Colloquium Series on Integrated Systems Physiology

The Endocannabinoid System in Local and Systemic Inflammation

Melanie E.M. Kelly • Christian Lehmann • Juan Zhou

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ABSTRACT

This book focuses on the role of the endocannabinoid system in local and systemic inflammation, with individual chapters written by experts in the field of cannabinoid research and medicine. The topics explore the actions of the endocannabinoid system on the immune system, including neuroinflammation in autoimmune disorders such as multiple sclerosis, and in neurodegenerative disorders such as Huntington's and Alzheimer's, as well as local and systemic inflammatory conditions affecting organs including the eye (uveitis and corneal inflammation), the bladder (interstitial cystitis), pancreas (diabetes), cardiovascular system (stroke), joints (arthritis), and sepsis. The objective of this book is to provide knowledge transfer on the use of cannabinoids in inflammatory disease by critically examining preclinical and clinical research on the immunomodulatory actions of the endocannabinoid system, with specific emphasis on the actions of cannabinoids in diseases where inflammation is a prominent component. By drawing these results together, we seek to provide further understanding of the complexities of endocannabinoid system modulation of immune function and identify potential uses and limitations for cannabinoid-based therapeutics.

KEY WORDS

Alzheimer's disease, amyloid β, arthritis, cannabinoids, cannabinoid receptors, cannabis, CNS injury, diabetic retinopathy, dronabinol, endocannabinoids, endocannabinoid system, endotoxin-induced uveitis, experimental autoimmune encephalomyelitis, experimental autoimmune uveoretinitis, Huntington's disease, immune system, immune cells, inflammation, marijuana, multiple sclerosis, Nabiximols, neuroinflammation, neurodegeneration, neuroprotection, ocular inflammation, pain, phytocannabinoids, proliferative vitreoretinopathy, spasticity, Sativex®

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Preface

Melanie E. M. Kelly

Cannabis use globally, both for recreational and medical purposes, currently outstrips other drugs (Bostwick, 2012). As cannabis moves into a new era of legalization in many parts of the world, it becomes imperative to carry out and disseminate basic and clinical research that provides a deeper understanding of the actions of this complex plant. In particular, information on the use of cannabis for therapeutic purposes, including its individual constituent phytocannabinoids, as well as synthetic cannabinoid derivatives, is critical to establish the potential for cannabis and cannabinoid drugs to be effectively used to alleviate human disease and suffering.

The cannabis plant contains a plethora of bioactive phytochemicals including >100 phytocannabinoids (Russo, 2007). The primary phytocannabinoid responsible for the psychoactive effects of cannabis following ingestion is Δ^9-tetrahydrocannabinol. Two cannabinoid receptors, cannabinoid type 1 receptor (CB_1R) and cannabinoid type 2 receptor (CB_2R) mediate many of the actions of Δ^9-tetrahydrocannabinol, with CB_1R responsible for the pyschoactivity of Δ^9-tetrahydrocannabinol (reviewed in, Mechoulam et al., 2014; Pertwee, 2010). The endogenous ligands for cannabinoid receptors are lipids called endocannabinoids that are produced in a Ca^{2+}-dependent manner by biosynthetic enzymes and released "on-demand" before being rapidly broken down by degradative enzymes. This system of endogenous ligands, biosynthetic and degradative enzymes, and cannabinoid receptors has been coined the endocannabinoid system (reviewed in Hillard, 2015; Pertwee, 2015; Maccarone et al., 2015).

Substantive evidence now indicates that the endocannabinoid system is an important modulator of numerous biological systems including the immune system, where activation of the endocannabinoid system, particularly CB_2R, may be a sentinel against inflammation (reviewed in, Turcotte et al., 2016; Chiurchiu et al., 2015; Cabral et al., 2015a). Elements of the endocannabinoid system, including endocannabinoids and cannabinoid receptors, are present in a diverse array of circulating and resident immune cells, and activation of cannabinoid receptors on immune cells by exogenous cannabinoids or endocannabinoids results in alterations in immune function (reviewed in Turcotte et al., 2016). Evidence indicates that in contrast to the more ubiquitous CB_1R expression, CB_2R expression is, for the most part, highly localized to immune cells. In addition to Δ^9-tetrahydrocannabinol, research also supports immunomodulatory actions of other phytocannabinoids in cannabis including the major non-pyschoactive phytocannabinoid, cannabidiol, which

may act differentially at cannabinoid receptors as well as non-cannabinoid receptors to produce its anti-inflammatory actions (Burstein, 2015).

The last few decades have seen significant advances in our understanding of the endocannabinoid system. Armed with this knowledge, the research community has begun to decipher the actions of the endocannabinoid system in regulating important biological functions. Furthermore, we are now beginning to have a better understanding of the complex pharmacology of compounds that modulate the endocannabinoid system, including plant cannabinoids, and endocannabinoids. The present book highlights several key areas where this information may be applied to develop new endocannabinoid system targeted therapeutics that could help to both understand and alleviate human disease.

CHAPTER 1

Inflammation and the Endocannabinoid System

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Abstract

Local inflammation is launched by trigger events such as microbial invasion or environmental factors and results in immune cell recruitment at the primary site of injury. In the case of systemic inflammation, the immune response is dysregulated, and the activation of endothelial cells and leukocytes occurs at multiple sites. The endocannabinoid system plays an important role in the modulation of the immune response. Increasing evidence supports upregulation of cannabinoid type 1 and type 2 (CB_1R and CB_2R) receptors and release of endocannabinoids from macrophages, dendritic cells, platelets and parenchymal cells in response to inflammatory stimuli. This chapter will summarize current knowledge regarding involvement of cannabinoid receptors and their ligands in both local and systemic inflammation.

Key Words

immune system, inflammation, infection, cannabinoid type 1 receptor, cannabinoid type 2 receptor

1.1 INTRODUCTION

The cardinal signs of inflammation—heat, redness, swelling, and pain—represent basic processes that define local inflammation. Inflammation is launched by trigger events such as cytokine secretion by cells challenged by microbes or microbial products, or environmental factors that result in degranulation of certain leukocytes. The initial wave of mediator release is followed by vasodilatation, increased vascular permeability, leukocyte margination, extravasation and tissue infiltration, and activation of proteases that cleave bradykinins. Vasodilation in the microvasculature contributes to the heat and redness, while plasma and cells accumulating in the tissue contribute to the swelling. Pain is a product of the kinins, which also modulate other aspects of inflammation (Moreau et a., 2005). In the case of systemic inflammation, the same events are occurring but without the

road map provided by chemokines or anaphylatoxins from local tissue sites. Consequently, endothelial cell and leukocyte activation become systemic and leukocytes marginate in multiple sites. This margination is mediated by specific adhesion molecule and integrin interactions between the endothelial cells and leukocytes though there may not be directed extravasation.

One of the prototypes of inflammatory triggers during local and systemic inflammation, is lipopolysaccharide, which, in turn, is primarily detected through Toll-like receptor 4 (TLR4) on multiple cell types. Thus, the activation of cells through TLR4 has continued to draw considerable attention as a means to understanding the inflammation and impact of inflammation on the immune system (Rosadini and Kagan, 2017). Notwithstanding the importance of the TLR4 response, there is great redundancy and synergy among the different mediators and cascades that become activated during inflammation, and which lead to cell death and further activation, that has made dissecting the pathophysiology and immune activation so elusive (Delano and Ward, 2016; Mira et al., 2016; Napier et al., 2016). The impact of the endocannabinoid system in this network offers a new perspective in the control of inflammatory processes.

1.2 CANNABINOID TYPE 1 RECEPTOR

Cannabis sativa has been used for recreational, religious and medicinal properties for several thousand years (reviewed in Russo, 2007). The cannabis plant contains >100 phytocannabinoids including Δ^9-tetrahydrocannabinol (THC) and cannabidiol. The first phytocannabinoids, cannabinol (CBN) and cannabidiol (CBD) were isolated prior to the 1950's (reviewed in Mechoulam and Hanus, 2000), however it was not until 1964 that THC, the primary phytocannabinoid responsible for the psychoactive effects seen after cannabis ingestion, was isolated by Gaoni and Mechoulam (1964). Subsequent to this, it was discovered that cannabinoids exert behavioral effects via high affinity binding to a receptor in the central nervous system (Devane et al., 1988). This receptor named the cannabinoid 1 receptor (CB_1R) was found to be a member of the 7 transmembrane Family A G-protein coupled receptors that transduce their actions via coupling to a G protein (Howlett et al., 2002). Development of high affinity synthetic cannabinoids (Table 1.1) was a key contributory factor in the identification and cloning of CB_1R (Matsuda et al., 1990). CB_1R is highly expressed in the brain and throughout the nervous system with expression also in peripheral tissues.

Following the identification of the "THC receptor," high-affinity endogenous ligands for CB_1R were discovered. These included arachidonoyl ethanolamide (AEA) (Devane et al., 1992) and 2-arachidonoyl–glycerol (2-AG) (Mechoulam et al., 1995). AEA and 2-AG both bind and activate CB_1R with 2-AG being a full agonist (Howlett et al., 2002). Cumulative research has now indicated that endocannabinoids are generated "on-demand" from cells, including immune cells, via enzymatic production from membrane lipids (Figure 1.1; reviewed in Hillard, 2015). The lifetime of endocannabinoids is limited by degradative enzymes including fatty acid amide hydrolase (Cravatt et al., 2001).

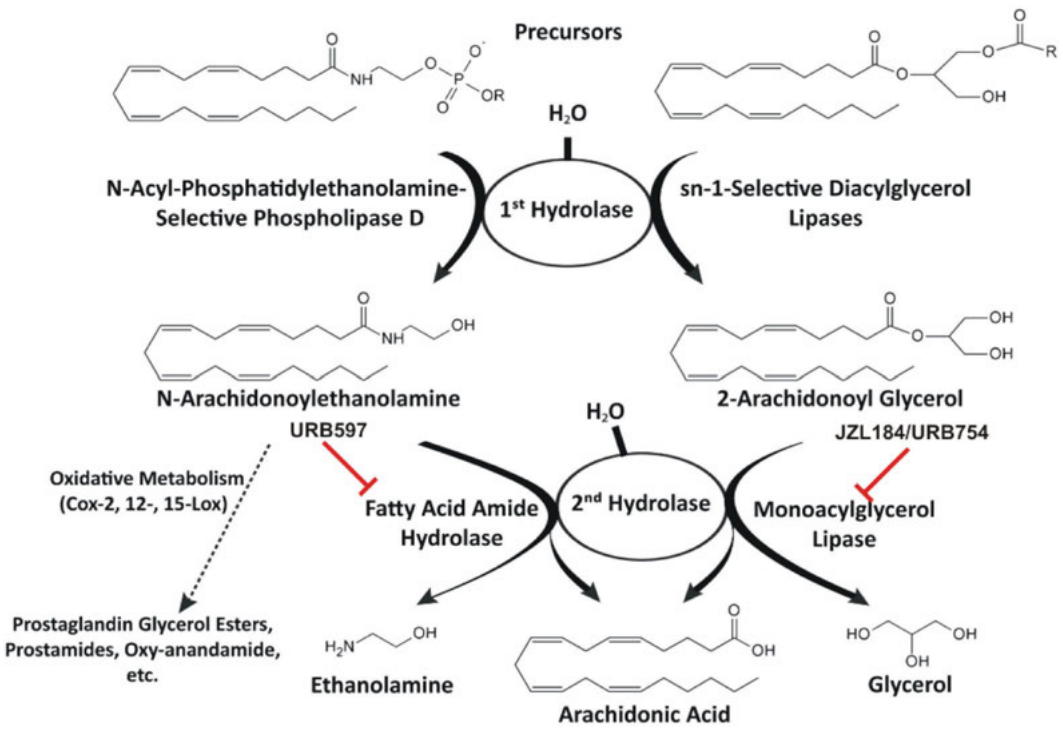

FIGURE 1.1: Biosynthetic and degradative pathways for AEA and 2-AG. URB597 inhibits fatty acid amide hydrolase (FAAH) while JZL184/URB754 inhibits monoacylglycerol lipase (MAGL) (adapted from Di Marzo et al., 2009).

Central nervous system (CNS) activation of CB_1R is associated with signaling via $G_{\alpha i}$-coupled downstream signaling pathways including adenylyl cyclase and cAMP and mitogen activated protein kinase (reviewed in Pertwee, 2010). In the CNS, activation of CB_1R by endocannabinoids or exogenous phyto- or synthetic cannabinoids is associated with a reduction in neurotransmitter (NT) release at central synapses via a retrograde signaling mechanism involving inhibition of presynaptic voltage-dependent Ca channels (Hillard, 2015). Both pre- and postsynaptic neuronal CB_1R activation has been demonstrated to be neuroprotective in various neurodegenerative CNS disorders and may involve, in part, a reduction in excitotoxic NT release, modification in glial release of pro-inflammatory mediators and improved blood flow to the damaged brain (Golech et al., 2004; Fernández-Ruiz et al., 2015). Validation of anti-inflammatory and neuroprotective effects in the CNS is supported by research using CB_1R antagonists or genetic loss of CB_1R. However, despite evidence of decreased neuroinflammation and neuroprotective efficacy, there are a few issues related to targeting CB_1R for CNS neuroinflammatory and neurodegenerative disease. Namely, the

behavioral actions of ligands that modulate CB_1R and the loss of neuronal CB_1R in neurodegenerative disorders (Fernández-Ruiz et al., 2015; McCaw et al., 2004).

1.3 CANNABINOID TYPE 2 RECEPTOR

Cloning of a second cannabinoid receptor, cannabinoid 2 receptor, with 44% amino acid homology to CB_1R was reported by Munro et al. (1993) and as the expression of this receptor, unlike CB_1R, was highly localized to immune cells, CB_2R was referred to as "the peripheral receptor". However, later studies have also indicated that this receptor may also be found in select areas of the CNS (Van Sickle et al., 2005). CB_2R is activated by the phytocannabinoid, THC, synthetic cannabinoids, and also by endocannabinoids such as 2-AG (Howlett et al., 2002; Pertwee, 2010). A number of other phytocannabinoids, including cannabidiol, activate CB_2R and have reported immunomodulatory actions (Pertwee, 2010). Additionally, cannabidiol has been reported to antagonize the actions of THC and other cannabinoids that act at CB_1R and can also bind to non-cannabinoid receptors including serotonin 1A (5-HT1A), as well as transient receptor potential receptor 1 (TRPV1) (Devinsky et al., 2014). The collective actions of constituent phytocannabinoids from cannabis is sometimes referred to as the "entourage effect," a description used to describe the effects of interactions between cannabis constituents (Russo, 2011). Several cannabinoid derivatives have now been developed that selectively activate CB_2R and alter immune function (Table 1.1).

TABLE 1.1: CB_2R agonists and antagonists (Turcotte et al., 2016)

Agonists	Ki (nM)	Other targets	References
AM 1241	3.4	TRPA1	Ibrhim et al., 2003; Akopian et al., 2008
JWH 133	3.4	TRPV1	Huffman et al., 1999; McDougall et al., 2008
GW 405833	3.6–3.92		Valenzano et al., 2005
JWH 015	13.8		Showalter et al., 1996
HU 308	22.7		Hanus et al., 1999
L-759,633	6.4		Ross et al., 1999
L-759,656	11.8		Ross et al., 1999
SER 601	6.3		Pasquini et al., 2008
GP 1a	0.037		Murineddu et al., 2006
GP 2a	7.6		Murineddu et al., 2006
CB 65	3.3		Manera et al., 2006
HU 210	0.061–0.5	CB_1R, GPR55, 5-HT2	Felder et al., 1995; Ryberg et al., 2007; Cheer et al., 1999
CP 55,940	0.6–5.0	CB_1R, GPR55	Ryberg et al., 2007; Thomas et al., 1998
WIN 55, 212-2	62.3	CB_1R,TRPA1	Akopian et al., 2008; Felder et al., 1995; Thomas et al., 1998

Antagonists	Ki (nM)	Other targets	References
SR144528	0.6–4.1		Ross et al., 1999; Rinaldi-Carmona et al., 1998
AM 630	5.6–31.2	TRPA1	Ross et al., 1999; Patil et al., 2011
JTE907	35.9		Buckley et al., 2000

In contrast to CB_1R, activation of CB_2R by phytocannabinoids and synthetic cannabinoid ligands is non-psychoactive. Activation of CB_2R promotes coupling of the receptor to $G_{\alpha i}$-signaling pathways, resulting in inhibition of adenylate cyclase (AC) and decreased cAMP, together with activation of mitogen-activated protein kinase (MAPK) signaling (Devane et al., 1988). CB_2R agonists attenuate the inflammatory response by inhibiting production of pro-inflammatory mediators and decreasing neutrophil chemotaxis and extravasation (Fernández-Ruiz et al., 2015; Cabral et al., 2015). In injury models, levels of CB_2R expression, along with endogenous cannabinoid levels (see Table 1.2), are increased, suggesting that this receptor may function in an "auto-protective role" to limit inflammation (Rom and Persidsky, 2013; Steffens and Pacher, 2012; Toguri et al., 2016). Accordingly, in preclinical models, activation of CB_2R has been associated with a reduction in inflammation (Toguri et al., 2015, 2014; Gómez-Gálvez et al., 2016; Wright et al., 2008).

TABLE 1.2: CB_2R-mediated effects of endocannabinoids on immune cell functions (Turcotte et al., 2016)

Cell type	Species	Endocannabinoid	Effects	References
Anti-inflammatory Effects				
Astrocytes	Rat	AEA	↓TNF	Ortega-Gutiérrez et al., 2005
Dendritic cells	Human	AEA	↓IL-6, IL-12, IFN	Chiurchiù et al., 2013
Microglia	Mouse	AEA	↓NO	Elijaschewitsch et al., 2006
	Mouse	AEA	↑IL-10, IL-12p70, IL-23	Correa et al., 2011
	Rat	AEA	↑NO	Malek et al., 2015
Neutrophils	Human	2-AG	↓ migration	Kurihara et al., 2006
Splenocytes	Human	AEA	↓antibody formation	Eisenstein et al., 2007
T cells	Human	AEA	↓ proliferation	Cencioni et al., 2010
		2-AG	↓ migration	Coopman et al., 2007
CD4+ T cells	Human	AEA	↓ IL-17, IFN, TNF	Cencioni et al., 2010
CD8+ T cells	Human	AEA	↓ IFN, TNF	Cencioni et al., 2010
	Human	AEA	↓ migration	Joseph, et al., 2004

Cell type	Species	Endocannabinoid	Effects	References
Pro-inflammatory Effects				
B cells	Human	2-AG	↑migration	Rayman et al., 2004
	Mouse	2-AG	↑migration	Tanikawa et al., 2007
Dendritic cells	Human	2-AG	↑migration	Maestroni, 2004
Eosinophils	Human	2-AG	↑migration	Kishimoto et al., 2006
	Human	2-AG	↑migration, LTC4, EXC4	Larose et al., 2014
Macrophages	Mouse	2-AG	↑phagocytosis	Shiratsuchi, et al., 2008
	Human	2-AG	↑actin polymerization	Kishimoto et al., 2003
Microglia	Mouse	2-AG	↑migration	Walter et al., 2003
Monocytes	Human	2-AG	↑migration, adhesion	Kishimoto et al., 2003; Gokoh et al., 2005
NK cells	Human	2-AG	↑migration	Kishimoto et al., 2005
T cells	Human	2-AG	↑adhesion, transmigration	Gasperi et al., 2014

1.4 OTHER RECEPTORS AND LIGANDS

Several other GPCRs and nuclear receptors have also been proposed as targets for cannabinoids that exert immunomodulatory effects. These include GPR55 as well as PPARγ respectively (Pertwee, 2015). The putative roles of these non-cannabinoid receptors have been outlined in several excellent comprehensive reviews (Pertwee, 2010; Golech et al., 2004; Fernández-Ruiz et al., 2015; McCaw et al., 2004; Munro et al., 1993; Van Sickle et al., 2005; Devinsky et al., 2014; Russo, 2011; Cabral et al., 2015; Rom and Persidsky, 2013; Steffens and Pacher, 2012; Toguri et al., 2016; Toguri et al., 2015; Toguri et al., 2014; Gómez-Gálvez et al., 2016; Wright et al., 2008; Pertwee, 2015; Macki and Stella, 2006). GPR55 was originally thought to be a third putative cannabinoid receptor but is now known to be the receptor for the endogenous ligand, Lysophosphatidylinositol (LPI). Additionally, a number of endogenous fatty acid ethanol amides and fatty amino-acid amides have been found to either bind to cannabinoid receptors, or their actions are blocked in cannabinoid receptor null mice, implying that interactions with cannabinoid receptors contribute to their actions. These interactions may be mediated via allosteric binding to binding sites at cannabinoid receptors that are distinct from the orthosteric site that endocannabinoids, synthetic cannabinoids, or THC bind to. It may even occur via interactions with distinct receptors that may form dimerized complexes with cannabinoid receptors with resultant allosteric modulation of cannabinoid receptor signaling when activated by orthosteric ligands (Hudson et al., 2010; Laprairie et al., 2015).

Extensive preclinical research has demonstrated that cannabis and cannabinoids have therapeutic potential in ameliorating symptoms for several diseases (reviewed in Pertwee, 2015). However, in contrast to preclinical studies, the number of clinical trials to determine safety and efficacy of cannabis and cannabinoids at this time still remains relatively limited. One area that shows considerable promise for the development of cannabinoid therapeutics is for inflammatory disease, as described below.

1.5 MODULATION OF THE INFLAMMATORY IMMUNE RESPONSE BY THE ENDOCANNABINOID SYSTEM

The endocannabinoid system (ECS) plays an important role in immune system modulation, and increasing evidence supports upregulation of the ECS during both local and systemic inflammation. Endocannabinoids released from macrophages, dendritic cells, platelets, and parenchymal cells in response to inflammatory stimuli and oxidative stress, are present in elevated levels in the sera of patients and animals with systemic inflammation (Varga et al., 1998; Wagner et al., 1998; Pacher et al., 2005; Orliac et al., 2003).

Examination of cannabinoid receptors function has revealed that CB_2R are present on macrophages, neutrophils, and lymphocytes, and activation of these receptors has generally been associated with anti-inflammatory effects, including reduced macrophage and neutrophil numbers at the site of infection and decreases in pro-inflammatory cytokines (Caldwell et al., 2010). The use of CB_2R agonists in experimental models of systemic inflammation and infection reduced the continued recruitment of neutrophils to the site of infection, while increasing phagocytosis and clearance of bacteria (Tschöp et al., 2009).

With respect to the contribution of CB_1R to inflammation and infection, several studies suggested that activation of CB_1R located on the presynaptic terminals of autonomic nerves or the vascular walls may contribute to vasodilation (Varga et al., 1998; Wagner et al., 1998; Pacher et al., 2005; Orliac et al., 2003).

Additionally, a number of *in vitro* studies have examined the effects of endocannabinoids on the levels of pro-inflammatory cytokines, including: TNF-α, interleukin-1beta (IL-1β), interleukin-6 (IL-6), and interleukin-2 (IL-2). Both the endocannabinoids, 2-AG and AEA, decreased LPS-mediated increases of pro-inflammatory cytokines, including TNFα from macrophages and microglial cells and 2-AG inhibited IL-2 secretion in activated murine splenocytes (Facchinetti et al., 2003; Gallily et al., 2000; Ouyang et al., 1998). Consistent with these findings, a study using the selective fatty acid amide hydrolase (FAAH) enzyme inhibitor, URB597, to augment levels of endogenous AEA, reported a reduction in LPS-stimulated microglial expression of inflammatory mediators, including nitric oxide, in LPS-stimulated rat cortical microglia (Tham et al., 2007). URB597 treatment also attenuated levels of pro-inflammatory cytokines, TNFα and IL-1β, in LPS-treated paws in a rat endotoxemia model of inflammatory pain (Naidu et al., 2010).

1.6 CONCLUSIONS

The ECS is upregulated in local inflammation and during systemic inflammatory responses. There is an increasing body of evidence on how endocannabinoids affect inflammatory reactions, which cannabinoid receptor subtypes and cell targets are involved, and the functional outcomes of modulating endocannabinoid signaling during different stages and severity grades of inflammation (see Figure 1.2).

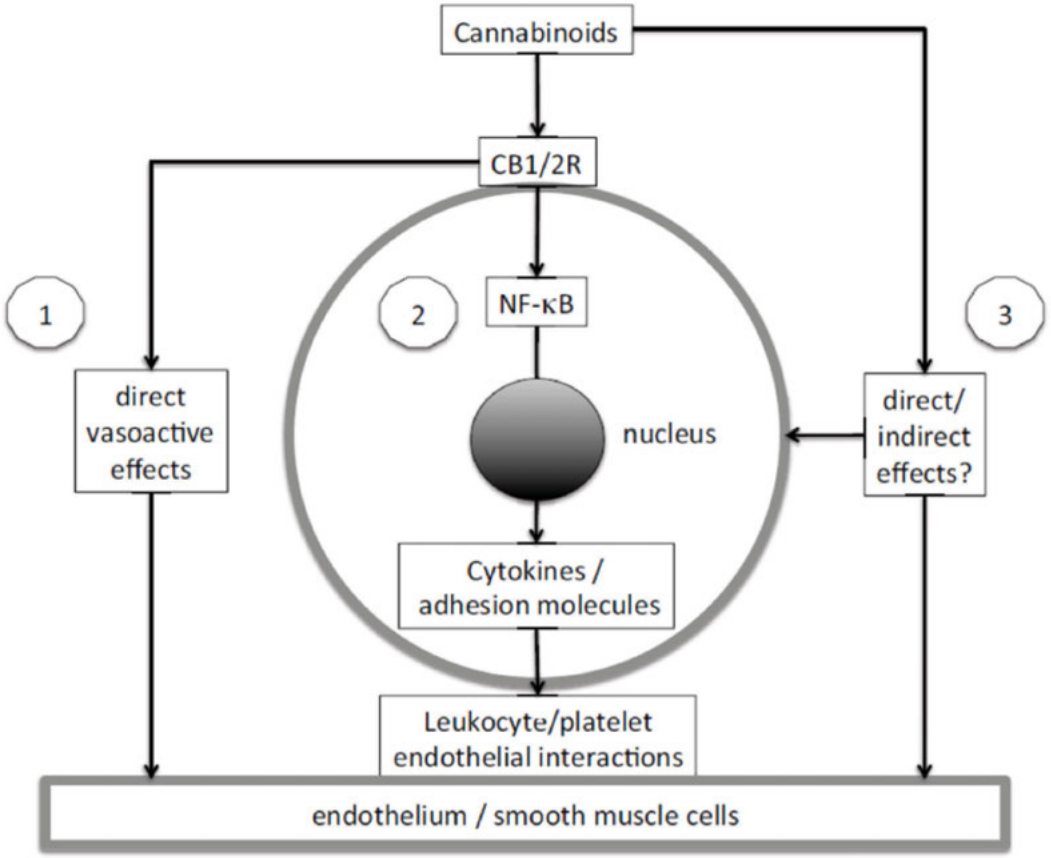

FIGURE 1.2: Hypothesized mechanisms. (1) direct vasoactive effects (vasodilation/vasoconstriction); (2) effects on cytokine and adhesion molecule expression, e.g. via NF-kB; and (3) direct/indirect effects mediated by non-CB_1R/CB_2R receptors, e.g. PPARγ, GPR55, or GPR18.

CHAPTER 2

Cannabinoids in Multiple Sclerosis

Contributing Author
Anna-Maria Szczesniak

Abstract

Multiple sclerosis (MS) is an autoimmune and neurodegenerative disorder of the central nervous system (CNS) that predominantly affects young adults. The current treatments for MS are not always effective in the management of symptoms and disease progression, may produce significant side effects, and are also not curative. The endocannabinoid system has been shown to modulate inflammatory and neurodegenerative processes in a number of CNS pathologies, including MS. In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, both endogenous and exogenous cannabinoids reduce symptomatic features associated with the disease, and are neuroprotective. This effect is primarily mediated by the CB_1R, since pharmacological inhibition or genetic deletion of this receptor results in a more severe disease. While the clinical evidence for the effectiveness of cannabinoids is less conclusive, there is sufficient evidence for symptomatic relief of spasticity and pain that are associated with MS. This chapter reviews the clinical and experimental data on the efficacy of cannabinoids in the treatment of MS.

Key Words

Dronabinol, endocannabinoids, experimental autoimmune encephalomyelitis, multiple sclerosis, Nabiximols, neuroinflammation, neuroprotection, pain, spasticity, Sativex®

Abbreviations

2-AG	2-arachidonoylglycerol
AEA	N-arachidonoylethanolamine; anandamide
CB_1R	cannabinoid type 1 receptor
CB_2R	cannabinoid type 2 receptor
CBD	cannabidiol
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase

MAGL	monoacylglycerol lipase
MS	Multiple Sclerosis
OEA	N-oleoylethanolamide
PEA	N-palmitoylethanolamide
PPMS	primary-progressive multiple sclerosis
RRMS	relapsing-remitting multiple sclerosis
SPMS	secondary-progressive multiple sclerosis
Δ^9THC	Δ^9 tetrahydrocannabinol
TNF-α	tumor necrosis factor -alpha

2.1 MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is the most common autoimmune, inflammatory disorder of the central nervous system (CNS), affecting around 2–3 million people worldwide (Browne et al., 2014). The onset of the disease usually presents itself in young adults (20–40 years of age), and is more common in females than males, with a ratio approaching 3:1. Historically, a higher incidence of MS has been reported in people of northern European background (MS Society, Canada; Compston and Coles, 2008), with the highest incidence of MS reported in Northern hemispheres and Southeastern Australia. However, more current epidemiological studies suggest that geographic location and incidence of MS are not as well correlated as previously thought, and other factors, including environmental influence and lifestyle, play a prominent role in the development of MS (Koch-Henriksen and Sorensen, 2010).

2.1.1 ETIOLOGY

There is evidence of genetic and environmental components that underlie the susceptibility of the development of MS. The involvement of genetic components is supported by the higher occurrence of MS in monozygotic twins as compared to dizygotic twins (Hansen et al., 2005), with concordance of approximately 25–30% (Ebers et al., 1986, 1996; Hansen et al., 2005). Furthermore, the genetic susceptibility to MS appears to be polygenic, with a number of loci affected. The association of major histocompatibility complex type II alleles, particularly HLA-DRB1*1501 and HLA-DRB5, is well established (International Multiple Sclerosis Genetics Consortium et al., 2007; Jersild et al., 1973; Okuda et al., 2009). The environmental factors that have been associated with CNS autoreactivity and development of MS includes infection with Epstein-Barr virus (Handel et al., 2010b), which has been detected in the majority, if not all, patients with MS (Ascherio and Munger, 2007), deficiency in sunlight/vitamin D, which affects immune responses (Smolders et al., 2008, 2011; Smolders, 2010), and cigarette smoking (Hedstrom et al., 2009). The link between environment and genetics can be partially explained by epigenetic modification, where gene expression is altered in a

heritable, but reversible, manner with environmental or biological factors, with no effect on DNA sequence. With respect to MS, unique alterations in DNA methylation have been shown in cell-free plasma DNA (cfpDNA) from patients with relapse and remitting multiple sclerosis (RRMS), as compared to healthy individuals (Liggett et al., 2010). Because HLA-DRB1*1501 is correlated to the clinical course of MS (Okuda et al., 2009), Handel and colleges (2010a) investigated whether the disease severity is correlated with DNA methylation status for HLA-DRB1*1501 and HLA-DRB5, but found no evidence for it. However, DNA hypomethylation for the key regulatory genes involved in the immune response and cell differentiation have been reported (Janson et al., 2011). As the field of epigenetic contribution to neurodegenerative disorders is still in the early stages, future research will shed more light on the impact it plays in the development and progression of diseases, including MS.

2.1.2 PATHOLOGY AND SYMPTOMS OF MULTIPLE SCLEROSIS

The pathology of MS is characterized by mononuclear cell infiltration of the CNS. The primary cells involved in MS are peripheral CD4+ autoreactive lymphocytes (T-helper 1; Th1), which are activated by autoantigenic peptides, including myelin basic proteins. Then, the Th1 cells trans-migrate into CNS and initiate a cascade of events that cumulate in inflammatory response and neurodegeneration. This pathogenic process involves the release of proinflammatory cytokines and chemokines, as well as the recruitment of other cells of innate and adaptive immunity, including CD8+ lymphocytes, CD4+ Th17 cells, antibody producing B cells, and monocytes. The activation of microglia and macrophages and the release of the inflammatory mediators further potentiate and sustain the inflammation. The consequence of the inflammatory cascade is the loss of oligo-dendrocytes and myelin sheath, as well as axonal loss and astrocyte proliferation. The formation of MS lesions (or plagues) affects neuronal transmission and results in the clinical manifestation of the disease.

The neurological deficits in MS can involve any part of the CNS and affect autonomic, sensory and motor functions. Furthermore, the symptoms can resolve completely, partially, or not at all. Initially, the majority of MS patients present themselves with an episode of acute neurological symptoms (defined as clinically isolated syndrome), with the most common symptoms including optic neuritis and/or incomplete myelitis (which may present itself with muscle weakness, lower back pain, abnormal sensations in toes and feet, and may progress to paralysis)(Milo and Miller, 2014; Miller et al., 2005). The common chronic symptoms of MS include spasticity, which affects movement and results in reduced walking ability (Oreja-Guevara et al., 2013) and falls (Gunn et al., 2013), bladder dysfunction, and pain (Pollmann and Feneberg, 2008). Interestingly, in patients where sensory symptoms predominate, complete recovery during remission is common. On the other hand, those presenting with motor impairments have much poorer prognosis (Eriksson et al.,

2003). As the disease progresses, the symptoms tend to worsen, which increases the burden of the disease for MS patients as well as their caregivers.

In terms of the clinical course of MS, in the majority of patients (approximately 80%), the disease initially presents itself with episodes of relapse and remission (RRMS), which is more common in early adulthood and often advances to a secondary progressive phase (SPMS). Another 10-15% of MS patients are diagnosed with primary progressive MS (PPMS), with a sustained neurodegenerative course of the disease; although minor and transitory improvement may occur (Milo and Miller, 2014). The onset of PPMS usually occurs later in life, and affects approximately equal numbers of women and men. PPMS is characterized by fewer brain lesions and much less inflammation as compared to RRMS and SPMS (Miller and Leary, 2007).

2.1.3 CURRENT TREATMENTS FOR MULTIPLE SCLEROSIS

The pathologic features associated with MS have been recognized for centuries, but only in the early 1990s did immune response-modifying therapies become available. Although they do not offer a cure for MS, they do modify the disease progression and improve symptoms in the majority, but not all, of patients (Wingerchuk and Weinshenker, 2016). Immune-modifying therapies are aimed at the relapsing stages of MS and have immunomodulatory/ immunosuppressive properties. First line treatments include interferon β (IFN-β) and glatiramer acetate, both of which induce a switch from pro-inflammatory Th1 leukocytes to anti-inflammatory Th2 phenotype, and also result in an increased number of regulatory T cells (for review, please see Wingerchuk and Weinshenker, 2016). One of the most potent drugs available is natalizumab, a monoclonal antibody directed against α4β1 integrin, expressed on the surface of lymphocytes that is critical for lymphocyte vascular endothelial adhesion and migration into the CNS (Polman et al., 2006). Natalizumab reduces the occurrence of relapses by 68% and decreases the rate of new CNS lesions by 83% (Butzkueven et al., 2014; Spelman et al., 2016). However, natalizumab is associated with numerous serious complications, including progressive multifocal leukoencepalitis and increased risk of opportunistic infections, which result in a higher morbidity and mortality rates (Butzkueven et al., 2014; Langer-Gould et al., 2005). Other, more currently approved therapies that aim to suppress the immune system include fingolimod (Gilenya), which sequesters autoreactive T and B cells within lymph nodes, and terifluonamide (Aubgio), which decreases T and B cell activation and proliferation. Tecfidera (BG-12) is another drug used in MS patients, and is thought to activate antioxidative pathways and be neuroprotective. Each of these medications comes with its own set of side effects (for review, please see McCoyd, 2013), and none of them cure the disease, or directly address symptoms such as spasticity, pain, gait problems, or tremor. Therefore, other pharmaceuticals are included and required in the treatment of MS in order to alleviate these symptoms and improve patient quality of life.

The current treatments for spasticity in MS patients include drugs such as baclofen, tizanidine gabapentin, or botulinum toxin, but many patients do not respond adequately, or become resis-

tant to these agents (Beard et al., 2003). Pain is treated with antiepileptic, tricyclic antidepressants, opioid analgesics, and anaesthetics (Pollmann and Feneberg, 2008; Solaro and Messmer Uccelli, 2010), but yet again, in many patients, the pain control is inadequate (Nick et al., 2012) and results in significant side effects. Finally, while patients diagnosed with RRMS respond well to immunomodulatory therapies, those with SPMS and PPMS are often refractory to these treatments (Miller and Leary, 2007; Solaro and Messmer Uccelli, 2010). Therefore, there is an urgent need for new pharmacotherapeutics, especially for those who do not respond well to current agents.

2.2 CANNABINOIDS IN MULTIPLE SCLEROSIS

Cannabinoids are chemical compounds that act at the receptors of the endocannabinoid system (ECS). The ECS consists of two G protein-coupled receptors, CB_1Rs and CB_2R; endogenous lipid ligands (anandamide (AEA) and 2-arachidonoyl glycerol (2-AG)) and enzymes responsible for endocannabinoid synthesis and degradation. Exogenous cannabinoids include phytocannabinoids, derived from the plant *Cannabis sativa*, and synthetic compounds that bind and modulate the activity of cannabinoid receptors (Pacher et al., 2006). CB_1R is expressed in the CNS and periphery, and its activation modulates synaptic transmission, while CB_2R is primarily located on the cells of the immune system, and plays a role in innate and adaptive immunity.

A growing body of evidence now indicates neuroprotective and immunomodulatory roles for cannabinoid compounds in the treatment of CNS pathologies, including MS. Both MS patients and animals induced with experimental autoimmune encephalomyelitis, when treated with cannabinoid agonists, show improvements in symptoms associated with the disease, supporting the use of cannabinoids in the treatment of MS (Pryce et al., 2003; Pryce and Baker, 2015).

2.2.1 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS ANIMAL MODELS

Experimental autoimmune encephalomyelitis (EAE) is the most widely used animal model of MS. It is also the major preclinical model used for developing therapeutic strategies and testing novel pharmacological treatments for MS prior to clinical studies (Baker et al., 2000, 2001). EAE is induced by a variety of immunological and neuropharmacological interventions, ultimately resulting in the development of a disease phenotype and in many aspects resembles the human condition (Constantinescu et al., 2011). Animals in the chronic phase of EAE show neurodegeneration, inflammatory lesions, loss of neuronal function, as well as experience tremor, hind-limb spasticity, and paralysis (Baker et al., 2000). Importantly, in the chronic phase of EAE, animals have increased levels of endocannabinoids, including AEA, 2-AG, and PEA, in the CNS as compared to control animals (Baker et al., 2001), an effect that is thought to be neuroprotective.

The beneficial effect of endocannabinoids is supported by the pharmacological inhibition of hydrolytic enzymes responsible for their degradation, and consequent elevation in endocannabi-

noids levels. For example, EAE-induced spasticity can be decreased by the inhibition of fatty acid amide hydrolase (FAAH) and consequent increase in AEA—an effect which is mediated by the activation of CB_1R, since CB_1R inverse agonist SR141716A (Rimonabant®) negates the anti-spastic action of FAHH inhibitor. This finding is further confirmed with the use of FAAH deficient animals, where the beneficial effect of enzyme inhibition is lost (Pryce et al., 2013). Beneficial effects of increased AEA levels have also been reported with a selective AEA uptake inhibitor, UCM-707, which reduces the symptoms of the EAE, and decreases microglia activation and immune cell infiltration into the CNS (Ortega-Gutierrez et al., 2005), or by the inhibition of the AEA transporter (de Lago et al., 2004, 2006; Ligresti et al., 2006). Inhibition of monoacylglycerol lipase (MAGL) has also been reported to ameliorate the disease progression of EAE, an effect that is accompanied by increased levels of 2-AG in the spinal cord of animals, as well as decreased leukocyte migration and microglia activity (Hernandez-Torres et al., 2014). Likewise, administration of the exogenous cannabinoid agonists, WIN55,212-2 or CP55,940, also reduce the symptoms of EAE and reduce neurodegenerative processes (Pryce et al., 2003). This latter effect is mediated by activation of CB_1R, since administration of SR141617A (Rimonabant®) increases hindlimb spasticity (Pryce et al., 2003; Pryce and Baker, 2007). In support of the involvement of CB_1R, the induction of EAE in mice lacking CB_1R leads to more pronounced neuronal damage than in wild-type (WT) animals and faster and more severe progression of EAE, including more prominent immobility and permanent hidlimb paralysis. In addition, higher mortality rates in CB_1R deficient mice are observed (Pryce et al., 2003).

The reduction in neuroinflammation and symptomatic relief produced by cannabinoids in EAE are mediated in part by the activation of CB_1R; Maresz et al. (2007) showed that the beneficial effects of Δ^9-THC on the clinical symptoms and the onset of EAE are abolished in knockout mice with neuronal CB_1R deficiency. CB_1Rs are expressed on the presynaptic terminals, and their activation regulates Ca^{2+} channels (Twitchell et al., 1997), and modifies the synaptic input through the inhibition of glutamate release, dampening the excitotoxic damage. In *in vitro* experiments, Pryce et al. (2003) showed that when cerebellar neurons obtained from either WT or CB_1R knockout animals were stimulated with NMDA agonists, the Ca^{2+} influx was more pronounced in CB_1R deficient cells. Thus, suggesting that CB_1R modulates NMDA glutamate receptor activity, an effect that has been reported by others (Nagayama et al., 1999).

The key players in the pathology of MS, neurodegeneration and inflammation, are T cells and microglia, both of which express CB_2Rs that are up-regulated during inflammatory states (Sagredo et al., 2009). The activation of CB_2Rs modulates the behavior of infiltrating T cells, as well as microglia. Maresz et al. (2007) showed that adoptive transfer of CB_2R deficient T cells into WT EAE animals results in a higher infiltration rate of these cells into inflamed CNS, and increased pro-inflammatory cytokines production, including IL-2 and IFN-γ. *In vitro* assays are consistent with these findings and show that the activation of CB_2R with the selective agonist JWH-133 reduces T cell proliferation and cytokines production, an effect which was absent in CB_2R deficient

T cells (Arevalo-Martin et al., 2003). Consistent with these findings, CB_2R deficient mice are more prone to disease induction, develop more severe EAE symptoms, and have higher mortality rates than WT controls (Maresz et al., 2007). The exacerbated EAE symptoms in CB_2R deficient mice are accompanied by axonal loss, and T lymphocyte and microglia activation (Palazuelos et al., 2008). The activation of microglia is associated with the increased levels of proinflammatory cytokines, including IL1β, IL-6, IFNγ and TNFα (Muzio et al., 2007), as well as generation of reactive oxygen species, and may account for the increased levels of cytokines in the CSF fluid of MS patients (Baraczka et al., 2003, 2004; Rovaris et al., 1996) and in the CNS of EAE animals (Murphy et al., 2010). TNFα released from microglia is inhibited by cannabinoid agonists, and therefore cannabinoids may modulate disease progression (Ortega-Gutierrez et al., 2005). Taken together, the experimental data in EAE demonstrates that the cannabinoid agonists are effective as neuroprotective and immunosuppressive agents, effects that are largely mediated through the activation of CB_1R; although CB_2R modulates some of the inflammatory responses.

2.2.2 CLINICAL DATA

Alterations in endocannabinoid levels have been reported in MS. Di Filippo et al. (2008) showed that the endocannabinoids levels, including AEA, 2-AG, PEA, and OEA are decreased in CSF of patients with MS, as compared to controls. Interestingly, the same study also showed that in the patients with RRMS, AEA and PEA levels, although still below control levels, are increased during relapse phase (Di Filippo et al., 2008). Contrary to these findings, Centonze et al. (2007) reported a significant increase in the level of AEA, but not 2-AG, in the CSF of relapsing MS patients. This latter study also showed elevated synthesis and reduced degradation of AEA in lymphocytes derived from these patients, suggesting that inflammatory cells infiltrating the active lesions may be the source of increased AEA in the CNS (Centonze et al., 2007). These results are also in line with another report by Jean-Giles et al. (2009), which showed a significant increase in levels of AEA in plasma from RRMS, SPMS, and PPMS patients, as compared to control subjects. In addition, in the SPMS group, PEA and OEA were also elevated. This study also reported a reduction in the mRNA expression for FAAH, an enzyme responsible for degradation of AEA, in SPMS, but not in RRMS or PPMS groups (Jean-Gilles et al., 2009). While findings from these clinical studies are inconsistent, the results likely reflect the different MS disease subtypes, i.e., relapse vs. remission phases, or methodological variables. Nevertheless, the alterations in the endocannabinoid system reported may reflect a neuroprotective role for endocannabinoids in MS disease progression, especially in patients diagnosed with PPMS. In keeping with this, dysfunction in ECS may add to disease severity.

Spasticity and pain are the most common symptoms in patients with MS, and are inadequately controlled with current pharmacological therapies. As new treatments have emerged, cannabinoid preparations have shown therapeutic benefits in symptom alleviation, and have now

been introduced into MS treatment in a number of countries, including Canada. Nabiximols (Sativex®), an oromucosal spray of an approximate 1:1 ratio of Δ^9-THC and cannabidiol (CBD), and Dronabinol, a synthetic Δ^9-THC, have been approved for the treatment of patients with moderate to severe MS, who do not respond well to standard therapies. However, as the clinical trials assess the effectiveness of cannabinoids, conflicting data emerges.

In the Cannabinoids in MS (CAMS) trial, involving over 600 patients with progressive MS, dronabinol had no significant effect on spasticity over a 15-week trial, as assessed by the Ashworth scale; although objective improvement in patients' mobility and subjective improvement in muscle spasm, pain, and sleep were reported (Zajicek et al., 2003). Interestingly, at the 1-year follow-up phase, a significant beneficial effect of dronabinol on muscle spasticity was evident (Zajicek et al., 2005). This latter finding led to the double-blind study, which investigated the neuroprotective effects of dronabinol, assessed by a degree of progression of MS in PPMS and SPMS patients with limited ability to walk, over a 3-year period (Zajicek et al., 2013). The researchers used neurological assessments of patients, as well as subjective responses to questionnaires, and showed no beneficial effect of dronabinol on the progression of the disease. However, the subgroup analysis revealed significant benefits in MS patients with lesser disability scores, quantified by the Extended Disability Status Scale (Zajicek et al., 2013). As this group of patients was relatively small, further investigation is needed in order to draw meaningful conclusions.

A number of clinical trials also evaluated the anti-spastic effect of nabiximols (Sativex®), a cannabis extract of 1:1 THC:CBD. In the enriched study design, Novotna et al, (2011) used Sativex® as an oromucosal spray and reported a reduction in spasticity, as assessed by a numeric rating scale (NRS, 0-10), as well as global improvement in function in MS patients. The beneficial effects of Sativex® on the amelioration of spasticity was also shown in the MOVE 2 Study (Flachenecker et al., 2014), SA.FE. study (Patti et al., 2016), and by the Marinelli group (2016), who, in addition to the Ashworth scale and NRS, evaluated stretch reflex, which validated the effect of the drug. As spasticity affects movement, Coghe et al. (2015) looked at the effects of the nabiximols, Sativex®, on this outcome. Objective assessment of movement function in response to nabiximols treatment, in patients who initially responded to the drug, revealed a significant improvement in speed velocity, as a function of lesser spasticity, and consequently greater and faster joint movements and improved walking ability (Coghe et al., 2015).

Another symptom that that is commonly associated with MS and responds to treatment with cannabinoids is pain, a clinical symptom experienced by 40–70% of patients (Osterberg et al., 2005; Solaro et al., 2004). The effect of dronabinol in the amelioration of pain, as a secondary outcome to spasticity, has been evaluated in a large double-blind randomized trial (Zajicek et al., 2003). Interestingly, while improvement in pain perception was reported by the majority of patients, around 20% of patients reported worsening of pain while on cannabinoid treatment (Zajicek et al., 2003). As neuropathic pain is the most common pain syndrome experienced by MS patients, effectiveness of Sativex® in neuropathic pain control has been investigated by focusing on this particular

symptom. More specifically, the beneficial effect of oromucosal nabiximols on neuropathic pain relief was reported by Rog and colleges (2007) in an open–labeled, 2-year extension trial, with mild to moderate side effects being reported. Similar findings were also reported by Russo et al. (2016) who evaluated the effect of Sativex® in the management of neuropathic pain in 20 MS patients (10 with and 10 without neuropathic pain). The study showed improvement in neuropathic pain and in the subjective and objective spasticity scores. Nabiximols (Sativex®), as an add-on therapy, was also evaluated in a larger cohort of MS patients who did not respond adequately to standard analgesics; however, the results from the two phases of the study were conflicting. The initial phase A of the study showed a large number of responders in both placebo and Sativex® treatment groups, and therefore no significant difference between those two groups; the second phase B resulted in more promising results and showed a significant improvement in pain relief, as well as quality of sleep, in the cannabinoid treated group (Langford et al., 2013).

While the majority of data support the beneficial effects of cannabinoids in the symptomatic relief of pain associated with MS, the findings from clinical trials are not as consistent as the experimental data. Among a number of different variables, the inconsistencies we see may reflect differences in study design, compound tested, or dosing schedule. Clinical trials evaluating the effectiveness of cannabinoid preparations in MS range from the uncontrolled open-label trials to trials with an initial enrichment phase, followed by second phase of a double-blind design. The enrichment phase allows the identification of "responders," who are then randomized into placebo or treatment groups, while non-responders discontinue the trial. The enriched trials that test the effectiveness of cannabinoids result in a smaller therapeutic effect, as compared to double-blind, placebo-controlled parallel trials (Collin et al., 2007, 2010; Wade et al., 2004). Furthermore, the majority of cannabinoid trials evaluat the effect of these agents in a population of MS patients with moderate to severe symptoms, who are refractory to current treatments. It is, therefore, possible that cannabinoid treatments may be more effective in the earlier stages of MS, as seen in a subgroup of patients from Zajicek et al. (2013) trial. In addition, many studies use subjective measures to assess the given outcomes, and patient expectancy of treatment effect can influence response. Indeed, the placebo effect varied significantly across the published studies ranging from 10% to as high as 50%, again, depending on the study design and compound tested, suggesting that findings from clinical trials should be evaluated with caution (Di Marzo and Centonze, 2015).

2.2.3 TOLERABILITY OF CANNABINOIDS

As the clinical trials continue to evaluate the efficacy of cannabinoids in the treatment of symptoms associated with MS, safety data for these compounds is also emerging. The retrospective analysis of risk-benefit profile for Sativex® from over 900 patients across the United Kingdom, Germany, and Switzerland, has shown that clinical outcomes, especially the sustained reduction in spasticity, outweigh the adverse effects of the drug (Fernandez, 2016). The most common adverse effects re-

ported by patients from these trials, as well as others, were nausea, dizziness, fatigue, and weakness (Fernandez, 2016; Flachenecker et al., 2014; Novotna et al., 2011; Rog et al., 2007; Zajicek et al., 2003). In addition, psychiatric events (including depression) are reported, albeit in a small number of patients. There is no evidence of tolerance or addiction/abuse of Sativex® in these studies (Fernandez, 2016; Rog et al., 2007).

2.3 CONCLUDING REMARKS AND FUTURE DIRECTIONS

The experimental and clinical data on the use of cannabinoid agonists in the treatment of MS suggests a potential benefit for these compounds in the symptomatic treatment of the disease, especially spasticity. Evidence for the neuroprotective effects, which may modulate MS progression, is supported by experimental studies, with clinical data being less conclusive. The beneficial effects of cannabinoids are mediated by the activation of both cannabinoid receptors, CB_1R and CB_2R, which modulate inflammatory and pain responses, and may confer neuroprotective benefit. As activation of CB_1R may produce side effects, including behavioral effects, another approach that may be useful in the future treatment of MS is the activation of ECS by pharmacological inhibition of degradative enzymes for AEA and 2-AG (Baker et al., 2001; Hernandez-Torres et al., 2014; Ligresti et al., 2006; Pryce et al., 2013). This strategy is especially attractive because it is localized and does not affect motor function or produce any overt psychotropic effects. Taken together, the existing preclinical and clinical data for MS suggest that activation of the ECS plays a protective role against inflammatory and neuronal damage in MS, while dysfunction in the ECS system may contribute to pathology.

CHAPTER 3

Huntington's Disease and the Endocannabinoid System

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Abstract

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by the expression of mutant huntingtin protein (mHtt) leading to preferential loss of striatal medium-sized spiny neurons (MSNs), excitotoxicity, mitochondrial damage, free radicals, and neuroinflammation. Neuroinflammation is mediated by the endogenous effects of mHtt within glia and sustained activation of microglia and astrocytes in response to neuronal damage. Dysregulation of the endocannabinoid system (ECS) contributes to HD pathogenesis. Specifically, early in HD progression, levels of the cannabinoid type 1 receptor (CB_1R), expressed on neurons, are reduced in the MSN, while later in HD levels of cannabinoid type 2 receptors (CB_2R), expressed on glia cells, increase. Cannabinoids that activate CB_2R might exert neuroprotective effects by reducing neuroinflammation. CB_2R selective agonists represent a promising means to slow HD disease progression. Future studies should focus on the positive and negative effects of cannabinoid receptor-selective agonists on neuroinflammation and HD progression.

Key Words

cannabinoids, neuroinflammation, neurodegeneration, Huntington's disease

Abbreviations

Δ^9–THC	Δ^9–tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
AA	arachidonic acid
ABHD6	α/β-hydrolase domain protein 6
ABHD12	α/β-hydrolase domain protein 12
AEA	arachidonoyl ethanolamide/anandamide

BBB	blood-brain barrier
CB_1R	cannabinoid type 1 receptor
CB_2R	cannabinoid type 2 receptor
CBD	cannabidiol
CCL5	chemokine chemokine (C-C motif) ligand 5
CNS	central nervous system
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
DAGL	diacylglycerol lipase
ECS	endocannabinoid system
ERK	extracellular signal-regulated kinase
FAAH	fatty-acid amide hydrolase
GFAP	glial fibrillary acidic protein
GLT-1	glutamate transporters
GPCR	G-protein-coupled receptor
HD	Huntington's disease
Htt	normal huntingtin protein
IL-1 β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin-8
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MAPK	mitogen activated protein kinase
mHtt	mutant huntingtin protein
MSN	medium spiny projection neuron
NAT	N-acyl-phosphatidylethanolamine
NAPE-PLD	NAPE specific phospholipase D
NSAID	nonsteroidal anti-inflammatory drug
PGE2G	prostaglandin E2 glyceryl;
PGG2	prostaglandin G2
PI3K	phosphatidylinositide 3-kinases
RANTES	regulated on activation normal T cell expressed and secreted
ROS	reactive oxygen species
TFC	Total Functional Capacity
TNF-α	tumor necrosis factor alpha
TRPV1	transient receptor potential cation channel subfamily V member 1
UHDRS	Unified Huntington's Disease Rating Scale

3.1 HUNTINGTON'S DISEASE

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder. The prevalence of HD is approximately 1 in 10,000 in individuals of European descent (Huntington's Disease Collaborative Research Group (HDCRG), 1993). Although the etiology of HD varies on a case-to-case basis, individuals will generally experience progressive decline in motor, cognitive, and affective function (HDCRG, 1993; Kirkwood et al., 2001; Waldvogel et al., 2014). While both juvenile and late-onset forms of the disease are known, the majority of HD patients have symptoms by the fourth or fifth decade of life, with symptoms gradually worsening until death (HDCRG, 1993; Walker 2007).

Tests to predict the risk of inheriting HD, including linkage analysis, have been available since the mid 1980's; however, definitive identification of HD gene-positive individuals, prior to symptom onset, was possible following the discovery of the genetic mutation responsible for HD (HDCRG, 1993). As such, early intervention may be feasible for gene-positive HD patients. The *huntingtin* gene encodes an essential and evolutionarily conserved 348 kDa huntingtin protein (Htt) which is ubiquitously expressed throughout the brain and body. The Htt serves an essential scaffolding function for a large number of proteins (cell body and nucleus) (Zuccatto and Cattaneo, 2014). HD is caused by the inheritance of a mutant copy of the *huntingtin* gene (HDCRG, 1993; Zuccatto and Cattaneo, 2014). Expansion of the CAG repeat within exon 1 of the *huntingtin* gene leads to the production of a mutant Htt (mHtt) protein with an expanded polyglutamine region within the N terminus of the protein. Normally, individuals express two copies of the *huntingtin* gene, each with 10-29 CAG repeats. In HD patients, at least one of the *huntingtin* alleles has greater than 36 CAG repeats (Kremer et al., 1990; HDCRG, 1993; Landwehrmeyer et al., 1995). The amino terminus of the mHtt protein is a substrate for caspase-1, -2, -3, -6, and calpain-mediated cleavage, which releases an N-terminal fragment (Hermel et al., 2004). Expression of mHtt is associated with both loss of normal Htt protein functions and gain of toxic functions of the mHtt protein and N-terminal protein fragments. mHtt negatively affects many cellular functions including gene expression, proteosomal and autophagic processes, mitochondrial function, energy production, control of excitotoxicity, and molecular trafficking (reviewed in Zuccatto and Cattaneo, 2014).

The age of onset and severity of HD is inversely proportional to the number of CAG repeats in the mutant *huntingtin* gene (HDCRG, 1993; Möller, 2010). However, there is considerable variability in the age of onset for HD patients especially for patients who have common mutant allele lengths (36–50 CAG repeats). Up to 60% of the observed variability in the age of onset is related to polygenic and environmental effects (Waldvogel et al., 2014). Even monozygotic twins with identical CAG repeat lengths within the mutant *huntingtin* gene show variability in HD progression, especially in affective and cognitive domains (Gómez-Esteban et al., 2007). Such variability may be due to environmental factors or polymorphisms in modifier genes. Somatic expansion of the CAG repeat within particular tissues is linked to differential appearance of symptoms, the age of onset,

and rate of disease progression suggesting that alterations in DNA maintenance and repair or local tissue environment also contribute to phenotypic variability (Swami et al., 2009).

While experiencing HD, there is widespread cellular dysfunction and apoptosis throughout the brain and periphery. The medium spiny projection neurons (MSNs) of the striatum within the basal ganglia are especially vulnerable to the neurodegenerative effects of mHtt (Vonsattel and Difiglia, 1998; Glass et al., 2000; Walker, 2007; Waldvogel et al., 2014). Many HD animal models exhibit pronounced neuronal and synaptic dysfunction in MSNs before the occurrence of neuronal death (Ross et al., 2014; Waldvogel et al., 2014). Specifically, neuronal dysfunction is first observed in MSNs of the indirect motor pathway, which project from the striatum to the external globus pallidus. This population of MSNs express dopamine receptor type 2 (D_2), cannabinoid type 1 receptors (CB_1R) and produce enkephalin. Neuronal dysfunction is followed by neuronal loss. These neuropathological changes are evident as much as 10 years before clinical diagnosis and are believed to be the prime cause of the chorea commonly seen in HD patients (Waldvogel et al., 2014; Ross et al., 2014). As HD progresses, neurodegeneration is also observed in MSNs of the direct motor pathway, leading to more severe motor impairment such as bradykinesia and hypokinesia (Ross et al., 2014).

In addition to the neurodegeneration observed in HD, alterations in the function of glial cells also occur. Altered glial activity in HD is the direct effect of intracellular expression of mutant *huntingtin* in glia cells and in response to mutant *huntingtin*-induced neuronal dysfunction. The phenomenon of neuroinflammation is widely accepted as a precursor and inducer of different neurodegenerative conditions including HD (Gao and Hong, 2008; Silvestroni et al., 2009; Möller, 2010; Ross et al., 2014). This chapter will discuss the role of neuroinflammation in HD progression and neurodegeneration, and the endocannabinoid system (ECS) as it relates to animal models of HD and the clinical treatment of HD-induced neuroinflammation.

3.2 NEUROINFLAMMATION IN HUNTINGTON'S DISEASE

A state of chronic neuroinflammation is observed in several neurodegenerative diseases including HD (Gao and Hong, 2008). Chronic neuroinflammation contributes to excitotoxicity, impairment of metabolic function, and oxidative stress, which further exacerbates HD pathogenesis (Gao and Hong, 2008; Silvestroni et al., 2009; Soulet and Cicchetti, 2011). Neuroinflammation is mediated by the actions of the glial cells, including microglia and astrocytes of the central nervous system (CNS), and the inflammatory mediators released by these activated cells (Streit 2002; Gao and Hong, 2008; Glass et al., 2010; Soulet and Cicchetti, 2011; Crotti and Glass, 2015).

Although neurons are profoundly affected by the expression of mHtt, there is a growing body of evidence suggesting that expression of mHtt in microglia and astrocytes and the subsequent alteration in immune functions also contribute to HD progression. Microglia isolated from the brains of YAC128 and BACHD transgenic HD mice models showed impaired transmigration in

Boyden Chamber Assays compared to wild-type control mouse tissue. Furthermore, time-lapse *in vivo* imaging using 2-photon microscopy demonstrated a ~20% decrease in microglial response and an inability of reactive microglia to fully encompass the site of focal laser ablation induced injury in BACHD transgenic HD mice (Kwan et al., 2012). Therefore, abnormal microglia activation could contribute to the early neuroinflammation observed in HD.

Post-mortem human HD brain tissue at various disease stages show an accumulation of reactive microglia, which contributes to reactive gliosis observed in HD (Sapp et al., 2001). Specifically, an increase in thymosin β4 immunostaining, a marker for reactive microglia, was observed in the medial caudate, cortex, and basal ganglia of *post-mortem* tissue derived from HD patients compared to age-matched controls (Sapp et al., 2001). In addition, positron emission tomography of HD patients showed increased binding of radioligand [11C](R)-PK11195, a marker of activated microglia, in the striatum, frontal, and parietal cortex of HD patients that correlates with clinical severity of the disease (Pavese et al., 2006). Microglial activation is positively correlated with HD progression and observed up to 15 years before HD symptomatic onset, implicating a role for activated microglia in HD pathogenesis (Tai et al., 2007; Soulet and Cicchetti, 2011).

mHtt-expressing microglia have increased expression of transcription factors that regulate the expression of proinflammatory genes, which further contribute to the neuroinflammation observed in HD (Crotti and Glass, 2015). Specifically, expression of mHtt in the microglia increases expression and transcriptional activities of the myeloid lineage-determining transcription factors, PU.1 and CCAAT/enhancer-binding protein α and β (C/EBPα,β), compared to normal microglia (Crotti et al., 2014). Subsequently, enhanced binding of the transcription factor PU.1 to PU.1 recognition elements leads to increases in proinflammatory cytokine levels, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). The increases in PU.1 gene expression correlated directly with the increased proinflammatory cytokine profile in mHtt expressing microglia even in the absence of proinflammatory stimuli, indicating that mHtt primes microglial cells for inflammatory activation prior to any actual inflammatory activity in the brain. This priming capability would be consistent with the findings of early microglial activation noted in asymptomatic HD patients (Sapp et al., 2001; Pavese et al., 2006; Tai et al., 2007; Soulet and Cicchetti, 2011). Overall, these observations indicate that mHtt alters the function of microglia during HD pathogenesis via cell intrinsic and extrinsic effects.

Astrocyte dysfunction has also been implicated in HD neuroinflammation. mHtt and mHtt aggregates were reported in glial fibrillary acidic protein (GFAP)-positive astrocytes, a hallmark of reactive astrocytes, derived from transgenic R6/2 HD models and in *post-mortem* brains of patients with HD (Shin et al., 2005). mHtt disrupts the transcription of essential astrocyte functional proteins, such as glutamate transporters (GLT-1) and inward-rectifying potassium channel 4.1 (Kir4.1), early in HD progression (Shin et al., 2005; Tonget al., 2014; Estrada-Sánchez and Rebec, 2013). Clearance of the extracellular glutamate neurotransmitter occurs mainly by GLT-1, which is expressed on astrocytes. In early stages of HD progression, mHtt accumulation in astro-

cytes decreases the expression of GLT-1, reducing glutamate uptake into astrocytes and resulting in MSN excitotoxicity observed in HD (Shin et al., 2005; Estrada-Sánchez and Rebec, 2013). Kir4.1 is a potassium channel that regulates extracellular K^+ levels. Loss of Kir4.1 in striatal astrocytes in HD mouse models led to higher ambient K^+ and MSN excitability (Tonget al., 2014).

mHtt also affects the transcription and secretion of chemokines in astrocytes derived from transgenic HD models (Shin et al., 2005; Chou et al., 2008). Expression of mHtt reduced steady-state levels of the chemokine chemokine (C-C motif) ligand 5 (CCL5)/regulated on activation normal T cell expressed and secreted (RANTES) mRNA in astrocytes isolated and cultured from prenatal R6/2 transgenic HD mice and reduced the amount of the CCL5/RANTES secreted by cultured astrocytes (Chou et al., 2008). In addition, immunofluorescence staining of neurons cultured in R6/2 mouse astrocyte-conditioned media displayed markedly shorter and less branched neuronal processes (Chou et al., 2008). Treatment of the cultures with a CCL5/RANTES neutralizing antibody blocked the negative effect of the R6/2 astrocyte-conditioned media on neuron growth (Chou et al., 2008). In addition, above-average levels of IκB kinase activity in R6/2 transgenic mice was attributed to the hyper-activation of proinflammatory NF-κB pathway in astrocytes, producing greater levels of proinflammatory cytokines and higher levels of nitric oxide (NO) compared to wild-type controls, leading to increased neuronal toxicity (Hsiao et al., 2013). Together, these findings indicate that mHtt-expressing astrocytes play a significant role in the neuroinflammatory and neurodegenerative processes observed in HD.

Reactive microglia directly activate astrocytes. Pro-inflammatory cytokines such as IL-6, TNF-α, interleukin 1 beta (IL-1β), and reactive oxygen species (ROS) produced from reactive microglia promote astrocyte activation, which in turn produces more inflammatory cytokines and ROS, further activating microglia (Glass et al., 2010). With mHtt exacerbating reactive gliosis, a feed forward neuroinflammatory cycle begins that turns the innate neuroprotective response of microglia and astrocytes into one that promotes neurodegeneration (Gao and Hong, 2008).

There is evidence that the patency of the blood-brain barrier (BBB) is compromised in both animal models of HD and in patients with HD (Franciosi et al., 2012; Drouin-Ouellet et al., 2015). Increased BBB permeability was observed following lipopolysaccharide (LPS)-induced neuroinflammation in young YAC128 transgenic HD mouse model (Franciosi et al., 2012). Similarly, BBB leakage was also observed in the striatum of R6/2 transgenic HD mouse model (Shin et al., 2005). In humans, it was observed that the expression levels of the BBB tight junction proteins, occludin and claudin-5 were significantly lower in *post-mortem* caudate-putamen tissue of HD patients compared to control subjects (Drouin-Ouellet et al., 2015). Additionally, inflammatory mediators associated with increased BBB permeability such as hepatocyte growth factor, interleukin-8 (IL-8), and tissue inhibitor metalloproteinase 1 were increased in HD patients (Drouin-Ouellet et al., 2015). Dynamic contrast-enhanced magnetic resonance imaging showed increased BBB permeability in mild-to-moderate stage HD patients, which correlated with HD progression (Drouin-Ouellet

et al., 2015). Therefore, the immune response in the brain observed in HD may be mediated by CNS-resident microglia and astrocytes, as well as, infiltrating immune cells from the periphery.

3.3 THE MANAGEMENT OF NEUROINFLAMMATION IN HUNTINGTON'S DISEASE

There is currently no cure for HD, and available pharmacological therapies provide modest relief of HD symptoms, but do not slow disease progression (Frank, 2014; Mason and Barker, 2016). The currently available treatment options include dopamine-depleting agents, dopamine receptor blockers, anxiolytic, and anti-depressant agents (Frank, 2014; Mason and Barker, 2016). Current research aiming at treating HD involves pharmacological approaches that modulate neuroinflammation, which may assist in delaying or slowing HD progression.

Several anti-inflammatory drugs have been evaluated in both HD animal models and in HD patients for neuroprotective effects. For example, minocycline is an antibiotic that exerts neuroprotective properties through inhibition of caspase-1 and caspase-3, and by decreased inducible nitric oxide synthase activities (Chen et al., 2000; Wang et al., 2003, Tikka et al., 2001). Additionally, minocycline exerts anti-inflammatory properties by inhibiting the production of TNF-α, IL-1β and IL-6 from activated microglial cells (Tikka et al., 2001). This compound was found to delay disease progression in the R6/2 mouse model (Chen et al., 2000; Wang et al., 2003). A case study and a small pilot study in HD patients demonstrated safety and modest efficacy (Denovan-Wright, et al., 2002; Thomas et al., 2004). A clinical trial was conducted to evaluate the efficacy of minocycline to improve Total Functional Capacity (TFC) and the Unified Huntington's Disease Rating Scale (UHDRS) motor score. Regardless of the dose tested, minocycline did not alter TFC or UHDRS scores (Thomas et al., 2004). The effect of minocycline on neuroinflammation was not assessed. The neuroprotective effects of nonsteroidal anti-inflammatory drugs (NSAIDs) were evaluated in HD animal models. The anti-inflammatory effects of NSAIDS are mediated through inhibiting cyclooxygenase-1 (COX-1) and/or cyclooxygenase-2 (COX-2), a prostaglandin-synthesizing enzyme. The COX-1 and COX-2 inhibitors, acetylsalicylate, and rofecoxib, were tested in R6/2 and N171-82Q transgenic mouse models of HD. No improvement in striatal neurodegenerative or motor behavior was observed in either mouse models (Norflus et al., 2004). The effects of these drugs on modulation of neuroinflammation were not evaluated in this study. Another approach to modulate neuroinflammation involves the administration of pharmacological compound that inhibits the proinflammatory cytokine TNF-α, such as etanercept. Etanercept is a biopharmaceutical approved for the treatment of several autoimmune diseases. Etanercept does not normally cross the BBB due to its size, however this molecule might gain access to the CNS in HD depending on the state of the BBB (Boado et al., 2010). The potential benefit of immunomodulatory compound, fingolimod (FTY720), has been evaluated in HD animal models. FTY720, a sphingosine 1-phosphate receptor modulator, is indicated and approved for the treatment of multiple sclerosis. Chronic administra-

tion of FTY720 improved memory function and reduced reactive astrocyte in R6/1 mice (Miguez et al., 2015). Although these drugs have not demonstrated clinical efficacy in HD, these findings contribute to the evidence that neuroinflammation plays an important role in the mechanisms of neuropathology of HD, and that the modulation of neuroinflammation could be beneficial to slow disease progression. Many of these newer treatments focus on decreasing neuroinflammation. One potential therapeutic target that could modulate neuroinflammation as well as the symptoms of HD is the ECS.

3.4 ENDOCANNABINOID SYSTEM IN HUNTINGTON'S DISEASE

The ECS is comprised of G-protein-coupled receptors (GPCR), including CB_1R and CB_2R cannabinoid receptors, the endogenous ligands arachidonoylglycerol (2-AG) and arachidonoyl ethanolamide (anandamide/AEA), and the anabolic and catabolic enzymes that maintain the levels of endogenous ligands. In the CNS, endocannabinoids levels are highly regulated as they are formed on-demand from precursors in the cell membrane and are rapidly degraded by specialized enzymes (for a detailed review see, Alexander and Kendall, 2007). The synthesis of AEA is catalyzed by the sequential activity of N-acyltransferase (NAT) and N-acyl-phosphatidylethanolamine (NAPE) specific phospholipase D (NAPE-PLD), while 2-AG is synthesized through the activity of diacylglycerol lipase (DAGL). Degradation of the endocannabinoids AEA and 2-AG occurs locally by fatty-acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Howlett et al., 2002: Atwood and Mackie, 2010). Cannabinoid receptors are among the most abundant GPCRs in the brain, with CB_1R showing widespread expression in neurons, and CB_2R being expressed in microglia and astrocytes, microvasular endothelial cells, and possibly neurons (Matsuda et al., 1990; Munro et al., 1993; Gong et al., 2006; Persidsky et al., 2015; Zhang et al., 2014).

Alterations in the ECS functions have been documented in several neurodegenerative disorders including HD. Changes in the ECS in HD were first documented in 1993 (Glass et al., 1993). Autoradiography studies using [^{3}H] CP55,940 revealed a 97.5% reduction in CB_1R levels in the substantia nigra of *post-mortem* human HD patients (Glass et al., 1993). Subsequent work demonstrated that CB_1R mRNA and protein levels begin to decline in GABAergic MSNs of the indirect motor pathway prior to cell loss in young mice of different transgenic HD mouse models (Denovan-Wright and Robertson, 2000; Lastres-Becker et al., 2003; McCaw et al., 2004; Dowie et al., 2009; Blázquez et al., 2011; Bari et al., 2013). mHtt appears to interfere directly with the transcription of the *CNR1* gene within MSNs (McCaw et al., 2004; Blázquez et al., 2011; LaPrairie et al., 2013). mHtt-dependent loss of CB_1R in MSN disinhibits GABA neurotransmission and triggers an imbalance in glutamate homeostasis in the basal ganglia and initiates exitotoxicity (Mievis et al., 2011; Chiarlone et al., 2014; Naydenov et al., 2014; Blázquez et al., 2011, 2015). Even though

CB_1R mRNA and protein levels decline in HD relative to age-matched wild-type mice, CB_1R are widely distributed at low levels (McCaw et al., 2004).

CB_2R expression is significantly increased in microglia during periods of CNS stress and neuroinflammation (Carlisle et al., 2002; Maresz et al., 2005; Mukhopadhyay et al., 2006). In HD, CB_2R expression routinely shows a dramatic increase in brain tissue in caudate and putamen tissue obtained from patients with mid-to-advanced stages of HD pathology (Bari et al., 2013; Laprairie et al., 2014). Elevation in CB_2R expression was also observed in different HD animal models. Increase in CB_2R levels was observed in striatal microglia of transgenic R6/2 HD mouse models and in the striatal lesion models of HD (Palazuelos et al., 2009; Sagredo et al., 2009). While reduction in CB_1R levels occurs early in HD progression as a direct effect of mHtt (McCaw et al., 2004), increases in CB_2R levels occurs later in HD progression and may be compensatory mechanisms to mitigate the negative effects of mHtt (Dowie et al., 2010; Naydenov et al., 2014). Increased CB_2R expression and activation may assist in striatal neuroprotection from neuroinflammatory insults produced by reactive microglia (Carlisle et al., 2002; Maresz et al., 2005; Mukhopadhyay et al., 2006). These neuroprotective effects of CB_2R overexpression may be mediated through the release of neurotrophins and anti-inflammatory cytokines (Palazuelos et al., 2009, Sagredo et al., 2009). In support of the hypothesis that CB_2R function may be protective in HD, CB_2R-deficient R6/2 mice showed faster HD symptom progression, enhanced microglial activation, and a reduced lifespan (Palazuelos et al., 2009). Modulation of CB_2R function might be a useful in the management of HD neuroinflammation and may slow disease progression.

Changes in the endocannabinoid levels have also been observed in HD. In a rat model of HD, AEA and 2-AG levels were decreased in the striatum, while there was an increase in AEA level in the substantia nigra (Lastres-Becker et al., 2001). These changes in endocannabinoid levels are similar to those found in the brain of HD patients (reviewed in Laprairie et al., 2015a).

3.5 MODULATION OF THE ENDOCANNABINOIDAL SYSTEM TO MINIMIZE NEUROINFLAMMATION AND NEURODEGENERATION IN HUNTINGTON'S DISEASE

Modulation of the ECS, primarily by cannabis, has been used anecdotally in historical medicine; however, recent research has demonstrated that the ECS could be a potential target for the treatment of chronic neurodegenerative disorders including HD (Mackie and Katona, 2009; Marchalant et al., 2009; Shohami et al., 2011). Modulation of ECS can be achieved by administering exogenous phytocannabinoids, synthetic cannabinoids, or through modulation of endogenous cannabinoid levels (2-AEA or 2-AG) by administering compounds that inhibit either the endocannabinoid anabolic or catabolic enzymes. Phytocannabinoids and synthetic cannabinoids have shown to be neuroprotective in experimental HD models (Chiarlone et al., 2014; Sagredo et al., 2007). The observed beneficial and detrimental effects of cannabinoids in experimental HD models are believed

to be mediated through multiple mechanisms including: CB_1R-dependent, CB_2R-dependent, and non-cannabinoid receptor-dependent mechanisms (Sagredo et al., 2012).

The activation of CB_1Rs are associated with the stimulation of intracellular signalling pathways, including RAS-mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositide 3-kinases (PI3K)-Akt pathways, which are involved in neuronal proliferation, differentiation, and survival (Fernández-Ruiz et al., 2010; Sagredo et al., 2012). Moreover, CB_1Rs are localized on presynaptic glutamatergic neurons, and activation of CB_1Rs results in the inhibition of glutamate release (Ohno-Shosaku T et al., 2012). The increase of glutamate release from cortical afferents and over activation of the postsynaptic glutamate receptors on striatal projection neurons have been implicated in excitoxicity in HD (Behrens et al., 2002; Benn et al., 2007; Estrada-Sanchez et al., 2009). Therefore, activating CB_1Rs may be effective in reducing glutamate excitotoxicity observed in HD. Activation of CB_2Rs *in vivo* had been shown to exert neuroprotective effects in different animal models of acute and chronic brain toxicity and neuroinflammatory damage (reviewed by Mackie, 2006; Palazuelos et al, 2009; Sagredoet al., 2009). Activation of CB_2Rs reduces the levels of activated microglia and the release of pro-inflammatory cytokines such as the TNF-α, IL-1β, IL-6, and NO, while promoting the production of post-survival molecules such a neurotrophin (e.g., glial cell-derived neurotrophic factor) and anti-inflammatory cytokines (e.g., IL-1 receptor antagonists; reviewed in Fernández-Ruiz et al., 2010; Sagredo et al., 2012). Other cannabinoids such as the phytocannabinoid cannabidiol (CBD) may act as an allosteric modulator that influences the activity of cannabinoids at CB_1Rs and CB_2Rs (Laprairie at al., 2015c). CBD, also activates different pathways independently of CB_1Rs and CB_2Rs; CBD can inhibit the AEA-metabolizing enzyme FAAH, increasing the level of AEA (Bisogno et al., 2001). In experimental studies, CBD exerts neuroprotective and anti-oxidant effects (Iuvone et al., 2009). Taken together, these neuroprotective, anti-inflammatory and anti-oxidant properties of cannabinoids make them attractive agents for developing new therapeutics useful to treat HD given that dysregulation of the ECS may play a role in HD pathogenesis.

In experimental models of HD, cannabinoid treatments have been shown to ameliorate HD symptoms. The phytocannabinoid, Δ^9-tetrahydrocannabinol (THC)—which activates both CB_1Rs and CB_2Rs; reduces hyperkinetic movement, striatal atrophy, and peripheral inflammation (Sagredo et al., 2011; Blázquez et al., 2011; Bari et al., 2013); but increased incidence of seizure in transgenic R6/2 HD mice (Dowie et al., 2009, 2010; Scotter et al., 2010). In the context of striatal lesion models of HD, select cannabinoids have neuroprotective and anti-inflammatory effects (Sagredo et al., 2007; Valdeolivas et al., 2012, 2015; Scotter et al., 2010). The clinically available cannabinoid preparation Sativex®, which contains equimolar Δ^9-THC with CBD, was tested for its neuroprotective effect in striatal lesions induced by the mitochondrial toxin malonate. Sativex® reversed the neurodegeneration induced by malonate, reduced microglia and astrocyte activation, and reduced the expression of inducible nitric oxide synthase. The observed beneficial effects of Sativex® were completely blocked when selective antagonists for both CB_1R and CB_2R types (i.e., SR141716 and

AM630) were co-administered prior to Sativex® administration (Sagredo et al., 2007; Valdeolivas et al., 2012, 2015; Scotter et al., 2010). Together, these observations indicated that the observed beneficial effects of Sativex® are mainly mediated through CB_1R- and CB_2R-dependent mechanisms, but that does not exclude the possibilities of CB_1R and CB_2R independent mechanisms. These studies provide preclinical evidence in support of the beneficial effects of using Sativex® in delaying disease progression in HD.

Selective CB_2R agonists are showing increasing therapeutic promise in the fight against HD. Activation of CB_2Rs *in vivo* using selective CB_2R agonists does not induce undesirable psychotropic actions. The CB_2R agonist, HU-308, decreased levels of TNF-α to control levels in a malonate-induced striatal injury in a rat model of HD (Sagredo et al., 2009). HU-308 protected striatal neurons from malonate-induced cell death, which was ablated when animals were pretreated with the CB_2R antagonist SR-144,528 (Sagredo et al., 2009). Palazuelos et al. (2009) reported that the CB_2R agonist, HU-308, inhibits overactive microglia, and reduces the chronic neuroinflammatory actions and striatal neurodegeneration in quinolinic-acid-lesioned mice. These studies demonstrate that stimulation of the CB_2Rs are neuroprotective against quinolinic acid and malonate-induced toxicity. The neuroprotective action of CB_2R agonists has yet to be confirmed in transgenic HD animal models.

Although endocannabinoids acting via CB_2Rs may promote ECS function to limit inflammation, endocannabinoid metabolites can be proinflammatory. The metabolism of 2-AG results in bioactive compounds including arachidonic acid (AA) and other prostaglandins, which mediate the inflammatory response. 2-AG is synthesized through the activity of DAGL, while 2-AG is primarily metabolized by MAGL and to a lesser extent by α/β-hydrolase domain proteins 6 and 12 (ABHD6 and ABHD12). The hydrolysis of 2-AG produces both AA and glycerol. 2-AG and AA are substrates of the oxidizing enzyme COX-2, which oxidizes 2-AG to prostaglandin E2 glyceryl ester (PGE2G) and oxidizes AA to prostaglandin G2 (PGG2); both PGE2G and PGG2 are known to be neurotoxic (reviewed in Janssen and van der Stelt, 2016). The DAGL inhibitor, O-3841, was neuroprotective in a malonate model of HD, attenuating malonate-induced GABA and the brain-derived neurotrophic factor deficiencies and glial activation. In contrast, the MAGL-inhibitor, JZL184, exacerbated malonate-induced striatal damage (Valdeolivas et al., 2013). This finding suggests that reducing 2-AG levels by blocking synthesis of 2-AG leads to decreases in levels in downstream prostaglandins, which limits inflammation resulting in neuroprotective in HD (Valdeolivas et al., 2013). Modulation of endocannabinoid levels and the cumulative effects of exogenously administered cannabinoids should be considered in future studies due to altered metabolism of cannabinoids in HD.

Pre-clinical studies modulating the ECS in models of HD present positive findings for the potential use of targeting the ECS as a therapy for HD. The potential benefits of targeting the CB_1R, attenuating excitotoxicity; the CB_2R reducing inflammation; and cannabinoid receptor-independent processes reducing oxidation injury. In a very early trial, CBD was found to be safe and

well tolerated in HD patients but did not reduce chorea (Consroe et al., 1991). Cesamet ® (nabilone), a synthetic Δ^9-THC analog, and partial agonist at CB_1Rs and CB_2Rs was evaluated in two clinical trials (Müller-Vahl et al.1999; Curtis el al., 2009). The UHDRS was used to evaluate total motor score, chorea, cognition and behavior, and neuropsychiatric outcomes. There was evidence of improvement in cognitive outcomes, but not in chorea (Müller-Vahl et al.1999; Curtis el al., 2009). In 2011, a double blind, randomized, cross-over, phase 2 clinical trial was conducted to assess the neuroprotective effects of Sativex® in HD. Although safe, no differences in motor, cognition, behavioral, or functional outcomes were detected during treatment with Sativex(®) compared to placebo (López-Sendón et al., 2016). All trials to date have enrolled symptomatic patients and had relatively short trials with a very limited number of agents. It is possible that earlier treatment, different agents, different doses, and longer treatment duration could limit damage. It is also possible that administration of cannabinoids in critical phases of brain maturation could be highly detrimental to HD patients.

There is a need for novel pharmacological approaches to modulate the underlying pathology of HD. The ECS provides a novel target for modulating the neuroinflammation associated with HD. In the context of animal models of HD, CB_1R/CB_2R agonists have resulted in: decreasing hyperkinetic movement; striatal atrophy protecting striatal neurons; and having neuroprotective and anti-inflammatory effects that include reducing the levels of TNF-α in models of HD (Sagredo et al., 2009, 2011; Blázquez et al., 2011; Bari et al., 2013, Valdeolivas et al., 2013). Although the available results are confined to a limited number of studies conducted in transgenic and legion-induced models of HD, they have shown promise for modifying some aspects of HD. To date, however, the clinical data has not shown any significant changes in UHDRS motor scores or biomarkers using Sativex® in HD or CBD (López-Sendón et al., 2016). Selective cannabinoids hold promise to modulate the ECS to control neuroinflammation but have yet to be thoroughly investigated in HD.

CHAPTER 4

Alzheimer's, Neuroinflammation and the Endocannabinoid System

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Abstract

Alzheimer's disease (AD) is a devastating neurodegenerative condition for which there is currently no effective treatment. Endocannabinoids are known to regulate the release of neurotransmitter in the central nervous system (CNS), and play a crucial role in activation of microglia, permeability of the blood brain barrier (BBB), and cognition. This chapter will highlight the inflammatory mechanisms that underlie AD pathology and describe how cannabinoid receptors and the endocannabinoid system may be exploited for therapeutic potential.

Keywords

Alzheimer's disease, amyloid β, neuroinflammation, endocannabinoids

4.1 INTRODUCTION TO ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the leading cause of dementia, globally accounting for up to 70% of all dementia cases. It is an age-associated neurodegenerative condition, manifesting in the progressive loss of cognitive function. While known genetic variants have been ascribed, the potential to predispose an individual to acquiring the condition, the familial (FAD) or inherited form of the disease accounts for <5% of all cases worldwide. FAD is most commonly an early-onset condition, with symptoms manifesting before the age of 65. However, it is the sporadic (SAD), or late-onset form of the condition, that constitutes an overwhelming 95% of AD cases, and for which the contribution of inherited factors has not been determined. Symptoms initially present as mild cognitive impairment, which worsen progressively within approximately 10 years, to the advanced cognitive dysfunction characteristic of late-stage AD.

Although they may originate from different bases, the two forms of AD share the same neuropathological mechanisms. The AD brain is characterized by the deposition of amyloid-β

peptide (Aβ) into extra-neuritic plaques. Aβ is a toxic peptide, produced by abnormal processing of amyloid precursor protein (APP). Cleavage of APP by the enzymes β- and γ-secretase most commonly generates two primary species of Aβ, consisting of either 40 or 42 amino acids; the latter being the more hydrophobic and prone to aggregation (Glabe, 2005). The other major hallmark of AD is the formation of neurofibrillary tangles composed of hyperphosphorylated tau causing disruption of microtubules. This results in neuronal dysfunction and subsequent neurodegeneration, evidenced by a significant loss of brain tissue volume in patients with late-stage AD. Typically, the cognitive deterioration is ascribed to aberrant neuronal network activity, caused by disruption of activity-dependent synaptic communication and neuronal hyperexcitability. To date this has largely been characterized within the hippocampus; a region significantly impacted by AD pathology. Originally, plaques of aggregated Aβ protein were thought to be the main neurotoxic conformation of this protein, however soluble Aβ oligomers are now known to be highly neurotoxic, disrupting synaptic plasticity (Walsh and Selkoe, 2004) with plaques likely acting as a sink for Aβ. Recently it has been proposed that Aβ clearance via the blood brain barrier (BBB) is reduced by up to 30% in AD patients (Krohn et al., 2011), mainly due to diminished Aβ transport (Mawuenyega, et al., 2010; Castellano et al., 2011).

4.2 ALZHEIMER'S DISEASE TREATMENTS AND THE ENDOCANNABINOID SYSTEM

Presently, acetylcholinesterase inhibitors (AchEIs) are used to treat mild to moderate cases of AD. These agents increase the availability of acetylcholine at cholinergic synapses. However, more recent trials of AchEIs have failed in phase III trials (Galimberti and Scarpini, 2016). The more severe cases of AD are treated with Memantine, an NMDAR2b antagonist, which is thought to improve the signal to noise ratio at glutamatergic/NMDAR synapses thereby improving conditions for synaptic plasticity (Danysz and Parsons, 2012). Unfortunately, both of these agents, AchEIs and the NMDAR2b antagonist, offer only short term improvement in the condition with no change in the progression of the disease. Additionally, pharmaceutical companies have also developed experimental agents targeting Aβ and the "Amyloid Hypothesis" of AD in the hope of modifying disease progression. These drugs, including the monoclonal antibody Solanezumab, have been designed to bind to Aβ thereby effectively lowering the concentration to prevent the formation of plaques. Beta secretase inhibitors such as Verubecestat, also lower the production of Aβ; this agent is presently in phase III trial, however, it is likely to alter levels of other proteins in addition to Aβ. Small molecule inhibitors such as CSP-1103 (Porrini et al., 2015) that can bind to and inhibit the APP intracellular domain (AICD) (Branca et al., 2014) are also under phase II trial, due to their ability to restore microglial function.

There is presently no treatment available to slow the progression of the disease. As incidences of dementia are expected to rise in line with the global aging population, identification of strategies

to effectively alleviate symptoms, delay progression, and lessen the socio-economic burden posed by AD have become paramount. Recently, drugs that target the endocannabinoid system (ECS), an endogenous signalling system which consists of cannabinoid receptors, endogenous endocannabinoid ligands and cognate enzymes that produce and degrade endocannabinoids, have shown therapeutic potential in experimental neuroinflammatory and neurodegenerative disease (Bedse et al., 2015). While research into the role of the ECS in AD is still in its infancy, the neuroprotective and anti-inflammatory actions of drugs that directly activate cannabinoid receptors suggest that targeting the ECS should be considered with the search for novel AD therapies.

4.3 CANNABINOID RECEPTORS

Cannabinoid receptors fall into two main categories, namely cannabinoid type 1 receptor (CB_1R) and cannabinoid type 2 receptor (CB_2R). Both of these receptors are members of the Family A G protein coupled receptors and are $G_{i/o}$ coupled receptors (Pertwee et al., 2010). CB_1R is most abundant in the CNS, and in addition to $G_{i/o}$, has been reported to signal via multiple systems including Gαs, Gαq11, β-Arrestin (Soethoudtr et al., 2017). In contrast to CB_1R, CB_2R expression, under non-pathological conditions, is highly localized to immune cells and is limited in CNS tissue primarily to glia, including microglia, (Bisogno et al., 2016). In addition to CB_1R and CB_2R, several other receptors have also been reported to bind cannabinoids, including GPR55, GPR-119, and TRPV1 (Begg et al., 2005; Baker et al., 2006; Overton et al., 2006). Activation of CB_1R in the CNS causes depolarization induced suppression of inhibition and excitation which has been reported to modulate hippocampal synaptic signaling (Straiker and Mackie, 2009). Recent evidence also demonstrates that CB_1R can negatively regulate synaptic plasticity and learning via a hyperpolarization-activated cyclic nucleotide gated (HCN) channel that underlies the h-current (Maroso et al., 2016). Given these actions, targeting CB_2R, which is expressed on microglial cells and known to be involved in neuroinflammation, provides a more credible target for the treatment of AD.

4.4 NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

The neuronal dysfunction seen in AD is primarily attributed to the inflammatory environment in the CNS. This was first indicated by the over-expression of the pro-inflammatory cytokine interleukin-1 β (IL-1β) in the brain of AD patients (Griffin et al., 1989), and the association of reactive glial cells with the expression and formation of Aβ plaques (Sheng et al., 1998). Subsequent investigation, largely through the use of animal models and *in vitro* systems, has consolidated our understanding of AD as an inflammatory-related pathology (Heneka et al., 2015). The role of pro-inflammatory cytokines in regulating neuronal function is widely acknowledged. IL-1β, a prominent contributor to AD pathology (Mrak and Griffin, 2001), is known to promote inflammatory signalling in neurons under acute conditions, and impair hippocampal long-term potentiation (LTP) (Vereker et al., 2000), likely via regulation of AMPA receptor trafficking (Lai et al., 2006).

Prolonged exposure to IL-1β *in vitro* can also induce neurotoxicity, through glial-mediated caspase activity (Thornton et al., 2006). Neuronal dysfunction has been reported in response to tumour necrosis factor α (TNFα), promoting hyper-excitability through enhanced calcium and sodium channel current (Furukawa and Mattson 1998; Gudes et al., 2015), modulation of glutamate receptor activity (Furukawa and Mattson 1998; Stellwagen and Malenka, 2006), and impaired synaptic plasticity (Lyons et al., 2012). When coupled with a complex inflammatory state, the chronic and sustained overexpression of cytokines characteristic of AD pathology likely contribute significantly to the progressive neuronal dysfunction and toxicity associated with cognitive decline.

4.5 MICROGLIA IN ALZHEIMER'S DISEASE

Microglia are the resident immune cells of the CNS, with a primary role in the development and refinement of neuronal networks and the maintenance of cellular homeostasis. This occurs through the synthesis and release of growth factors, neuromodulators and neuropeptides, essential for neuronal function and survival (Tremblay, 2011; Panatier and Robitaille, 2012; Parkhurst et al., 2013). In their quiescent, ramified state, they act as the sentinels of the CNS, surveying their environment for indicators of potential threat, damage or disruption (Nimmerjahn et al., 2005). In response to perceived challenge, however, they react by adopting a hypertrophic, amoeboid morphology. Cell-surface expression of immune-related molecules is enhanced to facilitate damage recognition, microglial mobility, phagocytic function, antigen presentation, and cellular interactions along with the production and secretion of cytokines, chemokines, reactive oxygen and nitrogen species, and other immuno-regulatory factors (Lynch, 2009).

Within the aged brain, microglia have a dystrophic appearance, enhanced sensitivity to inflammatory stimuli and reduced ability to adopt a resolving, anti-inflammatory state. This is thought to be due in part to cellular senescence but also through prolonged exposure to the basal inflammatory environment created largely by the sustained release of inflammatory mediators which accompanies age (Patterson, 2015). In the AD brain there is chronic activation of microglial cells by Aβ. *In vitro* studies have shown that Aβ is a potent activator of microglia, producing cytokines including TNFα, IL-1β and IL-6, chemokines including MCP-1 and IP-10 (Lyons et al., 2012; Barrett et al., 2015a), and modulation of phagocytic function in attempt to promote Aβ clearance (Koenigsknecht-Talboo and Landreth, 2005). A multitude of microglial sensors and innate immune receptors facilitate this interaction, including Toll-like receptors (TLRs), scavenger receptors, nucleotide-binding oligomerization domain-like receptors (NLRs), and the receptor for advanced glycosylation end-products (RAGE); this results in the activation transcription factors such as AP-1 and NFκB, and regulation of inflammatory-associated genes (Doens and Fernandez, 2014; Yu and Ye 2015). The sustained response to persistent activation by Aβ in AD leads to the over expression of inflammatory mediators and compromised microglial phagocytic capacity, further facilitating plaque deposition and thus rendering neurons increasingly vulnerable to damage.

Strategies which target the inflammatory response have to date been the primary method of treatment offered to AD patients. Regrettably, therapies such as non-steroidal anti-inflammatory drugs (NSAIDS), and more specific inflammatory modulators, have proven inconsistent in their ability to attenuate neuroinflammation and alleviate the consequences of AD pathology. Given the combined influence of chronic microglial and immune cell activation on AD pathology, it is tempting to propose that manipulation of their activation state might alleviate the inflammatory impact on the brain. Experimental strategies which attenuate microglial activation by antagonism of inflammatory receptors such as IL-1R (Schmid et al., 2009; Costello et al., 2011) and TLRs (Costello et al., 2011; Barrett et al., 2015a), and application of endogenous anti-inflammatory mediators like IL-4 (Lyons et al., 2007) and fractalkine (Lyons et al., 2009), can reverse the inflammatory- and Aβ-induced impairment in synaptic plasticity. In addition, restoring the quiescent state of microglia, through enhancing the interaction of CD200 with its cognate receptor (Lyons et al., 2012) and promoting the expression of SIGIRR, which attenuates Aβ-mediated inflammatory changes (Barrett et al., 2015a), can alleviate the inflammatory response and subsequent impairment in neuronal function.

4.6 HIPPOCAMPAL LONG-TERM POTENTIATION IN MODELS OF ALZHEIMER'S DISEASE

Long-term potentiation (LTP) is an activity dependent form of synaptic plasticity that is used as a cellular correlate of learning (Bliss and Lomo, 1973). The increased levels of Aβ found in AD cause neuronal dysfunction, leading to both *in vitro* and *in vivo* impairment of LTP and memory formation. Consequently, this form of synaptic plasticity is often used experimentally to investigate potential therapies that may be neuroprotective against Aβ. Attenuation of LTP can be observed *in vitro* following acute Aβ exposure (Freir et al., 2001; Costello et al., 2005; O'Nuallain et al., 2010; Nicoll et al., 2013; Barrett et al., 2015a) and *in vivo* following icv injection of Aβ (Freir and Herron 2003; Schmid et al., 2008). More recently, transgenic mice expressing genes associated with FAD have shown progressive Aβ deposition and attenuation of LTP (Gureviciene et al., 2004; Volianskis et al., 2010; Kelly et al., 2013; Metais et al., 2014). Advanced pathology in these animals, leading to marked reduction in synaptic density and neuronal cell mass, has been widely reported and reviewed (Wirths and Bayer, 2010; Naert and Rivest, 2012). The mechanisms through which neuroinflammatory processes can mediate neuronal dysfunction and death remain to be fully determined.

Glycogen synthase kinase 3β (GSK3β) is known to be involved in hippocampal long-term depression (Peineau et al., 2007) and meta-plasticity (Costello et al., 2012). Reduced expression of inactivated (phosphorylated) GSK3β accompanies the characteristic deficit in LTP observed in transgenic mouse models of Aβ deposition (Martin-Moreno et al., 2012; Metais et al., 2014). This enzyme is also known as tau kinase and is associated with increased levels of hyperphosphorylated

tau that are found in AD. The non-specific general agonist WIN 55,212 was shown to improve levels of inactive (phosphorylated) GSK3β; however, the CB_2R agonist JWH-133 had no effect, suggesting that CB_1Rs may be involved in this signalling cascade (Martin-Moreno et al., 2012).

4.7 CANNABINOID RECEPTORS AS A THERAPEUTIC TARGET IN ALZHEIMER'S DISEASE

Targeting CB_2R activation may be a useful strategy for the treatment of AD. CB_2Rs are expressed predominantly on cells of the immune system, including microglia, where their therapeutic potential has been highlighted (Aso and Ferrer, 2016). In postmortem brain tissue from AD patients and in an AD mouse model, CB_2R expression is upregulated in microglia associated with neuritic plaques (Benito et al., 2003; Savonenko et al., 2015). Due to the increase in microglial CB_2R and the association of microglial processes forming engulfment of synapses with Aβ plaques, it has been proposed that CB_2R expression, assessed through targeted PET scanning, may provide a novel biomarker of neuroinflammation in the early preclinical stages of AD, prior to significant neuronal loss (Savonenko et al., 2015). In contrast, however, a recent study using kinetic modeling of the CB_2R tracer [^{11}C] NE40-PET has suggested that there is a lower availability of CB_2R in AD patients (Ahmad et al., 2016). This decrease may be due to an overall loss of brain tissue volume at the later stage of the disease.

Characterization of the microglial phenotype in AD has demonstrated increased proinflammatory activation, accompanied by indicators of enhanced phagocytosis, including CD68 expression and reduced mobility (Minett et al., 2016). This suggests that despite their attempts at clearance of damaged cellular material, this chronically activated state renders microglia ineffective. In other models of neurodegeneration, treatment with a CB_2R agonist has proven to effectively reduce CD68 expression (Gomez-Galvez et al., 2016). The CB_2R agonist MDA7 (1-((3-benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl) carbonyl) piperidine) also reduced Aβ-induced neuroinflammation in rat hippocampus, along with markers of astrogliosis and inflammation. These changes were accompanied by restoration of synaptic plasticity, cognition, and memory (Wu et al., 2013).

Knock out of CB_2Rs in mice produces a decrease in synaptic transmission, impaired LTP, and a reduction in dendritic spine density (Li and Kim 2016a). CB_2R knock-out mice also display deficits in passive avoidance (Ortega-Alvaro et al., 2011; Garcia-Gutierrez et al., 2013) and contextual fear memory (Li and Kim 2016b). This suggests that tonic activity of this receptor is required for normal synaptic plasticity. Activation of the CB_2R is known to stimulate kinases including Akt and extracellular signal regulated kinase (ERK) (Demuth and Molleman, 2006; Fernandez-Ruiz et al., 2007). Alterations in levels of endocannabinoids in AD could therefore impact on the normal function of the CB_2R to alter the inflammatory response.

CB_2R activation can also reduce the deposition of Aβ, potentially through inhibition of APP cleavage by β-secretase 1 (Chen et al., 2012). Alternatively, this may be due to improved

clearance of Aβ (Tolon et al., 2009) as a result of increased trafficking at the level of the BBB. In the APP2576 mouse model of AD, long-term oral treatment with the non-specific cannabinoid agonist WIN 55212 and/or CB_2R agonist JWH-133 increased the transport of Aβ across the Blood-CSF barrier, thus reducing Aβ deposition (Martin-Moreno et al., 2012). Activation of CB_2Rs may then provide a mechanism to decrease the vicious cycle of microglial dysfunction and offer neuroprotection in AD.

4.7.1 ANANDAMIDE

The neuroprotective effects of the anandamide analogue Dipotassium N-stearoyltyrosinate (NSTK) have been examined in the triple transgenic mouse model of AD. Accompanying an improvement in spatial memory, NSTK decreased the level of Aβ42; reduced oxidative stress; up-regulated Bcl2; and decreased levels of BAX, caspase 3 and the inflammatory cytokines, IL-1β, IL-6, and TNF-α (Liu et al., 2016). Studies in the 5xFAD mouse model of AD have shown that genetic deletion of fatty acid amide hydralase (FAAH) reduced the production of APP and decreased levels of soluble $Aβ_{40/42}$ accompanied by decreased Aβ plaque density. There was, however, no change in the level of activated microglial cells, but an increase in the inflammatory cytokine IL-1β, indicative of an increased inflammatory response (Vazquez et al., 2015). In contrast, however, it has also been shown that in pathological AD brain, FAAH activity is decreased in frontal cortex—an effect that is mimicked by $Aβ_{1\text{-}40}$ (Pascual et al., 2014). In a cellular model of amyloid proteotoxicity, elevation of endogenous AEA is known to impair hippocampal LTP and learning and memory in mice, via activation of the CB_1R (Basavarajappa et al., 2014). It is therefore interesting that in 3xTG AD mice there is a significant decrease in CB_1R immunoreactivity in the dorsal hippocampus and basolateral amygdale at 12 months, with no change in other brain regions, suggesting the involvement of ECS in AD pathology (Bedse et al., 2014).

4.7.2 TETRAHYDROCANNABINOL AND CANNABIDIOL

The potential therapeutic effects of the psychoactive component of cannabis, THC, have also been investigated in a cellular model of AD expressing Aβ. THC was shown to inhibit Aβ aggregation and reduce levels of GSK3β and p-GSK3β (Cao et al., 2014). While this is interesting, the effects of THC at the CB_1R are more likely to produce cognitive deficits due to alteration in neurotransmitter release.

CBD may be an excellent therapeutic agent for AD. In a study of APPswe/PS1dE9 mice, long-term treatment with this naturally occurring phytocannabinoid, CBD, was shown to prevent deficits in social recognition memory, suggesting that this agent may be a useful therapy, particularly in the context of social withdrawal and loss of facial recognition (Cheng et al., 2014). Following icv injection of Aβ, CBD reduces neuroinflammation (Esposito et al., 2011). CBD also rescued spatial memory deficits in the Morris Maze and promoted microglial migration in AD models (Martin-Moreno et al., 2011). In support of the potential therapeutic benefits of CBD, it was also

shown to rescue Aβ-mediated attenuation of LTP in both acute and transgenic models of AD (Hughes and Herron, 2015).

4.8 CANNABINOIDS AND THE BLOOD-BRAIN BARRIER IN ALZHEIMER'S DISEASE: HEMI-CHANNELS AND ASTROCYTES

Systemic inflammatory challenge, such as that posed by pathogens of bacterial and viral origin, has a significant influence on brain function and behavior. It has also been shown that fungal DNA is found in both intracellular and extracellular domains of the AD brain (Pisa et al., 2015). These pathogens are likely to promote microglial activation and the neurodegeneration associated with the disease (Teeling and Perry, 2009; Perry and Teeling, 2013). Evidence accumulating in recent years has highlighted the contribution of infiltrating peripheral immune cells and mediators to neuroinflammation. The presence of T cells has been widely reported in the brain parenchyma of AD patients (Town et al., 2005; McManus et al., 2015), and their infiltration has been shown to promote pathology in models of the disease (Browne et al., 2013; McManus et al., 2014; Laurent et al., 2017). Macrophages also display enhanced sensitivity to inflammatory stimuli in aged and AD animal models, and those which infiltrate the brain parenchyma are thought to exacerbate the inflammatory environment and contribute to the characteristic neuronal and cognitive dysfunction (Barrett et al., 2015a, 2015b; Costello et al., 2016; Martin et al., 2016). This is likely facilitated by a progressive deterioration in the integrity of the BBB, under chronic inflammatory conditions such as those seen in AD and models of AD-like pathology (Ryu and McLarnon, 2009; Viggars et al., 2011; Kelly et al., 2013; Minogue et al., 2014).

While initial studies could not identify CB receptors on astrocytes, more recent studies have provided overwhelming evidence that both CB_1R and CB_2R are present (Molina-Holgado et al., 2002; Sheng et al., 2005; Oliveira da Cruz et al., 2016). Cannabinoid receptors are also known to modulate the activity of astrocytic hemi-channels. These plasma membrane channels act as aqueous pores that are permeable to ions and small molecules, providing a pathway for diffusional exchange of small molecules between intracellular and extracellular compartments. These channels comprise a 6-fold ring of connexin monomers. Activation of microglia by inflammatory stimuli including lipopolysaccaride (LPS) is reported to cause opening of CX43 hemi-channels (Froger et al., 2009). Aβ is known to induce neuronal death by causing excitotoxic release of ATP and glutamate via hemi-channel opening in glial cells (Orellana et al., 2011). It has been shown recently that the cannabinoid agonist WIN 55,212, 2AG and methanandamide can prevent Aβ-mediated hemi-channel activity and the inflammatory profile in astrocytes to prevent the release of excitotoxic glutamate and ATP. In addition, the cannabinoid agonists also reduce Aβ-mediated production of NO, IL1-β, and TNFα associated with hemi-channel activity in astrocytes and decrease neuronal damage caused by Aβ in acute hippocampal slices (Gajardo-Gomez et al., 2017). Aβ-mediated opening of

astroglial hemi-channels releasing ATP and glutamate may facilitate neuronal degeneration via a mechanism linked to activation of P2x7 and NMDA receptors and subsequent opening of Panx1 hemi-channels in neurons (Orellana et al., 2011).

4.9 THERAPEUTIC IMPLICATIONS FOR CANNABINOIDS

Targeting the ECS for the treatment of AD, especially the CB_2R, may offer a novel approach. Activation or inhibition of CB_1R is likely to cause further memory disruption due to the modulatory effects of this receptor on the release of excitatory and inhibitory neurotransmitters. However, activation of CB_2R on microglia and astrocytes, in addition to the use of non-psychoactive components of cannabis, e.g., cannabidiol, may offer novel therapies for this devastating disease. As CB_2Rs are involved in the immune response, further research to determine how modulation of this receptor and the impact it may have *in vivo* will be required prior to clinical trials.

CHAPTER 5

The Endocannabinoid System's Role in Ocular Inflammation

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Abstract

The endocannabinoid system (ECS) plays a role in the modulating of physiological, pathological, and homeostatic functions including the immune system and its response. Components of the ECS are localized within the eye; this includes the cognate enzymes, receptors, and endogenous ligands. Recent evidence has implicated the ECS in ocular inflammatory disease, including uveitis, proliferative vitreoretinopathy, and diabetic retinopathy. Current clinical treatments for these ocular diseases can have limited efficacy, be refractory, and have significant side-effects. The ECS presents a potential novel target to mitigate the ocular inflammatory response. This chapter will discuss ocular inflammatory disease and review recent evidence which supports therapeutic targeting of the ECS in ocular inflammation.

Key Words

endocannabinoids, ocular inflammation, experimental autoimmune uveoretinitis, endotoxin-induced uveitis, diabetic retinopathy, proliferative vitreoretinopathy

Abbreviations

2-AG	2-arachidonoylglycerol
AEA	anandamide
AH	aqueous humor
AP-1	activator protein-1
CBD	cannabidiol
CB_1R	cannabinoid 1 receptor
CB_2R	cannabinoid 2 receptor
$CB_2R^{-/-}$	cannabinoid 2 receptor knockout

CNS	central nervous system
DAGL	diacylglycerol lipase
DAMPs	danger-associated molecular patterns
DCs	dendritic cells
DR	diabetic retinopathy
EAU	experimental autoimmune uveoretinitis
eCBs	endocannabionids
ECS	endocannabinoid system
EIU	endotoxin-induced uveitis
FAAH	fatty acid amide hydrolase
HI	high glucose
HREC	human retinal endothelial cells
ICAM-1	intracellular adhesion molecule-1
IL	interleukin
IL-1R α	interleukin-1 receptor α
i.p.	intraperitoneal
i.v.	intravenous
IOP	intraocular pressure
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MHC	major histocompatibility complex
MIF	macrophage inhibitory factor
NAEs	N-acylethanolamines
NAPE-PLD	N-acyl-phosphatidylethanolamine-hydrolyzing-phospholipase-D
NF-κB	nuclear factor-kappa B
NPDR	non-proliferative diabetic retinopathy
PDR	proliferative diabetic retinopathy
PLC	phospholipase C
PNS	peripheral nervous system
PPAR	peroxisome proliferator-activated receptor;
PVR	proliferative vitreoretinopathy
ROS	reactive oxygen species
RPE	retinal pigmented epithelial
TGF-β2	transforming growth factor-β2
TNF-α	tumor necrosis factor-α
TRPV1	transient receptor potential vanilloid 1
TUNEL	terminal deoxynucleotidyltransferase-mediated nick-end labeling

VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

5.1 THE ENDOCANNABINOID SYSTEM AND MARIJUANA

While identification of the ECS and the actions of marijuana at cannabinoid receptors has only been documented in the last half a century, the use of marijuana for therapeutic purposes is not novel (Berdyshev, 2000; Pacher et al., 2006; Vemuri et al., 2008; Yazulla, 2008). *Cannabis sativa* and *Cannabis indica*, collectively referred to as cannabis, are two distinct species of marijuana (Pearce et al., 2014). Cannabis is widely known for its psychoactive properties and illicit abuse, and has documented medicinal use as far back as Ancient China, 5,000 years ago (Hanuš, 2009; Tomida et al., 2004). The effects of ingesting and inhaling cannabis are known to induce euphoria, sedation, analgesia, reduce cognitive function and motor coordination, as well as increase appetite (Howlett et al., 2004). The analgesic and potential anti-inflammatory properties of cannabis were beginning to be investigated by the 19th century. Therapeutic efficacy was demonstrated in tetanus associated muscle spasms, insomnia, dysmenorrhea, gonorrhea, migraine, and rheumatic disease (Mikuriya, 1969; Hanuš, 2009). Nevertheless, the increased use of opiates, providing superior analgesia compared to cannabis along with the increased recreational misuse of cannabis, led to cannabis being removed from the national formulary and pharmacopedia in the U.S. in 1941 (Mikuriya, 1969). Cannabis-based medicine continues to remain a controversial subject, with few cannabinoids approved for use worldwide (Gaoni and Mechoulam, 1964; Pertwee et al., 2010; Yazulla, 2008). However, the identification of Δ^9-tetrahydrocannabinol (Δ^9-THC) in the 1960's (Gaoni and Mechoulam, 1964), along with cannabidiol and the identification of the ECS (Devane et al., 1988; Matsuda et al., 1990) (reviewed in Pertwee et al., 2010), continues to pave the way for further discoveries that highlight the importance of the ECS and the therapeutic benefits of targeting this system.

In the eye, the role of the ECS to date has largely been focused on the ocular hypotensive effects of cannabinoids; ingestion of cannabis and exposure to THC reduces intraocular pressure (IOP). Lowering of IOP reduces disease progression in glaucoma, a blinding eye-disease, in which loss of retinal ganglion cells results in vision loss (reviewed in Yazulla, 2008; Cairnes et al., 2016a; Kokona et al., 2016). More recently, there has been additional interest in the role of the ECS in the modulation of ocular inflammatory diseases (reviewed in Toguri et al., 2016). This chapter will discuss evidence for the localisation of components of the ECS within the eye, the ocular inflammatory response, ocular inflammatory diseases, and the therapeutic implications of modulating the ECS in animal models of ocular inflammation.

5.2 ENDOCANNABINOID SYSTEM IN OCULAR TISSUE

Elements of the ECS are localized to ocular tissue: this includes enzymes required to biosynthesize (N-acylethanolamines (NAEs), phospholipase C (PLC), diacylglycerol lipase (DAGL)) and

degrade (fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL)); endogenous cannabinoids (reviewed by Wang and Ueda, 2009; Di Marzo et al., 2015; Hillard, 2015), as well as key endocannabinoids (eCBs), 2-arachidonoylglycerol (2-AG), and N-arachidonoyl ethanolamine (anandamide; AEA); and receptors that bind these eCBs. The cannabinoid receptors, which bind the eCBs, AEA, and 2-AG, are CB_1R and CB_2R. However, eCBs can also bind to and activate non-CB_1R/CB_2R cannabinoid-associated receptors (G-protein receptor 18 (GPR18); GPR55; transient receptor potential cation channel; subfamily V; member 1 (TRPV1); peroxisome proliferator-activated receptor α (PPAR α); and PPAR β/δ).

The presence of the ECS in the eye has been reviewed in detail by others (Yazulla., 2008; Cairns et al., 2016b; Kokona et al., 2016). Endocannabinoids are found extensively throughout the mammalian eye (rat, pig, human, bovine), including the iris, trabecular meshwork, ciliary body, and retina (Cairns et al., 2016b). Interestingly, the cognate enzymes, DAGL, N-acyl-phosphatidylethanolamine-hydrolyzing-phospholipase-D (NAPE-PLD), FAAH, and MAGL have only been identified in the trabecular meshwork and the retina (Njie et al., 2008; Yazulla, 2008; Hu et al., 2010; Cécyre et al., 2013; Cairns et al., 2016b). CB_1R has been localized throughout the eye, including the retina, and activation of CB_1R has been demonstrated to modulate retinal synaptic transmission (Straiker et al., 1999a; Bouskila et al., 2012; Cécyre et al., 2013, 2014; reviewed in Yazulla, 2008). Additionally, increases in the eCB AEA, in the retina following treatment with the FAAH inhibitor, URB597 is associated with increased neuronal survival following optic nerve injury and this action was mediated primarily via CB_1R (Slusar et al., 2013). Cannabinoids that activate CB_1R, such as HU210, as well as Δ^9-THC and WIN55212-2, have also been reported to provide retinal neuroprotection and decrease neuroinflammation (Pryce et al., 2003; Rossi et al., 2011a, 2011b). In addition to retina, CB_1R mRNA and protein are found in the cornea, iris, trabecular meshwork, Schlemm's canal, ciliary body, choroid (Porcella et al., 2000; Stamer et al., 2001; Straiker et al., 1999a,b; reviewed in Yazulla., 2008; Tomida et al., 2004; Nucci et al., 2007; Cairns et al., 2016a,b), and activation of CB_1R in anterior tissue results in decreases in IOP, mediated in part via decreased adrenergic tone with a resultant decrease in AH secretion and/or increased AH outflow (Hudson et al., 2011; Caldwell et al., 2013; reviewed in Cairns et al., 2016a,b).

In contrast to broad distribution of CB_1R, expression of CB_2R in the ocular tissues eye is limited. CB_2R is expressed by macrophages, dendritic cells and microglia, the resident immune cells in the eye, and retinal glia in primates (Bouskila et al., 2013). In the rat retina, CB_2R mRNA has been reported as localized in the photoreceptors, inner nuclear layer, and ganglion cell layer, while immunohistochemistry identified CB_2R protein in the retinal pigmented epithelial (RPE) cells, photoreceptors, horizontal, and amacrine cells (López et al., 2011). As immunomodulatory activity has been reported for both CB_1R and CB_2R, drugs that directly or indirectly target these receptors may have potential in modifying the ocular inflammatory response.

5.3 THE OCULAR INFLAMMATORY RESPONSE AND THE ENDOCANNABINOID SYSTEM

Dysregulated ocular inflammation can lead to tissue damage resulting in decreased visual acuity and even loss of vision (Caspi, 2010). The eye is one of a limited number of organs that has immune privilege which is protective from vision loss resulting from tissue damage caused by the immune response (Caspi, 2006; Streilein, 2003a; Taylor, 2009, 2016; Taylor and Kaplan, 2010). In the eye, immune privilege is attributable to both physical boundaries and immunological factors. The physical barriers which contribute to the ocular immune privilege include the blood-aqueous barrier, blood retinal barrier, and the lack of lymphatic vessels (Taylor, 2009). The immunological factors include the release of anti-inflammatory mediators (macrophage inhibitory factor (MIF), interleukin-1 receptor α (IL-1R α), transforming growth factor β2 (TGF-β2) (Streilein, 2003b)) into the anterior chamber to suppress the immune response (Streilein, 2003a).

In addition to reducing immune cell recruitment, some cells, such as corneal epithelial cells, lack or have decreased expression of the major histocompatibility complex (MHC) I and II. The MHC recognizes antigens of foreign pathogens and contributes to initiating the immune response (McMenamin, 1997; Streilein, 2003b). The eye contains components of both the peripheral nervous system (PNS) and the central nervous system's (CNS). Immune cells of PNS are localised to the cornea, iris, ciliary body, choroid, and sclera, while the immune cells of the CNS are found in the retina. Peripheral immune cells include macrophages, mast cells, lymphocytes (T cell $CD5^{+}$), dendritic cells (DCs) and Langerhans cells, and epithelial dendritic cells that are located in the cornea (McMenamin, 1997; Hamrah et al., 2002). The immune cells of the retinal tissue include DCs, and microglia, and specialized macrophages. Macrophages and DCs are antigen presenting cells, which act as sentinels activating the immune system when stimulated by antigens or damage-associated molecular patterns (DAMPs) released from cells following tissue injury (Bianchi, 2007; Wakefield et al., 2010). Macrophages and DCs phagocytize foreign bodies, damaged or infected host cells marked for apoptosis, release inflammatory mediators, and stimulate other immune cells to proliferate and differentiate through antigen presentation (Akpek and Gottsch, 2003).

Activation of tissue resident immune cells results in the release of pro-inflammatory mediators stimulating the innate or adaptive immune system. These inflammatory mediators include cytokines, chemokines, and adhesion molecules. Together, these factors activate, recruit, and play an integral role in leukocyte-endothelial cell interactions, one of the initial processes during inflammation. Following the recruitment of immune cells, they undergo transmigration into the tissue to interact and phagocytise pathogens. During this leukocyte transendothelial migration process, tissue damage can occur due to the breakdown of the endothelium and the release of chemical compounds.

The CB_2R has been identified throughout the immune system. Each immune cell subset has been shown to have varying levels of CB_2R mRNA. Galiègue et al. (1995) describes the expression

of CB_2R mRNA levels in B-cells > natural killer cells > monocytes > polymorphonuclear cells > CD 8 T cells > CD 4 T cells. Additionally, CB_2Rs are found on APCs, including macrophages and dendritic cells, which play an intrinsic role in the immune response (Adhikary et al., 2012; Matias et al., 2002). In several models of inflammation, the expression of CB_2R has shown to be increased on immune cells and throughout inflamed tissue (Carlisle et al., 2002; Mukhopadhyay et al., 2006; Kimball et al., 2010; Concannon et al., 2015). As such, the ECS could be an important target since it could influence the immune response of both tissue resident immune cells and those recruited during inflammation. The role of the ECS and modulation of this system could be of potential therapeutic benefit in ocular inflammatory diseases; this will be discussed below with respect to specific ocularpathologies.

5.4 DISEASES ASSOCIATED WITH OCULAR INFLAMMATION

5.4.1 UVEITIS

Uveitis is a diverse group of ocular inflammatory conditions affecting the middle layer of the eye known as the uvea. The uvea is comprised of the iris, ciliary body, and choroid. Inflammation is not limited to the uvea and may spread to the vitreous and retina. Uveitis is diagnosed and named according to which anatomical location of the eye is inflamed: anterior (iris, ciliary body), intermediate (ciliary body, vitreous), posterior (choroid), or panuveitis (iris, ciliary body, vitreous, choroid, retina) (Prete et al., 2016). Severity and symptoms vary in degree depending on type and location of the inflammation. These symptoms can include decreased vision, pain, red eye, photophobia (sensitivity to light), and epiphoria (increased tearing) (Durrani et al., 2004a; Forrester, 1991). Unlike other sight-threatening disorders that dramatically increase with age, uveitis affects all ages from childhood and peaks in the working adult age group, severely impacting quality of life (Durrani et al., 2004b; Williams et al., 2007). Uveitis can be acute or chronic in nature, depending on the underlying etiology. Underlying causes of uveitis include infectious and idiopathic systemic autoimmune disease, such as rheumatoid arthritis, sarcoidosis, and multiple sclerosis (Durrani et al., 2004a). The first-line treatments for uveitis are corticosteroids; the route of administration can vary from systemic, to topical, to intraocular injection, depending on disease severity (Prete et al., 2016). Unfortunately, uveitis can be highly tolerant to corticosteroid therapies that are associated with significant side effects such as cataracts, decreased wound healing, and increased intraocular pressure. Each of these side effects have their own risk factors affecting sight and ocular health resulting in a patient population who do not receive adequate treatment for their uveitis. Due to significant sight threatening side effects, several novel therapies are being explored, including cannabinoids. To date, there are no cannabinoid-based therapy studies or case reports involving humans and uveitis. However, recently the use of cannabinoids as a therapeutic treatment for uveitis has been investigated in

several animal models (Altinsoy et al., 2011; El-Remessy et al., 2006, 2011; Szczesniak and Kelly, 2012; Toguri et al., 2014, 2015; Xu et al., 2007).

Endotoxin-Induced Uveitis and Experimental Autoimmune Uveoretinitis

Uveitis is one of the most extensively studied ocular inflammatory diseases. Animal models of uveitis have been created in mice, rats, and rabbits (reviewed by Caspi, 2006a). Models vary in the anatomical region affected and the duration and course of inflammation, depending on how uveitis is induced. A model of chronic uveitis, experimental autoimmune uveoretinitis (EAU) involves activation of the adaptive immune system. The ocular autoimmune response is generated by the injection of complete Freund's adjuvant and retinal antigens (reviewed in Bose et al., 2016). A model of acute uveitis, endotoxin-induced uveitis (EIU), is generated by the injection of lipopolysaccharide (LPS), a cellular wall component of Gram-negative bacteria, which results in an anterior or panuveitis that, depending on the extent of the inflammatory response of the animal, can resolve over time (Baatz et al., 1995; McMenamin and Crewe, 1995; Taylor, 2009).

Cannabinoids for the Treatment of Uveitis

Cannabinoid-based therapies for the treatment of uveitis have only recently been explored, however, several animal models appear to be promising. CB_1R and CB_2R ligands for the treatment of uveitis have been examined in mice (Xu et al., 2007), rats (Toguri et al., 2014, 2015) and rabbits (Altinsoy et al., 2011). Using a chronic uveitis model, Xu et al. (2007) reported the beneficial effects of the CB_2R agonist, JWH 133, in a model of EAU. Following intravenous administration of JWH 133, leukocyte infiltration was significantly diminished in the retinal microvasculature and there was a decrease in cytokine and chemokine production. The clinical score and histological score of EAU were decreased following JWH 133 administration. Experiments conducted *in vitro* provided insight into the potential mechanism of JWH 133. When applied to stimulated T cells, JWH 133 produced decreased proliferation and antigen presentation (Xu et al., 2007).

In an alternative acute model of uveitis, EIU, both pro-inflammatory and anti-inflammatory effects were reported following exposure to cannabinoids. In rabbits that received intraocular LPS, the co-administration of AEA and LPS was reported to result in a significant increase in the clinical score associated with ocular inflammation, the number of leukocytes, and the amount of protein observed in the AH (Altinsoy et al., 2011). This pro-inflammatory effect of AEA was attributed to CB_1R activation. However, in combination with the CB_1R antagonist, AM251, AEA now inhibited the recruitment of leukocytes to the AH to levels comparable to those produced by LPS alone. Administration of AM251 did not result in a change of the clinical grade following EIU (Altinsoy et al., 2011). These results suggest that in the presence of a block of CB_1R, the reduction in leukocyte recruitment may result from AEA activation of non-CB_1R targets. For example, in addition to CB_1R, AEA is also an agonist at CB_2R (Davis, 2014), TRPV1 (Ross et al., 2001), and GPR55 (Ryberg et al., 2007). In contrast to Altinsoy and colleagues, Toguri et al. (2014) demonstrated

an anti-inflammatory effect of cannabinoids in experimental uveitis. The authors determined that CB_2R agonist, HU308, decreased ocular inflammation induced by an intravitreal LPS injection in rats. Topical administration of HU308 significantly decreased leukocyte-endothelial adhesion in the iridial microvasculature. The decrease in leukocyte adhesion was attributed to CB_2R mediated inhibition of mRNA transcription of NF-κB and AP-1, with subsequent decreases in the pro-inflammatory mediators tumor necrosis factor-α (TNF-α), IL-1β, and IL-6. Administration of the CB_2R antagonist, AM630, exacerbated leukocyte-adhesion during EIU and increased expression of NF-κB mRNA; however, protein levels of pro-inflammatory mediators, which were measured, were not increased compared with animals treated with LPS alone. Taken together, the results of Xu et al. (2007) and Toguri et al. (2014) provide compelling evidence that CB_2R activation during uveitis may be therapeutically beneficial in reducing ocular inflammation.

CB_1R and CB_2R activation was also found to be anti-inflammatory in uveitis induced by systemic LPS administration (Toguri et al., 2015). Here, the non-specific cannabinoid agonist WIN55212-2—alone, or in combination with the CB_2R antagonist, AM630, or the CB_1R antagonist, AM281—decreased leukocyte adhesion in the iridial microcirculation. These anti-inflammatory effects appeared to be affected in part by microvasculature diameter as the activation of either cannabinoid receptor resulted in different levels of leukocyte adhesion in microcirculatory vessels of greater or less than 25 μm. While WIN55212-2, alone or in combination with AM281, resulted in activation of CB_2R and reduced leukocyte adhesion throughout the microvasculature, CB_1R activation only reduced leukocyte adhesion in vessels of less than 25 μm. This effect was attributed to vasodilation and a decrease in shear forces within these smaller vessels of the microcirculation. Changes in the iridial microvascular blood flow were not caused by cannabinoid alterations in systemic hemodynamics. Collectively, these results implicate a promising cannabinoid-based target for the treatment of uveitis. The anti-inflammatory actions appear to be complex and involve predominately CB_2R, however, CB_1R and non-CB targets may also play a role.

5.4.2 PROLIFERATIVE VITREORETINOPATHY

Proliferative vitreoretinopathy (PVR) is a non-specific complication of retinal reattachment surgery or ocular trauma, which is associated with poor visual outcomes due to scarring of the retina (decreased visual field and acuity; Pastor et al., 2002). PVR occurs when the retina fails to attach or reattach and has an over exaggerated inflammatory response (Pastor, 1998). Retinal breaks commonly caused by vitreous traction can promote both anterior and posterior proliferation (Pastor, 1998, Kwon et al., 2016). Treatment for PVR is mostly surgical (Adelman et al., 2013, Storey et al., 2014, Kwon et al., 2016); however, currently there are several clinical trials evaluating pharmacologic approaches to reduce cellular proliferation that have seen some success with intravitreal 5-fluorouracil and heparin (Asaria et al., 2001, Kwon et al., 2016). Surgical success for the treatment of PVR is poor, with approximately 25% of patients having a recurring retinal detachment (Kwon et al., 2016). Furthermore, current treatments do not address the increase in inflammatory mediators

that promote cell proliferation and poor visual outcomes. For these reasons there is a need for the development of both primary and adjunctive therapies that target the full pathogenesis of PVR (Pastor et al., 2002, Kwon et al., 2016, Garweg et al., 2013).

Animal Models of Proliferative Vitreoretinopathy

Proliferative Vitreoretinopathy (PVR) has been modeled in rabbits (Frenzel et al., 1998), rats (Zheng et al., 2009), and mice (Cantó Soler et al., 2002). These models are generated by surgical interventions or injection of cellular (fibroblasts, RPE cells) or non-cellular components (platelet-derived growth factors, fibronectin or dispase) into the intravitreal cavity. Intraocular injection of these factors results in different time-courses and pathologies. *In vivo* models of PVR have been extensively reviewed by Agrawl et al. (2007).

Cannabinoids for the Treatment of PVR

Recently, synthetic cannabinoid treatments have been tested by Szczesniak et al. (2016) using a dispase model of PVR in the mouse. Two experimental protocols were used to investigate the acute (24 hours) and chronic (one week) effects of cannabinoid administration during PVR. Animals in the acute study arm were injected with the CB_2R agonist, HU308, or the CB_2R antagonist, AM630, at 0 and 12 hr. Animals in the chronic study received HU308 (intraperitoneal), or AM630 for one week following induction of PVR. Intravital microscopy of the iridial microcirculation was used to quantify leukocyte-endothelial adhesion (24 hr after dispase). Following administration of the CB_2R agonists, HU308, leukocyte adhesion was significantly reduced compared to those animals with vehicle treatment, while the CB_2R antagonist, AM630, severely exacerbated the immune response (Szczesniak et al., 2016). Chronic PVR experiments investigated gross changes in histological morphology, immunohistochemical staining (Iba-1 for microglia and macrophages, and anti-GFAP for astrocytes), and mRNA levels of inflammatory markers, including Iba-1, GFAP, CD68 (monocytes/macrophages), Ly6G (neutrophils), IL-1β, and IL-6. Treatment with the CB_2R agonist, HU308, decreased clinical histological scoring and retinal glia activation compared to dispase injection alone; and these actions were blocked by the CB_2R antagonist, AM630 (Szczesniak et al., 2016). Pharmacological block of CB_2R was associated with a significantly more severe inflammatory response. Consistent with this, genetic knockout of $CB_2R^{-/-}$ resulted in exacerbated retinal pathology, as per histopathological score and the number of activated microglia and macrophages within the retina, in PVR. Cannabinoid 2 receptor knockout ($CB_2R^{-/-}$) mice also displayed increased expression of mRNA for Ly6G and IL-1β (Szczesniak et al., 2016). While these results are promising, further investigation of the use of cannabinoids must also occur in alternative models of PVR to corroborate these findings.

5.4.3 DIABETIC RETINOPATHY

Diabetic retinopathy (DR) is a leading cause of blindness worldwide affecting approximately 60% of adults with diabetes mellitus (Stewart 2016; Bolinger and Antonetti, 2016, American Academy of Ophthalmology, 2016). DR remains a significant world health problem as the number of individuals living with diabetes is expected to grow to 642 million people by the year 2040 (*IDF Diabeties Atlas 2015*-7th Edition, 2015 at http://www.diabetesatlas.org/resources/2015-atlas.html). The clinical manifestations of DR depend on their classification as non-proliferative (NPDR) or proliferative (PDR) and are driven by inflammation (Antonetti et al., 2006). NPDR is characterized by microaneurysms, exudate deposits, basement membrane thickening, and microhemorrhages. PDR includes all of the signs of NPDR in addition to pathologic neovascularization of the iris and retina (Bolinger and Antonetti, 2016; Davidson et al., 2007; American Academy of Ophthalmology, 2016). This pathology results in DR being a significant sight-threatening disease, with loss of vision occurring from: macular edema (accumulation of fluid and/or blood in a normally avascular area primarily responsible for visual acuity), vitreous hemorrhages, or retinal detachment (Davidson et al., 2007; Gardner et al., 2009; American Academy of Ophthalmology, 2016). Treatment of DR includes: laser photocoagulation, intravitreal anti-vascular endothelial growth factors (VEGF) treatment, and to a lesser extent, corticosteroids (Bolinger and Antonetti, 2016; Gardner et al., 2009; Gibson and McGinnigle, 2016; Gross et al., 2015; Morello, 2007; Olsen, 2015; American Academy of Ophthalmology, 2016; Stewart, 2016). Despite the success of anti-VEGF treatment, there remains a population refractory to this therapy (Bolinger and Antonetti, 2016). There are several promising potential therapeutic targets for DR, including TNF-α agents, Kinin-Kallikrein system inhibitors, renin-angiotensin system inhibitors, and cannabinoids (reviewed by Bolinger and Antonetti, 2016; Kokona et al., 2016; Yazulla et al., 1999). The implication of the ECS in DR treatment can be, in part, attributed to a study conducted by Matias et al. (2006), which showed increased levels of AEA and 2-AG in patients with DR.

Animal Models of Diabetic Retinopathy

Several animal models for diabetic mellitus exist. These models include diet-induced diabetic mellitus, genetic manipulation, and streptozotocin–induced diabetes (reviewed by Jiang et al., 2015).As in DR or PDR in humans, the animal models of diabetes mellitus are characterized by elevated levels of inflammatory mediators including eicosanoids, lipids, adhesion molecules, integrins, VEGF, cytokines and chemokines, complement activation, and pro-inflammatory transcription factors (Liou et al., 2009; Brucklacher et al., 2008; Tang and Kern, 2011).

Cannabinoids for the Treatment of DR

Several studies have been conducted to evaluate the potential of cannabinoids for the treatment of DR. El-Remessy et al. (2006) evaluated the effect of (-)-cannabidiol (CBD) administration on retinal cell survival in a streptozotcin-induced model of diabetes. CBD is a non-psychotropic phytocannabinoid that acts as a negative allosteric modulator of CB_1R and an agonist at CB_2R

(LaPrairie et al., 2015), with additional actions at adenosine A2A, TRPV1, GPR55 and 5-HT receptors (reviewed by Burstein, 2015; Pertwee et al., 2010). Chronic administration with CBD decreased vascular permeability in the retinal parenchyma when measured at two weeks, and retinal cell apoptosis by four weeks. CBD also reduced both oxidative and nitrative stress as measured by lipid peroxide levels and the concentration of malondialdehyde, respectively. Increases in VEGF and intracellular cellular adhesion molecule-1 (ICAM-1) expression, which are associated with DR, were decreased at weeks two and four following the treatment of CBD. To investigate the mechanism of action El-Remessy et al. (2006) tested the effects of CBD on p38 MAP kinase, which modulates neuronal cell death and vascular permeability. Protein expression for phosphorylated p38 MAP kinase in diabetic retinas was decreased by CBD treatment at two and four weeks.

El-Remesy et al. (2011) investigated the role of the CB_1R in mice using the streptozotocin DR model, with either the CB_1R antagonist, SR141716A (rimonabant 10 mg/kg/day i.p.), or in cannabinoid receptor 1 knockout mice ($CB_1R^{-/-}$). No difference in blood sugar levels or weight was observed when wild type and $CB_1R^{-/-}$ mice were compared. Retinal cell death, termed apoptosis, following the induction of diabetes was significantly inhibited in $CB_1R^{-/-}$ mice, as indicated by terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) staining. Treatment of diabetic animals with CB_1R antagonist, SR141716A, also decreased TUNEL staining, as compared to vehicle treated animals. To investigate the mechanism by which CB_1R inhibition might be eliciting its protective effects, El-Remessy et al. (2011) measured markers of oxidative and nitrative stress. Both 3-nitrotyrosine and reactive oxygen species (ROS) were elevated in diabetic mouse retinas and were reduced following treatment by SR141716A. These results—the reduction of 3-nitrotyrosine and ROS following inhibition of CB_1R—were confirmed *in vitro* in human retinal endothelial cells (HREC) stimulated by high glucose-induced oxidative stress (El-Remessy et al., 2011). *In vivo* endothelial cells were co-localized with staining for NF-κB following induction of diabetes. The observed increase in NF-κB was inhibited by the SR141716A. CB_1R inhibition also reduced glial cell activation throughout retinas from animals with diabetes. Vascular components of DR involve increases in adhesion molecules, including ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), in retinal capillaries. Following SR141716A treatment, the relative levels of adhesion molecules in the retina did not significantly differ compared to animals without diabetes. The inhibition of ICAM-1 and VCAM-1 release by CB_1R inhibition was confirmed using HRECs in high-glucose media. The reduction of adhesion molecules from HRECs in high-glucose conditions was attributed to SR141716A inhibiting the phosphorylation of both p38 MAP kinase and JNK demonstrated by Western blot (El-Remessy et al., 2011).

Lim et al. (2012) investigated the role of FAAH, the enzyme that degrades AEA, and CB_1R on apoptosis of RPE cells caused by high glucose (HG) concentration. Following RPE death, tight junctions, and thus the retinal blood barrier, are broken, exacerbating DR. The human RPE cell line, ARPE-19, exposed to HG media, had significantly decreased FAAH I at the three time-points investigated (12, 24, and 48 hr) while CB_1R mRNA and protein were increased. HG induced CB_1R

internalization by endocytosis in HEK cells, which was inhibited by overexpression of FAAH I. The internalization of CB_1R protein was inhibited by treatment with the CB_1R antagonist AM251. FAAH overexpression inhibited endocytosis of the transcription and translation of CB_1R. The transfection of FAAH I or application of AM251 to ARPE-19 cells inhibited the generation of ROS, which was induced by HG media. Exposing cells to HG media, resulted in an increase of cytochrome c release, a measure of mitochondrial injury which was inhibited by the overexpression of FAAH I. This indicates that overexpression of FAAH I and CB_1R antagonism is beneficial in reducing cell death caused by HG formation of ROS and mitochondrial injury. The proposed mechanism of action occurs by HG decreasing FAAH I, and results in elevated levels of AEA which activate CB_1R and results in receptor internalization. These results could explain the increase in AEA and 2-AG that are seen in human ocular tissue from individuals who had DR (Matias et al., 2006).

5.5 CONCLUSION

Current experimental evidence indicates that the ECS could be a potential target for the treatment of ocular inflammation. CB_2R activation (which reduces immune cell activation, migration, and proliferation) inhibits activation of pro-inflammatory cellular cascades and decreases pro-inflammatory transcription factors and resultant cytokines, chemokines, and adhesion molecules. In contrast to CB_2R, evidence for efficacy for CB_1R activation in mitigating ocular inflammation is less clear with both anti-inflammatory (Toguri et al., 2015) and pro-inflammatory ocular actions (Altinsoy et al., 2011) reported. Furthermore, experimental evidence from models of DR indicates that inhibition of CB_1R may in fact be beneficial; use of the CB_1R antagonist, SR141716 (rimonabant), reduced apoptosis of retinal cells, ROS formation, and NF-κB activation (El-Remessy et al., 2011). These findings are supported by data from *in vitro* models, where use of the CB_1R antagonist, AM251, or overexpression of FAAH enzyme, with consequential reduction in the endocannabinoid, AEA, inhibited upregulation of CB_1R, ROS, and lipid peroxide formation and apoptosis following HG exposure. While some cannabinoids, such as CBD, may exert their ocular anti-inflammatory actions via both CBR and non-CBR targets, these targets still remain to be clearly identified, and the composite actions of these non-pyschotropic cannabinoids determined.

Although the ocular hypotensive actions of THC have been known since the 1970's, the field of ocular cannabinoid therapeutics is still in its infancy, and further investigation of the effects of the pharmacological effects of cannabinoids on ocular inflammation must occur (Yazulla et al., 2008; Tomida et al., 2004; Nucci et al., 2007; Cairns et al., 2016a,b). Current results appear promising. However, to achieve successful and efficacious treatment of ocular inflammation by ECS modulation, further research must identify the cell-specific molecular mechanisms that underlie both beneficial and detrimental effects of manipulating the ECS. Furthermore, it is imperative that research is conducted to explore efficacy, dosing, toxicity, tachyphylaxis, and appropriate formulation for ocular ECS targeted drugs in order to best tailor therapy to disease.

CHAPTER 6

Cannabidiol as a Potential Clinical Therapeutic Agent for the Reduction of Pancreatic Inflammation in Early Type 1 Diabetes Mellitus

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Abstract

Type 1 diabetes (T1D) prevalence is rapidly increasing and is placing a greater strain on the health care system than ever before in recorded history. T1D is an autoimmune disorder, initiated by the activation of T-cells, resulting in the destruction of insulin-producing beta cells in the pancreas. Cannabidiol (CBD), a non-psychoactive cannabinoid, has been shown to suppress cell-mediated autoimmune responses and has been reported to show therapeutic potential in the prevention of T1D. Histological analyses revealed that CBD administration could decrease the severity of pancreatic inflammation and maintained beta cell function. Prophylactic CBD treatment has shown a reduction in the incidence of T1D onset in experimental animal studies; however, continuous CBD administration is likely required for long-term prevention of T1D. Cannabinoids have been found to elicit far fewer severe adverse effects than conventional immunosuppressive therapies. Due to the safety and effectiveness, the use of CBD for the prevention or early treatment of T1D should be considered in experimental and clinical studies.

Key Words

type 1 diabetes, cannabidiol, intravital microscopy, rolling leukocyte, adhering leukocyte, functional capillary density

6.1 INTRODUCTION

Type 1 diabetes (T1D) is becoming more prevalent and is placing a greater strain on the health care system than ever before in recorded history. It has been reported that nearly 1.25 million Americans are living with T1D (JDRF, 2015). Complications due to diabetes were listed as the seventh leading cause of death in America and represent a major contributor in the development of kidney, liver, and cardiovascular diseases (American Diabetes Association, 2015). Currently, it is speculated that T1D is caused by a combination of environmental and genetic factors. Ramondetti et al. (2012) have shown that contraction of mumps or rubella is significantly associated with T1D onset. In another study, the T1D prevalence and incidence of children (0–14 years of age) from Sardinia, continental Italy, and Germany was compared (Ehehalt et al., 2009). Migrant children who had originated from regions of Sardinia and continental Italy displayed higher incidence rates of T1D, which were more similar to that of their regions of origin than to German-originated children. This finding suggests that genetic factors elicit a greater involvement in the pathogenesis of T1D than environmental factors.

6.2 PATHOGENESIS OF TYPE 1 DIABETES

T1D is an autoimmune disorder, mediated by the activation of T-cells, resulting in the destruction of the insulin producing beta cells of the pancreas. It has been shown that there are specific T-cell subpopulation differences between patients recently diagnosed with T1D and healthy non-diabetic patients (Skowera et al., 2015). CD8 T-cells attack and destroy invading antigens during early pancreatic inflammation in T1D. CD4 T-cells assist the immune system in both cell-mediated and antibody-mediated immune responses. Interleukin-2 is secreted by helper T-cells and stimulates further proliferation of CD4 and CD8 T-cells; inevitably leading to the activation of natural killer (NK) cells (Jahng et al., 2001). NK cells attack any cell in the body that is suspected of possessing any abnormal or unusual plasma membrane proteins. When NK cells bind to suspicious cells, the NK cells release toxic granules that proliferate the plasma membrane of surrounding cells with the same self-recognizing antigens in their membrane. The proliferation of surrounding cells from the integration of NK cells results in cell death.

Neutrophils are integral components of the immune response, which defend the body against invading pathogens and aid in the repair of damaged tissues (Schoenborn and Wilson, 2007). Neutrophils are highly involved in inflammatory processes; this includes rolling and adhesion to the surface of the microvascular endothelium, inevitably leading to the transmigration across the endothelial layer into the tissues (Granger and Senchenkova, 2010).

When the pancreas becomes inflamed, neutrophils migrate to the site of injury and adhere within the microvasculature. Mediator proteins, L-selectin, P-selectin, and E-selectin, control leukocyte rolling (Kansas, 1996). These mediator proteins are associated with P-selectin glycoprotein ligand 1 (PSGL1) and other glycosylated ligands (McEver and Cummings, 1997). L-selectin is

primarily expressed by leukocytes, in contrast to P-selectin and E-selectins, which are expressed by inflamed endothelial tissue (Ley et al., 2007). Integrins are activated through cell surface signaling pathways such as G-protein-coupled receptors, and involved in the adhesion of leukocytes to the endothelium (Ley et al., 2007).

Once symptoms of T1D are observed, approximately 80-90% of the pancreatic beta cells have been destroyed. If T1D is left untreated, severe and even life-threatening damage to various organ systems (such as the renal and cardiovascular systems) is likely to arise (Farrar et al., 2011). Current treatment options for patients with T1D include multiple daily insulin injections (MDI), insulin pump therapy, or pancreas/islet cell transplantation. Additionally, several prophylactic methods of delaying T1D pathogenesis are being studied at present (Gandhi et al., 2008; Lehmann et al., 2016; Weiss et al., 2006, 2008).

6.3 CURRENT THERAPIES

Administration of insulin via MDI is a standard and intensive method of insulin replacement therapy (IRT) and is not as effective as continuous insulin pump therapy (CIPT) at maintaining long term blood glucose control (Farrar et al., 2011). Even though MDI's have been used as a standard method of lowering blood glucose in patients with T1D for many decades, they are not an effective method of achieving blood glucose stability and can result in hypoglycemia, ketoacidosis, and coma (Lu et al., 2016). In a 2012 study, which compared the effectiveness of MDI vs. CIPT, it was reported that CIPT is a more effective method of lowering HbA_{1C} (a three month average blood glucose measurement) than MDI in adult patients with T1D (Golden, et al., 2012). The effectiveness of lowering HbA_{1C} in adult patients with T1D, who were using CIPT and a continuous blood glucose monitoring device (CMD) was found to be even more effective than CIPT alone.

Islet cell transplantation (ICT) was first conducted in 2000, through the transplantation of cadaver-isolated pancreatic islet cells into the liver of patients with T1D (Shapiro et al., 2000). Since then, methods for ICT have been improved upon, but not perfected. This is because of a lack of donor islet cells and the high probability that the patient's immune system will attack and destroy the donor cells (Lu et al., 2016).

Although various forms of IRT and ICT have been utilized to date to treat T1D, there is still an unmet need for prophylactic treatment options. Because of the primary autoimmune defect in T1D, several therapeutic attempts are directed toward immunological targets. The endocannabinoid system (ECS) is an important modulator of inflammation. Therefore, animal studies were performed to evaluate the potential of ECS manipulation to prevent pancreatic destruction in T1D. Our group confirmed earlier findings from Weiss et al. (2006, 2008), and showed that cannabidiol (CBD) significantly decreased parameters of pancreatic inflammation in early T1D (Figure 6.1, A-C) and delayed T1D onset in a mouse model of spontaneous T1D (female NOD mice; Figure 6.2; Lehmann et al., 2016).

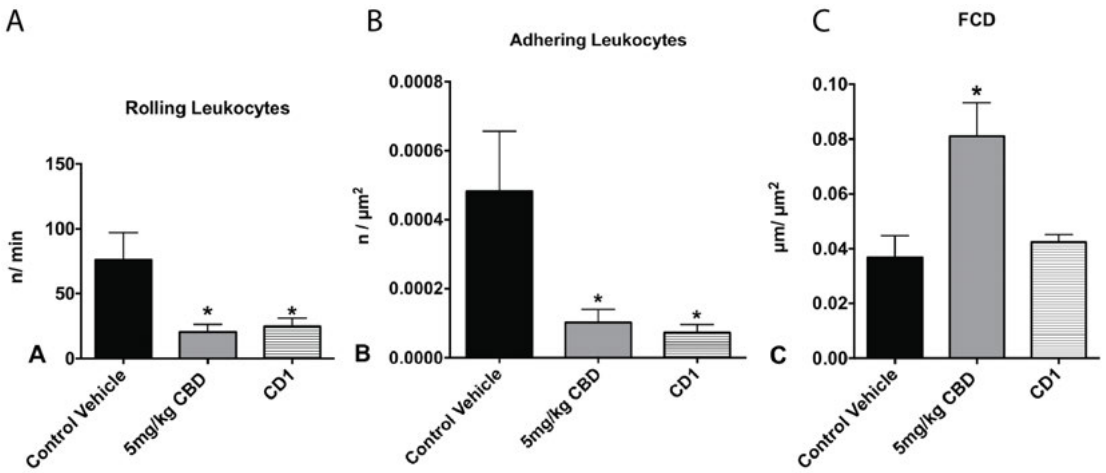

FIGURE 6.1: Number of rolling (A) and adhering (B) leukocytes and functional capillary density (FCD; C) within the pancreatic microcirculation of NOD mice treated with vehicle or 5 mg/kg CBD and CD1 control animals. Data presented as mean ± SEM. * $p < 0.05$ vs. control vehicle. (Lehmann et al., 2016).

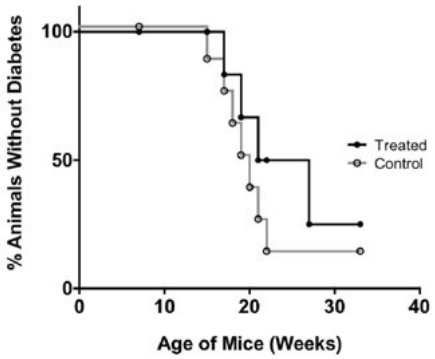

FIGURE 6.2: T1D onset: Kaplan-Meier plot showing the percentage of CBD treated and untreated control NOD mice without T1D during the observation time of 32 weeks (Lehmann et al., 2016).

6.4 CANNABIDIOL PHARMACOLOGY

Endocannabinoids are endogenous cannabinoid substances, which can activate one or both cannabinoid receptor subtypes, cannabinoid type 1 receptor (CB_1R) and cannabinoid type 2 receptor (CB_2R) (Di Marzo, 1998). CB_1Rs are located throughout the central nervous system (CNS) and the peripheral nervous system (PNS). CB_1R activation mediates the release of neurotransmitters, which affects specific G-protein-coupled receptors and their corresponding mechanisms (Horvath et al., 2012). CB_2Rs are mostly expressed within immune and hematopoietic cells and modulate immune activities (Horvath et al., 2012). Cannabidiol is one of many cannabinoids, which are derived from the plant, *Cannabis sativa*.

CBD is a non-psychoactive cannabinoid and has been shown to suppress cell-mediated autoimmune responses, such as the autoimmune response responsible for the onset of TD1 (Weiss et al., 2006). Cannabis-related fatalities, to date, have not been reported in humans. In a study conducted by Bergamaschi et al. (2011), it was reported that acute administration of CBD does not disrupt psychomotor functions in humans. It was additionally reported that doses of CBD (up to 600 mg) do not interfere with heart rate, blood pressure, or involuntary breathing. Since CB_1Rs are not located in the brain stem, ingestion of marijuana is unlikely to cause fatal overdose (Armentano et al., 2009). Due to the safety of CBD, even at elevated doses and potencies, more research needs to be conducted in the areas of study associated with autoimmune diseases and inflammation.

CBD has shown therapeutic potential in experimental diabetes. For example, CBD inhibits oxidative stress, NMDA receptor activation and inflammation in diabetic animals through activities that may involve inhibition of p38 MAP kinase (El-Remessy and Al-Shabrawey, 2006). The anti-inflammatory actions of CBD in models of inflammatory diseases are consistent with CBD suppression of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), (Malfait et al., 2000). The mechanisms underlying these effects of CBD may involve distinct cannabinoid receptor and non-cannabinoid receptor targets (Pertwee et al., 2010). CBD has been shown to bind to both CB_1Rs and CB_2Rs, acting as a negative allosteric modulator at CB_1R and an agonist at CB_2R (LaPrairie et al., 2015). However, the specific mechanisms of action that give rise to the anti-inflammatory actions of CBD still require clarification. CBD inhibits the enzyme fatty acid amide hydrolase (FAAH), which is responsible for the degradation of the endogenous cannabinoid anandamide (AEA) (Watanabe et al., 1996). The current literature suggests that the inhibitory actions of CBD on FAAH and AEA degradation may lead to activation of transient receptor potential vanilloid type 1 (TRPV1) and cannabinoid receptors, with subsequent suppression of TRPV1 signaling (Pertwee et al., 2010). Additionally, CBD is known to be an antagonist for GPR55, the lipid receptor for lysophosphatidylinositol, resulting in anti-inflammatory effects (Pertwee et al., 2010; Yang et al., 2016). Recently, the role of adenosine receptor activation by CBD has been reported to be linked with a marked reduction in ischemia/reperfusion (I/R)-induced disorders. Gonca and Darici (2015) were able to demonstrate that CBD has an antiarrhythmic

effect against I/R-induced arrhythmias in a rat model of I/R-induced ventricular arrhythmia, and that the associated effects of CBD are likely linked to the activation of the adenosine A_1 receptor (Gonca and Darici, 2015).

The anti-inflammatory actions of CBD together with the added benefits of being non-psychoactive and having low toxicity in animal and human models suggest therapeutic potential for CBD to decrease disease severity in diabetes, a pathology where chronic inflammation is present.

6.5 CANNABIDIOL FOR PROPHYLAXIS OF TYPE 1 DIABETES

Weiss et al. were the first to show that prophylactic administration of CBD to 6–12 week old female NOD mice was capable of significantly reducing the incidence of T1D onset from 86% in non-treated control animals to 30% in subjects who had received five intraperitoneal (IP) injections of 5 mg/kg CBD, over a 6-week experimental period (Weiss et al., 2006). CBD administration also reduced plasma concentrations of TNF-α and IFN-γ in this study.

In a second study in 2008, Weiss and colleagues administered CBD to 11–14 weeks old female NOD mice, at the same dose as in their 2006 study, over a four-week experimental period. The authors reported that T1D was only diagnosed in 32% of subjects who had been treated with CBD; whereas, control vehicle-treated and untreated subjects developed T1D at incidences of 86% and 100% respectively. Histological analysis of pancreatic tissues from CBD-treated, control-vehicle-treated and untreated NOD mice revealed that CBD administration could decrease the severity of islet cell destruction.

To further investigate CBD's effects in this model, we administered 5 mg/kg CBD to 7–17 week old female NOD mice and assessed time of T1D onset and leukocyte activation at time of T1D onset (via intravital microscopy, IVM). We observed that CBD-treated animals developed T1D later than control-treated subjects (Figure 6.2). CBD administration was also able to significantly reduce leukocyte rolling and adhesion, and improve functional capillary density (FCD) in pancreatic microvasculature, when compared to vehicle-treated control animals (Figure 6.1A-C; Lehmann et al., 2016). Histological analysis of pancreatic tissues from control-vehicle (Figure 6.3A) and CBD-treated (Figure 6.3B) female NOD mice as well as untreated female CD-1 control mice (Figure 6.3C) showed that CBD administration appears to maintain pancreatic beta cell and acinar cell functionality and density.

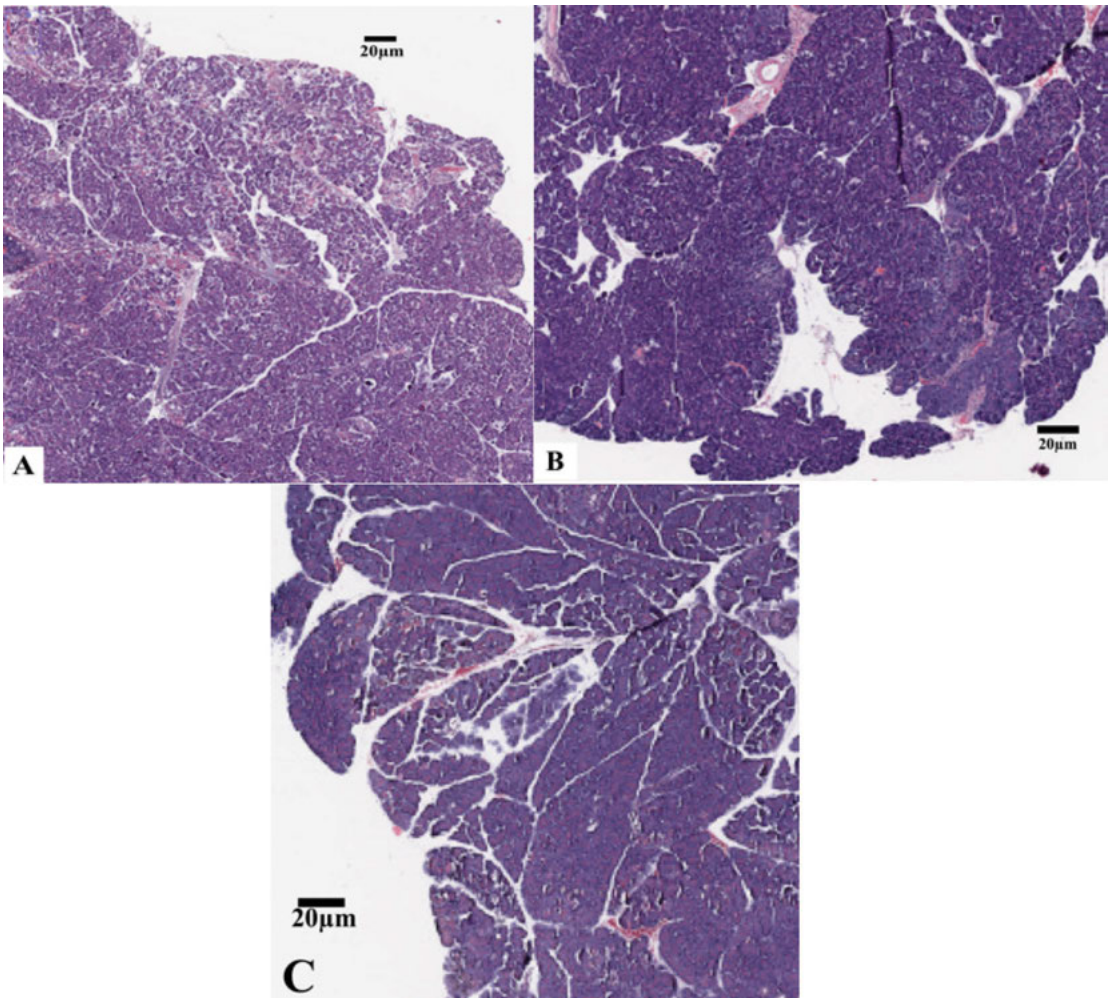

FIGURE 6.3: Histology of pancreatic tissues from female NOD mice, following ten weeks of either control-vehicle (A) or 5 mg/kg CBD treatment (B) and diagnosis of T1D. For comparison: pancreatic tissue from healthy, non-diabetic female CD-1 mice (C). Hemotoxylin and eosin staining.

These findings strongly correlate with our findings that CBD administration can significantly decrease parameters of leukocyte rolling and adhesion, in addition to increasing FCD in CBD-treated animals, when compared to animals treated with control-vehicle. Even though we did not find significant differences in T1D onset between control-vehicle and CBD-treated NOD mice, T1D diagnosis was consistently observed later in CBD-treated animals, when compared to control animals (Figure 6.2).

As mentioned by Weiss et al. (2006), early prophylactic intervention using CBD was able to significantly reduce plasma levels of TNF-α and IFN-γ. TNF-α is a pro-inflammatory cytokine, associated with E-selectin up-regulation on the surface of endothelium (Kansas, 1996). E-selectin is an endothelial adhesion molecule, and becomes activated upon interaction with inflammatory cytokines (Laferriere et al., 2001). IFN-γ is produced by natural killer (NK) cells. It is an inflammatory cytokine, associated with immune defense against intracellular pathogens (Schoenborn and Wilson, 2007). Reduction in TNF-α and IFN-γ levels, as reported in the research conducted by Weiss et al. (2006), has been shown to decrease inflammatory responses involved in autoimmune disorders. Inhibition of TNF-α and IFN-γ results in a decline in leukocyte adhesion to the endothelium and can suppress the response of NK cells during their assault on healthy tissues or organ systems of the body throughout the duration of autoimmune responses.

In summary, the present evidence shows a reduction in the incidence of T1D onset in experimental animals that had received early CBD prophylactic therapy. Although a reduction in T1D severity was observed in older animals, early intervention with CBD produced the best results for prevention or delay of T1D in the NOD mouse model. In our study, CBD administration was terminated following ten weeks of treatment. After this period, the rate of T1D onset in CBD-treated animals was found to increase faster than in animals without treatment. This could suggest that either continuous CBD administration is required for prevention of T1D or the optimal age of T1D onset could have occurred just before or slightly after cessation of CBD treatment.

6.6 CONCLUSION

Currently clinical research with CBD in T1D patients is lacking. However, compared with conventional immunosuppression therapies, cannabinoids have been found to elicit far fewer severe adverse effects. To date, various immunosuppressive therapies for delaying or preventing the onset of T1D have been assessed in clinical settings. Compounds such as cyclosporine and methotrexate have been studied in patients at risk of developing T1D; however, these treatments have been reported to either be ineffective or their beneficial immunosuppressive properties decline and become absent following cessation (Chase et al., 1990; Buckingham and Sandborg, 2000). In a previous clinical study, 43 patients with newly diagnosed T1D were treated with either cyclosporine A or a placebo for 4 months (Chase et al., 1990). It was reported that of the 22 cyclosporine A treated and 21 of the placebo treated patients, 6 and 4 (respectively) patients went into remission of T1D pathogenesis following 1 year of intervention. Of the 6 patients who had entered T1D remission, 5 were less than 19 years of age, indicating that intervention at both an early age and time of onset could be key to reversing T1D pathogenesis. As time progressed following cyclosporine A cessation, T1D onset in individuals that had been in remission began to regress and T1D pathogenesis was reinitiated. In another clinical study, the benefit of low-dose methotrexate immunosuppressive therapy on the prevention of early T1D pathogenesis was examined (Buckingham and Sandborg,

2000). It was reported that low-dose methotrexate administration to patients at the time of T1D onset was not beneficial at reversing T1D pathogenesis, and treatment with methotrexate led to earlier requirement for insulin therapy, compared to patients in the control group.

Although animal models of CBD treatment for the delay or prevention of T1D have used intraperitoneal injections as the route of administration, CBD can additionally be administered to patients either through oral or inhalation routes. This makes cannabinoid administration to patients cost effective and gives many possible options for dose administration in clinical settings. For a detailed comparison of adverse effects of CBD and cyclosporine treatments in human patients, see Table 6.1.

In summary, CBD has been found to be effective in preventing and delaying early inflammation and damage of the pancreas in experimental T1D. The detailed mechanism of action of CBD in T1D is still required to be elucidated. Experimental and clinical studies are needed for optimization of dosing and treatment regimen.

TABLE 6.1: Adverse effects of CBD therapy (Bergamaschi et al., 2011) versus common immunosuppressive cyclosporine (Novartis Pharmaceutical, 2015) therapy on the various organ systems in human patients

Organ System	Adverse Effects of CBD Administration	Adverse Effects of Cyclosporine Administration
Digestive	NS	Diarrhea, heartburn, gas, increased oral tissue growth, abdominal cramping
Central Nervous System	NS	Depression, sleep disturbances, seizures, loss of consciousness, mood/behavioral changes, difficulty controlling/moving part of the body, vision changes
Endocrine	Immunosuppression	Increased risk of lymphoma, increased risk of infection, immunosuppression, enlargement of breast (in men)
Urinary	NS	Kidney damage
Circulatory and Cardiovascular	NS	Unusual bleeding/bruising, hypertension, edema of peripheral extremities
Dermatological	NS	Yellowing of skin, purple blotching of the skin, rash, acne, pale skin, flushing of skin, increased hair growth on face, arms, or back
Ocular	NS	Yellowing of eyes, vision changes
Auditory	NS	Ear problems
General	NS	Burning/tingling sensations in extremities, muscle/joint pain, facial pain or pressure

Abbreviations: NS= no significant adverse effects reported in patients

CHAPTER 7

Role of the Endocannabinoid System in Interstitial Cystitis

Contributing Author
Juan Zhou

Abstract

Interstitial cystitis is a chronic inflammatory disorder of the bladder with uncertain etiology. Recent research has identified that the endocannabinoid system (ECS) is a key regulator of immune function and activation of cannabinoid receptors is anti-inflammatory. This chapter reviews the pathophysiology of interstitial cystitis and discusses experimental evidence for the involvement of cannabinoid receptors in bladder inflammation. Identification of the role of the endocannabinoid system in bladder function may facilitate the development of potential new treatments for interstitial cystitis and offer alternative and/or adjunct treatment options for patients with this chronic, painful syndrome.

Key Words

interstitial cystitis, bladder pain syndrome, bladder inflammation, cannabinoid receptors

7.1 INTRODUCTION

Interstitial cystitis (IC), also referred to as bladder pain syndrome, is a chronic inflammatory disorder of the bladder that predominantly occurs in women (Colaco and Evans, 2015). The clinical symptoms of interstitial cystitis are often associated with increased urinary frequency, urgency, and chronic pelvic or bladder pain (Cox et al., 2016; Hannoa and Dmochowski, 2009). The etiology of IC has been suggested to involve infectious agents, lymphovascular obstruction, neurologic or autoimmune pathologic features, and inflammatory conditions (Davis et al., 2014; McLennan, 2014). Due to the uncertain etiology, diverse presentation, and episodic nature of the disease, there is no effective treatment available for IC at the present time.

Currently, the US Food and Drug Association approved therapies for interstitial cystitis are oral pentosan polysulfate and intravesical dimethyl sulfoxide (DMSO), but the mechanisms for the action are not very clear (Mayer, 2007). The American Urological Association also suggested guidelines on management of IC, including: conservative therapy, such as education, behavioral modifi-

cation and stress control; oral pharmacological, intravesical, and surgical therapy; as well as the use of complementary and alternative treatments to improve the patient's pain and urinary symptoms. For example, oral amitriptyline is a tricyclic antidepressant that has been shown to be effective in controlling neuropathic pain (Colaco and Evans, 2015). Hydroxyzine and cimetidine may affect IC by preventing mast cell degranulation and histamine release (one of the mechanisms that have been suggested in the pathophysiology of IC) (Barr, 2014). Intravesical instillation by direct introduction of various treatment agents including heparin, steroid, DMSO, or hyaluronic acid into the bladder via a catheter has shown some effects on reducing nocturia and pain in some patients (Leong, 2014; Barr, 2014). Unfortunately, none of these treatments proved to be very effective.

Recently, the endogenous cannabinoid system (ECS) was found to have anti-inflammatory properties and the cannabinoids were studied as new targets in experimental cystitis (Walczak and Cervero, 2011; Wang et al., 2014; Mukerji et al., 2010). Treatment of cystitis with cannabinoids was reported in experimental and small clinical studies (Tambaro et al., 2014; Martin et al., 2015; Krenn et al., 2003). This evidence suggests that cannabinoids may have a potential therapeutic benefit for IC. In this chapter, the pathophysiology of IC and the effects of cannabinoids on IC are described in experimental and clinical studies.

7.2 INTERSTITIAL CYSTITIS

Interstitial cystitis was described as a condition characterized by urinary symptoms and markedly reduced bladder capacity with or without cystoscopic findings of glomerulations or bladder ulcers (Hunner's lesions)(Kim, 2016). Due to the complexity of the symptoms and overlap with other urinary disorders, the definition and diagnosis of IC has been modified several times. Since pain is the fundamental characteristic of the condition, the name of IC was also suggested to be changed to bladder pain syndrome (BPS) (Kim, 2016). The latest definition of IC/BPS by the American Urological Association is: "An unpleasant sensation (pain, pressure, or discomfort) perceived to be related to the urinary bladder, and associated with lower urinary tract symptoms of more than six weeks duration in the absence of infection or other identifiable cause" (Hannoa and Dmochowski, 2009).

Several experimental models of IC have been established in animals by injection of inflammatory substances or mediators systemically (i.e., intraperitoneally) or locally (i.e., intravesical) to generate conditions similar to clinical interstitial cystitis. For example, intraperitoneal injection of lipopolysaccharide (LPS, a major component of Gram-negative bacterial membrane) or cyclophosphamide (CYP) in mice or rats results in visceral pain with increased bladder contractility, bladder wall edema, and inflammation (Tambaro et al., 2014; Pessina et al., 2014). Systemically applied CYP is metabolized by the liver to acrolein that is accumulated in the urine and responsible for CYP-induced cystitis (Wang et al. 2015b). Intravesical administration of acrolein, LPS, or nerve

growth factor (NGF, a mediator of inflammatory pain) can also generate interstitial cystitis (Merriam et al., 2008; Jordan et al., 2007).

7.3 PHYSIOLOGY AND PATHOPHYSIOLOGY OF INTERSTITIAL CYSTITIS

The bladder is a hollow muscular organ, which primarily consists of mucosal, smooth muscular layers (detrusor) and surrounding connective tissue (Figure 7.1). The mucosal layer consists of transitional epithelia cells, termed urothelium, lining the lumen of the bladder and a lamina propria beneath the epithelial cells (Merrill et al., 2016). On top of urothelium, there is a glycosaminoglycan layer providing a defense mechanism for the urothelium. The urothelium not only acts as an impermeable barrier, but also functions as a sensory component capable of responding to multiple stimuli. The urothelium possesses ion channels and various receptors associated with neurotransmission, releases signaling molecules, and plays a critical role in the physiological and pathophysiological process of the bladder (Merrill et al., 2016; Daly et al., 2011)

The primary function of the bladder is to store and eliminate urine regulated by a complex interplay between efferent and afferent neural mechanisms (Daly et al., 2011). Afferent nerves consist of myelinated Aδ fibers and unmyelinated C fibers, which travel in the pelvic and hypogastric nerves. These cell bodies are in the dorsal root ganglia and pass into lumbrosacral spinal cord. These afferent nerves convey information about the extent of bladder wall distension; volume, pressure and the presence of noxious agents; and project it to the brain through spinal cord. A cross-talk between the urothelium and the sensory nerves involves multi-factorial process. Stimulation or inhibition of receptors on the urothelium can alter the bladder afferent fibers signaling and regulate bladder afferent transmission (Daly et al., 2011; Keay, 2008).

The etiology of IC is not clear and may involve multiple factors, including loss of epithelial integrity and dysfunction, inflammatory cell infiltration and vascular abnormalities, subclinical infection, neurogenic inflammation, autoimmune disorder, mast-cell activation, and increase of sensory nerve fibers in the bladder (Kim, 2016; Mayer, 2007). In addition, injury or dysfunction of the glycosaminoglycan layer caused by bacterial cystitis, pelvic surgery, urological instrumentation, and the factors that provoke sensory nerve activation can also contribute to IC etiology (Flores-Carreras et al., 2015).

Hunner's lesions occur in 5–10% of patients with IC or BPS and this is referred to as ulcerative IC. It is a distinctive inflammatory lesion in the bladder mucosal that presents patches of reddened mucosal area with small vessels radiating from a central pale scar with bleeding or a fibrin deposition (Figure 7.2) (Fall et al., 2014; van de Merwe et al., 2008). Severe inflammation with an increased number of mast cells, lymphocytes, neutrophils, and plasma cells in the bladder was reported in ulcerative IC (Kim, 2016). Most patients with nonulcerative IC have little or no inflammation, but edema and vascular congestion are often seen.

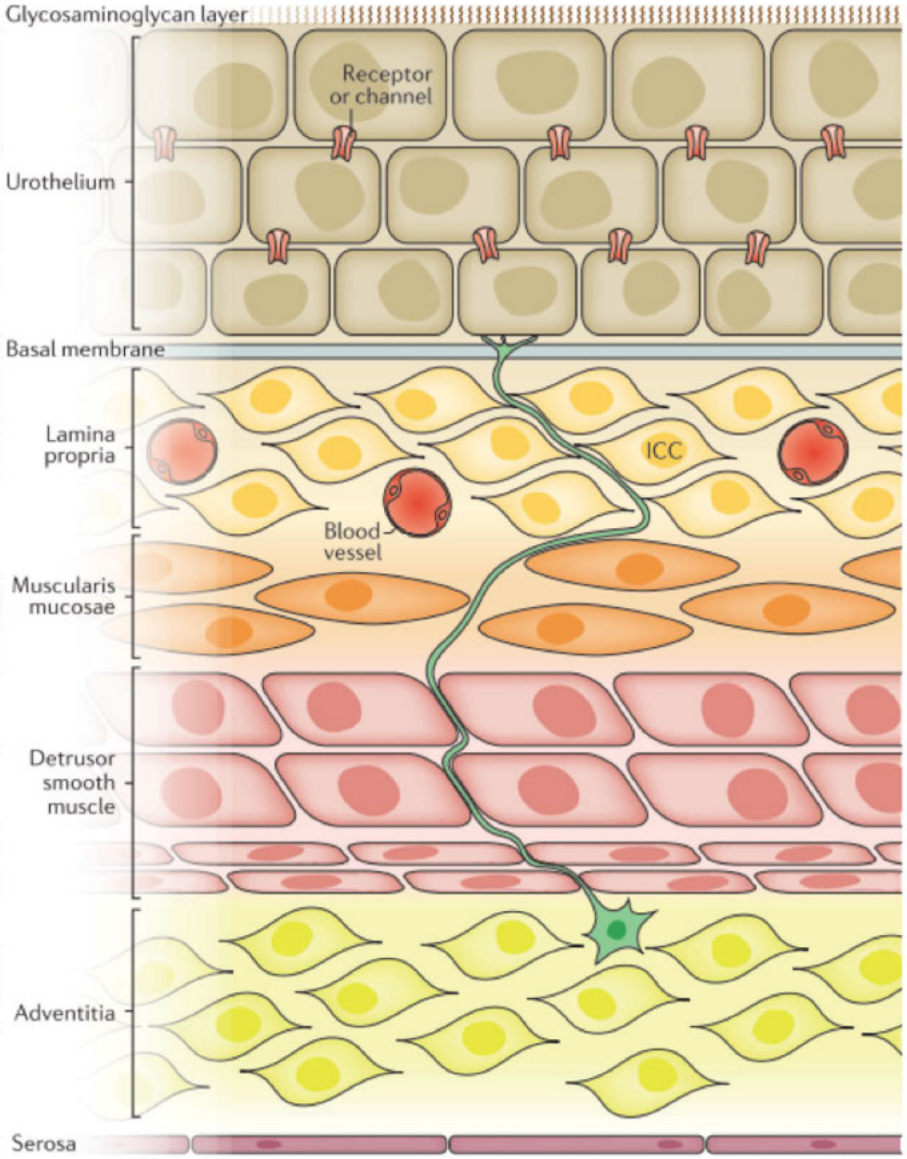

FIGURE 7.1: Cell layers of the wall of the urinary bladder. Adapted from Merrill et al. (2016).

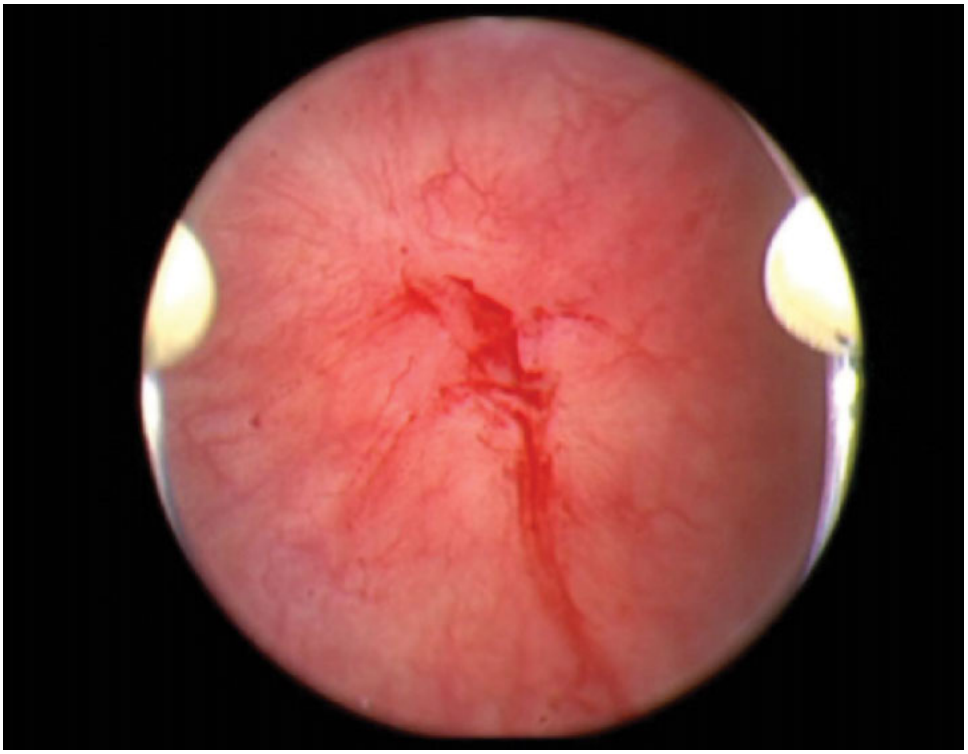

FIGURE 7.2: Lesion in classic IC, viewed though a resectoscope during bladder distension: small vessels radiating towards discrete central scar, with superficial rupture and waterfall like bleeding. Adapted from Fall et al. (2014).

7.4 THE ENDOCANNABINOID SYSTEM

The ECS is involved in a variety of physiological processes including: metabolism, pain-sensation, neurotransmission, and inflammation (De Petrocellis and Di Marzo, 2009; Pandey et al., 2009). The ECS consists of cannabinoid receptors, endogenous cannabinoids (EC), and endocannabinoid metabolizing enzymes (De Petrocellis and Di Marzo, 2009). Two cannabinoid receptors, cannabinoid 1 (CB_1R) and cannabinoid 2 (CB_2R) have been cloned (Matsuda et al., 1990; Munro et al., 1993). CB_1R are predominately expressed in the central nervous system (CNS) and some non-neural peripheral tissues (Pertwee and Ross, 2002). CB_2R are mainly expressed on immune cells but are also identified in selected CNS areas and some peripheral tissues, such as intestine and bladders (Hayn et al., 2008; Klein, 2005). Both CB_1R and CB_2R are G-coupled proteins that when activated couple to $G_{i/o}$ G proteins to inducing signal pathways, including inhibition of adenylyl cyclase and activation of mitogen-activated protein kinases (Devane et al., 1988; Howlett et al., 2002).

The most common endogenous cannabinoid ligands or endocannabinoids are N-arachidonylethanolamine or anandamide (AEA) and 2 arachidonoylglycerol (2-AG). These endocannabinoids are rapidly synthesized from postsynaptic membrane-lipid precursors in the cell membrane and act on presynaptic cannabinoid receptors (Zajicek and Apostu, 2011). Both AEA and 2-AG have higher affinities for CB_1R than CB_2R, but 2-AG also binds with high affinity to CB_2R (Reggio, 2002). AEA and 2-AG are degraded by hydrolyzing enzymes, fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL), respectively (Luchicchi and Pistis, 2012). Inhibition of the hydrolyzing enzymes results in increased tissue content of the endocannabinoid AEA and/or 2-AG. For example, AEA exerts potent analgesic and anti-inflammatory effects, but the lifetime of this endocannabinoid is relatively short as AEA is rapidly degraded by FAAH. Pharmacological inhibition or genetic deletion of FAAH enhances analgesic and anti-inflammatory effects by increasing tissue content of AEA (Luchicchi and Pistis, 2012) .

Several synthetic ligands have been developed that selectively bind to CB_1R and CB_2R and act as agonists or antagonists of the CB receptors. For example, arachidonyl-2'-chloroethylamide (ACEA) and O-1812 are selective CB_1R agonist, and JWH-133 and HU308 are selective CB_2R agonist. In addition, AM251 and AM630 are well-known CB_1R- and CB_2R-selective antagonists, respectively (Pertwee, 2006a, 2006b). These receptor ligands have been widely used in the studies of cannabinoid effects on inflammatory disorders, such as inflammatory bowel disease, sepsis, IC, and pain-related diseases (Toguri et al., 2015; Küster et al., 2012; Tambaro et al., 2014; Zajicek and Apostu, 2011).

7.5 THE ENDOCANNABINOID SYSTEM IN EXPERIMENTAL INTERSTITIAL CYSTITIS

Both CB_1R and CB_2R have been reported in the bladder of various species, including rats, mice, monkeys, and humans (Gratzke et al., 2009; Tyagi et al., 2009; Hayn et al., 2008; Walczak et al., 2009). Application of ajulemic acid (AJA), a mixed CB_1R/CB_2R agonist, to the isolated rat bladder inhibited chemically evoked releases of sensory neuropeptide calcitonin gene-related peptide (CGRP), suggesting a role of CB_1R and CB_2R in inhibition of the sensory neuronal activity generated from afferent nerve fibers in the bladder (Hayn et al., 2008). The inhibitory effect of AJA was mediated through both CB_1R and CB_2R since the effect was attenuated by application of both the CB_1R or CB_2R antagonist, AB251 or AM630 (Hayn et al., 2008).

Pharmacological experiments have demonstrated that cannabinoid agonists can modulate bladder contractility in isolated bladders due to pre- and post-synaptic effects (Pertwee and Fernando, 1996; Martin et al., 2015). In addition, systemic administration of AEA or palmitoylethanolamide (PEA) attenuated local NGF-induced bladder hyper-reflexia and reduction of micturition threshold in rats (Farquhar-Smith et al., 2002). Application of CB_1R and CB_2R antagonist indicated that the action of AEA was mediated by both CB_1R and CB_2R, whereas the

effect of PEA was via CB_2R (Farquhar-Smith et al., 2002). The CB_2R mediated effect of PEA was also reported in turpentine-induced bladder hyper-reflexia (Jaggar et al., 1998). However, in a CYP-induced bladder inflammation, the inhibitory effect of PEA was suggested to be mediated indirectly through CB_1R and peroxisome proliferator-activated receptor alpha (PPARα, one of the main pharmacological targets of PEA) (Pessina et al., 2015). These data suggested that cannabinoids are capable of modulating experimental cystitis and that this modulation is mediated by CB_1R and/or CB_2R and each mechanism may depend on the experimental model used.

7.6 CANNABINOID 1 RECEPTOR AND BLADDER PATHOPHYSIOLOGY

CB_1R was identified in the urothelium and afferent nerve fibers of the mouse bladder and in L6 dorsal root ganglion (Pertwee and Fernando, 1996; Walczak et al., 2009; Wang et al., 2015a). Increased CB_1R expression was found in response to CYP-induced inflammation in rat bladder (Pessina et al., 2015). Activation of CB_1R reduced the electrically evoked contraction of mouse bladder (Pertwee and Fernando, 1996; Martin et al., 2000) and suppressed enhanced afferent nerve activity induced by mechanical stimulation (Walczak et al., 2009) or by bladder inflammation (Walczak and Cervero, 2011). These data suggest an involvement of CB_1R in bladder pathophysiology.

In an *in vivo* experimental study, systemic administration of exogenous PEA attenuated CYP-induced pain behavior, bladder inflammation and voiding dysfunction. CB_1R and a PPARα antagonist, but not a CB_2R antagonist, reversed the PEA effect on gross damage, suggesting a role for CB_1R and PPARα in the anti-inflammatory effect of PEA (Pessina et al., 2015). It was proposed that although PEA does not directly activate CB_1R, it may involve enhancement of action with another endocannabinoid to act on CB_1R (Pessina et al., 2015). The mechanism of PEA on inhibition of pain behavior and voiding dysfunction was not clear.

Since systemically applied cannabinoids may act at multiple sites, including the CNS, studies with local (intravesical) treatment were established to explore the peripheral response. Intravesical application of the CB_1R agonist, ACEA, or the nonselective cannabinoid agonist, AZ12646915, reduced bladder activity in NGF or CYP induced cystitis (Walczak and Cervero, 2011; Wang et al., 2015a). CB_1R, but not CB_2R antagonism counteracted the effect of both agonists, ACEA and AZ12646915, suggesting that local activation of CB_1R is capable of inhibiting bladder activity (Wang et al., 2015a; Walczak and Cervero, 2011). The inhibitory effect of CB_1R agonists on bladder activity was suggested to mediate through peripheral modulation of bladder afferent information. In fact, greater pain relief was observed when the cannabinoid was given through local route than through systemic administration (Dmitrieva and Berkley, 2002).

The effects of endocannabinoids on cystitis were also studied in FAAH knockout ($FAAH^{-/-}$) mice, which have increased AEA levels in various tissues, including the bladder (Schlosburg et al., 2009; Wang et al., 2015b). Compared to wild type mice, $FAAH^{-/-}$ mice showed increased bladder

function and reduced severity of edema and inflammation in CYP-induced cystitis (Wang et al., 2015b). However, in NGF-induced cystitis, intravesical NGF failed to affect bladder activity in $FAAH^{-/-}$ mice, but local treatment with a CB_1R antagonist, AM251, restored NGF's effect on bladder activity, suggesting that inhibition of NGF-induced responses in $FAAH^{-/-}$ mice may be mediated, at least in part, by CB_1R within the bladder wall (Wang et al., 2015a). In addition, it has been reported that pharmacological inhibition or genetic deletion of FAAH has the capacity to ameliorate pain associated with bladder inflammation (Merriam et al., 2011; Wang et al., 2015b). As described above, CB_1R are present in the urothelium, and modulation of CB_1R in the urothelium may influence bladder function and pain sensation during bladder inflammation. In support of this, FAAH inhibition with resultant increased AEA, reducing bladder afferent nerve activity via activation of CB_1R and CB_2R (Aizawa et al., 2015).

However, contradictive data also exist for cannabinoids in the bladder. In an acrolein induced cystitis model in rats, the levels of CB_1R expression were not changed in the inflammatory bladders (Merriam et al., 2008). Although intrathecal administration of ACEA prevented the cystitis-associated hyperalgesia, ACEA did not ameliorate the increased bladder contractility and bladder inflammation associated with acrolein-induced cystitis. These findings suggest that the spinal signal of CB_1R activation inhibits cystitis-induced pain, but has little or no effect on local tissue response to inflammation and contractility of the bladder. The mechanism of action of spinal CB_1R activation on acrolein-induced cystitis is not clear and requires further investigation.

7.7 CANNABINOID 2 RECEPTOR AND BLADDER PATHOPHYSIOLOGY

Although both CB_1R and CB_2R were detected in the bladder (Gratzke et al., 2009; Tyagi et al., 2009; Walczak et al., 2009; Hayn et al., 2008), spinal cord, and dorsal root ganglia (Merriam et al., 2008; Wang et al., 2013), higher expression of CB_2R than CB_1R was reported in sensory nerves in the urothelium and detrusor in rats, monkeys, and humans in normal conditions (Gratzke et al., 2009). The level of CB_2R, not CB_1R, significantly increased acrolein-induced acute and chronic cystitis in the rat bladder, suggesting that CB_2R may play a prominent role in response to bladder inflammation (Merriam et al., 2008). Similar results of increased CB_2R, not CB_1R, were observed in LPS-induced cystitis bladder in mice (Tambaro et al., 2014).

In contrast, other studies showed that CB_2R expression was not changed in the bladder after exposure to acrolein, and neither the CB_2R agonist GP1a, or the CB_2R antagonist AM630, affected CB_2R expression in the acrolein-induced cystitis in mice (Wang et al., 2013, 2014). Systemic injection of the CB_2R agonist GP1a significantly decreased the severity of bladder inflammation, inhibited increased peripheral sensitivity to mechanical stimuli, and reduced the increased urinary frequency associated with cystitis (Wang et al., 2013, 2014). The protective effects were diminished

by pretreatment with a selective CB_2R antagonist, AM630, confirming that the protective effects were mediated through CB_2R (Wang et al., 2013).

A protective role of CB_2R in bladder inflammation was further demonstrated in an LPS-induced cystitis model in mice. Systemic administration of the CB_2R agonist JWH015, but not the CB_1R agonist ACEA, significantly reduced bladder contractile activity, decreased neutrophil and leukocyte infiltration, and decreased the expression of inflammatory cytokines, including IL-1α, IL-1β and TNF-α, in the bladder of the LPS challenged mice (Tambaro et al., 2014). This protection was mediated by CB_2R since AM630 reversed JWH015 effects and CB_1R agonist did not show the protective effect. The data suggests that CB_2R agonists have therapeutic efficacy in bladder inflammation and can provide pain relief in the interstitial cystitis.

The mechanism by which CB_2R activation reduces inflammation and provides protection in IC is not currently well established. However, CB_2R are predominately present on the immune cells and play a critical role in immune regulation. It has been reported that CB_2R activation suppresses neutrophil differentiation and migration (Nilsson et al., 2006), inhibits macrophage proliferation and phagocytosis (Chuchawankul et al., 2004), as well as inhibits leukocyte activation and pro-inflammatory cytokine production in inflammation (Lehmann et al., 2012). The presence of CB_2R on urothelial cells, which are capable of secreting various molecules such as nitric oxide, NGF, prostaglandin E2, and cytokines, may significantly influence bladder inflammation, bladder function, and pain sensation (Birder and Andersson, 2013). CB_2R activation may suppress the production of pro-inflammatory mediators from urothelial cells and inhibit the local inflammatory response in the bladder. Although controversial finding on the levels of endogenous cannabinoid AEA and 2-AG in bladder inflammation have been reported (Dinis et al., 2004; Merriam et al., 2011; Wang et al., 2015b), the increase in CB_2R in the bladder after inflammation may drive the suppression of inflammatory response in the bladder.

Recently, mast cells have been suggested to play an important role on the pathophysiology of IC (Choi et al., 2014; Wang et al., 2016); CB_2R are present on mast cells (Facci et al., 1995) and may regulate mast cell activation in the IC. Additionally, CB_2R activation may have a direct inhibitory action on afferent nerves given that CB_2R activation suppressed the release of CGRP from afferent nerve fibers in bladder (Hayn et al., 2008). Evidence that CB_2R agonists can increase the micturition interval and threshold pressure also supports a role for CB_2R in bladder afferent signals (Gratzke et al., 2009). Therefore, activation of CB_2R in the experimental IC may suppress bladder inflammation, improve bladder function, and reduce hyperalgesia through combined effects of reduction of inflammation and inhibition of afferent nerve activity.

7.8 THE ENDOCANNABINOID SYSTEM AND CANNABINOIDS IN CYSTITIS PATIENTS

In human bladder, both CB_1R and CB_2R have been identified in the urothelium and detrusor muscle (Gratzke et al., 2009; Tyagi et al., 2009) with a higher expression of CB_1R in both tissues (Tyagi et al., 2009). A selective CB_1R and CB_2R agonist ACEA and GP1a, respectively, attenuated detrusor strip contraction evoked by electrical stimulation in a dose-dependent manner, suggesting an inhibitory effect of the endocannabinoid system on contraction in the human bladder (Tyagi et al., 2009). Increased CB_1R positive nerve fibers were found in the urothelium in patients with BPS and the density of the CB_1R positive fibers was correlated with pain scores, indicating a role for CB_1R agonists in pharmacotherapy for bladder pain syndrome (Mukerji et al., 2010). The effect of cannabinoids on bladder dysfunction has been reported in multiple sclerosis patients (Podda and Constantinescu, 2012; Andersson, 2016), however, at this time there is only one case report suggesting a therapeutic effect of cannabinoids in chronic cystitis (Krenn et al., 2003). Further investigation of the effect of cannabinoids in clinical BPS is clearly warranted.

7.9 CONCLUSION

In summary, these studies suggest that the ECS in the bladder may play a modulatory role in sensory afferent signaling and inflammatory responses. Cannabinoid agonists show efficacy in alleviating inflammation and pain in experimental models of bladder pathophysiology and may be useful in treatment of IC, a debilitating and painful inflammatory bladder disorder in humans. However, the role of cannabinoid receptors in bladder physiology and pathophysiology is not yet fully elucidated and published data in some cases are in part contradictory. Therefore, further investigation of the ECS and the differential roles of CB_1R and CB_2R in IC remains critical to facilitate new therapeutic strategies for treatment of IC and bladder disorders.

CHAPTER 8

Arthritis and the Endocannabinoid System

Contributing Authors
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Jason J. McDougall

Abstract

A functional endocannabinoid system has only recently been discovered in synovial joints. This chapter will summarize the involvement of the endocannabinoid system in arthritis and localization in the joint. Moreover, this chapter will explore how direct action on or modification of the system can be used as a pharmacological target for inflammation, arthritis pain, and disease progression.

Key Words

endocannabinoid, arthritis, inflammation, pain

Abbreviations

2-AG	2-arachidonoylglycerol
ACEA	CB_1R agonist
AEA	anandamide
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CADUMS	Canadian Alcohol and Drug Monitoring Survery
CB_1R	cannabinoid type 1 receptor
CB_2R	cannabinoid type 2 receptor
CBD	cannabidiol
CNS	central nervous system
DAG	diacylglycerol
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
GPR	G-protein receptor
GW405833	CB_2R receptor agonist

HU–308	CB_2R receptor agonist
JA	juvenile arthritis
MAGL	monoacylglycerol lipase
MIA	sodium monoiodoacetate
NAPE	N-acylphosphatidylethanolamines
NSAID	non-steroidal anti-inflammatory drugs
O1812	CB_1R receptor agonist
OA	osteoarthritis
OEA	N-oleoylethanolamide
PEA	N-palmitoylethanolamide
PNS	peripheral nervous system
RA	rheumatoid arthritis
THC	Δ^9-tetrahydrocannabinol
TNF-α	tumor necrosis factor-alpha
TRPV1	transient receptor vanilloid 1 channel
URB597	fatty acid amide hydrolase enzyme inhibitor

8.1 INTRODUCTION TO ARTHRITIS

Arthritis is not a single disease. In fact, there are over 100 different types of arthritis with various aetiology, pathophysiology, and treatment strategy. Arthritis can involve one or many synovial joints within the body, but is most common in the weight-bearing joints such as the knees, hips, and ankles. Since some types of arthritis are systemic diseases, non-articular organs such as the heart and kidneys can also be affected by the disease (Brooks, 2006; The Arthritis Society of Canada, 2013). The symptoms of arthritis include joint pain, stiffness, loss of function, swelling, and chronic fatigue. There are many different conditions also included under the general term arthritis, such as tendonitis, gout, and spondyloarthropathy. Musculoskeletal diseases that also have a global pain phenotype, such as fibromyalgia, are members of the arthritis family. The commonality between this large group of conditions is joint and musculoskeletal pain. Chronic pain can be categorized as either nociceptive, inflammatory, or neuropathic. Nociceptive pain is caused by acute damage to the tissues and is usually described as sharp, throbbing, and aching. Inflammatory pain is caused by the release of inflammatory mediators into the damaged tissue which sensitize joint nerves (McDougall, 2006). Inflammation presents as pain, swelling, warmth, and redness, and is associated with many forms of arthritis (Poole, 1999; Rahmati et al., 2016; Kidd, 2001). Around 30% of arthritis patients suffer from neuropathic pain which occurs due to damage of the nerves innervating the affected joint and is described as tingling or shooting pain, like an electrical shock (Sofat et al., 2014). Neuropathic pain can range from mild to severe, and can present as spontaneous, increased response to pain (hyperalgesia), or increased response to a non-noxious stimulus (allodynia) (Zimmermann, 2001). Pain

and inflammation cause the joint to lose its normal function, often rendering it stiff or completely immobile, leading to a dramatic decline in a person's quality of life and ability to be active.

Musculoskeletal disorders are the number one cause of disability worldwide and can affect people at any age. Arthritis affects 1 in 6 people over 15 years of age in Canada alone (The Arthritis Society of Canada, 2013). Gender, age, genetics, and co-morbidities, such as obesity and traumatic joint injury, are all factors that can increase a person's risk of developing arthritis. The demographic of musculoskeletal disease is clear with two-thirds of people affected by arthritis being women, and it is more prevalent in the elderly population. The average annual cost of arthritis to the Canadian economy, due to health care costs, access to tertiary care and loss of productivity, is $33 billion and is expected to double within the next 20 years (Brooks, 2006; The Arthritis Society of Canada, 2013).

Establishing an early diagnosis and implementing a suitable treatment regimen is vital to combatting arthritis and inhibiting disease progression. Current therapies are primarily aimed at symptom relief (e.g., decreasing pain and improving joint function). For less severe cases, patients are usually recommended acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen and naproxen, or topical capsaicin cream, as needed (Matthews and Hunter, 2011, Schuelert et al., 2010). Patients with persistent or severe symptoms are usually prescribed more potent medications like opioids (e.g., codeine, oxycodone, hydrocodone) and corticosteroids (e.g., prednisone, dexamethasone) (Matthews and Hunter, 2011; Zamora-Legoff et al., 2016). Medicinal cannabis is primarily used for symptom relief and has anti-inflammatory properties, although there are psychoactive properties associated with its use. According to the Canadian Alcohol and Drug Monitoring Survey (CADUMS), 50% of people surveyed reported that they use cannabis for chronic pain, while around 60% of medical cannabis users do so for the management of musculoskeletal disease symptoms. Since more than half of chronic pain patients in Canada suffer from a rheumatic disease, this indicates that the majority of medical cannabis users are arthritis sufferers (Statistics Canada, 2011). Although there are multiple pharmacological treatment strategies, all drugs come with many unwanted and dose-limiting side effects such as gastrointestinal bleeding, hepatotoxicity, renal toxicity, and sedation. A further limitation of analgesics is that they treat only the symptoms of the disease and have minimal effect on disease progression. Therefore, development of novel therapeutics that have nominal adverse effects, but are still efficacious, and slow or reverse disease progression, are imperative.

8.2 COMMON TYPES OF ARTHRITIS

8.2.1 OSTEOARTHRITIS

Osteoarthritis (OA) is a painful and degenerative disease of synovial joints. OA is the most common type of arthritis, and usually affects the larger, weight-bearing joints like the hip and knee but is also common in hands. Traditionally OA was thought to affect primarily the articular cartilage;

however, it is now known that OA affects the entire joint including the joint capsule, synovium, menisci, articular ligaments, and subchondral bone. The prevalence of OA rises with age, and is more common in women than in men (Aigner et al., 2004). Cartilage degeneration can occur from aging, trauma, low-grade local or systemic inflammation, metabolic disorders, obesity, and genetic predispositions (Buckwalter and Mankin, 1998). In OA, chondrocytes are exposed to abnormal conditions due to the damage of the surrounding extracellular matrix. This breakdown in the cellular environment allows cytokines and growth factors to readily diffuse through the damaged matrix and into the chondrocytes more easily (Aigner et al., 2007) resulting in chondrocyte death, cartilage breakdown, and poor repair (Krustev et al., 2015). Cartilage loss and subsequent erratic subchondral bone remodeling leads to osteophyte formation, causing patients to present with varying levels of synovitis (Figure 8.1A). OA was historically classified as a non-inflammatory arthritis; however, there is evidence that some inflammation can occur in response to pro-inflammatory mediators being released into the joint (Poole, 1999; Robinson, et al., 2016). Additionally, a subset of OA patients (approximately 30%) report neuropathic pain-like symptoms such as burning, tingling ("pins and needles"), shooting pain, and numbness (Hochman et al., 2011; Sofat et al., 2014). Thus, OA is a complex disease with multiple phenotypes and symptomatic subsets.

8.2.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease which is characterized by episodic and recurrent inflammatory flares within the joint (Scott et al., 2010). RA causes pain, stiffness, swelling, and limited mobility of the joint. Although RA can affect any synovial joint in the body, it is typically the small joints in the hands and feet that are most often affected. In addition to articular manifestations, inflammation associated with RA can affect other organs, such as the eyes, heart, and lungs (Krustev et al., 2015). Epidemiological evidence indicates that the disease most often begins in the later years of life, but RA can occur at any age. Among the people affected with RA, about 75% of them are women (Mbvundula et al., 2005). Many people with RA experience acute, intermittent inflammatory episodes, where their joints become hot to the touch and their symptoms worsen. Inflammatory flares associated with RA are characterized by hyperaemia, edema, and an increase in pain levels. Infiltrating immune cells release pro-inflammatory mediators such as cytokines and proteinases (Krustev et al., 2015; Fernandes et al., 2002), which can exacerbate the inflammatory response and worsen the pain. If left unchecked, chronic inflammation can develop, which can ultimately lead to erosion of the joint cartilage and bone (Figure 8.1B) (McInnes and Schett, 2007).

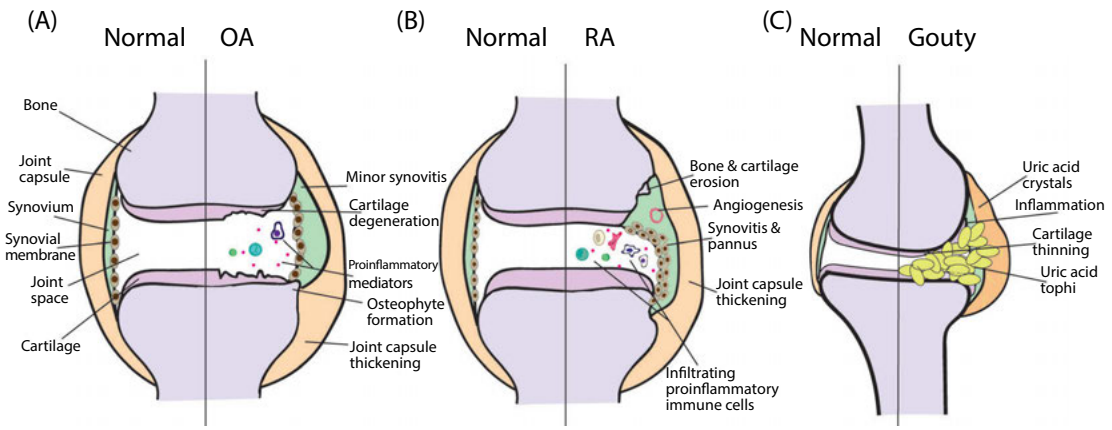

FIGURE 8.1: Schematic diagram illustrating the pathology of osteoarthritic (A), rheumatoid arthritic (B) and gouty arthritic (C) joints. Osteoarthritic joints are characterized by a mild synovitis, cartilage destruction, and bone remodeling including the formation of osteophyte protrusions. Rheumatoid arthritic joints have a more severe inflammatory component with synovial hyperplasia (pannus), bone, and cartilage erosion. Gouty joints contain an increased level of uric acid which can form solid bodies (tophi) which accumulate in the joint space. Inflammation is present and cartilage thickness is compromised.

8.2.3 GOUT

Gout is caused by a high serum urate concentration, which leads to the deposition of uric acid crystals and eventually uric acid mass build up (tophi) within the joint space causing inflammation and severe pain (Figure 8.1C). Initially, gout is acute and episodic with rapid accumulation of these crystals within the joint causing swelling, redness, and intense pain (Wilson and Saseen, 2016). A typical flare is monoarticular, and usually affects the metatarsophalangeal joint (big toe), but can also be present in the ankles, insteps of the feet, heels, knees, wrists, hands, and elbows. These acute flares may at first occur infrequently, but over time can become recurrent, with less time between episodes. Additionally, some patients can experience atypical gout episodes where the disease becomes polyarticular (Fravel et al., 2014). The prevalence of gout is increasing and there are many lifestyle traits that contribute to an increased risk of acquiring gout. Poor dietary patterns and comorbidities associated with high serum urate (e.g., obesity, hypertension, Type II diabetes, and chronic kidney disease) can all contribute to the generation of a gouty arthritis. Additionally, certain medications (e.g., diuretics or immunosuppressive agents) that are known to increase serum urate can potentiate gout (Khanna et al., 2012). Gout is currently treated using NSAIDs, colchicine, corticosteroids, or a combination of these. Treatment choice is usually based on severity of symptoms and medical history of the patient (Wilson and Saseen, 2016).

8.3 OVERVIEW OF CANNABINOIDS

The endocannabinoid system (ECS) is an endogenous system comprised of cannabinoid receptors and ligands, and is found throughout the central (CNS) and peripheral (PNS) nervous systems. The ECS is also found in many other tissues (e.g., blood vessels, lung, gut), and in each tissue, it is responsible for different tasks, with the ultimate goal being cellular homeostasis and communication between distinct organ systems (Battista et al., 2012). The ECS consists primarily of the classic cannabinoid receptors type 1 and type 2 (CB_1R and CB_2R). Other receptors are known to be associated with the ECS including the ionotropic transient receptor vanilloid 1 channel (TRPV1), and the orphan G-protein-coupled receptors, GPR55 and GPR18 (Pacher, 2006, Battista et al., 2012; Bradshaw et al., 2009). In addition to the receptor targets, the ECS also consists of endogenous ligands such as anandamide (AEA) and 2-aracydonylglycerol (2-AG). Overall, ECS tone depends on the physiological or pathological status of the organism, but is only "turned on" following injury or in the presence of a disease (Russo, 2016).

The biosynthetic pathways of the endocannabinoid ligands have been well worked out and involve multiple different precursor molecules, metabolic, and catabolic enzymes (DiMarzo, 2008). AEA and 2-AG are formed "on demand" as a consequence of increased intracellular calcium (Ca^{2+}) following cell depolarization or mobilization of internal Ca^{2+} stores. Precursor molecules for AEA and 2-AG are the phospholipid family derivatives, N-acylphosphatidylethanolamines (NAPEs) and diacylglycerols (DAGs), respectively (Deutsch and Chin, 1993; DiMarzo, 2008). AEA is produced by hydrolysis of the precursor NAPEs and inactivated by fatty acid amide hydrolase (FAAH), which cleaves the amide bond resulting in its constituent parts arachidonic acid and ethanolamine. 2-AG is produced by the hydrolysis of DAGs by DAG lipases and inactivated by monoacylglycerol lipase (MAGL) into free fatty acids and glycerol (Deutsch and Chin, 1993; DiMarzo, 2008; Aigner et al., 2007). CB receptors can be directly manipulated by their endogenous ligands or indirectly by drugs that modulate endocannabinoid levels (e.g., inhibitors of FAAH or MAGL), resulting in accumulation of tissue endocannabinoids.

Other *N*-acylethanolamides, *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), are co-synthesized with AEA. PEA lacks affinity for CB_1Rs and CB_2Rs, but has been shown to act on GPR55. OEA acts independently of the cannabinoid pathway, but instead acts largely to regulate a lipolysis pathway. There is evidence suggesting that even though these endocannabinoid-like ligands don't act directly on the cannabinoid signaling pathway, they are able to enhance the effects of AEA through the "entourage effect" (Jonsson et al., 2001; Ho et al., 2008); meaning they act in concert with endocannabinoids to augment physiological responses.

Several exogenous cannabinoids have been isolated that are derived from the cannabis plant (phytocannabinoids), which act on cannabinoid receptors. Δ^9-tetrahydrocannabinolic acid is a chemical found in the cannabis plant which when heated undergoes decarboxylation resulting in psychoactive Δ^9-tetrahydrocannabinol (THC) (Sharma et al., 2012). THC acts as a partial agonist

at CB_1Rs in the CNS, and CB_2Rs in peripheral immune cells. THC has been shown to act less selectively than endogenous cannabinoids (Schrott and Hubbard, 2016). Another major phytocannabinoid, cannabidiol (CBD), makes up about 40% of the cannabis plant, but lacks any psychoactive effects. It is not entirely clear what the mechanism of CBD is, but there is evidence to suggest that CBD acts as an agonist at CB_2Rs (Petitet et al., 1998; Thomas et al., 2009), an antagonist at GPR55 (Ryberg et al., 2009), and an antagonist at CB_1Rs (Petitet et al., 1998; Thomas et al., 2009). A number of synthetic compounds (synthetocannabinoids) have been produced which act as selective potent agonists at the various cannabinoid receptors (Schrot and Hubbard, 2016). Examples of synthetic CB_1R agonists include ACEA (Vera et al., 2013) and O1812 (McDougall, 2011), while HU-308 (Gui et al., 2015) and GW405833 (Schuelert et al., 2010) are known CB_2R agonists.

8.3.1 LOCALIZATION OF THE ENDOCANNABINOID SYSTEM IN JOINTS

As mentioned previously, under normal physiological conditions, the ECS has low tonic activity; the ligands are not stored, rather they are produced through an on-demand synthesis mechanism. Following release from nerve terminals, the endocannabinoids diffuse retrogradely where they activate cannabinoid receptors located on the same nerve ending. CB_1Rs are found primarily in the CNS and in the periphery. In contrast, CB_2Rs are predominantly expressed by immune cells in the periphery and glial cells in the CNS. Recent immunohistological evidence has shown that CB_1Rs and CB_2Rs are expressed on neurones innervating the rat knee joint (Schuelert et al., 2010; McDougall, 2011, 2009). Interestingly, CB_2Rs are co-localized with pronociceptive TRPV1 channels on small diameter neurones, where they act together to modulate joint pain (Schuelert et al., 2010; McDougall, 2011, 2009). McPartland et al. (2008) showed that CB_1Rs and CB_2Rs are expressed at low levels on fibroblast-like synoviocytes. These cells also showed expression of AEA and 2-AG catabolic enzymes, suggesting that endocannabinoid signaling may occur in an autocrine fashion within the joint (McPartland, 2008). In contrast, AEA and 2-AG are not found in the synovial fluid of normal joints (Richardson et al., 2008).

There has been increasing evidence to show that the articular ECS plays an important role in joint inflammation and pain. The functional roles of the endocannabinoid system have been shown in joint tissues of animals (Schuelert et al., 2010) as well as humans (Richardson et al., 2008). Schuelert and McDougall were the first to show that there is local tonic activation of the ECS in joints of rodents with monoiodoacetate (MIA)-induced OA (Schuelert et al., 2010). Furthermore, spinal cord levels of AEA, 2-AG, and their metabolic enzymes were increased in rats with MIA (Sagar et al., 2010). Downregulation of CB_1Rs and CB_2Rs was demonstrated in the ipsilateral lumbar spinal cord of mice with MIA-induced OA, likely in response to an increase in local endocannabinoid levels (La Porta et al., 2013). Additional animal studies showed that synthetic CB_1R and CB_2R agonists did not have any effects on synovial blood vessels in an acute inflammatory arthritis model, but the CB_2R agonist produced a marked increase in synovial blood flow in normal joints (Baker and McDougall, 2004; McDougall et al., 2008). The lack of a vasomotor effect in acutely

inflamed joints could be related to a downregulation of vascular CB_1Rs and CB_2Rs, although this has not been confirmed.

Synovial biopsies obtained from patients undergoing total knee arthroplasty revealed that the main components of the endocannabinoid signaling system, including CB receptors and ligands, increase in diseased joints. There was also an increase in the total amount of FAAH and MAGL compared to basal levels measured in healthy volunteers. AEA and 2-AG were detected in synovial fluid of both OA and RA patients, but again, not in healthy controls (Richardson et al., 2008), corroborating the "on demand" nature of ECS engagement. Interestingly, 2-AG was expressed at higher levels in OA synovial samples compared to RA synovial samples (Richardson et al., 2008) suggesting that drugs which target the ECS could be more effective in degenerative arthritis rather than in inflammatory joint disease.

8.3.2 EFFECTS OF ENDOCANNABINOIDS ON ARTHRITIS PATHOLOGY

The ECS has been recognized as playing an important role in many physiological processes and the involvement of the ECS in arthritis disease progression has been gaining increasing interest in recent years. Several groups have shown that the endocannabinoid system is a main regulator of bone metabolism, including bone mass, bone loss, and bone cell function (Idris and Ralston, 2010). Tam et al. showed that endocannabinoids are produced endogenously in bone and synovial joints. CB_1R, CB_2R, and GPR55 are all expressed by bone and synovial cells, and 2-AG and AEA are produced within the bone microenvironment (Tam et al., 2006; Idris and Ralston, 2010). Rossi et al. (2009) showed that 2-AG and AEA are expressed at detectable levels in cultured human osteoclasts, and their production increased when a FAAH inhibitor was applied. The cannabinoid receptors, along with the endocannabinoid ligands and their synthesizing enzymes, are also found in osteoblasts, osteoclasts, bone marrow stromal cells, and macrophages (Whyte et al., 2009; Tam et al., 2006, 2007; Idris and Ralston, 2010). More specifically, CB_1Rs modulate osteoblast differentiation by regulating intracellular cyclic adenosine monophosphate AMP (cAMP) levels (Tam et al., 2006). CB_1Rs and CB_2Rs have been implicated in the regulation of osteoclast differentiation and activity (Tam et al., 2006; Idris and Ralston, 2010). Since some cannabinoids can act through non-canonical signalling pathways, a portion of bone metabolism may not involve CB_1Rs or CB_2Rs. Rossi et al. (2009) showed evidence that TRPV1 is expressed by bone cells, which may be responsible for some of the effects of AEA seen on bone formation and differentiation. Elsewhere, GPR55 has been shown to be involved in regulating bone resorption (Whyte et al., 2009). Chondrocytes maintain cartilage homeostasis, regulating the balance between synthesis and degradation (Krustev et al., 2015). In cartilage, proteoglycan loss occurs early in the degradation process and is a major histopathological feature in rheumatic diseases. In later stages, there is significant breakdown of collagen, which is suggested to be the point of irreversible cartilage damage (Mbvundula et al., 2005, Little et al., 2002). *In vitro* studies have identified CB_1R and CB_2R expression on chondrocytes, and have shown that endocannabinoids and synthetic cannabinoid compounds have a direct effect on chon-

drocyte metabolism, resulting in an inhibition of proteoglycan breakdown and cartilage protection (Mbvundula et al., 2005). Thus, cannabinoids could be protective against joint degeneration.

The synovium is central to joint inflammation and is a major source of articular cytokine production (Maini and Feldmann, 1998). *In vitro* studies have demonstrated that exposure of naïve synoviocytes to pro-inflammatory cytokines leads to an upregulation of CB_1Rs, but a downregulation of CB_2Rs (McPartland, 2008). Additional *in vitro* studies showed that synovial cells from mice with inflammatory arthritis produced large amounts of tumor necrosis factor-alpha (TNF-α) (Malfait et al., 2000), which is a main contributor to inflammation in arthritis (Maini and Feldmann, 1998). Synovial cells from mice treated with exogenous CBD produced significantly less TNF-α in culture (Malfait et al., 2000). CBD had a dose-dependent therapeutic effect on disease progression in mice with inflammatory arthritis. The CBD treatment not only suppressed clinical signs of the disease, but there were no obvious side effects noted with chronic treatment (Malfait et al., 2000). Furthermore, hind paws from these mice were protected by CBD treatment from joint destruction in both acute and chronic disease states, when compared to control (Malfait et al., 2000).

The above evidence supports the novel therapeutic target and/or drug potential of cannabinoids in the progression of rheumatic disease states.

8.3.3 EFFECT OF ENDOCANNABINOIDS ON JOINT PAIN

The primary complaint of arthritis patients is chronic pain, which is not effectively managed and is often undertreated across the lifespan of the patient. Thus, the recent focus on the ECS as a safe and effective means of alleviating joint pain has become intriguing. Very few studies have investigated the potential benefits of phytocannabinoids in a scientific setting. In one of the few such studies, phytocannabinoids such as THC and CBD were able to decrease pain responses when administered to animals with inflammatory arthritic pain (Hammell et al., 2015).

Evidence is accumulating which shows that cannabinoids and cannabinoid receptors play an important role in the modulation of OA-associated pain (La Porta et al., 2013). The first indication that CBs could have a local effect in the joint came from a study in which intra-articular injection of the CB_1R agonist ACEA reduced nociceptor firing rate in a rat model of OA (Schuelert et al., 2010). Interestingly, this study also found that local injection of a CB_1R antagonist caused a moderate increase in joint afferent firing rate. This outcome suggests that in the OA knee there is a significant endocannabinoid tone that could be modifiable with endocannabinoid hydrolysis inhibitors. Indeed, later studies in which a FAAH inhibitor was introduced into the joint space of OA animals profoundly reduced joint mechanonociception and pain (Schuelert et al., 2011). These findings provided the first preclinical evidence that joints have a rich ECS that could be harnessed to manage OA pain at source in the periphery.

In other studies, mice lacking the CB_2R demonstrated exacerbated responses to mechanical stimulation in the MIA model of OA compared to wild type mice injected with MIA (La Porta et al., 2013). The mechanical allodynia was reduced in transgenic mice overexpressing CB_2Rs in the

CNS, suggesting that CB_2Rs play a central role in the modulation of joint nociception (La Porta et al., 2013). Contrasting results were observed in CB_1R knockout mice where mechanical allodynia was unaltered, suggesting that globally CB_1Rs have a lesser role in joint pain control compared to CB_2Rs. The pain behaviors correlated well with endocannabinoid gene expression changes, further confirming the functional relevance of receptor turnover to the pathogenesis of arthritis pre-clinically (La Porta et al., 2013).

Both endogenous and synthetic CB_1R agonists, like AEA or ACEA, have primarily been shown to produce analgesia in pre-clinical animal models of arthritis. Many of these studies use systemic administration that can produce centrally mediated tetrad effects. The tetrad includes four components, which are analgesia, hypothermia, hypomobility, and catalepsy; these can all be mediated through cannabinoid receptors. While cannabinoid-induced analgesia is optimal, these other side-effects are generally considered adverse and unwanted (Ameri, 1999). There has been conflicting evidence regarding CB_2R involvement in joint pain, with some studies showing that CB_2R agonists produce analgesia (Vera et al., 2013; Burston et al., 2013); however, a study by Schuelert et al. (2010) found that a particular CB_2R agonist GW405833 was pronociceptive in OA joints. The main limitation of these studies is the poor selectivity of the CB_2R agonists used. Further experiments are still required to clarify the role of these receptors in joint pain control.

Cannabinoids have been shown to be effective in reducing pain in acute inflammatory models of arthritis (Krustev et al., 2014; Guindon et al., 2011; Ignatowska-Jankowska et al., 2014). MAGL inhibition was shown to produce greater analgesia when delivered chronically at lower doses, rather than acutely at higher doses (Ghosh et al., 2013) which may relate to a slow adaptation of the ECS to drug intervention. Interestingly, studies using different compounds for MAGL inhibition saw that the compound underwent tolerance after chronic systemic administration, but acute doses did not (Ignatowska-Jankowska et al., 2014; Burston et al., 2016). Recently, a group developed a peripherally restricted FAAH inhibitor, and tested it in neuropathic and inflammatory pain models. Upon systemic administration of the compound, pain behavior was decreased yet there was no effect on brain levels of FAAH or FAAH activity (Clapper et al., 2010), highlighting a peripheral site of action.

Since there are many pathways and mechanisms involved in cannabinoid processing, combination therapies have become an attractive potential treatment strategy. These therapies intervene with multiple signal transduction pathways instead of just one. FAAH inhibition has been shown to have great pre-clinical potential, but unfortunately has failed in multiple clinical trials (Huggins et al., 2012), which may in fact be linked to other overlapping pathways involved in pain processing. For example, TRPV1 channels have been shown to be activated by endocannabinoids and may be contributing to pain symptoms (Chu et al., 2011). A study using a dual compound which inhibits both FAAH and TRPV1 showed antihyperalgesic effects in a rat model of OA (Malek et al., 2015). Other studies have used a dual FAAH/MAGL inhibitor (JZL195). This compound has been shown to decrease pain behavior in a neuropathic pain model (Barnes et al., 2015) and an inflam-

matory pain model (Anderson et al., 2014). These combination therapies were more efficacious than the enzyme inhibitors on their own, and they reduced the frequency and severity of unwanted cannabinoid tetrad side effects with the added benefit of an absence of tolerance (Barnes et al., 2015; Anderson et al., 2014). Combination therapies provide a novel strategy for treating arthritis at safer doses and for a longer therapeutic window, with minimal side effects and low tolerance. A summary of cannabinoid compounds used for pre-clinical treatment of arthritis can be found in Table 8.1.

8.3.4 EFFECT OF ENDOCANNABINOIDS ON INFLAMMATION

In recent years, there has been a shift in the understanding of the pathophysiological mechanisms underlying the progression of rheumatic diseases. All types of arthritis are viewed as multifactorial diseases, with chronic inflammation at center-stage. Both high-grade and low-grade inflammation results in local tissue damage and degenerative changes within the joint, metabolic dysregulation, and are main driving factors behind disease progression (Robinson et al., 2016). Endocannabinoid and cannabinoid compounds have the potential to modulate and regulate functional activities of a variety of immune cells. Endocannabinoid signaling events lead to immune cell migration and the production of cytokines and chemokines. CB_1Rs and CB_2Rs are both expressed on immune cells, but it is thought that cannabinoids primarily impart their immunocellular actions via CB_2Rs (Ghosh et al., 2013; Naidu et al., 2010). CB_2Rs have been implicated specifically in the pro-inflammatory cascade involving mediators such as cytokines, neuropeptides, chemokines, and nitric oxide. CB_2R knockout mice express higher levels of pro-inflammatory biomarkers than control, wild type mice (Maresz et al., 2007). Interestingly, an *in vitro* study showed that activation of both CB_1Rs and CB_2Rs, instead of CB_2Rs alone, on microglia, macrophages, and astrocytes blocked release of inflammatory mediators during an immune challenge (Sheng et al., 2004). An important central role for activated microglia and astrocytes in the development of injury and pain has been established, and is discussed below (Thakur et al., 2012). This evidence suggests that CB_1Rs and CB_2Rs play an equally important role in reducing inflammation, and not just CB_2Rs, as originally thought.

Both CB_1R and CB_2R agonists, synthetic and endogenous cannabinoids, have been shown to be anti-inflammatory in many pre-clinical arthritis models (Krustev et al., 2014; Schwarz et al., 1994). Oral administration of AEA, accompanied by the entourage molecule, PEA, decreased paw edema and mast cell degranulation in a carrageenan-induced inflammatory arthritis model (Richardson et al., 1998). FAAH and MAGL inhibition decrease inflammation as demonstrated by a reduction in paw edema in inflammatory pain models (Guindon et al., 2011; Ignatowska-Jankowska et al., 2014). Furthermore, a study using transdermal administration of CBD, in a chronic inflammatory arthritis model, showed a dose-dependent decrease in knee joint circumference, lower immune cell invasion into the spinal cord, and reduced synovial membrane thickening (Hammell et al., 2015).

In states of acute and chronic inflammation, immune cells undergo extravasation from the vasculature into surrounding inflamed tissues. Before extravasation, leukocytes begin to slow

TABLE 8.1: A summary of the pharmacological action of synthetic cannabinoid, phytocannabinoid, and endocannabinoid modulating compounds on pre-clinical models of osteoarthritis, inflammatory arthritis, and neuropathy

Drug	Delivery Method	Target	Selectivity (IC50)	Effect	Model	Reference
ACEA	local	CB1 agonist	4.52 nM	Decrease afferent nerve fiber sensitivity	MIA	McDougall et al. 2008
JWH133	Systemic	CB_2R agonist	N/A	Reduced mechanical allodynia	Cisplatin-induced neuropathy	Vera et al. 2013
				Decreased hyperalgesia	MIA	Burston et al. 2013
	Local			Reduced hyperaemia	Kaolin/Carrageenan, CFA	McDougall et al. 2008
JWH015	Local	CB_2R agonist		Reduced hyperaemia	Kaolin/Carrageenan, CFA	McDougall et al. 2008
GW405833	Local	CB_2R agonis		Increased OA pain	MIA	Schuelert et al. 2010
URB597	local & systemic	FAAH inhibitor	N/A	Decreased nociception and anti-inflammatory	MIA & aged guinea pigs	Schuelert et al. 2011 and Krustev et al. 2015
				Decrease in spontaneous activity of joint afferents	Aged guinea pigs	Schuelert et al. 2011
URB937	Systemic	Peripherally restricted FAAH inhibitor	N/A	Anti-hyperalgesic and anti-inflammatory	CCI, carrageenan paw edema	Clapper et al. 2010
MJN110	Systemic	MAGL inhibitors	9.1 nM	Anti-nociceptive	MIA	Burston et al. 2016
JZL184			8 nM	Anti-inflammatory and analgesic	Carrageenan inflammatory pain	Ghosh et al. 2013
JZL195	local	Dual FAAH/MAGL inhibitor	2 nM & 4 nM	Analgesic	CFA	Anderson et al. 2014
OMDM-198	Systemic	Dual TRPV1/FAAH inhibitor	3.36 uM	Analgesic	MIA	Malek et al. 2015
LY 2183240	Systemic	AEA reuptake inhibitor/FAAH inhibitor	270 pM	Antinociceptive	Formalin induced pain	Maione et al. 2008
CBD	Systemic	GPR55 antagonist, weak CB_1R antagonist, CB_2R inverse agonist, AEA reuptake inhibitor	0.445 uM, 3.35 uM, 27.5 uM	Immunosuppressive, anti-inflammatory, and anti-arthritic	Collagen-induced arthritis	Malfait et al. 2000
THC	Oral, systemic	Partial agonist at CB_1R & CB_2R	Non-selective binding	Anti-inflammatory and analgesic	Adjuvant-induced arthritis, carrageenan paw edema	Sofia et al. 1973

down and roll along the endothelial wall, then firmly adhere to the intimal surface of the venule before eventually transmigrating through intra-endothelial gaps into the surrounding tissue. Using intravital microscopy techniques, the number of rolling and adherent leukocytes is a quantifiable inflammation parameter *in vivo*. Articular FAAH inhibition using URB597 decreased leukocyte rolling and adhesion as well as inflammation-induced hyperemia in a pre-clinical model of acute synovitis at low, but interestingly not high, doses (Krustev et al., 2014). A summary of cannabinoid compounds used for the treatment of pre-clinical arthritis can be found in Table 8.1.

Our shift in the understanding of the role inflammation plays in the progression and pathogenesis of arthritis has revealed a novel target for therapy. Perhaps targeting inflammation early, or during intermittent flare-ups, could lead to the modification and dampening of the inflammatory response, which would subsequently slow disease progression and chronic pain. Further elucidation of the mechanisms of inflammation in arthritis and how to intervene in these processes with endocannabinoid promotion could yield new therapies for preventing the persistence of joint inflammation.

8.3.5 EFFECT OF ENDOCANNABINOIDS ON NEUROPATHIC PAIN

As mentioned previously, there is a subset of arthritis patients, about 30%, who experience neuropathic pain symptoms. These patients typically do not find conventional therapies such as NSAIDS or opioids effective for relieving their pain (Zimmermann, 2001). Previously, cannabinoid compounds have only been tested in nerve injury models or other demyelinating disease states such as multiple sclerosis (MS) (Ignatowska-Jankowska et al., 2014; Kinsey et al., 2009; Arevalo-Martin et al., 2003). A non-selective CB_1R/CB_2R agonist (WIN55-212,2), as well as an AEA reuptake inhibitor (AM404), reduced neuropathic pain symptoms in sciatic nerve ligation and chronic constriction nerve injury models (La Rana, et al., 2008). FAAH and MAGL inhibitors were also able to effectively reduce neuropathic pain symptoms in mice with nerve injury (Ignatowska-Jankowska et al., 2014; Kinsey et al., 2009) confirming that the ECS may be an effective way of treating this type of pain. Synthetic cannabinoid agonists, for CB_1Rs and CB_2Rs, given in a murine MS model, improved motor function and decreased inflammation; these results correlated with significant remyelination of nerves (Arevalo-Martin et al., 2003). Cannabinoids can therefore be neuroprotective and show great promise as a novel therapeutic target for related neuropathic pain states. Further studies are required to establish cannabinoids as neuropathic pain inhibitors and specifically help treat the subset of arthritis patients who are insensitive to first order pain therapies. Summary of pre-clinical studies using cannabinoid compounds can be found in Table 8.1.

Recently, MIA, a well-established pre-clinical OA model, has been shown to have a neuropathic pain component. Intra-articular injection of MIA induced expression of activating transcription factor-3 (ATF-3), a marker for peripheral nerve injury, in the dorsal root ganglia (DRGs), as well as microgliosis around the spinal cord, another indicator of neuronal injury (Thakur et al., 2012). Recognizing that this pre-clinical arthritis model includes a neuropathic pain component

directly links pre-clinical studies to arthritis patient symptoms, and can be used to validate the use of cannabinoid compounds for both neuropathic pain and arthritis.

8.4 ON CONSIDERING A VARIETY OF THERAPEUTIC IMPLICATIONS FOR CANNABINOIDS

Cannabinoid compounds are promising for the modulation of arthritis symptoms and disease progression based on their pharmacological properties and their implication in disparate pre-clinical pain studies. The alleviation of chronic inflammation is at the forefront of arthritis research, and is implicated as a contributor to symptom development in different types of arthritis. Focusing on the blockade of inflammation during patient flare ups could be the key to slowing the progression of many facets of the disease. As discussed earlier in this chapter, immune mediators are responsible for the destruction of joint tissues and nerves innervating the joint. Dampening this degeneration with the use of cannabinoid compounds would modify disease progression and alleviate the symptoms imparted by the inflammatory processes.

Most pre-clinical evidence uses systemic administration of cannabinoid compounds to produce their desired outcomes, and while not all of these compounds facilitate centrally-mediated effects, there is the possibility that cannabinoids can have a central mode of action which could complement peripheral drivers of disease activity. Gearing our focus on developing treatments that are peripherally restricted, or on drugs that are administered locally with a minimal-psychoactive profile, will be key to the future use of cannabinoids for alleviation of joint disease while avoiding unwanted highs.

Juvenile arthritis (JA) refers to the autoimmune and inflammatory conditions that occur in patients that are 16 years or younger. JA shares many of the same symptoms as other types of arthritis such as joint swelling, pain, redness, and loss of function. JA also has accompanying symptoms that affect organs outside of the musculoskeletal system such as eyes, skin, and the gastrointestinal tract (Lovell et al., 2008). Similar to arthritis in the more elderly population, JA has been increasingly difficult to treat with current therapies that target symptoms rather than disease progression. Safety issues and development of cannabinoid compounds that do not act in the CNS could become a viable option to treat JA patients while escaping some of the deleterious effects of cannabinoids in the developing brain. There is evidence that the cannabinoid system interferes with neurological development in adolescents and that many of the cognitive effects associated with cannabis can be detrimental in adolescent populations (Whiting et al., 2015; Crocker and Tibbo, 2015;Renard et al., 2016). Specifically, cannabinoids can affect prefrontal cortical functions such as memory recall, motor function, and can contribute to addictive behaviors development in later life (Fride, 2004). A neuroimaging study carried out on young adults who are recreational cannabis users showed a reduction in grey matter volume, density, and cerebral conformation when compared with older

subjects (Gilman et al., 2014). Therefore, any modification to the endocannabinoid system at the central level at a young age might have irreversible consequences and requires special consideration.

While cannabinoids have not been studied in JA, there is a small body of evidence which supports the use of cannabinoid preparations such as nabiximols for the treatment of refractory epilepsy in children. Nabiximols are a 50:50 mixture of THC and CBD extracted from the cannabis plant, which have been approved in the UK and Canada under the tradename Sativex. THC and CBD are known to be anti-convulsant in animal models of epilepsy, but tolerability is limited with THC because of its psychotropic effects; CBD appears to be safer and better tolerated (Devinsky et al., 2014). Unfortunately, there are no studies thus far that have examined the efficacy or safety of cannabis extracts in pediatric chronic pain patients. There has, however, been some evidence of adult MS patients using cannabis products for spasticity and centrally mediated chronic pain, as well as for several different neurological disorders (Koppel et al., 2014; Wade et al., 2004). Their use in juvenile chronic pain states requires further testing and safety validation.

Most medicinal cannabis users use smoking as the preferred route of administration, which is not an ideal method of drug delivery in any pain patient. To avoid centrally mediated effects of cannabinoid compounds, peripheral or local routes of administration should be considered, such as therapeutics administered subcutaneously, intra-articularly, or applied in topical formulations. A peripheral drug delivery system would allow for better control of dosing and concentrations of cannabinoid compounds compared to smoking. Using non-psychoactive compounds, as well as compounds that modify the endocannabinoid system instead of directly acting on it, would be a more robust way of diminishing the unwanted adverse effects of cannabis while sustaining their analgesic, anti-inflammatory, and disease-modifying potential.

Chronic pain and physical disability are often associated with anxiety, depression, and alterations in mood and cognitive function. The endocannabinoid system has been implicated in rheumatic disease states and has also been implicated in the regulation of behavioral responses to stress. Chronic pain and physical disability can lead to stress development and chronic fatigue syndromes. An interesting study evaluated the involvement of the endocannabinoid system in anxiety-like behaviors that are associated with osteoarthritis of the knee (Porta et al., 2015). PainDETECT scores qualify pain intensity and neuropathic pain-like symptoms correlated with depression and anxiety levels. The study showed a correlation between mice with MIA-induced OA and an increase in anxiety-like behaviors. There was a marked increase in anxiety-like behavior in CB_1R knockout mice compared to CB_2R knockout mice, injected with MIA. Moreover, systemic injection of the CB_1R and CB_2R agonists, ACEA or JWH133, respectively, improved pain behavior while also decreasing anxiety-like behaviors in OA mice (Porta et al., 2015). These data suggest that the endocannabinoid system is important in the emotional manifestations of OA pain and may be a promising therapeutic target for the psychosocial, as well as the physical, aspects of rheumatic diseases.

8.5 CONCLUSION

The endocannabinoid system is a critical regulator of many physiological functions, and is a highly plastic system which can change function and cellular signaling during disease or following injury. A plethora of pre-clinical evidence exists suggesting that the pharmacological interventions directed towards the endocannabinoid system are promising approaches, not only for symptom relief, but also for improving disease progression. Further research is required to increase the translatability of these many pre-clinical findings into the common practice.

CHAPTER 9

Cannabinoid 2 Receptor Activation in Sepsis

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Abstract

Sepsis represents a severe dysregulation of the immune system with organ dysfunction in response to infection. Despite a variable disease course, an initial hyper-inflammatory response is frequently followed by a state of immunosuppression. There are no sepsis therapies targeted to the immune dysregulation available to date, however, the role of the endocannabinoid system in immunomodulation suggests promise. Specifically, activation of the cannabinoid type 2 receptor (CB_2R) has been shown to have anti-inflammatory action. This might suggest that activation of CB_2R could improve sepsis outcomes by dampening the initial hyper-inflammatory immune state. Knockout of CB_2R decreased survival in animal models of sepsis confirming the complexity in the interaction between CB_2R and progression of sepsis. Other avenues of endocannabinoid system (ECS) modulation in sepsis may include blockade of cannabinoid type 1 receptor (CB_1R), with anti-inflammatory and blood pressure stabilizing effects, as well as antagonism of the endocannabinoid-activated G-protein coupled receptor 55 (GPR55), which has anti-inflammatory actions.

Key Words

sepsis, endocannabinoid system, immune dysfunction, inflammation, immunosuppression, CB_2R, CB_1R, GPR55

9.1 INTRODUCTION

Sepsis represents a severe dysregulation of the immune system. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) defines sepsis as life-threatening organ dysfunction caused by a dysregulated host response to an infection (Singer et al., 2016). Initially, the immune system is over-activated, whereas in later stages, immune-paralysis can occur. It is important to note that sepsis disease course varies greatly among patients. Sepsis can progress to severe sepsis or septic shock, a subset of sepsis characterized by profound circulatory, cellular, and metabolic

abnormalities associated with an increased risk of mortality (Singer et al., 2016). Together, sepsis and septic shock represent the leading cause of surgical ICU mortality internationally (Vincent et al., 2009). For sepsis treatment, the timing of potential immune modulation is essential to provide benefit and mitigate risk. A lack of reliable and specific sepsis biomarkers complicates the delivery of immunomodulatory therapy at particular time points in disease progression. Further, the lack of specific sepsis treatments underlines the need and potential value of exploring promising targets, such as the ECS. The ECS is of interest due to evidence that the CB_2R is expressed by various immune cells (Klein, 2005), and activation of the ECS is anti-inflammatory (Orliac et al., 2003). This chapter will summarize current knowledge of ECS function in sepsis, with focus on CB_2R activation as a potential target for immune modulation. In addition to CB_2R, potential benefits of modulation through CB_1R and GPR55 will also be discussed.

9.2 CANNABINOID 2 RECEPTOR, AN IMMUNOSUPPRESSIVE TARGET IN SEPSIS

In contrast to the predominant presence of CB_1R in the CNS, CB_2R is for the most part restricted to a range of immune cells in the periphery, listed here per descending level of expression: natural killer cells, monocytes, polymorphonuclear leukocytes, $CD4^+$ and $CD8^+$ lymphocytes (Galiègue et al., 1995). To date, there is a finite amount of research assessing the effects of CB_2R modulation in sepsis, and this has generated conflicting findings due in part to the necessity of understanding CB_2R signaling in the context of the complex immune pathophysiology present in sepsis. Activating CB_2R is associated with decreases in macrophage and neutrophil recruitment, as well as with reduced generation of pro-inflammatory cytokines (Orliac et al., 2003). This and other work suggests an overall anti-inflammatory action of CB_2R.

The pathophysiology of sepsis centers on a dysregulated systemic immune response to an infection. Initial hyper-activation of the immune system is accompanied by a robust release of anti-inflammatory mediators (Figure 9.1). Consequently, a state of immunosuppression may result, conferring a susceptibility to secondary infections and late mortality. Due to the dynamic changes in immune states during sepsis, immune modulation is a logical and promising target for therapeutics. Activation of CB_2R during the hyper-activation state may be beneficial through attenuation of the pro-inflammatory immune response. Activation in this phase may also decrease the magnitude of anti-inflammatory compensation, therefore preventing an ensuing state of immunosuppression. Alternately, blockade of CB_2R during an immunosuppressed state may lead to an overall strengthening of a patient's immune status. Accurate identification of the patient's immune status is critical to prevent either mistakenly bolstering a hyperactive state or further weakening an immunosuppressed state.

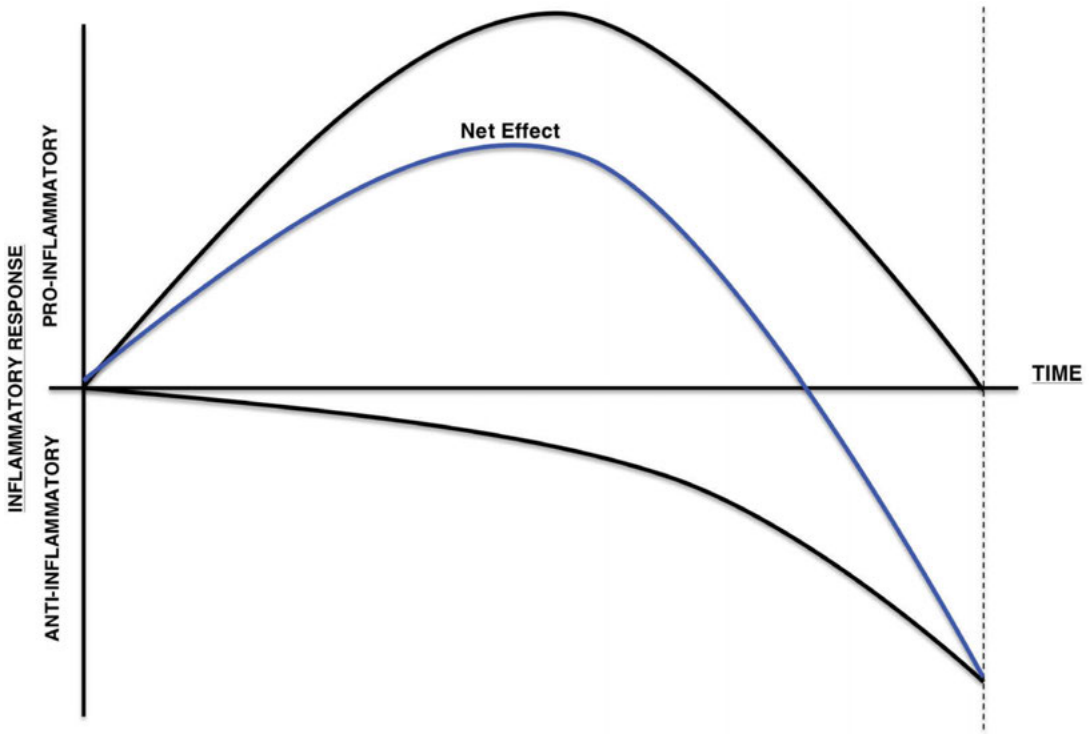

FIGURE 9.1: Generalized immune response in sepsis characterized by simultaneous release of pro-inflammatory and anti-inflammatory mediators, with a net effect resulting in an initially hyper-immune activated state, followed by a state of immunosuppression.

9.3 CANNABINOID TYPE 2 RECEPTOR SIGNALING IN SEPSIS

CB_2R signals through a $G_{\alpha i}$-linked GPCR (Oldham and Hamm, 2008; Pertwee et al., 2010). Activation of $G_{\alpha i}$ proteins leads to a decrease in adenylyl cyclase activity, resulting in a decrease in intracellular cyclic adenosine monophosphate (cAMP) levels (Simon et al., 1991; Bari et al., 2006). cAMP plays a key regulatory role in myeloid cell NF-kB and p38 signaling pathways. An increase in cAMP levels has been reported to lead to diminished phagocytic abilities and attenuated oxidative burst in neutrophils (Kreth et al., 2009). Studies of CB_2R signaling in neurotrophils indicate that activation of CB_2R decreases the recruitment, chemotaxis, and respiratory burst in neutrophils. At the same time, CB_2R signaling increases neutrophil phagocytosis, p38 activity, and expression of the activation marker CD11b.

Overall, studies have indicated an anti-inflammatory role of CB_2R activation (Kasten et al., 2010). However, an important consideration in sepsis is that CB_2R modulation may have different net effects based on the severity of the sepsis and the timing of intervention (Kasten et al., 2010).

In an experimental abdominal sepsis study utilizing CB_2R knockout mice, an increase in neutrophil recruitment and decrease in functionality, such as decreased p38 activity at the site of infection, was observed which is consistent with a constitutive anti-inflammatory role for this receptor (Tschöp et al., 2009). This same study also demonstrated that activation of CB_2R was associated with a decrease in neutrophil recruitment, increased phagocytosis, and p38 activity (Tschöp et al., 2009).

In a contradictory line of evidence, CB_2R inactivation was shown to decrease the release of IL-6, MIP-2, and splenic NF-kB activation (Csóka et al., 2009). Of interest was the finding of a significant decrease in levels of the anti-inflammatory cytokine IL-10 in both serum and peritoneal lavage in the CB_2R knockout mice. This may indicate the attenuation of the compensatory anti-inflammatory response to early phase immune hyper-activation in sepsis. The potential utility of this dampened compensatory immune suppression may assist in improving clinical outcomes. Csóka et al. also found that CB_2R knockout had a protective role against apoptosis in lymphoid organs and increased the leukocyte activity marker CD11b+, as well as cells with the B cell hallmark CD19 in experimental abdominal sepsis (Csóka et al., 2009).

Using intravital microscopy to study leucocyte-endothelial interactions in the microvasculature during experimental endotoxemia as marker of immune cell recruitment in sepsis, our group found that activation of CB_2R reduced leukocyte adherence to the endothelium within the intestinal microcirculation (Sardinha et al., 2014). Another study in the iridial microvasculature in a model of endotoxin-induced uveitis confirmed decreased leukocyte recruitment with CB_2R agonist treatment and a reduction in ocular inflammmation (Toguri et al., 2014).

9.4 CANNABINOID 2 RECEPTOR AND SEPSIS SURVIVAL

At present, only a few animal studies are available regarding CB_2R modulation and sepsis survival: three publications suggest a beneficial role of CB_2R activation, and one suggests a detrimental role. Using experimental cecal ligation and puncture (CLP) as a model of abdominal sepsis in mice, decreased survival was observed with CB_2R knockout mice compared to wild type animals (Tschöp et al., 2009). This group also found increased levels of the pro-inflammatory cytokine, IL-6, as well as bacteremia and lung injury at 24 hr after initiation of sepsis in CB_2R knockouts (Tschöp et al., 2009).

Another study by Gui et al. confirmed significantly decreased survival in CB_2R knockout mice in a different sepsis model—experimental endotoxemia (Gui et al., 2013). This group also found dose-dependent inhibition of pro-inflammatory cytokine release with CB_2R agonist treatment. However, a study by Csóka et al. contradicts these findings and reported that CB_2R knockout mice with CLP-induced sepsis had improved survival (Csóka et al., 2009). In this study, CB_2R

knockout mice had significantly lower mortality rates compared with wild type mice, which became apparent on the second day of observation. On the seventh day following CLP, the mortality rate of CB_2R knockout mice was markedly (by more than 40%) lower than that of CB_2R wild type mice. These authors also found that CB_2R knockout in CLP-induced sepsis had a protective effect on sepsis-induced muscle and renal injury and decreased translocation of bacteria into the blood.

The variation in results for CB_2R modulation in sepsis might be related to the differences in the experimental sepsis models. Tschöp et al., who observed a beneficial role for CB_2R activation, used CLP to induce poly-microbial sepsis and used CB_2R knockout mice and C57BL/6J wild type mice (Tschöp et al., 2009). Similarly, Csóka et al. induced poly-microbial sepsis by CLP (Csóka et al., 2009). In contrast to a smaller (23G) needle used by Tschöp et al., Csóka et al. used a larger (20G) needle to puncture the cecum. Due to the small size of a mouse cecum, the larger incision (20G) could lead to a significant increase in release of cecal contents into the peritoneal cavity. This is likely to potentiate a more severe infection through increased bacteremia. It is therefore reasonable to expect a more severe sepsis in the model used by Csóka's group. Furthermore, Csóka et al. recorded survival over 7 days post-CLP procedure. In contrast, Tschöp et al. recorded survival over 14 days post-CLP with chronic CB_2R agonist, GP1a, treatment. For the chronic treatment, osmotic minipumps were implanted in the upper back of the animals. Due to the complexity of the immunopathology of sepsis, this variation between experimental models may have elicited different processes resulting in a differing state of immune dysregulation. Despite this, CB_2R agonist treatment by Tschöp et al. and CB_2R knockout by Csóka et al. may in fact provide insight into CB_2R action in varied states of sepsis pathophysiology (see Figures 9.2 and 9.3).

9.5 OTHER ENDOCANNABINOID SYSTEM TARGETS IN SEPSIS

9.5.1 CB_1R

In contrast to CB_2R, blockade of CB_1R in the hyperinflammatory phase of sepsis has been reported to be beneficial. Blockade of CB_1R at 4 hr post-CLP induction of sepsis was associated with significantly increased survival compared to vehicle (Leite-Avalca et al., 2016). This group looked at both early and late phase sepsis, and found that CB_1R blockade in late phase increased survival, lowered body temperature, and increased circulating levels of arginine vasopressin.

Intestinal ileus is a frequent postoperative source of sepsis, resulting from gastrointestinal hypomotility in the absence of a mechanical obstruction. Agonists at both CB_1R and CB2R were shown to have a detrimental effect in septic ileus through reduction of myoelectric activity, and therefore reduction of GI motility (Li et al., 2010). This group also found that CB_1R blockade increased motility, and that pre-treatment with either a CB_1R antagonist or a CB_2R inverse-agonist exerted a protective role against LPS-induced reduction in motility.

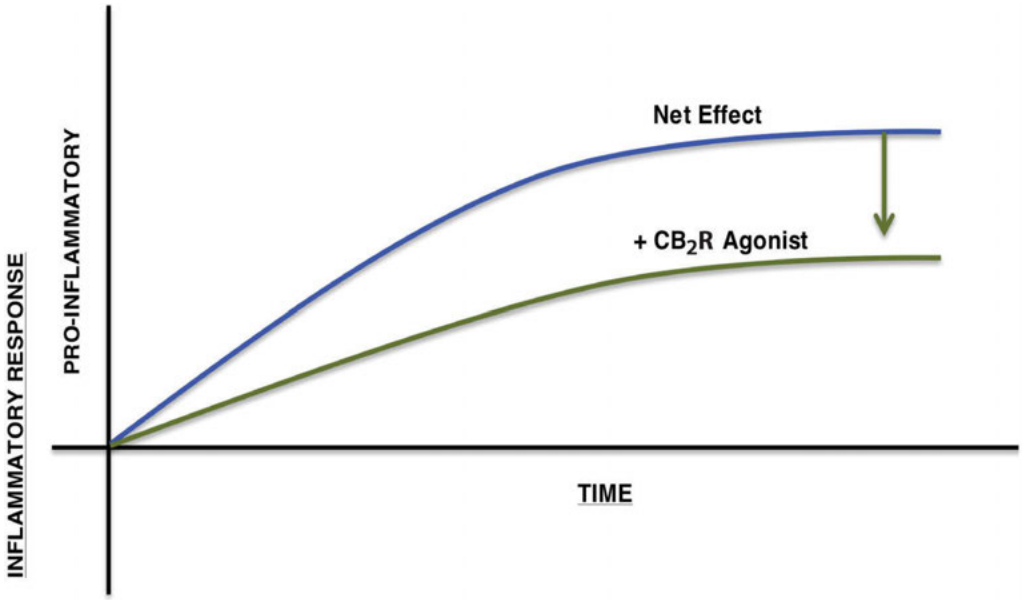

FIGURE 9.2: Immune response in a moderate model of sepsis, with immunosuppression from addition of CB_2R agonist.

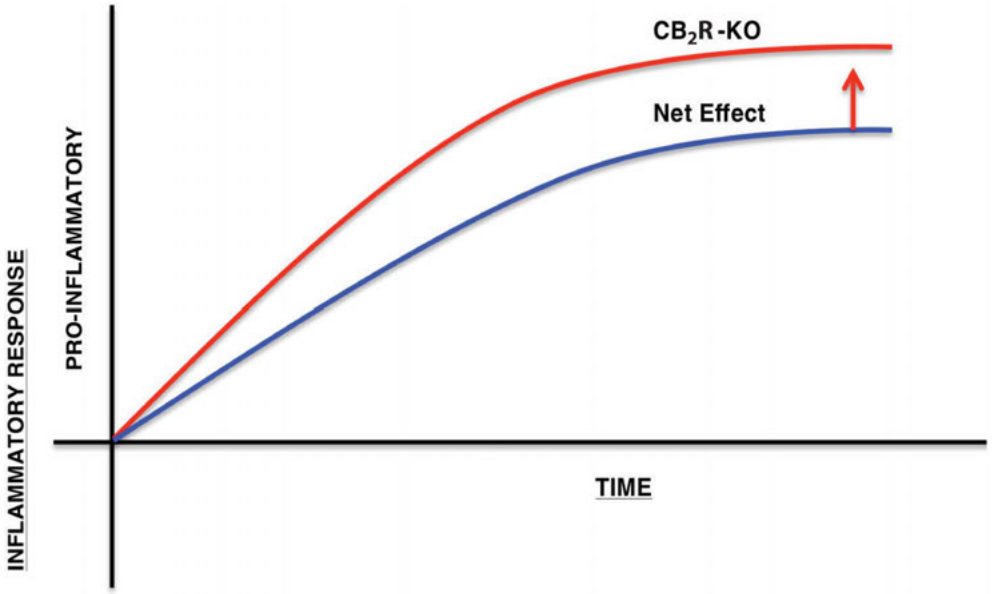

FIGURE 9.3: Immune response in a severe model of sepsis, with immune-activation resulting from knockout of CB_2R.

It is important to consider the effects of potential novel treatments on blood pressure, due to the important role of hypotension in sepsis pathology. Activation of CB_1R has been shown to result in normalization of blood pressure in hypertensive anesthetized animals (Bátkai et al., 2004). A similar experiment demonstrated a CB_1R-mediated bradycardic response in hypertensive mice (Wheal et al., 2007). These effects have yet to be examined in humans; however, there is evidence that the CB_1R antagonist Rimonabant® had minimal effects on blood pressure in normotensive humans (Ruilope et al., 2008). At the same time, other studies indicate that CB_1R blockade may prevent the drop in blood pressure associated with hemorrhagic shock (Wagner et al. 1997), as well as in cardiogenic shock (Wagner et al., 2001) and endotoxemic shock (Varga et al., 1998). Sepsis can progress to septic shock, characterized by poor tissue perfusion, and there is a requirement for vasopressors to maintain systemic blood pressure. Despite a lack of sepsis-specific evidence, it does appear that CB_1R activation has an overall blood pressure lowering effect, with CB_1R antagonism associated with an increase in blood pressure. It is important, however, to note that profound organ hypoperfusion may exist in severe sepsis and septic shock in the absence of systemic hypotension. While this role of microcirculatory dysfunction in sepsis is an important focus of understanding sepsis pathophysiology and treatment, systemic hypotension in septic shock must be corrected rapidly to prevent further decline.

9.5.2 GPR55

A more recently characterized endocannabinoid-activated GPCR is GPR55 (Pertwee et al., 2010). Despite a high affinity for cannabinoid ligands, GPR55 has limited sequence similarity to cannabinoid receptors and is therefore not classified "CB_3R" (Pertwee, 2007; Baker et al., 2006). Both the endogenous lysophospholipid, L-α-lysophosphatidylinositol (LPI), and 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-AGPI), have been shown to activate GPR55 (Kotsikorou et al., 2011). GPR55 has also been reported to be activated by the CB_1R antagonists AM251 and Rimonabant® (Henstridge et al., 2010). GPR55 has wide expression in the CNS peripheral tissues including the gastrointestinal tract and adrenal gland, and the immune system, with particularly high expression in the spleen and on leukocytes (Stančić et al., 2015). GPR55 is coupled to Gα12 and to Gα13 proteins and signals through activation of Rho-associated protein kinase, as well as Ras homolog gene family member A and the phospholipase C pathway. Activation of GPR55 is associated with leukocyte cytokine production, chemotaxis, and proliferation resulting from ERK phosphorylation due to increased intracellular Ca^{2+} level-activated rhoA, Rac, and cdc42 (Ryberg et al., 2007; Henstridge et al., 2010).

There is a lack of studies on the role of GRP55 in sepsis. However, a growing body of evidence indicates that GPR55 is involved in immune responses, raising the potential for GPR55 as a useful novel target for immunomodulation in sepsis. Furthermore, expression of GPR55 on immune cells is widespread, with evidence indicating roles in both innate and adaptive immune responses (Lin et al., 2011; Chiurchiù et al., 2015). Upon LPS challenge, activation of GPR55 on monocytes

and natural killer cells was found to increase the release of pro-inflammatory cytokines, decrease monocyte-mediated endocytosis, and increase cell cytotoxicity (Chiurchiù et al., 2015). Similarly, increased expression of GPR55 was observed in the gastrointestinal tract during sepsis (Lin et al., 2011). This suggests that decreasing GPR55 activity may be beneficial as anti-inflammatory strategy in sepsis. In support of this, use of a newly identified GPR55 antagonist (CID16020046) in an experimental colitis model decreased inflammation through reduction of pro-inflammatory cytokine levels (Stančić et al., 2015). Furthermore, a reduction in intestinal inflammation, significantly below that of either the CB_1R-knockout or CB_2R-knockout mice, was noted in GPR55 knockout mice (Schicho and Storr, 2012). These lines of evidence indicate a potential anti-inflammatory action of GPR55 blockade that may be useful during the acute phase of sepsis.

9.6 CONCLUSION

Modulation of the ECS is a novel and potentially powerful tool in the manipulation of dysregulated immune responses in sepsis. There are potential benefits to immunosuppressive therapy during an initial hyper-inflammatory response in sepsis patients, such as through CB_2R activation. An important balance lies between damage resulting from an over-active immune response, and damage resulting from the invading pathogen. Modulation of the ECS, including activation of CB_2R, can lead to decreased inflammation and may be valuable tools in future sepsis treatment. However, due to the complexities of the sepsis disease time-course and the changing immune status of patients, attention must be paid to assure anti-inflammatory and immunosuppressive therapies are not given during a phase of sepsis, which is characterized by immunosuppression. As real-time and specific biomarkers of immune status play an important role in guiding immunomodulatory therapy in sepsis, any sepsis treatment, including ECS targeted drugs, must be considered in the context of these read-outs given that in those sepsis patients with a secondary immunosuppressed state recovery of immune function may play a key role in preventing relapse of infection.

CHAPTER 10

Immune Modulation by Cannabinoids During Central Nervous System Injury-induced Neuroinflammation

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Abstract

The pathophysiology of central nervous system (CNS) injury is very dynamic and complex. The outcome of such injury depends on the interplay between the immune system and the CNS. Evidence suggests that the endocannabinoid system (ECS), an endogenous system of lipid mediators and cannabinoid receptors, may play an integral part in the onset, progression, and resolution of acute CNS injury. This chapter provides a review of the current findings regarding the ECS and immunomodulation following acute CNS injury. Additionally, the potential benefits and the limitations of cannabinoid compounds, both endogenous and exogenous, as candidates for therapeutic intervention for acute CNS injury are also discussed.

Key Words

endocannabinoid system, CNS injury, inflammation

Abbreviations

2-AG	2-arachidonylglycerol
AEA	anandamide
AM630	CB_2 antagonist
AraS	arachidonoyl serine
BBB	blood-brain barrier
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CB_1R	cannabinoid type 1 receptor

CB_2R	cannabinoid type 2 receptor
CBD	cannabidiol
CIDS	CNS injury-induced immunodepression
CNS	central nervous system
eCB	endocannabinoid
ECS	endocannabinoid system
Epac1	exchange protein directly activated by cAMP
FAAH	fatty acid amide hydrolase
GW405833	CB_2R agonist
ICAM-1	intracellular adhesion molecule 1
IFNγ	interferon gamma
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-6	interleukin 6
JWH-133	CB_2R agonist
LPS	lipopolysaccharide
NAAA	N-acyl amino acid
O-1966	CB_2 agonist
p-PKA	phosphorylated cAMP-dependent protein kinase
TBI	traumatic brain injury
Th	T-helper cell
TNF-α	tumor necrosis factor alpha
URB597	fatty acid amide hydrolase enzyme inhibitor
UTI	urinary tract infection
WAY100635	5-HT(1A) antagonist
WWL70	inhibitor of α/β-hydrolase domain 6 (ABHD6)

10.1 CENTRAL NERVOUS SYSTEM INJURY

The broad term "central nervous system (CNS) injury" includes a variety of different pathologies such as stroke, traumatic brain injury (TBI), or spinal cord injury. CNS injury is one of the leading causes of death worldwide and represents a major cause of long-term disability. Looking at the global statistics of just one pathology—stroke, there are more than 15 million cases and about 5 million deaths every year. This puts stroke as the second most common cause of death and a major cause of long-term disability. It is estimated that about 25% of people older than 85 years will develop a stroke (Macrez et al., 2011). The prognosis of patients surviving with CNS injury is mainly dependent on the occurrence of medical complications (Davenport et al., 1996). In a prospective study, up to 85% of stroke patients experienced complicating events, specifically infections, during

acute care (Langhorne et al., 2000), with pneumonia and urinary tract infections (UTI) being most common (Weimar et al., 2002). It has now been recognized that acute CNS injury dysregulates the normally well-balanced interplay between the CNS and immune system. This disruption in immune function has been termed as CNS injury-induced immunodepression (CIDS) and is thought to be responsible for neurological complications and worsened patient outcome (Dirnagl et al., 2007; Meisel et al., 2005).

While the mechanisms giving the rise to CIDS are still relatively unclear, several groups have begun to explore the changes in peripheral immunity that occur in response to a CNS injury, as well as the consequences of peripheral immunosuppression for further CNS injury exacerbation (Iadecola and Anrather, 2011). Currently, our understanding is that CNS injury induces local activation of the immune system within the brain. In order to limit inflammation, the damaged brain, in turn, promotes suppression of the immune response. The systemic consequence of immunodepression is an increased risk of infections such as pneumonia or UTI, further threatening the survival of patients after CNS injury (Iadecola and Anrather, 2011). Animal studies have shown that within the first days after CNS injury, experimental animals develop spontaneous infections, such as pneumonia (Prass et al., 2003). In addition, CNS injury has been shown to induce an extensive apoptotic loss of lymphocytes, atrophy of secondary lymphatic organs such as spleen and thymus, as well as compromised counts and function of monocytes (Dirnagl et al., 2007), all contributing to the pathophysiology of CIDS.

10.2 THE ENDOCANNABINOID SYSTEM

Isolation of plant-derived chemical constituents of the cannabis plant laid the foundation for investigative research looking for endogenous cannabinoid binding sites within the human body. The concept of the ECS was introduced shortly after the discovery of cannabinioid receptors, as well as identification of endogenous cannabinoid (eCB) compounds such as anandamide and 2-arachidonylglycerol (2-AG), with associated pathways of biosynthesis and degradation (reviewed in Howlett, 2005; Mechoulam and Parker, 2013; Pertwee, 2015; Pertwee et al., 2010). This signaling system contains 2 G protein coupled cannabinoid receptors—cannabinoid type 1 receptor (CB_1R) and cannabinoid type 2 receptor (CB_2R). Briefly, CB_1R is highly expressed within the CNS and is the molecular target for the known psychoactive effects of marijuana (Gui et al., 2015; Mechoulam and Parker, 2013; Pertwee, 2015). In the CNS and periphery, presynaptic CB_1R activation inhibits Ca^{2+} influx and neurotransmitter release (Daniel and Crepel, 2001; Kim and Thayer, 2000; Pertwee, 2015). Postsynaptic CB_1Rs are also present in a variety of non-neural peripheral tissues and cells, including the vasculature and gut, and activation of these receptors can produce hypotension and regulate emesis and feeding. The peripheral effects of cannabis such as analgesia, anti-inflammation, and immunosuppression are largely attributed to CB_2R. Unlike CB_1R, CB_2R is predominantly expressed in the cells of the immune and hematopoietic systems, under non-pathological conditions

(Munro et al., 1993), but has also been identified in select CNS areas (Van Sickle et al., 2005), in nonparenchymal cells of the cirrhotic liver (Julien et al., 2005), in the endocrine pancreas (Juan-Pico et al., 2006), and in bone (Idris et al., 2005; Ofek et al., 2006). Upregulation of CB_2R expression may be a feature of pathology in some tissues, and is seen following an acute CNS injury event, such as stroke (Hillard, 2008; Orgado et al., 2009).

A number of studies have now demonstrated that ECS modulation affects disease outcome for a diverse array of conditions involving systemic inflammation (Krustev et al., 2014; Pacher et al., 2008; Toguri et al., 2015; Zhou et al., 2016). Throughout the last decade, there has been an increasing experimental focus on the ECS, resulting in several cannabinoid drugs entering into clinical trials and being introduced to the market. Some examples of these include cannabinoid drugs for obesity, pain, emesis, and multiple sclerosis (Pacher et al., 2006; Pandey et al., 2009). Therapeutic benefits reported for the cannabinoids include a reduction in cytotoxic stimuli, such as excitotoxicity, inflammation, and oxidative stress (Coyle and Puttfarcken, 1993; Lutz, 2004; McNamara, 1999). While some of the cannabinoid agents introduced into the clinic have subsequently been withdrawn due to undesirable side-effects (e.g., Rimonabant, (Topol et al., 2010)), there is undeniable potential for the development of novel ECS modulatory drugs and finding new drug targets within ECS for therapeutic intervention for various CNS diseases and injuries (Croxford, 2003; Mechoulam et al., 2002).

The dynamic and complex changes in a body's immune system during and after CNS injury are poorly characterized in the literature. Studies have demonstrated that the pro-inflammatory immune response after CNS injury may be detrimental to patient outcome via secondary damage and exacerbated CNS injury (Gadani et al., 2015; Gyoneva and Ransohoff, 2015; Kong et al., 2014; Zhou et al., 2014). Since the ECS has emerged as an important regulator of the systemic immune response (Pandey et al., 2009), it provides novel drug targets to pharmacologically address the pathologic consequences associated with CNS injury (Mechoulam et al., 2002; Onaivi et al., 2012; Shohami et al., 2011).

10.3 THE ENDOCANNABINOID SYSTEM, CENTRAL NERVOUS SYSTEM INJURY AND INFLAMMATION

Several lines of evidence suggest that the local upregulation of the ECS following CNS injury represents an adaptive mechanism—significant increases in CBR expression and eCB levels have been detected in the brain with CNS injury. Activation of CB_1-related pathways has been reported to decrease CNS excitability and cell death by controlling glutamate homeostasis and reducing glutamate toxicity, and activation of CB_2R on cerebral immune cells limits post-ischemic neuroinflammation (Hillard, 2008). However, the ECS involvement in CNS injury is complex and while the experimental data, for the most part, suggests beneficial CNS actions, less is known about the consequences of altered cannabinoid signaling in the periphery following CNS injury. The role of

CB_2R signaling after CNS injury may be particularly interesting in the context of CIDS; activation of CB_2R is associated with a reduced immune response (Merighi et al., 2012). Therefore, it follows that increased CB_2R signaling may decrease local CNS neuroinflammation as one of the neuroprotective mechanisms employed by the brain, while systemically inducing peripheral immunosuppression or CIDS and increasing the risk of fatal infection.

With respect to ECS regulation of the immune response, there is evidence suggesting upregulation of the ECS during both local and systemic inflammation (Greineisen and Turner, 2010). Examination of ECS function has revealed that CB_2R are present on macrophages, neutrophils, and lymphocytes and activation of these receptors has been generally associated with anti-inflammatory effects including reduced macrophage and neutrophil numbers at the site of infection and decreased pro-inflammatory cytokines (Croxford and Miller, 2003; Merighi et al., 2012; Toguri et al., 2014, 2015). The use of CB_2R agonists in experimental models of moderate sepsis reduced the continued recruitment of neutrophils to the site of infection (Varga et al., 1998). It is well known that CNS injury induces excessive release of glutamate as a direct result of energy supply failure, as well as reversal and collapse of ion gradients, leading to a dramatic change in glutamate transporter activity, which in turn allows influx of Ca^{2+} ions via NMDA receptors and the inevitable cell necrosis and apoptosis. Perhaps one of the roles that the CB_1R contributes to during the onset of CNS injury is the suppression of synaptic glutamate release, which helps with reducing cytotoxic cellular death (Gerdeman and Lovinger, 2001).

Clinical data indicates that infections are one of the leading causes of death for stroke patients after day one admission to the hospital (Dirnagl et al., 2007). In general, immunodepression after stroke can be detected within a few hours after ischemic trauma, and can last for several weeks. Within the first days after cerebral ischemia, experimental animals develop spontaneous pneumonia and sepsis, which is mainly due to an extensive apoptotic loss of lymphocytes and a shift from T helper cell (Th)1 to Th2 cytokine production (Prass et al., 2003). Adoptive transfer of T lymphocytes and natural killer cells from wild-type into mice with cerebral ischemia was found to greatly decrease the bacterial burden (Prass et al., 2003). Furthermore, the same study suggested interferon gamma (IFNγ) pathway involvement, since the benefit of the adoptive transfer was not present when the cells were transferred from IFNγ deficient mice. Atrophy of lymphatic organs such as spleen and thymus with reduction of splenocyte numbers and T cell proliferation responses were also observed at three days after focal cerebral ischemia (Offner et al., 2006). Parallel to the changes in the adaptive immune system, monocyte counts and function are compromised as well (Prass et al., 2003). Involvement of ECS in regulating the immune function suggests that drugs that target the ECS may be useful in modifying both the time-course and the severity of post-CNS injury complications and potentially decreasing mortality.

A number of *in vitro* studies have examined the effects of endocannabinoids on the levels of proinflammatory cytokines including: TNF-α, interleukin-1beta (IL-1β), interleukin-6 (IL-6), and interleukin-2 (IL-2). Both the endocannabinoids, 2-AG and AEA, decreased lipopolysaccharide

(LPS)-mediated increases of pro-inflammatory cytokines, including TNFα, from macrophages and microglial cells. Additionally, 2-AG inhibited IL-2 secretion in activated murine splenocytes (Godlewski et al., 2004; Orliac et al., 2003; Pacher et al., 2005; Varga et al., 1998; Wagner et al., 1998). Consistent with these findings, a study using the fatty acid amide hydrolase (FAAH) enzyme inhibitor, URB597, to augment levels of endogenous AEA, reported a reduction in LPS-stimulated microglial expression of inflammatory mediators, including nitric oxide, in LPS-stimulated rat cortical microglia (Tham et al., 2007). URB597 treatment also attenuated levels of proinflammatory cytokines, TNFα and IL-1β, in LPS-treated paws in a rat endotoxemia model of inflammatory pain (Naidu et al., 2010). From the work cited above, we can conclude that the ECS appears to be an effective immunomodulator and plays a role during the pathophysiological development after CNS injury.

10.4 ENDOCANNABINOID THERAPIES FOR CENTRAL NERVOUS SYSTEM INJURY

Endocannabinoid and endocannabinoid-like compounds have been evaluated for their effectiveness as a potential therapy for TBI. Arachidonoyl serine (AraS), a member of N-acyl amino acid (NAAA) family, exerted eCB-mediated neuroprotection, which was evident in numerous aspects related to the secondary damage characterizing TBI (Hanuš et al., 2014; Panikashvili et al., 2006). Interestingly, AraS is structurally similar to 2-AG, which was also previously shown to be beneficial in the recovery in a closed head injury model of TBI (Panikashvili et al., 2001).

Other studies have focused on increasing endocannabinoid levels in the brains after CNS injury using enzyme inhibitors of endocannabinoid metabolism (Tchantchou and Zhang, 2013). This approach has the benefit of local activation of cannabinoid receptors and avoids the potential psychotropic side effects of exogenous cannabinoids, related to global activation of the CB_1R (Mackie, 2005). In line with this, Tchantchou and Zhang (2013) reported that inhibition of 2–AG degradation with inhibitor of α/β-hydrolase domain 6 WWL70 leads to a decrease in brain edema, lesion volume, blood-brain barrier (BBB) dysfunction, neuronal death, and overall improvement in behavioral performance after TBI. The tested compound also decreased pro-inflammatory molecules, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX2), that are usually expressed by glial cells after CNS injury. The authors also found that chronic administration of the WWL70 reversed the increase in post-TBI lesion volume and concluded that enhancing endocannabinoid (2-AG) levels in the brain has the potential to decrease BBB breakdown and alleviate secondary neuronal injury (Tchantchou and Zhang, 2013).

10.5 TARGETING CANNABINOID RECEPTORS FOR CENTRAL NERVOUS SYSTEM INJURY

A number of experimental studies have examined the role of cannabinoid receptors as druggable targets in ameliorating CNS injury (Mann et al., 2015; Panikashvili et al., 2001; Scotter et al., 2010). In support of a constitutive role for CB_2R in tempering the neuroinflammatory response, a study by Amenta et al. (2014) reported that genetic knockout of CB_2R exacerbated the increase in pro-inflammatory mediators, including TNF-α, mRNA, and intercellular adhesion molecule 1 (ICAM-1), seen following TBI. The same study also showed that treatment with CB_2R agonists (0-1966 and JWH-133) attenuated TNF-α protein levels, reduced ICAM-1 expression, and prevented increases in iNOS mRNA expression, thus confirming the immunosuppressive effects of CB_2R stimulation in relation to post-CNS injury inflammation (Amenta et al., 2014).

Microglial cells are resident CNS immune cells that emerge from erythro-myeloid precursors in the embryonic sac and migrate to the brain mesenchyme before the formation of the BBB. Microglia are unique in that they are both supportive for neuronal homeostasis as well as fully immunocompetent defense cells (Streit, 2002). The involvement of CB_2R signaling during the early onset of CNS injury and its functional expression on microglia (Mecha et al., 2016) suggests that the neuroprotective actions associated with CB_2R activation may be mediated, in part, through microglia (Palazuelos et al., 2009); CB_2R stimulation attenuated microglial accumulation and brain injury (Tang et al., 2015). Furthermore, JWH–133, a selective CB_2R agonist, reduced the pro-inflammatory cytokine levels and promoted transition of microglia from the more cytotoxic M1 phenotype to M2 phenotype, responsible for immuno-resolution and repair (Loane and Kumar, 2016; Navarro et al., 2016). This was mediated via facilitated synthesis of cyclic adenosine monophosphate (cAMP) and its downstream effectors, phosphorylated cAMP-dependent protein kinase (p-PKA), as well as the exchange protein activated by c-AMP 1 (Epac1) (Tao et al., 2016).

In terms of prophylaxis and treatment of the acute phase of CNS injury, there are currently limited therapeutic options available. The acute phase of CNS injury is dramatically exacerbated by a robust inflammatory response and BBB dysregulation, which introduces an influx of blood-borne cells with production of inflammatory mediators, chemokines, cytokines, proteases, reactive oxygen species, and vascular adhesion molecules (Fernández-Ruiz et al., 2015). For ischemic stroke, currently, the only pharmacological treatment is the recanalization of the occluded vessel with thrombolytic therapy with tissue plasminogen activator. This treatment, due to the narrow time window, is available to less than 5% of patients with CNS injury (Fernández-Ruiz et al., 2015). Nonetheless, this is a crucial window of time, and any therapeutic interventions during the acute phase can be very beneficial to the outcome of the patient, reducing their neurological impairment. Cannabinoid therapy could play a role during the acute phase; stimulation of CB_2R if activated earlier in anticipation of damage or during the acute phase, could potentially prevent or decrease

the initial inflammatory response and theoretically prevent the size of penumbra and extent of reperfusion damage.

Despite multiple studies reporting the positive neuroprotective effect of CB_2R agonists in various models of cerebral ischemia, there is a paucity of data on the long-term benefits of these interventions. One study examined behavioral outcomes 15 days after CNS injury with the use of CB_2R agonist GW405833, and found that the drug failed to provide any neuroprotection (Rivers-Auty et al., 2014). There are, however, a number of important caveats to consider with this study. A single dose of drug was used and may not have been optimal to maintain efficacy and the authors did not evaluate any other CB_2R ligands. It is possible that a different drug regimen might be needed to obtain long-term neuroprotection following CNS injury. Additional studies are urgently needed to evaluate long term outcomes of CB_2R agonists in different models of CNS injury to clarify the specific indications and benefits of this therapeutic approach.

In addition to studies using selective synthetic CB_2R agonists, the potential neuroprotective effect of the phytocannabinoid, cannabidiol (CBD) has also been explored (Ceprián et al., 2017; Mishima et al., 2005). CBD has been reported to act as a negative allosteric modulator at CB_1R and an agonist at CB_2R (Laprairie et al., 2015; Pertwee, 2008). The neuroprotective effects observed with CBD have been attributed to modulation of excitotoxicity, reduction of oxidative stress, and decrease in neuroinflammation (Hind et al., 2016; Marsicano et al., 2003; Mishima et al., 2005; Pazos et al., 2013). Further, a study by Pazoz et al. (2013) not only reported CBD-mediated neuroprotection but also provided evidence of functional recovery of brain activity in newborn pigs with hypoxic-ischemic brain injury (Pazos et al., 2013). Interestingly, CBD-mediated neuroprotection was reversed by co-administration of 5-HT(1A) antagonist, WAY100635, or CB_2R antagonist, AM630, supporting the involvement of CB_2R and 5HT(1A) receptors (Pazos et al., 2013). A more recent publication, though, challenges the efficacy of CBD for neuroprotection; Garberg et al. (2016) also used a hypoxia-ischemia model in newborn piglets and treated animals with CBD. The authors reported that CBD failed to produce significant neuroprotective effects, although the short experimental end-point of 9.5 hr limits the conclusions of this study (Garberg et al., 2016).

Despite current lack of evidence for long-term neuroprotective benefits, CB_2R activity may be beneficial after the acute phase of CNS injury; nevertheless, the treatment of acute CNS injury, as in the case of stroke, is complicated by CIDS onset and progression (Meisel et al., 2005). The impaired immune function in CIDS is thought to come as a side effect of the compensatory homeostatic countermeasure against the initial CNS inflammatory response in order to prevent any further CNS damage from its own immune system (Figure 10.1). This impairment, unfortunately, also causes systemic susceptibility to infection. The current line of CIDS therapy is very limited as it only provides prophylactic treatment with antibiotics (Nagashima et al., 2004). However, there is

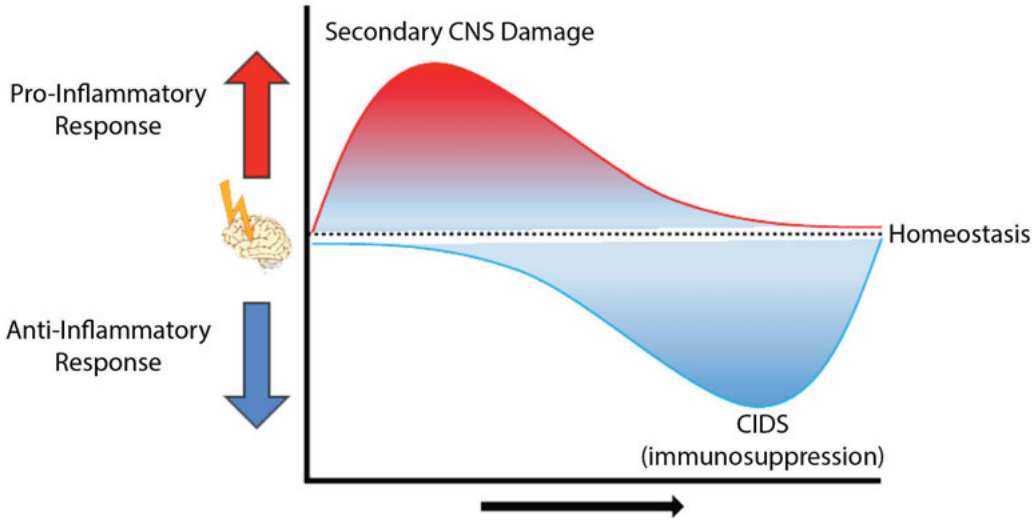


FIGURE 10.1: Graphic representation of the immune response after CNS injury. Graph represents the dynamic changes in the immune status of patients after an acute CNS injury. It is thought that the initial trauma (i.e., traumatic brain injury, stroke, etc.) causes a strong pro-inflammatory response—influx on immune cells, activation of resident microglia, production of inflammatory cytokines, and other pathophysiologic changes, throwing the immune system away from homeostasis. Combined with the dysfunction of the BBB, the strong inflammatory response causes secondary CNS damage and exacerbates the injury size. As a compensatory and neuroprotective mechanism, the brain is thought to defend itself by sending out anti-inflammatory modulators via the hypothalamus-pituitary-adrenal axis and sending the patient into an immunosuppressed state (CIDS). CIDS is known to compromise and expose patients to basic infections such as pneumonia and urinary tract infection, without the ability to give an adequate immune response, making those infections worsen patient outcome. The severity of immunosuppression is dependent on the initial size of CNS injury and the general background status of the patient prior to the injury.

emerging experimental evidence that judicious use of cannabinoid therapy in-line with assessment of the patient's immune status may also be useful during CIDS. The idea behind this therapeutic approach is to exploit the natural anti-inflammatory property of the CB_2R by using CB_2R agonists during the early acute phase of CNS injury while pharmacologically inhibiting CB_2R activity during CIDS when the patient is already immunocompromised (Lehmann et al., 2014). A study by Burkovskiy et al. (2016) examined this premise in mice with CNS injury induced via hypoxia-ischemia model. The authors reported two important findings; first, animals that were given an immunochallenge following a prior CNS injury had a severely compromised immune response, confirming that CIDS was a preserved phenomenon in rodents; second, inhibition of CB_2R with the antagonist, AM630, reversed the immunosuppression associated with CIDS and restored some of the immune function back to the animal (Burkovskiy et al., 2016). While no data has yet been reported on the long-term effect of this therapy, this study does provide additional evidence for the involvement of ECS in the immune response following a CNS injury and identifies the candidacy of CB_2Rr as a valid therapeutic target for future exploitation.

10.6 CONCLUSION

Taken together, the evidence above suggests that release of endocannabinoids and activation of cannabinoid receptors occurs during local and systemic inflammation associated with CNS injury. Manipulation of the ECS, in particular CB_2R signaling, may represent an important therapeutic target in managing CNS injury, complications, and outcome (Table 10.1). However, the exact parameters of the interface between the brain, the immune system, and the ECS still requires thorough investigation and validation. This especially applies to the complex pathophysiology of CIDS where a more comprehensive understanding of ECS-related regulation of physiological functions after CNS injury is urgently required to develop appropriate and effective therapy.

TABLE 10.1: Summary of studies exploring the neuroprotective and neuro-recovery potential of the endocannabinoid system

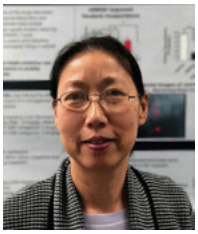
Compound Name	Receptor Target / Action	Model / Type of CNS Injury	Findings	Study
JWH 133	Selective CB_2R agonist	Germinal Matrix Hemorrhage, rat	Suppression of neuroinflammation by regulating microglial M1/M2 polarization through the cAMP/PKA pathway	Tao et al., 2016
AraS (Arachidonoyl serine)	Weak affinity to CB_1Rs and CB_2Rs	Closed head injury, mice	Neuroprotective, beneficial in recovery from CNS injury	Hanus et al., 2006
AM 630	Selective CB_2R inverse agonist	Hypoxia-ischemia injury, mice	Improvement in immune function, partial reversal of CIDS	Burkovskiy et al., 2016
O-1966 / JWH 133	Selective CB_2R agonists	Controlled cortical impact, mice	Attenuation of TNF-α protein levels, reduction of ICAM-1 expression and prevention of iNOS mRNA expression increase.	Amenta et al., 2014
Genetic CB_2R knockout	Removal of CB_2R	Controlled cortical impact, mice	Exacerbation of neuroinflammation - TNF-α mRNA and ICAM-1 level increase, worse outcome	Amenta et al., 2014
GW405833	Selective CB_2R agonist	Hypoxia-ischemia injury, rats	Failure to provide neuroprotection (15-day behavioral outcome)	Rivers-Auty et al., 2014
CBD (cannabidiol)	Negative allosteric modulator of CB_1R/CB_2R agonist	Hypoxia-ischemia injury, piglets	No significant protective effect found, higher dose siggested	Garberg et al., 2016
CBD (cannabidiol)	Negative allosteric modulator of CB_1R/CB_2R agonist	Hypoxia-ischemia injury, piglets	CBD exerted robust neuroprotective effects *in vivo* – modulating excitotoxicity, oxidative stress and inflammation. Both CB_2R and 5HT(1A) receptors are implicated.	Pazos et al., 2013

Lead Author Biographies

Melanie E.M. Kelly, Ph.D., is Professor of Pharmacology, Ophthalmology and Visual Sciences, and Anesthesia, Perioperative Medicine and Pain Management at Dalhousie University. Professor Kelly received her B.Sc. (Hons) in Physiology and Pharmacology from Southampton University, UK, in 1981 and, in 1984, completed a Ph.D. in Neurophysiology at Southampton University. Professor Kelly's research is in molecular and translational pharmacology, with expertise in G protein coupled receptors and ocular and vascular pharmacology. During the last decade, her research has centered on the pharmacology of the endocannabinoid system. Her laboratory, together with collaborators, has published ground-breaking research on the role of the endocannabinoid system in ocular disease and the therapeutic potential of cannabinoid-based drugs. Professor Kelly is actively engaged in "bench to bedside" research to identify novel drug targets and bring new cannabinoid-based medications to the clinic to treat human disease. She has published over 120 scientific peer-reviewed papers, reviews, book chapters, and books, and is an inventor on patents to use cannabinoid-based drugs to treat inflammatory disease and neuropathic pain. Professor Kelly's research is funded by the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council of Canada, and the National Institutes of Health, USA. Professor Kelly is also one of the founders of a Nova Scotia-based biopharmaceutical company that is developing phytocannabinoid therapies to treat human inflammation and chronic pain and she is on the Executive Board of the Canadian Consortium for the Investigation of Cannabinoids (CCIC). She is a licensed National Pro-rally Co-driver and enjoys running and hiking the beautiful Nova Scotia Coast.

Dr. Christian Lehmann is Professor of Anesthesia, Pharmacology, Microbiology and Immunology, and Physiology and Biophysics at Dalhousie University, Halifax, NS, Canada. He is also Staff Anesthesiologist in the Department of Anesthesia, Nova Scotia Health Authority, Halifax. Dr. Lehmann has 25 years of experience in experimental and clinical inflammation research. He established experimental intravital imaging laboratories in Berlin and Greifswald, Germany. His focus of research is the microcirculation, i.e., capillary blood flow, cell-cell-interactions, and endothelial pathology. He participated in diverse German and international clinical multicenter trials, e.g., in sepsis. He joined Dalhousie University in 2007 and currently supervises research in three laboratories with modern equipment to study the

microcirculation under various conditions experimentally and clinically. He is the author of more than 150 original publications, 13 book chapters and almost 200 published abstracts. He is a member of the Dalhousie Inflammation Group (DIG), the Microcirculation Diagnostics and Applied Studies (MiDAS) research group and the Cystic Fibrosis Translational Research (CFTR) group. In 2009, he founded the RESIST (Research in the Endocannabinoid System in Inflammation, Sepsis and Trauma) group with Dr. Melanie Kelly and others at Dalhousie University.



Dr. Juan Zhou is Associate Professor of Anaesthesia, Pain Management, and Perioperative Medicine at Dalhousie University. She received her M.D. from Xian Medical University in 1986, and practiced as an ophthalmologist at the Shaanxi Provincial Hospital in China. After moving to Canada, Dr. Zhou obtained her M.Sc. in Biology in 1993 and her Ph.D. in Immunology in 2001 from Dalhousie University. Dr. Zhou's principal research interests are in immunomodulation in transplantation tolerance and inflammation. Her current research focus is investigating the role of the endocannabinoid system in inflammatory disorders including experimental sepsis, central nerve system injury-induced immunosuppression, and interstitial cystitis. Dr. Zhou has over 60 peer-reviewed scientific journal publications, books, and book chapters. She is an avid badminton player, and she enjoys canoeing through the lakes of Nova Scotia.

Contributing Author Biographies

Shawn Adderley graduated with a B.Sc. honors with a concentration in Molecular Biology in 2013 from Saint Mary's University in Halifax, NS. He is currently working with the Denovan-Wright Laboratory.

Amina Bagher received her Doctor of Pharmacy degree from King Abdul Aziz University, Saudi Arabia in 2007. Subsequently she worked as a teaching assistant at the same university. In 2010, Amina joined the laboratory of Dr. Denovan-Wright and Dr. Kelly at Dalhousie University, Canada, as a graduate student. Amina received her M.Sc. degree in Pharmacology in 2012 and currently she is in her final year of a doctoral program. Her doctoral research focuses on the physical and functional interaction between cannabinoid receptors and dopamine receptors.

Ian Burkovskiy has received a B.S. in Neuroscience at Dalhousie University. Throughout his studies, he was involved in various research projects ranging from involvement of tau protein in Alzheimer's disease to physiology of retinal ganglion cells. Currently, he is in the final year of a doctoral program at Dalhousie University, focusing his research in the fields of Pharmacology and Neuroscience. The main subject of his thesis work revolves around post CNS injury pathology. His research interests include CNS injury, neuroinflammation, and cannabinoids. In parallel to his earlier studies, he produced a TV show on Bell's network that brought the latest scientific and technological advances to the general public. In addition, he published work on improvement of various *in vivo* methods by utilizing 3D manufacturing.

Meggie Caldwell was awarded a B.Sc. in Psychology and French, then a M.Sc. in Clinical Vision Science from Dalhousie University. She is a Canadian Certified Orthoptist and is the current Vice-President of the Canadian Orthoptic Society. Her research interests involve both endocannabinoid pharmacology and paediatric ophthalmology. Her research in the past has focused on the pharmacology of cannabinoids in the eye, specifically looking at intraocular pressure, pain and inflammation.

Derek Costello is an Assistant Professor in Neuroscience and Pharmacology at University College Dublin. He graduated with a B.S. in Physiology from University College Dublin in 2000. His research career began with an investigation of the mechanisms underlying the adverse effects of amyloid-β peptide (Aβ) on synaptic plasticity, for which he was awarded a Ph.D. in 2004 by the same institution. Dr. Costello undertook postdoctoral research in the laboratory of Prof. Paola Pedarzani at University College London, which primarily focused on examining the ionic and cellular mechanisms regulating intrinsic neuronal and synaptic function, and assessed the further influence of their disruption. Returning to Ireland in 2008, Dr. Costello took the position of Research Fellow in Neuroinflammation, under the mentorship of Prof. Marina Lynch at Trinity College Dublin. This enabled him to integrate his research interests: evaluating the dysregulation of neuronal function which results from inflammatory processes characteristic of neurodegenerative disease states. It was during this period that he developed his primary interest in manipulating endogenous immuno-modulatory mechanisms in the CNS, as a potential strategy to alleviate the detrimental consequences of neuroinflammation. In 2015, he returned to his alma mater to take up his current position as Assistant Professor and Principal Investigator. His current research team is concerned with understanding the factors which convey susceptibility to inflammatory-related neuronal dysfunction.

Dr. Eileen Denovan-Wright received her B.Sc. (Hons) and Ph.D. from Dalhousie University. She was a Postdoctoral Fellow in Pharmacology from 1995-2000. In 2000, she was appointed as an Assistant Professor of Pharmacology. Currently, Dr. Denovan-Wright is a Full Professor of Pharmacology at Dalhousie University Halifax. She also holds a cross-appointment as Associate Dean of the Faculty of Graduate Studies. Dr. Denovan-Wright has an active research program in Huntington's disease and Cannabinoid Pharmacology and teaches in the Faculty of Medicine and Faculty of Science at Dalhousie.

Nicholas Fisher has been working in the department of Pharmacology at Dalhousie University since 2013. He received his B.Sc. in Biology from Dalhousie University in June 2016. Nicholas' areas of research are involved with Type 1 diabetes prevention and the endocannabinoid system. As an undergraduate student, Nicholas's academic interests were molecular, physiological, developmental, and pharmacological biology. As a patient with Type 1 diabetes mellitus since 2007, Nicholas' passion for diabetes prevention was sparked by his own experiences with this life-long autoimmune disorder, which drives him to work towards finding a cure so that other people do not have to experience the complications associated with diabetes.

Caroline Herron is an Associate Professor of Neuroscience in the School of Biomolecular Biomedical Sciences, Conway Institute, University College Dublin. Caroline was awarded her B.S. and Ph.D. from the University of Southampton (UK) and has since worked in research on NMDA receptor mediated synaptic transmission, Long Term Depression (LTD), and Long Term Potentiation (LTP). Recently her main research focus has been on alterations in synaptic plasticity in models of Alzheimer's Disease. Caroline's main interest has been using electrophysiological methods to investigate mechanisms associated with the neurotoxic effects of beta amyloid peptide and also using Transgenic models of AD. Caroline is a member of the Physiological Society, The British Neuroscience Association, and Neuroscience Ireland.

J. Daniel Lafreniere is working as a research student in the Pharmacology Department and Retina and Optic Nerve Research Lab at Dalhousie University. He is in the final stages of B.Sc. degrees in Microbiology and Immunology and Biology. Dan's current thesis research work examines the potential role of anti-VEGF therapy as an adjunct treatment in ocular infection.

Jason J. McDougall was born in South Shields and educated in Scotland. He received his Ph.D. in Joint Physiology from the University of Glasgow and subsequently undertook postdoctoral training in Canada, Germany and Spain. Prof. McDougall was awarded postdoctoral fellowships from the Alberta Heritage Foundation for Medical Research (AHFMR), the Medical Research Council of Canada and was the recipient of the Ernst and Young Joint Injury and Arthritis Research Fellowship. In 2001, he joined the Faculty of Medicine at the University of Calgary where he held an AHFMR Senior Scholarship as well as an Arthritis Society Investigator award. He transferred to Dalhousie University in 2011, where he is currently a Professor of Pharmacology and Anaesthesia.

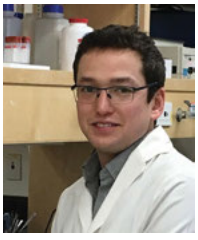
Prof. McDougall's research focuses on the neurobiology of pain and inflammation in the development of arthritis. His research is currently examining the role of cannabinoids and proteinases in the control of arthritis pain and inflammation. His research goal is to identify novel drug targets and develop new treatments which will help alleviate chronic pain and resolve joint inflammation. He currently receives project funding from CIHR, The Arthritis Society of Canada, and The Nova Scotia Health Research Foundation. He is the Chief Scientific Officer for GenCanBio and has been a consultant for Antibe Pharmaceuticals (Canada), AstraZeneca (UK), Eli Lilly and Company (U.S.) and Pfizer (UK). Prof. McDougall is an editor for *Inflammation Research* and

BMC Anaesthesiology as well as the Chair of the Scientific Advisory Committee at the Arthritis Society. He has also won numerous awards for his homebrewed beers which have subsequently been commercially produced.

Holly T. Philpott received a B.S. in Microbiology and Immunology from Dalhousie University. She is currently undergoing Masters' training in Pharmacology at Dalhousie University under the supervision of Dr. Jason McDougall. Her current project involves the investigation of exogenous cannabinoids and the modulation of the endocannabinoid system in osteoarthritis pain and disease progression.

Andrew W. Stadnyk is a Professor of Pediatrics and Microbiology and Immunology, Faculty of Medicine, Dalhousie University. Dr. Stadnyk's principal research interests are in mucosal immunology, particularly mediator networks connecting epithelial cytokines and split complement molecules in the intestinal inflammatory response. He brings expertise in cellular and molecular mechanisms of inflammation to help advance our understanding of endocannabinoids and inflammation.

Anna-Maria Szczesniak received her Ph.D. from Dalhousie University in Halifax, Nova Scotia. Her doctoral research project focused on the role of cannabinoid compounds in the modulation of ocular physiology. After her initial post-doctoral fellowship in stem cell research and neurodegenerative disorders, she joined the laboratory of Dr. Kelly, where she pursues her interest in the rapidly expanding field of cannabinoid research. Her research focuses on the therapeutic potential of cannabinoid compounds in the modulation of innate and adaptive immunity and neuroprotection.



James Thomas Toguri was awarded a B.S. in Biology, then a Ph.D. in Pharmacology from Dalhousie University. His research focus is investigating the modulation of the endocannabinoid system during ocular inflammation. Dr. Toguri has a strong interest in the role of cannabinoids with respect to pain and inflammation and the development of novel cannabinoid therapeutic agents.

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Preface

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Chapter 5

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Chapter 6

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