use.

Currently there are two recognised cannabinoid receptors, but there is pharmacological evidence (Howlett et al., 2002; Breivogel et al., 2001; Hajos et al., 2002; Baker et al., 2006; Oz, 2006; Brown, 2007), some of which is disputed (Kawamura et al., 2006; Takahashi and Castillo, 2006), for additional receptors or pathways that mediate cannabimimetic effects. Although the use of gene knockout technology is not without its own limitations, it provides an important tool in target validation. The loss of anti-spastic activity of *R*(+) WIN55,212-2 and CP55,940, both full CB_1/CB_2 agonists, in CB_1-deficient mice supports the indication that CB_1 and not CB_2 is mediating the therapeutic anti-spastic effect. Nevertheless anti-spastic control is feasible in CB_1-deficient animals as shown previously with arvanil (Brooks et al., 2002). Arvanil, a potent transient receptor potential vanilloid type 1 (TRPV1) receptor and weak CB_1 agonist, can also inhibit spasticity in the presence of CB_1/CB_2 antagonists and high doses of the TRPV1 antagonist capsazepine (Brooks et al., 2002). It can also induce cannabimemetic "tetrad"-type responses, such as hypothermia, hypomotility, in wildtype and *Cnr1*-/- mice (Brooks et al., 2002). However, capsazepine is a weak TRPV1 antagonist in mice (Correll et al., 2004) and the hypothermia and the marked hypomotility induced by 0.5mg/kg i.v.

Arvanil is lost in *Trpv1*-/- mice, further indicating the value of receptor knockout animals in target validation. However, cannabinoid receptors can exist as homodimers and novel heterodimer formations between CB_1 receptors and other G Protein coupled receptors are assumed or are generated (Wager-Miller et al., 2002; Kearn et al., 2005; Rios et al., 2006). Therefore CB_1/CB_2 receptor heterodimers or heterodimers between CB_1R and any other molecule to which the CB_2R agonists may bind would not exist in *Cnr1* -/- mice and this may have accounted for the loss of activity of RWJ400065 in CB_1R-deficient mice. Therefore similar studies in *Cnr2*-/- mice will be required to definitively exclude a role for CB_2R in the control of spasticity.

CHAPTER FIVE

CONTROL OF SPASTICITY BY TARGETING THE DEGRADATION OF ENDOCANNABINOIDS BY FATTY ACID AMIDE HYDROLASE AND MONOACYLGLYCEROL LIPASE.

5.1. *INTRODUCTION*

Based on the results in previous chapters, there is a suggestion that our previous data showing control of spasticity with endocannabinoid degradation inhibitors (Baker et al., 2001) may need to be more cautiously interpreted. Many of these pharmacological inhibitors, often based on the structural modifications of anandamide, have low affinity for CB_1 receptors and are inactive in "tetrad" tests, just as CB_2-selective agonists appear to be. Previously, it has been shown that compounds believed to inhibit the putative anandamide transporter, including; AM404, VDM11 (Baker et al., 2001), OMDM-1, OMDM-2 (de Lago et al., 2004), UCM707 (de Lago et al., 2006), 0-2093 and 0-3246 (Ligresti et al., 2006), all exhibit anti-spastic activity. However, many of these agents have activity on additional molecules such as TRPV1 vanilloid receptors and the cannabinoid degrading enzyme: fatty acid amide hydrolase (FAAH), which could account for their biological activity (Ralevich et al., 2001; Fowler et al., 2004). Although a site for membranous diffusion of endocannabinoids has been suggested (Moore et al., 2005), the existence of a specific transporter for anandamide, independent of FAAH, has been questioned (Glaser et al., 2003; Ortega-Gutierrez et al., 2004; Kaczocha et al., 2006) and is probably unlikely to exist (Di Pasquale et al., 2009; Kaczocha et al., 2009). Therefore, until the putative endocannabinoid transporter(s) are identified and cloned, it must be considered likely that the therapeutic, anti-spastic effect of cannabinoid re-uptake inhibitors may be explained by alternative mechanisms. The lack of the true understanding of the diversity of the cannabinoid system and importantly the lack of absolute specificity of current cannabinoid agonists and antagonists (Pertwee, 1999), means that it may be difficult to correctly interpret results, particularly *in vivo,* if using a purely pharmacological approach.

Although there has been recent progress in elucidating the biosynthetic and breakdown pathways of 2-AG (Blankman et al., 2007; Yates and Barker, 2009), few specific compounds have been generated until recently (Long et al,, 2009). Few genetic knockouts involving targets regulating 2-AG production and degradation have been reported until recently, where Diacylglycerol lipase α and β knockout

mice have been generated (Gao et al., 2010, Tanimura et al., 2010). These mice reveal that the major biosynthetic pathway for 2-AG is DAGLα and that retrograde endocannabinoid-mediated signaling is lost in DAGLα knockout animals, whilst being relatively unaffected in DAGLβ knockout mice, which display a lower reduction in 2-AG generation. In addition, adult neurogenesis in both DAGLα and β knockout mice is reduced, compared to wild type animals (Gao et al., 2010). Also recently, inhibitors of MAG lipase, the enzyme responsible for the majority of the degradation of 2-AG have been developed, which elevate CNS levels of 2-AG 8 fold in mice (Long et al., 2009) and a MAG lipase knockout mouse strain has recently been reported which displays significantly elevated levels of 2-AG in the CNS (Chanda et al., 2010). The ability of this additional endocannabinoid pathway to influence the treatment of spasticity is examined here.

The mechanism(s) for anandamide production is unclear compared to its breakdown (Yates & Barker, 2009). Whilst it had been suggested that NAPE would a major player in anandamide biosynthesis, the observation that genetic depletion of NAPE does not particularly influence anandamide levels (Leung et al., 2006; Simon & Cravatt, 2006), suggests that there are other compensatory pathways for anandamide production and so this molecule may not be particularly "drugable". In contrast, the observation that genetic depletion of FAAH results in elevated levels of anandamide but not 2-AG suggests that this may be an important target for control of anandamide-sensitive functions (Cravatt et al., 2001; Saario et al., 2006). We have previously reported that AM374, which is an inhibitor of FAAH can inhibit spasticity (Baker et al. 2001). Using *Faah* gene knockout mice (ABH.*Faah* -/-) it will be possible to verify the activity of some FAAH inhibitors (Boger et al., 2000) as anti-spastic agents.

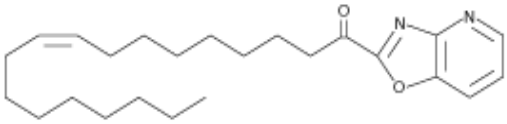
5.2. *RESULTS*

5.2.1. *Amelioration of experimental spasticity by inhibition of anandamide degradation by FAAH inhibitors.*

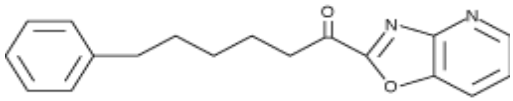
A number of FAAH reversible and irreversible FAAH inhibitors have been described (Boger et al. 2000). To date the most potent inhibitor is CAY10402 (Ki hFAAH = 0.0001μM Boger et al. 2000. Figure 5.1). This and CAY10400 ((Ki hFAAH = 0.001μM Boger et al. 2000. Figure 5.1) both inhibited spasticity (Figure 5.2A,B) at doses that did not induce any hypothermia (Table 5.1). On a dose/weight basis CAY10402 was marginally more potent than CAY10400 which is reflective of the increased potency of CAY10402 at inhibiting FAAH (Boger et al. 2000).

Figure 5.1. *Structure of Fatty Acid amide hydrolase inhibitors.*

CAY10400



CAY10402



URB 597

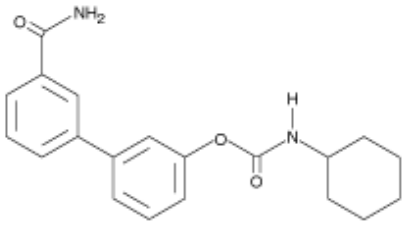
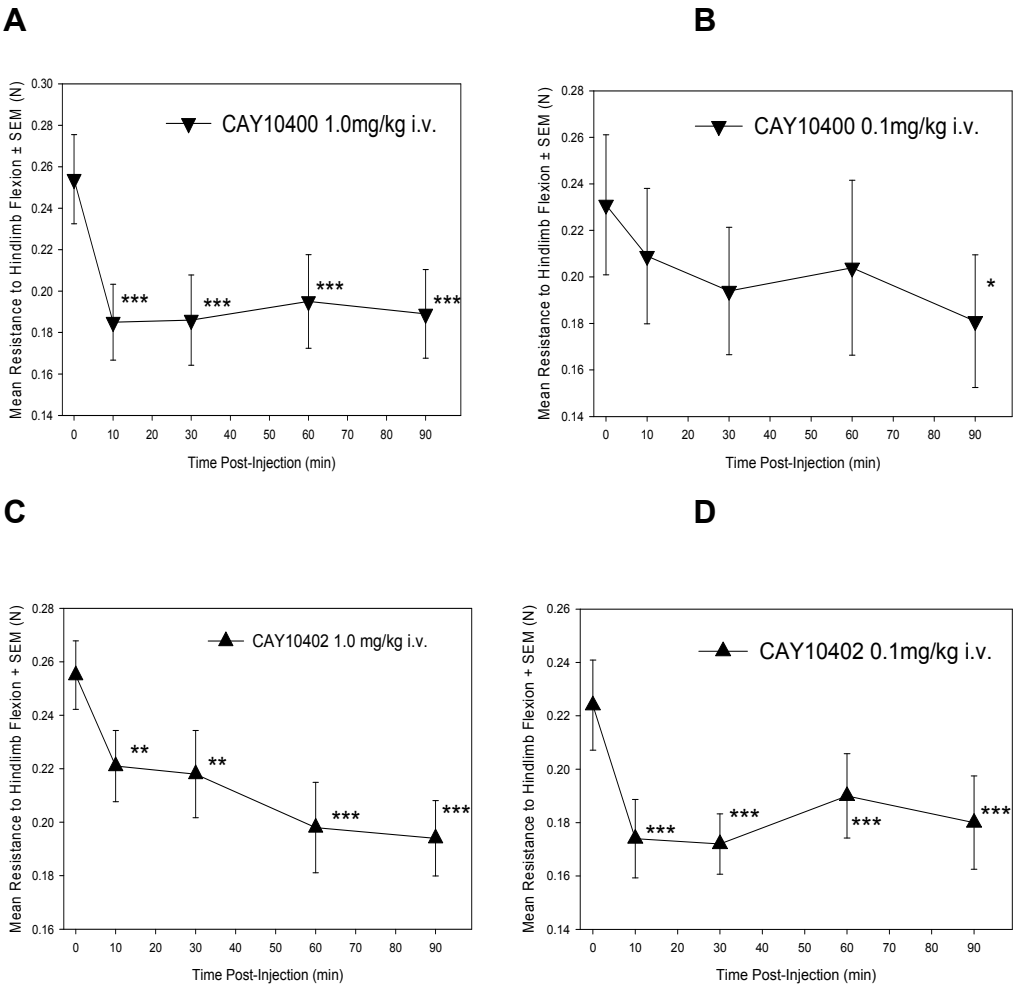


Table 5.1. *Fatty Acid Amide Hydrolase Inhibitors do not induce "tetrad" effects at therapeutic doses compared to the fully CNS penetrant CB1 agonist SAB722.*

			Temperature Change°C	
Compound	Dose	n	Mean ± SD	p
CAY10402	1.0mg/kg	7	0.47 ±0.57	n.s.
	0.1mg/kg	7	0.27 ±0.23	n.s.
CAY10400	1mg/kg	7	-0.36 ± 0.40	
	0.1mg/kg	7	0.10 ± 0.15	n.s.
SAB722	1mg/kg	7	-3.66 ± 0.84	P<0.001
	0.1mg/kg	7	0.63 ± 1.25	n.s.
	5mg/kg in *Cnr1*-/-	4	- 0.38 ± 0.25	n.s.

The temperature was measured under the hindlimb and then the compound was injected and the body temperatures of ABH mice were measured 20minutes later. The change in temperature was assessed using paired t tests.

Figure 5.2. *Inhibition of Spasticity with Fatty Acid Amide Hydrolase Inhibitors.*

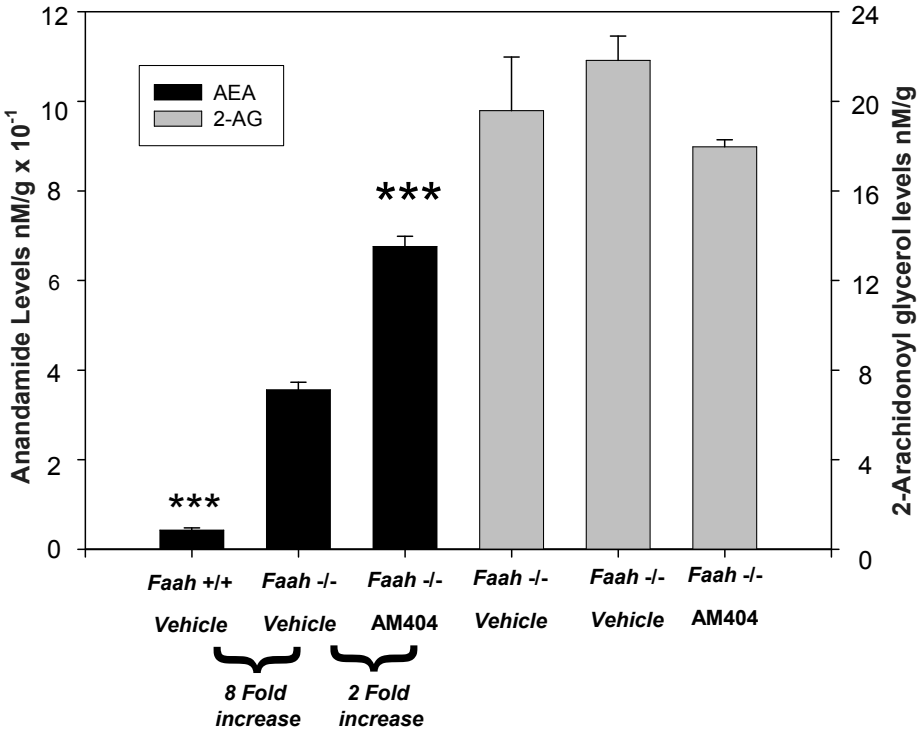


Following the development of spasticity ABH mice were injected i.v. with either: (**A, B**) CAY10400 or (**C,D**) CAY10402. These received (**A**) 0.1mg/kg (n=10 limbs from 7 animals), (**B**) 1.0 mg/kg (n=10 limbs from 7 animals) CAY10400 or (**C**) 1.0mg/kg (n=12 limbs from 7 animals) or (**D**) 0.1mg/kg (n=12 limbs from 7 animals) CAY10402 in intralipid. The resistance to flexion was measured against a strain gauge.*P<0.05, **P<0.01, ***P<0.001 compared to baseline.

Fatty acid amide hydrolase-deficient animals were used to verify whether FAAH was a realistic target for the control of spasticity. C57BL/6.*Faah*$^{-/-}$ were backcrossed with ABH mice to produce ABH.*Faah*$^{-/-}$. These mice demonstrated over an 8 fold increase in the levels of anandamide in the brains of ABH.*Faah*$^{-/-}$ mice as shown by liquid crystallography mass spectroscopic analysis (Baker et al. 2001) of the endocannabinoid anandamide (AEA) levels (Figure 5.3). Levels of 2-AG were unchanged. (Figure 5.3). Interestingly, there was a further increase in anandamide levels following the injection of AM404 (10mg/kg i.v.), a putative anandamide re-uptake inhibitor. This demonstrates that AM404 has a biological activity that is independent of FAAH, even if there is no specific transport molecule. However, there appeared to be compensation at the level of the CB_1 receptor as there was;

(a) no evidence of altered CB_1 receptor expression as assessed by ligand binding (Table 5.2) or *in situ* hybridization (Table 5.3 and importantly (b) little evidence of altered CB_1 receptor signaling (Table 5.4) in the brains of FAAH-deficient mice.

Figure 5.3. *Anandamide levels are elevated in FAAH deficient mice.*



Endocannabinoid levels were measured in the spinal cords (expelled from the spinal column using hydrostatic pressure and frozen in liquid nitrogen within 60s from death) of wildtype and FAAH (ABH.Faah-/-) knockout mice. This was performed as described previously (Baker et al. 2001). Mice were injected with 0.1ml of vehicle (ECP) or with 10mg/kg i.v. of the anandamide re-uptake inhibitor AM404 (Tocris, Bristol, UK) 30 minutes earlier. The results represent the mean ± SEM (n=4-5/group). This demonstrates that AM404 has a biological activity that is independent of FAAH and that knockout of the *Faah* gene enhances anandamide, but not 2-AG, levels. *** P<0.001 compared to vehicle treated FAAH knockout mice. **Analysis was performed by Tizania Bisogno and Vincenzo Di Marzo, Naples, Italy.**

Table 5.2. *CB_1 receptor mRNA levels (arbitrary units of optical density) in several brain regions of wildtype and FAAH knockout mice.*

Brain Region	Anatomical Location	Relative CB_1 Expression (AU) Wildtype	ABH.*Faah*$^{-/-}$
Cerebral cortex	Superficial layer (II-III)	0.149 ± 0.008	0.179 ± 0.019
	Deep layer (V-VI)	0.108 ± 0.006	0.105 ± 0.006
Basal ganglia	Lateral caudate-putamen	0.325 ± 0.021	0.349 ± 0.019
	Medial caudate-putamen	0.168 ± 0.011	0.182 ± 0.014
Hippocampus	Ammon's horn	0.157 ± 0.012	0.177 ± 0.011
	Dentate gyrus	0.231 ± 0.015	0.236 ± 0.013
Cerebellum	Granular layer	0.555 ± 0.015	0.589 ± 0.032

Radioactive i*n situ* hydridization to detect [^{35}S]-labeled oligonucleotide reactive with *Cnr1* mRNA was performed on coronal 20µm brain sections from either wildtype (WT) or FAAH knockout mice as described previously (Cabranes et al. 2006). Values are means ± SEM of 6-7sections per group. Data were assessed by Student's t-test. **This was performed by Anna Cabranes, Madrid, Spain.**

Table 5.3. *CB_1 receptor binding (fmol/mg of tissue), analyzed by [^{3}H]CP55,940 autoradiography in several brain regions of wildtype and FAAH knockout mice.*

Brain Region	Anatomical Location	CB_1 Expression (fmol/mg of Tissue) Wildtype	ABH.*Faah*$^{-/-}$
Cerebral cortex	Superficial layer (I)	111.4 ± 5.5	115.1 ± 6.5
	Deep layer (VI)	82.7 ± 4.6	73.9 ± 4.1
Basal ganglia	Lateral caudate-putamen	153.8 ± 9.0	166.2 ± 7.5
	Medial caudate-putamen	120.5 ± 8.0	135.1 ± 6.6
	Globus pallidus	206.8 ± 7.0	212.6 ± 5.7
	Entopeduncular nucleus	204.8 ± 21.6	218.1 ± 15.2
	Substantia nigra	426.5 ± 22.0	438.4 ± 21.0
Hippocampus	Ammon's horn	127.9 ± 5.2	123.3 ± 5.4
	Dentate gyrus	102.9 ± 4.8	95.6 ± 4.1
Cerebellum	Molecular Layer	217.5 ± 4.1	224.0 ± 7.6

Autoradiography of [^{3}H]CP55,940 ligand binding was performed on coronal 20µm brain sections from either wildtype (WT) or FAAH knockout mice as described previously (Cabranes et al. 2006). Values are means ± SEM of 6-7sections per group. Data were assessed by Student's t-test. **This was performed by Anna Cabranes, Madrid, Spain.**

Table 5.4. *WIN-55,212-2- Stimulated [^{35}S]GTPγS binding (% of stimulation over basal binding) in several brain regions of wildtype and FAAH knockout mice.*

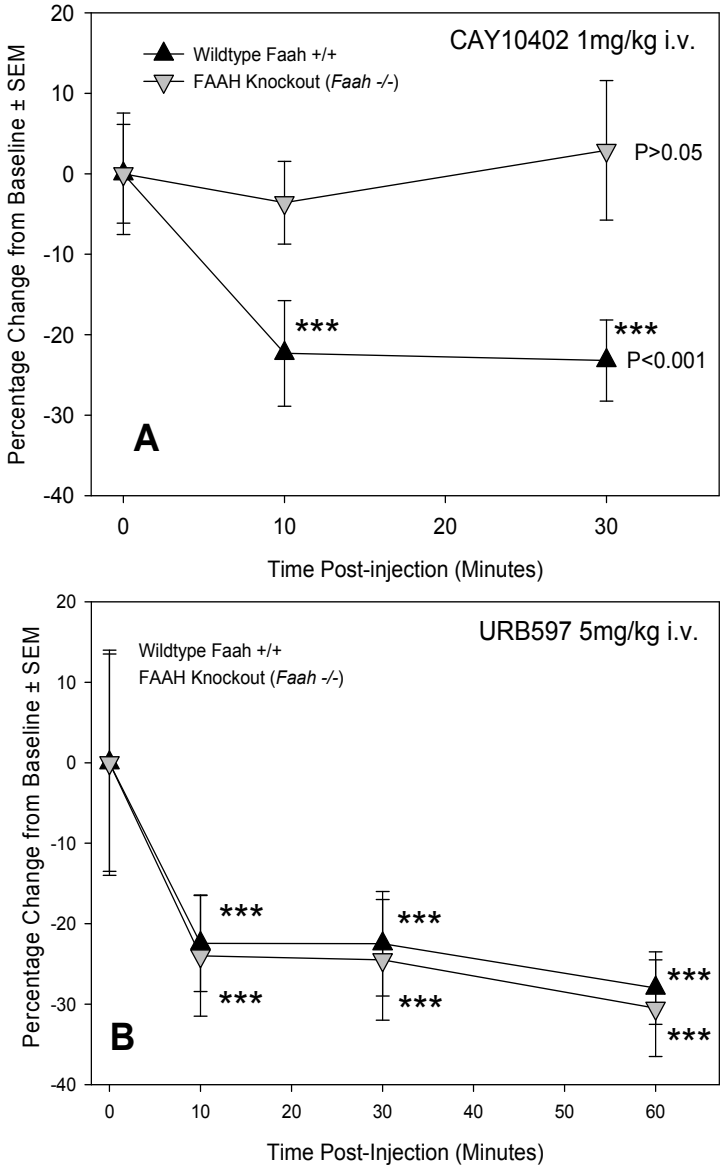
Brain Region	Anatomical Location	Relative CB_1 Expression (AU) Wildtype	ABH.*Faah*$^{-/-}$
Cerebral cortex	Superficial layer (I)	163.4 ± 18.0	156.9 ± 7.6
	Deep layer (VI)	150.4 ± 8.2	141.2 ± 7.7
Basal ganglia	Lateral caudate-putamen	174.0 ± 12.6	154.6 ± 6.0
	Medial caudate-putamen	166.7 ± 9.8	154.0 ± 5.9
	Globus pallidus	203.5 ± 5.0	185.9 ± 12.4
	Entopeduncular nucleus	377.2 ± 23.9	374.1 ± 28.6
	Substantia nigra	403.8 ± 20.0	334.5 ± 10.4*
Hippocampus	Ammon's horn	190.5 ± 7.9	195.6 ± 13.2
	Dentate gyrus	180.1 ± 11.0	180.4 ± 14.5
Cerebellum	Molecular Layer	252.9 ± 13.3	222.7 ± 4.5*

WIN-55,212-2-stimulated autoradiography of [^{35}S]GTPγS binding was performed on 20μm coronal brain sections from either wildtype (WT) or FAAH knockout mice as described previously (Cabranes et al. 2006). GTPγS binding was assessed in the presence and absence of the cannabinoid receptor agonist. Values are means ± SEM of 6-7sections per group. Data were assessed by Student's t-test. *P,0.05 compared to wildtype controls. **This was performed by Anna Cabranes, Madrid, Spain.**

5.2.2 *Anti-spastic activity of CAY10402 is lost in FAAH deficient mice whilst the anti-spastic activity of URB 597 is retained.*

It was found that the anti-spasticity activity of CAY10402 was lost when the same dose of compound was injected into FAAH-deficient mice (Fig 5.4A). This validated FAAH as a target for therapy. However, CAY10400 and CAT10402 are unlikely to be developed as therapeutic drugs because these drugs exhibit poor pharmacokinetics (Iain Janes, Novartis London. Personal Communication to D.Baker). URB597 is also a potent (IC_{50} = 4.6 μM FAAH) FAAH inhibitor which exhibits good pharmacokinetics (Piomelli et al. 2006). However whilst this compound also inhibited spasticity (Fig 5.4B) at a dose of 5mg/kg i.v. it was also active in FAAH deficient mice (Fig 5.4B), indicating that this compound had additional off-target specificites. This further demonstrated the importance of the use of knockout mice in validating targets for therapy.

Figure 5.4. *Inhibition of Spasticity with FAAH Inhibitors in wildtype and FAAH-deficient mice.*

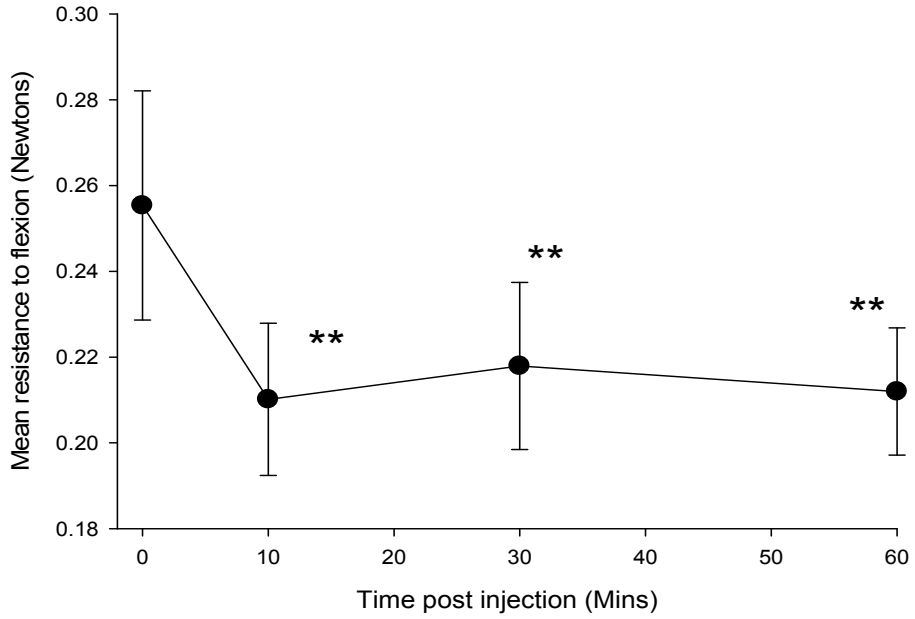


Wildtype or FAAH-deficient mice (*Faah* -/-) were injected with (A) 1mg/kg CAY10402 i.v. in intralipid or (B) 5mg/kg i.v. URB597 in Ethanol:Cremophor:PBS (1:1:18) and the resistance to hindlimb flexion was assessed using a strain gauge at baseline and following injection of drug (n=12-14/group). To facilitate visualization of differences between groups, results are expressed as the mean ± SEM percentage change in the resistance to hindlimb flexion compare to baseline and 10-60 minutes after the injection of compound. ***P<0.001 compared to baseline by paired *t* tests.

5.2.3 *2-AG-mediated inhibition of spasticity by inhibition of 2-AG degradation by MAG Lipase inhibition.*

Inhibition of spasicity in CREAE mice was also seen with the administration of the 2-AG degradation inhibitor JZL184, which targets the 2-AG degradation enzyme MAG Lipase, to animals displaying spastic hind limbs. This anti spastic effect persisted for at lest 60 minutes after administration and identifies the 2-AG pathway as another potential therapeutic taget for therapeutic intervention in spasticity in addition to targeting anandamide degradation pathways (Fig 5.5).

Figure 5.5. *Inhibition of Spasticity with the MAG lipase inhibitor JZL184.*



Following the development of spasticity ABH mice were injected i.v. with 5 mg/kg JZL184 in vehicle Ethanol:Cremophor:PBS (1:1:18) and the resistance to hindlimb flexion was assessed using a strain gauge at baseline and following injection of drug (n=16 hind limbs from 8 animals). The resistance to flexion was measured against a strain gauge.*P<0.05, **P<0.01, ***P<0.001 compared to baseline.

5.3. *DISCUSSION*

Although it has been previously shown that endocannabinoid degradation inhibitors can limit spasticity during EAE (Baker et al. 2001, de Lago et al 2006, Ligresti et al 2006), using FAAH-deficient mice it has been possible to provide definitive evidence that pharmacological manipulation of the FAAH degradative enzyme for endocannabinoids resulting in increased endocannabinoid levels such as anandamide, can be a target for symptom control such as spasticity.

It has previously been shown that there are elevated levels of anandamide in the spinal cord during spastic EAE and that CB_1 receptor antagonism transiently increased the level of spasticity (Baker et al. 2000, 2001). This may indicate that receptor tolerance may operate in the lesional areas or the endogenous elevated levels of anandamide in these areas are still not sufficient to control spasticity but it also suggested that the endocannabinoids were providing an endogenous tonic control mechanism. Anandamide levels are increased in a number of experimental models of CNS pathological events such as traumatic brain injury, cerebral ischaemia, seizure, Parkinson's disease and multiple sclerosis (Baker et al, 2001, Hwang et al 2009) and have been implicated as an endogenous neuroprotective mechanism in conditions of CNS insult. Genetic deletion of FAAH in SOD-1 mice, a model of motor neurone disease, results in a delayed onset of signs of disease in these animals (Bilsland et al 2006). Furthermore, FAAH knockout mice showed an improved clinical outcome after EAE induction with a reduction in clinical score in remission despite equivalent levels of inflammatory infiltrates compared to wild type controls, indicating the protective effect of elevated anandamide levels on neuronal survival during CNS inflammation (Webb et al 2008).

Whereas the degradative pathway for anandamide appears to be mediated almost exclusively by the enzyme FAAH, it appears that the situation for the endocannabinoid 2-AG appears more complex. Although FAAH can degrade 2-AG *in vitro*, 2-AG levels were essentially unaltered in FAAH-deficient mice *in vivo*. It appears that FAAH is responsible for only less than 1% of degradation of 2-AG (Blankman et al.2009), whereas monoacylglycerol (MAG) lipase appears to be responsible for approximately 85% of 2-AG degradation with the remaining 15% mediated by the uncharacterised enzymes ABHD6 and ABHD12 (Blankman et al., 2009). These three enzymes are reported to display distinct, subcellular profiles, possibly indicatiing that different pools of 2-AG are controlled in the CNS by these enzymes (Blankman et al., 2009). Whilst the tools such as selective inhibitors and knockout mice are available to investigate the actions of anandamide have been generated as shown here, specific inhibitors of MAG lipase are only beginning to be

reported (Long et al. 2009). However, in contrast to the lack of cannabimimetic effects following inhibition of FAAH and elevation of anandamide (Baker et al 2000; 2001), the observation that the concomitant increase in brain 2-AG levels following inhibition of MAG lipase was accompanied by cannabimimetic effects, which were blocked by CB_1 receptor antagonism, in the mouse tetrad tests, showing significant hypothermia and hypomotility (Long et al., 2009). Tetrad effects have not been reported for FAAH inhibitors, except for analgesia (Cravatt and Lichtman, 2003). This may reflect the observation that anandanide is a partial agonist at the CB_1 receptor with a lower intrinsic efficacy than 2-AG, which is a full agonist at CB_1 receptors and the fact that 2-AG is about a thousand fold more abundant in the brain than anandamide (Gonsiorek et al.,2000; Baker et al., 2001). Furthermore, repeated augmentation of 2-AG by MAG-lipase inhibition resulted in; cannabinoid receptor tolerance, loss of analgesic activity, impaired endocannabinoid-dependent synaptic plasticity and physical dependence which is not observed with the inhibition of FAAH and the elevation of anandamide levels (Schlosburg et al., 2010). CB_1 receptor desensitization and a reduction in cannabimimetic activity of CB_1 receptor agonists is also seen in mice with genetic deletion of MAG lipase and a concomitant increase in 2-AG levels in the CNS (Chanda et al., 2010). These results suggest that pharmacological elevation of 2-AG levels may have similar drawbacks to the use of CB_1 receptor agonists in patients, including psychoactive effects resulting from CB_1 receptor agonism, which may limit their usefulness as a clinically therapeutic target.

Compound CAY10402 was selected for these studies as it was reported to be the most potent FAAH inhibitor (Boger et al., 2000). Other compounds such as OL-135 and URB597 also display potent FAHH inhibitory activity, OL-135 is reversible (Boger et al, 2005), whereas URB597 binds irreversibly to FAAH (Kathuria et al, 2003). The submaximal efficacy of reversible FAAH inhibitors with transient increases of anandamide such as Ol-135 *in vivo* may reflect rapid metabolism of these compounds, which must be coupled with the observation that approximately 85% inhibition of FAAH is required for sustained anandamide elevation (Fegley et al 2005). Whilst poor pharmacokinetics will limit the utility of CAY10402 (Personal communication Iain James, Novartis, London UK to D. Baker) using i.v. administration it was possible to demonostrate that both CAY10402 and CAY10400 inhibited spasticity in a FAAH-dependent manner. URB597 is an orally-active, potent inhibitor of FAAH that gives long term elevation of anandamide (Kathuria et al, 2003). Whilst this compound could inhibit spasticity it was also active in FAAH-deficient mice indicating that this compound has off-target specificity. This is further evidenced by reported analgesic effects of URB597 via PPAR α nuclear receptor stimulation via increased endocannabinoid levels (Sagar et al, 2008)).

Whilst OL-135 and CAY-10402 have also been reported to have off target effects by the reported inhibition of serine hydrolases in peripheral tissues (Ahn et al., 2009) but experiments in FAAH-deficent mice allows the confirmation of the value of FAAH as a target for control of spasticity. The observation that URB597 reduces limb stiffness in FAAH knockout mice indicates that this compound also has effects on targets other than FAAH. In contrast the anti spastic effects of CAY10402 are lost in FAAH deficient animals suggesting a much greater selectivity for FAAH than URB597. FAAH inhibitors can also increase the levels of other fatty acid amides such as oleoylethanolamide (OEA) and N-palmitoyl ethanolamine (PEA), which act on non-cannabinoid receptors such as GPR119 and may act to enhance or antagonise the effects of anandamide (Farrell and Merkler, 2008; Godlewski et al., 2009).

It is interesting that whilst FAAH-deletion results in elevated levels of anandamide as shown here and in previous studies (Cravatt et al., 2001), it is clear that this triggers compensatory mechanisms as the CB_1 receptors do not signal substantially more than wild type animals despite the presence of about ten-fold elevated levels of anandamide. However, the role of anandamide in control of synaptic transmission may be more important during pathology. Whilst short term synaptic signalling appears to regulate synaptic transmission via iontrophic induction of 2-AG production, chronic glutamate release as occurs during pathology leads to stimulation of metabotrophic glutatemate receptors and anandamide control of synaptic neurotransmission (Maejima et al., 2001). This suggests that FAAH and anandamide may be more important therapeutic targets than 2-AG in pathological events in the CNS, although elevation of 2-AG by MAG Lipase inhibition, shown here is also effective in the amelioration of spasticity in mice, indicating that this pathway is also a potential therapeutic target for the development of anti-spastic agents. However, the reported central role of 2-AG in retrograde inhibition of synaptic transmission suggests that the therapeutic elevation of 2-AG levels may come with significant cannabimimetic side effects (Long et al., 2009), which may limit their therapeutic usefulness.

Despite caveats as to the potential side-effects such as fertility problems resulting from the inhibition of FAAH (Maccarone et al., 2002a; 2002b; Wang et al., 2006; Sun et al., 2009), drug induced increases in anandamide levels shows a potential therapeutic benefit not only in the treatment of neurological resulting from nerve damage, but also may limit this damage via the neuroprotective properties of the endogenous cannabinoid system. Therefore, FAAH degradation inhibitors may prove to be a novel class of compounds that can be used for the therapy of human disease.

CHAPTER SIX

CONTROL OF SPASTICITY BY CNS-EXCLUDED CB_1 RECEPTOR AGONISTS: PRODUCTION OF VSN16- A NOVEL PUTATIVE GPR55 MODULATOR.

6.1. *INTRODUCTION*

Using experimental allergic encephalomyelitis (EAE) models of MS, we have shown that the cannabinoid system exhibits tonic control of spasticity (Baker et al., 2000; 2001). Tetrahydrocannabinol (THC) and CB_1R are the important mediators for the control of spasticity by cannabis (Wilkinson et al., 2003; Chapter 4). Unfortunately, THC also mediates the unwanted, psychotrophic effects of cannabis due to CB_1R stimulation in certain cognitive-control centres in the brain (Howlett et al., 2002; Varvel et al., 2005). Plant-derived cannabinoid compounds are extremely hydrophobic molecules and rapidly enter the CNS. Therefore, as cannabis has no mechanism with which to selectively target the motor-centres that control spasticity, its use will be invariably be associated with psychoactive effects. Whilst these may be avoided through dose-titration, it means that these doses will be suboptimal and that cannabis will have a narrow therapeutic window in terms of its effect versus side-effect profile. This may in part account for the modest clinical effects of medicinal cannabis in trials, where the drug was dose-titrated to limit psychoactive effects (Zajicek et al., 2003; Wade et al., 2004; Zajicek et al., 2005). This therapeutic window may be greatly enlarged if CB_1R stimulation in cognitive-control centres of the brain is avoided.

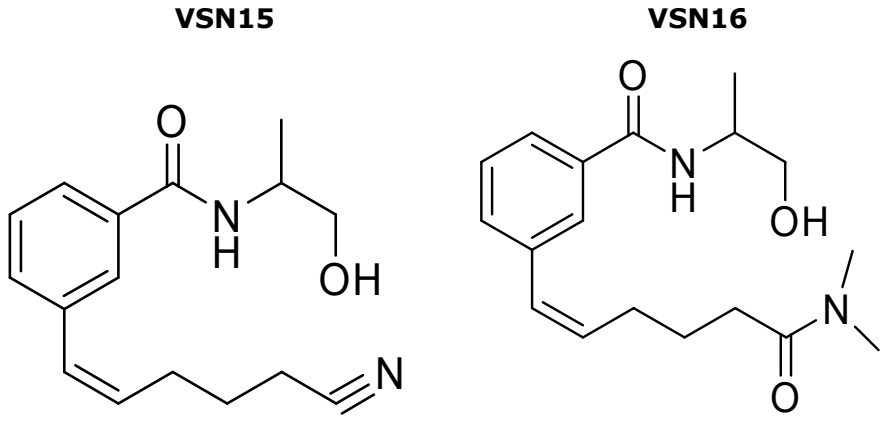
The blood:brain barrier (BBB) excludes molecules from entering the CNS and this is formed from the actions of astrocytes and specialized CNS endothelial cells (Colabufo et al., 2009; Wolburg et al., 2009). These endothelial cells have tight junctions, which exclude hydrophilic molecules, and a number of multi-drug resistance exclusion pumps (Feher et al,, 2000; Dallas et al., 2006; Colabufo et al., 2009; Wolburg et al., 2009). Exclusion of cannabinoids from the brain therefore, may be achieved by synthesizing compounds that are polar and/or targeted to ABC transporters.

6.2. *RESULTS*

6.2.1. *VSN16 is a hydrophilic water soluble compound which does not induce CNS cannabimimetic effects.*

In an attempt to make CNS-excluded, CB_1R agonists, a number of compounds based around monocyclic alkyl amide cannabinoid receptor ligands were made (Berglund et al., 2000; Hoi et al., 2007). These were synthesised by Cristina Visintin and David Selwood at the Wolfson Institute, University College London, to contain polar elements that would promote exclusion from the CNS via the blood:brain barrier (Feher et al., 2000). One of these compounds, VSN16 was found to be water soluble (at least 30mg/ml. Figure 6.1).

Figure 6.1. *Chemical Structure of VSN15 and VSN16.*

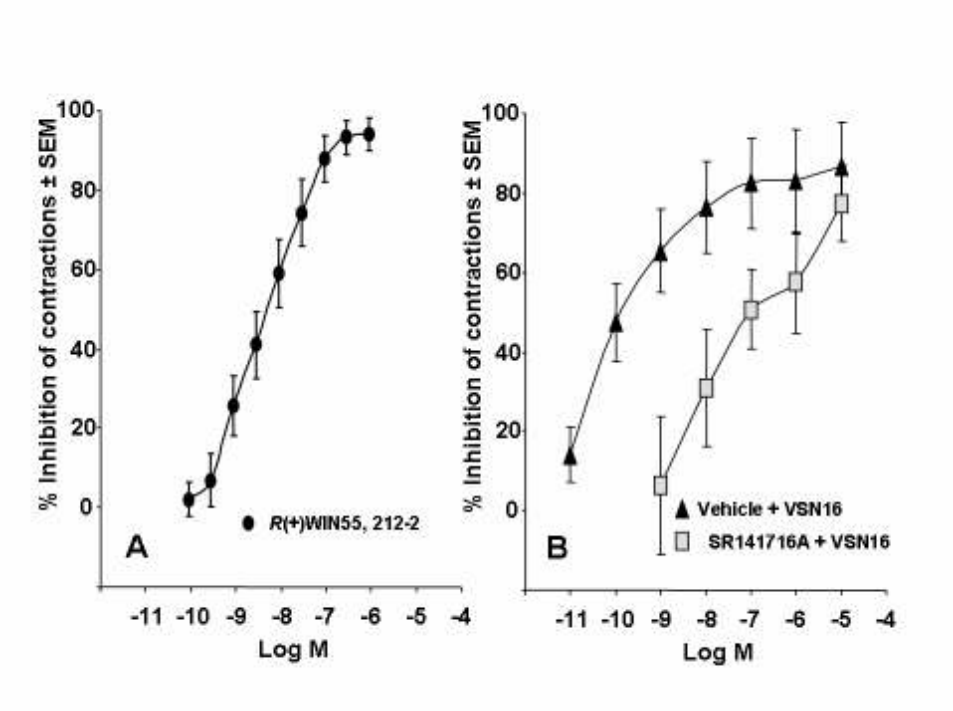


The CB_1R affinity of VSN16 (racemate) was first assessed using the mouse vas deferens contraction assay (Pertwee et al., 1992). VSN16 exhibited potent inhibitory activity with a IC_{50} in the low nM range, which compared favourably with the potent CB_1R/CB_2R agonist *R*(+)WIN55-212 (Figure 6.2A,B). The activity of VSN16 was inhibited by incubation with the CB_1R antagonist SR141617A (Figure 6.2B). The *in vivo* activity of VSN16 was assessed in "tetrad tests" to detect cannabimimetic effects (Howlett et al. 2002). Initially, the intravenous route was used as this avoids any issues of first pass metabolism. Although VSN16 was at least as potent as *R*(+)WIN55-212 in the vas deferens assay (Figure 6.2), it failed to induce any visible signs of sedation and did not induce significant hypomotility or hypothermia (Figure 6.3) that are indicative of cannabimimetic effects in rodents (Howlett et al. 2002). Further behavioural testing, performed by MDS Pharma, Taiwan indicated that rats treated with 120mg/kg p.o. did not display any adverse behavioural (Alertness, Passivity, Stereotypy, Vocalizations, Transfer Reactivity, Touch, Escape, Tail-Pinch, Toe-Pinch, Pinna Reflex, Corneal Reflex, Startle

Response, Visual Placing responses); *neurological* (Body Elevation, Limb Position, Tail Elevation, Limb Tone, Grip Strength, Body Tone, Abdominal Tone, Change in Gait, Catalepsy, Righting Reflex, Twitches, Convulsion (Clonic, Tonic)) or *autonomic* (Palpebral Size, Excretion (Urination, Diarrhea), Secretion (Salivation, Lacrimation), Piloerection, Body Temperature, Skin Colour (Blanch, Flush, Cyanosis), Respiration (Fast, Slow, Deep, Irregular) or Death effects.

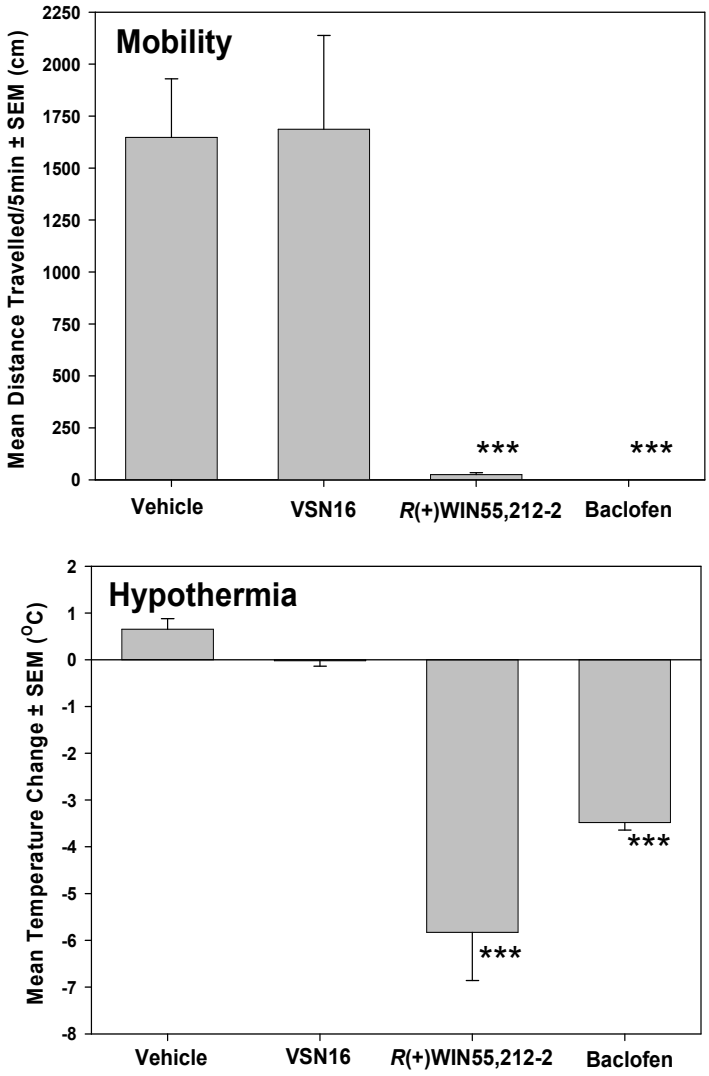
The chemical structure can be used to predict brain permeability, where a logBB brain:blood coefficient of -1.0 would indicate that compounds are excluded from the brain [Feher et al., 2000]. VSN16 has a logBB of -0.66, which would predict approximately 80% CNS exclusion and pharmacokinetic studies demonstrated a plasma:blood ratio at C_{max} of 0.16 (Figure 6.4). This suggested initially that VSN16 may represent a CNS-excluded, CB_1R agonist.

Figure 6.2. *Inhibition of contractions in the vas deferens by VSN16 racemate.*



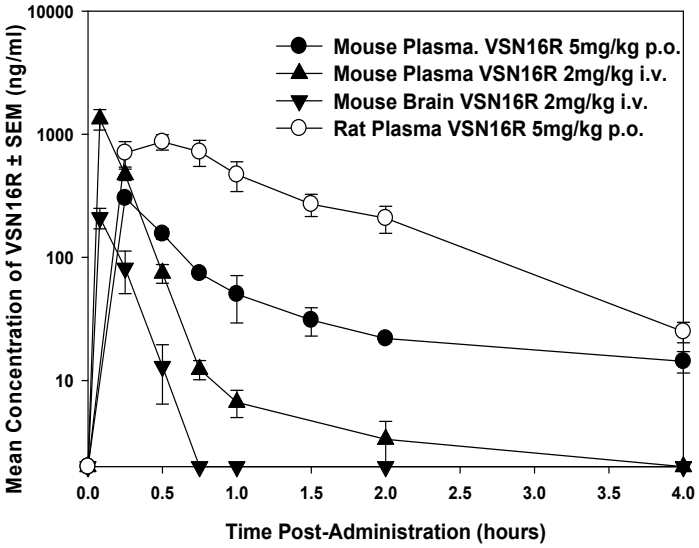
The mouse vas deferens were isolated and contractions were monitored (Pertwee et al., 1992) following incubation with various concentrations of either: (A) R(+)WIN55,212 or (B) VSN16. In some instances tissues were pretreated 30min earlier with either dimethylsulphoxide vehicle or 31.6nM SR141617A in DMSO. The results represent the mean ± SEM of 5-6 replicates. **The studies were performed by the group of Prof. Roger Pertwee, University of Aberdeen.**

Figure 6.3. *Absence of cannabimimetic effects induced by VSN16.*



ABH mice were injected iv. with either 1mg/kg VSN16 racimate or 1mg/kg i.v. *R*(+)WIN55,212-2 or ECP vehicle or 5mg/kg i.v. of Baclofen in PBS. Mobility in an open field activity chamber and temperature change were assessed 20min after injection. The results represent the mean ± SEM (n=5) distance traveled and the degree of temperature change. ***P<0.001 compared to vehicle. 20mg/kg i.v. VSN16 likewise failed to induce hypothermia.

Figure 6.4. *Pharmacokinetics of VSN16R.*

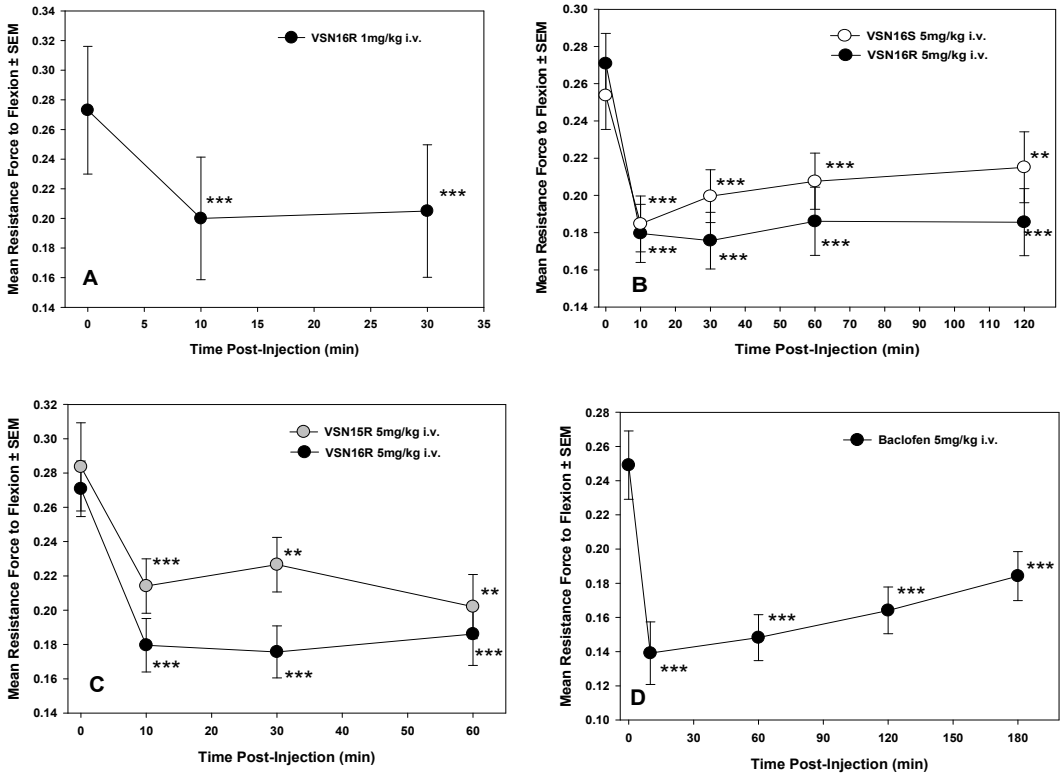


Blood (plasma) and in some instances brain samples were obtained prior to and following intravenous and oral administration of VSN16R into mice (CD-1®) and rats (Sprague Dawley). The results represent the mean ± SEM (n=3) amount of VSN16R is tissues as assessed using liquid chromatography-mass spectrometric assays. **This study was performed by Inpharmatica, Cambridge, UK.**

As CB_1R agonists can inhibit the spasticity that develops as a result of nerve damage caused by autoimmune attack of the CNS (Baker et al. 2000), it was investigated whether VSN16 could inhibit spasticity during EAE. It was found that VSN16 (racemate) inhibited spasticity in EAE at doses that failed to induce cannabimimetic effects (Figure 6.5A). Both of the S(-) and R(+) enantiomers of VSN16 were synthesised and both were found to be active in the inhibition of spasticity, although VSN16R appeared slightly more active than VSN16S (Figure 6.5B). Likewise, VSN16R appeared slightly more active than VSN15R (Figure 6.5C), which is consistent with the *in vitro* data in arteriole relaxation (Hoi et al., 2007). Baclofen is a $GABA_B$ receptor agonist that is an anti-spastic therapeutic agent used in humans. This inhibited spasticity (Figure 6.5D), however just as can occur in people, this dose of baclofen tended to cause flaccidity in the animals, hypomotility and hypothermia (Figure 6.3), probably due to neurotransmitter inhibitory effects. Therefore VSN16 induced a comparable inhibition of spasticity as Baclofen but exhibited a better side-effect profile, which is the chief reason for non-compliance with many anti-spastic agents in patients.

6.2.2. *VSN16 inhibits spasticity associated with chronic EAE*

Figure 6.5. *Intravenous VSN15 and VSN16 inhibit spasticity in CREAE.*



ABH mice were immunized with spinal cord antigens in Freund's adjuvant to induce relapsing EAE. Following the development of spasticity, animals were injected intravenously with (**A**) 1mg/kg VSN16 racimate (n=6 animals, n=11 limbs), (**B**) 5mg/kg of either the VSN16R or VSN16S enantiomers (n=7 animals, n=13 limbs) or (**C**) 5mg/kg VSN15R or VSN16R (n=7 animals, n=13 limbs). The results represent the mean ± SEM forces to bend hindlimbs to full flexion against a strain gauge. * P<0.05, ** P<0.01, ***P<0.001 compared to baseline.

The metabolic stability of VSN16 was assessed first *in vitro* (Table 6.1) *a*nd then *in vivo* (Table 6.2.). The compounds VSN15, VSN16R, VSN16S were stable against degradation by liver microsomes and plasma compared to positive controls (Table 6.1). When the *in vivo* pharmacokinetic responses were assessed in both mice and rats, clearance of compound administered via the intravenous route was relatively fast (Half-life of 7-11min. Table 6.2) compared with delivery via the oral route (Half-life of 43-89min. Table 6.2). Oral absorption was rapid and C_{Max} was detected within 15min from administration in mice (Figure 6.2), indicating good absorption from the gastrointestinal tract and good oral bioavailability was evident (Table 6.2). Therefore the action of oral VSN16R was analysed (Figure 6.6).

Table 6.1. *In vitro pharmacokinetic stability of VSN16-related compounds.*

Compound	Half-life (min) of Compound	
	Liver microsomes	Plasma
VSN16R	>100	>100min
VSN16S	>100	>100min
VSN15R	>100	>100min
Midazolam	17	n.t.
Bisacodyl	n.t.	2

The metabolic stability of 1μM VSN15 and VSN16 to hepatic and plasma degradation was assessed *in vitro*. Compounds with poor stability have a half life of less than 25 min. The upper limit for assessment is 100min. n.t. =not tested. **This study was performed by MDS Pharma, Taiwan.**

Table 6.2. *In vivo pharmacokinetic profile of VSN16R in Mice and Rats.*

Species	Route of Administration (Dose)	C_{max}	Half-Life	Oral Bioavailability
Mouse	i.v. (2mg/kg)	1335ng/ml	7min	n.a.
Rat	i.v. (5mg/kg)	7018ng/ml	42 min	n.a.
Mouse	p.o. (5mg/kg)	304ng/ml	89min	22%
Rat	p.o. (5mg/kg)	869ng/ml	43min	31%

VSN16R was administered to outbred laboratory mice and rats via the oral and intravenous route and the amount of VSN16 in plasma was assessed. The maximum concentration (C*max*) was present at the first time point assessed 5min for i.v. and 15-30min for the oral route (Figure 6.4). The half-life and oral bioavailability by comparing areas under plasma concentration/time curves were assessed using PK solutions software. **This study was performed by Inpharmatica, Cambridge, UK**.

Whilst 0.5mg/kg p.o. VSN16R failed to produce muscular relaxation and inhibition of spasticity (Figure 6.6A), therapeutic activity was evident within 10min following administration of 5mg/kg VSN16R p.o. ($P<0.001$. Figure 6.6B). This activity was long-lasting following a single dose of 40mg/kg p.o. where inhibition of spasticity lasted over 6 hours (Figure 6.6C). Following single administration of cannabinoids, the level of spasticity returns to baseline levels with hours (de Lago et al. 2006). The therapeutic effect was sustained after repeated daily 40mg/kg p.o. VSN16R administration for 7 days, suggesting the lack of significant receptor tolerance and even suggested some cumulative benefit as following repeated administration there was a reduced spasticity ($P<0.001$) compared to starting values. However, when

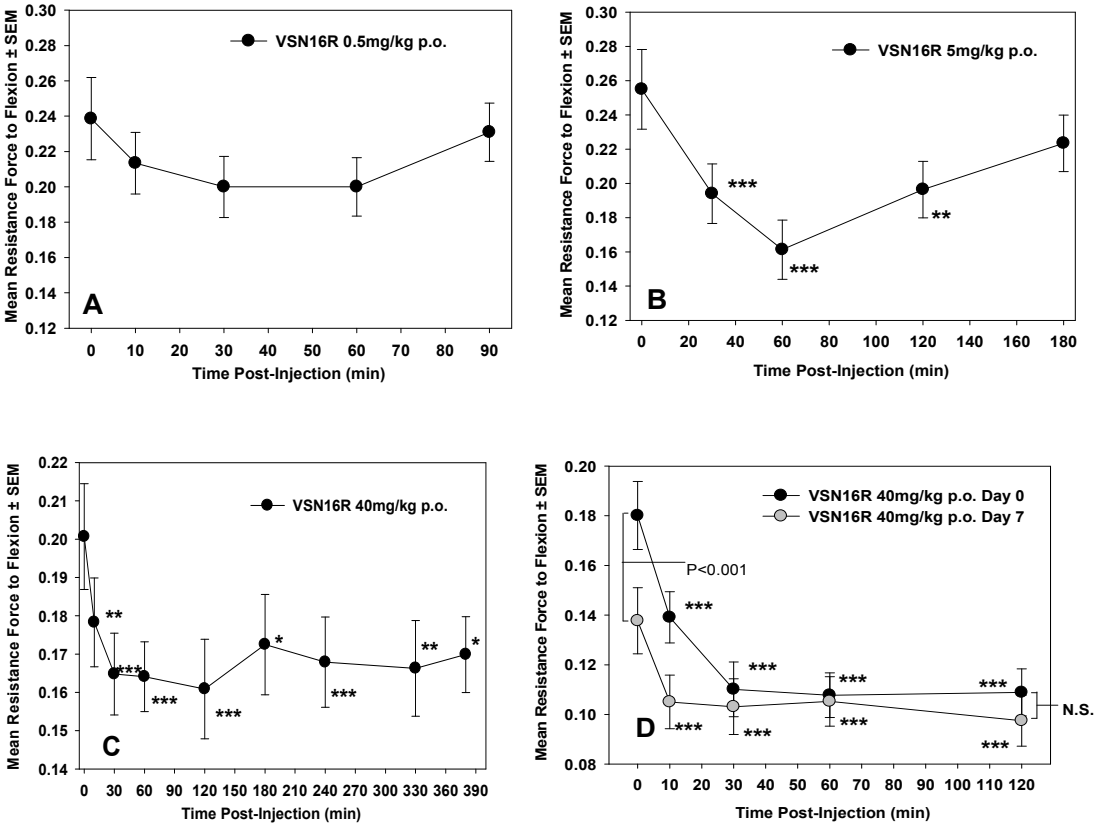
the level of spasticity was assessed 2 weeks after the cessation of treatment the level of spasticity was not different from the initial levels of spasticity prior to treatment (Resistance to Flexion Force Baseline 0.174 ± 0.033N. 1 week later, 24 hour after last treatment with VSN16R 0.127 ± 0.045N ($P<0.001$ compared to baseline); 3 weeks later 0.157 ± 0.045N $P>0,05$ compared to baseline and $P<0.02$ compared to week 1. n=10 limbs, 6 animals. Therefore VSN16R is a water-soluble, orally-active agent that is well tolerated (no toxicity has been noted when tested up to 50mg/kg i.v. and 150mg/kg p.o.) that can inhibit spasticity in experimental models of MS.

6.2.3. *VSN16 does not influence all signs associated with chronic EAE.*

Animals with limb spasticity also tend to exhibit neuropathic bladder abnormalities, which are associated with voiding problems (Al-Izki et al. 2009). Ultrasonic detection of urine volume demonstrated that anti-spastic doses of VSN16 (40mgkg p.o.) and R(+) WIN55,212 (5mgkg i.p.) did not influence urine volume within 60 minutes of drug administration in contrast to the significant voiding induced by bethanecol chloride administration (20mg/kg i.p.and p.o) (Figure 6.7). This indicated a selectivity of action on signs during EAE. Likewise daily treatment of mice with 40mg/kg VSN16 p.o. did not inhibit the development of paralysis EAE (Figure 6.8), indicating that the VSN16, at this dosing schedule was not inducing immunosuppressive responses (Figure 6.8). However, that this treatment regime was well tolerated indicated that repeated long-term dosing of animals with VSN16R was not toxic.

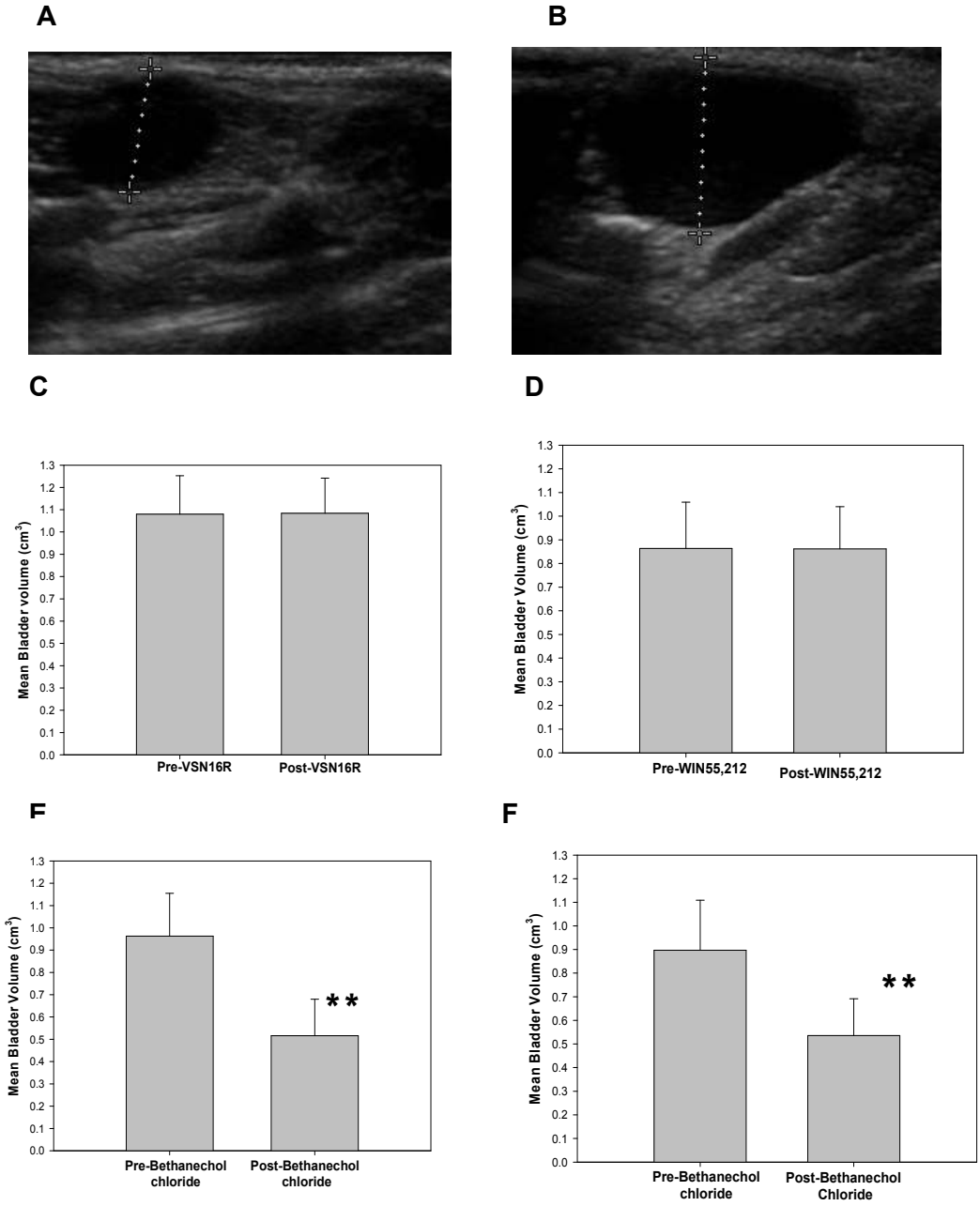
Gut motility is reported to show hypomotility following peripheral CB_1R agonism (Fride et al. 2006). Gut motility as assessed by number and weight of faecal pellets was inhibited following the administration of a centrally-active (R(+) WIN55,212. Figure 6.9A.B) and peripherally-active (CT3 Figure 6.9 C,D) CB_1R agonist (see chapter 7). This was shown to be dependent on CB_1R expression on peripheral nerves as the inhibitory effect of R(+) WIN55,212 on gut motility was substantially reduced in mice lacking CB_1R in central and peripheral nerves following cre-mediated deletion under control of the nestin gene promoter and was of a comparable level to peripheral nerve deletion using the peripherin gene promoter. (Figure 6.9.A.B). Doses of VSN15 and VSN16R that inhibit spasticity failed to influence normal gut motility (Figure 6.10 A,B). Therefore, VSN16 did not behave in a similar manner to a CB_1R agonist in this assay.

Figure 6.6. *Oral VSN16R inhibits spasticity in EAE.*



ABH mice were immunized with spinal cord antigens in Freund's adjuvant to induce relapsing EAE. Following the development of spasticity, animals were received a single oral administration of: (**A**) 0.5mg/kg p.o. VSN16R (**B**) 5mg/kg p.o.VSN16R or (**C**) 40mg/kg p.o. VSN16R in water or (**D**) repeated daily administrations of 40mg/kg p.o. VSN16R for one week (n=8 animals n=15 limbs) and spasticity was assessed at day 0 and on day 7, 24 hours after the previous treatment. Analysis of available animals (n=10) two weeks after the cessation of treatment reveal a level of spasticity that was not significantly different from that at day 0 of VSN16 treatment. The results represent the mean ± SEM forces to bend hindlimbs to full flexion against a strain gauge. * P<0.05, ** P<0.01, ***P<0.001 compared to baseline.

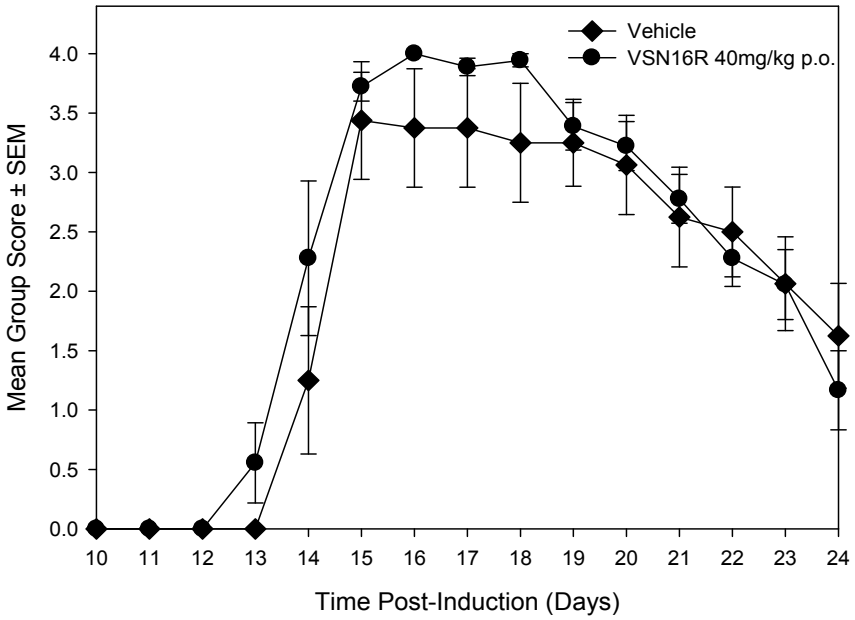
Figure 6.7. *Neither VSN16R or a CB_1 Receptor agonist promote voiding of an underactive (detrusor hyporeactive) bladder during EAE.*



Ultrasonogram of a bladder from a (A) normal animal (volume= 0.21cm3) and a (**B**) animal with EAE (day 60 p.i. Volume = 0.63cm3). The volume of the bladder was assessed before and 60 minutes after anti-spastic doses of (**C**) 40mg/kg. p.o. VSN16R (n=7), (**D**) 5mg/kg. i.p. R(+)WIN55,212-2 (n=5). The bladder did respond to muscarinic receptor agonism using oral (E) 20mg/kg p.o. and intraperitoneal injection (F) 20mg/kg i.p. of bethanecol chloride (n=7 /group). **This study was performed in collaboration with Dr.Sarah Al-Izki, QMUL, London, UK.**

Figure 6.8. *Oral VSN16R does not inhibit the development of autoimmunity.*

A

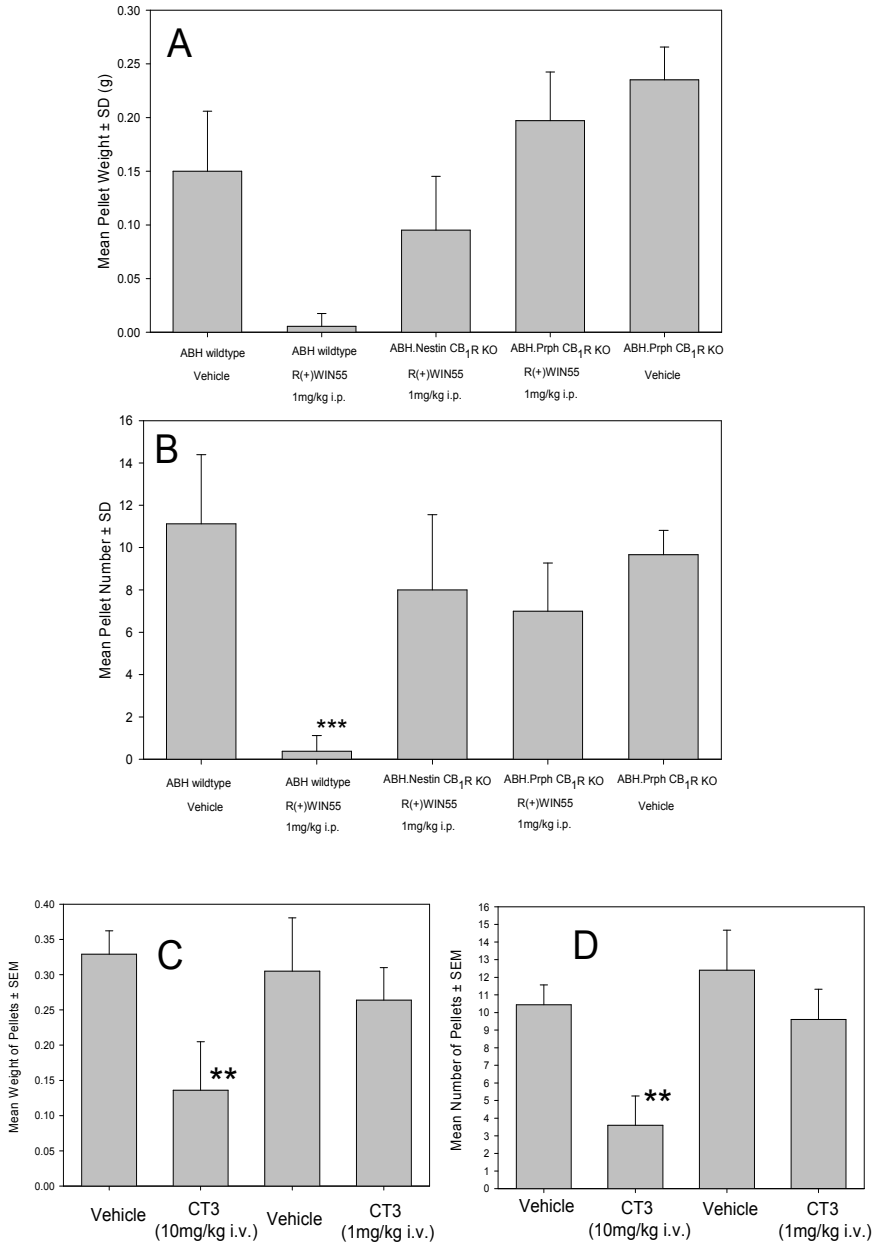


B

	Untreated	Vehicle (H_20)	VSN16 (40mg/kg p.o.)
Complete Paralysis	12	8	10
Partial Paralysis	-	-	-
Impaired Right Reflex	-	-	-
Limp Tail	1	-	-
Normal	-	1	-
No. EAE/Total	13/13	8/9	10/10
Mean Group Score ±SEM	3.7 ± 0.2	3.5 ± 0.4	4.0 ± 0.0
Mean EAE Score ±SEM	3.7 ± 0.2	3.9 ± 0.1	4.0 ± 0.0
Day of Onset ±SD	14.6 ± 2.8	15.0 ± 1.7	13.6 ± 1.5

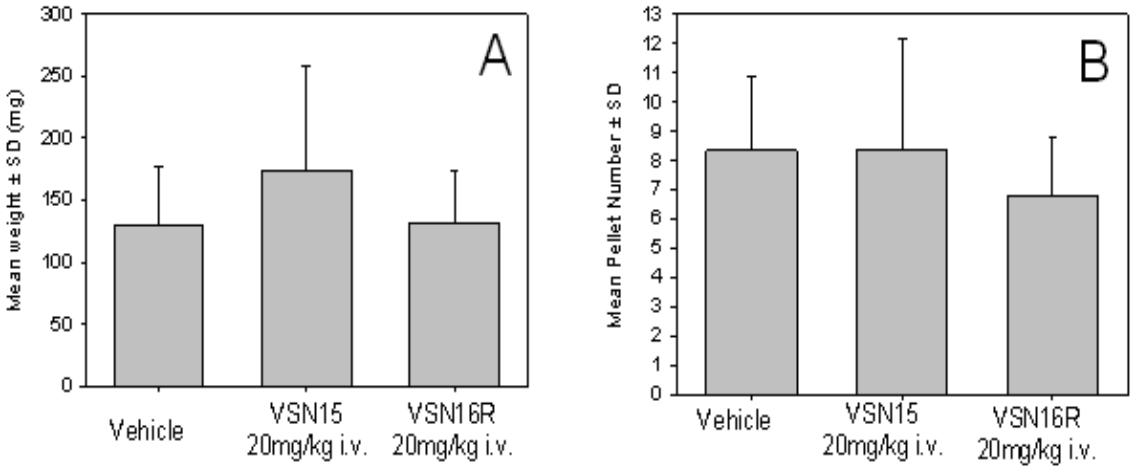
ABH mice were injected with 1mg spinal cord homogenate in Freund's adjuvant on day 0 & 7. Animals were treated daily with 40mg/kg p.o. VSN16R in water. (**A**) The results represent the mean daily clinical score of animals ± of susceptible animals, excluding one VSN16 treated animal which developed disease on day 10p.i. (**B**). The clinical scores during EAE. **This was performed in collaboration with Sofia Sisay, QMUL, London, UK.**

Figure 6.9. *Cannabinoid Control of Gut Motility.*



ABH mice or CB_1R conditional knockout mice were injected with (**A,B**) vehicle or 1mg/kg i.p. R(+) WIN55,212-2 (n=7-8/group) or (**C,D**) vehicle or 1-10mg/kg i.v. CT3 in ECP (n=8/group). in DCP, VSN15 racimate (n=8) or VSN16R (n=9) intravenously in ECP. The (**A, C**) weight and (**B, D**) number of faecal pellets was assessed after 3 hours. The differences in weights were assessed by students t tests and the faecal number was assessed by Mann Whitney U statistics. *P<0.05, **P<0.01, P<0.001 compared to control.

Figure 6.10. *VSN16 does not affect normal gut motility.*



ABH mice were injected with vehicle (n=9), VSN15 racemate (n=8) or VSN16R (n=9) intravenously in ECP. The (A) weight and (B) number of faecal pellets was assessed after 3 hours. The differences in weights were assedd by students t tests and the faecal number was assessed by Mann Whitney U statistics.

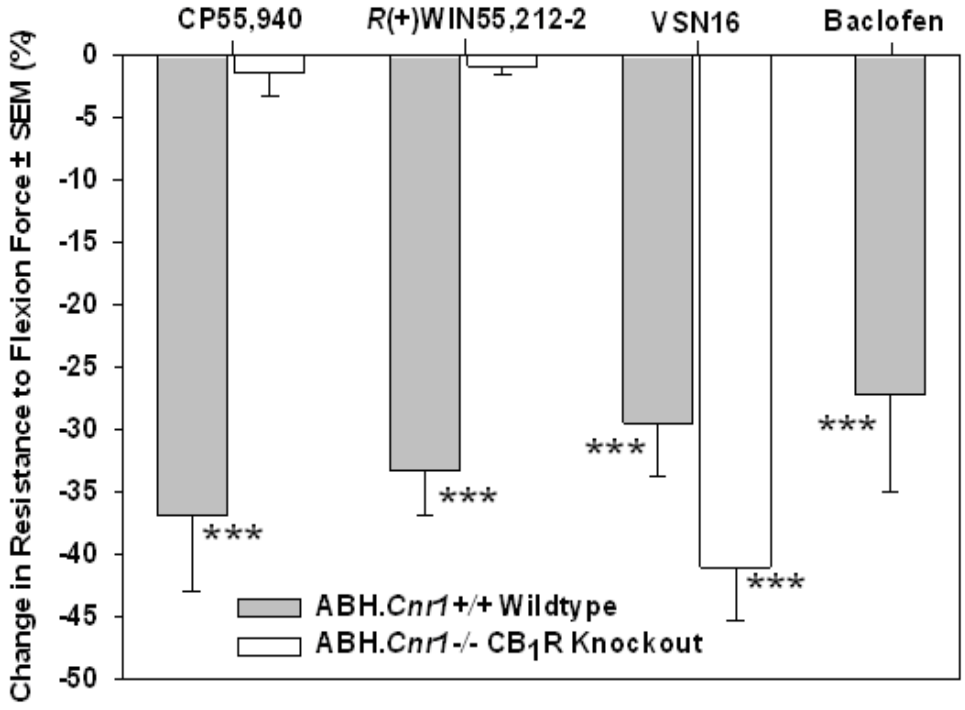
6.2.4. *The target for VSN16 is not the CB_1 Receptor.*

To establish whether the action of VSN16 was mediated via the CB_1 receptor, spasticity was assessed in CB_1R-deficient mice. Whilst the anti-spastic effect of the CB_1R/CB_2R agonists CP55,940 and R(+)WIN55, 212 was not evident at 10min (Chapter 4) or 30minutes after treatment, surprisingly VSN16 still displayed anti-spastic activity (Figure 6.11), indicating efficacy via a non-CB_1R-dependent mechanism. This also indicated that VSN16 could induce comparable anti-spastic activity as cannabinoids and Baclofen (Figure 6.11), but exhibited a much better side-effect profile (Figure 6.3).

Importantly, VSN16 does not directly bind to CB_1R *in vitro*. VSN16 (tested up to 300μM) failed to inhibit binding of ^{3}H-CP55,940 (Positive control Ki=0.36nM) to rat cerebellar membranes and demonstrated (tested up to 100μM) no appreciable CB_1R agonist activity in human CB_1R transfected cells as demonstrated by GTPγS-binding activity (Positive control CP55,940. EC_{50} = 24nM Table. 6.3). The *in vitro* activity of relaxation of the vas deferens was still present in CB_1 and CB_2 (C57BL.*Tm1.Cnr2*^{Delt}) deficient mice (Figure 6.12). In addition to a lack of significant binding to CB_2R, TRPV1 vanilloid receptors, VSN16 (tested to 10μM) failed to exhibit affinity for a number of other receptors including; human A1, A2A, A3, α1(non-selective), α2(non-selective), β1, AT1, BZD, β2, CCKA, D1, D2S, ETA, GABA (non-selective), GAL2, CXCR2, CCR1, H1, H2, MC4, ML1, M1, M2, M3, NK2, NK3, Y1, Y2,NT1,δ2, κ, μ, ORL1, 5-HT1A, 5-HT1B, 5-HT2A, 5-HT3, 5-HT5A, 5-HT6, 5-HT7, sst(non-selective), VIP1, V1a, Ca^{2+} channel (L verapamil site), K^+ V channel, SK^+ Ca

channel, Na^+ channel (site 2), Cl^- channel, NE transporter, DA transporter, Nav 1.5 Na^+ channel, hERG Kv11.1 K^+ channel, GPR3, GPR6, GPR12, and GPR119. There was some weak agonist activity on (EC_{50} 1-10μM) on LPA1, LPA2, LPA3, LPA4, and LPA5 receptors (D. Selwood, Multispan Inc, CA, USA; J. Chung, Scripps Institute USA. Unpublished)

Figure 6.11. *The anti-spastic effect of VSN16 is not dependent on the expression of the CB_1 receptor.*



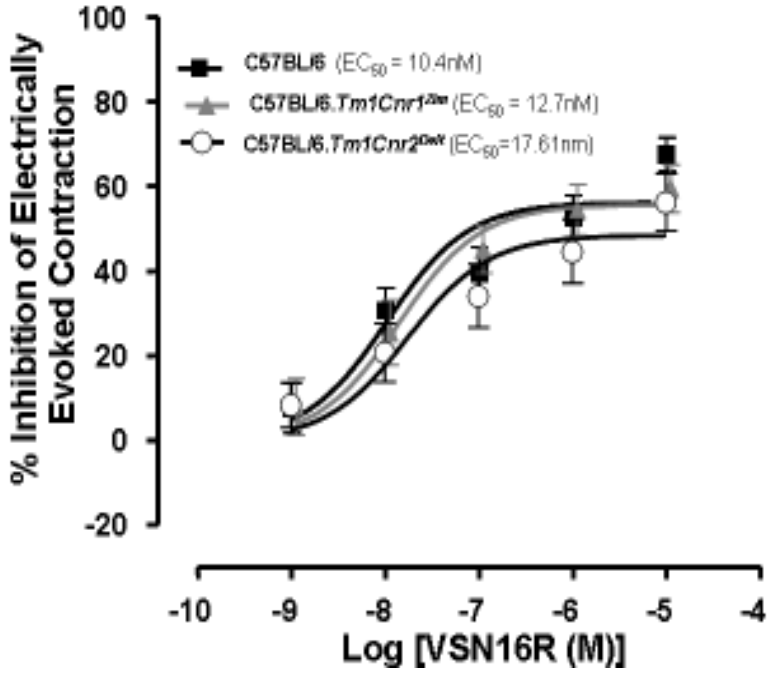
Wildtype or CB_1-deficient mice (ABH.*Cnr1* -/-) were injected intraperitoneally with the full CB_1/CB_2 agonists CP-55,940 (n=8/group) or *R*(+)WIN-55,212-2 (n=14/group) or intravenously with 5mg/kg i.v. of VSN16 racemate. To facilitate visualisation of differences between groups, results are expressed as the mean ± SEM percentage change in the resistance to hindlimb flexion compare to baseline, 30 minutes after the injection of compound. ***P<0.001 compared to baseline by paired *t* tests.

Table 6.3. *VSN16 is not a cannabinoid receptor binding compound.*

Receptor	Activity of VSN16R (Maximum dose tested)	Positive Control	CRO
CB_1 RECEPTOR			
rCB_1 Receptor (Cerebellar membranes)	No Activity (>300μM)	CP55,940 (IC_{50} = 0.36nM competitive Ligand Binding)	C.R. Hiley, Cambridge
hCB_1 Receptor (CHO.CNR1)	No Activity (>10μM)	CP55,940 (EC_{50} =24nM cAMP Assay)	CEREP
hCB_1 Receptor (HEK293.CNR1)	No Activity (>10μM)	Anandamide (IC_{50} =344nM GTPγS Binding assay)	MDS Pharma
		(R)+WIN55,212 (IC_{50} =32nM GTPγS Binding assay)	MDS Pharma
CB_2 RECEPTOR			
hCB_2 Receptor (HEK293T.*CNR2*)	No Activity (>10μM)	CP55,940 (EC_{50} =1nM cAMP assay)	Multispan Inc.
hCB_2 Receptor (CHO-K1.*CNR2*)	No Activity (>10μM)	(R)+WIN55,212 (IC_{50} =5.2nM GTPγS Binding assay)	MDS Pharma
		CP55,940 (IC_{50} =2.37nM GTPγS Binding assay)	MDS Pharma

Cell Lines that were stably transfected with mouse (m), human (h) or rat (r) cannabinoid receptors were incubated with various concentrations of VSN16R and functional activity was assessed using a variety of different read-outs by Contract Research Organizations (CRO) or collaborators.

Figure 6.12. *VSN16R induces relaxation of the mouse vas deferens, independent of CB_1R.*

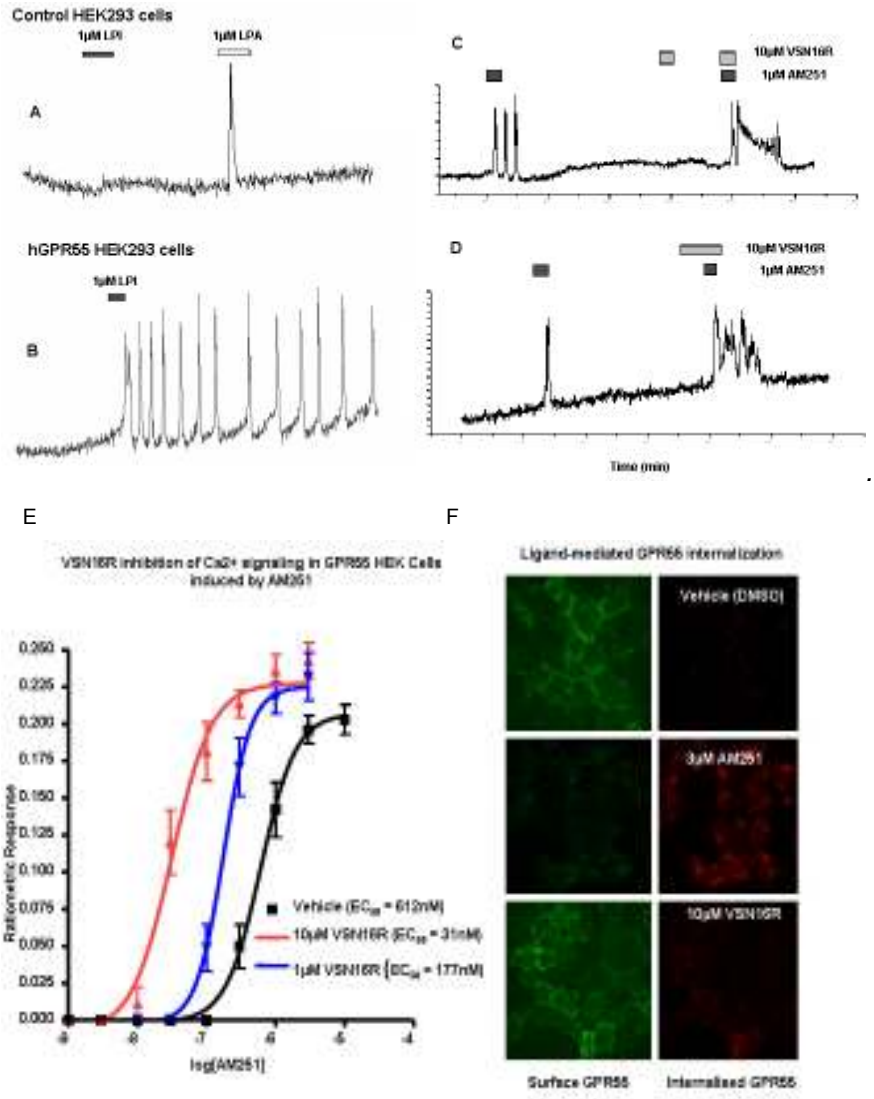


The mouse vas deferens was isolated and contractions were monitored following incubation with various concentrations of VSN16R in water. The results represent the mean ± SEM of 5-6 replicates. **The study was performed by Carolyn Tanner and Prof. Ruth Ross, University of Aberdeen.**

6.2.5. *VSN16 is a modulator of GPR55.*

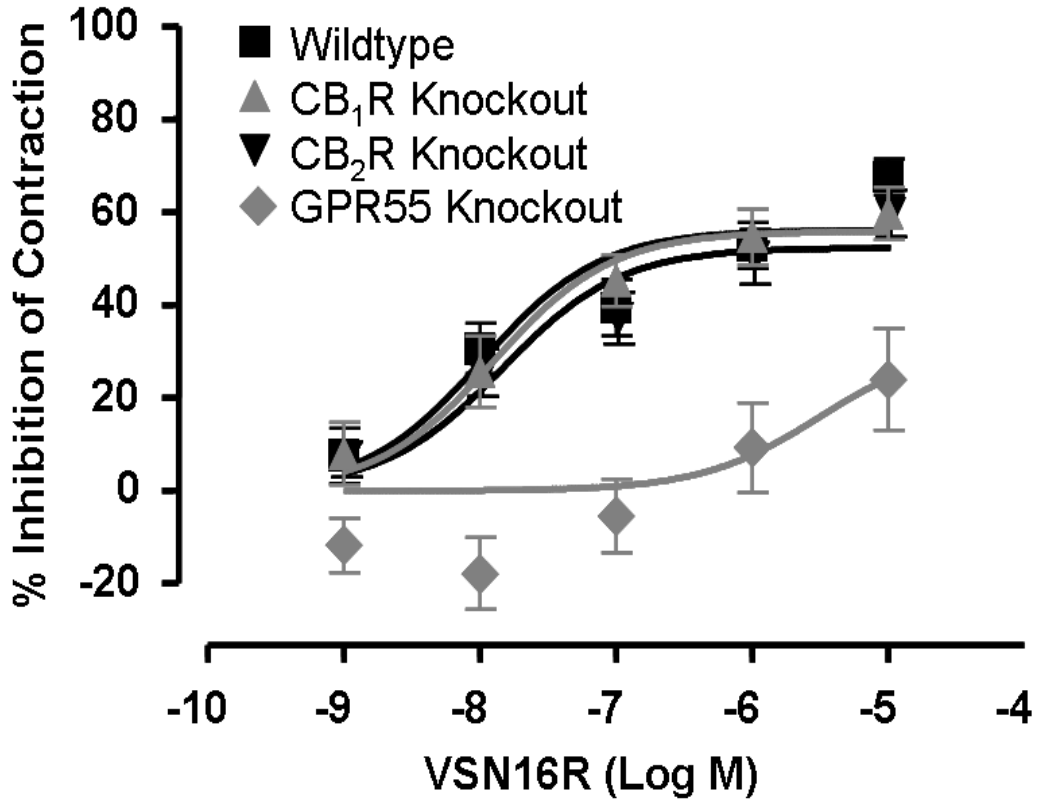
The pharmacological activities of VSN16 in rat and mouse mesenteric or retinal arterial beds are inhibited by AM251, SR141617A and 0-1918, the antagonist of the receptor stimulated by abnormal cannabidiol and lysophosphoinositol (LPI) receptor currently known as GPR55 (Baker et al., 2006; Hoi et al., 2007; Ross, 2009). Although it has been reported that the Multispan C1113 (stably human GPR55 transfected HEK cells) cell line gave a calcium response to 10μM VSN16, that was inhibited by SR141617A (Hoi et al., 2007), this result was not reproduced in calcium influx assays in the same Multispan C1113 cell line (LPI Response EC_{50} =2.73μM. Performed by Chris Henstridge and Andrew Irving, University of Dundee) or three other HEK293T.*GPR55* cells lines (Henstridge et al., 2009). LPI response EC_{50} =2.99μM. Performed by Chris Henstridge and Andrew Irwing, University of Dundee; (Johns et al., 2007). LPI response EC_{50} =49nM performed by Glaxo Smith Kline, (A. Brown Personal communication to D.Baker) and the multispan H1113 cell line performed by Multispan Inc, USA. Figure 6.13). Likewise VSN16 (up to 10μM) did not stimulate a cell line transfected with mouse GPR55 (Nephi Stella, University of Washington, Seattle USA. Personal communication to. D. Baker). However, VSN16 did modulate the calcium response induced by AM251 at 1 and 10μM (Figure 6.14) suggesting that it can modulate GPR55 responses. This was definitively shown using *Gpr55* gene deficient mice, where it was found that the vas deferens responses of both LPI (D. Baker. Personal communication) and importantly VSN16 were attenuated in GPR55-deficient mice (Figure 6.15). Therefore, it suggests that VSN16 may be a modulator of GPR55.

Figure.6.14. *VSN16R does not stimulate GPR55, but can modify the activity of AM251 on the GPR55 receptor.*



HEK 293 cells, which expresss lysophophotidyl acid (LPA) receptors were transfected with a haemagglutinin protein tagged hGPR55 and stable lines were selected (Henstridge et al. 2009). These cells demonstrated calcium signalling following incubation with (**A, B**) lysophosphoinositol (LPI) and (**C,D,E**) AM251. **(C, D**) The calcium signalling induced by AM251 could be augmented by co-incubation with 10μM VSN16, although **(C,E)** it did not produce any calcium responses by itself. It did not promote (**F**) GPR55 receptor internalization detected by immunoassaying for haemagglutinin protein (Henstridge et al. 2009), which is evident following GPR55 agonism with AM251. **This study was performed by A. Irving, University of Dundee, UK.**

Figure.6.15. *VSN16R is a modulator of GPR55.*



The vas deferens from C57BL/6 wildtype and C57BL/6.*Tm1Cnr1*^{Zim}, C57BL.6.*Tm1Cnr2*^{Delt}, and C57BL/6.*Tm1Gpr55*^{Lex} knockout mice were isolated and contractions were monitored following incubation with various concentrations of VSN16R in water. The results represent the mean ± SEM of 5-6 replicates. **The study was performed by Carolyn Tanner and Ruth Ross, University of Aberdeen, UK.**

6.3. *DISCUSSION*

In an attempt to generate a CNS excluded CB_1 receptor agonist VSN16 was synthesised. This is an: orally-active, bioavailable, water soluble and seemingly safe, CNS-excluded compound that modulated GPR55 function that inhibited spasticity in chronic EAE. This exhibited anti-spastic activity but did not induce any sedative effects associated with central CB_1R or $GABA_B$ receptor stimulation. No form of toxicity has been detected and the compound was tolerated up to doses of 120mg/kg and whilst it did not inhibit the autoimmune response in EAE, it was safely administered repeatedly for over 3 weeks. Although active, all current anti-spastic agents can induce marked adverse effects which limit their clinical use (Shakespeare et al. 2003). This will tend to favour poor compliance in drug use and favour use only in late stages of disease. A drug that lacks such side-effects may be used earlier in disease course, during the day and thus could have commercial advantages over competitor compounds in the treatment of limb spasticity in MS and other diseases such as occurs in spinal cord injury.

Although the pharmacological activity of VSN16 can be inhibited by SR141617A and AM251 (Hoi et al., 2007), which can antagonise the CB_1R, the compound failed to bind or agonize the CB_1R and drug activities were maintained in CB_1R-deficient mice. However, the pharmacological profile, in terms of antagonistic compounds that inhibit VSN16, notably 0-1918, was suggestive that GPR55 may be the target for VSN16 (Baker et al., 2006; Ryberg et al., 2007; Johns et al., 2007). This was supported by some initial studies in GPR55-transfected HEK239 cells (Ho et al., 2009). However, repetition of these data has proved elusive and a number of independent-laboratories using different cell lines have been unable to show any agonistic or antagonistic effect of VSN16 at human and mouse GPR55. Whilst it is relatively consistent that LPI behaves as an agonist at GPR55, there is much contradiction over *in vitro* responses of GPR55 to endogenous and exogenous cannabinoids in over-expressing, GPR55 transfected cell lines (Johns et al. 2007; Ota et al. 2007; Ryberg et al., 2007; Lauckner et al. 2008; Godlewski et al. 2009; Henstridge et al. 2009; Ota et al. 2009; Ross 2009; Yin et al. 2009). This may relate to the lack of appropriate or sufficient signalling molecules in the transfected cells. VSN16 induces responses in the low nM range in tissue based assays such as relaxation of the vas deferens and mesenteric artery bed (Hoi et al., 2007) and using GPR55-deficient mice, that delete the whole coding region of *Gpr55,* it was possible to demonstrate that GPR55 modulates the response of VSN16. Although AM251 antagonises the effect of VSN16 in tissue assays (Hoi et al., 2007), VSN16 appeared to augment the GPR55-mediated response to AM251. This could suggest that this difference is due to the use of GPR55 over-expressing cells, or

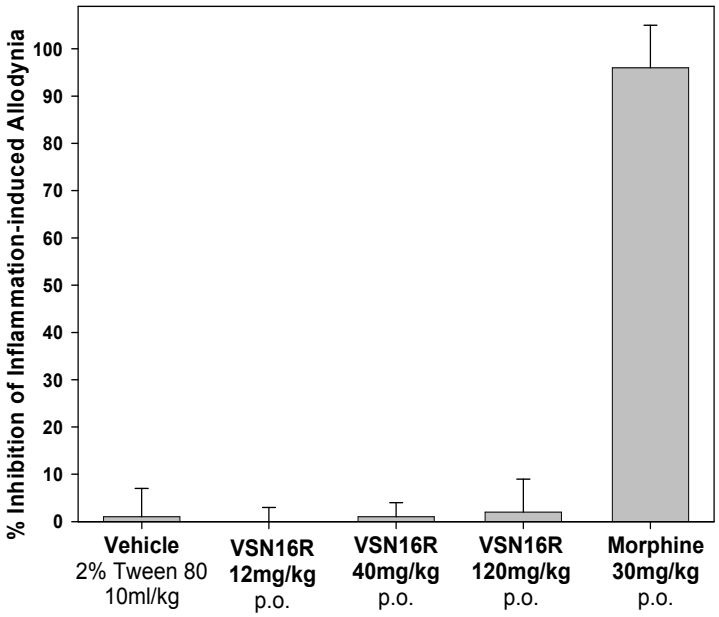
alternatively VSN16 may be stimulating another receptor such as the LPA receptors expressed by HEK293 cells, which mediate the augmentation. As such this could account for the loss of this activity below 1μM *in vitro,* compared to activity of VSN16 is active in tissue assays at concentrations in the low nM range. Whilst studies may argue against direct activity at GPR55, the activity of VSN16 was markedly attenuated in relaxation of the vas deferens in GPR55 deficient mice. This suggests that VSN16 may be a modulator of GPR55. This could result from binding to an allosteric site, as occurs with some CB_1 receptor binding compounds (Griffin et al., 1999), or VSN16 may stimulate a co-receptor such as seen with CB_1 receptor dimerisation with dopamine D2 receptors (Kearn et al., 2005) and may account for the inability to detect direct binding to GPR55-transfected cell lines. However, the lack of direct agonism at the GPR55 may limit receptor internalisation and tolerance and account for the consistent, repeated therapeutic activity following repeated administration.

Currently the function of GPR55 remains enigmatic. It has been suggested that GPR55 may be involved in fat metabolism and blood pressure (Baker et al., 2006; Brown, 2007), although the blood pressure in GPR55 knockout mice is normal (Johns et al., 2007, http://www.informatics.jax.org/external/ko/lexicon/261.html) and VSN16 does not induce significant changes in either normotensive (Hoi et al. 2007) or spontaneously hypertensive rats (D. Selwood & D. Baker Personal Communication). This adds further to safety data for the compound. More recently it has been reported that GPR55 may modulate pain pathways (Staton et al., 2008) and GPR55-deficient mice do not appear to develop mechanical hyperalgesia following inflammatory and neuropathic pain stimuli (Staton et al., 2008). Following administration of VSN16, no obvious pain behaviours such as vocalisations, freezing or locomotor reduction have been detected despite high doses of VSN16 administration and there is no influence on Freund's adjuvant-induced hyperalgesia in rats even up to 120mg/kg p.o. was noted (Figure 6.16). Further studies will be needed to assess further whether VSN16 has any value in analgesia.

To date VSN16 has been associated with relaxation in spasticity of limbs, the vas deferens and the mesenteric and retinal artery bed (Hoi et al., 2007, Dong et al., 2009) and it is perhaps not surprising that VSN16, and CB_1R agonism, did not induce enhanced gut motility or voiding of the bladder during chronic EAE, as this latter function required detrusor contraction, which was stimulated by muscarinic acetyl choline receptor stimulation. Whilst urine retention may be treated by catheterization, incontinence due to detrusor hyperactivity is also a problem in MS and spinal injury (McCombe et al., 2009). Rodents do not have the social restraints concerning incontinence and have no need to control urination as occurs with

humans. Rodents frequently urinate, often when handled, therefore studies on incontinence in EAE, although possible (Altuntas et al., 2008) may be less amenable to study than studies on lack of voiding. However, cystometric investigations in conscious normal, female Sprague-Dawley rats has indicated that systemic (8mg/kg i.v.) and intravesical (100μM) VSN could increase the micturition interval, micturation volume and bladder capacity by up to about 50%, without affecting bladder pressure (Gratzke et al., 2009). GPR55 was detected in the urothelium and it was suggested that VSN16 may modulate afferent pathways of the micturition reflex (Gratzke et al., 2009). This suggests that VSN16 may be useful for the control of limb and bladder (hyperactivity) spasticity.

Figure.6.16. *VSN16R does not inhibit the development of mechanical hyperalgesia during inflammatory pain.*



Male Sprague Dawley rats were injected in the plantar surface of the foot with 0.1 ml of Freund's complete adjuvant and 24 hrs later mechanical hyperalgesia to pressure from a #12 supertip was measured using an ITC electronic von Frey anaesthesiometer model 2390 (ITC, USA). The foot withdrawal response was measured at baseline and 60 minutes after drug administration. The results represent the mean ± SEM (n=5/group). **This study was performed by MDS Pharma Services Taiwan.**

Although VSN16 is largely excluded from the CNS, probably due to its polarity, it is not clear if the mechanism of action of VSN16 is at central or peripheral sites. Whilst CNS-exclusion was thought to be important to limit potential cannabimimetic effects, as GPR55 modulation does not seem to induce behavioural effects even at

high doses, where some CNS penetration will be likely, especially during chronic EAE where blood:brain barrier dysfunction and interference with drug exclusion mechanisms may occur. This suggests that this CNS permeability is unlikely to represent an issue in drug development. The fact that VSN16 is water soluble however, offers a considerable advantage over the hydrophobic cannabinoids in relation to drug formulation for clinical delivery. GPR55 has been found to be expressed within the CNS and within the dorsal root ganglion (Sawzdargo et al., 1999; Baker et al., 2006; Lauckner et al., 2008) and can be co-localised with and modify the function of the CB_1 receptor (Waldeck-Weiermair et al., 2008) and may be ideally placed to modify altered neurotransmission during spasticity and therefore the activity may be both central and peripheral, as hypothesized to occur with the cannabinoid receptor agonists. However should central activation of GPR55 receptors be required for activity, it may be possible to investigate this using VSN compounds which display low aqeous solubility and thus likely to be CNS penetrant.

VSN16 represents a novel class of compounds, which modulate GPR55 function that may have therapeutic utility in the control of spasticity. This compound fails to bind to CB_1 receptors and thus different compounds will need to be employed to examine the role of peripheral CB_1 receptors in the control of spasticity.

CHAPTER SEVEN

CONTROL OF SPASTICITY BY CNS EXCLUDED CB_1 RECEPTOR AGONISTS.

7.1. *INTRODUCTION*

Exclusion of cannabinoids from the brain may be achieved by synthesizing compounds that are polar and/or targeted to ABC drug-exclusion transporters. Some CNS-excluded, CB_1R agonists have been generated and recently there has been an increasing amount of data that indicates that both inflammatory and neuropathic pain can be controlled within the peripheral compartment of the nervous system (Fox et al., 2001; Fride et al., 2004; Agarwal et al., 2007; Dziadulewicz et al., 2007; Worm et al., 2007; Yu et al., 2007). Similarly, although bladder hyper-reflexia is usually controlled by micturation centres within the brain, reflex arcs between the bladder and spinal cord become more prominent following spinal transections (Kalsi and Fowler, 2005). These pathways may both be controlled by cannabinoids, which can limit bladder dysfunction (Brady et al., 2004; Freeman et al., 2005). Thus, although muscle spasticity has been thought to be controlled at spinal and supraspinal sites within the CNS (Brown, 1994; Nielson et al., 2007) this may also occur with in the periphery. This is supported by the observations that spinal motor outputs that signal through efferent nerves via neuromuscular junctions to the muscle and sensory inputs that signal via the dorsal root ganglia, both traverse nerves expressing CB_1R (Howlett et al., 2002; Sanchez-Pastor et al., 2007). Indeed, peripheral CB_1R-mediated control of spasticity was indicated using novel synthetic cannabinoid receptor agonists.

7.2. *RESULTS*

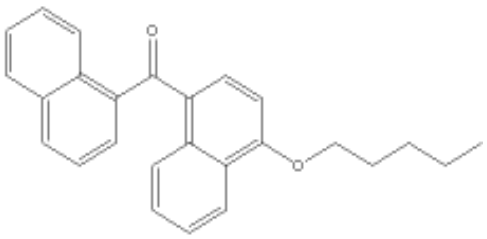
7.2.1. *Modelling of CNS permeability.*

Chemical structure can be used to predict CNS permeability. Using one such algorithm a logBB brain:blood coefficient of -0.5 would predict compounds to be excluded from the CNS (Feher et al, 2000). This was performed by Dr. Cristina Visintin, UCL, London. To date (+)-cannabidiol 1,1-dimethylheptyl (logBB 0.488) and recently SAB378 have been reported to be CNS-excluded, CB_1R agonists that exhibit analgesia in inflammatory and neuropathic pain models (Fride et al., 2004; Dziadulewicz et al., 2007). SAB378 (Figure 7.1) exhibits marked hydrophobic properties with a logBB of 0.837, compared to logBB of 0.18 for R(+)WIN-55212-2,

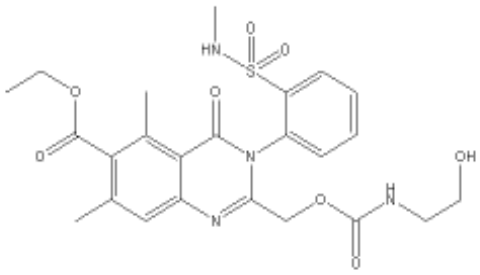
which is a well known CNS-penetrant cannabinoid (Dyson et al., 2005; Adam-Worrall et al., 2007). This suggested that SAB378 must be targeted to a CNS-exclusion pump to avoid CNS penetration. CT3 (Figure 7.1) has been reported be an analgesic agent without the "high" (Burstein et al., 2004). This has a logBB of -0.44 and has been reported to be 70% excluded from the CNS (Dyson et al., 2005). As this molecule has significant CB_1R affinity (Rhee et al. 1987; Dyson et al. 2005), it was hypothesized that this agent may also be a CNS excluded, CB_1R agonist. However, through examination of the patent literature, SAD448 (Figure 7.1) was identified (Brain et al., 2003; Adam-Worrall et al. 2007). This may be one of the most CNS-excluded, CB_1R agonist yet described. This highly polar molecule has a logBB of -2.76 and exhibits some water solubility (Adam-Worrall et al., 2007). This is 98% excluded from the CNS following administration of 1.6mg/kg i.v. SAD448 (Adam-Worrall et al., 2007). This compound was synthesised and its capacity to inhibit spasticity in EAE was examined.

Figure 7.1. *Structure of CNS-Excluded CB_1R Agonists.*

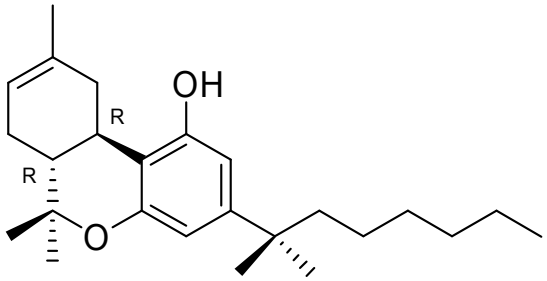
SAB378



SAD448



CT3

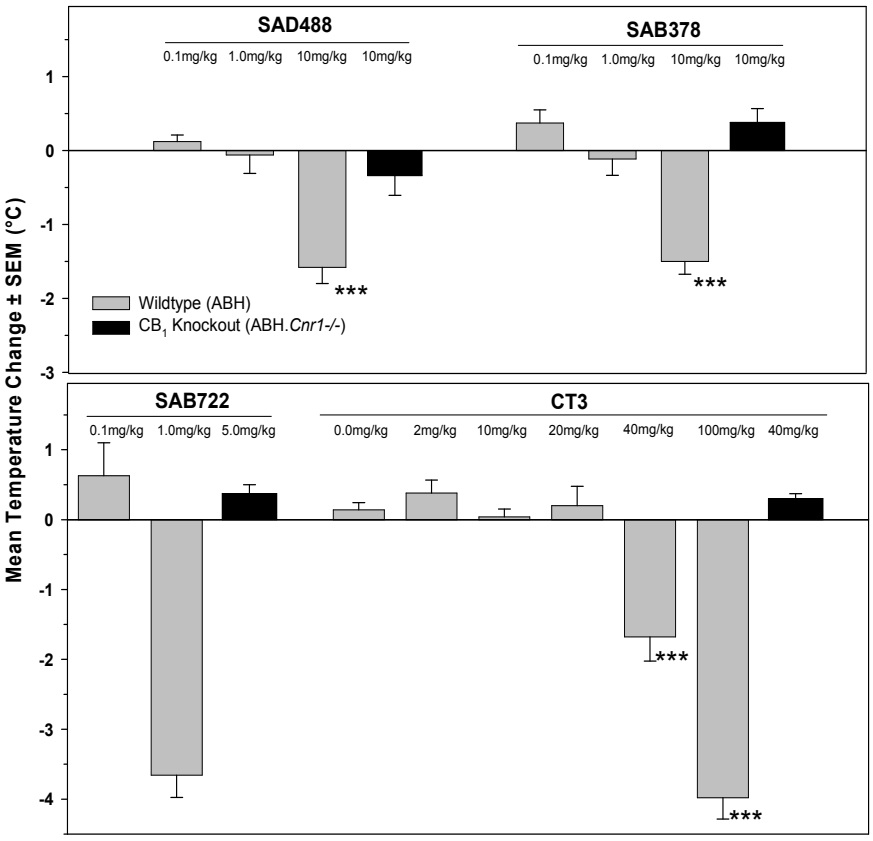


7.2.2. *Peripheralized CB_1 receptor agonists inhibit spasticity.*

7.2.2.1. *SAD488.*

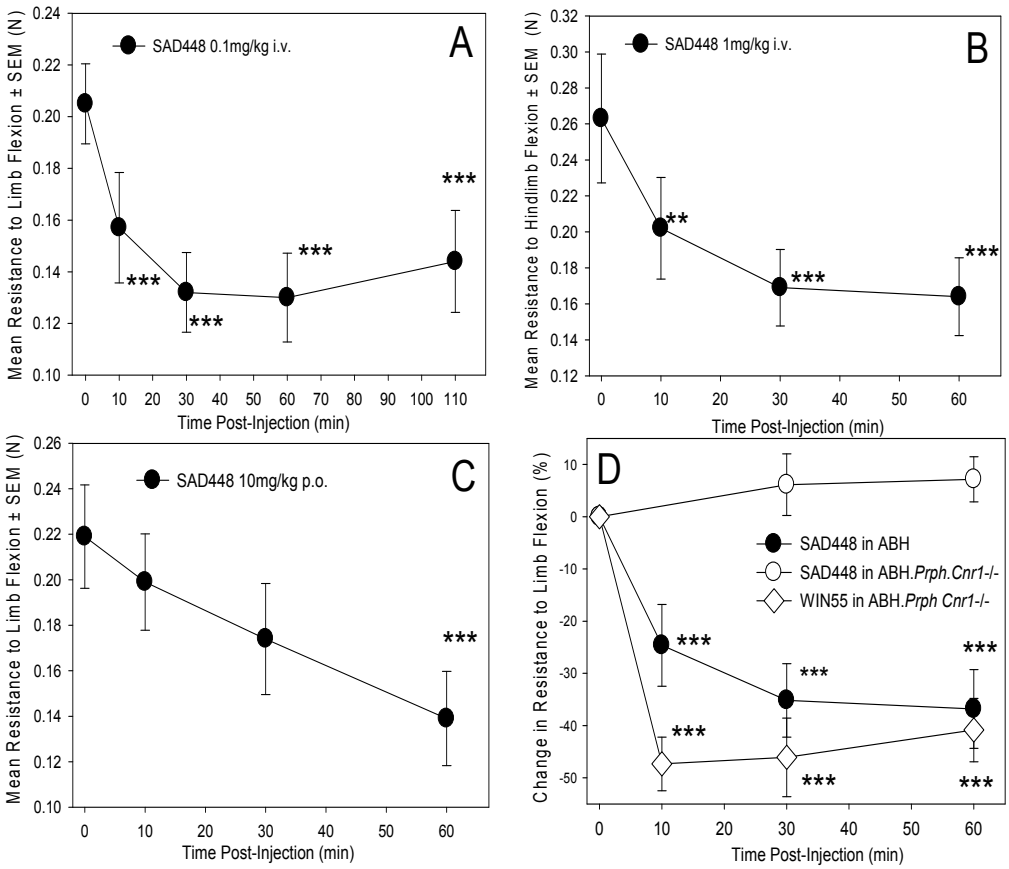
It has been reported that the tail-flick response to noxious stimuli is a centrally-controlled, CB_1R-dependent activity (Howlett et al., 2002; Fride et al., 2004). Although active in inflammatory and neuropathic pain at doses of less than 0.5mg/kg i.v., SAD448 has been reported to inhibit tail-flick responses at doses ≥16mg/kg i.v. (Adam-Worrall et al., 2007). This is suggestive of a central effect and indeed a CB_1R-dependent mild, transient, hypothermia was evident following injection of 10mg/kg i.v. in ABH mice (Figure 7.2). These mice demonstrated a significant ($P<0.001$) reduction (-1.58 ± 0.22°C n=5) in temperature 20 minutes post administration, which was not was evident 1 hour after administration (-0.06 ± 0.29°C SAD488 vs. 0.02 ± 0.32°C vehicle control). Likewise, no hypothermic response was detected following administration of 1mg/kg i.v. SAD448, which was consistent with previous data showing CNS exclusion of the compound at this dose and route (Adam-Worrall et al., 2007). Delivery of SAD448 either via the intravenous (0.1mg/kg (Figure 7.3A), 1mg/kg (Figure 7.3B) or oral route (Figure 7.3C) inhibited spasticity in EAE in the absence of marked cannabimimetic effects. As would be predicted the rate of action was faster following i.v. delivery (Figure 7.2), but within 60min after administration both i.v. and oral routes of delivery had induced a similar reduction, which was probably the maximum achievable with those animals (Brooks et al., 2002), in the force required to bend the limb (-36.5% for intravenous (Figure 7.2A) and -37.6% for oral administration (Figure 7.3C) compared to 0.09% for 0.1ml i.v. of ECP vehicle. Mass spectrometric analysis of tissues from orally treated mice demonstrated that the compound was not detected in 5/6 (sensitivity 5ng/ml) brain lysates of spastic animals within 2 hours following oral administration where it was detectable in the 6/6 plasma samples (Dr.T.Hart, Personal communication to D.Baker, Novartis, London). This study suggests that polar cannabinoids, which are excluded from the CNS, can be used to inhibit spasticity. In addition, the observation that the anti-spastic activity of SAD448 was lost in animals where the CB_1 receptor was conditionally deleted from peripheral nerves, using *Peripherin1*-promoter-driven excision of CB_1 genes, provides futher evidence for the therapeutic potential of peripherally active CNS excluded CB_1 agonists in the treatment of spasticity (Figure 7.3D). A further observation of interest was that the anti-spastic activity of the CNS-penetrant CB_1 receptor agonist, WIN 55 was maintained in peripherally-deleted CB_1 receptor knockout mice (Figure 7.3D), in contrast to a global CB_1 receptor knockout, where the anti-spastic activity of WIN 55 is lost (Figure 4.3), indicating that the control of spasticity can be achieved both centrally and peripherally by therapeutic agents.

Figure 7.2. *Hypothermic effects of CNS-excluded cannabinoids.*



The temperature of wildtype (n=5-7) or CB_1 receptor-deficient (n=4-5) mice was measured before and after the intravenous administration of: SAD448, SAB378 or SAB722 and CT3 in Ethanol:Cremophor:PBS. The results represent the mean ± SEM temperature (ºC) change from baseline 20minutes after cannabinoid administration. ***P<0.001 compared to baseline.

Figure 7.3. *SAD448 inhibits spasticity in CREAE and inhibition is lost in peripherally deleted CB_1 deficient spastic CREAE mice.*



ABH mice were immunized with spinal cord antigens in Freund's adjuvant on day 0 and 7 to induce relapsing EAE. Following the development of spasticity, ABH mice were treated with: (A) 0.1mg/kg i.v SAD488 (14 limbs, 8 animals) (B) 1mg/kg i.v. SAD448 (8 limbs from 6 animals) or (C) 10mg/kg p.o. SAD488 supplied by Novartis (8 limbs from 6 animals) in ECP and limb stiffness was assessed. (D) 0.1mg/kg i.v SAD448 was injected into wildtype (7 limbs, 6 animals) or peripheral nerve CB_1-deficent mice (13 limbs, 7 animals) or 5mg/kg i.p. WIN55 in ECP (11 limbs, 6 animals). The results represent the mean ± SEM forces to bend hindlimbs to full flexion against a strain gauge. * P<0.05, ** P<0.01, ***P<0.001 compared to baseline.

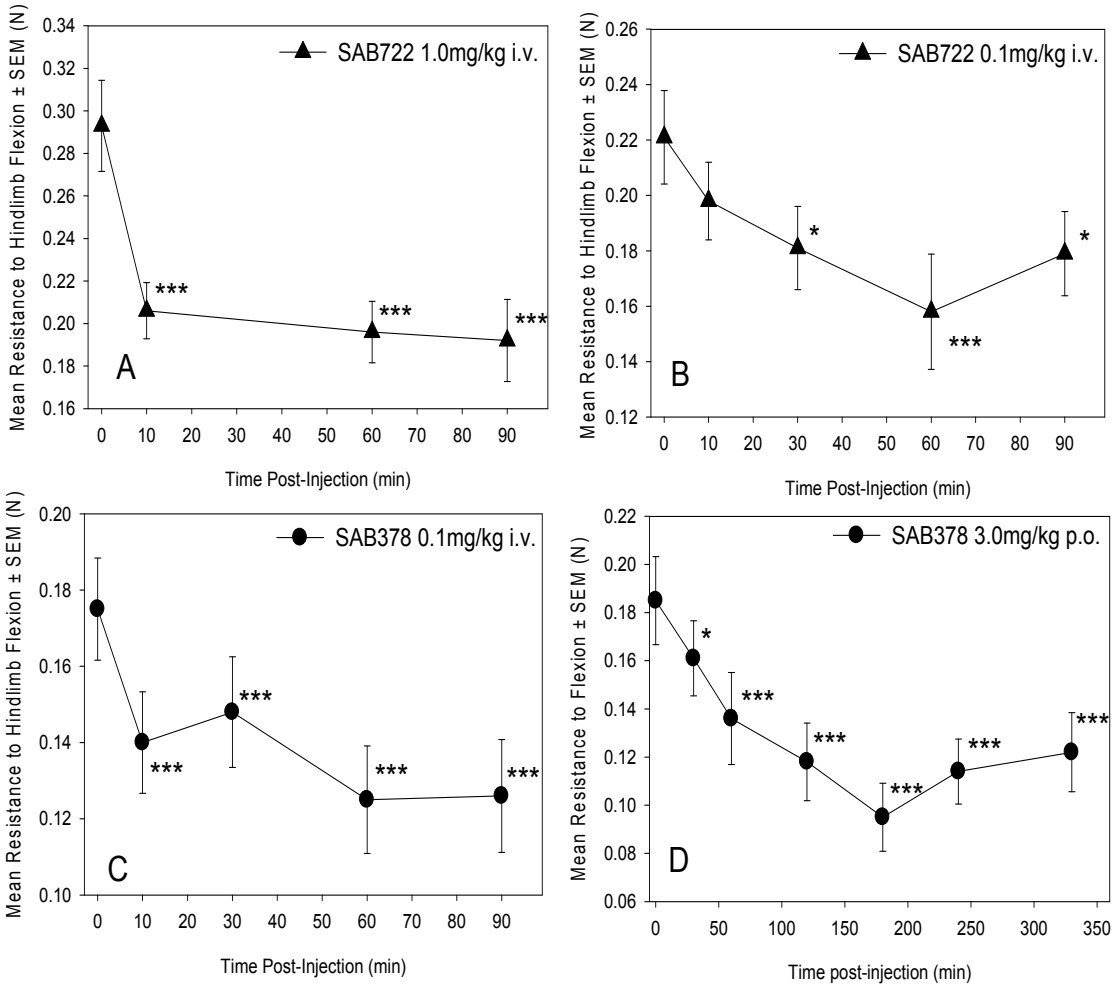
7.2.2.2. *SAB378.*

Recently it has been reported that SAB378 is a CNS-excluded, CBR agonist (Dziadulewicz et al., 2007). The CNS-penetrant CB_1R agonist, SAB722 exhibits comparable CB_1R affinity to SAB378, yet induced CB_1R-dependent hypothermic effects at least 10 times lower doses than that observed with SAB378 (Figure 7.2). Both SAB722 (Figure 7.4A) and SAB378 (Figure 7.4B, C) inhibited spasticity at doses that did not induce hypothermia, but the efficacy profile of 0.1mg/kg i.v. SAB722 appeared less significant than that seen with 0.1mg/kg SAB378 (Figure 7.4B, C). The capacity of SAB378 to affect spasticity following oral dosing was assessed, at doses (3mg/kg p.o.) used previously in pain without adverse physiological effects (Dziadulewicz et al., 2007). SAB378 was active for many hours after oral administration (Figure 7.4D).

7.2.2.3. *CT3 (Ajulemic Acid).*

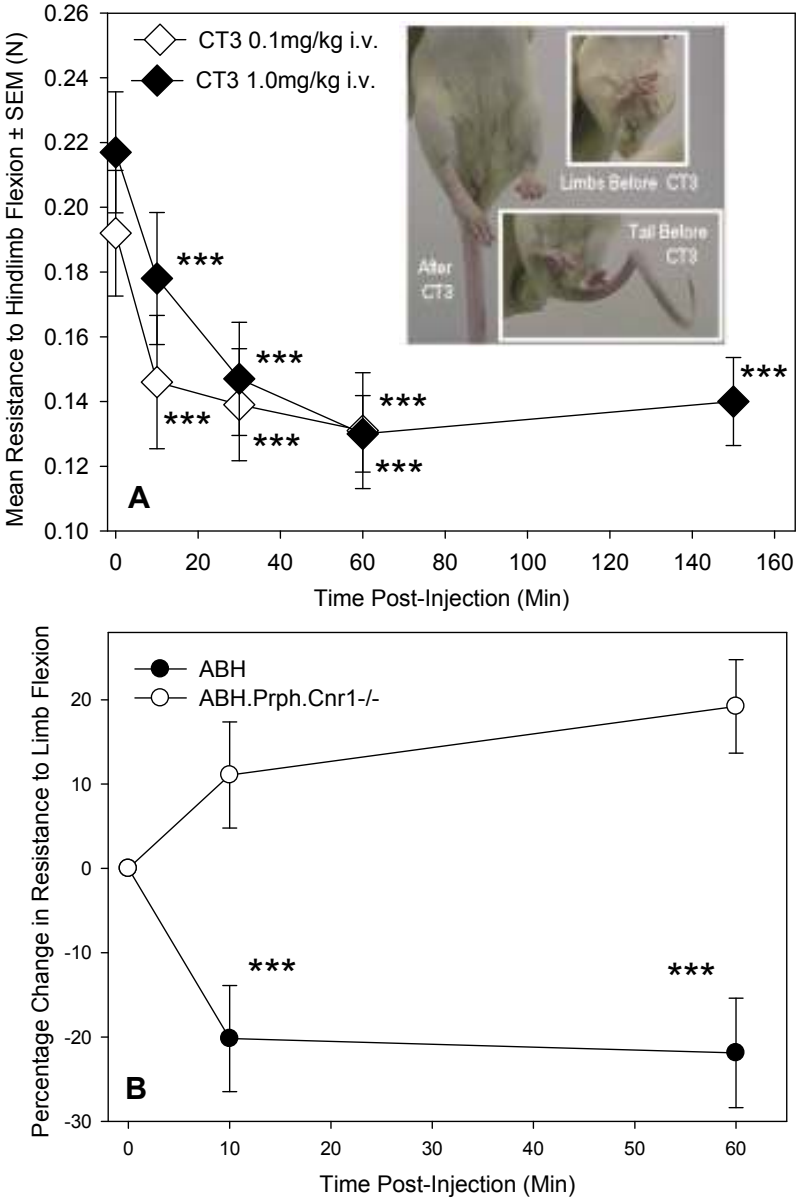
Although the mode of action has been under debate (Burstein et al., 2004; Vann et al., 2007), it appears that CT3 is likewise a CB_1/CB_2 agonist that is CNS excluded (Dyson et al., 2005). Therefore, the ability of CT3 to inhibit spasticity and to induce hypothermia was assessed. CT3-induced hypothermia was not evident 20min after injection of 2, 10 or even 20mg/kg i.v. in ABH mice (Figure 7.2). A CB_1R-dependent hypothermia was detectable at 40mg/kg and 100mg/kg i.v. (Figure 7.2) and a small but significant (-0.88 ± 0.31°C. $P<0.05$. $n=5$) hypothermia was evident 2 hours after injection of 20mg/kg i.v. However, as low doses (0.1-1mg/kg i.v.) of CT3 could inhibit hindlimb and tail spasticity without obvious signs of cannabimimetic effects (Figure 7.5A). This indicated that the therapeutic dose can be observed at least a 200 fold dose lower than marginal cannabimimetic effects in normal mice. Furthermore, the anti-spastic activity of CT3 was lost in animals where the CB_1 receptor was conditionally deleted from peripheral nerves (Figure 7.5B). Therefore the "peripheralization" of CB_1 receptor agonists may increase the therapeutic window compared to fully CNS-penetrant compounds.

Figure 7.4 *SAB378 inhibits spasticity in CREAE.*



ABH mice were immunized with spinal cord antigens in Freund's adjuvant on day 0 and 7 to induce relapsing EAE. Following the development of spasticity, ABH mice were treated with: (**A**) 1mg/kg i.v. SAB722 (11 limbs from 7 animals), (**B**) 0.1mg/kg i.v. SAB722 (11 limbs from 7 animals), (**C**) 0.1mg/kg i.v. SAB378 (14 limbs from 7 animals) or (**D**) 3mg/kg p.o. SAB378 (n=12 limbs from 6 animals) in ECP and limb stiffness was assessed. The results represent the mean ± SEM forces to bend hindlimbs to full flexion against a strain gauge. * P<0.05, ** P<0.01, ***P<0.001 compared to baseline.

Figure 7.5. *CT3 inhibits spasticity in CREAE and inhibition is lost in peripherally deleted CB_1 deficient spastic CREAE mice*.



ABH mice were immunized with spinal cord antigens in Freund's adjuvant on day 0 and 7 to induce relapsing EAE. Following the development of spasticity, ABH mice were treated i.v. with CT3 (16 limbs from 8 animals/group) in ECP and limb stiffness was assessed. The results represent the mean ± SEM forces to bend hindlimbs to full flexion against a strain gauge. In some instances mice exhibited a spastic tail and leg crossing and poor limb posture that prevented them being used in strain gauge analysis (insert). These signs could be seen to be relieved following CT3 administration (insert). (B) 0.1mg/kg i.v. CT3 in ECP was injected into either wildtype (n= 10 limbs n=8 animals) or peripherin conditional CB_1 receptor deficient mice (n= 12 limbs. n =6 mice) and the degree of spasticity was assessed before and following drug treatment. The results represent the mean ± SEM percentage change compared to baseline. *** P<0.001 compared to baseline.

7.2.3. *Exclusion pumps limit the entry of "peripheralised" cannabinoids into the CNS.*

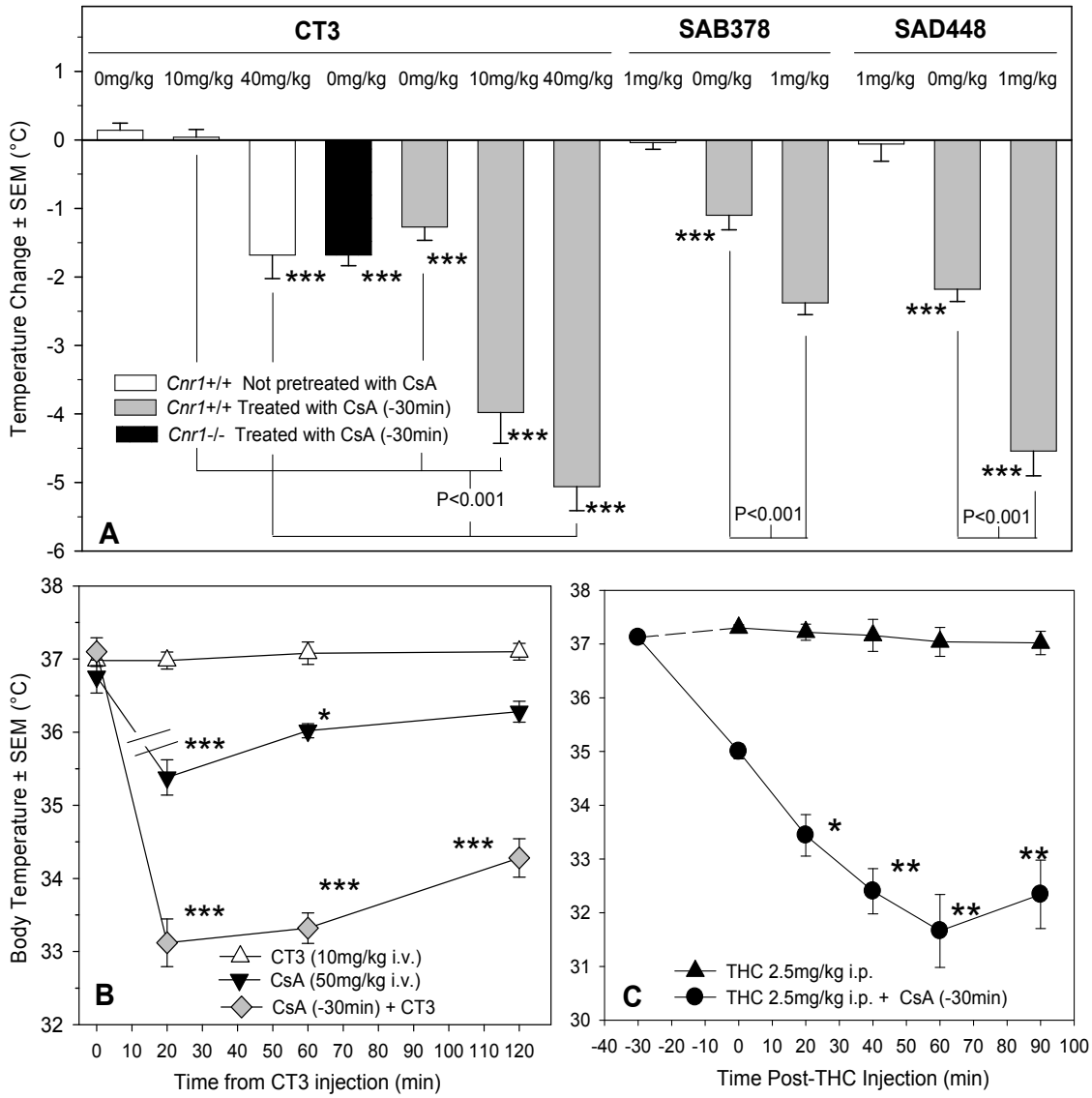
As SAB378 and to some extent CT3 were sufficiently hydrophobic to necessitate the use of ECP to get the compounds into solution for *in vivo* dosing and should therefore penetrate the CNS, it suggested to us that they must be targeted to a CNS-exclusion pump. The most documented pump is P-glycoprotein, which can be inhibited *in vivo* following injection of 50mg/kg i.v. CsA (Hendrikse et al., 1998). Whilst CsA induced a mild and transient hypothermia, which was not greater than -2.0°C over 40-60min period, this was markedly and significantly ($P<0.001$) augmented with subsequent administration of cannabinoid compounds (Figure 7.6A,B). In contrast to the cannabinoid compounds the hypothermia induced by CsA was independent of the CB_1 receptor as it was also evident in $CB_1R^{-/-}$ mice (Figure 7.6A). In addition to hypothermia, animals treated with sub-cannabimimetic doses of CT3, SAB378 and SAD448 were visibly sedated following CsA pretreatment, which was consistent with a marked cannabimimetic effect. Whilst CsA augmented CNS penetration of CT3 and SAB378, it also induced cannabimimetic effects of SAD448 to induce a marked hypothermia (Figure 7.6A). Therefore targeting cannabinoids to CNS-exclusion pumps is an additional and perhaps more important approach than enhancing polarity, for excluding cannabinoid compounds from the CNS. Interestingly CsA pretreatment also augmented the cannabimimetic effects of tetrahydrocannabinol (THC. Figure 7.6C), suggesting that CNS-exclusions pumps influence the CNS penetration of plant-based cannabinoid compounds.

Although it was initially thought that the cannabinoid exclusion pump was likely to be P-glycoprotein (Abcb1). It was found that CT3 (10nM-100μM) did not inhibit the exclusion of rhodamine 123, a Abcb1/p-glycoprotein substrate in CMEC/D3 human brain endothelial cells (Carl et al. 2010) *in vi*tro. (Figure 7.7A). Furthermore, no CT3-induced hypothermia occurred in P-glycoprotein-deficient, FVB.*Abcb1a/b*$^{-/-}$ or FVB wildtype mice (0.7 ± 0.2°C and 0.5 ± 0.1°C respectively n=3). Likewise, THC did not induce hypothermia in P-glycoprotein knockout mice *in vivo* (2.5mg/kg i.p.), in contrast to that seen following CsA pretreatment (Figure 7.6C. -0.7 ± 0.1°C and -0.5 ± 0.2°C respectively n=3). This indicated that the exclusion pump associated with cannabinoid exclusion was surprisingly not P-glycoprotein.

However, Cyclosporin A is reported also to influence breast cancer resistance protein one (BCRP1/ABCG2) and multidrug resistance protein one (MRP1/ABCC1), in addition to effects on P-glycoprotein (Pawarode et al., 2007). Injection i.p. with 5mgkg mitoxantrone (ABCG2 substrate/inhibitor) induced a transient hypothermia,

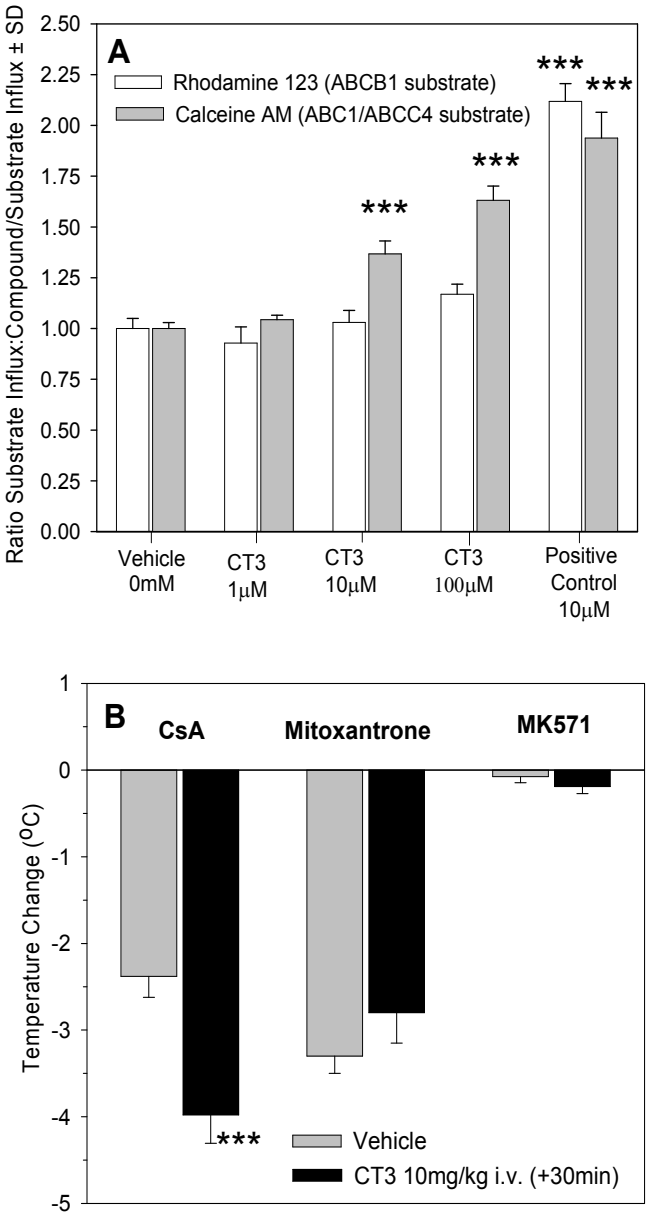
but in contrast to the effect with CsA, no additional hypothermia was induced by subsequent administration of CT3 (Figure 7.7B). This suggested that CT3 was not an ABCG2 inhibitor. However, i*n vitro* analysis indicated that high concentrations (10μM) of CT3 had a small inhibitory influence on casein AM accumulation in brain endothelial cells, suggestive of a weak activity on ABCC2 (Figure 7.7A). However, the *in vivo* inhibition of this, and ABCC1/ABCG4, with 20mg/kg i.p. MK571 failed to induce hypothermia by itself (n=8) or when administered in conjunction with 10mg/kg CT3 (n=8) suggesting that ABCG2 may not be the major mechanism of CNS-exclusion of CT3 (Figure 7.7B). As MRP1/ABCC1 expression is reported to be at the basolateral side of mouse brain endothelial cells, it is unlikely to have a role in resistance to drug entry to the CNS in mice (Löscher and Potschka, 2005). Therefore the nature of the CT3-exclusion pump is unclear.

Figure 7.6. *Cannabimimetic effects of CNS-excluded cannabinoids following blockade of CNS-exclusion pump function with cyclosporin A.*



The temperature of ABH (n=5-7) or ABH.CB_1R-deficient (n=4-5) mice was measured before and after the intravenous administration of (**A**) SAD448, SAB378 or CT3 in ECP. The results represent the mean ± SEM temperature (ºC) change from baseline 20minutes after cannabinoid administration. In some instances these mice were treated with 50mg/kg i.v. CsA 30min previously and the influence on temperature following administration of sub-hypothermic doses of (B) 10mg/kg i.v. CT3 or (C) 2.5mg/kg i.p. THC was assessed. * P<0.05, **P<0.01, ***P<0.001 compared to baseline.

Figure 7.7. *CNS exclusion pumps influence permeability of cannabinoids into the CNS.*

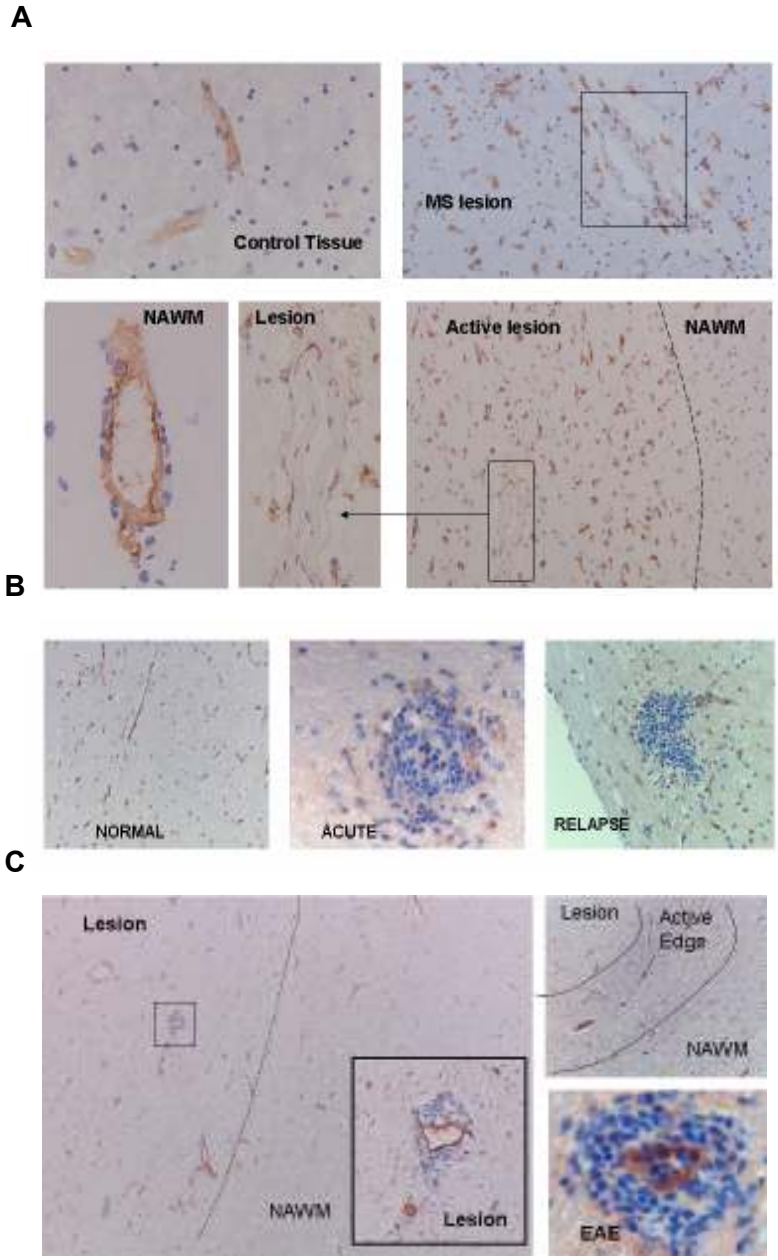


(A) Human brain endothelial cells were incubated for 45min with 2μM of either rhodamine 123 or calceine AM fluorescent substrates of ABC transporters. This was performed 60 minutes after incubation of the cells with CT3 or positive control, reservin 121 and MK571, inhibitor compound. The results represent the mean ± SEM ratio of influx of reporter compound with and without test inhibitor compound. n=5/group. **This was performed by Gijs Kooij and Helga De Vries. Free University Amsterdam, NL.** Alternatively (B), ABH mice were injected with either 50mg/kg i.v. CsA in ECP, 5mg/kg i.p. mitoxantrone or 20mg/kg i.p. MK571 in saline 30 minutes before the administration of 10mg/kg i.v. CT3 in ECP. Temperature was measured 20minutes later. The results represent the mean ± SEM temperature change compared to pre-drug treatment levels (n=8).

7.2.4. *CNS exclusion pump expression during multiple sclerosis and EAE.*

Whilst disease in both MS and EAE is associated with blood:brain barrier dysfunction that facilititates entry of cells and plasma proteins into the CNS (Butter et al., 1991a; Compston and Coles, 2002). The nature of this dysfunction is not well documented and at the time of undertaking the study, surprisingly there were no reports of alterations in ABC transporter expression in either MS or EAE. Therefore, immuohistochemical expression of the three CsA sensitive ATP transporters was investigated. ABCB1 and ABCG2 expression could be detected in blood vessels in both normal appearing white matter in MS and normal mouse tissue (Figure 7.8 A, B, C). Although ABCC1 is present on the CMEC/D3 human brain endothelial cells used *in vitro* (Carl et al., 2010), ABCC1 is not expressed on the lumen of blood vessels in human tissue (Aronica et al., 2004) and was weakly expressed in mice (Figure 7.9). ABCB1 expression was decreased and lost on blood vessels within mononuclear cell-rich lesions in chronic active MS lesions (Figure 7.8A) and in both acute and relapsing ABH mouse EAE lesions (Figure 7.8B). Consistent with loss of cell cuffing during remission in mice, ABCB1 expression was evident as found in normal white matter. In chronic MS lesions, although ABCB1 expression on blood vessels decreased, there was increased expression in astrocytic cells (Figure 7.8A). In contrast to that seen with ABCB1, there was no apparently loss of ABCG2 protein in both MS and EAE lesions (Figure 7.10C). Work to address the expression of other ABC transporters is ongoing. However, the status of the pumps during EAE are of functional significance as administration of 10mg/kg i.v. of CT3 into mice with chronic EAE resulted in the development of hypothermia (Figure 7.10), which will serve to reduce the therapeutic window of some CNS-excluded cannabinoids.

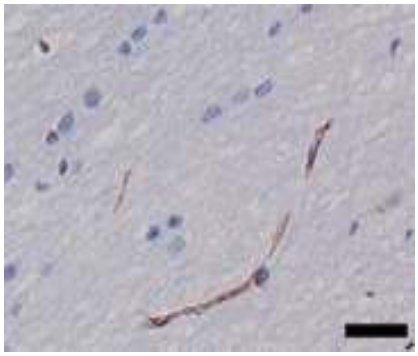
Figure 7.8. *Brain endothelial cell expression of CNS exclusion pumps during multiple sclerosis and EAE.*



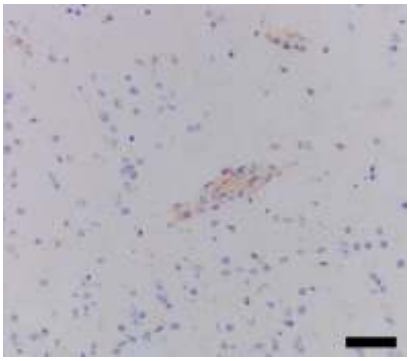
Immunoperoxidase staining of chronic active MS lesions and acute EAE lesion with an (A) mouse IgG2a ABCB1/Abcb1 (p-glycoprotein) or (B) Rat IgG ABCG2/Abcg2 (BCRP) specific antibodies in paraffin embedded tissue, counterstained with haematoxylin. Endothelial cells within lesions and normal appearing white matter (NAWM) express ABCG2/Abcg2. In contrast there is relative loss of ABCB1/Abcb1 on blood vessels in lesion areas in both MS and EAE lesions compared to normal appearing white matter and control non-MS tissue. In contrast there is a relative increase in astrocytic expression of p-glycoprotein in MS lesions. **Staining was performed by Wouter Gerritson and Sandra Amor, Amsterdam, The Netherlands.** Use of human material was ethically reviewed in accordance with the both the NL and UK law.

Figure 7.9. *Spinal cord expression of CNS exclusion pumps in the mouse.*

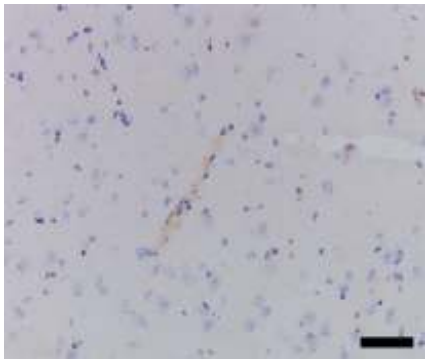
ABCB1 (MDR-1)



ABCC1 (MRP1)

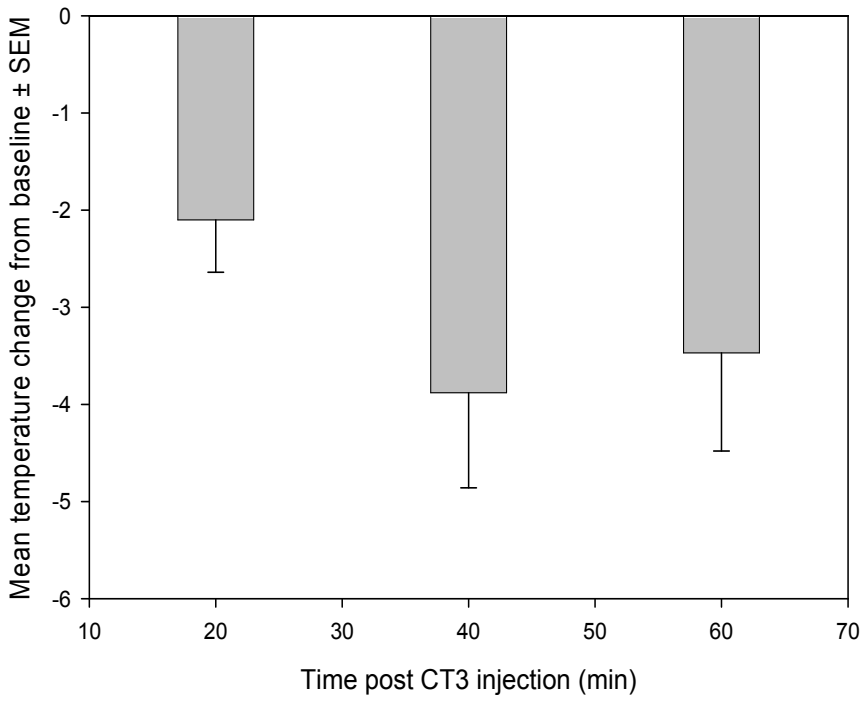


ABCC3 (MRP3)



Immunoperoxidase staining of normal mouse spinal cord tissue with rat antibodies specific for ABCC1 and ABCC3.
Staining was performed by Wouter Gerritson, Victoria Perkins and Sandra Amor, Amsterdam, The Netherlands. Bar = 10μm.

Figure 7.10. *CT3 is excluded less from the CNS in chronic stage spastic ABH CREAE mice.*



Following the development of spasticity in animals induced to develop EAE using spinal cord homogenate in Freunds adjuvant, animals were injected with 10 mg/kg CT3 i.v. in ECP, a dose which does not produce hypothermia in normal control ABH. The temperature was measured 20, 40 and 60 minutes after CT3 administration using a thermocouple placed under the hindlimb. The results represent the mean ± SEM temperature change from baseline over the monitoring period.

7.2.5. *The Cannabinoid (CT3) Exclusion pumps are polymorphic in mice.*

7.2.5.1. *Polymorphic responses to CT3 in CD-1® mice.*

Recently, it has been reported that CT3 exhibits marked cannabimimetic effects within its therapeutic dose range (Vann et al., 2007). Yet in contrast to that previously reported in outbred ICR mice from Harlan USA (Vann et al., 2005), as shown here, comparable doses of CT3 did not induce hypothermia in ABH mice. This suggested to us that CT3 could be a substrate for a polymorphic CNS-exclusion pump. Indeed some outbred Crl:CD-1®/ICR mice developed hypothermic effects following administration of 10mg/kg i.v. CT3 whereas, similar to ABH mice, other Crl:CD-1® mice did not (Figure 7.11A). The sedating effect of 10mg/kg i.v. CT3 was confirmed in another set (n=5/10) of CD-1® mice bred at QMUL from founders obtained from commercial (Charles Rivers UK) stock three years apart. CT3-induced hypothermia was present in Crl:CD-1® ex-breeders animals (n=1/10) and with

high frequency n=13/13 in Hsd:ICR(CD-1®) mice (Harlan UK) obtained directly from commercial sources within the UK. This is consistent with the hypothermic effects of CT3 in Hsd:ICR(CD-1®) from the USA (Vann et al., 2007).

7.2.5.2. *CD-1® mice do not express the Abcb1a^{mds} genotype.*

A P-glycoprotein deficiency has been reported to occur in Crl:CF-1 mice (Lankas et al., 1997). Genotyping of CD-1® mice that developed hypothermia following CT3 administration, failed to demonstrate evidence for the either the presence of the recessive mutant *Abcb1a^{mds}* allele or the long terminal repeat of mouse leukaemia virus within *Abcb1a^{mds}* (Figure 7.11B,C), which has been previously associated with loss of P-glycoprotein function in Crl:CF-1 mice (Lankas et al., 1997; Jun et al., 2000; Pippert and Umbenhauer, 2001). This excluded the possibility that the albino CD-1® stock had been contaminated by albino CF-1 mice.

7.2.5.3. *Microarray analysis of mice susceptible and resistant to the hypothermic effect of CT3.*

Microarray analysis of intestinal expression of ten CD-1®) mice suggested that there may be variation in ABC transporters such as Abcb1b and Abcc7, as the deviation of expression was as large as the mean suggesting that some mice express the protein where others do not (Mutch et al., 2004). Analysis of Illumia® ref 8 microarrays hybridized with message from the whole brains of 4 different CT3 responder and 4 CT3-non-responder mice demonstrated some differences in the expression of ABC transport message with a significant under-expression of Abcc8 and an over expression of Abcf1 when comparing responder to non-responder mice (Table 7.1). However, the gene difference between the two groups of mice resulted in the detection of 514 probes that were over expressed and 575 probes that were under expressed ($P<0.05$ of false positive) and 2 probes over expressed and 5 probes under expressed ($P<0.01$ of false positives,Table 7.2) between non-responder and responder mice. The under-expression of keratin 12 was the most differentially expressed gene product (Table 7.2). However, the differences between ABC transporter message were marginal and thus the nature of the gene which controls exclusion of cannabinoids (CT3) from the CNS may reside in one of the about 1100 genes, whose expression was altered. There were seven loci (*Odgh*, *Chst10*, *C2*, *Rpl38*, *Ccdc117*, *Rapgefl1*, *Krt12*) that detected significant ($P<0.01$) differences between responder and non-responder mice. However, based on the limited information of the function of most of these genes it is unclear how they may be involved in drug transport, such as keratin 12 which is a keratin present in corneal epithelium. Interestingly many of the most differentially expressed loci

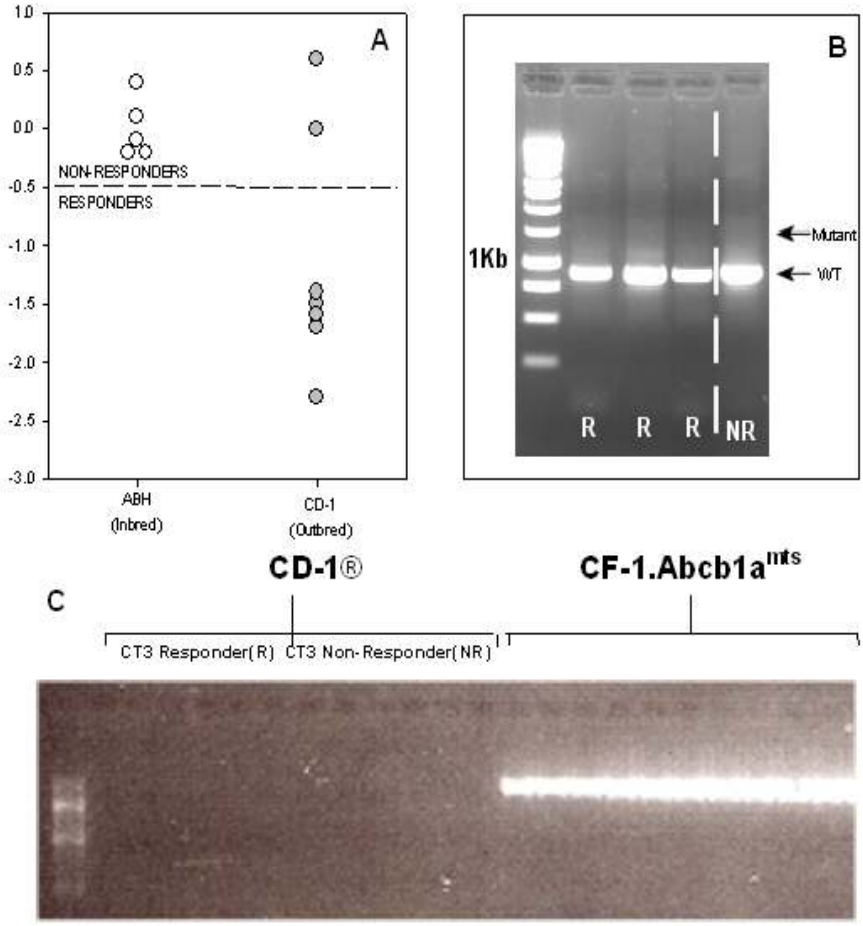
were on chromosome 11 (Table 7.2). Therefore, it is feasible that the CT-3 induced hypothermia gene was localized on this chromosome and had co-segregated with the detected loci.

7.2.5.4. *Polymorphic responses to CT3 in mice.*

The CD-1® mice strain originated from two male and seven female mice imported from Switzerland to the USA (Chia et al 2005). These Swiss mice have given rise to a variety of different: outbred (Hds:ICR(CD-1®), Hds:NIH(S), Hds:ND4, Swiss Webster); inbred (NOD, NIH, FVB, SJL, SWR) and inbred then outbred (Crl:CFSW, NIH:PI to Hds:NIMR) lines of mice (Beck et al., 2000; Chia et al., 2005). That the hypothermic phenotype was detected with high frequency in lines that have been divergent for many years indicates that the cannabinoid–induced hypothermia genotype is highly endemic in outbred, albino, Swiss (USA) laboratory mice (Figure 7.12). Interestingly, although BALB/cJ did not develop hypothermia (n=0/5), C57BL/6 mice developed a transient hypothermia (C57BL/6J n=4/4, -2.5 ± 0.3°C and C57BL/6JOlacHsd. n=3/3 -1.7 ± 0.3°C at 20min after 10mg/kg i.v. CT3. This indicates that the cannabinoid CNS-exclusion pump deficiency was also present in other mouse lines in addition to Swiss mice.

To determine the mode of inheritance of the CNS-exclusion pump deficiency, a single CD-1® male, which exhibited CT-3-induced hypothermia, was mated with a number of female ABH mice and the resultant F1 offspring (a single male and multiple females) were backcrossed with their parents. It was found that 10/18 (CD1® x ABH)F1, 14/20 male and female CD1® x (CD1® x ABH)F1 backcross and 6/18 male and female (CD1® x ABH)F1 x ABH backcross mice developed hypothermia (>1°C over 20 min) following injection of 10mg/kg i.v. CT3. This suggests that the male CD1® parent was heterozygous for a single autosomal dominant allele ($\chi 2$ = 2.07. 5.d.f (N.S.) for these 3 sets of data) that excludes cannabinoids from the CNS. This should be amenable to genetic mapping in further studies (Table 7.3).

Figure 7.11. *CD-1® mice do not express the Abcb1a^{mds} genotype.*



CD-1® mice were injected with 10mg/kg i.v. CT3 and the temperature was assessed 20 minutes later and (A) mice that developed hypothermic (>1.5ºC temperature drop) responses (responder) were separated from non-responder, non-hypothermic mice. (B, C) DNA was prepared and PCR and gel electrophoresis performed to detect the (B) wild type *Abcb1a* or mutant *Abcb1a^{mts}* or (C) the pro viral integration in *Abcb1a^{mts}*. **Prof. Alison Hardcastle, UCL, London, UK is thanked for primer design.**

Table 7.1. *Differential expression of ABC transporter RNA in the brains of CD-1® mice, which were either responders or non-responders to the hypothermia-inducing properties of CT3.*

PROBE	Locus	Mean Expression Level ± SD Non-Responders	Responders
ILMN_1226851	Abca1	51.13 ± 1.88	54.54 ± 4.04
ILMN_1216987	Abca2	159.44 ± 23.08	152.67 ± 10.16
ILMN_3150233	Abca3	382.12 ± 36.14	374.59 ± 21.07
ILMN_2829604	Abca4	54.18 ± 3.45	53.77 ± 2.60
ILMN_2998335	Abca5	54.47 ± 1.61	55.15 ± 2.76
ILMN_2965613	Abca6	50.68 ± 2.19	51.81 ± 3.07
ILMN_2716039	Abca7	52.12 ± 1.36	53.82 ± 5.93
ILMN_2896639	Abca7	67.49 ± 3.26	61.44 ± 3.06
ILMN_2686700	Abca8a	66.61 ± 8.86	56.54 ± 2.88
ILMN_2956871	Abca8b	54.74 ± 1.29	55.07 ± 2.13
ILMN_1253984	Abca9	61.26 ± 1.40	63.02 ± 4.85
ILMN_1228438	Abca12	48.96 ± 4.77	52.00 ± 2.37
ILMN_2931277	Abca13	49.42 ± 1.91	53.49 ± 2.48
ILMN_1258096	Abca14	58.80 ± 1.92	56.07 ± 3.40
ILMN_2598661	Abca15	55.55 ± 5.47	54.04 ± 1.15
ILMN_1228982	Abca16	52.33 ± 2.41	56.03 ± 2.50
ILMN_3153157	Abca17	53.59 ± 1.87	52.63 ± 3.73
ILMN_2768563	Abcb1a	51.14 ± 3.41	52.50 ± 3.43
ILMN_2918499	Abcb1b	49.42 ± 3.84	50.30 ± 2.08
ILMN_1250409	Tap1(Abcb2)	58.17 ± 2.18	61.99 ± 7.81
ILMN_2686721	Tap2(Abcb3)	99.86 ± 5.28	92.06 ± 7.10
ILMN_2648742	Abcb4	97.93 ± 2.48	85.87 ± 6.84
ILMN_2833596	Abcb6	100.70 ± 10.72	98.63 ± 6.44
ILMN_2864834	Abcb8	188.88 ± 23.71	189.67 ± 18.13
ILMN_1239687	Abcb9	57.14 ± 4.30	60.16 ± 3.29
ILMN_3142789	Abcb10	58.71 ± 1.00	57.07 ± 2.14
ILMN_2758509	Abcb11	54.89 ± 2.24	56.22 ± 1.97
ILMN_1248173	Abcc1	56.35 ± 3.40	60.04 ± 3.22
ILMN_2606719	Abcc2	54.89 ± 1.28	54.26 ± 0.64
ILMN_2685157	Abcc3	53.94 ± 6.90	54.69 ± 2.07
ILMN_2702171	Abcc5	54.09 ± 3.59	55.10 ± 2.96
ILMN_2934941	Abcc6	54.32 ± 2.47	57.64 ± 1.73
ILMN_2645526	Abcc8	86.84 ± 7.86	73.10 ± 5.91*
ILMN_3104271	Abcc9	54.98 ± 1.72	54.51 ± 4.99
ILMN_2660048	Abcc10	61.50 ± 6.20	63.04 ± 3.72
ILMN_1246917	Abcc12	48.84 ± 2.66	50.53 ± 1.98
ILMN_1245447	Abcd1	70.75 ± 4.32	71.76 ± 7.53
ILMN_1245831	Abcd2	60.99 ± 4.08	59.50 ± 3.41
ILMN_2925281	Abcd3	366.69 ± 29.01	389.07 ± 32.77
ILMN_2607474	Abcd4	61.40 ± 1.06	59.08 ± 6.11
ILMN_2982652	Abce1	73.88 ± 6.10	73.13 ± 3.24
ILMN_2760415	Abcf1	72.77 ± 2.20	84.93 ± 5.19*
ILMN_2768926	Abcf1	694.49 ± 22.54	754.00 ± 40.47
ILMN_2789544	Abcf2	600.43 ± 75.87	602.05 ± 40.58
ILMN_2677696	Abcf3	471.58 ± 92.81	430.34 ± 41.89
ILMN_2441335	Abcg1	139.92 ± 12.56	130.37 ± 19.12
ILMN_2728879	Abcg2	50.65 ± 2.49	52.76 ± 0.87
ILMN_2649306	Abcg3	51.97 ± 2.81	54.39 ± 4.75
ILMN_1225587	Abcg4	474.16 ± 31.13	499.66 ± 98.31
ILMN_2725781	Abcg5	60.24 ± 4.29	61.55 ± 2.87
ILMN_2789904	Abcg8	47.33 ± 1.86	47.73 ± 1.05

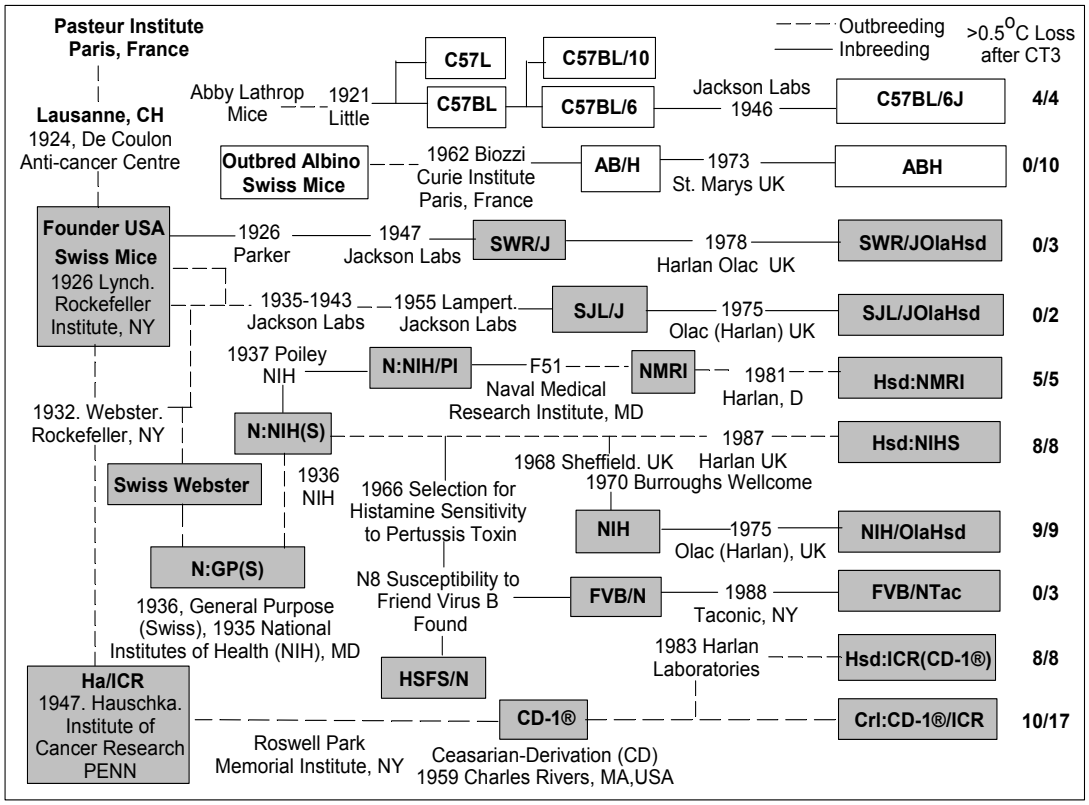
CD-1® mice were injected with 10mg/kg i.v. CT3 and the temperature was assessed 20 minutes later and mice that developed hypothermic (>1.5ºC temperature drop) responses (responder) were separated from non-responder, non-hypothermic mice. At least two weeks later the brains were rapidly dissected from the mice following euthanasia and RNA prepared. This level of gene expression was assessed using Illumia Ref 8 microarrays. The results represent the mean ± SD signal of n=4/group. * = P<0.05 of a false positive result. **This was performed by the Genome Centre, QMUL, London, UK by Lia de Faveri and Charles Mein.**

Table 7.2. *Differential expression of RNA in the brains of CD-1® mice, which were either responders or non-responders to the hypothermia-inducing properties of CT3*.

Probe	Locus	Chromosome	Mean Expression ± SD			Gene
			Non-Responder	Responder	Difference	
Over expression in CT3 responder mice						
ILMN_2639805	Ogdh	11	386.13 ± 28.34	541.30 ± 21.56	82.30783	Oxoglutarate dehydrogenase (lipoamide) nuclear gene encoding mitochondrial protein
ILMN_2595863	Chst10	1	88.04 ± 17.76	154.85 ± 17.46	66.49313	Carbohydrate sulfotransferase 10
ILMN_3161626	Prkag2	4	61.03 ± 2.97	113.80 ± 19.31	64.36749	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit
Under expression in CT3 responder mice						
ILMN_2603581	Aurkaip1	19	2066.25 ± 316.34	1126.94 ± 196.55	-63.39143	Aurora kinase A interacting protein 1
ILMN_2612895	C2	17	151.07 ± 7.64	110.23 ± 6.66	-70.85784	Complement component 2
ILMN_2594971	Rpl38	11	513.67 ± 42.49	341.05 ± 45.83	-73.99739	Ribosomal protein L38, transcript variant 1
ILMN_1259355	Ccdc117	11	134.07 ± 8.64	74.44 ± 10.86	-128.9300	Coiled-coil domain containing 117
ILMN_1250569	Rapgefl1	11	701.51 ± 43.47	405.28 ± 41.46	-156.5910	Rap guanine nucleotide exchange factor (GEF)-like 1
ILMN_2865527	Krt12	11	413.47± 39.57	177.67 ± 15.24	-273.1947	Keratin 12

CD-1® mice were injected with 10mg/kg i.v. CT3 and the temperature was assessed 20 minutes later and mice that developed hypothermic (>1.5ºC temperature drop) responses (responder) were separated from non-responder, non-hypothermic mice. At least two weeks later the brains were rapidly dissected from the mice following euthanasia and RNA prepared. This level of gene expression was assessed using Illumia Ref 8 microarrays. The results represent the mean ± SD signal of n=4/group. Differences of more than 65 had a $P<0.01$ of being a false positive result. **This was performed by the Genome Centre, QMUL, London, UK by Lia de Faveri and Charles Mein.**

Figure 7.12. *Polymorphic response in the hypothermic effects of CT3 in laboratory mice***.**



The genealogy of mouse strains and their hypothermic response to injection with 10mg/kg i.v. CT3 in ECP. The (>0.5°C) hypothermic response was assessed in a variety of mouse laboratory strains that were derived from founder Swiss albino mice imported to the USA by Carla Lynch in 1926. The date and researcher or Institution when sublines were generated are indicated as mice became commercial stock at companies such as the Jackson laboratories (J) or Olac (Ola) that became Harlan Sprague Dawley (Hsd).

Table 7.3. *Mode of Inheritance of CT3-induced hypothermia.*

	Expected Result for Mode of Inheritance			
Strain	Recessive Trait (*cep/cep*)	Dominant Trait (*Cep/Cep*)	Dominant Trait (Cep/Wt)	(Responders/Total)
CD-1®	100%	100%	100%	100% (1/1)
ABH	0%	0%	0%	0% (0/4)
(CD-1 x ABH)F1 F1	0%	100%	50%	56% (10/18)
CD-1 x (CD1 x ABH)F1	50%	100%	75%	70% (14/20)
(CD1 x ABH)F1 x ABH N1	0%	50%	50%	50% (9/18)
ABH x (CD1 x ABH)N1 N2	0%	50%	50%	50% (9/18)
(CD1 x ABH)N2 x ABH N3	0%	50%	50%	44% (8/18)
(CD1 x ABH)N3 x ABH N4	0%	50%	50%	41% (7/17)

The mode of inheritance of 10mg/kg i.v. CT3-induced hypothermia was assessed in selective crosses between a single CD-1 male and Biozzi ABH mice. The F1 mice used for breeding were CT3-responder mice and these were backcrossed with their parents. For N2 backcrosses ABH male mice were used to mate with female (CD1 x ABH) x ABH N2 to eliminate CD-1-derived sex chromosomes from the gene pool. The frequency of responder mice was estimated based on the male CD-1 parents harboring either recessive cannabinoid exclusion pump (*cep*) genes or being either homozygous (*Cep/Cep*) or heterozygous (Cep/Wt-wildtype (wt) for dominant cannabinoid exclusion pump genes.

7.3. *DISCUSSION*

Spasticity in EAE is controlled by the binding of Δ^9THC within the cannabis plant to neuronal CB_1R receptors, which also induce the adverse behavioural and physiological affects of cannabis (Wilkinson et al., 2003; Varvel et al., 2007). Although the adverse effects are associated with activation of CB_1R receptors in brain (Howlett et al., 2002), CNS-excluded, CB_1R agonists can inhibit spasticity as shown here and can inhibit inflammatory and neuropathic pain (Fox et al., 2001; Fride et al., 2004; Agarwal et al., 2007; Dziadulewicz et al., 2007; Worm et al., 2007; Yu et al., 2007). Although SAD448 (Brain et al., 2003; Adam-Worrall et al., 2007), SAB378 (Dziadulewicz et al., 2007) and CT3 (Rhee et al., 1997) bind and have some selectivity towards CB_2R, there is no good evidence that CB_2R plays a role in the control of spasticity (Chapter 4). This study demonstrates that by excluding CB_1R-stimulating chemicals from the brain, using their physicochemical properties, it is possible to increase the therapeutic window at least 10-100 fold, of CB_1R agonists. This study also shows that spasticity may be controlled both centrally and peripherally by CB_1 receptor stimulation. This probably occurs in the CNS and at peripheral nerve terminals at the neuromuscular junction. This may allow the exploitation of the medicinal properties that the cannabinoid system has to offer whilst limiting the well known adverse effects.

Polar molecules that are CNS-excluded can inhibit spasticity. Although SAD448 is a polar molecule that is less sedative than some CNS-penetrant compounds, such as SAB722, some water-soluble compounds can induce significant cannabimimetic effects (Pertwee et al., 2000; Martin et al., 2006). These include 0-1057 (Pertwee et al., 2000), a water-miscible compound that forms micelles in aqueous solution (Billy Martin. Virginia Commonwealth University, USA. Personal communication to D. Baker) and the water-soluble compound, 0-2545 (logBB=-0.08 Martin et al., 2006). Thus, limiting entry of compounds to the CNS may best be achieved by targeting compounds to exclusion pumps as was also evident following administration of SAD448. This activity was found to be a mechanism for exclusion from the CNS of CT3 and SAB378. Whilst SAB378 is reported to be a CB_1R agonist (Dziadulewicz et al., 2007; Cluny et al., 2010), there has been controversy concerning the cannabimimetic effects of CT3 (Burstein et al., 2004; Karst, 2007). Recently it has been suggested that CT3 exhibits analgesia within the dose range that exhibits adverse cannabimimetic effects (Vann et al., 2007). These are detected in rodents using a tetrad of tests, including the capacity to induce hypothermia within 15-20min following injection of the test compound (Howlett et al., 2002; Vann et al., 2007). CT3 failed to induce hypothermia within 60min following administration of up to 10mg/kg CT3 p.o. in ICR mice (Vann et al., 2007)

and Sprague Dawley rats at a time when CT3 exhibited analgesia at 1mg/kg p.o via a CB_1R dependent mechanism (Dyson et al., 2005). However, 10mg/kg p.o. CT3 did induce tetrad effects three hours after administration in rats (Dyson et al., 2005). This suggested that either a metabolite of CT3 was mediating the cannabimimetic effects or that it took a significant amount of time for sufficient CT3 to accumulate within the CNS. Similarly, high doses (>10mg/kg p.o. in rats) of SAB378 (Dziadulewicz et al., 2007) and (+)CBD-DMH could induce tetrad effects but their onsets were significantly delayed compared to their analgesic actions (Raphael Mechoulam personal communication, Hebrew University, Jerusalem, Israel). Although doses of around 30mg/kg p.o. CT3 or greater were required to induce hypothermia within 15min of administration in ICR mice, hypothermic effects of CT3 were detected at doses as low as 0.3mg/kg CT3 i.v. and were marked at 3mg/kg i.v. (Vann et al., 2007). This dose-response was distinctly different from that observed here in ABH mice. Although it has been suggested that CT-3 mediates actions via the peroxisome proliferator-activated receptor gamma (Ambrosio et al., 2007), these differences can be reconciled by the hypothesis that CT-3 is a CNS-excluded, CB_1R agonist. As such the actions of CT3 *in vivo* have been consistently inhibited by CB_1R antagonists (Dyson et al., 2005; Hiragata et al., 2007; Vann et al., 2007,). CT3 is a substrate for a polymorphic exclusion pump expressed at the blood:brain barrier. CNS exclusion pumps such as P-glycoprotein are also present in gut epithelial cells, which can result in poor oral bioavailability (Sparreboom et al., 1997). However, as both CT3 and SAB378 are orally available/active in rodents and humans there is sufficient discrimination between intestinal and CNS exclusion to allow therapeutic concentrations of drug to be achieved (Dyson et al., 2005; Dziadulewicz et al., 2007; Karst, 2007; Gardin et al., 2009).

This current study used CD-1®/ICR (caesarian-derived (CD)) mice which originated from a colony of outbred mice from the Institute of Cancer Research (ICR) mice (Chia et al., 2005) CD-1 mice are routinely used for toxicology studies as they are inexpensive and considered to be heterogeneous, which is confirmed by a genome-wide analysis of gene variation (Aldinger et al., 2009), and support a previous observation that i.v. administration of CT3 induces cannabimimetic events in ICR mice (Vann et al., 2007). However, ABH mice required significantly greater doses to induce such effects, unless pretreated with CsA. This is known to target the multi-drug resistance exclusion pumps that block drug entry into the CNS and other tissues (Hendrikse et al., 1998). Some CD-1/ICR mice used in these studies lacked a pump that excludes CT3 from the CNS. Outbred mice are seldom genetically monitored and variation exists between CD-1® colonies (Inoue et al., 1999). It is possible that the incidence of the hypothermic phenotype in CD-1® mice was high

in QMUL-derived mice compared to the many closed colonies of commercially-derived CD-1 mice. Importantly however, the CT3-sedating phenotype was evident in animals derived from commercial stock years apart. Furthermore, as the CT3-sedating phenotype also appears to be detected in a colony of ICR mice derived from Harlan Olac, USA (Dyson et al., 2005), whose founders originated from Charles Rivers, Willmington, Mass, USA, this suggests that there may be widespread expression of a polymorphism that can exclude drugs from the CNS in CD-1® mice. As ICR/CD-1®, other albino outbred Swiss mouse strains and presumably obese C57BL/6 such as the Pound Mouse^{TM} (C57BL/6NCrl-Lep^{rdb-lb}/Crl) and other C57BL/6 transgenic mice are often used by the pharmaceutical industry in toxicological and pharmacokinetic studies, it provides a warning that drug-studies in these strains may need to be interpreted with caution. Ivermectin is a p-glycoprotein substrate that have been used to treat onchocerciasis in over 40 million humans and endo and ecto-parasitic infestations in over five billion livestock and pets [Omura 2008]. Had ivermectin been first screened *in vivo* in mice (CF1) that lacked p-glycoprotein, therapeutic doses would have caused fatalities due to neurotoxicity and may have hampered or terminated drug development (Lankas et al., 1997). It is conceivable, especially as inbred NIH mice were generated at Burroughs Wellcome UK, that the development of some compounds may have been halted, due to pharmacokinetic and importantly toxicity issues following initial screening in such mouse strains

The CD-1® mice strain originated from two male and seven female mice imported in 1926 to the USA, by Carla Lynch at the Rockefeller Institute, from Lausanne, Switzerland (Chia et al., 2005). That the hypothermic phenotype was detected in many mouse strains that were derived from these Swiss mice and have been divergent since 1932 suggested that this phenotype was endemic in many albino Swiss laboratory mice and was probably present in the original Swiss mouse stock imported into the USA. This phenotype is controlled by a single dominant gene and provides a good explanation why the presence of this phenotype has remained high in outbred CD1® mouse stocks. Microarray of intestinal expression of ten CD-1®) suggested that there may be variation in ABC transporters such as Abcb1b and Abcc7, as the deviation of expression was as large as the mean suggesting that some mice express the protein where others do not (Mutch et al., 2004). However, the CT3-exclusion pump appeared not to be P-glycoprotein and Abcc7 was not detected in brain microarray of CT-3 responder and non-responder mice. Analysis of expression ABC transporters in brains did detect differences between CT-3 responder and non-responder mice, however ABCC7 has not been reported to be a drug efflux pump and ABCF1, has no transmembrane domains and so unlikely to behave as a drug efflux pumps. However, the expression of the causative gene may

have been masked by differential expression of CNS cells types, as whole brain and not endothelia was analyzed. Furthermore the observed differences between CT3-responder and non-responder mice may be dysregulated due to the consequences of the lack of function of the drug pump. As the inheritance of the genetic effect is dominant it may be that there is a loss of function of the protein due to interference of the normal translated protein via the products of the mutant allele down stream from RNA producton and would account for lack of any obvious difference in known ABC transporters between the normal and mutant line. Interestingly, the most significant over represented (*Ogdh*) and 4 most significant under represented (*Rpl38, Ccdc117, Rapgefl, Krt12*) RNA species between CT-3 responder and non-responder mice were coded by genes on chromosome 11. Although physical mapping indicates that these five genes are located in very different locations on chromosome 11, it is possible that the *CT3-induced hypothermia* gene may be located on chromosome 11 and co-segregate with the above genes. This could be supported by genomic mapping of the hypothermic trait using microsatellites or single nucleotide polymorphisms (SNP) in future studies.

Although, P-glycoprotein deficiency has been investigated and has not been detected in outbred CD-1 mice (Lankas et al., 1997), a recessive mutant (*Abcb1a*^{mds}) causing the loss of Abcb1a function had been identified in outbred CF-1 (Lankas et al., 1997; Jun et al., 2000; Pippert and Umbenhauer, 2001). However, no evidence for the expression of *Abcb1a*^{mds} was found in the CD-1 mice exhibiting CT3-induced hypothermia and it is clear that p-glycoprotein was not the target for the exclusion of cannabinoids based on *in vitro* data and the lack of influence of P-glycoprotein deficiency on CT3-induced "tetrad" responses *in vivo.* For many years it has been recognised that neuroinflammation is associated with blood brain barrier dysfunction, yet it is surprising that the nature of the drug pumps, even in microarray analysis, has not been investigated. This is particularly surprising as drug exclusion could be central to therapeutic activity. Cyclosporin A, which is so effective at preventing tissue rejection, had a very limited efficacy in MS (The Multiple Sclerosis Study Group 1990; Steck et al., 1990), because it is excluded from the CNS by a number of drug efflux pumps. Mitoxantrone is an MS drug, which has the properties of an ABCG2 substrate. Mitoxantrone is an immunomodulatory drug that is more immunosuppressive if it enters the CNS during EAE (Baker et al., 1992; Cotte et al., 2009). However, mitoxantrone can be potentially neurotoxic, when delivered to the CNS (Siegal et al., 1988). Therefore it is of interest that ABCG2 expression was maintained in lesions during MS and EAE. However, the level of CNS permeability may vary between individuals as it has been shown that *ABCG2* genotypes influence levels of drug efflux (Cotte et al., 2009). The activity of ABCG2 has also been reported to be inhibited *in* vitro by

cannabinoids THC, cannabidiol and cannabinol, leading to an increase in the accumulation of mitoxantrone (Holland et al., 2007). The same group also reported the inhibition of the mutidrug transporter ABCC1 (MRP1) with an order of efficacy: cannabidiol>cannabinol>THC as measured by the accumulation of the ABCC1 substrates Fluo3 and vincristine in ovarian cancer cells overexpressing ABCC1 (Holland et al., 2008). There are many polymorphisms in ABC transporters with functional consequences on drug uptake (Cascorbi, 2006). These undoubtedly influence response to therapy and probably influence the variability in the capacity of individuals to tolerate cannabinoid administration such as the reported association between a polymorphism in the drug pump ABCB1 (p-glycoprotein) and cannabis dependence (Benyamina et al., 2009). In contrast to effects on ABCG2, expression of P-glycoprotein was lost from vessels in active lesions in both active lesions in MS and mouse EAE. Others such as as ABCC2 (MRP-2) and ABCC6 (MRP-6) may likewise be down regulated in MS lesions (Victoria Perkins, QMUL unpublished). Likewise, the CT3-cannabinoid drug pump is down regulated during chronic EAE. Although this may limit the therapeutic value of some cannabinoids, lesions are often not in cognitive centres during MS and therefore psychoactivity may not occur during blood-brain barrier leakage elsewhere in the CNS. This may be used to selectivity target drugs to lesions using ABCB1 substrates. This observation has been recently confirmed, where it appears that as lymphocytes penetrate the CNS endothelium, and crosslink intracellular adhesion molecule one (ICAM-1,CD54) on the endothelial surface, an N-FAT mediated signalling cascade is triggered that induces the down-regulation of ABCB1 (Kooji et al., 2010). Whilst the loss of ABC transporters has been linked to the process of infiltration (Kooij et al., 2010), this persists in chronic-inactive MS lesions and in chronic EAE lesions. As these lesions are in areas of progressive neurodegeneration (Pryce et al., 2005), through the use of drugs that are substrates for ABC transporters missing in lesions, it may be possible to selectively deliver drugs to areas where therapy is required. We have demonstrated that THC is partially excluded by CNS drug pumps. Although the p-glycoprotein shows limited action of THC *in vitro* (Holland et al., 2006), *in vivo* P-glycoprotein has been shown to exclude about 50% of the THC using wildtype and P-glycoprotein-deficient CF-1 mice (Bonhomme-Faivre et al. 2008). In addition, the cannabinoids THC, cannabidiol and cannabinol are a substrate for ABCG2 and ABCC1 *in vitro* (Holland et al., 2007; Holland et al., 2008). Potential polymorphisms in these genes may explain the variation in the tolerability of cannabis-based therapeutics in some patients.

As THC is a neuroprotective agent (Pryce et al., 2003,. and Chapter 3), it may deliver neuroprotective and symptom control benefit at lesion sites, due to loss of p-glycoprotein function. Further studies are warranted to systematically investigate drug pump function during MS and EAE. Although elegantly shown in models of

pain (Agarwal et al., 2007), a definitive demonstration that SAD448, SAB378 and CT3 have a solely peripheral mode of action in spasticity is difficult. This is because of disease-related events that affect BBB function and that the actions of exclusion pumps are not absolute. As such they may only produce 10-100 fold exclusion of compounds (Schinkel and Jonker, 2003). Therefore, small amounts of compounds may enter the CNS, as seen with the increased CNS penetrance of a normally sub hypothermic dose of CT3 in chronic CREAE mice. This may reflect the downregulation of drug-efflux pumps responsible for the exclusion of CT3 at the blood:brain barrier as a result of disease pathology in these animals. However, results suggest that for certain compounds such as SAD448, deletion of the CB_1 receptor from peripheral nerves is sufficient to nullify the anti-spastic properties of this compound, indicating that selective targeting of CB_1 agonists to peripheral nerves can be efficacious in the amelioration of experimental spasticity. Furthermore, disease-related pathologies can cause the CNS-expression of molecules, such as Nav 1.8 sodium channels and peripherin (as used in this study), which can be used to conditionally deplete CB_1R from peripheral nerves (Troy et al., 1990; Mathew et al., 2001; Zhou et al., 2002; Craner et al., 2003; Agarwal et al., 2007). However, irrespective of whether the action of these compounds is solely peripheral, or peripheral and central, this study indicates that by excluding cannabinoids from the CNS it is possible to increase the therapeutic window of CB_1R agonists, such that they may be suitable for clinical development for the treatment of spasticity in MS.

CHAPTER EIGHT

FINAL CONCLUSIONS AND FUTURE DIRECTIONS

This study and abundant experimental data from other groups indicate the important and pleiotropic roles that the cannabinoid system plays in the maintenance of physiological processes under normal physiological conditions, but also in pathological processes. In the CNS, the neuroprotective nature of the cannabinoid system in events of CNS damage such as; neuroinflammation, ischaemia, brain trauma and neurodegenerative disease is becoming increasingly well established. That these conditions may be ameliorated by CB_1 receptor stimulation over and above endogenous levels via the endocannabinoid system, further points to a central role for cannabinoids as neuroprotective agents in episodes of CNS damage.

In experimental models of MS both in this study and elsewhere, exogenous CB_1 receptor stimulation can significantly slow the rate of disease progression and the development of disability that arises from the degeneration of axons. This is particularly the case in the spinal cord, where the pathology leads to the worsening of disability and the concomitant development of spasticity, tremor and bladder dysfunction, which are common symptoms associated with progressive disease in MS. This has lead to recent licensing of cannabis-based drugs for the treatment of MS and ongoing clinical investigations (CUPID), within the University and elsewhere, to study the potential benefits of cannabinoids (THC) as neuroprotective agents to slow the rate of disability development in progressive MS. Although, we have been unable to find a role for CBD for the inhibition of autoimmunity or control of spasticity (Baker et al., 2000), we have demonstrated the neuroprotective properties of the CBD, which is the non-psychoactive cannabis constituent of Sativex®. The mechanism(s) of the neuroprotective properties of cannabidiol have yet to be established, but merit further study as cannabidiol does not have any of the psychoactive properties of cannabinoid agonists such as THC, which make their clinical therapeutic use problematic. Our experimental systems could be used to investigate the optimum ratio of THC:CBD in medicinal cannabis extracts as there is no compelling evidence that a 1:1 mixture as in Sativex® is optimum. The data presented here support our previous studies indicating a neuroprotective role for the cannabinoid system (Pryce et al., 2003). In contrast we can find less compelling evidence for a role of cannabinoid therapy in autoimmunity.

A definitive explanation of the role of CB_1 versus CB_2 receptors in this process remains to be elucidated. Whilst CB_2-deficient mice in this study showed an enhanced level of susceptibility to the induction of EAE, no immunosuppressive effect on the development of autoimmunity by CB_2 receptor agonists has yet been demonstrated. In contrast, a robust immunosuppression of EAE is seen with CB_1 receptor agonists but only at doses that produce significant cannabimimetic effects in treated animals that would preclude their use in a clinical setting. The ability of CB_1 agonists to induce immunosuppressive effects is lost in global CB_1 receptor-deficient mice and also in mice where the CB_1 receptor is conditionally deleted from (CNS) nerve cells, but is absent using CNS-excluded agonists. This indicated that CB_1 receptor stimulation in the CNS is necessary for the immunosuppressive properties of cannabinoid receptor agonists, which may be as a result of downstream production of inflammation-suppressing mediators such as glucocorticoids, which are produced in response to CNS, CB_1 receptor stimulation. A clearer picture as to the role of the CB_2 receptor as an immune-modulator may be provided by the administration of immunosuppressive compounds into CB_2 deficient mice bred onto the ABH background. To date, all studies purporting to demonstrate and influence of CB_2 receptor on autoimmune function, have used transgenic mice on the C57BL/6 mouse background. This is a low EAE susceptibility strain compared to ABH mice and disease in C57BL/6 mice is highly variable in incidence and severity. EAE induction on the ABH background strain is, in contrast, highly reproducible with high incidence and of consistent disease severity. As such it may be more difficult to inhibit disease in ABH mice with weakly immunosuppressive compounds, compared to that induced in C57BL/6 mice. We expect that immunosuppressive cannabinoids such as high-dose THC will still produce downregulation of EAE in these ABH.CB_2 deficient mice. This will indicate further that the immunosuppressive properties of CB_1 agonists are mediated by CNS-expressed CB_1 receptors. Unfortunately, the N6 ABH.*Cnr2-/-* backcross colony of mice that I had generated for such studies were lost due to mistakes by our animal technicians. The backcrossing into the ABH mouse background to perform this experiment is currently ongoing such that these experiments can be performed in the future.

In summary, I believe that the use of cannabinoids as potential modulators of autoimmunity in the CNS will be precluded in human disease due to the high doses of agonists needed to produce this effect, leading to unacceptable psychotropic side-effects. In contrast, low dose cannabinoid-mediated CB_1 receptor stimulation can produce a significant neuroprotective benefit in inflammatory CNS disease at a level where the side-effect profile may be more acceptable to MS patients. Although

disease modification of MS is research for the future, cannabinoid therapy for symptom control of MS is now a reality.

The amelioration of spasticity and licensing of medicinal cannabis for the treatment of symptoms of MS has translated our earlier observations in chronic EAE (Baker et al., 2000) into the clinic. Unfortunately, our work shown here using CB_1 receptor knockout animals and THC-deficient cannabis (Wilkinson et al., 2003) indicate that the mediators of therapy are also the same components that mediate the adverse effects of cannabis use (Varvel et al. 2007). We therefore seek to utilise knowledge of the cannabinoid system to exploit the therapeutic benefits that the cannabinoid system has to offer, whilst limiting the adverse effects.

Although CB_2 agonists were shown to inhibit spasticity due to their weak cross-reactivity with the CB_1 receptor, future work should demonstrate that these agents are active during spasticity in CB_2-deficient mice. However, as CB_2 agonists have low affinity for the CB_1 receptor it may be easier to dose-titrate such agents in clinical use. We believe that high affinity CB_1 agonists are unlikely to be of therapeutic use due to the potential for psychoactivity, receptor tolerance considering the wide variety of the capacity of individuals to tolerate cannabis. Amelioration of spasticity can also be achieved by modulation of the endocannabinoid system by administration of inhibitors of the degradative enzymes for the endocannabinoids anandamide (FAAH inhibition) and 2-AG (MAG-lipase inhibition). The reduction in spastic tone in the hind limbs of CREAE mice was significantly reduced by FAAH inhibition although subsequent analysis in spastic FAAH-deficient mice revealed that whilst the anti-spastic activity of CAY10402 was lost in these animals, the anti-spastic activity of URB597 was retained. This indicates that either URB597 has additional off-target effects at other components of the endocannabinoid system or its metabolites also target these components. However, because the full extent of the endocannabinoid system remains to be discovered and the fact that essentially no cannabinoid-related agent is specific for its target, a combination of drug with cannabinoid system knockout mice has proved invaluable in defining therapeutic targets. A significant reduction of spastic hind-limb tone was also seen by the inhibition of MAG-lipase by JZL-184 and enhancement of 2-AG levels in CREAE mice. The specificity of the anti-spastic effects of JZL-184 at MAG-lipase cannot be confirmed until access to a MAG-lipase knockout mouse strain is obtained. Whilst the administration of FAAH inhibitors produced no obvious cannabimimetic effects in CREAE mice, (as seen with conventional CB_1 receptor agonists) and CB_1 receptor desensitisation has not been reported, the observation that repeated MAG-lipase inhibition produces cannabimimetic effects and rapid CB_1 receptor desensitisation suggests that therapeutically, in the clinical situation, FAAH inhibitors will have more utility than

MAG-lipase inhibitors. The inhibition of FAAH may not be without negative consequences however, as this enzyme is widely expressed throughout the body and particularly in the liver where it may be important for lipid metabolism and thus may have negative hepatotoxic consequences. It is of interest that some FAAH-deficient mice bred in this laboratory have fatty livers at post mortem (unpublished observation).

The negative side-effect profile of CNS-penetrant CB_1 receptor agonists is well established and the use of such agents to treat symptoms such as spasticity will always be accompanied, if used optimally, by a degree of cannabimmetic effects due to the stimulation of these receptors in the CNS. This will limit the use of these agents as some patients will find these psychotropic effects intolerable and the therapeutic window of symptom relief before the development of negative side-effects is narrow. It has been long-held neurological dogma that limb spasticity is a purely CNS-mediated problem and thus CNS-penetrant agents (such as baclofen) are required for its treatment. It is shown here, that this is probably not the case and in experimental MS at least, it is possible to reduce hind limb spastic tone by the stimulation of peripherally-expressed CB_1 receptors using cannabinoid receptor agonists that are not CNS-penetrant and which will not have psychoactive side-effects. We have identified novel cannabinoid agents that form this new class of agents and have defined mechanisms of drug-exclusion. It has also become clear during this study that CNS drug-resistance pumps operating at the endothelial cells of the blood:brain barrier are responsible for the non-penetrance of peripheralised cannabinoids to the CNS in addition to influencing the CNS-penetrance of conventional cannabinoids such as THC. Genetic polymorphisms in these pumps may influence drug-responsiveness and the capacity to tolerate cannabinoid drugs. Interestingly, we have identified the presence of a dominant cannabinoid drug exclusion pump with mice strains commonly used in pharmacological and toxicological drug studies. The identity of this locus is currently being examined in genome wide screens of CT-3 responder and non-responder mice. Once a chromosome location is indicated, gene sequencing, transfection of mutants into brain endothelial cells to block transport of substrates, expression profiling in EAE/MS will be performed and studies in knockout mice can be performed to identify the causative gene.

A further novel observation of interest, which could influence current and future treatment modalities, is the observation that there is drug pump dysregulation during MS and EAE. The level of expression of drug-resistance pumps can be influenced not only by ongoing neuroinflammation but also in chronic inactive lesions where inflammation has long resolved. Downregulation of drug-resistance

pumps at these lesional areas can facilitate the entry of potentially neuroprotective compounds such as cannabinoids, which may be normally excluded by the blood:brain barrier. This situation may also allow the penetrance of peripheralised cannabinoid agonists to these lesional areas which, as the drug entry will be focal may not produce global cannabimimetic effects seen with CNS-penetrant CB_1 agonists.

VSN16 was developed to be a peripheralised CB_1 receptor agonist, based on early pharmacology studies. However, intriguingly although it is: hydrophilic, water soluble, excluded from the CNS, inhibited by CB_1 receptor antagonist compounds and has a robust anti-spastic activity both via intravenous and oral routes with good pharmacokinetics, it has no activity at the CB_1 receptor. The target for the anti-spastic activity of VSN16 at present remains elusive, although VSN16 can modulate the actions of a GPR55 receptor agonist. To determine whether the anti-spastic activity of VSN16 is mediated via the GPR55 receptor, experiments will be performed to determine if spasticity reduction is observed in spastic, GPR55-deficient CREAE mice, which are currently ongoing. Furthermore, in contrast to cannabinoids, the expression profile of GPR55 is unclear and the mechanisms of symptom control need explanation. Nevertheless, VSN16 represents a novel class of compounds with a novel target. Although, it is as active as current anti-spastic agents, it appears to lack the side-effect potential of competitor compounds, such as baclofen or CNS-penetrant cannabinoids. Therefore, due to an apparent superior side-effect profile it could compete favourably with current anti-spastic drugs and could be prescribed earlier in the disease course as VSNB16 appears to lack the intolerable side-effects of current anti-spastic compounds. VSN16 has been patented and is in development for clinical use.

FUTURE AIMS

Chapter 3: Autoimmunity suppression/neuroprotection

(a) Test the ability of THC to modulate EAE in CB_2 knockout mice on ABH background (ongoing).
(b) Further investigate mechanisms of CBD-mediated neuroprotection.

Chapter 4: Anti-spastic effects of CB1-mediated agonists

Determine whether the anti-spastic activity of CB_1/CB_2 active compounds is maintained in CB_2-deficient spastic mice on ABH background (ongoing).

Chapter 5: FAAH Inhibition

(a) Test lower doses of UCM579 in FAAH knockout mice to demonstrate specificity.
(b) Test competitive and non-competitive FAAH inhibitors compounds.

Chapter 6: VSN16

(a) Antagonise the action of VSN16 with compounds reported to have GPR55 antagonist activity such as cannabidiol.
(b) Test VSN16 in spasticity in GPR55 knockout mice.
(c) Further identify the target for VSN16 action.

Chapter 7: Peripheral CB_1 receptor agonisits.

(a) Further identify the CT3 Drug exclusion pump using gene mapping/sequencing.
(b) Identify the nature of drug exclusion pumps in chronic EAE.
(d) Examine expression of ABCB1(MDR-1), ABCC1 (MRP1) and ABCC3 (MRP3) in EAE and MS.

Additional study.

Investigate the role of the novel putative cannabinoid receptor GPR18 in the modulation of immune responses with particular respect to the role of microglial activation on neuroinflammation in the CNS and whether stimulation or antagonism of this receptor can influence the development of EAE in the ABH mouse.

REFERENCES

Abood, M.E., Rizvi, G., Sallapudi, N. and McAllister SD. (2001). Activation of the CB1 cannabinoid receptor protects cultured mouse spinal neurons against excitotoxicity, *Neurosci Lett.* 309:197-201.

Adams, M.M. and Hicks, A.L. (2005). Spasticity after spinal cord injury. *Spinal Cord* 43:577-586.

Adam-Worrall, J., Hill, D.R. and Cotttney, J. (2007) Synergistic combination for the treatment of pain cannabinoid receptor agonist and opiod receptor agonist. *World Int. Prop. Org.* WO2007006732.

Agarwal, N., Pacher, P., Tegeder, I., Amaya, F., Constantin, C.E., Brenner, G.J., Rubino, T., Michalski, C.W., Marsicano, G., Monory, K., Mackie, K., Marian, C., Batkai, S., Parolaro, D., Fischer, MJ., Reeh, P., Kunos, G., Kress, M., Lutz, B., Woolf, C.J. and Kuner, R. (2007). Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat Neurosci.* 10:870-879.

Aguado, T., Romero, E., Monory, K., Palazuelos, J., Sendtner, M., Marsicano, G., Lutz, B., Guzman, M., and Galve-Roperh, I. (2007). The CB1 cannabinoid receptor mediates excitotoxicity-induced neural progenitor proliferation and neurogenesis. *J Biol Chem.* 282:23892-23898.

Agurell, S., Halldin, M., Lindgren, J.E., Ohlsson, A., Widman, M., Gillespie, H. and Hollister, L. (1986). Pharmacokinetics and metabolism of delta 1-tetrahydrocannabinol and other cannabinoids with emphasis on man, *Pharmacol Rev.* 38:21-43.

Ahn, K., Johnson, D.S. and Cravatt BF. (2009). Fatty acid amide hydrolase as a potential therapeutic target for the treatment of pain and CNS disorders. *Expert Opin. Drug Discov.* 4:763-784.

Ahmed, Z., Doward, A.I., Pryce, G., Taylor, D.L., Pocock, J.M., Leonard, J.P., Baker, D. and Cuzner ML. (2002). A role for caspase-1 and -3 in the pathology of experimental allergic encephalomyelitis : inflammation versus degeneration. *Am J Pathol.*161:1577-1586.

Alexander, S.P.H. and Kendall, D.A. (2007).The complications of promiscuity: endocannabinoid action and metabolism.*Br J Pharmacol.*152:602-23.

Aldinger, K.A., Sokoloff, G., Rosenberg, D.M., Palmer, A.A. and Millen, K.J. (2009). Genetic variation and population substructure in outbred CD-1 mice: implications for genome-wide association studies. PLoS One. 4:e4729.

Al-Izki, S., Pryce, G., Giovannoni, G. and Baker D. (2009). Evaluating potential therapies for bladder dysfunction in a mouse model of multiple sclerosis with high-resolution ultrasonography. *Mult Scler*.15:795-801.

Altuntas, C.Z., Daneshgari, F., Liu, G., Fabiyi, A., Kavran, M., Johnson, J.M., Gulen, M.F., Jaini, R., Li, X., Frenkl, T.L. and Tuohy, V.K. (2008). Bladder dysfunction in mice with experimental autoimmune encephalomyelitis. *J Neuroimmunol.* 203:58-63.

Amato, M.P. and Ponziani, G. (2000). A prospective study on the prognosis of multiple sclerosis, *Neurol Sci.* 21:S831-838.

Ambrosio, A.L., Dias, S.M., Polikarpov, I., Zurier, R.B., Burstein, S.H, and Garratt, R.C. Ajulemic acid, a synthetic nonpsychoactive cannabinoid acid, bound to the ligand binding domain of the human peroxisome proliferatoractivatedreceptor gamma. *J Biol Chem.* 282:18625-33.

Amor, S., Smith, P.A., t' Hart, B. and Baker D. (2005). Biozzi mice: of mice and human neurological diseases. *J Neuroimmunol.* 165:1-10.

Andersson, P.B., Waubant, E., Gee, L,. and Goodkin, D.E. (1999). Multiple sclerosis that is progressive from the time of onset: clinical characteristics and progression of disability. *Arch Neurol.* 56:1138-1142.

Arevalo-Martin, A., Vela, J.M., Molina-Holgado, E., Borrell, J. and Guaza, C. (2003).Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *J Neurosci*. 23:2511-2516.

Aronica, E., Gorter, J.A., Ramkema, M., Redeker, S., Ozbas-Gerçeker, F., van Vliet, E.A., Scheffer, G.L., Scheper, R.J., van der Valk, P., Baayen, J.C. and Troost, D. (2004). Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Epilepsia.* 45:441-451.

Ascherio, A. and Munger, K.L. (2010). Epstein-Barr virus infection and multiple sclerosis: a review. *J Neuroimmune Pharmacol.* 5:271-217.

Ashton, J.C., Rahman, R.M., Nair, S.M., Sutherland, B.A., Glass, M. and Appleton, I. (2007).Cerebral hypoxia-ischemia and middle cerebral artery occlusion induce expression of the cannabinoid CB2 receptor in the brain. *Neurosci Lett.* 412:114-117.

Baker, D., O'Neill, J.K., Gschmeissner, S.E., Wilcox, C.E., Butter, C. and Turk, J.L. (1990). Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. J *Neuroimmunol.* 28:261-270.

Baker, D., O'Neill, J.K., Davison, A.N. and Turk, J.L. (1992). Control of immune-mediated disease of the central nervous system requires the use of a neuroactive agent: elucidation by the action of mitoxantrone. *Clin Exp Immunol.* 90:124-128.

Baker, D., Pryce, G., Croxford, J.L., Brown, P., Pertwee, R.G., Huffman, J.W. and Layward, L. (2000). Cannabinoids control spasticity and tremor in a multiple sclerosis model. *Nature*. 404:84-7.

Baker, D., Pryce, G., Croxford, J.L., Brown, P., Pertwee, R.G., Makriyannis, A., Khanolkar, A., Layward, L., Fezza, F., Bisogno, T. and Di Marzo, V. (2001). Endocannabinoids control spasticity in a multiple sclerosis model. *FASEB J* 15:300-302.

Baker, D., Pryce, G., Giovannoni, G. and Thompson A.J. (2003). The therapeutic potential of cannabis. *Lancet Neurol*. 2:291-298.

Baker, D., Pryce, G., Davies, W.L. and Hiley C.R. (2006). In silico patent searching reveals a new cannabinoid receptor. *Trends Pharmacol Sci.* 27:1-4.

Baker, D. and Jackson. S.J. (2007). Models of multiple sclerosis. *Adv Clin Neurosci Rehab*. 6: 10-12.

Baker, D., Jackson, S.J. and Pryce, G. (2007). Cannabinoid control of neuroinflammation related to multiple sclerosis. *Br J Pharmacol.* 152:649-654.

Barann, M., Molderings, G., Bruss, M., Bonisch, H., Urban, B.W. and Gothert, M. (2002). Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site. *Br J Pharmacol.* 137:589-596.

Barna, I., Zelena, D., Arszovszki, A.C. and Ledent, C. (2004). The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: in vivo and in vitro studies in CB1 receptor knockout mice. *Life Sci.* 75:2959-2970.

Barnett, M.H. and Prineas, J.W. (2004). Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol.* 55:458-468.

Basavarajappa, B.S. (2007).Critical enzymes involved in endocannabinoid metabolism. *Protein Pept Lett.* 14:237-246.

Bayewitch, M., Avidor-Reiss, T., Levy, R., Barg, J., Mechoulam, R. and Vogel, Z. (1995). The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS Lett.* 375:143-147.

Bayewitch, M., Rhee, M.H., Avidor-Reiss, T., Breuer, A., Mechoulam, R. and Vogel. Z. (1996). (-)-Delta9-tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem.* 271:9902-9905.

Bechtold, D.A., Miller, S.J., Dawson, A.C., Sun, Y., Kapoor, R., Berry, D. and Smith, K.J. (2006). Axonal protection achieved in a model of multiple sclerosis using lamotrigine. *J Neurol.* 253:1542-1551.

Begg, M., Pacher, P., Batkai, S., Osei-Hyiaman, D., Offertaler, L., Mo, F.M., Liu, J. and Kunos, G. (2005). Evidence for novel cannabinoid receptors. *Pharmacol Ther* 106:133-145.

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.

Benito, C., Romero, J.P., Tolon, R.M., Clemente, D., Docagne, F., Hillard, C.J., Guaza, C.. and Romero, J. (2007). Cannabinoid CB1 and CB2 receptors and fatty acid amide hydrolase are specific markers of plaque cell subtypes in human multiple sclerosis. *J Neurosci.* 27:2396-2402.

Benyamina, A., Bonhomme-Faivre, L., Picard, V., Sabbagh, A., Richard, D., Blecha, L., Rahioui, H., Karila, L., Lukasiewicz, M., Farinotti, R., Picard, V., Marill, C. and Reynaud, M. (2009). Association between ABCB1 C3435T polymorphism and increased risk of cannabis dependence.Prog *Neuropsychopharmacol Biol Psychiatry.* 33:1270-1274.

Berghuis, P., Dobszay, M.B., Wang, X., Spano, S., Ledda, F., Sousa, K.M., Schulte, G., Ernfors, P., Mackie, K., Paratcha, G., Hurd, Y.L. and Harkany, T. (2005). Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. *Proc Natl Acad Sci U S A.* 102:19115-19120.

Berghuis, P., Rajnicek, A.M., Morozov, Y.M., Ross, R.A., Mulder, J., Urban, G.M., Monory, K., Marsicano, G., Matteoli, M., Canty, A., Irving, A.J., Katona, I., Yanagawa, Y., Rakic, P., Lutz, B., Mackie, K. and Harkany, T. (2007). Hardwiring the brain: endocannabinoids shape neuronal connectivity. *Science.* 316:1212-1216.

Berglund, B.A., Fleming, P.R., Rice, K.C., Shim, J.Y., Welsh, W.J. and Howlett, A.C. (2000). Development of a novel class of monocyclic and bicyclic alkyl amides that exhibit CB1 and CB2 cannabinoid receptor affinity and receptor activation. *Drug Des Discov.*16:281-294.

Bettelli, E., Baeten, D., Jäger, A., Sobel, R.A. and Kuchroo V.K. (2006). Myelin oligodendrocyte glycoprotein-specific T and B cells cooperate to induce a Devic-like disease in mice. *J Clin Invest.* 116:2393-2402.

Bilsland, L.G., Dick, J.R., Pryce, G., Petrosino, S., Di Marzo, V., Baker, D. and Greensmith, L. (2006). Increasing cannabinoid levels by pharmacological and genetic manipulation delay disease progression in SOD1 mice. *FASEB J.* 20:1003-1005.

Bisogno. T., Maurelli, S., Melck, D., De Petrocellis, L. and Di Marzo, V. (1997). Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J Biol Chem.* 272:3315-3323.

Bisogno, T., Melck, D., Bobrov, M., Gretskaya, N.M., Bezuglov, V.V., De Petrocellis, L. and Di Marzo, V. (2000). N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. *Biochem J*. 351 Pt 3:817-824.

Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M.G., Ligresti, A., Matias, I., Schiano-Moriello, A., Paul, P., Williams, E.J., Gangadharan, U., Hobbs, C., Di Marzo, V. and Doherty, P. (2003). Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J Cell Biol.* 163:463-468.

Bjartmar, C., Kidd, G., Mork, S., Rudick, R. and Trapp, B.D. (2000). Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients. *Ann Neurol.* 48:893-901.

Bjartmar, C., Wujek, J.R. and Trapp, B.D. (2003). Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *J Neurol Sci.* 206:165-71.

Bjartmar, C. and Trapp, B.D. (2003). Axonal degeneration and progressive neurologic disability in multiple sclerosis. *Neurotox Res*. 5:157-64.

Black, J.A., Dib-Hajj, S., Baker, D., Newcombe, J., Cuzner, M.L. and Waxman, S.G. (2000). Sensory neuron-specific sodium channel SNS is abnormally expressed in the brains of mice with experimental allergic encephalomyelitis and humans with multiple sclerosis. *Proc Natl Acad Sci U S A .* 97:11598-11602.

Black, J.A. and Waxman, S.G. (2008). Phenytoin protects central axons in experimental autoimmune encephalomyelitis. *J Neurol Sci.* 274:57-63.

Blankman, J.L., Simon, G.M. and Cravatt B.F. (2007). A comprehensive profile of brain enzymes that hydrolise the endocannabinoid 2-arachidonoylglycerol. *Chem Biol.* 14:1347-1356.

Boger, D.L., Sato, H., Lerner, A.E., Hedrick, M.P., Fecik, R.A., Miyauchi, H., Wilkie, G.D., Austin, B,J., Patricelli, M.P. and Cravatt, B.F. (2000). Exceptionally potent inhibitors of fatty acid amide hydrolase: the enzyme responsible for degradation of endogenous oleamide and anandamide. *Proc Natl Acad Sci USA* 97:5044-5049.

Boger, D.L., Miyauchi, H., Du, W., Hardouin, C., Fecik, R.A., Cheng, H., Hwang, I., Hedrick, M.P., Leung, D., Acevedo, O., Guimarães, C.R., Jorgensen, W.L. and Cravatt, B.F. (2005). Discovery of a potent, selective, and efficacious class of reversible alpha-ketoheterocycle inhibitors of fatty acid amide hydrolase effective as analgesics. *J Med Chem*. 48:1849–1856.

Bolton, C., O'Neill, J.K., Allen, S.J., and Baker, D. (1997). Regulation of chronic relapsing experimental allergic encephalomyelitis by endogenous and exogenous glucocorticoids. *Int Arch Allergy Immunol.* 1997;114:74-80.

Bolton, C. and Paul, C. (2006). Glutamate receptors in neuroinflammatory demyelinating disease. *Mediators Inflamm.* 2006:1-12.

Bondarenko, A., Waldeck-Weiermair, M., Naghdi. S., Poteser, M., Malli, R. and Graier, W.F. (2010). GPR55-dependent and -independent ion signalling in response to lysophosphatidylinositol in endothelial cells. *Br J Pharmacol.* 161:308-320.

Bonhaus, D.W., Chang, L.K., Kwan, J. and Martin, G.R. (1998). Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J Pharmacol Exp Ther.* 287:884-888.

Bonhomme-Faivre, L., Benyamina, A., Reynaud, M., Farinotti, R. and Abbara, C. (2008). Disposition of Delta tetrahydrocannabinol in CF1 mice deficient in mdr1a P-glycoprotein. *Addict Biol.* 13:295-300.

Borner, C., Hollt, V., Sebald, W. and Kraus, J. (2007).Transcriptional regulation of the cannabinoid receptor type 1 gene in T cells by cannabinoids. *J Leukoc Biol.* 81:336-343.

Bouaboula, M., Rinaldi, M., Carayon, P., Carillon, C., Delpech, B., Shire, D., Le Fur, G. and Casellas, P. (1993). Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem.* 214:173-180.

Bouaboula, M., Bourrie, B., Rinaldi-Carmona, M., Shire, D., Le Fur, G. and Casellas, P. (1995a). Stimulation of cannabinoid receptor CB1 induces krox-24 expression in human astrocytoma cells. *J Biol Chem.* 270:13973-13980.

Bouaboula, M., Poinot-Chazel, C., Bourrie, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G. and Casellas, P. (1995b). Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J.* 312:637-641.

Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrie, B,, Rinaldi-Carmona, M., Calandra, B., Le Fur, G. and Casellas, P. (1996). Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression, *Eur J Biochem.* 237:704-711.

Bouaboula, M., Hilairet, S., Marchand, J., Fajas, L., Le Fur, G. and Casellas, P. (2005). Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. *Eur J Pharmacol.* 517:174-181.

Brady, C.M., DasGupta, R., Dalton, C., Wiseman, O.J., Berkley, K.J. and Fowler, C.J. (2005). An open-label pilot study of cannabis-based extracts for bladder dysfunction in advanced multiple sclerosis. *Mult Scler* 10: 425-433.

Brain, C.T., Dziadulewicz, E.K. and Hart, T.W. (2007) Quinazolione derivatives and their use as CB agonists. *World Int. Prop. Org.* WO03066603.

Bredt, B.M., Higuera-Alhino, D., Shade, S.B., Hebert, S.J., McCune, J.M. and Abrams, D.I. (2002). Short-term effects of cannabinoids on immune phenotype and function in HIV-1-infected patients. *J Clin Pharmacol.* 42:82S-89S.

Breivogel, C.S., Sim, L.J. and Childers, S.R. (1997). Regional differences in cannabinoid receptor/G-protein coupling in rat brain. *J Pharmacol Exp Ther.* 282:1632-1642.

Breivogel, C.S., Griffin, G., Di Marzo, V. and Martin, B.R. (2001). Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol*. 2001; 60:155-63.

Brooks, J.W., Pryce, G., Bisogno, T., Jagger, S.I., Hankey, D.J.R., Brown, P., Bridges, D., Ledent, C., Bifulco, M., Rice, A.S., Di Marzo, V. and Baker, D. (2002). Arvanil-induced inhibition of spasticity and persistent pain: evidence for therapeutic sites of action different from the vanilloid VR1 receptor and cannabinoid CB(1)/CB(2) receptors. *Eur J Pharmacol.* 439:83-92.

Brown, P. (1994). Pathophysiology of spasticity. *J Neurol Neurosurg Psychiatry* 57:773-777.

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

Buckley, N.E., McCoy, K.L., Mezey, E., Bonner, T., Zimmer, A., Felder, C.C., Glass, M. and Zimmer, A. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur J Pharmacol. 396*:141-149.

Buckley, N.E. The peripheral cannabinoid receptor knockout mice: an update. (2008). *Br J Pharmacol.* 153:309-318.

Burstein, S.H., Rossetti, R.G., Yagen, B. and Zurier, R.B. (2000a). Oxidative metabolism of anandamide. *Prostaglandins Other Lipid Mediat.* 61:29-41.

Burstein, S.H. Ajulemic acid (CT3): a potent analog of the acid metabolites of THC. (2000b). *Curr Pharm Des.* 6:1339-1345.

Burstein, S.H., Karst, M., Schneider, U. and Zurier, R.B. (2004). Ajulemic acid: A novel cannabinoid produces analgesia without a "high". *Life Sci.* 75:1513-22.

Burstein, S. (2005). PPAR-gamma: a nuclear receptor with affinity for cannabinoids. *Life Sci.* 77:1674-1684.

Butter, C., Baker, D., O'Neill, J.K. and Turk, J.L. (1991a). Mononuclear cell trafficking and plasma protein extravasation into the CNS during chronic relapsing experimental allergic encephalomyelitis in Biozzi AB/H mice. *J Neurol Sci.* 104:9-12.

Butter, C., O'Neill, J.K., Baker, D., Gschmeissner, S.E. and Turk, J.L. (1991b). An immunoelectron microscopical study of the expression of class II major histocompatibility complex during chronic relapsing experimental allergic encephalomyelitis in Biozzi AB/H mice. *J Neuroimmunol.* 33:37-42.

Butovsky, E., Juknat, A., Goncharov, I., Elbaz, J., Eilam, R., Zangen, A. and Vogel, Z. (2005). In vivo up-regulation of brain-derived neurotrophic factor in specific brain areas by chronic exposure to Delta-tetrahydrocannabinol. J Neurochem. 93;802-811.

Cabral, G.A., Harmon, K.N. and Carlisle, S.J. (2001). Cannabinoid-mediated inhibition of inducible nitric oxide production by rat microglial cells: evidence for CB1 receptor participation. *Adv Exp Med Biol*. 493:207-214.

Cabranes, A., Venderova, K., de Lago, E., Fezza, F., Sanchez, A., Mestre, L., Valenti, M., Garcia-Merino, A., Ramos, J.A., Di Marzo, V. and Fernandez-Ruiz, J. (2005).Decreased endocannabinoid levels in the brain and beneficial effects of agents activating cannabinoid and/or vanilloid receptors in a rat model of multiple sclerosis. *Neurobiol Dis.* 20:207-17.

Cabranes, A., Pryce, G., Baker, D. and Fernandez-Ruiz, J. (2006).Changes in CB1 receptors in motor-related brain structures of chronic relapsing experimental allergic encephalomyelitis mice. *Brain Res*. 1107:199-205.

Carl, S.M., Lindley, D.J., Couraud, P.O., Weksler, B.B., Romero, I., Mowery, S.A. and Knipp, G.T. (2010). ABC and SLC transporter expression and pot substrate characterization across the human CMEC/D3 blood-brain barrier cell line. *Mol Pharm.* 7:1057-1068.

Carrier, E.J., Kearn, C.S., Barkmeier, A.J., Breese, N.M., Yang, W., Nithipatikom, K., Pfister, S.L., Campbell, W.B. and Hillard, C.J. (2004). Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. *Mol Pharmacol.* 65:999-1007.

Cascorbi, I. (2006). Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacol Ther.* 112:457-473.

Centonze, D., Bari M., Rossi S. Prosperetti, C., Furlan, R., Fezza F., Chiara V.D., Battistini, L., Bernardi, G,. Bernardini, S., Martino, G. and Maccarrone, M. (2007). The endocannabinoid system is dysregulated in multiple sclerosis and in experimental autoimmune encephalomyelitis. *Brain.* 130:2543-2553.

Chanda, P., Gao, Y., Mark, L., Btesh, J., Strassle, B., Lu, P., Piesla, M., Zhang, M.Y., Bingham, B., Uveges, A., Kowal, D., Garbe, D., Kouranova, E., Ring, R., Bates, B., Pangalos, M., Kennedy, J., Whiteside, G. and Samad, T. (2010). Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. *Mol Pharmacol.* (Epub ahead of print).

Chang, A., Smith, MC., Yin, X., Fox, R.J., Staugaitis, S.M. and Trapp, B.D. (2008).Neurogenesis in the chronic lesions of multiple sclerosis. *Brain* 131:2366-2375.

Chemin, J., Monteil, A., Perez-Reyes, E., Nargeot, J. and Lory, P. (2001).Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide, Embo J 2001, 20:7033-7040.

Chen, J., Errico, S.L. and Freed, W.J. (2005). Reactive oxygen species and p38 phosphorylation regulate the protective effect of Delta9-tetrahydrocannabinol in the apoptotic response to NMDA. *Neurosci Lett.* 389:99-103.

Chia, R., Achilli, F., Festing, M.F. and Fisher, E.M. (2005). The origins and uses of mouse outbred stocks.*Nat Genet.* 37:1181-1186.

Childers, S.R. and Deadwyler, SA. (1996). Role of cyclic AMP in the actions of cannabinoid receptors. *Biochem Pharmacol.* 52:819-827.

Cluny, N.L, Keenan, C.M., Duncan, M., Fox, A., Lutz, B. and Sharkey, K.A. (2010). Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (SAB378), a peripherally restricted, cannabinoid CB1/CB2 receptor agonist inhibits gastrointestinal motility, but has no effect on experimental colitis in mice. *J Pharmacol Exp Ther.* 334:973-980.

Colabufo, N.A., Berardi, F., Perrone, M.G., Cantore, M., Contino, M., Inglese, C., Niso, M. and Perrone, R. (2009). Multi-drug-resistance-reverting agents: 2-aryloxazole and 2-arylthiazole derivatives as potent BCRP or MRP1 inhibitors. *Chem Med Chem.* 4:188-195.

Coles, A.J., Wing, M.G., Molyneux, P., Paolillo, A., Davie, C.M., Hale, G., Miller, D., Waldmann, H. and Compston, A. (1999). Monoclonal antibody treatment exposes three mechanisms underlying the clinical course of multiple sclerosis. *Ann Neurol.* 46:296-304.

Coles, A., Deans, J. and Compston, A. (2004). Campath-1H treatment of multiple sclerosis: lessons from the bedside for the bench. *Clin Neurol Neurosurg.* 106:270-274.

Coles, A.J., Cox, A., Le Page, E., Jones, J., Trip, S.A., Deans J., Seaman, S., Miller, D.H., Hale, G., Waldmann, H. and Compston, D.A. (2006). The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy. *J Neurol.* 253:98-108.

Coles, A.J, Compston, D.A, Selmaj, K.W., Lake, S.L., Moran, S., Margolin, D.H., Norris, K. and Tandon, P.K. CAMMS223 Trial Investigators.(2008). Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. *N Engl J Med.* 359:1786-1801.

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

Compston, A. and Coles, A. (2002). Multiple Sclerosis. *Lancet.* 359:1221-1231.

Compston, A. and Coles, A. (2008). Multiple sclerosis. *Lancet.* 372:1502-1517.

Compton, D.R., Gold, L.H., Ward, S.J., Balster, R.L. and Martin, B.R. (1992). Aminoalkylindole analogs: cannabimimetic activity of a class of compounds structurally distinct from delta 9-tetrahydrocannabinol. *J Pharmacol Exp Ther.* 263:1118-1126.

Confavreux, C., Vukusic, S., Moreau, T. and Adeleine, P. (2000). Relapses and progression of disability in multiple sclerosis. *N Engl J Med.* 343:1430-1438.

Confavreux, C. and Vukusic, S. (2006). Accumulation of irreversible disability in multiple sclerosis: from epidemiology to treatment. *Clin Neurol Neurosurg.* 108:327-332.

Consroe, P., Musty, R., Rein, J., Tillery, W. and Pertwee, R. (1997). The perceived effects of smoked cannabis on patients with multiple sclerosis. *Eur Neurol.* 38:44-48.

Correa, F., Docagne, F., Mestre, L., Loria, F., Hernangomez, M., Borrell, J. and Guaza, C. (2007). Cannabinoid system and neuroinflammation: implications for multiple sclerosis. *Neuroimmunomodulation* 14:182-187.

Correll, C.C., Phelps, P.T., Anthes, J.C., Umland, S. and Greenfeder, S. (2004). Cloning and pharmacological characterization of mouse TRPV1. *Neurosci Lett* 370:55-60.

Cotte, S., von Ahsen, N., Kruse, N., Huber, B., Winkelmann, A., Zett,l U.K., Starck, M., König, N., Tellez, N., Dörr, J., Paul, F., Zipp, F., Lühder, F., Koepsell, H., Pannek, H., Montalban, X., Gold, R. and Chan, A. ABC-transporter gene-polymorphisms are potential pharmacogenetic markers for mitoxantrone response in multiple sclerosis. *Brain.* 132:2517-2530.

Craner, M.J., Kataoka, Y., Lo, A.C., Black, J.A., Baker, D. and Waxman, S.G. (2003). Temporal course of upregulation of Na(v)1.8 in Purkinje neurons parallels the progression of clinical deficit in experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol.* 62:968-975.

Craner, M.J., Lo, A.C., Black, J.A. and Waxman, S.G. (2003). Abnormal sodium channel distribution in optic nerve axons in a model of inflammatory demyelination, Brain 2003, 126:1552-1561.

Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A. and Gilula, N.B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384: 83–87.

Cravatt, B.F., Demarest, K., Patricelli, M.P., Bracey, M.H., Giang, D.K., Martin, B.R. and Lichtman, A.H. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci U S A.* 98:9371-9376.

Cravatt, B.F. and Lichtman, .AH. (2003). Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. *Curr Opin Chem Biol.* 7:469-475.

Croxford, J.L. and Miller, S.D. (2003). Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. *J Clin Invest.* 111:1231-1240.

Curran, H.V., Brignell, C., Fletcher, S., Middleton, P. and Henry, J. (2002).Cognitive and subjective dose-response effects of acute oral Delta 9-tetrahydrocannabinol (THC) in infrequent cannabis users. *Psychopharmacology (Berl)* 164:61-70.

Curran, N.M., Griffin, B.D., O'Toole, D., Brady, K.J., Fitzgerald, S.N. and Moynagh, P.N. (2005). The synthetic cannabinoid R(+)WIN 55,212-2 inhibits the interleukin-1 signaling pathway in human astrocytes in a cannabinoid receptor-independent manner. *J Biol Chem*. 280:35797-35806.

Dainese, E., Oddi, S., Bari, M. and Maccarrone, M. (2007). Modulation of the endocannabinoid system by lipid rafts. *Curr Med Chem.*14:2702-2715.

Dallas, S., Miller, D.S. and Bendayan, R. (2006). Multidrug resistance-associated proteins: expression and function in the central nervous system. *Pharmacol Rev.* 58:140-161.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A. and Sheardown, S.A. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 405(6783):183-187.

Day, T.A, Rakhshan, F., Deutsch, D.G. and Barker, E.L. (2001). Role of fatty acid amide hydrolase in the transport of the endogenous cannabinoid anandamide. *Mol Pharmacol.* 591369-1375.

de Lago, E., Ligresti, A., Ortar, G., Morera, E., Cabranes, A., Pryce, G., Bifulco, M., Baker, D., Fernandez-Ruiz, J. and Di Marzo, V. (2004). In vivo pharmacological actions of two novel inhibitors of anandamide cellular uptake. *Eur J Pharmacol*. 484:249-257.

de Lago, E., Fernández-Ruiz, J., Ortega-Gutiérrez, S., Cabranes, A., Pryce, G., Baker, D., López-Rodríguez, M. and Ramos, J.A. (2006). UCM707, an inhibitor of the anandamide uptake, behaves as a symptom control agent in models of Huntington's disease and multiple sclerosis, but fails to delay/arrest the progression of different motor-related disorders. *Eur Neuropsychopharmacol*. 16:7-18.

Demuth, D.G. and Molleman, A. (2006). Cannabinoid signalling. *Life Sci.* 78:549-563.

Denic, A., Johnson, A.J., Bieber, A.J., Warrington, A.E., Rodriguez, M. and Pirko, I. (2010). The relevance of animal models in multiple sclerosis research. *Pathophysiology.* May 25. [Epub ahead of print]

De Petrocellis, L., Bisogno, T., Davis, J.B., Pertwee, R.G. and Di Marzo, V. (2000). Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett.* 483:52-56.

De Petrocellis, L., Bisogno, T., Maccarrone, M., Davis, J.B., Finazzi-Agro, A. and Di Marzo, V. (2001). The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. *J Biol Chem.* 276:12856-12863.

Di Pasquale, E., Chahinian, H., Sanchez, P. and Fantini, J. (2009). The insertion and transport of anandamide in synthetic lipid membranes are both cholesterol-dependent. *PLoS ONE.* 4:e4989.

De Jager, P.L., Baecher-Allan, C., Maier, L.M., Arthur, A.T., Ottoboni, L., Barcellos, L., McCauley, J.L., Sawcer, S., Goris, A., Saarela, J., Yelensky, R., Price, A., Leppa, V., Patterson, N., de Bakker, P.I., Tran, D., Aubin, C., Pobywajlo, S., Rossin, E., Hu, X., Ashley, C.W., Choy, E., Rioux, J.D., Pericak-Vance, M.A., Ivinson, A., Booth, D.R., Stewart, G.J., Palotie, A., Peltonen, L., Dubois, B., Haines, J.L., Weiner, H.L., Compston, A., Hauser, S.L., Daly, M.J., Reich, D., Oksenberg, J.R. and Hafler, D.A. (2009).The role of the CD58 locus in multiple sclerosis. *Proc Natl Acad Sci U S A.* 106:5264-5269.

Deutsch, D.G., Glaser, S.T., Howell, J.M., Kunz, J.S., Puffenbarger, R.A., Hillard, C.J. and Abumrad, N. (2001). The cellular uptake of anandamide is coupled to its breakdown by fatty-acid amide hydrolase. *J Biol Chem.* 276:6967-6973.

Deutsch, D.G., Ueda, N. and Yamamoto, S. (2002). The fatty acid amide hydrolase (FAAH). *Prostaglandins Leukot Essent Fatty Acids*. 66:201-210.

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

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.

Di Filippo, M., Pini, L.A., Pelliccioli, G.P., Calabresi, P. and Sarchielli, P. (2008). Abnormalities in the cerebrospinal fluid levels of endocannabinoids in Multiple Sclerosis. *J Neurol Neurosurg Psychiatry* 79:1224-1229.

Dinh, T.P., Carpenter, D., Leslie, F.M., Freund, T.F., Katona, I., Sensi, S.L., Kathuria, S. and Piomelli, D. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci U S A* 99:10819-10824.

Dinh, T.P., Kathuria, S. and Piomelli, D. (2004). RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. *Mol Pharmacol.* 66:1260-1264.

Dinis, P., Charrua, A., Avelino, A., Yaqoob, M., Bevan, S., Nagy, I. and Cruz, F. (2004). Anandamide-evoked activation of vanilloid receptor 1 contributes to the development of bladder hyperreflexia and nociceptive transmission to spinal dorsal horn neurons in cystitis. *J Neurosci.* 24:11253-11263.

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.

Di Marzo, V. (2008). Endocannabinoids: synthesis and degradation. *Rev Physiol Biochem Pharmacol.* 160:1-24.

Docagne, F., Muneton, V., Clemente, D., Ali, C., Loria, F., Correa, F., Hernangomez, M., Mestre, L., Vivien, D. and Guaza, C. (2007). Excitotoxicity in a chronic model of multiple sclerosis: Neuroprotective effects of cannabinoids through CB1 and CB2 receptor activation. *Mol Cell Neurosci.* 34:551-561.

Dong, A.X., Kelly, M.E.M. and Howlett, S.E. (2009). Vasoactive Actions of Abnormal Cannabidiol and 3-(5-Dimethylcarbamoyl-pent-1-enyl)-N-(2-hydroxy-1-methyl-ethyl)benzamide in Retinal Arterioles.*19th Symposium of the International Cannabinoid Research Society, Burlingame, Vermont USA*

Dowie, M.J., Bradshaw, H.B., Howard, M.L., Nicholson, L.F., Faull, R.L., Hannan, A.J., and Glass, M. (2009).Altered CB1 receptor and endocannabinoid levels precede motor symptom onset in a transgenic mouse model of Huntington's disease. *Neuroscience.* 163:456-465.

Dowie, M.J., Howard, M.L., Nicholson, L.F., Faull, R.L., Hannan, A.J. and Glass, M. (2010). Behavioural and molecular consequences of chronic cannabinoid treatment in Huntington's disease transgenic mice. *Neuroscience.* 170:324-336.

Dutta, R., McDonough, J., Yin, X., Peterson, J., Chang, A., Torres, T., Gudz, T., Macklin, W.B., Lewis, D.A., Fox, R.J., Rudick, R., Mirnics, K. and Trapp, B.D. (2006). Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann Neurol* 59:478-489.

Dutta, R. and Trapp, B.D. (2007).Pathogenesis of axonal and neuronal damage in multiple sclerosis. *Neurology* 68:S22-31.

Dyson, A., Peacock, M., Chen, A., Courade, J.P., Yaqoob, M., Groarke, A., Brain, C., Loong, Y. and Fox, A. (2005). Antihyperalgesic properties of the cannabinoid CT-3 in chronic neuropathic and inflammatory pain states in the rat. *Pain.*116:129-137.

Dziadulewicz, E.K., Bevan, S.J., Brain, C.T., Coote, P.R., Culshaw, A.J., Davis, A.J., Edwards, L.J., Fisher, A.J., Fox, A.J., Gentry, C., Groarke, A., Hart, T.W., Huber, W., James, I.F., Kesingland, A., Vecchia, L.L., Loong, Y., Lyothier, I., McNair, K., O'farrell, C., Peacock, M., Portmann, R., Schopfer, U., Yaqoob, M.. and Zadrobilek, J. (2007). Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone: A potent, orally bioavailable human CB(1)/CB(2) Dual agonist with antihyperalgesic properties and restricted central nervous system penetration. *J Med Chem.* 50:3851-3856.

Ehrhart, J., Obregon, D., Mori, T., Hou, H., Sun, N., Bai, Y., Klein, T., Fernandez, F., Tan, J. and Shytle, D. (2005). Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *J. Neuroinflammation.* 2:29.

Eljaschewitsch, E., Witting, A., Mawrin, C., Lee, T., Schmidt, P.M., Wolf, S., Hoertnagl, H., Raine, C.S., Schneider-Stock, R., Nitsch, R. and Ullrich, O. (2006). The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. *Neuron* 49:67-79.

Ellmerich, S., Mycko, M., Takacs, K., Waldner, H., Wahid, F.N., Boyton, R.J., King, R.H., Smith, P.A., Amor, S., Herlihy, A.H., Hewitt, R.E., Jutton, M., Price, D.A., Hafler, D.A., Kuchroo, V.K. and Altmann, D.M. (2005). High incidence of spontaneous disease in an HLA-DR15 and TCR transgenic multiple sclerosis model. *J Immunol.* 174:1938-1946.

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, GI. (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.

El-Remessy, A.B., Al-Shabrawey, M., Khalifa, Y., Tsai, N.T., Caldwell, R.B. and Liou, G.I. (2006).Neuroprotective and blood-retinal barrier-preserving effects of cannabidiol in experimental diabetes. *Am J Pathol.* 168:235-244.

Esposito, G., De Filippis, D., Maiuri, M.C., De Stefano, D., Carnuccio, R. and Iuvone, T. (2006). Cannabidiol inhibits inducible nitric oxide synthase protein expression and nitric oxide production in beta-amyloid stimulated PC12 neurons through p38 MAP kinase and NF-kappaB involvement. *Neurosci Lett.* 399:91-95.

Farrell, E.K. and Merkler, D.J. (2008).Biosynthesis, degradation and pharmacological importance of the fatty acid amides. *Drug Discov. Today* 13:558-568.

Fegley, D., Gaetani, S., Duranti, A., Tontini, A., Mor, M., Tarzia, G. and Piomelli, D. (2005). Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. *J Pharmacol Exp Ther.* 313:352-358.

Feher, M., Sourial, E., and Schmidt, J.M. (2000). A simple model for the prediction of blood-brain partitioning. *Int J Pharm.* 201:239-247.

Felder, C.C., Joyce, K.E., Briley, E.M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A.L.. and Mitchell, R.L.(1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol..* 48:443-450.

Ferguson, B., Matyszak, M.K., Esiri, M.M. and Perry, V.H. (1997). Axonal damage in acute multiple sclerosis lesions. *Brain* 120:393-399.

Fezza, F., Bisogno, T., Minassi, A., Appendino, G., Mechoulam, R. and Di Marzo, V. (2002). Noladin ether, a putative novel endocannabinoid: inactivation mechanisms and a sensitive method for its quantification in rat tissues. *FEBS Lett.* 513:294-298.

Fimiani, C., Liberty, T., Aquirre, A.J., Amin, I., Ali, N. and Stefano, G.B. (1999). Opiate, cannabinoid, and eicosanoid signaling converges on common intracellular pathways nitric oxide coupling. *Prostaglandins Other Lipid Mediat.* 57:23-34.

Fiskerstrand, T., H'mida-Ben Brahim, D., Johansson, S., M'zahem, A., Haukanes, B.I., Drouot, N., Zimmermann, J., Cole, A.J., Vedeler, C., Bredrup, C., Assoum, M., Tazir, M., Klockgether, T., Hamri, A., Steen, V.M., Boman, H., Bindoff, L.A., Koenig, M. and Knappskog, P.M. (2010). Mutations in ABHD12 Cause the Neurodegenerative Disease PHARC: An Inborn Error of Endocannabinoid Metabolism. *Am J Hum Genet.* 87:410-417.

Fowler, C.J., Tiger, G., Ligresti, A., Lopez-Rodriguez, M.L. and Di Marzo, V. (2004). Selective inhibition of anandamide cellular uptake versus enzymatic hydrolysis--a difficult issue to handle. *Eur J Pharmacol.* 492:1-11.

Fox, A., Kesingland, A., Gentry, C., McNair, K., Patel, S., Urban, L, and James, I. (2001).The role of central and peripheral Cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain.* 92:91-100.

Freeman, R.M., Adekanmi, O., Waterfield, M.R., Waterfield, A.E., Wright, D. and Zajicek, J. (2006). The effect of cannabis on urge incontinence in patients with multiple sclerosis: a multicentre, randomised placebo-controlled trial (CAMS-LUTS). *Int Urogynecol J Pelvic Floor Dysfunct*. 17:636-641.

Freedman, D.M., Dosemeci, M. and Alavanja, M.C. (2000). Mortality from multiple sclerosis and exposure to residential and occupational solar radiation: a case-control study based on death certificates*. Occup Environ Med* 57*:*418–421*.*

Freund, T.F., Katona, I. and Piomelli, D. (2003). Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev.* 83:1017-1066.

Fride, E., Feigin, C., Ponde, D.E., Breuer, A., Hanus, L., Arshavsky, N. and Mechoulam, R. (2004). (+)-Cannabidiol analogues which bind cannabinoid receptors but exert peripheral activity only. *Eur J Pharmacol.* 506:179-188.

Friese, M.A., Montalban, X., Willcox, N., Bell, J.I., Martin, R. and Fugger, L. (2006).The value of animal models for drug development in multiple sclerosis. *Brain.* 129:1940-1952.

Fujiwara, M. and Egashira, N. (2004). New perspectives in the studies on endocannabinoid and cannabis: Abnormal behaviors associate with CB(1) cannabinoid receptor and development of therapeutic application. *J Pharmacol Sci*. 96:362-366.

Fuller, K.G., Olson, J.K., Howard, L.M., Croxford, J.L. and Miller, S.D. (2004). Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods Mol Med.* 102:339-361.

Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G. and Casellas, P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* . 232:54-61.

Galve-Roperh, I., Aguado, T., Rueda, D., Velasco, G. and Guzman, M. (2006). Endocannabinoids: a new family of lipid mediators involved in the regulation of neural cell development. *Curr Pharm Des.* 12:2319-2325.

Galve-Roperh, I., Aguado, T., Palazuelos, J. and Guzman, M. (2007). The endocannabinoid system and neurogenesis in health and disease. *Neuroscientist* 13:109-114.

Gantz, I., Muraoka, A., Yang, Y.K., Samuelson, L.C., Zimmerman, E.M., Cook, H. and Yamada, T. (1997).Cloning and chromosomal localization of a gene (GPR18) encoding a novel seven transmembrane receptor highly expressed in spleen and testis. *Genomics.* 42:462-466.

Gao, Y., Vasilyev, D.V., Goncalves, M.B., Howell, F.V., Hobbs, C., Reisenberg, M., Shen, R., Zhang, M.Y., Strassle, B.W., Lu, P., Mark, L., Piesla, M.J., Deng, K., Kouranova, E.V., Ring, R.H., Whiteside, G.T., Bates, B., Walsh, F.S., Williams, G., Pangalos, M.N,, Samad, T.A. and Doherty, P. (2010). Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. *J Neurosci.* 30:2017-2024.

Gaoni, Y. and Mechoulam, R. (1964). Isolation, structure and partial synthesis of an active component of hashish., *J Am Chem Soc*. 86:1646-1647.

Garcia, D.E., Brown, S., Hille, B. and Mackie, K. (1998). Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB1 cannabinoid receptor. J Neurosci. 18:2834-2841.

Gardin, A., Kucher, K., Kiese, B. and Appel-Dingemanse, S. (2009). Cannabinoid receptor agonist 13, a novel cannabinoid agonist: first in human pharmacokinetics and safety. *Drug Metab Dispos.* 37:827-833.

Gebremedhin, D., Lange, A.R., Campbell, W.B., Hillard, C.J. and Harder, D.R. (1999). Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca2+ channel current. *Am J Physiol.* 276:H2085-2093.

Gardin, A., Kucher, K., Kiese, B. and Appel-Dingemanse, S. (2009). Cannabinoid receptor agonist 13, a novel cannabinoid agonist: first in human pharmacokinetics and safety. *Drug Metab Dispos.* 37:827-833.

Gilbert, S.L., Zhang, L., Forster, M.L., Anderson, J.R., Iwase, T., Soliven, B., Donahue, L.R., Sweet, H.O., Bronson, R.T., Davisson, M.T., Wollmann, R.L. and Lahn, B.T. (2006). Trak1 mutation disrupts GABA(A) receptor homeostasis in hypertonic mice. *Nat Genet.* 38:245-250.

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.

Glaser, S.T., Abumrad, N.A., Fatade, F., Kaczocha, M., Studholme, K.M. and Deutsch, D.G. (2003). Evidence against the presence of an anandamide transporter. *Proc Natl Acad Sci U S A* 100:4269-4274.

Glass, M. and Felder, C.C. (1997). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *J Neurosci.* 17:5327-5333.

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.

Godlewski, G., Offertáler, L., Wagner, J.A. and Kunos, G. (2009). Receptors for acylethanolamides-GPR55 and GPR119. *Prostaglandins Other Lipid Mediat.* 89:105-111.

Gonsiorek, W., Lunn, C., Fan, X., Narula, S., Lundell, D. and Hipkin, R.W. (2000). Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. *Mol Pharmacol.* 57:1045-1050.

Gratzke, C., Weinholt, P., Selwood, D., Stief, C.G., Andersson, K-E. and Hedlund, P. (2009). Distribution of the g-protein-coupled receptor 55 (gpr55) in the rat bladder and urodynamic effects of a selective gpr55-agonist (vsnr16) in conscious rats. *Eur Urol Suppl.* 8:271.

Griffin, G., Fernando, S.R., Ross, R.A., McKay, N.G., Ashford, M.L., Shire, D., Huffman, J.W., Yu, S., Lainton, J.A. and Pertwee, R.G. (1997). Evidence for the presence of CB2-like cannabinoid receptors on peripheral nerve terminals. Eur J Pharmacol. 339:53-61.

Griffin, G., Wray, E.J., Martin, B.R., and Abood, M.E. (1999). Cannabinoid agonists and antagonists discriminated by receptor binding in rat cerebellum.*Br J Pharmacol.* 128:684-688.

Grotenhermen, F. (2003). Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet.* 42:327-360.

Hafler, D.A., Compston, A., Sawcer, S., Lander, E.S., Daly, M.J., De Jager, P.L., de Bakker, P.I., Gabriel, S.B., Mirel, D.B., Ivinson, A.J., Pericak-Vance, M.A., Gregory, S.G., Rioux, J.D., McCauley, J.L., Haines, J.L., Barcellos, L.F., Cree, B., Oksenberg, J.R. and Hauser, S.L. (2007) International Multiple Sclerosis Genetics Consortium, Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med.* 357:851-862.

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.

Hampson, R.E., Mu, J, and Deadwyler, S.A. (2000). Cannabinoid and kappa opioid receptors reduce potassium K current via activation of G(s) proteins in cultured hippocampal neurons. *J Neurophysiol.* 84:2356-2364.

Handel, A.E., Williamson, A.J., Disanto, G., Handunnetthi, L., Giovannoni, G. and Ramagopalan, S.V. (2010). An Updated Meta-Analysis of Risk of Multiple Sclerosis following Infectious Mononucleosis. *PLoS One.* 5: pii: e12496.

Hansen, H.H., Schmid, P.C., Bittigau, P., Lastres-Becker, I., Berrendero, F., Manzanares, J., Ikonomidou, C., Schmid, H.H., Fernandez-Ruiz, J.J. and Hansen, H.S. (2001). Anandamide, but not 2-arachidonoylglycerol, accumulates during in vivo neurodegeneration. *J Neurochem.* 78:1415-1427.

Hansen, H.H., Azcoitia, I., Pons, S., Romero, J., Garcia-Segura, L.M., Ramos, J.A., Hansen, H.S. and Fernandez-Ruiz, J. (2002). Blockade of cannabinoid CB(1) receptor function protects against in vivo disseminating brain damage following NMDA-induced excitotoxicity. *J Neurochem.* 82:154-158.

Hanus, L., Abu-Lafi, S., Fride, E., Breuer, A., Vogel, Z., Shalev, D.E., Kustanovich, I. and Mechoulam, R. (2001). 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci U S A* 98:3662-3665.

Hardin-Pouzet, H., Krakowski, M., Bourbonniere, L., Didier-Bazes, M., Tran, E. and Owens, T. (1997). Glutamate metabolism is down-regulated in astrocytes during experimental allergic encephalomyelitis. *Glia* 20:79-85.

Hashimotodani, Y., Ohno-Shosaku, T. and Kano, M. (2007). Endocannabinoids and synaptic function in the CNS. *Neuroscientist* 13:127-137.

Hasseldam, H. and Johansen, F.F. (2010). Neuroprotection without immunomodulation is not sufficient to reduce first relapse severity in experimental autoimmune encephalomyelitis. *Neuroimmunomodulation.* 17:252-264.

Hauser, S.L., Bhan, A.K., Gilles, F., Kemp, M., Kerr, C. and Weiner, H.L. (1986). Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann Neurol.* 19:578-587.

Hayes, C.E., Cantorna, M.T. and DeLuca, H.F. (1997). Vitamin D and multiple sclerosis. *Proc Soc Exp Biol Med. 216*:21-27.

Hendrikse, N.H., Schinkel, A.H., de Vries, E.G., Fluks, E., Van der Graaf, W.T., Willemsen, A.T., Vaalburg, W. and Franssen, E.J. (1998). Complete in vivo reversal of P-glycoprotein pump function in the blood-brain barrier visualized with positron emission tomography. *Br J Pharmacol.* 124:1413-1418.

Hennet, T., Hagen, F.K., Tabak, L.A., and Marth, J.D. (1995). T-cell-specific deletion of a polypeptide N-acetylgalactosaminyl-transferase gene by site-directed recombination. *Proc Natl Acad Sci U S A.* 92:12070-12074.

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.

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., de Costa, B.R. and Rice K.C. (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci.* 11:563-583.

Hiragata, S., Ogawa, T., Hayashi, Y., Tyagi, P., Seki, S., Nishizawa, O., de Miguel, F., Chancellor, M.B. and Yoshimura, N. (2007). Effects of IP-751, ajulemic acid, on bladder overactivity induced by bladder irritation in rats. *Urology.* 70:202-208.

Hoi, P.M., Visintin, C., Okuyama, M., Gardiner, S.M., Kaup, S.S., Bennett, T, Baker, D., Selwood, D.L. and Hiley CR. (2007). Vascular pharmacology of a novel cannabinoid-like compound, 3-(5-dimethylcarbamoyl-pent-1-enyl)-N-(2-hydroxy-1-methyl-ethyl)benzamide (VSN16) in the rat. *Br J Pharmacol.*152:751-764.

Holland, M.L., Panetta, J.A., Hoskins, J.M., Bebawy, M., Roufogalis, B.D., Allen, J.D. and Arnold, J.C. (2006). The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochem Pharmacol.* 71:1146-1154.

Holland, M.L., Lau, D.T., Allen, J.D. and Arnold, J.C. (2007). The multidrug transporter ABCG2 (BCRP) is inhibited by plant-derived cannabinoids. *Br J Pharmacol.* 152:815-824.

Holland, M.L., Allen, J.D. and Arnold, J.C. (2008). Interaction of plant cannabinoids with the multidrug transporter ABCC1 (MRP1). *Eur J Pharmacol.* 591:128-131.

Hoppenbrouwers, I.A., Aulchenko, Y.S., Janssens, A.C., Ramagopalan, S.V., Broer, L., Kayser, M., Ebers, G.C., Oostra, B.A., van Duijn, C.M. and Hintzen, R.Q. (2009). Replication of CD58 and CLEC16A as genome-wide significant risk genes for multiple sclerosis. *J Hum Genet.* 54:676-680.

Howlett, AC. (1984). Inhibition of neuroblastoma adenylate cyclase by cannabinoid and nantradol compounds. *Life Sci.* 35:1803-1810.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R. and Pertwee, R.G. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev*. 54:161-202.

Howlett, A.C., Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E. and Porrino, L.J. (2004). Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* 47 Suppl 1:345-358.

Huang, S.M., Bisogno, T., Petros, T.J., Chang, S.Y., Zavitsanos, P.A., Zipkin, R.E., Sivakumar, R., Coop, A., Maeda, D.Y., De Petrocellis, L., Burstein, S., Di Marzo, V. and Walker J.M. (2001).Identification of a new class of molecules, the arachidonyl amino acids, and characterization of one member that inhibits pain. *J Biol Chem.* 276:42639-42644.

Huffman, J.W., Yu, S., Showalter, V., Abood, M.E., Wiley, J.L., Compton, D.R., Martin, B.R., Bramblett, R.D. and Reggio, P.H. (1996). Synthesis and pharmacology of a very potent cannabinoid lacking a phenolic hydroxyl with high affinity for the CB2 receptor. *J Med Chem* 39:3875-3877.

Huffman, J.W., Liddle, J., Yu, S., Aung, M.M., Abood, M.E., Wiley, J.L. and Martin, B.R. (1999). 3-(1',1'-Dimethylbutyl)-1-deoxy-delta8-THC and related compounds: synthesis of selective ligands for the CB2 receptor. *Bioorg Med Chem*. 7:2905-2914.

Hwang, J., Adamson, C., Butler, D., Janero, D.R., Makriyannis, A. and Bahr, B.A. (2010). Enhancement of endocannabinoid signaling by fatty acid amide hydrolase inhibition: A neuroprotective therapeutic modality. *Life Sci.* 86:615-623.

Inglese, M., Mancardi, GL., Pagani, E., Rocca, M.A., Murialdo, A., Saccardi, R., Comi, G. and Filippi, M; Italian GITMO-NEURO Group on Autologous Hematopoietic Stem CellTransplantation (2004).. Brain tissue loss occurs after suppression of enhancement in patients with multiple sclerosis treated with autologous haematopoietic stem cell transplantation. *J Neurol Neurosurg Psychiatry.* 75:643-644.

Inoue, Tl, Coitinho, H., Pochintesta, E., Baltar, J. and Goto, N. (2000) Genetic Relationships between CD-1 Stocks of Mice in Uruguay. *JARQ* 34;265-270.

Iuvone, T., Esposito, G., Esposito, R., Santamaria, R., Di Rosa, M. and Izzo, A.A. (2004). Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells. *J Neurochem.* 89:134-141.

Ivanhoe, C.B. and Reistetter, T.A. (2004). Spasticity: the misunderstood part of the upper motor neuron syndrome. *Am J Phys Med Rehabil*. 83 (Suppl):S3-9.

Jackson, S.L., Pryce, G., Diemel, D.T. and Baker, D. (2005). Cannabinoid receptor null mice are susceptible to neurofilament damage and caspase 3 activation. *Neuroscience.* 2005. 134:261-268.

Járai, Z., Wagner, J.A., Varga, K., Lake, K.D., Compton, D.R., Martin, B.R., Zimmer, A.M., Bonner, T.I., Buckley, N.E., Mezey, E., Razdan, R.K., Zimmer, A. and Kunos, G. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci U S A.* 96:14136-14141.

Jarrahian, A., Manna, S., Edgemond, W.S., Campbell, W.B. and Hillard, C.J. (2000). Structure-activity relationships among N-arachidonylethanolamine (Anandamide) head group analogues for the anandamide transporter. *J Neurochem*. 74:2597-2606.

Jin, K., Xie, L., Kim, S.H., Parmentier-Batteur, S., Sun, Y., Mao, X.O., Childs, J. and Greenberg, D.A. (2004). Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Mol Pharmacol.* 66:204-208.

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., Shabon, U., Bao, W., Aiyar, N., Yue, T.L., Brown, A.J., Morrison, A.D. and Douglas, S.A. (2007). The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. *Br J Pharmacol.* 152:825-831.

Johnson, M.R., Melvin, L.S., Althuis, T.H., Bindra, J.S., Harbert, C.A., Milne, G.M. and Weissman, A. (1981). Selective and potent analgetics derived from cannabinoids. *J Clin Pharmacol*. 21:271S-282S.

Jones, J.L. and Coles, A.J. (2010). New treatment strategies in multiple sclerosis. *Exp Neurol.* 225:34-39.

Jones, J.L., Anderson, J.M., Phuah, C.L., Fox, E.J., Selmaj, K., Margolin, D., Lake, S,L., Palmer, J., Thompson, S.J., Wilkins, A., Webber, D.J., Compston, D.A. and Coles, A.J. (2010). Improvement in disability after alemtuzumab treatment of multiple sclerosis is associated with neuroprotective autoimmunity. *Brain.* 133:2232-2247.

Jones, N.A., Hill, A.J., Smith, I., Bevan, S.A., Williams, C.M., Whalley, B.J. and Stephens, G.J. (2010). Cannabidiol displays antiepileptiform and antiseizure properties in vitro and in vivo. *J Pharmacol Exp Ther.* 332:569-577.

Jun, K., Lee, S.B. and Shin, H.S. (2000). Insertion of a retroviral solo long terminal repeat in mdr-3 locus disrupts mRNA splicing in mice. *Mamm Genome.* 11:843-848.

Jung, K.M., Astarita, G., Zhu, C., Wallace, M., Mackie, K. and Piomelli, D. (2007). A key role for diacylglycerol lipase-alpha in metabotropic glutamate receptor-dependent endocannabinoid mobilization. *Mol Pharmacol.* 72:612-621.

Kaczocha, M., Hermann. A., Glaser, S.T., Bojesen, I.N. and Deutsch, D.G. (2006) Anandamide uptake is consistent with rate-limited diffusion and is regulated by the degree of its hydrolysis by fatty acid amide hydrolase. *J Biol Chem*. 281:9066-9075.

Kaczocha, M., Glaser, S.T. and Deutsch, D.G. (2009). Identification of intracellular carriers for the endocannabinoid anandamide. *Proc Natl Acad Sci U S A.* 106:6375-6380.

Kalsi, V. and Fowler. C.J. (2005). Therapy Insight: bladder dysfunction associated with multiple sclerosis. *Nat Clin Pract Urol.* 2:492-501.

Kapoor, R., Davies, M., Blaker. P.A., Hall, S.M. and Smith, K.J. (2003). Blockers of sodium and calcium entry protect axons from nitric oxide-mediated degeneration. *Ann Neurol.* 53:174-180.

Kapoor, R., Furby, J., Hayton, T., Smith, K.J., Altmann, D.R., Brenner, R., Chataway, J., Hughes, R.A. and Miller, D.H. (2010). Lamotrigine for neuroprotection in secondary progressive multiple sclerosis: a randomised, double-blind, placebo-controlled, parallel-group trial. *Lancet Neurol.* 9:681-688.

Kapur, A., Zhao, P., Sharir, H., Bai, Y., Caron, M. G., Barak, L. S. and Abood, M. E. (2009). Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. *J BiolChem* 284, 29817−29827.

Karanian, D.A., Brown, Q.B., Makriyannis, A. and Bahr, B.A. (2005a). Blocking cannabinoid activation of FAK and ERK1/2 compromises synaptic integrity in hippocampus. Eur J Pharmacol. 508:47-56.

Karanian, D.A., Brown, Q.B., Makriyannis, A., Kosten, T.A. and Bahr, B.A. (2005b). Dual modulation of endocannabinoid transport and fatty acid amide hydrolase protects against excitotoxicity. *J Neurosci*. 25:7813-7820.

Karin, N., Mitchell, D.J., Brocke, S., Ling, N. and Steinman, L. (1994). Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon gamma and tumor necrosis factor alpha production. *J Exp Med.* 180:2227-2237.

Karsak. M., Gaffal, E., Date, R., Wang-Eckhardt. L., Rehnelt, J., Petrosino, S., Starowicz, K., Steuder, R., Schlicker, E., Cravatt, B., Mechoulam, R., Buettner, R., Werner, S., Di Marzo, V., Tüting, T. and Zimmer, A. (2007). Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science.* 316:1494-1497.

Karst M. (2007). Comments on "cannabimimetic properties of ajulemic Acid". *J Pharmacol Exp Ther.* 322:420-421.

Katona S., Kaminski, E., Sanders, H. and Zajicek, J. (2005). Cannabinoid influence on cytokine profile in multiple sclerosis. *Clin Exp Immunol.* 140:580-585.

Kathuria, S., Gaetani, S., Fegley, D., Valiño, F., Duranti, A., Tontini, A., Mor, M., Tarzia, G., La Rana, G., Calignano, A., Giustino, A., Tattoli, M., Palmery, M., Cuomo, V. and Piomelli, D. (2003). Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med.* 9:76-81.

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.

Kearn, C.S., Blake-Palmer, K., Daniel, E., Mackie, K. and Glass, M. (2005). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk? *Mol Pharmacol* 67:1697-1704.

Khaspekov, L.G., Brenz Verca, M.S., Frumkina, L.E., Hermann, H., Marsicano, G. and Lutz, B. (2004). Involvement of brain-derived neurotrophic factor in cannabinoid receptor-dependent protection against excitotoxicity. *Eur J Neurosci*. 19:1691-1698.

Killestein, J., Hoogervorst, E.L., Reif, M., Blauw, B., Smits, M., Uitdehaag, B.M., Nagelkerken, L. and Polman, C.H. (2003). Immunomodulatory effects of orally administered cannabinoids in multiple sclerosis. *J Neuroimmunol.* 137:140-143.

Killestein, J., Hoogervorst, E.L., Reif, M., Kalkers, N.F., Van Loenen, A.C., Staats, P,G., Gorter, R.W., Uitdehaag, B.M. and Polman, C.H. (2002). Safety, tolerability, and efficacy of orally administered cannabinoids in MS. *Neurology*. 58:1404-1407.

Kim, K., Moore, D.H., Makriyannis, A. and Abood M.E. (2006a). AM1241, a cannabinoid CB2 receptor selective compound, delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Eur J Pharmacol. 542*:100-105.

Kim, S.H., Won, S.J., Mao, X.O., Ledent, C., Jin, K. and Greenberg, D.A. (2006b). Role for neuronal nitric-oxide synthase in cannabinoid-induced neurogenesis. *J Pharmacol Exp Ther.* 319:150-154.

Kita, M., and Goodkin, D.E. (2000). Drugs used to treat spasticity. *Drugs* 59:487-495.

Klegeris, A., Bissonnette, C.J. and McGeer, P.L. (2003). Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. *Br J Pharmacol.* 139:775-786.

Klein, T.W., Newton, C. and Friedman, H. (1998). Cannabinoid receptors and immunity. *Immunol Today*. 19:373-381.

Klein, T.W., Newton, C., Larsen, K., Lu, L., Perkins, I., Nong, L. and Friedman, H. (2003). The cannabinoid system and immune modulation. *J Leukoc Biol.* 74:486-496.

Klein, T.W. (2005). Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat Rev Immunol*. 5:400-411.

Klein, T.W. and Cabral, G.A. (2006). Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. *J Neuroimmune Pharmacol.* 1:50-64.

Kohno, M., Hasegawa, H., Inoue, A., Muraoka, M., Miyazaki, T., Oka, K., and Yasukawa, M. (2006). Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochem Biophys Res Commun.* 347:827-832.

Kooij, G., van Horssen, J., de Lange, E.M.C., Reijerkerk, A., Susanne, M.A., van der Polm S.M.A., van het Hof, B., Drexhage, J., Scheffer, G., Oerlemans, R., Scheper, R., van der Valk, P., Dijkstra, C.D. and de Vries, H.E. (2010). T lymphocyte mediated loss of P-glycoprotein function during neuroinflammation. *J. Autoimm.* 34:416-425.

Kornek, B., Storch, M.K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M. and Lassmann, H. (2000). Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am J Pathol*. 157:267-276.

Kraft, B. and Kress, H.G. (2004). Cannabinoids and the immune system. Of men, mice and cells. *Schmerz*. 18:203-10.

Kreitzer, A.C., Carter, A.G. and Regehr, W.G. (2002). Inhibition of interneuron firing extends the spread of endocannabinoid signaling in the cerebellum. *Neuron* 34:787-796.

Kurtzke, J.F. (1993). Epidemiologic evidence for multiple sclerosis as an infection. *Clin Microbiol Rev.* 6:382-427.

Lankas, G.R., Cartwright, M.E. and Umbenhauer, D. (1997). P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicol Appl Pharmacol.* 143:357-365.

Lauckner, J.E., Jensen, J.B, Chen, H.Y., Lu, H.C., Hille, B. and Mackie, K. (2008), GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc Natl Acad Sci U S A*.105:2699-2704.

Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J.F., Beslot, F., Bohme, G.A, Imperato, A., Pedrazzini, T., Roques, B.P., Vassart, G., Fratta, W. and Parmentier, M. (1999). Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science*. 283:401-404.

Ledgerwood, C.J., Greenwood, S.M., Brett, R.R., Pratt, J.A. and Bushell, T.J. (2010). Cannabidiol inhibits synaptic transmission in rat hippocampal cultures and slices via several receptor pathways. *Br J Pharmacol.* (Epub ahead of print).

Leray, E., Yaouanq, J., Le Page, E., Coustans, M., Laplaud, D., Oger, J. and Edan, G. (2010). Evidence for a two-stage disability progression in multiple sclerosis. *Brain.*133:1900-1913.

Leung, D., Saghatelian, A., Simon, G.M. and Cravatt, B.F. (2006). Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry.* 45:4720-4726.

Lichtman, A.H., Shelton, C.C., Advani, T. and Cravatt, B.F. (2004). Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. *Pain* 109:319-327.

Ligresti, A., Cascio, M.G., Pryce, G., Kulasegram, S., Beletskaya, I., De Petrocellis, L., Saha, B., Mahadevan, A., Visintin, C., Wiley, J.L., Baker, D., Martin, B.R., Razdan, R.K. and Di Marzo, V. (2006). New potent and selective inhibitors of anandamide reuptake with antispastic activity in a mouse model of multiple sclerosis. *Br J Pharmacol* 147:83-91.

Liu, G.Y., Baker, D., Fairchild, S., Figueroa, F., Quartey-Papafio, R., Tone, M., Healey, D., Cooke, A., Turk, J.L. and Wraith, D.C. (1993). Complete characterization of the expressed immune response genes in Biozzi AB/H mice: structural and functional identity between AB/H and NOD A region molecules. *Immunogenetics* 37:296-300.

Liu, J., Li, H., Burstein, S.H., Zurier, R.B. and Chen, J.D. (2003). Activation and binding of peroxisome proliferator-activated receptor gamma by synthetic cannabinoid ajulemic acid. *Mol Pharmacol.* 63:983-992.

Lo, A.C., Black, J.A. and Waxman, S.G. (2002). Neuroprotection of axons with phenytoin in experimental allergic encephalomyelitis. *Neuroreport* 13:1909-1912.

Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A.. and Piomelli, D. (2005). The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol*. 67:15-19.

Long, J.Z., Li, W., Booker, L., Burston, J.J., Kinsey, S.G., Schlosburg, J.E., Pavón, F.J., Serrano, A.M., Selley, D.E., Parsons, L.H., Lichtman, A.H. and Cravatt, B.F. (2009). Selective blockade of 2-arachidonylglycerol hydrolysis produces cannabinoid behavioural effects.*Nat Chem Biol.* 2009 5:37-44.

Lombard, .C, Nagarkatti, M. and Nagarkatti, P. (2007). CB2 cannabinoid receptor agonist, JWH-015, triggers apoptosis in immune cells: potential role for CB2-selective ligands as immunosuppressive agents. *Clin Immunol.* 122:259-270.

Lopez-Rodriguez, M.L., Viso, A., Ortega-Gutierrez, S., Lastres-Becker, I., Gonzalez, S., Fernandez-Ruiz, J. and Ramos, J.A. (2001). Design, synthesis and biological evaluation of novel arachidonic acid derivatives as highly potent and selective endocannabinoid transporter inhibitors. *J Med Chem.* 44:4505-4508.

Loría, F., Petrosino, S., Hernangómez, M., Mestre, L., Spagnolo, A., Correa, F., Di Marzo, V., Docagne, F., and Guaza, C. (2010).An endocannabinoid tone limits excitotoxicity in vitro and in a model of multiple sclerosis. *Neurobiol Dis.* 37:166-176.

Löscher, W. and Potschka, H. (2005). Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx.* 2:86-98.

Lu, F., Selak, M., O'Connor, J., Croul, S., Lorenzana, C., Butunoi, C. and Kalman, B. (2000). Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. *J Neurol Sci*. 177:95-103.

Lunn, C.A., Fine, J.S., Rojas-Triana, A., Jackson, J.V., Fan, X., Kung, T.T., Gonsiorek, W., Schwarz, M.A., Lavey, B., Kozlowski, J.A., Narula, S.K., Lundell, D.J., Hipkin, R.W. and Bober, L.A. (2006). A novel cannabinoid peripheral cannabinoid receptor-selective inverse agonist blocks leukocyte recruitment in vivo. *J Pharmacol Exp Ther.* 316:780-788.

Lyman, W.D., Sonett, J.R., Brosnan, C.F., Elkin, R. and Bornstein, M.B. (1989). Delta 9-tetrahydrocannabinol: a novel treatment for experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 23:73-81.

Maccarrone, M., Falciglia, K., Di Rienzo, M. and Finazzi-Agrò, A. (2002a). Endocannabinoids, hormone-cytokine networks and human fertility. *Prostaglandins Leukot Essent Fatty Acids.* 66:309-17.

Maccarrone, M., Bisogno, T., Valensise, H., Lazzarin, N., Fezza, F., Manna, C., Di Marzo, V. and Finazzi-Agrò, A. (2002b). Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. *Mol Hum Reprod.* 8:188-195.

Mackie, K., Lai, Y., Westenbroek, R. and Mitchell, R. (1995). Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J Neurosci.* 15:6552-6561.

Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A. and Kano, M. (2001) Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. *Neuron.* 31:463-475.

Maingret, F., Patel, A.J., Lazdunski, M. and Honore, E. (2001). The endocannabinoid anandamide is a direct and selective blocker of the background K(+) channel TASK-1. *Embo J*. 20:47-54.

Makara, J.K., Katona, I., Nyiri, G., Nemeth, B., Ledent, C., Watanabe, M., de Vente, J., Freund, T.F. and Hajos, N. (2007). Involvement of nitric oxide in depolarization-induced suppression of inhibition in hippocampal pyramidal cells during activation of cholinergic receptors. *J Neurosci.* 27:10211-10222.

Malfait, A.M., Gallily, R., Sumariwalla, P.F., Malik, A.S., Andreakos, E., Mechoulam, R. and Feldmann, M. (2000). The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A.* 97:9561-9566.

Malfitano, A.M., Matarese, G., Pisanti, S., Grimaldi, C., Laezza, C., Bisogno, T., Di Marzo, V., Lechler, R.I. and Bifulco, M. (2006). Arvanil inhibits T lymphocyte activation and ameliorates autoimmune encephalomyelitis. *J Neuroimmunol.* 171:110-119.

Marchetti, B., Morale, M.C., Brouwer, J., Tirolo, C., Testa, N., Caniglia, S., Barden, N, Amor S., Smith, P.A. and Dijkstra, C.D. (2002). Exposure to a dysfunctional glucocorticoid receptor from early embryonic life programs the resistance to experimental autoimmune encephalomyelitis via nitric oxide-induced immunosuppression. *J Immunol.* 168:5848-5859.

Maresz, K., Carrier, E.J., Ponomarev, E.D., Hillard, C.J. and Dittel, B.N. (2005) Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. *J Neurochem* 95:437-445.

Maresz, K., Pryce, G., Ponomarev, E.D., Marsicano, G., Croxford, J.L., Shriver, L.P., Ledent, C., Cheng, X., Carrier, E.J., Mann, M.K., Giovannoni, G., Pertwee, R.G., Yamamura, T., Buckley, N.E., Hillard, C.J., Lutz, B., Baker, D. and Dittel, B.N. (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB_1 on neurons and CB_2 on autoreactive T Cells. *Nat Medicine* 13:492-497.

Marquez, N., De Petrocellis, L., Caballero, F.J., Macho, A., Schiano-Moriello, A., Minassi, A., Appendino, G., Munoz, E. and Di Marzo, V. (2006). Iodinated N-acylvanillamines: potential "multiple-target" anti-inflammatory agents acting via the inhibition of t-cell activation and antagonism at vanilloid TRPV1 channels. *Mol Pharmacol.* 69:1373-1382.

Maron, D. M. and Ames, B. N. (1983) Revised Methods for the Salmonella Mutagenicity Test. *Mutat. Res.* 113, 173-215.

Marrs, W.R., Blankman, J.L., Horne, E.A., Thomazeau, A., Lin, Y.H., Coy, J., Bodor, A.L., Muccioli, G.G., Hu, S.S., Woodruff, G., Fung, S., Lafourcade, M., Alexander, J.P., Long, J.Z., Li, W., Xu, C., Möller, T., Mackie, K., Manzoni, O.J., Cravatt, B.F. and Stella, N. (2010). The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. *Nat Neurosci.* 13:951-957.

Marsicano, G., Moosmann, B., Hermann, H., Lutz, B. and Behl, C. (2002). Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. *J Neurochem.* 80:448-456.

Marsicano, G., Wotjak, C.T., Azad, S.C., Bisogno, T., Rammes, G., Cascio, M.G., Hermann, H., Tang, J., Hofmann, C., Zieglgansberger, W., Di Marzo, V. and Lutz, B. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature.* 418:530-534.

Marte, A., Cavallero, A., Morando, S., Uccelli, A., Raiteri, M. and Fedele, E. (2010).Alterations of glutamate release in the spinal cord of mice with experimental autoimmune encephalomyelitis. *J Neurochem*. (Epub ahead of print)

Martin, B.R., Wiley, J.L., Beletskaya, I., Sim-Selley, L.J., Smith, F.L., Dewey, W.L., Cottney, J., Adams, J., Baker, J., Hill, D., Saha, B., Zerkowski, J., Mahadevan, A. and Razdan, R.K. (2006). Pharmacological characterization of novel water-soluble cannabinoids. *J Pharmacol Exp Ther.* 318:1230-1239.

Matarese, G., Di Giacomo, A., Sanna, V., Lord, G.M., Howard, J.K. Di Tuoro, A, Bloom, S. R. Lechler, R.I., Zappacosta, S. and Fontana, S. (2001). Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. *J Immunol.* 166:5909-5916.

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.

Mattes, R.D., Shaw, L.M., Edling-Owens, J., Engelman, K. and Elsohly, M.A. (1993). Bypassing the first-pass effect for the therapeutic use of cannabinoids. *Pharmacol Biochem Behav.* 44:745-747.

Mathew, J.S., Westmoreland, S.V., Alvarez, X., Simon, M.A., Pauley, D.R., MacKey, J.J. and Lackner, A.A. (2001). Expression of peripherin in the brain of macaques (Macaca mulatta and Macaca fascicularis) occurs in astrocytes rather than neurones and is associated with encephalitis. *Neuropathol Appl Neurobiol.* 27:434-443.

McAllister, S.D., Tao, Q., Barnett-Norris, J., Buehner, K., Hurst, D.P., Guarnieri, F., Reggio, P.H., Nowell Harmon, K.W., Cabral, G.A. and Abood, M.E. (2002). A critical role for a tyrosine residue in the cannabinoid receptors for ligand recognition. *Biochem Pharmacol.* 63:2121-2136.

McCombe, P.A., Gordon, T.P. and Jackson, M.W. (2009). Bladder dysfunction in multiple sclerosis. *Expert Rev Neurother.* 9:331-340.

McHugh, D., Hu, S.S., Rimmerman, N., Juknat, A., Vogel, Z., Walker, J.M. and Bradshaw, H.B. (2010). N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC Neurosci.* 11:44.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., Pertwee, R.G., Griffin, G., Bayewitch, M., Barg, J. and Voge,l Z. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol.* 50:83-90.

Mestre, L., Correa, .F, Arevalo-Martin, A., Molina-Holgado, E., Valenti, M., Ortar, G., Di Marzo, V. and Guaza, C. (2005). Pharmacological modulation of the endocannabinoid system in a viral model of multiple sclerosis. *J Neurochem.* 92:1327-1339.

Metz, I., Lucchinetti, C.F., Openshaw, H., Garcia-Merino, A., Lassmann, H., Freedman, M.S., Atkins, H.L., Azzarelli, B., Kolar, O.J. and Brück, W. (2007). Autologous haematopoietic stem cell transplantation fails to stop demyelination and neurodegeneration in multiple sclerosis. *Brain.* 130:1254-1262.

Miller, D.H., Khan, O.A., Sheremata, W.A., Blumhardt, L.D., Rice, G.P., Libonati, M.A., Willmer-Hulme, A.J., Dalton, C.M., Miszkiel, K.A. and O'Connor, PW; International Natalizumab Multiple Sclerosis Trial Group.(2003). A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med.* 348:15-23.

Miller, D.H. and Leary, S.M. (2007). Primary-progressive multiple sclerosis. *Lancet Neurol.* 6:903-912.

Milman, G., Maor, Y., Abu-Lafi, S., Horowitz, M., Gallily, R., Batkai, S., Mo, F.M., Offertaler, L., Pacher, P,, Kunos, G., and Mechoulam,.R. (*2006*). N-arachidonoyl *l*-serine, an endocannabinoid-like brain constituent with vasodilatory properties. *Proc Natl Acad Sci U S A* 103**:** 2428-2433.

Moldrich, G. and Wenger, T. (2000). Localization of the CB1 cannabinoid receptor in the rat brain. An immunohistochemical study. *Peptides* 21:1735-1742.

Molina-Holgado, F., Rubio-Araiz, A., Garcia-Ovejero, D., Williams, R.J., Moore, J.D., Arevalo-Martin, A., Gomez-Torres, O. and Molina-Holgado, E. (2007). CB2 cannabinoid receptors promote mouse neural stem cell proliferation. *Eur J Neurosci.* 25:629-634.

Moore, S.A., Nomikos, G.G., Dickason-Chesterfield, A.K., Schober, D.A., Schaus, J.M., Ying,.B.P., Xu, Y.C., Phebus, L., Simmons, R.M., Li, D., Iyengar, S. and Felder, C.C. (2005). Identification of a high-affinity binding site involved in the transport of endocannabinoids. *Proc Natl Acad Sci USA* 102:17852-17857.

Muller-Vahl, K.R., Prevedel, H., Theloe, K., Kolbe, H., Emrich, H.M. and Schneider, U. (2003). Treatment of Tourette syndrome with delta-9-tetrahydrocannabinol (delta 9-THC): no influence on neuropsychological performance. *Neuropsychopharmacology* 28:384-388.

The Multiple Sclerosis Study Group. (1990). Efficacy and toxicity of cyclosporine in chronic progressive multiple sclerosis: a randomized, double-blinded, placebo-controlled clinical trial. *Ann Neurol.* 27:591-605.

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

Murphy, L.L., Munoz, R.M., Adrian, B.A. and Villanua, M.A. (1998). Function of cannabinoid receptors in the neuroendocrine regulation of hormone secretion. *Neurobiol Dis.* 6:432-446.

Mutch, D.M., Anderle, P., Fiaux, M., Mansourian, R., Vidal, K., Wahli, W., Williamson, G. and Roberts, M.A. (2004). Regional variations in ABC transporter expression along the mouse intestinal tract. *Physiol Genomics.* 17:11-20.

Nagayama, T., Sinor, A.D., Simon, R.P., Chen, J., Graham, S.H., Jin, K. and Greenberg, D.A. (1999). Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci.* 19:2987-2995.

Natarajan, C. and Bright, J.J. (2002). Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. *Genes Immun.* 3:59-70.

Nemeth, J., Helyes, Z., Than, M., Jakab, B., Pinter, E. and Szolcsanyi, J. (2003). Concentration-dependent dual effect of anandamide on sensory neuropeptide release from isolated rat tracheae. *Neurosci Lett.* 336:89-92.

Newton, C.A., Lu, T., Nazian, S.J., Perkins, I., Friedman, H. and Klein, T.W. (2004). The THC-induced suppression of Th1 polarization in response to Legionella pneumophila infection is not mediated by increases in corticosterone and PGE2. *J Leukoc Biol.* 76:854-61.

Ni X., Geller, E.B. Eppihhimer M.J. Eisenstein, T.K., Adler, M.W. and Tuma, R.F. (2004). Win 55212-2, a cannabinoid receptor agonist, attenuates leukocyte/endothelial interactions in an experimental autoimmune encephalomyelitis model. *Mult Scler* 10:158-64.

Nicholson, R.A., Liao, C., Zheng, J., David, L.S., Coyne, L., Errington, A.C., Singh, G. and Lees, G. (2003). Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. *Brain Res.* 978:194-204.

Nielsen, J,B., Crone, C. and Hultborn, H. (2007). The spinal pathophysiology of spasticity--from a basic science point of view. *Acta Physiol (Oxf).* 189:171-180.

Niino, M., Iwabuchi, K., Kikuchi, S., Ato, M., Morohashi, T., Ogata, A., Tashiro, K. and Onoe, K. (2001). Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by an agonist of peroxisome proliferator-activated receptor-gamma. *J Neuroimmunol.* 116:40-48.

Noe, S.N., Newton, C., Widen, R., Friedman, H. and Klein, T.W. (2001). Modulation of CB1 mRNA upon activation of murine splenocytes. *Adv Exp Med Biol.* 493:215-21.

Ohgoh, M., Hanada, T., Smith, T., Hashimoto, T., Ueno, M., Yamanishi, Y., Watanabe, M. and Nishizawa, Y. (2002). Altered expression of glutamate transporters in experimental autoimmune encephalomyelitis. *J Neuroimmunol*. 125:170-178.

Oka S., Wakui, J., Gokoh, M., Kishimoto, S. and Sugiura, T. (2006). Suppression by WIN55212-2, a cannabinoid receptor agonist, of inflammatory reactions in mouse ear: Interference with the actions of an endogenous ligand, 2-arachidonoylglycerol. *Eur J Pharmacol*. 538:154-162.

Oka, S., Nakajima, K., Yamashita, A., Kishimoto, S. and Sugiura, T. (2007). Identification of GPR55 as a lysophosphatidylinositol receptor.*Biochem Biophys Res Commun.* 362:928-934.

Oka, S., Toshida, T., Maruyama, K., Nakajima, K., Yamashita, A. and Sugiura, T. (2009). 2-Arachidonoyl-sn-glycero-3-phosphoinositol: a possible natural ligand for GPR55. *J Biochem.* 145:13-20.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. and Ueda, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem.* 279:5298-5305.

Okonkwo, D.O. and Povlishock, J.T. (1999). An Intrathecal Bolus of Cyclosporin A Before Injury Preserves Mitochondrial Integrity and Attenuates Axonal Disruption in Traumatic Brain Injury. *Journal of Cerebral Blood Flow & Metabolism* 19:443-451.

Omura, S. (2008). Ivermectin: 25 years and still going strong. *Int J Antimicrob Agents.* 31:91-98.

O'Neill, J.K., Butter, C., Baker, D., Gschmeissner, S.E., Kraal, G., Butcher, E.C. and Turk, J.L. (1991). Expression of vascular addressins and ICAM-1 by endothelial cells in the spinal cord during chronic relapsing experimental allergic encephalomyelitis in the Biozzi AB/H mouse. *Immunology* 72:520-525.

O'Neill, J.K., Baker, D., Davison, A.N., Maggon, K.K., Jaffee, .BD. and Turk, J.L. (1992). Therapy of chronic relapsing experimental allergic encephalomyelitis and the role of the blood-brain barrier: Elucidication by the action of brequinar sodium. *J. Neuroimmunol*. 38:53-62.

O'Neill, J.K., Baker, D., Morris, M.M., Gschmeissner, S.E., Jenkins, H.G., Butt, A.M., Kirvell, S.L. and Amor, S. (1998). Optic neuritis in chronic relapsing experimental allergic encephalomyelitis in Biozzi ABH mice: demyelination and fast axonal transport changes in disease. *J Neuroimmunol.* 82:210-218.

Ortar, G., Ligresti, A., De Petrocellis, L., Morera, E.. and Di Marzo, V. (2003). Novel selective and metabolically stable inhibitors of anandamide cellular uptake. *Biochem Pharmacol*. 65:1473-1481.

Ortega-Gutierrez, S., Hawkins, E.G., Viso, A., Lopez-Rodriguez, M.L. and Cravatt, B.F. (2004). Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry* 43:8184-8190.

Ortega-Gutierrez, S., Molina-Holgado, E., Arevalo-Martin, A., Correa, F., Viso, A., Lopez-Rodriguez, M.L., Di Marzo, V. and Guaza, C. (2005). Activation of the endocannabinoid system as therapeutic approach in a murine model of multiple sclerosis *Faseb J.* 19:1338-1340.

O'Sullivan, S.E. (2007). Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. *Br J Pharmacol.* 152:576-582.

Oz, M. (2006). Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids.*Pharmaco Ther* 111:114-144.

Pacheco, M., Childers, S.R., Arnold, R., Casiano, F. and Ward, S.J. (1991). Aminoalkylindoles: actions on specific G-protein-linked receptors. *J Pharmacol Exp Ther.* 257:170-183.

Pacifici, R., Zuccaro, P., Pichini, S., Roset, P.N., Poudevida, S., Farre, M., Segura, J. and De la Torre, R. (2003). Modulation of the immune system in cannabis users. *Jama* 289:1929-1931.

Pahan, K. and Schmid, M. (2000). Activation of nuclear factor-kB in the spinal cord of experimental allergic encephalomyelitis. *Neurosci Lett.* 287:17-20.

Paisley, S., Beard, S., Hunn, A. and Wight, J. (2002). Clinical effectiveness of oral treatments for spasticity in multiple sclerosis: a systematic review. *Mult Scler.* 8:319-329.

Palaszynski, K.M., Loo, K.K., Ashouri, J.F., Liu, H.B. and Voskuhl, R.R. (2004a). Androgens are protective in experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *J Neuroimmunol.* 146:144-152.

Palaszynski, K.M., Liu, H., Loo, K.K. and Voskuhl, R.R. (2004b). Estriol treatment ameliorates disease in males with experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *J Neuroimmunol*. 149:84-89.

Palazuelos, J., Davoust, N., Julien, B., Hatterer, E., Aguado, T., Mechoulam, R., Benito, C., Romero, J., Silva, A., Guzmán, M., Nataf, S. and Galve-Roperh, I. (2008).The CB(2) cannabinoid receptor controls myeloid progenitor trafficking: involvement in the pathogenesis of an animal model of multiple sclerosis. *J Biol Chem.* 283:13320-13329.

Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A., Mechoulam, R. and Shohami, E. (2001). An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 413:527-531.

Panikashvili, D., Mechoulam, R., Beni, S.M., Alexandrovich, A. and Shohami, E. (2005). CB1 cannabinoid receptors are involved in neuroprotection via NF-kappa B inhibition. *J Cereb Blood Flow Metab*. 25:477-484.

Panikashvili, D., Shein, N.A., Mechoulam, R., Trembovler, V., Kohen, R., Alexandrovich, A. and Shohami, E. (2006). The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. *Neurobiol Dis*. 22:257-264.

Parmentier-Batteur, S., Jin, K., Mao, X.O., Xie, L. and Greenberg, D.A. (2002). Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *J Neurosci*. 22:9771-9775.

Pawarode, A., Shukla, S., Minderman, H., Fricke, S.M., Pinder, E.M., O'Loughlin, K.L., Ambudkar, S.V. and Baer, M.R. (2007). Differential effects of the immunosuppressive agents cyclosporin A, tacrolimus and sirolimus on drug transport by multidrug resistance proteins. *Cancer Chemother Pharmacol.* 60:179-188.

Perry, V.H. and Anthony, D.C. (1999). Axon damage and repair in multiple sclerosis. *Philos Trans R Soc Lond B Biol Sci.* 354:1641-1647.

Pershadsingh, H.A., Heneka, M.T., Saini, R., Amin, N.M., Broeske, D.J. and Feinstein, .DL. (2004). Effect of pioglitazone treatment in a patient with secondary multiple sclerosis. *J Neuroinflammation* 1:3.

Pertwee, R.G. (1972). The ring test: a quantitative method for assessing the "cataleptic" effect of cannabis in mice. *Br. J. Pharmacol*. 1972; 46: 753-763.

Pertwee, R.G. (1974). Tolerance to the effect of delta1-tetrahydrocannabinol on corticosterone levels in mouse plasma produced by repeated administration of cannabis extract or delta1-tetrahydrocannabinol. *Br J Pharmacol.* 51:391-397.

Pertwee, R.G., Stevenson, L.A., Elrick, D.B., Mechoulam, R. and Corbett, A.D. (1992). Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br J Pharmacol.* 105:980-984.

Pertwee, R.G., Stevenson, L.A. and Griffin, G. (1993). Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide. *Brit J Pharmacol*. 110:1483-1490.

Pertwee, R.G. (1999). Pharmacology of cannabinoid receptor ligands. *Curr Med Chem* 6:635-664.

Pertwee, R.G., Gibson, T.M., Stevenson, L.A., Ross, R.A., Banner, W.K., Saha, B., Razdan, R.K. and Martin, B.R. (2000). O-1057, a potent water-soluble cannabinoid receptor agonist with antinociceptive properties. *Br J Pharmacol.* 129:1577-1584.

Pertwee, R.G. (2002). Cannabinoids and multiple sclerosis. *Pharmacol Ther*; 95:165-174.

Pertwee, R.G. (2005a). Pharmacological actions of cannabinoids. Edited by RG Pertwee. *Heidelberg, Springer-Verlag* p.pp. 1-51.

Pertwee, R.G. (2005b). Inverse agonism and neutral antagonism at cannabinoid CB1 receptors *Life Sci.* 76:1307-1324.

Pertwee, R.G. (2007). Cannabinoids and multiple sclerosis. *Mol Neurobiol.* 36:45-59.

Pertwee, R.G. (2008). CB1and CB2 Receptor Pharmacology. In *Cannabinoids and the brain Edited by Attila Kofalvi. Springer p*. pp. 91-99.

Petzold, A., Baker, D., Pryce, G., Keir, G., Thompson, E.J. and Giovannoni, G. (2003). Quantification of neurodegeneration by measurement of brain-specific proteins. *J Neuroimmunol.* 138:45-48.

Piomelli, D., Tarzia, G., Duranti, A., Tontini, A., Mor, M., Compton, T.R., Dasse, O., Monagham, E.P., Parrott, J.A. and Putman, D. (2006). Pharmacological profile of the selective FAAH inhibnitor KDS-4103 (URB597). *CNS Drugs Rev* 12: 21-38.

Pitt, D., Werner, P. and Raine, C.S. (2000). Glutamate excitotoxicity in a model of multiple sclerosis. *Nat Med.* 6:67-70.

Polman, C.H., O'Connor, P.W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D.H., Phillips, J.T., Lublin, F.D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M.A. and Sandrock, A.W.; AFFIRM Investigators. (2006). A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med.* 354:899-910.

Polster, B.M. and Fiskum, G. (2004).Mitochondrial mechanisms of neural cell apoptosis. *J Neurochem.* 90:1281-1289.

Porter, A.C., Sauer, J.M., Knierman, M.D., Becker, G.W., Berna, M.J., Bao, J., Nomikos, G.G., Carter, P., Bymaster, F.P., Leese, A.B. and Felder, C.C. (2002). Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther*. 301:1020-1024.

Pippert, T.R. and Umbenhauer, D.R. (2001). The subpopulation of CF-1 mice deficient in P-glycoprotein contains a murine retroviral insertion in the mdr1a gene. *J Biochem Mol Toxicol.* 15:83-89.

Prat, E., Tomaru, U., Sabate,r L., Park, D.M., Granger, R., Kruse, N., Ohayon, J.M., Bettinotti, M.P. and Martin, R. (2005). HLA-DRB5*0101 and -DRB1*1501 expression in the multiple sclerosis-associated HLA-DR15 haplotype. *J Neuroimmunol.* 167:108-119.

Pryce, G., Ahmed, Z., Hankey, D.J., Jackson, S.J., Croxford, J.L., Pocock, J.M., Ledent, C., Petzold, A., Thompson, A.J., Giovannoni, G., Cuzner, M.L. and Baker, D. (2003a). Cannabinoids inhibit neurodegeneration in models of multiple sclerosis. *Brain*.126 2191-202.

Pryce G., Giovannoni, G. and Baker, D. (2003b). Mifepristone or inhibition of 11beta-hydroxylase activity potentiates the sedating effects of the cannabinoid receptor-1 agonist Delta(9)-tetrahydrocannabinol in mice. *Neurosci Lett.* 3412:164-166.

Pryce, G., O'Neill, J.K., Croxford, J..L, Amor, S., Hankey, D.J., East, E., Giovannoni, G. and Baker, D. (2005a). Autoimmune tolerance eliminates relapses but fails to halt progression in a model of multiple sclerosis. *J. Neuroimmunol.*165:41-52.

Pryce, G., and Baker, D. (2005b). Emerging properties of cannabinoid medicines in management of multiple sclerosis. 2005b.*Trends Neurosci.* 28:272-6.

Pryce, G. and Baker, D. (2007). Control of Spasticity in a Multiple Sclerosis Model is mediated by $CB_{1,}$ not $CB_{2,}$ cannabinoid receptors. *Brit J Pharmacol.* 150:519-525.

Rachelefsky, G.S., Opelz, G., Mickey, M.R., Lessin, P., Kiuchi M., Silverstein, M.J. and Stiehm. E.R. (1976). Intact humoral and cell-mediated immunity in chronic marijuana smoking. *J Allergy Clin Immunol.* 58:483-490.

Ralevic, V., Kendall, D.A., Jerman, J.C., Middlemiss, D.N. and Smart, D. (2001). Cannabinoid activation of recombinant and endogenous vanilloid receptors. *Eur J Pharmacol* 424:211-219.

Ramagopalan, S.V., Maugeri, N.J., Handunnetthi, L., Lincoln, M.R., Orton, S.M., Dyment, D.A., Deluca, G.C., Herrera, B.M., Chao, M.J., Sadovnick, A.D., Ebers, G.C. and Knight, J.C. (2009). Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D. *PLoS Genet.* 5:e1000369.

Ramagopalan, S.V., Heger, A., Berlanga, A.J., Maugeri, N.J., Lincoln, M.R., Burrell, A., Handunnetthi, L., Handel, A.E., Disanto, G., Orton, S.M., Watson, C.T., Morahan, J.M., Giovannoni, G., Ponting, C.P., Ebers, G.C. and Knight, J.C. (2010). A ChIP-seq defined genome-wide map of vitamin D receptor binding: Associations with disease and evolution. *Genome Res.* 20:1352-1360.

Ramirez, B.G., Blazquez, C., Gomez del Pulgar, T., Guzman, M. and de Ceballos, M.L. (2005). Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J Neurosci*. 25:1904-1913.

Rhee, M.H., Vogel, Z., Barg, J., Bayewitch, M., Levy, R., Hanus, L., Breuer, A. and Mechoulam, R. (1997). Cannabinol derivatives: binding to cannabinoid receptors and inhibition of adenylylcyclase. *J Med Chem.* 40:3228-3233.

Rhee, M.H. and Kim, S.K. (2002). SR144528 as inverse agonist of CB2 cannabinoid receptor. *J Vet Sci.* 3:179-184.

Rice, G.P., Filippi, M. and Comi, G. (2000). Cladribine and progressive MS: clinical and MRI outcomes of a multicenter controlled trial. Cladribine MRI Study Group. *Neurology*. 54:1145-1155.

Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Néliat G, Caput D, Ferrara P, Soubrié P, Brelière JC. and Le Fur G (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* 350:240-244.

Rinaldi-Carmona, M., Barth, F., Millan, J., Derocq, J.M., Casellas, P., Congy, C., Oustric, D., Sarran, M., Bouaboula, M., Calandra, B., Portier, M., Shire, D., Breliere, J.C. and Le Fur, G.L. (1998). SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther.* 284:644-650.

Rios, C., Gomes, I. and Devi, L.A (2006). mu opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. *Br J Pharmacol* 148:387-395.

Rockwell, C.E., Snider, N.T., Thompson, J.T., Vanden Heuvel, J.P. and Kaminski, N.E. (2006). Interleukin-2 suppression by 2-arachidonyl glycerol is mediated through peroxisome proliferator-activated receptor gamma independently of cannabinoid receptors 1 and 2. *Mol Pharmacol.* 70:101-111.

Rog, D.J., Nurmikko, T.J., Friede, T. and Young, C.A. (2005). Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. *Neurology.* 65:812-819.

Ross, R.A. The enigmatic pharmacology of GPR55. (2009). *Trends Pharmacol Sci.* 30:156-163.

Roth, M.D., Baldwin, G.C. and Tashkin, D.P. (2002). Effects of delta-9-tetrahydrocannabinol on human immune function and host defense. *Chem Phys Lipids* 121:229-239.

Rubio-Araiz, A., Arevalo-Martin, A., Gomez-Torres, O., Navarro-Galve, B., Garcia-Ovejero, D., Suetterlin, P., Sanchez-Heras, E., Molina-Holgado, E. and Molina-Holgado, F. (2008). The endocannabinoid system modulates a transient TNF pathway that induces neural stem cell proliferation. *Mol Cell Neurosci.* 38:374-380.

Rubovitch, V., Gafni, M. and Sarne, Y. (2002). The cannabinoid agonist DALN positively modulates L-type voltage-dependent calcium-channels in N18TG2 neuroblastoma cells. *Brain Res Mol Brain Res*. 101:93-102.

Runmarker, B. and Andersen, O. (1993). Prognostic factors in a multiple sclerosis incidence cohort with twenty-five years of follow-up. *Brain* 116 :117-134.

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.

Ryberg, E., Larsson, N., Sjögren, 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.

Saario, S.M., Palomäki, V., Lehtonen, M., Nevalainen, T., Järvinen, T. and Laitinen, J.T. (2006). URB754 has no effect on the hydrolysis or signaling capacity of 2-AG in the rat brain. *Chem Biol.* 13:811-814.

Sadovnick, A.D. and Ebers. G.C. (1993). Epidemiology of multiple sclerosis: a critical overview. *Can J Neurol Sci.* 20:17-29.

Sagar. D.R., Kendall, D.A. and Chapman. V. (2008). Inhibition of fatty acid amide hydrolase produces PPAR-alpha-mediated analgesia in a rat model of inflammatory pain.*Br J Pharmacol.* 155:1297-1306.

Salim, K., Schneider, U., Burstein, S., Hoy, L. and Karst, M. (2005). Pain measurements and side effect profile of the novel cannabinoid ajulemic acid. *Neuropharmacology.* 48:1164-1171.

Samuelson, L.C., Swanberg, L.J. and Gantz, I. (1996). Mapping of the novel G protein-coupled receptor Gpr18 to distal mouse chromosome 14. *Mamm Genome.* 7:920-921.

Sanchez, A.J., Gonzalez-Perez, P., Galve-Roperh, I, and Garcia-Merino, A. (2006). R-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1naphtalenylmethanoneameliorates experimental autoimmune encephalomyelitis and induces encephalitogenic T cell apoptosis: partial involvement of the CB_2 receptor. *Biochem Pharmacol.* 72:1697-1706.

Sanchez-Pastor, E., Trujillo, X., Huerta, M. and Andrade, F. (2007). Effects of cannabinoids on synaptic transmission in the frog neuromuscular junction. *J Pharmacol Exp Ther.* 321:439-445.

Sanna, V., Di Giacomo, A., La Cava, A., Lechler, R.I., Fontana, S., Zappacosta, S. and Matarese, G. (2003). Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses. *J Clin Invest.* 111:241-250.

Sarchielli, P., Pini, L.A., Coppola, F., Rossi, C., Baldi, A., Mancini, M.L. and Calabresi, P. (2007). Endocannabinoids in chronic migraine: CSF findings suggest a system failure. *Neuropsychopharmacology* 32:1384-1390.

Savinainen, J,R., Kokkola, T., Salo, O.M.H., Poso, A., Jarvinen, T. and Laitinen. J.T. (2005). Identification of WIN55212-3 as a competitive neutral antagonist of the human cannabinoid CB2 receptor. *Brit. J. Pharmacol.* 145: 636-645.

Sawzdargo, M., Nguyen, T., Lee, D.K., Lynch, K.R., Cheng, R., Heng, H.H., George, S,R. and O'Dowd, B.F. (1999). Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain Res Mol Brain Res.* 64:19319-8.

Schabitz, W.R., Giuffrida, A., Berger, C., Aschof, A., Schwaninger, M., Schwab, S. and Piomelli, D. (2002). Release of fatty acid amides in a patient with hemispheric stroke: a microdialysis study. *Stroke* 33:2112-2114.

Schinkel, A.H. and Jonker, J.W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev.* 55:3-29.

Schlosburg, J.E., Blankman, J.L., Long, J.Z., Nomura, D.K., Pan, B., Kinsey, S.G., Nguyen, P.T., Ramesh, D., Booker, L., Burston, J.J., Thomas, E.A., Selley, D.E., Sim-Selley, L.J., Liu, Q.S., Lichtman, A.H. and Cravatt, B.F. (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci.* 13:1113-1119.

Schweitzer, P. (2000). Cannabinoids decrease the K(+) M-current in hippocampal CA1 neurons, J Neurosci 2000, 20:51-58.

Selley, D.E., Rorrer, W.K., Breivogel, C.S., Zimmer, A.M., Zimmer, A., Martin, B.R. and Sim-Selley, LJ. (2001). Agonist efficacy and receptor efficiency in heterozygous CB1 knockout mice: relationship of reduced CB1 receptor density to G-protein activation. *J Neurochem.* 77:1048-57.

Shakespeare, D.T., Boggild, M. and Young, C. (2003). Anti-spasticity agents for multiple sclerosis. *Cochrane Database Syst Rev.* 2003;(4):CD001332.

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.

Sheng, W.S., Hu, S., Min, X., Cabral, G.A., Lokensgard, J,R. and Peterson, .PK. (2005). Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta-stimulated human astrocytes. *Glia* 49:211-219.

Siegal, T., Melamed, E., Sandbank, U. and Catane, R. (1988). Early and delayed neurotoxicity of mitoxantrone and doxorubicin following subarachnoid injection. *J Neurooncol.* 6:135-140.

Siegmund, S.V., Seki, E., Osawa, Y., Uchinami, H., Cravatt, B.F. and Schwabe, R.F. (2006). Fatty acid amide hydrolase determines anandamide-induced cell death in the liver. *J Biol Chem* 281: 10431–10438.

Siegmund, S.V., Qian, T., de Minicis, S., Harvey-White, J., Kunos, G., Vinod, K.Y., Hungund, B. and Schwabe, R.F. (2007). The endocannabinoid 2-arachidonoyl glycerol induces death of hepatic stellate cells via mitochondrial reactive oxygen species. *FASEB J* 21: 2798–2806.

Siffrin, V., Radbruch, H., Glumm, R., Niesner, R., Paterka, M., Herz, J., Leuenberger, T., Lehmann, .SM., Luenstedt, S., Rinnenthal, J.L., Laube, G., Luche, H., Lehnardt, S., Fehling, H.J., Griesbeck, O, and Zipp, F. (2010). In vivo imaging of partially reversible th17 cell-induced neuronal dysfunction in the course of encephalomyelitis. Immunity. 33:424-436.

Simon, G.M. and Cravatt. B.F. (2006). Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for alpha/beta-hydrolase 4 in this pathway. *J Biol Chem.* 281:26465-26472.

Singh Tahim, A., Santha, P. and Nagy, I. (2005). Inflammatory mediators convert anandamide into a potent activator of the vanilloid type 1 transient receptor potential receptor in nociceptive primary sensory neurons. *Neuroscience* 136:539-548.

Sipe, J.C., Chiang, K., Gerber, A.L., Beutler, E. and Cravatt, B.F. (2002). A missense mutation in human fatty acid amide hydrolase associated with problem drug use. *Proc Natl Acad Sci U S A.* 99:8394-8399.

Sipe, J.C., Waalen, J., Gerber, A. and Beutler, E. (2005). Overweight and obesity associated with a missense polymorphism in fatty acid amide hydrolase (FAAH). *Int J Obes (Lond).* 29:755-759.

Smith, K.J., Kapoor, R., Hall, .SM. and Davies, M. (2001). Electrically active axons degenerate when exposed to nitric oxide. *Ann Neurol* 49:470-476.

Smith, T., Groom, A., Zhu, B. and Turski, L. (2000). Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat Med.* 6:62-66.

Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A.H., Smit, J.W., Meijer, D.K., Borst, P., Nooijen, W,J., Beijnen, J. and van Tellingen, O. (1997). Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci U S A.* 94:2031-5.

Srinivasan, R., Sailasuta, N., Hurd, R, Nelson, S, and Pelletier, D. (2005). Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T. *Brain* 128:1016-1025.

Stahel, P.F., Smith, W.R., Bruchis, J. and Rabb, C.H. (2008). Peroxisome proliferator-activated receptors: "key" regulators of neuroinflammation after traumatic brain injury. *PPAR Res.* 2008:538141.

Staton, P.C., Hatcher, J.P., Walker, D.J., Morrison, A.D., Shapland, E,M., Hughes, J,P., Chong, E., Mander, P.K., Green, P.J., Billinton, A., Fulleylove, M., Lancaster, H,C., Smith, J.C., Bailey, L.T., Wise, A., Brown, A.J., Richardson, J.C. and Chessell, I.P. (2008).The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *Pain.*139:225-236.

Steck, A.J., Regli, F., Ochsner, F, and Gauthier, G. (1990). Cyclosporine versus azathioprine in the treatment of multiple sclerosis: 12-month clinical and immunological evaluation. *Eur Neurol.* 30:22422-8.

Steffens, S., Veillard, N.R., Arnaud, C., Pelli, G., Burger, F., Staub, C., Zimmer, A., Frossard, J,L, and Mach, F. (2005). Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature.* 434:78278-6.

Stella, N., Schweitzer, P. and Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388:773-778.

Storer, P.D., Xu J, Chavis, J. and Drew, P.D. (2005). Peroxisome proliferator-activated receptor-gamma agonists inhibit the activation of microglia and astrocytes: implications for multiple sclerosis. *J Neuroimmunol*. 161:113-122.

Stover, J.F, Pleines, U.E, Morganti-Kossmann, M.C., Kossmann, T., Lowitzsch, K. and Kempski, O.S. (1997). Neurotransmitters in cerebrospinal fluid reflect pathological activity. *Eur J Clin Invest.* 27:1038-1043.

Sugiura, T., Kodaka, T., Kondo, S., Tonegawa, T., Nakane, S., Kishimoto, S., Yamashita, A. and Waku, K. (1996). 2-Arachidonoylglycerol, a putative endogenous cannabinoid receptor ligand, induces rapid, transient elevation of intracellular free Ca2+ in neuroblastoma x glioma hybrid NG108-15 cells. *Biochem Biophys Res Commun.* 229:58-64.

Sugiura, T. and Waku, K. (2000). 2-Arachidonoylglycerol and the cannabinoid receptors. *Chem Phys Lipids* 108:89-106.

Sugiura, T., Kobayashi, Y., Oka, S. and Waku, K. (2002). Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot Essent Fatty Acids* 66:173-192.

Sulkowski. G., Dabrowska-Bouta, B., Kwiatkowska-Patzer, B. and Struzyńska, L. (2009).Alterations in glutamate transport and group I metabotropic glutamate receptors in the rat brain during acute phase of experimental autoimmune encephalomyelitis. *Folia Neuropathol.* 47:329-337.

Sun, D., Whitaker, J.N., Huang, Z., Liu, D., Coleclough, C., Wekerle, H. and Raine, C.S. (2001). Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol.* 166:7579-7587.

Sumaya, C.V., Myers, .LW. and Ellison, G.W. (1980). Epstein-Barr virus antibodies in multiple sclerosis. *Arch Neurol.* 37:94-96.

Sun, X., Wang, H., Okabe, M., Mackie, K., Kingsley, P.J., Marnett, L.J., Cravatt, B.F. and Dey, S.K. (2009). Genetic loss of Faah compromises male fertility in mice. *Biol Reprod.* 80:235-242.

Suryani, S, and Sutton, I. (2007). An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *J Neuroimmunol.* 183:96-103.

Tagliaferro, P., Javier Ramos, A., Onaivi, E.S., Evrard, S.G., Lujilde, J. and Brusco, A. (2006). Neuronal cytoskeleton and synaptic densities are altered after a chronic treatment with the cannabinoid receptor agonist WIN 55,212-2. *Brain Res.* 1085:163-176.

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

Tanimura, A., Yamazaki, M., Hashimotodani, Y., Uchigashima, M., Kawata, S., Abe, M., Kita, Y., Hashimoto, K., Shimizu, T., Watanabe, M., Sakimura, K. and Kano, M. (2010). The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. *Neuron.* 65:320-327.

't Hart, B.A., Bauer, J., Brok, H.P. and Amor, S. (2005). Non-human primate models of experimental autoimmune encephalomyelitis: Variations on a theme. *J Neuroimmunol.* 168:1-12.

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.

Trapp, B.D. and Nave, K.A. (2008). Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci*. 31:247-269.

Traugott, U., Reinherz, E.L. and Raine, C.S. (1983a). Multiple sclerosis: distribution of T cell subsets within active chronic lesions. *Science* 219:308-310.

Traugott, U., Reinherz, E.L. and Raine, C.S. (1983b). Multiple sclerosis. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. *J Neuroimmunol.* 4:201-221.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R. and Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet*. 23:99-103.

Troy, C.M., Muma, N.A., Greene, L.A., Price, D.L. and Shelanski, M.L. (1990). Regulation of peripherin and neurofilament expression in regenerating rat motor neurons. *Brain Res.* 529:232-238.

van den Broek, H.H., Damoiseaux, J.G., De Baets, M.H. and Hupperts, R.M. (2005). The influence of sex hormones on cytokines in multiple sclerosis and experimental autoimmune encephalomyelitis: a review. *Mult Scler.* 11:349-359.

van der Stelt, M., Veldhuis, W.B., Bar, P.R., Veldink, G.A., Vliegenthart, J.F. and Nicolay, K. (2001a). Neuroprotection by Delta9-tetrahydrocannabinol, the main active compound in marijuana, against ouabain-induced in vivo excitotoxicity. *J Neurosci.* 21:6475-6479.

van der Stelt, M., Veldhuis, W.B., van Haaften, G.W., Fezza, F., Bisogno, T., Bar, P.R., Veldink, G.A., Vliegenthart, J.F., Di Marzo, V. and Nicolay, K. (2001b). Exogenous anandamide protects rat brain against acute neuronal injury in vivo. *J Neurosci.* 21:8765-8771.

Vaney, C., Heinzel-Gutenbrunner, M., Jobin, P., Tschopp, F., Gattlen, B., Hagen, U., Schnelle, M. and Reif, M. (2004). Efficacy, safety and tolerability of an orally administered cannabis extract in the treatment of spasticity in patients with multiple sclerosis: a randomized, double-blind, placebo-controlled, crossover study. *Mult Scler.* 10:417-424.

van Oosten B.W., Killestein, J., Mathus-Vliegen, E.M. and Polman, C.H. (2004). Multiple sclerosis following treatment with a cannabinoid receptor-1 antagonist. *Mult Scler.* 10:330-331.

Vann, R.E., Cook, C.D., Martin, B.R. and Wiley, J.L. (2007). Cannabimimetic properties of ajulemic acid. *J Pharmacol Exp Ther.* 320:678-86.

van Sickle, M.D., Duncan, M., Kingsley, P.J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J.S., Marnett, L.J., Di Marzo, V., Pittman, Q.J., Patel, K.D. and Sharkey, K.A. (2005). Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science.* 310:329-332.

Varvel, S.A., Bridgen. D.T., Tao, Q., Thomas, B., Martin, B. and Lichtman, A. (2005). ∆9-Tetrahydrocannabinol accounts for the antinociceptive, hypothermic, and cataleptic effects of marijuana in mice. *J Pharmacol Exp Ther*. 314:329-337.

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., Robson, P., House, H., Makela, P. and Aram, J. (2003). A preliminary controlled study to determine whether whole-plant cannabis extracts can improve intractable neurogenic symptoms. *Clin Rehabil*. 17:21-29.

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.

Wager-Miller, J., Westenbroek, R. and Mackie, K. (2002). Dimerization of G protein-coupled receptors: CB1 cannabinoid receptors as an example. *Chem Phys Lipids* 121:83-89.

Waksman, Y., Olson, J.M., Carlisle, S.J. and Cabral, G.A. (1999). The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. *J Pharmacol Exp Ther.* 288:1357-1366.

Waldeck-Weiermair, M., Zoratti, C., Osibow, K., Balenga, N., Goessnitzer, E., Waldhoer, M., Malli, R. and Graier, W.F. (2008). Integrin clustering enables anandamide-induced Ca2+ signaling in endothelial cells via GPR55 by protection against CB1-receptor-triggered repression. *J Cell Sci.*121:1704-1717.

Walker, J.M., Krey, J.F, Chu, C.J. and Huang, S.M. (2002). Endocannabinoids and related fatty acid derivatives in pain modulation. *Chem Phys Lipids* 121:159-172.

Walter, L., Franklin, A., Witting, A., Wade, C., Xie, Y., Kunos, G., Mackie, K. and Stella, N. (2003). Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci.* 2003;23:1398-1405.

Wang, H., Xie, H., Guo, Y., Zhang, H., Takahashi, T., Kingsley, P.J., Marnett, L.J., Das, S.K., Cravatt, B.F. and Dey, S.K. (2006). Fatty acid amide hydrolase deficiency limits early pregnancy events. *J Clin Invest.* 116:2122-2131.

Wang, J. and Ueda, N. (2009). Biology of endocannabinoid synthesis system. *Prostaglandins Other Lipid Mediat*. 89:112-119.

Ware, M.A., Adams, H. and Guy. G.W. (2005). The medicinal use of cannabis in the UK: results of a nationwide survey. *Int J Clin Pract.* 59:291-295.

Waxman, S.G. (2001). Acquired channelopathies in nerve injury and MS. *Neurology* 56:1621-1627.

Webb, M., Luo, L., Ma, J.Y. and Tham, C.S. (2008). Genetic deletion of Fatty Acid Amide Hydrolase results in improved long-term outcome in chronic autoimmune encephalitis. *Neurosci Lett.* 439:106-110.

Weinshenker, B.G., Bass, B., Rice, G.P., Noseworthy, J., Carriere, W., Baskerville, J. and Ebers, G.C. (1989). The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. *Brain* 112:133-146.

White, S.C., Brin, S.C. and Janicki, B.W. (1975). Mitogen-induced blastogenic responses of lymphocytes from marihuana smokers. *Science.* 188:71-72.

Whyte, L.S., Ryberg, E., Sims, N.A., Ridge, S.A., Mackie, K., Greasley, P.J., Ross, R,A. and Rogers, M.J. (2009).The putative cannabinoid receptor GPR55 affects osteoclast function in vitro and bone mass in vivo. *Proc Natl Acad Sci U S A.* 106:16511-16516.

Wilkinson, J.D., Whalley, B.J., Baker, D., Pryce, G., Constanti, A., Gibbons, S. and Williamson, E.M. (2003). Medicinal cannabis: is Δ^9-tetrahydrocannabinol necessary for all its effects*? J. Pharm Pharmacol* 55:1687-1694.

Willer, C.J., Dyment, D.A., Sadovnick, A.D., Rothwell, P.M., Murray, T.J. and Ebers, G.C; Canadian Collaborative Study Group. Timing of birth and risk of multiple sclerosis: population based study. *BMJ.* 2005 Jan 15;330(7483):120-123.

Wilson, R.I. and Nicoll, R.A. (2002). Endocannabinoid signalling in the brain. *Science* 296, 678-68.

Wirguin, I., Mechoulam, R., Breuer, A., Schezen, E., Weidenfeld, J. and Brenner, T. (1994). Suppression of autoimmune encephalomyelitis by cannabinoids. *Immunopharmacology* 28: 209-214.

Wise, .LE., Shelton, C.C., Cravatt, B.F., Martin, B.R. and Lichtman, A.H. (2007). Assessment of anandamide's pharmacological effects in mice deficient of both fatty acid amide hydrolase and cannabinoid CB1 receptors. *Eur J Pharmacol*. 557:44-48.

Witting, A., Chen, L., Cudaback, E., Straiker, A., Walter, L., Rickman, B., Möller, T., Brosnan C. and Stella, N. (2006). Experimental autoimmune encephalomyelitis disrupts endocannabinoid-mediated neuroprotection. *Proc Natl Acad Sci U S A.* 103:6362-6367.

Wolburg, H., Noell, S., Mack, A., Wolburg-Buchholz, K. and Fallier-Becker, P. (2009). Brain endothelial cells and the glio-vascular complex. *Cell Tissue Res.* 335:75-96.

Worm, K., Zhou, Q.J., Stabley, G.J., DeHaven, R.N., Conway-James, N., LaBuda, C.J., Koblish, M., Little, P.J. and Dolle, R.E. (2007) Non-psychotropic biaryl cannabinoid agonists. *17th Annual symposium on the Cannabinoids, Burlington Vermont, International Cannabinoid Research Society.* Pg 2.

Wotherspoon, G., Fox, A., McIntyre, P., Colley, S., Bevan, S. and Winter, J. (2005). Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* 135:235-245.

Wujek, J.R., Bjartmar, C., Richer, E., Ransohoff, R.M., Yu, M., Tuohy, V.K. and Trapp, B.D. (2002). Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J Neuropathol Exp Neurol.* 61:23-32.

Xu, H., Cheng, C.L., Chen, M., Manivannan, A., Cabay, L., Pertwee, R.G., Coutts, A. and Forrester, J.V. (2007). Anti-inflammatory property of the cannabinoid receptor-2-selective agonist JWH-133 in a rodent model of autoimmune uveoretinitis. *J Leukoc Biol.* 82:532-541.

Yang, C., Hader, W., and Zhang, X. (2006)Therapeutic action of cannabinoid on axonal injury induced by peroxynitrite. *Brain Res*. 1076:238-242.

Yates, M.L. and Barker, E. (2009). Inactivation and Biotransformation of the Endogenous Cannabinoids Anandamide and 2-Arachidonoylglycerol. *Mol Pharmacol.* 76:11-17.

Yau, J.L., Noble, J., Thomas, S., Kerwin, R., Morgan, P.E., Lightman, S., Seckl, J.R. and Pariante, C.M. The Antidepressant Desipramine Requires the ABCB1 (Mdr1)-Type p-Glycoprotein to Upregulate the Glucocorticoid Receptor in Mice. *Neuropsychopharmacology.* 32:2520-2529.

Yiangou, Y., Facer, P., Durrenberger, P., Chessell, I.P., Naylor, A., Bountra, C., Banati, R.R. and Anand, P. (2006) COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. *BMC Neurol*. 6:12.

Yin, H., Chu, A., Li, W., Wang, B., Shelton, F., Otero, F., Nguyen, D.G., Caldwell, J.S. and Chen, Y.A. (2009). Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. *J Biol Chem.* 284:12328-12338.

Yoshida, T., Fukaya, M., Uchigashima, M., Miura, E., Kamiya, H., Kano, M. and Watanabe, M. (2006). Localization of diacylglycerol lipase-alpha around postsynaptic spine suggests close proximity between production site of an endocannabinoid, 2-arachidonoyl-glycerol, and presynaptic cannabinoid CB1 receptor. *J Neurosci.* 26:4740-4751.

Yu, X.H., Martino, J,. Puma, C. St-Onge, S., Lessard, É., Perkins, M. and Laird, J.M.A. (2007). Activation of peripheral CB1 receptors produces robust analgesia in rodent inflammatory and neuropathic pain models. *17th Annual symposium on the Cannabinoids, Burlington Vermont, International Cannabinoid Research Society.* Pg 164.

Yuan, M., Kiertscher, S.M., Cheng, Q., Zoumalan, R., Tashkin, D.P. and Roth, M.D. (2002). Delta 9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. *J Neuroimmunol.* 133:124-131.

Zajicek, J., Fox, P., Sanders, H., Wright, D., Vickery, J., Nunn, A. and Thompson A., UK MS Research Group. (2003). Cannabinoids for treatment of spasticity and other symptoms related to multiple sclerosis (CAMS study): multicentre randomised placebo-controlled trial. *Lancet.* 362:1517-1526.

Zajicek, J.P., Sanders, H.P., Wright, D.E., Vickery, P.J., Ingram, W.M., Reilly, S.M., Nunn, A.J., Teare, L.J., Fox, P.J. and Thompson, A.J. (2005). Cannabinoids in multiple sclerosis (CAMS) study: safety and efficacy data for 12 months follow up. *J Neurol Neurosurg Psychiatry.* 76:1664-1669.

Zhang, M., Martin, B.R., Adler, M.W., Razdan, R.J., Kong, W., Ganea, D. and Tuma, R.F. (2009). Modulation of cannabinoid receptor activation as a neuroprotective strategy for EAE and stroke. *J Neuroimmune Pharmacol*. 4:249-259.

Zhang, X., Maor, Y., Wang, J.F., Kunos, G. and Groopman, J.E. (2010). Endocannabinoid-like N-arachidonoyl serine is a novel pro-angiogenic mediator. *Br J Pharmacol.* 160:1583-1594.

Zhou, L., Nepote, V., Rowley, D.L., Levacher, B., Zvara, A., Santha, M., Mi, Q.S., Simonneau, M. and Donovan, D.M. (2002). Murine peripherin gene sequences direct Cre recombinase expression to peripheral neurons in transgenic mice. *FEBS Lett.* 523:68-72.

Zimmer, A., Zimmer, A.M., Hohmann, A.G., Herkenham, M. and Bonner, T.I. (1999) Increased mortality, hypoactivity and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc Natl Acad Sci U S A.* 96:5780-5785.

Zygmunt, P.M., Petersson, J., Andersson, D.A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D. and Hogestatt, E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400:452-457.

PUBLICATIONS ARISING FROM THIS STUDY

G. Pryce and D. Baker. (2007). Control of spasticity in a multiple sclerosis model is CB1, not CB2, cannabinoid receptor mediated. *B. J. Pharmacol.* 150:519-25. 2007.

Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP, Ledent C, Cheng X, Carrier E, Mann MK, Giovannoni G, Pertwee RG, Yamamura T, Buckley NE, Hillard CJ, Lutz B, Baker D, Dittel BN. (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB_1 on neurons and CB_2 on autoreactive T Cells. *Nat Medicine* 13:492-97. (Joint 1st Author).

David Baker, Samuel Jackson and Gareth Pryce. (2007). Cannabinoid control of neuroinflammation related to multiple sclerosis. *Br. J. Pharmacol.* 152:649-654.

J. Ludovic Croxford, Gareth Pryce, Samuel J. Jackson, Catherine Ledent, Gavin Giovannoni, Roger G. Pertwee, Takashi Yamamura and David Baker. (2008). Cannabinoid-mediated neuroprotection, not immunosuppression, may be more relevant to multiple sclerosis. *J. Neuroimmunol.* 193:120-129. (Joint 1st Author).

Al-Izki S, Pryce G, Giovannoni G, Baker D. (2009). Evaluating potential therapies for bladder dysfunction in a mouse model of multiple sclerosis with high resolution ultrasonography. *Mult Scler*. 5:795-801.