Handbook of Experimental Pharmacology 231

Roger G. Pertwee *Editor*

Endocannabinoids

Springer

Handbook of Experimental Pharmacology

Volume 231

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-319-20824-4 ISBN 978-3-319-20825-1 (eBook)
DOI 10.1007/978-3-319-20825-1

Library of Congress Control Number: 2015951984

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

For Teresa

Preface

The Endocannabinoid System: A Look Back and Ahead

The major neurotransmitter systems were discovered many decades ago. It is strange that the endocannabinoid system—certainly one of major physiological importance—remained unknown until the early 1990s. This was due, in part at least, to the lack of research on the plant cannabinoids, made difficult by legal constraints. Indeed, many of the major plant cannabinoids were not isolated, and their structures were not elucidated until the 1960s. Later, their chemistry, metabolism, biochemistry, and pharmacology were extensively investigated. However, the mechanism of action of Δ^9-tetrahydrocannabinol (THC), the major psychoactive constituent, remained elusive for nearly 20 years. It was originally believed that it acts nonspecifically on neural membranes. Gradually, pharmacological and chemical data surfaced, which were inconsistent with the nonspecificity of THC action. Indeed, in the late 1980s and early 1990s, two specific cannabinoid receptors, CB_1 and CB_2, were identified. Stimulation of CB_1 leads to the well-known marijuana effects; CB_2 is apparently part of a major, new protective system. Two endogenous cannabinoids (endocannabinoids), anandamide and 2-AG, were isolated in the 1990s. Anandamide is arachidonoyl ethanolamide and 2-AG is the arachidonoyl ester of glycerol. Thereafter, enzymes involved in their biosynthesis and metabolism were found. Many thousands of papers on this novel system have been published [see Di Marzo et al. (2015), Maccarrone et al. (2014), and Mechoulam et al. (2014), for recent reviews on the endocannabinoid system]. However, we are still far away from fully understanding this system, or, indeed, plant cannabinoids. Thus, although there are thousands of publications on THC and many hundreds on cannabidiol (CBD), the rest of the plant cannabinoids have barely been investigated. And neither anandamide nor 2-AG has ever been administered to humans. By comparison, insulin was administered to humans almost immediately after its discovery in the 1920s and became a medicine within months. Are we missing something?

Several additional endogenous fatty acid ethanol amides, in which the fatty acid moiety is not derived from arachidonic acid as in anandamide, but from other fatty acids, have been found to bind to the endocannabinoid receptors. Are they relevant to endocannabinoid activity? There are also some chemically related endogenous fatty acid–amino acid amides, which lower brain trauma damage, but do not bind to cannabinoid receptors. However, their activity can be blocked by CB_1 or CB_2 antagonists and they do not act on CB_2 knockout mice. Is the endocannabinoid system actually much more complex than was first thought?

Recently, two synthetic cannabinoid enantiomers were both reported to be CB_2 agonists; however, an inverse relationship was found between binding affinity and biological potency, which was cannabinoid-based (Smoum et al. 2015). A molecular-modeling analysis suggested that these enantiomers have two different binding conformations within the CB_2 receptor, with one of them possibly responsible for the affinity difference. Hence, different cannabinoid ligands may have different orientations relative to the same binding site. Thus, cannabinoid orientation-targeted ligands, at least for the CB_2 receptor, may have promising potential for the pharmacological activation of distinct processes. Is this a general phenomenon?

Many patients claim that plant extracts work better than pure THC in many disease states. It is widely believed that *Cannabis sativa* extracts differ in their activity from *Cannabis indica* extracts, regardless of the level of THC. And, in spite of the wide use of various medicinal cannabis products, which are mixtures of cannabinoids and other plant constituents, pure THC has remained a minor drug. Is this due to modification of THC actions via "entourage effects"? CBD is well known to modify THC activity in human users. Do additional plant cannabinoids and terpene constituents also affect THC activity?

Over the last few years, several research groups have noted that both THC and CBD, as well as anandamide, cause epigenetic modifications by methylation, leading to distinct therapeutic effects. Does epigenetics play a major role in the functioning of the endocannabinoid system? If it does, then cannabinoids may affect the bases of at least some diseases rather than their symptoms—a major difference from the activity of many drugs used today.

There have been very few clinical trials with THC or with CBD or with cannabis extracts containing fixed ratios of the major constituents. Such trials on small numbers of patients have shown very positive results in epilepsy, post-trauma, Parkinson's disease, Crohn's disease, and a few other disorders. Somewhat larger clinical trials on schizophrenia and graft-versus-host-disease (GVHD) have led to statistically significant therapeutic effects. But, where are the clinical trials in various cancer diseases where anecdotal evidence points to possible therapeutic effects?

We have made major advances in understanding the chemistry, biochemistry, and pharmacological effects of the plant cannabinoids and the endocannabinoids. The present book provides an outstanding summary of a wide variety of important endocannabinoid findings that will help generate novel ideas for future research.

Thus, I have no doubt that further research with endocannabinoids will throw additional light on the actions of these compounds. However, I believe that the most important future steps in the endocannabinoid area are to advance cannabinoid-based clinical trials in many disease states where strong anecdotal evidence already exists.

Jerusalem, Israel Raphael Mechoulam

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Endocannabinoids and Their Pharmacological Actions

Roger G. Pertwee

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_1

Abstract

The endocannabinoid system consists of G protein-coupled cannabinoid CB_1 and CB_2 receptors, of endogenous compounds known as endocannabinoids that can target these receptors, of enzymes that catalyse endocannabinoid biosynthesis and metabolism, and of processes responsible for the cellular uptake of some endocannabinoids. This review presents in vitro evidence that most or all of the following 13 compounds are probably orthosteric endocannabinoids since they have all been detected in mammalian tissues in one or more investigation, and all been found to bind to cannabinoid receptors, probably to an orthosteric site: anandamide, 2-arachidonoylglycerol, noladin ether, dihomo-γ-linolenoylethanolamide, virodhamine, oleamide, docosahexaenoylethanolamide, eicosapentaenoylethanolamide, sphingosine, docosatetraenoylethanolamide, *N*-arachidonoyldopamine, *N*-oleoyldopamine and haemopressin. In addition, this review describes in vitro findings that suggest that the first eight of these compounds can activate CB_1 and sometimes also CB_2 receptors and that another two of these compounds are CB_1 receptor antagonists (sphingosine) or antagonists/inverse agonists (haemopressin). Evidence for the existence of at least three allosteric endocannabinoids is also presented. These endogenous compounds appear to target allosteric sites on cannabinoid receptors in vitro, either as negative allosteric modulators of the CB_1 receptor (pepcan-12 and pregnenolone) or as positive allosteric modulators of this receptor (lipoxin A_4) or of the CB_2 receptor (pepcan-12). Also discussed are current in vitro data that indicate the extent to which some established or putative orthosteric endocannabinoids seem to target non-cannabinoid receptors and ion channels, particularly at concentrations at which they have been found to interact with CB_1 or CB_2 receptors.

Keywords

2-Arachidonoylglycerol • Anandamide • Cannabinoid receptors • Dihomo-γ-linolenoylethanolamide • Docosahexaenoylethanolamide • Docosatetraenoylethanolamide • Eicosapentaenoylethanolamide • Endocannabinoid pharmacology • Haemopressin • Lipoxin A_4 • *N*-arachidonoyldopamine • Noladin ether • *N*-oleoyldopamine • Oleamide • Pepcan-12 • Pregnenolone • Sphingosine • Virodhamine

Abbreviations

5-HT	5-hydroxytryptamine
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
CHO	Chinese hamster ovary
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate

HEK	Human embryonic kidney
NMDA	*N*-methyl-D-aspartate
TRP	Transient receptor potential
TRPA1	TRP channel of ankyrin type 1
TRPM8	TRP channel of melastatin type 8
TRPV1	TRP channel of vanilloid type 1
TRPV4	TRP channel of vanilloid type 4

1 Introduction

The endocannabinoid system is currently thought to consist of cannabinoid CB_1 and CB_2 receptors, of endogenous ligands for these two G protein-coupled receptors and of the enzymes and processes responsible for the biosynthesis, cellular uptake or metabolism of these "endocannabinoid" ligands (Pertwee et al. 2010).

As discussed in greater detail elsewhere by Pertwee et al. (2010), CB_1 receptors are found mainly at the terminals of central and peripheral neurons, where they usually mediate inhibition of the release of a range of different neurotransmitters. However, they are also expressed by immune cells and by certain other types of non-neuronal cells. CB_2 receptors are located primarily in immune cells and, when activated, can modulate immune cell migration and cytokine release, both outside and within the brain. Some neurons have also been found to express CB_2 receptors, although the role of these neuronal CB_2 receptors has yet to be identified. CB_1 and CB_2 receptors both signal through $G_{i/o}$ proteins in a manner that, for example, inhibits adenylyl cyclase and activates mitogen-activated protein kinases. In addition, CB_1 receptor $G_{i/o}$ proteins can mediate activation of A-type and inwardly rectifying potassium channels, and inhibition of N- and P/Q-type calcium currents, and CB_1 receptors can also signal through G_s proteins. Drugs that activate CB_1 and CB_2 receptors do so by targeting orthosteric sites on these receptors. However, it is now generally accepted that the CB_1 receptor also contains allosteric sites with which certain ligands can interact to enhance or inhibit its activation by direct "orthosteric" agonists. The presence of an allosteric site on the CB_2 receptor has also recently been reported (Feng et al. 2014). As to endocannabinoids, the first two of these to be discovered were *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (Fig. 1), both of which are synthesized on demand in response to elevations of intracellular calcium (Pertwee et al. 2010). Evidence has also emerged for the existence of additional endocannabinoids.

This article reviews (1) current in vitro evidence that there are at least 15 endogenous compounds that can target cannabinoid receptors either orthosterically (Sect. 2) or allosterically (Sect. 4) and (2) current knowledge about the extent to which some of these compounds also target non-cannabinoid receptors or ion channels, particularly at those concentrations at which they seem to interact with cannabinoid receptors (Sect. 3).

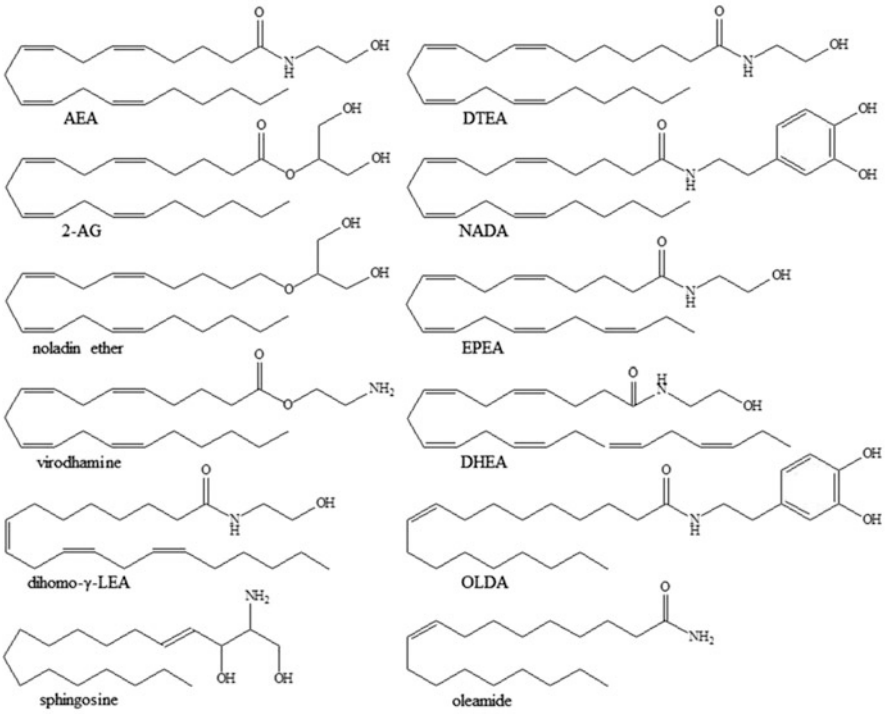

Fig. 1 The structures of anandamide (AEA), 2-arachidonoylglycerol (2-AG), noladin ether (2-arachidonyl glyceryl ether), virodhamine (*O*-arachidonoylethanolamine), dihomo-γ-linolenoylethanolamide (dihomo-γ-LEA), sphingosine, docosatetraenoylethanolamide (DTEA), *N*-arachidonoyldopamine (NADA), eicosapentaenoylethanolamide (EPEA), docosahexaenoylethanolamide (DHEA), *N*-oleoyldopamine (OLDA) and oleamide

2 Evidence That Certain Endogenous Compounds Target Cannabinoid Receptors Orthosterically

2.1 Evidence from Binding Assays

Several endogenous compounds have been found to bind to cannabinoid CB_1 receptors in vitro at concentrations in the low or mid nanomolar range, in at least some investigations. These compounds are anandamide, 2-arachidonoylglycerol, dihomo-γ-linolenylethanolamide, 2-arachidonyl glyceryl ether (noladin ether), docosatetraenoylethanolamide, *N*-arachidonoyldopamine, docosahexaenoylethanolamide and eicosapentaenoylethanolamide (Fig. 1), the first three of which have also been reported to display significant affinity for the CB_2 receptor (Table 1). Thus, as summarized in Table 1, there is evidence that, at such concentrations, each of these compounds can compete for specific binding sites on CB_1 and/or CB_2

Table 1 Affinity for cannabinoid CB_1 and CB_2 receptors displayed by established or putative endogenous compounds in competitive binding assays performed in vitro with tritium-labelled ligands for these receptors

Target	Endogenous compound	Displaced ligand	Tissue	K_i or IC_{50}	Reference
CB_1	AEA	[^{3}H]HU-243[a]	CB_1 COS-7 cell membranes	$K_i = 252$ nM[b]	Mechoulam et al. (1995)
	AEA	[^{3}H]HU-243[a]	CB_1 COS-7 cell membranes	$K_i = 350$ nM[b]	Mechoulam et al. (1997)
	AEA	[^{3}H]HU-243[a]	Rat CB_1 CHO cell membranes	$K_i = 37$ nM[b]	Vogel et al. (1993)
	AEA	[^{3}H]HU-243[a]	Rat CB_1 COS-7 cell membranes	$K_i = 155$ nM[b]	Barg et al. (1995)
	AEA	[^{3}H]CP55940[c]	hCB_1 CHO cell membranes	$K_i = 359.6$ nM[b]	Rinaldi-Carmona et al. (1996a)
	AEA	[^{3}H]CP55940[c]	hCB_1 L cell membranes	$K_i = 543$ nM[b]	Felder et al. (1993)
	AEA	[^{3}H]CP55940[c]	hCB_1 L cell membranes	$K_i = 400$ nM[d]	Felder et al. (1995)
				$K_i = 805$ nM[b]	
	AEA	[^{3}H]CP55940[a]	hCB_1 CHO cell membranes	$K_i = 631$ nM[b]	Bonhaus et al. (1998)
	AEA	[^{3}H]WIN55212-2[a]	hCB_1 HEK293 cell membranes	$K_i = 115.6$ nM[b]	Song and Bonner (1996)
	AEA	[^{3}H]CP55940[c]	hCB_1 HEK293 cell membranes	$K_i = 321$ nM[d]	Tao and Abood (1998)
CB_1[e]	AEA	[^{3}H]CP55940[c]	Human neocortical synaptosomal membranes	$K_i = 25.7$ nM[d]	Steffens et al. (2005)
				$K_i = 209$ nM[b]	
	AEA	[^{3}H]HU-243[a]	Rat brain synaptosomal membranes	$K_i = 39.2$ nM[b]	Sheskin et al. (1997)
	AEA	[^{3}H]HU-243	Rat brain synaptosomal membranes	$K_i = 52$ nM[b]	Devane et al. (1992) and Hanuš et al. (1993)
	AEA	[^{3}H]SR141716A[c]	Rat brain synaptosomal membranes	$IC_{50} = 266$ nM[b]	Rinaldi-Carmona et al. (1996b)
	AEA	[^{3}H]CP55940[c]	Rat brain membranes	$K_i = 89$ nM[d]	Adams et al. (1995)
				$K_i = 5400$ nM[b]	
	AEA	[^{3}H]CP55940[c]	Rat brain membranes	$K_i = 101$ nM[d]	Smith et al. (1994)
				$K_i = 5400$ nM[b]	
	AEA	[^{3}H]CP55940[c]	Rat brain membranes	$K_i = 12$ nM[d]	Pinto et al. (1994)
				$K_i > 2000$ nM[b]	

(continued)

Table 1 (continued)

Target	Endogenous compound	Displaced ligand	Tissue	K_i or IC_{50}	Reference
	AEA	[^{3}H]SR141716A[c]	Rat brain membranes	$K_i = 800$ nM[d]	Bezuglov et al. (2001)
	AEA	[^{3}H]CP55940[c]	Rat brain membranes[a]	$K_i = 428$ nM[b]	Leggett et al. (2004)
	AEA	[^{3}H]CP55940	Rat brain membranes	$K_i = 80$ nM[b,f]	Boring et al. (1996)
	AEA	[^{3}H]CP55940[c]	Rat cerebellar plasma membranes	$K_i = 129$ nM[d]	Felder et al. (1995)
				$K_i = 1950$ nM[b]	
	AEA	[^{3}H]WIN55212-2[c]	Rat cerebellar membranes	$IC_{50} = 90$ nM[d]	Childers et al. (1994)
				$IC_{50} > 1000$ nM[b]	
	AEA	[^{3}H]WIN55212-2[c]	Rat cerebellar membranes	$IC_{50} = 15$ nM[d]	Petitet et al. (1997)
				$IC_{50} = 1500$ nM[b]	
	AEA	[^{3}H]CP55940[c]	Rat cerebellar membranes	$K_i = 71.7$ nM[d]	Hillard et al. (1999)
	AEA	[^{3}H]CP55940[c]	Rat forebrain membranes	$K_i = 61$ nM[d]	Lin et al. (1998)
				$K_i = 5810$ nM[b]	
	AEA	[^{3}H]CP55940[c]	Mouse brain membranes	$K_i = 48$ nM[d,g]	Lichtman et al. (2002)
				$K_i = 61$ nM[d]	
				$K_i = 52$ nM[b,g]	
				$K_i = 794$ nM[b]	
CB_1	2-AG	[^{3}H]HU-243[a]	CB_1 COS-7 cell membranes	$K_i = 472$ nM[b]	Mechoulam et al. (1995)
	2-AG	[^{3}H]HU-243[a]	CB_1 COS-7 cell membranes	$K_i = 34.6$ nM[d]	Ben-Shabat et al. (1998)
				$K_i = 58.3$ nM[b]	
CB_1[e]	2-AG	[^{3}H]HU-243[a]	Rat brain synaptosomal membranes	$K_i = 5850$ nM[b]	Mechoulam et al. (1995)
	2-AG	[^{3}H]CP55940[c]	Human neocortical synaptosomal membranes	$K_i >> 10$ μM[d]	Steffens et al. (2005)
				$K_i >> 10$ μM[b]	
	2-AG	[^{3}H]CP55940[c]	Rat neocortical synaptosomal membranes	$K_i = 13.49$ μM[d]	Steffens et al. (2005)
				$K_i = 10.72$ μM[b]	
	2-AG	[^{3}H]CP55940[c]	Mouse brain membranes	$K_i = 2100$ nM[b,g]	Lichtman et al. (2002)
				$K_i = 1890$ nM[b]	

CB_1[e]	Noladin ether	[^{3}H]HU-243[a]	Rat brain membranes	$K_i = 21.2$ nM[b]	Hanuš et al. (2001)
	Noladin ether	[^{3}H]CP55940[c]	Human neocortical synaptosomal membranes	$K_i = 102$ nM[d]	Steffens et al. (2005)
				$K_i = 97.7$ nM[b]	
CB_1	DTEA	[^{3}H]HU-243[a]	Rat CB_1 COS-7 cell membranes	$K_i = 253.4$ nM[b]	Barg et al. (1995)
CB_1[e]	DTEA	[^{3}H]HU-243[a]	Rat brain synaptosomal membranes	$K_i = 34.4$ nM[b]	Hanuš et al. (1993) and Sheskin et al. (1997)
CB_1	Dihomo-γ-LEA	[^{3}H]HU-243[a]	Rat CB_1 COS-7 cell membranes	$K_i = 244.8$ nM[b]	Barg et al. (1995)
	Dihomo-γ-LEA	[^{3}H]CP55940[c]	hCB_1 L cell membranes	$K_i = 598$ nM[b]	Felder et al. (1995)
CB_1[e]	Dihomo-γ-LEA	[^{3}H]HU-243[a]	Rat brain synaptosomal membranes	$K_i = 53.4$ nM[b]	Hanuš et al. (1993) and Sheskin et al. (1997)
CB_1[e]	NADA	[^{3}H]SR141716A[c]	Rat brain membranes	$K_i = 250$ nM[d]	Bisogno et al. (2000)
	NADA	[^{3}H]SR141716A[c]	Rat brain membranes	$K_i = 380$ nM[d]	Bezuglov et al. (2001)
	NADA	[^{3}H]SR141716A[c]	Rat brain membranes	$K_i = 500$ nM[d]	Chu et al. (2003)
CB_1[e]	Virodhamine	[^{3}H]CP55940[c]	Human neocortical synaptosomal membranes	$K_i = 912$ nM[d]	Steffens et al. (2005)
				$K_i = 1738$ nM[b]	
CB_1[e]	OLDA	[^{3}H]SR141716A[c]	Rat brain membranes	$K_i = 1600$ nM[d]	Chu et al. (2003)
CB_1	Oleamide	[^{3}H]CP55940[c]	hCB_1 HEK-293 T cell membranes	$K_i = 8140$ nM[b]	Leggett et al. (2004)
CB_1[e]	Oleamide	[^{3}H]CP55940[c]	Rat brain membranes[a]	$K_i = 1140$ nM[b]	Leggett et al. (2004)
	Oleamide	[^{3}H]SR141716A[c]	Rat brain membranes[a]	$K_i = 2630$ nM[b]	Leggett et al. (2004)
	Oleamide	[^{3}H]CP55940	Rat brain membranes	$K_i = 44$ μM[b,f]	Boring et al. (1996)
	Oleamide emulsified	[^{3}H]CP55940[c]	Rat cerebellar membranes	$IC_{50} = 9.7$ μM[d]	Cheer et al. (1999)
				$IC_{50} = 11.1$ μM[b]	
	Oleamide in ethanol	[^{3}H]CP55940[c]	Rat cerebellar membranes	$IC_{50} = 10.8$ μM[d]	Cheer et al. (1999)
				$IC_{50} = 20.8$ μM[b]	
	Oleamide	[^{3}H]CP55940[c]	Mouse brain membranes	$K_i > 10$ μM[b,g]	Lichtman et al. (2002)
				$K_i > 10$ μM[b]	
CB_1	DHEA	[^{3}H]CP55940[c]	hCB_1 L cell membranes	$K_i = 12.2$ μM[b]	Felder et al. (1993)

(continued)

Table 1 (continued)

Target	Endogenous compound	Displaced ligand	Tissue	K_i or IC_{50}	Reference
CB_1[e]	DHEA	[^{3}H]HU-243[a]	Rat brain synaptosomal membranes	$K_i = 324.1$ nM[b]	Sheskin et al. (1997)
	DHEA	[^{3}H]CP55940[c]	Mouse brain membranes	$K_i = 124$ nM[d]	Brown et al. (2010)
				$K_i = 633$ nM[b]	
CB_1[e]	EPEA	[^{3}H]CP55940[c]	Rat brain membranes	$K_i = 1470$ nM[d]	Adams et al. (1995)
	EPEA	[^{3}H]HU-243[a]	Rat brain synaptosomal membranes	$K_i = 162.3$ nM[b]	Sheskin et al. (1997)
	EPEA	[^{3}H]CP55940[c]	Mouse brain membranes	$K_i = 55$ nM[d]	Brown et al. (2010)
				$K_i = 2000$ nM[b]	
CB_1	Sphingosine	[^{3}H]SR141716A[c]	Mouse CB_1 CHO cell membranes	$K_i = 347$ nM[b]	Paugh et al. (2006)
	Sphingosine	[^{3}H]CP55940[c]	Mouse CB_1 CHO cell membranes	$K_i = 3090$ nM[b]	Paugh et al. (2006)
	Sphingosine	[^{3}H]CP55940[c]	hCB_1 HEK293 cell membranes	$K_i = 5888$ nM[b]	Paugh et al. (2006)
CB_1[e]	Sphingosine	[^{3}H]CP55940[c]	Mouse cerebellar membranes	$K_i = 4571$ nM[b]	Paugh et al. (2006)
CB_1[e]	Haemopressin[h]	[^{3}H]SR141716A[c]	Rat striatal membranes	$IC_{50} = 0.35$ nM[b]	Heimann et al. (2007)
CB_2	AEA	[^{3}H]HU-243[a]	CB_2 COS-7 cell membranes	$K_i = 581$ nM[b]	Mechoulam et al. (1995)
	AEA	[^{3}H]CP55940[c]	hCB_2 AtT-20 cell membranes	$K_i = 1760$ nM[d]	Felder et al. (1995)
				$K_i = 1650$ nM[b]	
	AEA	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 371$ nM[d]	Showalter et al. (1996)
	AEA	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 3500$ nM[b]	Gonsiorek et al. (2000)
	AEA	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 348$ nM[b,i]	Gonsiorek et al. (2000)
	AEA	[^{3}H]CP55940[c]	hCB_2 Sf9 insect cell membranes	$K_i = 795$ nM[b]	Gonsiorek et al. (2000)
CB_2[e]	AEA	[^{3}H]CP55940[c]	Rat spleen membranes	$K_i = 279$ nM[d]	Hillard et al. (1999)
	AEA	[^{3}H]CP55940[c]	Rat spleen membranes	$K_i = 1890$ nM[d]	Felder et al. (1995)
				$K_i = 5020$ nM[b]	
	AEA	[^{3}H]CP55940[c]	Mouse spleen membranes	$K_i = 1930$ nM[b]	Lin et al. (1998)

CB_2	2-AG	[^{3}H]HU-243[a]	CB_2 COS-7 cell membranes	$K_i = 1400$ nM[b]	Mechoulam et al. (1995)
	2-AG	[^{3}H]HU-243[a]	CB_2 CHO cell membranes	$K_i = 1640$ nM[b]	Ben-Shabat et al. (1998)
	2-AG	[^{3}H]HU-243[a]	CB_2 COS-7 cell membranes	$K_i = 145$ nM[b]	Ben-Shabat et al. (1998)
	2-AG	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 650$ nM[b]	Gonsiorek et al. (2000)
	2-AG	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 474$ nM[b,i]	Gonsiorek et al. (2000)
	2-AG	[^{3}H]CP55940[c]	hCB_2 Sf9 insect cell membranes	$K_i = 949$ nM[b]	Gonsiorek et al. (2000)
CB_2	Noladin ether	[^{3}H]HU-243[a]	hCB_2 COS-7 cell membranes	$K_i > 3000$ nM[b]	Hanuš et al. (2001)
CB_2	Dihomo-γ-LEA	[^{3}H]CP55940[c]	hCB_2 AtT-20 cell membranes	$K_i = 857$ nM[b]	Felder et al. (1995)
CB_2[e]	NADA	[^{3}H]WIN55212-2[c]	Rat spleen membranes	$K_i = 12$ μM[d]	Bisogno et al. (2000)
CB_2	Oleamide	[^{3}H]CP55940[c]	hCB_2 HEK-293 T cell membranes	$IC_{50} > 100$ μM[b]	Leggett et al. (2004)
CB_2	DHEA	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 2141$ nM[d]	Brown et al. (2010)
				$K_i = 3843$ nM[b]	
CB_2	EPEA	[^{3}H]CP55940[c]	hCB_2 CHO cell membranes	$K_i = 3440$ nM[d]	Brown et al. (2010)
				$K_i = 9027$ nM[b]	

COS-7, African green monkey kidney
CHO Chinese hamster ovary, *EC* endocannabinoid, *h* human, *HEK* human embryonic kidney, K_i inhibition constant. See Fig. 1 legend for definitions of other abbreviations
[a]Centrifugation-based binding assay
[b]In the absence of phenylmethylsulphonyl fluoride (PMSF) (or PMSF not mentioned in cited paper)
[c]Filtration-based binding assay
[d]In the presence of 10–200 μM PMSF
[e]At least in part CB_1 or CB_2
[f]In the presence of an irreversible inactivator of serine proteases
[g]Fatty acid amide hydrolase genetically deleted
[h]Haemopressin is a putative endogenous compound (Sect. 2.1)
[i]In the presence of 0.3 nM GTPγS

receptors with one or other of four tritium-labelled ligands: [^{3}H]HU-243, [^{3}H]CP55940 and [^{3}H]WIN55212-2, each of which binds with high potency to both CB_1 and CB_2 receptors, and [^{3}H]SR141716A which possesses much higher affinity for CB_1 than for CB_2 receptors (Pertwee 2005). Haemopressin, an α-haemoglobin-derived peptide, has also been reported to bind to the CB_1 receptor with high potency (Table 1). This peptide contains nine amino acids in the following order: proline, valine, asparagine, phenylalanine, lysine, phenylalanine, leucine, serine and histidine (Heimann et al. 2007).

Importantly, there have also been reports that it has proved possible to detect

- Anandamide, initially in porcine brain (Devane et al. 1992), and subsequently in human hippocampus, in rat and mouse brain, and in mouse spinal cord (Artmann et al. 2008; Di Marzo et al. 2000; Porter et al. 2002; Stella et al. 1997), as well as in additional mammalian and invertebrate tissues (Artmann et al. 2008; Bradshaw and Walker 2005);
- 2-Arachidonoylglycerol in canine small intestine (Mechoulam et al. 1995), and also, for example, in rat brain at a level much higher than that of anandamide (Stella et al. 1997), and in rat small intestine (Artmann et al. 2008);
- Dihomo-γ-linolenoylethanolamide and docosatetraenoylethanolamide in porcine brain (Hanuš et al. 1993);
- Noladin ether in porcine brain (Hanuš et al. 2001) and in rat brain, particularly in the thalamus and hippocampus (Fezza et al. 2002);
- *N*-arachidonoyldopamine, both in bovine dorsal root ganglia, and in various rat brain areas, particularly the striatum, hippocampus and cerebellum (Huang et al. 2002);
- Docosahexaenoylethanolamide, and sometimes also eicosapentaenoylethanolamide, in mammalian tissues (Artmann et al. 2008; Brown et al. 2013; Kim et al. 2011);
- Haemopressin in rodent tissues (Heimann et al. 2007), albeit not in all investigations (Heimann et al. 2007; Gomes et al. 2009).

There has, however, also been a report that one of these compounds, noladin ether, could not be detected in rat, mouse, hamster, guinea-pig or porcine brain (Oka et al. 2003). It is noteworthy too that the extent to which eicosapentaenoylethanolamide is present in mammalian tissues is likely to be "diet-dependent". Thus, this compound, which is a metabolite of the omega-3 fatty acid, eicosapentaenoic acid, and hence of a constituent of fish oils that form part of some human diets (Brown et al. 2013), has been reported by Artmann et al. (2008) only to be detectable in rat small intestine when this is obtained from animals that had first received a fish oil diet. It was not, however, detectable in brain or liver tissue even when this had been taken from rats that had received such a diet. Docosahexaenoylethanolamide (see above) is also a metabolite of an omega-3 fatty acid (docosahexaenoic acid), and this too is present in fish oils (Brown et al. 2013).

Other endogenous compounds that have already been reported to bind to cannabinoid receptors, albeit only at concentrations in the micromolar or high nanomolar

range (Table 1 and Fig. 1), are (1) oleamide, which has, for example, been detected in the cerebrospinal fluid of sleep-deprived cats (Cravatt et al. 1995), and can be produced by mouse brain microsomes (Sugiura et al. 1996), (2) *N*-oleoyldopamine, which has been detected in bovine striatum (Chu et al. 2003), (3) *O*-arachidonoylethanolamine (virodhamine), which has been detected in human hippocampus and in many areas of rat brain (Porter et al. 2002) and (4) sphingosine which is produced endogenously in cell membrane lipid rafts from ceramide (Paugh et al. 2006). There is evidence as well that oleamide displays lower CB_2 than CB_1 affinity (Table 1) and that, at least at concentrations of up to 10 μM, sphingosine does not displace [^{3}H]CP55940 from human CB_2 CHO cell membranes (Paugh et al. 2006).

When performing binding assays with an endocannabinoid, it is important to bear in mind that some cells or membranes used in such assays contain enzymes that will affect the apparent K_i value of the compound under investigation by catalysing its conversion to a metabolite. One strategy that has been used to avoid this problem has been to include an inhibitor of fatty acid amide hydrolase, an enzyme that metabolizes the catabolism of anandamide and certain other endogenous compounds. One such inhibitor that has been widely used for this purpose is phenylmethylsulphonyl fluoride, and there have been several reports that anandamide binds to CB_1 receptors much more potently in the presence of this inhibitor than in its absence (Table 1). Such potentiation has also been observed in CB_1 binding experiments with virodhamine (Steffens et al. 2005; Table 1), and with docosahexaenoylethanolamide and eicosapentaenoylethanolamide (Brown et al. 2010; Table 1). There has been a report too that anandamide, although not 2-arachidonoylglycerol, displaced [^{3}H]CP55940 much more potently from brain membranes when these were obtained from mice from which fatty acid amide hydrolase had been genetically deleted rather than from mice still expressing this enzyme (Lichtman et al. 2002; Table 1). It is also noteworthy that it was found by Cheer et al. (1999) that phenylmethylsulphonyl fluoride increased the potency with which oleamide displaced [^{3}H]CP55940 from rat cerebellar membranes to a greater extent when this amide was administered as an ethanolic solution than when it was added as a water emulsion (Table 1). There is evidence too that although metabolism by brain membranes of some endocannabinoids occurs in ligand binding assays when binding is terminated using a filtration method, it does not when binding is terminated using a centrifugation method (Sheskin et al. 1997; Table 1). Such metabolism has also been reported not to occur in some cannabinoid receptor binding assays performed with mouse spleen membranes (Lin et al. 1998) or with membranes of cultured cells that have been transfected with CB_1 or CB_2 receptors (Felder et al. 1995).

Finally, there is evidence that for most of the endogenous compounds mentioned in this section, the displacement of tritium-labelled ligands from CB_1 or CB_2 receptors they reportedly produced was from orthosteric rather than from allosteric sites on these receptors. Thus, for example, with the exception of *N*-arachidonoyldopamine and *N*-oleoyldopamine, all of the compounds mentioned in Table 1 have been found capable of producing a complete displacement of these tritiated ligands from CB_1 receptors, and sometimes also from CB_2 receptors, in some of the investigations that yielded the data summarized in this table.

2.2 Evidence from Two Functional Bioassays

This section describes results obtained using one or other of two well-established functional bioassays: the [^{35}S]GTPγS binding assay, which exploits the fact that cannabinoid receptors signal through $G_{i/o}$ proteins, and the cyclic AMP assay which exploits the fact these receptors also signal further downstream through adenylyl cyclase (Sect. 1).

The measured response in the first of these two bioassays is agonist-induced stimulation of binding to G proteins of the hydrolysis-resistant GTP analogue, [^{35}S]GTPγS. It is performed in the presence of guanosine diphosphate (GDP) in order to minimize the amount of [^{35}S]GTPγS binding to G protein-coupled receptors that occurs in the absence of any added agonist (Pertwee 1999, 2005). GDP increases the ratio of agonist-stimulated to basal [^{35}S]GTPγS binding in this bioassay (Breivogel et al. 1998; Selley et al. 1996; Sim et al. 1995) and has also been found to increase any differences in efficacy displayed in this bioassay by full and partial agonists (Savinainen et al. 2001). It is noteworthy, however, that GDP decreases both agonist-stimulated and basal [^{35}S]GTPγS binding, to the extent that, at high concentrations, it can cause [^{35}S]GTPγS binding to fall to a level that is too low to be measured reproducibly (Selley et al. 1996).

The cyclic AMP assay exploits the negative coupling of CB_1 and CB_2 receptors to adenylyl cyclase (Pertwee 1999, 2005), the measured response usually being agonist-induced inhibition of forskolin-stimulated cyclic AMP production, for example, by CB_1- or CB_2-transfected whole cells or by neurons or brain synaptosomes that contain naturally expressed cannabinoid receptors. It is expected to be more sensitive than the [^{35}S]GTPγS assay at detecting signs of cannabinoid receptor activation since (1) adenylyl cyclase is located further along the signalling cascade than G protein, and hence (2) the cannabinoid receptor signal is more amplified when the measured response is inhibition of cyclic AMP production rather than stimulation of [^{35}S]GTPγS binding (Pertwee 2005). It is noteworthy too, however, that the cyclic AMP assay provides a measure of the activation of just one particular cannabinoid receptor effector process, adenylyl cyclase inhibition. In contrast, the [^{35}S]GTPγS assay provides a total measure of G protein-mediated cannabinoid receptor activation and should, therefore, be independent of any variations between tissues in the relative contributions made by different G protein-coupled effector processes.

2.2.1 [^{35}S]GTPγS Binding Assay

A number of endogenous compounds that behave as cannabinoid receptor ligands in binding assays (Sect. 2.1) have been found to induce signs of cannabinoid receptor agonism in at least some [^{35}S]GTPγS binding assays at nanomolar or micromolar concentrations. These are anandamide, 2-arachidonoylglycerol, oleamide, noladin ether, virodhamine, docosahexaenoylethanolamide and eicosapentaenoylethanolamide (Table 2). It has also been found in several investigations that anandamide displays significantly lower efficacy than the established CB_1/CB_2 receptor agonists WIN55212-2, CP55940 or HU-210 in at

Table 2 Potency displayed in vitro by endogenous compounds as stimulators of [^{35}S]GTPγS binding to membranes expressing cannabinoid receptors

Target	Endogenous compound	Tissue	Potency	Reference
CB_1	AEA	hCB_1 HEK-293 cell membranes	$EC_{50} = 31$ nM[a]	Ryberg et al. (2007)
CB_1[b]	AEA	Rat cerebellar membranes	$EC_{50} \geq 5100$ nM[c]	Selley et al. (1996)
	AEA	Rat cerebellar membranes	$EC_{50} = 540$ nM[c]	Petitet et al. (1997)
			$EC_{50} = 2300$ nM[a]	
	AEA	Rat cerebellar membranes	$EC_{50} = 390$ nM[c]	Breivogel et al. (1998)
			$EC_{50} = 1750$ nM[a]	
	AEA	Rat cerebellar membranes	$EC_{50} = 276$ nM[c]	Hillard et al. (1999)
	AEA	Mouse brain membranes	$EC_{50} = 846$ nM[a]	Burkey et al. (1997)
	AEA	Rat cerebellar membranes	$EC_{50} = 5012$ nM[a,d]	Savinainen et al. (2003)
	AEA	Rat brain membranes[e]	$EC_{50} = 10{,}430$ nM[a]	Leggett et al. (2004)
CB_1	2-AG	hCB_1 HEK-293 cell membranes	$EC_{50} = 519$ nM[a]	Ryberg et al. (2007)
CB_1[b]	2-AG	Rat cerebellar membranes	$EC_{50} = 3162$ nM[a]	Savinainen et al. (2001)
	2-AG	Rat cerebellar membranes	$EC_{50} = 1000$ nM[a,d]	Savinainen et al. (2003)
CB_1	Noladin ether	hCB_1 HEK-293 cell membranes	$EC_{50} = 37$ nM[a]	Ryberg et al. (2007)
CB_1[b]	Noladin ether	Rat cerebellar membranes	$EC_{50} = 7943$ nM[a]	Savinainen et al. (2001)
	Noladin ether	Rat cerebellar membranes	$EC_{50} = 6310$ nM[a,d]	Savinainen et al. (2003)
CB_1	Virodhamine	CB_1 Sf9 cell membranes	$EC_{50} = 1906$ nM[a]	Porter et al. (2002)
	Virodhamine	hCB_1 HEK-293 cell membranes	$EC_{50} = 2920$ nM[a]	Ryberg et al. (2007)
CB_1[b]	Oleamide	Rat brain membranes	$EC_{50} > 1000$ μM	Boring et al. (1996)
	Oleamide	Rat brain membranes[e]	$EC_{50} = 1640$ nM[a]	Leggett et al. (2004)
CB_1[b]	DHEA	Mouse brain membranes	$EC_{50} = 50$ nM[a]	Brown et al. (2010)
CB_1[b]	EPEA	Mouse brain membranes	$EC_{50} = 1361$ nM[a]	Brown et al. (2010)
CB_2	AEA	hCB_2 Sf9 cell membranes	$EC_{50} = 121$ nM[a]	Gonsiorek et al. (2000)
	AEA	hCB_2 CHO cell membranes	$EC_{50} = 261$ nM[a]	Gonsiorek et al. (2000)

(continued)

Table 2 (continued)

Target	Endogenous compound	Tissue	Potency	Reference
	AEA	hCB_2 HEK-293 cell membranes	$EC_{50} = 27$ nM[a]	Ryberg et al. (2007)
CB_2	2-AG	hCB_2 Sf9 cell membranes	$EC_{50} = 38.9$ nM[a]	Gonsiorek et al. (2000)
	2-AG	hCB_2 CHO cell membranes	$EC_{50} = 122$ nM[a]	Gonsiorek et al. (2000)
	2-AG	hCB_2 HEK-293 cell membranes	$EC_{50} = 618$ nM[a]	Ryberg et al. (2007)
CB_2	Noladin ether	hCB_2 HEK-293 cell membranes	$EC_{50} > 30{,}000$ nM[a]	Ryberg et al. (2007)
CB_2	Virodhamine	CB_2 Sf9 cell membranes	$EC_{50} = 1401$ nM[a]	Porter et al. (2002)
	Virodhamine	hCB_2 HEK-293 cell membranes	$EC_{50} = 381$ nM[a]	Ryberg et al. (2007)
CB_2	DHEA	hCB_2 CHO cell membranes	$EC_{50} = 42$ nM[a]	Brown et al. (2010)
CB_2	EPEA	hCB_2 CHO cell membranes	$EC_{50} = 397.1$ nM[a]	Brown et al. (2010)

CHO Chinese hamster ovary, *h* human, *HEK* human embryonic kidney. See Fig. 1 legend for definitions of other abbreviations

[a]In the absence of phenylmethylsulphonyl fluoride (PMSF) (or PMSF not mentioned in cited paper)

[b]At least in part CB_1 or CB_2

[c]In the presence of 50 or 150 μM PMSF

[d]In the presence of 10 μM methyl arachidonoyl fluorophosphonate (MAFP)

[e]Minus brainstem

least some [^{35}S]GTPγS binding assays performed with rat or mouse brain membranes (Breivogel et al. 1998; Burkey et al. 1997; Hillard et al. 1999) or with human CB_1 or CB_2 CHO or HEK-293 cell membranes (Gonsiorek et al. 2000; Ryberg et al. 2007), suggesting it to be a partial CB_1 and CB_2 receptor agonist. Anandamide has, however, been reported to stimulate [^{35}S]GTPγS binding to brain membranes with greater efficacy than the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (Breivogel et al. 1998; Burkey et al. 1997). As to 2-arachidonoylglycerol, there is evidence that it displays higher efficacy than anandamide in vitro as both a CB_1 and a CB_2 receptor agonist. Thus, 2-arachidonoylglycerol has been reported to stimulate [^{35}S]GTPγS binding to human CB_1 HEK-293 and human CB_2 CHO cell membranes with similar efficacy to HU-210 (Gonsiorek et al. 2000; Ryberg et al. 2007) and to rat cerebellar membranes with even greater efficacy than CP55940 (Savinainen et al. 2001, 2003). It has also been found to display greater efficacy than anandamide at stimulating such binding to membranes obtained from rat cerebellum (Savinainen et al. 2003), from human CB_1- or CB_2-transfected HEK-293 cells (Ryberg et al. 2007), and from human CB_2-transfected CHO cells (Gonsiorek et al. 2000).

There have been reports as well that in [^{35}S]GTPγS binding assays:

- Oleamide either lacks detectable activity as an agonist of brain membrane CB_1 receptors (Boring et al. 1996) or displays similar efficacy to anandamide at such receptors (Leggett et al. 2004);
- Noladin ether displays similar efficacy to CP55940 in rat cerebellar membrane experiments (Savinainen et al. 2001, 2003), but less efficacy than CP55940 in human CB_1 HEK-293 cell membrane experiments (Ryberg et al. 2007), and lacks any detectable efficacy as an agonist in human CB_2 HEK-293 cell membrane experiments, even at 30 μM (Ryberg et al. 2007);
- Virodhamine displays less CB_1 efficacy than WIN55212-2 or 2-arachidonoylglycerol, but either greater or less CB_1 efficacy than anandamide in cannabinoid receptor-expressing Sf9 or HEK-293 cells, and CB_2 receptor efficacy similar to that of WIN55212-2, 2-arachidonoylglycerol and anandamide, or greater than that of anandamide (Porter et al. 2002; Ryberg et al. 2007).

There has been a report too that the endogenous compound, sphingosine, is a CB_1 receptor competitive antagonist. Thus, at 1 μM or above, and hence at concentrations at which it can bind to the CB_1 receptor (Table 1), sphingosine has been found not to stimulate [^{35}S]GTPγS binding to mouse CB_1 CHO cell membranes but, instead, both to reduce the ability of WIN55212-2 to produce such stimulation, and at 6 μM, to produce a dextral parallel shift in the log concentration–response curve of WIN55212-2 for its stimulation of [^{35}S]GTPγS binding to these membranes, with an apparent K_B value of 1738 nM (Paugh et al. 2006). There is evidence as well that the α-haemoglobin-derived peptide, haemopressin, shares the ability of SR141716A to behave as a CB_1 receptor antagonist/inverse agonist in both [^{35}S]GTPγS binding and adenylyl cyclase assays, performed with rat striatal membranes (Heimann et al. 2007).

Since brain membranes contain non-cannabinoid as well as cannabinoid G protein-coupled receptors, it is also noteworthy that, at 1 μM or less, the CB_1-selective antagonist/inverse agonist, SR141716A, has been found to prevent anandamide, 2-arachidonoylglycerol, noladin ether and oleamide from stimulating [^{35}S]GTPγS binding to membranes obtained from rat cerebellum or rat or mouse whole brain (Burkey et al. 1997; Leggett et al. 2004; Petitet et al. 1997; Savinainen et al. 2001). Finally, docosahexaenoylethanolamide and eicosapentaenoylethanolamide each appear to stimulate [^{35}S]GTPγS binding to both mouse brain membranes and human CB_2 CHO cell membranes with rather low efficacy (Brown et al. 2010). However, the efficacy of neither of these compounds has yet been compared with any other compound that can activate CB_1 or CB_2 receptors in experiments performed in parallel.

2.2.2 Cyclic AMP Assay

Of the endogenous compounds that reportedly bind to cannabinoid receptors with significant potency (Table 1), those that have been found to behave as CB_1 receptor agonists in cyclic AMP assays, often but not always at submicromolar concentrations, are anandamide, 2-arachidonoylglycerol, oleamide, noladin ether, virodhamine, dihomo-γ-linolenoylethanolamide and docosahexaenoylethanolamide (Table 3). It has also been found that when the cyclic AMP assay is

Table 3 Activity displayed in vitro by endogenous compounds as inhibitors of cyclic AMP production by cells or tissues expressing cannabinoid receptors

Target	Endogenous compound	Tissue	Potency or active concentration	Reference
CB_1	AEA[a]	hCB_1 CHO cells	$IC_{50} = 322$ nM[b]	Felder et al. (1995)
	AEA[a]	hCB_1 CHO cells	$IC_{50} = 444$ nM[b]	Hillard et al. (1999)
	AEA[a]	hCB_1 CHO cells	$IC_{50} = 160$ nM[b]	Felder et al. (1993)
	AEA[a]	hCB_1 CHO cells	$IC_{50} = 1995$ nM[b]	Bonhaus et al. (1998)
	AEA[c]	hCB_1 CHO cells	$IC_{50} = 7943$ nM[b]	Bonhaus et al. (1998)
	AEA[a]	hCB_1 CHO cells	$IC_{50} = 108.5$ nM[b]	Rinaldi-Carmona et al. (1996a)
	AEA[a]	hCB_1 HEK-293 cells	$IC_{50} = 81.8$ nM[d]	Song and Bonner (1996)
	AEA[a]	hCB_1 CHO cells	$IC_{50} = 201$ nM[b]	Vogel et al. (1993)
CB_1[e]	AEA[a]	Neuroblastoma $N_{18}TG_2$ cells	$IC_{50} = 540$ nM[b]	Vogel et al. (1993)
	AEA[a]	Human neocortical synaptosomes	$IC_{50} = 69.2$ nM[b]	Steffens et al. (2005)
	AEA[a]	Rat cortical neurons	$IC_{50} = 1200$ nM[b]	Stella et al. (1997)
	AEA[a]	Human myometrial smooth-muscle cells	$IC_{50} = 229$ nM[b]	Brighton et al. (2011)
CB_1	2-AG[a]	CB_1 COS-7 cells	IC_{50} 428 nM[d]	Ben-Shabat et al. (1998)
			IC_{50} 1463 nM[b]	
	2-AG[c]	hCB_1 CHO cells	$EC_{50} = 83.2$ nM[b]	Bauer et al. (2012)
CB_1[e]	2-AG[a]	Human neocortical synaptosomes	$IC_{50} > 10$ μM[d]	Steffens et al. (2005)
			$IC_{50} > 10$ μM[b]	
	2-AG[a]	Rat neocortical tissue	$IC_{50} = 12.59$ nM[b]	Steffens et al. (2005)
	2-AG[a]	Rat cortical neurons	$IC_{50} = 800$ nM[b]	Stella et al. (1997)
CB_1[e]	Noladin ether[a]	Human neocortical synaptosomes	$IC_{50} = 427$ nM[b]	Steffens et al. (2005)
CB_1	Dihomo-γ-LEA[a]	hCB_1 CHO cells	$IC_{50} = 682$ nM[b]	Felder et al. (1995)
CB_1[e]	Virodhamine[a]	Human neocortical synaptosomes	$IC_{50} > 10$ μM[d]	Steffens et al. (2005)
			$IC_{50} > 10$ μM[b]	
CB_1[e]	Oleamide[a]	Mouse neuroblastoma NIE 115 cells	10 μM[b]	Leggett et al. (2004)
CB_1	DHEA[a]	hCB_1 CHO cells	$IC_{50} = 6000$ nM[b]	Felder et al. (1993)
CB_2	AEA[a]	hCB_2 CHO cells	$IC_{50} = 957$ nM[b]	Felder et al. (1995)
	AEA[a]	hCB_2 CHO cells	$IC_{50} > 10$ μM[b]	Hillard et al. (1999)
	AEA[a]	hCB_2 CHO cells	$IC_{50} > 30$ μM[b]	Gonsiorek et al. (2000)

(continued)

Table 3 (continued)

Target	Endogenous compound	Tissue	Potency or active concentration	Reference
CB_2	2-AG[a]	CB_2 COS-7 cells	$IC_{50} = 1884$ nM[d]	Ben-Shabat et al. (1998)
			$IC_{50} = 2724$ nM[b]	
	2-AG[a]	hCB_2 CHO cells	$IC_{50} = 1300$ nM[b]	Gonsiorek et al. (2000)
CB_2	Dihomo-γ-LEA[a]	hCB_2 CHO cells	$IC_{50} = 1160$ nM[b]	Felder et al. (1995)

COS-7, African green monkey kidney
CHO Chinese hamster ovary, *h* human. See Fig. 1 legend for definitions of other abbreviations
[a]Inhibition of forskolin-stimulated cyclic AMP production by whole cells
[b]In the absence of phenylmethylsulphonyl fluoride (PMSF) (or PMSF not mentioned in cited paper)
[c]Inhibition of basal cyclic AMP production by whole cells
[d]In the presence of 50, 100 or 200 μM PMSF
[e]At least in part CB_1 or CB_2

performed with human CB_1-transfected cells, anandamide displays lower efficacy as an agonist than the CB_1/CB_2 agonists, HU-210, WIN55212-2 or CP55940 (Bonhaus et al. 1998; Rinaldi-Carmona et al. 1996a; Vogel et al. 1993). It should be noted, however, that in one investigation in which this assay was performed with human CB_1-transfected HEK-293 cells, anandamide displayed greater apparent efficacy as an agonist than HU-210, WIN55212-2 or CP55940 (Song and Bonner 1996). It has been found as well from experiments carried out using the cyclic AMP assay, first, that the efficacy that noladin ether displayed as an agonist in human neocortical synaptosomes matched that of anandamide (Steffens et al. 2005), and second, that the efficacy 2-arachidonoylglycerol displayed as an agonist in human CB_2-transfected CHO cells was the same as that of the high-efficacy CB_1/CB_2 receptor agonist, HU-210 (Gonsiorek et al. 2000).

Confirmatory evidence supporting the hypothesis that anandamide can inhibit forskolin-stimulated cyclic AMP production by activating CB_1 receptors has come from experiments both with CB_1-selective antagonists/inverse agonists and with untransfected cells. Thus, it has been found first that this effect of anandamide can be prevented by SR141716A ($IC_{50} = 143$ nM) in human CB_1 but not in human CB_2-transfected cells (Felder et al. 1995), and by AM251, at 1 μM, both in human neocortical synaptosomes (Steffens et al. 2005) and in human myometrial smooth-muscle cells (Brighton et al. 2011), and second that this effect of anandamide is undetectable in untransfected CHO cells (Bonhaus et al. 1998; Felder et al. 1993; Vogel et al. 1993). SR141716A or AM251 (1 μM) has also been found to prevent inhibition of forskolin-stimulated cyclic AMP production induced in rat cortical neurons by 2-arachidonoylglycerol (Stella et al. 1997), in mouse neuroblastoma NIE 115 cells by oleamide (Leggett et al. 2004) and in human neocortical synaptosomes by noladin ether (Steffens et al. 2005). It has been found too that pertussis toxin can prevent the inhibitory effects on forskolin-stimulated cyclic AMP production of anandamide in human CB_1 CHO cells or $N_{18}TG_2$ cells (Felder et al. 1993; Rinaldi-Carmona et al. 1996a; Vogel et al. 1993), of 2-arachidonoylglycerol in rat cortical neurons (Stella et al. 1997) and of oleamide

in mouse neuroblastoma NIE 115 cells (Leggett et al. 2004). This is an indication that these inhibitory effects are mediated by $G_{i/o}$-type and not by G_s-type G proteins of the CB_1 receptor (Felder et al. 1993; Leggett et al. 2004; Rinaldi-Carmona et al. 1996a).

Finally, it should be noted that 2-arachidonoylglycerol can undergo non-enzymatic isomerization to 1(3)-arachidonoylglycerol, and that the latter compound has been found to display slightly greater potency than 2-arachidonoylglycerol as an inhibitor of forskolin-stimulated cyclic AMP production by rat cortical neurons (Stella et al. 1997). However, there is no evidence that this 1(3) isomer can directly target either CB_1 or CB_2 receptors.

3 Non-Cannabinoid Receptors and Channels Targeted by Endogenous Cannabinoid Receptor Ligands at Submicromolar Concentrations

The data summarized in Tables 1, 2 and 3 suggest that, with the exception of oleamide, all of the endogenous compounds listed in these Tables can interact with cannabinoid receptors at concentrations in the submicromolar range. This section focuses on non-cannabinoid receptors and ion channels that have been found to be activated or blocked by at least one of these compounds at a submicromolar concentration (Table 4). Additional receptors and ion channels reportedly targeted by some established and putative endocannabinoids, at micromolar concentrations, are listed in Table 5 but not discussed in this review.

3.1 Non-Cannabinoid G Protein-Coupled Receptors

3.1.1 GPR55

In experiments in which the [^{35}S]GTPγS binding assay (Sect. 2.2.1) was performed with membranes obtained from HEK-293 cells that had been transfected with the deorphanized G protein-coupled receptor, GPR55, Ryberg et al. (2007) obtained evidence that human GPR55 can be activated by four established/putative endocannabinoids: anandamide, 2-arachidonoylglycerol, noladin ether and virodhamine. The EC_{50} values of these compounds were 18, 3, 10 and 12 nM, respectively, evidence that each of them is more potent at activating GPR55 than at activating CB_1 or CB_2 receptors (Table 2), at least in the [^{35}S]GTPγS binding assay. The corresponding E_{max} values that they displayed in this bioassay were 73 %, 99 %, 95 % and 160 %, respectively, suggesting that virodhamine activates GPR55 with particularly high efficacy. There have also been reports that anandamide induces apparent GPR55-mediated mobilization of intracellular calcium, albeit with lower potency. It produced this effect with an EC_{50} of 7.3 μM in GPR55-expressing human umbilical vein-derived endothelial EA.hy926 cells (Waldeck-Weiermair et al. 2008), and at a concentration of 5 μM in human GPR55-transfected HEK-293 cells, in an investigation in which, however, neither

Table 4 Apparent non-cannabinoid receptor-mediated pharmacological actions displayed in at least some in vitro investigations by certain established and putative endocannabinoids at submicromolar concentrations

Target class	Target name	Endogenous compounds	Section
GPCR	GPR55[a]	AEA[b], 2-AG[b], noladin ether[b], virodhamine[b]	3.1.1
Ligand-gated ion channel	5-HT_3[c,d]	AEA[b]	3.2.1
	Nicotinic acetylcholine[c]	AEA[b], 2-AG[b]	3.2.2
	Glycine[e,f]	AEA[b], NADA[g]	3.2.3
	Glycine[c]	AEA[b], 2-AG[b]	3.2.3
	Glutamate NMDA[e]	AEA[b]	3.2.4
TRP channel	TRPV1[a]	AEA[b], 2-AG[b], NADA[b], noladin ether[b]	3.3.1
	TRPM8[c]	AEA[b], NADA[b]	3.3.2
Voltage-gated ion channel	T-type calcium Ca_v3[c]	AEA[b], NADA[b], DTEA[g]	3.4.1
	2TM potassium K_{ATP}[c]	AEA[b], 2-AG[g]	3.5.1
	6TM potassium K_v[c]	AEA[b], 2-AG[b], DHEA[g], dihomo-γ-LEA[g]	3.5.2
	6TM potassium K_{Ca}[a]	AEA[b]	3.5.2
	6TM potassium K_{Ca}[c]	Virodhamine[b]	3.5.2

GPCR G protein-coupled receptor, *TRP* transient receptor potential. See Fig. 1 legend for definitions of other abbreviations
[a]Agonism
[b]<1 μM, for an effective concentration, EC_{50} or IC_{50}
[c]Inhibition or antagonism
[d]Possible negative allosteric modulation
[e]Potentiation
[f]Possible positive allosteric modulation
[g]1–10 μM, for an effective concentration, EC_{50} or IC_{50}

2-arachidonoylglycerol (5 μM) nor virodhamine (3 μM) produced any detectable GPR55 activation (Lauckner et al. 2008).

3.2 Ligand-Gated Ion Channels

3.2.1 5-HT_3 Receptors

It has been reported that activation of 5-HT_3/5-HT_{3A} receptors by 5-HT can be directly antagonized by anandamide both at 100 nM (Xiong et al. 2012) and with IC_{50} values in the low nanomolar or low micromolar range: 130 nM (Barann et al. 2002), 190 nM (Fan 1995), 239 nM (Xiong et al. 2008) and 3.7 μM (Oz et al. 2002). Results obtained in these investigations suggest that this

Table 5 Some apparent non-cannabinoid receptor-mediated pharmacological actions displayed in vitro by certain established and putative endocannabinoids at micromolar concentrations

Target class	Target name	Endogenous compounds	Reference
GPCR	Muscarinic M_1 & M_4[a]	AEA[b] or [c]	[d]
	Adenosine A_3[e]	AEA[b], 2-AG[b]	[d]
	5-HT_1 & 5-HT_2[a]	AEA[b] or [c]	[d]
	$S1P_1$ (edg1)[f]	AEA[g], 2-AG[g]	Yin et al. (2009)
	GPR18[f]	AEA[b]	McHugh et al. (2012)
	GPR119[f]	AEA[c], oleamide[b], OLDA[b]	Chu et al. (2010) and Overton et al. (2006)
Ion channel	Ligand-gated $GABA_A$[h]	AEA[b], 2-AG[b], noladin ether[b]	Sigel et al. (2011)
	TRPA1[f]	AEA[b]	[d]
	L-type calcium Ca_v1[a]	AEA[b], 2-AG[b]	[d]
	P-type calcium $Ca_v2.1$[i]	AEA[b], 2-AG[b]	[d]
	N-type calcium $Ca_v2.2$[i]	AEA[b]	[d]
	4TM potassium TASK[i]	AEA[b]	[d]
	4TM potassium TREK[i]	AEA[b]	[d]
	Sodium Na_v[a,e]	AEA[c], 2-AG[c], NADA[c], noladin ether[c]	[d]
	Sodium Na_v[i]	AEA[b], 2-AG[b], NADA[b], noladin ether[b]	Duan et al. (2008)[d]
Nuclear receptor	PPARα[a,f]	AEA[b], noladin ether[b], virodhamine[b]	Sun et al. (2007)[d]
	PPARγ[a]	AEA[b], 2-AG[b]	Bouaboula et al. (2005)[d]
	PPARγ [f]	AEA[b], 2-AG[b], noladin ether[c]	Bouaboula et al. (2005) and Rockwell et al. (2006)
	PPARδ[f]	AEA[b], 2-AG[b], noladin ether[b]	Ghosh et al. (2007)

GPCR G protein-coupled receptor, *TRP* transient receptor potential, *PPAR* peroxisome proliferator-activated receptor. See Fig. 1 legend for definitions of other abbreviations

[a]Displacement of a radioligand from a binding site

[b]1–10 μM, for an effective concentration, EC_{50} or IC_{50}

[c]>10–100 μM, for an effective concentration, EC_{50} or IC_{50}

[d]See review by Pertwee et al. (2010)

[e]Possible negative allosteric modulation

[f]Agonism

[g]Unspecified μM, for an effective concentration, EC_{50} or IC_{50}

[h]Potentiation of γ-aminobutyric acid

[i]Inhibition or antagonism

antagonism is non-competitive, and hence possibly allosteric, in nature. It is also noteworthy, however, that in two other in vitro investigations, an anandamide concentration of 100 or 300 nM was found not to produce any detectable antagonism of 5-HT-induced activation of the 5-HT_3 receptor (Oz et al. 2004, 2005). Moreover, at concentrations of up to 10 μM, anandamide has been found not to affect binding of [^{3}H]GR65630 to human 5-HT_{3A} receptors expressed in HEK-293 cell membranes (Barann et al. 2002), although it has been reported to reduce specific [^{3}H]5-HT binding to bovine brain synaptic membranes at concentrations of 1, 10 and 100 μM (Kimura et al. 1998).

3.2.2 Nicotinic Acetylcholine Receptors

As discussed in greater detail elsewhere (Oz et al. 2014), it has been found that both anandamide and 2-arachidonoylglycerol can antagonize the activation of α7 nicotinic acetylcholine receptors by nicotine or acetylcholine in a non-competitive and cannabinoid receptor-independent manner, anandamide with an IC_{50} of 230 or 238 nM (Oz et al. 2003, 2005) and 2-arachidonoylglycerol with an IC_{50} of 168 nM (Oz et al. 2004). Arachidonic acid, a metabolite of anandamide, was also found to block the activation of α7 nicotinic acetylcholine receptors, albeit with an IC_{50} value of 1.2 μM, and hence with lower potency than anandamide (Oz et al. 2004). It seems unlikely, therefore, that it was responsible for the antagonism of these receptors induced by the administration of anandamide. There has also been one report that anandamide can antagonize the activation of α4β2 nicotinic acetylcholine receptors in SH-EP1 cells both non-competitively and in a cannabinoid receptor-independent manner ($IC_{50} = 900$ nM; Butt et al. 2008) and another report that it can antagonize these receptors with an IC_{50} of approximately 300 nM (Spivak et al. 2007). It has been found too that anandamide does not inhibit specific binding of [^{3}H]nicotine either to human frontal cortical membranes at 50–300 μM (Lagalwar et al. 1999) or to mouse thalamic membranes at 30 μM (Butt et al. 2008).

3.2.3 Glycine Receptors

Results obtained (1) by Hejazi et al. (2006) using either *Xenopus* oocytes transfected with human α1 homomeric or α1β1 heteromeric glycine receptors, or rat isolated ventral tegmental area neurons that express glycine receptors naturally, and (2) by Yang et al. (2008) using HEK-293 cells transfected with human α1 or α1β glycine receptors show that activation of these receptors by glycine can be enhanced by anandamide with EC_{50} values ranging from 38 to 319 nM, through a mechanism that is cannabinoid CB_1 receptor-independent and possibly allosteric in nature. There has also been a report first that at 1 μM, anandamide enhances the activation of glycine receptors in rat cultured spinal neurons in a CB_1, CB_2 and vanilloid receptor-independent manner, and second that at 1 or 10 μM, it enhances activation both of human α1 and α1β1 and of rat α2 and α3 glycine receptors that had been transfected into HEK-293 cells (Xiong et al. 2012). In addition, Yévenes and Zeilhofer (2011) have detected the production of apparent allosteric enhancement by *N*-arachidonoyldopamine, as well as by anandamide, each at 10 μM, of

glycine-induced activation of α1, α2 and α3 glycine receptors expressed by HEK-293 cells. Evidence has also emerged, however, from experiments with rat isolated hippocampal neurons that, at 0.2–2 μM, both anandamide and 2-arachidonoylglycerol can inhibit the activation of glycine receptors, that this inhibition is not mediated by TRPV1 channels and that the inhibitory effect of 2-arachidonoylglycerol is not mediated by cannabinoid CB_1 receptors (Lozovaya et al. 2005). It is noteworthy as well that Yang et al. (2008) found that, at concentrations of up to 30 μM, anandamide did not enhance glycine-induced activation of human α2 or rat α3 glycine receptors that had been transfected into HEK-293 cells.

3.2.4 Ionotropic Glutamate NMDA Receptors

There is evidence that anandamide can act through a CB_1 receptor-independent mechanism to enhance activation of ionotropic glutamate *N*-methyl-D-aspartate (NMDA) receptors by NMDA, at 100 nM in rat brain cortical slices, at 1 μM in rat cerebellar and hippocampal slices and at both nanomolar and micromolar concentrations in NMDA-expressing *Xenopus* oocytes (Hampson et al. 1998). This it was found to do, at least in rat cortical slices and *Xenopus* oocytes, in a manner that did not depend on its metabolic conversion to arachidonic acid.

3.3 Transient Receptor Potential Channels

3.3.1 TRPV1

N-arachidonoyldopamine has been found to increase calcium influx in human TRPV1-transfected HEK-293 cells with considerable potency ($EC_{50} = 26$ nM) and efficacy (De Petrocellis et al. 2000). Evidence has also emerged that it can produce TRPV1-mediated calcitonin gene-related peptide release from rat cultured trigeminal ganglion neurons with an EC_{50} of 760 nM (Price et al. 2005b). In addition, *N*-arachidonoyldopamine has been found to increase intracellular calcium in cultures of rat dorsal root ganglia and trigeminal ganglia with EC_{50} values of 1.6 μM and 794 nM, respectively, and also to evoke calcitonin gene-related peptide release from these ganglia with EC_{50} values of 1.6 μM and 4.8 μM, respectively, and with an efficacy slightly or considerably less than that of capsaicin (McDonald et al. 2008).

As indicated in greater detail elsewhere (Pertwee et al. 2010; Starowicz et al. 2007), anandamide has also been found to target TRPV1 receptors, albeit with less potency than *N*-arachidonoyldopamine. Thus, for example, this endocannabinoid has been reported to

- Displace the established TRPV1 ligand, [^{3}H]resiniferatoxin, from membranes of rat TRPV1 receptor-transfected CHO cells, with a K_i value of 1.7 μM, in experiments performed in the presence of 200 μM phenylmethylsulphonyl fluoride (Ross et al. 2001);

- Stimulate $^{45}Ca^{2+}$ uptake into rat TRPV1 receptor-transfected CHO cells with an EC_{50} value of 1.6 μM (Ross et al. 2001);
- Elicit outwardly rectifying cationic currents in TRPV1-transfected HEK-293 cells with an EC_{50} of 4.9 μM, and in a manner that could be completely and rapidly antagonized by the TRPV1 antagonist, capsazepine, at 100 nM (Zygmunt et al. 1999);
- Produce outward currents from mouse isolated trigeminal sensory neurons with an EC_{50} of 2.5 μM, with less efficacy than the established TRPV1 agonist, capsaicin, and in a manner that was blocked by 30 μM capsazepine, but not by the CB_1-selective antagonist, SR141716A, at 1 μM (Roberts et al. 2002);
- Increase intracellular calcium levels in human TRPV1-transfected HEK-293 cells with an EC_{50} of 1.15 μM, with similar efficacy to capsaicin, and in a manner that could be antagonized by the TRPV1 antagonist, capazepine, with a K_B value of 39.8 nM but not by the CB_1-selective antagonist, AM281, or the CB_2-selective antagonist, AM630, at 100 pM to 10 μM (Smart et al. 2000);
- Increase intracellular calcium levels in human TRPV1-transfected HEK-293 cells with an EC_{50} of 520 or 630 nM, with similar efficacy to capsaicin, and in a manner that could be completely antagonized by both SR141716A and capsazepine (De Petrocellis et al. 2000, 2001);
- Increase calcium influx in rat TRPV1-expressing HEK-293 cells with an EC_{50} of 6.9 μM (Qin et al. 2008);
- Produce TRPV1-mediated calcitonin gene-related peptide release from rat cultured trigeminal ganglion neurons with an EC_{50} of 7.4 μM (Price et al. 2005b);
- Induce calcium influx into human cultured keratinocytes at concentrations of 3, 10 and 30 μM, an effect that was prevented by the TRPV1 antagonists, capsazepine and iodoresiniferatoxin, and interestingly, also by the CB_1 antagonist, AM251, although not by the CB_2 antagonist, AM630 (Tóth et al. 2011).

It is noteworthy that in two of the above investigations, it was found that the apparent activation of TRPV1 receptors by anandamide could be antagonized by the CB_1 antagonists, SR141716A or AM251, albeit possibly at least in part through a CB_1 receptor-independent mechanism (De Petrocellis et al. 2001; Tóth et al. 2011). However, it is noteworthy as well that in two of the other investigations, no such antagonism was found to be induced by SR141716A or AM281 (Roberts et al. 2002; Smart et al. 2000).

The abilities of 2- and 1-arachidonoylglycerol, and of noladin ether, to interact with TRPV1 receptors have also been investigated. 2-arachidonoylglycerol has been found to increase calcium influx in rat TRPV1-expressing HEK-293 cells ($EC_{50} = 8.4$ μM) (Qin et al. 2008), but to display only marginal efficacy at producing such an effect in human TRPV1-expressing HEK-293 cells (De Petrocellis et al. 2000). It has been reported as well that 2- and 1-arachidonoylglycerol can each increase currents in rat TRPV1-expressing CHO cells at 10 μM (Zygmunt et al. 2013). When applied at concentrations of 0.32–10 μM, noladin ether has been found to share the abilities of 2-arachidonoylglycerol (0.32–10 μM) and

1-arachidonoylglycerol (1–10 μM) to produce apparent TRPV1-mediated vasorelaxation in rat mesenteric arteries (Zygmunt et al. 2013).

3.3.2 TRPM8 Channels

It has been discovered that anandamide and *N*-arachidonoyldopamine can each reduce the ability of icilin and of menthol to induce calcium entry into HEK-293 cells overexpressing the rat TRPM8 channel with IC_{50} values of 150 nM and 740 nM, respectively, versus icilin, and of 3.1 μM and 2.0 μM, respectively, versus menthol (De Petrocellis et al. 2007, 2012). Anandamide has also been reported to oppose icilin-induced calcium entry into rat dorsal root ganglia sensory neurons at concentrations of 10–100 μM (De Petrocellis et al. 2008).

3.4 Voltage-Gated Calcium Channels

3.4.1 T-Type Ca_v3 Calcium Channels

Chemin et al. (2001, 2007) have obtained evidence that anandamide can target T-type calcium channels. Thus, for example, they have found that this endocannabinoid can inhibit Ca^{2+} currents generated by human cloned $Ca_v3.1$ (α_{1G}), $Ca_v3.2$ (α_{1H}) and $Ca_v3.3$ (α_{1I}) calcium channels expressed by cultured cells with IC_{50} values of 4.15 μM, 330 nM and 1.1 μM, respectively (Chemin et al. 2001). They have also obtained evidence that, at 10 μM, both docosatetraenoylethanolamide and the anandamide metabolite, arachidonic acid, can inhibit $Ca_v3.1$, $Ca_v3.2$ and Ca_v3 currents (Chemin et al. 2007). It is noteworthy, therefore, that other results obtained in this investigation suggest that the T-current inhibition induced by in vitro administration of anandamide did not depend on its fatty acid amide hydrolase-catalysed conversion to an active metabolite.

Another putative endocannabinoid that has been reported to inhibit T-type calcium channels is *N*-arachidonoyldopamine. Thus, evidence has been obtained that this compound can inhibit T-type Ca^{2+} currents with quite high efficacy both in $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ channels expressed in HEK-293 cells, with IC_{50} values of 513 nM, 1.1 μM and 355 nM, respectively, and in mouse isolated trigeminal ganglion neurons at 300 nM (Ross et al. 2009). In a more recent investigation, however, it was found that 3 μM *N*-arachidonoyldopamine displayed rather low efficacy as an inhibitor of $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ currents, compared, for example, with anandamide, and also that it could antagonize inhibition of $Ca_v3.3$ currents induced by the selective T-channel inhibitor, TTA-A2 (Cazade et al. 2014). It was also found in the latter investigation that *N*-arachidonoyldopamine can displace [^{3}H]TTA-A1 from $Ca_v3.3$ binding sites both with significant potency ($K_i = 170$ nM) and in a competitive manner.

3.5 Potassium Channels

3.5.1 ATP-Sensitive Inward Rectifier K_{ATP} Channels of the 2TM Domain Family

Oz et al. (2007) have obtained evidence from experiments with *Xenopus* oocytes that anandamide can act independently of CB_1 or CB_2 cannabinoid receptors as a non-competitive inhibitor of K_{ATP} channels ($IC_{50} = 8.1$ μM) and that this endocannabinoid also inhibits [^{3}H]glibenclamide binding to such channels ($IC_{50} = 6.3$ μM), again in a non-competitive manner. It has been reported too that K_{ATP} channels in mouse R7T1 β-cells can be inhibited by 2-arachidonoylglycerol with an IC_{50} value of 1 μM and through a CB_1 receptor-independent mechanism (Spivak et al. 2012). More recently, evidence has emerged, from experiments with rat isolated cardiac ventricular myocytes, that at concentrations of 10 and 100 nM, anandamide can act through CB_2 receptors to increase potassium currents in K_{ATP} channels (Li et al. 2012). It was also found in this investigation that, at 10 and 100 nM, anandamide can inhibit transient outward potassium currents in these myocytes in an apparent CB_1 and CB_2 receptor-independent manner.

3.5.2 Voltage-Gated K_v and K_{Ca} Channels of the 6TM Domain Family

There is good evidence that certain delayed rectifier K_v channels can be blocked by anandamide, 2-arachidonoylglycerol, docosahexaenoylethanolamide and/or arachidonic acid with significant potency. Thus, there have been reports that it is possible to block

- K_v channels expressed in rat arterial myocytes both with anandamide ($IC_{50} = 600$ nM) and with 1 μM arachidonic acid, albeit only in the presence of tetraethylammonium chloride (Van den Bossche and Vanheel 2000);
- K_v channels expressed by rat cultured cortical astrocytes with anandamide ($IC_{50} \sim 300$ nM) (Vignali et al. 2009);
- $K_v1.1$ channels expressed by *Xenopus* oocytes with both anandamide ($IC_{50} = 1.3$ μM) and arachidonic acid ($IC_{50} = 1.5$ μM) (Decher et al. 2010);
- $K_v1.2$ channels transfected into mouse fibroblasts with anandamide ($IC_{50} = 2.7$ μM), docosahexaenoylethanolamide ($IC_{50} = 1.5$ μM), dihomo-γ-linolenoylethanolamide (at 6 μM) and arachidonic acid ($IC_{50} = 6.6$ μM) (Poling et al. 1996);
- Human cardiac $K_v1.5$ channels expressed by mouse fibroblasts with anandamide ($IC_{50} = 0.9$ μM), 2-arachidonoylglycerol ($IC_{50} = 2.5$ μM) and arachidonic acid ($IC_{50} = 1.5$ μM) (Barana et al. 2010);
- Human $K_v1.5$ channels transfected into HEK-293 cells with anandamide (extracellular $IC_{50} = 2.1$ μM; intracellular $IC_{50} = 213$ nM) (Moreno-Galindo et al. 2010);
- $K_v3.1$ channels expressed by *Xenopus* oocytes with both anandamide and arachidonic acid at 3 μM (Oliver et al. 2004);

- Human cardiac $K_v4.3$ channels expressed by CHO cells with anandamide ($IC_{50} = 400$ nM), 2-arachidonoylglycerol ($IC_{50} = 300$ nM) and arachidonic acid (at 1 μM) (Amorós et al. 2010).

Evidence has been obtained in several of these investigations that endocannabinoid-induced blockade of at least some of these K_v channels (1) is not mediated by cannabinoid CB_1 or CB_2 receptors (Amorós et al. 2010; Barana et al. 2010; Poling et al. 1996; Van den Bossche and Vanheel 2000; Vignali et al. 2009), or indeed either by other G_i-coupled receptors or by TRPV1 or TRPV4 channels (Poling et al. 1996; Vignali et al. 2009), and (2), is not dependent on the formation of arachidonic acid from anandamide (Barana et al. 2010; Van den Bossche and Vanheel 2000; Vignali et al. 2009). Some evidence has been obtained too that anandamide blocks $K_v1.1$ channels with greater efficacy and speed (Decher et al. 2010), and $K_v1.5$ channels with greater potency (Moreno-Galindo et al. 2010; see above), when it is applied to intracellular rather than to extracellular regions of these channels.

Evidence has also been obtained that anandamide increases outward K^+ currents in BK and SK K_{Ca} channels. Thus, it has been found that this endocannabinoid induces such an increase in BK-$\alpha\beta_1$ channels expressed by HEK-293 cells, at 0.3, 1 and 3 μM (Sade et al. 2006), in human BK K_{Ca} channels expressed by HEK-293 cells, with an EC_{50} value of 4.8 μM (Godlewski et al. 2009), and in small conductance calcium-activated SK K_{Ca} channels expressed naturally by rat cultured hippocampal neurons, at 1–100 nM (Wang et al. 2011). Evidence was obtained as well in some of these investigations that anandamide did not increase K^+ currents in BK or SK K_{Ca} channels by acting on CB_1 or CB_2 receptors or indeed on TRPV1 channels (Sade et al. 2006; Wang et al. 2011). There has been a report too that outward K^+ currents in BK K_{Ca} channels expressed by HEK-293 cells are unaffected by 2-arachidonoylglycerol (10 μM) and that they are decreased by virodhamine with an IC_{50} value of 447 nM (Godlewski et al. 2009).

4 Allosteric Endocannabinoids

Whereas some endogenous compounds appear to activate or block cannabinoid receptors by targeting orthosteric sites on these receptors (Sect. 2), there are at least three other endogenous compounds that seem to interact with allosteric sites on these receptors as positive or negative allosteric modulators. These three compounds are pepcan-12, lipoxin A_4 and pregnenolone.

Turning first to pepcan-12, this is a member of the same family of α-haemoglobin-derived peptides as the potent CB_1 receptor antagonist/inverse agonist, haemopressin (Sects. 2.1 and 2.2.1), has been detected in mouse and hamster brain and in human and mouse plasma and contains 12 amino acids in the following order: arginine, valine, aspartic acid, proline, valine, asparagine, phenylalanine, lysine, leucine, leucine, serine and histidine (Bauer et al. 2012). Pepcan-12 has been found to behave as a potent negative allosteric modulator (NAM) of the CB_1 receptor as indicated by

its ability (1) at 1 and 100 nM to decrease the maximal stimulatory effects of the CB_1 receptor agonists, HU-210 and 2-arachidonoylglycerol, on [^{35}S]GTPγS binding to mouse cerebellar membranes, (2) at 1, 100 and 1000 nM to decrease the ability of 2-arachidonoylglycerol and the CB_1 receptor agonist,WIN55212-2, to increase cyclic AMP levels in human CB_1-transfected CHO cells, (3) not to display any activity in either of these assays when administered by itself, and (4) at 300 nM to increase the rate of dissociation of [^{3}H]CP55940 from human CB_1 CHO cell membranes (Bauer et al. 2012). These signs of negative allosteric modulation were all produced by concentrations of pepcan-12 that had been found to be present in samples of brain and plasma and not to inhibit the metabolic degradation of anandamide or 2-arachidonoylglycerol (Bauer et al. 2012). In line with its classification as an allosteric modulator of the CB_1 receptor, pepcan-12 can only partially displace [^{3}H]CP55940 and [^{3}H]WIN55212-2 from human CB_1 receptors present in CHO cell membranes (Bauer et al. 2012). This it did with K_B values of 4.68 and 8.13 nM, respectively, and hence with a potency similar to its apparent allosteric potency. Both pepcan-12 and two other haemoglobin-derived endogenous peptides have, in addition, been reported to produce only partial displacement of [^{3}H]CP55940 from specific binding sites on mouse cerebellar membranes, again with significant potency (Gomes et al. 2009). It was also found in this investigation that although two of these three haemoglobin-derived peptides, including pepcan-12, did not share the reported ability of many established cannabinoid receptor agonists to stimulate [^{35}S]GTPγS binding to G protein in mouse brain membranes, they did both seem to produce signs of CB_1 and CB_2 receptor activation in other in vitro bioassays. Finally, it is noteworthy that in vitro data described in a recent preliminary report suggest that pepcan-12 may also be a potent positive allosteric modulator (PAM) of the CB_2 receptor (Petrucci et al. 2014).

Moving on to lipoxin A_4 (LXA_4; Fig. 2), this endogenous compound is released in brain tissues during ischaemia and is an agonist of the formyl peptide receptor, FPR2/ALX (Pamplona et al. 2012; Ye et al. 2009). LXA_4 has been found to behave as a potent PAM of the CB_1 receptor as indicated by its ability at 100 nM (1) to increase the potency with which anandamide and 2-arachidonoylglycerol inhibit forskolin-stimulated cyclic AMP production by mouse CB_1-transfected HEK-293 cells and (2) to decrease the rate of WIN55212-2-induced dissociation of [^{3}H]CP55940 from mouse whole brain membranes (Pamplona et al. 2012). In contrast, 100 nM LXA_4 did not potentiate the stimulatory effect of anandamide on [^{35}S]GTPγS binding to such brain membranes, an indication that it may be a signalling-dependent

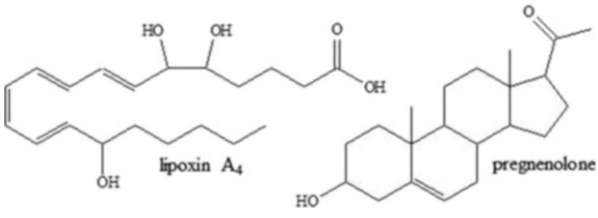

Fig. 2 The structures of lipoxin A_4 and pregnenolone

allosteric modulator of the CB_1 receptor. It was also found that 100 nM LXA_4 reduced the efficacy with which 2-arachidonoylglycerol inhibited forskolin-stimulated cyclic AMP production (Pamplona et al. 2012). When administered by itself at concentrations of up to 1 or 10 μM, LXA_4 did not display any activity as an inhibitor of forskolin-stimulated cyclic AMP production, as a stimulator of [^{35}S]GTPγS binding, or, in mouse whole brain homogenates, as an inhibitor of fatty acid amide hydrolase or monoacyl glycerol lipase. Moreover, at concentrations above 1 or 10 nM, LXA_4 displaced [^{3}H]SR141716A from binding sites in mouse whole brain membranes, albeit only partially even at 10 μM, and increased the affinity of [^{3}H]CP55940 for these binding sites (Pamplona et al. 2012). Similar results were obtained in previous binding experiments performed with the synthetic NAM of the CB_1 receptor, ORG27569 (Price et al. 2005a).

As to pregnenolone (Fig. 2), some evidence has emerged that this endogenous precursor of steroid hormones is a negative allosteric modulator of the CB_1 receptor. Thus, pregnenolone has been reported to reduce the maximal stimulatory effect of the CB_1 receptor agonist, Δ^9-tetrahydrocannabinol, on extracellular signal-regulated kinase (ERK) phosphorylation in human CB_1 CHO cells (Vallée et al. 2014). This it did at 1 and 5 μM, concentrations at which it did not displace [^{3}H]CP55940 or [^{3}H]WIN55212-2 from membranes obtained from these cells. Pregnenolone did not affect the ability of Δ^9-tetrahydrocannabinol to decrease cyclic AMP production in human CB_1 CHO cells (Vallée et al. 2014), suggesting that its apparent negative allosteric modulation of the CB_1 receptor may be signalling-dependent. Importantly, although pregnenolone is normally undetectable in rat and mouse brains, it was found by Vallée et al. (2014) that its levels in these brains can be greatly increased by Δ^9-tetrahydrocannabinol, particularly in rat nucleus accumbens, prefrontal cortex, striatum and thalamus, and that this effect is CB_1 receptor mediated, at least in rat nucleus accumbens.

The findings described in this section have clearly created a need for further research directed at identifying any physiological and/or pathophysiological consequences of allosteric modulation by endogenously released pepcan-12, LXA_4 or pregnenolone of cannabinoid receptor activation induced by endogenously released orthosteric endocannabinoids such as anandamide, or indeed, particularly with regard to pregnenolone, by exogenously administered cannabinoid receptor agonists, including Δ^9-tetrahydrocannabinol, the main psychoactive constituent of cannabis.

5 Conclusions

It would appear that most, or even all, of the 12 compounds whose structures are shown in Fig. 1 should be classified as orthosteric endocannabinoids. Thus, first, they have each been detected in one or more mammalian tissue in at least some investigations (Sect. 2.1), second, there is evidence that they can all bind to orthosteric sites on cannabinoid receptors (Table 1) and third, with the exception of oleamide and *N*-oleoyldopamine, they can bind to these receptors with K_i or IC_{50}

values in the submicromolar range, and hence with significant potency. Furthermore, eight of these compounds have been found, again in at least some investigations, to behave as cannabinoid receptor agonists, one of them only in the [^{35}S]GTPγS binding assay (Sect. 2.2.1 and Table 2), another of them only in the cyclic AMP assay (Sect. 2.2.2 and Table 3) and six of them in both of these bioassays. Some of these compounds have been reported also to interact with other pharmacological targets at submicromolar or micromolar concentrations, sometimes in an allosteric manner (Sect. 3 and Tables 4 and 5). In addition, evidence has emerged first that sphingosine is an endogenous competitive antagonist of the CB_1 receptor (Sect. 2.2.1) and second, for the existence of endogenous allosteric modulators of this receptor (Sect. 4.): for example, pregnenolone (Fig. 2) and pepcan-12, which behave as CB_1 negative allosteric modulators, and lipoxin A_4 (Fig. 2), which behaves as a CB_1 positive allosteric modulator (PAM). Interestingly, preliminary evidence has emerged as well that pepcan-12 can also target CB_2 receptors, in this case as a PAM.

The findings described in this review, all gleaned from papers published by the end of October 2014, prompt a need for further research directed at (1) seeking out any other orthosteric or allosteric endocannabinoids, (2) characterizing the pharmacological profiles of all endocannabinoids more fully and (3) identifying all endocannabinoids that reach active concentrations at cannabinoid receptors, either following their release in health or disease, or in response to an administered medicine or recreational drug, or after the ingestion of food that contains an endocannabinoid precursor such as docosahexaenoic acid. Moreover, it will be important when considering possible strategies for developing medicines that exploit an "autoprotective" effect of an endogenously released endocannabinoid (Pertwee 2014) to establish the extent to which this effect is CB_1 or CB_2 receptor-mediated. Thus, one such strategy, to increase the endogenous concentration of one or more released endocannabinoid, for example, with an inhibitor of its metabolism (Pertwee 2014), would be expected to increase endocannabinoid-induced targeting of all of the receptors and channels at which such a concentration increase occurs. This strategy would, in addition, be expected to produce an increase in the concentrations of any pharmacologically active endogenously released non-cannabinoid receptor ligands whose metabolism is reduced by this same inhibitor. In contrast, another strategy, to boost the strength with which endogenously released endocannabinoids activate cannabinoid receptors by administering a CB_1- or CB_2-selective PAM, would of course be expected only to augment autoprotective effects that are CB_1- or CB_2-mediated. Importantly, since PAMs of some receptors can display "probe selectivity", it may also prove both possible and therapeutically beneficial to develop a CB_1- or CB_2-selective PAM that does not enhance CB_1 or CB_2 receptor activation by all endocannabinoids, but instead, selectively enhances such activation only by endocannabinoids that produce a particular sought-after CB_1- or CB_2-mediated autoprotective effect.

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Biosynthesis and Fate of Endocannabinoids

Maria Grazia Cascio and Pietro Marini

Contents

Abstract

Since the discovery of the two cannabinoid receptors, CB_1 and CB_2, several molecules, commonly defined as endocannabinoids, able to bind to and functionally activate these receptors, have been discovered and characterized. Although the general thought was that the endocannabinoids were mainly derivatives of the n-6 fatty acid arachidonic acid, recent data have shown that also derivatives (ethanolamides) of n-3 fatty acids may be classified as endocannabinoids. Whether the n-3 endocannabinoids follow the same biosynthetic and metabolic routes of the n-6 endocannabinoids is not yet clear and so warrants further investigation. In this review, we describe the primary biosynthetic and metabolic pathways for the two well-established endocannabinoids, anandamide and 2-arachidonoylglycerol.

Keywords

2-arachidonoylglycerol • Anandamide • Biosynthesis • Degradation • Endocannabinoids • Uptake

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_2

Abbreviations

2-AG	2-Arachidonoylglycerol
2-AGE	2-Arachidonoylglyceryl ether
AA	Arachidonic acid
Abh4	Alpha/beta hydrolase 4
ABHD	Alpha/beta hydrolase domain
AEA	Anandamide
Asp	Aspartic acid
CB	Cannabinoid
Cis	Cysteine
COX-2	Cyclooxygenase-2
DAG	Diacylglycerol
DGL	Diacylglycerol lipase
DHA	Docosahexaenoic acid
DHEA	Docosahexaenoyl-ethanolamide
DTT	Dithiothreitol
EMT	Endocannabinoid membrane transporter
EPA	Eicosapentaenoic acid
EPEA	Eicosapentaenoyl-ethanolamide
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
FLAT	FAAH-like anandamide transporter
GpAEA	Glycerophospho-arachidonoylethanolamide
GPR	G-protein coupled receptor
GSH	Glutathione
HEK	Human embryonic kidney
His	Histidine
Hsp	Heat shock protein
LOX	Lipoxygenase
Lys	Lysine
MAFP	Methylarachidonoylfluorophosphonate
MGL	Monoacylglycerol lipase
NAAA	*N*-acylethanolamine-selective acid amidase
NADA	*N*-arachidonoyldopamine
NAE	*N*-acyl-ethanolamine
NAGly	*N*-arachidonoylglycine
NAM	*N*-arachidonoylmaleimide
NAPE	*N*-acylphosphatidylethanolamine
NArS	*N*-arachidonoylserine
NAT	*N*-acyltransferase
OEA	Oleoyethanolamide
OLDA	*N*-oleoyl dopamine

pAEA	Phosphoanandamide
PE	Phosphatidylethanolamine
PEA	Palmitoylethanolamide
PHARC	Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, cataract
PL	Phospholipase
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
SEA	Stearoylethanolamide
Ser	Serine
TRPM	Transient receptor potential melastatin
TRPV	Transient receptor potential vanilloid

1 Endocannabinoids and Endocannabinoid-Like Compounds

The discovery, in 1988, of a high-affinity, stereoselective and pharmacologically distinct cannabinoid receptor in rat brain tissue (Devane et al. 1988), led to a continuous search for natural endogenous ligands. Since then, several molecules have been identified and collectively named as "endocannabinoids". Endocannabinoids are defined as derivatives (amides, esters and ethers) of a long-chain polyunsaturated fatty acid (PUFA), mainly arachidonic acid (AA), capable of binding and functionally activating the cannabinoid receptors (Di Marzo et al. 2004). Although anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids (an *N*-acylethanolamine and a monoacylglycerol, respectively), other endogenous compounds that may also bind to the cannabinoid receptors have been discovered and suggested to be endocannabinoids: *N*-dihomo-γ-linolenoyl ethanolamine and *N*-oleoyl dopamine (OLDA) (Pertwee 2005), 2-arachidonoylglycerol ether (noladin ether, 2-AGE) (Hanus et al. 2001), *O*-arachidonoylethanolamine (virodhamine) (Porter et al. 2002), and *N*-arachidonoyldopamine (NADA) (Huang et al. 2002). It is now well established that the two most studied endocannabinoids (AEA and 2-AG) do not interact only with cannabinoid CB_1 and CB_2 receptors, and exhibit instead a degree of promiscuity that applies also to the less-studied arachidonic acid-derived endocannabinoids (Zygmunt et al. 1999; Hanus et al. 2001; Huang et al. 2002; Porter et al. 2002; Rozenfeld and Devi 2008; den Boon et al. 2012). Anandamide (AEA), from the Sanskrit word *ananda*, which means *bliss*, was the first endogenous cannabinoid identified (Devane et al. 1992; Hanus 2007). This is the ethanolamide of arachidonic acid and behaves as a partial agonist at both cannabinoid CB_1 and CB_2 receptors (Pertwee et al. 2010). Interestingly, it is now well established that AEA possesses an ability to interact also with other receptors, such as the transient receptor potential vanilloid 1 (TRPV1) (Zygmunt et al. 1999) and the peroxisome proliferator-activated receptor (PPAR) family (O'Sullivan 2007). Indeed, some of the effects of AEA are non-CB_1/non-CB_2 receptor mediated (Breivogel et al. 2001;

Monory et al. 2002). 2-Arachidonoylglycerol (2-AG) is the arachidonate ester of glycerol that was isolated from peripheral tissues. This molecule can activate both CB_1 and CB_2 receptors with similar potency and efficacy (Mechoulam et al. 1995; Sugiura et al. 1995) as well as γ-aminobutyric acid receptors (Sigel et al. 2011). 2-Arachidonoy-glyceryl ether (noladin ether) binds to CB_1 receptors, and very weakly to CB_2 receptors, and also affects AEA uptake (Fezza et al. 2002; Páldyová et al. 2008). Recently, its classification as an endocannabinoid has been questioned because of its very low concentration in the brain (Oka et al. 2003). Virodhamine (from the Sanskrit word *virodha*, which means *opposite*) is the ester of arachidonic acid, and it has been reported to behave as a full agonist at CB_2 receptors and as both a partial agonist/antagonist at CB_1 receptors and a weak inhibitor of AEA uptake (Porter et al. 2002). This molecule can also interact with PPAR-α receptors (Sun et al. 2006) and GPR55 receptors (Sharir et al. 2012). *N*-arachidonoyl-dopamine (NADA), like AEA, behaves both as an endovanilloid and an endocannabinoid (Bisogno et al. 2000; Huang et al. 2002). It also interacts with PPAR-γ receptors (O'Sullivan 2007) and can antagonize the melastatin type-8 (TRPM8) cation channel (De Petrocellis et al. 2007).

We recently discovered that in addition to n-6 long-chain PUFA endocannabinoids, the ethanolamides of two n-3 fatty acids derived mainly from fish oils in the human diet, DHA (C22:6) and EPA (C20:5), should also be classified as endocannabinoids (Brown et al. 2010; Cascio 2013). These n-3 fatty acid ethanolamides are docosahexaenoyl-ethanolamide (DHEA) and eicosapentaenoyl-ethanolamide (EPEA), both of which bind to and partially activate CB_1 and CB_2 receptors and are produced both in vivo and in vitro after administering fish oil or individual n-3 long-chain PUFA (Sugiura et al. 1996; Bisogno et al. 1999; Berger et al. 2001; Brown et al. 2010, 2011; Maccarrone et al. 2010; Cascio 2013). These n-3 endocannabinoids show anti-inflammatory properties in macrophages and adipocytes (Fezza et al. 2014) and can inhibit cell growth in breast cancer by triggering autophagy via PPAR-γ receptors (Fezza et al. 2014). Interestingly, we recently reported evidence that both DHEA and EPEA possess cannabinoid receptor-dependent and -independent anti-proliferative effects in androgen receptor-positive and -negative prostate cancer cell lines (Brown et al. 2010).

Finally, endocannabinoids are produced together with cannabinoid receptor-inactive saturated and mono- or di-unsaturated compounds that are defined as endocannabinoid-like compounds. These compounds have been reported to exert their cannabimimetic effects by acting as "entourage molecules" that prevent endocannabinoids being degraded by specific metabolic enzymes (Cascio 2013). Palmitoylethanolamide (PEA) possesses both anti-inflammatory and analgesic activity, likely mediated by the TRPV1 and PPAR-α receptors (Costa et al. 2008; Ho et al. 2008; Di Cesare et al. 2013; Esposito et al. 2014), and it also interacts with GPR55 receptors (Moriconi et al. 2010). Stearoylethanolamide (SEA) produces anti-inflammatory, immunomodulatory as well as anorexic effects (Maccarrone et al. 2002; Dalle Carbonare et al. 2008; Ghafouri et al. 2013). Oleoyethanolamide (OEA) can activate both GPR119 and GPR55 receptors (Overton et al. 2008; Moriconi et al. 2010), and it regulates food intake in rodents by a mechanism that

involves the activation of PPAR-α receptors (Rodríguez de Fonseca 2004). Oleamide is an unsaturated fatty acid amide isolated from the cerebrospinal fluid of sleep-deprived cats (Cravatt et al. 1995) which behaves as a full cannabinoid CB_1 receptor agonist (Leggett et al. 2004). Other compounds that have been recently classified as endocannabinoids-like compounds are *N*-arachidonoylglycine (NAGly) and *N*-arachidonoylserine (NArS). NAGly interacts with both GPR18 and GPR92 receptors (Kohno et al. 2006; Oh et al. 2008; Fezza et al. 2014; McHugh et al. 2014) and behaves as a FAAH inhibitor (Cascio et al. 2004). The chemical structures of the endocannabinoids can be found in this volume in Pertwee "Endocannabinoids and Their Pharmacological Actions".

2 Biosynthesis of the Endocannabinoids

It is generally accepted that endocannabinoids are not stored in cells awaiting release, but are rather synthesized on demand in a Ca^{2+}-dependent manner in response to physiological and pathological stimuli (Di Marzo and Deutsch 1998). However, recent data suggest that AEA can also be stored inside the cell (Oddi et al. 2008). AEA as well as other *N*-acyl-ethanolamines were initially considered as terminal products of *post mortem* tissue degradation, and their physiological role remained controversial until the identification of their biosynthetic and metabolic pathways (Piomelli 2014).

The principal pathway of AEA biosynthesis includes a first step, catalysed by a calcium-dependent *N*-acyltransferase (NAT), in which an acyl chain is transferred from the *sn*-1 position of a glycerophospholipid to the amino group of the hydroxyethyl moiety of phosphatidylethanolamine (PE), and a second step in which the generated *N*-acylphosphatidylethanolamine (NAPE) is hydrolysed to NAE and phosphatidic acid, through a reaction catalysed by a phosphodiesterase of the phospholipase D-type (NAPE-PLD) (Fig. 1). NAPE-PLD, that is chemically and enzymatically distinct from other known PLDs, is a member of the β-lactamase family of zinc-metal hydrolases, is highly conserved in mouse, rat and human, is stimulated by calcium, is highly expressed in the brain as well as in kidney, spleen, lung, heart and liver and is involved in the formation of other, cannabinoid-receptor inactive, *N*-acyl-ethanolamines (C16:0, C18:0 and C18:1) (Petersen and Hansen 1999; Ueda et al. 2001a; Liu et al. 2002; Okamoto et al. 2004). Interestingly, studies performed using NAPE-PLD knockout mice suggested that while an increase in endogenous levels of NAPEs with saturated and monounsaturated *N*-acyl chains was observed, few or no changes were observed in the levels of polyunsaturated NAPEs and NAEs, thus suggesting the existence of alternative AEA biosynthetic pathways (Brown et al. 2013; Cascio 2013; Fonseca et al. 2013). There is evidence too that: (1) AEA is formed from *N*-acyl-lysophosphatidylethanolamine by a lysophospholipase-D-enzyme (lyso-PLD) (Sun et al. 2004) (Fig. 1); (2) AEA is also formed in a pathway in which a crucial role is played by an additional enzyme, α/β-hydrolase 4 (Abh4), which can act on either NAPE or lyso-NAPE to generate glycerophospho-arachidonoylethanolamide (GpAEA), that is subsequently

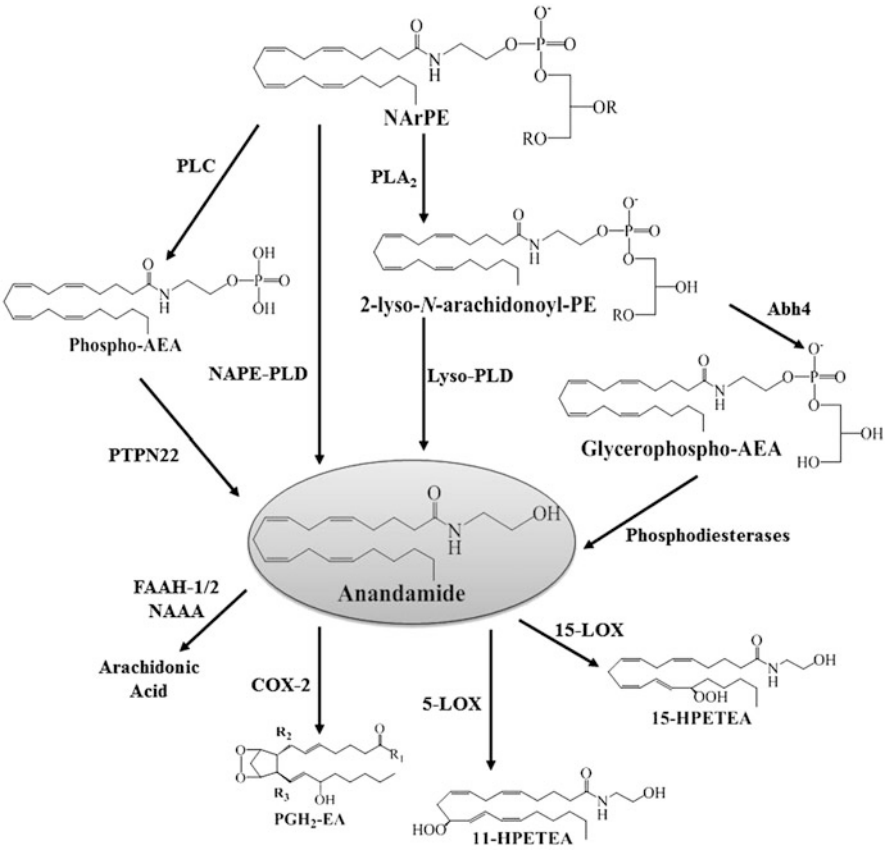

Fig. 1 Schematic representation of anandamide biosynthesis and degradation. *NArPE* *N*-arachidonoylphosphatidyl-ethanolamine, *PLC* phospholipase C, *PTPN22* protein tyrosine phosphatase, *PLA*$_2$ phospholipase A$_2$, *PE* phosphatidyl-ethanolamine, *PLD* phospholipase D, *Abh4* α/β-hydrolase 4, *PG* prostaglandin, *HPETEA* hydroxyperoxyeicosatetraenoylethanolamide, *LOX* lypoxygenase, *COX* cyclooxygenase, *FAAH* fatty acid amide hydrolase, *NAAA* *N*-acylethanolamine-hydrolysing acid amidase, *R*^{1} ethanolamine

converted to AEA in the presence of a phosphodiesterase (Simon and Cravatt 2006) (Fig. 1) and finally (3) that NAPE can also be hydrolysed, by phospholipase C, to phosphoanandamide (pAEA) which, in turn, is dephosphorylated by phosphatases to AEA (Liu et al. 2006) (Fig. 1). Interestingly, an alternative biosynthetic pathway for AEA might also exist that involves direct condensation of free arachidonic acid and ethanolamine, catalysed by an AEA synthase. However, this pathway requires high "non-physiological" concentrations of both substrates (Sugiura et al. 1996; Ueda et al. 1996).

For 2-AG, the most accepted biosynthetic pathway is the hydrolysis of membrane phospholipids that is catalysed by phospholipase C (PLC) and that produces 1,2-diacylglycerol (DAG), which in turn is converted to 2-AG by diacylglycerol

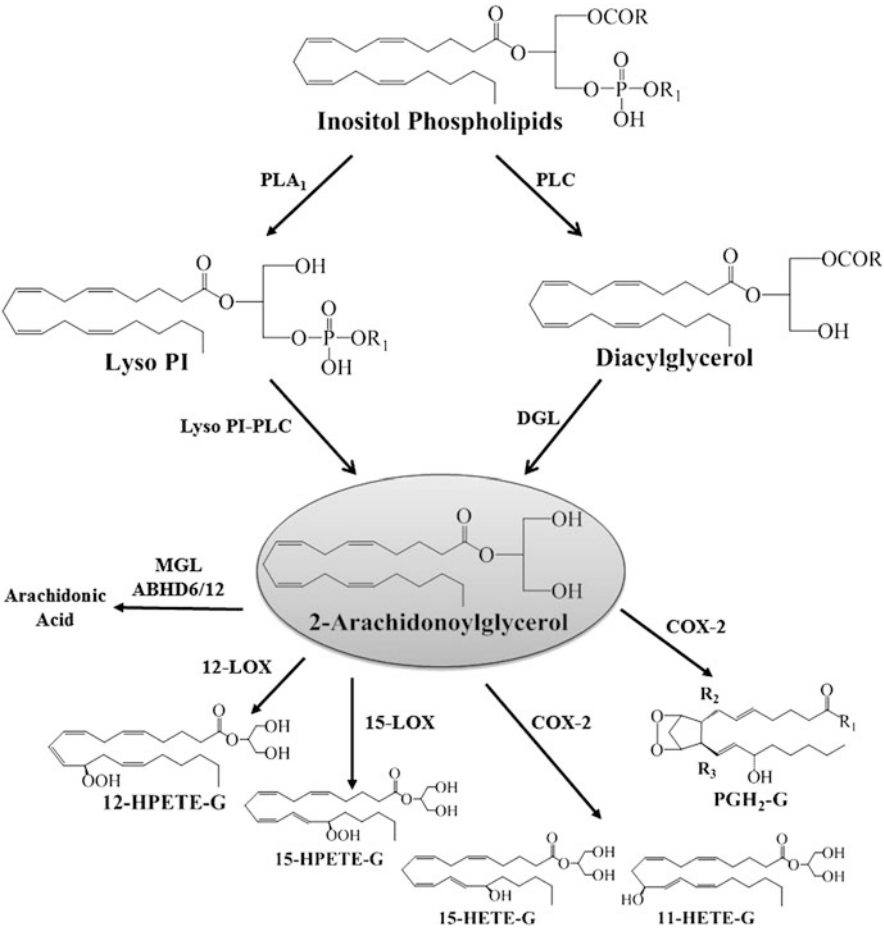

Fig. 2 Main pathways for 2-arachidonoylglycerol biosynthesis and degradation. *PLC* phospholipase C, *PLA1* phospholipase A_1, *PI* phospatidyl-inositol, *DGL* diacylglycerol lipase, *HETE-G* hydroxyeicosatetraenoyl-glycerol, *HPETE-G* hydroxyperoxyeicosatetraenoyl-glycerol, *LOX* lypoxygenase, *COX* cyclooxygenase, *MGL* monoacylglycerol lipase, *ABHD* α/β-hydrolase domain, *R1* glycerol

lipase (DGL) (Bisogno et al. 2003) (Fig. 2). DGL exists in two closely related forms designated α and β, that are both active at pH 7, both stimulated by calcium and glutathione (GSH) and both inhibited by inhibitors of Ser/Cis-hydrolases, such as *p*-hydroxy-benzoate-mercuric and $HgCl_2$ but not by phenylmethylsulphonyl fluoride (Bisogno et al. 2003). Both enzymes are also inhibited by RHC80267, which is able to block the formation of 2-AG by intact cells (Bisogno et al. 2003). Pharmacological studies have revealed that during neuronal development, localization of DGLα and DGLβ changes from pre- to post-synaptic elements, i.e. from axonal tracts in the embryo to dendritic fields in the adult, suggesting a different need for 2-AG

synthesis from the pre- to the post-synaptic compartment during brain development (Bisogno et al. 2003; Williams et al. 2003). Furthermore, there is evidence too that DGLα plays an essential role in the regulation of retrograde synaptic plasticity and neurogenesis (Gao et al. 2010; Tanimura et al. 2010; Savinainen et al. 2012). Like AEA, 2-AG can also be synthesized via other pathways. However, the physiological importance of these proposed pathways is not yet clear.

3 Uptake of the Endocannabinoids: Proposed Mechanisms

Once released into the extracellular space, endocannabinoids exert the majority of their effects by acting, as retrograde messengers, at CB_1 cannabinoid receptors present on the surface of presynaptic nerve terminals (Piomelli 2014). So far, it is not clear how the endocannabinoids access their metabolic enzymes. Indeed, while monoacylglycerol lipase (MGL, the main metabolic enzyme of 2-AG) is localized pre-synaptically, fatty acid amide hydrolase (FAAH, the main metabolic enzyme of AEA) is localized post-synaptically, thus at a certain distance from the site of action of AEA (Piomelli 2014). To explain the mechanism(s) by which AEA would be taken up by cells, several interesting hypotheses have been proposed (Fowler 2012, 2013). Briefly, AEA is a lipophilic molecule and as such it could easily diffuse through the cell membrane. However, a simple diffusion through the membrane would cease once the equilibrium in the AEA gradient between the extracellular and intracellular environment is reached, unless this equilibrium is prevented by intracellular metabolism induced by FAAH (Fowler 2012, 2013). However, although FAAH may, of course, influence the uptake of AEA (at least the speed with which this process takes place), the uptake is clearly distinct from FAAH. Indeed, (1) compounds able to selectively inhibit the cellular uptake of AEA, but not FAAH, have been identified (De Petrocellis et al. 2000; Di Marzo et al. 2001, 2002; López-Rodríguez et al. 2001; Ortar et al. 2003); (2) inhibitors of FAAH increase, and inhibitors of AEA uptake decrease, the accumulation of AEA within cells (Kathuria et al. 2003); (3) cells that do not express FAAH rapidly internalize AEA (Di Marzo et al. 1999; Deutsch et al. 2001); (4) NADA and noladin, although they are not FAAH substrates, are rapidly internalized by cells (Fezza et al. 2002; Huang et al. 2002) and (5) a saturable AEA accumulation was observed in synaptosomes and cells prepared from genetically modified mice that do not express FAAH (Fegley et al. 2004; Ligresti et al. 2004).

In addition, since endocannabinoid uptake is rapid, temperature-dependent, selective for anandamide over other acylethanolamides and saturable, the hypothesis that AEA uptake may occur through a facilitated transport mechanism has also been proposed (Di Marzo et al. 1994; Hillard and Jarrahian 2000; Fezza et al. 2008). Unfortunately, the protein responsible for this transport, better known as "Endocannabinoid Membrane Transporter" or EMT, has not yet been cloned and its existence is supported only by indirect evidence. More recent studies have shown the existence of carrier proteins that would facilitate diffusion of AEA through the plasma membranes. Examples are the fatty acid binding proteins FABP5 and FABP7, but not FABP3 (Kaczocha et al. 2009), the heat shock protein

70 (Hsp70) and albumin (Oddi et al. 2009), and the most recently identified FAAH-like anandamide transporter (FLAT). FLAT is a dimeric protein lacking the membrane-anchoring domain of the FAAH dimer, and its overexpression in HEK-293 cells increases AEA uptake (Fu et al. 2012). Other hypotheses have also been proposed (Oddi et al. 2008; Di Pasquale et al. 2009; Fowler 2013).

Several synthetic compounds that are able to inhibit the cellular uptake of AEA have been developed so far, some examples being AM404, VDM11, UCM707, OMDM1, OMDM2 and LY21832110 (Pertwee 2014). These compounds have been reported to possess, at least in animals, promising pharmacological properties for the treatment of cancer, pain, multiple sclerosis, Parkinson's disease, Huntington disease and anxiety (Pertwee 2014). Interestingly, AM404 has also been reported to be effective against nicotine-seeking behaviour and obsessive compulsive disorders (Pertwee 2014).

4 Degradation of the Endocannabinoids

Two main metabolic pathways have been identified so far: one hydrolytic and the other oxidative (Figs. 1 and 2). AEA is mainly hydrolyzed by FAAH (Cravatt et al. 1996; Giang and Cravatt 1997; Bracey et al. 2002), while 2-AG is mainly hydrolyzed by MGL (Dinh et al. 2002) and also by FAAH. In addition to these two enzymes, an *N*-acylethanolamine-selective acid amidase (NAAA) (Ueda et al. 1999) and, more recently, a second FAAH (FAAH-2) (Wei et al. 2006), as well as two other enzymes ABHD6 and ABHD12, (Blankman et al. 2007) have been reported to participate in the degradation of several endocannabinoids. Both AEA and 2-AG can also be degraded by enzymes of the arachidonate cascade, such as cyclooxygenase-2 (COX-2), lipoxygenases (LOXs) as well as cytochrome P450 enzymes, to produce the corresponding hydroxy- (in the case of lipoxygenases) and epoxy- (in the case of cytochrome P450 monooxidases) derivatives or to produce prostamides and prostaglandin glycerol esters (in the case of cyclooxygenases and prostaglandin synthases) (Piscitelli and Di Marzo 2012) (Figs. 1 and 2). While both hydroxy- and epoxy-endocannabinoids have been reported to act at both cannabinoid CB_1 and CB_2 receptors as well as at the vanilloid receptors, TRPV1 (hydroxy-endocannabinoids) and TRPV4 (epoxy-endocannabinoids), both prostamides and prostaglandin-glycerol esters are inactive at cannabinoid receptors. It has been suggested that they act at new, not yet identified, receptors (Piscitelli and Di Marzo 2012). Below, we report a brief description of the enzymes involved in the hydrolysis of endocannabinoids.

4.1 FAAH and NAAA

FAAH, which was first cloned by Cravatt et al. (1996), is an integral membrane protein widely distributed in various tissues of rat (Desarnaud et al. 1995; Cravatt et al. 1996; Katayama et al. 1997), mouse (Sun et al. 2005), and human (Giang and

Cravatt 1997). This enzyme, active at pH 8–10, is mainly localized on microsomal membranes and contains 597 amino acids, with a short "amidase" sequence enriched in glycine and serine residues. The isolation of FAAH was made possible by the previous development of potent transition state inhibitors, one of which was used to carry out affinity chromatography purification (Cravatt et al. 1996; Petrosino and Di Marzo 2010). A covalent inhibitor, instead, was used to facilitate the formation of crystals of a slightly modified, soluble form of FAAH and to obtain its structure by X-ray crystallography (Bracey et al. 2002; Petrosino and Di Marzo 2010). FAAH catalytic triad is composed of Ser-Ser-Lys, in which Ser241 plays a critical role as both acid and base in the hydrolytic cycle, whereas Lys142 is the activator of Ser241, and Ser217 participates in the catalytic mechanism of FAAH by facilitating the nucleophile attack and the exit of the leaving group (Petrosino and Di Marzo 2010). Importantly, it has been reported that the promoter region of the FAAH gene is up-regulated by progesterone and leptin and down-regulated by estrogens and glucocorticoids (Puffenbarger et al. 2001; Waleh et al. 2002; Maccarrone et al. 2003a, b). Ergetova and co-workers (Egertová et al. 1998) analysed the distribution of FAAH in rat brain and compared its cellular localization with CB_1-type cannabinoid receptors using immunocytochemistry. High concentrations of FAAH were detected in the cerebellum, hippocampus and neocortex, which are enriched with cannabinoid receptors. Immunocytochemical analysis of these brain regions revealed a complementary pattern of FAAH and CB_1 expression with CB_1 immunoreactivity occurring in fibres surrounding FAAH-immunoreactive cell bodies and/or dendrites (Egertová et al. 1998). In the cerebellum, FAAH was expressed in the cell bodies of Purkinje cells and CB_1 was expressed in the axons of granule cells and basket cells, neurons which are presynaptic to Purkinje cells (Egertová et al. 1998).

FAAH is also able to metabolize other fatty acid amides such as *N*-arachidonoyldopamine and a large number of mono-unsaturated and saturated compounds (Ueda 2002; Fegley et al. 2005; Ho and Hillard 2005; Lo Verme et al. 2005). Examples are PEA (De Petrocellis et al. 2001; Ueda et al. 2001b; Ueda 2002; Lo Verme et al. 2005), oleoylethanolamide, *N*-arachidonoylserine and *N*-arachidonoylglycine, the latter two of which have also been reported to be FAAH inhibitors (Sheskin et al. 1997; Bradshaw and Walker 2005; Ho and Hillard 2005). Recently, a second isoform of FAAH, FAAH-2, has been identified. It shows ~20 % sequence similarity with FAAH at the amino acid level and is expressed in several species, including human, primates, frog, chicken, pufferfish and zebrafish, but not in rodents (Wei et al. 2006). FAAH-1 and FAAH-2 are located on the cytosolic and luminal sides of intracellular membranes, respectively. Both FAAH enzymes have distinct tissue distribution. Indeed, FAAH-2 was detected in the heart and ovary, but not in the brain, small intestine or testis, which are known to express FAAH-1. However, FAAH-1 and FAAH-2 were both detected in the prostate, lung, kidney and liver (Wei et al. 2006). FAAH is also involved in the hydrolysis of 2-AG (Di Marzo and Deutsch 1998), although it has been observed that levels of 2-AG, unlike those of AEA, are not increased in FAAH-knockout mice (Lichtman et al. 2002). Interestingly, recent reports have shown that FAAH is involved in the production of

symptoms of a variety of disorders and that FAAH inhibitors may be effective at ameliorating acute, inflammatory, visceral and neuropathic pain as well as osteoarthritic pain and hyperalgesia induced by bladder inflammation (Pertwee 2014). Importantly, unlike direct CB_1 agonists, FAAH inhibitors produce antinociception in mice at doses that do not induce hypomotility, hypothermia, catalepsy and hyperphagia or signs of physical or psychological dependence (Pertwee 2014). A few examples of FAAH inhibitors are URB597, OL135, O-1887, URB532, AM374 (palmitylsulphonyl fluoride), *N*-arachidonoylglycine and *N*-arachidonoyl serotonin, JNJ1661010 and CAY10401, AM3506 and AM5206, ST4070, PF3845 and PF04457845 (Pertwee 2014).

One other enzyme involved in AEA hydrolysis is NAAA. This enzyme is a cysteine hydrolase belonging to the *N*-terminal nucleophile hydrolase superfamily, is present in cellular lysosomes or in the Golgi apparatus of cells, is active only at acidic pH and shows higher selectivity for PEA than for AEA (Brown et al. 2013; Ueda et al. 2013). Millimolar concentrations of dithiothreitol (DTT) as well as non-ionic detergents such as Triton X-100 and Nonidet P-40 are required to promote its full activity (Brown et al. 2013; Ueda et al. 2013). NAAA is highly expressed in a number of blood cell lines, as well as in macrophages in various rodent tissues. In humans, NAAA mRNA is expressed most abundantly in prostate followed by leukocytes, liver, spleen, kidney and pancreas (Ueda et al. 2010). Prostate cancer cell lines like PC3, LNCaP and DU-145 also express high levels of NAAA (Ueda et al. 2010). Interestingly, due to its selectivity towards PEA, selective NAAA inhibitors that can increase local levels of endogenous PEA are expected to be anti-inflammatory and analgesic drugs (Petrosino et al. 2010; Ueda et al. 2013).

4.2 MGL, ABHD6 and ABHD12

MGL is a serine hydrolase responsible for about 85 % of the 2-AG hydrolyzing activity of mouse brain (Blankman et al. 2007). This enzyme of about 303 amino acids is present in both membrane and cytosolic subcellular fractions and can recognize other unsaturated monoacylglycerols also as substrates, which in some cases compete with 2-AG inactivation (Ben-Shabat et al. 1998; Di Marzo and Deutsch 1998). MGL is sensitive to sulphydryl-specific reagents, and comparison models strongly suggest that cysteine residues present near its binding site play a role in the catalytic mechanism (Saario et al. 2005), although the catalytic triad of this enzyme also involves Ser122, Asp239 and His269 (Karlsson et al. 1997). The distribution of MGL was studied in rat, and it was shown to be ubiquitous (Karlsson et al. 1997). Specifically, MGL mRNA was reported to be present in adrenal gland, heart, adipose tissue, kidney, ovary, testis, spleen, lung, liver, skeletal muscle and brain (particularly in hippocampus, cortex, thalamus and cerebellum, where CB_1 receptors are highly expressed) (Dinh et al. 2002). Ultrastructural localization studies show that MGL is mainly pre-synaptic and often co-localizes with CB_1 receptors in the axon terminals (Savinainen et al. 2012). The complimentary

localization in the brain for MGL and FAAH, pre-synaptic and post-synaptic, respectively, has suggested different roles for the two main endocannabinoids in the central nervous system (Gulyas et al. 2004). Several MGL inhibitors have been developed so far. Methylarachidonoylfluorophosphonate (MAFP) inhibits MGL irreversibly but lacks selectivity, since it inhibits most metabolic serine hydrolases (Saario et al. 2004; Savinainen et al. 2010, 2012). *N*-arachidonoylmaleimide (NAM) selectively, but only partially (85 %), inhibits MGL (Saario et al. 2005; Blankman et al. 2007; Savinainen et al. 2012). Other MGL inhibitors include the non-competitive/irreversible inhibitors, URB602 and JZL184, and the reversible inhibitor, OMDM169 (Petrosino and Di Marzo 2010). Like FAAH inhibitors, MGL inhibitors have been found to have potential therapeutic applications, as indicated, for example, by results obtained from experiments using animal models of acute, visceral, inflammatory, neuropathic or bone cancer pain (Pertwee 2014). Interestingly, MGL inhibitors have also been reported to be efficacious against signs of breast, ovarian, skin and prostate cancer in animal models (Pertwee 2014). Recent data have shown that MGL inhibitors such as JZL184 and URB602 can protect neurons from β amyloid peptide-induced neurodegeneration and apoptosis, suggesting a therapeutic potential for the treatment of Alzheimer's disease (Pertwee 2014). Unfortunately, it has been reported that JZL184 shares the ability of direct CB_1 agonists to induce both physical and psychological dependence in mice as well as tolerance to their antinociceptive effects (Schlosburg et al. 2010; Ghosh et al. 2013; Pertwee 2014).

2-AG metabolism is also catalysed by two integral membrane proteins, α/β-hydrolase domain containing protein-6 (ABHD6) and -12 (ABHD12). Both enzymes belong to the α/β-hydrolase superfamily, with the postulated catalytic triad serine-aspartic acid-histidine (Savinainen et al. 2012). ABHD6, in neurones, is localized at sites of 2-AG generation, including post-synaptic dendrites of principal glutamatergic neurones as well as some GABAergic interneurons (Savinainen et al. 2012). ABHD12 is highly expressed in microglia, macrophages and osteoclasts (Fiskerstrand et al. 2010). Interestingly, it was observed that mutations in the ABHD12 gene are causally linked to a neurodegenerative disease called PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract) (Fiskerstrand et al. 2010; Savinainen et al. 2012).

5 Conclusions

Over the past 20 years, substantial progress has been made in the understanding of the endocannabinoid system. In particular, new molecules have been classified as endocannabinoids (e.g., the ethanolamides of two omega-3 fatty acids), and new targets other than cannabinoid CB_1 and CB_2 receptors have been identified and held accountable for some of the effects of the endocannabinoids. Moreover, substantial progress has also been made in the identification as well as in the characterization of the enzymes responsible for both the biosynthesis and the metabolism of the main endocannabinoids, AEA and 2-AG. It still remains to be established whether these

enzymes catalyse the formation or degradation of other, less studied, endocannabinoids. In addition, several molecules have been developed that are able to interact more or less selectively or more or less potently with enzymes or uptake processes of the endocannabinoid system. Many of these molecules, such as FAAH and MGL inhibitors as well as endocannabinoid uptake inhibitors, have been discovered, albeit only in animal models, to possess notable therapeutic potential for the treatment of diseases such as cancer, pain, neurodegenerative diseases and so on. Unfortunately, some of these molecules, such as MGL inhibitors, have also been shown to share the ability of direct CB_1 cannabinoid receptor agonists to cause physical and psychological dependence. This problem still needs to be overcome. Finally, as recently and elegantly discussed by Piomelli (Piomelli 2014), one important question about the endocannabinoids that still remains unresolved is how such lipophilic molecules are able to cover the distance between their site(s) of action and the site(s) of their enzymatic degradation. This distance is quite short for 2-AG, whose main metabolic enzyme (MGL) is localized presynaptically, and thus close to the pre-synaptic CB_1 receptors on which 2-AG acts, but longer for anandamide, which after acting on presynaptic CB_1 receptors must travel trans-synaptically in order to be metabolized by FAAH, which is primarily postsynaptic. The research on the understanding of the endocannabinoid system never ends.

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Distribution of the Endocannabinoid System in the Central Nervous System

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology
231, DOI 10.1007/978-3-319-20825-1_3

Abstract

The endocannabinoid system consists of endogenous cannabinoids (endocannabinoids), the enzymes that synthesize and degrade endocannabinoids, and the receptors that transduce the effects of endocannabinoids. Much of what we know about the function of endocannabinoids comes from studies that combine localization of endocannabinoid system components with physiological

or behavioral approaches. This review will focus on the localization of the best-known components of the endocannabinoid system for which the strongest anatomical evidence exists.

Keywords
CB_1 cannabinoid receptor • Immunocytochemistry • In situ hybridization

Abbreviations

2-AG	2-Arachidonoylglycerol
ABHD4/6/12	α/β-hydrolase domain 4/6/12
AEA or anandamide	*N*-arachidonoylethanolamine
BLA	Basolateral amygdala
CB_1 and CB_2	Cannabinoid receptor 1 and 2
CRIP1a	Cannabinoid receptor interacting protein 1a
DAGLα/β	Diacylglycerol lipase α/β
DRG	Dorsal root ganglion
FAAH	Fatty acid amide hydrolase
GDE1	Glycerophosphodiesterase 1
M1	Muscarinic cholinergic receptor
MAGL	Monoacylglycerol lipase
mGluR5	Metabotropic glutamate receptor 5
MSNs	Striatal medium spiny neurons
NAAA	*N*-acylethanolamine-hydrolyzing acid amidase
NAPE-PLD	*N*-acyl phosphatidylethanolamine phospholipase D
PAG	Periaqueductal gray
SN	Substantia nigra
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area

1 Introduction

1.1 Overview

Studies of the distribution of the protein components of the endocannabinoid system (ECS) are motivated by the notion that we can gain important insights into the function of the ECS by understanding the location of its component enzymes and receptors. This review has been written with that concept in mind, with an emphasis on studies that integrate function and location. Because of space limitations, this review will focus on components of the ECS in the CNS with the highest quality localization data. Thus, the emphasis will be on the cannabinoid

CB_1 receptors and on the enzymes *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (Di Marzo et al. 1994; Okamoto et al. 2004), fatty acid amide hydrolase (FAAH) (Cravatt et al. 2001), diacylglycerol lipase (DAGL) (Bisogno et al. 2003; Gao et al. 2010), monoacylglycerol lipase (MAGL) (Dinh et al. 2002, 2004), and α/ß-hydrolase domain 6 (ABHD6) (Blankman et al. 2007; Marrs et al. 2010). The interesting topic of neuronal expression of CB_2 receptors is complicated by the high inducibility of CB_2 in pathological conditions, the low levels of CB_2 compared to CB_1, the presence of CB_2 in microglia and endothelial cells, and nonspecific antibodies. Nonetheless, CB_2 may be present on a limited population of neurons with functional consequences, for example: (Viscomi et al. 2009; den Boon et al. 2012; Zhang et al. 2014). The interested reader can refer to the literature for additional details and consideration (Marsicano and Kuner 2008; Atwood and Mackie 2010). We will also focus on ECS component localization in the mature brain in this review. The ECS and its components are subject to dynamic regulation in the developing CNS, a topic that has been well covered in recent reviews (Harkany et al. 2008; Maccarrone et al. 2014).

A variety of techniques are available to determine protein localization in tissues. These include autoradiography (labeled ligands or GTPγS), in situ hybridization, and antibody-based techniques. Each of these techniques gives complementary information, which taken together, can enhance our understanding of ECS function—autoradiography requires high affinity binding of a probe to the protein, *in situ* hybridization detects mRNA (often useful for identifying cell types synthesizing a protein), and antibody-based techniques detect a (protein) epitope resembling the epitope that antibody was raised against. As antibody-based techniques are most commonly used for ECS localization, it is important to be aware of some of their caveats. The issue of spurious results from antibody studies has received considerable attention in the neuroscience community (Saper 2005; Rhodes and Trimmer 2006; Michel et al. 2009; Manning et al. 2012), though numerous examples of poor practice continue to be published. “Best practices” have been discussed and implemented by several journals (Saper 2005; Rhodes and Trimmer 2006; Manning et al. 2012) and are summarized in Table 1. As much as is possible, studies that have adhered to those practices will be emphasized in this review.

1.2 Cells Expressing Components of the ECS

While the ECS in neurons has received the most attention due to the prominent effects of endogenous and exogenous cannabinoids on neuronal function, it is important to appreciate that glial cells are a major component of the ECS, sometimes acting independently of neurons and sometimes in concert. Strong evidence supports the presence of CB_1 receptors in some astrocytes and microglia (Rodriguez et al. 2001; Stella 2010; Bosier et al. 2013), and these cells as well as oligodendrocytes are prodigious synthesizers and degraders of endocannabinoids (Walter et al. 2002; Stella 2009); however, their complement of enzymes vary somewhat from those in neurons (Marrs et al. 2010). In addition, the CNS

Table 1 Antibody controls for immunocytochemistry

Approach	Strength
Block with immunizing protein	Weak (block with immunizing protein is a necessary, but not sufficient condition to establish antibody specificity)
Lack of staining with preimmune serum	Weak (immune response can induce the expression of many serum proteins that can interact with extraneous epitopes)
Lack of staining in knockout (KO)	Strong (requires knockout; need to understand how the KO was made, articularly if the exon which the antibody was raised against remains; knockout studies should be conducted in parallel (identical tissue processing, incubation times, etc.) with experiments performed with tissue that expresses the genetically deleted protein
Identical staining with antibody directed against independent epitopes	Strong (for alternatively spliced proteins need to ascertain that the appropriate exons are expressed)
Lack of staining in knockdown	Strong (need independent verification of knockdown; stronger if knockdown from limited population of cells so "controls" are adjacent)
Detection of protein in transfected cells	Medium (demonstrates antibody can detect protein, but not necessarily in tissue; stronger if conducted in a mixed population of cells (expressing and non-expressing) and target protein is epitope-tagged to allow its unequivocal detection; difficult to apply if target protein is present in the cell line used)
Detection of appropriate band on western blot	Can be helpful correlation (however, the conformation of protein in fixed tissue and denatured gel is quite different; many examples where antibody won't detect a specific band in Western blots and works for immunocytochemistry and vice versa)
Correlation with *in situ* hybridization	Useful at the level of cell populations (assumes mRNA is translated to protein)
Correlation with function	Helpful (e.g., CB_1-mediated responses and CB_1 receptors detected; however can lead to circular reasoning)

vasculature also participates in endocannabinoid signaling (Gebremedhin et al. 1999; Schley et al. 2009; Zhang et al. 2009; Dowie et al. 2014).

1.3 Subcellular Localization of CB_1 Cannabinoid Receptors

Due to their prominent effects on presynaptic calcium channels and synaptic transmission, it is not surprising that high levels of CB_1 receptors are found on some presynaptic terminals and preterminal axon segments (Katona et al. 1999; Nyiri et al. 2005a). The ability of endocannabinoids to suppress spiking in low threshold spiking cortical interneurons and some pyramidal cells suggests that CB_1 receptors are also located on neuron somata (Marinelli et al. 2009). Within neurons,

CB_1 receptors are sometimes associated with specialized structures (e.g., multilamellar bodies (Katona et al. 1999)) that may be involved in their trafficking. A recent though not uncontroversial finding (Benard et al. 2012; Hebert-Chatelain et al. 2014a, b; vs. Morozov et al. 2013) is that CB_1 receptors are associated with some mitochondria, including mitochondria found in astrocytes, where they contribute to energy balance and may play a role in synaptic plasticity.

2 Retina

2.1 Receptors

Using the immunocytochemical approach, CB_1 receptors are found in subsets of amacrine cells and horizontal cells and densely expressed in the inner plexiform layer (Straiker et al. 1999a; Yazulla et al. 1999). CB_1 are also present in rod and cone photoreceptor terminals in a wide range of vertebrate retinas, including human (Straiker et al. 1999a, b; Hu et al. 2010), as well as in rod bipolar cells in rat retina (Yazulla et al. 1999).

2.2 Synthetic Enzymes

While the presence of synthetic enzymes for *N*-arachidonoylethanolamine (anandamide or AEA) has not yet been examined in retina, two isoforms of the major synthetic enzyme for 2-arachidonoylglycerol (2-AG), diacylglycerol lipase-α and -β (DAGLα/β) (Bisogno et al. 2003; Gao et al. 2010), were both found in the mouse retina. DAGLα appears in the two synaptic layers, the outer plexiform layer and inner plexiform layer, whereas DAGLβ immunoreactivity is limited to retinal blood vessels. Furthermore, DAGLα is present in postsynaptic type 1 OFF cone bipolar cells juxtaposed to CB_1-containing cone photoreceptor terminals (Hu et al. 2010). These findings suggest that retrograde 2-AG signaling exists at type 1 OFF bipolar cell-cone photoreceptor synapses, consisting of presynaptic CB_1 receptors and postsynaptic DAGLα.

2.3 Degradative Enzymes

Both degradative enzymes for anandamide and related *N*-acylethanolamines (NAEs), fatty acid amide hydrolase (FAAH) (Cravatt et al. 2001) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Guo et al. 2005; Tsuboi et al. 2005), are present in retina. FAAH immunoreactivity was first detected in large ganglion cells, in large dopaminergic amacrine cells, in the dendrites of star-burst amacrine cells, and in the somata of horizontal cells of the rat retina (Yazulla et al. 1999). In the mouse retina, FAAH is widely expressed in the inner segments, outer nuclear layer, ganglion cell layer, and in the axon terminals of photoreceptors in the outer plexiform layer, and also co-localizes with CB_1 in subpopulation of amacrine cells

in the inner nuclear layer and in cells in the ganglion cell layer (Hu et al. 2010). The most notable difference between mouse and rat FAAH staining is the absence of staining in horizontal cells of the mouse, possibly a function of species difference. Finally, NAAA immunoreactivity is limited to retinal pigment epithelium in the mouse retina (Hu et al. 2010).

Among five candidate degradative enzymes for 2-AG, monoacylglycerol lipase (MAGL) and α/β-hydrolase domain 6 (ABHD6) (Blankman et al. 2007) were found in the mouse retina. Similar to DAGLα, MAGL staining was found in the outer and inner plexiform layers and additionally in the ganglion cell layer (Hu et al. 2010). While MAGL is not co-localized with DAGLα in the inner plexiform layer, it is distal to DAGLα in the outer plexiform layer. MAGL is present in photoreceptor terminals, including the rod spherules and cone pedicles (Hu et al. 2010), where it is well positioned to break down 2-AG after retrograde release onto both rod and cone terminals.

On the other hand, ABHD6 is widely distributed in the inner plexiform layer, inner nuclear layer, and ganglion cell layer. ABHD6 is localized to the calbindin- and GAD67-positive amacrine cells in the inner nuclear layer and to the dendrites of ganglion or displaced amacrine cells in the proximal inner plexiform layer (Hu et al. 2010). The postsynaptic staining of ABHD6 suggests its potential role in the breakdown of extrasynaptic 2-AG that has diffused beyond its intended target synapses.

3 Cerebral Cortex

3.1 Neocortex

3.1.1 Receptors

CB_1 receptors are densely expressed in all regions of the cortex, with high levels found in cingulate gyrus, frontal cortex, secondary somatosensory, and motor cortex, reviewed in (Mackie 2005). An immunocytochemical study found a heterogeneous distribution of CB_1-immunoreactive axons across neocortex in macaque monkeys and humans. Most neocortical association regions, such as the prefrontal and cingulate cortex, contain a higher density of CB_1-immunoreactive axons compared to the primary motor and somatosensory cortices (Eggan and Lewis 2007). Furthermore, in many cortical regions, CB_1-immunoreactivity displays a distinctly laminar pattern of expression, corresponding to the cytoarchitectonic boundaries. Although the distribution of CB_1 immunoreactivity across monkey neocortical regions is broadly similar to that observed in the rat using immunocytochemical (Egertova and Elphick 2000; Hajos et al. 2000; Katona et al. 2001; Bodor et al. 2005) and autoradiographic (Herkenham et al. 1991) approaches, there are several species differences in the laminar distribution of CB_1-immunoreactive axons. For example, CB_1-immunoreactive axons were reported to be most densely expressed in layers 2–3 and 6 and least densely expressed in layer 4 of the rat frontal and cingulate cortex (Egertova and Elphick 2000). In contrast, the highest density of CB_1-immunoreactive axons is localized in layer 4 of the same regions in monkey

(Eggan and Lewis 2007). In the primary somatosensory cortex, CB_1-immunoreactive axons are most densely localized in layer 5A in rat (Bodor et al. 2005), whereas a relatively similar density of CB_1-immunoreactive axons exists in layers 2–3 and 5–6, and a sparse axonal labeling presents in layer 4 of monkey (Eggan and Lewis 2007). Despite these differences, the laminar distribution of CB_1-immunoreactivity is quite similar within primates. For example, both autoradiographic (Glass et al. 1997) and immunocytochemical (Eggan and Lewis 2007) methods yield similar laminar distribution of CB_1 receptors in human and monkey neocortex, respectively.

In forebrain, high levels of CB_1 receptors have been primarily found on large cholecystokinin (CKK)-containing basket interneurons, with lesser levels found in non-CCK-expressing neurons (Marsicano and Lutz 1999). For example, CB_1 immunoreactivity is absent in nonadapting multipolar interneurons, such as the parvalbumin or bi-tufted adapting somatostatin-expressing interneurons (Tsou et al. 1999; Bodor et al. 2005). Despite original studies suggesting lack of expression of CB_1 immunoreactivity in principal glutamatergic neurons (Tsou et al. 1998a; Freund et al. 2003), more sensitive in situ hybridization studies revealed low but detectable levels of CB_1 mRNA in the great majority of glutamatergic neurons in many cortical regions including neocortex (Monory et al. 2006). Moreover, a single-cell real-time polymerase chain reaction (qPCR) study revealed that at least 50% of neocortical glutamatergic pyramidal neurons contain CB_1 mRNA (Hill et al. 2007), which is consistent with functional data showing that the CB_1 receptor agonist WIN-55212-2 decreased the intracortical electrical stimulation-evoked excitatory postsynaptic currents (EPSCs) in a CB_1 antagonist-dependent fashion (Hill et al. 2007). In summary, CB_1 receptors are densely expressed in multiple cortical regions. While CB_1 expression is highest on CCK-positive interneurons, functionally important CB_1 receptors are present on multiple neuron populations.

3.1.2 Synthetic Enzymes

Studies examining the protein and mRNA distribution of the anandamide synthesizing enzymes, NAPE-PLD, in neocortex have found it to be widespread, but at relatively low levels (Egertova et al. 2008). On the other hand, in situ hybridization revealed that moderate levels of DAGLα and DAGLβ mRNA are expressed in mouse cerebral cortex (Yoshida et al. 2006). DAGLα is typically dendritic, as shown for a cultured cortical neuron in Fig. 1.

3.1.3 Degradative Enzymes

FAAH-immunoreactive neuronal somata and dendrites are present throughout the transitional and neocortical regions of the mouse cerebral cortex and are often surrounded by CB_1-immunoreactive fibers. Except for layer 1, FAAH immunostaining is evident in all cortical layers, especially in the large cells in layer 5 (Egertova et al. 2003).

In situ hybridization revealed that high levels of MAGL mRNA exist throughout the rat brain cortex, especially in layers 4, deep 5, and 6 (Dinh et al. 2002).

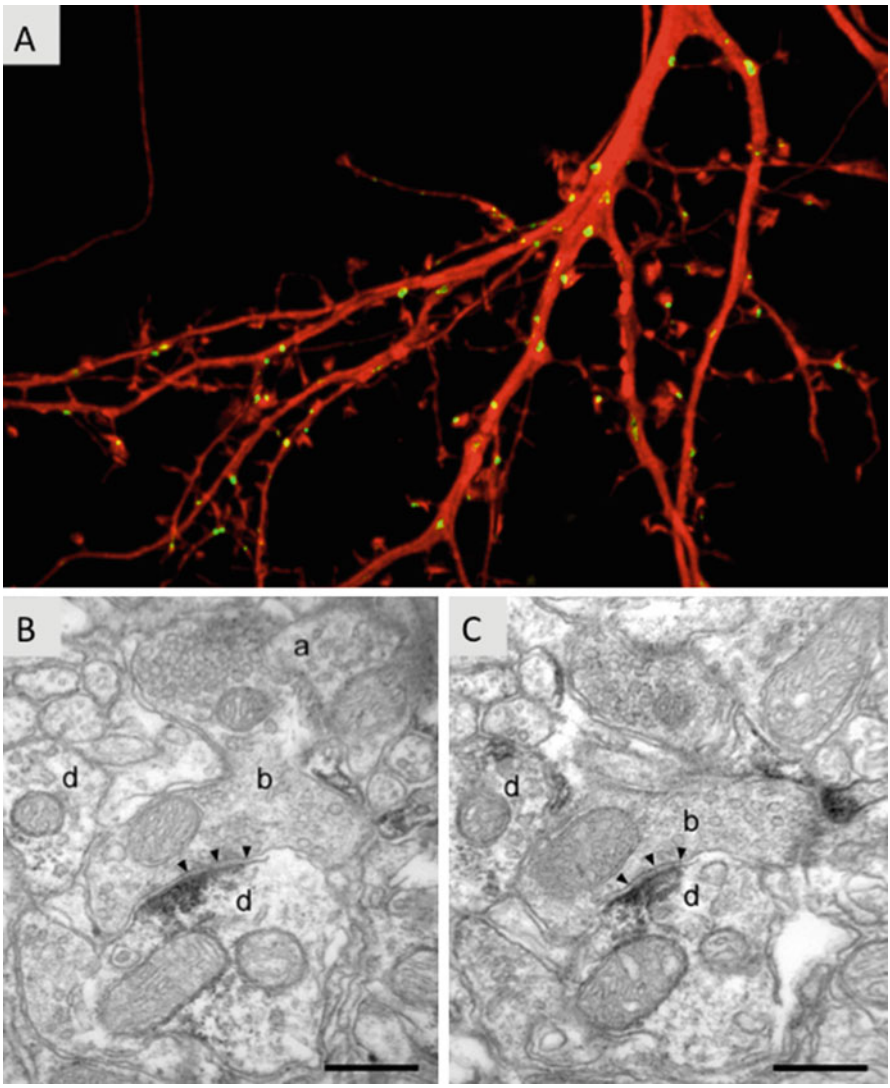

Fig. 1 Postsynaptic expression of diacylglycerol lipase alpha (DAGLα). (**A**) Dual immunofluorescent staining for DAGLα (*green*) and MAP2 (*red*). DAGLα as detected by a C-terminal antibody in a cultured neuron was found in close proximity to many dendritic spines. (**B** and **C**) Two consecutive ultrathin sections from mouse spinal cord demonstrate that the electron-dense reaction product representing DAGLα immunoreactivity (*arrowheads*) is present in the dendrite (*d*) close to the asymmetric postsynaptic density across from an excitatory terminal (*b*). Scale bar = 200 nm. Original figures provided by Barna Dudok and Istvan Katona (**A**) and Rita Nyilas and Istvan Katona (**B**, **C**)

In the mouse prefrontal cortex, ABHD6 immunoreactivity is predominantly localized to the postsynaptic dendritic spines, which is juxtaposed to the CB_1-positive presynaptic terminals (Marrs et al. 2010). Moreover, selective inhibition of ABHD6 allowed the induction of CB_1-mediated long-term depression by the subthreshold stimulation, suggesting this enzyme is a *bona fide* member of the endocannabinoid signaling system (Marrs et al. 2010).

3.2 Olfactory Areas (Olfactory Bulb, Piriform Cortex, Associated Regions)

3.2.1 Receptors

In the olfactory bulb, CB_1 receptors are highly expressed in the inner granular cell layer, followed by the inner plexiform layer, while less are expressed in the external plexiform layer, the glomerular layer, and the accessory olfactory bulb (Herkenham et al. 1991; Tsou et al. 1998a; Egertova and Elphick 2000). However, a detailed examination revealed that CB_1 receptor immunoreactivity is abundant in the periglomerular processes of GAD65-positive interneurons and the inner granular cell layer (A. Straiker, personal communication). Furthermore, CB_1 receptors are expressed uniformly by most neurons in the anterior olfactory nucleus and the anterior commissure, which connect the olfactory bulbs (Herkenham et al. 1991; Matsuda et al. 1993; Glass et al. 1997; Tsou et al. 1998a; Egertova and Elphick 2000). Moreover, most neurons in the piriform cortex contain CB_1 mRNA (Marsicano and Lutz 1999). Finally, CB_1 receptor immunoreactivity is present on dendritic processes in the olfactory epithelium of *Xenopus laevis* tadpoles, where it mediates cannabinoid modulation of odor-induced spike-associated currents in individual olfactory receptor neurons (Czesnik et al. 2007).

3.2.2 Synthetic Enzymes

NAPE-PLD mRNA expression has been detected in several olfactory areas. For example, NAPE-PLD mRNA is present in granule and periglomerular cells in the olfactory bulb and in neuronal cell body layers in the olfactory tubercle and the piriform cortex (Egertova et al. 2008). Interestingly, the immunostaining of NAPE-PLD is very intense in glomeruli of the accessory olfactory bulb and in the vomeronasal axons projecting into the accessory olfactory bulb (Egertova et al. 2008). Moderate expression of DAGLα and DAGLβ mRNA was found in mouse olfactory bulb by in situ hybridization (Yoshida et al. 2006).

3.2.3 Degradative Enzymes

Despite the overall low levels of CB_1 receptor expression in the olfactory bulb, FAAH immunoreactivity is intense, especially in fibers of the olfactory nerve and in the olfactory glomeruli, as well as in the somata and dendrites of mitral cells (Egertova et al. 2003). Moreover, FAAH-immunoreactive neuronal somata are evident in the majority of cortical olfactory regions receiving direct input from the olfactory bulb, including the anterior olfactory nucleus, the piriform cortex,

the tenia tecta, and the indusium griseum (Egertova et al. 2003). Importantly, in all of these regions, FAAH-immunoreactive postsynaptic neuronal somata are surrounded by a complementary network of CB_1-immunoreactive fibers, which supports the hypothesis that anandamide and other acyl amides may function as transynaptic signaling molecules (Egertova et al. 2000).

3.3 Hippocampal Formation

3.3.1 Receptors

The hippocampus is highly involved in cognitive functions such as the spatial and declarative learning and memory, thereby drawing much attention as a site of action of endogenous and exogenous cannabinoids due to their effects on memory. Early autoradiographic studies found very high levels of CB_1 receptors in all subfields of the hippocampus as well as the dentate gyrus (Herkenham et al. 1991; Jansen et al. 1992). In situ hybridization studies revealed that most CB_1 receptor expression arose from a restricted subset of interneurons (Matsuda et al. 1990, 1993; Mailleux and Vanderhaeghen 1992). Immunocytochemical studies showed high levels of CB_1 receptors on large CKK-positive basket and Schaffer collateral-associated interneurons in the hippocampal pyramidal cell layer, as well as the molecular layer and at the base of the granule cell layer in the dentate gyrus (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999; Egertova and Elphick 2000) (e.g., Fig. 2a), while few or no CB_1 receptors were found on parvalbumin-positive interneurons. Agonist activation of CB_1 receptors on these interneurons was found to decrease power in theta, gamma, and ripple oscillations in the hippocampus (Robbe et al. 2006). Actions of cannabinoids such as these appear to be widespread in cortical and subcortical circuits (Sales-Carbonell et al. 2013) and may contribute to the effects of cannabinoids on memory (Chen et al. 2003; Freund et al. 2003; Klausberger et al. 2005; Robbe et al. 2006).

Building on earlier studies that detected CB_1 mRNA, but not protein in CA1 and CA3 pyramidal neurons, studies in which CB_1 receptors were selectively deleted from either GABAergic or glutamatergic neurons allowed the conclusive anatomical identification of low levels of CB_1 protein in glutamatergic hippocampal pyramidal neurons (Marsicano et al. 2003; Lutz 2004). Indeed, several groups independently identified CB_1 protein in glutamatergic pyramidal neurons in the CA1 and CA3 regions (Degroot et al. 2006; Katona et al. 2006) (examples of CB_1 expression in hippocampal inhibitory and excitatory terminals are shown in Fig. 2A–D). However, among glutamatergic neurons, the highest CB_1 levels are present in dentate gyrus mossy cells (Kawamura et al. 2006; Monory et al. 2006). Interestingly, many CB_1-positive hilar mossy cells also contain dopamine D_2 receptors, suggesting that this region might be involved in the interactions of these two neuromodulatory systems (Degroot et al. 2006). Moreover, CB_1 immunoreactivity was also identified in the majority of hippocampal cholinergic nerve terminals, where presynaptic CB_1 receptors control acetylcholine release in vitro (Gifford and Ashby 1996) and in vivo (Degroot et al. 2006). On the other hand, CB_1

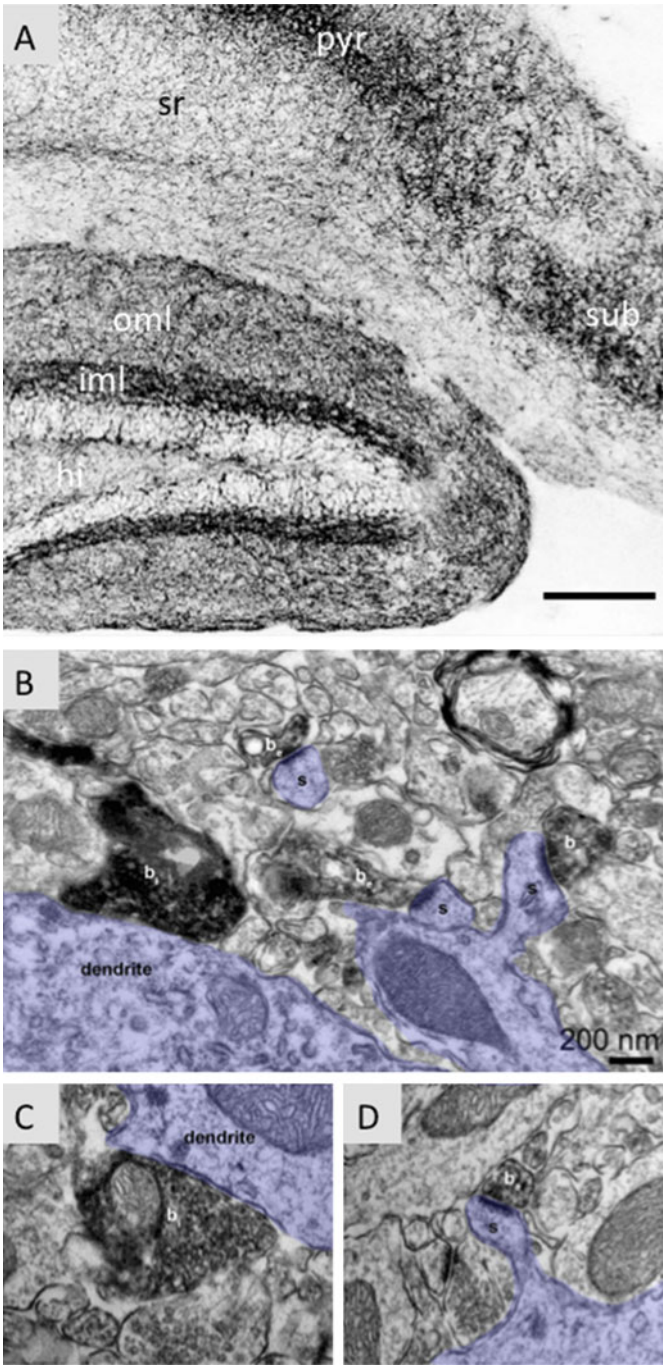

Fig. 2 CB_1 expression in rodent brain is primarily presynaptic. (**A**) CB_1 receptors in mouse hippocampal formation were detected by an antibody directed to its C-terminus. A dense meshwork of CB_1-expressing axons is evident. Levels are particularly high in the inner molecular layer

receptors appeared to be absent from granule cells of the dentate gyrus. Finally, low but detectable levels of CB_1 receptors were found in a subset of progenitor cells in the subgranular zone of the dentate gyrus, which regulate proliferation, survival, and differentiation of these adult progenitor cells (Aguado et al. 2005; Galve-Roperh et al. 2007).

3.3.2 Synthetic Enzymes

NAPE-PLD mRNA, as revealed by in situ hybridization, is most intensely expressed in the dentate gyrus granule cell layer, followed by the pyramidal cell layer of the hippocampus throughout all three fields (CA1-CA3) (Cristino et al. 2008; Egertova et al. 2008; Nyilas et al. 2008). Moreover, NAPE-PLD immunoreactivity was detected in granule cell axons (Egertova et al. 2008; Nyilas et al. 2008) as well as in many neurons of the hilus region in mouse dentate gyrus (Cristino et al. 2008; Nyilas et al. 2008). Therefore, in contrast to 2-AG's prominent role as a retrograde signaling messenger (Kano et al. 2009), anandamide and related NAEs generated by NAPE-PLD in axons may act as anterograde synaptic signaling molecules to regulate the activity of postsynaptic neurons (Egertova et al. 2008).

High levels of DAGLα mRNA and protein have been found in postsynaptic dendritic spines of the majority of hippocampal pyramidal neurons, including the spine head, neck, or both (Katona et al. 2006; Yoshida et al. 2006). A similar pattern for DAGLα was found in the postmortem human hippocampus, with highest levels in strata radiatum and oriens of the cornu ammonis and in the inner third of stratum moleculare of the dentate gyrus (Ludanyi et al. 2011). The juxtaposition of DAGL-α-immunoreactive postsynaptic dendritic spines to CB_1-expressing presynaptic terminals at excitatory glutamatergic synapses highlights the prominence of 2-AG as a retrograde messenger at many synapses (Katona et al. 2006).

3.3.3 Degradative Enzymes

FAAH is highly expressed in the somata and proximal dendrites of the pyramidal cells, which are densely innervated by CB_1-positive axon terminals (Egertova et al. 1998, 2003; Tsou et al. 1998b; Gulyas et al. 2004). However, it is important to note that FAAH is more frequently present on the membrane surface of intracellular organelles (e.g., mitochondria, smooth endoplasmic reticulum) than on somatic or dendritic plasma membranes (Gulyas et al. 2004). In addition, FAAH

Fig. 2 (continued) of the dentate gyrus and the pyramidal neuron layer: *hi* hilus, *iml* inner molecular layer, *oml* outer molecular layer, *pyr* pyramidal cell layer, *sr* stratum radiatum, *sub* subiculum. (**B**–**D**) Expression of CB_1 receptors at the ultrastructural level in the mouse hippocampus assessed using a C-terminal CB_1 antibody. (**B**) Electron-dense reaction product is present in both inhibitory (b_i) and excitatory (b_e) boutons. Note that the excitatory boutons synapse onto spines (*s*) while the inhibitory boutons form synapses on a dendritic shaft. Dendritic structures are pseudocolored blue for ease of identification. (**C**) Higher magnification image showing a CB_1-positive inhibitory terminal synapsing onto the shaft of a dendrite. (**D**) Higher magnification image showing a CB_1-positive excitatory bouton forming an asymmetric synapse onto a dendritic spine. Scale bar = 200 nm in **B**. Original figures provided by Jim Wager-Miller (**A**) and Chris Henstridge and Istvan Katona (**B**–**D**)

immunostaining is also evident in the somata of mouse dentate gyrus granule cells (Egertova et al. 2003), which contrasts to the absence of FAAH immunoreactivity in granule cells of the rat dentate gyrus (Tsou et al. 1998b; Gulyas et al. 2004). An in situ hybridization study showed that MAGL mRNA is abundantly expressed in the CA3 field of rat hippocampus (Dinh et al. 2002). Additional immunocytochemical studies revealed that MAGL protein is particularly prominent in axon terminals of granule cells, CA3 pyramidal cells, and some interneurons of rat hippocampus (Gulyas et al. 2004), as well as in axon terminals of glutamatergic neurons in both rodent and human hippocampus (Yoshida et al. 2006; Ludanyi et al. 2011) (Fig. 3A, B).

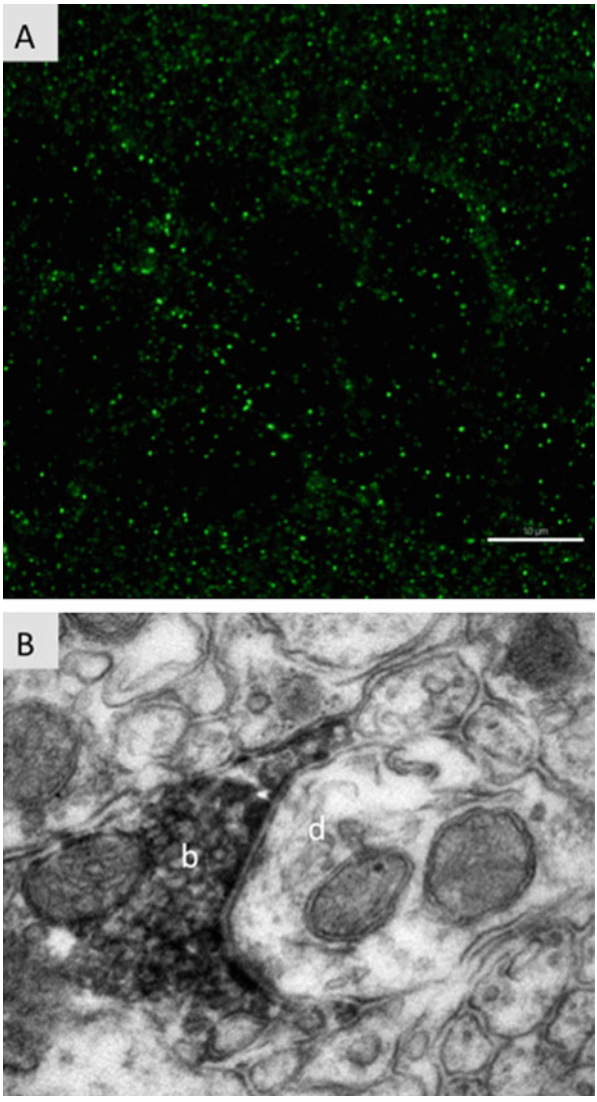

Fig. 3 Presynaptic localization of monoacylglycerol lipase (MAGL) detected with an antibody recognizing residues positioned in the middle of mouse MAGL. (**A**) Punctate expression of MAGL immunoreactivity in the mouse hippocampus pyramidal cell layer. Scale bar = 10 μm. (**B**) Electron-dense reaction product representing MAGL immunoreactivity is present in an inhibitory terminal (*b*) synapsing onto a dendritic shaft (*d*). Original figures provided by Stephen Woodhams and Istvan Katona

3.4 Cortical Subplate (Other Amygdala Nuclei)

3.4.1 Receptors

The amygdala is divided into a cortical component (e.g., the basolateral, lateral, and basomedial nuclei) and a striatal component (e.g., the central and medial nuclei) (Swanson and Petrovich 1998). This subdivision corresponds to the different structural organization and neurochemical properties of the two components. For example, while most principal neurons in the cortical subdivision of the amygdala are glutamatergic, the great majority of neurons in the striatal subdivision are GABAergic. Therefore, similar to cortex, high levels of CB_1 receptors are primarily expressed in the CCK-positive GABAergic basket cells (Marsicano and Lutz 1999; Katona et al. 2001). For example, in the basal (but not lateral) nucleus of the basolateral amygdala (BLA), high levels of CB_1 receptors are localized to presynaptic CCK-positive GABAergic terminals at invaginating synapses (Yoshida et al. 2011). On the other hand, as in cortex, low but functionally significant levels of CB_1 receptor are present in glutamatergic neurons in the cortical part of amygdala (Monory et al. 2006; Yoshida et al. 2011). Consistent with the above anatomical data, functional studies suggest that CB_1 receptors and endocannabinoids facilitated extinction of fear conditioning *via* inhibiting GABA release in the BLA (Marsicano et al. 2002). Finally, in contrast to earlier studies finding very weak CB_1 immunoreactivity within the central amygdala (Katona et al. 2001; Kamprath et al. 2011), a recent study using a highly sensitive CB_1 receptor antibody showed the presence of functional CB_1 receptors in the central amygdala (Ramikie et al. 2014).

3.4.2 Synthetic Enzymes

In the amygdaloid complex, NAPE-PLD mRNA is expressed in neurons of the cortical and medial amygdaloid nuclei, while less staining has been found in the basal and lateral nuclei (Egertova et al. 2008). At invaginating synapses, a unique type of perisomatic synapses in the basal nucleus of the BLA, DAGLα is recruited to somatic membrane of postsynaptic pyramidal neurons, juxtaposed to the CB_1-, MAGL-, and CCK-containing presynaptic terminals (Yoshida et al. 2011). In the central amygdala, DAGLα is localized to postsynaptic dendritic spine heads and dendritic shafts, juxtaposed to the CB_1-containing presynaptic terminals at glutamatergic synapses (Ramikie et al. 2014).

3.4.3 Degradative Enzymes

FAAH-immunoreactive neuronal somata are also present throughout the basolateral complex of the amygdala, which includes the lateral, basolateral (BLA), and basomedial nuclei. In all of these nuclei, the FAAH-immunoreactive somata are surrounded by CB_1-immunoreactive fibers (Egertova et al. 2003).

As mentioned above (Sect. 3.4.2), MAGL is co-expressed with CB_1 receptors in the presynaptic CCK-positive terminals at invaginating synapses in the basal nucleus of the BLA. Together with the postsynaptic DAGLα expression, this constitutes a molecular convergence for 2-AG-mediated retrograde signaling.

This contrasts with the flat perisomatic synapses made by parvalbumin-positive interneurons (Yoshida et al. 2011) where no such arrangement is present.

4 Subcortical Nuclei (Striatum, Basal Ganglia)

4.1 Striatum (Dorsal, Caudate)

4.1.1 Receptors

The subcortical nuclei with the highest level of CB_1 receptor expression are the basal ganglia. In situ hybridization studies showed that many striatal medium spiny neurons (MSNs) express CB_1 receptors, while adult pallidal and nigral neurons contain little or no CB_1 mRNA (Matsuda et al. 1993; Julian et al. 2003). Due to its axonal terminal localization, the high levels of pallidal and nigral CB_1 receptor binding and protein observed in autoradiographic and immunocytochemical studies mostly arose from GABAergic neurons projecting from the caudate putamen (Tsou et al. 1998a; Egertova and Elphick 2000). The staining for CB_1 in axons is denser in the globus pallidus than in the caudate putamen, while both show a gradient in the staining intensity increasing from medial to lateral (Tsou et al. 1998a; Egertova and Elphick 2000). Moreover, CB_1 receptors are present on both the striatonigral and striatopallidal projection pathways; thus, they are well positioned to modulate both the direct and indirect striatal output pathways (Hohmann and Herkenham 2000). In addition, a study utilizing a high-sensitivity CB_1 receptor antibody showed CB_1 protein to be intensely expressed on GABAergic axon terminals of striatal MSNs and parvalbumin-positive interneurons (Uchigashima et al. 2007). Finally, CB_1 protein was found on excitatory corticostriatal afferents (Gerdeman and Lovinger 2001; Rodriguez et al. 2001; Uchigashima et al. 2007), GABAergic aspiny interneurons (Hohmann and Herkenham 2000) and neurons in the subthalamic nucleus (Matsuda et al. 1993), with important functional implications (Kreitzer and Malenka 2007).

4.1.2 Synthetic Enzymes

The immunostaining of NAPE-PLD is present in the caudate putamen (Egertova et al. 2008). On the other hand, because 2-AG is synthesized by DAGL after membrane depolarization and G_q-coupled receptor activation, an immunocytochemical study was carried out to examine the subcellular distribution of DAGLα, metabotropic glutamate receptor 5 (mGluR5), and muscarinic cholinergic receptor 1 (M1) in mouse striatum (Uchigashima et al. 2007). Even though all three proteins were present on somatodendritic membranes of MSNs, only DAGLα and mGluR5 were present in spines and the perisynaptic region, while M1 receptors were absent in these domains (Uchigashima et al. 2007). This subcellular arrangement may account for the differential involvement of mGluR5 and M1 in endocannabinoid-mediated depolarization-induced suppression of inhibition and depolarization-induced suppression of excitation (Uchigashima et al. 2007).

4.1.3 Degradative Enzymes

In the mouse caudate putamen, FAAH-immunoreactive oligodendrocytes are present in fiber tracts (white matter) (Egertova et al. 2003). FAAH protein is also localized to the myelin sheath surrounding the unstained axons of large neurons (Egertova et al. 2003). This is consistent with the findings that FAAH mRNA is present in white matter of the rat brain (Thomas et al. 1997). However, the functional significance of FAAH expression in these non-neuronal cells is not yet understood. Moreover, although FAAH and CB_1 receptors are anatomically associated in many brain regions (Egertova et al. 1998), FAAH-expressing neurons are present in some brain areas such as thalamus and midbrain that express few or no CB_1 receptors (Egertova et al. 2003). Conversely, there is a population of striatal GABAergic MSNs where CB_1 receptors are present in their axonal terminals projecting to globus pallidus, entopeduncular nucleus, and substantia nigra, but FAAH-expressing neurons are absent (Egertova et al. 2003). In these situations, FAAH's biological role may be to degrade related acyl amides other than anandamide, for example, oleoylethanolamine and palmitoylethanolamine (Melis et al. 2008).

4.2 Striatum (Ventral, Accumbens)

4.2.1 Receptors

CB_1 receptors are expressed at low to moderate levels in the nucleus accumbens, with a pattern reminiscent of the striatum. CB_1 receptor protein is also localized on the terminals of the prefrontal glutamatergic efferents projecting to the nucleus accumbens as well as on the GABAergic axon terminals of accumbens MSNs and parvalbumin-positive interneurons (Robbe et al. 2001; Uchigashima et al. 2007). However, CB_1 receptors seem to be absent in the dopaminergic terminals projecting from the ventral tegmental area (VTA) to the accumbens. Therefore, cannabinoid stimulation of dopamine release in nucleus accumbens is likely mediated by inhibition of GABA release (either from within the nucleus accumbens or in the VTA) (Tanda et al. 1997; Szabo et al. 1999, 2002).

4.2.2 Synthetic Enzymes

DAGLα immunostaining, similar to that of mGluR5, is intense in spines and the perisynaptic region of the somatodendritic surface of striatal MSNs (Uchigashima et al. 2007).

4.2.3 Degradative Enzymes

FAAH immunoreactivity is absent in the nucleus accumbens (Egertova et al. 2003). However, moderate amounts of MAGL mRNA were found in the nucleus accumbens shell, islands of Calleja, and pontine nuclei by in situ hybridization (Dinh et al. 2002).

4.3 Striatum Medial (Lateral Septum, Septohippocampal, etc.)

4.3.1 Receptors

Moderate levels of CB_1 protein and mRNA are present in the basal forebrain including the medial and lateral septum and the nucleus of the diagonal band (Herkenham et al. 1991; Mailleux and Vanderhaeghen 1992; Matsuda et al. 1993; Marsicano and Lutz 1999). In situ hybridization studies showed the presence of CB_1 mRNA in cholinergic territories, especially the medial septum in mice. An immunocytochemical study revealed that a dense network of CB_1-positive fibers is present in the tenia tecta, ventral pallidum, and substantia innominate, whereas a fine network of CB_1-positive fibers is localized to the medial septum, diagonal bands, and nucleus basalis (Harkany et al. 2003). In the same study, no CB_1 immunoreactivity was detected in the cell bodies of basal forebrain cholinergic cells; instead these cells contain high levels of FAAH (Harkany et al. 2003). This is consistent with CB_1 receptors being synthesized and rapidly transported to axon terminals. Indeed, a later study showed that CB_1 protein is expressed in at least one-third of cholinergic neuron somata when axonal protein transport was blocked by cholchicine (Nyiri et al. 2005b). In rat medial septum, at least two types of cholinergic cells were identified, one with large somata, expressing CB_1 and $GABA_B$ receptors and projecting to the hippocampus, whereas the other had smaller somata and lacked these two receptors (Nyiri et al. 2005b). Therefore, endocannabinoid signaling is implicated in the function of a population of septohippocampal cholinergic neurons, including cognition as well as generation of hippocampal theta rhythms.

4.3.2 Synthetic Enzymes

The immunostaining of NAPE-PLD is localized to the lateral nucleus of the septum (Egertova et al. 2008).

4.3.3 Degradative Enzymes

Neuronal FAAH-immunoreactivity has been detected in the lateral septum and the triangular septal nucleus (Egertova et al. 2003), as well as in the basal forebrain cholinergic neurons (Harkany et al. 2003).

4.4 Striatum Caudal (Striatum-like Amygdala Nuclei, Central Amygdala, Bed Nucleus, Medial Amygdala, Etc.)

4.4.1 Receptors

In contrast to the cortical component of the amygdala, the striatal component of amygdala (e.g., central and medial nuclei) displays much lower levels of CB_1 receptors. CB_1 mRNA in the striatal amygdala was revealed by sensitive in situ hybridization with the absence of signal in the same region of CB_1 knockout mice (Marsicano and Lutz 1999).

5 Cerebellum and Associated Nuclei

5.1 Cerebellar Cortex

5.1.1 Receptors

The patterns of CB_1 receptor expression in the cerebellum are striking. While autoradiographic and immunocytochemical studies showed intense labeling of CB_1 protein in the molecular layer, in situ hybridization studies yielded robust CB_1 mRNA in the granule cell layer (Matsuda et al. 1990; Herkenham et al. 1991; Glass et al. 1997; Tsou et al. 1998a; Egertova and Elphick 2000). Purkinje neurons are devoid of CB_1 protein labeling, while the axon terminals of basket cells surrounding the Purkinje cell axon initial segment display extremely strong CB_1 labeling. Putting this together, CB_1 receptors are mainly expressed in the axon terminals of the climbing fibers, parallel fibers, and (some) basket cells, suggesting a prominent presynaptic localization of CB_1 receptors, mediating modulatory effects of (endo)cannabinoids at glutamatergic and GABAergic inputs onto Purkinje neurons. Consistent with this pattern of expression, several elegant electrophysiological studies demonstrated a role for endocannabinoid inhibition of glutamatergic and GABAergic neurotransmission onto Purkinje neurons (Kreitzer and Regehr 2001; Maejima et al. 2001; Diana et al. 2002; Brenowitz and Regehr 2003). However, additional electrophysiological studies supported the functional somatic expression of CB_1 receptors (Kreitzer et al. 2002).

5.1.2 Synthetic Enzymes

NAPE-PLD mRNA has been found in the granule cell layer and Purkinje cells, but not in the molecular layer or white matter (Egertova et al. 2008). However, NAPE-PLD immunoreactivity is localized in the pre- and post-synaptic areas of the Purkinje neurons and in the somata of the basket cells in the molecular layer (Cristino et al. 2008; Suarez et al. 2008).

DAGLα immunoreactivity is highly concentrated at the base of the spine neck of cerebellar Purkinje cells. In contrast to its distribution in hippocampal pyramidal cells, DAGLα is excluded from the main body of spine neck and head (Yoshida et al. 2006). However, there are no DAGLα-immunoreactive neurons in the granular layer and in any subdivisions of the inferior olive, suggesting that DAGLα is not present in parallel and climbing fibers (Suarez et al. 2008).

High levels of DAGLβ mRNA have been found in the mouse cerebellar granular layer by in situ hybridization (Yoshida et al. 2006). However, DAGLβ immunostaining in the cerebellar cortex is less intense than that of DAGLα. DAGLβ protein is localized to cell bodies of Purkinje neurons and in the molecular layer. In contrast to DAGLα, DAGLβ-containing neuropil of the molecular layer probably represents parallel and climbing fibers from the granular cells and inferior olive neurons, respectively (Suarez et al. 2008).

5.1.3 Degradative Enzymes

FAAH immunoreactivity is present in the somata and dendrites of the Purkinje cells, which are innervated by CB_1-positive axon terminals (Egertova et al. 1998, 2003; Tsou et al. 1998b; Gulyas et al. 2004). Weak FAAH immunostaining is also evident in the somata of granule cells (Egertova et al. 2003), which is consistent with the detection of FAAH mRNA in rat cerebellar granule cells (Thomas et al. 1997).

Both Northern blot and in situ hybridization analyses revealed that MAGL mRNA is present in rat cerebellum (Dinh et al. 2002), whereas MAGL immunoreactivity is localized to the axon terminals in the molecular layer but absent in the FAAH-positive Purkinje cell dendrites in rat cerebellum (Gulyas et al. 2004). Interestingly, a recent immunocytochemical study found that MAGL is heterogeneously expressed in mouse cerebellum, with highest levels in parallel fiber terminals, weak levels in Bergman glia, and complete absence in other synaptic terminals (Tanimura et al. 2012). Even though the expression of MAGL is limited to a subset of nerve terminals and astrocytes in the cerebellum, MAGL still regulates 2-AG retrograde signaling broadly at parallel fiber or climbing fiber to Purkinje cell synapses (Zhong et al. 2011; Tanimura et al. 2012).

5.2 Deep Cerebellar Nuclei (Fastigial, Interpos, Dentate Nucleus)

5.2.1 Receptors

All cerebellar nuclei (medial, lateral, and interposed nuclei) contain very weak CB_1-immunoreactivity throughout the neuropil. However, intense CB_1 immunostaining was found in the neuropil of the dorsal part of the principal nucleus of the inferior olive (Suarez et al. 2008).

5.2.2 Synthetic Enzymes

Most cerebellar nuclei showed intense neuropil NAPE-PLD immunoreactivity and a number of moderately NAPE-PLD-labeled neurons. On the other hand, both the posterior parvicellular part of the interposed cerebellar and lateral cerebellar nuclei have considerably fewer NAPE-PLD-labelled neurons and a less intense neuropil immunoreactivity (Suarez et al. 2008).

DAGLα immunoreactivity is absent in cell bodies of cerebellar and vestibular nuclei and in other regions with mossy fiber projections in the granular layer such as the pontine nuclei or the spinal cord (Suarez et al. 2008). In contrast, DAGLβ immunoreactivity is associated mainly with cell bodies embedded in a network of fibers. As with NAPE-PLD immunostaining, the posterior parvicellular parts of the interposed cerebellar and lateral cerebellar nuclei contain fewer DAGLβ-positive neurons when compared with the remaining cerebellar nuclei (Suarez et al. 2008).

5.2.3 Degradative Enzymes

The strong FAAH immunoreactivity observed in all cerebellar nuclei is related mainly to the presence of a dense meshwork of fibers, consisting of FAAH-positive

punctate labeling that contain immunoreactive somata (Egertova et al. 2003; Suarez et al. 2008). These cerebellar nuclei are devoid of CB_1 immunoreactivity and receive synaptic input mainly from Purkinje cell axons. In white matter surrounding the cerebellar nuclei, FAAH-immunoreactive oligodendrocytes are conspicuous (Egertova et al. 2003).

Cerebellar and functionally related vestibular nuclei have numerous MAGL-immunoreactive neurons, showing a perikaryal and dendritic Golgi-like labeling, similar to that of DAGLβ (Suarez et al. 2008).

6 Brainstem

6.1 Diencephalon

6.1.1 Thalamus (All Nuclei, Including Reticular Thalamic Nucleus, Habenula)

Receptors

CB_1 receptor expression is very low in most areas of the thalamus, with the exception of strong labeling in the lateral habenular nucleus, the anterior dorsal thalamic nucleus, and the reticular thalamic nucleus (Herkenham et al. 1991; Mailleux and Vanderhaeghen 1992; Tsou et al. 1998a; Marsicano and Lutz 1999). Since CB_1 mRNA is quite abundant in the lateral habenular nucleus, which has extremely diverse projections, it is likely that (endo)cannabinoids, acting through CB_1 receptors, significantly affect the diverse functions of the lateral habenula (Herkenham and Nauta 1977; Herkenham et al. 1991).

Synthetic Enzymes

NAPE-PLD mRNA is found in several thalamic nuclei such as lateral posterior nuclei and the medial geniculate nucleus, albeit at quite low intensity in mice (Egertova et al. 2008; Nyilas et al. 2008). In contrast, the highest levels of NAPE-PLD activity, protein and mRNA, were identified by enzyme assay, western blotting, and qPCR in rat thalamus, among nine different brain regions examined (Morishita et al. 2005). In addition, in situ hybridization revealed that moderate levels of DAGLα mRNA are expressed in mouse thalamus (Yoshida et al. 2006).

Degradative Enzymes

FAAH immunoreactivity has been detected in neuronal somata in the majority of thalamic nuclei, including the anterodorsal, the anteroventral, the anteromedial, the ventroanterior, the paratenial, the mediodorsal, the reticulothalamic, the ventrolateral, the ventroposterior, the ventromedial, the posterior, the lateral geniculate, and the medial geniculate (Egertova et al. 2000, 2003). Interestingly, FAAH protein is much more abundant in these nuclei than the cannabinoid CB_1 receptor, suggesting its role may be to degrade acyl amides other than anandamide in these regions.

An in situ hybridization study revealed that MAGL mRNA is abundantly expressed in the anterior thalamus, particularly in the anterodorsal nucleus, whereas it is sparse in other thalamic nuclei (Dinh et al. 2002).

6.1.2 Hypothalamus (All Nuclei)

Receptors

High levels of CB_1 immunoreactivity have been found in the arcuate, paraventricular, ventromedial, dorsomedial nuclei, and the external zone of the median eminence (Wittmann et al. 2007), as well as in the infundibular stem and lateral hypothalamic area (Tsou et al. 1998a). Further analysis revealed that CB_1 immunoreactivity is detectable in the preterminals of approximately equal numbers of symmetric and asymmetric synapses, suggesting the occurrence of retrograde signaling by endocannabinoids in both excitatory and inhibitory hypothalamic neuronal networks (Wittmann et al. 2007). An in situ hybridization study suggests that CB_1 mRNA is primarily present on glutamatergic neurons in the hypothalamus (Marsicano and Lutz 1999). Despite the relatively low levels of CB_1 receptors in the hypothalamus, functional GTPγS assays revealed these receptors are more efficiently coupled to G proteins than in many other regions (Breivogel and Childers 1998). Finally, a recent study showed that mice with viral-mediated knockdown of the CB_1 receptor gene (~60 % decrease of the mRNA level) in the hypothalamus, while maintained on a normocaloric standard diet, displayed decreased body weight gain over time, subsequent to increased energy expenditure and elevated β_3-adrenergic receptor expression in brown adipose tissues (Cardinal et al. 2012). This result suggests that hypothalamic CB_1 receptor signaling plays an important role in energy expenditure under basal conditions, contributing to the antiobesity effect of CB_1 receptor antagonism.

Synthetic Enzymes

The staining of NAPE-PLD mRNA is evident in cells of the ventromedial nucleus (Egertova et al. 2008).

6.1.3 Mesencephalon (Colliculi, VTA, PAG, SN, Raphe)

Receptors

A. Substantia Nigra

Both autoradiographic and immunocytochemical studies showed extremely high levels of CB_1 receptor protein in the substantia nigra (SN) pars reticulata (Herkenham et al. 1991; Egertova and Elphick 2000). In contrast, in situ hybridization studies showed very low amounts of CB_1 mRNA in the SN (Matsuda et al. 1993), suggesting the high levels of CB_1 protein are restricted to incoming axonal projections from other brain regions. For example, CB_1 receptor protein is restricted to the GABAergic axonal terminals from the putamen MSNs and the glutamatergic terminals from the subthalamic nucleus, which may be involved in

the control of locomotility by CB_1 activation in the SN (Mailleux and Vanderhaeghen 1992; Sanudo-Pena and Walker 1997; Sanudo-Pena et al. 1999a). On the other hand, very low levels of CB_1 receptors exist in sparse intrinsic nigral neurons, which may exert a direct control on dopaminergic transmission (Matsuda et al. 1993; Julian et al. 2003). Finally, in rat striatal nerve terminals, a low but significant percentage of CB_1–immunoreactivity is co-localized with tyrosine hydroxylase (TH), a marker for both noradrenergic and dopaminergic terminals (Kofalvi et al. 2005).

B. Ventral Tegmental Area

Both cannabinoids and endocannabinoids modulate the primary rewarding effects of many abused drugs, including exogenous cannabinoids, *via* regulation of drug-induced increases in dopaminergic neural activity in the VTA (Maldonado et al. 2006). Therefore, it is of interest to elucidate the expression and function of CB_1 receptors in the VTA. A sensitive CB_1 polyclonal antibody (Fukudome et al. 2004) revealed a dense neuropil labeling of CB_1 receptors in the VTA (Matyas et al. 2008). The CB_1 immunoreactivity is restricted to presynaptic axon terminals of symmetric synapses, which may belong to local intrinsic GABAergic neurons (Matyas et al. 2008). Moreover, CB_1-immunoreactivity is co-localized with vesicular glutamate transporter in presynaptic terminals near dopamine neuron dendrites in the VAT, indicating the presence of CB_1 receptors in glutamatergic terminals (Kortleven et al. 2011). Interestingly, co-localization of CB_1 receptor and TH has been revealed in several brain areas including VTA, thereby pointing to a possible direct influence of CB_1 receptor activation on dopaminergic neurons (Wenger et al. 2003). However, further studies are required to clarify this possibility.

C. Periaqueductal Gray

Low to moderate levels of CB_1 receptors have been found in the midbrain periaqueductal gray (PAG), where the ECS is involved in the control of pain sensation, including stress-induced analgesia (Walker et al. 1999; Hohmann et al. 2005; Gregg et al. 2012). In contrast to opiate receptors on GABAergic aqueductal neurons, CB_1 receptors are preferentially, but not exclusively, localized in the dorsal portion of the PAG (Tsou et al. 1998a; Azad et al. 2001). In addition, moderate levels of CB_1 mRNA and protein have been found in the reticular formation and raphe nucleus, the latter being the main neuronal source of serotonin in the brain (Glass et al. 1997; Haring et al. 2007), which might have functional implications in emotion/mood modulation.

Synthetic Enzymes

A. Ventral Tegmental Area

Moderate to high levels of DAGLα are expressed in most neurons of the VTA (Matyas et al. 2008). High-resolution electron microscopy further revealed that DAGLα is accumulated in postsynaptic plasma membrane of glutamatergic and GABAergic synapses, of both TH-positive and negative dendrites. The finding that

DAGLα is present in postsynaptic dendrites juxtaposed to presynaptic CB_1 receptors suggests that 2-AG-CB_1-mediated retrograde synaptic signaling may modulate the drug-reward circuitry at multiple types of synapses in the VTA.

B. Periaqueductal Gray
DAGLα protein is co-localized with mGluR5 within the same dendritic spine heads at postsynaptic excitatory synapses in rat dorsolateral PAG, which is involved in 2-AG-mediated stress-induced analgesia (Gregg et al. 2012).

Degradative Enzymes

A. Substantia Nigra
FAAH-immunoreactive neurons are not evident in the pars reticulata of the SN, which contains a very high concentration of CB_1 immunoreactivity in both mouse and rat (Egertova et al. 2000, 2003).

B. Other Nuclei
FAAH-immunoreactive neurons are present in the superior and inferior colliculus, the rhabdoid nucleus, and several mesencephalic raphe nuclei and are also intensely stained in the mesencephalic trigeminal nucleus (Egertova et al. 2003). FAAH-immunoreactive oligodendrocytes associated with fiber tracts are abundant in the midbrain. For example, FAAH immunostaining was found to be localized in the myelin sheath surrounding the unstained axons of the mesencephalic trigeminal tract (Egertova et al. 2003).

6.2 Hindbrain

6.2.1 Medulla (Area Postrema, Cochlear Nuclei, Nucleus of the Solitary Tract, Trigeminal Nuclei, Various Other Cranial Nerve Nuclei)

Receptors
Expression of CB_1 receptors, in contrast to the opioid receptors, in the medullary respiratory control centers is relatively low (Herkenham et al. 1991; Glass et al. 1997). This likely explains the low mortality caused by cannabinoid intoxication in humans and animals. However, relatively high levels of CB_1 receptors are present in the medullary nuclei associated with emesis and vagal control of gut motility, which may underlie the inhibition of emesis and gastrointestinal motility by cannabinoids (Krowicki et al. 1999; Van Sickle et al. 2001, 2003). For example, high to moderate levels of CB_1 receptors were found in the area postrema, the dorsal motor nucleus of the vagus, as well as the medial subnucleus and the subnucleus gelatinosus of the nucleus of the solitary tract (Van Sickle et al. 2001, 2003; Mackie 2005; Storr and Sharkey 2007).

In mouse dorsal cochlear nucleus, CB_1 receptors are highly expressed in glutamatergic terminals of the parallel fibers, at intermediate levels in glycinergic terminals, and completely absent in the auditory nerve inputs innervating to the same DCN principle neurons—fusiform and cartwheel cells (Zhao et al. 2009). Therefore, CB_1 receptors are well positioned to mediate short- and long-term plasticities exhibited at parallel fiber synapses, but not at auditory nerve inputs (Zhao et al. 2011; Zhao and Tzounopoulos 2011).

Synthetic Enzymes

Both DAGLα and DAGLβ proteins were identified in the somata of fusiform and cartwheel cells of mouse DCN, while they were present only in the dendritic spines of cartwheel cells (Zhao et al. 2009). These findings suggest that the synthesis of 2-AG is more distant from parallel fiber synapses in fusiform than cartwheel cells.

7 Spinal Cord (Dorsal, Ventral, Dorsal Root Ganglion)

7.1 Receptors

Intrathecal application of cannabinoids has been found to suppress pain in various pain models (Smith and Martin 1992; Welch et al. 1995), which is consistent with their suppression of noxious stimulus-evoked neuronal firing (Hohmann and Herkenham 1998) and Fos protein expression in the spinal dorsal horn (Hohmann et al. 1999b). Moreover, cannabinoids inhibited glutamate release from afferents in lamina I of dorsal horn in a CB_1 receptor-dependent fashion (Jennings et al. 2001; Morisset and Urban 2001). Compatible with these functional studies, moderate levels of CB_1 receptors were found in the superficial layers of the dorsal horn, the dorsolateral funiculus, and lamina X, all regions of the spinal cord associated with analgesia (Farquhar-Smith et al. 2000; Nyilas et al. 2009).

However, only a small percentage of CB_1 receptors are localized at central terminals of primary afferent C fibers, with many more present on large, myelinated Aβ and Aδ fibers, as well as postsynaptic interneurons (Hohmann and Herkenham 1998, 1999; Hohmann et al. 1999a; Farquhar-Smith et al. 2000). A dorsal rhizotomy produced time-dependent 50 % losses in cannabinoid binding densities in the dorsal horn since rhizotomy destroyed the terminals of both small- and large-diameter fibers (Hohmann et al. 1999a). However, a quantitative autoradiographic study showed a modest (16 %) decrease in cannabinoid binding sites in the superficial dorsal horn by neonatal capsaicin-mediated destruction of sensory C fibers (Hohmann and Herkenham 1998). Moreover, another study showed little decrease in CB_1 receptor immunoreactivity following dorsal rhizotomy or hemisection of the spinal cord, suggesting CB_1 receptors are primarily expressed on interneurons (Farquhar-Smith et al. 2000). Similarly, CB_1 receptors are only minimally co-localized with markers for C primary afferents both in the superficial dorsal horn and dorsal root ganglion (DRG) (Farquhar-Smith et al. 2000; Bridges et al. 2003). The above data suggest that the majority of CB_1 receptors are not

localized at the presynaptic terminals of nociceptive primary afferents, but rather may exist on postsynaptic interneurons (Farquhar-Smith et al. 2000; Salio et al. 2002), a CB_1 distribution which differs from the strong presynaptic axon terminal localization of CB_1 receptors in most other brain regions (Katona et al. 1999; Nyiri et al. 2005a). Indeed, research showed that CB_1 receptors localized at dorsal horn inhibitory postsynaptic interneurons mediate C-fiber-induced pain sensitization. However, it is important to emphasize the unexpected pro-nociceptive role of endocannabinoids is specific for C-fiber-mediated activity-dependent hyperalgesia, in contrast to the anti-nociceptive effect of these endogenous lipid molecules in models of inflammatory and neuropathic pain (Pernia-Andrade et al. 2009). Moreover, a conditional nociceptor-specific loss of CB_1 was found to reduce spinal CB_1-specific ligand binding by approximately 20 % only and did not substantially decrease CB_1-immunoreactivity in spinal laminae I and II (Agarwal et al. 2007). However, this conditional knockout of CB_1 in peripheral nociceptive neurons led to a substantial reduction of analgesia produced by local and systemic delivery of cannabinoids, suggesting that low levels of CB_1 expression did not necessarily mean lack of functional significance (Agarwal et al. 2007). Therefore, it is likely that the interplay between cannabinoid actions on peripheral primary afferents, interneurons, and descending pathways collectively contributes to the analgesic effects of CB_1 receptor activation in the spinal cord.

Interestingly, despite the earlier reports of minimal localization of CB_1 receptors in nociceptive DRG neurons (Hohmann and Herkenham 1999; Bridges et al. 2003), more recent studies suggest a much broader (40–80 %) distribution of CB_1 receptors in nociceptive neurons of DRG and trigeminal ganglia (Mitrirattanakul et al. 2006; Agarwal et al. 2007). While the possible reasons for these discrepancies have been well discussed elsewhere (Marsicano and Kuner 2008), it is also important to note that the expression and peripheral transport of CB_1 receptors in the DRG can be upregulated by peripheral inflammation (Amaya et al. 2006). Finally, some immunocytochemical evidence suggests CB_1 receptors are also present in the ventral horn (Tsou et al. 1998a; Sanudo-Pena et al. 1999b), a spinal cord area associated with movement.

7.2 Synthetic Enzymes

DAGLα mRNA is widely expressed in spinal dorsal horn neurons (Nyilas et al. 2009). Similar to CB_1 receptors, high levels DAGLα protein have been found to be localized at the superficial dorsal horn. High-resolution electron microscopy demonstrated a postsynaptic localization of DAGLα at nociceptive synapses, which is juxtaposed to the presynaptic CB_1-containing excitatory primary afferents (Nyilas et al. 2009) (Fig. 1B, C). Interestingly, postsynaptic DAGLα is co-localized with mGluR5, whose activation induces 2-AG biosynthesis (Nyilas et al. 2009).

7.3 Degradative Enzymes

FAAH has been found in the cell bodies of ventral horn neurons (Tsou et al. 1998b). The presence of FAAH and CB_1 receptors in circuits involved in spinal reflexes may underlie the antispastic effects of (endo)cannabinoids.

8 Summary, Concluding Thoughts, and Future Directions

As this brief review has shown, the components of the endocannabinoid system (ECS) are widespread throughout the CNS. Endocannabinoids have a major role as retrograde transmitters in many brain regions, although often with region-specific specialization as discussed above. Thus, in most brain regions, the highest levels of CB_1 receptors are found presynaptically, while endocannabinoid synthesizing enzymes are present postsynaptically. Interestingly, some endocannabinoid degrading enzymes are found presynaptically (e.g., MAGL) and others postsynaptically (e.g., ABHD6 and FAAH), suggesting specialized roles of each in endocannabinoid degradation (Gulyas et al. 2004; Blankman et al. 2007), and introducing an extra layer of complexity in endocannabinoid metabolism. A variation on the presynaptic localization of CB_1 is the somatic expression of CB_1, most commonly in some cortical and cerebellar neurons, where endocannabinoid signaling is cell autonomous. Future studies are needed to better define the distribution of some less-well studied (putative) ECS components, including α/β-hydrolase domain 4 (ABHD4) (Simon and Cravatt 2006), glycerophosphodiesterase 1 (GDE1) (Simon and Cravatt 2006, 2010), ABHD6 (Blankman et al. 2007; Marrs et al. 2010), α/β-hydrolase domain 12 (ABHD12) (Blankman et al. 2007), cannabinoid receptor interacting protein 1a (CRIP1a) (Niehaus et al. 2007), and DAGLβ (Gao et al. 2010).

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The Potential of Inhibitors of Endocannabinoid Metabolism for Drug Development: A Critical Review

Christopher J. Fowler

Contents

Abstract

The endocannabinoids anandamide and 2-arachidonoylglycerol are metabolised by both hydrolytic enzymes (primarily fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL)) and oxygenating enzymes (e.g. cyclooxygenase-2, COX-2). In the present article, the in vivo data for compounds inhibiting endocannabinoid metabolism have been reviewed, focussing on inflammation and pain. Potential reasons for the failure of an FAAH inhibitor in a clinical trial in patients with osteoarthritic pain are discussed. It is concluded that there is a continued potential for compounds inhibiting endocannabinoid metabolism in terms of drug development, but that it is wise not to be unrealistic in terms of expectations of success.

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology
231, DOI 10.1007/978-3-319-20825-1_4

Keywords
2-Arachidonoylglycerol • Anandamide • Cyclooxygenase-2 • Drug development • Fatty acid amide hydrolase • Monoacylglycerol lipase • Pain

Abbreviations

Δ^9-THC	Δ^9-Tetrahydrocannabinol
2-AG	2-Arachidonoylglycerol
AA-5-HT	*N*-Arachidonoyl-serotonin
AEA	Arachidonoyl ethanolamide, anandamide
(e)CB	(Endo)cannabinoid
FAAH	Fatty acid amide hydrolase
FABP5	Fatty acid binding protein 5
[^{35}S]GTPγS	Guanosine 5′-O-(3-[^{35}S]thio)triphosphate
IL-1ß	Interleukin-1ß
MAG	Monoacylglycerol
MGL	Monoacylglycerol lipase
NAAA	*N*-Acylethanolamine-hydrolyzing acid amidase
NAE	*N*-Acylethanolamine
NSAID	Nonsteroidal anti-inflammatory drug
PEA	Palmitoylethanolamide
OEA	Oleoylethanolamide
TRPV1	Transient receptor potential (vanilloid) 1 ion channels

1 Introduction

There are several enzymes involved in the metabolism of the endocannabinoids (eCBs, for a schematic, see Fig. 1). The hydrolytic enzymes (fatty acid amide hydrolase, FAAH; monoacylglycerol lipase, MGL; α/ß-hydrolase domain 6 and 12, ABHD6 and ABHD12) have been the most studied, but oxidative pathways, in particular the cyclooxygenase-2 (COX-2) pathway, are gaining increasing attention both in view of the properties of the derived products and also in respect to their ability to impact eCB levels in inflamed tissues (see reviews by Seierstad and Breitenbucher 2008; Ueda et al. 2010, 2011; Fowler 2012a, b; Piscitelli and Di Marzo 2012; Alhouayek and Muccioli 2014; Hermanson et al. 2014). The related *N*-acylethanolamines (NAEs) palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are also metabolised by FAAH, but PEA is also a substrate for *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (for a review, see Ueda et al. 2010). All of these pathways have evoked interest in terms of drug development.

Here, the in vivo properties of compounds with known mechanisms of action on targets involved in the accumulation and metabolism of eCBs and related

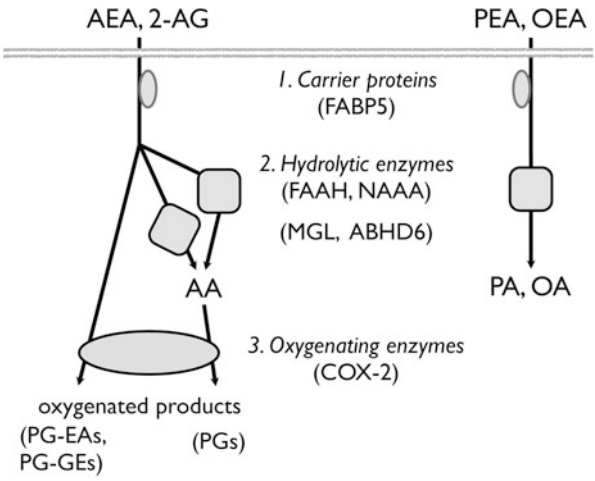

Fig. 1 Schematic representation of the metabolic pathways for AEA, 2-AG and the related *N*-acylethanolamines PEA and OEA. Abbreviations: *AA* arachidonic acid, *PA* palmitic acid, *OA* oleic acid, *PG* prostaglandin, *EAs* ethanolamides, *GEs* glyceryl esters

N-acylethanolamines are discussed. The phrase "known mechanisms of action" is pertinent in particular with respect to compounds reducing the uptake and release of the eCBs. The arachidonate and oleate derivatives AM404, VDM11, UCM707 and OMDM-2 have been shown to have pharmacological effects in vivo in a wide range of animal models of diseases, including cancer (Bifulco et al. 2004), multiple sclerosis (Baker et al. 2001), opioid withdrawal (Del Arco et al. 2002) and pain (Costa et al. 2006; La Rana et al. 2006). However, these compounds target FAAH and FABP5 in addition (or not) to the putative transporter protein (Fowler et al. 2004; Kaczocha et al. 2012), and so it is difficult to know to which target (s) the biological activities of the compounds can be ascribed.[1]

A different issue concerns ARN272, a compound reported to inhibit a putative FAAH-like anandamide transporter (FLAT) and which produces CB receptor-mediated effects in vivo (Fu et al. 2012; O'Brien et al. 2013). In this case, the role of FLAT has been disputed (Leung et al. 2013), and thus, it may be premature to use this compound as an experimental tool until this issue has been settled.

Attrition rates during clinical development of novel drugs are high, with only about 10 % of drugs entering clinical programmes being approved. Indeed, only

[1] Phenotypical screening (here inhibition of uptake) is of course a highly valid route to drug discovery, and it has been argued that it is a more appropriate route to first-in-class drugs than a target-centric approach (Enna and Williams 2009; Swinney and Anthony 2011). Follow-up drugs tend to be discovered more frequently on the basis of target-based approaches than phenotypic assays (Swinney and Anthony 2011). If it is argued that cannabis-based medicines are first in class, the target-based approach discussed in the present article makes sense. On the other hand, if cannabinoid-based medicines do not represent the same biological realm as inhibitors of NAE and MAG metabolism, then a phenotypical approach is well warranted. Identification of uptake inhibitors with considerably improved potencies to those currently available may well lead to useful drugs. For a review on the therapeutic potential of endocannabinoid uptake inhibitors, see Di Marzo (2008).

about 20 % reach phase 3, lack of efficacy being a major reason for discontinuation (Kola and Landis 2004). Drugs targeting central nervous system disorders are a case in point, and target-based approaches have not exactly excelled themselves (see Enna and Williams 2009). Thus, it is prudent not to be too effervescent in describing the clinical possibilities for inhibitors of the synthesis and metabolism of the two main eCBs, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and this review has deliberately tried to avoid such effervescence.

2 Carrier Proteins (FABP5)

$FABP^{-/-}$ mice show higher levels of AEA in whole brain than wild-type mice (Yu et al. 2014). SB-FI-26 binds to FABP5 and reduces AEA uptake, but does not inhibit FAAH activity (Berger et al. 2012). At a dose of 20 mg/kg i.p., SB-FI-26 increased brain levels of AEA, but not PEA, OEA or 2-AG, and reduced both phases of the formalin test of prolonged pain in C57BL/6J mice. This dose of SB-FI-26 also reduced thermal nociception and oedema following intraplantar (i.pl.) administration of λ-carrageenan in a manner blocked by combined administration of the CB_1 and CB_2 receptor antagonists/inverse agonists rimonabant and SR144528. The compound was active in the acetic acid model of visceral pain, an effect blocked by rimonabant and the peroxisome proliferator-activated receptor α (PPARα) antagonist GW6471. No overt cannabinoid-like effects upon locomotion were seen (Berger et al. 2012; Kaczocha et al. 2014). The effects of SB-FI-26 upon neuropathic pain were less convincing: in the chronic constriction injury (CCI) model of neuropathic pain in male Fisher 344 rats, it produced a short-term effect upon thermal hyperalgesia, but was without effect upon mechanical hyperalgesia (Kaczocha et al. 2014). It is not known if SB-FI-26 produces the same sort of memory and learning deficits as those seen in $FABP5^{-/-}$ mice (Yu et al. 2014).

3 Hydrolytic Enzymes (FAAH, MGL and NAAA)

The use of inhibitors of FAAH and MGL and, more recently, of NAAA and ABHD6, has greatly increased our understanding of the roles played by the eCB system in the body. Some of the key areas are described below from the point of view of drug development.

3.1 Cannabimimetic Effects (or Lack Thereof) of FAAH and MGL Inhibitors

FAAH inhibitors that penetrate the brain produce increases in brain levels of NAEs (AEA, OEA, PEA) without affecting 2-AG levels, whereas the reverse is seen for selective MGL inhibitors (see, e.g. Kathuria et al. 2003; Ahn et al. 2009, 2011; Long et al. 2009a, b; Ignatowska-Jankowska et al. 2014).

Cannabimimetic effects have been assessed in two main ways: (a) Δ^9-THC-like behaviour in the classic tetrad test (catalepsy, decreased body temperature, decreased locomotion and thermal antinociception) and (b) drug discrimination/self-administration tests using Δ^9-THC as a reference. Sedative effects of the compounds have also been investigated using a rotarod apparatus. Selective FAAH inhibitors do not produce either sedative/motor effects or the full gamut of behavioural effects reminiscent of Δ^9-THC (although individual components of the tetrad test can be affected, the most robust being a modest degree of analgesia in the hotplate test) (see, e.g. Kathuria et al. 2003; Solinas et al. 2007; Justinova et al. 2008; Karbarz et al. 2009; Ahn et al. 2011; Stewart and McMahon 2011; Chobanian et al. 2014). In humans, PF-04457845, at doses producing increases in plasma AEA, OEA and PEA, did not show any effects on cognitive function using a test battery of tasks assessing spatial memory, problem solving, psychomotor function, attention and learning after either 1, 8 or 14 days of treatment (Li et al. 2011).

In the case of MGL inhibitors, JZL184 is without significant effect upon either catalepsy or rectal temperature, but does decrease locomotor activity and produces a similar degree of antinociception in the hotplate test to that seen with PF-3840, following a dose of 40 mg/kg i.p. (Long et al. 2009b). The more selective compound KML29 (40 mg/kg i.p.) does not affect any of these parameters other than thermal antinociception and does not substitute for Δ^9-THC in a drug discrimination test (Ignatowska-Jankowska et al. 2014). Both compounds, however, produce cannabimimetic effects in mice lacking FAAH activity (either by genetic deletion or treatment with the FAAH inhibitor PF-3845), as does the combined FAAH/MGL inhibitor JZL195. These effects are blocked by rimonabant and, in the case of JZL184, are seen at doses of 20–40 mg/kg i.p. (Long et al. 2009b; Wise et al. 2012; Ignatowska-Jankowska et al. 2014). The combination of a low dose (8 mg/kg i.p.) of JZL184 with PF-3840 does not produce the full tetrad response, nor does the dual FAAH/MGL inhibitor SA-57 at doses of 1.25–5 mg/kg i.p. However, the dose of 12.5 mg/kg i.p. SA-57 does produce tetrad effects (Ramesh et al. 2013), so it is hard to envisage such a narrow band of selectivity being translated to the clinic and repeated use of the inhibitor. These data would suggest that selective blockade of either FAAH or MGL, but not concomitant blockade of both, may avoid the type of cannabimimetic unwanted effects that are an issue with cannabis-based medicines.

3.2 Pain

Electrical stimulation of the periaqueductal grey region (PAG) produces analgesia coupled with release of AEA (Walker et al. 1999). The authors concluded that "The spontaneous and stimulated release of AEA in a pain-suppression circuit suggests that drugs that inhibit the reuptake of AEA or block its degradation may form the

basis of a modern pharmacotherapy for pain, particularly in instances where opiates are ineffective". Studies performed with mice lacking FAAH, or with mice expressing FAAH in the nervous system alone ($FAAH^{NS}$ mice), supported the hypothesis that this enzyme, at least in the nervous system, is a potential target for development of novel analgesic drugs (Cravatt et al. 2004; Lichtman et al. 2004b). Since then, a large body of work has demonstrated that inhibitors of FAAH, MGL and, more recently, NAAA are indeed effective in many different experimental pain models. The data are summarised in Tables 1, 2, 3, 4 and 5. A number of conclusions can be drawn from these data:

- In the case of FAAH, the potentially beneficial effects are a class effect. Most data with respect to MGL has been obtained using JZL184, but other compounds are emerging which have corroborated the picture, so it is reasonable to talk about class effects here as well. In contrast, the positive effects of NAAA inhibition in the animal models are currently restricted to a single compound.
- Mechanistic studies indicate a plurality of pathways involved, a perhaps unsurprising result given both that AEA has multiple targets and that FAAH inhibition will not only increase AEA levels (and 2-AG levels in some tissues) but also related NAEs such as PEA and OEA, which have their own biological targets. Thus, depending upon the model and inhibitor used, antagonistic effects of rimonabant, AM251 and AM281 (CB_1 receptors); SR144528 and AM630 (CB_2 receptors); capsazepine, iodoresiniferatoxin, AMG9810 and parvanil (transient receptor potential vanilloid 1, TRPV1 receptors); GW6471 MK886 (peroxisome proliferator-activated receptor α, PPARα, with which SR144528 also interacts); naloxone and naltrexone (opioid receptors); and AP-18 and HC030031 (TRPA1 receptors) (Tables 1, 2, 3, 4 and 5) have been seen.
- FAAH inhibitors can be given repeatedly without induction of tolerance. Tolerance to MGL inhibitors occurs, dependent upon the dose used.
- Almost invariably the gender of the experimental animals used has been male, the only exception being the study of Merriam et al. (2010, summarised in Table 1). This is an unsatisfactory state of affairs given that in humans, there are clear gender differences in pain sensitivity and possibly responsiveness to treatment (Bartley and Fillingim 2013).
- Many of the studies use models based on measurement of the reflex responses of the animals to a mechanical or thermal stimulus. Rice et al. (2008) have argued that the "predominant symptom in neuropathic pain, and therefore the primary efficacy measure in nearly all RCTs[2] is not evoked pain, but spontaneous pain

[2] Randomised clinical trials.

Table 1 Effects of inhibitors of eCB hydrolysis in models of inflammatory pain

Model	cpds	Dose range	Behavioural effect	References
LPS model, ♂ C57BL/6J mice	*URB597*	Single dose: 1–10 mg/kg i.p. 23 h after LPS Triple dose: 10 mg/kg i.p. 1, 6 and 23 h after LPS	Single dose: 10 mg/kg dose, not others, ↓ thermal hyperalgesia Triple dose: ↓ thermal hyperalgesia and paw levels of IL-1ß and TNFα. Antihyperalgesic effect not seen in $CB_1^{-/-}$ mice but retained in $CB_2^{-/-}$ mice and in wt mice treated with capsazepine	Naidu et al. (2010)
LPS model, ♂ C57BL/6J mice	*PF-3845* *URB597* *OL135*	1–10 mg/kg i.p. 0.1–10 μg i.pl. 1–10 mg/kg i.p. 1–30 mg/kg i.p.	i.p.: ↓ allodynia at highest doses of all compounds. 10 mg/kg PF-3845 effects seen in $FAAH^{NS}$, $CB_1^{+/+}$ and $CB_2^{+/+}$ mice, not in $CB_1^{-/-}$ or $CB_2^{-/-}$ mice. Not blocked by naltrexone, I-RTX or PPARα antagonist MK866 i.pl. PF-3845: partial reduction after 1, 3 and 10 but not 0.1 μg doses	Booker et al. (2012)
CFA model, ♂ SD rats	*URB597*	0.03–0.3 mg/kg i.p.	↓ mechanical allodynia at 0.1 and 0.3 mg/kg doses. Partial block by AM251 and SR144528, complete block with both. ↓ thermal hyperalgesia, but to less extent than HU210	Jayamanne et al. (2006)
CFA model, ♂ SD rats	*PF-3845*	1–30 mg/kg p.o.	Dose-dependent ↓ mechanical allodynia, significant at ≥3 mg/kg. At dose 10 mg/kg, effect is seen at 2–6 h and is reduced by both rim and SR144528	Ahn et al. (2009)
CFA model, ♂ SD rats	*PF-04457845*	0.003–10 mg/kg p.o.	Partial ↓ mechanical allodynia (to same level as naproxen) at doses ≥0.1 mg/kg. "All or nothing" type of response curve. Effect of 1 mg/kg seen over 1–24 h. Effect of 3 mg/kg blocked by rim + SR144528, not by either compound alone	Ahn et al. (2011)

(continued)

Table 1 (continued)

Model	cpds	Dose range	Behavioural effect	References
CFA model, ♂ CD1 mice	*URB597* *URB937*[a] *PF-04457845*	1–30 mg/kg p.o. 1–30 mg/kg p.o. 1–30 mg/kg p.o. Compounds given as single doses either on day 3, 7 or 14	Dose-dependent ↓ mechanical and thermal hyperalgesia for all compounds on all three days for 10 and 30 mg/kg doses, for URB937 and PF-04457845 at 3 mg/kg and for URB937 at 1 mg/kg dose	Sasso et al. (2012)
CFA model, ♂ SD rats	*MK-4409*	10 and 30 mg/kg p.o. Repeat regime: 30 mg/kg up to 10 days	↓ mechanical allodynia 1 and 3 h after administration. No sign of tachyphylaxis after repeated dosing, in contrast to that seen for WIN55,212-2 (2 mg/kg/day i.p.)	Chobanian et al. (2014)
Carrageenan-induced thermal hyperalgesia, ♂ C57BL/6J mice	***URB602***	1–10 mg/kg i.p.	↓ thermal hyperalgesia seen 5 (5 and 10 mg/kg) and 24 h (10 mg/kg) after carrageenan. Effect of 10 mg/kg at 5 h blocked by SR144528, not rim	Comelli et al. (2007)
Carrageenan-induced hyperalgesia, ♂ SD rats	*URB597*	25 μg i.pl.	↓ expansion of dorsal horn WDR neuron receptive field size. Effect reduced by PPARα antagonist GW6471, but not by AM251. Mechanically evoked responses of WDR neurons not affected	Sagar et al. (2008)
Carrageenan-induced hyperalgesia, ♂ SD rats	*URB597*	25 and 100 μg i.pl.	25 μg but not 100 μg ↓ difference in weight bearing between ipsi- and contralateral paws. Larger effect of 50 μg i.pl. nimesulide. Effect of 25 μg dose and of nimesulide blocked by PPARα antagonist GW6471 but not by PPARγ antagonist GW9662	Jhaveri et al. (2008)
Carrageenan-induced hyperalgesia, ♂ SD rats	*JNJ-1661010*	50 mg/kg i.p.	↓ thermal hyperalgesia	Karbarz et al. (2009)
Carrageenan-induced hyperalgesia, ♂ mice	URB597 URB937[a]	1 mg/kg i.p. 1 mg/kg i.p.	↓ mechanical hyperalgesia and allodynia, ↓ thermal hyperalgesia. Effect of URB937 blocked by rim, not by AM630 or by the PPARα antagonist MK886	Clapper et al. (2010)

Carrageenan-induced hyperalgesia, ♂ C57BL/6J mice	*URB597*	0.3–3 mg/kg i.p.	Small partial reversal with URB597	Costa et al. (2010)
Carrageenan-induced mechanical hyperalgesia, ♂ CD1 mice	*URB937*[a]	0.1–3 mg/kg p.o.	Dose-dependent ↓ mechanical and thermal hyperalgesia. Effects seen at ≥0.3 mg/kg, between 2 and 24 h (3 mg/kg at 48 h; none at 72 h).	Sasso et al. (2012)
Carrageenan-induced nociception, ♂ SD rats	**JZL184**	25–100 μg topically onto spinal cord	Naïve rats: ↓ mechanically evoked firing rates of dorsal horn WDR neurons. Carrageenan-treated animals: 100 μg dose prevents expansion of WDR receptive fields	Woodhams et al. (2012)
Carrageenan-induced hyperalgesia, ♂ SD rats	*URB597*	0.3 mg/kg i.p. either acute or repeated for 4 days	Acute: ↓ difference in weight bearing between ipsi- and contralateral paws. Not seen after repeated treatment. Effect of acute treatment blocked by AM251 and the PPARα antagonist GW6471	Okine et al. (2012)
Carrageenan-induced mechanical allodynia, ♂ C57BL/6J mice. $CB_1^{-/-}$ and $CB_2^{-/-}$ mice	*PF-3845* **JZL184**	1–10 mg/kg i.p. (acute) 1.6–40 mg/kg i.p. (acute or repeated [6 days])	Acute PF-3845 (10, not 1, 3 mg/kg) and JZL184 (4, 16, 40 not 1.6 mg/kg) ↓ mechanical allodynia. JZL184 16 mg/kg effect blocked by rim or SR144528 and not seen in $CB_1^{-/-}$ or $CB_2^{-/-}$ mice. Tolerance seen with repeated treatment with 40 mg/kg but not with 4 mg/kg JZL184	Ghosh et al. (2013)
Carrageenan-induced thermal hyperalgesia, ♂ CD1 mice; PPARα$^{-/-}$	ARN077	1–30 % topical application; 0.005–50 μg i.pl.	All i.pl. doses ↓ thermal hyperalgesia at $t = 2$ h, 0.5–50 μg doses at 6 and 24 h, not 48 h. Topical ARN077 effective at $t = 4$ h at 10 and 30 %, topical doses, blocked by PPARα antagonist GW6471, not AM251 nor AM630. Effect of 30 % topical dose not seen in PPARα$^{-/-}$ mice	Sasso et al. (2013)

(continued)

Table 1 (continued)

Model	cpds	Dose range	Behavioural effect	References
Carrageenan-induced mechanical allodynia, ♂ C57BL/6J mice. ♀ and ♂ $CB_1^{-/-}$ and $CB_2^{-/-}$ mice	**JZL184** **KML29**	40 mg/kg i.p. (acute) 5–40 mg/kg i.p. (acute) 40 mg.kg. i.p. (repeated [6 days])	↓ mechanical allodynia (JZL184; KML29 20 and 40 mg/kg doses). Effect of 40 mg/kg KML29 blocked by rim and SR144528, and not seen in $CB_1^{-/-}$ or $CB_2^{-/-}$ mice. Tolerance seen after repeated treatment	Ignatowska-Jankowska et al. (2014)
Carrageenan-induced mechanical allodynia, ♂ C57BL/6J mice	*PF-3845*	1–10 mg/kg i.p.	↓ mechanical allodynia at 10 mg/kg. Threshold dose of 3 mg/kg + threshold dose of diclofenac also gave ↓ mechanical allodynia. Effect of combination partially blocked by rim and by SR144528	Grim et al. (2014)
Acrolein-induced bladder inflammation, ♀ Wistar rats	*URB597*	0.3 mg/kg i.p.	↓ referred mechanical hyperalgesia in hind paws at 4 and 24 h. No effect of this dose in control animals	Merriam et al. (2010)

Throughout the tables, FAAH inhibitors are indicated in italics, MGL inhibitors are in bold and the NAAA inhibitor is underlined. JZL195 and MAFP inhibit both FAAH and MGL and are thus shown as bold italic. URB602 is also listed as such since it shows no selectivity for inhibition of MGL vs. FAAH (Vandevoorde et al., 2007). It is, however, functionally selective since the % inhibition required to ↑ 2-AG levels is considered to be lower than that required to ↑ AEA levels. Insufficiently characterised compounds have not been included

Abbreviations: CFA, complete Freund's adjuvant; con, contralateral; cpds, compounds; i.paw., subcutaneous injection into the dorsal surface of the appropriate paw; I-RTX, iodoresiniferatoxin (TRPV1 antagonist); LPS, lipopolysaccharide; rim, rimonabant (SR141716A); SD, Sprague-Dawley; TPA, 12-O-tetradecanoylphorbol 13-acetate; WDR, wide dynamic range; wt, wild type

[a]Peripherally restricted compound. Dual-action compounds like AA-5-HT are not discussed in the table. The knockout mice were backcrossed onto a C57BL/6J background

Table 2 Effects of eCB hydrolysis inhibitors in models of visceral pain

Animals	cpd	Dose range	Behavioural effect	References
♂ C57BL/6J mice	*URB597* *OL135*	1–10 mg/kg s.c. 30 mg/lkg s.c.	↓ AAS for 10 mg/kg (URB597) and for OL135. Effects of compounds blocked by rim, not by SR144528 or naltrexone. Effect of URB597 synergises with that of diclofenac	Naidu et al. (2009)
C57BL/6J mice	**JZL184**	16 mg/kg i.p.[a]	↓ AAS, blocked by rim	Long et al. (2009a)
FAAH $^{+/+}$ and FAAH$^{-/-}$ mice	**JZL184** ***JZL195***	16 mg/kg s.c. 20 mg/kg s.c.	JZL184: ↓ AAS in FAAH$^{+/+}$ and FAAH$^{-/-}$ mice. JZL195: ↓ AAS in FAAH$^{+/+}$ mice, blocked by rim and not seen in FAAH$^{-/-}$ mice	Long et al. (2009b)
♂ Swiss-Webster mice; FAAH$^{+/+}$ and FAAH$^{-/-}$, PPARα$^{+/+}$ and $^{-/-}$ mice	*URB597* *URB937**	1 mg/kg s.c. 1 mg/kg.s.c.	↓ AAS. Effect of URB937 blocked by rim, AM251, but not AM630 or the PPARα antagonist MK886. Effect of URB937 not seen in FAAH$^{-/-}$ or PPARα$^{-/-}$ mice	Clapper et al. (2010)
♂ mice[b]	*URB597* **JZL184**	1 mg/kg i.p. either acute or repeated for 6 days 8 mg/kg i.p. either acute or repeated for 6 days	↓ AAS for both compounds, without appearance of tolerance after repeated administration	Busquets-Garcia et al. (2011)
♂ C57BL/6J mice	*URB597*	0.32–10 mg/kg s.c.	↓ AAS, with ID_{50} value 1.3 (95 % CI 0.92–1.84) mg/kg, reduced by rim. Additive interaction with morphine. Acetic acid ↓ feeding, wheel running and schedule-controlled behaviour. Feeding and wheel running effects attenuated by 1–10 mg/kg URB597	Miller et al. (2012)
♂ C57BL/6N mice	**JZL184**	40 mg/kg s.c. daily for 6 days	↑ AAS. Same result seen for MGL$^{-/-}$ mice	Petrenko et al. (2014)
♂ CD1 mice; FAAH$^{-/-}$ mice	*PF-3845*	10–30 mg/kg i.p. 30 μg i.c.v.	10 mg/kg i.p. and 30 μg i.c.v. ↓ AAS. i.c.v. effect blocked by i.c.v. AM251; 10 mg/kg i.p. ↓ pain behaviours following i.c. mustard oil, blocked by AM251 and not seen in FAAH$^{-/-}$ mice	Fichna et al. (2014)

(continued)

Table 2 (continued)

Animals	cpd	Dose range	Behavioural effect	References
♂ SD rats	*URB597*	1, 3.2 and 10 mg/kg i.p.	10 mg/kg dose ↓ lactic acid-induced abdominal stretching, blocked by rim, AM251, but not AM630. Effect seen when URB597 was given either 60 or 240 min before lactic acid. Lactic acid per se ↓ intracranial self-stimulation. URB597 10 mg/kg dose mitigated this effect when given 260 min, but not 60 min, prior to lactic acid. Effect not blocked by rim or SR144528	Kwilasz et al. (2014)

[a]Route of administration listed as i.p. (Fig. 6 legend of the paper), but as s.c. in the supplementary methods section of the paper
[b]Strain used not explicitly stated
Abbreviations (other than those listed in Table 1): *AAS* acetic acid-induced abdominal stretching, *i.c.v.* intracerebroventricular

Table 3 Effects of eCB hydrolysis inhibitors in the formalin model of persistent pain

Animals	cpd	Dose range	Behavioural effect	References
C57BL/6J mice	*OL135*	3–30 mg/kg i.p.	10 and 30 mg/kg doses ↓ both phases of the formalin test. Effects of 10 mg/kg dose blocked by rim, not by SR144528	Lichtman et al. (2004a)
mice	**OMDM-169**	1.25–5 mg/kg i.p.	↓ nocifensive behaviour at 5 mg/kg (both phases) and 2.5 mg/kg (phase 2). 5 mg/kg dose blocked by AM251 and partially blocked by AM630	Bisogno et al. (2009b)
♂ C57BL/6J mice	**JZL184**	40 mg/kg p.o.	↓ both phases of nocifensive behaviour, blocked by rim	Long et al. (2009a)
ERK phosphorylation in the spinal cord, anaesthetised ♂ C57/BL6 mice	*URB597*	10–100 μg i.pl.	↓ phosphorylation of ERK at 100, not 30 or 10 μg. Effect blocked by AM251	Thors et al. (2010)
♂ SD rats	*URB937**	1 mg/kg i.p.	↓ phase 2 nocifensive behaviour and spinal cord Fos immunoreactivity. Both responses blocked by rim	Clapper et al. (2010)
♂ SD rats	**JZL184** ***URB602***	1 ng–300 μg i.paw. 1 ng–600 μg i.paw.	Both phases ↓ by compounds. ID_{50} values (μg) for phases 1 and 2, respectively: JZL184 0.06±0.028 and 0.03±0.011; URB602 120±51 and 66±24. Effects of 10 μg JZL184 on both phases blocked by AM251 and AM630. No effects of high dose of either compound when given contralaterally	Guindon et al. (2011)
♂ Lister-hooded rats	*URB597*	0.3 mg/kg i.p.	↓ composite pain score in fear-conditioned animals (which is analgesic per se). No effect upon non-fear-conditioned rats	Butler et al. (2012)
♂ C57BL/6N mice	**JZL184**	40 mg/kg s.c. daily for 6 days	↑ second phase nocifensive response. Same result seen for $MGL^{-/-}$ mice	Petrenko et al. (2014)

[a]Species not indicated
For clarification of explanation of the classification of the compounds, see legend to Table 1.
Abbreviation (other than those listed in Table 1): *ERK* extracellular signal-regulated kinase

Table 4 Effects of eCB hydrolysis inhibitors in models of neuropathic pain

Model	cpd	Dose range	Effect	References
CCI model, ♂ Wistar rats	*OL135* *URB597*	3 mg/kg s.c. 3 mg/kg s.c. All compounds 3- and 7-day treatments	↓ mechanical allodynia at 7 but not 3 days for OL135; URB597 not significant. ↓ thermal hyperalgesia for both compounds at 3 and 7 days	Maione et al. (2007)
CCI model, ♂ Swiss mice	*URB597*	1–50 mg/kg p.o. for 7 days 10 mg/kg p.o. acute after 7 days	7 days of treatment: dose-dependent ↓ mechanical hyperalgesia. Effect at doses ≥3 mg/kg. 10 mg/kg dose also ↓ mechanical allodynia, thermal hyperalgesia and plasma extravasation, all blocked by rim. SR144528 reduces all effects of URB597 except mechanical hyperalgesia. Smaller effect upon mechanical hyperalgesia after single dose of URB597	Russo et al. (2007)
CCI model, ♂ C57BL/6J mice; $FAAH^{+/+}$ and $FAAH^{-/-}$ mice	*URB597* *OL135* **JZL184**	1–10 mg/kg i.p. 10 mg/kg i.p. 16 mg/kg i.p.	URB597 10 mg/kg, but not 1–5 mg/kg, OL135 and JZL184 ↓ mechanical and cold allodynia. Blocked by rim and SR144528. Effects of OL135 not blocked by naltrexone or capsazepine. JZL184 retained its effects in $FAAH^{-/-}$ mice, whereas URB597 (10 mg/kg) and OL135 did not	Kinsey et al. (2009)
CCI model, CB_1 and $CB_2^{+/+}$ and $^{-/-}$ mice	*PF-3845* **JZL184**	10 mg/kg i.p. 40 mg/kg i.p.	Both compounds ↓ mechanical and cold allodynia in the $CB_1^{+/+}$ and $CB_2^{+/+}$ but not $CB_1^{-/-}$ mice. PF-3845 but not JZL184 effect lost in $CB_2^{-/-}$ mice. Gabapentin effective regardless of mouse genotype	Kinsey et al. (2010)
CCI model of neuropathic pain, ♂ C57BL/6J mice	*PF-3845* **JZL184**	10 mg/kg i.p. 40 mg/kg i.p. Both compounds acute or repeated (6 days)	Acute treatment: both compounds ↓ mechanical and cold allodynia. Repeated treatment: PF-3845 effects retained, tolerance to JZL184. JZL184 also produces cross-tolerance to effects of PF-3845 and WIN55,212	Schlosburg et al. (2010)
CCI model, ♂ CD1 mice	*ST4070* *OL135* *URB597*	10–100 mg/kg p.o. 100 mg/kg i.p. 50 mg/kg p.o.	All compounds ↓ mechanical allodynia in CCI mice (ST4070 30 mg/kg and URB597 0.5–48 h; OL135 0.5–2 h); no effect in contralateral paws. ST4070 30 mg/kg dose blocked by rim, SR144528 and PPARα antagonist GW6471	Caprioli et al. (2012)

CCI model, ♂ C57BL/6J mice	*OL135*	30 mg/kg i.p.	↓ cold allodynia	Otrubova et al. (2013)
CCI model, ♂ Wistar rats	*URB597*	200 μg i.t.	↓ mechanical and cold allodynia, blocked by TRPV1 antagonist I-RTX, not AM251; ↓ thermal hyperalgesia blocked by AM251 and I-RTX (all compounds given i.t.)	Starowicz et al. (2013)
CCI model, ♂ CD1 mice	ARN077	1–30 % topical application once daily on days 3. 7 or 14 after CCI	All doses ↓ mechanical allodynia on all three treatment days. 10 and 30 % (all days) and 3 % (days 7 and 14) ↓ thermal hyperalgesia	Sasso et al. (2013)
CCI model, ♂ C57BL/6J mice	**JZL184**	4 and 40 mg/kg i.p. either acute or repeated (6 days)	Acute treatment: both doses ↓ mechanical and cold allodynia to same degree. Repeated treatment: tolerance seen with 40 mg/kg (and cross-tolerance to Δ^9-THC) but not with 4 mg/kg dose. Repeated treatment with 16 and 40 mg/kg, but not with 4 and 8 mg/kg doses, produce cannabinoid withdrawal signs upon rim treatment of naïve animals	Kinsey et al. (2013)
CCI model, ♂ C57BL/6J mice	**JZL184** **KML29**	40 mg/kg i.p. 40 mg/kg i.p.	Both compounds ↓ mechanical and cold allodynia. Effects of KML29 reduced by rim, not affected by SR144528	Ignatowska-Jankowska et al. (2014)
CCI model, ♂ C57BL/6J mice	*PF-3845*	1–10 mg/kg i.p.	↓ mechanical allodynia at 10 mg/kg. Threshold dose of 5 mg/kg + threshold dose of diclofenac also gave ↓ mechanical allodynia	Grim et al. (2014)
PNL model, ♂ SD rats	*URB597*	0.3 mg/kg i.p.	No effect on mechanical allodynia. HU210 was effective in the model	Jayamanne et al. (2006)
PNL model, ♂ Wistar rats	*URB597* ***URB602***	25 μg i.paw. 0.1–1000 μg i.paw.	Both compounds ↓ mechanical allodynia and thermal hyperalgesia. ID_{50} values for URB602 127±83 and 86±52 μg, respectively. URB602 (300 μg) effects blocked by AM251 and AM630; URB597 effects blocked by AM251 not AM630. No effects of the compounds (125 and 300 μg doses for URB602) given to contralateral paw	Desroches et al. (2008)

(continued)

Table 4 (continued)

Model	cpd	Dose range	Effect	References
PNL model, ♂ C57BL/6J mice, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice	*URB597* ***URB602***	100 μg i.paw 100 μg i.paw	URB597: ↓ mechanical allodynia and thermal hyperalgesia in wt and $CB_2^{-/-}$ mice, but not in $CB_1^{-/-}$ mice. URB602: ↓ mechanical allodynia in wt and $CB_1^{-/-}$ mice, but not in $CB_2^{-/-}$ mice. ↓ thermal analgesia in all three. No effects seen when the compounds were given to the contralateral paw	Desroches et al. (2013)
SNL model, ♂ SD rats	*URB597*	25 and 100 μg i.pl.; 10–50 μg spinally	i.pl.: ↓ WDR responses to mechanical stimulation in sham rats (25 μg) and SNL rats (100 μg, not 25 μg); both blocked by AM251. Naloxone blocked effect in sham, not SNL rats. Spinal: ↓ responses in both sham and SNL rats, blocked by AM251 and naloxone	Jhaveri et al. (2006)
SNL model, ♂ SD rats	*OL135*	2–50 mg/kg i.p.	Dose-dependent ↓ mechanical allodynia. 20 mg/kg: blocked by SR144528 and naloxone, not by rim	Chang et al. (2006)
SNL model, ♂ SD rats	*JNJ-1661010* *OL135* *URB597*	2 and 20 mg/kg i.p. 20 mg/kg i.p. 3 mg/kg i.p.	All three compounds (but not low dose of JNJ-1661010) ↓ tactile allodynia. Not blocked by rim, but blocked by SR144528 and naloxone	Karbarz et al. (2009)
SNL model, ♂ Swiss-Webster mice	*URB937**	1 mg/kg i.p. either acute or repeated for 7 days	↓ mechanical hyperalgesia and allodynia, ↓ thermal hyperalgesia, blocked by rim, not by AM630 or by the PPARα antagonist MK886	Clapper et al. (2010)
SNL model, ♂ CD1 mice	*URB937**	0.3–3 mg/kg p.o. given as single dose either on day 3, 7 or 14	Dose-dependent ↓ mechanical allodynia, thermal hyperalgesia and mechanical hyperalgesia. Effects seen on all three treatment days with 1 and 3 mg/kg doses. No effects of 0.3 mg/kg dose.	Sasso et al. (2012)
SNL model, rats	*JNJ-40413269*	2–10 mg/kg[b]	↓ mechanical allodynia, max effect at 30 min. Robust responses seen with ≥6 mg/kg doses	Keith et al. (2014a)

SNL model, rats	*JNJ-42119779* *JNJ-40355003*	20 mg/kg p.o. 60 mg/kg p.o.	JNJ-42119779 ↓ mechanical allodynia. Smaller effect than for JNJ-40355003, but lower dose	Keith et al. (2014b)
SNL model, rats	*MK-4409* *URB597*	3–100 mg/kg p.o. 3–50 mg/kg p.o.	Both compounds ↓ mechanical allodynia	Chobanian et al. (2014)
Vincristine-induced neuropathic pain, ♂ SD rats	*ST4070*	50 mg/kg p.o.	↓ mechanical allodynia	Caprioli et al. (2012)
Cisplatin-induced peripheral neuropathy, ♂ SD rats	*URB597* *URB937** **JZL184**	0.1 and 1 mg/kg i.p. 0.1 and 1 mg/kg i.p. 1, 3, 8 mg/kg i.p.	All three compounds ↓ mechanical and cold allodynia at all doses. High-dose effects blocked by AM251 and AM630 (all three compounds) and TRPV1 antagonist AMG9810 (URBs 597 and 937), not by TRPA1 antagonist HC030031	Guindon et al. (2013)
STZ-induced diabetic neuropathic pain, ♂ CD1 mice	*ST4070*	10–100 mg/kg p.o.	↓ mechanical allodynia at all doses (effect 100 > 30 > 10 mg/kg)	Caprioli et al. (2012)
Spinal cord injury pain, ♂ SD rats	*URB597* *PF-3845*	3 mg/kg i.p. b.i.d. 7 days 3, 10 mg/kg p.o. (single dose)	URB597: no effect on below-level cutaneous hypersensitivity (in contrast to large effect of WIN55,212-2) on days 1, 3 or 7. PF-3845: no effect of low dose, modest effect of high dose on this measure	Hama et al. (2014)

[a]CB_1 receptor functionality determined ex vivo using agonist-stimulated [^{35}S]GTPγS binding
[b]Administration route not given for the SNL data. However, the other pharmacodynamic in vivo data (on NAE levels) was with oral administration.
For clarification of classification of compounds, see legend to Table 1.
*URB937 is a peripherally restricted compound.
Abbreviations (other than those listed in Table 1): *CCI* chronic constriction injury, *i.t.* intrathecal, *PAG* periaqueductal grey, *PNL* partial sciatic nerve ligation, *RVM* rostral ventromedial medulla, *SNL* spinal nerve ligation, *STZ* streptozotocin

Table 5 Effect of eCB hydrolysis inhibitors in other pain models

Model	cpd	Dose range	Effect	References
Bone cancer pain model ♂ C3H/HeN mice	*URB597*	9 μg i.pl.	↓ mechanical hyperalgesia, blocked by co-injection of AM281 (10 μg i.pl.)	Khasabova et al. (2008)
Bone cancer pain model ♂ C3H/HeNCr MTV$^-$ mice	**JZL184**	4–40 μg i.pl.	↓ mechanical hyperalgesia at 40 μg dose, blocked by AM630 but not AM281	Khasabova et al. (2011)
Mild thermal injury-induced hypersensitivity, ♂ SD rats	*OL135*	2–50 mg/kg i.p.	Dose-dependent ↓ mechanical allodynia, clear effect at doses ≥10 mg/kg. 20 mg/kg: not blocked by rim, SR144528 or combination, but blocked by naloxone	Chang et al. (2006)
Mild thermal injury-induced hypersensitivity, ♂ C57BL/6 mice and FAAH$^{-/-}$ mice	*OL135*	100 mg/kg i.p.	↓ allodynia in C57Bl/6 mice, but not in FAAH$^{-/-}$ mice. Ibuprofen ↓ allodynia in both C57Bl/6 and FAAH$^{-/-}$ mice	Chang et al. (2006)
Mild thermal injury-induced hypersensitivity, ♂ B6. SJL-Ptprca Pep3b/BoyJ mice	*OL135*	3–100 mg/kg i.p.	Dose-dependent ↓ mechanical allodynia, clear effect at doses ≥10 mg/kg. Peak effect at $t = 30$–60 min	Palmer et al. (2008)
Mild thermal injury-induced hypersensitivity, ♂ SD rats; C57BL/6 and FAAH$^{-/-}$ mice	*JNJ-1661010* *OL135* *URB597*	2–20 mg/kg i.p. (rats) 100 mg/kg i.p. (mice) 20 mg/kg i.p. 3 mg/kg i.p.	JNJ-1661010 (6 mg/kg at $t = 30$ min; 20 mg/kg at 30–90 min) and OL135 ↓ tactile allodynia in the rats. UBR597 effect not significant. JNJ-1661010 effect not blocked by rim or SR144528, but blocked by naloxone. ↓ allodynia in wt mice at 30 min (not 1 h). This was not seen in FAAH$^{-/-}$ mice	Karbarz et al. (2009)
UV-B-induced thermal hyperalgesia, SD rats	ARN077	3–30 % topical application	10 and 30 % doses ↓ thermal hyperalgesia blocked by i.pl GW6471 (PPARα antagonist) not by i.pl. AM251 or AM630	Sasso et al. (2013)
MIA-induced arthritic pain. ♂ SD rats	*URB597*	10–50 μg spinal administration	25 and 50 μg ↓ WDR neuron responses to mechanical punctate stimuli	Sagar et al. (2010)
MIA-induced arthritic pain ♂ SD rats	*PF-04457845*	0.03 and 0.3 mg/kg p.o. for 1 or 3 days	Partial ↑ joint compression threshold in affected knee (to same level as celecoxib) at 2 and 4 h following 1 or 3 days of treatment	Ahn et al. (2011)
Collagen-induced arthritis, ♂ DBA1/J mice	*URB597*	10 mg/kg i.p.	↓ thermal hyperalgesia, blocked by rim, not SR144528	Kinsey et al. (2011a)

Stress-induced analgesia, ♂ SD rats	*URB597* ***URB602*** ***MAFP***	0.3 mg/kg i.p. 0.1 nmol into PAG 0.1 nmol into PAG 2.6 and 26 nmol into PAG	URB597 either i.p. or into dorsolateral PAG, URB602 into either dorsolateral or lateral/ventrolateral PAG or MAFP (both doses) into dorsolateral PAG ↑ tail-flick latency, blocked by rim	Hohmann et al. (2005)
Stress-induced analgesia, ♂ SD rats	*URB597* ***URB602***	1 nmol i.t. 1 nmol i.t.	↑ tail-flick latency, blocked by rim	Suplita et al. (2006)
Postoperative pain model (plantar incision surgery), ♂ Swiss mice	*URB937** **JZL184**	0.01–1 mg/kg i.p. 0.16–16 mg/kg i.p.	Both drugs ↓ mechanical hyperalgesia at the highest doses. URB597 0.1 mg/kg and JZL184 1.6 mg/kg prolonged the analgesia produced by ankle joint mobilisation (which per se is blocked by i.p. and i.th. but not i.pl. AM281)	Martins et al. (2013)
Capsaicin (i.paw.)-induced nocifensive behaviour, mechanical allodynia and thermal hyperalgesia, ♂ SD rats	*URB597* **JZL184**	5 and 75 μg i.pl. 1–100 μg i.pl.	75 μg (not 5 μg) URB597 ↓ mechanical allodynia blocked by i.pl. AM251 not i.pl. AM630. No effect upon nocifensive behaviour or thermal hyperalgesia. 100 μg (not 1 or 10 μg) JZL184 ↓ nocifensive behaviour and thermal hypersensitivity, blocked by i.pl. AM251 and AM630. Mechanical allodynia not affected by JZL184. Administration of URB597 and JZL184 to the contralateral side did not produce the changes seen after ipsilateral administration	Spradley et al. (2010)
Capsaicin (i.paw)-induced mechanical hyperalgesia, C57BL/6J mice	*URB597*	0.1–10 nmol i.t.	↑ hyperalgesia (ΔAUC for 2–4 h) at 1 nmol but not other doses. AM251 is analgesic in this model, and capsaicin does not produce hyperalgesia in $CB_1^{-/-}$ mice. 1 nmol URB without effect in naïve mice	Pernía-Andrade et al. (2009)
Nerve growth factor (NGF)-induced pain, ♂ C57BL/6J mice	*URB597* **JZL184**	1 mg/kg/ i.p. daily 8 mg/kg i.p. daily both for 6 weeks; first dose 2 weeks prior to first NGF test; NGF administered on three occasions in a 23-day time frame	In control animals, no effects of either compound on mechanical analgesia. In animals subjected to chronic unpredictable stress, the animals had a lower threshold and were not responsive to NGF; both URB597- and JZL184-treated animals showed higher withdrawal thresholds than vehicle-treated animals	Lomazzo et al. (2015)

For explanation of classification of compounds, see legend to Table 1.
Abbreviations (other than those listed in Table 1): *MIA* monosodium iodoacetate, given intra-articularly

(either continuous or paroxysmal), a feature that is not generally explored in animal studies". Whiteside et al. (2013) have argued there is a large variation in the predictive validity for different animal models. This may contribute to the failure of PF-04457845 in the only so far reported clinical trial of an FAAH inhibitor in pain (Huggins et al. 2012, see Box 1).[3] These is thus a clear need for more preclinical data on the potential of inhibitors of eCB metabolism in models that more closely resemble the human condition, difficult though this task may be. In this respect, a very recent study using the monosodium iodoacetate model of osteoarthritis in rats, where the end-point was reversal of the deficit in spontaneous burrowing behaviour, the FAAH inhibitor PF-04457845 was ineffective (Bryden et al. 2015).

Box 1 Clinical Trial of PF-04457845 in Patients with Osteoarthritic Pain (Huggins et al. 2012)

Patients diagnosed with osteoarthritis of the knee with X-ray confirmation within the previous year were either given the active drug (4 mg q.d. PF-04457845 or 500 mg b.i.d. naproxen) for two weeks and then placebo following a washout period or given the placebo first followed by the active drug (protocol: ClinicalTrials.gov NCT00981357). The baseline Hospital and Anxiety Depression Scale scores were the same for the different groups, and exclusion criteria included body mass index >40 kg/m^2 and concurrent cardiovascular and psychiatric disorders. The primary endpoint was the pain subscale of the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). The study was designed with decision rules to limit the number of patients required, based upon prior probabilities obtained with previous studies with naproxen. The key figure to be met either in interim or in final analyses was ≥ 80 % certainty that PF-04457845 gave a reduction in the WOMAC pain score >1.8 units. The number of patients who received placebo (as one arm of the crossover), PF-04457845 and naproxen were 70, 37 and 36, respectively. FAAH inhibition by PF-04457845 was almost complete, and robust increases in AEA, OEA, PEA and linoleoyl ethanolamide were seen (presumably lymphocyte and plasma measures, respectively, although it is not explicitly stated in the paper), and the drug was well tolerated. For the full analysis set, the difference in WOMAC scores between PF-04457845 and the corresponding placebos was 0.04 (80 % CI -0.63, 0.71). Stiffness and physical function measures were also not affected, whereas all the measures

(continued)

[3] To my knowledge, the only other completed efficacy study of an FAAH inhibitor in patients with pain was a compound from Ironwood Pharmaceuticals, a single dose study in patients undergoing third molar extraction (ClinicalTrials.gov identifier NCT01107236). The outcome of this study has not been reported. However, cannabinoids are not that efficacious in acute clinical pain (Beaulieu and Ware 2007), and the compound is not mentioned as far as I can see on the Ironwood website (as of May 2014) so the auguries are not good.

were reduced by naproxen. It should be noted, however, that naproxen only reached −1.13 (80 % CI −1.79, −0.47), i.e. also under the key figure of >1.8 units set in the decision rules. Patients were also allowed rescue medication, if needed, and 59 % of the PF-04457845 patients (the same percentage as seen for the placebo arms of the trial) declared that they had utilised this possibility. The corresponding percentage for naproxen was 39 %. The authors concluded that the trial "found that in OA patients with chronic pain, naproxen but not PF-04457845 demonstrated analgesic activity according to predefined decision criteria".

In mice, injection of the mast cell degranulating agent compound 48/80 elicits a scratching response in mice, reduced by URB597 and OL135. The scratching behaviour is also lower in $FAAH^{-/-}$ mice than for $FAAH^{+/+}$ mice but not for $FAAH^{NS}$ mice, suggesting a neural effect. The effect of URB597 (and the reduced behaviour in the $FAAH^{+/+}$ mice) was blocked by rimonabant (Schlosburg et al. 2009). Scratching behaviour in mice and rats following intradermal serotonin administration into the rostral part of the back is reduced by both URB597 and JZL184 (Spradley et al. 2012; Tosun et al. 2014). In contrast, the two compounds increased, rather than decreased, the scratching behaviour following serotonin injection into the cheek of the rats (Spradley et al. 2012).

3.3 Inflammation

Carrageenan instillation into the paw not only results in hyperalgesia and allodynia but also a pronounced oedema. Both genetic deletion of FAAH and systemic administration of FAAH and/or MGL inhibitors reduce the oedema (Lichtman et al. 2004b; Holt et al. 2005; Comelli et al. 2007; Clapper et al. 2010; Costa et al. 2010; Naidu et al. 2010; Sasso et al. 2012; Ghosh et al. 2013; Maione et al. 2013; but see Okine et al. 2012 and Woodhams et al. 2012). URB597 is not efficacious when given locally (Jhaveri et al. 2008; Sagar et al. 2008), and neither JZL184 nor URB602 given into the paw reduces the oedema produced by formalin injection at doses which are antinociceptive in this model (Guindon et al. 2011). I.p. lipopolysaccharide (LPS) also produces neuroinflammatory responses in the mouse brain, and these are reduced by pretreatment with JZL184. The effect of the compound upon the LPS-induced increase in cytokine levels is not prevented by either rimonabant or AM630 and may be secondary to the drop in prostaglandin levels produced by inhibition of 2-AG hydrolysis to their precursor, arachidonic acid (Nomura et al. 2011).

The eCB system plays important regulatory roles in the gastrointestinal tract. Colitis produced by 2,4-dinitrobenzene sulphonic acid (DNBS) or by dextran sulphate sodium is more severe in mice treated with rimonabant or in mice lacking CB_1 receptors and less severe in $FAAH^{-/-}$ mice than in $FAAH^{+/+}$ animals (Massa

et al. 2004). A partial reduction of colitis following FAAH inhibition either with URB597, with PF-3845 or with a novel compound (compound 39) is seen in the 2,4,6-trinitrobenzene sulphonic acid (TNBS) model (Storr et al. 2008; Andrzejak et al. 2011; Sałaga et al. 2014). A similar result was seen with JZL184, an effect blocked by both rimonabant and AM630 (Alhouayek et al. 2011). The effects of PF-3845 upon the colon macroscopic score and ulcer score were blocked by AM251 but not AM630 (Sałaga et al. 2014), and the effects of URB597 were not seen in $CB_1^{-/-}$ or $CB_2^{-/-}$ mice (Storr et al. 2008). The peripherally restricted CB receptor agonist SAB378 was not efficacious in either the TNBS or the dextran sulphate sodium model (Cluny et al. 2010). Positive results have been reported for PF-3845 given either i.p. or i.c.v. in stress-, neostigmine- and mustard oil-induced hypermotility in the mouse (Fichna et al. 2014), but not in dextran sulphate sodium-induced colitis (Sałaga et al. 2014). Systemic (i.p.) administration of LPS causes an increased upper gastrointestinal transit and total stool weight that is blocked by the FAAH inhibitor AM3506 in an AM251-sensitive manner (Bashashati et al. 2012). This route of LPS administration increases the mRNA for the inflammatory cytokine IL-1ß in the cerebellum and the lung, and these increases are reduced by prior treatment with the ABHD6 inhibitor WWL70 (Alhouayek et al. 2013). I.v. administration of LPS increases the number of leukocytes adhering to intestinal post-capillary venules, and this is prevented by URB597 in a manner blocked by AM630 (Kianian et al. 2013). Thus, in a wide range of acute inflammatory models, inhibitors of eCB hydrolysis produce beneficial effects. Whether such effects translate into clinically useful actions awaits elucidation. The same is true for the effects of FAAH inhibitors in collagen-induced arthritis (Kinsey et al. 2011a), in bladder overactivity secondary to either intravesical prostaglandin E_2 treatment or partial urethral obstruction (Füllhase et al. 2013; Gandaglia et al. 2014), and for the apparent beneficial effect of JZL184 upon LPS-induced lung injury (Costola-de-Souza et al. 2013).

Local application of the NAAA inhibitor (S)-OOPP to carrageenan-containing sponges implanted under the skin of mice reduced neutrophil migration in response to the carrageenan in $PPAR\alpha^{+/+}$ but not $PPAR\alpha^{-/-}$ mice, an effect mimicked by PEA (Solorzano et al. 2009). Local application of the NAAA inhibitor ARN077 also reduces the oedema produced by carrageenan injection into the paw. The effect of the topical administration of ARN077 is blocked by the PPARα antagonist GW6471, but not by AM251 or AM630 (Sasso et al. 2013). Topical application of ARN077 also reduces 12-O-tetradecanoylphorbol 13-acetate-induced ear oedema (Sasso et al. 2013).

Both FAAH and MGL inhibitors have been found to reduce the incidence of gastric haemorrhaging and/or ulceration in mice treated with either diclofenac sodium or indomethacin (Naidu et al. 2009; Kinsey et al. 2011b; Sasso et al. 2012; Ignatowska-Jankowska et al. 2014). This is unlikely to be the result of a pharmacokinetic interaction, since URB597 and JZL184 do not produce gastroprotective effects in $CB_1^{-/-}$ mice (whereas they still do in $CB_2^{-/-}$ mice), since the effects of JZL184 and KML29 are blocked by rimonabant but not by SR144528 and since the ulcerative propensity of diclofenac sodium is lower in

$FAAH^{-/-}$ mice than in $FAAH^{+/+}$ mice (Naidu et al. 2009; Kinsey et al. 2011b; Ignatowska-Jankowska et al. 2014). The gastroprotective property of JZL184 underwent tolerance following repeated dosing of 40 mg/kg (but not 4 and 8 mg/kg) i.p. for six days (Kinsey et al. 2013).

3.4 Other Potential Indications

Given the multitude of functions of the eCB system in the brain, it is not surprising that eCB inhibitors have been investigated in models of neurological and psychiatric disorders as well as in cancer. For reasons of space, I have not considered these indications. However, a brief analysis of the available data would suggest that some of these indications are of potential interest for drug development, the main caveats being the predictive validity of the animal models in question and the focus, as above, upon male animals in the studies. A placebo-controlled phase 2 trial with PF-04457845 and patients with Tourette syndrome is currently recruiting with improvement in tic severity as primary outcome measure (ClinicalTrials.gov identifier NCT02134080), as is a phase 2 study investigating the potential of this compound for the treatment of cannabis withdrawal (ClinicalTrials.gov Identifier: NCT0 1618656), and so hopefully, information will become available concerning the clinical potential of FAAH inhibitors in these disorders.

4 Oxygenating Enzymes (COX-2)

The ability to inhibit COX underlies the therapeutic action of NSAIDs such as the profens (Vane 1971). However, the eCB system is involved in the antinociceptive effects produced in animals by spinally administered NSAIDs (Gühring et al. 2002; Ates et al. 2003; Staniaszek et al. 2010; Telleria-Diaz et al. 2010). In the formalin test, locally administered ibuprofen potentiates the effect of locally administered AEA (Guindon et al. 2006). The profens are racemates, and their ability to block arachidonic acid oxygenation is associated with the (*S*)-enantiomers. However, (*R*)-flurbiprofen is analgesic in humans with modest enantioconversion in vivo and has a better gastrointestinal safety profile than the (*S*)-enantiomer (Lötsch et al. 1995; Holzer et al. 2001). Bishay et al. (2010) found that systemically administered (*R*)-flurbiprofen alleviated mechanical hyperalgesia, cold allodynia and cold hyperalgesia in the spared nerve injury model of neuropathic pain accompanied by a normalisation of AEA levels in the dorsal root ganglia, dorsal horn and forebrain. (*R*)-flurbiprofen inhibits FAAH (Fowler et al. 1999), but a key mechanism of action is its ability to block the oxygenation of eCBs at lower concentrations than are required for inhibition of arachidonic acid (Duggan et al. 2011). This is also the case for an analogue of indomethacin, LM-4131, which increases brain AEA and to a lesser extent 2-AG concentrations. OEA, PEA and stearoyl ethanolamide (SEA) levels are not affected. LM-4131 also increases AEA (but not 2-AG) in peripheral tissues. The compound is active in several

models of anxiety, but does not affect brain arachidonic acid or prostaglandin levels. Further, it does not produce gastrointestinal haemorrhages or produce cannabimimetic-like effects on body temperature, catalepsy or thermal nociception (Hermanson et al. 2013).

5 Designed Multiple Ligands

The failure of the FAAH inhibitor PF-04457845 in a clinical trial of osteoarthritis is naturally a setback for the field, and it has been argued above that this may be the result of a choice of indication where preclinical data is not sufficiently predictive. However, an additional explanation may be that upon blockade of FAAH, AEA and/or other NAEs are metabolised by alternative pathways. Benson et al. (2014) have modelled the expected changes in plasma NAE levels following inhibition of FAAH by a single dose of 10 mg of PF-04457485. They found that a model whereby FAAH inhibition was the only route of AEA clearance did not adequately describe the observed data. In other words, upon inhibition of FAAH by this compound, an additional enzyme (or process) becomes an important route of AEA metabolism (Benson et al. 2014). The fact that PEA was also inadequately modelled resulted in the suggestion by the authors that NAAA may be "the most likely FAAH-independent route" (Benson et al. 2014).

The conclusion from the study above underlines an important point, namely, that compounds with multiple actions may be more useful than highly selective FAAH (or MGL) inhibitors. Current possibilities include the combination of FAAH inhibitors with TRPV1 antagonist, prostamide $F_{2\alpha}$ receptor antagonist or substrate-selective COX-2 inhibition (see reviews by Fowler et al. 2009; Starowicz and Di Marzo 2013).

The most well-studied FAAH/TRPV1 dual-action compound is arachidonoyl-serotonin (AA-5-HT), which is active in models of inflammatory, persistent (formalin) and neuropathic pain (Ortar et al. 2007; Maione et al. 2007; Costa et al. 2010). The compound also reduces gastrointestinal transit time (Capasso et al. 2005), and colitis produced by DNBS is reduced, but not completely reversed, by AA-5-HT (D'Argenio et al. 2006). The concept of an FAAH inhibitor cum prostamide $F_{2\alpha}$ receptor antagonist is based on the tenet that the beneficial effects of FAAH inhibition may be mitigated by COX-2-derived prostamide $F_{2\alpha}$ production. COX-2-derived eCB metabolites have a number of actions, with respect to both inflammation and pain (Gatta et al. 2012; Alhouayek and Muccioli 2014), and both AGN21135 and AGN21136, which block FAAH and prostamide $F_{2\alpha}$ receptors, reduce nocifensive behaviour in the second phase of the formalin test (Ligresti et al. 2014). The compounds have similar potencies at TP receptors as at the prostamide $F_{2\alpha}$ receptors (Ligresti et al. 2014), but this is probably not a confounding factor in view of the fact that $TP^{-/-}$ mice have a normal phenotype in the formalin test (Popp et al. 2009).

Synergistic/enhanced effects of FAAH inhibitors and NSAIDs in models of inflammatory, visceral and neuropathic pain have been reported (Naidu

et al. 2009; Grim et al. 2014). When combined with the ability of FAAH inhibitors to reduce NSAID-induced gastrointestinal damage (see above), the notion of a combined FAAH-eCB-selective COX inhibitor is an attractive one, albeit one subject to the same hurdles of translational predictivity as the other compounds described in this review. Such compounds are starting to appear, and ibu-am5 has both antinociceptive activity in a visceral pain model and a low ulcerogenic activity. However, involvement of CB receptors in the antinociceptive effect of this compound could not be demonstrated (Cocco et al. 2003; Fowler et al. 2013), and so further optimisation may be needed. Most recently, ARN2508, a compound incorporating elements of URB597 and flurbiprofen, has been described. The compound inhibits both FAAH and COX and reduces TNBS-induced colon inflammation in mice whilst protecting against flurbiprofen-induced gastrointestinal damage (Sasso et al. 2015).

6 Conclusions

The present review has discussed the potential for drug development of compounds affecting eCB metabolism. The predictive usefulness of the animal models is an issue, as is the gender bias of the studies. The use of young animals for disorders that more commonly affect the aged may also be misleading (see Bishay et al. 2013).

Throughout the review, the focus has been on compounds enhancing eCB signalling, but there are of course situations where a reduction of eCB signalling by blockade of eCB synthesis may be better (for a review, see Di Marzo 2008). Most work in this area has focussed upon identifying the eCBs involved in physiological processes such as retrograde signalling, neuronal development (see review by Oudin et al. 2011), pain (Ho et al. 2011; see also Gregg et al. 2012) and food intake (Bisogno et al. 2009a, 2013). However, blockade of eCB synthesis may also be neuroprotective in certain circumstances (Valdeolivas et al. 2013).

Attrition rates in the clinical development of drugs are high, particularly during phases 1 and 2. Lack of efficacy is an important reason for drug discontinuation, but others include clinical safety, toxicology and commercial considerations (Kola and Landis 2004). Little is known about the long-term consequences of treatment with eCB-modulating agents of the sort discussed here, other than a study investigating the effects of perinatal treatment with URB597 upon behavioural patterns of the offspring (Wu et al. 2014). It is thus prudent to be cautious in considering the therapeutic value of compounds with global effects on the breakdown not only of the eCBs but on related endogenous substrates with biological actions of their own. Will the development of inhibitors of eCB hydrolysis be thwarted by unwanted effects on food intake (Soria-Gómez et al. 2007), memory (Busquets-Garcia et al. 2011), alcohol consumption (Basavarajappa et al. 2006; Hansson et al. 2007; Vinod et al. 2008 but see Cipitelli et al. 2008), insulin resistance (Brown et al. 2012) and/or clearance of triglyceride-rich lipoproteins (Ruby et al. 2008) [examples are for FAAH]? Will the failure of rimonabant, of

PF-04457845 in the osteoarthritic pain clinical trial, and the lack of clinical progress with CB_2 receptor agonists in the pain field result in a loss of interest in eCB-based medicines? Finally, will COX-2-based approaches be thwarted by a reticence to target this enzyme in the post-Vioxx era? Time will tell.

Acknowledgements The author thanks the Swedish Science Council (Grant no. 12158, medicine) and the Research Funds of the Medical Faculty, Umeå University, for their financial support of his research.

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Genetic Manipulation of the Endocannabinoid System

Andreas Zimmer

Contents

Abstract

The physiological and pathophysiological functions of the endocannabinoid system have been studied extensively using transgenic and targeted knockout mouse models. The first gene deletions of the cannabinoid CB_1 receptor were described in the late 1990s, soon followed by CB_2 and FAAH mutations in early 2000. These mouse models helped to elucidate the fundamental role of endocannabinoids as retrograde transmitters in the CNS and in the discovery of many unexpected endocannabinoid functions, for example, in the skin, bone and liver. We now have knockout mouse models for almost every receptor and enzyme of the endocannabinoid system. Conditional mutant mice were mostly developed for the CB_1 receptor, which is widely expressed on many different neurons, astrocytes and microglia, as well as on many cells outside the CNS. These mouse strains include "floxed" CB_1 alleles and mice with a conditional re-expression of CB_1.

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology
231, DOI 10.1007/978-3-319-20825-1_5

The availability of these mice made it possible to decipher the function of CB_1 in specific neuronal circuits and cell populations or to discriminate between central and peripheral effects. Many of the genetic mouse models were also used in combination with viral expression systems. The purpose of this review is to provide a comprehensive overview of the existing genetic models and to summarize some of the most important discoveries that were made with these animals.

Keywords
Conditional knockouts • Genetic disease models • Transgenic mice • Viral expression systems

Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	Anandamide
bp	Base pairs
CMV	Cytomegalovirus
CNS	Central nervous system
DAGL	Diacylglycerol lipase
DNBS	Dinitrobenzene sulfonic acid
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
ES cells	Embryonic stem cells
FAAH	Fatty acid amide hydrolase
frt	FLP recombinase recognition target
GDE1	Glycerophosphodiester phosphodiesterase 1
LTP	Long-term potentiation
mPFC	Medial prefrontal cortex
mRNA	Messenger RNA
NAE	*N*-Acylethanolamine
NAPE-PLD	*N*-Acyl phosphatidylethanolamine phospholipase D
NAPEs	*N*-Acyl phosphatidylethanolamines
neo	Geneticin-resistance gene (aminoglycoside phosphotransferase)
NKT cell	Natural killer T cell
PGK	Phosphoglycerokinase
RNA	Ribonucleic acid
siRNA	Small interfering RNA
THC	Tetrahydrocannabinol
tTA	Tetracycline-dependent transcriptional activator

1 Introduction

Genetic animal models, including transgenic mice and mice with targeted gene deletions (knockouts), are instrumental in the analysis of gene functions and pathomechanisms. Basically, knockout mice are good for two things: as a discovery tool and to test specific hypotheses. They are discovery tools, because mouse mutants often reveal hitherto unknown gene functions by their phenotype. Several phenotyping centres, such as the German Mouse Clinic, have capitalized on the discovery potential of these genetically manipulated animals, by using the combined expertise of specialists in different fields to comprehensively search for novel molecular, physiological and behavioural changes (Fuchs et al. 2012). There are numerous instances where unexpected phenotypes in mutant mouse strains have opened up completely novel avenues of research. Initially, research on cannabinoids was largely driven by neuroscience questions, due to the profound effects of cannabinoids on the central nervous system (CNS). Today, we know that endocannabinoid signalling affects the physiology and pathology of many organs and physiological systems. This growth of the field would not have been possible without the discovery of relevant phenotypes in the mutant mouse models.

The second important function of mouse mutants is the testing of specific hypotheses. In the cannabinoid field, one of the first hypotheses to be addressed with knockouts was related to the molecular target of cannabinoids. Thus, the discovery of CB_1 and the elucidation of its expression pattern suggested that this receptor mediates most of the effects of THC. However, only knockout mice provided an unequivocal demonstration that this is indeed the case, because most of the effects of THC were not present in mice lacking CB_1 or CB_2. The issue of receptor specificity has not been resolved entirely, however, because several reports also demonstrated residual effects of cannabinoids in receptor knockout mice (Sect. 2.1).

As shown below (Sect. 2.1), most of the mutations that affect gene function globally were obtained by homologous recombination in embryonic stem (ES) cells, where a critical part of the target gene was removed or replaced by a "selection cassette", which encodes an antibiotic resistance gene to select genetically modified ES cell clones (Zimmer 1992). Cell- or tissue-specific mutant mice were generated for several cannabinoid genes by introducing two loxP sequences, which are recognition sites for the Cre recombinase from bacteriophage P1, upstream and downstream of essential exons. Alleles carrying such loxP sequences are referred to as "floxed" alleles. When a Cre enzyme is introduced into cells with a floxed allele, for example, by transgenic expression of Cre under a cell-type-specific promoter, it will mediate a recombination event between the two loxP sites. If they have a head-to-head orientation, the recombination event will result in an inversion of the sequence. If they have a head-to-tail orientation, which is more common, Cre-mediated recombination will remove the loxP-flanked sequence, leaving only one loxP element behind (Gavériaux-Ruff and Kieffer 2007).

This review describes different genetic mouse models that have been generated to probe the endocannabinoid system. I also briefly summarize the main

physiological, pharmacological and behavioural consequences of the genetic manipulations. Please note that the strain designations used in this review do not correspond to the official strain nomenclature. The adaptation was made in an attempt to simplify the strain names as much as possible in order to enhance the readability of the manuscript.

2 CB_1 Receptor

The CB_1 receptor gene, *Cnr1*, is located on mouse chromosome 4 at position 16.28 cM (position 33,924,593–33,948,831, + strand). The entire coding region is contained within a single exon, but *Cnr1* splice variants with different untranslated 5′ exons have been described. Several genetic animal models have been developed to delete the receptor either globally or in specific cell populations.

2.1 Global CB_1 Deletions

Several labs have independently generated mouse strains with a globally disrupted *Cnr1* gene (Table 1). The first report was published by Ledent and co-workers in 1999 (Ledent et al. 1999). In their targeting strategy, they replaced the first 233 codons of the gene with a PGK-neo (phosphoglycerokinase promoter driving aminoglycoside phosphotransferase expression) cassette in ES cells from a 129 mouse strain. The mutant mice (CB_1-KO^{Led}) were then crossed to outbreed CD1 mice, thus resulting in a genetically heterogeneous mouse stock with a *Cnr1* deletion. Maintaining a mutant allele on an outbred genetic background can offer advantages, because an outbred population may reflect the genetic diversity of the human population more appropriately than an inbred strain. However, it should be noted that such outbred stocks also create problems (Chia et al. 2005). They are genetically not well defined and require large breeding colonies to maintain the genetic heterogeneity. Without appropriate genetic quality control and breeding concepts, it is difficult to avoid founder effects, which refer to the manifestation of specific phenotypic features in a small genetically isolated population. It is thus likely that the genetic heterogeneity differs substantially between the different breeding colonies of this knockout strain. The genetic background of these mutant mice could have therefore differed from one study to the next. It is also not always clear from the description of the breeding strategies in the corresponding publications if knockouts and wild-type controls were littermates and thus had the same heterogeneous genetic background.

The first publication about the CB_1-KO^{Led} line was closely followed by the description of another mutant mouse strain, in which the CB_1 coding region was disrupted after amino acid 32 with a neo cassette (Steiner et al. 1999; Zimmer et al. 1999). These mutant mice (CB_1-KO^{Zim}) were backcrossed to C57BL/6J animals for more than ten generations, thus resulting in mutation on a pure congenic C57BL/6J background. The laboratory of Beat Lutz recently published another

Table 1 Global CB_1 deletions or mutation

Strain designation	Genetic background	Phenotypes and systems studied	References
CB_1-KO^{Map}	Unique CD1 derived	Response to cannabinoids	Ledent et al. (1999), Derkinderen et al. (2001), Haller et al. (2002), Köfalvi et al. (2003), Köfalvi et al. (2005), Takahashi and Castillo (2006), Mato et al. (2009), Yoshida et al. (2010)
		Behavioural phenotypes	Ledent et al. (1999), Maccarrone et al. (2001), Martin et al. (2002), Maccarrone et al. (2002), Haller et al. (2002), Haller et al. (2004a), Urigüen et al. (2004), Haller et al. (2004b), Jardinaud et al. (2005), Aso et al. (2008), Thiemann et al. (2009), Schechter et al. (2013), Rodriguez-Arias et al. (2013), Burokas et al. (2014)
		Neuronal development, transmitter physiology and synaptic plasticity	Böhme et al. (2000), Hájos et al. (2001), Maccarrone et al. (2001), Varma et al. (2001), Jin et al. (2004), Urigüen et al. (2005), Mato et al. (2007), Aso et al. (2009), Álvaro-Bartolomé et al. (2010), Madroñal et al. (2012), Straiker et al. (2012), Atwood et al. (2012), Keimpema et al. (2013), Sylantyev et al. (2013), Schechter et al. (2013)
		Learning and memory	Martin et al. (2002), Thiemann et al. (2007), Madroñal et al. (2012)
		Reward, drug effects and addiction	Ledent et al. (1999), Cossu et al.(2001), Castañé et al.(2002), Naassila et al.(2004), Houchi et al.(2005), Thanos et al.(2005), Ballesteros-Yáñez et al. (2007), Corbillé et al. (2007), Touriño et al. (2008), Guegan et al.

(continued)

Table 1 (continued)

Strain designation	Genetic background	Phenotypes and systems studied	References
			(2013), Álvaro-Bartolomé and García-Sevilla(2013)
		Pain	(Valverde et al.(2000), Castañé et al.(2006), Pol et al.(2006), Paldy et al.(2008), La Porta et al.(2013), Deng et al.(2014)
		Neuroinflammation and degeneration	Parmentier-Batteur et al.(2002), Jackson et al.(2005), Bilsland et al.(2006), Pérez-Rial et al.(2011), Mievis et al.(2011), Cutando et al.(2013)
		Reproductive system and embryo development	Wang et al.(2003a), Ricci et al.(2007), Cobellis et al.(2010), Chioccarelli et al.(2010), Cacciola et al.(2013)
		Liver and metabolism	Teixeira-Clerc et al.(2006), Wasmuth and Trautwein(2007), Kola et al.(2008), Jourdan et al.(2013), Malenczyk et al.(2013), Kola et al.(2013)
		Bone	Idris et al.(2005), Tam et al.(2006), Idris et al.(2009)
CB_1-KO^{Zim}	C57BL/6J	Response to cannabinoids	Zimmer et al. (1999), Jarai et al. (1999), Selley et al.(2001), Breivogel et al.(2001), Varvel and Lichtman(2002), Lichtman et al.(2002), Fride et al.(2003), Baskfield et al.(2004), Takahashi and Castillo(2006), Navarrete and Araque(2008), Sibaev et al.(2009), Brusberg et al.(2009), Sain et al.(2009), Tam et al.(2010), Hegde et al.(2010), Song et al.(2011), Xiong et al.

(continued)

Table 1 (continued)

Strain designation	Genetic background	Phenotypes and systems studied	References
			(2012), Lazenka et al. (2014)
		Behavioural phenotypes	Steiner et al. (1999), Zimmer et al. (1999), Degroot and Nomikos (2004), Pava et al.(2014), Friemel et al.(2014)
		Synaptic plasticity and transmitter physiology	Kathmann et al.(2001), Wilson et al.(2001), Yoshida et al.(2002), Gerdeman et al.(2002), Ohno-Shosaku et al. (2002), Safo and Regehr (2005), Grueter et al. (2006), Carey et al.(2011), Chen et al.(2011), Schulte et al.(2012), Péterfi et al. (2012)
		Learning and memory	Varvel and Lichtman (2002), Varvel et al. (2005), Degroot et al. (2005), Bilkei-Gorzo et al. (2005), Kishimoto and Kano(2006), Crombag et al.(2010), Albayram et al.(2011), Albayram et al.(2012), Zádor et al. (2012)
		Reward, drug effects and addiction	Lichtman et al.(2001), Hungund et al.(2003), Wang et al. (2003b), Racz et al. (2003), Xi et al. (2011), Guegan et al. (2012), Hiranita et al. (2014), Nagre et al. (2014)
		Pain	Zimmer et al. (1999), Fioravanti et al. (2008), Brusberg et al. (2009), Sain et al. (2009), Xiong et al. (2012)
		Neuroinflammation and degeneration	Panikashvili et al. (2005), Touriño et al. (2010), Albayram et al. (2011), Piyanova et al. (2013)
		Development and cancer	Steiner et al. (1999), Gerald et al. (2006), Wolf et al. (2010), Jiang et al. (2010), Baireddy

(continued)

Table 1 (continued)

Strain designation	Genetic background	Phenotypes and systems studied	References
			et al. (2011), Marshall et al. (2011), Bilkei-Gorzó et al. (2012)
		Reproductive system and embryo development	Paria et al. (2001), Fride et al. (2003), Wang et al. (2004), Wang et al. (2008b), Sun et al. (2009), Sun et al. (2010), Xie et al. (2012)
		Liver, metabolism and food intake	Di Marzo et al. (2001), Jeong et al. (2008), Osei-Hyiaman et al. (2008), Ruby et al. (2008), Kim et al. (2011), Mukhopadhyay et al. (2011a), Song et al. (2011), Liu et al. (2012), Liu et al. (2013), Bowles et al. (2014)
		Bone	Tam et al. (2006), Tam et al. (2008), Bab et al. (2008), Wasserman et al. (2015)
		Cardiovascular system	Jarai et al. (1999), Jarai et al. (2000), Szekeres et al. (2012, Molica et al. (2013)
		Gastrointestinal system	Carai et al. (2006), Wang et al. (2008a), Sibaev et al. (2009), Gyires et al. (2014)
		Skin	Karsak et al. (2007), Bilkei-Gorzó et al. (2012), Sugawara et al. (2012), Gaffal et al. (2013)
CB_1-KO^{Ltz}	C57BL/6N	Response to cannabinoids	Azad et al. (2003), Agarwal et al. (2007), Monory et al. (2007), Azad et al. (2008), Bénard et al. (2012), Vallée et al. (2014)
		Behavioural phenotypes	Marsicano et al. (2002b), Cannich et al. (2004), Cota et al. (2007), Kamprath et al. (2009), Plendl and Wotjak (2010),

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Table 1 (continued)

Strain designation	Genetic background	Phenotypes and systems studied	References
			Dubreucq et al. (2010), Metna-Laurent et al. (2012), Dubreucq et al. (2012b), Jacob et al. (2012), Pava et al. (2014), Silvani et al. (2014b)
		Synaptic plasticity	Marsicano et al. (2002b), Azad et al. (2003), Azad et al. (2004), Azad et al. (2008), Lourenço et al. (2010), Navarrete and Araque (2010), Bénard et al. (2012), Kato et al. (2012), Glangetas et al. (2013)
		Learning and memory	Marsicano et al. (2002b), Hölter et al. (2005), Kamprath et al. (2006), Plendl and Wotjak (2010)
		Drug effects and addiction	Sanchis-Segura et al. (2004), Melis et al. (2014)
		Pain	Agarwal et al. (2007), Pernía-Andrade et al. (2009), Duncan et al. (2013), Desroches et al. (2014b), Desroches et al. (2014a)
		Neuroinflammation and degeneration	Marsicano et al. (2002a), Marsicano et al. (2003), Khaspekov et al. (2004), Maresz et al. (2007)
		Neuronal development and transmitter physiology	Aguado et al. (2006), Deshmukh et al. (2007), Cota et al. (2007), Aguado et al. (2007), Mulder et al. (2008), Trazzi et al. (2010), Dubreucq et al. (2010), Wu et al. (2010), Díaz-Alonso et al. (2012), Fitzgerald et al. (2012), Tortoriello et al. (2014)
		Liver and metabolism	Cota et al. (2003), Tedesco et al. (2008), Bellocchio et al. (2010),

(continued)

Table 1 (continued)

Strain designation	Genetic background	Phenotypes and systems studied	References
			Quarta et al. (2010), Massa et al. (2010), Cluny et al. (2010)
		Cardiovascular, gastrointestinal system, bladder	Massa et al. (2004), Capasso et al. (2005), Li et al. (2013b), Silvani et al. (2014a), Füllhase et al. (2014)
CB_1-$Stop^{Ltz}$	C57BL/6J	Behavioural phenotypes	Ruehle et al. (2013), Soria-Gómez et al. (2014)
CB_1-KO^{Ojm}	C67BL/6	Synaptic plasticity	Robbe et al. (2002)
		Behavioural phenotypes	Ravinet Trillou et al. (2003), Poncelet et al. (2003)
		Metabolism	Ravinet Trillou et al. (2004)
CB_1-KO^{Dgen}	129SvJ	Response to cannabinoids	Ibrahim et al. (2003), Roberts et al. (2014)
		Synaptic plasticity	Pan et al. (2008b), Pan et al. (2008a)
CB_1-KO^{Pch}	C57BL/6 × 129	None reported	Vianna et al. (2012)
CB_1-KO^{Lex}	C57BL6/J × 129/SvEvBrd	Metabolism	Brommage et al. (2008)
Unclear origin		Synaptic plasticity, cardiac functions, muscle development, ethanol effects, effects of JWH-081, gastrointestinal functions	Rajesh et al. (2012), Xu et al. (2012), Subbanna et al. (2013), Chen et al. (2013), Fichna et al. (2014), Basavarajappa and Subbanna (2014), Iannotti et al. (2014), Basavarajappa et al. (2014)
CB_1-S426A/S430A-KI	C57BL/6J	More sensitive to THC, delayed tolerance, increased dependence	Morgan et al. (2014)

globally acting *Cnr1* mutation on a C57BL/6J genetic background (Ruehle et al. 2013). This mouse line (CB_1-$Stop^{Ltz}$) was intended for the conditioned rescue of CB_1 expression (see below) and contained a transcriptional STOP cassette flanked by loxP sequences inserted into the 5′ untranslated region of the *Cnr1* gene, just upstream of the CB_1 open reading frame. Mice homozygous for the CB_1-$STOP^{Ltz}$ allele showed much reduced CB_1 mRNA levels, but the mutation was not completely able to inhibit transcription of the *Cnr1* locus, for unknown reasons.

Nevertheless, no CB_1 protein was detected in CB_1-$STOP^{Ltz}$ mice, which therefore also represents a complete knockout.

The same laboratory had previously described a global *Cnr1* mutation on a C57BL/6N genetic background. This line, CB_1-KO^{Ltz}, was derived from a floxed allele for the conditional deletion of CB_1 (Sect. 2.2). It was crossed to ubiquitously Cre-expressing mice, thus deleting the coding region globally and subsequently to C57BL/6N animals. A further *Cnr1* mutant mouse line on a C57BL/6 background was developed by Robbe et al., who replaced parts of the 5′ untranslated region plus most of the CB_1 open reading frame with a neo cassette (Robbe et al. 2002). These mice, CB_1-KO^{Ojm}, were also backcrossed to C57BL/6, but it is not clear to which substrain of the C57BL/6 lineage (Mekada et al. 2009; Matsuo et al. 2010). C57BL/6J and C57BL/6N mice are closely related as they originate from the same parental strain established at The Jackson Laboratory, but they do display a number of important genetic, behavioural and physiological differences (Bryant et al. 2008; Mekada et al. 2009; Simon et al. 2013).

A second floxed line was generated in the laboratory of Pierre Chambon for Sanofi Aventis (Vianna et al. 2012). They were also used to generate a global gene deletion (CB_1-KO^{Pch}) on a mixed C57BL/6 × 129 genetic background. Deltagen developed a further CB_1 knockout mouse line, CB_1-KO^{Dgen} on a pure 129/SvJ genetic background by replacing the major part of the coding region with an IRES–LacZ–Neo–pA cassette (Ibrahim et al. 2003; Pan et al. 2008b; Pan et al. 2008a). Even though this cassette was probably designed to direct expression of the LacZ reporter gene under the control of the CB_1 promoter, to the best of my knowledge such an expression has never been reported. Finally, Lexicon also developed a CB_1 knockout mouse line, CB_1-KO^{Lex}, by homologous recombination on a mixed C57BL6/J × 129/SvEvBrd genetic background.

Thus, at least eight different global CB_1 knockout mouse models have been independently generated. These knockout strains differ in the nature of the mutation and the genetic background. It is therefore important to provide precise information about the mutant mouse strains used in any study. Surprisingly, several reports failed to do this (Wang et al. 2003a; Hegde et al. 2010; Marongiu et al. 2012; Xu et al. 2012; Rajesh et al. 2012; Duff et al. 2013; Keimpema et al. 2013; Desroches et al. 2014a; Iannotti et al. 2014; Chen et al. 2013; Busquets-Garcia et al. 2013), or referred to previous publications that, however, do not contain the strain information (Basavarajappa and Subbanna 2014; Basavarajappa et al. 2014).

The availability of so many mutant lines for the same gene strongly increases the confidence that shared phenotypes are really due to the invalidation of CB_1. The knockout strains and the major phenotypes, physiological functions and organ systems studied with these animals are listed in Table 1. Most studies reported that the CNS effects of THC or synthetic cannabinoid receptor agonists were mostly absent in CB_1 knockouts (please see Table 1 for references). Nevertheless, some authors also reported the persistence of neuronal cannabinoid effects in CB_1-KO mice, demonstrating that cannabinoids also act on other brain receptors. It is now known that AEA modulates the activity of membrane receptors such as TRPV1,

5-HT_3 (Racz et al. 2008) and GPR55, as well as peroxisome proliferator-activated receptors (Maccarrone et al. 2014).

CB_1-deficient mice were instrumental in probing the physiology of synaptic endocannabinoid signalling and helped to establish the role of endocannabinoids as retrograde messengers. They also highlighted the importance of CB_1 receptors for neuronal signalling, as CB_1-KO mice displayed several behavioural deficits such as increased anxiety-like behaviours and fear responses and altered cognitive functions. CB_1-KO mice were also more prone to develop depression-like behaviours. The latter phenotype already indicated that depressive episodes could be a potential side effect of medications that antagonize CB_1. Indeed, rimonabant, a CB_1 antagonist clinically used for the treatment of the metabolic syndrome, was withdrawn from the market due to its pro-depressant effects.

Interestingly, CB_1-KO mice were relatively resistant to the rewarding and addictive properties of drugs of abuse. In this respect they are similar to mice lacking μ-opioid receptors (Contet et al. 2004; Le Merrer et al. 2009). These findings indicate that the opioid and cannabinoid systems are both essential modulators of neuronal mechanisms in the addiction process. It is important to note that global CB_1 knockouts display a number of CNS developmental alterations, and deficits in adult neurogenesis, and are more prone to develop seizures. It is unclear, however, to what extend these deficits contributed to the behavioural phenotypes. Interestingly, many phenotypes of CB_1-KO mice were different in young and old animals (Maccarrone et al. 2002; Wang et al. 2003b; Bilkei-Gorzo et al. 2005; Albayram et al. 2011; Albayram et al. 2012; Bilkei-Gorzó 2012). CB_1-KO mice also showed an increased mortality (Zimmer et al. 1999) and an increased vulnerability to neuronal loss in animal models of neurodegeneration. It has therefore been suggested that brain ageing was accelerated in mice lacking CB_1 receptors (Di Marzo et al. 2014).

Considering the strong focus of the field on CNS effects of cannabinoids, it was perhaps most surprising to see that CB_1-KO animals displayed so many phenotypes outside the CNS. It is now clear that endocannabinoid signalling via CB_1 influences cardiovascular, gastrointestinal, immune, metabolic (Di Marzo et al. 2001; Cota et al. 2003; Kola et al. 2008; Tedesco et al. 2008; Osei-Hyiaman et al. 2008; Brommage et al. 2008; Bellocchio et al. 2010; Quarta et al. 2010; Massa et al. 2010; Kim et al. 2011; Kola et al. 2013; Soria-Gómez et al. 2014), liver, reproductive (Paria et al. 2001; Wang et al. 2004; Ricci et al. 2007; Wang et al. 2008b; Cobellis et al. 2010; Sun et al. 2010; Chioccarelli et al. 2010; Cacciola et al. 2013), skin, muscle (Iannotti et al. 2014) and bone (Idris et al. 2005; Tam et al. 2006; Tam et al. 2008) physiology and pathophysiology. Thus, CB_1-KO mice helped to discover novel functions of endocannabinoid signalling.

In addition to the gene deletions, the Mackie lab has recently described an allele, where two serine phosphorylation sites in the C-terminal domain have been converted into nonphosphorylatable alanines by a gene replacement strategy (Morgan et al. 2014). CB_1 protein levels in these animals were largely unchanged, but they showed altered acute responses to THC, as well as reduced tolerance and increased dependence.

2.2 Conditional CB_1 Deletions

A more detailed analysis of endocannabinoid signalling via CB_1 in specific cell populations requires genetic approaches to delete CB_1 receptors from specific brain areas or cell populations, or to specifically restore CB_1 activity at the site of interest. Both approaches have been realized using interesting genetic and viral tools. Two laboratories have independently generated floxed *Cnr1* alleles for the cell-specific deletion of ablation of the gene via Cre-mediated recombination.

Two floxed lines, CB_1-$Flox^{Ltz}$ and CB_1-$FLox^{Pch}$, were generated by the Lutz and the Chambon laboratories, respectively, using virtually identical strategies (Marsicano et al. 2003; Vianna et al. 2012). Both groups introduced one loxP site upstream of the CB_1 coding exon and another one downstream. A neo selection cassette flanked by two FLP recombinase recognition target (frt) sites was inserted at the downstream lox site for the selection of recombinant ES cell clones. This was later removed by breeding the animals with a mouse strain expressing FLP recombinase in the germ cell lineage. The final floxed allele thus contained only the two loxP sites and a single remaining frt site, which apparently did not interfere with expression. The main difference between these two lines was the genetic background, which was C57BL/6N for the CB_1-$Flox^{Ltz}$ strain and C57BL/6×129 for the CB_1-$Flox^{Pch}$ strain.

The CB_1-$Flox^{Ltz}$ line in particular has been crossed with many different Cre-expressing transgenic mouse lines to delete CB_1 in specific cell populations (see Table 2 and references therein). As CB_1 receptors are widely expressed in many neuronal populations, these mice were extremely useful for assessing the role of CB_1 signalling in specific behaviours or physiological functions in specific cell populations. Thus, CamKII–CB_1-KO^{Ltz} mice have a relatively wide deletion of CB_1 receptors in principal forebrain neurons, which include excitatory glutamatergic neurons and inhibitory GABAergic neurons (Monory et al. 2007). The deletion of CB_1 receptors from glutamatergic neurons was accomplished with mice expressing Cre under the control of a Nex promoter (Nex–CB_1-KO^{Ltz}), whereas the Dlx5/6-Cre line was used to delete CB_1 from GABAergic neurons (Dlx5/6–CB_1-KO^{Ltz}) (Monory et al. 2007). Using these different mouse strains, Monory et al. found that, surprisingly, CB_1 receptors on GABAergic neurons were of minor importance for the locomotor, cataleptic, hypothermic and analgesic effects of THC. In contrast, CamKII–CB_1-KO^{Ltz} mice showed strongly reduced responses to THC (Monory et al. 2007). Deletion of CB_1 receptors from GABAergic neurons nevertheless produced several phenotypes, such as a deficit in postsynaptic target selection by cortical interneurons (Berghuis et al. 2007), a reduced behavioural inhibition when encountering novel palatable food (Lafenêtre et al. 2009) or an increased active coping behaviour in a fear-conditioning paradigm (Metna-Laurent et al. 2012). Interestingly, similar to global CB_1-KO mice, GABA–CB_1-KO^{Ltz} animals also showed signs of accelerated brain ageing (Albayram et al. 2011). Conditional CB_1 knockout mice were also used to dissect the roles of CB_1 in peripheral systems, particularly with relevance to metabolic and

Table 2 Conditional CB_1 deletions

Strain designation	Cell types affected	Phenotypes and systems studied	References
CamKII–CB_1-KO^{Ltz}	Forebrain projection neurons	Increased kainate toxicity, longer seizure duration	Marsicano et al. (2003), Monory et al. (2006), von Rüden et al. (2015)
		WIN55,212-2 effects on glutamatergic transmission and synaptic plasticity in the amygdala absent, no CP55,940 effects on thalamocortical high-voltage spindles or fast cortical oscillations	Domenici et al. (2006), Azad et al. (2008), Sales-Carbonell et al. (2013)
		THC effects on open field activity reduced, on catalepsy, hypothermia and analgesia abolished, sustained freezing response to footshock	Monory et al. (2007), Kamprath et al. (2009)
		Lean phenotype and resistance to diet-induced obesity	Quarta et al. (2010)
Dlx5/6–CB_1-KO^{Ltz}	GABAergic neurons	Impaired postsynaptic target selection of cortical interneurons	Berghuis et al. (2007)
		Behaviour: altered locomotor activity of stressed animals, increased active coping in fear conditioning, normal forced-swim immobility, increased social interaction, no cognitive deficits after chronic THC exposure	Puighermanal et al. (2009), Metna-Laurent et al. (2012), Puighermanal et al. (2013), Häring et al. (2013), Dubreucq et al. (2013), Terzian et al. (2014)
		Feeding and metabolism: increased palatable food consumption, increased novel object interaction, less behavioural inhibition, partially resistant to diet-induced obesity	Lafenêtre et al. (2009), Bellocchio et al. (2010), Massa et al. (2010)
		Neurophysiology: no train-induced depression of inhibition, plasticity at CA3–CA1 synapse preserved, no CP55,940-induced increase of thalamocortical high-voltage spindles	Lourenço et al. (2010), Han et al. (2012), Sales-Carbonell et al. (2013)
		Neurology: accelerated brain ageing and inflammation, not sensitive to quinolinic acid	Puente et al. (2010), Albayram et al. (2011),

(continued)

Table 2 (continued)

Strain designation	Cell types affected	Phenotypes and systems studied	References
		excitotoxic damage, shorter seizure duration, mapping of CB_1 terminals in the bed nucleus of the stria terminalis	Chiarlone et al. (2014), von Rüden et al. (2015)
Nex–CB_1-KO^{Ltz}	Glutamatergic neurons	Behaviour: THC effects on open field activity, hypothermia reduced, no anxiety phenotype, reduced novel object and social exploration, sustained freezing response to footshock, increased coping in fear conditioning, reduced forced-swim immobility, reduced social interaction, decreased palatable food consumption, decreased novel object interaction, more behavioural inhibition	Monory et al. (2007), Kamprath et al. (2009), Jacob et al. (2009), Lafenêtre et al. (2009), Bellocchio et al. (2010), Metna-Laurent et al. (2012), Dubreucq et al. (2012b), Häring et al. (2013), Terzian et al. (2014)
		Neurology: deficits in neuronal progenitor proliferation, aberrant fasciculation and pathfinding in corticothalamic and thalamocortical axons, sensitive to quinolinic acid excitotoxic damage, unbalanced neurogenic fate determination, alterations in corticospinal motor neuron generation and subcerebral connectivity, normal brain ageing and inflammation	Mulder et al. (2008), Wu et al. (2010), Albayram et al. (2011), Díaz-Alonso et al. (2012), Chiarlone et al. (2014)
		Neurophysiology: plasticity at CA3–CA1 synapse preserved, acute stress-induced changes of synaptic plasticity in the bed nucleus of the stria terminalis disrupted, no CP55,940-induced fast ECoG oscillations	Han et al. (2012), Sales-Carbonell et al. (2013), Glangetas et al. (2013)
		Large reduction in CB_1–G protein signalling	Steindel et al. (2013)
D_1–CB_1-KO^{Ltz}	Neurons expressing dopamine D_1 receptors	Normal THC effects on hypothermia, reduced or abolished THC effects on catalepsy	Monory et al. (2007)

(continued)

Table 2 (continued)

Strain designation	Cell types affected	Phenotypes and systems studied	References
		Mild anhedonia, increased contextual and auditory-cued fear	Terzian et al. (2011)
		No CP55,940-induced increase of thalamocortical high-voltage spindle	Sales-Carbonell et al. (2013)
Nestin–CB_1-KO^{Ltz}	All neurons	No THC suppression of symptoms in an experimental autoimmune encephalitis model	Maresz et al. (2007)
Nav1.8–CB_1-KO^{Ltz}	Dorsal root ganglia neurons	Reduced cannabinoid analgesia, normal capsaicin secondary mechanical hyperalgesia, R-flurbiprofen antinociception partially lost	Agarwal et al. (2007), Pernía-Andrade et al. (2009), Bishay et al. (2010)
		Reduced long-term depression at dorsal horn nociceptor synapses	Kato et al. (2012)
		Normal allergic contact dermatitis	Gaffal et al. (2013)
LCK–CB_1-KO^{Ltz}	T cells	No phenotype in experimental autoimmune encephalitis	Maresz et al. (2007)
Alb–CB_1-KO^{Ltz}	Hepatocytes	Delayed liver regeneration	Mukhopadhyay et al. (2011a)
		Resistant to ethanol-induced steatosis	Jeong et al. (2008)
		High-fat diet did not induce hepatic insulin resistance	Liu et al. (2012)
		Normal dyslipidaemic effects of glucocorticoid exposure	Bowles et al. (2014)
ptf1a–CB_1-KO^{Ltz}	Doral horn interneurons	Capsaicin secondary mechanical hyperalgesia abolished	Pernía-Andrade et al. (2009)
Gabra6–CB_1-KO^{Ltz}	Cerebellar granule cells	No long-term depression and reduced short-term plasticity at parallel fibres	Carey et al. (2011)
		Enhanced cerebellar inflammation	Cutando et al. (2013)
K14–CB_1-KO^{Ltz}	Epidermal keratinocytes	Increased allergic contact dermatitis	Gaffal et al. (2013, Gaffal et al. (2014)
Gfap–CB_1-KO^{Ltz}	Astrocytes, tamoxifen inducible	Plasticity at CA3–CA1 synapse abolished	Han et al. (2012)

(continued)

Table 2 (continued)

Strain designation	Cell types affected	Phenotypes and systems studied	References
Phox2b–CB_1-KO^{Pch}	Efferent branch of the vagus nerve	Normal body weight homeostasis, increased gastrointestinal motility	Vianna et al. (2012)
Tph2–CB_1-KO^{Ltz}	Serotonergic neurons	Higher anxiety, decreased cued fear expression (freezing)	Dubreucq et al. (2012b)
Rgs9–CB_1-KO^{Ltz}	Medium spiny neurons	Cannabinoid effects on plasticity of medium spiny neuron synapses abolished	Mathur et al. (2013)
Pvalb–CB_1-KO^{Ltz}	Parvalbumin neurons	Cannabinoid modulation of synaptic plasticity altered	Mathur et al. (2013)
Sim1–CB_1-KO^{Ltz}	Hypothalamus, mediobasal amygdala	Changes in activity, unconditioned anxiety, cued fear expression and stress responses	Dubreucq et al. (2012a)
		Decreased adiposity, improved insulin sensitivity and increased energy expenditure on high-fat diet	Cardinal et al. (2014b)
SF1–CB_1-KO^{Ltz}	Hypothalamic ventromedial nucleus	Decreased adiposity by increased sympathetic activity and lipolysis, leptin resistance and adiposity on high-fat diet	Cardinal et al. (2014a)
Prph1–CB_1-KO^{Ltz}	Peripheral nerve	No effect of peripherally restricted CB_1 agonists on spasticity	Pryce et al. (2014)

inflammatory processes. Table 2 provides an overview of the different CB_1 conditional mutant mouse strains as well as major phenotypes.

In addition to deleting CB_1 from specific cells, researchers have also addressed its functional roles by re-expressing this receptor in specific cells using transgenic methods (Table 3). For example, a construct containing the CB_1 gene under the control of an albumin promoter on a global CB_1-KO genetic background resulted in a mouse line expressing CB_1 receptors exclusively on hepatocytes (Liu et al. 2012). CB_1 receptors were also re-expressed using a doxycycline-inducible system (Marongiu et al. 2012). For this purpose transgenic mice were generated, in which a tetracycline-dependent transcriptional activator (tTA) was expressed in forebrain neurons under the control of the Ca^{2+}/calmodulin-dependent kinase IIa (CamKII) promoter (IRh-CB-KO). The tTA is constitutively active, but becomes inactive in the presence of doxycycline. A second transgenic line was generated that carried a construct, in which a bidirectional cytomegalovirus (CMV) promoter was driving CB_1 expression in one direction and ß-galactosidase (lacZ) expression in the other direction. The CMV promoter can be inactivated by binding of the tTA to

Table 3 Transgenic re-expression of CB_1

Strain designation	Purpose	Phenotypes and systems studied	References
Albumin$CB_1 \times CB_1$-KO^{Zim}	Re-expression of CB_1 in hepatocytes	Hyperinsulinaemia as a result of reduced insulin clearance, increased hepatic glucose production	Liu et al. (2012)
		Rescue: high-fat diet did not induce hepatic insulin resistance	Liu et al. (2013)
Nex–CB_1-RS^{Ltz}	Re-expression of CB_1 in glutamatergic neurons	Partial rescue of anxiety phenotype in the elevated plus maze, rescue of increased kainate seizure sensitivity, rescue of depolarization-induced suppression of excitation in CA1 and DG, more sustained conditioned freezing response	Ruehle et al. (2013)
		Rescued hypophagic in fasting–re-feeding conditions	Soria-Gómez et al. (2014)
IRh-CB_1-KO	Doxycycline-inducible re-expression of CB_1 in the forebrain	Doxycycline administration decreased pre-pulse inhibition of the startle reflex	Marongiu et al. (2012)
R26-Stop-CB_1-TG	Expression of CB_1 can be activated in all cells after activation with Cre recombinase	None reported	Naydenov et al. (2014)
R26-CB_1-TG $\times$ CB_1-KO^{Ltz}	CB_1 transgene expressed in all cells, no endogenous CB1 expression	Normal CB_1 signalling, normal behavioural response to CP55,940	Naydenov et al. (2014)
PGK-CB_1-TG	Increased expression of CB_1	Proteinuria	Hsu et al. (2014)

central tetracycline operator sequences in the presence of doxycycline. Thus, the expression of lacZ and CB_1 is constitutively active in double-transgenic mice and can be reduced in forebrain neurons by doxycycline administration (Marongiu et al. 2012). The Stella lab has recently described a mouse strain (Naydenov et al. 2014), where a CB_1 open reading frame preceded by a floxed Stop cassette was inserted into a ubiquitously active Rosa26 locus (R26-Stop-CB_1-TG^{Nst}). After deleting the Stop cassette, the mice were crossed to a CB_1 knockout background. CB_1 signalling in these mice was similar to that of wild-type animals.

Finally, the mouse strain CB_1-Stop-KI^{Ltz}, already mentioned above, represents a global knockout, but can be used for the re-expression of CB_1 in specific cells using Cre-expressing transgenic mice (Ruehle et al. 2013). This strain carries a

transcriptional STOP cassette flanked by loxP sequences in the 5′ untranslated region of the *Cnr1* gene, which blocks expression. The animals homozygous for this allele showed typical phenotypes associated with the CB_1 deletion such as increased anxiety-related behaviours in the elevated plus maze, impairment of conditioned fear extinction and increased kainate sensitivity (Ruehle et al. 2013). The anxiety-related behaviours and kainate hypersensitivity were restored when these animals were bred to a mouse line expressing Cre in dorsal telencephalic glutamatergic neurons under the control of a Nex promoter (Ruehle et al. 2013). These mice also showed normal food intake after fasting, in contrast to the hyperphagia observed in global CB_1 knockouts (Soria-Gómez et al. 2014).

Besides transgenic methods, viral technologies have been used to inactivate CB_1 receptors from specific brain regions, or to re-express them in brain regions of global knockouts (Table 4). This strategy is particularly useful for brain regions where specific Cre-expressing lines are not available.

Thus, for the deletion of CB_1, an AAV1/2 was generated that expressed Cre recombinase under the control of globally active or cell-specific promoters. Injection of these viruses into the hippocampus, hypothalamus, anterior olfactory nucleus or anterior piriform cortex of mice carrying a floxed *Cnr1* allele resulted in a profound reduction of CB_1 expression in the corresponding brain regions (Monory et al. 2006; Soria-Gómez et al. 2014). The Guzman group has investigated neuronal populations in which CB_1 receptors mediate protective effects in a Huntington's disease model. Using a recombinant AAV vector expressing Cre recombinase under the control of a CamKII promoter, they were then able to delete CB_1 specifically in principal forebrain neurons. By directing the site of injection either to the striatum or the cortex, they were able to detangle the corresponding contribution of these neurons to the aggravated disease symptoms (Chiarlone et al. 2014). Finally, different lentiviral constructs were developed that expressed small hairpin ribonucleic acid (RNA) under the control of a U6 promoter targeted against different regions of CB_1 transcripts. Injection of these viral constructs into the hippocampus resulted in deficits in conditioned eye blink responses and corresponding fEPSPs that were similar to those seen in CB_1 knockout animals (Madroñal et al. 2012). Silencing of CB_1 expression by lentiviral delivery of small interfering RNA (siRNA) into the nucleus accumbens was also used in rats to investigate the role of CB_1 signalling in the rewarding effects of cocaine (Ramiro-Fuentes et al. 2010).

For the re-expression of CB_1, Metna-Laurent developed an AAV1/2 virus that expressed haemagglutinin-tagged CB_1 receptors under a chicken ß-actin promoter (Metna-Laurent et al. 2012). This vector was used to express CB_1 in the amygdala of global knockout mice, which restored the normal switch between different fear-coping strategies. Soria-Gómez also used the CB_1-$Stop^{Ltz}$ strain to restore CB_1 expression in specific olfactory circuits using an AAV-Cre virus (Soria-Gómez et al. 2014).

Table 4 Viral inactivation and re-expression of CB_1

Aim	Animals	Virus	Site of virus injection	Phenotypes and systems studied	References
CB_1 deletion	CB_1-$Flox^{Ltz}$	AAV-CBA-WPRE-Cre	Dentate gyrus, CA1, CA3	Increased kainate seizure sensitivity	Monory et al. (2006)
	CB_1-$Flox^{Ltz}$	AAV-CBA-WPRE-Cre	Hypothalamus	Decreased body weight, increased energy expenditure, leptin insensitive	Cardinal et al. (2012)
	CB_1-$Flox^{Ltz}$	AAV-CamKIIα-Cre	Anterior olfactory nucleus, anterior piriform cortex	Hypophagic phenotype in fasting–re-feeding conditions	Soria-Gómez et al. (2014)
	R6/2L × CB1-$Flox^{Ltz}$	AAV-CamKIIα-Cre	Dorsolateral striatum, motor cortex	Aggravates Huntington symptoms, rescued by MK-801	Chiarlone et al. (2014)
CB_1 silencing	C57BL/6J	Lenti-CB_1-siRNA	CA1	Reduced eyeblink conditioning and synaptic plasticity	Madroñal et al. (2012)
	Wistar rats	Lenti-CB_1-siRNA	Nucleus accumbens	Place aversion to cocaine	Ramiro-Fuentes et al. (2010)
CB_1 rescue	CB_1-$Stop^{Ltz}$	AAV-CamKIIα-Cre	Anterior olfactory nucleus, anterior piriform cortex	Partially rescued hypophagic in fasting–re-feeding conditions	Soria-Gómez et al. (2014)
CB_1 expression	CB_1-KO^{Ltz}	AAV-CBA-WPRE-CB_1	Amygdala	Restored normal switch of fear-coping strategies	Metna-Laurent et al. (2012)

3 CB_2 Receptor and Double Knockout Mice

The CB_2 receptor gene, *Cnr2*, is also located on chromosome 4 at position 68.01 cM (135895394–135920207 bp, + strand). Similar to *Cnr1*, the entire coding region of the 347 amino acids protein is located within a single exon, although different

splice variants have been described (Liu et al. 2009). To date, two global knockout mouse lines have been described (Table 5). In the first line, CB_2-KO^{Zim}, 341 base pairs (bp) of the coding exon were replaced with a neo selection cassette (Buckley et al. 2000). To the best of my knowledge, details about the mutation in the second global knockout, CB_2-KO^{Dgen}, were never published. However, the strain information available from The Jackson Laboratory states that 334 bp were deleted due to homologous recombination of a "Neo555T" construct. CB_2-KO^{Zim} mice are on a congenic C57BL/6J genetic background and CB_2-KO^{Dgen} animals on a mixed C57BL/6J × C57BL/6N background. It should be noted, however, that some investigators have unfortunately crossed the CB_2-KO^{Zim} strain to outbreed Swiss or CD1 mice (Zarruk et al. 2012; La Porta et al. 2013; García-Gutiérrez et al. 2013; Navarrete et al. 2013; Zoppi et al. 2014), thus yielding an ill-defined and poorly controlled genetic background.

Both strains have been used to elucidate the role of CB_2 signalling in various immune processes, inflammatory disorders, tissue remodelling and fibrosis, metabolic processes, nociception, neurodegenerative disorders and bone remodelling (Table 5). The most prominent phenotype of these animals is perhaps a striking age-related osteoporosis (Ofek et al. 2006; Bab and Zimmer 2008; Sophocleous et al. 2011). While bone mass in young adult mice was normal, they displayed a massive bone loss as they grew older. The loss of bone mass was accompanied by a high bone turnover, resembling symptoms of postmenopausal osteoporosis. Interestingly, several genetic studies in humans have demonstrated that cannabinoid receptor 2 (*Cnr2*) polymorphisms are associated with low bone mass and osteoporosis (Safo and Regehr 2005; Yamada et al. 2007; Karsak et al. 2009). CB_2 knockout mice therefore represent an interesting animal model for human osteoporosis. CB_2-KO^{Zim} mice are also larger due to an enhanced elongation of the appendicular and axial skeleton. This finding led to the discovery of functional endocannabinoid signalling in the epiphyseal growth cartilage (Wasserman et al. 2015). CB_2-deficient mice have also been used to study a possible involvement of CB_2 receptors in neuronal signalling, a topic that has attracted a high interest.

The Manzanares lab has generated a transgenic mouse strain expressing CB_2 under the control of a prion promoter (CB_2xP) in the CNS, particularly in the spinal cord {Racz:2008hw}. Expression was detected in neurons and in microglia, but not in astrocytes. These mice showed a number of phenotypes (Table 5), which demonstrate that the activation of CB_2 on these cells produces physiological effects.

Combinatorial genetic animal models with a simultaneous deletion of CB_1 and CB_2 have also been generated by interbreeding of the single knockouts (Table 5). The *Cnr1* and *Cnr2* genes are both located on chromosome 4, separated by a distance of approximately 50cM. This distance results in a 50 % crossover frequency and makes the breeding of double knockouts relatively efficient. These mice were used to ascertain whether or not effects of cannabinoid compounds are mediated by CB_1/CB_2 receptors (Jarai et al. 1999; Paria et al. 2001; Racz et al. 2008; Springs et al. 2008; Wenzel et al. 2013). They were also used to investigate cannabinoid modulation of inflammatory responses and carcinogenesis

Table 5 CB_2 deletions and $CB_1 \times CB_2$ double knockouts

Strain designation	Phenotypes and systems studied	References
CB_2-KO^{Zim}	Immune system: no cannabinoid immunomodulatory effects, reduction in the number of splenic B cells, altered dendritic cell migration, enhanced damage by cisplatin or oxidized low-density lipoproteins, improved outcome in sepsis model	Buckley et al. (2000), Gertsch et al. (2008), Freeman-Anderson et al. (2008), Csóka et al. (2009), Mukhopadhyay et al. (2010), Basu et al. (2011), Adhikary et al. (2012), Basu et al. (2013)
	Skin: increased allergic contact dermatitis, enhanced fibrosis, no AM1241-stimulated endorphin release, more sensitive to bleomycin-induced fibrosis	Ibrahim et al. (2005), Karsak et al. (2007), Akhmetshina et al. (2009), Servettaz et al. (2010)
	Development: abnormal eye-specific segregation of retinal projections, resistant to lipopolysaccharide-driven preterm birth	Duff et al. (2013), Sun et al. (2014))
	Neurodegeneration: more severe Huntington symptoms, more sensitive to malonate injury, increased experimental autoimmune encephalitis, neuroprotective effect of JWH-133 abolished, impaired neuronal progenitor proliferation	(Palazuelos et al. (2006), Maresz et al. (2007), Palazuelos et al. (2008), Sagredo et al. (2009), Murikinati et al. (2010), Álvaro-Bartolomé et al. (2010), Zarruk et al. (2012), Bouchard et al. (2012), Sisay et al. (2013)
	Bone: high-turnover osteoporosis, bone formation after traumatic brain injury normal, elongated appendicular and axial skeleton, no effects of HU308 or AM630 on bone cells, *N*-oleoyl-L-serine effects intact	Ofek et al. (2006), Bab and Zimmer (2008), Tam et al. (2008), Bab et al. (2008), Idris et al. (2008), Smoum et al. (2010), Ofek et al. (2011), Sophocleous et al. (2011), Wasserman et al. (2015)
	Cardiovascular: more widespread ischaemic injury, exacerbated fibrosis, adverse remodelling, increased apoptosis of cardiomyocytes, increased balloon-induced neointima formation, increased macrophage adherence and migration, no effect of THC on atherosclerosis	Steffens et al. (2005), Defer et al. (2009), Molica et al. (2012), Duerr et al. (2014b), Duerr et al. (2014a), Heinemann et al. (2014)
	Pain: contralateral hyperalgesia in neuropathic and joint pain models, enhanced γ-interferon response, no effect of ß-caryophyllene, normal cannabidiol analgesia, altered opioid receptor expression	Paldy et al. (2008), Xiong et al. (2012), La Porta et al. (2013), Klauke et al. (2013)
	Liver: increased hepatic ischaemia–reperfusion or CCl4-induced injury, delayed regeneration, enhanced alcohol-induced liver steatosis,	Julien et al. (2005), Bátkai et al. (2007a), Avraham et al. (2008), Deveaux et al. (2009), Teixeira-Clerc

(continued)

Table 5 (continued)

Strain designation	Phenotypes and systems studied	References
	enhanced M1 gene signature, reduced high-fat diet-induced insulin resistance and hepatic steatosis	et al. (2010), Trebicka et al. (2011), Louvet et al. (2011)
	Addiction: stronger ethanol withdrawal score and conditioned place preference and increased motivation to drink ethanol, no nicotine-induced conditioned place preference and reduced nicotine self-administration, no effect of JWH-133 on cocaine self-administration or dopaminergic activity	Xi et al. (2011), Navarrete et al. (2013), Zhang et al. (2014), Ortega-Álvaro et al. (2015)
	Behaviour: impaired aversive memory consolidation, schizophrenia-related behavioural phenotypes, greater increases in food intake and body weight with age	Agudo et al. (2010), Ortega-Álvaro et al. (2011), García-Gutiérrez et al. (2013)
CB_2-KO^{Dgen}	Pain: CP55,940 analgesia normal, reduced morphine analgesia, no upregulation of IB4 after neuropathic injury, no effect of AM1710 on paclitaxel-induced allodynia, 2-AG-induced anti-allodynia reduced	Wotherspoon et al. (2005), Sain et al. (2009), Desroches et al. (2014b), Desroches et al. (2014a), Deng et al. (2014)
	Immune system: THC stimulation of CD11b1Gr-11 cell expansion reduced, increased endothelial–leukocyte adhesion, more severe experimental autoimmune encephalitis, reduced numbers of CD1d-high B cells and impaired retention of immature B cells in bone marrow sinusoids	Pereira et al. (2009), Hegde et al. (2010), Muppidi et al. (2011), Sisay et al. (2013), Rom et al. (2013), Kong et al. (2014)
	Nervous system: increased prefrontal corticosterone levels after stress, delayed depolarizing response in mPFC neurons absent, no JWH-133 effects on synaptic activity	Hill et al. (2011), den Boon et al. (2012), Kim and Li (2014)
	No changes in atherogenesis, diabetic nephropathy worsened	Willecke et al. (2011), Barutta et al. (2014)
CB_2-KO of unclear origin	Sepsis model, exacerbated stress-induced neuroinflammatory responses, no nicotine-induced conditioned place preference, anxiolytic effects of JZL184 abolished, colitis model, retinal physiology	Tschöp et al. (2009), Busquets-Garcia et al. (2011), Ignatowska-Jankowska et al. (2013), Fichna et al. (2014), Zoppi et al. (2014), Cecyre et al. (2013)

(continued)

Table 5 (continued)

Strain designation	Phenotypes and systems studied	References
CB_1-$KO^{Zim} \times CB_2$-KO^{Zim}	Cannabinoid effects: no cardiovascular HU-210 effects, no THC effects on humoral T-cell responses, 2-AG reduced locomotor activity, protective effect of O-1602 in colitis model normal, effect of URB597 and AEA on pulmonary vasoconstriction unchanged, residual analgesic effect of AEA, no analgesic effect of THC, effects of O-1602 on the ileum and colon not altered, THC effect on allergen-induced airway inflammation intact, no effect of AM6545 on food intake, no effect of THC on MDMA toxicity, effects of 2-AG on suppression of IFN-γ responses unchanged, rimonabant effects on μ-opioid receptors intact, NADA-induced killing of hepatic stem cells intact	Jarai et al. (1999), Kaplan et al. (2005), Racz et al. (2008), Springs et al. (2008), Touriño et al. (2010), Cluny et al. (2010), Braun et al. (2011), Schicho et al. (2011), Sigel et al. (2011), Wojtalla et al. (2012), Zádor et al. (2012), Li et al. (2013a), Wenzel et al. (2013)
	Asynchronous embryo development	Paria et al. (2001)
	Inflammation and immune responses: increased allergic contact dermatitis, enhanced inflammation after influenza infection, resistant to UVB-induced inflammation and carcinogenesis, augmented cell-mediated immune responses	Karsak et al. (2007), Buchweitz et al. (2008), Zheng et al. (2008), Petrosino et al. (2010), Karmaus et al. (2013), Simkins et al. (2014)
	Susceptible to the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine	Simkins et al. (2012)
CB_2xP	Reduced hyperalgesia in a neuropathic pain model or a joint pain model	La Porta et al. (2013)
	Protected in a 6-OHDA model	Ternianov et al. (2012)
	Increased glutamate uptake, anti-inflammatory or neuroprotective actions similar to JWH-133	Zoppi et al. (2014)
	Decreased motor responses to cocaine and decreased locomotor sensitization	Aracil-Fernández et al. (2012)
	Hyperglycaemia, lean phenotype	Romero-Zerbo et al. (2012)
	Reduced anxiety-like behaviours, depression resistant	García-Gutiérrez et al. (2010), García-Gutiérrez and Manzanares (2011)

(Table 5). However, the only phenotype that was clearly enhanced in double knockouts vs. single knockouts was an increased sensitivity to contact allergic dermatitis (Karsak et al. 2007). Such a lack of increased phenotype severity may by an indicator of nonredundant functions of both receptors, but it should be noted that only a few studies have compared double vs. single knockouts.

4 Biosynthetic Enzymes: NAPE-PLD, GDE1 and DAGLs

N-Acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) catalyses the hydrolytic cleavage of *N*-acyl phosphatidylethanolamines (NAPEs) to generate *N*-acylethanolamines, including AEA. The enzyme is encoded by the *Napepld* gene on chromosome 5, position 9.97 cM (21662901–21701396 bp,—strand). The transcript has 5 exons. Exons 2–5 contain the open reading frame of the 396 amino acid NAPE-PLD protein. Knockout mice were created in the Palmiter lab (Napepld-KO^{Rpa}) using a targeting construct that introduced loxP site upstream and downstream of exon 3 (Liu et al. 2008). The neo selection cassette in this construct was flanked by frt sites and removed by breeding with FLP expressing mice. To generate a null allele, mice were subsequently bred with a mouse strain expressing Cre in the germ cell lineage (Table 6) (Tallquist and Soriano 2000). Tsuboi and colleagues described an almost identical knockout mouse strategy to generate a mouse strain with a global deletion of the *Napepld* exon 3 (Napepld-KO^{Itl}) (Tsuboi et al. 2011). The Cravatt lab generated a *Napepld* knockout line (Napepld-KO^{Crv}) by removing exon 4, which encoded amino acids 98 to 313 (Simon and Cravatt 2010).

The knockout mice showed reduced NAPE-PLD activity and reduced levels of saturated and monosaturated *N*-acylethanolamines (NAEs). However, the levels of polyunsaturated NAEs, including AEA, were not significantly (Leung et al. 2006; Liu et al. 2008) or only slightly reduced (Tsuboi et al. 2011). This result was surprising and indicated the existence of alternative AEA biosynthetic pathways. One possible pathway involves glycerophospho-NAEs as intermediary products, which are converted to NAEs by glycerophosphodiester phosphodiesterase 1 (GDE1). This enzyme is encoded by the *Gde1* gene on chromosome 7 position 63.52 cM (118688545–118705778 bp, − strand). Two mutant mouse strains for this gene have been generated by the Helmholtz Centre in Munich and by the Cravatt lab, but only data for the Cravatt strain (Gde1-KO^{Crv}) have been published so far (Simon and Cravatt 2010). It was generated by replacing exon 2 (the gene has six coding exons) with a neo selection cassette. These mice also showed an almost complete loss of glycerophospho-NAE phosphodiesterase activity in brain tissues, but virtually no change in the level of NAEs. Furthermore, Gde1-KO^{Crv} × Napepld-KO^{Crv} double knockout mice were generated that show an almost complete inability to convert NAPE to NAE. Nevertheless, NAEs in the brains of these mice were almost unchanged (Simon and Cravatt 2010). These knockout studies therefore show that alternative AEA biosynthetic pathways must exist, which still remain to be elucidated.

Table 6 Deletions of biosynthetic and metabolic enzymes

Gene locus	Strain designation	KO type	Genetic background	Phenotypes and systems studied	References
Napepld	Napepld-KO^{Rpa}	Global	C57BL/6 × 129SV	Reduced generation of AEA from exogenous NAPE	Liu et al. (2008)
	Napepld-KO-Itl	Global	C57BL/6	Decreased *N*-acetylethanolamine levels, including AEA	Tsuboi et al. (2011)
	Napepld-KO^{Crv}	Global	129SvJ × C57BL/6	Reduced levels of saturated and monosaturated NAEs, reduced NAPE-PLD activity, normal AEA levels, normal levels of polyunsaturated NAEs	Leung et al. (2006)
				Deregulated palmitoylethanolamide production in macrophages, altered response to carrageenan	Zhu et al. (2011)
Gde1	Gde1-KO^{Crv}	Global	C57BL/6	No change in GP-NAE levels	Simon and Cravatt (2010)
Dagla	Dagla-KO-Kano	Global	C57BL/6N	Faster habituation to an odour, enhanced theta burst-stimulated long-term potentiation	Sugaya et al. (2013)
				DSE at mossy cell–granule cell synapses abolished	Uchigashima et al. (2011)
				2-AG level reduced, retrograde synaptic suppression abolished	Tanimura et al. (2010)
				No change in CB_1–G protein signalling	Aaltonen et al. (2014)
	Dagla-KO^{Pado}	Global	C67BL/6	80% lower brain 2-AG levels, hippocampal synaptic plasticity impaired, reduced hippocampal neurogenesis	Gao et al. (2010)
	Dagla-GT^{Lex}	Gene trap	Unclear	Lower brain 2-AG levels, small reduction in hippocampal AEA levels, depolarization-induced suppression of inhibition abolished	Yoshino et al. (2011)

Daglb	Daglb-GT^{Lex}	Gene trap	Unclear	Normal brain 2-AG levels, small reduction in brain AEA	Yoshino et al. (2011)
			C57BL/6	2-AG levels lower: 50% in the brain, 90% in the liver, parallel decrease of arachidonic acid	Gao et al. (2010)
	Daglb-KO^{Kano}	Global	C57BL/6N	Brain 2-AG level normal, retrograde synaptic suppression intact	Tanimura et al. (2010)
				No change in CB_1–G protein signalling	Aaltonen et al. (2014)
Dagla/ Daglb	Dagla-GT^{Lex} × Daglb-GT^{Lex}	Double KO	Unclear	Brain 2-AG further reduced compared to single KO	Yoshino et al. (2011)
Faah	Faah-KO^{Crv}	Global	129SvJ × C57BL/6	Endocannabinoids: increased levels of AEA and other NAEs, supersensitivity to AEA, decreased AEA transport, lower post-mortem accumulation of AEA, reduced level of 2-AG, increased affinity of AEA for CB_1	Cravatt et al. (2001, Lichtman et al. (2002, Ortega-Gutierrez et al. (2004, Patel et al. (2005, Saghatelian et al. (2006, Mulder and Cravatt (2006, Long et al. (2011)
				Synaptic plasticity: inhibitory GABAergic synaptic transmission in the amygdala enhanced, changes at medium spiny neurons	Azad et al. (2004, Mathur et al. (2013)
				Embryo development: normal placentation, impaired preimplantation development and oviductal transport, altered transcription profiles in blastocysts, retarded embryo development, reduced fertilization capacity	Wang et al. (2006, Sun et al. (2009, Sun et al. (2010, Xie et al. (2012)
				Liver: hepatocytes with increased AEA-induced reactive oxygen species formation and susceptibility to AEA-mediated death, more prone to diet-	Siegmund et al. (2006), Brown et al. (2012), Siegmund et al. (2013)

(continued)

Table 6 (continued)

Gene locus	Strain designation	KO type	Genetic background	Phenotypes and systems studied	References
				induced hepatic insulin resistance, reduced expression of thyroid-stimulating hormone, hepatic cell death induced by 2-AG	
				Cardiovascular: decreased age-related cardiac dysfunction myocardial nitrative stress, inflammatory gene expression and apoptosis, enhanced balloon-induced neointima formation, increased responsiveness to AEA-induced hypotension and cardiodepression, reduced hypoxic pulmonary vasoconstriction, doxorubicin-induced myocardial stress increased	Pacher et al. (2005), Bátkai et al. (2007b), Mukhopadhyay et al. (2011b), Molica et al. (2013), Wenzel et al. (2013)
				Immune system and inflammation: aberrant NKT cell activation, reduced allergic contact dermatitis, reduced DNBS-induced colitis, improved remission from experimental autoimmune encephalitis	Massa et al. (2004), Karsak et al. (2007, Webb et al. (2008), Freigang et al. (2010)
				Neurological: altered TRPV1 signalling in the striatum, pro-convulsant activity of AEA, increased neurogenesis	Clement et al. (2003), Aguado et al. (2005), Aguado et al. (2006), Musella et al. (2009)
				Behaviour: accelerated acquisition rates in Barnes maze, reduced anxiety, increased ethanol consumption and preference, attenuated morphine withdrawal, hypoalgesia, no acetaminophen analgesia, enhanced effects of JZL184, lower	Lichtman et al. (2004), Basavarajappa et al. (2006), Moreira et al. (2008), Vinod et al. (2008), Wise et al. (2009), Mallet et al. (2010), Ramesh et al. (2011), Schlosburg et al. (2014)

				sensitivity to acute ethanol effects and withdrawal	
	Faah-KO^{Crv} × FAAH-NS	Global, except neurons	C57BL/6	Reduced inflammation	Cravatt et al. (2004)
				No effect of FAAH inhibitors on lipopolysaccharide-induced allodynia	Booker et al. (2012)
Mgll	Mgll-GT^{Lex}	Gene trap	Unclear	Increased 2-AG levels, impaired endocannabinoid-dependent synaptic plasticity, desensitized CB_1 receptors	Schlosburg et al. (2010)
			129SvEv × C57BL/6J	Altered synaptic plasticity, enhanced theta burst stimulation of long-term depression, enhanced learning	Pan et al. (2011)
				Reduced neuroinflammation, protected in Parkinson model	Nomura et al. (2011)
				Prolonged DSE at parallel or climbing fibre to Purkinje cell synapses	Zhong et al. (2011)
	Mgll-KO^{Mwa}	Global	C57BL/6N	Used for expression analysis	Uchigashima et al. (2011)
				Prolonged cerebellar 2-AG signalling	Tanimura et al. (2010)
				Augmented acute somatic and visceral tonic pain	Petrenko et al. (2014)
	GluN2C–Mgll-KO^{Mwa}	Granule cells	Unclear	Prolonged cerebellar 2-AG signalling but less than in global KO	Tanimura et al. (2012)
	Mgll-KO^{Rzim}	Global	C57BL/6	Impaired lipolysis, pharmacological tolerance to CB_1 agonists, attenuated diet-induced insulin resistance	Taschler et al. (2011)

2-Arachidonoylglycerol (2-AG) is generated by hydrolysis of diacylglycerol through an sn-1-specific diacylglycerol lipase (DAGL). Two isoforms of this enzyme, DAGLα and DAGLß, are encoded by different genes (*Dagla* and *Daglb*) located on chromosomes 19 and 5, respectively (*Dagla*: Chr19 6.55 cM, 10245265–10304877 bp, − strand; *Daglb*: Chr5: 82.19 cM, 143472947–143504442 bp, + strand).

The Kano lab has generated knockouts for both genes, by inserting a neo cassette and lox sequences into introns 2 and 4 of the *Dagla* or into introns 9 and 11 of the *Daglb* genes, respectively. The exons flanked by the lox sequences were then removed by crossing the animals with a "Cre-deleter" strain (Tanimura et al. 2010). The Doherty lab has also generated a *Dagla* knockout by replacing parts of exon 1 with a GFP–neo cassette (Gao et al. 2010). This mutation resulted in a complete loss of function deletion, but expression of the GFP reporter has not been shown yet. In addition to these targeted gene deletions, mutant mice were also generated from Lexicon Omnibank gene trap clones. For *Dagla*, an ES cell clone (OST-288027) was identified in which the gene trap vector was inserted in intron 4. This insertion resulted in a null allele, as *Dagla* messenger RNA (mRNA) was no longer detectable (Yoshino et al. 2011). For *Daglb*, Gao et al. and Yoshino et al. independently generated a mutant mouse strain (Daglb-GT^{Lex}) from the same gene trap ES clone (OST195261) (Gao et al. 2010; Yoshino et al. 2011). This clone contained an insertion of the gene trap vector in exon 1, just downstream of the translational start.

DAGLα is the most important enzyme for the generation of 2-AG in the brain, as knockout mice showed a vast reduction in brain 2-AG levels whereas brain 2-AG levels were unchanged in DAGLß knockouts. Retrograde synaptic suppression was also completely abolished in DAGLα-deficient mice, but not affected in DAGLß knockouts. However, brain 2-AG levels were further reduced in DAGLα × DAGLß double knockout mice (Yoshino et al. 2011), indicating that DAGLß also plays some role in the brain. The physiological role of these enzymes seems to be different in the liver, where DAGLß knockouts showed a substantial decrease of 2-AG levels, which were not altered in DAGLα-deficient mice (Gao et al. 2010).

5 Metabolic Enzymes: FAAH and MGLL

AEA is mainly degraded by fatty acid amid hydrolase (FAAH), a protein of 579 residues. The *Faah* gene is, like the cannabinoid receptors, also located on chromosome 4 at position 53,08 cM (115967145–116017926 bp, − strand). It has at least 15 exons and produces a transcript of approximately 3.8 kb. Mouse mutants for this gene have been established by Lexicon Genetics and the Cravatt lab. However, I am not aware of any publication with the Lexicon strain and therefore will describe only the Cravatt strain (Cravatt et al. 2001). The first coding exon of this gene and approximately 2 kb of upstream sequences was replaced by a neo selection cassette, thus producing a null allele. Mutant mice show a strong reduction in their ability to degrade anandamide and a concomitant elevation of anandamide

levels. They show a number of behavioural phenotypes that are consistent with an enhanced CB_1 signalling tone (see Table 6). The Cravatt lab has also generated a mouse strain expressing FAAH under the control of the neuron-specific enolase promoter and crossed these to global FAAH knockouts (Cravatt et al. 2004). The resulting mouse strain showed FAAH activity exclusively in neurons. These mice had wild-type levels of AEA in the brain and spinal cord, but elevated AEA levels in peripheral tissues.

The gene *Mgll* encoding monoacylglycerol lipase, the main degrading enzyme of 2-AG, is located on mouse chromosome 6 (39,51 cM, position 88724412–88828360 bp, + strand). It consists of at least eight exons, with seven protein-coding exons. Two targeted gene deletions and one gene trap mutant have been reported for this gene. The first mutation has been produced by a gene trapping approach (Schlosburg et al. 2010). It contained a gene trap cassette insertion downstream of exon 3, which resulted in a complete loss of function mutation. Mgll-GT^{Lex} mice show a vastly decreased reduction of 2-AG hydrolytic activity of brain lysates and a tenfold elevation of brain 2-AG levels (Table 6).

Uchigashima and colleagues described a targeted deletion of *Mgll*, in which exon 3 was flanked by two loxP sites. The upstream neo selection cassette was flanked by frt sites and removed by FLP recombination. Global knockouts were generated by mating of the floxed allele to mice expressing Cre recombinase in the germ cell lineage (Uchigashima et al. 2011). This floxed strain was also used to delete *Mgll* specifically from cerebellar granule cells (Tanimura et al. 2012). Taschler et al. also described a targeted mutation, in which exons 3 and 4 were deleted (Taschler et al. 2011). *Mgll*-deficient mice showed elevated 2-AG levels, with concomitantly prolonged 2-AG signalling at different synapses and desensitized CB_1 receptors.

6 Combinatorial Genetic Models with Cannabinoid Receptors

To investigate the role of cannabinoid receptors in specific pathogenic processes, a number of investigators have crossed CB_1 or CB_2 knockout mice with mutant or transgenic animals that develop a pathology of interest (Table 7). For example, the Guzman laboratory has shown that CB_1-KO mice expressing a human Huntington disease allele (R6/2) exhibit aggravated Huntington-like symptoms (Blázquez et al. 2011). The Stella lab used an elegant transgenic approach to rescue expression of CB_1 in medium spiny neurons, of R6/2 mice, which would otherwise be reduced during disease progression (Naydenov et al. 2014). This rescue ameliorated several aspects of the Huntington pathology. Hoyer et al. have shown that CB_2-deficient animals show a more severe atherosclerosis when crossed to ApoE4-deficient mice and placed on a high-cholesterol diet (Hoyer et al. 2011). Schmöle

Table 7 Combinatorial genetic models

Strains	Phenotypes and systems studied	References
CB_1-KO^{Zim} × ob/ob	More growth retardation, stronger glucose intolerance, similar leptin sensitivity	Li et al. (2013c)
CB_1-KO^{Map} × A2a-KO	Increased anxiety	Berrendero et al. (2003)
CB_1-KO^{Ltz} × R6/2-TG	Aggravated Huntington symptoms, rescued by MK-801	Blázquez et al. (2011, Chiarlone et al. (2014)
Gpr88-R26CB_1-TG × R6/2-TG	Rescue of the loss of excitatory striatal synapses	Naydenov et al. (2014)
CB_1-KO^{Map} × N171-82Q-TG	Aggravated Huntington symptoms	Mievis et al. (2011)
CB_1-KO^{Ltz} × Thy1–eYFP-TG	YFP selectively expressed in layer 5 neurons, thus allowing visualization of corticospinal tracts, aberrant phenotype of subcerebral projection neurons	Díaz-Alonso et al. (2012)
CB_1-KO^{Ltz} × APP23-TG	Increased mortality, reduced plaque load and neuroinflammation, cognitive performance worsened	Stumm et al. (2013)
CB_1-HET × Fmr1-KO	Amelioration of the Fmr1 phenotype	Busquets-Garcia et al. (2013)
CB_2-KO^{Zim} × APP23/PS1-TG	Reduced neuroinflammation	Schmöle et al. (2014)
CB_2-KO^{Dgen} × J20-APP-TG	Increased amyloid pathology altered Tau processing	Koppel et al. (2013)
CB_2-KO^{Zim} × ApoE-KO	More severe atherosclerosis	Hoyer et al. (2011)
CB_2-KO-Zim × R6/2-TG	Aggravated Huntington symptoms	Palazuelos et al. (2009)
Faah-KO^{Crv} × ApoE-KO	Enhanced neointima formation	Molica et al. (2013)
Faah-KO^{Crv} × Sod1-TG	Delay of superoxide dismutase (SOD) phenotype, but normal life span	Bilsland et al. (2006)

et al. investigated the role of CB_2 receptors and Stumm et al. the role of CB_1 receptors in an APP23 transgenic animal model of Alzheimer's disease (Schmöle et al. 2014; Stumm et al. 2013). Other studies used cannabinoid receptor knockouts in combination with other gene knockouts to investigate gene–gene interactions (Table 7). Finally, cannabinoid receptor knockouts were crossed to transgenic reporter strains or used in combination with viral reporter constructs, in order to visualize pathological processes in these animals (Díaz-Alonso et al. 2012).

7 Conclusion

The use of genetic animal models has clearly enhanced our knowledge about the physiological and pathophysiological relevance of endocannabinoid signalling. Thus, phenotypes observed in these mouse models have helped to formulate new hypotheses about the endocannabinoid system, and many follow-up studies, often using conditional knockouts, have been performed to refine them. In these studies, pharmacology and genetics often went hand in hand. As each method is prone to artefacts, overlapping findings with both methods greatly improves confidence in the validity of the findings. Pharmacological substances may show off-target effects, particularly when a high dose is used. Conversely, they may lack effects if they are administered inappropriately, or at sub-effective doses. Genetic manipulations suffer from two major problems. First, they can (and often do) induce compensatory changes in the expression of other genes. It is thus difficult to ascertain if the observed phenotype was due to the change of the expression in the gene of interest or instead due to compensatory mechanisms. Other sources for artefacts are inappropriate genetic controls. Experimenters should also be aware of the many problems that are inevitable when mutations are studied on complex genetic backgrounds, which include outbred genetic backgrounds (Chia et al. 2005). It is often impossible to discern if the observed phenotype is due to the genetic manipulation, or to unspecific effects of such genetic backgrounds.

On the other hand, when used appropriately and interpreted with caution, genetic and pharmacological methods offer tremendously powerful tools for dissecting physiological processes and disease mechanisms. Moreover, these tools will become ever more powerful with the advent of novel methods for genetic manipulations. The Crispr/Cas technology, for example, has greatly simplified our ability to genetically manipulate not only mice but also other species (Gennequin et al. 2013). It is thus likely that cannabinoid research will also benefit in the future from genetic models using other species, for example, zebrafish or rats.

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Endocannabinoids and the Immune System in Health and Disease

Guy A. Cabral, Gabriela A. Ferreira, and Melissa J. Jamerson

Contents

Abstract

Endocannabinoids are bioactive lipids that have the potential to signal through cannabinoid receptors to modulate the functional activities of a variety of immune cells. Their activation of these seven-transmembranal, G protein-

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_6

coupled receptors sets in motion a series of signal transductional events that converge at the transcriptional level to regulate cell migration and the production of cytokines and chemokines. There is a large body of data that supports a functional relevance for 2-arachidonoylglycerol (2-AG) as acting through the cannabinoid receptor type 2 (CB2R) to inhibit migratory activities for a diverse array of immune cell types. However, unequivocal data that supports a functional linkage of anandamide (AEA) to a cannabinoid receptor in immune modulation remains to be obtained. Endocannabinoids, as typical bioactive lipids, have a short half-life and appear to act in an autocrine and paracrine fashion. Their immediate effective action on immune function may be at localized sites in the periphery and within the central nervous system. It is speculated that endocannabinoids play an important role in maintaining the overall "fine-tuning" of the immune homeostatic balance within the host.

Keywords

Anandamide • Antigen presentation • 2-Arachidonoylglycerol • Astrocyte • Basophil • Cannabinoid receptor • Chemokine • Cytokine • Dendritic cell • Endocannabinoid • Interferon • Interleukin • Lymphocyte • Macrophage • Mast cell • Microglia • Monocyte • Natural killer (NK) cell • Neutrophil • Nitric oxide

Abbreviations

2-AG	2-Arachidonoylglycerol
Abn-CBD	Abnormal cannabidiol
AEA	Anandamide
ALIA	Autacoid local inflammation antagonism
ApoE	Apolipoprotein E
Arg-1	Arginase 1
BBB	Blood–brain barrier
CB1R	Cannabinoid receptor type 1
CB2R	Cannabinoid receptor type 2
ConA	Concanavalin A
CNS	Central nervous system
COX	Cyclooxygenase
ECM	Extracellular matrix
FAAH	Fatty acid amide hydrolase
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus type 1
HUVECs	Human umbilical vein endothelial cells
IL	Interleukin
IRAK1BP1	IL-1 receptor-associated kinase 1 binding protein
iNOS	Inducible nitric oxide synthase
IFN	Interferon

LC-APCI-MS	Liquid-chromatography-atmospheric pressure chemical ionization-mass spectrometry
L-NAME	L-NG-nitroarginine methyl ester
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
mBSA	Methylated bovine serum albumin
MCP-1	Monocyte chemoattractant protein 1
mDCs	Myeloid dendritic cells
MHC	Major histocompatibility complex
MKP-1	Mitogen-activated protein kinase phosphatase 1
MS	Multiple sclerosis
NADA	*N*-Arachidonoyldopamine
NAGly	*N*-Arachidonoyl glycine
NK cell	Natural killer cell
NMDA	*N*-Methyl-D-aspartate
NO	Nitric oxide
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PMN	Polymorphonuclear
PPARγ	Peroxidase proliferator-activated receptor γ
PSGL1	P-selectin glycoprotein ligand 1
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
TAK1	TGF-β-activated kinase 1
Tat	Trans-activator of transcription
Tc	Cytotoxic T cells
TCR	T-cell receptor
Th cells	T helper cells
TLRs	Toll-like receptors
TMEV	Theiler's murine encephalomyelitis virus
TMEV-IDD	Theiler's murine encephalomyelitis virus-induced demyelinating disease
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
T_{regs}	Regulatory T cells
VCAM	Vascular cell adhesion molecules

1 Introduction

The immune system in mammals contains a diverse array of cells. These cells function coordinately to drive their maturation, direct antimicrobial activities, and promote repair as well as damage of tissues (Table 1). Many of these activities are

Table 1 Cell markers and functional attributes associated with immune cells

Cell type	Key cell markers	Functional attributes
Astrocyte	CD44, GFAP, S100B Glast	Maintains brain homeostasis Defines brain micro-architecture Synaptic transmission
B lymphocyte (B cell)	CD19, CD20, CD21, CD40, MHC class II	Antigen presentation Antibody production
Basophil	CD13, CD107a, CD123, FcεRIα	Responds to parasitic infections Mediates allergic responses
Dendritic cell	CD11c, CD141, CD303	Antigen presentation
Eosinophil	CD23, CD88, FcεRIα	Responds to parasitic infections Mediates allergic responses
Macrophage	CD14, CD11b, CD68, CD86, MAC-1/MAC-3,	Phagocytosis Stimulation of lymphocytes and other immune cells
Mast cell	CD117, CD23 FcεRIα, CD203c	Mediates allergic responses
Microglia	CD11b, CD40, CD45, B7, ICAM-1	Brain homeostasis Scavenging Phagocytosis Antigen presentation
Monocyte	CD11c, CD14, CD45, CD68, CD163	Phagocytosis Macrophage precursor
Neutrophil	CD121a	Initial responder to acute inflammation
Natural killer (NK) cell	CD16, CD56	Destroys tumors and virus-infected cells
T lymphocyte (T cell)	CD2, CD3, CD4, CD8, TCR	Attacks infected or cancerous cells Regulates immune responses

CD cluster of designation, *GFAP* glial fibrillary acid protein, *ICAM* intercellular adhesion molecule, *MHC* major histocompatibility complex, *TCR* T-cell receptor

mediated through soluble factors, such as chemokines and cytokines (Table 2) that are released from immune cells and bind to cognate receptors and other cellular targets, setting in motion signaling cascades that culminate in the activation of select genes. This cross talk between different cell types occurs in a fashion that maintains overall homoeostatic balance of the immune system. Bioactive lipid molecules participate in the interplay of proinflammatory and anti-inflammatory factors. Included among these are endocannabinoids, principal among which are the amide and ester of the long chain polyunsaturated fatty acids anandamide (2-arachidonoylethanolamine, AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995). To date, two receptors that meet strict pharmacological and biochemical characterization for designation as endocannabinoid receptors have been identified. The first of these, the cannabinoid receptor type 1 (CB1R), is found in the central nervous system (CNS) and testis and at lower levels in some immune cells (Matsuda et al. 1990 ; Herkenham et al. 1990; Galiegue et al. 1995; Gerard et al. 1991). The second of these, the cannabinoid receptor type 2 (CB2R), is localized primarily in immune cells. The CB2R is found at highest levels in B lymphocytes, followed in order by natural

Table 2 Select chemokines/cytokines and their functional attributes

Cytokine	Property	Cells that produce cytokines	Functional attributes
IL-1 RA	Proinflammatory	Monocytes, macrophages, neutrophils, hepatocytes	Natural antagonist to IL-1
IL-1α	Proinflammatory	Macrophages, microglia, neutrophils, endothelial cells	Activates other cells, chemotactic, inflammatory reaction
IL-1β	Proinflammatory	Leukocytes, endothelial cells, macrophages	Inflammatory reaction, cell proliferation, cell differentiation, apoptosis
IL-2	Proinflammatory	T cells	Development and maturation of T cells, stimulates or activates other cells
IL-4	Anti-inflammatory	T cells, basophils, mast cells	Stimulates B cells
IL-6	Anti-/ proinflammatory	T cells, macrophages, microglia	Fever, stimulates acute phase response
IL-8	Proinflammatory	Eosinophils, macrophages, microglia, endothelial cells	Activates other cells, chemotactic, inflammatory reaction; kills parasites and amplifies the inflammatory reaction.
IL-10	Anti-inflammatory	T cells, macrophages, microglia	Stimulates or activates other cells, activates other cells, chemotactic, inflammatory reactions
IL-12	Proinflammatory	Macrophages, dendritic cells, B cells	Activates NK cells, T-cell differentiation
IL-17A	Proinflammatory	T helper cells	Inflammatory reaction against pathogen
IL-23	Proinflammatory	Dendritic cells, macrophages	Proliferation of memory T cell, increased IFN-γ production
MIP-1α	Proinflammatory	Eosinophils, mast cells	Inflammatory reaction in allergic reactions, stimulates other cells, kills parasites
MIP-2	Proinflammatory	Monocytes, macrophages	Chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells
Eotaxin (CCL11)	Proinflammatory	Eosinophils, monocytes, lymphocytes	Kills parasites and amplifies inflammatory reactions, chemotaxis of eosinophils
RANTES (CCL5)	Proinflammatory	Eosinophils, T cells	Kills parasites and amplifies inflammatory reaction
IFN-γ	Proinflammatory	T cells, NK cells	Stimulates or activates cells

(continued)

Table 2 (continued)

Cytokine	Property	Cells that produce cytokines	Functional attributes
KC	Proinflammatory	Macrophages, neutrophils, epithelial cells	Neutrophil chemotactic activity
MCP-5	Proinflammatory	Lymph node, thymus	Allergic reactions, immune response to pathogens
TNF-α	Proinflammatory	T cells, NK cells, mast cells, microglia	Stimulates or activates other cells, inflammatory reaction, chemotaxis

IL interleukin, *MIP* macrophage inflammatory protein, *RANTES* regulated upon activation normal T cell expressed and presumably secreted, *IFN* interferon, *KC* keratinocyte chemoattractant, *MCP* monocyte chemoattractant protein, *TNF* tumor necrosis factor

killer (NK) cells, monocytes/macrophages/microglia, and T lymphocytes (Galiegue et al. 1995; Schatz et al. 1997). The ordered distribution of these receptors suggests that certain immune cell subpopulations may be more responsive to endocannabinoids.

Endocannabinoids have been identified in immune cells such as monocytes/macrophages, basophils, lymphocytes, and dendritic cells (Matias et al. 2002). It has been suggested that 2-AG is the cognate functionally relevant endocannabinoid for the CB2R (Sugiura et al. 2000; Parolaro et al. 2002). Lee et al. (1995) reported that 2-AG suppressed the lymphoproliferation of splenocytes to bacterial lipopolysaccharide (LPS) and anti-CD3, an antibody that induces T-lymphocyte activation. However, this suppression occurred only at concentrations greater than 10 μM. Sugiura et al. (2000) also examined the effect of 2-AG on intracellular free Ca^{2+} concentrations in human HL-60 macrophage-like cells and found that this endocannabinoid induced a rapid transient increase in levels of intracellular free Ca^{2+}. The induced Ca^{2+} transient was blocked by a CB2R antagonist, consistent with the involvement of the CB2R in this response. In contrast, AEA was found to be a weak partial agonist for the CB2R. Based on these results, it was proposed that the CB2R was originally a 2-AG receptor and that 2-AG constituted the native cognate ligand.

On the other hand, Stefano et al. (2000) reported that 2-AG stimulated the release of nitric oxide (NO) from human immune and vascular tissues and invertebrate immunocytes, but that it did so through the activation of the CB1R. Berdyshev et al. (1997) reported that AEA diminished levels of the cytokines interleukin (IL)-6 (IL-6) and IL-8 from human monocytes, while Valk et al. (1997) indicated that AEA acted through the CB2R as a synergistic growth factor for hematopoietic cells. Derocq et al. (1998), using IL-3-dependent and IL-6-dependent mouse cell lines, proposed that AEA exerted a growth-promotion effect. However, both Berdyshev et al. (1997) and Derocq et al. (1998) concluded that the growth-promoting effect of AEA was cannabinoid receptor independent. Facci et al. (1995) reported that mast cells, bone marrow-derived cells found in mucosal and connective tissues and in the nervous system that play a role in tissue inflammation and neuroimmune interactions, expressed a peripheral cannabinoid receptor that was differentially sensitive to AEA. These cells reportedly expressed the CB2R that exerted negative

regulatory effects on mast cell activation. However, AEA did not down-modulate mast cell activation in vitro.

2 Effects of Endocannabinoids In Vitro

A variety of mammalian cell systems have been used as experimental models for documenting the in vitro effects of endocannabinoids on immune function. Early experiments involved the exogenous introduction of endocannabinoids to cultures of transformed immune cells or to immune cell subpopulations obtained from mice and humans. Such studies were complemented with those using mixed cell populations that putatively replicated more closely an in vivo condition that integrated cross talk between different immune cell types. The use of mixed cell populations allowed for the conducting of depletion and reconstitution studies in which selected immune cell subpopulations were removed from, or added to, the culture system in an attempt to identify the immune cell subpopulation targeted by a particular endocannabinoid. While early studies focused on the relevance of endocannabinoids in modulating the function of immune cells associated with the peripheral immune system, recent studies have centered on the effects of endocannabinoids on immune functionality within the central nervous system (CNS).

3 Immune Cells in the Peripheral Immune System

3.1 Basophils

Basophils are a type of polymorphonuclear (PMN) cell or "white" blood cell and make up less than 1 % of this blood cell type. When activated, basophils degranulate to release histamine, proteoglycans (e.g., heparin and chondroitin), and proteolytic enzymes (e.g., elastase and lysophospholipase). They also secrete lipid mediators such as leukotrienes and several cytokines such as IL-4. Basophils have receptors on their cell surface that bind IgE, an immunoglobulin involved in macroparasite defense and allergy. It is the bound IgE antibody that confers a selective response of these cells to environmental substances, for example, pollen proteins or helminth antigens. Vannacci et al. (2002) reported that 2-AG diminished the expression of CD63, a cell differentiation marker that is used to identify activated basophils. The inhibitory effect was found to be concentration dependent and reversed by the CB2R antagonist SR144528 and the NO synthase inhibitor L-NG-nitroarginine methyl ester (L-NAME). In guinea pig mast cells, the antigen-mediated release of histamine was found to be decreased by 2-AG in a dose-dependent fashion. The release of histamine was returned to control values by the CB2R antagonist SR144528. However, because L-NAME abrogated the inhibitory effects of 2-AG and reduced the immunological activation of both human basophils

and guinea pig mast cells, it was questioned whether the activation of cannabinoid receptors was linked directly to the modulation of CD63 expression.

3.2 Dendritic Cells

Dendritic cells are antigen-processing and antigen-presenting cells that are critical for the induction of primary immune responses, induction of immunological tolerance, and regulation of T-cell-mediated immune responses. Matias et al. (2002) reported that human dendritic cells harbored a constitutive endocannabinoid system. Using a liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) method, lipids extracted from immature dendritic cells were shown to contain 2-AG and AEA. The amounts of 2-AG were increased following cell maturation induced by LPS or by the major mite fecal allergen Der p 1. The investigators found that dendritic cells also expressed the CB1R, the CB2R, and fatty acid amide hydrolase (FAAH). Do et al. (2004) examined the effect of endocannabinoids on murine bone marrow-derived dendritic cells. Addition of AEA to dendritic cell cultures induced their apoptosis. The dendritic cells expressed the CB1R and CB2R. It was found that engagement of both receptors was necessary to trigger apoptosis. Chiurchiù et al. (2013) compared the level of cytokine production by myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) in healthy human subjects and multiple sclerosis (MS) patients, following in vitro stimulation of Toll-like receptors (TLRs)7/8 that play a key role in the innate immune system. They also evaluated the effect of AEA on these dendritic cell subsets and correlated cytokine levels with defects in the endocannabinoid system. It was found that mDCs obtained from MS patients produced higher levels of IL-12 and IL-6, whereas pDCs produced lower levels of interferon (IFN)-α compared to healthy subjects. AEA inhibited cytokine production from healthy mDCs and pDCs, as well as their ability to induce T-lymphocyte Th-1 and Th-17 lineages. Th1 cells produce IFN-γ, IL-2, and tumor necrosis factor (TNF)-β and mediate delayed type hypersensitivity (DTH) responses. Th-17 cells produce IL-17, IL-17F, and IL-22 and have been linked to inflammation and tissue injury in autoimmune disease. It was suggested that AEA had an immunomodulatory effect on mDCs and pDCs from MS patients, possibly as a reflection of an alteration of the expression of FAAH.

3.3 Eosinophils

Eosinophils comprise approximately 6 % of white blood cells (i.e., PMNs) in the bloodstream and multiply in response to parasitic infections or allergic reactions. Oka et al. (2004) reported that 2-AG induced the migration of human eosinophilic leukemia EoL-1 cells. The migration evoked by 2-AG was abolished in the presence of the CB2R antagonist SR144528 or by pretreatment of the cells with pertussis toxin, an exotoxin produced by the bacterium *Bordetella pertussis* that

prevents G_i signaling by G protein-coupled receptors. The inhibition of 2-AG-stimulated migration by the CB2R antagonist was consistent with involvement of the CB2R. It was suggested that 2-AG served as a chemoattractant for human peripheral blood eosinophils, but had no effect on neutrophils. It has also been demonstrated that 2-AG induces chemotaxis, a directed movement of cells toward a gradient of a chemical stimulus, of EoL-1 cells and human peripheral blood eosinophils in a CB2R-dependent manner (Kishimoto et al. 2006).

3.4 Lymphocytes

T lymphocytes play a central role in cell-mediated immunity and are distinguished from other lymphocytes, such as B lymphocytes and NK cells, by the presence of a T-cell receptor (TCR) on the cell surface. There are several subsets of T lymphocytes, each exhibiting a distinctive functional capability. These include T helper cells (T_h cells), CD4+ cells, cytotoxic T lymphocytes (T_c cells), CD8+ cells, memory T lymphocytes that persist long-term after an infection has resolved, regulatory T lymphocytes (T_{reg} cells), natural killer T (NKT)-lymphocytes, and gamma-delta (γδ) T cells. B lymphocytes function to produce antibodies against antigens, act as antigen-presenting cells, and develop into memory B cells following activation by antigen interaction. Schwarz et al. (1994) indicated that AEA caused an inhibition of mitogen-induced T- and B-lymphocyte proliferation. The effects of AEA on DNA synthesis in T and B lymphocytes occurred rapidly as exposure of the cells during the final 4 h of culture was sufficient to achieve greater than 40 % inhibition. Low concentrations of AEA that were shown to inhibit lymphocyte proliferation also caused DNA fragmentation. Lee et al. (1995) investigated the immunomodulatory effects of AEA and 2-AG in splenocytes obtained from $B_6C_3F_1$ mice. 2-AG produced a concentration-related inhibition of the mixed lymphocyte response that is used as a barometer of an individual's response to transplanted tissue or organ. It also inhibited the anti-CD3 monoclonal antibody-induced T-cell proliferation and LPS-induced B-cell proliferation. However, it had no inhibitory effect on phorbol-12-myristate-13-acetate/ionomycin-induced cell proliferation. Similarly, in vitro primary immunoglobulin M antibody-forming cell responses were found to be enhanced by 2-AG. Conversely, AEA exhibited no inhibitory effects on cell proliferative responses to stimulation by anti-CD3 monoclonal antibody, LPS, or phorbol-12-myristate-13-acetate/ionomycin treatment. AEA also showed no effect on the in vitro sheep erythrocyte antibody-forming cell response, while 2-AG exhibited no effect on basal adenylate cyclase activity in splenocytes. In addition, AEA showed negligible inhibitory effects on forskolin-stimulated adenylate cyclase activity and had no effect on basal adenylate cyclase activity in splenocytes. Coopman et al. (2007) found that 2-AG elicited the activation of downstream biochemical effectors based on assessment of phosphorylation of ERK1/2 MAP kinases in T lymphocytes. Since 2-AG inhibited CXCL12-induced chemotaxis, it was suggested that it played a modulatory role in activated T lymphocytes.

It has been demonstrated also that a cyclooxygenase (COX)-2 metabolite of 2-AG inhibits IL-2 secretion in activated T cells through peroxisome proliferator-activated receptor-γ (PPARγ) activation independent of the CB1R and CB2R. Rockwell et al. (2008) investigated the role of COX-catalyzed metabolism in the inhibition of IL-2 secretion by 2-AG. Pretreatment with nonselective and COX-2-selective inhibitors resulted in a complete abrogation of 2-AG-mediated suppression of IL-2 secretion. In contrast, pretreatment with COX-1-selective inhibitors had no effect upon 2-AG-mediated inhibition of IL-2 secretion. It was also demonstrated that while the potency of 2-AG was comparable between human Jurkat T cells and murine splenocytes, AEA was more potent in suppressing IL-2 production in Jurkat T cells compared to murine splenocytes. The COX-2 protein was detected in resting Jurkat T cells but not in resting mouse splenocytes. Furthermore, levels of COX-2 mRNA and protein were increased over basal levels 2h following the activation of Jurkat cells, whereas increases in COX-2 protein in murine splenocytes were not observed until 4h after cellular activation. The collective results suggested that the potency of AEA in the suppression of IL-2 secretion correlated with levels of COX-2 protein in T cells and that the 2-AG-mediated inhibition of IL-2 secretion was dependent upon COX-2-catalyzed metabolism.

Gasperi et al. (2014) found that 2-AG was able to initiate and complete the leukocyte adhesion cascade, by modulating the expression of selectins, cell adhesion molecules that are involved in lymphocyte homing and in chronic and acute inflammatory processes. A short exposure of primary human umbilical vein endothelial cells (HUVECs) to 2-AG was sufficient to prime them toward an activated state. Within 1 h of treatment, endothelial cells showed a time-dependent plasma membrane expression of P- and E-selectins, which both trigger the initial steps (i.e., capture and rolling) of leukocyte adhesion. The effect of 2-AG was mediated by the CB1R and CB2R and was long lasting, because endothelial cells incubated with 2-AG for 1 h released the proinflammatory cytokine TNF-α for up to 24 h. Consistently, TNF-α-containing medium was able to promote leukocyte recruitment. That is, human Jurkat T cells grown in conditioned medium derived from 2-AG-treated HUVECs showed enhanced L-selectin and P-selectin glycoprotein ligand-1 (PSGL1) expression, as well as increased efficiency of adhesion and transmigration. It was concluded that the in vitro data indicated that 2-AG, by acting on endothelial cells, could indirectly promote leukocyte recruitment.

3.5 Mast Cells

Mast cells are bone marrow-derived immune cells that are resident to several tissues in the body, including connective and mucosal tissues. These cells are involved in a variety of processes such as allergic inflammation, immune regulation, innate immunity, and parasite rejection (Metcalfe et al 1997). Mast cells (also known as mastocytes and labrocytes) contain granules rich in histamine and heparin. Facci et al. (1995) reported that mast cells contained the CB2R gene and expressed functional CB2R protein that was linked to negative regulatory effects on mast

cell activation. Although AEA bound to the CB2R, it was shown not to down-modulate mast cell activation in vitro. It was suggested that modulatory activities on mast cells supported the existence of an autacoid local inflammation antagonism (ALIA) mode of action. Lau and Chow (2003) reported that AEA, when used at concentrations higher than micromolar, induced the release of histamine. However, when mast cells were activated with anti-IgE, the histamine release that was induced was not affected by AEA. Furthermore, the histamine-releasing action of AEA on anti-IgE-induced histamine release was not reduced by the selective CB1R antagonist AM281 or the selective CB2R antagonist AM630. It was concluded that AEA, rather than suppressing mast cell activation, enhanced its activation. However, the high concentrations required, and the failure of cannabinoid receptor antagonists to reverse these effects, brought into question whether a functional linkage to a cannabinoid receptor existed. On the other hand, Vannacci et al. (2004) reported that the antigen-induced release of histamine from sensitized guinea pig mast cells was dose dependently reduced by 2-AG. The inhibitory action afforded by 2-AG was reversed by the selective CB2R antagonist SR144528 and unaffected by the selective CB1R antagonist AM251. The inhibitory action of 2-AG was reduced by the unselective NO synthase inhibitor L-NAME and reinstated by L-arginine, the physiological substrate. The inhibitory action of 2-AG also was reduced by the unselective cyclooxygenase (COX) inhibitor indomethacin and the selective COX-2 blocker rofecoxib. 2-AG also increased the production of nitrite from mast cells, an increase that was abrogated by the selective inducible NO synthase (iNOS) inhibitor L-NAME and N-(3-(aminomethyl)benzyl)acetamidine (1400W). 2-AG increased the generation of PEG_2 from mast cells, an increase that was abrogated by indomethacin and rofecoxib. In addition, mast cell challenge with antigen was accompanied by a net increase in intracellular calcium levels. 2-AG decreased the intracellular calcium level, an effect that was reversed by the CB2R antagonist SR144528 and the NO synthase inhibitor L-NAME. In unstimulated mast cells, 2-AG increased cGMP levels. This increase was abrogated by SR144528, L-NAME, indomethacin, and rofecoxib. The collective results suggested that 2-AG decreased mast cell activation in a manner that was linked functionally to the CB2R.

More recently, Sugawara et al. (2012) examined the effect of the activation of the CB1R on the biology of human skin mast cells in situ. The mast cell-rich connective tissue sheath of organ-cultured human scalp hair follicles was examined using quantitative immunohistomorphometry, ultrastructural analysis, and quantitative polymerase chain reaction (PCR). These assessments were conducted in concert with the use of CB1R agonists or antagonists, knockdown of CB1R expression, and the use of CB1R knockout mice. Mast cells within the connective tissue sheath of human hair follicles were shown to express functional CB1Rs. Their pharmacological blockade or gene silencing resulted in a stimulation of degranulation and the maturation of mast cells from resident progenitor cells in situ. This enhancement was shown to be, at least in part, stem cell factor dependent. CB1R agonists counteracted the mast cell-activating effects of classical mast cell secretagogues, substances that cause other substances to be secreted. A similar

outcome was obtained using CB1R knockout mice. It was suggested that locally synthesized endocannabinoids served to limit excessive activation and maturation of mast cells from resident progenitors through the mediation of a "tonic" CB1R stimulation.

3.6 Monocytes, Macrophages, and Macrophage-Like Cells

Monocytes constitute 2–10 % of leukocytes in humans and play multiple roles in immune function. These cells and their macrophage and dendritic cell progeny function in phagocytosis, antigen presentation, and cytokine production. Early studies indicated that endocannabinoids, principally AEA, inhibited the production of proinflammatory cytokines by macrophage-like cells. Cabral et al. (1995) reported that AEA inhibited mouse RAW264.7 macrophage-like cell killing of TNF-sensitive mouse L929 fibroblasts. Berdyshev et al. (1997) investigated the effects of AEA on the production of TNF-α, IL-4, IL-6, IL-8, IL-10, IFN-γ, p55, and p75 TNF-α soluble receptors by stimulated human peripheral blood mononuclear cells as well as [^{3}H]arachidonic acid release by non-stimulated and formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe, fMLP)-stimulated human monocytes. AEA was shown to diminish IL-6 and IL-8 production at low nanomolar concentrations but to inhibit the production of TNF-α, IFN-γ, IL-4, and p75 TNF-α soluble receptors at higher concentrations. The effect of AEA on IL-6 and IL-8 production disappeared when used at a higher concentration. AEA had no effect on IL-10 synthesis. The release of [^{3}H]arachidonate was stimulated only by high concentrations of AEA. Chang et al. (2001) compared the effects of AEA and 2-AG on LPS-induced NO, IL-6, and PGE_2 (prostaglandin E2) release from mouse J774 macrophage-like cells. AEA diminished LPS-induced NO and IL-6 production, while 2-AG inhibited the production of IL-6 but slightly increased inducible nitric oxide synthase (iNOS)-dependent NO production. AEA and 2-AG had no effect on LPS-induced PGE_2 production and COX-2 induction. It was proposed that the discrepant results of 2-AG on iNOS and COX-2 induction were due to its bioactive metabolites, arachidonic acid, and PGE_2, since incubation with these potentiated the induction of both iNOS and COX-2. The AEA metabolite, PGE_2-ethanolamide, had no effect on the production of the LPS-induced NO or IL-6. It was suggested that the results were consistent with a direct cannabinoid receptor activation leading to anti-inflammatory action through the inhibition of macrophage function. It was indicated, in addition, that 2-AG also served as a substrate for COX-catalyzing PGE_2 production, which in turn modulated the action of the CB2R. Ribeiro et al. (2010) investigated whether treatment in vivo with a low dose of AEA immediately prior to sensitization with ovalbumin that was injected at the base of the tail would have an immunosuppressive or immunostimulatory effect on the Th1 cell-mediated immune response in mice. It was reported that AEA, administered prior to sensitization, increased the Th1 response to ovalbumin in vivo and ex vivo. AEA increased the DTH response, splenocyte proliferation, and IFN-γ production in a co-culture of adherent and non-adherent splenocytes. Moreover,

administration of AEA prior to sensitization increased both the expression of dendritic cell co-stimulatory molecules (CD80/CD86) and IL-12/IL-23 (p40) production ex vivo. The direct effects of AEA in the IFN-γ/IL-4 balance of Concanavalin A (ConA)-stimulated splenocytes in vitro were also assessed. AEA at nanomolar concentrations increased the production of IFN-γ, but this production decreased at micromolar range. Thus, AEA induced both the increment of dendritic cell activation and IFN-γ production, considered likely factors involved in the increase of Th1 immune response.

Stefano et al. (2000) found that 2-AG caused human monocytes and immunocytes from the blue mussel, *Mytilus edulis*, to assume a round shape and become immobile, a transformation that was postulated to correlate with decreased production of cytokines and adhesion molecules associated with an immunosuppressive response. In addition, exposure of these cells to 2-AG resulted in NO release, which was blocked by the NO synthase inhibitor L-NAME and the CB1R antagonist SR141716A, but not by the CB2R antagonist SR144528. Similar results were obtained using cells related to the human vascular system. Treatment of human saphenous veins and atria with 2-AG stimulated basal NO release, which was antagonized by L-NAME and the CB1R antagonist. Kishimoto et al. (2003) examined the effects of 2-AG on the motility of human promyelocytic leukemia cells (HL-60 cells) that had been differentiated into macrophage-like cells. It was found that 2-AG induced the migration of these cells in a mode that was blocked by treatment with the CB2R antagonist SR144528 or pertussis toxin, consistent with a functional linkage to the CB2R and G_i/G_o proteins. In contrast, AEA did not induce migration by these cells. The 2-AG-induced migration was observed also for human monocytic leukemia U937 cells, human monocytic leukemic (THP-1) cells, and peripheral blood monocytes. Gokoh et al. (2005) reported that 2-AG enhanced the adhesion of HL-60 cells, differentiated into macrophage-like cells, to fibronectin and vascular cell adhesion molecule (VCAM)-1. The CB2R, G_i/G_o, intracellular free Ca^{2+}, and phosphatidylinositol 3-kinase were shown to be involved in 2-AG-induced augmented cell adhesion. 2-AG also enhanced the adhesion of U937 cells and peripheral blood monocytes. These results suggested that 2-AG played an essential role in inflammatory reactions and immune responses by inducing adhesion to extracellular matrix (ECM) proteins and adhesion molecules in several types of inflammatory cells and immune-competent cells. Gokoh et al. (2007) also examined the effect of 2-AG on the phagocytosis of opsonized zymosan by HL-60 cells that had been differentiated into macrophage-like cells. 2-AG augmented the phagocytosis of opsonized zymosan. Treatment of the HL-60 cells with the CB2R antagonist SR144528 or pertussis toxin abolished the effect of 2-AG, indicating that the CB2R and G_i/G_o were involved in the augmented phagocytosis. It was suggested that phosphatidylinositol 3-kinase and extracellular signal-regulated kinase were involved in this process since treatment of the HL-60 cells with wortmannin or PD98059 abrogated the 2-AG-augmented phagocytosis. More recently, Montecucco et al. (2009) assessed levels of endocannabinoids and related molecules during atherosclerosis development in mice. It was found that endocannabinoid-degrading enzymes were expressed by macrophages within

atherosclerotic lesions. In vitro, 2-AG induced monocyte migration, which corresponded to the levels observed in aortas. It was suggested that enhanced 2-AG levels in advanced atherosclerotic lesions triggered the inflammatory process by recruiting inflammatory cells and inducing ECM degradation through the CB2R. However, while this possibility was supported in vitro, in vivo experiments using the CB2R antagonist SR144528 failed to provide confirmation.

3.7 Natural Killer Cells

Natural killer (NK) cells are a type of cytotoxic lymphocyte that provides rapid responses to virally infected cells and tumor cells. NK cells are capable of recognizing stressed cells in the absence of antibodies and markers of the major histocompatibility complex (MHC). That is, they do not require activation in order to kill cells that are missing "self" markers of the MHC class 1. The functional role of NK cells is important since cells that are missing MHC 1 markers cannot be detected and destroyed by other immune cells, such as T cells. Kishimoto et al. (2005) examined the effects of 2-AG on the motility of human NK cells. It was found that 2-AG induced the migration of a line of natural killer leukemia cells (KHYG-1 cells) and human peripheral blood NK cells. The migration of NK cells induced by 2-AG was abolished by treating the cells with SR144528, a CB2R antagonist, suggesting that the CB2R was involved in the 2-AG-induced migration. In contrast, AEA did not induce migration of these cells.

3.8 Neutrophils

Neutrophils constitute approximately 60 % of PMNs and contain lysosomal enzymes in their cell granules that break down bacterial cells. In this capacity, they play a critical role in acute inflammation and provide a first line of defense against microbes. Chemotactic signals include IL-8, the streptococcal peptidase C5a, the bacterial protein derivative fMLP, and leukotriene B4. Kraft and Kress (2005) investigated the respiratory burst reaction of human whole-blood PMNs under the influence of cannabinoids using flow cytometry. In their natural whole-blood milieu, a CB2R-dependent stimulation of the PMN respiratory burst was found at nanomolar concentrations of methanandamide, a synthetic relatively stable derivative of AEA, whereas the short-living and rapidly hydrolyzed endogenous ligand AEA did not alter the burst reaction of whole-blood PMNs. However, the stimulatory cannabinoid effect was absent in isolated PMNs but could be transferred onto isolated polymorphonuclear leukocytes by adding the cell-free low-molecular mass plasma fraction (<5000 Da) of cannabinoid-incubated blood, consistent with an indirect mode of action that was dependent on humoral products or mediators. It was suggested that products of arachidonic acid metabolism acted as mediators of the cannabinoid-induced enhancement of the respiratory burst reaction of whole-blood PMNs. Kurihara et al. (2006) found that upon stimulation

with 2-AG, HL60 cells rapidly extended and retracted one or more pseudopods containing F-actin in different directions instead of developing a front/rear polarity typically exhibited by migrating leukocytes. Activity of Rho-GTPase RhoA decreased in response to CB2R stimulation, whereas that of Rac1, Rac2, and Cdc42 increased. Moreover, treatment of cells with the RhoA-dependent protein kinase (p160-ROCK) inhibitor Y27632 yielded a cytoskeletal organization similar to that observed for CB2R-stimulated cells. In human neutrophils, 2-AG did not induce motility or morphologic alterations. Pretreatment of neutrophils with 2-AG disrupted fMLP-induced front/rear polarization and migration and suppressed fMLP-induced RhoA activity. The 2-AG results were replicated using JWH015, a CB2R-selective agonist. The collective results suggested that the CB2R played a role in regulating excessive inflammatory responses by controlling RhoA activation that, in turn, resulted in a suppression of neutrophil migration.

On the other hand, there have been reports that receptors other than the CB2R, or pathways not involving this receptor, are involved in the modulation of neutrophil functional activities. McHugh et al. (2008) found that certain endogenous lipids, including AEA, inhibited human neutrophil migration at nanomolar concentrations in a biphasic manner. These investigators implicated a pharmacological target that was distinct from the CB1R and CB2R that was antagonized by the endogenous compound *N*-arachidonoyl l-serine. Balenga et al. (2011) suggested that GPR55 modulated CB2R-mediated responses. It was found that GPR55 was expressed in human blood neutrophils and that its activation augmented their migratory response toward 2-AG while concomitantly inhibiting neutrophil degranulation and reactive oxygen species (ROS) production. Chouinard et al. (2011) postulated that arachidonic acid released by 2-AG and AEA hydrolysis was metabolized into eicosanoids and that these metabolic products could, in turn, serve as mediators of some of the effects of endocannabinoids. It was concluded that while 2-AG activated human neutrophils, it did so indirectly as a result of 2-AG hydrolysis, de novo LTB_4 biosynthesis, and an autocrine activation loop that involved the LTB_4 receptor 1. In a subsequent series of studies, Chouinard et al. (2013) reported that 2-AG and arachidonic acid-stimulated neutrophils released a variety of antimicrobial effectors. Supernatants of neutrophils activated with nanomolar concentrations of 2-AG and arachidonic acid inhibited the infectivity of herpes simplex virus type 1 (HSV-1) and respiratory syncytial virus (RSV). In addition, these supernatants impaired the growth of *Escherichia coli* and *Staphylococcus aureus*. The impairment of bacterial growth correlated with the release of α-defensins, as well as a limited amount of the antimicrobial peptide LL-37 (cathelicidin). The effects of arachidonic acid and 2-AG were prevented by inhibiting LTB_4 biosynthesis or by blocking that of BLT_1. CB2R agonists or antagonists could neither mimic nor prevent the effects of 2-AG. It was suggested that the rapid conversion of 2-AG to arachidonic acid and their metabolism into LTB_4 (leukotriene B4) promoted 2-AG and arachidonic acid as multifunctional activators of neutrophils, exerting their effects primarily by activating the BLT_1 receptor.

4 Immune Cells in the Central Nervous System

The CNS harbors an endogenous "immune" system that includes astrocytes, microglial cells, and perivascular macrophages. Endocannabinoids have been linked to the modulation of these cells' functional capabilities and play a role in regeneration of damaged CNS tissue. For example, studies performed using the medicinal leech have provided insights into the regeneration of the CNS following mechanical trauma. Using an electrochemical NO-selective electrode to measure NO levels, Arafah et al. (2013) found that the time course of NO release in the injured leech CNS was partially under the control of AEA and 2-AG. The results showed that after injury concurrent with ATP production, purinergic receptor activation, NO production, microglial recruitment, and accumulation to the lesion site, an imbalance occurred in the endocannabinoid system.

4.1 Astrocytes

Astrocytes, or astroglia, are the most abundant cell type in the human CNS. These cells provide nutrients to nervous tissue, maintain extracellular ion balance, and play a role in the repair and scarring of the brain and spinal cord following traumatic injuries. They also elicit a plethora of proinflammatory cytokines upon secondary activation by inflammatory factors released from microglia. Molina-Holgado et al. (1997) showed that primary cultures of neonatal mouse cortical astrocytes stimulated with LPS (BALB/c mice strain) or Theiler's murine encephalomyelitis virus (TMEV) (SJL/J mice strain), used as an encephalomyelitis model for MS, released increased amounts of nitrites (NO_2-) and TNF-α. AEA blocked the release of NO_2- and TNF-α that was induced by LPS. In TMEV-stimulated astrocytes, AEA also suppressed the stimulatory effects of TMEV on both NO_2- and TNF-α. Molina-Holgado et al. (1998) also investigated whether AEA could modify IL-6 production by primary cultures of mouse brain cortical astrocytes infected with TMEV. Infection of a susceptible strain of mice resulted in virus persistence in the brain and chronic primary immune-mediated demyelination, which resembled MS. Astrocytes from susceptible (SJL/J) and resistant (BALB/c) strains of mice infected with TMEV released higher levels of IL-6. AEA caused an enhancement of the release of IL-6 by the TMEV-infected astrocytes. Treatment of TMEV-infected astrocytes with arachidonyl trifluoromethyl ketone, a potent inhibitor of the amidase that degrades AEA, potentiated the effects of AEA on IL-6 release. The selective CB1R antagonist SR141617A blocked the enhancing effects of AEA on IL-6 release by TMEV-infected astrocytes, suggesting a linkage to this cannabinoid receptor in the AEA-mediated enhancement of IL-6 release. Molina-Holgado et al. (2002) extended these studies regarding the role of the CB1R in mediating the actions of AEA. Coincubation of primary mouse astrocyte cultures with AEA resulted in an inhibition of LPS-induced release of NO. The inhibitory effect was abolished by the CB1R antagonist SR141716A. Furthermore, SR141716A alone increased NO release in response to LPS, suggesting that endocannabinoids

modified inflammatory responses. In addition, coincubation of astrocytes with the CB2R antagonist SR144528 abolished the inhibitory effects of AEA on LPS-induced NO release. Ortega-Gutiérrez et al. (2005) studied whether UCM707, a potent and selective AEA uptake inhibitor, was able to inhibit the production of proinflammatory mediators by LPS-stimulated astrocytes. UCM707 reduced NO release, iNOS expression, and the production of the proinflammatory cytokines TNF-α and IL-1β, while producing a slight increase in IL-6 levels.

4.2 Microglia

Microglia are resident macrophages in the brain and spinal cord. These cells exhibit many of the functional properties of macrophages in tissues at nonneuronal sites and upon stimulation release chemokines and proinflammatory cytokines. Kreutz et al. (2009) investigated whether the activation of the "abnormal cannabidiol" (abn-CBD) receptor, a receptor that is coupled to a G_i/G_o protein and is sensitive to abn-CBD (a synthetic isomer of CBD) that is inactive at the CB1R or CB2R, contributed to 2-AG-mediated neuroprotection. In their studies, they used an organotypic hippocampal slice culture system that included an excitotoxic lesion induced by *N*-methyl-D-aspartate (NMDA) that causes neuronal damage and accumulation of microglia within the granule cell layer. Application of abn-CBD or 2-AG to lesioned slice cultures resulted in a decrease in the number of microglia and neurons in the dentate gyrus. 2-AG was reported to exert a neuroprotective effect through the activation of abn-CBD-sensitive receptors on the microglia. It was indicated that 2-AG modulated migration and proliferation of microglia that then were rapidly activated following introduction of the brain lesion. Furthermore, these modulatory effects were attributed to the activation of CB2R and abn-CBD-sensitive receptors. McHugh et al. (2010) investigated the relationship between *N*-arachidonoyl glycine (NAGly), an endogenous metabolite of AEA that reportedly acts as an agonist at the G protein-coupled receptor GPR18. It was found that NAGly acted as an effective lipid recruiter of mouse BV-2 microglial-like cells and that it mimicked the effects of abn-CBD. NAGly exhibited marked potency for acting on GPR18 to elicit directed migration and proliferation. Based on these data, it was suggested that GPR18, also referred to as the *N*-arachidonoyl glycine receptor, was the "abn-CBD" receptor.

Navarrete et al. (2009) examined the role of AEA and *N*-arachidonoyldopamine (NADA) in the regulation of PGE2 synthesis in primary glial cells. It was demonstrated that NADA exerted a robust inhibition of PGE2 synthesis in LPS-stimulated cells, without modifying the expression or enzymatic activity of COX-2 and the production of PGD2 (prostaglandin D2). It was shown also that NADA prevented free radical formation in primary microglia. Thus, AEA and NADA exerted opposite effects on glial cells. The results suggested that NADA had the potential to serve as an antioxidative and anti-inflammatory agent acting through a mechanism that involved reduction in the synthesis of microsomal PGE in LPS-activated microglia.

Correa et al. (2009) investigated the effects of AEA on the inducible expression of IL-12p70 and IL-23 and their forming subunits in activated human and mouse microglia. They also studied the signaling pathways involved in the regulation of IL-12p70/IL-23 expression. It was reported that AEA inhibited the production of biologically active IL-12p70 and IL-23 and their subunits. Treatment of these activated cells with inhibitors of mitogen-activated protein kinases (MAPK) revealed that AEA acted through the ERK1/2 and JNK pathways to downregulate IL-12p70 and IL-23 and that these effects were mediated, at least in part, by the activation of the CB2R. These investigators (Correa et al. 2010) subsequently showed that AEA enhanced LPS/IFN-γ-induced IL-10 production in microglia by targeting the CB2R through the activation of ERK1/2 and JNK MAPKs. AEA also inhibited NF-κB activation by interfering with the phosphorylation of IκBα, an action that was postulated to account for the increase of IL-10 production. It was suggested that, by altering the cytokine network, AEA indirectly modified the type of immune responses within the CNS. Hernangomez et al. (2012) reported that AEA protected neurons from microglial-induced neurotoxicity through a CD200–CD200R interaction. CD200 is a membrane glycoprotein expressed in neurons that suppresses immune activity through its receptor CD200R that is located mainly in macrophages/microglia. It was indicated that the AEA-mediated increase of the expression of CD200R1 in LPS/IFN-γ-activated microglia was due to the activation of the CB2R. The neuroprotective effect of AEA disappeared when microglia were obtained from CD200R1$^{-/-}$ mice, lending support for a functional linkage to the CB2R. In the chronic phases of Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), used as a model of MS, the expression of CD200 and CD200R1 was reduced in the spinal cord. AEA-treated animals exhibited an upregulation in the expression of CD200 and CD200R1 to levels comparable to those found in sham animals. The AEA-treated animals also exhibited an increase in the expression of IL-10 and a reduction in that of IL-1β and IL-6. Because AEA upregulated the expression of CD200R1 in microglia, but failed to enhance CD200 in neurons, it was suggested that the AEA-induced upregulation of CD200 in the TMEV-IDD paradigm was mediated through the action of IL-10 as the level of this anti-inflammatory cytokine was shown to be increased in CD200-positive neurons.

5 Effects of Endocannabinoids In Vivo

Experimental models using animals such as guinea pigs and mice have been employed for nearly a century to document effects of various toxic and infectious agents on host resistance. These in vivo models have offered unique advantages for assessment of effects of drugs on infection and immunity due to their well-defined immune systems. Furthermore, the use of animal models has allowed for the definition of factors of host resistance that are targeted by drugs under stringently controlled conditions. As a result, acquisition of statistically significant data with minimal confounding variables has been possible, a condition that is difficult to

attain for human populations as a result of potential environmental toxic exposures and multiple drug use. The conducting of in vivo studies using endocannabinoids has been challenging, partially because these substances are readily degraded, necessitating that they be applied experimentally at relatively high doses. Furthermore, their intracellular fate, compartmentation, and processing within the host may be distinctive from that of exogenously introduced synthetic cannabinoids. Nevertheless, given these caveats, the results obtained with endocannabinoids through in vivo studies are in general agreement with those that have been derived from in vitro studies.

Maestroni (2004) suggested that 2-AG acted as a chemotactic substance that recruited dendritic cells or their precursors during the innate immune response. These cells, in the presence of a Toll-like receptor (TLR) agonist, display a T helper-1 (Th1)-shifted adaptive response. It was demonstrated that 2-AG injected intradermally in mice together with a soluble protein and a T helper-2 (Th2) priming TLR agonist during primary immunization shifted the memory response to the Th1 type. This effect was demonstrated by the enhanced hypersensitivity response and by the Th1 pattern of cytokines that were produced, a result that was abolished by the CB2R antagonist SR144528. It was postulated that 2-AG operated during the innate immune response by increasing the number of dendritic cells migrating to draining lymph nodes. Oka et al. (2005) investigated the pathophysiological roles of the CB2R and 2-AG in acute inflammation in the mouse ear that was induced by the topical application of 12-O-tetradecanoylphorbol-13-acetate. It was found following treatment that the amount of 2-AG was markedly augmented while that of AEA was not affected. The 12-O-tetradecanoylphorbol-13-acetate-induced ear swelling was blocked by the CB2R antagonist SR144528. In addition, the application of 2-AG to the mouse ear-evoked swelling was abolished by the CB2R antagonist SR144528. It was suggested that NO was involved in the ear swelling induced by 2-AG, implicating a role for neutrophils in this process, and that the CB2R and 2-AG played crucial stimulative roles during the course of the inflammatory reaction. Mimura et al. (2012) using the ear dermatitis model showed that the level of 2-AG increased upon serial 2,4-dinitrofluorobenzene challenges and was correlated with ear weight gain. The increased ear thickness in this allergy model was clearly suppressed in CB2R knockout mice, suggesting that generated endocannabinoids induced ear thickness through aberrant inflammatory responses and remodeling mediated through the CB2R. In addition, in an allergic bronchitis model induced by ovalbumin, the 2-AG level in bronchoalveolar lavage was increased and sustained during the elevation of inflammatory cell infiltration. DNA microarray analysis of human HL-60 cells revealed that the 2-AG ether, noladin ether, induced the expression of inflammatory chemokines/cytokines and cell growth factors. The data suggested that endocannabinoids that served as endogenous CB2R ligands that were upregulated upon disease progression in allergic models were involved in aberrant alterations of both inflammatory responses and tissue cell growth. Ribeiro et al. (2010) investigated whether treatment in vivo with a low dose of AEA immediately prior to in vivo sensitization with ovalbumin had an immunosuppressive versus an immunostimulatory effect on cell-

mediated immunity (i.e., Th1 response) in mice. Administration of AEA prior to sensitization resulted in an increase in the Th1 response to ovalbumin in vivo and ex vivo. AEA administration resulted in an increase in the DT response, splenocyte proliferation, and IFN-γ production in a co-culture of adherent and non-adherent splenocytes. Moreover, AEA administration prior to sensitization resulted in an increase in both the expression of dendritic cell co-stimulatory molecules (CD80/CD86) and IL-12/IL-23 (p40) production ex vivo. Direct effects of AEA in the IFN-γ/IL-4 balance of ConA-stimulated splenocytes in vitro also were assessed. AEA at nanomolar concentrations increased the production of IFN-γ. Thus, AEA induced both dendritic cell activation and IFN-γ production, key factors involved in the increase of the Th1 response. Jackson et al. (2014) observed that a single intraperitoneal administration of AEA caused rapid induction of myeloid-derived suppressor cells. This heterogeneous population of cells consisted of a mixture of granulocytic and monocytic subtypes and expressed arginase-1 (Arg-1), a binuclear manganese metalloenzyme that catalyzes the conversion of L-arginine into L-ornithine and urea, and iNOS. The myeloid-derived suppressor cells inhibited T-cell proliferation in vitro mediated through iNOS. In addition, mice were sensitized by a subcutaneous injection of methylated bovine serum albumin (mBSA). Twelve hours before mBSA rechallenge, mice were injected with myeloid-derived suppressor cells. Adoptive transfer of myeloid-derived suppressor cells led to suppression of methylated bovine serum albumin (mBSA)-induced DTH. Through the use of pharmacological inhibition, as well as genetic knockout mice, it was found that the induction of myeloid-derived suppressor cells by AEA was dependent on the CB1R. The induction of myeloid-derived suppressor cells by AEA was reduced in mast cell-deficient mice, while maintained in LPS-insensitive mice, showing that the induction of myeloid-derived suppressor cells by AEA was dependent, at least in part, on mast cells and independent of Toll-like receptor 4 (TLR4), a receptor that detects LPS from gram-negative bacteria and leads to the activation of the immune system. Chemokine analysis of AEA-treated wild-type mice showed an early spike of monocyte chemoattractant protein-1 (MCP-1). This spike was decreased in Kit (W/W-sh) mast cell-deficient mice, implicating a role of mast cells in the secretion of MCP-1 in response to AEA. Use of antibodies against MCP-1 or mice deficient in MCP-1 confirmed the linkage to MCP-1. It was concluded that endocannabinoids activated the CB1R on mast cells to induce MCP-1, which facilitated recruitment of monocytic myeloid-derived suppressor cells.

There have been a limited number of studies that have addressed the effects of endocannabinoids on peripheral, nonneuronal pathological processes. Sugamura et al. (2009) sought to determine whether the endocannabinoid system was involved in human atherosclerosis in which plaque builds up inside the arteries. They investigated whether pharmacological blockade of the CB1R could modulate proinflammatory activity in macrophages. Patients with coronary artery disease demonstrated the activation of the endocannabinoid system that was accompanied by elevated levels of blood endocannabinoids and increased expression of CB1R in coronary atheroma, an accumulation of fatty material within the inside lining of the arteries. It was indicated that blockade of the CB1R in macrophages with the

CB1R-selective antagonist rimonabant (SR141716A) led to anti-inflammatory effects on the part of these cells. Lenglet et al. (2013) assessed atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) and $ApoE^{-/-}FAAH^{-/-}$ mice. These investigators observed enhanced recruitment of neutrophils, but not monocytes, to large arteries of $ApoE^{-/-}$ mice treated with the FAAH inhibitor URB597. Spleens of $ApoE^{-/-}FAAH^{-/-}$ mice had reduced CD4+FoxP3+regulatory T-cell content, and in vitro stimulation of splenocytes revealed significantly elevated IFN-γ and TNF-α production in the case of FAAH deficiency. It was concluded that increased AEA and related FAAH substrate levels were associated with the development of smaller atherosclerotic plaques with high neutrophil content that was accompanied by an increased proinflammatory immune response. Rettori et al. (2012) investigated the role of AEA in experimental periodontitis, an infectious disease leading to inflammation and destruction of tissue surrounding and supporting the tooth. In this disease, the progress of the inflammatory response depends on the host's immune system and risk factors such as stress. Experimental periodontitis was induced by a ligature around the first inferior molars and immobilization stress for 2 h twice daily for 7 days in a rat model. AEA was shown to diminish the inflammatory response in periodontitis even during a stressful situation. Donovan and Grundy (2012) examined cannabinoid modulation of sensory signaling from the gastrointestinal tract following an acute inflammatory response triggered by systemic administration of LPS. A segment of proximal jejunum was intubated to allow for measurement of intraluminal pressure in anesthetized rats. Afferent impulse traffic was recorded from a single isolated paravascular nerve bundle supplying the jejunal loop. It was found that the AEA transport inhibitor, VDM11, but not the FAAH inhibitor URB597, caused an increase in afferent activity. The VDM11 response was found to be linked to mediation by both the CB1R and CB2R. LPS evoked an increase in afferent activity that was reduced by the blockade of the CB1R and CB2R. It was concluded that endocannabinoids played a role in modulating afferent signaling and that, in this context, the endocannabinoid system represented a target for the treatment of visceral hypersensitivity. However, inhibition of the breakdown of endocannabinoids through the use of URB597 had no effect on baseline or LPS-induced afferent firing. These observations suggested that the uptake of endocannabinoids rather than their breakdown mediated by FAAH terminated their action in the gastrointestinal tract.

6 Effects of Endocannabinoids on Immune Function in the CNS

Since endocannabinoids have been shown to ablate immune functional activities, they have been proposed as candidate agents for treatment of a variety of pathological processes in the CNS that are characterized by a hyperimmune response. In this context, these substances afford several advantages. They have low cytotoxicity and readily cross the blood–brain barrier (BBB) due to their lipophilicity. Furthermore, as in the case of 2-AG, they target the CB2R that is expressed on selective

cell types that have immune function capability within the CNS. The selective targeting of the CB2R is important since the activation of the CB2R obviates untoward psychotropic effects that could be engendered if signal transduction were effected through the CB1R. Finally, due to the short half-life of endocannabinoids, their effects are postulated to be short-lived, thereby minimizing the potential for long-term perturbation of immune functional capability within the CNS. It is now apparent that resident immune cells within the CNS and eye harbor an endocannabinoid system (Suárez et al. 2010; Hu et al. 2010). Recently, Krishnan and Chatterjee (2014) suggested that endocannabinoids had potential as neurotherapeutic agents for select conditions linked to human immunodeficiency virus (HIV-1)-induced inflammation. They investigated whether the innate immune response in human retinal Muller glia could be modulated to combat inflammation since an increased inflammatory response can cause visual impairment during HIV infection in spite of successful antiretroviral therapy. AEA and 2-AG were used to alleviate cytotoxicity induced by the HIV-specified nonstructural transactivating protein Tat (trans-activator of transcription) and to rescue retinal cells. The neuroprotective effect of these endocannabinoids involved suppression of the production of proinflammatory cytokines and augmentation of that of anti-inflammatory cytokines. This altered expression was effected mainly through the activation of the MAPK pathway and regulated primarily by mitogen-activated protein kinase phosphatase-1 (MKP-1). Both endocannabinoids regulated cytokine production of the transcriptional level of the NF-κB complex, including that of IL-1 receptor-associated kinase 1 binding protein 1 (IRAK1BP1) and TGF-β-activated kinase 1/MAP3K7 binding protein 2 (TAB2), a signal transducer that acts as an adaptor molecule of TNF receptor-associated factor 6 (TRAF6) and TGF-β-activated kinase 1 (TAK1) and mediates the activation of TAK1 (a ubiquitin-dependent kinase of mitogen-activated protein kinase and IκB kinase). Nevertheless, there has been a paucity of studies that have addressed from an immunological perspective the effect of endocannabinoids on neuropathological processes in animal models and humans. The studies that have been conducted have focused on experimentally induced Alzheimer's disease (Piro et al. 2012), multiple sclerosis (Bittner et al. 2009; Rossi et al. 2011; Correa et al. 2011; Mestre et al. 2011; Lourbopoulos et al. 2011; Sanchez Lopez et al. 2014), Parkinson's disease (Fernandez-Suarez et al. 2014), and traumatic brain injury (Katz et al. 2014).

7 Summary and Conclusions

Lipid-derived messengers and their cognate receptors cooperate with other signaling molecules to modulate the functional activities of immune cells at nonneuronal and neuronal sites. Included among these bioactive lipids are endocannabinoids, endogenous cannabinoids that have the potential to signal through the seven-transmembranal, G protein-coupled CB1R and CB2R. Their ligation to, and activation of, these receptors sets in motion a series of signal transductional events that converge at the transcriptional level to regulate cell migration and the production of

cytokines and chemokines. There is a large body of data that supports a functional relevance for 2-AG as acting through the CB2R to inhibit migratory activities for a diverse array of immune cell types, and it has been suggested that 2-AG is the cognate functionally relevant endocannabinoid for the CB2R (Sugiura et al. 2000; Parolaro et al. 2002). AEA has been reported to inhibit immune functional activities, particularly the production of proinflammatory cytokines. However, unequivocal evidence that supports a functional linkage of AEA to a cannabinoid receptor in mediating these effects remains to be obtained. Endocannabinoids, typical of bioactive lipids, have a short half-life intracellularly and extracellularly and appear to act in an autocrine and paracrine fashion. Thus, it appears that their immediate effective action on immune function is at localized sites in the periphery and CNS. It is speculated that, in this context, endocannabinoids play an important role in maintaining the overall "fine-tuning" of the immune homeostatic balance within the host. Recognition that immune functional activities can be mediated through interaction of lipid ligands with specified receptors leading to the activation of signaling cascades should provide unique and novel insights into the development of therapeutics for the manipulation and ablation of untoward immunological events, including possibly those associated with infection with the HIV and other pathogens. Furthermore, definition of the functional relevance of lipid bio-effector molecules in the immune system could lead to the development of strategies for therapeutic intervention related to the use of illicit drugs that may interfere with endogenous immune homoeostatic and modulatory processes.

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Endocannabinoids in Multiple Sclerosis and Amyotrophic Lateral Sclerosis

Gareth Pryce and David Baker

Contents

Abstract

There are numerous reports that people with multiple sclerosis (MS) have for many years been self-medicating with illegal street cannabis or more recently medicinal cannabis to alleviate the symptoms associated with MS and also amyotrophic lateral sclerosis (ALS). These anecdotal reports have been confirmed by data from animal models and more recently clinical trials on the ability of cannabinoids to alleviate limb spasticity, a common feature of progressive MS (and also ALS) and neurodegeneration. Experimental studies into the biology of the endocannabinoid system have revealed that cannabinoids have efficacy, not only in symptom relief but also as neuroprotective agents which may slow disease progression and thus delay the onset of symptoms. This review

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_7

discusses what we now know about the endocannabinoid system as it relates to MS and ALS and also the therapeutic potential of cannabinoid therapeutics as disease-modifying or symptom control agents, as well as future therapeutic strategies including the potential for slowing disease progression in MS and ALS.

Keywords
Amyotrophic lateral sclerosis • Endocannabinoid • Experimental autoimmune encephalomyelitis • Multiple sclerosis • Neurodegeneration • Neuroinflammation • Neuroprotection • Symptom management

Abbreviations

2-AG	2-Arachidonoyl glycerol
AEA	Anandamide
ALS	Amyotrophic lateral sclerosis
EDSS	Expanded disability status scale
EPSC	Excitatory post-synaptic current
FAAH	Fatty acid amide hydrolase
FTD	Fronto-temporal dementia
GABA	Gamma aminobutyric acid
MAG lipase	Monoacylglycerol lipase
MS	Multiple sclerosis
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
SOD-1	Superoxide dismutase 1
THC	Δ^9-tetrahydrocannabinol

1 Introduction

Multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) are two relatively common neurological conditions which are major causes of disability in adults (and also some children in the case of paediatric MS). The course of disease in MS is typically slow, with eventual increasing disability leading to death whereas the course of disease in ALS is typically rapid from diagnosis, with rapidly evolving disability and typically death within 2 years. This review will discuss what is known about the influence of the endocannabinoid system in these diseases, what is the potential influence of endocannabinoids in disease progression and what these findings may hold for the potential exploitation of the endocannabinoid system as a therapeutic strategy in MS and ALS.

2 Multiple Sclerosis

2.1 Natural History

Multiple sclerosis (MS) is an inflammatory, autoimmune, demyelinating disease of the central nervous system (CNS) and is the most common cause of non-traumatic neurological disability in young adults of northern European descent (Compston and Coles 2002, 2008). This disease affects approximately 100,000 people within the United Kingdom. The absolute number of cases of MS around the world has gradually increased, which may be as a result of improved diagnosis, as well as other factors, and currently affects 2–3 million people worldwide (Kurtzke 1993). The incidence of MS is geographically restricted and particularly occurs with a high incidence in Northern Europe and in regions colonized by white Northern Europeans, such as Canada and Northern USA, Australia and New Zealand, with a gradient of higher incidence further from the equator (Compston and Coles 2002). MS is more common in females compared to males, with an increasing ratio of historically 2:1–3:1 today, with a more pronounced female incidence in younger MS patients with relapsing-remitting disease (RRMS) (Runmarker and Andersen 1993). The highest incidence of MS reported is in the Orkney Isles with an incidence of 1 in 170 females (Visser et al. 2012). The susceptibility to the development of MS is influenced by genetics, as shown by an increased concordance of MS in monozygotic twins (~30 %) compared to dizygotic twins (~5 % concordance rate), and this susceptibility is polygenically controlled (Compston and Coles 2002, 2008). MS is associated with the expression of certain MHC haplotypes such as HLADRB1*1501 and is also influenced by over 150 other immune-related, susceptibility genes (Prat et al. 2005; Sawcer et al. 2011). However, the discrepancy in concordance of disease incidence in identical twins demonstrates that other, environmental, factors may influence susceptibility. Migration studies from low to high incidence areas suggest that the environmental trigger is acquired before the age of 15 (Compston and Coles 2002). Some have suggested that it may relate to age of infection and there is a long-standing hypothesis that this could relate to Epstein Barr Virus (EBV) infection (Ascherio and Munger 2010). The vast majority of people with MS, if not all, have been shown to have been infected with EBV compared to 90 % of the general population, and there is increased frequency of MS in people who developed glandular fever (Handel et al. 2010). This is indirectly supported by the geographic distribution of people with MS (Ebers and Sadovnick 1993). Vitamin D levels can influence the immune response and may even be important in utero (Willer et al. 2005), and a "month of birth" effect has been reported in a number of studies where in the northern hemisphere, there was a 5 % excess of cases among patients born in April and 5–8 % reductions in MS risk associated with birth in October or November. This suggests that ambient UV radiation and hence maternal vitamin D levels are prenatal environmental modulators of MS risk. Importantly, a number of genes associated with MS, such as certain human leucocyte antigen (HLA) haplotypes, contain vitamin D responsive elements in their promoter regions that can influence

expression and may link environmental and genetic susceptibility elements (Ramagopalan et al. 2009, 2010). MS most commonly (approximately 80 %) presents as a series of relapsing-remitting episodes of loss of neurological function due to conduction block in axons that eventually develops into a chronic, secondary progressive MS (SPMS) phase with no remission and with increasing disability over time, which correlates with CNS atrophy and axonal loss, particularly in the spinal cord (Bjartmar et al. 2000). In addition, a subtype of MS, primary progressive MS (PPMS) presents as a progressive degenerative phenotype in 10–15 % of patients after an initial bout of CNS inflammation (particularly in those with a disease onset later in life), which along with secondary progressive MS is largely refractory to currently available MS therapies such as immunomodulation (Miller and Leary 2007) and where neuroprotective strategies are urgently indicated. Clinically, PPMS develops at a later age than RRMS, with onset in the fourth decade rather than the third decade as seen in RRMS (Andersson et al. 1999) and with a lower female preponderance. As such, about 80 % of people with MS will be severely disabled within 25 years from disease onset.

2.2 Pathology, Symptoms and Disability

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

As the disease evolves, inflammatory attacks in the CNS increase the burden of demyelination and a dystrophic environment that leads to eventual neuronal and axonal loss, which impairs normal levels of neurotransmission. As a consequence of neuronal/axonal loss, there is the increasing development of additional distressing symptoms such as incontinence, limb tremor, pain, spasms, fatigue and spasticity, which have a major negative impact on quality of life indices (Compston and Coles 2002; Confavreux and Vukusic 2006). The time taken to convert to a secondary progressive neurodegenerative phenotype can vary widely between individuals and may reflect differences in an individual's ability to cope with episodes of neuronal insult, perhaps consistent with genetic control and heterogeneity of disease (Compston and Coles 2002). In approximately a quarter

of cases, neurological disability does not reach a level where it impinges on daily living, but conversely, in around 15 % of cases the progression to disability is rapid. The prognosis for patients is better in cases where sensory symptoms dominate the course of disease, and there is a complete recovery from these symptoms at remission whereas the prognosis is poorer when there is motor involvement such as deficits of pyramidal, visual, sphyncteric and cerebellar systems (Amato and Ponziani 2000). Frequent relapses and incomplete recovery plus a short time period between the initial neurological event and the subsequent relapse also have a poorer prognosis. There is also a poorer prognosis for the disease in older men who develop MS (Compston and Coles 2002). However, once a threshold of disability has been reached, disability progression is remarkably uniform (Confavreux et al. 2000), and approximately 90 % of RRMS patients will develop progressive disease after 25 years of clinical follow-up (Weinshenker et al. 1989). It may be that given enough time, all RRMS patients will eventually convert to the progressive phase of the disease. A recent study demonstrated that disability progression seems to follow a two stage course. The first stage, corresponding to clinical disease onset to irreversible Kurztke expanded disability status scale (EDSS) level 3, is dependent on ongoing focal neuroinflammation. There is a second stage, from irreversible disability scale 3 to irreversible disability scale 6, which is independent of ongoing focal neuroinflammation where neuroprotective strategies are indicated, rather than immunomodulatory therapies which are indicated for the phase one stage of MS (Leray et al. 2010).

Whilst immune-mediated conduction block and destruction of CNS myelin, followed by lesion resolution and limited myelin repair, may account for the relapsing-remitting nature of the disease, what is less clear are the mechanisms that account for the conversion to the chronic neurodegenerative secondary phase, which appears to be independent of, though worsened by, the accumulated neuronal dysfunction accompanying relapses (Bjartmar et al. 2003). A gradual degeneration of predominantly the pyramidal and cerebellar systems evolves which is often accompanied by sphincter and sexual dysfunction (Amato and Ponziani 2000). Axonal pathology during MS has been re-examined in recent years, a shift away from the predominant focus on demyelination, and it has been established that CNS atrophy and axonal loss occurs, coincidentally with inflammatory lesion formation, early in the relapsing-remitting phase. This may be accommodated initially by utilisation of spare neuronal capacity in the CNS, remodelling of neuronal circuits (neural plasticity) or an increase in the number of neural precursors in some lesioned areas contiguous with subventricular zones (Chang et al. 2008). However, as the disease continues, a threshold is reached, beyond which permanent impairment and increasing disability are established (Bjartmar et al. 2000, 2003; Confavreux et al. 2000; Confavreux and Vukusic 2006). This suggests that axonal loss rather than myelin damage is the key determinant of progressive disability in MS. In addition, a doubling in the levels of glutamate, an excitatory amino acid that has been shown to be neurotoxic in excess, is seen in the CSF of MS patients undergoing an inflammatory episode (Stover et al. 1997).

In experimental allergic encephalomyelitis (EAE), an animal model of MS induced by the development of autoimmunity against myelin antigens, 15–30 % of spinal cord axons can be lost before permanent locomotor impairment is noted (Bjartmar et al. 2000). After a number of relapse events, permanent disability develops with significant axonal loss in the spinal cord (40–80 %, as also occurs in MS), and the development of hind limb spasticity and tremor (Baker et al. 2000), which may reflect preferential loss of inhibitory circuits in certain locations of the spinal cord and their influence on signalling to skeletal muscles. Whilst inflammatory events are associated with axonal transections, chronic demyelination may contribute to a slow degenerative process.

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

2.3 Endocannabinoids in Multiple Sclerosis

2.3.1 Experimental Evidence

The first evidence for the involvement of the endocannabinoid system in multiple sclerosis was obtained from a mouse model of multiple sclerosis (EAE). Here it was shown that mice that had entered the chronic phase of disease, accompanied by profound neurodegeneration leading to the development of hind-limb spasticity, had elevated levels of the endocannabinoids anandamide [arachidonoylethanolamide (AEA)], the anandamide congener palmitoylethanolamide (PEA) and 2-arachidonoyl glycerol (2-AG) in the brain and spinal cord, compared to non-spastic EAE mice and normal controls. Spasticity was ameliorated by CB_1 receptor agonists but also by elevation of the endogenous levels of endocannabinoids via inhibition of their uptake or degradation (Baker et al. 2000, 2001). Importantly, spasticity was transiently increased after the administration of Rimonabant®, a CB_1 receptor inverse agonist/antagonist and strongly suggested the presence of an endogenous endocannabinoid tone which was elevated in response

to the development of limb spasticity. This amelioration of hind-limb spasticity is CB_1-dependent (Pryce and Baker 2007) and can be achieved by the elevation of the endogenous levels of endocannabinoids via inhibition of the putative AEA transporter (Baker et al. 2001; de Lago et al. 2004, 2006), inhibition of the degradation of AEA via fatty acid amide hydrolase (FAAH) inhibition (Pryce et al. 2013) or inhibition of the degradation of 2-AG via the inhibition of monoacylglycerol lipase (MAG lipase) (Pryce et al. 2013).

Endocannabinoids also have an important role in neurodegenerative processes arising from neuroinflammation, which was confirmed by the observation that CB_1-deficient mice show an increased rate of neurodegeneration compared to wild-type mice undergoing EAE or experimental autoimmune uveitis (Jackson et al. 2005; Pryce et al. 2003).

In another study, EAE-induced alterations of glutamate-mediated cortico-striatal excitatory postsynaptic current (EPSC) frequencies were exacerbated in mice lacking CB_1 receptors on glutamatergic neurons (Glu-CB1R-KO), indicating that this subset of receptors controls the effects of inflammation on glutamate release and mediates the potential excitotoxic effects of enhanced glutamate levels (Musella et al. 2014).

The rate of neurodegeneration in EAE can be decreased by the exogenous administration of either CB_1 agonists (Croxford et al. 2008), by exogenous 2-AG administration (Lourbopoulos et al. 2011), via pharmacological inhibition of endocannabinoid (AEA) degradation/uptake (Cabranes et al. 2005) or by the genetic ablation of FAAH, the enzyme that degrades AEA (Webb et al. 2008; Rossi et al. 2011; unpublished observation from this laboratory). Also, oligodendrocyte (the cell responsible for the production of myelin) excitotoxicity and white matter damage have been reported to be ameliorated by the administration of a MAG lipase inhibitor via enhancing endogenous levels of 2-AG, in contrast to the inhibition of FAAH, which had no effect in this animal model of MS (Bernal-Chico et al. 2015). In another study, EAE was also ameliorated via the selective inhibition of MAG lipase (Hernández-Torres et al. 2014).

Endocannabinoid (AEA and 2-AG) levels have been reported to be decreased in EAE in response to neuroinflammation (Cabranes et al. 2005) and in another study 2-AG was also found to be decreased (Witting et al. 2006). However, in contrast, an increase in AEA but not 2-AG has also been reported in EAE (Centonze et al. 2007). Excessive glutamate-mediated synaptic transmission and secondary excitotoxicity have been proposed as key determinants of the neurodegenerative damage in MS arising from neuroinflammation (Smith et al. 2000), and the primary action of neural CB_1 receptors is to regulate synaptic transmission via regulation of glutamate release from synaptic vesicles (Marsicano et al. 2003). Further evidence that supports this hypothesis is the observation that mice with genetically deleted CB_1 receptors show enhanced neurodegeneration as a result of neuroinflammation (Pryce et al. 2003), via enhanced neuronal apoptosis mediated by caspase-3 (Jackson et al. 2005) and loss of the neuroprotective effect of pharmacological potentiation of CB_1 signalling during neuroinflammation (Croxford et al. 2008). This protective action of CB_1 receptors may be via the suppression of tumour

necrosis factor α (TNFα)-mediated potentiation of striatal spontaneous glutamate-mediated EPSCs, which may contribute to the inflammation-induced neurodegenerative damage observed in EAE mice (Rossi et al. 2011). Decreases in CB_1 receptor levels in certain brain regions (striatum and cortex) in rat EAE have been reported, but this is accompanied by an increased coupling to GTP-binding protein-mediated signalling pathways, indicating a potential compensatory mechanism is in play here (Berrendero et al. 2001).

The role of the endocannabinoids in CB_2 receptor-mediated modulation of cells of the immune system in neuroinflammation is more contentious. It has been reported that selective stimulation of CB_2 receptors in C57Bl/6 mouse EAE can ameliorate disease (Kong et al. 2014). However, in a study with CB_2-deficient mice using a genetic strain (Biozzi ABH) that shows far more robust disease than C57Bl/6, it was shown that immunosuppression obtained by administration of high-dose Δ^9-tetrahydrocannabinol (THC) is maintained in CB_2-deficient mice. This indicates that THC-induced suppression of neuroinflammation does not result from CB_2 receptor activation but probably, rather from CB_1 receptor-mediated stimulation of glucocorticoid release via the hypothalamic/pituitary/adrenal axis (Sisay et al. 2013), with no immunomodulatory effect induced by a CB_2 selective receptor agonist in wild-type mice. That this immunosuppressive activity of THC is CB_1 receptor mediated is confirmed by the observation that THC-induced immunosuppression is lost in either global CB_1 knockout animals or where CB_1 is conditionally deleted in nerve cells (Maresz et al. 2007).

In summary, experimental studies have revealed that the endocannabinoid system is actively involved in protection against neurodegeneration arising from neuroinflammation and also positive benefits from CB_1 agonists such as THC and Sativex® in symptom control in MS point to the potential benefits of endocannabinoid modulation in these conditions.

2.3.2 Clinical Evidence

Endocannabinoids have been reported to be implicated in the pathophysiology of MS. AEA has been reported to be elevated in the cerebrospinal fluid (CSF) of MS patients compared to controls. Elevated levels of AEA were also seen in peripheral blood lymphocytes in this patient cohort (Centonze et al. 2007). In another study, increased plasma levels of AEA, oleoylethanolamide (OEA) and PEA were detected in MS patients, and increased AEA was seen in relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS) MS (Jean-Gilles et al. 2009). An increase in PEA was seen in RRMS and SPMS plasma but not PPMS. OEA was increased in the plasma of SPMS patients only. The levels of 2-AG was unchanged compared to controls across all three MS variants. A caveat here is that blood levels of endocannabinoids may not correlate to the levels of endocannabinoids that pertain to the more clinically relevant setting of the CNS. However, it has also been reported that both AEA and 2-AG levels are significantly reduced in the CSF of MS patients versus control subjects with lower values detected in the SPMS group. Higher levels of AEA and PEA, although below those of controls, were found in the CSF of relapsing-remitting MS patients during

a relapse and increased levels of AEA, 2-AG and OEA were found in patients with active neuroinflammatory lesions (Di Filippo et al. 2008). Further evidence for the potential reduction of endocannabinoids in MS was provided by the observation that enhanced FAAH expression was detected in active lesions in MS brain tissue (Benito et al. 2007).

To date there are no reports of clinical trials on the efficacy of the modulation of endocannabinoid levels in multiple sclerosis for either symptom relief or neuroprotection. However, the efficacy of cannabinoid agonists (THC, Sativex®) in the amelioration of spasticity has now been proven in a number of clinical studies (Fernández et al. 2014; Flachenecker et al. 2014; Lorente Serpell et al. 2013; Zajicek et al. 2005, 2012). Sativex® has been licensed for the treatment of spasticity in MS. There has also been one study investigating the neuroprotective potential of THC to slow the development of disability in MS (Zajicek et al. 2013). This stemmed from follow-up studies in symptomatic trials that suggested a neuroprotective effect of THC (Zajicek et al. 2005). Participants in the neuroprotective trial were randomly assigned to receive THC capsules or placebo capsules, to be taken by mouth over a period of 3 years. 329 people were allocated to receive the THC capsules and 164 were allocated to the placebo group. For each participant, the first 4 weeks of the trial were devoted to establishing the best tolerated dose of study treatment. For the remainder of the study period, participants remained on a stable dose of trial treatment, as far as possible, before the dose was gradually reduced to zero at the end of the treatment period. The study was "double-blind", meaning that neither the participants nor the doctors and nurses involved at the study sites knew which treatment group they were in. Despite the abundant experimental evidence that cannabinoid therapy has a neuroprotective role in a spectrum of neurological diseases, overall the study found no evidence that THC had an effect on MS progression in either of the main outcomes [the EDSS neurological assessments conducted by doctors at the study clinics or the 29-item multiple sclerosis impact scale (MSIS-29) questionnaire responses provided by the participants]. The EDSS and MSIS-29 scores showed little change over the course of the study, and no difference was found between the active and placebo groups. A confounding finding was that the placebo group had not progressed as expected, which complicates assessing the value of the trial. However, and potentially importantly, there was some evidence from subgroup analysis that THC might have a significant ($p < 0.01$) beneficial effect in participants at the lower end of the EDSS disability scale (<5.5 EDSS) where people tend to progress more rapidly (Leray et al. 2010). There was also some evidence from the two main study assessments (EDSS and MSIS-29) that participants with less disability had some slowing of MS progression, but the number of people in this category was too small (in statistical terms) to conclude with any certainty that THC is effective in slowing MS progression. More research will be needed to investigate these findings, and patients will need to be selected at the lower end of the disability spectrum before meaningful conclusions on the neuroprotective ability of cannabinoids or endocannabinoids to slow the rate of disease progression in MS can be drawn.

3 Amyotrophic Lateral Sclerosis

3.1 Natural History

ALS is a fatal progressive neurodegenerative condition predominantly of later life that primarily affects motor neurons in the spinal cord, brainstem and motor cortex, leading to complete paralysis and death usually within 3–5 years from diagnosis. The majority of cases of ALS are sporadic, but there are also familial cases which are now increasingly identified. One is the inheritance of autosomal dominant mutations in the superoxide dismutase (SOD-1) gene encoding for the antioxidant enzyme Cu/Zn-superoxide dismutase (Rosen et al. 1993). Another more recently discovered cause of hereditary ALS is the inheritance of an autosomal dominant hexanucleotide repeat expansion in the non-coding region of the gene C9ORF72 (De Jesus-Hernandez et al. 2011; Renton et al. 2011), which is also associated with fronto-temporal dementia (FTD).

The annual incidence rate of ALS in Europe is 2.16 per 100,000 person years, with similar incidence rates across all countries studied. The incidence was higher among men (3.0 per 100,000 person years) than among women (2.4 per 100,000 person years), and incidence decreases dramatically after 80 years of age (Logroscino et al. 2010). Both upper motor neurons and lower motor neurons degenerate in ALS and the communication between the neuron and muscle is lost, prompting progressive muscle weakening and the appearance of fasciculations (persistent muscle twitches). In the later disease stages, the patients become progressively paralyzed and up to 50 % of people with ALS can show cognitive impairment, particularly involving more severe executive dysfunction and mild memory decline.

To date the anti-glutamatergic agent Riluzole (Rilutek®) is the only licensed medication for ALS whose mechanism of action is by blockade of voltage-dependent sodium channels on motor neurons (Cheah et al. 2010). However, only a modest increase in survival time is seen with Riluzole, indicating the urgent need for better therapeutics for ALS. The major problem with ALS currently is that at the time of diagnosis, the disease progression is already well advanced, with significant motor neuron loss, and so the administration of therapeutics is unlikely to significantly affect disease course. However, particularly in familial ALS, those at risk of developing ALS could be identified well before significant pathology has developed, and so neuroprotective agents may be administered prophylactically to hopefully delay or prevent the development of ALS.

3.2 Pathology, Symptoms and Disability

The pathogenesis of ALS is still incompletely understood, but a number of mechanisms have been implicated including neurofilament accumulation leading to cellular inclusions via defective protein processing, disruption of axonal transport, neurotransmitter-mediated excitotoxicity, oxidative stress, mitochondrial

dysfunction and also neuroinflammation with extensive microglial activation (Bilsland et al. 2008; Malik et al. 2013; Rao and Weiss 2004; Ström et al. 2008; Zhao et al. 2008). An established hallmark of ALS is the presence of various inclusion bodies in degenerating neurones and surrounding reactive astrocytes. Ubiquitinated inclusions are the most common and specific type of inclusion in ALS and are found in lower motor neurons of the spinal cord and brainstem (Matsumoto et al. 1993). ALS typically presents as muscle weakness and/or fasciculations, which gradually worsen, bulbar symptoms (speech problems and difficulty swallowing) and eventually respiratory problems leading to failure. Spasticity can develop in weakened atrophic limbs affecting dexterity and gait, and at the later stages of disease flexor spasms may develop due to excessive activation of the flexor arc in a spastic limb (Wijesekera and Leigh 2009).

Many of these mechanisms and symptoms may be amenable to manipulation via the pharmacological action of cannabinoid receptor agonists or by the manipulation of the levels of endogenous endocannabinoids.

3.3 Endocannabinoids in ALS

3.3.1 Experimental Evidence

Compared to MS, there is a relative paucity of studies on endocannabinoids and ALS. The main source of evidence for the potential role of endocannabinoids and cannabinoids in the amelioration of ALS comes from studies conducted in the G93A-SOD1 mouse, which is a transgenic mouse strain expressing the human mutated SOD-1 autosomal dominant gene expressed in familial cases of ALS (Tu et al. 1996). This mouse model displays many of the clinical and pathological hallmarks of ALS, and evidence for the involvement of the endocannabinoid system has been obtained by several groups using this model system.

Increases in the levels of both AEA and 2-AG have been reported in the lumbar regions of the spinal cords of G93A-SOD 1 mice, which is the first portion of the spinal cord to show neurodegeneration before overt motor impairment (Witting et al. 2004). It is postulated that the increase in endocannabinoid levels is an endogenous neuroprotective mechanism in response to neurodegenerative processes. Supportive evidence for this was provided by the observation that genetic ablation of FAAH in G93A-SOD 1 mice, thereby increasing AEA levels, significantly delayed disease progression, which was also demonstrated by treating G93A-SOD 1 mice with the CB_1/CB_2 agonist WIN 55,212 (Bilsland et al. 2006). However, WIN 55,212-2, or elevation of endocannabinoid levels by FAAH ablation, had no effect on life span. Genetic depletion of the CB_1 receptor, in contrast, had no effect on disease onset in G93A-SOD 1 mice but significantly extended life span. These results showed that cannabinoids have significant neuroprotective effects in this model of ALS but suggested that these beneficial effects may be mediated by non-CB_1 receptor mechanisms such as the activation of CB_2 receptors (also activated by WIN 55-212) that putatively suppress neuroinflammatory processes. Further evidence for the involvement of the CB_2 receptor was demonstrated

by the observation that CB_2 receptors are dramatically upregulated in the spinal cords of G93A-SOD 1 mice. This is presumably in activated cells of the immune system such as microglia, and administration of the selective CB_2 agonist AM-1241 at onset of neurological signs increased the survival interval after disease onset by 56 % (Shoemaker et al. 2007).

The sensitivity of cannabinoid CB_1 receptors controlling both glutamate and gamma aminobutyric acid (GABA) transmission was potentiated in ALS mice, indicating that adaptations of the endocannabinoid system might be involved in the pathophysiology of ALS (Rossi et al. 2010). Excitatory and inhibitory synaptic transmission was investigated in the striatum of G93A-SOD1 ALS mice, along with the sensitivity of these synapses to cannabinoid CB_1 receptor stimulation. There was a reduced frequency of glutamate-mediated EPSCs and increased frequency of GABA-mediated spontaneous inhibitory postsynaptic currents recorded from striatal neurons of ALS mice, possibly due to presynaptic defects in neurotransmitter release via CB_1 receptor overactivity (Rossi et al. 2010). In hippocampal neuron cultures, there was a blocking of the TNF-α (a proinflammatory cytokine), induced increase of the expression of AMPA glutamate receptors via CB_1 receptor stimulation and a subsequent protection from excitotoxic death (Zhao et al. 2010).

Increases in *N*-acylphosphatidylethanolamine phospholipase D (one of the enzymes responsible for the generation of AEA) and CB_2 receptors were detected in the spinal cords of male G93A-SOD 1 mice but in female mice only increases in CB_2 receptors were reported, pointing to an increase in neuroinflammation in these animals (Moreno-Martet et al. 2014). Treatment of these mice with a Sativex®-like combination of phytocannabinoids only produced weak improvements in the progression of neurological deficits and survival, particularly in females (Moreno-Martet et al. 2014). These experimental data point to the potential role of the endocannabinoid system as a promising therapeutic avenue for the treatment of ALS although much further work needs to be done.

3.3.2 Clinical Evidence

To date, there is little clinical evidence on endocannabinoids or cannabinoid therapy in ALS, but the preclinical experimental data indicate that there may be a clinical state of endocannabinoid deficiency involved. People with ALS have reported that cannabis can alleviate some of the symptoms associated with ALS such as pain and muscle spasms, appetite improvement and alleviation of depression and excessive drooling (sialorrhea) due to the reduction in saliva production (Amtmann et al. 2004). Patients who were able to get access to cannabis found it preferable to prescribe medication for their symptoms (Carter et al. 2010). In addition to pain, spasticity is also a major problem for patients with ALS and patients report that cannabis can subjectively improve spasticity (Amtmann et al. 2004). However, a small scale study on cramps in ALS did not demonstrate a subjective improvement in cramp intensity in 27 ALS patients in a randomised double-blind crossover trial with 5 mg THC twice daily (Weber et al. 2010). The situation for cannabis and ALS is analogous to that of MS at the beginning of this century, with anecdotal patient reports of potential efficacy that needs to be

followed up by properly designed randomised clinical trials that can properly examine the therapeutic benefit of cannabis or the endocannabinoids in symptom management or their potential for disease modification.

4 Summary

The beneficial therapeutic effects of cannabinoids and potentially the endocannabinoids in symptom management in MS can now be said to be proven, particularly for symptoms such as spasticity. The potential for cannabis in slowing disease progression in MS is less clear, but experimental evidence clearly suggests that cannabis and the endocannabinoids are definitely neuroprotective, and the findings of a single clinical trial performed with THC capsules, though certainly not definitive, do suggest that there was a neuroprotective benefit in a sub-group of patients with a lower initial level of disability (although the numbers in this group were too low for a definitive conclusion). Such a clinical trial needs to be repeated in a larger group of MS patients with lower levels of disability on entering the study. In ALS, the field is some years behind that of MS. Experimental studies do point to a potential role of cannabis and the endocannabinoids in the management of this disease, particularly with regard to symptoms such as pain and spasticity, but also potentially in the modification of disease progression, and the need for clinical trials in this area to investigate this is indicated.

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Endocannabinoids and Neurodegenerative Disorders: Parkinson's Disease, Huntington's Chorea, Alzheimer's Disease, and Others

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_8

Abstract

This review focuses on the role of the endocannabinoid signaling system in controlling neuronal survival, an extremely important issue to be considered when developing new therapies for neurodegenerative disorders. First, we will describe the cellular and molecular mechanisms, and the signaling pathways, underlying these neuroprotective properties, including the control of glutamate homeostasis, calcium influx, the toxicity of reactive oxygen species, glial activation and other inflammatory events; and the induction of autophagy. We will then concentrate on the preclinical studies and the few clinical trials that have been carried out targeting endocannabinoid signaling in three important chronic progressive neurodegenerative disorders (Parkinson's disease, Huntington's chorea, and Alzheimer's disease), as well as in other less well-studied disorders. We will end by offering some ideas and proposals for future research that should be carried out to optimize endocannabinoid-based treatments for these disorders. Such studies will strengthen the possibility that these therapies will be investigated in the clinical scenario and licensed for their use in specific disorders.

Keywords

Alzheimer's disease • Cannabinoids • Endocannabinoids • Huntington's disease • Neurodegeneration • Neuroprotection • Parkinson's disease

Abbreviations

2-AG	2-Arachidonoyl-glycerol
3NP	3-Nitropropionate
$5HT_{1A}$	Serotonin 1A receptor type
AD	Alzheimer's disease
AEA	Anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BACE1	β-site amyloid precursor protein cleaving enzyme 1
BBB	Blood brain barrier
CAG	Cytosine-adenine-guanine
CB	Cannabinoid
CB_1	Cannabinoid receptor type 1
CB_2,	Cannabinoid receptor type 2
CBD	Cannabidiol
CBN	Cannabinol
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
DAGL	Diacylglycerol lipase
eCB	Endocannabinoid
FAAH	Fatty acid amide hydrolase
HD	Huntington's disease

HU-211	Dexanabinol
IL-10	Interleukin-10
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
PD	Parkinson's disease
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SCA	Spinocerebellar ataxia
TNF-α	Tumor necrosis factor-α
TRPV1	Transient receptor potential vanilloid type 1
Δ^9-THC	Δ^9-tetrahydrocannabinol
Δ^9-THCV	Δ^9-tetrahydrocannabivarin

1 Compounds Targeting Endocannabinoids as Neuroprotective Agents

The homeostastic regulation of cells is one of the key functions assigned to the endocannabinoid (eCB) system, and it extends to the decision of cells to die or survive in certain unfavorable conditions. This influence of eCBs on death/survival explains the notable cytoprotective properties exhibited by compounds that target specific elements in the eCB system in distinct pathological conditions (Fernández-Ruiz et al. 2010, 2014). These properties are especially relevant in the central nervous system (CNS), in which neuron loss is extremely difficult to overcome given that neurons are post-mitotic cells incapable of replicating their DNA and dividing. Lost neurons may be replaced by new neurons, although orchestrating the generation of these neurons from neural cell progenitors in the adult brain is still limited by our lack of understanding (Ziemka-Nałęcz and Zalewska 2012). These limitations make the preservation of the original neurons generated during brain development or naturally replaced during the lifetime of the individual, a key objective to ensure the correct functioning of the different brain structures. Numerous compounds have been investigated to achieve such preservation and to facilitate the development of novel neuroprotective therapies for neurodegenerative disorders (e.g., antioxidants, anti-inflammatory or anti-excitotoxic agents, inhibitors of apoptosis, enhancers of autophagy, neurotrophic factors, etc.. . .), although most have failed to reproduce in humans the positive effects seen in experimental models (Athauda and Foltynie 2014; Berk et al. 2014; Sampaio et al. 2014).

In the past 10–15 years, the capacity of compounds targeting the eCB system to protect neurons and some glial cell sub-populations (e.g., astrocytes, oligodendrocytes, and their precursor cells) against different types of cytotoxic insult has been

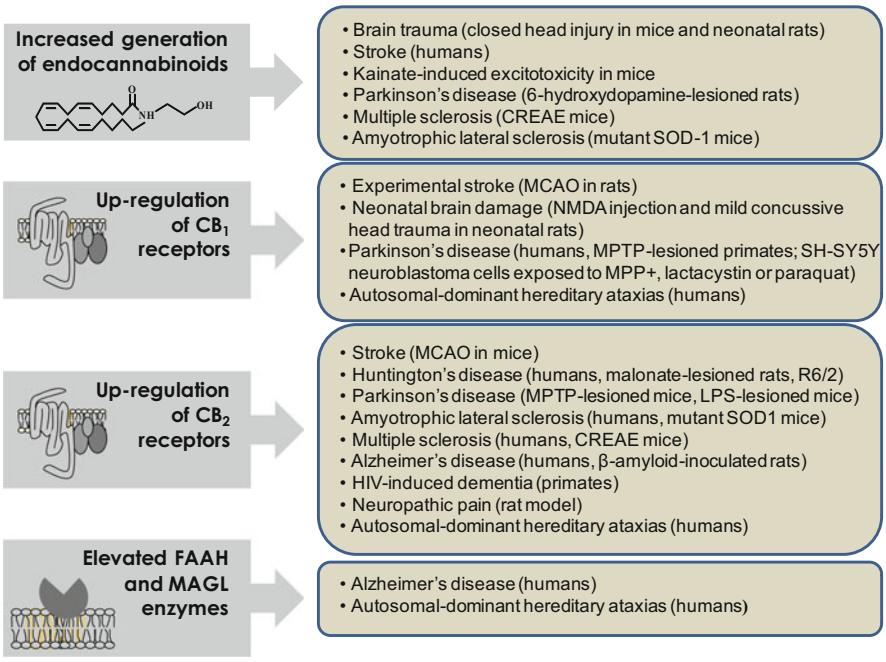

Fig. 1 Alterations to eCB ligands, their receptors, and enzymes in neurodegenerative disorders

investigated by different research groups (Fernández-Ruiz et al. 2014). These studies concentrated on identifying the targets within the eCB system, such as the cannabinoid (CB) receptors or eCB enzymes that can be pharmacologically activated or inhibited, provoking neuroprotection in experimental models of the most prevalent neurodegenerative disorders (Fernández-Ruiz et al. 2010, 2014). These exhaustive studies have placed the eCB system in a promising position, since the results they have generated indicate that specific CBs or other compounds (alone or in combination) may serve as therapies for neurodegenerative disorders that modify disease progression (Fernández-Ruiz et al. 2010). These therapies might provide notable advantages over other agents with proposed neuroprotective activity, for example, by mimicking the endogenous protective response of this signaling system to stimuli that damage the brain (Fernández-Ruiz et al. 2010; Pacher and Mechoulam 2011). In other words, the well-defined alterations to eCB ligands, their receptors, and/or their signaling pathways evident in neurodegenerative disorders (see Fig. 1) may reflect an endogenous response of this system to combat the brain damage caused by inflammatory, excitotoxic, infectious, traumatic, or oxidative insults, and this response may be replicated by administering compounds that interact with the eCB system, producing beneficial results (Fernández-Ruiz et al. 2014). Indeed, the elevation of eCBs that is typically associated with neurodegenerative conditions can be further enhanced with inhibitors of their inactivation (e.g., UCM707: Marsicano et al. 2003), as well as by treatment with eCBs (Panikashvili et al. 2001), and this enhancement is expected to

be neuroprotective. However, it is also possible that the efficacy of compounds acting on the eCB system may be due to the ability of these compounds to correct any potential dysregulation of eCB signals that might be instrumental in the pathogenesis of these disorders (Fagan and Campbell 2014). Indeed, far from being mutually exclusive, both types of responses may occur concomitantly: the mimicking of endogenous protection and the correction of dysregulated signals.

The second singular feature of CBs as neuroprotectants is their broad-spectrum activity as opposed to a greater potency. Indeed, their potency is relatively similar to those neuroprotective agents studied more frequently: N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists (dizocilpine); calcium channel blockers (nimodipine); antioxidants (coenzyme Q10, *N*-acetylcysteine); and anti-inflammatory compounds (minocycline). However, their advantage is that they combine all these properties in a single molecule or in a mixture of two or more CBs. This is extremely important in neurodegenerative disorders as neuronal damage also results from the concerted influence of different cytotoxic events: energy failure, excitotoxicity, mitochondrial dysfunction, failures in proteostasis, inflammation, and oxidative stress. Accordingly, it appears difficult to effectively control brain damage with compounds or strategies that affect only one of these cytotoxic events. Thus, a reliable therapy is likely to require the use of a broad-spectrum strategy, employing "multi-target" drugs or the combination of different therapeutic agents (Geldenhuys and Van der Schyf 2013). Compounds acting on the eCB system may display such broad-spectrum activity as they can influence different elements within the eCB signaling system, for example, the type 1 (CB_1) and type 2 (CB_2) cannabinoid receptor, or fatty acid amide hydrolase (FAAH), and these elements might fulfill specific roles in the neuroprotective responses (Fernández-Ruiz et al. 2014). In addition to the possibility of combining compounds with such profiles, novel neuroprotective targets within the eCB system or in its vicinity are being identified. For example, activation of the GPR55 orphan receptor was recently found to be beneficial against excitotoxic insults in an in vitro model of cultured rat hippocampal slices (Kallendrusch et al. 2013). CBs can also act on other non-eCB system targets that might play key roles in controlling neuronal homeostasis and survival, and, hence, a well-demonstrated neuroprotective potential, such as: (1) the nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family (Fidaleo et al. 2014); (2) transcription factors like NrF-2, NFκB (Iuvone et al. 2009; Fernández-Ruiz et al. 2013); (3) the serotonin 1A receptor type ($5HT_{1A}$: Pazos et al. 2013); and (4) components of the adenosine signaling pathway (Castillo et al. 2010).

A last advantageous feature of compounds that act on the eCB system compared to other neuroprotectant agents is the key location of the molecular targets in the different cell substrates in the CNS (e.g., neurons, astrocytes, resting and reactive microglia, perivascular microglial cells, oligodendrocytes and oligodendrocyte precursor cells, and neural progenitor cells) and even in key CNS structures [e.g., the blood–brain barrier (BBB)]. These specific locations enable such compounds to exert selective control over the specific functions fulfilled by these cells in degeneration, protection, and/or repair (Fernández-Ruiz et al. 2014). This is particularly relevant to the CB receptors, for example, CB_1 receptors that are involved in

controlling excitotoxic damage are mainly located in neurons and, particularly, in glutamatergic neurons. As indicated in Fig. 1, a few studies have demonstrated that the CB_1 receptor is upregulated in chronic neurodegenerative conditions (Lastres-Becker et al. 2001; Rodríguez-Cueto et al. 2014a). However, CB_1 receptors are not generally a target for neuroprotection, precisely due to the progressive loss, in neurodegenerative disorders, of specific groups of neurons in which CB_1 receptors are expressed, thereby provoking a premature and rapid reduction in the availability of these receptors. In contrast, CB_2 receptors are generally expressed weakly in the healthy brain, but they are dramatically upregulated in glial elements in conditions of neurodegeneration in reactive microglia and activated astrocytes (Fernández-Ruiz et al. 2007, 2010), making them a particularly interesting and promising target from a therapeutic point of view. An additional and interesting discovery, in line with the idea that eCB elements may be located in structures crucial for maintaining the integrity of the CNS, is the association of CB receptors with preserving the integrity and function of the BBB (Vendel and de Lange 2014). This barrier is essential for neuroprotection, and recent studies demonstrated that it is under the control of both CB_1 and CB_2 receptor-mediated signals (Fujii et al. 2014), which maintain the integrity of tight junctions, inhibit leukocyte infiltration, and facilitate β-amyloid clearance (Vendel and de Lange 2014).

In this article, we will review the information that supports the promising therapeutic potential of compounds acting on the eCB system for preserving neurons and glial cells in conditions of brain damage. This article will be divided into three parts. First, we will review the different cellular and molecular mechanisms underlying the neuroprotective effects derived from the pharmacological manipulation of key elements of the eCB signaling system (e.g., the CB receptors) or of additional targets indirectly related to this signaling system (e.g., PPARs, transcription factors). Second, we will concentrate on three important chronic neurodegenerative disorders in which the neuroprotective effects of CBs have been studied in most depth, Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD), as well as on a few less well-studied disorders like autosomal-dominant hereditary ataxias, Down's syndrome, and prion-related disorders. Lastly, we will conclude with some ideas about the steps that should be taken to extrapolate these findings, mainly obtained in preclinical models, to the clinical arena.

2 Mechanisms Involved in the Neuroprotection Afforded by Compounds That Target the eCB System

As mentioned above (Sect. 1), compounds that target the eCB system display a wide range of effects when compared to other neuroprotectants, which may be important in neurodegenerative disorders where nerve cell damage is the consequence of a combination of cytotoxic events. To oppose such a "collaborative strategy" for killing neurons, any attempt to provide neuroprotection should be based on the idea of using broad-spectrum compounds or combinations of different therapeutic agents (Geldenhuys and Van der Schyf 2013), a profile that CBs fit

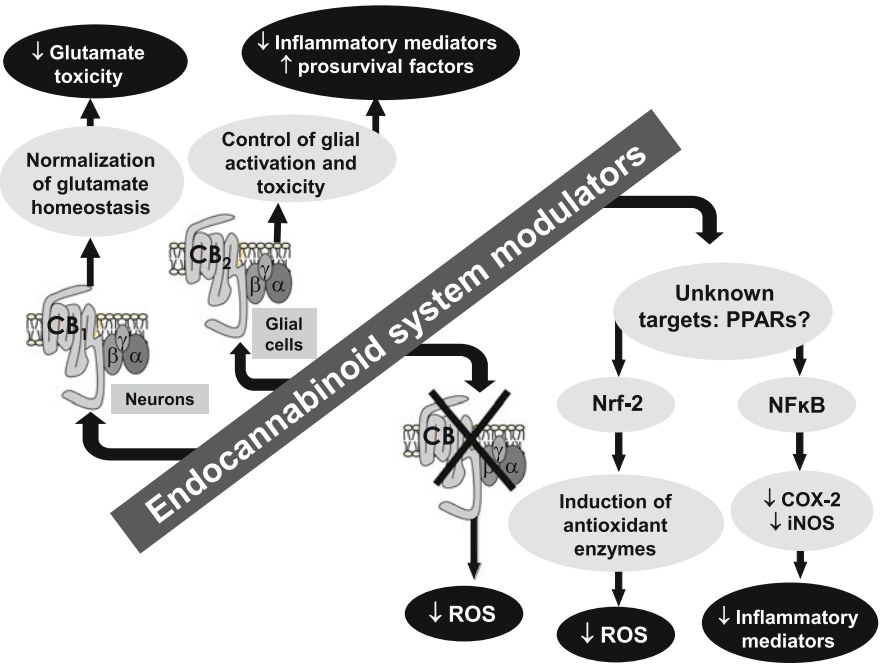

Fig. 2 Molecular and cellular mechanisms proposed for the neuroprotective effects of compounds that act on the eCB system

perfectly. Their wide range of effects are likely to be beneficial because the neuroprotective mechanisms they target are quite diverse and frequently complementary (see Fig. 2). They include not only the activation of CB_1 and/or CB_2 receptors but also they extend to CB receptor-independent mechanisms, such as the blockade of NMDA receptors, or the activation of nuclear receptors of the PPAR family, NFκB and/or Nrf-2 signaling (Fernández-Ruiz et al. 2010; Iuvone et al. 2009; Fidaleo et al. 2014).

2.1 The Effects Mediated by CB_1 Receptors

The participation of eCBs and their receptors in a retrograde signaling system in glutamatergic synapses (Ohno-Shosaku and Kano 2014) enables the eCB system to be pharmacologically targeted for diseases characterized by abnormal glutamate homeostasis, such as neurodegenerative disorders. Thus, compounds that can normalize glutamate homeostasis by targeting the CB_1 receptor (see Fig. 2) act as anti-excitotoxic agents (Fernández-Ruiz et al. 2010), although such effects may also be elicited by cannabinoids that do not activate the CB_1 receptor. One example of such a compound is dexanabinol (HU-211), which is the inactive enantiomer of HU-210, a potent CB_1/CB_2 receptor agonist (Shohami and Mechoulam 2000). HU-211 does not bind to CB receptors, but it affords neuroprotection by blocking

NMDA receptors (Nadler et al. 1993). The anti-glutamatergic properties of HU-211 have been investigated extensively in preclinical models, yet such benefits have not been demonstrated in patients (Klein and Newton 2007).

The anti-excitotoxic effects mediated by CB_1 receptors are apparently exerted at two neuronal sites: (1) through presynaptic CB_1 receptors in glutamatergic terminals where CBs would reduce excess glutamate release and (2) through postsynaptic CB_1 receptors on neurons containing NMDA receptors, in which CBs may reduce the excessive intracellular levels of calcium by closing voltage-dependent calcium channels, thereby dampening the over-activation of calcium-dependent destructive pathways. All this information has been obtained in numerous studies conducted in vitro on cultured neurons (Shen and Thayer 1998; Abood et al. 2001) or on rat brain slices (Hampson et al. 1998), as well as in vivo using rodent models of ischemic damage (Nagayama et al. 1999) or following the induction of excitotoxicity (van der Stelt et al. 2001; Marsicano et al. 2003). In all cases, the participation of CB_1 receptors was demonstrated with CB_1 receptor antagonists or using mice genetically deficient in CB_1 receptors (Fernández-Ruiz et al. 2005, 2010).

Another neuroprotective effect mediated by CB_1 receptors is improvement of the blood supply to the injured brain, which is particularly relevant to stroke or traumatic injuries (Fernández-Ruiz et al. 2005). This effect would be exerted through CB_1 receptors located in the brain microvasculature, which would reduce the generation of endothelin-1 and other endothelium-derived mediators (Mechoulam et al. 2002). In ischemic conditions, it is these factors that cause vasoconstriction and that limit the blood supply to the injured area, thereby aggravating brain damage. This effect was reversed by rimonabant, supporting the contribution of CB_1 receptors (Chen et al. 2000), although more recent evidence also suggests the involvement of CB_2 receptors (Choi et al. 2013).

2.2 The Effects Mediated by CB_2 Receptors

Based on their key location in glial elements, CB_2 receptors may be activated to control the influence of glial cells on neuronal homeostasis and survival, particularly when they become reactive (Fernández-Ruiz et al. 2007, 2010). Although CB_2 receptors are the main elements contributing to this control, we cannot rule out a neuroprotective effect of CB_1 receptors that involves glia (Chung et al. 2011). This may be particularly relevant to the regulation of astrocyte activity in conditions of brain damage, which could be affected exclusively by the activation of CB_2 receptors, or might involve CB_1 receptors, either alone or in conjunction with CB_2 receptors (Fernández-Ruiz et al. 2007, 2010; Stella 2010). Irrespective of the CB receptor type involved, the benefits that CBs may provide appear to be associated with the trophic role exerted by these glial cells, including improvements in the supply of metabolic substrates to neurons (lactate or ketone bodies: Duarte et al. 2012). They could also enhance the generation of neurotrophins or anti-inflammatory mediators that could potentially rescue damaged neurons (e.g.,

interleukin-10 [IL-10], and of IL-1 receptor antagonists, or pro-survival factors like transforming growth factor-β [TGF-β]: Smith et al. 2000; Molina-Holgado et al. 2003). Finally, CBs could also inhibit the production of chemokines by astrocytes (e.g., fractalkine), an effect that would be predominantly mediated by the activation of CB_2 receptors (Sheng et al. 2009). CB_2 receptors have also been identified in oligodendrocytes and their precursor cells (Gómez et al. 2010, 2011), in which they play a role in key functions of these glial cells, which are also crucial for neurons.

In contrast to astrocytes, microglial cells are greatly affected by CB_2 receptors in the CNS, particularly when these cells are activated. This has been studied intensively over the past 10 years after microglial cells surrounding senile plaques were seen to be immunostained for CB_2 receptors in postmortem AD brains (Benito et al. 2003). Indeed, CB_2 receptors appear to play an important role in the proliferation and migration of these cells at lesion sites (Walter et al. 2003; Carrier et al. 2004). In addition, the activation of CB_2 receptors dampens the generation of a variety of neurotoxic factors by microglial cells (Fernández-Ruiz et al. 2007, 2010), for example, tumor necrosis factor-α (TNF-α), a major contributor to the pathophysiology of brain injury (Stella 2010). Activation of CB_2 receptors apparently inhibits the production of TNF-α by inhibiting NFκB (see Fig. 2: Oh et al. 2010), a transcription factor that plays a key role in the regulation of pro-inflammatory responses. However, the inhibition of NFκB by CBs that do not activate the CB_2 receptor leads to an anti-inflammatory scenario too (e.g., cannabidiol [CBD] or HU-211: Kozela et al. 2010; Shohami and Mechoulam 2000). This may be achieved by activating the nuclear receptors of the PPAR family (Fidaleo et al. 2014) that regulate NFκB signaling (Stahel et al. 2008). Therefore, CBs may control inflammatory responses by working through two well-differentiated molecular mechanisms, consistent with the idea that their principal added value is their broad-spectrum profile.

Recent studies have also shown the CB_2 receptor to be present in certain neuronal subpopulations in the CNS (Lanciego et al. 2011; Rodríguez-Cueto et al. 2014a; García et al. 2015), supporting a possible role of this receptor in synaptic activity, although this has not yet been conclusively demonstrated. There is no evidence that the activation of these "neuronal CB_2 receptors" may be neuroprotective, although they may serve as a potential biomarker for neuronal loss in specific neurodegenerative disorders like PD (García et al. 2015) or spinocerebellar ataxias (SCAs: Rodríguez-Cueto et al. 2014a).

Therefore, the presence of CB_2 receptors in reactive microglia, activated astrocytes, oligodendrocytes, and in some neuronal subpopulations places these receptors in a promising position for their use as a target for neuroprotection (Fernández-Ruiz et al. 2010). Such pharmacological manipulations may be the best way to reproduce the endogenous response provoked by these receptors, which are upregulated in activated astrocytes and reactive microglia in response to inflammatory, excitotoxic, and traumatic insults, such as those that occur in neurodegenerative disorders (see Fig. 2). In addition, those CBs that selectively target the CB_2 receptor do not provoke the psychotropic side effects elicited by CBs

that activate the CB_1 receptor, indicating that they may be safe and well tolerated in clinical applications.

2.3 Effects Not Mediated by CB_1 or CB_2 Receptors

The neuroprotection provided by CBs includes some responses that are not mediated by CB_1 or CB_2 receptors (Fernández-Ruiz et al. 2010, 2013). For example, the antioxidant properties of phytocannabinoids and some synthetic CBs have been found not to depend on CB_1 receptor activation (Marsicano et al. 2002). An interesting compound with such a CB_1/CB_2 receptor-independent antioxidant profile is CBD, although a relatively similar antioxidant activity has also been found with other structurally similar compounds, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN), nabilone, and HU-211 (Marsicano et al. 2002). This potential has been frequently related to the ability of CBD to act as a scavenger of reactive oxygen species (ROS) rather than to its capability to activate intracellular targets and specific signaling pathways. Indeed, CBD has a poor affinity for the CB_1 and CB_2 receptors (Fernández-Ruiz et al. 2013), although CBD does display certain activity as a CB_1/CB_2 antagonist in vitro (Pertwee 2008), and it can activate CB_2 receptors in the immature brain in conditions of neonatal ischemia (Castillo et al. 2010). A priori, this lack of CBD activity at the classic CB receptors would suggest that this phytocannabinoid does not control excitotoxicity by directly activating CB_1 receptors or by reducing microglial toxicity via direct CB_2 receptor activation. However, CBD is no less active against the brain damage produced by altered glutamate homeostasis than CBs that do target the CB_1 receptor (El-Remessy et al. 2003) or those targeting the CB_2 receptor against local inflammatory events (Ruiz-Valdepeñas et al. 2011). It is possible that these anti-glutamatergic and anti-inflammatory effects of CBD may be mediated indirectly by cannabinoid receptors, especially given the capability of CBD to inhibit the inactivation of eCBs (e.g., by inhibiting the FAAH enzyme [Leweke et al. 2012] or the eCB transporter [Bisogno et al. 2001]). Such effects may enhance the action of eCBs at CB_1 and CB_2 receptors (Fernández-Ruiz et al. 2013) and also at other receptors that may be activated by eCBs, such as transient receptor potential vanilloid type 1 (TRPV1) receptors (Bisogno et al. 2001) or nuclear PPAR receptors (Fidaleo et al. 2014). In fact, PPAR receptors, as well as other unknown intracellular targets, have recently been proposed to mediate the antioxidant and anti-inflammatory actions of CBD, Δ^9-THC, and other CBs (Iuvone et al. 2009; Fernández-Ruiz et al. 2013; see Fig. 2). These CBs could regulate the intracellular pathways that control endogenous antioxidant defenses, in particular the signaling triggered by Nrf-2 (Fernández-Ruiz et al. 2013; see Fig. 2). This transcription factor plays a major role in controlling antioxidant response elements located in genes encoding different phase II antioxidant enzymes, and it was recently linked to the activity of certain CBs in an experimental model of the infarcted heart, although a major role for CB_2 receptors was proposed in these effects (Wang et al. 2014). Recent studies proved that the eCB anandamide (AEA), which given its eicosanoid

structure is not a ROS scavenger, can nevertheless reduce oxidative stress through a mechanism that might involve CB_1 receptors (e.g., a reduction in Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2: Jia et al. 2014). Thus, together these data implicate the two classic cannabinoid receptors in the control of oxidative stress, a process frequently regarded as being CB receptor-independent.

CBD is also anti-inflammatory, reducing microglial cell migration (Walter et al. 2003) and the pro-inflammatory mediators they produce (Esposito et al. 2007) to a similar extent as CBs that target the CB_2 receptor. However, its effects are likely to be associated with its ability to bind and activate the nuclear receptors of the PPAR family, in particular PPAR-γ (Esposito et al. 2011), and to the regulation of their downstream signals, including the NFκB signaling that controls several genes encoding pro-inflammatory enzymes (e.g., inducible nitric oxide synthase [iNOS], cyclooxygenase-2 [COX-2], metalloproteases) and cytokines (e.g., IL-1β: see Fig. 2; Esposito et al. 2006a, 2007). These PPAR-mediated anti-inflammatory effects are also elicited by other CBs, although with some differences. For example, the activation of PPAR-γ receptors by Δ^9-THC protected differentiated SH-SY5Y neuroblastoma cells exposed to relevant parkinsonian toxins (e.g., MPP+, lactacystin, paraquat), whereas other antioxidant CBs did not (e.g., CBD, nabilone: Carroll et al. 2012).

2.4 eCB-Derived Molecules May Enhance Neurotoxicity

Despite the well-demonstrated neuroprotective properties of certain CBs that directly or indirectly target the eCB system, there is evidence that some CBs may also enhance brain damage in certain circumstances (Fowler et al. 2010). Such an enhancement has been found to be produced by the two major eCBs, each of which may help generate new lipid mediators via COX-2 metabolism (e.g., prostaglandin-glyceryl-esters, prostamides), which may be toxic to neurons (Sang et al. 2007; Valdeolivas et al. 2013). When such a detrimental enhancement of brain damage occurs, inhibiting eCB synthesis, for example, with diacylglycerol lipase (DAGL) inhibitors, would be neuroprotective as it would reduce the availability of eCBs for COX-2 metabolism, and indeed, such benefits have been attributed to COX-2 inhibitors (Valdeolivas et al. 2013). In contrast, inhibiting eCB hydrolysis by monoacylglycerol lipase (MAGL) aggravated brain damage by augmenting the eCBs available (Valdeolivas et al. 2013). In other circumstances, toxicity may derive from the excess arachidonic acid generated through eCB hydrolysis (by MAGL or FAAH) and its oxygenation to prostaglandins by COX-2 promoting inflammatory damage. In this case, inhibitors of FAAH and MAGL may be neuroprotective due to mechanisms independent of CB_1 and CB_2 receptors (Nomura et al. 2011; Piro et al. 2012). Significantly, MAGL-deficient mice were less vulnerable to the induction of experimental parkinsonism than wild-type animals (Nomura et al. 2011). These mechanisms are all described in Fig. 3.

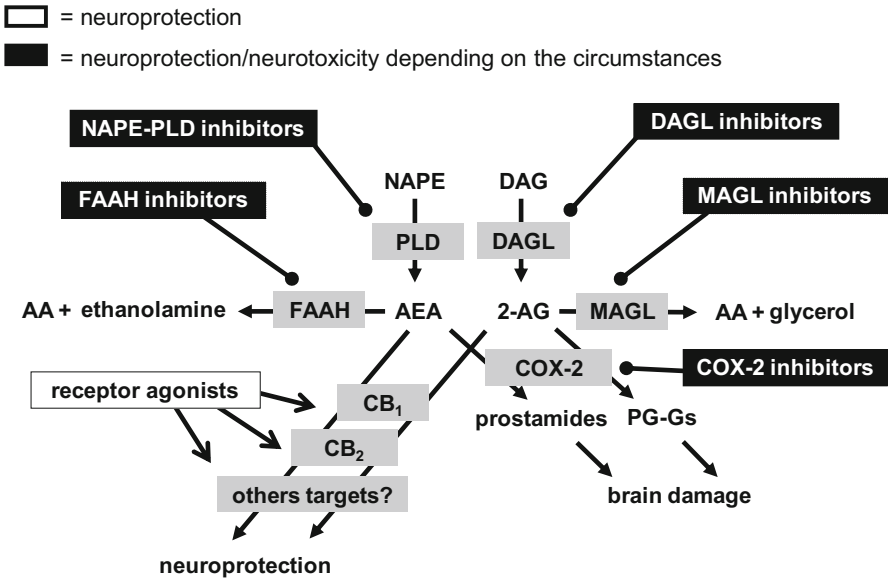

Fig. 3 Dual effects of some compounds acting on the eCB system in relation to the metabolism of the two major eCB ligands. Open rectangle = neuroprotection. Filled rectangle = neuroprotection/neurotoxicity depending on the circumstances

3 Cannabinoids in HD

HD is a chronic progressive disorder caused by an excessive number of repeat citosine-adenine-guanine (CAG) triplets in the gene encoding the regulatory protein huntingtin, and the potential use of CBs as a novel neuroprotective treatment in HD has been investigated intensively (see Fig. 4 for details: Sagredo et al. 2012). These studies have been predominantly carried out in preclinical models of HD in vivo, for example, in a rat model that relies on quinolinate-induced excitotoxic damage, in which compounds targeting the CB_1 receptor preserved striatal neurons (Pintor et al. 2006). The relevance of CB_1 receptors in HD was also demonstrated in the R6/2 mutant mouse model of the disease in which CB_1 receptor activation again preserved striatal neurons from death, whereas striatal damage was aggravated in double mutants: CB_1 receptor-deficient R6/2 mutant mice (Blázquez et al. 2011). It is noteworthy, however, that early defects have been found in CB_1 receptor signaling, and that there is a progressive loss of these receptors prior to neuronal death and the onset of choreic symptoms (Glass et al. 2000; Lastres-Becker et al. 2002). Although it is possible that early stimulation of CB_1 receptors may dampen their impairment, thereby maintaining their capacity to inhibit the excitotoxic events that initiate the damage to striatal neurons (Pintor et al. 2006), such an approach is unlikely to work at later symptomatic stages that are

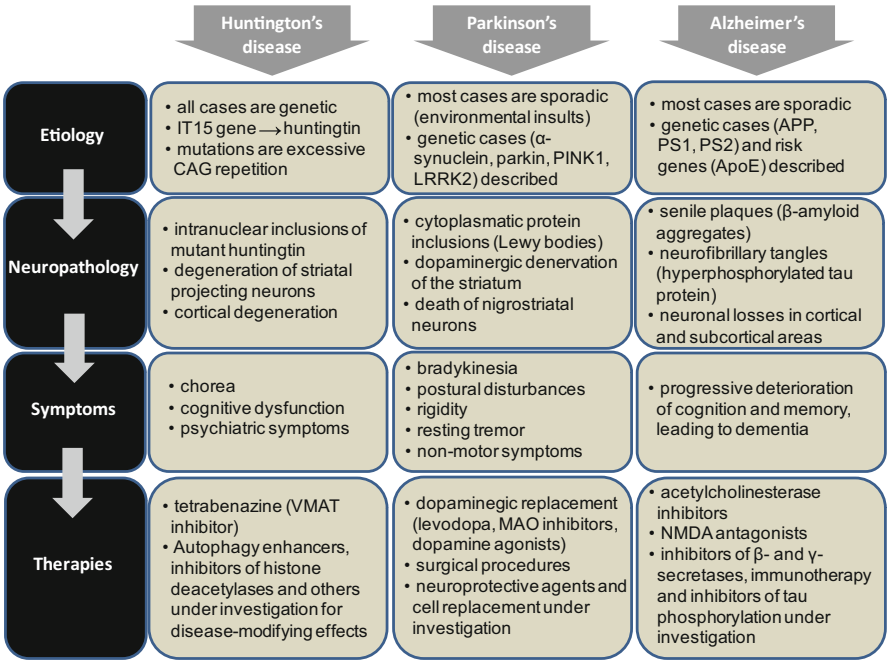

Fig. 4 Clinical, neuropathological, and pharmacological aspects of HD, PD, and AD

characterized by an important loss of CB_1 receptor-containing striatal neurons (Fernández-Ruiz et al. 2014). However, it was recently demonstrated unequivocally that CB_1 receptor-dependent neuroprotective activity in HD is predominantly derived from a restricted population of these receptors on cortical glutamatergic neurons that project to the striatum and that are preserved during the progression of HD, rather than from the CB_1 receptors located on striatal projection GABAergic neurons that are progressively lost during disease progression (Chiarlone et al. 2014). This is highly relevant as it supports the putative efficacy of treatments targeting CB_1 receptors irrespective of their loss as HD progresses.

Compounds that selectively activate the CB_2 receptor also appear to be effective in HD, preferentially ameliorating the inflammatory events and microglial activation that occurs after the striatum is damaged with malonate (a complex II inhibitor) in rats (Sagredo et al. 2009), in R6/2 mice (Palazuelos et al. 2009), and following the excitotoxicity induced by striatal lesion with quinolinate in mice (Palazuelos et al. 2009). As mentioned above, these benefits may be facilitated by overexpression of the CB_2 receptor in the striatal parenchyma, an effect that was first detected when striatal damage was provoked in rats with malonate (Sagredo et al. 2009) and subsequently, in R6/2 mice (Palazuelos et al. 2009) and other genetic mouse models of HD (Bouchard et al. 2012), as well as in postmortem tissues from HD patients (Palazuelos et al. 2009). This upregulation appears to occur in astrocytes (Sagredo et al. 2009), although no CB_2 receptor expression was

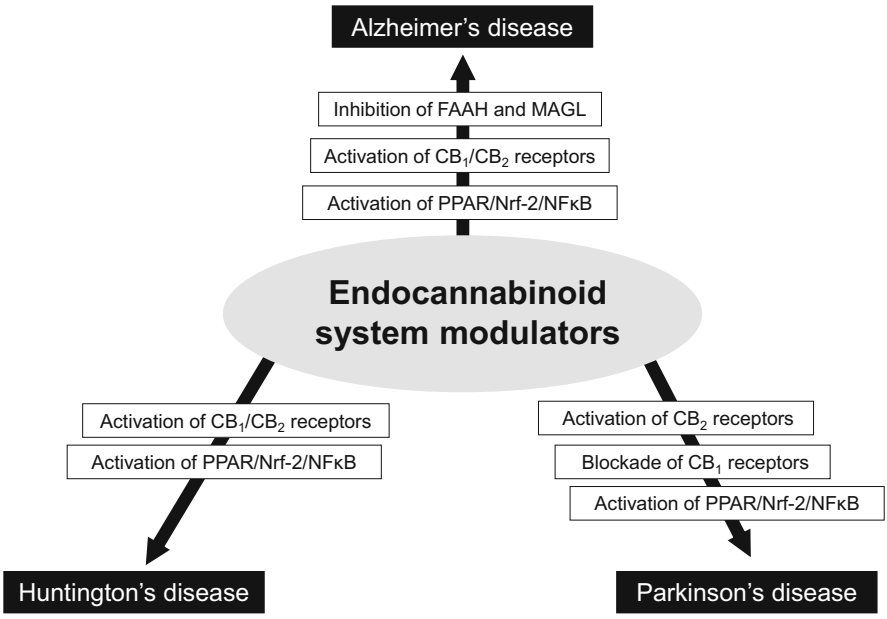

Fig. 5 Pharmacological targets and cannabinoid combinations that appear to be most adequate for clinical evaluation as neuroprotective therapies in HD, PD, and AD

found in these glial cells in human HD tissues (Dowie et al. 2014), and particularly in reactive microglia (Fernández-Ruiz et al. 2007, 2010). Hence, targeting CB_2 receptors in these glial cells may enhance the positive responses and/or reduce the negative influences exerted by these cells on striatal neurons (Sagredo et al. 2009; Palazuelos et al. 2009).

CBD has also been investigated in experimental models of HD, even though its effects are independent of CB_1/CB_2 receptors. CBD was very active in animal models characterized by mitochondrial damage, oxidative stress, and calpain activation, such as rats intoxicated with the complex II inhibitor 3-nitropropionate (3NP; Sagredo et al. 2007), yet it was inactive in pro-inflammatory models like malonate lesioned rats (Sagredo et al. 2009). The efficacy of CBD in 3NP-lesioned rats was independent of CB_1, TRPV1, and adenosine receptors (Sagredo et al. 2007), yet possibly related to the activation of PPARs (although this was not investigated). However, these nuclear receptors have been found to participate in the beneficial effects induced in 3-NP-lesioned and R6/2 mice by cannabigerol, another plant-derived CB that does not bind to CB_1/CB_2 receptors (Valdeolivas et al. 2014). CBD combined with Δ^9-THC, as in the cannabinoid-based medicine Sativex®, has also been studied in animal models of HD given the wide spectrum of pharmacological actions produced by this combination (see Fig. 5). This combination preserved striatal neurons in malonate lesioned mice (Valdeolivas et al. 2012) and in 3-NP lesioned rats (Sagredo et al. 2011), but not in R6/2 mice (unpublished results) in which the presence of CBD may possibly limit the benefits produced by

Δ^9-THC alone (Blázquez et al. 2011). We also participated in a clinical trial carried out on HD patients in Spain to determine the potential of Sativex® as a disease-modifying therapy. This was the first clinical trial aimed at validating a CB-based neuroprotective therapy in HD, as previous clinical studies focused only on the alleviation of symptoms (e.g., chorea: reviewed in Fernández-Ruiz et al. 2014). This clinical trial successfully demonstrated that Sativex® was safe and well tolerated in HD patients, as previously found in controls, yet unfortunately, it failed to provide any evidence that it may slow down disease progression in HD (García-Caldentey et al. 2015).

4 Cannabinoids in PD

Compounds acting on the eCB system are being studied to establish whether they could be used to develop disease-modifying therapies for PD (García-Arencibia et al. 2009), the most important neurodegenerative disorder affecting the basal ganglia (see Fig. 4 for details). As for HD, the different lines of research with CBs have been prompted by the changes in the eCB signaling system found in this disease in basal ganglia, as detected in animal models (Lastres-Becker et al. 2001; Gubellini et al. 2002; Price et al. 2009; García et al. 2011) or postmortem tissues (Lastres-Becker et al. 2001) and in biological fluids (Pisani et al. 2005) from PD patients. These changes include: (1) the upregulation of CB_1 receptors in striatal neurons under the control of dopaminergic neurons that degenerate in PD, as observed in postmortem tissue from patients and in different experimental models of the disease (García-Arencibia et al. 2009); (2) the elevation of CB_2 receptors in glia recruited to the lesion sites of mice suffering nigrostriatal neural damage caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Price et al. 2009) or lipopolysaccharide (LPS) (García et al. 2011); and (3) the loss of neuronal CB_2 receptors in postmortem tissues of PD patients due to the degeneration of nigrostriatal dopaminergic neurons (García et al. 2015).

Most pharmacological studies performed with CBs in experimental models of PD have concentrated on antioxidant plant-derived CBs, given that oxidative stress is a major hallmark in the pathogenesis of PD. These studies include the assessment of Δ^9-THC (Lastres-Becker et al. 2005), CBD (Lastres-Becker et al. 2005; García-Arencibia et al. 2007; García et al. 2011), and Δ^9-tetrahydrocannabivarin (Δ^9-THCV) (García et al. 2011) in rats carrying 6-hydroxydopamine lesions, a model in which the death of dopaminergic neurons is related to mitochondrial dysfunction and oxidative damage. Neuroprotection has also been provided by synthetic CBs like the AEA analogue AM404 (García-Arencibia et al. 2007) or the CB_1/CB_2 receptor agonist CP55,940 (Jiménez-Del-Rio et al. 2008), again acting through CB receptor-independent mechanisms. In contrast, CBs that selectively target the CB_2 receptor were active in MPTP-lesioned mice, a model with a modest glial response (Price et al. 2009), as well as in a more pro-inflammatory model generated by lesions with LPS (García et al. 2011), although they were inactive in 6-hydroxydopamine lesioned rats (García-Arencibia et al. 2007). In addition, CB_2 receptor-knockout mice were more susceptible to LPS than 6-hydroxydopamine

(García et al. 2011), although overexpression of CB_2 receptors in mice was recently shown to protect against 6-hydroxydopamine (Ternianov et al. 2012).

CBs selectively targeting CB_1 receptors have also been studied but with controversial results. No effects were observed in 6-hydroxydopamine lesioned rats (García-Arencibia et al. 2007), while nigrostriatal dopaminergic neurons were preserved in MPTP lesioned mice through an effect that surprisingly involves the inhibition of microglial activation (Chung et al. 2011). Nevertheless, a neuroprotective strategy based on targeting CB_1 receptors might have some disadvantages in PD, since the hypokinetic effects associated with the activation of this receptor may worsen specific parkinsonian symptoms, such as bradykinesia (García-Arencibia et al. 2009). In contrast, blockade of CB_1 receptors reduces parkinsonian akinesia (Fernández-Ruiz 2009). Therefore, it would seem that a future CB-based neuroprotective therapy for PD would have to be based on an adequate combination of compounds to ensure: (1) the antioxidant activity that would be exerted by CB receptor-independent mechanisms, possibly involving activation of the nuclear PPAR receptor family whose activators are also beneficial in experimental parkinsonism (e.g., thiazolidinedione as a PPAR-γ agonist: Carta and Simuni 2014); (2) CB_2 receptor activation and the control of inflammatory events; and (3) the blockade of CB_1 receptors to improve akinesia and reduce motor inhibition. The phytocannabinoid Δ^9-THCV has such a profile, making it an interesting compound to be used therapeutically in PD, alone or in combination with CBD (see Fig. 5), and highlighting the need for a formulation that can be further evaluated in patients.

5 Cannabinoids in AD

AD is the most prevalent chronic progressive neurodegenerative disorder (see Fig. 4 for details), and it is being intensively investigated to determine the benefits of potential CB-based therapies against β-amyloid toxicity, tau protein hyperphosphorylation, and other cytotoxic processes associated with this disease (Karl et al. 2012). As in other disorders, numerous studies have characterized the changes experienced by specific elements of the eCB signaling system during the progression of this disease, particularly in the two most affected brain structures: the hippocampus and cerebral cortex. These studies revealed: (1) an increase in 2-arachidonoylglycerol (2-AG) levels in association with β-amyloid protein-induced hippocampal degeneration and gliosis (Van Der Stelt et al. 2006); (2) a significant upregulation of CB_2 receptors in microglial cells surrounding the β-amyloid plaques (Benito et al. 2003; Ramírez et al. 2005); (3) a reduction in CB_1 receptors associated with neuronal loss, particularly in the hippocampus and basal ganglia (Westlake et al. 1994; Ramírez et al. 2005); and (4) an elevation of the FAAH enzyme in astrocytes associated with senile plaques, thereby enhancing eCB hydrolysis, elevating arachidonic acid levels, and contributing to the destructive inflammatory process that accompanies AD (Benito et al. 2003). These alterations have been interpreted in two ways. On the one hand, the loss of neuronal CB_1

receptors and the elevation of FAAH may contribute to the progression of AD pathogenesis by enhancing the vulnerability of specific groups of cortical and subcortical neurons to different neurotoxic stimuli (D'Addario et al. 2012; Manuel et al. 2014). On the other hand, the increase in 2-AG and the upregulation of microglial CB_2 receptors may protect against β-amyloid-induced neuroinflammation and neuronal injury (Benito et al. 2003; Ramírez et al. 2005).

A neuroprotective therapy for AD based on compounds that act on the eCB system may be effective in reducing classic neurotoxic events, such as excessive glutamatergic transmission, prolonged calcium influx, oxidative stress, and inflammation (Gowran et al. 2011; Karl et al. 2012), although it may also cause more specific effects on the processing, aggregation, and clearance of β-amyloid protein (Tolón et al. 2009; Martín-Moreno et al. 2012; Scuderi et al. 2014). One example of a drug which acts classically through CB_1 and CB_2 receptors and also through mechanisms independent of CB receptors is Δ^9-THC (Gowran et al. 2011). Thus, this compound can prevent β-amyloid protein aggregation and thereby hinder plaque formation by inhibiting acetylcholinesterase activity (Eubanks et al. 2006). Studies on mice bearing five amyloid-related mutations (5xFAD) demonstrated that Δ^9-THC decreases the density of neuritic plaques by increasing the expression of neprilysin, an enzyme in the β-amyloid degradation cascade, but not by inhibiting the expression of the β-site amyloid precursor protein cleaving enzyme 1 (BACE1: Chen et al. 2013). Importantly, this beneficial effect of Δ^9-THC was not reversed by COX-2 inhibition, in contrast to other damaging effects linked to the administration of this phytocannabinoid that were effectively reduced after pharmacological or genetic blockade of COX-2 (such as the impairment of hippocampal long-term synaptic plasticity, and of spatial working and fear memories, and the decrease in dendritic spine density of hippocampal neurons, all induced by Δ^9-THC: Chen et al. 2013). These observations pave the way for the development of novel therapies in which Δ^9-THC might be combined with COX-2 inhibitors, thereby preserving the putative beneficial effects of this CB while avoiding its deleterious ones.

CBD has also been studied extensively in AD, both in vitro (Iuvone et al. 2004) and in vivo (Esposito et al. 2006b, 2007), revealing a notable capacity to: (1) reduce the levels of ROS and the magnitude of lipid peroxidation; (2) prevent glutamate-induced toxicity, as well as β-amyloid-induced glial activation and pro-inflammatory responses; and (3) inhibit β-amyloid-induced tau protein hyperphosphorylation by glycogen synthase kinase-3β. These effects may be at least partially related to the activation of PPAR nuclear receptors (Fakhfouri et al. 2012; Scuderi et al. 2014), whose classic activators are also active in experimental models of this disease (e.g., pioglitazone: Yamanaka et al. 2012). Synthetic compounds targeting CB_1 and/or CB_2 receptors also effectively improve cognitive impairment, preserving neuronal cells, and preventing β-amyloid protein-induced microglial activation and the generation of pro-inflammatory mediators, as well as removing pathological deposits in different in vivo and in vitro models of AD (Ramírez et al. 2005; Tolón et al. 2009; Fakhfouri et al. 2012; Aso et al. 2012, 2013). The benefits of Δ^9-THC, CBD, and synthetic agonists of CB receptors in

preclinical models of AD support the interest that has developed in carrying out clinical studies directed at investigating whether the recently licensed phytocannabinoid-based medicine Sativex® could be a novel disease-modifying therapy for AD patients (see Fig. 5). In fact, a Sativex®-like phytocannabinoid combination was recently evaluated in a preclinical model of an AD-related disorder (frontotemporal dementia), producing a decrease in gliosis and oxidative stress, an improvement in chaperone function, a reduction in the severity of the tau and β-amyloid pathology, and the induction of autophagy (Casarejos et al. 2013).

Some recent studies have also highlighted the interest in preventing eCB inactivation in AD (see Fig. 5). For example, the development of amyloid pathology in PS1/APP$^+$ mice increased the brain levels of monoacylglycerols, *N*-acylethanolamines, free fatty acids, eicosanoids, and other lipid species, as well as the levels of some cytokines (IL1β, IL6, and TNF-α: Piro et al. 2012). Blocking MAGL with JZL184, or genetic inactivation of MAGL, improved behavioral (spatial learning and memory) parameters, decreased the density of amyloid plaques, and reduced astro- and microgliosis, as well as the production of inflammatory cytokines. Interestingly, none of these effects were prevented by CB_1 or CB_2 antagonists, suggesting that they were effects mediated primarily by alterations in arachidonic acid and/or prostaglandin signaling. In addition, the treatment of 5xFAD mice with JZL184 markedly decreased the appearance of neuritic plaques by inhibiting BACE1 expression (Chen et al. 2012). Furthermore, these changes also affected the gliotic process, with decreases in GFAP+ and CD11b+ cells in the cortex and hippocampus of JZL184-treated mice. An impact of the treatment on synaptic function was also evident, with a preservation of dendritic spine density, and a prevention of the amyloid-linked decrease in the expression of glutamate receptor subunits and of PSD-95 (a marker of post-synaptic integrity). These changes had a behavioral correlate in terms of the improved performance of JZL184-treated mice in the Morris water maze test. Finally, the anti-inflammatory effects linked to MAGL inhibition were not mediated by CB_1 or CB_2 receptors, which is consistent with previous studies (Piro et al. 2012), suggesting that other targets of 2-AG may be involved in these effects (e.g., PPAR).

The contribution of FAAH to amyloid pathology could be more complex. As mentioned above, we found that FAAH expression and activity is enhanced in the brains of AD patients (Benito et al. 2003), which is consistent with the recent observation of a significant decrease in AEA levels in the absence of changes in 2-AG in the postmortem human cortex (Jung et al. 2012). Moreover, we have recently found that the effects resulting from a reduction of FAAH activity are not the same when this reduction is induced by genetic manipulation as when it is induced by pharmacological inhibition (Benito et al. 2012; Vázquez et al. 2015). This is best appreciated by comparing the effects of URB597, a FAAH inhibitor, with the genetic ablation of FAAH. Since both manipulations significantly affect the inflammatory milieu in vitro (primary astrocytes) and in vivo (5xFAD mice), the long-term inhibition of FAAH would appear to be associated with the development of a pro-inflammatory phenotype. However, this situation may provide beneficial effects at the behavioral level in vivo (improved memory in the Morris water

maze) as well as in terms of decreased neuritic plaque density and gliosis. Although still preliminary, these data support the idea that targeting eCB degradation may be an attractive therapeutic strategy for AD, a strategy that certainly deserves more attention in the near future.

6 Cannabinoids in Other Less Well-Studied Chronic Neurodegenerative Disorders

Cannabinoid-based therapies may also be useful for other chronic neurodegenerative disorders in which CBs have been less well studied due to the lower incidence of these disorders or the lack of useful preclinical models. Such disorders include autosomal-dominant hereditary SCAs, Down's syndrome, and prion-related disorders. SCAs are a family of chronic progressive neurodegenerative diseases characterized by a loss of balance and motor coordination due to degeneration in the cerebellum, and a loss of its afferent and efferent connections (Rossi et al. 2014). They are rare diseases, although the most prevalent are all polyglutaminopathies like HD. Only very recently has interest been shown in the eCB system in relation to SCAs, and all the available data come from the analysis of postmortem cerebellar tissues from SCA patients (Rodríguez-Cueto et al. 2014a, b). These data include the notably higher presence of CB_1 and CB_2 receptors in the granular layer, in Purkinje cells, in dentate gyrus neurons, and in the white matter of the cerebellum of patients (Rodríguez-Cueto et al. 2014a). These CB_2 receptors appear to be located in Purkinje neurons, as well as in glial elements in the granular layer and the white matter of SCA patients (Rodríguez-Cueto et al. 2014a), a similar profile to that found for FAAH and MAGL (Rodríguez-Cueto et al. 2014b).

Down's syndrome is sometimes referred to as a human model of AD-like β-amyloid deposition. In postmortem human samples, immunohistochemical analysis produced similar findings to those obtained from postmortem tissues of AD patients (Benito et al. 2003; Núñez et al. 2008). Thus, in Down's syndrome, the expression of both CB_2 receptors and FAAH is greater than normal in β-amyloid plaque-associated microglia and astroglia, respectively (Núñez et al. 2008), suggesting that their induction may contribute to, or be a result of, β-amyloid deposition and subsequent plaque formation. This issue has not been investigated at the pharmacological level, in part due to the lack of useful models that reproduce this disease in laboratory animals.

Prion diseases are transmissible neurodegenerative disorders characterized by the accumulation of the protease-resistant prion protein in the CNS (Takada and Geschwind 2013). CBs have also been investigated in prion disease studies, and in a prion mouse model, in which brain levels of 2-AG but not of AEA were found to be elevated (Petrosino et al. 2011). CB_2 receptor expression was also upregulated in this model, with no changes in other cannabinoid receptors, consistent with the fact that microglial cell activation is a common feature of prion diseases (Petrosino et al. 2011). All these alterations were already evident in early stages of the disease, prior to the appearance of the major clinical symptoms (Petrosino et al. 2011). It has

also been found that CBD is effective at inhibiting the accumulation of protease-resistant prion protein in mouse and sheep scrapie infected cells in vitro, an effect that AEA, Δ^9-THC, and methanandamide did not reproduce (Dirikoc et al. 2007). In addition, treatment of scrapie infected mice with CBD also limited the cerebral accumulation of protease-resistant prion protein, significantly increasing the survival of infected mice (Dirikoc et al. 2007). The benefits of CBD were more closely related to the reduction in the cytotoxic events elicited by prion infection (e.g., microglial cell migration), than to any direct interaction of CBD with the prion protein that may alter its stabilization, sub-cellular localization, or the formation of aggregates (Dirikoc et al. 2007).

7 Concluding Remarks and Future Perspectives

Because of the nature of their pharmacological properties, compounds acting on the eCB system are potentially useful and clinically promising neuroprotectants. In this article, we have reviewed the cellular and molecular mechanisms that might be involved in these neuroprotective effects, putting emphasis on those mediated by the activation of CB_1 receptors (e.g., the reduction of excitotoxic stimuli induced either by inhibiting glutamate release or by reducing NMDA receptor-mediated calcium influx) and/or by the activation of CB_2 receptors (e.g., the decrease of local inflammatory events derived from the activation of glial elements). We have also considered the neuroprotective effects apparently mediated by CB receptor-independent mechanisms (e.g., the reduction of oxidative injury by scavenging ROS or by inducing antioxidant defenses), again emphasizing the contribution of alternative intracellular targets in these effects, such as PPARs or transcription factors. Through one or more of these processes, compounds acting on the eCB system may delay/arrest the progression of neurodegeneration in chronic diseases affecting cognitive processes like AD or motor control or performance like PD and HD. We have reviewed the knowledge accumulated over the years regarding these three disorders, as well as the incipient knowledge being generated for a few less well-studied disorders (SCAs, Down's syndrome-related dementia, and prion disorders). It is important to note that most of the studies carried out with CB-based therapies in these diseases have been preclinical. Even so, some of them have provided enough solid evidence to justify the study of these molecules or their combinations in clinical investigations. In this regard, we have proposed some ideas about the route(s) that should be followed to extend this research into the clinical area.

Acknowledgements This work was supported by grants from CIBERNED (CB06/05/0089), MINECO (SAF2012/39173), and CAM (S2011/BMD-2308).

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Endocannabinoids and Mental Disorders

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Contents

Abstract

Preclinical and clinical data fully support the involvement of the endocannabinoid system in the etiopathogenesis of several mental diseases. In this review we will briefly summarize the most common alterations in the endocannabinoid system, in terms of cannabinoid receptors and endocannabinoid levels, present in mood disorders (anxiety, posttraumatic stress disorder, depression, bipolar disorder, and suicidality) as well as psychosis

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_9

(schizophrenia) and autism. The arising picture for each pathology is not always straightforward; however, both animal and human studies seem to suggest that pharmacological modulation of this system might represent a novel approach for treatment.

Keywords
Autism • Bipolar disorder and suicidality • Depression • Endocannabinoid system anxiety • Posttraumatic stress disorder • Schizophrenia

List of Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	Anandamide
CB1r	Cannabinoid type 1 receptor(s)
CB2r	Cannabinoid type 2 receptor(s)
CBD	Cannabidiol
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAGL	Diacylglycerol lipase
dlPAG	Dorsolateral periaqueductal gray
EC	Endocannabinoid
ECS	Endocannabinoid system
EPM	Elevated plus-maze
FAAH	Fatty acid amide hydrolase
HPA	Hypothalamus–pituitary–adrenal
MAGL	Monoacylglycerol lipase
NAc	Nucleus accumbens
OEA	Oleoylethanolamide
PCP	Phencyclidine
PEA	Palmitoylethanolamide
PET	Positron emission tomography
PFC	Prefrontal cortex
PPI	Prepulse inhibition
PTSD	Posttraumatic stress disorder
THC	Delta-9-tetrahydrocannabinol
VPA	Valproic acid
VTA	Ventral tegmental area

1 Introduction

The endocannabinoid system (ECS) is highly represented in brain areas implicated in processing of emotionally salient information as well as learning and memory, such as the hippocampus, cortex, and amygdala circuit. Abnormal emotional

processing and cognitive deficits are core features of various neuropsychiatric conditions, including mood disorders and schizophrenia, thus implying that the ECS might play a role in the neurobiology of these disorders.

2 Mood Disorders

2.1 Anxiety

The ECS has been extensively studied in the context of anxiety. The first evidence for the involvement of the ECS in anxiety comes from the observation that cannabis consumption dose-dependently affects anxiety behaviors, with low doses being anxiolytic and high doses ineffective or possibly anxiogenic. The bidirectional effect of cannabinoids observed in humans can be mimicked in laboratory animals. In models predictive of anxiolytic-like activity, low doses of CB1 agonists tend to be anxiolytic and high doses tend to increase anxiety-related behaviors (Viveros et al. 2005).

Cannabinoid modulation of anxiety occurs through CB1 as well as non-CB1 receptors. The involvement of the CB1 receptor in anxiety has been demonstrated by comparing the behavior of CB1 knockout (ko) and wild-type control mice in standard behavioral measures of anxiety, including the elevated plus-maze (EPM) and light–dark box. Under basal conditions CB1 deletion causes no effect (Haller et al. 2002; Jacob et al. 2009; Marsicano et al. 2002), or anxiety (Jacob et al. 2009; Litvin et al. 2013; Maccarrone et al. 2002; Martin et al. 2002; Uriguen et al. 2004), whereas it always causes anxiety under aversive conditions (Haller et al. 2004; Mikics et al. 2009). Furthermore, CB1 ko mice appear to be more resistant to the anxiolytic effects of benzodiazepines (Uriguen et al. 2004).

Further genetic studies have revealed that the location of CB1 receptors (CB1r) on specific neuronal subtypes is one of the most important factors accounting for the biphasic effect of the ECS on anxiety. Conditional mutant mice have shown that CB1r located on glutamatergic neurons are critical for the anxiolytic effects of low doses of exogenous CB1r agonists, while CB1r expressed by GABAergic neurons are required for the anxiogenic effect induced by high doses of CB1 agonists (Rey et al. 2012). Genetic deletion of CB1 in glutamatergic neurons does not affect anxiety under basal non-aversive conditions, but increases anxiety upon repeated exposure to the open field under high light conditions, suggesting an anxiolytic role for CB1 on glutamatergic neurons after exposure to an aversive environment (Jacob et al. 2009).

Pharmacological studies on the role of CB1r in anxiety appear to be more inconsistent. CB1r blockade, through systemic SR141716, AM251, and AM281 administration, has been found to exert anxiogenic-like effects in several preclinical models (Arevalo et al. 2001; Dono and Currie 2012; Dubreucq et al. 2012; Gamble-George et al. 2013; Haller et al. 2004; Kamprath et al. 2006, 2009; Lin et al. 2011; Litvin et al. 2013; Navarro et al. 1997; O'Brien et al. 2013; Patel and Hillard 2006; Plendl and Wotjak 2010; Rodgers et al. 2005; Sink et al. 2010a, b; Thiemann

et al. 2009; Varga et al. 2012). In contrast, a few other studies have demonstrated either no effect (Bortolato et al. 2006; Kathuria et al. 2003) or an anxiolytic-like activity of CB1r antagonists (Akinshola et al. 1999; Degroot and Nomikos 2004; Griebel et al. 2005; Haller et al. 2002; Rodgers et al. 2003; Rubio et al. 2008). These discrepancies may partly reflect variations in the dosage or behavioral tests (performed under basal or aversive conditions). Concerning the dose, in the studies in which no effect was observed, relatively low doses of the antagonists were used (Griebel et al. 2005), whereas higher doses of rimonabant and its analogues may induce anxiogenic-like responses because of their activity as inverse agonists. Accordingly, systemic administration of the neutral antagonists, AM4113 and NESS0327, did not induce any effect in either conditioned or unconditioned animal models of anxiety (Sink et al. 2008, 2010a, b; Meye et al. 2013).

Data that are both few in number and controversial have been obtained when investigating the role of the CB2 receptor (CB2r) in anxiety. CB2 ko mice have been shown to present a more pronounced anxiogenic-like phenotype compared with wild-type controls (Ortega-Alvaro et al. 2011), suggesting that CB2 activation could be of potential therapeutic importance. However, pharmacological investigations using CB2 antagonists and/or agonists have given contradictory results, showing anxiolytic/anxiogenic effects, depending on the regimen of administration used (García-Gutiérrez et al. 2012; Onaivi et al. 2008). The discrepant findings between pharmacological and genetic inhibition of CB2 signaling could be attributed to differences in dosage and treatment duration, as well as to compensatory mechanisms that could develop in mutant mice.

As a whole, available data indicate that endocannabinoid (EC) signaling plays an important role in modulating anxiety responses particularly under aversive or stressful environmental contexts, while it may contribute to the constraint of anxiety under basal non-aversive conditions. Hence, aversive stimuli or stressful conditions could induce EC release in specific brain regions to maintain emotional homeostasis, mainly acting via presynaptic CB1r activation.

In this context, the pharmacological enhancement of EC signaling, either by inhibiting EC degradation or by blocking EC cellular uptake, has been widely tested as a possible therapeutic tool to manage anxiety. Systemic treatment with fatty acid amide hydrolase (FAAH) inhibitors has been reported to exert anxiolytic-like effects in several independent studies (Bluett et al. 2014; Busquets-Garcia et al. 2011; Gunduz-Cinar et al. 2013a; Kathuria et al. 2003; Kinsey et al. 2011; Micale et al. 2009; Moise et al. 2008; Moreira et al. 2008; Naidu et al. 2007; O'Brien et al. 2013; Patel and Hillard 2006; Rubino et al. 2008a; Scherma et al. 2008). In line with pharmacological studies, FAAH ko mice exhibit reduced anxiety (Moreira et al. 2008; Naidu et al. 2007). Unequivocally, the anxiolytic effects of pharmacological FAAH inhibition or genetic deletion are blocked by CB1r antagonists (Kathuria et al. 2003; Moreira et al. 2008), indicating that FAAH inhibition reduces anxiety through augmentation of anandamide (AEA) signaling at the CB1r. Similar results have been obtained using the AEA uptake inhibitor, AM404 (Bortolato et al. 2006; Braida et al. 2007; Gomes et al. 2011; Naderi et al. 2008).

Although FAAH inhibitors have been generally considered as putative anxiolytic drugs, there are also papers reporting conflicting results of no anxiolytic responses (Haller et al. 2009; Naderi et al. 2008; Naidu et al. 2007) or anxiogenic effects (Roohbakhsh et al. 2009). Conflicting data can be explained by differences in test conditions, considering that FAAH inhibition does not affect anxiety in nonstressful circumstances but may modulate emotionality resulting from stress induced by environmental conditions (Haller et al. 2009; Hill et al. 2013a). Furthermore, the controversial outcome that has been reported following pharmacological enhancement of EC signaling could be due to the ability of AEA to activate not only CB1r but also TRPV1 receptors. Indeed, unlike CB1r, genetic deletion or pharmacological blockade of TRPV1 receptors reduces anxiety (Aguiar et al. 2009; Kasckow et al. 2004; Marsch et al. 2007; Micale et al. 2009; Santos et al. 2008; Terzian et al. 2009). These findings suggest that CB1 and TRPV1 receptor stimulation may elicit opposite effects on anxiety responses, CB1r being anxiolytic and TRPV1 channel activation being anxiogenic. It is therefore plausible that while physiological increases of AEA promote an anxiolytic response through activation of CB1r, excessive tissue levels of AEA may stimulate TRPV1 channels, thus leading to anxiogenic responses. In line with this hypothesis, Scherma et al. (2008) reported that low doses of AEA and URB597 had anxiolytic effects when injected singly but anxiogenic effects when combined. Similarly, the anxiogenic response induced by high doses of AEA was enhanced after URB597 administration (Scherma et al. 2008). Accordingly, the anxiogenic and anxiolytic responses triggered by different doses of methanandamide were counteracted by the administration of TRPV1 and CB1r antagonists, respectively (Rubino et al. 2008a).

In this context, the use of dual FAAH/TRPV1 inhibitors may represent a promising approach for treating anxiety, by promoting the anxiolytic effect associated with CB1r activation and simultaneously preventing the anxiogenic response mediated by TRPV1 activation. Accordingly, one such compound, *N*-arachidonoyl-serotonin (AA-5-HT), has been proven to exert anxiolytic-like effects in the EPM in both rats and mice (John and Currie 2012; Micale et al. 2009).

Although the role of AEA in the control of anxiety-like behaviors has been well documented, little is known about the specific effect of 2-arachidonoylglycerol (2-AG) on anxiety-like responses. The monoacylglycerol lipase (MAGL) inhibitor, JZL-184, has been reported to reduce anxiety in highly aversive conditions (Busquets-Garcia et al. 2011; Kinsey et al. 2011; Sciolino et al. 2011), but not standard conditions (Aliczki et al. 2012). Both CB1r and CB2r have been implicated in the anxiolytic effects of JZL-184 (Busquets-Garcia et al. 2011; Kinsey et al. 2011; Sciolino et al. 2011). Although more studies are clearly needed, these preliminary data suggest that enhancement of 2-AG could also represent an innovative approach to obtain anxiolytic-like effects. Finally several papers have tried to identify the role of the ECS in specific brain areas associated with the modulation of anxiety behavior.

In the prefrontal cortex (PFC), enhancement of AEA levels, through local URB597 infusions, reduces anxiety-like behaviors, whereas overexpression of FAAH reduces AEA levels and increases anxiety in the EPM (Rubino

et al. 2008a). Besides activation of CB1r, activation of TRPV1 channels may also contribute to the anxiety-modulating effects of local AEA signaling enhancement, as blockade of TRPV1 receptors in the PFC elicits an anxiolytic-like response (Aguiar et al. 2009).

Anxiety has been typically associated with reduced activity in the PFC and enhanced activity in the amygdala (Shin and Liberzon 2010). Interestingly, several data have demonstrated that activation of EC signaling increases neuronal activity in the PFC, by suppressing GABA release (Hill et al. 2011; Kiritoshi et al. 2013), while it decreases amygdala activation, by inhibiting glutamate neurotransmission (Azad et al. 2003; Huang et al. 2003; Perra et al. 2008). Accordingly, inhibition of FAAH activity in the basolateral amygdala reduces anxiety (John and Currie 2012) while blockade of CB1r signaling in this brain region increases it (Dono and Currie 2012).

In addition, CB1r within the ventral hippocampus may be involved in the modulation of anxiety behaviors. Facilitation of EC signaling in the ventral hippocampus has been proven to modulate anxiety-like behaviors (Campos et al. 2010; Roohbakhsh et al. 2009; Rubino et al. 2008b), being either anxiolytic or anxiogenic depending on previous stress experience (Campos et al. 2010).

Furthermore, CB1 and TRPV1 receptors in the dorsolateral periaqueductal gray (dlPAG) have also been implicated in the perception of behaviors related to anxiety (Moreira et al. 2007; Terzian et al. 2009). Local administration of AEA into this midbrain region induces CB1-dependent anxiolytic-like effects (Lisboa et al. 2008; Moreira et al. 2007). Besides AEA, 2-AG has also been shown to play a role in modulating anxiety-related responses within the dlPAG since its local administration exerts anxiolytic-like effect in the EPM. This effect seems to be mediated by both CB1r and CB2r activation and was also observed after inhibition of 2-AG hydrolysis through local infusions of URB602 (Almeida-Santos et al. 2013).

In conclusion, the available data indicate that the ECS is clearly implicated in the modulation of anxiety behaviors. Importantly, the effects of cannabinoids on anxiety strongly depend upon stress conditions, suggesting that the ECS may act as a bridge between these two phenomena and hence that elevation of AEA could be an effective treatment for stress-related psychopathology. Overall, it can be suggested that enhancing EC signaling could represent a novel approach to the treatment of anxiety-related disorders whereas dysregulating the ECS may result in anxiety and stress-related disorders.

2.2 Posttraumatic Stress Disorder

There is growing evidence that abnormalities in the ECS are implicated in stress-related/anxiety disorders, such as posttraumatic stress disorder (PTSD).

A recent in vivo imaging study using positron emission tomography (PET) demonstrated that CB1r expression was increased in the brain of individuals with PTSD compared to controls (Neumeister et al. 2013). Interestingly, increased CB1r binding was paralleled by a significant reduction of both peripheral AEA and

cortisol concentrations, suggesting that abnormal CB1 receptor-mediated AEA signaling could be implicated in the etiology of PTSD (Neumeister et al. 2013). In line with this, it has been recently shown that baseline anxiety inversely correlates with peripheral AEA content in healthy individuals (Dlugos et al. 2012) and that among individuals with PTSD, those with lower peripheral AEA levels exhibit more intrusive symptoms (Hill et al. 2013b). Thus, reduced peripheral AEA levels together with a compensatory upregulation of CB1r suggest the presence of a reduced EC signaling in PTSD.

As far as 2-AG levels are concerned, controversial findings have been obtained, reporting either reduced (Hill et al. 2013b), increased (Hauer et al. 2013), or unchanged (Neumeister et al. 2013) circulating levels of 2-AG among individuals with PTSD. Variables such as duration, acuity, and nature of stress exposure may account for the observed discrepancies, and further research will be needed to clarify the role of 2-AG.

Taken together, the available data suggest that reduced EC signaling could be a feature of PTSD. Notably, elevated rates of cannabis consumption have been reported among individuals with PTSD (Cornelius et al. 2010; Vetter et al. 2008). Such observations could partly explain emerging evidence indicating that synthetic cannabinoid receptor agonists may produce some benefits in individuals with PTSD by helping to relieve haunting nightmares and other symptoms (Cameron et al. 2014; Fraser 2009).

Moreover, there is a large body of evidence indicating that the ECS also plays a pivotal role in the regulation of other important aspects of PTSD, such as emotional memory extinction and recall as well as hypothalamus–pituitary–adrenal (HPA) axis function (for extensive reviews, see Hill and Tasker 2012; Ruehle et al. 2012; Trezza and Campolongo 2013). Thus, a pharmacological modulator of the ECS may provide a useful tool for relieving different clinical conditions related to this pathology.

2.3 Depression

The idea that the ECS could play a role in mood disorders is rooted in the observation that consumption of *Cannabis sativa* may improve mood. Indeed, for centuries, cannabis has been known by humans for its mood-elevating and euphoric effects. However, the first paper that openly discussed a possible role for the ECS in depression was published only ten years ago (Hill and Gorzalka 2005). As the authors claimed, their hypothesis at that time was entirely speculative. However, since then, papers providing evidence for an involvement of the ECS in the neurobiology of mood disorders have accumulated.

A large amount of data comes from preclinical studies. For example, in animal models, manipulations that lead to reduced EC signaling result in depressive-like symptoms. This was demonstrated for CB1r-deficient mice: these animals display depressive-like symptoms such as passive coping behavior (Aso et al. 2008; Steiner et al. 2008) and anhedonia (Martin et al. 2002; Sanchis-Segura et al. 2004).

Moreover, in accordance with the presence of cognitive dysfunction in depression, CB1 ko mice also had cognitive impairments, including memory and learning deficits that appear, however, only in adult/mature mice (Varvel and Lichtman 2002; Martin et al. 2002; Bilkei-Gorzo et al. 2005), as well as impairment in the extinction of aversive memories (Marsicano et al. 2002). Finally, they have higher corticosterone serum levels after exposure to stress, suggesting hyperactivity of the HPA axis (Uriguen et al. 2004), one of the most consistent findings in major depressive disorder. Similarly, chronic treatment with a CB1r antagonist in rats induced passive coping behavior and anhedonia together with alterations in biochemical markers of depression (Beyer et al. 2010). Accordingly, when used in humans for its efficacy at reducing body weight, the CB1r antagonist, rimonabant, was soon withdrawn from the market due to psychiatric complications including depressed mood disorders, anxiety, and suicidal ideation (Mitchell and Morris 2007). These signs were present also when the CB1 antagonist was given to healthy subjects (Christensen et al. 2007; Horder et al. 2010).

As a whole these data suggest that a lack or a reduction in EC signaling may be crucial for the development of mood disorders. In support of this hypothesis, it has been demonstrated that long-lasting reductions in CB1r density and AEA levels, induced in the PFC of adult female rats by adolescent exposure to delta-9-tetrahydrocannabinol (THC), triggered the development of a complex depressive-like phenotype characterized by passive coping behavior, anhedonia, social withdrawal, and cognitive deficits (Rubino et al. 2008a, 2009). In this phenotype the reduction in AEA levels seems to play a crucial role since the administration of a drug able to block AEA degradation is sufficient to restore normal behavior except for cognitive impairments (Realini et al. 2011).

On the other hand, validated rodent models of depression display dysregulation in EC signaling. For example, chronic stress represents a significant risk factor for the development of depression, and in animals chronic stress paradigms successfully replicate certain behavioral features of human depression. Animals exposed to chronic stress protocols display a significant reduction in CB1r levels in several limbic structures including the hippocampus (Hill et al. 2005, 2008; Reich et al. 2009), as well as in AEA content (Gorzalka and Hill 2011). Apparently in contrast, chronic stress has also been shown to induce an increase in CB1r density or expression in the PFC (McLaughlin et al. 2013; Bortolato et al. 2007; Hill et al. 2008). However, this increase seems to be important for the promotion of active coping, thus suggesting once again that increasing EC signaling can mitigate against the effects of stress (McLaughlin et al. 2013). Accordingly, the administration of drugs that inhibit AEA degradation in chronically stressed rats exerts antidepressant-like effects (Bortolato et al. 2007). A very recent paper reported that chronic treatment with an inhibitor of 2-AG hydrolysis is also able to reverse chronic stress-induced biochemical and behavioral abnormalities (Zhong et al. 2014). Moreover, in a genetic animal model of depressive behavior, the Wistar Kyoto rat, lower AEA levels in the frontal cortex and hippocampus paralleled by higher FAAH level and activity have been found (Vinod

et al. 2012). Again, pharmacological inhibition of the FAAH enzyme in these animals produced antidepressant-like effects (Vinod et al. 2012).

As a whole, data coming from preclinical studies clearly suggest that hypofunctioning of the ECS might be involved in the etiopathogenesis of depressive disorders, and treatments that increase its signaling may be effective at restoring normal mood.

Dysfunctions in the ECS have also been observed in humans suffering from mood disorders. Indeed, in women diagnosed with depression, serum content of AEA and 2-AG was decreased, suggesting that hypoactivity of the EC system may also be present in patients (Hill et al. 2009). Unfortunately, data regarding CB1r density in the frontal cortex of patients with major depression appear to be inconsistent: there have been reports of decreased CB1 density (Koethe et al. 2007), no alterations (Eggan et al. 2010), or even upregulation (Hungund et al. 2004; Vinod et al. 2010; Choi et al. 2012). Possible reasons for this complexity include ante- and postmortem factors such as medication, substance use, brain pH, and different methodological approaches for evaluating receptor levels. The recent feasibility of in vivo imaging of CB1 in the human brain using quantitative positron emission tomography (PET) with the CB1-selective radioligand [18F] MK-9470 (Burns et al. 2007) could help to resolve this issue.

However, the observation that polymorphisms in genes coding for components of the ECS seem to be associated with vulnerability to mood disorders further supports a role for this system in the etiopathogenesis of this pathology. The most studied gene is the one encoding the CB1r, the CNR1 gene. Different studies have now provided evidence for a role of this gene per se or in combination with life events or other genes in the development of depression (Mitjans et al. 2013; Bagdy et al. 2012; Agrawal et al. 2012; Monteleone et al. 2010; Juhasz et al. 2009; Domschke et al. 2008). Few studies have explored the consequences of polymorphic changes in the FAAH gene; nonetheless the data that have been obtained point toward the importance of this gene in stress reactivity and thus susceptibility to mood disorders (Monteleone et al. 2010; Gunduz-Cinar et al. 2013b). Recently, the presence of a significant association between bipolar disorders and a polymorphism in the CNR2 gene was reported (Minocci et al. 2011), supporting the hypothesis that the CB2r may be involved in the pathogenic mechanism underlying this affective disorder.

Finally, it has been reported that an enhancement in CB1r signaling occurs after some, although not all, antidepressant treatments (see Gorzalka and Hill 2011 for review). The observation that the ECS appears to be only partially involved in current antidepressant treatments does not weaken the hypothesis of its crucial role in the etiopathogenesis of depressive disorders. On the contrary, it should drive its exploitation as a new target, a target that maybe acts upstream if compared to the available therapies, in the still not completely known pathway that tunes mood, thus possibly providing another implement for the therapist's toolbox. However, strangely enough, despite these promising findings, to our knowledge no clinical trial that builds on them is currently ongoing.

3 Psychotic Disorders

3.1 Schizophrenia

While the debate on the correlation between cannabis use and psychosis is still ongoing, several preclinical and clinical data reveal that disturbance of the ECS can have a role in the pathophysiology of schizophrenia. Indeed, findings from genetic, postmortem, and neuroimaging studies, as well as information obtained from samples of blood and cerebrospinal fluid, fully support this hypothesis.

The preclinical results on CB1 alterations in different experimental models of schizophrenia-like disorders are often controversial. Indeed, increases in cannabinoid receptor binding, as well as decreases or no alterations, were found in several brain areas using different animal models (for review, see Zamberletti et al. 2012b). A confused picture also arises from postmortem studies in humans. Accordingly, in cortical areas, either upregulation (Dean et al. 2001; Newell et al. 2006; Zavitsanou et al. 2004) or downregulation (Eggan et al. 2008, 2010) or even no alterations (Koethe et al. 2007) of CB1r have been reported. Interestingly, Uriguen et al. (2009) showed that the immunodensity of CB1r was significantly decreased in antipsychotic-treated subjects but not in drug-free schizophrenic subjects. Cannabis consumption, treatment with antipsychotic drugs, and the different techniques used for determining CB1r levels could explain the apparent inconsistent results obtained in these studies.

Moreover, a different contribution of the CB1r in different subtypes of schizophrenia was also suggested, since a significant increase in CB1r binding in the dorsolateral PFC in a subgroup of patients who suffered from paranoid schizophrenia compared to normal controls and patients with non-paranoid schizophrenia has been recently reported (Dalton et al. 2011). Collectively, a clear picture of the alteration in CB1r in postmortem studies of schizophrenics is still missing, and methodological reasons as well as medication state and cannabis use could explain the current controversy. Interestingly, studies reporting increased CB1r density employed receptor autoradiography, while studies that reported lower levels measured mRNA expression or protein levels.

Recently, Volk et al. (2014) performed both techniques with the same postmortem brain specimens and found a reduced CB1 mRNA and higher CB1r binding levels in the PFC of schizophrenics. They suggest that this discrepancy may be a result of higher affinity of the CB1r or its altered trafficking into the membrane.

More recent studies based on in vivo brain imaging techniques in schizophrenics revealed an increase of CB1 binding, localized in the pons, nucleus accumbens, and cingulate and insular cortex (Ceccarini et al. 2013; Horti et al. 2006; Wong et al. 2010). Specifically, Ceccarini et al. (2013) found that the increased CB1r binding is present in medicated as well as in antipsychotic-free patients but that it is negatively associated with negative symptoms and depression only in antipsychotic-free patients.

Another approach to clarify the CB1r's role in schizophrenia is the use of ko mice. Acute phencyclidine (PCP) administration is a well-accepted preclinical

model reproducing some aspects of schizophrenia, since it induces locomotor activation, stereotyped behaviors, and reduced social interactions in wild-type animals. Interestingly, PCP-induced behavioral alterations were different in CB1 ko mice (Haller et al. 2005), since reduced locomotion, greater enhancement in ataxia, and stereotypies as well as no alterations in social behavior were observed. As social disruption and stereotypy are believed to model, respectively, negative and positive symptoms of schizophrenia, it can be hypothesized that the CB1r may contribute differently to these two types of symptoms, possibly inhibiting positive but facilitating negative ones.

Human genetic studies too suggest the presence of an association of CB1r gene with a particular subtype of schizophrenia, the hebephrenic one (Chavarría-Siles et al. 2008; Ujike et al. 2002), but other studies refute this link (Seifert et al. 2007; Tsai et al. 2000; Zammit et al. 2007).

Finally, in the last years, some evidence has emerged pointing to a role for central CB2r in schizophrenia. CB2 deletion in mice caused decreased motor activity, enhanced motor responses to acute cocaine, a prepulse inhibition (PPI) deficit, and cognitive impairment (Ortega-Alvaro et al. 2011), suggesting that pharmacological manipulation of CB2r could be a potential therapeutic tool for the treatment of schizophrenia-related disorders. Accordingly, recent clinical evidence seems to support a role for CB2r in schizophrenia. A close correlation between a polymorphism of the CB2r gene and increased susceptibility to schizophrenia has been reported (Ishiguro et al. 2010). Moreover, at the peripheral level, decreased CB2r mRNA was associated with a clinical remission of schizophrenia (De Marchi et al. 2003) whereas increased CB2r on lymphocytes was significantly associated with a worsening of cognitive performance (Ferretjans et al. 2014). This is the first evidence highlighting that alterations in cognitive performance in schizophrenia are associated with altered CB2r on peripheral immune cells. Therefore, these cells could reflect changes in central cannabinoid receptors in cognitive brain areas or in microglial cells that when activated can contribute to the worsening of cognitive function.

Both human and animal studies have yielded data suggestive of changes in EC levels and in related enzymes in schizophrenia. Preclinical measurement of EC levels (AEA and 2-AG) in brain regions relevant for schizophrenia in different animal models produced controversial results depending on the experimental model used and the time of EC measurement (before or immediately after the behavioral test). Specifically, using an animal model based on PCP administration, Viganò et al. (2009) found no changes in AEA levels in the PFC and hippocampus, whereas 2-AG content was increased in the PFC. On the contrary, in a similar pharmacological model, Seillier et al. (2010) found increases in AEA levels in the nucleus accumbens and ventral tegmental area (VTA) whereas 2-AG levels were increased only in the VTA. The different levels may depend on the PCP protocol used (chronic-intermittent vs. sub-chronic) and on whether or not animals were submitted to behavioral testing prior to measurements. More recently, the same group (Seillier et al. 2013) found reduced AEA level in prefrontal cortex and amygdala when PCP-treated rats underwent a social interaction test.

Finally, Robinson et al. (2010) found that mRNA levels of different components of the ECS were increased in the brains of rats by post-weaning social isolation, an environmental model of schizophrenia. The strength of this study is mainly in its quantitative determination of the relevant transcript species in a single study, but the lack of information on EC levels reduces its impact. More interestingly, Zamberletti et al. (2012a) found that disruption of the PPI response in rats reared in isolation was paralleled by significant alterations in 2-AG content in specific brain regions. Chronic AM251 treatment completely restored normal PPI responding in isolated rats and normalized the 2-AG levels.

Alterations in EC levels in schizophrenia were also found in humans using peripheral blood samples, cerebrospinal fluid (CSF), or, more recently, postmortem brain specimens. De Marchi et al. (2003), measuring AEA levels from blood, found significantly more AEA in schizophrenic patients in comparison to controls, and clinical remission was accompanied by a significant drop in the levels of AEA and the mRNA transcript for FAAH. Thus, EC signaling might be altered during the acute phase of schizophrenia not only in the central nervous system but also in the blood, although it should be considered that this difference could also represent a consequence of the modified immune response observed in the course of schizophrenia (De Marchi et al. 2003). In fact, one of the major criticisms concerning peripheral blood samples is whether alterations in peripheral blood reflect changes in the brain and vice versa. However, studies investigating levels in CSF have found similar changes. Koethe et al. (2009) reported elevated levels of AEA in the CSF in early psychosis, with higher AEA levels correlated to delayed transition to psychosis in those at risk. Leweke and colleagues (1999) reported an increase in the concentrations of AEA and palmitoylethanolamide (PEA) in CSF from patients with schizophrenia when compared to healthy controls, whereas no such data were obtained for 2-AG, as it was not present at a detectable level. The same group further found an increase of AEA in CSF from patients with drug-naive first-episode schizophrenia and even in prodromal stages of psychosis (Giuffrida et al. 2004). However, AEA levels were normalized in patients prescribed with typical antipsychotics but remained elevated following atypical antipsychotics (Giuffrida et al. 2004).

As a whole, these data suggest a protective role for AEA in psychosis and are in line with the above-cited experimental data suggesting that pharmacological treatment that increases AEA may be useful in schizophrenia. A major question is whether these alterations in AEA are causally linked to psychosis or a consequence of the disease process. Nevertheless, the intriguing hypothesis that altered peripheral EC levels might parallel changes in the brain could be an indication that EC levels are biomarkers of illness and/or treatment response in psychosis. However, the recent paper by Muguruza et al. (2013) seems to invalidate this hypothesis. Indeed, when a quantification of ECs was performed in postmortem brain of schizophrenics, it was shown that the levels of 2-AG were higher in the cerebellum, hippocampus, and PFC when compared to matched controls. AEA levels were instead lower in all brain regions of schizophrenic subjects. These data seem to invalidate the hypothesis that alterations of AEA levels in the biological fluids reflect those in the CNS. Finally, the higher levels of 2-AG observed by Muguruza

et al. deserve a comment since they could represent an adaptive response to lower the glutamatergic hyperactivity present in the brain of the schizophrenic patients. Indeed, Melis et al. (2004) demonstrated that 2-AG selectively depresses the increase in firing and bursting activity evoked in dopamine neurons by PFC stimulation. Accordingly, significantly higher levels of 2-AG and lower levels of AEA were found in the PFC and hippocampus in two different experimental models of schizophrenia (Viganò et al. 2009; Zamberletti et al. 2012a).

3.2 Pharmacological Modulation of the ECS as a New Opportunity for Schizophrenia Treatment

The potential contribution of the ECS to the physiopathology of schizophrenia prompted testing cannabinoid compounds as possible medicines for its treatment. Several preclinical papers reported the effects of both acute and chronic manipulation of the ECS on hyperlocomotion and stereotypies induced by psychotomimetic agents, two behaviors considered as indicators of positive signs of schizophrenia. The majority of these studies highlight the ability of both natural and synthetic cannabinoid agonists to reduce locomotor activation induced by dopaminergic agents, although some controversial data are also present (see for review Zamberletti et al. 2012b). It is worth noting that the efficacy of cannabinoid receptor agonists agrees with the hypothesis of a protective role of AEA in schizophrenia, which is also supported by the beneficial effect of indirect agonists (URB597 and AM404) in reversing hyperlocomotion and social withdrawal (Beltramo et al. 2000; Seillier et al. 2013). Accordingly, the non-psychoactive compound, cannabidiol (CBD), has an antipsychotic effect in both experimental animals and humans partly because of its ability to inhibit FAAH and thus increase AEA levels (see for review Parolaro et al. 2014). In contrast, the administration of the CB1 antagonist/inverse agonist, rimonabant, either augmented, attenuated, or did not alter locomotor responses or stereotyped behaviors induced by dopaminergic agents (for review, see Zamberletti et al. 2012b). Negative symptoms of schizophrenia are social withdrawal and aggressive behaviors that can be assessed in animals through the social interaction test. In this regard, CB1 stimulation by direct/indirect agonists improves social withdrawal in different animal models (Seillier et al. 2010, 2013; Almeida et al 2014). However, the chronic administration of AM251, a CB1 receptor antagonist, also counteracted the increase in aggressive behaviors observed in rats reared in isolation (Zamberletti et al. 2010). Accordingly, genetic deletion of the CB1 receptor prevented PCP-induced social deficits in mice (Haller et al. 2005).

So far only a few studies have examined the effect of a pharmacological modulation of the ECS on cognitive deficits in animal models of schizophrenia. Treatment with direct or indirect agonists as well as antagonists exerted a beneficial effect in different cognitive tasks (Black et al. 2011; Guidali et al. 2011; Seillier et al. 2010; Spano et al. 2010; Zamberletti et al. 2010).

The normal sensorimotor gating response is typically impaired in schizophrenic patients and can be measured both in humans and in animals. The data obtained

from investigations into the effects of cannabinoid agonists on PPI disruption that has been induced pharmacologically or non-pharmacologically appear to be inconsistent and inconclusive. Thus a cannabinoid receptor agonist can evoke opposite effects on the PPI response, the direction of its effect depending on the dose used, the duration of its administration, and the environmental conditions, as well as on the animals' genetic background (Levin et al. 2014; for review, see Zamberletti et al. 2012b). The same controversial picture is present also for antagonists that reversed the PPI disruption induced by pharmacological agents (cannabinoid drugs included) but did not recover the PPI deficit induced by adverse environment. Recently, PPI responses have been reported to be disrupted in CB2 ko mice (Ortega-Alvaro et al. 2011), suggesting that CB2 receptor activation could represent a promising target for the rescue of PPI deficits.

Although there is still a debate on whether cannabis use is an independent risk factor for schizophrenia and on whether high prevalence of cannabis use in patients denotes self-medication for its neuroprotective effect (Potvin et al. 2008), some clinical investigations aimed at verifying the potentiality of cannabinoid drugs in treating schizophrenics have been performed. The most interesting results were obtained with CBD. In healthy subjects, CBD inhibits THC-induced psychotic episodes as well as the psychotic symptoms induced by subanesthetic doses of ketamine (Bhattacharyya et al. 2010; Bosi et al. 2003; Englund et al. 2013). Interestingly, the first paradigm is consistent with the finding that people smoking cannabis with a high CBD/THC ratio seem to be less prone to develop psychosis than people smoking cannabis with a low CBD/THC ratio (Di Forti et al. 2009; Schubart et al. 2011). Moreover, CBD showed the same efficacy as amisulpride and was even better than amisulpride in reducing negative symptoms in schizophrenic patients (Leweke et al. 2012). The beneficial effect of CBD may rely on its ability to inhibit FAAH thus increasing AEA level. Its safety and tolerability compared to traditional antipsychotics makes it an ideal candidate for schizophrenia treatment. However, long-term, double-blind, placebo-controlled trials in larger samples are strictly necessary to firmly address its potentiality.

Based on the possibility that hyperactivity of the ECS in schizophrenia could be related to the development of clinical symptoms, rimonabant was tested in a controlled trial in schizophrenic patients (Meltzer et al. 2004). It did not improve clinical scores. In contrast, more recently, Kelly et al. (2011) found a significant improvement in clinical scores in a rimonabant group. However, the small sample size and its premature termination (rimonabant marketing was suspended in 2008) are the major limitations of this study that consequently shows limited power to detect efficacy or rare adverse events.

In conclusion, ECS dysfunction is certainly involved in the pathogenesis of schizophrenia, but the real meaning of the observed alterations is far from being elucidated. The contrasting data arising from the experimental approaches could be partially explained by the use of different animal models that only reproduce single or some elements of the disease, whereas human studies often suffer from confounding factors linked to the pharmacotherapy and/or to cannabis self-medication. Nevertheless pharmacological modulation of the system produced positive results overall when it was directed at enhancing AEA levels although

even antagonists seem to be efficacious. Further studies are therefore needed to better understand ECS dysfunctions in schizophrenia, in order to design more appropriate pharmacological interventions.

4 Autism

There is increasing evidence that the ECS has a role in social and emotional processing. However, there is a paucity of studies directly examining the role of this system in autism. Using the valproic acid (VPA) model of autism, Kerr et al. (2013) showed that levels of AEA and 2-AG in the hippocampus, frontal cortex, or cerebellum were not altered in VPA-exposed rats, whereas diacylglycerol lipase (DAGL) mRNA expression was reduced in the cerebellum of these animals. Furthermore, while the expression of mRNA for MAGL was reduced, the activity of this enzyme was increased in the hippocampus of VPA-exposed animals. CB1 or CB2 receptor expression was not altered in any of the regions examined; however, VPA-exposed rats exhibited reduced PPAR alpha and GPR55 expression in the frontal cortex and hippocampus. Finally, tissue levels of the FAAH substrates, AEA, oleoylethanolamide, and PEA, were higher in the hippocampus of VPA-exposed rats immediately following social exposure. As a whole, these data indicate that prenatal VPA exposure is associated with alterations in the brain's ECS and support the hypothesis that EC dysfunction may underlie behavioral abnormalities observed in autism spectrum disorders.

5 Conclusions

In conclusion, a dysregulation of the ECS seems to play a role in the neurobiology of both mood disorders and psychosis. Data already published seem to indicate that enhancing EC signaling could represent a novel approach for the treatment of anxiety and depression, since a hypofunctionality of the ECS has been reported in these illnesses. More difficult is to depict the rationale for ECS manipulation in psychosis, inasmuch as positive results have been described after manipulations aimed at increasing AEA levels or blocking CB1 signaling. Clinical trials are now urgently needed to test the validity of ECS-related experimental hypotheses.

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Cannabis and Endocannabinoid Signaling in Epilepsy

István Katona

Contents

Abstract

The antiepileptic potential of *Cannabis sativa* preparations has been historically recognized. Recent changes in legal restrictions and new well-documented cases reporting remarkably strong beneficial effects have triggered an upsurge in exploiting medical marijuana in patients with refractory epilepsy. Parallel research efforts in the last decade have uncovered the fundamental role of the endogenous cannabinoid system in controlling neuronal network excitability raising hopes for cannabinoid-based therapeutic approaches. However, emerging data show that patient responsiveness varies substantially, and that cannabis administration may sometimes even exacerbate seizures. Qualitative and quantitative chemical variability in cannabis products and personal differences in the etiology of seizures, or in the pathological reorganization of epileptic networks, can all contribute to divergent patient responses. Thus, the

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_10

consensus view in the neurologist community is that drugs modifying the activity of the endocannabinoid system should first be tested in clinical trials to establish efficacy, safety, dosing, and proper indication in specific forms of epilepsies. To support translation from anecdote-based practice to evidence-based therapy, the present review first introduces current preclinical and clinical efforts for cannabinoid- or endocannabinoid-based epilepsy treatments. Next, recent advances in our knowledge of how endocannabinoid signaling limits abnormal network activity as a central component of the synaptic circuit-breaker system will be reviewed to provide a framework for the underlying neurobiological mechanisms of the beneficial and adverse effects. Finally, accumulating evidence demonstrating robust synapse-specific pathophysiological plasticity of endocannabinoid signaling in epileptic networks will be summarized to gain better understanding of how and when pharmacological interventions may have therapeutic relevance.

Keywords

2-Arachidonoylglycerol • Anticonvulsant • Cannabidiol • Cannabis • CB_1 cannabinoid receptor • Diaclyglycerol lipase-α • Epilepsy • Glutamate • GPR55 receptor • Hippocampus • Metabotropic glutamate receptor • Perisynaptic machinery • Seizure • Synapse • Synaptic circuit-breaker • Δ^9-tetrahydrocannabinol

Abbreviations

2-AG	2-Arachidonoylglycerol
CBD	Cannabidiol
CBDV	Cannabidivarin
DAG	Diacylglycerol
DEA	Drug Enforcement Administration
DGL-α	Diacylglycerol-lipase-α
EEG	Electroencephalogram
GABA	γ-aminobutyric acid
MGL	Monoacylglycerol lipase
mGluR	Metabotropic glutamate receptor
PLC-β	Phospholipase-β
THC	Δ^9-tetrahydrocannabinol
THCV	Δ^9-tetrahydrocannabivarin
TRP	Transient receptor potential channel
UKCIA	United Kingdom Cannabis Internet Activists
WHO	World Health Organization

1 Cannabis in Epilepsy

Cannabis sativa is one of our most ancient medical plants. It has been used for over 5000 years in many ancient cultures for various therapeutic purposes (Mechoulam 1986). Its recreational use and abuse became widespread in the 1960s in Western societies, about the same time, as the underlying scientific explanation for the behavioral and therapeutic effects of *Cannabis* began to unfold. The chemical identification of Δ^9-tetrahydrocannabinol (THC) as the major bioactive and psychoactive constituent of cannabis (Gaoni and Mechoulam 1964; Mechoulam and Gaoni 1967; Mechoulam et al. 1970) and several other structurally related, but non-psychoactive, cannabinoids such as cannabidiol (CBD; Mechoulam and Shvo 1963); cannabidivarin (CBDV; Vollner et al. 1969) or Δ^9-tetrahydrocannabivarin (THCV; Gill et al. 1970) opened the way for hypothesis-driven medical experiments to fully characterize the widespread pharmacological effects of the chemical class of natural products called phytocannabinoids. More than 100 chemical species of C_{21} terpenophenolic compounds, which are uniquely produced by the *Cannabis* plant, have been identified so far (Elsohly and Slade 2005; Husni et al. 2014). However, due to its robust and specific behavioral effects, intense research was primarily focused on THC, which thereby played the central role among these phytocannabinoids in the discovery of a new signaling system in our body known as the endocannabinoid system. Two metabotropic cannabinoid receptors, CB_1 and CB_2, were identified as mediating most of the biological effects of THC (Devane et al. 1988; Matsuda et al. 1990; Munro et al. 1993). Moreover, two lipid molecules, anandamide and 2-arachidonoylglycerol (2-AG), were recognized soon thereafter as being the endogenous ligands for these G-protein-coupled receptors (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995), and these, together with several metabolic enzymes precisely regulating tissue endocannabinoid levels, constitute the endocannabinoid system (Cravatt et al. 1996; Dinh et al. 2002; Bisogno et al. 2003; Okamoto et al. 2004; Blankman and Cravatt 2013). During the last two decades, it has become obvious that the therapeutic significance of the pharmacological activation of CB_1 and CB_2 cannabinoid receptors by THC or by synthetic cannabinoids is manifold (for review see Pertwee 2012). In addition, detailed investigations have also uncovered the fundamental role of the endocannabinoid system in numerous pathophysiological processes, paving the way for new approaches aiming to manipulate the activity of endocannabinoid signaling for therapeutic purposes (Pacher et al. 2006).

In the nervous system, CB_1 receptors are widespread and important regulators of neurotransmitter release probability (Kano et al. 2009). Retrograde activation of presynaptic CB_1 receptors on axon terminals is now widely accepted as the general signaling mode by which a postsynaptic neuron can temporarily or persistently limit the strength of its afferent inputs (Castillo et al. 2012). This negative feedback signaling pathway plays a general regulatory role in setting the threshold for neuronal circuit excitability and therefore has fundamental significance in several neurological disorders, including epilepsy (Katona and Freund 2008). In

accordance, the antiepileptic potential of cannabis preparations was recognized several hundred years ago. Perhaps the most famous anecdotal example is the case of the chamberlain's son of the Baghdad Caliphate in the fifteenth century, whose recurrent seizures could be kept under control with hashish (Mechoulam and Lichtman 2003). Another well-known account comes from William Brooke O'Shaughnessey working at the Medical College of Calcutta in India in the ninenteenth century, who wrote the first detailed description on therapeutic cannabis effects in the form of a 49-page monograph and highlighted its antiseizure effectiveness as a most notable feature:

> The preceding cases constitute an abstract of my experience on this subject, and which has led me to the belief that in Hemp the profession has gained an anti-convulsive remedy of the greatest value (O'Shaughnessy 1843).

In addition, a few papers are available in the modern medical literature, primarily presenting case reports, and these works have also generally found that the various cannabis products usually, though not always, behave as anticonvulsants (for reviews see Gordon and Devinsky 2001; Szaflarski and Bebin 2014; Dos Santos et al. 2015).

2 Case Reports and Clinical Data of Cannabis Use in Epileptic Patients

Given that the medicinal and recreational use of cannabis and its components in the modern era were until very recently illegal or are still banned in most countries (e.g., cannabimimetics are registered as Schedule I drugs in the USA), proper double-blind, randomized, placebo-controlled trials to establish the efficacy and safety of different cannabis preparations in the treatment of certain specific forms of epilepsies have not been permitted. In addition, legal and ethical issues have made epidemiological investigations nearly impossible. However, the importance of prospective studies should not be questioned, especially in light of the recent recognition of the strong link between cannabis use and the risk of developing psychotic syndromes, based on data primarily obtained in European countries. With the help of scrutinized epidemiological analysis, the bidirectionality of association has been demonstrated, i.e., regarding the self-medication hypothesis (higher frequency of cannabis use among schizophrenic patients, who may use cannabis to alleviate symptoms) and regarding the damage hypothesis (the use of cannabis increases the risk of schizophrenia-like psychosis) (Griffith-Lendering et al. 2013). Furthermore, the strong association between the specific chemical content of cannabis preparations and the risk of developing psychosis has also been established, revealing that high THC-, low CBD-containing, skunk-like cannabis significantly increases psychosis vulnerability (Di Forti et al. 2015). These data highlight the critical necessity of knowledge on the qualitative and quantitative chemical properties of medical marijuana aimed to be used for therapeutic effect, e.g., as an anticonvulsant.

Although in general accumulating data from these early reports does not allow one to draw strong conclusions, as in the case of association with schizophrenia and cannabis use, some preliminary observations can be made on the potentially very weak association between cannabis use and seizure disorders. These findings indicate the possibility that a small epileptic patient population, including only adult subjects, may use cannabis for self-medication. Most probably, the very first survey report dates back to 1976, when 13 out of 72 epileptic patients (18 %) responded to a questionnaire reporting cannabis use as a kind of self-medication. However, only one patient reported a significant improvement in his/her condition, whereas another patient noted that his/her seizures got worse (Feeney 1976). Later, in a more detailed, but indirect, case study examining illicit drug use in 308 patients versus 294 controls, significantly less men with unprovoked or provoked seizures admitted marijuana use in the preceding 3 months, which led the authors to conclude that cannabis may be protective against new onset seizures (Ng et al. 1990; Brust et al. 1992). Interestingly, a similar association was not reported for women, and one must be cautious considering that the lower rate of cannabis use among epileptic patients could also be an indication that adverse effects reduced the likelihood of cannabis intake. The general picture emerging from more recent informal surveys is also very complex. Among 215 US marijuana users with active epilepsy, only 7.4 % reported a beneficial effect on their seizure frequency, whereas the majority of patients (90 %) did not recognize an association between marijuana use and their epileptic condition (Gordon and Devinsky 2001). However, another Canadian survey study reported a clear association in 28 out of 165 patients (17 %) treated in tertiary care epilepsy centers, who responded to a phone interview by claiming to regularly use cannabis. Notably, 54 % of these patients reported a decrease in seizure frequency and 68 % felt that cannabis ameliorates seizure severity (Gross et al. 2004). In striking contrast, Hamerle and colleagues in Germany very recently reported that, although 20 % of 310 monitored epileptic patients used cannabis after epilepsy onset, most of them (84.1 %; 53 out of 63 patients) did not experience any change in seizure frequency or severity of epileptic convulsions. Moreover, seven subjects reported intensified seizures of higher frequencies and only three patients noticed improvements in their conditions (Hamerle et al. 2014). Although cannabis use for personal purposes in Germany is generally well-tolerated, only ~4 % of medical marijuana users used cannabis to alleviate their epilepsy symptoms (Schnelle et al. 1999). Thus, at this stage it is very difficult to draw a clear conclusion, but with the increasing legal availability of medical marijuana, especially in the USA, scientifically more established epidemiological analysis should be performed in different forms of epileptic disorders to determine any potential causality between cannabis use and the beneficial or adverse effects in either direction, i.e., supporting the self-medication hypothesis and/or the damage hypothesis. The precise selection of adequate control groups and cannabis consumption modes will be pivotal, as several other confounding factors can increase sampling variability. Parameters such as age at first use, duration of use, frequency of use, type of administration, type of cannabis used, as well as the specific type of epilepsy, the etiology, the severity, and the progression of disease

will all render any epidemiological analysis very difficult. However, in principle, considering that the World Health Organization (WHO) estimates the number of epileptic patients at around 50 million, whereas approximately 147 million people are calculated to regularly use cannabis preparations, it is expected that future studies will be feasible and will also gain important information for a large patient population.

Besides epidemiological studies at the population level, single case studies also add useful information about the potential benefits of cannabis in controlling seizures and about the possible caveats in using cannabis or cannabimetics in epilepsy. To introduce just a few of the notable examples available in the widespread epilepsy literature, Davis and Ramsey administered THC homologs as active cannabis substances to five children with intractable epilepsy as early as 1949. Two of the children responded very well, whereas the other three did not improve, and one even suffered a worsening of his/her condition (Davis and Ramsey 1949). Another early, but well-documented report comes from Consroe and colleagues, who described in detail the case of a young man, who did not respond to conventional antiepileptic treatments, but whose seizures could be effectively controlled by smoking marijuana. An important observation was that the seizures returned when cannabis use was briefly curtailed, indicating an acute anticonvulsant effect (Consroe et al. 1975). More recently, Mortati and coworkers published a clinical case report of a 45-year-old male patient with uncontrolled cerebral palsy and partial epilepsy, whose condition was successfully improved to an almost seizure-free status by taking marijuana (Mortati et al. 2007). Alongside these clinical reports, several internet sources present anonymous case examples such as the United Kingdom Cannabis Internet Activists (UKCIA) web page, which lists 36 patient testimonies all concluding that cannabis use alleviated their epilepsy symptoms (http://www.ukcia.org/medical/epilepsy.php). Nevertheless, it is very important to emphasize that patients with personal benign experiences are more likely to share their positive feelings than those who took medical marijuana and did not improve or even experienced adverse effects. In light of this consideration, it was already noted decades ago that cannabis consumption may even sometimes be proconvulsant, as exemplified by the case of a 29-year-old epilepsy patient whose seizure-free period was interrupted by the recurrence of generalized tonic-clonic seizures after initiating marijuana use (Keeler and Reifler 1967). Likewise, similar first-time onset of epileptic seizures were observed in 4 participants in a clinical trial aiming to test the effects of a medical cannabinoid-based medicine against spasticity and pain in multiple sclerosis (Wade et al. 2006). Importantly, this latter well-controlled clinical study allows a rough estimation of the rate of these adverse effects to be around 3 %, because 4 out of 137 patients experienced their first-ever seizures (Wade et al. 2006).

Another important consideration is that of how long-term cannabis administration may affect the unbalanced network excitability in epileptic patients. Some case reports suggest that this issue should not be neglected, as shown in the case of an epileptic patient who began to have his first focal seizures after cessation of regular marijuana smoking (Ellison et al. 1990). Notably, antiepileptic drug treatment and

reinstating marijuana use brought his condition back under control, but repeated trials of marijuana abstinence always resulted in the recurrence of seizures (Ellison et al. 1990). A recent case report by Hegde and colleagues in California also described the reappearance of seizures in two patients whose focal epilepsy had previously been controlled by medical marijuana, directly after stopping marijuana use due to admission to an epilepsy special care unit (Hegde et al. 2012). This study is the first well-documented case report including direct monitoring of epileptic activity, as the authors used electroencephalogram (EEG) video telemetry to detect a dramatic increase in seizure frequency together with underlying abnormal electrical brain activity (Hegde et al. 2012). The precipitation of epileptic seizures after marijuana cessation may be an indication that cannabis-induced tonic control of excess network excitability was required to keep these patients seizure-free, likely due to a general hypofunction of the protective endocannabinoid system (Katona and Freund 2008; Hegde et al. 2012). Interestingly, another case report describes a conceptually similar situation in an obese patient with a history of generalized childhood epilepsy, but who had been seizure-free for several decades. The patient was administered the CB_1 receptor antagonist rimonabant, which was on the market as an approved medicine to treat his obesity at the time of the study. This patient responded to global CB_1 receptor blockade with paroxysmal epileptic EEG activity and had nocturnal partial seizures, which disappeared immediately after ceasing rimonabant intake (Braakman et al. 2009), further underlying the critical importance of endocannabinoid tone in regulating network excitability and seizure threshold.

3 Cannabidiol and Cannabidiol-Enriched Cannabis in Epilepsy Treatment

While the studies mentioned in Sect. 2 reveal potential antiepileptic effects of cannabis in a select population of adult subjects, the high prevalence of childhood epilepsy and seizure disorders (Russ et al. 2012), together with the large number of patients with refractory forms of epilepsy not responding to current treatment regimes, makes it especially important to find new therapeutic avenues with increased safety and efficacy (Wheless 2009). In an observational study, Lorenz tested the effects of THC in four children with intractable epilepsy, and indeed, two of them responded with a decrease in seizure frequency (Lorenz 2004). However, the general concept of chronic administration of conventional cannabis strains to children is not feasible due to the unique susceptibility of the developing prenatal, postnatal, and adolescent brain to the effects of high THC-containing cannabis (Volkow et al. 2014; Hadland et al. 2015). Therefore, to circumvent this obstacle, recent efforts have focused on the alternative approach of using cannabis preparations enriched in CBD (Fig. 1). This alternative phytocannabinoid has also been shown to exhibit anticonvulsant effects in several preclinical and in some clinical studies, but lacks psychoactive side effects and does not behave as a CB_1 cannabinoid receptor agonist (for reviews see Pertwee 2008; Devinsky et al. 2014).

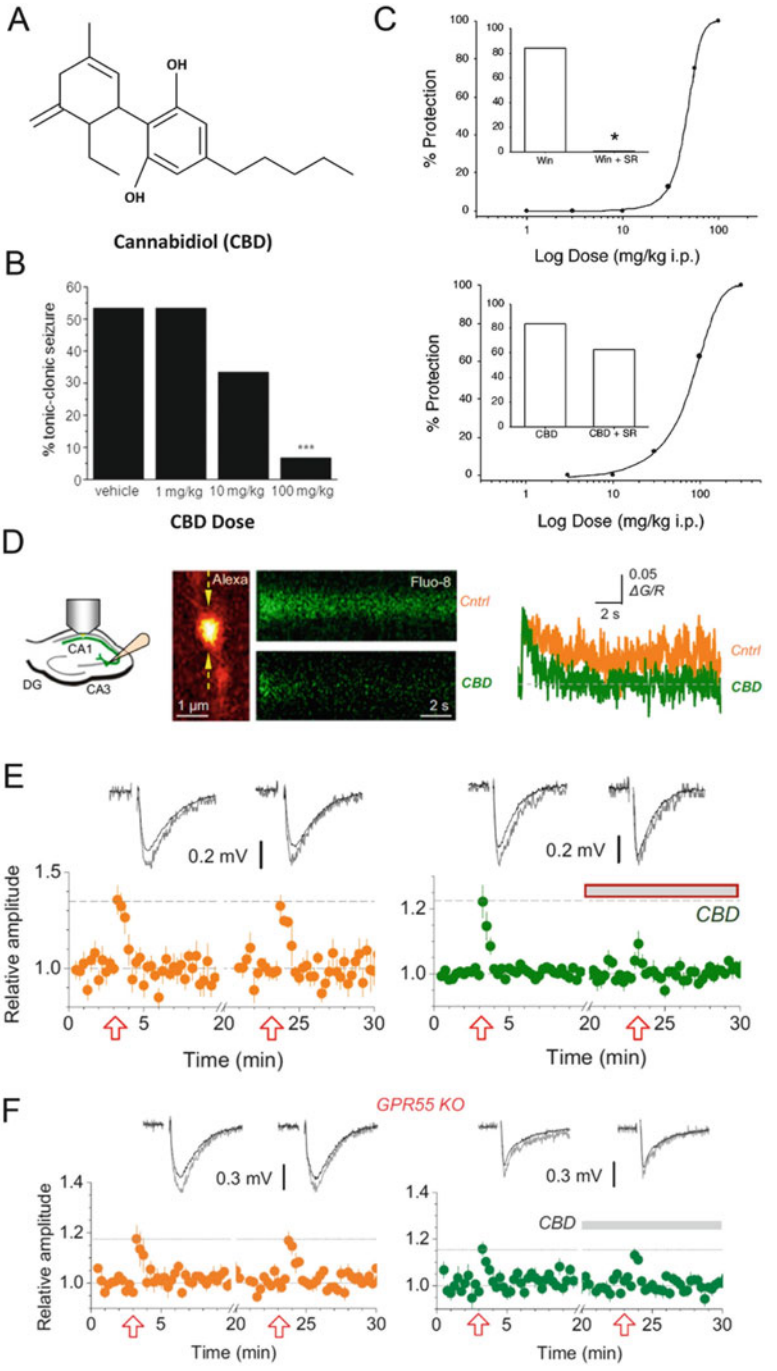

Fig. 1 Cannabidiol is a potent anticonvulsant cannabinoid and inhibits high frequency-induced excitatory synaptic potentiation. (**a**) The chemical structure of cannabidiol, a non-psychoactive

This alternative approach has been primarily prompted by two early reports on the beneficial effects of CBD in a small population of patients. The very first study was performed by Raphael Mechoulam and his colleagues in Israel on nine subjects with therapy-resistant uncontrolled temporal lobe epilepsy (Mechoulam and Carlini 1978). Patients were randomized into two groups, receiving daily administration of either 200 mg CBD, or placebo. While the placebo-treated patients showed no improvement during the three-month trial, the authors observed a major reduction in seizure frequency in three out of the four individuals receiving CBD (Mechoulam and Carlini 1978). Likewise, Cunha et al. found that four out of seven patients became seizure-free during a year-long trial with a similar dose of CBD, whereas only one out of eight individuals from the placebo-treated group showed improvement (Cunha et al. 1980).

With the increasing legal availability of medical marijuana in some US states, and with the help of improved information transfer via the internet, several parents have very recently started to apply CBD-enriched cannabis preparations as an adjunctive therapy to their children with very serious, debilitating forms of childhood epilepsies. Perhaps the most striking example of the antiepileptic potential of medical marijuana comes from the highly publicized recent case report of Charlotte Figi in Colorado, who suffers from Dravet syndrome—an especially severe form of usually intractable epilepsy caused by a mutation in the SCN1A gene encoding a voltage-gated sodium channel subunit (Maa and Figi 2014). After an unsuccessful treatment algorithm including all conventional anticonvulsant drugs and even the ketogenic diet, her parents are able to successfully control her seizures using a high CBD/low THC content cannabis oil made from a specific cannabis strain, now named after her as Charlotte's Web. Remarkably, her prior seizure frequency of

Fig. 1 (continued) compound in marijuana. (**b**) Cannabidiol (CBD) administration has a strong dose-dependent antiepileptic effect in vivo (Jones et al. 2010). Note that high doses of CBD almost completely prevented tonic-clonic seizures in the pentylenetetrazol model of epilepsy. (**c**) Interestingly, the anticonvulsant effect of CBD may not be mediated by CB_1 cannabinoid receptors (Wallace et al. 2001). While rimonabant (SR), a CB_1 receptor antagonist (10 mg/kg i.p.), could fully block the protective effects of the CB_1 agonist WIN55,212-2 (Win), its administration did not significantly change the antiepileptic effects of CBD in the maximal electroshock model, a model of partial seizures with secondary generalization. Both Win and CBD were applied at their ED_{84} doses (60 mg/kg i.p. and 160 mg/kg i.p., respectively). (**d**) GPR55 mediates glutamatergic synaptic facilitation at excitatory synapses, and it is inhibited by CBD pointing to an alternative candidate mechanism, which may contribute to the anticonvulsant effects of CBD (Sylantyev et al. 2013). Glutamatergic pyramidal neurons were stimulated in the hippocampal CA3 region at high frequency (10 stimuli at 100 Hz), and the concomitant presynaptic calcium increase was detected in excitatory Schaffer collaterals in the CA1 subfield. Note that CBD application potently inhibited postburst calcium increase (visualized by Fluo-8) within the same example bouton. (**e**) Presynaptic calcium increase leads to increased amplitude of postsynaptic current responses after high frequency stimulation (postburst synaptic potentiation; PBP), which was significantly reduced by CBD application at 0.2 μM in wild-type, but not in GPR55 knockout (KO) mice (illustrated in **f**). Moreover, baseline PBP in GPR55 KO mice was one-third of that in control animals. The individual figures have been modified from the originals with permission from the authors

~300 grand mal seizures a week has now dropped to three convulsions per month, a level consistently maintained over the last 3 years. At the same time, parents of children with treatment-resistant epilepsies, especially in the USA, have started to use similar cannabis preparations with high CBD content, and the very first observational reports have now been published. For example, in a survey organized by Stanford University among parents belonging to a Facebook group specializing in sharing information on the use of medical marijuana to treat pediatric epilepsy, 16 out of 19 parents reported significant improvement in their children, and 53 % (10 children) had more than an 80 % reduction in seizure frequency. Importantly, these CBD-enriched cannabis preparations were well-tolerated, with only fatigue and drowsiness mentioned as side effects (Porter and Jacobson 2013). In this study, some data were provided on the variable dosage of cannabinoids, with CBD ranging from 0.5 mg/kg/day to 28.6 mg/kg/day, whereas THC content usually remained below 0.8 mg/kg/day. A similar retrospective open-label analysis, also based on parental reports of using oral cannabis extracts, was conducted on 75 therapy-resistant epileptic subjects under 18 years of age in Colorado. Altogether 33 % of the patients experienced at least a 50 % reduction in seizures, as estimated by their parents (Press et al. 2015). However, a word of caution in interpreting these numbers has already been put forward by the same authors, recognizing that the 33 % responder rate is heavily biased due to a larger probability of positive association in families who had moved to Colorado to access medical marijuana (45 % responder rate), compared to those families who were established inhabitants (22 % responder rate) (Press et al. 2015). It is very important to take this into consideration in light of the fact that the average placebo responder rate in open-label studies for antiepileptic drugs such as clobazam or eslicarbazepine is around 20–30 % (Ng et al. 2011; Elger et al. 2009). Moreover, those patients who were monitored in epilepsy care units did not show improvements in their interictal EEG pattern, and the outcome is even further complicated by the observation that 13 % of these patients experienced increased seizure number after treatment with oral cannabis extracts (Press et al. 2015).

Certainly, it is critical that those children who cannot be helped by available conventional medical approaches should get a chance to try any potentially beneficial alternative treatment strategy. As is exemplified in the case of Charlotte Figi, there are patients who respond very well to medical marijuana without any apparent adverse side effects (Maa and Figi 2014). On the other hand, the emerging data summarized above also indicate that there is a significant proportion of patients who do not respond well to cannabis administration and even that CBD-enriched medical marijuana can worsen the condition of patients. However, as is often the case with new treatment options, especially any considered by the media to be miraculous, newsworthy developments, parents may expose their children to increased risk by replacing conventional and potentially helpful treatment regimes with medical marijuana. In this respect, the results of the recent poll organized by the journal Epilepsia, which summarized the opinions on medical marijuana of 776 neurologists, general physicians, and patients, are especially worrisome (Mathern et al. 2015). While only 28 % and 34 % of neurologists judge medical

marijuana use in epilepsy treatment as efficacious and safe, respectively, the public almost unanimously (98 %) considers it as a treatment option, with a similarly large proportion of general doctors and allied health professionals (83 %) agreeing with the public (Mathern et al. 2015). This paradoxical view can only be resolved if the legal environment changes to support high quality clinical trials, which allow the medical community to clearly establish the patient population for whom the beneficial effects of long-term medical marijuana use override the potential adverse effects, as well as revealing the appropriate approximate chemical composition (e.g., CBD/THC ratio), therapeutic dose range, and mode of administration.

The complexity of outcomes following medical marijuana administration in epileptic patients suggests that an obvious alternative in epilepsy treatment would be the specific use of pharmaceutical grade compounds. In principle, at low doses THC acts as an agonist of presynaptically located CB_1 receptors on excitatory axon terminals and thus may dampen seizure activity by reducing glutamate release (Katona et al. 2006; Kawamura et al. 2006). The dose range used to achieve the beneficial effects of Sativex results in ~10 ng/ml blood THC levels. Importantly, this dose does not evoke psychoactive side effects and may not induce the internalization and removal of CB_1 receptors from axon terminals after long-term treatment (Stott et al. 2013; Dudok et al. 2015), which is important as prolonged exposure to a high dose of receptor agonists such as THC causes downregulation of CB_1 and the development of tolerance to its anticonvulsant effects (Blair et al. 2009; Dudok et al. 2015). However, CB_1 receptors are also present on GABAergic axon terminals (Katona et al. 1999), and decreased GABAergic inhibition by high doses of THC may reduce seizure threshold (HÃjos et al. 2000; Hoffman and Lupica 2000). This is especially intriguing in light of the emerging abuse of synthetic cannabinoid receptor agonists. In contrast to THC, which is a partial agonist of CB_1 receptors, many of these cannabimetics are full agonists and evoke very robust behavioral effects. In parallel with their increased use, case reports have started to appear describing the proconvulsant effects of these "Spice" compounds (Tofighi and Lee 2012). One of the more well-known recent examples depicts the story of a young man who took his dog to a veterinary surgeon because it was experiencing convulsions. However, while waiting for his animal the man also suffered a serious generalized tonic-clonic seizure. Subsequent investigations revealed that both the dog and its owner were under the influence of a synthetic cannabinoid PB-22 nicknamed "Crazy Monkey" (Gugelmann et al. 2014). Similarly, robust proconvulsant effects were also observed with another synthetic cannabinoid called AM2201 or "Black Mamba" in Europe (McQuade et al. 2013). These observations highlight that the medicinal application of pure THC or other cannabimimetics would likely be very difficult due to the narrow therapeutic window and significant safety issues. Together with the complexity of the network functions of CB_1 receptors, these difficulties render the feasibility of this approach highly unlikely.

On the other hand, the remarkable successes reported in cases using cannabis extracts containing high concentrations of CBD and low doses of THC (Maa and Figi 2014), together with data from preclinical experiments testing CBD in animal models (for review see Devinsky et al. 2014), point to CBD as a potentially

beneficial compound in a select population of epileptic patients, especially children with refractory epilepsy (Cilio et al. 2014). For example, malignant migrating partial seizure is a severe form of usually pharmacoresistant epileptic encephalopathy, with unknown etiology in infants. Another remarkable case study recently reported successful reduction of migrating partial seizures in a 10-month-old infant in Iowa, using pure CBD as a therapeutic approach (Saade and Joshi 2015). Moreover, the most important advance in this research and development direction is likely the recent introduction of Epidiolex by GW Pharmaceuticals. This drug is also purified from cannabis extracts and contains about 98 % CBD and only trace amounts of other cannabinoids. Epidiolex is now approved as an investigational drug, and more than 300 pediatric patients with Dravet syndrome and Lennox–Gastaut syndrome were treated with this substance in a multicenter clinical trial. The very first results are highly encouraging, as reported on the 13th of April, 2015 at the 67th Annual Meeting of the American Academy of Neurology (Devinsky et al. 2015). In an open-label study, data were obtained from 213 patients with treatment-resistant epilepsies, who received Epidiolex liquid at a dose of up to 25 mg/kg/day for at least 12 weeks as an adjunctive therapy. On average, the data show ~50 % decrease in convulsive seizure frequency, and only 4 % of the patients discontinued treatment because of adverse effects (Devinsky et al. 2015). Although the actual proportion of responders was not available at the time of writing, these promising data suggest that conducting double-blind, placebo-controlled trials will be an important next step to fully establish the efficacy and safety of Epidiolex.

Finally, it is very important to mention that preclinical research indicates that other phytocannabinoid molecules besides THC and CBD may also hold therapeutic potential as anticonvulsant agents (for review see Hill et al. 2012a). Both in vitro and in vivo evidence, including chemical (e.g., the pentylenetetrazol and pilocarpine models) and electrical seizure paradigms (e.g., the audiogenic seizure model), converge on the notion that the two most compelling candidate cannabinoids are cannabidivarin and Δ^9-tetrahydrocannabivarin (Hill et al. 2010, 2012b, 2013; Amada et al. 2013; Bialer et al. 2015). These compounds are therefore also likely to be tested in human clinical experiments in the near future.

In conclusion, ample evidence suggests that cannabis and purified cannabinoid-based medicines may be beneficial in several patient populations, but may not be effective in other significantly large patient populations, and can even aggravate epileptic seizures in some cases. This highlights that further research is required to understand the neurobiological substrates of the beneficial and the adverse effects of cannabinoids in seizure disorders (detailed in Sect. 4). As the latest Cochrane review states “No reliable conclusion can be drawn at present regarding the efficacy of cannabinoids as a treatment for epilepsy. Further trials are needed” (Gloss and Vickrey 2014). This is in agreement with the view of the American Epilepsy Society which has stated that “at present, the epilepsy community does not know if marijuana is safe and effective treatment, nor do they know the long-term effects that marijuana will have on learning, memory, and behavior, especially in infants and young children” and has urged the DEA “to change marijuana from its current Schedule I status to allow researchers to conduct studies more efficiently”

(Benbadis et al. 2014). By helping intensified on-going research efforts and with new preclinical and clinical data appearing at a rapid pace, it is very likely that more solid information will soon emerge about how cannabis or, more likely, how cannabinoid-based medicines could be used in epilepsy treatment in the future.

4 Endocannabinoid Signaling in Epilepsy

The most important aim of neuroscience research is to provide insights into the neurobiological mechanisms intrinsic to brain function, and it is these processes that underlie the surprising complexity of the effects of cannabis preparations on human epilepsy. Elucidating these mechanisms will greatly facilitate the establishment of protocols determining which cannabinoid molecules, and at what dose, should be used in a given patient population with a certain form of epilepsy. The operational principles of endocannabinoid signaling and its complex cell-type-specific relationship to the regulation of network excitability are fairly well known and have been extensively summarized in excellent reviews (Hofmann and Frazier 2013; Alger 2014; Blair et al. 2015; Soltesz et al. 2015). It is expected that detailed investigations of other recently recognized, pharmacologically related messenger systems (e.g., GPR55 signaling: see Ryberg et al. 2007; Henstridge et al. 2009; Sylantyev et al. 2013) may also enhance our understanding of how cannabinoid-based medicines could be applied in epilepsy treatments.

Three principal reasons can be outlined to account for the divergent patient responses to cannabinoid preparations: chemical variability, biological variability, and pathological variability. First, and foremost, the absence of legally accepted cannabinoid-based medicines and proper clinical trials of their efficacy in seizure disorders renders it very difficult to monitor how the qualitative and quantitative chemical variability in different medical marijuana preparations affects patients. However, the emerging evidence clearly points to CBD as a highly important candidate underlying some of the beneficial effects of cannabis in epilepsy. In addition, low concentrations of THC are, most likely, usually anticonvulsant, whereas high doses may exacerbate seizures. Secondly, biological variability arises from both the polypharmacology of CBD and the contrasting effects of THC at CB_1 receptors located on different network elements. CBD effects are not confined to a single receptor, and thus may involve several biological targets, probably positioned on different cellular network elements at distinct subcellular domains. In contrast, THC primarily, though not exclusively, acts via presynaptic CB_1 receptors on axon terminals. However, CB_1 is present on both glutamatergic excitatory nerve endings, where it is likely to attenuate excess network excitability by reducing glutamate release, as well as on GABAergic inhibitory boutons, where its activation reduces GABA release and is therefore likely to have a proconvulsant effect. Thirdly, there are more than 40 different major forms of epilepsies with fundamentally distinct etiologies, and all of these complex genetic or acquired epilepsies may be associated with distinct cellular adaptations in brain circuits. Certain cell types

are lost during epileptogenesis; others have reduced axonal arbors, whereas sprouting of axon terminals characterizes additional cell types, all leading to a perturbed ratio of excitatory to inhibitory synapses in epileptic networks. Among the numerous molecular adaptations, epilepsy-relevant pathological plasticity of endocannabinoid signaling has been well studied in the last decade. In general, this further decreases seizure threshold via the disappearance of protective CB_1 receptors from excitatory axon terminals and the increased expression levels of proconvulsant CB_1 receptors on GABAergic boutons.

5 Potential Neurobiological Mechanisms of the Antiepileptic Effects of Cannabidiol

Considering the promising results of the first open-label clinical study (Devinsky et al. 2015), and the convincing evidence from preclinical experiments (Hill et al. 2012a), one of the most burning questions is what is the neurobiological substrate through which CBD exerts its striking anticonvulsant effects? CBD has only millimolar affinity for CB_1 receptors (Bisogno et al. 2001), but it may behave as a CB_1 antagonist, and it also bears surprisingly high potency antagonist/inverse agonist effects at CB_2 (Thomas et al. 2007). However, these properties are unlikely to explain the antiepileptic effects of CBD, as CB_1 activation is primarily protective against seizures and CB_1 antagonists exhibit proconvulsant effects (Blair et al. 2015). In this respect, further studies are required as some reports suggest that the anticonvulsant potential of CBD is CB_1-independent (Fig. 1) (Wallace et al. 2001; Jones et al. 2010; Hill et al. 2013), while others demonstrate CB_1-mediated effects. For example, Ledgerwood et al. describe reduced excitatory synaptic transmission in hippocampal slices treated with CBD, which was blocked by the CB_1 receptor antagonist AM251 (Ledgerwood et al. 2011). This raises the possibility that CBD may produce its beneficial effects in part via the same mechanism as low-dose THC.

In addition, several alternative molecular targets for CBD have been proposed. For example, CBD acts as a ligand of the serotonin receptor $5HT_{1A}$ (Russo et al. 2005; Magen et al. 2010) and may also reduce glutamatergic neurotransmission through this pathway. Indeed, the $5HT_{1A}$ receptor antagonist WAY100135 has been shown to reduce the inhibitory effect of CBD on hippocampal glutamatergic transmission (Ledgerwood et al. 2011). This is also entirely consistent with finding that $5HT_{1A}$ knockout mice show increased seizure activity (Sarnyai et al. 2000). A second possibility is that CBD as an adenosine uptake inhibitor (Carrier et al. 2006) may elevate endogenous adenosine levels, which activate presynaptic A_1 receptors and thereby reduce glutamate release and ultimately epileptic seizures (Dunwiddie and Masino 2001; Vianna et al. 2005; Rosim et al. 2011). On the other hand, CBD effects on adenosine have been mainly linked to facilitation of A_{2A} receptor activity (Liou et al. 2008; Magen et al. 2009; Ribeiro et al. 2012), which is generally considered a proconvulsive process (Rosim et al. 2011). Intriguingly, a very exciting interaction of elevated endogenous adenosine tone with synaptic

endocannabinoid signaling was recently reported by Hoffman and colleagues (Hoffman et al. 2010). The authors showed that increased endogenous adenosine tone via presynaptic A_1 receptor activation reduced the effects of the CB_1 receptor agonists WIN55,212-2 and THC on excitatory glutamatergic transmission in the hippocampus (Hoffman et al. 2010). On the one hand, the CBD-induced increase in endogenous adenosine tone may counteract the effects of THC on glutamatergic axon terminals and may have important relevance in the psychotic and antipsychotic actions of THC and CBD, respectively (Iseger and Bossong 2015). On the other hand, given that THC exerts its anticonvulsant effect via presynaptic CB_1 receptors on glutamatergic axon terminals, CBD should counteract the antiepileptic effects of THC, which does not seem to be the case. Another group of potential molecular targets of CBD, TRP channels, such as $TRPV_1$ have also been proposed as potential mediators of the antiepileptic effects of CBD (De Petrocellis and Di Marzo 2010). Although the precise role of $TRPV_1$ in the regulation of network excitability has not yet been fully elucidated, and may depend on the experimental paradigm (Manna and Umathe 2012; Kong et al. 2014; Jia et al. 2015; von Rüden et al. 2015a), it has been suggested that $TRPV_1$ is phosphorylated by increased neuronal activity and that the main function of CBD (and also cannabidivarin) is to trigger $TRPV_1$ dephosphorylation, thereby desensitizing the ion channel and thus reducing excitability to produce an antiepileptic effect (Iannotti et al. 2014). Finally, perhaps one of the most exciting recent development is the potential antagonistic effect of CBD on the recently characterized G-protein-coupled receptor GPR55 (Ryberg et al. 2007). Notably, GPR55 was recently found to be localized on excitatory axon terminals, where it facilitates glutamate release in an activity-dependent manner by triggering calcium release from boutonal intracellular calcium stores (Sylantyev et al. 2013). Because CBD efficiently blocks GPR55 activation in this experimental paradigm (Fig. 1) (Sylantyev et al. 2013), antagonists of this new receptor represent ideal candidates to be tested in the future for their anticonvulsant potential. Abnormal high frequency activity of excitatory neurons during epileptic seizures is very likely to recruit this endogenous calcium-releasing pathway, which also involves lysophosphatidylinositol signaling (Henstridge et al. 2009). Thus, in this scenario, CBD may selectively dampen excess presynaptic glutamate release from only the hyperactive excitatory neurons during epileptic seizures. Importantly, this mode of action does not require the endogenous activity of a signaling pathway under basal conditions, which may also contribute to the general safety of GPR55 antagonists, potentially including CBD, in the treatment of epilepsy. Since CBD effectively reduced epileptic electrical activity and decreased seizure-associated mortality within a wide dose range and in several chemical and electrical epilepsy paradigms (Jones et al. 2010, 2012), it is conceivable that there is a primary molecular target and a specific physiological mechanism underlying its antiepileptic effects. However, this argument certainly does not exclude the additive adjunct role of other molecular players.

6 Cell-Type-Specific Anticonvulsant and Proconvulsant Endocannabinoid Signaling

Interestingly, accumulating evidence indicates that low-dose THC also exerts its beneficial antiepileptic effects via toning down excess glutamatergic neurotransmission. In contrast, the proconvulsant effects of high THC dose may be mediated by the CB_1 receptors located at GABAergic synapses. Beat Lutz and his colleagues developed a series of elegant cell-type-specific CB_1 knockout models, and using these invaluable tools they recently provided direct evidence for this notion. They have shown that the behavioral effects of cannabinoids at low doses are mediated by CB_1 receptors present on excitatory axon terminals, but that the opposite behavioral reactions can be triggered by the activation of presynaptic CB_1 on GABAergic boutons (Rey et al. 2012). In accordance with these findings, and with special significance to the young developing brain, it was demonstrated that application of a low-dose of a CB_1 receptor agonist has a strong diminishing effect on seizure scores and seizure-related EEG activity (Rudenko et al. 2012). Conversely, administration of a high dose of the same agonist is proconvulsant (Rudenko et al. 2012). At first, this seems to be paradoxical in the light of sheer protein numbers, i.e., the density of CB_1 receptors on GABAergic terminals is much higher than on glutamatergic axons (Kawamura et al. 2006). However, indirect evidence indicates that a significant population of the CB_1 proteins on GABAergic terminals may represent a reservoir pool (Dudok et al. 2015). In addition, CB_1-positive interneurons only represent 13.9 % of all GABAergic interneurons (Bezaire and Soltesz 2013), which make up only about 10 % of all hippocampal neurons (Jinno and Kosaka 2006). Thus, assuming roughly equal efferent synapse numbers, there are approximately 50–100 times more CB_1-sensitive excitatory synapses than CB_1-sensitive GABAergic synapses in cortical networks. Finally, Steindel and colleagues revealed that coupling of CB_1 receptors to their downstream G-protein signaling pathway is several fold more efficient on excitatory axon terminals (Steindel et al. 2013).

The above data are in general agreement with several earlier pharmacological and genetic findings from preclinical experiments. First of all, it is now well accepted that the classical tetrad effects of THC are all mediated by the population of CB_1 receptors that are located on glutamatergic neurons (Monory et al. 2007), and this receptor pool also mediates the general neuroprotective effects of endocannabinoid signaling (Chiarlone et al. 2014). Moreover, administration of low doses of THC, application of endocannabinoids, or administration of synthetic cannabimimetics are all anticonvulsant in vitro and in vivo, and these effects can be blocked by CB_1 receptor antagonists (Wallace et al. 2001, 2003; Blair et al. 2006; Deshpande et al. 2007a; Rudenko et al. 2012). In contrast, when applied alone, CB_1 antagonists have usually been shown to increase seizure duration and frequency in several chemical and electrical epilepsy paradigms (Wallace et al. 2003; Deshpande et al. 2007b; Vinogradova et al. 2011; Arslan et al. 2014; Kow et al. 2014) and can even trigger unprovoked cryptogenic seizures in healthy, non-epileptic rats (van Rijn et al. 2011). Moreover, both global CB_1 knockout mice (i.e., CB_1 is deleted

from all cell types) and glutamatergic neuron-specific CB_1 knockout mice exhibit increased seizure severity in several epilepsy models (Marsicano et al. 2003; Monory et al. 2006; Kow et al. 2014; von Rüden et al. 2015a). Perhaps the most robust evidence supporting the critical role of these CB_1 receptors in setting seizure threshold derives from a sophisticated rescue mouse model, in which CB_1 receptors were selectively reintroduced into glutamatergic neurons in global CB_1 knockout mice (Ruehle et al. 2013). Thus, these mice express CB_1 receptors only on excitatory axon terminals, but this is sufficient to rescue the kainic acid-induced epileptic phenotype of the global CB_1 knockout mice (Ruehle et al. 2013). Interestingly, this pool of CB_1 receptors may have both chronic and acute protective functions. Selectively deleting CB_1 from glutamatergic neurons in mice fairly early during cortical development produces a highly increased density of excitatory synapses and reduces the threshold for long-term synaptic potentiation (Monory et al. 2015). On the other hand, acute adeno-associated virus-induced overexpression of CB_1 receptors in glutamatergic neurons significantly attenuates seizure severity and mortality (Guggenhuber et al. 2010). While these data unequivocally demonstrate that CB_1 receptors on glutamatergic axon terminals play a fundamental role in protection against epileptic seizures, it is not entirely clear how CB_1 receptors on the nearby GABAergic axon terminals may be recruited during excess network excitability. It may depend on the specific epilepsy model and may vary among patients with distinct etiologies, because selective deletion of CB_1 from GABAergic interneurons does not alter susceptibility in the kainic acid model (Monory et al. 2006), but shortens seizure duration in the kindling model of temporal lobe epilepsy (von Rüden et al. 2015a).

7 The Synaptic Circuit-Breaker Model

To explain the molecular mechanisms of endocannabinoid signaling at excitatory synapses, and their critical importance in the regulation of network excitability, we proposed the "synaptic circuit-breaker" model a few years ago (Fig. 2) (Katona and Freund 2008). This model stems from the original observation of Peter Somogyi, who discovered that postsynaptic glutamate receptors are not uniformly distributed within synapses (Somogyi et al. 1998). While ionotropic glutamate receptors are localized intrasynaptically, group I metabotropic glutamate receptors (mGluRs) such as $mGlu_1$ and $mGlu_5$ accumulate at the edge of synapses in a peculiar perisynaptic position (Baude et al. 1993; Lujan et al. 1996). This striking anatomical location suggests that perisynaptic mGluRs are located in an ideal position to oppose increased synaptic glutamate release and are only activated when glutamate is "spilled over." When presynaptic activity reaches a certain threshold, mGluR activation further facilitates basal neurotransmission by feed-forward excitation involving TRPC channels (Kim et al. 2003; Hartmann et al. 2008). However, when this excitatory signaling pathway overshoots, e.g., during epileptic seizures, then the synapse and the circuit in general must have a protective feed-forward as well as a negative feedback mechanism to defend itself

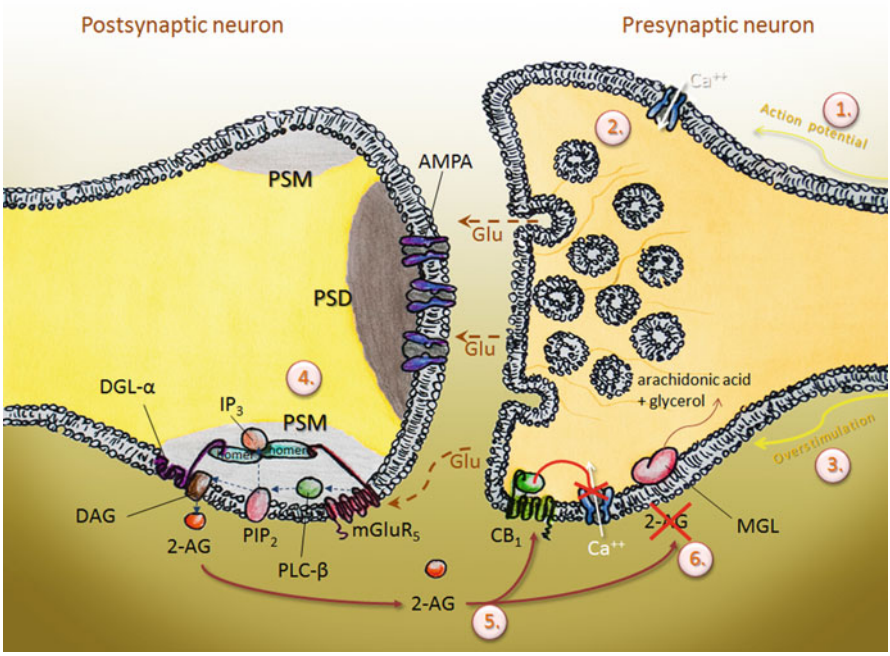

Fig. 2 The synaptic circuit-breaker model. Under basal conditions, a single action potential (*1*) triggers the opening of voltage-gated calcium channels, and the resulting calcium transient evokes glutamate release from synaptic vesicles into the synaptic cleft (*2*). The released glutamate primarily activates intrasynaptically located ionotropic AMPA-type glutamate receptors. In presynaptic hyperactivity, such as may occur during epileptic seizures (*3*), an exceedingly high concentration of glutamate spills over from the synaptic cleft and reaches perisynaptically located metabotropic glutamate receptors such as $mGluR_5$. This event triggers $G_{q/11}$ signaling and then PLC-β activity (*4*), both of which are also located at the perisynaptic zone and are integrated into a large macromolecular protein complex called the perisynaptic machinery (*PSM*) adjacent to the intrasynaptic postsynaptic density (*PSD*), a dense protein matrix at the postsynaptic membrane that contains ionotropic receptors. PLC-β splits phosphatidylinositol biphosphate (PIP_2) into inositol trisphosphate (IP_3) and DAG, and when a larger amount of DAG is produced, DGL-α, another perisynaptically accumulated enzyme, steps in and converts DAG to 2-AG. This endocannabinoid then travels backwards through the synapse (*5*) and mediates feedback inhibition of glutamate release via the activation of presynaptic CB_1 cannabinoid receptors and the subsequent closure of voltage-gated calcium channels (*6*). The whole process is tightly regulated by MGL, which inactivates 2-AG by catalyzing its hydrolysis into arachidonic acid and glycerol. The model was adapted from Katona and Freund (2008). The artwork is a courtesy of Mr. Benjamin Barti

(see in detail in Katona and Freund 2012). Therefore, the anatomical findings that all molecular components downstream of group I mGluR signaling are accumulated perisynaptically, as well as the observation that genetic deletion of these proteins always led to an epileptic phenotype, highlight the significance of an intact "synaptic circuit-breaker" system involving endocannabinoid signaling at excitatory synapses in epilepsy (Katona and Freund 2008). In short, it was discovered that

the G protein subunits G_q and G_{11}, which are necessary for group I mGluR signaling, are also distributed perisynaptically (Tanaka et al. 2000). Moreover, double G_q/G_{11} knockout mice exhibit impaired endocannabinoid production and are susceptible to epilepsy (Wettschureck et al. 2006). The next important protein in this signaling pathway is phospholipase β1 ($PLC\beta_1$), which is also found in a perisynaptic ring around excitatory synapses (Fukaya et al. 2008), and notably, $PLC\beta_1$-knockout mice also die from spontaneous epileptic seizures at a young age (Kim et al. 1997). A product of $PLC\beta_1$ is diacylglycerol (DAG), which is the precursor for the synthesis of the endocannabinoid molecule 2-arachidonoylglycerol (2-AG), a step catalyzed by diacylglycerol-lipase-α (DGL-α) (Bisogno et al. 2003). In agreement with the proposed model, DGL-α was found to be located within the perisynaptic machinery at excitatory synapses in the spinal cord, the hippocampus, and the neocortex of rodents (Nyilas et al. 2009; Katona et al. 2006; Yoshida et al. 2006; Lafourcade et al. 2007), as well as in the human brain (Ludanyi et al. 2011). Furthermore, recent data suggest that DGL-α knockout mice are especially sensitive to neuronal insults leading to epileptic seizures (Sugaya et al. 2014). This perisynaptic machinery is anchored together by the scaffold protein Homer (Katona and Freund 2008). In accordance with the prediction of the "synaptic circuit-breaker" model, an epileptic stimulus uncouples the perisynaptic machinery via increased Homer1a expression and disrupts mGluR-induced endocannabinoid signaling at excitatory synapses (Li et al. 2012). An independent line of evidence recently emerged from studies of fmrp knockout mice, a model of Fragile X syndrome, in which patients frequently have seizures. In these mice, the perisynaptic machinery is dismantled, likely due to impaired Homer and DGL-α subcellular targeting (Jung et al. 2012; Ronesi et al. 2012). As a result, group I mGluR-induced endocannabinoid mobilization and retrograde synaptic signaling are absent in the excitatory synapses of these mice (Jung et al. 2012; Tang and Alger 2015).

DGL-α may have a dual function, first reducing excess feed-forward signaling via termination of DAG signaling at TRPC channels, but also initiating negative feedback signaling mediated via its product 2-AG. This endocannabinoid travels back across excitatory synapses, activating presynaptic CB_1 receptors located on glutamatergic axon terminals to inhibit glutamate release in rodents (Katona et al. 2006; Kawamura et al. 2006) and also in the human hippocampus (Ludanyi et al. 2008). Accordingly, it has also been demonstrated that epileptiform activity induced by group I mGluR activation can be blocked by CB_1 receptor antagonism (Karr et al. 2010). A key control point in this pathway is the threshold for recruitment of mGluR-induced endocannabinoid signaling, and this may be dependent on the cell type. Importantly, excitatory neurons exhibit much higher DGL-α levels, and concomitantly a much lower threshold to trigger endocannabinoid signaling, than GABAergic interneurons (Peterfi et al. 2012), and neuronal insults triggering epileptic seizures consistently elevate 2-AG levels in many epilepsy paradigms (Wallace et al. 2003; Wettschureck et al. 2006; Fezza et al. 2014). After activation of presynaptic CB_1 receptors, 2-AG is inactivated by monoacylglycerol lipase (MGL) (Dinh et al. 2002), which is also a ubiquitous protein in

excitatory axon terminals throughout the rodent and human central nervous systems (Gulyas et al. 2004; Ludanyi et al. 2011; Uchigashima et al. 2011; Horvath et al. 2014). Thus, in principle, MGL inhibitors should have an anticonvulsant effect by increasing the lifetime of the protective 2-AG messenger. In fact, three independent recent studies have confirmed this prediction in chemical and electrical epilepsy paradigms (Fezza et al. 2014; Griebel et al. 2015; von Rüden et al. 2015b). Notably, von Rüden and colleagues even demonstrated that MGL inhibitors delay the development of generalized seizures and decrease seizure duration in wild-type, but not in glutamatergic neuron-specific CB_1 knockout mice (von Rüden et al. 2015b). These data further highlight the importance of intact endocannabinoid signaling at excitatory synapses as a "synaptic circuit-breaker" in the protection of brain circuits against neuronal insults (Katona and Freund 2008).

8 Pathophysiological Changes in Endocannabinoid Signaling in the Epileptic Brain

The critical role of the endocannabinoid system in setting the threshold for network excitability implies that it is crucial to characterize the cell-type-specific pathophysiological plasticity and reorganization of endocannabinoid signaling in epileptic brain circuits. Ample evidence shows strikingly different directions of change at both regional and cell-type-specific levels (Falenski et al. 2007, 2009; Bhaskaran and Smith 2010; van Rijn et al. 2010), which may reflect the opposing function of CB_1 receptors on excitatory and inhibitory terminals (von Rüden et al. 2015a). Abnormal endocannabinoid signaling may reflect either a molecular perturbation or may represent a molecular adaptation. In other words, changes in synaptic endocannabinoid signaling may causally contribute to increased seizure frequency and severity or, alternatively, may be the pathological consequence of epilepsy. In brief, emerging data support the concept that endocannabinoid signaling is first impaired in the epileptic brain as a consequence of seizures, but that these molecular changes may further reduce seizure threshold and thus have a proconvulsant effect. In their state-of-the art study, Goffin and colleagues monitored functional CB_1 receptor availability in the living human brain in both control subjects and patients with mesial temporal lobe epilepsy, via [^{18}F]MK-9470 radioligand binding and positron emission tomography (Goffin et al. 2011). Although the spatial resolution of this technology is insufficient to distinguish between different axon terminal types, it is likely that the majority of the labeling visualizes CB_1 on GABAergic boutons, as this receptor population represents ~90 % of all CB_1 receptors in forebrain areas (Steindel et al. 2013). Two important conclusions can be drawn from the observations of Goffin and colleagues, with the note of caution that antiepileptic treatments such as valproate can also alter CB_1 levels (Goffin et al. 2008). First, CB_1 receptors can be both up- and downregulated in a brain region-specific manner (Goffin et al. 2011). In particular, a massive decrease in CB_1 availability was observed in the superior insular cortex, whereas an upregulation was detected in the ipsilateral temporal lobe (Goffin et al. 2011). Second, the

magnitude of changes positively correlated with the number of seizures in the month before scanning, indicating a strong acute effect of abnormal epileptic activity on receptor number. This finding is nicely paralleled in another thorough anatomical study, which followed changes in CB_1 receptor number on GABAergic axon terminals using immunohistochemistry and the pilocarpine-induced epilepsy model of temporal lobe epilepsy (Karlocai et al. 2011). Notably, the extent of molecular alterations mirrored the severity of seizures. Only those animals which underwent strong tonic-clonic seizures and later developed sclerosis showed changes in CB_1 receptor density (Karlocai et al. 2011). Perhaps the most remarkable observation of Karlócai and coworkers was that, 2 h after pilocarpine-induced status epilepticus, CB_1 had almost completely disappeared from GABAergic axon terminals (Karlocai et al. 2011). A potential explanation for this observation is that epileptic seizures trigger massive endocannabinoid release (Wallace et al. 2003; Wettschureck et al. 2006; Fezza et al. 2014), and high ligand concentration results in robust receptor downregulation on GABAergic axon terminals (Dudok et al. 2015). One may speculate that the seemingly paradoxical prophylactic effect of CB_1 receptor antagonists may also be related to this phenomenon by counteracting excess activation and internalization of presynaptic CB_1 receptors. As discovered by the Soltesz lab, the administration of the otherwise proconvulsant CB_1 antagonist rimonabant prevented epileptogenesis in both febrile seizures and in a traumatic brain injury model of epilepsy (Chen et al. 2007; Echegoyen et al. 2009; for review see Armstrong et al. 2009). This finding may have special significance in the developing brain, though its significance in the adult brain is less clear (Dudek et al. 2010; Di Maio et al. 2015). One important future task will be to establish whether blockade by rimonabant of CB_1 receptors on GABAergic axon terminals immediately after the neuronal insult may counteract the degradation of these axon terminals themselves (Wyeth et al. 2010, 2012; Sun et al. 2014). The loss of GABAergic inhibitory terminals may have deleterious consequences on network excitability by shifting the excitatory/inhibitory balance. Furthermore, the remaining GABAergic terminals contain elevated levels of CB_1 receptors following the latent period of epileptogenesis, as observed in both human samples and in animal models (Fig. 3) (Chen et al. 2003; Falenski et al. 2009; Magloczky et al. 2010; Karlocai et al. 2011), which then leads to a further decrease in GABAergic inhibition (Chen et al. 2003, 2007). Interestingly, a similar upregulation of CB_1 receptors was also noted on normally CB_1-immunonegative astrocytes in the pilocarpine model (Meng et al. 2014). The appearance of CB_1 on astrocytes has also been observed in sclerotic hippocampi derived from human patients with temporal lobe epilepsy and may have a proconvulsant effect on the epileptic brain (Coiret et al. 2012.Meng et al. 2014). While these molecular changes are likely to further aggravate epileptic seizures, recent data demonstrate that CB_1

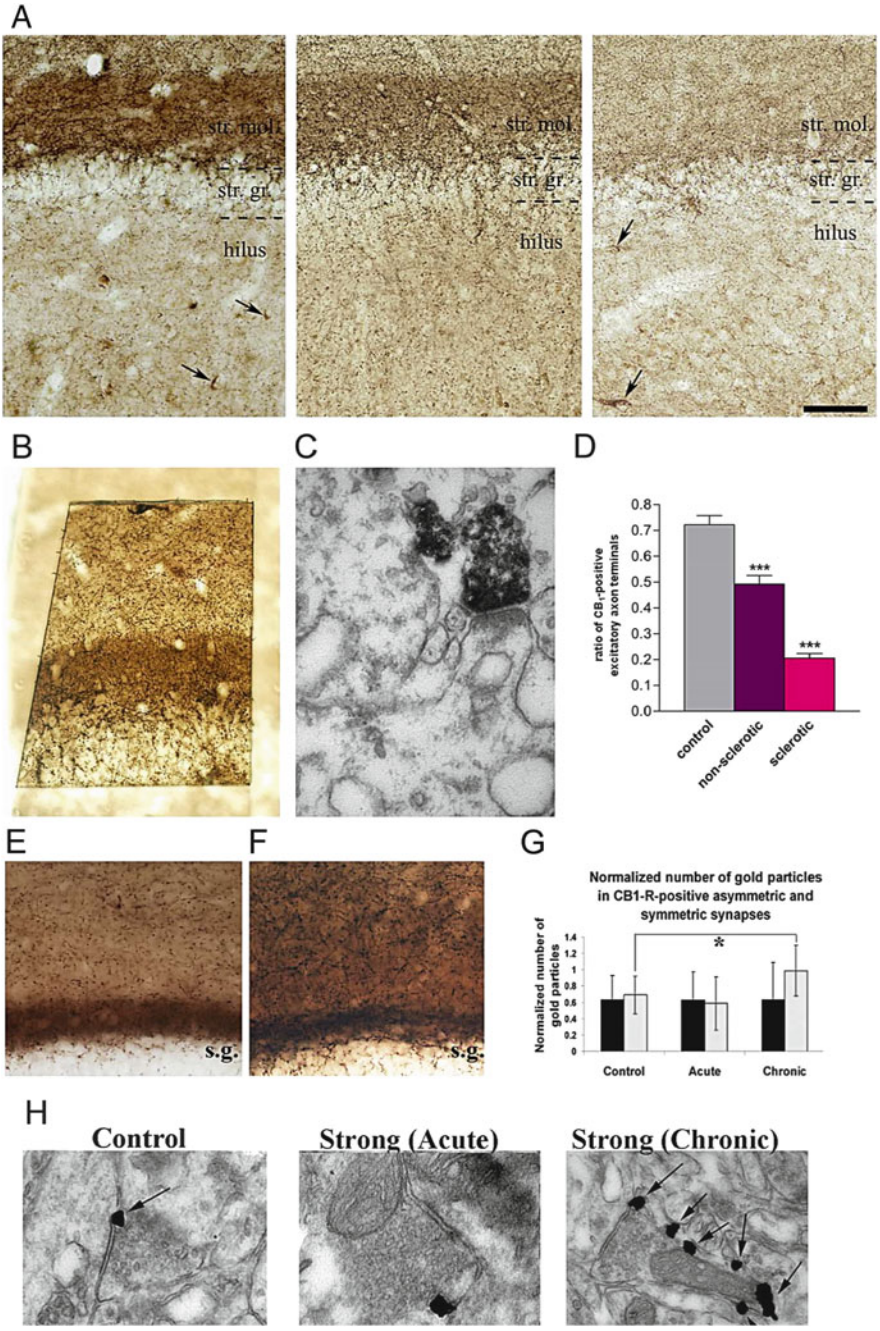

Fig. 3 Downregulation of the anticonvulsant CB_1 receptors at excitatory synapses and upregulation of proconvulsant CB_1 receptors at inhibitory synapses in the epileptic hippocampus. (**a**) Massive downregulation of CB_1 cannabinoid receptors was found in the dentate gyrus of patients with temporal lobe epilepsy (Ludanyi et al. 2008). *Left* panel shows dense CB_1-positive axon staining especially in stratum moleculare (str. mol.) in age-matched human control samples,

receptor expression on excitatory axon terminals and the "synaptic circuit-breaker" is also reduced in the epileptic human brain. Both DGL-α and CB_1 receptor expression levels are diminished at the mRNA level, and CB_1 receptors are largely absent from excitatory axon terminals at the protein level (Fig. 3). These data indicate the loss of the important negative feedback signaling pathway targeted by 2-AG at excitatory synapses in the hippocampus of patients with temporal lobe epilepsy (Ludanyi et al. 2008).

9 Conclusions

In summary, our knowledge of the molecular organization of endocannabinoid signaling and its critical physiological importance in regulating synaptic transmission and network excitability has significantly improved in the last decade. Moreover, it is becoming clear that there is a strong pathophysiological reorganization of this important messenger system in epileptic brain circuits. Further work should be aimed at characterizing these molecular changes and their effects on synaptic plasticity and seizures in greater detail at the cell-type-specific level. Such studies should also distinguish between the acute, latent, and chronic periods of epileptogenesis to better predict when and how molecular interventions may succeed as a prophylactic approach to epilepsy treatment (Armstrong et al. 2009; Di Maio et al. 2015). At the same time, more efficient symptomatic treatments of genetic and acquired epilepsies, with reduced side effect profiles, could emerge from a better understanding of the neurobiological mechanisms through which selected chemical constituents of the cannabis plant act on the epileptic brain (Hill et al. 2012b).

Fig. 3 (continued) which is reduced in samples from non-sclerotic epileptic patients (*middle panel*), and disappears in sclerotic patients (*right panel*). Scale bar is 100 μm. Str. gr. stands for stratum granulosum. (**b**) The same samples were re-embedded for electron microscopy and (**c**) excitatory synapses forming asymmetrical connections were analyzed for their CB_1 content (black endproduct of immunoreaction). (**d**) Notably, the loss of immunostaining at the light microscopic level in **a** is explained by the robustly reduced ratio of CB_1-containing excitatory synapses indicating that an important molecular component of the "synaptic circuit-breaker" is absent in epileptic patients. (**e**–**f**) In striking contrast, strong upregulation of CB_1 receptor levels was found on GABAergic axon terminals in the pilocarpine model of temporal lobe epilepsy (Karlocai et al. 2011). These mice exhibited increased CB_1-immunostaining throughout the dentate gyrus, which was due to an increased number of CB_1 receptors on GABAergic axon terminals. (**g**) In chronically epileptic animals, which showed consistent seizure activity (labeled as strong chronic), but not after status epilepticus (strong acute), the increased protein number was demonstrated by immunogold visualization of CB_1 receptors at symmetrical inhibitory synapses and by a thorough quantitative electron microscopic analysis (in **h**). *Scale bar* is 200 nm. The individual figures have been modified from the originals with permission from the authors.

Acknowledgements This work was supported by the Hungarian Academy of Sciences Momentum Program LP-54/2013 and by the Wellcome Trust International Senior Research Fellowship. The author is very grateful to Dr. S. Woodhams for his critical comments on the manuscript; to Mr. B. Barti for his help with artwork; to Profs. R. DeLorenzo, R. Karlócai, D. Rusakov, G. Stephens for permission to modify figures from their original work; and to Profs. T. Freund, Z. Maglóczky, and I. Soltesz for their generous support and advice on his research relevant to the topic of this review over the years.

Conflict of Interest

The author declares no conflict of interest.

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Endocannabinoids and the Endocrine System in Health and Disease

Cecilia J. Hillard

Contents

Abstract

Some of the earliest reports of the effects of cannabis consumption on humans were related to endocrine system changes. In this review, the effects of cannabinoids and the role of the CB1 cannabinoid receptor in the regulation of the following endocrine systems are discussed: the hypothalamic–pituitary–gonadal axis, prolactin and oxytocin, thyroid hormone and growth hormone, and the hypothalamic–pituitary–adrenal axis. Preclinical and human study results are presented.

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_11

Keywords
Corticosterone • Gonadotropin • Gonadotropin-releasing hormone • Growth hormone • HPA axis • Oxytocin • Prolactin • Testosterone • Thyroid hormone

Abbreviations

2-AG	2-Arachidonoylglycerol
ACTH	Adrenocorticotropic hormone
AEA	*N*-arachidonoylethanolamine
BLA	Basolateral amygdala
CB_1R	Type 1 cannabinoid receptor
CB_2R	Type 2 cannabinoid receptor
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DAG	Diacylglycerol
eCB	Endocannabinoid
ECS	Endocannabinoid signaling
FAAH	Fatty acid amide hydrolase
FGR	Fetal growth restriction
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
HPA	Hypothalamic–pituitary–adrenal
HPG	Hypothalamic–pituitary–gonadal
HPT	Hypothalamic–pituitary–thyroid
i.c.v.	Intracerebroventricular
LH	Luteinizing hormone
MGL	Monoacylglycerol lipase
MR	Mineralocorticoid receptor
OVX	Ovariectomized
OXT	Oxytocin
PLC	Phospholipase C
PVN	Periventricular nucleus
SON	Supraoptic nucleus
THC	Δ^9-Tetrahydrocannabinol
TRH	Thyrotropin-releasing hormone
TRß1	ß1 subtype of the thyroid hormone receptor
TSH	Thyroid-stimulating hormone
VTA	Ventral tegmental area

1 Introduction

Endocannabinoid signaling (ECS) plays a wide variety of modulatory roles throughout the central nervous system (CNS). The endocannabinoid system consists of two G protein-coupled receptors, CB1 receptor (CB_1R) and CB2 receptor (CB_2R), the vanilloid subtype of transient potential receptor, and members of the peroxisome proliferator-activated receptor family. Two endocannabinoid (eCB) ligands have been identified: *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). Both are synthesized from phospholipid precursors in an "on demand" manner and are metabolized by hydrolysis. AEA is hydrolyzed by fatty acid amide hydrolase (FAAH), while 2-AG is hydrolyzed by monoacylglycerol lipase (MGL) and by alpha–beta hydrolase 6 (Marrs et al. 2010). Both AEA and 2-AG are also substrates for cyclooxygenase 2, which converts them to ethanolamide and glycerol-substituted prostaglandins, respectively (Hermanson et al. 2014).

Within the CNS, ECS mediates activity-dependent, retrograde signaling in many brain regions, including the hippocampus, prefrontal cortex, amygdala, and cerebellum (Freund et al. 2003). In most cases, 2-AG is mobilized in postsynaptic neurons by receptors that activate phospholipase C (PLC), including the metabotropic glutamate family of receptors. The diacylglycerol (DAG) that is produced is further metabolized by DAG lipase to monoacylglycerol, including 2-AG. 2-AG acts on presynaptic CB_1Rs to inhibit neurotransmitter release, through inhibition of the opening of voltage-operated calcium channels.

The CB_1R is also present outside the CNS, including adipose tissue, liver, and the adrenal gland. The CB_1R in adipose and liver promotes the storage of fat and reduces fat utilization (Silvestri et al. 2011). There is little known about the sources of the eCBs that innervate the non-CNS CB_1R. However, the eCBs are present in the circulation, and recent data indicate that the circulating concentrations of 2-AG are nearly 4 times higher at noon than at 4 am in healthy humans (Hanlon et al. 2014), leading to the hypothesis that circulating eCBs activate these receptors and thereby coordinate adipose and liver function with caloric intake.

Formulations of the cannabis plant have been used by humans for thousands of years for the treatment of a variety of conditions, including pain and spasticity (Kumar et al. 2001). Δ^9-Tetrahydrocannabinol (THC) is a direct agonist of the CB receptors and is responsible for these medicinal effects as well as the feeling of euphoria or "high" that is sought by those using cannabis recreationally. There are many other chemicals in the plant that also have beneficial effects, but whose mechanisms are not as well understood (Devinsky et al. 2014).

CB_1Rs are present in the hypothalamus at relatively low density compared to other brain regions (Herkenham et al. 1991); however, it is argued that this population of cannabinoid receptors is highly active, given the broad range of endocrine effects of the cannabinoids (Fernandez-Ruiz et al. 1997). Within the hypothalamus, the CB_1R protein is heterogeneously distributed (Wittmann et al. 2007). CB_1Rs are present on both symmetrical and nonsymmetrical synapses, and most immunoreactivity is in preterminal and terminal portions of axons. There

is sparse CB_1R distribution within the suprachiasmatic and lateral mammillary nuclei, but other regions of the hypothalamus express significant amounts of CB_1R.

Hypothalamic CB_1R density differs between male and female rodents (Rodriguez de Fonseca et al. 1994) and this likely reflects important sex-related endocrine differences as well as differences in cannabinoid effects between male and female animals and humans (Craft et al. 2013). CB_1R mRNA has been identified in the external zone of the median eminence (Wittmann et al. 2007; Herkenham et al. 1991), and CB_1Rs are expressed at low levels in the intermediate and anterior lobes of the pituitary gland (Pagotto et al. 2001).

2 Cannabinoid Interactions with the Hypothalamic–Pituitary–Gonadal Axis

The first step in the regulation of the hypothalamic–pituitary–gonadal (HPG) axis involves the peptide hormone, gonadotropin-releasing hormone (GnRH), which is produced by neurons in the preoptic area of the hypothalamus. GnRH secretion is pulsatile and it affects the release of two pituitary hormones through receptors in the anterior pituitary: low-frequency GnRH pulses induce the release of follicle-stimulating hormone (FSH), while high-frequency pulses induce luteinizing hormone (LH) release. In males, the frequency of GnRH release is constant, while in females, frequency increases significantly at the time of ovulation, resulting in a surge of LH. FSH and LH regulate follicular growth, ovulation, and maintenance of the corpus luteum in females and spermatogenesis in males.

CB_1R activation inhibits the release of GnRH through effects in the hypothalamus in male rats. Studies in isolated hypothalamic tissue demonstrated THC-induced suppression of simulated but not basal GnRH release (Rettori et al. 1990) and inhibition of pulsatile GnRH release by other CB_1R agonists (Gammon et al. 2005). Mediobasal GnRH was increased by intracerebroventricular (i.c.v.) THC treatment, data consistent with a reduction in GnRH release in the pituitary (Wenger et al. 1987). However, another study demonstrated reduced concentrations of GnRH in the preoptic area and mediobasal hypothalamus following in vivo THC treatment (Kumar and Chen 1983). It is possible that the discrepancy between these two studies is the result of different stimulus durations.

Cells adjacent to GnRH-secreting cells express CB_1R mRNA (Gammon et al. 2005) and a subset of neurons forming symmetrical synapses with GnRH neurons express CB_1R protein (Farkas et al. 2010). CB_1R activation inhibits gamma-aminobutyric acid (GABA) release onto GnRH neurons (Glanowska and Moenter 2011; Farkas et al. 2010), data in agreement with the predominant effect of CB_1Rs being suppression of neurotransmitter release (Freund et al. 2003). Reduced release of GABA is associated with increased excitatory drive, so these data seem to contradict the findings described above that CB_1R agonists inhibit GnRH release. However, GABA can depolarize GnRH neurons under some circumstances (Herbison and Moenter 2011), and thus, inhibition of GABA release could paradoxically decrease GnRH neuronal activation. Alternative mechanisms have been

suggested; for example AEA-induced inhibition of GnRH release evoked by NMDA in mediobasal hypothalamic fragments was blocked by both a CB_1R antagonist and bicuculline, suggesting an increase in GABA release (Fernandez-Solari et al. 2004). Other data suggest that THC acutely suppresses norepinephrine stimulation of GnRH release (Steger et al. 1990; Murphy et al. 1990). Chronic treatment of male mice with bhang (a cannabis preparation) results in reduced expression of receptors for GnRH in the pituitary (Banerjee et al. 2011). Thus, while the effect of activation is consistently depression of GnRH release, currently available evidence suggests that multiple mechanisms are involved.

Given the differences in patterns of release of GnRH between male and female, it is not surprising that sex steroid status profoundly affects CB_1R regulation of GnRH release (Scorticati et al. 2004). In particular, AEA had no effect on GnRH release in ovariectomized (OVX) rats and increased GnRH release in hypothalamic tissues from OVX rats in which estrogen is replaced (Scorticati et al. 2004). In agreement with findings obtained in experiments with other systems (Craft et al. 2013), it seems that estradiol is an important contributor to differences in response to CB_1R activation. An earlier study also found opposite effects of in vivo THC treatment on hypothalamic GnRH in male and OVX female rats (Kumar and Chen 1983).

In accord with the evidence that CB_1R activation suppresses GnRH release, many studies also find that THC decreases circulating LH concentrations in male rats (Marks 1973; Murphy et al. 1990), intact female mice (Dalterio et al. 1983a), OVX female rats (Tyrey 1978), and OVX female monkeys (Smith et al. 1979). The effect of THC in monkeys was reversed by the administration of GnRH (Asch et al. 1981), which is consistent with THC-induced suppression of hypothalamic GnRH release. AEA treatment reduced circulating concentrations of LH in wild-type but not $CB_1R^{-/-}$ mice (Wenger et al. 2001), data supporting the CB_1R as the site of action for THC- and other cannabinoid-induced suppression of LH release. Administration of THC by i.c.v. administration also decreases LH but not FSH release, support for a CNS site of action (Wenger et al. 1987).

It is well known that stress dysregulates the HPG axis, resulting in negative consequences on reproduction. Stress elevates hypothalamic endocannabinoid concentrations (Patel et al. 2004), likely through glucocorticoid receptor activation (Evanson et al. 2010), leading to the hypothesis that ECS mediates stress-induced inhibition of the HPG axis. In support of this hypothesis, recent data demonstrate that immobilization stress-induced decrease in LH release in male rats is reversed by CB_1R antagonist treatment (Karamikheirabad et al. 2013).

There is considerable evidence that systemically administered THC and other cannabinoid agonists suppress testosterone production and circulating concentrations in animal models (Dalterio et al. 1977; Jakubovic et al. 1979) and chronic exposure induces regression of testes (Dixit et al. 1977; Kumar and Chen 1983). These data are consistent with an ability of THC to suppress LH release, secondary to reduced GnRH. However, $CB_1R^{-/-}$ mice exhibit decreased circulating testosterone (Battista et al. 2008), which is at odds with the inverse effect of CB_1R activation and HPG activation outlined above. However, there is

evidence that CB_1Rs are also expressed in the testes and play a role in the postnatal differentiation and maturation of Leydig cells (Cacciola et al. 2008). Thus, the reduction of testosterone in $CB_1R^{-/-}$ mice could be the result of abnormal Leydig cell development. Components of the ECS are also present in Sertoli cells (Maccarrone et al. 2003) and THC inhibits FSH-induced signaling in Sertoli cell cultures (Heindel and Keith 1989). Thus, ECS can alter the responsivity to testosterone in addition to its production.

Cannabinoid-mediated dysregulation of HPG activity has been found to have consequences on female reproduction as well. THC treatment blocks ovulation and the LH surge in rats (Nir et al. 1973) and high doses of cannabis extract decrease progesterone concentrations during the luteal phase of mice (Kostellow et al. 1980). THC treatment of monkeys in the follicular phase decreases both ovulation and LH, FSH, and estrogen concentrations in the circulation (Asch et al. 1981). On the other hand, THC has been shown to facilitate sexual receptivity in female rats, possibly as a result of direct effects on the progesterone receptor (Mani et al. 2001).

In spite of consistent findings of changes in HPG function by ECS in both male and female preclinical models, data from humans using cannabis are far less consistent (see Gorzalka et al. 2010 for an excellent review). A recent meta-analysis of the effects of cannabis use on male fertility concluded that THC can have negative effects on male fertility (Fronczak et al. 2012), but epidemiological studies do not support this conclusion in the population at large (Hall and Solowij 1998). It is possible that tolerance or sensitization develops to the effects of THC on reproduction in humans (Gorzalka and Dang 2012). In a study in Korean males in which cannabis effects on the ratio of urinary testosterone/epitestosterone was examined, an eightfold suppression was detected in 30-year-old cannabis users (total number studied was 18), but not in other age groups (Moon et al. 2014). More to the point, 3.7 % of couples presenting to an infertility clinic in Italy were positive for cannabis, which exceeds the incidence of cannabis use in the overall population (Pichini et al. 2012). These studies suggest that cannabis use can contribute to infertility in some couples.

3 Interaction of Cannabinoids with Hormones of Lactation

3.1 Prolactin

Prolactin, a peptide hormone secreted from lactotrophs of the anterior pituitary, is essential for lactation and its release is promoted by suckling. Prolactin release is also evoked by copulation, ovulation, and eating, and it plays roles in a diverse number of physiological processes in addition to milk production, including sexual satisfaction, immune regulation, and hematopoiesis (Majumdar and Mangal 2013). Prolactin release is tonically inhibited by dopamine, released from tuberoinfundibular neurons and acting through D2 dopamine receptors (Majumdar and Mangal 2013).

Preclinical studies consistently demonstrate that CB_1R agonists reduce prolactin concentrations in the circulation through an effect upstream of the pituitary. Both intravenous (Hughes et al. 1981) and i.c.v. (Rettori et al. 1988) administration of THC produce long-lasting inhibition of prolactin release in male rats. This effect is shared by AEA and inhibited by the CB_1R antagonist, rimonabant (Fernandez-Ruiz et al. 1997). THC has no effect when the pituitary is removed from hypothalamic influence (Hughes et al. 1981) and does not affect prolactin release when incubated directly with dispersed pituitary cells (Hughes et al. 1981; Rettori et al. 1988), suggesting an effect in the hypothalamus or CNS. Data from nonhuman primates are in accord with the rat findings; in particular, THC suppresses prolactin basally but does not inhibit prolactin release induced by thyrotropin-releasing hormone (TRH) (Asch et al. 1979). There is evidence that cannabinoids can also regulate prolactin secretion through effects in the pituitary. For example, 2-AG was found to potentiate forskolin- and adenosine-induced prolactin secretion from cultured pituitary cells from Syrian hamsters in a CB_1R-dependent manner (Yasuo et al. 2014). CB_1R antagonist treatment does not affect prolactin concentrations in rats (Black et al. 2011), evidence that the CB_1Rs involved in regulating prolactin release are not tonically active.

Cannabinoids have been shown to increase the release of dopamine in several brain regions, including the hypothalamus (Rodriguez De Fonseca et al. 1992; Hao et al. 2000; Murillo-Rodriguez et al. 2007, 2011). Given that dopamine exerts inhibitory control over prolactin release, cannabinoids could potentiate dopamine-mediated inhibition of prolactin through this mechanism. In support of this hypothesis, AEA inhibition of prolactin release in male rats is accompanied by an increase in dopamine turnover in the anterior pituitary (Scorticati et al. 2003) and the inhibitory effect of THC is occluded by dopamine antagonist treatment (Kramer and Ben-David 1978).

The effects of cannabinoid agonists on prolactin in female rats are more complicated. Administration of THC to female rats in the morning of estrus results in decreased prolactin and increased dopamine turnover in the hypothalamus, a pattern that parallels the changes seen in males (Bonnin et al. 1993). However, administration of THC in the afternoon of estrus, or in proestrus and diestrus, was without effect on prolactin and had variable effects on dopamine turnover. Similarly, while an i.c.v. injection of AEA decreased circulating prolactin in males, the same dose had no effect on prolactin in OVX female rats and very significantly increased prolactin in OVX estrogen-replaced rats (Scorticati et al. 2003). While AEA increased pituitary dopamine turnover in male rats, AEA treatment decreased this measure in both OVX and OVX estrogen-replaced females in a CB_1R-dependent manner. CB_1R blockade significantly reduced prolactin in the OVX estrogen-replaced but not OVX rats, suggesting an increase in tonic CB_1R activity in the OVX estrogen-replaced setting. These data are consistent with other evidence that estrogen increases the synthesis of eCBs in females (Huang and Woolley 2012). Interestingly, THC was found to reverse the stimulatory effect of estrogen on prolactin release in female rats in vitro (Murphy et al. 1991a) and in vivo (Murphy et al. 1991b). Since THC has low efficacy at the CB_1R (Kearn et al. 1999), it is

possible that it acts as an antagonist in this situation in which eCB tone is high. Several mechanisms have been suggested by which CB_1R activation alters prolactin release in females, including direct effects on CB_1Rs of dopaminergic terminals resulting in inhibition of dopamine release (Scorticati et al. 2003) or alterations in the sensitivity of lactotrophs to stimulation (Murphy et al. 1991a).

Cannabinoid effects on prolactin in humans parallel those seen in rodents. In a study carried out in young men, THC was found to produce a slight decrease in prolactin concentrations (Liem-Moolenaar et al. 2010). Two other studies in which THC was administered by inhalation to cannabis-experienced young men also found small but significant reductions in circulating prolactin concentrations measured 90 min after treatment (Klumpers et al. 2012; Kleinloog et al. 2012). Another study, which compared the effects of intravenous THC administration to experienced cannabis users and healthy controls, found no acute effect of THC on prolactin in either group (Ranganathan et al. 2009). On the other hand, these investigators found that baseline prolactin concentrations were very significantly lower in the cannabis users than controls, which could reflect dysregulation of prolactin release or be persistent effects arising from a significant body burden of THC.

Two studies have examined the role of dopamine signaling in the mechanism of action of THC. In one, haloperidol pretreatment abrogated the reduction in prolactin by THC (Liem-Moolenaar et al. 2010), while in the other, THC continued to reduce prolactin in olanzapine-pretreated individuals (Kleinloog et al. 2012). These limited data and the very large increase in prolactin that results from dopamine receptor inhibition make these studies difficult to interpret.

3.2 Oxytocin

The hypothalamic–neurohypophyseal axis consists of magnocellular neurons within the supraoptic (SON) and periventricular nuclei (PVN) of the hypothalamus. These neurons synthesize the neuropeptides oxytocin (OXT) and vasopressin (VP) and send axonal projections to the posterior pituitary. Activation of magnocellular neurons results in the release of OXT and VP from axon terminals in the posterior pituitary. OXT and VP regulate reproduction and body fluid homeostasis through effects in peripheral organs. The release of OXT and VP occurs in response to a wide variety of stimuli, including suckling, mating behavior, stress, fever, and infection (McDonald et al. 2008).

In addition to acting as a hormone, OXT is also released within multiple limbic and cortical brain regions (McGregor et al. 2008). OXT receptors are present in non-hypothalamic brain areas (Neumann et al. 1993), and centrally released OXT contributes to maternal behaviors and increases sexual and social interactions (McGregor et al. 2008).

A series of important and interesting papers have characterized a role for ECS in the regulation of activity in the magnocellular neurons of both the SON and PVN. CB_1R activation by endogenously produced eCBs reduces glutamate release onto

magnocellular neurons of the PVN and SON (Hirasawa et al. 2004; Di et al. 2003, 2005a, b; McDonald et al. 2008). A variety of mechanisms can evoke eCB release from magnocellular neurons, including glucocorticoids, acting via a membrane receptor (Di et al. 2003, 2005a, b), OXT itself (Hirasawa et al. 2004; McDonald et al. 2008), and alpha-melanocyte-stimulating hormone (Sabatier and Leng 2006). OXT also recruits ECS in layer V of the infralimbic region of the prefrontal cortex to decrease glutamate release (Ninan 2011). A recent in vitro study suggests that AEA can also decrease the release of OXT through a mechanism requiring increased nitric oxide synthase activity and CB_2Rs and vanilloid receptors but not CB_1Rs (Luce et al. 2014).

CB_1Rs are also present on GABA terminals in the hypothalamus (Wittmann et al. 2007) and several studies support a role for ECS in the suppression of tonic GABA release onto magnocellular neurons (Oliet et al. 2007; Di et al. 2009; Wang and Armstrong 2012). Low concentrations of OXT in the dendritic regions of the magnocellular neurons recruit ECS to produce a tonic inhibition of GABA release (Oliet et al. 2007). This process is hypothesized to provide a mechanism by which OXT itself can regulate inputs in an autocrine fashion that is coordinated and easily reversed when needed. The eCB that subserves this process is not known, although i.c.v. administration of the FAAH inhibitor, URB597, increases, while AM251 inhibits OXT release evoked by lipopolysaccharide (De Laurentiis et al. 2010). These data are consistent with an ability of AEA to inhibit GABAergic influence over the magnocellular SON neurons and thus potentiate OXT release.

Data from Tasker and colleagues suggest that 2-AG-mediated inhibition of GABA release is normally opposed by efficient buffering by astrocytes of 2-AG released from magnocellular neurons (Di et al. 2013). When astroglia are retracted, as during dehydration, or metabolically inactivated, 2-AG-mediated inhibition of GABA release is revealed. These data are very interesting and suggest that the primary role for 2-AG is to regulate glutamate inputs into magnocellular neurons, but that under certain circumstances, an effect on GABA release can also occur. These data also suggest different roles for 2-AG versus AEA in the regulation of magnocellular neuronal activation.

In vivo studies of the effects of cannabinoids on circulating OXT, lactation, and maternal behaviors suggest an inhibitory effect of the CB_1R over OXT release and are therefore in accord with the evidence that CB_1R activation inhibits glutamatergic drive onto magnocellular neurons. Early studies demonstrated that THC and a variety of cannabis extracts interfere with nest-building behavior in mice (Moschovakis et al. 1978) and rats (Sieber et al. 1980). In a more recent study, a synthetic CB_1R agonist was demonstrated to produce a very significant reduction in circulating OXT concentrations and to reduce maternal behaviors (Vilela and Giusti-Paiva 2014). Dexamethasone-induced disruption of suckling-induced secretion of OXT and maternal behavior is also blocked by CB_1R antagonism (Vilela et al. 2013). These data, together with the evidence that glucocorticoids mobilize ECS in the hypothalamus to inhibit glutamate release in the SON (Di et al. 2005a), are consistent with ECS-mediated suppression of OXT neurons.

Paradoxically, both dams treated with a CB_1R antagonist (Schechter et al. 2012) and $CB_1R^{-/-}$ dams (Schechter et al. 2013) also exhibit poor maternal care, as

measured by time to retrieve pups. However, circulating OXT concentrations are not different between wild-type and $CB_1R^{-/-}$ dams, suggesting that release of OXT is not affected by loss of CB_1R signaling. $CB_1R^{-/-}$ dams had significantly lower amounts of OXT receptor mRNA and protein in hippocampus than wild-type dams and did not exhibit a postpartum-mediated increase as occurred in the wild-type dams. These data indicate that the CB_1R is needed for proper increases in CNS sensitivity to OXT following delivery, through regulation of increased OXT receptor expression.

Receptors for OXT are expressed in many brain regions involved in reward and drug seeking, including the nucleus accumbens and ventral tegmental area (VTA) (Vaccari et al. 1998). OXT in these brain regions is thought to be involved in the production by cannabinoids of enhanced feelings of sociability (McGregor et al. 2008). The OXT system exhibits significant neuroplasticity, and it has been hypothesized that chronic exposure to rewarding drugs, including THC, can downregulate OXT-mediated signaling and that loss of OXT signaling contributes to withdrawal (McGregor et al. 2008). Indeed, the administration of a moderate dose of THC for 7 days resulted in a significant reduction in expression of mRNA and protein for OXT in nucleus accumbens and VTA of rats without any effect in the hypothalamus (Butovsky et al. 2006). Similarly, chronic THC exposure results in lasting dysregulation of social interactions in rodents (O'shea et al. 2004, 2006; Quinn et al. 2008). OXT itself is not a useful therapeutic, since it does not cross the blood–brain barrier. However, lithium was shown to alleviate all of the symptoms of precipitated withdrawal induced by CB_1R antagonism in CB_1R agonist-tolerant rats, and this effect was accompanied by a large increase in circulating OXT concentrations (Cui et al. 2001).

A small study demonstrated that lithium treatment reduced withdrawal signs and promoted abstinence in chronic cannabis users (Bowen et al. 2005). However, a larger clinical trial recently published did not find any overall effects of lithium on withdrawal, although some of the individual withdrawal symptoms, including loss of appetite, stomach aches, and nightmares, were reduced (Johnston et al. 2014). While this study concluded that there was no clear advantage of lithium over placebo, the timing of lithium administration was such that its concentrations may not have been in a therapeutic range during the period of greatest withdrawal and further studies are warranted.

4 Interaction of Cannabinoids with Hormonal Regulation of Growth, Development, and Metabolism

4.1 Thyroid Hormones

The thyroid hormones, 3,5,3′-triiodothyronine and L-thyroxin, regulate development and metabolism in many mammalian tissues. Receptors for the thyroid hormones function as transcription factors, regulating gene transcription through thyroid hormone response elements in promoter regions of multiple genes (Flamant

et al. 2007). Thyroid hormone release is the end product of a regulatory cascade that includes hypothalamic TRH and pituitary thyroid-stimulating hormone (TSH), defining a hypothalamic–pituitary–thyroid (HPT) axis.

Treatment of adult rats with THC reduces thyroid hormone concentrations in the circulation (Nazar et al. 1977; Rosenkrantz and Esber 1980; Hillard et al. 1984). Several possible mechanisms for the effect have been suggested. Data showing that THC does not inhibit TRH-induced increases in circulating thyroid hormone (Hillard et al. 1984) together with evidence that CB_1Rs are expressed on neurons innervating TRH-expressing neurons (Di et al. 2003; Deli et al. 2009) suggest that THC and other CB_1R agonists can inhibit TRH release through effects in the hypothalamus. It is interesting in this regard that glucocorticoid-induced mobilization of ECS has been shown to inhibit glutamate release onto TRH-positive neurons in the hypothalamus (Di et al. 2003), suggesting that eCBs link stress and suppression of the HPT axis. Cannabinoids have also been shown to suppress the HPT axis at the pituitary (Veiga et al. 2008) and thyroid gland (Porcella et al. 2002). However, inhibition at the first step in a cascade such as the HPT axis will have the greatest impact in vivo.

TSH and thyroid hormone concentrations were all within normal limits and did not correlate with concentrations of THC or its major metabolites in a study of chronic cannabis users (Bonnet 2013). These findings suggest that chronic exposure of adults to THC does not produce a long-lasting impact on HPT axis function in otherwise healthy adults. However, perinatal hypothyroidism can result in severe and irreversible cognitive deficits in later life (Bernal 2007), suggesting that cannabis use during pregnancy could have adverse effects on fetal development through dysregulation of the HPT axis. In support of this notion, treatment of a trophoblast cell line with THC results in inhibition of proliferation and a nearly threefold reduction in the expression of thyroid receptor β1 (TRβ1) (Khare et al. 2006). This effect on TRβ1 expression is similar to what occurs in fetal growth restriction (FGR) (Ohara et al. 2004). Since cannabis use has been associated with FGR (Zuckerman et al. 1989), it is possible that THC exposure during pregnancy could interfere with growth as a result of decreased expression of TRβ1 and, thus, a decrease in thyroid hormone effect.

One recent study indicates that thyroid hormone status also modulates ECS. Hypothyroid rats exhibit an increase in the inhibitory effect of CB_1R agonism on the formation of spatial memories (Gine et al. 2013). This defect was normalized by administration of thyroid hormone. There was no difference in hippocampal CB_1R expression between control and hypothyroid rats, suggesting that the effect of the thyroid hormone is to enhance CB_1R signaling. Hypothyroid rats have also been shown to have a 50 % reduction in the cerebral expression of G protein receptor kinase 2 (GRK2) (Penela et al. 2000, 2001), an enzyme that participates in the desensitization of a variety of G protein-coupled receptors, including the CB_1R (Kouznetsova et al. 2002). Thus, it is possible that loss of thyroid hormones results in dampening of a negative regulatory process that affects the CB_1R.

4.2 Growth Hormone

Growth hormone (GH) is a polypeptide released from somatotrophs of the anterior pituitary that stimulates growth and regulates energy homeostasis. GH secretion is negatively and positively regulated by the hypothalamic peptides somatostatin and growth hormone-releasing hormone (GHRH), respectively. Somatostatin and GHRH release are regulated by biogenic amines, metabolic status, sex hormones, and sleep. GH is released in a pulsatile manner, with the largest GH peak occurring about an hour after the onset of sleep (Takahashi et al. 1968). Surges in GH release occur during waking as well, with a frequency of approximately 3–5 h (Natelson et al. 1975).

Acute and chronic THC treatment of adult and adolescent rodents decreases basal circulating GH concentrations (Kokka and Garcia 1974; Dalterio et al. 1981, 1983b) and suppresses episodic release of GH in adult male rats (Falkenstein and Holley 1992). A synthetic CB_1R agonist also produces a dose-dependent suppression of GH in male rats (Martin-Calderon et al. 1998). Data showing that THC administration into the third ventricle also suppresses GH (Rettori et al. 1988) and THC increases somatostatin release from hypothalamic explants (Rettori et al. 1990) support the hypothesis that THC inhibits GH via increased somatostatin. CB_1Rs are also expressed by GH-secreting cells in the human pituitary, and CB_1R agonist treatment inhibits GH secretion from acromegaly associated pituitary adenomas in culture (Pagotto et al. 2001), although another study found no effect of THC on GH release from isolated pituitary cells (Rettori et al. 1988). The ability of ghrelin to increase GH release is not affected by CB_1R antagonist treatment (Kola et al. 2013).

5 Cannabinoids and the Hypothalamic–Pituitary–Adrenal Axis

The hypothalamic–pituitary–adrenal (HPA) axis contributes to the circadian regulation of physiological function and is an essential component of the stress response. HPA axis activation begins with the neuropeptide, corticotropin-releasing hormone (CRH), which is synthesized by PVN neurons that respond to and integrate inputs from the amygdala, prefrontal cortex (PFC), and hippocampus (Herman et al. 2003). CRH induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary which stimulates glucocorticoid synthesis and release from the adrenal cortex.

Outputs of the HPA axis, the glucocorticoids cortisol and corticosterone (CORT), have wide-ranging effects on the body, influencing metabolism, immune function, and behavior. The HPA axis is activated by both physical and psychological stress and glucocorticoids, acting via the glucocorticoid receptor (GR), are responsible for many of the homeostatic changes that follow stress, including increased food consumption and suppression of the immune system. However, the HPA axis also has "housekeeping" duties at basal concentrations that are

mediated by mineralocorticoid receptor (MR) activation, as these receptors have higher affinity for the corticosteroids than GRs. Circulating glucocorticoid concentrations are circadian and the highest concentrations of corticosterone are reached shortly after the beginning of the active period of the day.

As is the case for the other endocrine systems discussed, the ECS plays an important role in the regulation of the HPA axis at the level of the hypothalamus. Activation of CB_1Rs in the PVN inhibits the release of glutamate onto CRH neurons, data consistent with CB_1R-mediated inhibition of the HPA axis (Di et al. 2003, 2005b). ECS at this synapse is rapidly activated by CORT, leading to the hypothesis that ECS regulates CORT-mediated feedback inhibition (Evanson et al. 2010). In addition to effects in the PVN, CORT-mediated increases in 2-AG also contribute to feedback regulation of the HPA axis in the medial PFC (Hill et al. 2011) and hippocampus (Wang et al. 2012). As a result of actions in all of these brain regions, deficient or absent ECS results in prolonged activation of the HPA axis by restraint stress (Hill et al. 2011).

ECS in the basolateral amygdala (BLA) also regulates the HPA axis; in particular, it constrains the initiation of HPA axis activation by stress. Intra-BLA injections of a CB_1R agonist and antagonist decrease and increase, respectively, CORT responses to stress in male rats (Hill et al. 2009; Ganon-Elazar and Akirav 2009). Since stress exposure produces a rapid decrease in BLA AEA concentrations (Hill et al. 2009), it has been suggested that AEA concentrations in BLA are high at rest and function to inhibit spurious activation of the HPA axis (Patel et al. 2004; Hill et al. 2009). In order for a robust HPA axis activation to occur, the concentration of AEA in the BLA must decrease. This is accomplished via activation of FAAH in the amygdala (Hill et al. 2009), likely through CRH acting through the CRH-R1 receptor (Gray et al. 2013). In further support of this mechanism, low concentrations of direct CB_1R agonists and inhibition of FAAH inhibit activation of the HPA axis by restraint stress (Patel et al. 2004), while systemic administration of rimonabant increases circulating CORT concentrations in response to injection (Wade et al. 2006) and restraint stresses (Patel et al. 2004).

There is evidence that ECS negatively regulates basal and circadian HPA axis activation states as well. For example, i.c.v. administration of high doses of rimonabant increases circulating CORT and ACTH concentrations in rat, suggesting a tonic inhibition of HPA axis activation by the CB_1R (Manzanares et al. 1999). Female $CB_1R^{-/-}$ mice exhibit significantly elevated concentrations of both CORT and ACTH at the onset of the active period (i.e., dark phase) compared to wild-type mice (Cota et al. 2007). CB_1R antagonist treatment increased both ACTH and CORT concentrations in non-stressed rats; however, it had a far greater effect on CORT concentrations when administered during the diurnal trough than during the diurnal peak (Atkinson et al. 2010). These data suggest that endogenous tone at the CB_1R is higher in the early light period than in the early dark period. In accord with this notion, we have recently demonstrated that hypothalamic contents of AEA are highest at the times of 07:00 and 11:00 and are low between 15:00 and 03:00 (Liedhegner et al. 2014).

In vivo studies showed that low doses of CB_1R agonists other than THC reduced basal and stress-induced HPA axis responses in rodents (Patel et al. 2004; Saber-Tehrani et al. 2010), data that are consistent with the regulatory mechanisms discussed above. However, high doses of synthetic agonists (Patel et al. 2004), and THC treatment, increase circulating concentrations of CORT (Steiner and Wotjak 2008). A pharmacological study in rats suggests that the cannabinoid-induced increase in HPA axis activity is secondary to activation of monoaminergic hindbrain nuclei as both noradrenergic and serotonergic blockade reduced the stimulatory effects (McLaughlin et al. 2009). It is interesting that this circuit seems to be preferentially activated by THC, while higher-efficacy cannabinoids and AEA inhibit HPA axis through direct actions on limbic and hypothalamic circuitry as described above.

There is some evidence that CB_1R activation regulates the HPA axis via effects in the pituitary and adrenal gland as well as in the brain. Pituitary cells isolated from $CB_1R^{-/-}$ mice exhibited greater secretion of ACTH in response to both CRH and forskolin stimulation (Cota et al. 2007), suggesting an inhibitory role for the CB_1R in the pituitary. CB_1R mRNA is expressed in the adrenal gland of rodents (Buckley et al. 1998) and humans (Ziegler et al. 2010), and AEA-mediated activation of the CB_1R has been found to decrease basal and stimulated adrenocortical steroidogenesis (Ziegler et al. 2010). Additionally, CB_1R activation decreases epinephrine release from adrenal medullary cells (Niederhoffer et al. 2001). Therefore, ECS could decrease glucocorticoid synthesis within adrenocortical cells directly or via reduced sympathetic drive. This conclusion is supported by a study in which systemic administration of a CB_1R antagonist elevated circulating CORT concentrations without an effect on ACTH or pituitary c-fos expression, suggesting a direct effect of the antagonist on the adrenal gland (Newsom et al. 2012).

Human studies reproducibly demonstrate that acute consumption of cannabis (Cone et al. 1986) or THC (D'Souza et al. 2004, 2008; Klumpers et al. 2012; Ranganathan et al. 2009; Kleinloog et al. 2012) increases the secretion of cortisol in individuals who were either naïve to cannabis or infrequent users. The stimulatory effect of THC administration on cortisol levels was blunted in chronic cannabis users, suggesting that tolerance develops (D'Souza et al. 2008; Ranganathan et al. 2009). On the other hand, some (King et al. 2011; Somaini et al. 2012), but not all (Block et al. 1991), studies have reported that chronic cannabis users exhibit elevated basal cortisol levels, and other studies demonstrate that stress-induced activation of the HPA axis is blunted in chronic adult and adolescent cannabis users (Somaini et al. 2012; van Leeuwen et al. 2011). In adolescents with an early onset of use, chronic cannabis use is associated with altered diurnal cortisol rhythms such that cortisol concentrations are higher than normal at night and blunted in the morning (Huizink and Mulder 2006). Taken together, the human data suggest that chronic cannabis use has the potential to dysregulate basal, circadian, and stress-regulated HPA axis activity in a complex manner.

6 Summary

The hypothalamus is an important center for the regulation of metabolism, reproduction, and responses to stress. Although the density of the CB_1R in the hypothalamus is lower than in other brain regions, it is clear from a vast number of studies that ECS plays a highly significant role in hypothalamic function through regulation of neurotransmitter release from glutamatergic, GABAergic, and possibly other nerve terminals. The ECS is designed to act in a localized manner, and it is clear that it can regulate the activation state of several hypothalamic neuronal subtypes in an independent manner. However, stress produces an increase in eCBs in the hypothalamus, and it is possible, although not validated completely, that CB_1Rs mediate the effects of stress on multiple endocrine systems.

A consistent theme throughout all available studies is that THC has relatively inconsistent effects in humans, in spite of consistent effects seen in preclinical studies. It is possible that individual differences in the underlying ECS result in inconsistent effects of THC. While it is tempting to conclude that THC does not have significant endocrine effects in humans, it is possible that THC could have significant effects in some individuals that increase the risk of health problems, including infertility, hypothyroidism, or problems in stress responding.

Acknowledgments The author was supported during the writing of this review by NIH grant R01 DA26996 and the Research and Education Program, a component of the Advancing a Healthier Wisconsin endowment at the Medical College of Wisconsin.

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Endocannabinoids and Reproductive Events in Health and Disease

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_12

Abstract

The lasting research on the endocannabinoid system (ECS) has now provided solid and convincing evidence that proves the detrimental effects of recreational drug abuse (a growing habit among teenagers) on fertility. Endocannabinoids (eCBs) affect reproductive events from gametogenesis to fertilization, from embryo implantation to the final outcome of pregnancy and, thus, they have been proposed as suitable biomarkers to predict the reproductive potential of male and female gametes in clinical practice. Novel tools for reproductive medicine are highly sought after, and here we report the latest findings on the impact of the ECS on fertility, demonstrating how basic research can be translated into new medical strategies.

Keywords

AEA Anandamide • Biomarkers • Endocannabinoid system • Infertility • Reproduction

Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	*N*-arachidonoylethanolamine (anandamide)
AR	Acrosome reaction
BF	Blastocoelic fluid
CB_1	Type-1 cannabinoid receptor
CB_2	Type-2 cannabinoid receptor
CBR	Cannabinoid receptors
COX	Cyclooxygenase
CRH	Corticotrophic hormone
DAGL	Diacylglycerol lipase
E	Estrogen
eCB	Endocannabinoid
eCBs	Endocannabinoids
ECS	Endocannabinoid system
EMT	Endocannabinoid membrane transporter
ERE	Estrogen-responsive element
FAAH	Fatty acid amide hydrolase
FSH	Follicle-stimulating hormone
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LOX	Lipoxygenase
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
NAPE-PLD	*N*-arachidonoyl-phosphatidylethanolamine-specific phospholipase D
OEA	*N*-oleoylethanolamine

P	Progesterone
PEA	*N*-palmitoylethanolamine
PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
TC	Trophoblastic cells
THC	Δ^9-tetrahydrocannabinol
TRPV1	Transient receptor potential vanilloid-1
TS	Trophoblast stem
ZP	Zona pellucida

1 Introduction

The beginning of a new life is the culmination of a series of events finely tuned by endocrine signals, environmental, psychological, and lifestyle factors. Recreational use of marijuana is a habit that is widespread among people of reproductive age, and cannabis consumption is listed among the leading causes of both male and female infertility. The discovery of cannabinoid receptors (CBR), endocannabinoids (eCBs), and their metabolic enzymes and transporters has shed light on the relevance of endocannabinoid (eCB) signaling in the modulation of reproductive events under healthy and pathological conditions (Meccariello et al. 2014). *N*-Arachidonoyl-ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the main eCBs, and their biological actions are controlled by cellular mechanisms that include enzymes responsible for: (i) their synthesis, e.g., the *N*-acyl-phosphatidylethanolamines (NAPE)-specific phospholipase D (NAPE-PLD) and the *sn*-1-specific diacylglycerol lipase (DAGL), respectively and (ii) their degradation, e.g., fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Fezza et al. 2014). Besides these pathways, there are other metabolic routes for eCB metabolism (Fezza et al. 2014; Ueda et al. 2013). Thus, AEA and 2-AG are also susceptible to oxidative mechanisms catalyzed by lipoxygenases (LOXs) and cyclooxygenases (COXs) and can be enzymatically transformed into prostaglandin (PG) ethanolamine and PG glyceryl ester, respectively, through the sequential actions of COX-2 and several PG synthases (Rouzer and Marnett 2011). eCBs act principally through type-1 and type-2 cannabinoid (CB_1 and CB_2) receptors. GPR55, that acts like a putative "type-3 cannabinoid (CB_3)" receptor (Moriconi et al. 2010), is also one of their targets. eCBs are also able to interact with non-CBR targets, such as the transient receptor potential vanilloid type 1 (TRPV1) channel, which is activated by both AEA (Di Marzo and De Petrocellis 2010) and 2-AG (Zygmunt et al. 2013), and the peroxisome proliferator-activated receptor (PPAR) α and PPAR γ (Pistis and Melis 2010). The cellular uptake of eCBs from the extracellular to the intracellular space is ascribed to a purported "endocannabinoid membrane transporter (EMT)" that may well take up both AEA and 2-AG. However, the mechanisms through which eCBs are carried across plasma membranes

and transported within the cell are not yet fully understood and remain a matter of debate (Fowler 2013). *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA) are compounds structurally related to eCBs, known as "eCB-like" substances. They potentiate the effect of genuine eCBs by the so-called "entourage effect", that is by competitively inhibiting eCB degradation, or by allosterically modulating their receptor binding.

To date, the ECS is considered a master system deeply involved in the control of several physiological processes, including fertility. Alterations of eCBs and/or ECS components might affect negatively various reproductive stages from gametogenesis to fertilization, embryo implantation and development, and parturition. Therefore, these bioactive lipids have a huge potential for the diagnosis and/or therapy of female and male defects (Di Blasio et al. 2013; Maccarrone 2013; Meccariello et al. 2014; Rapino et al. 2014).

2 Gametogenesis

2.1 Ovary and Folliculogenesis

Female reproductive cells are produced in the ovary and their survival is regulated by gonadotrophic and sex hormones, which interact with each other to control the ovarian cycle. The first step of oogenesis occurs during intrauterine life, starting in the primary oocyte after the first meiotic division. At puberty meiotic division restarts, leading to the production of follicles that contain secondary oocytes and that will be released at ovulation, in order to be fertilized. The presence of AEA in the human follicular fluid (Schuel et al. 2002), and the observation that alteration of its plasma levels could be related to fertility/infertility in healthy women, identified eCBs as key mediators of reproductive events (Maccarrone et al. 2000, 2002; Lam et al. 2008). AEA, its metabolic enzymes, and its molecular targets have been recently localized in the human ovary (El Talatini et al. 2009a). In particular, it has been shown that both CB_1 and CB_2 are expressed at different time points in follicular cells and oocytes. Indeed, CB_2 is generally more highly expressed than CB_1 in the ovary and is detected in oocytes only at a late stage of development (El-Talatini et al. 2009a). Moreover, NAPE-PLD and FAAH are expressed in secondary and tertiary follicles and in the corpus luteum and albicans (El-Talatini et al. 2009a). Data on the regulation of NAPE-PLD protein expression between the proliferative and secretory phases of the endometrium are conflicting, probably because of differences between studied patients (Taylor et al. 2010; Gebeh et al. 2012; Scotchie et al. 2015). However, published data on increased expression in the middle and late secretory phases are more consistent (Taylor et al. 2010; Gebeh et al. 2012; Scotchie et al. 2015).

Interestingly, AEA measured in the follicular fluid of women undergoing controlled ovarian hyperstimulation for in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) is higher in follicles with mature oocytes than in those with immature oocytes (El-Talatini et al. 2009a) (Fig. 1a, b). These data support the

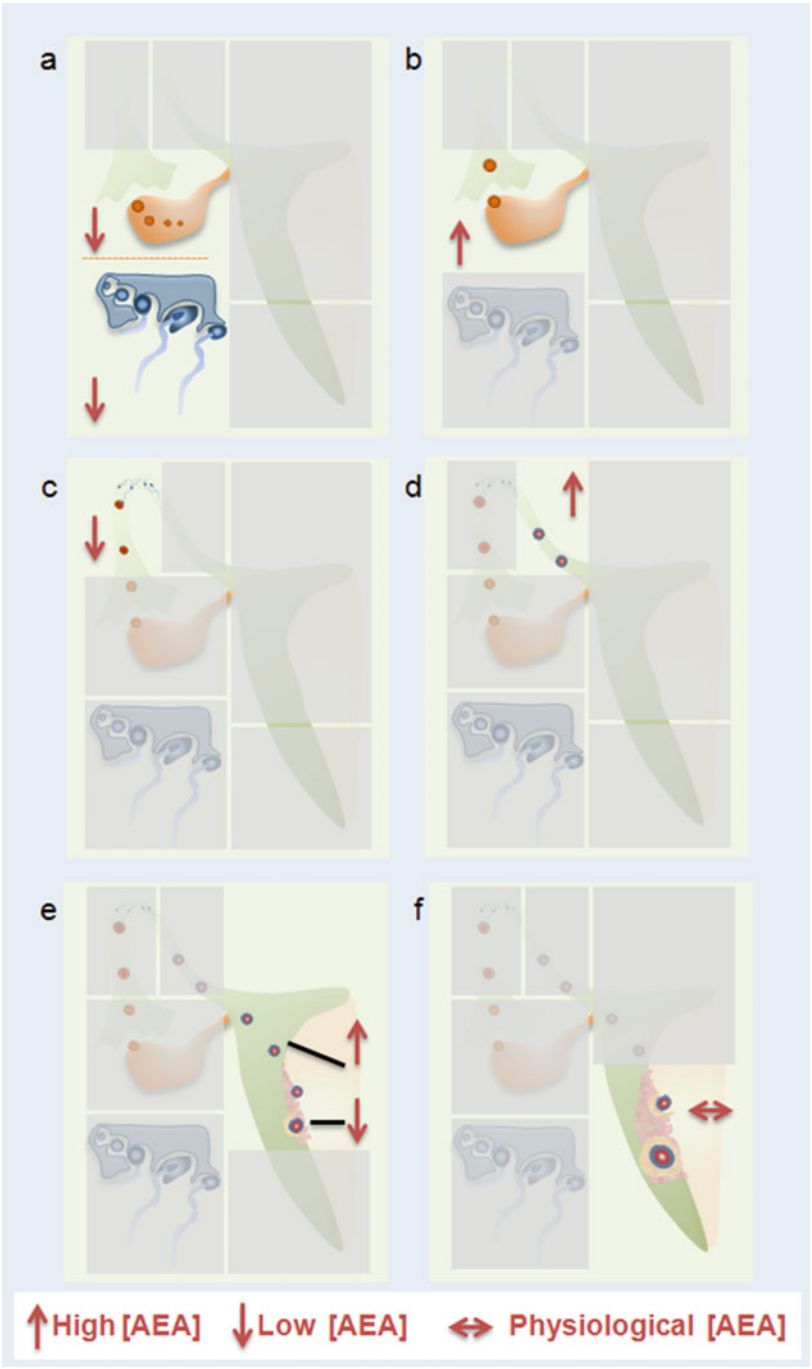

Fig. 1 Role of AEA in the early stages of reproduction: from gametogenesis to decidualization. Schematic representation of fluctuations of AEA levels during early stages of pregnancy. (**a**) *Gametogenesis:* Low AEA levels are required during all phases of this process to warrant

hypothesis that AEA in follicular and oviductal fluids may be involved in oocyte maturation (Schuel et al. 2002; El-Talatini et al. 2009a). On this basis, one can speculate that AEA exerts effects on fertility during the late phase of human folliculogenesis. In addition, these data are in keeping with recent observations that granulosa cells of the rat uterus lack CB receptors (Bagavandoss and Grimshaw 2010). Another recent study has monitored the expression of AEA and 2-AG catabolic enzymes in human endometrium during the menstrual cycle (Scotchie et al. 2015). Maximal FAAH expression was detected in the middle secretory phase, whereas MAGL expression reached its highest level in the early secretory phase (Scotchie et al. 2015). Furthermore, arachidonic acid, generated by the degradation of AEA and 2-AG, serves as a good substrate for COX-2, which is expressed in the cumulus oophorus but not in the egg cell. This leads to an increase in PGE_2 required for the maturation of the egg (Bayne et al. 2009; Feuerstein et al. 2007). In this context, increased mRNA levels of COX-2 were found during the proliferative phase, followed by a reduction in the secretory phases (Scotchie et al. 2015). In summary, regulation of ovarian physiology by eCB signaling is apparent (Cecconi et al. 2014).

2.2 Spermatogenesis and Spermiogenesis

Sertoli cells are supporting cells localized within the seminiferous tubules, where they create a unique protective environment for sperm development during spermatogenesis. Their main role is to provide hormones, growth factors, and other glycoproteins needed for sperm nourishment, as well as to form the blood–testis barrier. The biological function of Sertoli cells is controlled by follicle-stimulating hormone (FSH) that, by binding to its specific receptor on targeted cells, can trigger different signal transduction pathways, thus leading to cell proliferation and differentiation. Some ECS elements have been detected in Sertoli cells at different development stages (Maccarrone et al. 2003; Rossi et al. 2007). In this context, it has been demonstrated that FSH is able to specifically stimulate expression and

Fig. 1 (continued) maturation of both oocytes and sperm cells. (**b**) *Ovulation:* At a late stage of folliculogenesis and at ovulation, follicles and follicular fluid show high AEA levels. (**c**) *Fertilization:* Mature oocytes and sperm cells meet each other in the central portion of the Fallopian tube called the ampulla, where establishment of an increasing AEA gradient favors the journey of the fertilized egg. (**d**) *Oviductal transport:* To reach the implantation site in the uterus, the zygote must cross the isthmus, the end portion of the Fallopian tube, where a gradually increased AEA concentration prevents early extrauterine implantation. (**e**) *Implantation:* Regulated AEA levels are conducive to normal implantation, so that low AEA content must be kept in the uterine epithelium at the implantation site, whereas high AEA content is needed at inter-implantation sites. (**f**) *Decidualization:* The maternal–fetal relationship is definitively established by decidualization, where high AEA levels are often related to ectopic pregnancy, while low AEA levels trigger apoptosis. These findings suggest a dual role of AEA that depends on its concentration. See text for details on the various stages of reproduction

activity of FAAH (the main AEA hydrolase), by triggering protein kinase A- and/or aromatase-dependent pathways (Rossi et al. 2007). Indeed, estrogen (E) activates FAAH transcription by binding to its receptor at two proximal E-responsive element (ERE) sites that trigger demethylation of DNA and of histone H3 (Grimaldi et al. 2009a, 2012, 2013). This process might play a role in finely regulating the endogenous tone of AEA and thus the pro-apoptotic action of this eCB in Sertoli cells. As a consequence, E impacts on the number of spermatids in the adult, that indeed depends on the size of the Sertoli cell population produced during perinatal development (Orth et al. 1988) (Fig. 1a). The crosstalk among androgens, estrogens, and eCBs during murine postnatal testicular development, from puberty to adulthood, has also been documented (Gye et al. 2005; Cacciola et al. 2008a). Indeed, spatiotemporally distinct expression of CB_1 was revealed in Leydig cells, spermatogonia, and spermatocytes, suggesting a functional involvement of CB_1-mediated signaling in steroidogenesis and spermatogenesis. In this context, it has been reported that long-term use of HU-210, a synthetic analogue of Δ^9-tetrahydrocannabinol (THC) and a potent CB_1 agonist, impairs spermatogenesis by decreasing the number of Sertoli cells and by reducing spermatogenic efficiency through a CB_1-mediated mechanism (Lewis et al. 2012a).

Recently, it has been demonstrated that CB_2 also plays a crucial role in the mitotic and meiotic phases of spermatogenesis, but not during the late process of spermiogenesis, that is responsible for the transformation of spermatids into spermatozoa (Grimaldi et al. 2009b). Indeed, CB_2 mRNA and protein expression are detectable in male germ cells from mitotic spermatogonia to haploid spermatids, while they are absent in mature sperm cells (Grimaldi et al. 2009b). Accordingly, the endogenous content of 2-AG, but not of AEA, markedly decreased during the differentiation process and was paralleled by lower or higher mRNA levels of 2-AG synthesizing or degrading enzymes, respectively (Grimaldi et al. 2009b). In addition, it was suggested that a steady AEA tone in testes might activate TRPV1, thus protecting germ cells against testicular hyperthermia (Mizrak et al. 2008). In line with this, experiments performed with *Trpv1*$^{-/-}$ mice demonstrated that this receptor promotes apoptosis of spermatogonia and blocks the progression of germ cell meiosis, as a defensive mechanism against abnormally elevated testicular temperature (Mizrak et al. 2008). Results obtained in a number of studies performed with animal models and humans have further demonstrated that isolated spermatocytes and spermatids, as well as mature spermatozoa, possess both AEA and 2-AG metabolic enzymes, along with cannabinoid and vanilloid receptors (Maccarrone et al. 2005; Rossato et al. 2005; Francavilla et al. 2009; Grimaldi et al. 2009b; Gervasi et al. 2011). Interestingly, a correlation between the expression of some ECS elements, such as CB_1 (Cobellis et al. 2006; Meccariello et al. 2006; Cacciola et al. 2008b), FAAH (Cobellis et al. 2006) and NAPE-PLD (Chianese et al. 2012), and the progression of spermatogenic stages has also been established in non-mammalian vertebrates (Battista et al. 2012).

Altogether, these findings suggest a synchronized biological action of eCBs that, by a timely binding to their specific molecular targets, promote and orchestrate the different stages of spermatogenesis and spermiogenesis (Maccarrone 2009).

3 Fertilization

3.1 Sperm Motility and Interaction With the Oviduct

The fusion of gametes to produce a new organism is the culmination of a multitude of intricate cellular processes. Sperm cells become fertilization-competent after undergoing a maturation process, whereby they become motile and their plasma membrane is reorganized in order to interact with the oocyte. The ability of eCBs to regulate sperm functions required for fertilization is well established in non-mammals and mammals (for a review see Battista et al. 2012). Prior to ejaculation, sperm resides within the epididymis, where it acquires motility upon travelling from caput to cauda. Previous research with the *Rana esculenta* frog (Cobellis et al. 2006), and with *cnr1* (the gene encoding for CB_1) null mice (Ricci et al. 2007), demonstrated that any disruption of CB_1-dependent eCB signaling leads to precocious acquisition of sperm motility along the male reproductive tract. Further evidence has arisen from studies with epididymal spermatozoa of *faah* (the gene encoding for FAAH) null mice that compromised motility and impaired fertilizing ability result from elevated AEA levels in the male reproductive tract (Sun et al. 2009). On the other hand, it has been hypothesized that a decreasing 2-AG gradient from caput to cauda is required to promote sperm start-up and to maintain sperm motility during the journey through the epididymus (Cobellis et al. 2010).

Once sperm cells are released into the vagina, their swimming ability from cervix to oviduct seems to be finely tuned by a differential activation of CB_1 and CB_2. Indeed, binding of selective agonists to CB_1 increases the percentage of immobile cells, whereas activation of CB_2 shifts the sperm population from rapid to sluggish/slow progressing spermatozoa, thus regulating the in vivo proportion of motile sperm (Agirregoitia et al. 2010). In addition, indirect capacitation signals (like heparin) that promote sperm release might remodel the sperm surface and make sperm cells insensitive to AEA (Gervasi et al. 2011).

The involvement of eCB signaling is apparent also in the control of sperm energy homeostasis, which in turn affects critical sperm functions like motility, capacitation, and the acrosome reaction (Rossato 2008). Indeed, AEA negatively affects sperm motility in a CB_1-dependent manner that involves inhibition of mitochondrial activity (Rossato et al. 2005), cellular respiration (Badawy et al. 2009) and glycolysis (Barbonetti et al. 2010).

At physiological concentrations, AEA acts as a capacitating signal in boar (Maccarrone et al. 2005), frog (Cottone et al. 2008), mouse (Catanzaro et al. 2011) and human spermatozoa (Rossato et al. 2005; Francavilla et al. 2009). Consistently, capacitated mouse sperm cells are characterized by the presence of elevated levels of both AEA (due to reduced degradation by FAAH) and 2-AG (due to enhanced synthesis by DAGL). These distinct pathways might allow a differential regulation of the content of these two major eCBs that, through a differential activation of either extracellular CB_1 or intracellular TRPV1, maintain a suitable environment that makes it possible for the sperm to travel along the

uterine tract without activating the acrosome arsenal (Catanzaro et al. 2011). This finding is also consistent with boar (Maccarrone et al. 2005), bull (Gervasi et al. 2011) and human sperm data (Francavilla et al. 2009), showing that increased intracellular AEA levels, by activating TRPV1 receptors at an intracellular binding site during capacitation, promote the fertilizing ability of sperm. In addition, AEA present in both seminal plasma and uterine fluids plays a physiological role, via CB_1 receptors, in maintaining a quiescent, uncapacitated condition before sperm interacts with an oocyte, thus preventing premature capacitation in freshly ejaculated sperm (Battista et al. 2008; Lewis et al. 2012b). Overall, it can be proposed that an increased intracellular content of AEA can lead to the activation of TRPV1, and that by means of its export by a putative EMT, AEA can build up a sufficient extracellular concentration for the activation of CB_1. This regulation facilitates the sperm journey through the uterine tract. Further activation of CB_1 can also be induced by intracellular 2-AG, after this eCB is released from the cell (Catanzaro et al. 2011).

It should be remembered that the presence of an oviductal gradient of AEA might have an impact on the fertilizing properties of sperm cells during their storage within the female reproductive tract. These conditions are required to ensure the presence of enough competent sperm at the time of ovulation (Gervasi et al. 2009; Talevi et al. 2010). Bovine epithelial cells of the oviduct express NAPE-PLD mRNA, and could contribute to release AEA in the isthmus, further enhancing its local concentration (Gervasi et al. 2009). Higher levels of AEA in this region might prolong the fertile life of sperm cells and hence ensure their progression through the oviduct (Gervasi et al. 2009; Talevi et al. 2010).

3.2 Sperm Interaction With the Oocyte

Once capacitation has occurred, the second major stage of fertilization begins. In this second stage, which depends on a sperm cell–egg interaction, only capacitated sperm cells activate the series of processes required for fertilization, among which acrosome reaction (AR) is the key event. The highly specialized gametes begin their interactions by signaling to one another to ensure that fertilization occurs when they meet. The oocyte releases PGs to help to guide sperm cells to the site of fertilization, and these cells secrete a specific protein to trigger oocyte maturation and ovulation. After their encounter, sperm and oocyte fuse in a specific and tightly regulated manner.

The fertilizing ability of sperm and the sperm–oocyte interaction depend on AEA binding to either CB_1 or TRPV1 receptors (Fig. 2). Indeed, experimental data obtained with boar sperm have demonstrated that AEA acts via a CB_1-mediated mechanism to inhibit a physiological AR triggered by zona pellucida (ZP) proteins (Maccarrone et al. 2005). In contrast, activation of TRPV1 reduces the ability of sperm to react with ZP proteins, thus preventing an "out of place" acrosomal exocytosis or spontaneous AR (Maccarrone et al. 2005). This idea is supported by the finding that inhibition of TRPV1 by its selective antagonist, capsazepine,

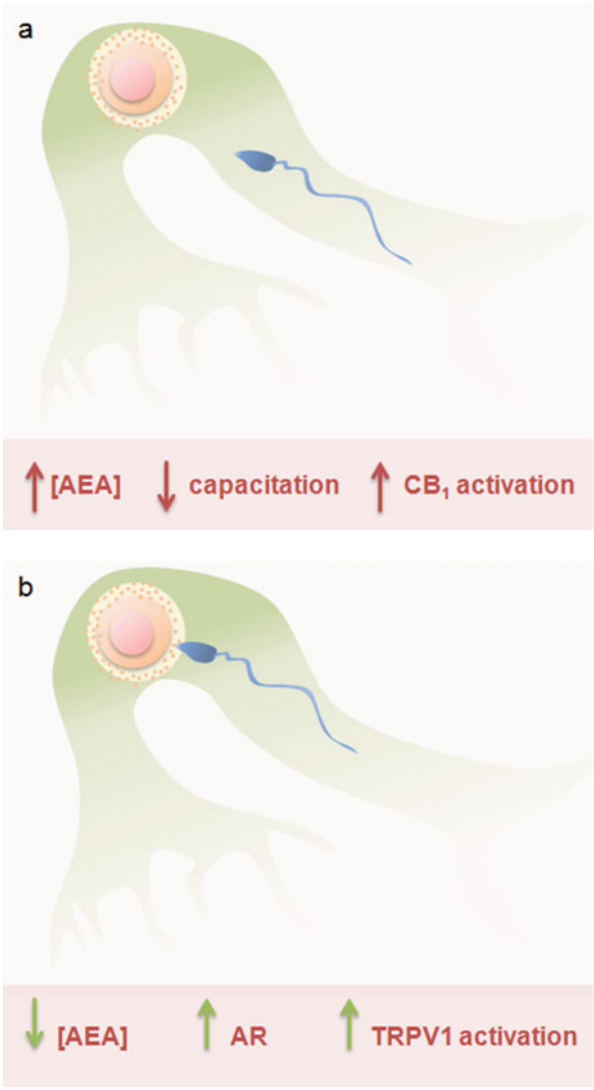

Fig. 2 Dual stage-dependent effect exerted by AEA to regulate sperm fertilizing ability. (**a**) AEA, present in both seminal plasma and uterine fluids, may prevent precocious capacitation in freshly ejaculated sperm via a CB_1-mediated mechanism in the uterine tract. (**b**) Once capacitation is completed, AEA stabilizes acrosome membranes by activating TRPV1 and thus reduces spontaneous acrosome reactions and promotes sperm–oocyte fusion

leads to premature AR in a high percentage of in vitro capacitated boar (Maccarrone et al. 2005) and human (Francavilla et al. 2009) sperm cells. In addition, prolonged exposure of human sperm cells to capsazepine significantly inhibited their ability to fuse with oocytes in response to progesterone (P), an effect prevented by the addition of a specific inhibitor of EMT (Francavilla et al. 2009). Therefore, increased intracellular AEA levels activate TRPV1, and thus maximize sperm responsiveness to physiological inducers of AR, by reducing sperm fusion with the oocyte membrane (Francavilla et al. 2009). Incidentally, in vitro evidence for TRPV1 translocation within sperm after capacitation further suggests a role of this

receptor in the fertilizing ability of sperm (Bernabò et al. 2010). Indeed, this event could be crucial for the completion of spontaneous AR and for preventing premature fusion between the outer acrosome membrane and the plasma membrane (Bernabò et al. 2010). Overall, AEA present in both seminal plasma and uterine fluids prevents premature capacitation in freshly ejaculated sperm via a CB_1-mediated mechanism. Once sperm cells reach the oviduct, they are exposed to a progressively reduced concentration of AEA in the proximal female genital tract (Schuel et al. 2002), and sperm capacitation may finally occur. At this time, increased intracellular AEA content activates TRPV1 and prevents spontaneous AR. Consequently, AR will result only from sperm–egg interactions, thus maximizing sperm fertilizing potential (Fig. 1c).

4 Embryogenesis

4.1 Oviductal Transport

At ovulation, the secondary oocyte at its metaphase II stage is released into the oviduct, where it can be fertilized thus completing meiosis. There has been an accumulation of evidence that, in order to allow normal tubal transport, an eCB gradient is established (Sun and Dey 2008). In mouse oviduct, an increasing AEA longitudinal gradient from ampulla to isthmus is achieved by a higher NAPE-PLD/FAAH ratio in the isthmus compared to the ampulla (Guo et al. 2005; Wang et al. 2006), as depicted in Fig. 1c, d. Although most of the studies in this area have been limited to mice for obvious ethical reasons, recently, in a framework of studies on ectopic pregnancies, the ECS has been characterized in the human Fallopian tube (Horne et al. 2008; Gebeh et al. 2012). Higher levels of AEA and its congeners (i.e., PEA and OEA) were found in oviductal tissues of women with ectopic pregnancy, compared to those of nonpregnant women undergoing hysterectomy for medical reasons (Gebeh et al. 2012). NAPE-PLD and FAAH protein expression was found in the epithelium of human Fallopian tubes, and a significant reduction of FAAH mRNA was observed in ectopic pregnancy compared to controls (Gebeh et al. 2012). These data are in keeping with previous observations of a down-regulation of CB_1, but not of CB_2, in Fallopian tubes in ectopic pregnancy (Horne et al. 2008), and of a negative CB_1-mediated effect of AEA on oviduct transport (Wang et al. 2006). It is not clear whether CB_1de-regulation is a cause or an effect of ectopic pregnancy in humans, yet CB_1 clearly plays a key role in pregnancy. Overall, the most relevant outcome of recent studies is that higher AEA levels are associated with: (1) low blastocyst development, (2) oviductal retention of embryos, and (3) implantation failure (Wang et al. 2006; Sun and Dey 2012). Two main processes are involved in normal tubal transport: smooth muscle contraction and cilia beating (Halbert et al. 1976). The former process is under adrenergic control and, since CB_1 was found to co-localize with adrenergic receptors, it has been hypothesized that eCB effects on muscle contraction could be mediated by a regulation of adrenergic signaling (Wang et al. 2004). Additionally,

recent data have suggested that ciliary beat frequency in Fallopian tube epithelial cells is the most critical event in controlling transport of both gametes and fertilized eggs (Shi et al. 2011; Ezzati et al. 2014). Unfortunately, no studies have yet explored the role of eCBs in this process.

4.2 Implantation

Following fertilization, the egg undergoes series of cell divisions to form the blastocyst, which will nestle in the receptive uterus to grow and develop. Implantation is a critical step in pregnancy and starts with the attachment of the blastocyst trophectoderm to the luminal side of the uterus that, by developing protrusions named "pinopodes," promotes the adhesion process. Finally, the blastocyst invades endometrial cells to successfully complete the implantation process. It is clear that blastocyst development, competence, and uterine receptivity are inevitably synchronized events that are needed for successful implantation. Increasing evidence suggests that AEA could play a key role in all of this. Appropriate levels of AEA are ensured by a tight regulation in the NAPE-PLD/FAAH ratio (Paria et al. 1998; Guo et al. 2005). Recently, it has been demonstrated that both too high and too low levels of AEA are deleterious for pregnancy (Sun and Dey 2009). In vitro experiments demonstrated that high AEA levels inhibit the development of the blastocyst and its zona hatching in the mouse. Moreover, they inhibit trophoblast differentiation, whereas low levels of AEA accelerate trophoblast differentiation and outgrowth (Wang et al. 2006). On the maternal side, AEA and 2-AG are present in mouse uterus, and their concentration gradient is finely tuned by NAPE-PLD/FAAH and DAGLα/MAGL ratios, respectively (Wang et al. 2007). As a result, higher AEA levels are found at inter-implantation than at implantation sites (Paria et al. 2001; Guo et al. 2005) (Fig. 1e). These data are paralleled by high expression and activity of NAPE-PLD in inter-implantation sites compared to the implantation site (Guo et al. 2005) and by lower FAAH expression and activity at inter-implantation sites than at the implantation site (Wang et al. 2007). Also the DAGLα/MAGL ratio is higher at inter-implantation than at implantation sites (Wang et al. 2007). In this context, a correlation between AEA uterine content and the autophagic state of the blastocyst has been recently documented (Oh et al. 2013). Overall, these findings have prompted the speculation that the embryo may play a role in the regulation of eCB tone. Indeed, implanting blastocysts decreases the NAPE-PLD/FAAH ratio by releasing a "FAAH activator" that reduces AEA levels at the implantation site (Maccarrone et al. 2004; Guo et al. 2005). Of note, a successful implantation is affected not only by the concentration of eCBs but also by CB_1 activation (Wang et al. 2006). Absence of embryonic and maternal CB_1 causes asynchronous development and oviductal retention of embryos (Wang et al. 2006). Notably, a complex network of hormones, cytokines, and eCBs must be well balanced to guarantee proper folliculogenesis, embryo implantation, and development (Maccarrone 2009; Bambang et al. 2012). Not surprisingly, ovarian hormones influence AEA metabolism during reproductive

events, so that P stimulates FAAH activity, whereas E down-regulates NAPE-PLD activity (Maccarrone et al. 2001; Guo et al. 2005; Battista et al. 2008). Even human plasma AEA levels are regulated by FSH in the follicular phase of the menstrual cycle and by LH and E during ovulation, implantation, and early pregnancy (El-Talatini et al. 2010). Interestingly, circulating lymphocytes also play a critical role in human embryo implantation. The addition of AEA to human lymphocytes in vitro inhibits the release of leukemia inhibitor factor (LIF), further highlighting the tight interaction between the many processes that support early pregnancy (Maccarrone et al. 2001; Melford et al. 2014). Taken together, it is apparent that eCB signaling plays a key role in all the early phases of embryo development, in order to ensure a proper pregnancy outcome.

4.3 Placentation and Parturition

A correct blastocyst implantation is followed by a sequence of events that include uterine decidualization and embryo trophoectodermal invasion of uterine stroma. These events are preliminary to the development of the placenta that will be the vital link between mother and fetus. In humans, decidualization occurs during luteal phases of the uterine cycle, independently of fertilization; in mice, the same event is stimulated after blastocyst implantation (Ramathal et al. 2010). Blastocyst implantation occurs in a very short time frame, that in turn is limited by decidualization triggered by the same blastocyst whose docking prevents implantation at other sites. When decidualization ends, placentation begins, a process in which the blastocyst is organized into outer trophoblastic cells (TCs), inner cell mass (ICM), and a blastocoelic fluid (BF). To work properly, the placenta requires a correct distribution of TCs, that all derive from trophoblast stem (TS) cells. Aberrations in trophoblast differentiation compromises normal placentation (Sun et al. 2010). In this context, it has been demonstrated that AEA controls the fate of TS cells via CB_1 (Sun et al. 2010). In particular, both hyper- and hypo- eCB signaling compromise normal pregnancy at different stages (Sun et al. 2010). Indeed, high AEA influences trophoblast invasion by causing decreased fibronectin-binding activity of the blastocyst; in contrast, low AEA activates mitogen-activated protein kinase (MAPK) signaling, suggestive of an important role in trophoblast behavior (Sun et al. 2010). These observations are in agreement with a recent study showing slower proliferation of CB_1/CB_2 null TS cells compared to wild types (Xie et al. 2012). CB_1 and AEA metabolic enzymes are also expressed during human placental development (Chamley et al. 2008; Taylor et al. 2011), and the whole ECS has been characterized in murine placentas (Sun et al. 2010). CB_1 and FAAH have also been identified in human amniotic epithelial cells, chorionic cytotrophoblasts, and syncytiotrophoblasts (Park et al. 2003; Habayeb et al. 2008). Evidence has emerged that the final outcome of pregnancy correlates with altered levels of CB_1, NAPE-PLD, and FAAH in first trimester placentas and, more generally, with high levels of AEA (Park et al. 2003; Trabucco et al. 2009; Meccariello et al. 2014) (Fig. 1f).

Higher levels of AEA are also detected in the plasma of nonviable than of viable pregnancies (Taylor et al. 2011). In line with this, *cnr1* and *faah* null mice showed pregnancy loss at different stages of midgestation (Sun and Dey 2012).

Interestingly, in the peak phases of rat decidua development, high NAPE-PLD activity elevates AEA levels that are probably involved in decidua remodeling (Fonseca et al. 2013, 2014). In contrast, FAAH activity prevails during placentation, corroborating the sound concept that this AEA-hydrolase is a key regulator of early pregnancy signaling (Fonseca et al. 2014). During endometrial development, other substances such as steroid hormones and cytokines (i.e., interleukin 11) are engaged to guarantee a correct decidual response (Dimitriadis et al. 2002; Paiva et al. 2011) and thus proper and timely pregnancy. All these findings confirm that a tight regulation of eCB signaling is essential for normal trophoblast development and placentation and hence for a healthy pregnancy.

In humans, successful full pregnancy occurs after ~40 weeks of gestation and pregnancy, whereas less than 37 weeks of pregnancy is considered preterm. Preterm birth is the leading cause of infant death in developed countries, and its causes remain unclear. However, many factors seem to affect this complication: genetic and environmental factors, viral infections, drug abuse, and alcohol consumption (Kourtis et al. 2014; Sowell et al. 2014). Normally, labor is the last stage of pregnancy where maternal inflammatory signals and fetal hormones cooperate to allow childbirth. P and corticotrophin hormone (CRH) are the main mediators in labour. P is essential for maintenance of myometrial quiescence, and its release induces labour to such an extent that it has been proposed for the prevention of preterm birth (Dodd and Crowther 2010; Tan et al. 2012; Areia et al. 2013). In agreement with this, a decreased P/E ratio has been reported to set the timing of parturition in rodents (Mesiano and Welsh 2007). Also placental CRH plays a critical role during pregnancy, by adjusting length of gestation and the time frame of delivery (Grammatopoulos 2007; Iliodromiti et al. 2012).

It should be recalled that in mice, genetic or pharmacological ablation of CB_1, but not of CB_2, causes preterm birth (Wang et al. 2008). Incidentally, *cnr1*$^{-/-}$ mice have altered P and E levels, leading to a decreased P/E ratio that impairs normal parturition. Premature birth in mice lacking CB_1 can be restored by subcutaneous injection of P just before they give birth (on day 18), further supporting the involvement of CB_1 in P and E regulation of pregnancy (Wang et al. 2008). Another interesting finding is the regulation of fetal PGE_2 production by eCBs via CB_1 activation (Mitchell et al. 2008). PGE_2 is involved in the early stage of labour, and its main source is the amnion. Since PGs are products of eCB hydrolysis (that releases arachidonic acid) and subsequent oxidative metabolism by COX-2, once again regulation of the eCB content impacts on late human pregnancy with implications for timing and progression of labour (Mitchell et al. 2008). In line with this, increased plasma AEA levels and low placental CB_1 expression have been observed in women in labour compared to women not in labour (Acone et al. 2009; Nallendran et al. 2010). Overall, alterations of eCB signaling are related to defects in various stages of reproduction that are clinically diagnosed as subfertile conditions. Therefore, eCBs must be kept at physiological levels from

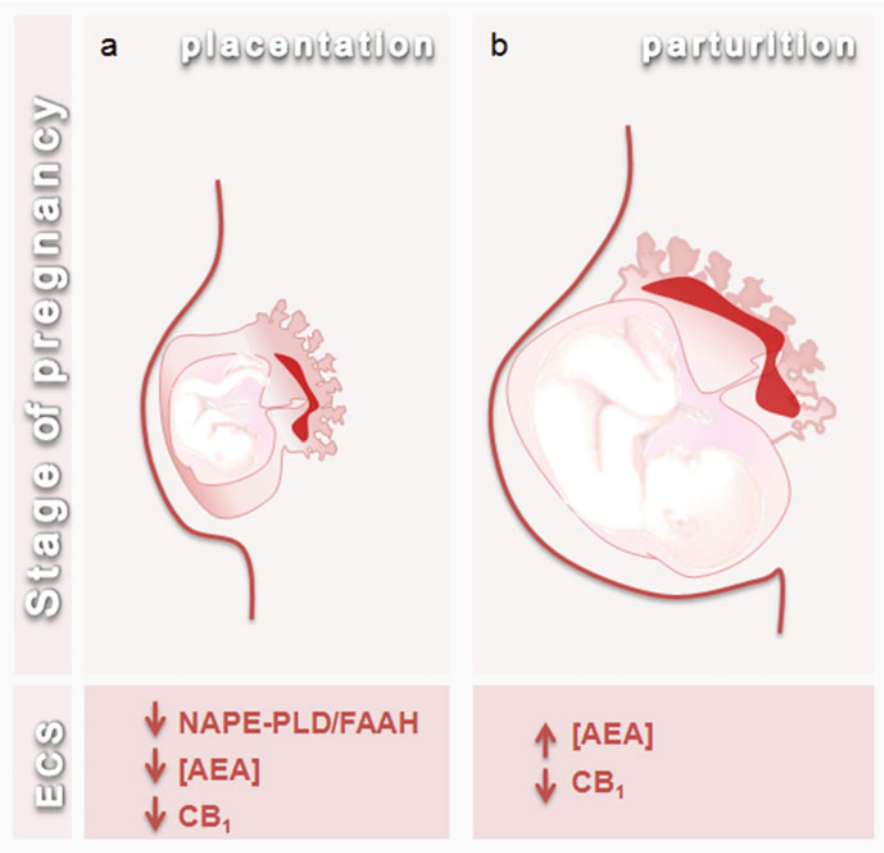

Fig. 3 Role of ECS elements in pregnancy. (**a**) *Placentation:* In the first trimester of pregnancy, a decreased NAPE-PLD/FAAH ratio and high CB_1 expression are required to ensure a successful pregnancy. (**b**) *Parturition:* In the late stage of pregnancy, high AEA levels and low CB_1 expression are essential for a timely onset of labour

the early phases of pregnancy to gestation and parturition, in order to warrant healthy pregnancy and successful delivery. The main alterations of ECS elements, that negatively affect placentation and parturition, are shown in Fig. 3.

5 The Endocannabinoid System as a Biomarker of Infertility

Data described in the previous sections highlight that alterations of distinct ECS components, and/or dysregulation of eCB tone in various stages of reproduction, may impair fertilization and the beginning of a new life. Therefore, they point to ECS elements/eCBs as biomarkers for the diagnosis of reproductive defects and/or

as therapeutic targets for the treatment of human infertility in clinical practice (Maccarrone 2009; Di Blasio et al. 2013; Rapino et al. 2014).

On the female side, follicular fluid withdrawn from women undergoing IVF/ICSI treatment contains higher AEA concentrations when obtained from follicles with mature oocytes than when obtained from follicles with immature oocytes (El-Talatini et al. 2009a), suggesting that higher AEA levels are required to guarantee proper follicular maturation and development (Bagavandoss and Grimshaw 2010). During early pregnancy, low levels of AEA, without differences between first and second trimesters with respect to the levels measured in the luteal phase of the menstrual cycle, are needed to promote uterine receptivity and pregnancy maintenance (El-Talatini et al. 2009b). Consistent with these data, elevated levels of AEA in blood, due to lower FAAH activity, were found in T lymphocytes of women who spontaneously miscarried in the first 8 weeks of pregnancy, compared to healthy controls (Maccarrone et al. 2000, 2001, 2002). Similarly, women who failed to achieve an ongoing pregnancy after IVF treatment, through either embryo transfer (Maccarrone et al. 2002) or ICSI (El-Talatini et al. 2009b), had higher plasma AEA concentration than women who became pregnant. Moreover, elevated AEA levels and reduced FAAH activity might also be predictive of early pregnancy complications due to incorrect attachment of the fertilized egg outside the womb, as in the case of ectopic pregnancy. Since other ECS elements, and notably the AEA biosynthetizing enzyme NAPE-PLD, are not affected in these pathological conditions, it can be suggested that AEA levels and/or FAAH activity could be a suitable biomarker to distinguish viable from nonviable pregnancies (Kuc et al. 2011; Kagan et al. 2012; Daponte et al. 2013; Nicolaides et al. 2013). Interestingly, it has been recently reported that, despite the involvement of the ECS in the control of nausea and emesis (Mechoulam and Parker 2013), plasma eCBs remain at physiological levels in women suffering from hyperemesis gravidarum (Gebeh et al. 2014). As a final note, a recent study by Abán and colleagues has shown differential expression of NAPE-PLD and FAAH in the syncytiotrophoblasts of normal and pre-eclamptic human placentas (Abán et al. 2013). Increased AEA could, via CB_1, affect nitric oxide synthase (NOS) activity, and thus NO production (Abán et al. 2013), as observed also in rat uterus during the peri-implantation period (Sordelli et al. 2011). Since NO is fundamental for implantation, and for maintaining low vascular resistance in the fetoplacental circulation, CB_1 could represent a novel target for the treatment of implantation deficiencies and preeclampsia.

On the male side, semen analysis, in which the concentration, motility, and morphology of sperm is monitored, has long represented the standard test for evaluating male fertility. The evaluation of the eCB content of human sperm cells and/or of seminal plasma could be predictive of sperm anomalies in humans, and thus could be used as a diagnostic tool in the field of reproductive medicine. In this context, a few studies have demonstrated that seminal plasma levels of eCBs and their cogeners decrease in infertile men with normozoospermia who were diagnosed with idiopathic infertility, asthenozoospermia, oligoasthenoteratozoospermia, and teratozoospermia (Lewis et al. 2012b; Amoako et al. 2013,

2014). Usually poor semen quality, such as a decreased sperm cell count and abnormal sperm motility, is directly associated with pathological semen subtypes (Lewis et al. 2012b; Amoako et al. 2013, 2014). However, these differences were not detected in these studies (Lewis et al. 2012b), pinpointing seminal plasma eCB content as a new biomarker of male reproductive defects. Interestingly, TRPV1 is the only eCB target receptor whose activity was markedly less in infertile than in fertile human sperm (Lewis et al. 2012b). These dysfunctions could lead to reduced fertilizing capacity of sperm in infertile subjects, and could be at least partly responsible for their oligospermia, as a consequence of TRPV1-triggered apoptosis (Mizrak et al. 2008). On this basis, one might speculate that reduction of AEA causes infertile sperm cells to lose their quiescent state and consequentially their ability to prevent premature capacitation. This condition could then precipitate a premature AR, making sperm infertile because of a reduced ability to penetrate the oocyte in vivo, as well as in assisted conceptions like IVF.

Incidentally, it should be recalled that semen analysis is not yet a perfect test, as it fails to accurately predict fertility status in certain situations (Guzick et al. 2001; Jequier 2010) nor does it take into account functional aspects of sperm, like the ability to fertilize the oocyte. Over the past few years, a number of tests have been developed to evaluate more specifically sperm DNA quality, which may be more informative and clinically relevant than semen analysis alone (Vasan 2011). In this context, recent studies have explored the possible involvement of CB_1 signaling in chromatin remodeling of mouse spermatids (Cacciola et al. 2013a, b). Genetic inactivation of *cnr1* appears to reduce both histone displacement and transient protein 2 (*Tnp*2) gene expression that have a direct role in maintaining DNA integrity (Chioccarelli et al. 2010). Against the concept that histone retention and poor chromatin quality in spermatids might negatively affect nuclear size elongation of mature sperm (Dadoune 2003; Johnson et al. 2011), it was reported that in $cnr1^{-/-}$, sperm nuclear length may become a valuable biomarker to identify morphologically normal sperm with good chromatin quality (Cacciola et al. 2013a). As yet, evaluation of sperm DNA integrity does not represent a useful criterion, in clinical practice of reproductive medicine, for predicting the final outcome of pregnancy through natural or artificial conception (Bartoov et al. 2002; Practice Committee of American Reproductive Society 2013).

On a final note, the AEA congener PEA also seems to play a role in the development of sperm hyperactivation during capacitation. Sperm from men with idiopathic infertility are more susceptible to PEA treatment and acquire a very energetic motility compared to controls. PEA-induced changes might help to switch sperm motility, from progressive to hyperactivated, and to provide sperm with more strength for penetrating the extracellular matrix of the egg (Ambrosini et al. 2005). These effects could be due to perturbations in the physicochemical properties of the lipid bilayer of membranes (Ambrosini et al. 2003) or to a direct interaction between PEA and membrane-bound enzymes.

Altogether, these data point to eCBs as new biomarkers of male and female defects and suggest ways in which they could help in the management of infertility

and in combating reproductive defects in humans, through the development of new ECS-oriented drugs.

6 Conclusions and Future Directions

In conclusion, endogenous levels of eCBs and activity/expression of distinct ECS elements (like FAAH, CB_1, and TRPV1) have the potential to provide useful diagnostic biomarkers and therapeutic targets of fertility defects (Maccarrone 2013; Rapino et al. 2014). Since some alterations of the ECS in blood cells mirror defects observed in reproductive organs, assays of FAAH activity/expression and of AEA content in easily accessible cells could be used in combination with other conventional diagnostic tests (e.g., progesterone or human chorionic gonadotropin assays) to track the progress of a normal pregnancy, to determine complications related to this process and to evaluate semen quality.

New perspectives on the therapeutic exploitation of PEA have recently emerged from studies with dietary supplements that contain this eCB-like compound in different amounts and are commercially available under different brand names (i.e., PeaVera or Normast). These medications are mainly painkillers for the treatment of neuropathies, but recently a PEA-containing cream appeared on the market as a useful treatment also for chronic vulvodynia (Keppel Hesselink et al. 2014). Indeed, some clinical cases of infertility *sine causa* (approximately 1 in 8 couples) might be due to unconsummated sexual relationships, caused by involuntary contractions of the pelvic floor muscles that provoke pain, burning, and stinging during intercourse. Therefore, the use of these PEA-containing products could help in the care of a problem (like infertility) that could be actually secondary to a central one (like vulvodynia/vaginismus).

Funding This investigation was partly supported by Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2010–2011 grant) to M.M.

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Endocannabinoids and Metabolic Disorders

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_13

Abstract

The endocannabinoid system (ECS) is known to exert regulatory control on essentially every aspect related to the search for, and the intake, metabolism and storage of calories, and consequently it represents a potential pharmacotherapeutic target for obesity, diabetes and eating disorders. While the clinical use of the first generation of cannabinoid type 1 (CB_1) receptor blockers has been halted due to the psychiatric side effects that their use occasioned, recent research in animals and humans has provided new knowledge on the mechanisms of actions of the ECS in the regulation of eating behavior, energy balance, and metabolism. In this review, we discuss these recent advances and how they may allow targeting the ECS in a more specific and selective manner for the future development of therapies against obesity, metabolic syndrome, and eating disorders.

Keywords

CB_1 receptor • CB_2 receptor • Eating disorder • Endocannabinoid • Metabolic disorder • Obesity

Abbreviations

2-AG	2-Arachidonoylglycerol
ACC1	Acetyl coenzyme-A carboxylase-1
AEA	Anandamide
AMPK	AMP-activated protein kinase
AN	Anorexia nervosa
BAT	Brown adipose tissue
BMI	Body mass index
BN	Bulimia nervosa
CB_1	Cannabinoid type 1
CB_1-KO	Mice with genetic deletion of CB_1
CB_2	Cannabinoid type 2
CNS	Central nervous system
DHA	Docosahexaenoic acid
ECS	Endocannabinoid system
EPA	Eicosapentaenoic acid
ERK 1/2	Extracellular-signal regulated kinase 1/2
FAK	Focal adhesion kinases
FAS	Fatty acid synthase
IRS	Insulin receptor substrate
MCH	Melanin-concentrating hormone
MEK 1/2	Mitogen-activated protein kinase kinase 1/2
MGL	Monoacylglycerol lipase
mRNA	Messenger ribonucleic acid

NAc	Nucleus accumbens
NTS	Nucleus of the solitary tract
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
PKB	Protein kinase B
PPARγ	Peroxisome proliferator-activated receptor-γ
PUFAs	Polyunsaturated fatty acids
PVN	Paraventricular nucleus
SF1	Steroidogenic factor 1
Sim1	Single-minded homolog 1
SNS	Sympathetic nervous system
TRPV1	Transient receptor potential vanilloid 1
VTA	Ventral tegmental area
WAT	White adipose tissue
WT	Wild-type
Δ^9-THC	Δ^9-tetrahydrocannabinol

1 Introduction

Obesity, a condition characterized by the pathological accumulation of fat mass, is a complex multifactorial disease in which several aspects spanning from the biological vulnerability of the individual to the interaction with the environment play a role. Obesity is a risk factor for several chronic pathologies, including metabolic disorders such as hyperlipidemia and type 2 diabetes, which nowadays represent a major medical, social, and economic burden (Smyth and Heron 2006). Obesity has now reached pandemic proportions, and it is estimated that ~58 % of the world population will become overweight or obese by the year 2030 (Kelly et al. 2008). However, only a few pharmacological therapeutic options are currently available for tackling this obesity pandemic (Yanovski and Yanovski 2014). Moreover, similar to obesity, eating disorders such as bulimia nervosa (BN) and anorexia nervosa (AN) are diseases for which novel and more efficient treatments are urgently needed (Hoek and van Hoeken 2003; Monteleone and Maj 2013). Thus, in recent years, scientific research has intensely focused on the unravelling of the biological mechanisms that regulate energy balance and metabolism in the hope of identifying suitable targets for therapy.

Within the mammalian brain, a complex and redundant series of circuits collect and integrate information from the environment and the peripheral organs in order to constantly match feeding behavior and peripheral metabolism with the caloric needs of the organism and guarantee its energy requirements. The endocannabinoid system (ECS) has been recognized to play a critical role in the regulation of energy balance and metabolism in mammals, since it exerts a regulatory control essentially on every aspect related to search, intake, metabolism and storage of calories. Activation of the ECS generally leads to changes that favor positive energy balance,

such as the promotion of the intake of food, particularly if palatable and calorically rich, the stimulation of fat mass expansion, and the inhibition of energy expenditure and thermogenesis. Consequently, ECS overactivity is a landmark feature of obesity and metabolic disorders (Quarta et al. 2011; Bermudez-Silva et al. 2012).

The best characterized endocannabinoids, *N*-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), are both known to increase food intake in animal models, generally through the activation of CB_1 receptors. The latter are expressed throughout the central nervous system (CNS) in neurons that regulate food intake, energy expenditure, and reward-related responses as well as in peripheral organs participating to the regulation of metabolic homeostasis, such as the gastrointestinal tract, adipose tissue, the liver, and muscle. On the other hand, inhibition of CB_1 receptor activity is beneficial for the treatment of obesity and related metabolic disorders (Quarta et al. 2011; Bermudez-Silva et al. 2012; Silvestri and Di Marzo 2013). Mice with genetic deletion of CB_1 (CB_1-KO) are lean and resistant to diet-induced obesity (Cota et al. 2003a, b; Ravinet Trillou et al. 2004). Accordingly, synthetic compounds like the CB_1 receptor antagonist rimonabant have shown potential for the therapy of obesity and diabetes in both animal models and patients (Cota et al. 2003a, b; Despres et al. 2005; Van Gaal et al. 2005; Pi-Sunyer et al. 2006; Scheen et al. 2006; Bermudez-Silva et al. 2012). However, due to the important psychiatric side effects related to its ability to pass the blood-brain barrier and exert its action on CNS CB_1 receptors, the early enthusiasm for the clinical use of rimonabant faded away and the drug, which was initially approved as an antiobesity treatment in several countries including the European Union, was withdrawn from the European market in January 2009. These events, while profoundly influencing the pharmaceutical industry, have also prompted further investigations into the mechanisms of action of the ECS in energy balance and metabolism with the aim of gaining new knowledge that would allow the targeting of this system in a more specific and selective manner.

2 Endocannabinoids and Their Role in Gustation and Olfaction

Sensory cues (the taste, the smell, the sight of food) are known to guide the organism towards food intake or food rejection (Scheen et al. 2006; Cornier et al. 2007; Herman and Polivy 2008). When food is introduced into the mouth, it is first sensed by the taste buds located on the papillae of the tongue. Gustatory neurotransmission from the oral cavity is carried by cranial nerves VII, IX, and X to the nucleus of the solitary tract (NTS) in the brainstem, which in turn sends projections to and receives information from the forebrain and peripheral organs (Grill and Hayes 2012). Gustatory signals from fats and sugar are thought to be particularly important for reward-related responses to food intake (DiPatrizio and Piomelli 2012). In the mouse, CB_1 receptor are to a great extent co-localized on the tongue with type 1 taste receptor 3, a putative sweet receptor (Montmayeur et al. 2001), and recent studies suggest that CB_1 receptor-dependent

endocannabinoid signaling enhances neural responses to sweet taste (Yoshida et al. 2010). In fact, peripheral administration of endocannabinoids increases the neural activity elicited in the chorda tympani by sweeteners, but not by bitter, umami, salty, or sour compounds. This effect is absent in CB_1-KO mice and can be observed also in vitro, after direct application of endocannabinoids to taste cells, implying that local endocannabinoid signaling in the oral cavity modulates sensitivity to sweet taste (Yoshida et al. 2010). Accordingly, CB_1 receptor signaling may also be involved in the plastic development of sweet preference (Yoshida et al. 2010). Of note, endocannabinoids (AEA and 2-AG) and related *N*-acylethanolamines produced together with endocannabinoids such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are quantifiable in human saliva (Matias et al. 2012b), and their levels are significantly higher in obese patients as compared to normal weight subjects (Matias et al. 2012b). Fasting salivary AEA and OEA levels are actually positively correlated with body mass index (BMI), waist circumference, and fasting insulin levels (Matias et al. 2012b). These data are preliminary and will require further investigation. Even so, it is possible that salivary endocannabinoids might participate to the modulation of orosensory information. In particular, since dietary fat can influence the endocannabinoid and *N*-acylethanolamines pool in different tissues (Artmann et al. 2008), the composition of the meal and the specific presence of fat might affect salivary endocannabinoid and *N*-acylethanolamines levels, which in turn might modulate taste signaling and orosensory processes. Even though evidence for a direct link between CB_1 receptor activation in the oral cavity and neural responses to fat is currently missing, it should be mentioned that a relation exists between dietary fat preference, fat ingestion, and endocannabinoids produced at the level of the gastrointestinal tract (see also subsequent section). Furthermore, recent studies suggest that oral detection and perception of dietary fats might be impaired in obese animal models and humans so that higher concentrations of nutritive fats might be required in order to initiate the consumption of food (Stewart et al. 2011; Stewart and Keast 2012; Chevrot et al. 2013).

A tight relationship also exists between perception of smell and food intake (Rolls 2005; Yeomans 2006). Accordingly, malfunctioning of the olfactory system can be found in diseases characterized by alteration of feeding behavior, such as AN (Rapps et al. 2010), and obesity also seems to be associated with altered food olfactory-driven behavior (Karine et al. 2014). In particular, it has been shown that chronic consumption of a high-fat diet causes structural and functional changes in olfactory circuits resulting in impairments in olfactory discrimination and olfactory learning in mice (Thiebaud et al. 2014). However, whether these altered responses causally participate in the development of obesity is at present not known. Of interest, Soria-Gomez and colleagues have recently demonstrated that olfactory circuits are regulated by endocannabinoid signaling, with the net result of increasing odor sensitivity and consequently food intake (Soria-Gomez et al. 2014). Prolonged fasting induces CB_1 receptor activation in olfactory circuits, thus determining olfactory performance and amount of food ingested once mice are reexposed to food. More specifically, fasting induces an increase in

endocannabinoid levels in the olfactory bulb, activating CB_1 receptors on olfactory cortex axon terminals and consequently reducing excitation of granular cells in the olfactory bulb, overall leading to an increase in odor detection and food intake (Soria-Gomez et al. 2014). Thus, published evidence available so far, although slim, suggests that endocannabinoid signaling through CB_1 activation enhances taste and olfactory responses to (certain types of) food, implying that deregulation of these endocannabinoid-dependent responses might have a role in obesity and eating disorders.

3 Gastrointestinal Endocannabinoids and Their Role in Food Intake and Metabolic Responses

When food is introduced into the mouth, anticipatory responses (also called cephalic phase responses) are elicited to enhance digestion and metabolism of a meal. Cephalic phase responses are initiated by the CNS to cues that reliably predict the opportunity to eat and digest nutrients (Smeets et al. 2010). These responses can be studied in rodents using, for example, the sham-feeding model in which the physiological effects of the orosensory properties of food can be separated from its post-ingestive qualities. Using this experimental model, it has been shown that gut-derived endocannabinoids regulate the intake of food and particular fat based on its orosensory properties (DiPatrizio et al. 2011, 2013). Sham-feeding rats with a high-fat liquid meal increases AEA and 2-AG levels specifically in the jejunal part of the small intestine, but not in other portions of the gastrointestinal tract or in the brain (DiPatrizio, et al. 2011; DiPatrizio et al. 2013). Changes in endocannabinoid levels are observed in response to fat intake and to a nutritionally complete diet, but not when animals are fed with meals containing significant amounts of proteins or carbohydrates (DiPatrizio et al. 2011). The intestinal increase in endocannabinoids in turn induces food consumption, since local blockade of CB_1 receptors in the small intestine with the CB_1 receptor antagonist rimonabant just before sham-feeding prevented food intake (DiPatrizio et al. 2011). Importantly, the sham-feeding effect on gastrointestinal endocannabinoids is lost after transection of the vagus nerve, implying that signals that originate in the oral cavity are transmitted to the brainstem, and then through the vagus to the intestine, where they induce the production of endocannabinoids (DiPatrizio et al. 2011; DiPatrizio and Piomelli 2012). It seems that certain types of fatty acids may be responsible for the cephalic phase of gut endocannabinoid production, since sham-feeding emulsions containing oleic acid or linoleic acid caused, on average, a nearly twofold accumulation of jejunal endocannabinoids (DiPatrizio et al. 2013; DiPatrizio 2014). Moreover, in a two-bottle-choice sham-feeding test, rats displayed strong preference for emulsions containing linoleic acid, which was blocked by pretreatment with a peripherally restricted CB_1 cannabinoid receptor antagonist (DiPatrizio et al. 2013; DiPatrizio 2014). These data therefore suggest that mobilization of endocannabinoids in the gut is essential for fat preference (DiPatrizio et al. 2013). In a recent article, DiPatrizio actually suggests that the intake of food rich in

linoleic acid rather than saturated fats might drive obesity, since the presence of linoleic acid in western diet has steadily increased over this past century and is associated with the increase in obesity rates (DiPatrizio 2014). However, whether the excessive consumption of food rich in linoleic acid may have a causative role in the obesity epidemic is unknown. Nevertheless, the evidence reviewed above suggests that endocannabinoids in the gut may be critically involved in favoring food intake and particularly the consumption of fatty, palatable food. Of note, in a recent study carried out in healthy, normal weight humans, Monteleone and colleagues have shown that the consumption of food for pleasure is associated with increased plasma levels of both ghrelin and the endocannabinoid 2-AG (Monteleone et al. 2012). The exact mechanisms linking such increases with the feeding behavior will require further investigations, but it is possible that gut-produced ******endocannabinoids might have a role.

Finally, recent evidence suggests that gut microbiota might impact gut endocannabinoids and consequently metabolic responses (Muccioli et al. 2010; Cani et al. 2014). Gut microbiota is known to influence energy balance and metabolism (Cani et al. 2014). Muccioli and colleagues have demonstrated that gut microbiota controls the intestinal ECS tone, which in turn modulates gut permeability (Muccioli et al. 2010). In particular, CB_1 receptor stimulation in the gut increases gut permeability, which in turn leads to increased lipopolysaccharide levels and exacerbates gut permeability and ECS tone in both the gastrointestinal tract and white adipose tissue (WAT), favoring body weight gain (Muccioli et al. 2010). However, seemingly in contrast with these initial observations, the same research group later demonstrated that beneficial gut microbiota protecting from fat mass gain and insulin resistance also induce increased 2-AG content in the ileum (Everard et al. 2013). Thus, further studies are needed in order to better clarify the relationship between gut microbiota and the ECS.

4 The Regulation of Energy Balance and Metabolism by Peripheral Non-gastrointestinal Endocannabinoids

Up to CB_1 receptors were thought to be located exclusively in the CNS. Seminal work published that year by Cota and colleagues and Bensaid and colleagues demonstrated for the first time the functional presence of CB_1 receptors on white adipocytes (Bensaid et al. 2003; Cota et al. 2003a, b). Since then, several studies have investigated the function of the ECS in peripheral organs substantiating the role of this system in the regulation of lipid and glucose metabolism. CB_1 receptor activation in adipocytes in vitro promotes adipogenesis by increasing the expression of adipogenic enzymes and the activity of lipoprotein lipase, favoring the build-up of triglycerides-rich droplets (Bensaid et al. 2003; Cota et al. 2003a, b; Bouaboula et al. 2005; Matias et al. 2006). Endocannabinoids induce the expression of genes associated with adipocyte differentiation, such as peroxisome proliferator-activated receptor-γ (PPARγ), while pharmacological or genetic blockade of adipocyte CB_1 receptors promotes mitochondrial biogenesis in white adipocytes via an

endothelial nitric oxide synthase mechanism (Tedesco et al. 2008), and transdifferentiation of white adipocytes into a mitochondria-rich, thermogenic brown fat phenotype (Perwitz et al. 2010). Thus, while activation of adipocyte CB_1 receptors in vitro generally leads to increased fatty acid synthesis and triglycerides accumulation, the opposite is usually observed when blocking these receptors. Whether these changes are relevant in vivo is at present unclear, since modulation of WAT and brown adipose tissue (BAT) function can be driven by CB_1-dependent signaling originating from the CNS and engaging the sympathetic nervous system (SNS) (see also below, Sect. 5). However, preliminary data obtained using adipocyte-specific CB_1-KO mice do suggest that adipocyte CB_1 receptors favor WAT expansion and the development of obesity and insulin resistance in vivo (Mancini et al. 2010). Furthermore, endocannabinoid production in white adipocytes is under the negative control of both insulin and leptin (Matias et al. 2006; Buettner et al. 2008; D'Eon et al. 2008). Therefore, conditions leading to leptin and insulin resistance, such as prolonged consumption of high-fat diets, might favor ECS overactivity in the WAT, which in turn would further support fat accumulation and body weight gain.

As in white adipocytes, activation of CB_1 receptors in hepatocytes favors lipid accumulation and causes liver steatosis by inducing the expression of lipogenic enzymes, such as acetyl coenzyme-A carboxylase-1 (ACC1) and fatty acid synthase (FAS), and increasing de novo fatty acid synthesis (Osei-Hyiaman et al. 2005). Mice with specific deletion of CB_1 receptors in hepatocytes, gain body weight when consuming a high-fat diet, but are protected against liver steatosis, hyperglycemia, dyslipidemia and insulin resistance (Osei-Hyiaman et al. 2008). Accordingly, the beneficial effects of a peripherally-restricted CB_1 receptor antagonist on liver steatosis and insulin resistance depend upon the action of the compound on hepatocyte CB_1 receptors (Tam et al. 2010, 2012). Thus, endocannabinoid-dependent CB_1 receptor signaling in hepatocytes may be particularly relevant in the context of the regulation of lipid metabolism and insulin sensitivity (Silvestri and Di Marzo 2013). As for the molecular mechanisms linking CB_1 receptor (over)activation to insulin resistance, it has been proposed that activation of hepatic CB_1 receptors causes the upregulation of the inhibitory phosphorylation of insulin receptor substrate (IRS) and of the inhibitory dephosphorylation of insulin-activated protein kinase B (PKB) through the recruitment of an endoplasmic reticulum stress-dependent pathway (Liu et al. 2012). Finally, expression of hepatic CB_2 receptors is also induced in obese mice and activation of these receptors favors liver steatosis (Deveaux et al. 2009), suggesting that, together with activation of hepatic CB_1 receptors, activation of hepatic CB_2 receptors causes deregulation of hepatic function during consumption of obesogenic diets.

Like the adipose tissue and the liver, skeletal muscle and the endocrine pancreas also produce endocannabinoids and express the different ECS components. However, the role of the ECS in these two organs is much less clear. Muscle endocannabinoids levels and muscle CB_1 receptor expression are altered by high-fat diet consumption and in obesity (Silvestri and Di Marzo 2013). Activation of the ECS in muscle inhibits oxidative pathways and mitochondrial biogenesis (Tedesco

et al. 2010). Moreover, in isolated soleus muscle, CB_1 receptor activation inhibits both basal and insulin-stimulated glucose transport, while pharmacological blockade of CB_1 has the opposite effect (Lindborg et al. 2010). Other studies have also demonstrated that activation of CB_1 receptor negatively impacts the responsiveness of skeletal muscle to insulin through the modulation of the PI 3-kinase/PKB and of the Raf-MEK1/2-ERK1/2 intracellular pathways (Lipina et al. 2010).

The endocrine pancreas plays a critical role in guaranteeing glucose homeostasis. Several studies have demonstrated the presence of both CB_1 and CB_2 receptors in rodent and human islets, with species-dependent degree of expression (Li et al. 2011), and recent evidence suggests that CB_1 receptor signaling regulates not only insulin signaling but also insulin release. In particular, it has been shown that activation of CB_1 receptors in β cells recruits focal adhesion kinases (FAK) that, by allowing cytoskeletal reorganization, lead to exocytosis of secretory insulin vesicles (Malenczyk et al. 2013). Conversely, published evidence suggests that pharmacological CB_1 receptor blockade reduces insulin secretion in vitro only when this is elevated above normal, as a consequence of obesity or prolonged exposure to a high-fat diet, implying that under these conditions there is higher endocannabinoid tone in the endocrine pancreas, as found in other organs (Silvestri and Di Marzo 2013). CB_1 receptor blockade also seems to ameliorate β cell function in obesity by increasing β cell proliferation and mass (Kim et al. 2011). Indeed, CB_1 receptor activation stimulates apoptotic activity and β cell death (Kim et al. 2012). Moreover, macrophage infiltration of pancreatic islets and consequent inflammation, which plays a role in the pathogenesis of diabetes, is a phenomenon recently recognized to be under the control of CB_1 receptor activity. Peripheral CB_1 receptor blockade, in vivo depletion of macrophages or macrophage-specific knockdown of the CB_1 receptor restores normoglycemia and glucose-induced insulin secretion (Jourdan et al. 2013).

Thus, the information reviewed above strongly suggests that pharmacological interventions aimed at targeting peripheral CB_1 receptors might represent new attractive avenues for the treatment of diabetes.

In addition, endocannabinoids can be detected in the circulation and measurement of circulating endocannabinoids has been a favorite strategy for the study of the ECS in humans (Matias et al. 2012a). Increased peripheral ECS activity (i.e., increased plasma AEA and increased CB_1 mRNA expression in blood) has been found in patients affected by eating disorders, such as AN and BN (Monteleone and Maj 2013). Several human studies have demonstrated that plasma endocannabinoid levels correlate positively with markers of obesity and metabolic disorder, such as the BMI, the waist circumference, visceral fat mass and insulin resistance (Engeli et al. 2005; Bluher et al. 2006; Cote et al. 2007; Gatta-Cherifi et al. 2012). Indeed it has actually been suggested that plasma endocannabinoids might work as biomarkers of WAT distribution and insulin resistance (Silvestri and Di Marzo 2013). However, further studies are needed in order to determine whether this is indeed the case. Moreover, several issues still need to be addressed, including the requirement for standardized methods dealing with the extraction and measurement of endocannabinoids and the establishment of reference levels for plasma

endocannabinoids in humans, which might be affected by gender, among other factors (Fanelli et al. 2012). Other points that will also require further investigation are the possible participation of circulating endocannabinoids in signaling events that might in turn affect feeding and metabolism and the actual origin(s) of circulating endocannabinoids. A few studies have shown that circulating levels of endocannabinoids change in healthy humans in relation to food intake (Matias et al. 2007; Gatta-Cherifi et al. 2012). In particular, we have demonstrated that both normal weight and obese subjects have a significant preprandial AEA peak, suggesting that AEA might be a physiological meal initiator (Gatta-Cherifi et al. 2012). Surprisingly, no meal-related changes were found for 2-AG, suggesting that AEA and 2-AG might have distinct roles in response to food intake in humans (Gatta-Cherifi et al. 2012). As to from where the observed changes in AEA and 2-AG plasma levels originate, we have proposed as a likely candidate the gastrointestinal tract (Gatta-Cherifi et al. 2012), since this organ is directly exposed to the nutrients introduced through the diet and produces endocannabinoids when food is ingested (DiPatrizio et al. 2011).

5 CNS Endocannabinoids and the Regulation of Food Intake, Energy Balance and Metabolism

Within the CNS, endocannabinoids generally work as retrograde neuromodulators, produced at the postsynaptic terminals and acting on CB_1 receptors located on the presynaptic terminals, to inhibit both excitatory and inhibitory neurotransmission (Wilson and Nicoll 2001). Endocannabinoids therefore critically control neuronal activity. This includes neuronal activity of relevance to the topic of this review, since they regulate neuronal circuits in the hypothalamus, the main brain structure overseeing energy balance and peripheral metabolism (Morton et al. 2006), in cortico-limbic structures, which modulate hedonic aspects of food intake (Berridge et al. 2010), and in the brainstem, a hub structure relaying bi-directional information going from the brain to the periphery and coming from the periphery to the brain (Grill and Hayes 2012).

In animals, variations of endocannabinoid levels in the hypothalamus are in agreement with the general orexigenic role of the ECS. In particular, hypothalamic endocannabinoid levels increase with fasting and decrease during refeeding (Kirkham et al. 2002), and administration of AEA to the hypothalamus increases food intake through a CB_1 receptor-dependent mechanism (Jamshidi and Taylor 2001). CB_1 receptor signaling plays a key role in modulating the activity of several hypothalamic neuronal populations known to regulate energy balance. For instance, within the hypothalamic paraventricular nucleus (PVN), glucocorticoid-dependent production of endocannabinoids and consequent CB_1 receptor activation suppresses excitatory glutamatergic synaptic input to PVN neuroendocrine cells (Hill and Tasker 2012). Thus, glucocorticoids, which have a known appetite-stimulating action, modulate neuroendocrine responses involved in stress and energy balance via the ECS. Similarly, in the lateral hypothalamus the ability of melanin-

concentrating hormone (MCH) and orexins to modulate neuronal excitability and, most likely, food intake and arousal, depends upon retrograde endocannabinoid release (Haj-Dahmane and Shen 2005; Jo et al. 2005). Moreover, recent studies have demonstrated that CB_1 receptor activation has opposite effects on food intake depending on whether CB_1 receptors are localized on presynaptic terminals of excitatory or inhibitory neurons (Bellocchio et al. 2010). In particular, while the well-known orexigenic effect of the endocannabinoids depends on actions at CB_1 receptors located at the terminals of cortical glutamatergic neurons, CB_1 receptor signaling in brain GABAergic neurons suppresses food intake (Bellocchio et al. 2010). Thus, in order to explain the well-known orexigenic effects of endocannabinoids and CB_1 receptor agonists and the anorexigenic effects of CB_1 receptor antagonists, it is likely that CB_1-dependent inhibition of glutamatergic signaling predominates over the effect on GABAergic signaling.

By using a genetic strategy, we have also recently demonstrated that virally mediated knockdown of CB_1 mRNA expression within the adult mouse hypothalamus does not alter basal food intake, but does increase energy expenditure, leading to a lean phenotype (Cardinal et al. 2012). In other studies, we have also shown that basal food intake is not altered in chow-fed mice lacking CB_1 in Single-minded homolog 1 (Sim1)-expressing neurons, which constitute the majority of neurons of the PVN (Cardinal et al. 2015), or in mice lacking CB_1 receptors in steroidogenic factor 1 (SF1)-expressing neurons (Cardinal et al. 2014), which represents a neuronal population of the ventromedial hypothalamic nucleus critically involved in energy balance regulation (Dhillon et al. 2006). Interestingly, however, SF1-CB_1-KO mice are characterized by hypophagia when reexposed to food after a prolonged fast (Bellocchio et al. 2013) and show hyperphagia when fed a high-fat diet, suggesting that the specific role played by CB_1 receptor signaling in SF1-expressing neurons depends upon an interaction with other signals (i.e., hormones and nutrients) relevant to the regulation of fasting-induced food intake and upon the diet consumed.

The relationship between endocannabinoids and feeding-regulated hormones, and in particular the anorexigenic hormone leptin and the orexigenic hormone ghrelin, have also been intensely studied. Leptin is known to decrease hypothalamic endocannabinoids levels (Di Marzo et al. 2001), while genetic models lacking leptin, such as ob/ob mice, or characterized by defective leptin receptor signaling, such as db/db mice, are obese and have increased hypothalamic endocannabinoid levels (Di Marzo et al. 2001). Treatment of these mice with a CB_1 receptor antagonist attenuates the hyperphagia and retards the weight gain, implying that ECS overactivity may be a contributing factor in some forms of genetic obesity (Di Marzo et al. 2001). Additionally, electrophysiological studies have shown that the CB_1-dependent suppression of inhibition of orexigenic MCH neurons in the lateral hypothalamus is prevented by leptin, which inhibits endocannabinoid synthesis by reducing intracellular calcium levels in MCH neurons (Jo et al. 2005). Similarly, leptin prevents glucocorticoid-mediated endocannabinoid release in the PVN (Malcher-Lopes et al. 2006). We have also demonstrated that leptin requires hypothalamic CB_1 receptor signaling to exert its anorexigenic effect, since deletion

of hypothalamic CB_1 mRNA leads to the inability of the hormone to decrease food intake in mice (Cardinal et al. 2012). Furthermore, a link exists between the action of leptin on SF1-expressing neurons and CB_1 receptor signaling in this neuronal population. Indeed, deletion of CB_1 from SF1-expressing neurons increases sensitivity to the anorexigenic and metabolic effects of leptin during consumption of regular chow, while it leads to molecular leptin resistance when a high-fat diet is consumed (Cardinal et al. 2014).

The diet consumed, like the feeding status, also affects the structural plasticity or rewiring of hypothalamic circuits. Such rewiring is the consequence of changes in leptin signaling, among other factors (Pinto et al. 2004). In this context, it has been shown that presynaptic inputs expressing CB_1 receptors change from being excitatory to inhibitory in orexin neurons of diet-induced obese mice (Cristino et al. 2013). This rewiring is caused by leptin signaling impairment and leads to elevated retrograde disinhibition and increased activation of orexin neurons, which in turn might contribute to increased food intake and cause obesity (Cristino et al. 2013). In addition, increased hypothalamic endocannabinoid levels due to impaired leptin action might also lead to peripheral insulin resistance. In fact, central CB_1 receptor activation is sufficient to impair glucose homeostasis by altering insulin action in both liver and adipose tissue. Conversely, central CB_1 receptor antagonism restores hepatic insulin sensitivity in a model of overfeeding-induced insulin resistance (O'Hare et al. 2011).

Several studies have investigated the possible interaction between the ECS and the hormone ghrelin, because of their similar function in the regulation of energy balance. In 2004, Tucci and colleagues demonstrated that a sub-anorectic dose of the CB_1 receptor rimonabant abolished the orexigenic effect of an intra-PVN injection of ghrelin in rats, implying that endocannabinoids might mediate ghrelin's action on food intake (Tucci et al. 2004). Indeed, later studies have demonstrated that ghrelin requires functional CB_1 receptor signaling, which in turn may lead to the activation of AMP-activated protein kinase (AMPK), an intracellular fuel gauge known to mediate ghrelin's action within the hypothalamus (Kola et al. 2008; Stark et al. 2013). Furthermore, peripheral administration of anandamide or of synthetic CB_1 receptor agonists in rats increases plasma ghrelin levels and ghrelin secretion from the stomach (Zbucki et al. 2008). Thus, a feed-forward mechanism exists where the ECS favors secretion of ghrelin and the latter in turn increases hypothalamic ECS activity, with the overall result of stimulating food intake and body weight gain.

As well as modulating the activity of hypothalamic circuits, endocannabinoids also regulate the activity of circuits that control hedonic and motivational aspects of food intake. In particular, they may regulate the liking and the motivation to consume palatable food, by specifically acting in the nucleus accumbens (NAc). Endocannabinoids levels in the NAc are increased by fasting (Kirkham et al. 2002), and anandamide infusion into the shell of the NAc increases the liking and the intake of a sucrose solution in rats in a CB_1 receptor-dependent fashion (Mahler et al. 2007). Within the NAc and the ventral tegmental area (VTA), the ECS interacts with both the opioid and the dopaminergic system, which have key roles

in the regulation of reward-related responses. Systemic administration of the CB_1 receptor antagonist rimonabant inhibits the increase in dopamine release in the NAc shell caused by the consumption of a novel palatable food (Melis et al. 2007), while administration of Δ^9-THC facilitates the increase in dopamine release in the NAc shell in response to sucrose (De Luca et al. 2012). Thus, consumption of palatable food might increase endocannabinoids levels in the NAc shell, an effect that in turn induces dopamine release in this brain area. Such an effect might depend upon activation of CB_1 receptors on glutamatergic terminals, which by inhibiting glutamate release, would inhibit GABAergic neurons that project from the NAc to the VTA, to produce an overall disinhibition of VTA dopaminergic neurons (Maldonado et al. 2006).

Finally, there is some evidence that alteration in the functioning of reward-related brain areas and of the ECS in these areas might have a role in the physiopathology of eating disorders, such as AN, BN, and binge eating disorder (Monteleone and Maj 2013; Scherma et al. 2014). For instance, global CB_1 receptor availability is significantly increased in cortical and subcortical brain areas in AN patients compared with healthy control subjects (Gerard et al. 2011). Accordingly, in experiments using a rodent model of AN, increased binding of a ligand to CB_1 receptors has been detected in cortical and subcortical brain areas (Casteels et al. 2014). Thus, it has been proposed that CB_1 receptor upregulation in AN patients is a possible long-term compensatory mechanism to an underactive ECS in the CNS under anorectic conditions (Gerard et al. 2011). Interestingly, this is opposite to what has been observed for the peripheral ECS, the activity of which is found usually to be enhanced in patients affected by AN and BN (Monteleone and Maj 2013).

Overall, the evidence reviewed above clearly demonstrates that the ECS regulates feeding behavior by acting upon neuronal circuits located in the hypothalamus and reward-related structures, favoring intake of calories, particularly from palatable food. However, the role of the ECS in the regulation of energy balance by the CNS may not be limited to the modulation of signaling of CB_1 receptors located at the level of the neuronal membrane. Recent studies have demonstrated that CB_1 is also located on mouse brain mitochondrial membranes, where it regulates neuronal energy metabolism and neuronal function (Benard et al. 2012). CB_1 receptor agonists decrease respiration in brain mitochondria, and mitochondrial CB_1 contributes to the endocannabinoid-dependent depolarization-induced suppression of inhibition, a classic effect of endocannabinoids on synaptic plasticity that can be studied in the hippocampus (Benard et al. 2012). Furthermore, CB_1 receptors are also expressed by astrocytes (Bosier et al. 2013), which have been increasingly recognized to have important functions in the regulation of energy balance (Garcia-Caceres et al. 2012). We recently demonstrated that astroglial CB_1 receptors directly interfere with leptin signaling and its ability to regulate glycogen storage, thus representing a novel mechanism for regulating brain energy storage and neuronal functions (Bosier et al. 2013). Whether and how such mechanisms may impact whole body energy balance regulation is currently unknown.

Lastly, it should be mentioned that the diet consumed can have an impact on the ECS and its function within the CNS, since endocannabinoids are products of phospholipid-derived arachidonic acid, whose levels can be modified in response to n-3 and n-6 polyunsaturated fatty acids (PUFAs) consumed in the diet (Di Marzo 2008). For instance, lifelong n-3 PUFAs dietary insufficiency abolishes long-term synaptic depression mediated by endocannabinoids in structures such as the nucleus accumbens and prevents presynaptic CB_1 receptors from responding to endocannabinoids due to the uncoupling of their effector $G_{i/o}$ proteins, thereby impairing emotional behavior (Lafourcade et al. 2011).

6 Endocannabinoids and the Central and Peripheral Integration of Energy Balance Regulation

The brain must continuously communicate with peripheral organs in order to integrate information coming from the periphery and appropriately coordinate intake, storage, and use of calories. Several studies have recently highlighted how central endocannabinoid-dependent mechanisms may impact peripheral processes controlled by the SNS, such as energy expenditure, thermogenesis, and lipolysis (Verty et al. 2009; Quarta et al. 2010; Bajzer et al. 2011; Cardinal et al. 2012, 2014). Mice lacking CB_1 receptors in the forebrain and sympathetic neurons are resistant to diet-induced obesity because they display increased lipid oxidation and thermogenesis as a consequence of enhanced sympathetic activity associated with a decrease in energy absorption (Quarta et al. 2010). Hypothalamic circuits under CB_1 receptor-mediated control actually play a major role in the regulation of energy expenditure via the SNS.

We have recently shown that virally mediated knockdown of CB_1 receptor mRNA in the adult mouse hypothalamus causes a decrease in body weight gain mainly because of increased energy expenditure, while not altering basal food intake (Cardinal et al. 2012). Furthermore, if CB_1 receptors are deleted from Sim1-expressing neurons, such deletion protects from diet-induced obesity by driving increased expression of thermogenic genes in BAT and by inducing energy expenditure. These modifications seem to be due to increased SNS activity, since pharmacological blockade of β-adrenergic receptors or chemical sympathectomy, respectively, inhibit energy expenditure responses and abolish the obesity-resistant phenotype of Sim1-CB_1-KO mice (Cardinal et al. 2015). Additionally, deletion of CB_1 receptors from SF1-expressing neurons protects chow-fed mice from body weight gain by inducing increased BAT activity and lipolysis in WAT through heightened SNS activity (Cardinal et al. 2014). CB_1 receptors on SF1-expressing neurons actually act as an adaptive molecular switch to diet change and affect energy balance via SNS modulation in a direction that depends on the diet consumed. In fact, while chow-fed SF1-CB_1-KO mice are leaner than WT littermates, SF1-CB_1-KO fed a high-fat diet and gained more weight than their littermates due, at least in part, to a decreased SNS-driven lipolysis in the WAT (Cardinal et al. 2014). Moreover, genetic induction of 2-AG hydrolysis in the forebrain, by

overexpression of the 2-AG hydrolyzing enzyme monoacylglycerol lipase (MGL), causes resistance to diet-induced obesity by increasing both SNS-mediated BAT thermogenesis and mitochondrial density (Jung et al. 2012). Of note, the rapid (within 1 h) hypophagic effect caused by the peripheral administration of rimonabant also seems to require SNS activity. We recently demonstrated that rimonabant-induced hypophagia is fully abolished by peripheral blockade of β-adrenergic transmission (Bellocchio et al. 2013). Peripheral blockade of sympathetic neurotransmission also blunted central effects of CB_1 receptor blockade, such as fear responses and anxiety-like behaviors, implying that, independently of their site of origin, important effects of CB_1 receptor antagonism are expressed via activation of peripheral sympathetic activity. Thus, the evidence provided so far clearly pinpoints the existence of a strong link between the ECS and the SNS in the regulation of energy balance. However, whether this link is relevant for the pathogenesis of human obesity and metabolic disorders is still matter for further investigation.

7 The ECS as a Target for the Treatment of Metabolic and Eating Disorders

The evidence that we have reviewed so far suggests that the major pharmacological target through which the ECS can be modulated in obesity and metabolic disorders is the CB_1 receptor. Indeed, soon after the discovery of this receptor, specific antagonists were developed. The first of these was rimonabant (Rinaldi-Carmona et al. 1994), a systemically penetrant CB_1 receptor inverse agonist whose chronic administration in obese rodents and humans reliably decreases body weight and fat mass, while improving glucose homeostasis, insulin sensitivity, and associated cardiometabolic risks (Silvestri and Di Marzo 2012). However, the concern over possible neuropsychiatric side effects, which were more common than had been initially estimated from the clinical trials, such that the overall benefits are lower than the risks, led to the withdrawal of rimonabant from the market and to the dismissal of research programs pursuing the production and entry into the market of similar CB_1 receptor antagonists. Yet, taking once more into account the strong evidence that the ECS is strategically positioned to regulate every step affecting the intake and use of calories, the development of novel CB_1 antagonists, particularly characterized by limited or null ability to pass the blood–brain barrier, is being intensely pursued (Silvestri and Di Marzo 2012). Recent work by Tam and colleagues has demonstrated that a new peripherally restricted CB_1 receptor neutral antagonist does not alter behavioral responses mediated by CNS CB_1 receptors, but does reduce obesity by reversing leptin resistance and improving glucose homeostasis, hepatic steatosis, and plasma lipid profile in mice with genetic or diet-induced obesity (Tam et al. 2010, 2012). Moreover, CB_2 receptors have also recently emerged as a potential target for the treatment of certain metabolic disorders. Indeed, as has been briefly mentioned already, CB_2 receptor activation favors hepatic steatosis and insulin resistance, most likely by reducing heightened

inflammatory responses, since CB_2 receptors are expressed on immune cells (Deveaux et al. 2009; Agudo et al. 2010). However, additional work is needed in order to exactly understand the function of CB_2 receptors or of other receptors that can be activated by endocannabinoids, such as transient receptor potential vanilloid 1 (TRPV1) and PPARs, in the context of endocannabinoid-dependent regulation of energy balance and metabolism.

Since obesity is characterized by an up-regulation of endocannabinoid tone, increasing endocannabinoid degradation or interfering with endocannabinoid synthesis are other viable therapeutic strategies. For instance, diacylglycerol lipase inhibitors able to decrease 2-AG synthesis can inhibit food intake and body weight in mice (Bisogno et al. 2009, 2013). Another possible therapeutic strategy for reducing up-regulated endocannabinoid tone could also be decreasing the availability of endocannabinoid precursors. This could be achieved by increasing the levels of n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the diet. Indeed the increased intake of dietary n-3 PUFAs decreases endocannabinoid levels in visceral adipose tissue, liver, and heart and therefore reduces ectopic fat and associated inflammation in obese rats (Batetta et al. 2009). This dietary strategy might be helpful in humans, since it has been shown for instance that sheep cheese naturally enriched in n-3 PUFAs decreases plasma AEA levels and improves the lipid profile of hypercholesterolemic subjects (Pintus et al. 2013). Thus, the higher dietary consumption of n-3 PUFAs, particularly of EPA and DHA, may be a relatively easy way of regulating endocannabinoid levels in a manner that helps to prevent as well as to treat metabolic disorders. Finally, given the complexity and the redundancy of the biological systems that participate in the regulation of energy balance, it would be extremely relevant to investigate pharmacological approaches that would allow modulating two or more different therapeutic targets at the same time. This combinational strategy is an extremely attractive approach for the treatment of obesity and associated metabolic disorders (Day et al. 2009) and could be used to target one or more components of the ECS or the ECS together with other systems known to regulate energy balance and metabolic responses.

Lastly, the ECS might also represent a potential treatment target for eating disorders such as binge eating, BN and AN. For instance, chronic administration of rimonabant reduced feeding behavior in an animal model of binge eating disorder (Scherma et al. 2013) and the same drug was able to decrease body weight and binge eating behavior in obese patients affected by binge eating disorder (Pataky et al. 2013). In contrast, Verty and collaborators were able to demonstrate that Δ^9-THC attenuated weight loss in a rodent model of activity-based anorexia through a mechanism that likely involves a decrease in energy expenditure (Verty et al. 2011). However, previous clinical studies have failed to show an effect on weight loss in AN patients treated with Δ^9-THC, while depression and perfectionism, a personality trait often found in AN patients, were ameliorated by the treatment (Gross et al. 1983; Berry 2006).

8 Concluding Remarks

The evidence that we have reviewed here has clearly expanded knowledge about the role of the ECS in the regulation of feeding behavior, energy balance, and metabolism. The main conclusion from this overview is that the ECS seems to generally act in the body to preserve and store energy. This signaling system is advantageous in conditions in which the availability of food is limited or cannot be predicted. However, when food is available at all times (day and night), it becomes one of the biological systems that causes the development of eating disorders, obesity, and metabolic syndrome.

We also need to point out that we have focused mainly on the functions of CB_1 receptors, and of AEA and 2-AG, since most of the available information concerning the role of the ECS in energy balance is related to this specific cannabinoid receptor and these CB receptor ligands. As previously mentioned, however, endocannabinoids can also affect metabolism through the activation of PPARs or TRPV1, and endocannabinoid-related compounds are known to play a role in the context of energy balance regulation. For instance, OEA, a lipid related to AEA that decreases appetite and favors weight loss and lipolysis by acting through PPAR-α receptors, opposes metabolic effects of endocannabinoids induced by CB_1 receptor activation (Piomelli 2013). Furthermore, recent studies have highlighted the existence of allosteric endogenous modulators that are capable of enhancing or inhibiting the activity of CB_1 receptors in the presence of their ligands. This new class of endogenous compounds includes the anti-inflammatory lipid lipoxin A4 that, by strengthening anandamide-induced CB_1 receptor activation, works as an endogenous allosteric enhancer of the CB_1 cannabinoid receptor (Pamplona et al. 2012). In addition, the small peptide endocannabinoids (called pepcans) and the neurosteroid pregnenolone have been identified as endogenous allosteric inhibitors of the CB_1 receptor (Bauer et al. 2012; Vallee et al. 2014). Thus, the ability of endocannabinoids to bind to receptors other than CB receptors, the existence of endocannabinoid-related compounds that exert actions opposite to those of endocannabinoids, and the identification of other lipid and peptide ligands that modulate CB_1 receptor activity constitute a rather complicated scenario that requires considerable additional investigation so that the impact of the ECS on feeding behavior, energy balance, and metabolism can be fully understood.

Finally, available evidence suggests that the ECS remains an attractive target for therapy. New therapeutic strategies, such as the use of peripherally restricted CB_1 receptor antagonists or inhibitors of endocannabinoid synthesis, are currently being tested in rodent models, and other pharmacological ways of targeting the ECS (e.g., with CB_1 allosteric modulators) might also soon be explored in such models. Testing in larger animals and clinical trials will then be required in order to assess both the efficacy and the unwanted effects of these strategies. Nevertheless, there is strong optimism that such therapeutic approaches might successfully help in the battle against metabolic and eating disorders.

Acknowledgments This work was supported by INSERM, Aquitaine Region, University of Bordeaux, and University hospital of Bordeaux. The authors declare no competing financial interest.

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Endocannabinoids and the Cardiovascular System in Health and Disease

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_14

Abstract

The endocannabinoid system is widely distributed throughout the cardiovascular system. Endocannabinoids play a minimal role in the regulation of cardiovascular function in normal conditions, but are altered in most cardiovascular disorders. In shock, endocannabinoids released within blood mediate the associated hypotension through CB_1 activation. In hypertension, there is evidence for changes in the expression of CB_1, and CB_1 antagonism reduces blood pressure in obese hypertensive and diabetic patients. The endocannabinoid system is also upregulated in cardiac pathologies. This is likely to be cardioprotective, via CB_2 and CB_1 (lesser extent). In the vasculature, endocannabinoids cause vasorelaxation through activation of multiple target sites, inhibition of calcium channels, activation of potassium channels, NO production and the release of vasoactive substances. Changes in the expression or function of any of these pathways alter the vascular effect of endocannabinoids. Endocannabinoids have positive (CB_2) and negative effects (CB_1) on the progression of atherosclerosis. However, any negative effects of CB_1 may not be consequential, as chronic CB_1 antagonism in large scale human trials was not associated with significant reductions in atheroma. In neurovascular disorders such as stroke, endocannabinoids are upregulated and protective, involving activation of CB_1, CB_2, TRPV1 and PPARα. Although most of this evidence is from preclinical studies, it seems likely that cannabinoid-based therapies could be beneficial in a range of cardiovascular disorders.

Keywords

Artery • Atherosclerosis • Blood • Blood pressure • Blood–brain barrier • Heart • Hypertension • Vein

Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	Anandamide
ARA-S	*N*-arachidonoyl-L-serine
BBB	Blood–brain barrier
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
COX	Cyclooxygenase

eNOS	Endothelial nitric oxide synthase
FAAH	Fatty acid amide hydrolase
MAGL	Monoacylglycerol lipase
NADA	*N*-arachidonoyl dopamine
NO	Nitric oxide
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
PPAR	Peroxisome proliferator-activated receptors
PTX	Pertussis toxin
SHR	Spontaneously hypertensive rat
TBI	Traumatic brain injury
THC	Delta-9-tetrahydrocannabinol
TRPV1	Transient receptor potential vanilloid 1

1 Introduction

Cannabinoid receptors are widely distributed throughout the cardiovascular system. The CB_1 receptor is expressed in myocardium, human coronary artery, endothelial and smooth muscle cells and on pre-synaptic sympathetic nerve terminals innervating the cardiovascular system. CB_2 receptors have also been identified in the myocardium and in human coronary endothelial and smooth muscle cells. Endocannabinoids are produced in endothelial and smooth muscle cells and in cardiac tissue, and circulating levels of endocannabinoids are detectable in blood. Despite this, under normal conditions, it is unlikely that the endocannabinoid system plays a major role in the regulation of cardiovascular function. The evidence for this is that animals in whom either CB_1 (Mukhopadhyay et al. 2007), CB_2 (Batkai et al. 2007) or fatty acid amide hydrolase (FAAH, the main endocannabinoid degradation enzyme) (Pacher et al. 2005) has been knocked down have no major changes in cardiovascular function. However, it is clear that in many pathological conditions of the cardiovascular system, the endocannabinoid system is upregulated and appears to play an important, possibly protective, role. For example, mice in which FAAH has been knocked-out (which will increase endocannabinoid levels due to decreased degradation) have a reduced decline in age-related cardiac dysfunction and increased susceptibility to atherosclerosis (Batkai et al. 2007). Mice in which the CB_1 receptor has been knocked-out are more susceptible to chronic heart failure (Liao et al. 2013), and stroke (Batkai et al. 2007). CB_2-deficient mice have increased susceptibility to atherosclerosis (Netherland et al. 2010; Hoyer et al. 2011), stroke (Zhang et al. 2008) and cardiomyopathy (Duerr et al. 2014).

In the following article, I will review the role for the endocannabinoid system in cardiovascular function in health and disease, starting from the in vivo haemodynamic (changes in blood pressure and heart rate) effects of endocannabinoids, the role of endocannabinoids in modulating cardiac function, vascular and haematological (blood) function as well as neurovascular function. I will also discuss the evidence

for endocannabinoid involvement in hypertension, cardiovascular shock, myocardial infarction (heart attack), atherosclerosis, stroke and traumatic brain injury.

2 In Vivo Haemodynamic Response to Endocannabinoids

2.1 Blood Pressure and Heart Rate

Endocannabinoids have a complex effect on blood pressure and heart rate in animal studies, and the response observed is dependent on whether the animal is anaesthetised or conscious. Differences between anaesthetised and conscious responses may be due to altered basal levels of sympathetic activity observed with anaesthesia (Neukirchen and Kienbaum 2008), as many of the in vivo responses to endocannabinoids are mediated by modulation of the autonomic nervous system, through changes in both vagal and sympathetic activity.

In anaesthetised animals, application of anandamide (AEA) causes a triphasic response; rapid and transient bradycardia (fall in heart rate), a rapid and transient pressor response (increase in blood pressure) and a prolonged hypotensive phase (see Malinowska et al. 2012 for a detailed review). The initial bradycardic response to AEA is absent in transient receptor potential vanilloid 1 (TRPV1) knockout mice (Pacher et al. 2004) and is brought about by vagal activation (Varga et al. 1995). The mechanisms behind the brief pressor response are more complex and are likely to involve TRPV1, *N*-methyl-D-aspartate (NMDA) and beta2 (β_2) adrenoceptors (Malinowska et al. 2012). The prolonged hypotensive response to AEA in anaesthetised animals has been best characterised. This response is absent in CB_1 knockout mice (Jarai et al. 1999), and the location of the CB_1 receptors involved is likely to be on nerve terminals of the sympathetic nervous system inhibiting function at the level of the heart and vasculature. Endocannabinoids inhibit noradrenaline release via CB_1 activation in arteries (Deutsch et al. 1997) and in the mesenteric arterial bed (Ralevic et al. 2002). 2-arachidonoylglycerol (2-AG) administration to anaesthetised rats also causes a fall in blood pressure, although accompanied by tachycardia. Unlike AEA, the response to 2-AG is CB_1-independent and more likely to involve cyclooxygenase-catalysed metabolism to other vasoactive compounds (Jarai et al. 2000).

In contrast to its effects on anaesthetised animals, in conscious rats, AEA causes profound bradycardia, with a transient hypotension followed by a longer lasting pressor effect accompanied by vasoconstriction of the renal and mesenteric vascular beds (Stein et al. 1996; Gardiner et al. 2001, 2002, 2009). This is also accompanied by a hindquarter β_2-adrenoceptor-mediated vasodilator response (Gardiner et al. 2002). Gardiner and colleagues (2001, 2002) showed this complex haemodynamic effect results from increased circulating adrenaline acting via β_2-adrenoceptors and a CB_1-mediated increase in sympathetic activity. In contrast to data obtained from anaesthetised rats, there appears to be no role for TRPV1 activation (Gardiner et al. 2009). Looking at other endocannabinoid agonists in conscious animals, *N*-arachidonoyl dopamine (NADA) causes a similar triphasic

response to that seen in anaesthetised animals but with tachycardia accompanying the hypotensive phase, mediated by TRPV1 (Wang and Wang 2007). Oleamide has no effect on haemodynamics (Huitron-Resendiz et al. 2001).

The endocannabinoid system appears to be involved in the central control of blood pressure via the brainstem baroreceptor complex. The nucleus tractus solitarius is one site of termination of baroreceptor afferent fibres from arterial baroreceptors and cardiac mechanoreceptors. Cannabinoid CB_1 receptors are functionally expressed in the nucleus tractus solitarius (Himmi et al. 1998), and microinjection of AEA prolongs reflex inhibition of renal sympathetic nerve activity, suggesting an increase in baroreflex sensitivity, probably due to inhibition of GABAergic tone (Rademacher et al. 2003). AEA concentrations in the nucleus tractus solitarius increase after a phenylephrine-induced rise in blood pressure, supporting the physiological relevance of the endocannabinoid control of baroreflex activity (Seagard et al. 2004). Interestingly, this effect of AEA is blunted in hypertensive rats, possibly contributing to impaired baroreflex sensitivity (Brozoski et al. 2009).

It is worth remembering that animals in whom the CB_1, CB_2 or FAAH proteins have been knocked out have normal cardiovascular function, suggesting the endocannabinoid system plays a minimal role in the regulation of blood pressure and cardiac function under normal conditions. Similarly antagonists of CB_1 or FAAH/monoacylglycerol lipase (MAGL) inhibitors do not affect blood pressure and heart rate in conscious, normotensive animals (later in this review I will discuss how this is different in hypertensive animals), also shedding doubt on the role of the endocannabinoid system in the regulation of haemodynamics. However, recent evidence has pointed to a role for CB_1 in modulating sleep–wake cardiorespiratory control (Silvani et al. 2014). Mice lacking the CB_1 receptor had a significantly enhanced blood pressure and heart rate response to changes in sleep–wake cycles, and irregular breathing rhythms during sleep, suggesting further research is required to fully understand the role of the endocannabinoid system in all aspects of cardiovascular control.

There is very little evidence for a potential role of the endocannabinoid system in regulating blood pressure in humans in non-pathological situations. To my knowledge, there are no studies that have examined the acute haemodynamic effects of endocannabinoids in humans, although administration of a CB_1 receptor antagonist does not affect resting blood pressure in normotensive humans (Ruilope et al. 2008). However, a FAAH gene variant is associated with lower blood pressure in young males, suggesting a potential endocannabinoid role (Sarzani et al. 2008).

2.2 Endocannabinoids and Hypertension

In anaesthetised hypertensive rats, the prolonged hypotensive effect of AEA is enhanced compared to normotensive rats (Lake et al. 1997). CB_1 receptor agonists or FAAH inhibition also decreases contractility and normalises blood pressure in anaesthetised hypertensive animals (Bátkai et al. 2004). However, a similar

experiment with conscious, freely moving animals showed only a modest response to AEA, although the hypertensive rats had a CB_1-mediated bradycardic response to AEA not seen in the normotensive animals (Wheal et al. 2007). Nonetheless, Bátkai and colleagues (2004) also showed that CB_1 expression was significantly greater in both cardiac tissue and the aortic endothelium of spontaneously hypertensive rats (SHRs) than in normotensive controls, indicating at least that the expression of the endocannabinoid system is altered in hypertension.

Clinical studies with the CB_1 antagonist, Rimonabant, in the Rimonabant in Obesity trials demonstrated minimal effects on blood pressure in normotensive subjects, but much greater reductions in blood pressure in obese hypertensives and patients with type II diabetes (Ruilope et al. 2008) suggesting that excessive endocannabinoid activation of CB_1 receptors could underlie the patients' hypertension. To support this theory, several studies have shown positive correlations between circulating endocannabinoid levels and blood pressure. One study looking at potential correlations between circulating AEA and obstructive sleep apnea found that circulating AEA was a stronger determinant of blood pressure than sleep apnea severity, obesity, insulin resistance or inflammation (Engeli et al. 2012). Another study in females with depression showed that diastolic and mean arterial blood pressures were positively correlated with serum levels of AEA and 2-AG (Ho et al. 2012).

2.3 Endocannabinoids in Shock

Shock is characterised by a reduction in cardiac output, significantly reduced blood pressure and poor tissue perfusion. In 1997, Wagner and colleagues showed that a CB_1 receptor antagonist could prevent the fall in blood pressure associated with haemorrhagic shock and that AEA and 2-AG synthesised by monocytes and platelets were responsible for CB_1 activation in shock (Wagner et al. 1997). The same group went on to show a similar role for CB_1 in endotoxic shock (Varga et al. 1998) and in cardiogenic shock after a myocardial infarction (Wagner et al. 2001). The effects of the CB_1 receptor antagonist were not observed when administered centrally, indicating a peripheral mechanism of action. Activation of CB_1 on arteries to directly produce vasodilatation (see Sect. 4), as well as inhibition of sympathetic neurotransmitter release (see Malinowska et al. 2008 for a review), brings about vasodilatation and the drop in blood pressure. It is suggested that the release of endocannabinoids may play an important role in mediating cardioprotection in shock (see Sect. 3.1), however, it has also been shown that CB_1 antagonists decrease mortality in models of shock (Kadoi et al. 2005).

2.4 Summary on Haemodynamic Effects of Endocannabinoids

Endocannabinoids play a minimal role in the regulation of cardiovascular function in normal conditions, with the exception of modulation of baroreflex sensitivity and sleep–wake cardiorespiratory control at a central level. In various forms of shock, there is a clear role for activation of the CB_1 receptor by endocannabinoids released within blood in mediating the associated hypotension. In hypertension, there is evidence for both upregulation (cardiac tissue and the aortic endothelium) and downregulation (nucleus tractus solitarius) of the CB_1 receptor involved in the maintenance of high blood pressure and reduced baroreflex sensitivity. Human studies have shown that CB_1 antagonism reduces blood pressure in obese hypertensive and diabetic patients.

3 Endocannabinoids and Cardiac Function

The endocannabinoid system is expressed throughout the myocardium, and endocannabinoids are detected in cardiac tissue (see Tuma and Steffens 2012 for a review), playing roles in various aspects of cardiac function including contractility and regulation of coronary tone. AEA decreases contractile performance in human atrial muscle via CB_1 receptors (Bonz et al. 2003). This may be related to the finding that CB_1 activation in the heart decreases noradrenaline release, so less β_1 receptors will then be activated (Molderings et al. 1999). It is also related to the fact that AEA inhibits the function of voltage-dependent Na^+ and L-type Ca^{2+} channels in rat ventricular myocytes (Al Kury et al. 2014). AEA causes endothelium-dependent vasorelaxation of rat or sheep coronary arteries via CB_1 activation, with no role for endocannabinoid metabolites, CB_2 or TRPV1 (White et al. 2001; Ford et al. 2002; Grainger and Boachie-Ansah 2001). In contrast, palmitoylethanolamide (PEA) did not relax precontracted rat coronary arteries (White et al. 2001).

3.1 Cardioprotective Effects of Endocannabinoids

In cardiac pathologies, the endocannabinoid system is altered, and the majority of evidence suggests the increases in endocannabinoid levels in cardiac disorders are protective. AEA levels are transiently increased during ischaemia/reperfusion (Duerr et al. 2014). Patients with aortic stenosis have higher concentrations of AEA (Duerr et al. 2013). Chronic heart failure patients have elevated AEA and 2-AG levels (Weis et al. 2010). Cardiac levels of 2-AG, but not AEA, are increased in preconditioning (Wagner et al. 2006). However, acute stress was recently shown to decrease cardiac endocannabinoid levels (Holman et al. 2014), which might indicate that an upregulation of the endocannabinoid system in the heart is a chronic effect. Upregulation of cannabinoid receptors has been shown in cardiac pathologies, particularly CB_2, which is upregulated in chronic heart failure (Weis

et al. 2010), in aortic stenosis (Duerr et al. 2013) and in ischaemia/reperfusion (Duerr et al. 2014). CB_2 expression is under the control of microRNA-665 (miR-665), whose expression is increased in heart failure (Mohnle et al. 2014).

The hypothesis that endocannabinoids are protective in cardiac dysfunction comes from multiple pieces of evidence. Exogenous application of 2-AG (Wagner et al. 2006), PEA (Lepicier et al. 2003) or AEA (Underdown et al. 2005; Hydock et al. 2009; Li et al. 2013a) confers cardiac protection after various stressors in animal models. The majority of studies suggest that this is a CB_2 receptor-mediated event, although AEA has been shown to also have cardioprotective actions through CB_1. The importance of CB_2 in cardioprotection was highlighted in a recent paper which found that CB_2-deficient mice showed greater damage in response to repetitive periods of ischaemia/reperfusion leading to cardiomyopathy (Duerr et al. 2014). This was because the hearts of the CB_2 knockout mice had increased inflammatory responses, adverse remodelling, increased rates of apoptosis and an inability to turn on anti-oxidative enzymes (Duerr et al. 2014). CB_1 knockout mice are also more susceptible to a chronic heart failure model (Liao et al. 2013). Similarly, mice in whom FAAH has been knocked-out have reduced age-related cardiac dysfunction, indicating a cardioprotective role for locally produced endogenous cannabinoids (Batkai et al. 2007). In humans, a polymorphism of FAAH is associated with an increased risk of a myocardial infarction (Chmelikova et al. 2014).

In the heart, mild stress confers protection leading to a reduction in infarct size in response to subsequent stressors. This is known as preconditioning. A role for the endocannabinoid system has been well established in mediating cardiac preconditioning. Endotoxin preconditioning (Lagneux and Lamontagne 2001) and heat stress preconditioning (Joyeux et al. 2002) are attenuated by CB_2 receptor blockade, suggesting a protective role for locally produced endocannabinoids. Delayed preconditioning is also sensitive to CB_1 receptor blockade (Wagner et al. 2006).

Cardiac protection is conferred not only by endocannabinoids locally synthesised in the heart but also by circulating endocannabinoids. Remote ischaemic preconditioning is defined as transient brief episodes of ischaemia at a remote site before a subsequent prolonged ischaemia/reperfusion injury of the target organ. In the heart, remote ischaemic preconditioning reduces subsequent infarct volume, and this was inhibited by a CB_2, but not CB_1, antagonist, implicating a role for circulating endocannabinoids (Hajrasouliha et al. 2008).

The mechanisms by which endocannabinoids are cardioprotective include decreased neutrophil infiltration, decreased inflammation, decreased oxidative stress and increased activation of cardioprotective signalling pathways, through activation of CB_1 and CB_2 (Tuma and Steffens 2012). The cardioprotective effects of AEA involve the induction of heat shock protein 72 through the PI3K/Akt signalling pathway via CB_2 (Li et al. 2013a). CB_2 activation also inhibits mitochondria-mediated apoptosis via PI3K/Akt signalling in the myocardium after ischaemia/reperfusion injury (Li et al. 2013b).

3.2 Cardiodeleterious Effects of Endocannabinoids

There are also studies suggesting CB_1 receptor activation has negative effects on cardiac function. For example, CB_1 receptor antagonism reduces, and AEA enhances, the cardiotoxic effects of the chemotherapy drug doxorubicin in human cardiomyocytes (Mukhopadhyay et al. 2010). CB_1 activation by AEA in human coronary artery endothelial cells activates cell death (Rajesh et al. 2010). Daily treatment with the CB_1 antagonist Rimonabant has also been shown to reduce infarct size, and this effect was absent in $CB_1^{-/-}$ mice (Lim et al. 2009). Daily treatment with Rimonabant also improves systolic and diastolic heart function after permanent ligation of the left coronary artery (Slavic et al. 2013).

3.3 Endocannabinoids and Arrhythmias

CB_2 receptor activation reduces the incidence of ventricular arrhythmias during coronary occlusion (Krylatov et al. 2001). AEA also reduces epinephrine-induced arrhythmias, although this was CB_1 and CB_2 independent (Ugdyzhekova et al. 2001). However, more recently, neither AEA nor 2-AG were found to affect ischaemia-induced ventricular fibrillation, although a CB_1 antagonist (but not CB_2 antagonist) alone did have some positive effects during the later stage of acute ischaemia (Andrag and Curtis 2013). In isolated sinoatrial node samples from rabbits, AEA shortens the action potential duration and amplitude via CB_1 (Zhang et al. 2013). A similar effect of AEA, that resulted from an inhibitory effect on the functioning of voltage-dependent Na^+ and L-type Ca^{2+} channels, has been observed on the action potential of rat ventricular myocytes, although in these cells, the effect was independent of CB_1 and CB_2 receptors (Al Kury et al. 2014).

3.4 Summary of Cardiac Effects of Endocannabinoids

There is much evidence that the endocannabinoid system is upregulated in cardiac pathologies. The majority of evidence indicates this is likely to be cardioprotective, mainly through CB_2 activation, but with a role also for CB_1 activation. However, the role of CB_1 is controversial because in some situations, CB_1 activation may be detrimental in the heart.

4 Endocannabinoids and the Vasculature

CB_1 and CB_2 are widely distributed in the vasculature, observed in vascular smooth muscle and endothelial cells (Sugiura et al. 1998; Liu et al. 2000; Rajesh et al. 2007; Rajesh et al. 2008). The first in vitro report of endocannabinoid-induced vasorelaxation of isolated arteries and arterial beds came from Ellis and colleagues (1995) who showed that AEA and Δ^9-tetrahydrocannabinol (THC) cause

vasorelaxation of rabbit cerebral arteries, associated with an increase in vasoactive prostanoids. Many studies have since shown acute vasorelaxant responses (within minutes of application) to other endocannabinoid and endocannabinoid-like compounds including 2-AG, NADA, oleoylethanolamine (OEA), PEA, *N*-arachidonoyl-L-serine (ARA-S), *N*-arachidonoyl glycine and oleamide in a range of different arterial beds from different species (see Stanley and O'Sullivan 2014a). The mechanisms underlying these responses involve the activation of some, but not necessarily all, of the following targets/actions: CB_1, TRPV1, a site on the endothelium and modulation of ion channels. Some endocannabinoids also cause a time-dependent (over hours) vasorelaxant effect mediated by peroxisome proliferator-activated receptors (PPARs; O'Sullivan et al. 2009; Romano and Lograno 2012). The evidence for each of the pathways involved will now be discussed.

4.1 Role for CB_1

A potential role for CB_1 activation is one of the most commonly investigated mechanisms of action for the vascular effects of cannabinoids, and we know this underpins the hypotensive effects of endocannabinoids in shock. Vasorelaxation to AEA is inhibited by CB_1 receptor antagonism in renal arterioles (Deutsch et al. 1997; Koura et al. 2004), rat mesenteric arteries (White and Hiley 1998; O'Sullivan et al. 2004a), the perfused mesenteric bed (Wagner et al. 1999), bovine ophthalmic arteries (Romano and Lograno 2006), cat cerebral arteries (Gebremedhin et al. 1999) and the rabbit aorta (Mukhopadhyay et al. 2002). However, other studies have shown that CB_1 antagonism does not affect AEA-induced vasorelaxation in rat mesenteric arteries (Plane et al. 1997), the rat mesenteric bed (Peroni et al. 2004), rat hepatic arteries or guinea pig basilar arteries (Zygmunt et al. 1999) or the rat aorta (O'Sullivan et al. 2005). AEA is also capable of causing vasorelaxation of the same magnitude in the mesenteric bed of $CB_1^{-/-}$ as $CB_1^{+/+}$ mice (Jarai et al. 1999), suggesting other pathways can compensate when CB_1 is blocked or absent. Vasorelaxation induced by NADA, OEA and oleamide are all at least partly mediated by CB_1 (see Stanley and O'Sullivan 2014a). The mechanism by which CB_1 activation brings about relaxation is likely to involve numerous pathways. Gebremedhin et al. (1999) showed that AEA decreases Ca^{2+} currents via CB_1 in smooth muscles cells from cat cerebral microvasculature. Other studies have shown that CB_1 activation in the vasculature is coupled to nitric oxide (NO) release (Deutsch et al. 1997; Poblete et al. 2005).

In humans, AEA-induced vasorelaxation of isolated mesenteric arteries is inhibited by CB_1 antagonism (Stanley and O'Sullivan 2012). However, in the same arteries, the vasorelaxant effect of 2-AG was not CB_1 mediated (Stanley and O'Sullivan 2014b). AEA and virodhamine-induced vasorelaxation of the human pulmonary artery is also not dependent on CB_1 (Kozlowska et al. 2007; Kozlowska et al. 2008; Baranowska-Kuczko et al. 2014).

4.2 Role for CB_2

Most studies have found that there is no involvement of CB_2 in mediating the vascular responses to endocannabinoids in animals or humans (see Stanley and O'Sullivan 2014a). However, there are a couple of exceptions to this. AlSuleimani and Hiley (2013) showed a role for CB_2 in OEA-induced vasorelaxation of small resistance arteries of the mesenteric bed. AEA also causes vasorelaxation of rat coronary arteries that is inhibited by CB_2 antagonism (Mair et al. 2010). It is more likely that CB_2 plays a role in other functions of the endothelium such as the regulation of adhesion molecules, monocyte adhesion and endothelial permeability (see Sect. 4.10).

4.3 Role for CB_e

Early indications of an endothelial cannabinoid receptor that is distinct from CB_1 and CB_2 came from the works of Jarai and colleagues (1999) who showed that AEA was able to cause endothelium-dependent vasorelaxation of the mesenteric vasculature equally in CB_1/CB_2 knockouts as in wild-type mice, suggesting the involvement of receptors other than CB_1 or CB_2 located on the endothelium. This has become known as the endothelial cannabinoid receptor, or CB_e. Activation of this receptor by AEA has been confirmed in numerous studies. In rabbit aortic rings, AEA causes vasorelaxation through a pertussis toxin (PTX)-sensitive endothelial receptor (Mukhopadhyay et al. 2002), and in the rat aorta, AEA-induced relaxation is sensitive to endothelium denudation, PTX and O-1918 (a proposed antagonist of CB_e that has no affinity for CB_1 or CB_2 receptors), but not to CB_1 or CB_2 antagonism (Herradon et al. 2007). Similar results have been obtained in rat resistance mesenteric arteries (O'Sullivan et al. 2004a). Other endocannabinoids or endocannabinoid-like compounds suggested to activate CB_e include NADA in rat mesenteric arteries (O'Sullivan et al. 2004b), OEA in rat mesenteric arteries and the aorta (Wheal et al. 2010; AlSuleimani and Hiley 2013), oleamide in rat mesenteric resistance arteries (Hoi and Hiley 2006) and ARA-S (Milman et al. 2006) and *N*-arachidonoyl glycine (Parmar and Ho 2010) in rat mesenteric arteries. However, there is no role for CB_e in the vasorelaxant effects of 2-AG (Kagota et al. 2001) or PEA (White and Hiley 1998). Vasorelaxation induced by the activation of CB_e may involve the release of endothelium-derived hyperpolarising factor (Jarai et al. 1999; O'Sullivan et al. 2004b), BK_{ca} channel modulation (Hoi and Hiley 2006) and NO production (Mukhopadhyay et al. 2002; Herradon et al. 2007; McCollum et al. 2007).

In human pulmonary and mesenteric arteries, AEA causes endothelium-dependent vasorelaxation that can be inhibited using the proposed CB_e antagonist O-1918 (Stanley and O'Sullivan 2012; Baranowska-Kuczko et al. 2014). Similarly, in the human pulmonary artery, the vasorelaxant effects of virodhamine are inhibited by O-1918 (Kozlowska et al. 2007, 2008). This suggests that this proposed

endothelial target site for endocannabinoids is also present and functional in human vasculature.

4.4 Role for Other Uncloned CB Receptors

Some pharmacological evidence suggests there may be other cannabinoid receptors in the vasculature that remain to be identified. For example, 2-AG-induced vasorelaxation of the rabbit mesenteric arteries is inhibited by 3 μM but not 1 μM Rimonabant and is not affected by removal of the endothelium. This is not consistent with a role for either CB_1 or CB_e and suggests that another target for 2-AG may exist on the vascular smooth muscle (Kagota et al. 2001). ARA-S-induced vasorelaxation of rat mesenteric arteries is inhibited by O-1918 (even in denuded arteries) but not PTX (Milman et al. 2006), which casts doubt on the specificity of action of O-1918 at CB_e if it inhibits responses in endothelial-denuded arteries. In the rat aorta, vasorelaxation by AEA or NADA is inhibited by PTX, but not by antagonism of either CB_1 or CB_2 or removal of the endothelium (O'Sullivan et al. 2005), again suggesting another receptor for these endocannabinoids is located on vascular smooth muscle. Similarly, vasorelaxation of the rat aorta by ARA-S is inhibited by PTX but not O-1918, or CB_1 or CB_2 antagonism (Milman et al. 2006). Together, these studies suggest that further sites of action for endocannabinoids may exist on vascular smooth muscle.

4.5 Role for TRPV1

Zygmunt and colleagues (1999) were the first to show that the vasorelaxant effects of AEA, but not 2-AG or PEA, could be blocked by capsaicin pre-treatment (to deplete sensory neurotransmitters) or inhibited by a TRPV1 antagonist. They showed this involves the release of calcitonin gene-related peptide (CGRP) causing vasorelaxation through activation of CGRP receptors (Zygmunt et al. 1999). AEA induced vasorelaxation though TRPV1 is also reported to be linked to NO production in the rat mesenteric vascular bed (Poblete et al. 2005). Many studies have confirmed the role of TRPV1 in AEA-induced vasorelaxation (Harris et al. 2002; Ho and Hiley 2003; O'Sullivan et al. 2004b; Peroni et al. 2004). Other endocannabinoids or endocannabinoid-like compounds that cause vasorelaxation through TRPV1 activation include NADA (O'Sullivan et al. 2004a) and OEA (Ho et al. 2008; Wheal et al. 2010; AlSuleimani and Hiley 2013). However, in rat coronary arteries and rat pulmonary arteries, AEA-induced vasorelaxation is not affected by incubation with capsaicin or a TRPV1 antagonist (White et al. 2001; Baranowska-Kuczko et al. 2012), which may reflect differences in sensory innervations or TRP expression between vascular beds. In isolated human mesenteric arteries and pulmonary arteries, capsaicin pre-treatment does not inhibit AEA-, 2-AG- or virodhamine-induced vasorelaxation (Kozlowska et al. 2008; Stanley and O'Sullivan 2014b; Baranowska-Kuczko et al. 2014), possibly suggesting species

differences in the role or expression of TRP channels in the vasculature or the ability of endocannabinoids to activate these sites.

4.6 Role for PPARs

In addition to the acute vascular responses to endocannabinoids, a time-dependent (over hours) vasorelaxant response can be seen after a single application of AEA and NADA, but not PEA (O'Sullivan et al. 2009). This effect was mediated by PPARγ. Romano and Lograno (2012) showed a similar time-dependent vasorelaxant response to AEA and PEA in the bovine ophthalmic artery that could be inhibited by a PPARα (but not PPARγ) antagonist. As PPAR activation in the vasculature mediates other effects such as anti-inflammatory and anti-atherosclerotic actions, the possibility exists that the endocannabinoid system and production of endocannabinoids, in endothelial or smooth muscle cells, could bring about some of these effects through PPAR activation.

4.7 Metabolic Products of Cannabinoids

Some of the vascular effects of endocannabinoids are mediated by their metabolic products. This is evidenced by the fact that the vasorelaxant effects of AEA and 2-AG can be inhibited by FAAH, MAGL, cyclooxygenase (COX) and cytochrome P450 inhibition (Ellis et al. 1995; Fleming et al. 1999; Gauthier et al. 2005; Herradon et al. 2007; Awumey et al. 2008; Czikora et al. 2012; Stanley and O'Sullivan 2014b). The metabolites produced include arachidonic acid, prostaglandins and epoxyeicosatrienoic acids (Pratt et al. 1998; Stanke-Labesque et al. 2004), which can themselves have direct vascular effects, or be further metabolised into vasoactive substances. For example, metabolic products of AEA metabolism activate the prostacyclin receptor in the rat and human pulmonary artery (Baranowska-Kuczko et al. 2012, 2014). It is likely that for some endocannabinoids, their vascular responses are brought about by a combination of effects of the compounds themselves (through CB_1, TRPV or PPAR activation) and vascular effects of their metabolites. Some of these metabolites formed from endocannabinoids or endocannabinoid-like compounds can also have vasoconstrictor effects. For example, metabolites of AEA can induce vasoconstriction in the rabbit lung via the prostanoid EP_1 receptor (Wahn et al. 2005), and metabolites of 2-AG (Stanke-Labesque et al. 2004) and OEA (Wheal et al. 2010) cause vasoconstriction via the thromboxane receptor. Therefore, it is worth considering that the vascular effects of endocannabinoids might be altered in pathologies where the expression of enzymes involved (FAAH, MAGL or COX) and of the receptors activated might be altered.

4.8 Vascular Responses to Endocannabinoids in Disease Situations

The vascular responses to endocannabinoids are altered in some disease situations. Wheal et al. (2007) showed an enhanced vasorelaxant response to AEA in perfused mesenteric beds of rats made hypertensive by chronic NO synthase inhibition. A subsequent study with this model showed this was abolished by capsaicin pre-treatment, suggesting an increased sensory nerve involvement (Wheal and Randall 2009). However, in the SHR, the vasorelaxant effects of AEA were reduced in the perfused mesenteric bed and were enhanced in aortic rings (Wheal and Randall 2009). The enhanced response in SHR aortae was endothelium-dependent (Wheal and Randall 2009). Hopps et al. (2012) also showed that the vasorelaxant response to oleamide was enhanced in the aorta of SHRs, and that this could be abolished by capsaicin pre-treatment, again suggesting an increased role for sensory nerve activation by endocannabinoids in hypertension. In contrast, the COX-sensitive component of the response to oleamide was lost in SHRs (Hopps et al. 2012).

Domenicali and colleagues (2005) showed that the vasorelaxant response to AEA was enhanced in cirrhotic rats, and that this was associated with an increase in CB_1 and TPRV1 receptor expression. Similarly, Moezi et al. (2006) showed that AEA increases mesenteric arteriole diameter in cirrhotic rats but not control rats, and that this was blocked by a CB_1 antagonist and associated with increased CB_1 and TPRV1 receptor protein. By contrast, the vasorelaxant responses to AEA are reduced in mesenteric arteries from young obese Zucker rats, and this is associated with decreased CB_1 and CB_2 expression (Lobato et al. 2013). We have also shown that the responses to AEA and 2-AG are reduced in the Zucker diabetic model, which appears to be brought about by enhanced metabolism of these endocannabinoids, including the production of vasoconstrictor metabolites acting at the thromboxane receptor (Wheal et al. 2012).

4.9 Endocannabinoids and Veins

Despite the wealth of literature on the direct effects of endocannabinoids on arteries, there are few studies on the effects of endocannabinoids in veins. Although many authors have used human umbilical vein endothelial cells, this has been as a model of endothelial cell function, rather than to examine the effects of endocannabinoid on venous function. Only two studies have looked at this. Stefano et al. (1998) showed that acute treatment with AEA increased NO release in human saphenous vein, and this was associated with decreased monocyte adherence. However, chronic treatment of human saphenous veins with AEA led to increased monocyte adherence because of a desensitisation to AEA-induced NO release (Stefano et al. 1998). In isolated rings of human umbilical vein (Pelorosso et al. 2009), 150 min (but not 15 min) exposure to AEA decreases the contractile response to bradykinin via the CB_1 receptor and not the CB_2 receptor.

4.10 Endocannabinoids and Atherosclerosis

Many studies have investigated the role of the endocannabinoid system in atherosclerosis (see Steffens and Pacher 2015; Carbone et al. 2014 for reviews). Increased expression of CB_1 has been observed in human coronary atherectomy samples and CB_1 expression was greater in lipid-rich atheromatous plaques than in fibrous plaques (Sugamura et al. 2009). Increased levels of 2-AG have also been observed in the aorta of a mouse model of atherosclerosis (Montecucco et al. 2009). Plasma levels of AEA and 2-AG are raised in patients with coronary artery disease (Sugamura et al. 2009). As in cardiac pathologies, the assumption is that up-regulation of the endocannabinoid system in atherosclerosis is protective. Accordingly, FAAH knockout mice show increased monocyte adhesion to endothelial cells (Batkai et al. 2007), and genetic deletion of CB_2 worsens atherogenesis in hyperlipidic mice (Hoyer et al. 2011).

Given the anti-inflammatory effects of CB_2 activation, it is not surprising that many studies have indicated a protective role of CB_2 agonists/activation in vivo in animal models of atherosclerosis. The effects of CB_2 activation in vivo include decreased plaque development, decreased vascular smooth muscle cell proliferation, improved endothelial function, decreased expression of adhesion molecules, decreased oxidative stress, and decreased macrophage infiltration (Steffens et al. 2005; Zhao et al. 2010; Hoyer et al. 2011). In endothelial cell studies, AEA and CB_2 agonists decrease TNFα and adhesion molecules, and chemotaxis and neutrophil adhesion (Rajesh et al. 2007). CB_2 agonists also decrease the proliferation and migration of human vascular smooth muscle cells (Rajesh et al. 2008).

The role of CB_1 in atherosclerosis is more controversial, with evidence suggesting both a pro- and anti-atherosclerotic effect of receptor activation. Rimonabant has been shown to reduce atherosclerotic lesions and decrease cytokine release in a mouse model (Dol-Gleizes et al. 2009), and cell studies have shown that CB_1 blockade decreases inflammatory cytokines in macrophages (Sugamura et al. 2009; Han et al. 2009). Also, CB_1 activation causes endothelial cell injury (Rajesh et al. 2007). In contrast to these studies, the STRADIVARIUS trial studying the effect of Rimonabant on atherosclerosis progression in patients with abdominal obesity and coronary artery disease did not see a significant difference in their primary outcome measure, atheroma volume (Nissen et al. 2008). Similarly, the AUDITOR study (Atherosclerosis Underlying Development assessed by Intima-media Thickness in patients On Rimonabant) saw no difference in atherosclerosis progression in patients receiving Rimonabant for 30 months (O'Leary et al. 2011), casting doubt on a contributory role for CB_1 activation in atherosclerosis. Furthermore, a screening of 2411 patients looking at 19 different polymorphisms of the gene encoding CB_1 did not reveal any association with coronary heart disease (de Miguel-Yanes et al. 2011). However, the G1359A polymorphism of CNR1 (the gene encoding CB_1) does contribute to the genetic risk of coronary artery disease in a Chinese Han population with type 2 diabetes (Wang et al. 2012).

4.11 Summary of Vascular Effects of Endocannabinoids

Endocannabinoids cause acute and time-dependent vasorelaxation of arteries in animal and human studies through activation of CB_1, CB_e, TRPV and PPARs, coupled to inhibition of calcium channels, activation of potassium channels, NO and vasoactive metabolite production and the release of other vasoactive substances such as CGRP. Changes in the expression of any of these components alters the vascular effects of endocannabinoids, with both enhancement and reductions in the response to endocannabinoids observed in hypertension, cirrhosis, obesity and diabetes. Endocannabinoids can have positive and negative effects on the progression of atherosclerosis. Most evidence suggests a protective role for CB_2 activation and a negative effect of CB_1 activation. However, any negative CB_1-mediated effects may not be consequential, as chronic CB_1 antagonism in large scale human trials was not associated with significant reductions in atheroma volume.

5 Endocannabinoids and Blood

Circulating levels of endocannabinoids are altered in a multitude of disorders including (but not limited to) obesity (Blüher et al. 2006), diabetes and insulin resistance (Cote et al. 2007; Abdulnour et al. 2014), obstructive sleep apnea (Engeli et al. 2012) and post-traumatic stress (Hauer et al. 2013). In many studies, it has been shown that plasma levels of AEA and 2-AG are correlated with metabolic and cardiovascular risks (Weis et al. 2010; Quercioli et al. 2011), although it is not clear whether there is a causal link between these factors. It is also not clear what the source of circulating endocannabinoids are, although in situations like cardiogenic shock, it is likely that endocannabinoids are derived from platelets and macrophages (Varga et al. 1998), while in obesity, it is suggested that they might arise from adipose tissue.

Looking first at the effects of endocannabinoids and endocannabinoid-like compounds on the formation of blood cellular components, AEA, 2-AG and PEA have been shown to stimulate mouse haematopoietic cell growth and differentiation into granulocyte, erythrocyte, macrophage and megakaryocyte colonies (Valk et al. 1997; Patinkin et al. 2008) through activation of the CB_2 receptor (Valk et al. 1997). 2-AG can also increase the formation and maturation of platelets from human megakaryoblasts (Gasperi et al. 2014).

AEA can easily pass through the cell membrane of red blood cells (erythrocytes) (Bojesen and Hansen 2005), and in red blood cells, AEA increases cytosolic Ca^{2+} activity, leading to cell shrinkage and cell membrane scrambling of mature erythrocytes, and this was inhibited by cyclooxygenase inhibitors (Bentzen and Lang 2007). This ability of AEA to stimulate red blood cell death is beneficial in infections in which erythrocytes get infected, and inducing cell death maintains a healthy red blood cell population (Bobbala et al. 2010).

Both AEA (Maccarrone et al. 1999) and 2-AG (Maccarrone et al. 2001) activate platelets, albeit at very high concentrations. However, the platelet levels of

endocannabinoids may also be very high, suggesting this activation is likely to be physiologically relevant. Activation of platelets by endocannabinoids has been ascribed to their metabolism to arachidonic acid (Braud et al. 2000) or to cannabinoid receptor activation (Maccarrone et al. 2001). Interestingly, CB_1 and CB_2 have been detected in human platelets, within the cell membrane (Catani et al. 2010a). More recently, virodhamine and 2-AG, but not AEA, were shown to share the ability of arachidonic acid to induce human platelet aggregation (Brantl et al. 2014). This could be blocked by inhibitors of their metabolism by MAGL or COX, and was not mimicked by CB_1 or CB_2 agonists, suggesting it is metabolites of virodhamine and 2-AG that mediate their effects. 2-AG can also increase platelet formation and maturation (Gasperi et al. 2014). Similarly, AEA can extend platelet survival through CB_1-dependent Akt signalling (Catani et al. 2010b), indicating that there are many aspects of platelet function that can be modulated by endocannabinoids.

In human peripheral blood mononuclear cells (lymphocytes, monocytes and macrophages), endocannabinoids decrease cytokine production and regulate many aspects of white blood cell function and immunity. Immune system modulation by endocannabinoids is discussed in detail in this volume in Cabral et al., "Endocannabinoids and the immune system in health and disease".

6 Endocannabinoids and Neurovascular Function

Endocannabinoids are neuroprotective, an effect brought about by decreased excitotoxicity, decreased oxidative stress, anti-inflammatory actions and the induction of hypothermia (see Fernández-Ruiz et al., "Endocannabinoids and neurodegenerative disorders: Parkinson's disease, Huntington's chorea, Alzheimer's disease, and others" in this volume). As well as these neurological actions, endocannabinoids also affect vascular function in the brain. As in other arteries, endocannabinoids cause vasorelaxation of cerebral arteries through the production of vasoactive prostanoids (Ellis et al. 1995). Activation of the CB_1 receptor in cat cerebral vascular smooth muscle cells inhibits the influx of Ca^{2+} through L-type Ca^{2+} channels, helping to bring about vasorelaxation (Gebremedhin et al. 1999). 2-AG reduces the effects of endothelin-1 and thus reduces cerebral vasoconstriction in human cerebral endothelial cells, mediated by CB_1 (Chen et al. 2000). AEA also inhibits the vasoconstrictor effects of endothelin-1 in rabbit basilar arteries (Dogulu et al. 2003). There appears to be a relationship between endocannabinoids and cerebral vasoconstriction, as another study showed that the thromboxane mimetic, U-46619, significantly increased AEA and 2-AG content of the middle cerebral artery, whereas serotonin decreased AEA and 2-AG content (Rademacher et al. 2005). U46619-induced contractions of the rat middle cerebral artery could also be enhanced by antagonism of the CB_1 receptor. This may help to explain the potential beneficial effects of endocannabinoids in migraine (see Greco et al. 2010 for a review).

The blood–brain barrier (BBB) is formed by brain endothelial cells that line the cerebral microvasculature, capillary basement membranes and astrocyte end feet, which surround 99 % of the BBB endothelia and play an important role in maintaining BBB integrity. Increased BBB permeability associated with multiple

sclerosis is decreased by AEA (Mestre et al. 2011). We recently investigated the effects of various endocannabinoids and endocannabinoid-like compounds on BBB permeability using an in vitro model in which human brain microvascular endothelial cells and human astrocytes were co-cultured (Hind et al. 2015). We found that only AEA and OEA affected BBB permeability in control conditions and that they both decreased BBB permeability (i.e. increased resistance). This was mediated by CB_2, TRPV1 and CGRP receptors (for AEA) and PPARα (for OEA). In contrast, oleamide has been shown to inhibit gap junction coupling in pig brain microvascular endothelial cells, thus increasing barrier permeability in vitro (Nagasawa et al. 2006). However, we saw no effect of oleamide on BBB permeability in our human in vitro model (Hind et al. 2015).

Given the knowledge that endocannabinoids are neuroprotective, cause cerebral vasorelaxation and reduce BBB permeability, it is not surprising that they have been shown to be protective in neurovascular disorders such as traumatic brain injury (TBI) and cerebral ischaemia/reperfusion injury (stroke).

6.1 Endocannabinoids and Traumatic Brain Injury

TBI occurs when an external force traumatically injures the brain. This type of brain injury has been shown to increase 2-AG levels up to tenfold within hours and to last for at least 24 h post-injury (Panikashvili et al. 2001). The hypothesis that this increase in 2-AG might be protective was proven when it was found that administration of 2-AG enhanced the recovery from TBI, associated with a decrease in infarct volume, neuronal loss and inflammation (Panikashvili et al. 2001). TBI is known to disrupt the BBB, and in this study, 2-AG limited the increase in BBB permeability, and thus reduced the associated oedema. The effect of 2-AG was inhibited by CB_1 receptor antagonism and absent in CB_1 knockout mice. The effects of TBI are worse in CB_1 knockout mice, suggesting a CB_1-mediated protective role for endogenous endocannabinoid production in TBI. However, there is probably also a contribution of the CB_2 receptor, as a synthetic CB_2-selective agonist can also ameliorate TBI outcomes, which can be inhibited by CB_2 antagonism (Elliott et al. 2011). The endocannabinoid-like substance *N*-arachidonoyl-L-serine also improves TBI outcomes, and for this compound, the effects were inhibited by antagonists of CB_2 and TRPV1, but not CB_1 (Cohen-Yeshurun et al. 2013). More recently, PEA has been shown to have a beneficial effect in reducing oedema and infarct size in TBI (mechanisms of action not probed) (Ahmad et al. 2012a).

6.2 Endocannabinoids and Cerebral Ischaemia/Stroke

The expression of cannabinoid receptors is upregulated in the rat brain following cerebral ischaemia (stroke), indicating that the endocannabinoid system may play an important role in the endogenous response to stroke (see Hillard 2008; Tuma and

Steffens 2012). Human and animal in vivo data have shown increases in neurological and circulating plasma levels of AEA, 2-AG, OEA and PEA after stroke (Schabitz et al. 2002; Hillard 2008; Naccarato et al. 2010). As in other cardiovascular disorders, the hypothesis is that upregulation of the endocannabinoid system is protective in stroke, and this is supported by numerous studies showing that 2-AG (Wang et al. 2009), AEA (Wang et al. 2009) as well as the endocannabinoid-like compounds, OEA (Sun et al. 2007; Zhou et al. 2012) and PEA (Schomacher et al. 2008; Garg et al. 2010; Ahmad et al. 2012b), offer protection against ischaemic/reperfusion injury. *N*-acylethanolamine compounds such as lauroylethanolamide and linoleoylethanolamide have also been shown to be protective against stroke (Garg et al. 2011). In a recent systematic review and meta-analysis, we reported that endocannabinoids significantly reduced infarct volume in several models of experimental stroke (England et al. 2015).

There are multiple target sites at which endocannabinoids may act in this regard. Mice that are lacking the CB_1 receptor are more susceptible to stroke (Parmentier-Batteur et al. 2002), and CB_1 has been shown to mediate the protective effects of AEA and 2-AG (Wang et al. 2009). CB_1 activation increases neurotrophic factors, reduces excitotoxicity, reduces oxidative stress and causes the induction of hypothermia (see Tuma and Steffens 2012 for a review). CB_2 activation is also important in cerebral ischaemic injury by decreasing the release of pro-inflammatory cytokines, decreasing neutrophil recruitment, decreasing leukocyte adhesion to cerebral vessels and increasing brain-derived neurotrophic factor (Choi et al. 2013). Mice that lack the CB_2 receptor are also more susceptible to stroke (Zhang et al. 2008). In addition, the protective effects of OEA have been shown to be mediated by PPARα (Sun et al. 2007), while the protective effects of PEA are independent of CB_1 or TRPV1 (Garg et al. 2010). We have found that OEA and PEA decreased ischaemia/reperfusion-induced increases in BBB permeability in vitro and that this was PPARα mediated (Hind et al. 2015). The vasodilatory effects of endocannabinoids in the cerebral vasculature may also play a role in maintaining and restoring blood flow after a stroke.

6.3 Summary

In neurovascular disorders such as TBI and stroke, endocannabinoids are produced and the endocannabinoid system is upregulated in a protective manner, as shown by the ability of various endocannabinoid agonists to reduce damage in TBI and stroke. This protection involves CB_1, CB_2, TRPV1 and PPARα activation, and both vascular tissue (vasorelaxation, inhibition of vasoconstriction and reductions of BBB permeability and oedema) and neuronal tissue.

7 Conclusions and Closing Comments

It is clear that the endocannabinoid system has important roles in the cardiovascular system, particularly in cardiovascular pathologies. However, although much research has been carried out with AEA and 2-AG, comparatively little is known about the role and effect of other endocannabinoids and endocannabinoid-like compounds in the cardiovascular system and cardiovascular pathologies. When probing possible mechanisms of action, many studies have focussed on the potential role of CB_1 and CB_2 activation, and less is therefore known about the impact on cardiovascular pathologies of the activation by endocannabinoids of other targets, such as CB_e, and the vascular receptors, PPARs, GPR55 and $5HT_{1A}$. Furthermore, the majority of work in this area has been carried out in animals, and more research is required in humans to establish the importance of the endocannabinoid system (including as yet unidentified targets on the endothelium and vascular smooth muscle), especially in cardioprotection and atherosclerosis, both areas of unmet medical needs. Despite this, is seems likely from the evidence presented in this review that greater understanding of the role and effects of the endocannabinoid system in cardiovascular regulation in humans will lead to new target sites of action for drug discovery.

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Endocannabinoids and the Digestive Tract and Bladder in Health and Disease

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_15

Abstract

Components of the so-called endocannabinoid system, i.e., cannabinoid receptors, endocannabinoids, as well as enzymes involved in endocannabinoid synthesis and degradation, have been identified both in the gastrointestinal and in the urinary tract. Evidence suggests that the endocannabinoid system is implicated in many gastrointestinal and urinary physiological and pathophysiological processes, including epithelial cell growth, inflammation, analgesia, and motor function. A pharmacological modulation of the endocannabinoid system might be beneficial for widespread diseases such as gastrointestinal reflux disease, irritable bowel syndrome, inflammatory bowel disease, colon cancer, cystitis, and hyperactive bladder. Drugs that inhibit endocannabinoid degradation and raise the level of endocannabinoids, non-psychotropic cannabinoids (notably cannabidiol), and palmitoylethanolamide, an acylethanolamide co-released with the endocannabinoid anandamide, are promising candidates for gastrointestinal and urinary diseases.

Keywords

2-Arachydonoylglycerol • Anandamide • Bladder • Cancer • Cannabidiol • Cannabinoid receptors • Cystitis • Fatty acid amide hydrolase • Inflammation • Monoacylglycerol lipase • Palmitoylethanolamide • Transient receptor potential channels

Abbreviations

2-AG	2-Arachidonoylglycerol
ABHD6	α/β-Hydrolase domain-containing protein 6
ACEA	Arachidonyl-2-chloroethylamide
AEA	Arachidonoyl ethanolamide
ATP	Adenosine triphosphate
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
CBC	Cannabichromene
CBD	Cannabidiol
CBDV	Cannabidivarin
CBG	Cannabigerol
CD	Crohn's disease

CGRP	Calcitonin gene-related polypeptide
CNS	Central nervous system
COX	Cyclooxygenase
DRG	Rat dorsal root ganglia
DVC	Dorsal vagal complex
EDTA	Ethylenediaminetetraacetic acid
EFS	Electric field stimulation
ENS	Enteric nervous system
EP1	Prostaglandin E receptor 1 (subtype EP1)
FAAH	Fatty acid amide hydrolase
GI	Gastrointestinal
GPR119	G protein-coupled receptor 119
GPR55	G protein-coupled receptor 55
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
IHC	Immunohistochemistry
LES	Lower esophageal sphincter
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
NAAA	N-acylethanolamine-hydrolyzing acid amidase
NAE	N-acylethanolamine
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
PPARs	Peroxisome proliferator-activated receptors
PPARα	Peroxisome proliferator-activated receptor α
PPARγ	Peroxisome proliferator-activated receptor γ
THC	Δ^9-Tetrahydrocannabinol
THCV	Δ^9-Tetrahydrocannabivarin
TNF-α	Tumor necrosis factor alpha
TRP	Transient receptor potential channels
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV1	Transient receptor potential vanilloid 1
TRPV4	Transient receptor potential vanilloid 4
UC	Ulcerative colitis
WB	Western blot

1 Introduction

Cannabis preparations have been used to treat a variety of gastrointestinal conditions that range from inflammatory and infective conditions to disorders of motility, secretion, abdominal pain, and emesis (Nocerino et al. 2000; Grinspoon and Bakalar 1995). Furthermore, anecdotal reports from patients with multiple

sclerosis have suggested that preparations from the plant *Cannabis sativa* might have a beneficial effect on lower urinary tract symptoms. The pharmacological basis of these empirical/traditional uses emerged after the discovery, in the early 1960s, of Δ^9-tetrahydrocannabinol (THC) as the major bioactive constituent of *Cannabis* and in the 1990s, with the identification of cannabinoid (CB_1 and CB_2) receptors, of the endogenous ligands that activate them, initially the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG), and of the enzymes involved in the biosynthesis and degradation of endocannabinoids.

This article presents an overview on the pharmacology and potential therapeutic applications of cannabinoids in the gut and in the lower urinary tract

2 The Endogenous Cannabinoid System in the GI Tract

The gastrointestinal (GI) tract is a complex system which requires the interaction of numerous different cell types including epithelial cells, glandular cells, muscle cells, neurons, and of course gut microbiota. Studies over the years have demonstrated that endocannabinoids play a pivotal role in maintaining the homeostasis of the GI tract (Izzo and Sharkey 2010; Alhouayek and Muccioli 2012).

Two lipid mediators, the *N*-acylethanolamine (NAE) anandamide and the acylglycerol 2-arachidonoylglycerol (2-AG), are at the center of the endocannabinoid system. They are both synthesized on demand from membrane lipid precursors. Although multiple pathways have been described, the key enzyme in anandamide production is phospholipase D (NAPE-PLD), whereas the key enzymes in 2-AG production are diacylglycerol lipase α and β (Muccioli 2010). Anandamide and 2-AG are both found throughout the intestinal tract. The cannabinoid (CB) receptors 1 and 2 are the main molecular targets for 2-AG and anandamide, although other receptors, such as transient receptor potential vanilloid 1 (TRPV1) and peroxisome proliferator-activated receptors (PPARs), can play important roles in mediating endocannabinoid effects (Izzo and Sharkey 2010). The CB_1 receptor is expressed in all the GI tract segments, with the highest levels of expression found in the stomach and the colon (Casu et al. 2003). In the colon, it is present in epithelial cells, smooth muscle, and submucosal–myenteric plexus. CB_2 receptor expression is found in immune cells but also in the enteric nervous system (ENS) (Wright et al. 2005; Duncan et al. 2008a).

Fatty acid amide hydrolase (FAAH) is responsible for anandamide catabolism. It is expressed throughout the GI tract, and at similar levels in the different sections of the intestine (Capasso et al. 2005). 2-AG catabolism is mainly mediated by monoacylglycerol lipase (MAGL). This enzyme is expressed from the epithelium to the muscle layers of the gut wall and is also expressed by the enteric neurons. Interestingly its activity decreases from the duodenum to the distal colon (Duncan et al. 2008b).

Although not stricto sensu endocannabinoids, at least two other NAEs—palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)—are important players in the GI tract. Their metabolism is to a certain degree similar to AEA

metabolism. However, PEA and OEA do not bind to the cannabinoid receptors, although they can indirectly activate them via the so-called entourage effect, i.e., the augmentation of the endocannabinoid levels and/or actions at cannabinoid receptors (De Petrocellis et al. 2002). Several PEA and OEA effects are mediated by peroxisome proliferator-activated receptor α (PPARα), including effects on the gut (Fu et al. 2003; Esposito et al. 2014). Nevertheless, PPARα activation is not the only mechanism underlying the effects of OEA and PEA. The G protein-coupled receptor GPR119, activated by OEA (Overton et al. 2006), is one example of the additional molecular targets that are progressively added to the list of players in the endocannabinoid field.

3 The Endogenous Cannabinoid System in the Urinary Tract

There has been great interest in the role of the endocannabinoid system in the regulation of urination since the publication of a beneficial effect of oral administration of *Cannabis* extract on incontinence episodes in patients with multiple sclerosis (Freeman et al. 2006). Because oral administration causes effects on the central nervous system (CNS) as well as on the peripheral structures involved in micturition, it is important to answer the question of whether the beneficial effects are mediated centrally or peripherally. Different in vitro responses to cannabinoid drugs between the rat, mouse, and human bladder suggest that the rat or mouse may not be ideal animal models for the effects of cannabinoid drugs on human bladder function at the end-organ level. It is clear that both CB_1 and CB_2 receptors, as well as FAAH, are expressed in the human bladder, and they may also be involved in sensory pathways governing micturition. The cannabinoid receptors and FAAH are expressed in bladder nerves, dorsal root ganglia, and spinal cord dorsal horn neurons that co-express calcitonin gene-related polypeptide (CGRP), TRPV1, transient receptor potential vanilloid 4 (TRPV4), and purinergic P2X3 receptors. It is not entirely clear whether differences also exist between human and rat or mouse animal models in these sensory mechanisms.

4 Endocannabinoids and the GI Tract: Pharmacology

4.1 Nausea and Emesis

Nausea and vomiting (emesis) are unpleasant conditions of diverse causes, such as infection, chemotherapy, or pregnancy, yet both conditions are highly amenable to cannabinoid treatment (Parker et al. 2011; Sharkey et al. 2014). The regions in the brainstem that propagate vomiting are located in the dorsal vagal complex (DVC) and express CB_1 and CB_2 receptors (Van Sickle et al. 2001, 2005; Mackie 2005). Enzymes of endocannabinoid synthesis are less abundant in these areas, but enzymes of endocannabinoid degradation, i.e., FAAH and MAGL, have been described in the DVC of the ferret (van Sickle et al. 2001) and the area postrema

of the rat, respectively (Suárez et al. 2010). It is widely known that *Cannabis* and exogenous ligands of CB_1 receptors are able to inhibit vomiting in humans and animal models of emesis (Parker et al. 2011; Izzo and Sharkey 2010). Likewise, anandamide and 2-AG were shown to reduce dose dependently emesis in the ferret (van Sickle et al. 2005). Anandamide, in particular, may play an important part in the control of vomiting by maintaining an "endocannabinoid tone" (Sharkey et al. 2007), a situation pharmacologically created by blockade of FAAH or endocannabinoid transporters, resulting in increased endocannabinoid levels (van Sickle et al. 2005). Thus, the endocannabinoid reuptake inhibitor VDM11 and the FAAH inhibitor URB597 reduced LiCl-induced emesis via CB_1 and CB_2 receptor activation (van Sickle et al. 2005). The MAGL inhibitors JZL184 and MJN110 were both able to reduce emesis in shrews (Parker et al. 2014; Sticht et al. 2012). In humans, in whom motion sickness was induced by parabolic flight maneuvers, sickness was associated with low levels of blood endocannabinoids and reduced expression of CB_1 receptors in leukocytes (Choukèr et al. 2010). In line with this study, dexamethasone prevented motion sickness symptoms in rats partly by increasing anandamide levels in the blood and CB_1 expression in the DVC and the stomach (Zheng et al. 2014).

The mechanisms by which endocannabinoids affect the development of nausea are less clear (reviewed in Rock et al. 2014). Blockade of FAAH, MAGL, or anandamide transporters was shown to reduce gaping reactions to LiCl via CB_1-dependent mechanisms (Cross-Mellor et al. 2007; O'Brien et al. 2013; Parker et al. 2014). Suppression of nausea was also seen after application of 2-AG; however, this effect was not prevented by CB_1 or CB_2 antagonists but was blocked by indomethacin (Sticht et al. 2012). In summary, endocannabinoids are important messengers in the neuronal networks that control vomiting and nausea. Pharmacological manipulation of endocannabinoid degradation may represent a valuable therapeutic approach against emesis.

4.2 Lower Esophageal Sphincter Relaxation

Lower esophageal sphincter (LES) relaxation is the chief mechanism for gastroesophageal reflux. Cannabinoid CB_1 receptor activation, via central and peripheral vagal mechanisms, has been shown to inhibit transient LES relaxations in dogs and ferrets (Lehmann et al. 2002; Partosoedarso et al. 2003; Beaumont et al. 2009), the effect being associated, at least in the dog, with the inhibition of gastroesophageal reflux (Lehmann et al. 2002; Beaumont et al. 2009). Consistent with animal studies, THC (10 and 20 mg) inhibited the increase in transient LES relaxations evoked by meal ingestion and reduced spontaneous swallowing as well as basal LES pressure in healthy volunteers (Beaumont et al. 2009). Intriguingly, the data indicating suppression of transient LES relaxation following CB_1 receptor activation were not confirmed in a subsequent clinical study, in which the CB_1 receptor antagonist rimonabant inhibited the meal-induced increase in transient LES relaxation and increased postprandial LES pressure leading to a lower number of acid reflux events

(Scarpellini et al. 2011). It should be noted, however, that rimonabant may exert potent CB_1-receptor-independent pharmacological effects (Bifulco et al. 2007).

4.3 Gastroprotection

The gastric antisecretory and antiulcer activity of cannabinoids was first observed more than 35 years ago when it was found that THC reduced gastric juice volume and ulcer formation after ligation of the pylorus (Shay rat test) (Sofia et al. 1978). Such early reports have been confirmed by a number of more recent experimental studies showing a decrease in acid production in rodents following CB_1 receptor activation. The site of action is on vagal efferent pathways to the gastric mucosa and not on parietal cells because CB_1 receptor activation results in a reduction in acid secretion induced by 2-deoxy-D-glucose (which increases acid secretion through the release of acetylcholine), but not histamine, which directly activates H_2 receptors on parietal cells (Adami et al. 2002). However, species differences likely exist since cannabinoid receptors have been identified on human parietal cells (Pazos et al. 2008).

In agreement with a gastric antisecretory action, direct or indirect—via FAAH or MAGL inhibition—cannabinoid CB_1 receptor activation results in protective effects in a number of rodent models of gastric ulceration (Dembiński et al. 2006; Naidu et al. 2009; Rutkowska and Fereniec-Gołebiewska 2009; Shujaa et al. 2009; Warzecha et al. 2011; Kinsey et al. 2011). The protective role of endocannabinoids is further supported by the observation that angiotensin II protects gastric mucosa via a mechanism involving CB_1 receptors and 2-AG biosynthesis (Gyires et al. 2014).

4.4 Gastrointestinal Motility

Motility of the gut is regulated by the ENS, which expresses key components of the endocannabinoid system, i.e., cannabinoid receptors, FAAH, and MAGL (Izzo and Sharkey 2010). CB_1 receptor agonists decrease contractility of the stomach, ileum, and colon in rodents and contractility of the ileum and colon in humans through inhibition of acetylcholine release (Aviello et al. 2008; Izzo and Sharkey 2010). In vivo, inhibition of FAAH and MAGL in mouse intestine was shown to lead to increased levels of anandamide and 2-AG, respectively, also resulting in reduced motility (Capasso et al. 2005; Duncan et al. 2008a; Alhouayek et al. 2011). Anandamide seems to play an important role in the regulation of peristaltic reflex pathways within the ENS of rodents (Grider et al. 2009). In contrast to CB_1, the CB_2 receptor may not be involved in the physiological control of gut motility, except in the inflamed gut (Duncan et al. 2008b). Using dronabinol (i.e. THC) in a human study, involvement of CB_1 receptors was shown in gastric accommodation (Ameloot et al. 2010) while small bowel and colonic transit were not influenced by dronabinol (Esfandyari et al. 2006); however, after applying a different dose

regimen of dronabinol, relaxation of the colon and a reduction of postprandial colonic motility and tone were observed (Esfandyari et al. 2007). It also needs to be taken into account that the brain–gut axis may mediate some cannabinoid effects on gut motility because intracerebroventricular activation of cannabinoid receptors by WIN55212-2 slowed down whole gut transit in mice (Izzo et al. 2000; Li et al. 2013). In addition, CB_1 receptor deficiency in the vagal nerves of $Cnr1^{flox/flox}$/Phox2b–Cre mice led to increased gastrointestinal motility in comparison with controls (Vianna et al. 2012).

Endocannabinoids may also be involved in the control of gut motility in experimental inflammatory bowel disease. CB_1 receptor knockout mice displayed disturbed electrophysiological functioning of the colon as compared to their wild-type littermates (Sibaev et al. 2006), while agonism of CB_1 with CP55,940 delayed intestinal hypermotility in murine intestinal inflammation (Izzo et al. 2001). In patients with irritable bowel syndrome (IBS) (with diarrhea and alternating diarrhea), dronabinol inhibited fasting colonic motility but was without effect on sensation and tone (Wong et al. 2011). Endocannabinoids, therefore, control physiological tone and excitability predominantly via CB_1 receptors; however, in pathophysiological disturbances, the endocannabinoid system seems to control only certain aspects of motility, also acting via non-CB_1 targets.

4.5 Intestinal Secretion

Although *Cannabis* preparations have been traditionally used to treat dysentery and diarrhea, only few studies have investigated the effect of cannabinoids on intestinal ion transport and fluid accumulation. Using short-circuit current (Isc) as an indicator of net electrogenic ion transport in Ussing chambers, it was shown that activation of CB_1 receptors may produce an antisecretory effect through a neuronal mechanism involving the inhibition of neurotransmitter(s) release from submucosal plexus neurons and extrinsic primary afferents (Tyler et al. 2000; MacNaughton et al. 2004). In vivo, CB_1 receptor activation reduces intestinal hypersecretion induced by cholera toxin in the mouse small intestine (Izzo et al. 2003).

4.6 Visceral Sensation

Visceral hypersensitivity is a common symptom in gastrointestinal diseases including IBS. There is still a large ongoing debate about the origin of this enhanced visceral perception, with putative causes including altered gas transit, altered gut microbiota composition, alterations in the enterochromaffin cell serotoninergic system, chronic inflammation, and alterations in the gut–brain axis, to name a few (Camilleri et al. 2012; Keszthelyi et al. 2012). Using synthetic agonists it was shown that activation of CB_1 and CB_2 cannabinoid receptors decreases visceral pain in rodent models of colorectal distention (Sanson et al. 2006). It is interesting to note that, upon colon inflammation, the administration of the CB_1 inverse agonist

rimonabant increased hypersensitivity in the same model (Sanson et al. 2006). Similar results were obtained using a model of visceral hypersensitivity induced by stress in rats. Administration of the CB_1 agonist ACEA reduced, while rimonabant increased, the response to colonic distension (Shen et al. 2010). The results obtained with rimonabant in several studies point to a homeostatic role of CB_1 receptors in this context (Sanson et al. 2006; Brusberg et al. 2009; Shen et al. 2010). However, it must be noted that the behavioral response to pain induced by intracolonic instillation of an irritant was reduced by administration of taranabant, another CB_1 inverse agonist (Fichna et al. 2013).

FAAH inhibition was able to reduce visceral pain in models in which stretching movements are monitored following i.p. injection of an irritant. Indeed, administration of FAAH inhibitors reduced the number of stretches, an effect blocked by rimonabant or AM251, thus suggesting the involvement of anandamide and of CB_1 receptors (Haller et al. 2006; Naidu et al. 2009; Fichna et al. 2014). CB_2 receptor antagonism was not able to reverse the analgesic effect of FAAH inhibition (Naidu et al. 2009). Of note, synergy was observed between FAAH inhibition and COX inhibition in this model (Naidu et al. 2009). Further supporting the critical role of anandamide, a recent study demonstrated that this endocannabinoid mediates some effects of serotonin on visceral nociception (Feng et al. 2014).

From a pharmacological standpoint, the antinociceptive effects of FAAH inhibition are of great interest, when compared to CB_1 agonists, as no clear sign of CNS side effects have been reported for FAAH inhibition. However, perhaps mitigating this positive view of cannabinoids in visceral pain is the fact that administration of Δ^9-THC (dronabinol) to healthy volunteers and to IBS patients did not reduce the rectal perception of distension (Esfandyari et al. 2007; Wong et al. 2011; Klooker et al. 2011).

4.7 Intestinal Inflammation

Inflammation of the intestine alters the expression of the cannabinoid receptors and of FAAH and MAGL. However, the changes in expression that are observed are quite different in magnitude and direction depending on the study. Endocannabinoid levels are usually less affected although, again, this depends on the study. For instance, intraperitoneal LPS administration induced an inflammation of the colon and altered cannabinoid receptor expression, but had no effect on 2-AG and NAE levels (Bashashati et al. 2012). Similarly, mustard oil administration did not alter endocannabinoid levels (Fichna et al. 2014). In inflammatory bowel disease (IBD) murine models, 2-AG levels were found to be unchanged (Alhouayek et al. 2011), decreased (Salaga et al. 2014), or increased (Borrelli et al. 2015), whereas AEA was more often increased (D'Argenio et al. 2006; Borrelli et al. 2015). A few studies also analyzed the human inflamed colon during ulcerative colitis (UC) or Crohn's disease (CD), but here again no clear conclusion can be drawn on the variation of endocannabinoid tone (Alhouayek and Muccioli 2012). This is likely due to the inevitable heterogeneity of the samples used in human studies.

Although the impact of inflammation on the endocannabinoid system is highly variable depending on the model and even the study, there is clear evidence supporting the ability of endogenous and exogenous cannabinoids to decrease intestinal inflammation. Indeed, limiting the degradation of NAEs, by inhibiting either FAAH or anandamide uptake, reduces the extent of colitis (D'Argenio et al. 2006; Storr et al. 2008; Salaga et al. 2014). A similar result was also obtained after blocking 2-AG's degradation through inhibition of MAGL (Alhouayek et al. 2011). These positive effects are partly mediated by both cannabinoid receptors as demonstrated using receptor antagonists (Alhouayek et al. 2011). Even though increasing endocannabinoid levels through inhibition of their hydrolysis has proven efficacious, it is important to consider the fact that FAAH controls the levels of numerous bioactive lipids, including in the GI tract (Long et al. 2011; Alhouayek and Muccioli 2014). Therefore, the effects observed upon its inhibition might not only be due to anandamide.

Activation of the cannabinoid receptors remains of course an excellent therapeutic option. This was first shown using nonselective CB_1 and CB_2 agonists (e.g., WIN55212, HU-210) but also using CB_1-selective agonists such as ACEA or CB_2-selective agonists such as JWH-133 (Alhouayek and Muccioli 2012). The efficacy of JWH-133 was shown in numerous IBD models (Singh et al. 2012; Storr et al. 2009; Kimball et al. 2006), further supporting the strategy of selectively activating the CB_2 receptor in the context of colon inflammation and prompting the development of novel CB_2 agonists with anti-inflammatory properties (Tourteau et al. 2013; El Bakali et al. 2012). The CNS side effects associated with CB_1 receptor activation are somewhat hampering the study of centrally active CB_1 agonists in the context of colon inflammation. Once available, peripherally restricted CB_1 receptor agonists should help understand which of the two cannabinoid receptors is the best target for reducing colon inflammation.

4.8 Intestinal Cancer

Cannabinoids are able to suppress proliferation and migration of various cancer cells (Velasco et al. 2012), suggesting an inhibitory role of the endocannabinoid system in tumor growth. In the gut, effects of endocannabinoids on tumor growth and metastasis are mediated primarily via CB_1 receptors (Izzo and Camilleri 2009). Anandamide, for instance, inhibits growth of Caco-2 (Ligresti et al. 2003) and migration of SW480 colon cancer cells via activation of CB_1 receptors (Joseph et al. 2004). Wang et al. (2008) demonstrated that knockout of the *CB1* gene in $Apc^{Min/+}$mice led to increased development of intestinal tumors. Furthermore, in 77 % of tumor samples from colon cancer patients, the *CB1* gene was hypermethylated (Wang et al. 2008). It is generally thought that the aim of an upregulated endocannabinoid system is to restore homeostasis (Schicho and Storr 2010). Accordingly, tumor biopsies from patients with colon cancer revealed increased levels of anandamide and 2-AG, but expression of cannabinoid receptors and FAAH were unchanged in comparison to healthy tissue (Ligresti et al. 2003).

High CB_1 immunoreactivity was described in stage II microsatellite stable colorectal cancer, but correlated with poorer survival rates (Gustafsson et al. 2011), while in another study, CB_1 receptor expression was lower in stage IV than stage I/II or III colon cancer (Jung et al. 2013). However, high *vs* low CB_1 expression in stage IV was associated with poorer survival rate following colorectal surgery (Jung et al. 2013). These observations suggest a more complex expression pattern of CB_1 during cancer disease than previously suspected.

CB_1 activation induces apoptosis in colon cancer cells via inhibition of RAS-MAP kinase and PI3-Akt pathways (Greenhough et al. 2007) or via downregulation of antiapoptotic factors like survivin (Wang et al. 2008). Anandamide inhibited cancer cell proliferation and has been also shown to cause a CB_1-dependent reduction in polyamine levels (Linsalata et al. 2010). In DLD-1 colon cancer cells, some reports describe an involvement of CB_2 receptors in the growth-inhibiting effect of cannabinoids (Ligresti et al. 2003; Romano et al. 2014), partly through TNF-α-induced ceramide synthesis (Cianchi et al. 2008). Interestingly, CB_1 antagonism by rimonabant (Santoro et al. 2009), but not by AM251 (Wang et al. 2008), produced antiproliferative effects in DLD-1 colon cancer cells.

Anandamide induces cannabinoid receptor-independent cell death in apoptosis-resistant colon cancer cells with high levels of COX-2, such as in SW480 cells (Patsos et al. 2005). Since high levels of COX-2 correlate with reduced survival in colorectal cancer patients (Soumaoro et al. 2004), anandamide may be a therapeutic option for apoptosis-resistant colon cancer. An increase in anandamide can be achieved by treatment with FAAH inhibitors, and indeed a study by Izzo et al. (2008) showed that the FAAH inhibitor *N*-arachidonoylserotonin increased colon endocannabinoids and reduced the number of early neoplastic lesions in an azoxymethane-induced colon cancer model. Collectively, endocannabinoids exert anticarcinogenic effects in gastrointestinal cancer through CB_1, CB_2, and CB receptor-independent pathways. In particular, inhibitors of FAAH and probably MAGL (Ye et al. 2011) represent promising future therapeutics for colon cancer.

5 Endocannabinoids and the Urinary Tract

5.1 Cellular Location of Cannabinoid Receptors and Metabolizing Enzymes in the Urinary Tract

In the human bladder, both CB_1 and CB_2 receptors can be localized with immunohistochemistry (IHC) to nerve fibers in the suburothelial region and between smooth muscle bundles (Gratzke et al. 2009; Mukerji et al. 2010; Weinhold et al. 2010; Veress et al. 2013). In biopsies from patients with bladder pain (painful bladder syndrome) or urinary urgency (idiopathic detrusor overactivity), the density of the CB_1 positive nerve fibers is increased (Mukerji et al. 2010). Although there are some inconsistencies in published IHC reports in human urothelium, the majority of findings indicate that both CB_1 and CB_2 receptor staining is higher in the urothelium than the muscle layer (Gratzke et al. 2009;

Tyagi et al. 2009; Mukerji et al. 2010; Weinhold et al. 2010; Bakali et al. 2013). Similar findings pertain to mouse (Hayn et al. 2008; Walczak and Cervero 2011) and rat (Walczak et al. 2009; Veress et al. 2013) bladder. Many of these reports confirmed immunohistochemical results with Western blots (WB) (Gratzke et al. 2009; Tyagi et al. 2009; Bakali et al. 2013; Veress et al. 2013).

FAAH is localized to the urothelium but not the detrusor muscle, using both IHC and WB in the human, rat, and mouse bladder, and FAAH is found to colocalize with CB_2 receptors in human and rat urothelium (Strittmatter et al. 2012). In the mouse bladder, CB_1 receptors colocalize with $P2X_3$ receptors in urothelial cells, primarily the umbrella cells exposed to the bladder lumen, as well as in some nerve fibers mainly in the muscular layer of the bladder wall (Walczak et al. 2009). It is known that with urothelial stretching during bladder filling, umbrella cells release ATP (Wang et al. 2005; Apodaca et al. 2007) which can activate sensory nerves by stimulating P2X receptors. Because most of the CB_1 positive nerve fibers were observed not to co-express $P2X_3$ receptors, any effects of cannabinoids on the purinergic sensory system might be mediated by reduction of urothelial ATP release as opposed to suppression of sensory nerve activity (Walczak et al. 2009). Similar studies in human urothelium of CB_1 and $P2X_3$ receptor colocalization have not been reported. In human bladder, CB_2 receptors and the vesicular acetylcholine transporter (VAChT) colocalize to nerve fibers between strands of detrusor smooth muscle cells. Most of the CB_2 positive nerve fibers and varicosities are observed to also express CGRP. In addition, slender nerve fibers that extended into the urothelium stain positive for both CB_2 and TRPV1 receptors (Gratzke et al. 2009). Because both TRPV1- and CGRP-labeled nerves are associated with sensory function, this co-labeling with CB_2 receptors suggests a possible role for cannabinoids in bladder afferent signaling. CB_1 receptor-positive primary sensory nerve cell bodies are found in the rat dorsal root ganglia (DRG) and spinal cord dorsal horn lamina I and II, subpopulations of which are colocalized with the nociceptive markers CGRP and non-peptidergic *Griffonia* (*Bandeiraea*) *simplicifolia* IB4 isolectin binding (Veress et al. 2013). An immuno-electron microscopic study indicates both a pre- and a postsynaptic localization of CB_1 receptors in dendrites and soma of the rat DRG and dorsal horn (Salio et al. 2002).

5.2 Role of Endocannabinoids in Bladder Function

Electric field stimulation (EFS) of in vitro bladder smooth muscle from all species tested induces transmitter release from intramural nerve endings, thus causing contraction indirectly because blocking sodium channels with tetrodotoxin virtually abolishes EFS-induced contractions. The cannabinoid receptor subtypes involved in these contractions are different in different species. Nerve-evoked (EFS-induced) contractions of in vitro mouse and rat detrusor strips are dose dependently inhibited by cannabinoid agonists (Pertwee and Fernando 1996). In the mouse bladder this is unaffected by the CB_2 antagonist AM630 but reversed by the CB_1 antagonist SR14176A with a potency similar to its K_i for inhibition of [^{3}H]-CP55,940 binding

to CB_1 receptors (Rinaldi-Carmona et al. 1994; Felder et al. 1995). In the rat bladder, both CB_1 and CB_2 antagonists (SR14176A and SR144528, respectively) inhibit with high potency the effects of the non-subtype-selective agonist WIN55212-2 or the CB_2-selective agonist JWH-015 (Martin et al. 2000). This is consistent with an involvement of CB_1 but not CB_2 receptors in the mouse bladder but both CB_1 and CB_2 receptors in the rat bladder. However, nerve-evoked contractions of bladder strips from the dog, pig, primate, or human bladder are completely resistant to activation of cannabinoid receptors with up to 3 μM of the non-subtype-selective full agonist WIN55212-2 (Martin et al. 2000). Based on these findings, the rat and mouse appear to be poor animal models for the effects of cannabinoids in the human bladder at the end-organ level, and any clinical benefit of cannabinoid therapy for human bladder dysfunction is not likely to be mediated by interaction with pre-neuromuscular junction receptors in the bladder wall unless the bladder dysfunction causes induction of bladder pre-junctional cannabinoid receptors.

In the rat urinary bladder, inhibition of FAAH with URB597 reduces the contraction produced by anandamide (Saitoh et al. 2007). The anandamide-induced contraction of the rat bladder is not blocked by CB_1 receptor antagonism with AM251 or CB_2 receptor antagonism with AM630 but virtually abolished by the cyclooxygenase inhibitor indomethacin and partially decreased by the EP1 receptor antagonist ONO9130. Thus, rat bladder anandamide-induced contractions are mediated partly by TRPV1 receptors, partly by increased prostaglandin production and EP1 receptor activation, and perhaps by other yet to be identified mechanisms but not by activation of CB_1 or CB_2 receptors (Saitoh et al. 2007). During filling cystometry in awake rats, intravesical infusion of the central and peripheral FAAH inhibitor oleoyl ethyl amide increases bladder capacity, and this is reversed by the CB_2 antagonist SR144528 but not the CB_1 antagonist rimonabant (Strittmatter et al. 2012). This may indicate that urothelial and suburothelial CB_2 receptors may be the targets for the endocannabinoids that are substrates for FAAH. The FAAH inhibitor URB937 does not readily cross the blood brain barrier since the intravenous IC_{50} for FAAH is 200-fold lower for rat liver than rat brain (Clapper et al. 2010). Intravenous URB937 decreases rat bladder distension-induced afferent nerve activity (both Aδ and C-fibers) in L6 dorsal roots, and this is reversed with either the CB_1 inverse agonist rimonabant or the CB_2 antagonist SR144528 (Aizawa et al. 2014).

The CB_1 agonist ACEA and the CB_2 agonist GP1a reduce nerve-evoked contractions of human bladder muscle strips; however, the paucity of available human tissue precluded quantitative analysis of the data (Tyagi et al. 2009). Results from another laboratory show that anandamide increases but CP55940 decreases nerve-evoked contractions of human, monkey, and rat bladder muscle strips (Gratzke et al. 2009). The anandamide effect in this instance may be a result of its activation of TRPV1 receptors as opposed to its activity at cannabinoid receptors; however, there was no effect of anandamide on baseline tonus of the human, monkey, or rat bladder strips (Gratzke et al. 2009), whereas anandamide was previously reported to induce contraction of rat bladder strips through a CB_1- and CB_2-independent, possibly TRPV1-mediated, mechanism (Saitoh et al. 2007).

The effect of activation of cannabinoid receptors on afferent nerve activity was observed in an ex vivo mouse bladder–nerve preparation in the same investigation (Sect. 5.1) as that in which CB_1 and $P2X_3$ receptors had been found to be colocalized in urothelial umbrella cells (Walczak et al. 2009). Intravesical administration of the CB_1 and CB_2 agonist AZ12646915 reduced the distension-evoked activity of bladder afferents in the pelvic nerve. This inhibition was prevented by previous administration of the CB_1-selective antagonist AM251 implicating CB_1 receptor involvement in the peripheral modulation of bladder afferent signaling. It is not known whether this apparent cannabinoid receptor-mediated suppression of afferent nerve activity induced by bladder distension is actually mediated by $P2X_3$-containing neurons. The mixed CB_1 and CB_2 agonist ajulemic acid has been reported to reduce the increased release of CGRP induced by capsaicin and ATP in the rat bladder. This effect was prevented by the CB_1 antagonist AM251 and the CB_2 antagonist AM630 (Hayn et al. 2008). Because nearly all bladder sensory fibers are immunoreactive for both the capsaicin receptor TRPV1 and CGRP (Avelino et al. 2002), capsaicin-induced CGRP release serves as a marker for measuring bladder afferent sensory nerve activity.

6 OEA and PEA and Their Actions in the Gastrointestinal and Urinary Tract

PEA and OEA are biosynthesized in the gastrointestinal tract, and their levels may change in response to noxious stimuli (Borrelli and Izzo 2009). Pharmacological studies have shown that these two acylethanolamides, particularly PEA, may exert relevant pharmacological effects, both in the gastrointestinal tract and in the urinary tract.

OEA reduces gastric emptying (which represents an important brake against overfilling the gut) and small intestinal transit. The effect of OEA on gastrointestinal motility does not involve PPARα, TRPV1, or cannabinoid receptors (Aviello et al. 2008; Capasso et al. 2005; Cluny et al. 2010). OEA blocked stress-induced accelerated upper GI transit at a dose that had no effect on physiological transit (Cluny et al. 2010), which may be relevant from a therapeutic viewpoint. Preliminary evidence suggests that OEA may exert anti-inflammatory and analgesic actions in the gut. Specifically, OEA reduces intestinal permeability in vitro and exerts—in a PPARα-insensitive way—analgesic properties, reducing the nociceptive responses produced by administration of acetic acid, an experimental model of visceral pain (Suardíaz et al. 2007).

PEA shares the ability of OEA to reduce upper gastrointestinal transit through a cannabinoid receptor-independent-mediated mechanism in physiological states (Capasso et al. 2001, 2005, 2014). In addition, by using a functional experimental model of accelerated transit that models some aspects of post-inflammatory IBS, it has recently been shown that this acylethanolamide normalized functional post-inflammatory accelerated intestinal transit, an effect which involves indirect CB_1 receptor activation and modulation of TRPV1 (Capasso et al. 2014).

Evidence exists that PEA exerts intestinal anti-inflammatory effects. Three independent research groups have recently shown that PEA attenuates murine colitis following intraperitoneal (Esposito et al. 2014; Borrelli et al. 2015; Alhouayek et al. 2015) or oral (Borrelli et al. 2015) administration. Protection can be attained not only by administering PEA exogenously but also by increasing its intestinal levels through *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) inhibition (Alhouayek et al. 2015). The protective effect of oral PEA was associated with changes in TRPV1 channels and GPR55 and CB_1 receptor mRNA expression; was pharmacologically mediated by multiple targets, including CB_2 receptors, GPR55, and PPARα; and was modulated by a TRPV1 channel antagonist (Borrelli et al. 2015). Additionally, PEA (partly via PPARα) has a positive effect on intestinal injury and inflammation caused by ischemia–reperfusion in mice (Di Paola et al. 2012) and reduced (in a mast cell-dependent manner) structural injury, intestinal wall thickness, collagen deposition, and intestinal injury associated with localized, fractionated intestinal irradiation (Wang et al. 2014). In human ulcerative colitis tissues, PEA counteracted enteroglial activation, inhibited macrophage and neutrophil infiltration, and downregulated the expression and release of pro-inflammatory markers (Esposito et al. 2014). Collectively, such results lend support for considering PEA as a new pharmaceutical tool for the treatment of intestinal inflammation, including ulcerative colitis.

PEA has been evaluated for its potential beneficial effects in bladder experimental diseases. Some papers published more than 10 years ago showed that exogenously administered PEA attenuated viscero-visceral hyperreflexia induced by intravesical instillation of nerve growth factor or turpentine (Jaggar et al. 1998; Farquhar-Smith and Rice 2001; Farquhar-Smith et al. 2002). In these models of bladder inflammation, the pharmacological action of PEA was attributed to activation of CB_2-like receptors, since it was counteracted by the selective CB_2 receptor antagonist SR144528. In more recent years, it has been shown that PEA is elevated in experimental models of cystitis induced by cyclophosphamide (Pessina et al. 2015) or acrolein, a cyclophosphamide metabolite (Merriam et al. 2011). More importantly, in the cyclophosphamide model of cystitis, PEA attenuated pain behavior, bladder inflammation, and voiding dysfunction with mechanisms involving CB_1 receptors and PPARα (Pessina et al. 2015).

7 Non-psychotropic Phytocannabinoids and Their Actions in the Gastrointestinal and Urinary Tract

The *Cannabis* plant contains, in addition to THC, non-psychotropic cannabinoids of potential therapeutic interest. These include cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), cannabidivarin (CBDV), and Δ^9-tetrahydrocannabivarin (THCV). Pharmacodynamic studies have shown that such phytocannabinoids may interact with specific targets (e.g., components of the so-called endogenous cannabinoid system, TRP channels) which play a key role in gastrointestinal and urinary diseases.

CBD represents the non-psychotropic phytocannabinoid most thoroughly investigated. CBD restored the increased permeability induced by EDTA or cytokines in a cell culture model of intestinal permeability (Alhamoruni et al. 2010, 2012), counteracted reactive enteric gliosis (De Filippis et al. 2011), induced autophagy in the intestinal epithelium (Koay et al. 2014), reduced the expression of S100B and iNOS proteins in IBD patients' biopsies (De Filippis et al. 2011), and attenuated the ability of IL-17A to elicit mucosal damage in a human colonic explant model (Harvey et al. 2014). In vivo, CBD, given intraperitoneally, was effective in experimental models of colitis (Borrelli et al. 2009; Jamontt et al. 2010) and normalized the hypermotility associated with intestinal inflammation (Capasso et al. 2008). Additionally, intrarectal CBD also has protective effects, suggesting that rectal application of CBD for the therapy of intestinal inflammation may be a practicable option (Schicho and Storr 2012). Finally, CBG and CBC share the ability of CBD to exert anti-inflammatory actions in murine models of colitis (Borrelli et al. 2013; Romano et al. 2013), and CBC also has the ability to reduce motility in the inflamed gut (Izzo et al. 2012).

The link between intestinal inflammation and colon cancer is well established. Consistent with their intestinal anti-inflammatory actions, CBG and CBD have been shown to hamper colon cancer progression in vivo (Aviello et al. 2012; Borrelli et al. 2014; Romano et al. 2014). Results from studies with colorectal carcinoma cells suggest that CBD exerts antiproliferative effects through multiple mechanisms that involve CB_1 receptors, TRPV1, and PPARγ, while CBG inhibits cell growth through TRPM8 antagonism.

Ultimately, CBDV and THCV have been shown to suppress LiCl-induced conditioned gaping, suggesting an anti-nausea potential for these two non-psychotropic phytocannabinoids (Rock et al. 2013). Few studies have investigated the actions of non-psychotropic phytocannabinoids in the urinary tract. Yamada and colleagues showed that CBD induces apoptotic cell death in human T24 bladder cancer cells (Yamada et al. 2010). In addition, CBD has been shown to attenuate acetylcholine receptor-mediated contractility in the rat and human bladder (Capasso et al. 2011), which is consistent with clinical studies showing the ability of Sativex to reduce incontinence episodes in multiple sclerosis patients (Freeman et al. 2006). Sativex is a cannabinoid-based medicine composed primarily of a 1:1 ratio of two *Cannabis sativa* extracts, a *Cannabis sativa* extract with high content of THC and a *Cannabis sativa* extract with high content of CBD. Finally, CBG was found to reduce acetylcholine-induced contractions in the human bladder (Pagano et al. 2015).

8 Potential Therapeutic Applications of Cannabinoids in Gut and Urinary Diseases

Disorders of the gastrointestinal and urinary tract are widespread and costly in terms of health as well as economically. There are extensive unmet medical needs for many conditions where these systems are affected primarily or as a secondary consequence of systemic disease. Experimental evidence suggests that

cannabinoids exert pharmacological actions in the gut potentially beneficial for a number of diseases, including gastrointestinal reflux disease, IBS, IBD, and colon cancer. Notably, preliminary clinical evidence suggests that *Cannabis* use is beneficial for IBD patients (Naftali et al. 2011, 2014; Lal et al. 2011; Ravikoff Allegretti et al. 2013). Cannabinoids are also beneficial in urinary diseases including chronic pelvic pain conditions such as interstitial cystitis and chronic nonbacterial prostatitis, as well as chronic neurological conditions that affect bladder function such as multiple sclerosis and Parkinson's and Alzheimer's diseases. Historically, the major limitation of *Cannabis* was its psychotropic side effects. There are different possible strategies for minimizing these unwanted side effects. First, the on-demand nature of endocannabinoid biosynthesis and degradation could provide a promising approach that would retain the positive effects of cannabinoids in the gut and the bladder. Evidence suggests that increasing endogenous cannabinoid tone, by using FAAH, MAGL, or NAAA inhibitors, results in beneficial effects in the gut (Storr et al. 2008; Alhouayek et al. 2011, 2015) and in the bladder (Merriam et al. 2011). A second strategy would be to selectively target the CB_2 receptors. This strategy is supported by experimental studies showing the ability of selective CB_2 receptor agonists to attenuate experimental intestinal inflammation (Storr et al. 2009), cancer (Cianchi et al. 2008), as well as experimental cystitis (Wang et al. 2013) or bladder emptying in animals with partial urethral obstruction (Gratzke et al. 2011). A third strategy would be to focus on the pharmacological actions of *N*-acylethanolamines, particularly PEA, which are devoid of central psychotropic effects. Apart from being a lipid mediator co-released with anandamide from membrane phospholipids, PEA is a safe plant-derived compound presently marketed as a food component for special medical purposes to alleviate bowel or bladder complaints. Notably, PEA exerts potent anti-inflammatory effects in the gut when given orally (Borrelli et al. 2015) and exerts anti-inflammatory and analgesic actions in experimental models of cystitis (Pessina et al. 2015). Finally, animal studies have clearly shown that non-psychotropic cannabinoids exert beneficial effects in experimental models of inflammatory bowel disease (Borrelli et al. 2009; Romano et al. 2013) and colon cancer (Aviello et al. 2012; Romano et al. 2014). In view of their safety records, such compounds appear to be promising therapeutic agents, at least for the digestive tract.

9 Conclusions and Future Directions

The studies outlined in this review clearly support the notion that the endogenous cannabinoid system is a potentially valuable therapeutic target for gastrointestinal and urinary diseases. However, there are still many questions to be answered. For example, endocannabinoids are metabolized by a large number of different enzymes and, once biosynthesized, act on targets which are not limited to cannabinoid receptors. Establishing the precise role of these enzymes and targets, such as GPR55, constitutes an important objective for future research, especially since some of the enzymes involved in endocannabinoid metabolism, such as the serine hydrolase ABHD6, which is involved in 2-AG degradation, have been largely

unexplored. Another direction for future research is the evaluation of the efficacy of cannabinoids compared to available therapies, a field that has so far generated few published findings. We propose that the potential of the endogenous cannabinoid system warrants further investment for the development of new therapeutic agents for the treatment of gastrointestinal and urinary disease.

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Endocannabinoids and Cancer

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_16

Abstract

A large body of evidence shows that cannabinoids, in addition to their well-known palliative effects on some cancer-associated symptoms, can reduce tumour growth in animal models of cancer. They do so by modulating key cell signalling pathways involved in the control of cancer cell proliferation and survival. In addition, cannabinoids inhibit angiogenesis and cell proliferation in different types of tumours in laboratory animals. By contrast, little is known about the biological role of the endocannabinoid system in cancer physiopathology, and several studies suggest that it may be over-activated in cancer. In this review, we discuss our current understanding of cannabinoids as antitumour agents, focusing on recent advances in the molecular mechanisms of action, including resistance mechanisms and opportunities for combination therapy approaches.

Keywords

Angiogenesis • Apoptosis • Autophagy • Cancer • Cannabinoid • Cell proliferation • Cell signalling • Combinational therapy

Abbreviations

2-AG	2-Arachidonoylglycerol
ALK	Anaplastic lymphoma kinase
ATF-4	Activating transcription factor 4
CB_1	Cannabinoid CB_1 receptor
CB_2	Cannabinoid CB_2 receptor
CBD	Cannabidiol
CHOP	C/EBP homologous protein
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
MDK	Midkine
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
THC	Δ^9-tetrahydrocannabinol
TRIB3	Tribbles-homologue 3
TRPV1	Transient receptor potential cation channel subfamily V member 1
VEGF	Vascular endothelial growth factor

1 Introduction

Preparations from *Cannabis sativa* L. (marijuana) have been used for many centuries both medicinally and recreationally. However, the chemical structures of their unique active components—the cannabinoids—were not elucidated until the 1960s. Three decades later the first solid clues on cannabinoid molecular action were established, which led to an impressive expansion of basic cannabinoid research and to a renaissance in the study of the therapeutic effects of cannabinoids in various fields, including oncology. Today, it is widely accepted that, out of the ~108 cannabinoids produced by *C. sativa*, Δ^9-tetrahydrocannabinol (THC) is the most relevant owing to its high potency and abundance in plant preparations (Gaoni and Mechoulam 1964; Pertwee 2008). THC exerts a wide variety of biological effects by mimicking endogenous substances—the endocannabinoids anandamide (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995)—that engage specific cell-surface cannabinoid receptors (Pertwee et al. 2010). So far, two major cannabinoid-specific receptors—CB_1 and CB_2—have been cloned and characterized from mammalian tissues (Matsuda et al. 1990; Munro et al. 1993). In addition, other receptors such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) and the orphan G protein-coupled receptor GPR55 have been proposed to act as endocannabinoid receptors (Pertwee et al. 2010). Most of the effects produced by cannabinoids in the nervous system and in non-neural tissues rely on CB_1 receptor activation. In contrast, the CB_2 receptor was initially described to be present in the immune system (Pertwee et al. 2010), but more recently it has been shown to be expressed as well in cells from other origins (Atwood et al. 2010; Fernandez-Ruiz et al. 2007). Of note, expression of CB_1 and CB_2 receptors has been found in many types of cancer cells, which does not necessarily correlate with the expression of these receptors in the tissue type of origin (Fernandez-Ruiz et al. 2007; Guzman et al. 2006; Sarfaraz et al. 2008).

The endocannabinoids, together with their receptors and the proteins responsible for their synthesis, transport and degradation, constitute the endocannabinoid system. Aside from its pivotal neuromodulatory activity (Katona and Freund 2008), the endocannabinoid system exerts other regulatory functions in the body such as the control of cardiovascular tone, energy metabolism, immunity and reproduction (Pacher et al. 2006; Pertwee 2009). This miscellaneous activity makes the pharmacological manipulation of the endocannabinoid system a promising strategy for the management of many different diseases. Specifically, cannabinoids are well known to exert palliative effects in cancer patients (Pacher et al. 2006; Pertwee 2009). The best-established use is the inhibition of chemotherapy-induced nausea and vomiting (Guzman 2003; Pertwee 2009). Today, capsules of THC (Marinol) and its synthetic analogue nabilone (Cesamet) are approved for this purpose. Cannabinoids also inhibit pain, and thus a standardized cannabis extract (Sativex) has been already approved in Canada and is currently subject of large-scale Phase III clinical trials for managing cancer-associated pain. Another potential palliative effect of cannabinoids in oncology,

supported by Phase III clinical trials, includes appetite stimulation and attenuation of wasting. In relation to this, Marinol can currently be prescribed for anorexia associated with weight loss in AIDS patients.

The therapeutic potential of cannabinoids in oncology may not be restricted to their aforementioned palliative actions. Thus, numerous studies have provided evidence that THC and other cannabinoids exhibit antitumour effects on a wide array of animal models of cancer (Guzman 2003; Sarfaraz et al. 2008; Velasco et al. 2012) Moreover, these observations have led to the development of two clinical studies to investigate the antitumour activity of cannabinoids in human patients (see Sect. 7). Nonetheless, a few studies have shown that, under certain conditions, cannabinoid treatment can stimulate cancer cell proliferation in vitro (Cudaback et al. 2010; Hart et al. 2004) and interfere with the tumour-suppressor role of the immune system (McKallip et al. 2002; Zhu et al. 2000). Likewise, there are conflicting reports regarding the role (tumour-suppressor or oncogenic) of the endocannabinoid system in cancer (Malfitano et al. 2011) (Box 1).

Box 1. Biological Role of the Endocannabinoid System in Tumour Generation and Progression

To date, little is known about the biological role of the endocannabinoid system in cancer physio-pathology. Although there are some exceptions that may be tumour type-specific, both cannabinoid receptors and their endogenous ligands are generally up-regulated in tumour tissue compared with non-tumour tissue (Caffarel et al. 2006; Guzman 2003; Malfitano et al. 2011; Sanchez et al. 2001). Additionally, different studies have associated the expression levels of cannabinoid receptors, endocannabinoids and/or endocannabinoid-metabolizing enzymes with tumour aggressiveness (Malfitano et al. 2011; Nomura et al. 2010; Thors et al. 2010), which suggests that the endocannabinoid system may be over-activated in cancer and hence pro-tumourigenic (Malfitano et al. 2011). In support of this, in mouse models of cancer, genetic ablation of CB_1 and CB_2 receptors reduces ultraviolet light-induced skin carcinogenesis (Zheng et al. 2008), and CB_2 receptor over-expression enhances the predisposition to leukaemia after leukaemia virus infection (Joosten et al. 2002).

Conversely, and in line with the evidence supporting the hypothesis that pharmacological activation of cannabinoid receptors reduces tumour growth (Guzman 2003; Sarfaraz et al. 2008), the up-regulation of endocannabinoid-degrading enzymes has been observed in aggressive human tumours and cancer cell lines (Nomura et al. 2010; Thors et al. 2010), indicating that endocannabinoid signalling can also have a tumour-suppressive role. In support of this, deletion of CB_1 receptors accelerates intestinal tumour growth in a genetic mouse model of colon cancer (Wang et al. 2008), increased endocannabinoid levels diminish azoxymethane-induced precancerous

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lesions in the mouse colon (Izzo et al. 2008), and a reduction in the expression of the endocannabinoid-degrading enzyme monoacylglycerol lipase reduces tumour growth in xenografted mice (Nomura et al. 2010).

Further studies, including those analysing the activation of the precise signalling mechanisms involved in the regulation of cannabinoid-induced cell death or cell proliferation upon genetic or pharmacological manipulation of the endocannabinoid system, are therefore needed to clarify which are the contextual determinants for this system to act as either a guardian or an inducer of tumourigenesis or tumour progression.

This review summarizes these observations and provides an integrated view of the molecular mechanisms responsible for cannabinoid antitumour activity. It also discusses the experimental evidence supporting the existence of mechanisms of resistance to the cell death-promoting actions of THC in certain types of cancer cells, the possible strategies that could be undertaken to overcome such resistance, and the preclinical data supporting that the combined administration of cannabinoids and other drugs could be useful in anticancer therapies.

2 The Endocannabinoid System and Cancer

To date, little is known about the precise biological role of the endocannabinoid system in cancer physio-pathology. Although there are some exceptions that may be tumour type-specific, both cannabinoid receptors and their endogenous ligands are generally up-regulated in tumour tissue compared with non-tumour tissue (Caffarel et al. 2006; Guzman 2003; Malfitano et al. 2011; Sanchez et al. 2001). Additionally, different studies have associated the expression levels of cannabinoid receptors, endocannabioids and/or endocannabinoid-metabolizing enzymes with tumour aggressiveness (Malfitano et al. 2011; Nomura et al. 2010; Thors et al. 2010), which suggests that the endocannabinoid system may be over-activated in cancer and hence pro-tumourigenic (Malfitano et al. 2011). In support of this, in mouse models of cancer, genetic ablation of CB_1 and CB_2 receptors reduces ultraviolet light-induced skin carcinogenesis (Zheng et al. 2008), and CB_2 receptor over-expression enhances the predisposition to leukaemia after leukaemia virus infection (Joosten et al. 2002).

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(Wang et al. 2008), increased endocannabinoid levels diminish azoxymethane-induced precancerous lesions in the mouse colon (Izzo et al. 2008), and a reduction in the expression of the endocannabinoid-degrading enzyme monoacylglycerol lipase reduces tumour growth in xenografted mice (Nomura et al. 2010).

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3 Preclinical Antitumour Activity

Since the late 1990s, a large body of evidence has accumulated demonstrating that various cannabinoids exert antitumour effects in a wide variety of experimental models of cancer, ranging from cancer cell lines in culture to genetically engineered mice [reviewed by Velasco et al. (2012)]. Multiple cannabinoids have shown this activity, including THC; the endocannabinoids 2-AG and anandamide; and different synthetic cannabinoid receptor agonists that have either comparable affinity for CB_1 and CB_2 receptors (e.g. WIN 55212-2 or HU-210), higher affinity for CB_1 (e.g. methanandamide) or higher affinity for CB_2 (e.g. JWH-133). These findings strongly support that, aside from the role played by the endogenous cannabinoid system in cancer, pharmacological stimulation of CB receptors is in most cases antitumourigenic. Nonetheless, a few reports have proposed a tumour-promoting effect of cannabinoids (Cudaback et al. 2010; Hart et al. 2004; McKallip et al. 2002; Zhu et al. 2000). These apparently conflicting observations are discussed below.

Cannabinoids impair tumour progression at different levels. Their most prevalent effect is the induction of cancer cell death by apoptosis and the inhibition of cancer cell proliferation. At least one of these actions has been demonstrated in virtually all cancer cell types tested (Velasco et al. 2012). In addition, in vivo experiments have shown that cannabinoids impair tumour angiogenesis and block invasion and metastasis.

4 Mechanisms of Antitumour Effects

4.1 Induction of Cancer Cell Death

A significant amount of the research conducted so far on the mechanism of cannabinoid antitumour activity has focussed on glioma cells. Initial studies showed that THC and other cannabinoids induce the apoptotic death of glioma cells via CB_1- and CB_2-dependent stimulation of the de novo synthesis of the pro-apoptotic sphingolipid ceramide (Blazquez et al. 2004; Galve-Roperh et al. 2000; Gomez del Pulgar et al. 2002; Sanchez et al. 2001). Further studies,

based on the analysis of the gene expression profile of THC-sensitive and resistant glioma cells, gave further insight into the specific signalling events downstream of ceramide that are activated in cancer cells by CB_1 and CB_2 cannabinoid receptor agonists (Carracedo et al. 2006b). THC acutely up-regulates the expression of the stress-regulated protein p8 (also named NUPR1), a transcriptional regulator that has been implicated in the control of tumourigenesis and tumour progression (Encinar et al. 2001), together with several of its downstream targets such as the endoplasmic reticulum (ER) stress-related transcription factors ATF4 and CHOP and the pseudokinase tribbles-homologue 3 (TRIB3) (Carracedo et al. 2006b) (Fig. 1).

ER stress, as induced by different anticancer agents, can also lead through different mechanisms (Verfaillie et al. 2010) to the stimulation of autophagy, an essential cellular process participating in a number of physiological functions within the cell (Mizushima et al. 2008; Verfaillie et al. 2010). During autophagy, organelles and other cytoplasmic components are engulfed within double-membrane vesicles designated autophagosomes. The maturation of these vesicles involves their fusion with lysosomes, which leads in turn to the degradation of the autophagosome components by lysosomal enzymes (Mizushima et al. 2008). Autophagy is primarily a cytoprotective mechanism, although its activation can also lead to cell death (Eisenberg-Lerner et al. 2009; Mizushima et al. 2008). Indeed, THC-triggered stimulation of the p8-regulated pathway enhances the inhibitory interaction of TRIB3 with a pro-survival kinase, AKT, which leads to the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) and the subsequent stimulation of autophagy-mediated cell death (Salazar et al. 2009, 2013) (Fig. 1). CB_1 and/or CB_2 cannabinoid receptor agonists induce autophagy in different types of cancer cells in culture, and pharmacological or genetic inhibition of autophagy prevents the antitumour action of these agents in different animal models of cancer (Fig. 1), thus demonstrating that autophagy is important for the antineoplastic activity of cannabinoid receptor agonists (Salazar et al. 2009; Vara et al. 2011). Moreover, autophagy blockade prevents cannabinoid receptor agonist-induced apoptosis and cell death whereas apoptosis blockade prevents cell death but not autophagy induced by these compounds (Salazar et al. 2009; Vara et al. 2011). This indicates that autophagy is upstream of apoptosis in the process of cannabinoid receptor agonist-induced cell death (Fig. 1).

The direct participation of the p8-mediated autophagy pathway in the antitumour action of THC and other CB_1 and/or CB_2 receptor agonists has been clearly demonstrated in glioma cells and pancreatic and hepatic cancer cells (Carracedo et al. 2006a; Carracedo et al. 2006b; Salazar et al. 2009; Vara et al. 2011). At least part of this signalling route has also been found to be up-regulated after cannabinoid treatment in other types of cancer cells. This suggests that—with some variations—this could be a general mechanism by which activation of CB_1 and CB_2 receptors promotes cancer cell death.

Additional mechanisms may nonetheless cooperate with the p8-mediated autophagy pathway to evoke cancer cell death (Fig. 1). For example, in hepatocellular carcinoma cells, THC and the CB_2 receptor agonist JWH-015 can trigger an ER stress-dependent activation of AMPK that cooperates with the TRIB3-mediated

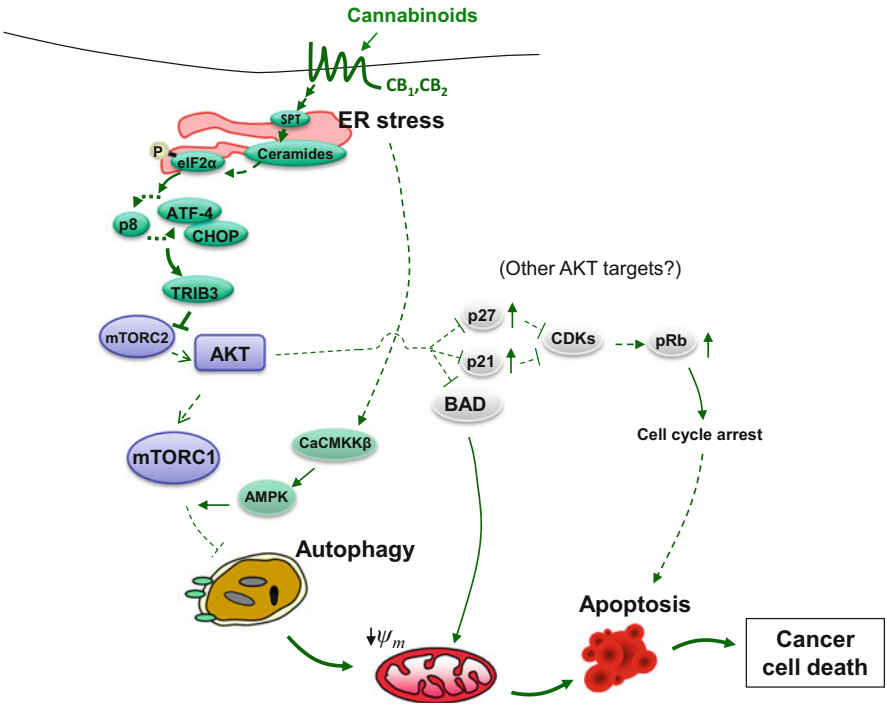

Fig. 1 Cannabinoid-induced apoptosis relies on the stimulation of ER stress and autophagy. Scheme depicting the mechanism of cannabinoid-induced apoptosis in glioma, pancreatic and hepatocellular carcinoma cells. This signalling route may constitute the main mechanism of cannabinoid-induced cell death, with some variations inherent to different types of cancer cells. Cannabinoid agonists bind to CB_1 and/or CB_2 receptors (CBR) to stimulate de novo synthesis of ceramide (Carracedo et al. 2006b; Galve-Roperh et al. 2000; Gomez del Pulgar et al. 2002; Herrera et al. 2006; Salazar et al. 2009), which triggers the induction of an endoplasmic reticulum (ER) stress-related response that promotes the up-regulation of the transcription factor p8 and several of its downstream targets, including the pseudokinase Tribbles 3 (TRIB3) (Carracedo et al. 2006b; Salazar et al. 2009). This favours the interaction of TRIB3 with AKT (Du et al. 2003; Salazar et al. 2009), thus leading to the inhibition of the AKT-mechanistic target of rapamycin C1 (mTORC1) axis and the subsequent induction of autophagy (Salazar et al. 2009). Autophagy is upstream of intrinsic mitochondrial apoptosis in the process of cannabinoid-induced cell death. The importance of this pathway is highlighted by the ability of different chemical and genetic manipulations to block cannabinoid-induced cell death. In hepatocellular carcinoma cells, the cannabinoid-evoked and ER stress-dependent activation of calcium/calmodulin-dependent protein kinase kinase 2-beta (CaCMKKβ) and AMP-activated protein kinase (AMPK) leads, together with the p8–TRIB3 pathway, to autophagy and apoptosis (Vara et al. 2011). The cannabinoid-evoked inhibition of AKT could promote cycle arrest in breast cancer and melanoma cells, as well as apoptosis, through additional mechanisms, including the decreased phosphorylation of the pro-apoptotic protein BCL2-associated agonist of cell death (BAD) (Ellert-Miklaszewska et al. 2005) and the activation of the cyclin-dependent kinase (CDK) inhibitory proteins p21 and p27 (Blazquez et al. 2006; Caffarel et al. 2008; Caffarel et al. 2006). This would lead to the subsequent decreased phosphorylation of the retinoblastoma protein (pRB), which thus would be active to arrest cell cycle. *ATF4* activating transcription factor 4, *CHOP* C/EBP homology protein, *eIF2α* eukaryotic translation initiation factor 2 alpha, *SPT* serine palmitoyltransferase

inhibition of the AKT–mTORC1 axis in the stimulation of autophagy–mediated cell death (Vara et al. 2011). In melanoma (Blazquez et al. 2006), breast carcinoma (Caffarel et al. 2006, 2012) and prostate carcinoma (Sarfaraz et al. 2006) cells, cannabinoid receptor agonists can induce cell cycle arrest in concert with apoptosis (Blazquez et al. 2006; Caffarel et al. 2006; Sarfaraz et al. 2006). Of note the antiproliferative action—at least in melanoma (Blazquez et al. 2006) and breast cancer (Caffarel et al. 2006) cells—of THC and CB_2 receptor agonists also relies on AKT inhibition.

Likewise, the effect of cannabinoid receptor agonists in hormone-dependent tumours may rely, at least in part, on their ability to interfere with the activation of growth factor receptors (Guzman 2003; Sarfaraz et al. 2008). This interference and other mechanisms (Guindon and Hohmann 2011) may participate in the cytotoxic action of cannabinoid receptor agonists in different types of cancer cells together with the autophagy-mediated cell death pathway. However, further investigation is required to clarify this issue (Box 2).

Box 2. Mechanism of Cannabinoid Receptor-Mediated Cancer Cell Death: Some Important Unanswered Questions

Research conducted during the last few years has shed light onto the intracellular signalling mechanisms underlying cannabinoid anticancer action. However, a number of important observations—in particular ones related to the role played by cannabinoid receptors in the triggering of these signals—remain to be clarified. For some examples of these, see below.

– Unlike the cell death-promoting action of cannabinoids on cancer cells, the viability of normal (non-transformed) cells is unaffected or—under certain conditions—even enhanced by cannabinoid challenge (Carracedo et al. 2006b; Galve-Roperh et al. 2000, 2008; Gomez del Pulgar et al. 2002; Salazar et al. 2009). For example, THC treatment of astrocytes (a cell type that expresses functional CB_1 receptors) does not trigger the activation of ER stress, the up-regulation of the p8 pathway, the inhibition of the AKT–mTORC1 axis or the stimulation of autophagy and apoptosis, even when concentrations of THC higher than those that promote glioma cell death are used (Carracedo et al. 2006b; Salazar et al. 2009). Similar results were obtained with primary embryonic fibroblasts (Carracedo et al. 2006b; Salazar et al. 2009) and other types of non-transformed cells expressing functional cannabinoid receptors when compared with their transformed counterparts (Blazquez et al. 2006; Caffarel et al. 2006; Casanova et al. 2003; Chan et al. 1996). Thus, stimulation of cannabinoid receptors seems to be coupled to the activation of different signalling mechanisms in transformed and non-transformed cells. The precise

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Box 2 (continued)

molecular reasons for this different behaviour remain as an important open question in the cannabinoid field.

- Another intriguing observation is that, in some types of cancer cells, such as glioma cells, pharmacological blockade of either CB_1 or CB_2 receptors prevents cannabinoid-induced cell death with similar efficacy (Galve-Roperh et al. 2000; Lorente et al. 2011), while in other types of cancer cells, for example, pancreatic (Carracedo et al. 2006a), breast (Caffarel et al. 2006) or hepatic (Vara et al. 2011) carcinoma cells, antagonists of CB_2 but not of CB_1 receptors inhibit cannabinoid antitumour actions. Why cannabinoids produce their antitumour actions through one or other of these receptor types depending on the type of cancer cell studied has yet to be established.
- Some cannabinoid receptor agonists promote cancer cell death more efficiently than other agonists that exhibit similar or even higher affinity for CB_1 or CB_2 receptors. For example, THC promotes cancer cell death in a CB_1 and/or CB_2-dependent manner at lower concentrations than the synthetic cannabinoid receptor agonist WIN-55,212-2, although the latter agent displays significantly higher affinity for CB_1 and CB_2 receptors in binding assays (Pertwee et al. 2010).

Further work aimed at investigating, for example, CB receptor homo or hetero-oligomerization in response to different cannabinoid agonists, their association with specific domains in the plasma membrane such as lipid rafts, changes in the subcellular location of CB receptors, and the selective coupling to different G proteins and other signalling proteins will be essential to answer these questions and precisely define the role played by each cannabinoid receptor type as an anticancer signalling platform.

Of note, cannabidiol (CBD), a phytocannabinoid with low affinity for cannabinoid receptors (Pertwee 2009), and other marijuana-derived cannabinoids (Ligresti et al. 2006) have also been proposed to promote the apoptotic death of cancer cells acting independently of CB_1 and CB_2 receptors. The mechanism by which CBD produces this effect has not been completely clarified as yet, but seems to rely—at least in part—on its ability to enhance the production of reactive oxygen species in cancer cells (Massi et al. 2008; Shrivastava et al. 2011). It has also been proposed that CBD may activate TRPV2 receptors to promote glioma cell death (Nabissi et al. 2012).

4.2 Inhibition of Angiogenesis, Invasion and Metastasis

In cancer cells, cannabinoid receptor agonists block the activation of the vascular endothelial growth factor (VEGF) pathway, an inducer of angiogenesis. Specifically, different elements of this cascade, such as the main ligand (VEGF) and the active forms of its main receptors (VEGFR1 and VEGFR2), are down-regulated upon treatment of skin carcinomas (Casanova et al. 2003), gliomas (Blazquez et al. 2003; Blazquez et al. 2004) and thyroid carcinomas (Portella et al. 2003) with CB_1 and/or CB_2 receptor agonists. In vascular endothelial cells, CB_1 and/or CB_2 receptor activation inhibits proliferation and migration and induces apoptosis (Blazquez et al. 2003; Pisanti et al. 2007). These and perhaps other cannabinoid-evoked actions result in a normalized tumour vasculature; that is, smaller and/or fewer vessels that are more differentiated and less leaky.

Likewise, CB_1 and/or CB_2 receptor agonists reduce the formation of distant tumour masses in animal models of both induced and spontaneous metastasis and inhibit adhesion, migration and invasiveness of glioma (Blazquez et al. 2008), breast (Grimaldi et al. 2006; Qamri et al. 2009), lung (Preet et al. 2008; Ramer and Hinz 2008), and cervical (Ramer and Hinz 2008) cancer cells in culture. These effects depend, at least in part, on the modulation of extracellular proteases [such as matrix metalloproteinase 2 (MMP2)] (Blazquez et al. 2008) and their inhibitors [such as tissue inhibitor of matrix metalloproteinases 1 (TIMP1)] (Ramer and Hinz 2008).

Of note, pharmacological inhibition of ceramide biosynthesis abrogates the antitumour and anti-angiogenic effect of CB_1 and/or CB_2 receptor agonists in glioma xenografts and decreases VEGF production by glioma cells in vitro and in vivo (Blazquez et al. 2004). Likewise, inhibition of MMP-2 expression and glioma cell invasion is prevented by blocking ceramide biosynthesis and by knocking down p8 expression (Blazquez et al. 2008). Although further research is still necessary to precisely define the molecular mechanisms responsible for these actions of cannabinoids, these observations indicate that the ceramide/p8-regulated pathway plays a general role in the antitumour activity of cannabinoids targeting CB_1 and CB_2 receptors.

It is worth noting that CBD, by acting independently of CB_1 and CB_2 receptors, produces a remarkable antitumour effect—including reduction of invasiveness and metastasis—in different animal models of cancer. This effect of CBD seems to rely—at least in part—on the down-regulation of the helix-loop-helix transcription factor inhibitor of DNA binding-1 (ID-1) (McAllister et al. 2011; Soroceanu et al. 2012).

4.3 Regulation of Antitumour Immunity

Of note, stimulation of cannabinoid receptors may lead to important changes in the processes that regulate antitumour immunity. Thus, for example, treatment of mice with THC triggers a shift (from Th1 to Th2) on the cytokine profile (Lu et al. 2006; McKallip et al. 2005; Newton et al. 2009; Steffens et al. 2005) and induces

mobilization of myeloid-derived suppressor cells (Hegde et al. 2010), two events that play a critical role in the suppression of antitumour immunity. In agreement with this notion, stimulation of CB_2 receptors has been proposed in some reports to enhance tumourigenesis by interfering with tumour surveillance by the immune system (McKallip et al. 2005; Zhu et al. 2000). By contrast, cannabinoids may also enhance immune system-mediated tumour surveillance in some contexts: the antitumour action of WIN 55212-2 (a CB_1/CB_2-mixed agonist) or JWH-133 (a CB_2-selective agonist) was more pronounced in melanoma xenografts generated in immunocompetent mice compared with those in immunodeficient mice (Blazquez et al. 2006). This also indicates that, at least in this model, stimulation of CB_2 receptors primarily inhibits tumour growth through direct effects on cancer cells rather than necessarily through interfering with the normal antitumour function of the immune system. In line with this idea, treatment for 2 years of immunocompetent rats with very high doses (50 mg/kg/day 5 times a week) of THC decreased the incidence of several types of tumours and enhanced the overall survival of these animals (Chan et al. 1996). These observations might be related to the ability of THC to reduce inflammation (Burstein and Zurier 2009; Liu et al. 2010), an effect that may prevent certain types of cancer (Liu et al.). For cannabinoid use to be clinically successful, antitumour effects will need to overcome immunosuppressive (potentially tumour-promoting) effects. Additional studies should clarify this issue. For example, it could be conceivable to study the effect of cannabinoid administration on the generation and progression of tumours exhibiting different sensitivity to CB_1 and/or CB_2 receptor agonists and generated in immunocompetent or immunodeficient mice in which the expression of CB_1 and/or CB_2 receptors in cells from the immune system has been genetically manipulated.

5 Resistance Mechanisms

Numerous studies have contributed to our appreciation of the heterogeneity of cancer, whereby each subtype of cancer, and even each individual tumour, exhibits a series of molecular characteristics that determines its behaviour and, in particular, its responsiveness to different anticancer drugs. In agreement with this line of reasoning, are results obtained in a recent investigation into the molecular features associated with the resistance of a collection of human glioma cell lines and primary cultures to cannabinoid antitumour action (Lorente et al. 2011). This study showed that, although the apoptotic effect of THC on glioma cells relied on the stimulation of cannabinoid receptors and the activation the p8-mediated autophagy pathway, the differences in the sensitivity to THC-induced cell death correlated with the enhanced expression of a particular set of genes in the THC-resistant glioma cells rather than with the presence of different expression levels of CB_1 or CB_2 receptors (Lorente et al. 2011). Of interest, up-regulation of one of these genes, midkine (*MDK*), that encodes a growth factor that has been previously associated with increased malignancy and resistance to anticancer

therapies in several types of tumours (Kadomatsu 2005; Mirkin et al. 2005), correlates with a lower overall survival of patients with glioblastoma (Lorente et al. 2011). Moreover, MDK plays a direct role in the resistance to THC action via stimulation of the anaplastic lymphoma kinase [ALK (Palmer et al. 2009)]. Thus, the stimulation of ALK by MDK inhibits the THC-evoked autophagy-mediated cell death pathway. Further research should clarify whether this mechanism could also be responsible for the resistance of cancer cells expressing high levels of MDK to other therapies. Interestingly, in vivo silencing of MDK or pharmacological inhibition of ALK in a mouse xenograft model abolishes the resistance to THC treatment of established tumours derived from cannabinoid-resistant glioma cells (Lorente et al. 2011).

Taken together, these findings support the idea that stimulation of the MDK–ALK axis promotes resistance to THC antitumour action in gliomas and could help to set the basis for the potential clinical use of THC in combination with inhibitors of this axis (Fig. 2). In line with this idea, ALK inhibitors have started to be used in clinical trials for the management of non-small-cell lung cancer and other types of tumours (de Bono and Ashworth 2010; Grande et al. 2011). Future research should clarify whether this mechanism of resistance to cannabinoid action operates in other types of tumours. In agreement with this possibility, MDK silencing enhanced the sensitivity of cannabinoid-resistant pancreatic cancer cells to THC-induced cell death (Lorente et al. 2011) .

The release by cancer cells of other growth factors has also been implicated in the mechanism of resistance to cannabinoid antitumour action. Thus, increased expression of the heparin-bound epidermal growth factor receptor (EGFR) ligand amphiregulin is associated with enhanced resistance to THC antitumour action in glioma xenografts (Lorente et al. 2009). Of note, illustrating that the dose of cannabinoids could be crucial for their optimal therapeutic effect, low (sub-micromolar) concentrations of THC or other synthetic cannabinoid agonists enhance the proliferation of several cancer cell lines in vitro. This effect relies on the activation of the protease ADAM17, the shedding of heparin-bound EGFR ligands, including amphiregulin, and the subsequent stimulation of extracellular signal-regulated kinase (ERK) and AKT pathways (Hart et al. 2004). In line with this idea, a recent report has shown that treatment with the synthetic cannabinoid, CP-55,940, increases the proliferation of murine glioma cells engineered to express CB_1 or CB_2 receptors only when these receptors were coupled to AKT activation (Cudaback et al. 2010). Although a pro-tumourigenic effect has not been observed on the growth of tumour xenografts generated with glioma cells and treated with low doses of THC (Torres et al. 2011), increased expression of amphiregulin promotes resistance to THC antitumour action through a mechanism that involves the EGFR-dependent stimulation of ERK and the subsequent inhibition of p8 and TRB3 expression. Likewise, pharmacological inhibition of EGFR, ERK (Lorente et al. 2009) or AKT (authors' unpublished observations) enhances the cell death-promoting action of THC in cultures of glioma cells. These observations suggest that targeting EGFR and the AKT and ERK pathways could enhance the antitumour effect of cannabinoids.

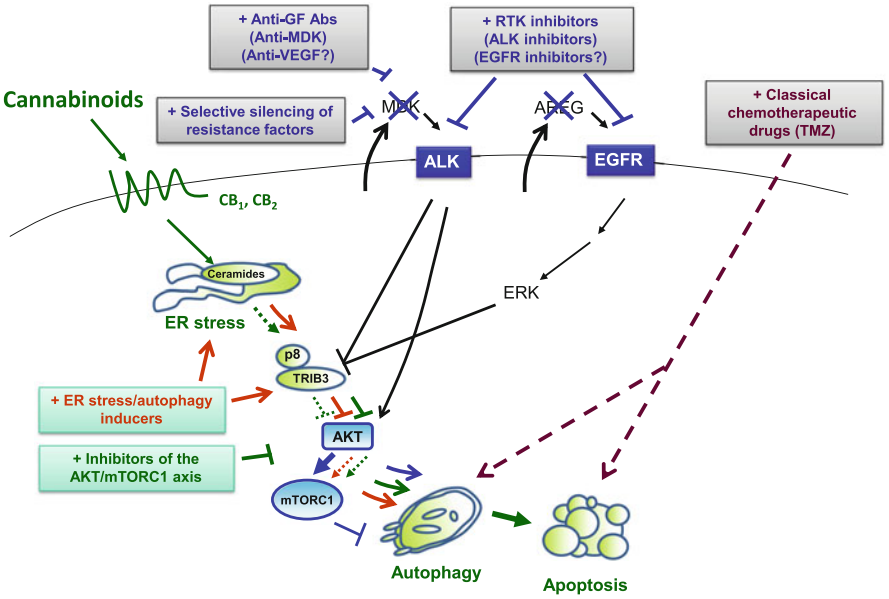

Fig. 2 Possible strategies aimed at optimizing cannabinoid-based therapies against gliomas. Glioblastoma is highly resistant to current anticancer therapies (Lonardi et al. 2005; Nieder et al. 2006; Purow and Schiff 2009). Specifically, resistance of glioma cells to cannabinoid-induced cell death relies, at least in part, on the enhanced expression of the growth factor midkine (MDK) and the subsequent activation of the anaplastic lymphoma receptor tyrosine kinase (ALK) (Lorente et al. 2011). Likewise, enhanced expression of the heparin-bound EGFR-ligand amphiregulin (AREG) can promote resistance to THC antitumour action via ERK stimulation (Lorente et al. 2009). Combination of THC with pharmacological inhibitors of ALK (or genetic inhibition of MDK) enhances cannabinoid action in resistant tumours, which provides the rationale for the design of targeted therapies capable of increasing cannabinoid antineoplastic activity (Lorente et al. 2011). Combinations of cannabinoids with classical chemotherapeutic drugs such as the alkylating agent temozolomide [TMZ; the benchmark agent for the management of glioblastoma (Lonardi et al. 2005; Stupp et al. 2005)] have been shown to produce a strong anticancer action in animal models (Torres et al. 2011). Combining cannabinoids and TMZ is thus a very attractive possibility for clinical studies aimed at investigating cannabinoids antitumour effects in glioblastoma. Other potentially interesting strategies to enhance cannabinoid anticancer action (still requiring additional experimental support from data obtained using preclinical models) could be combining cannabinoids with endoplasmic reticulum (ER) stress and/or autophagy inducers or with inhibitors of the AKT-mechanistic target of rapamycin C1 (mTORC1) axis. *Abs* antibodies, *EGFR* epidermal growth factor receptor, *ERK* extracellular signal-regulated kinase, *GF* growth factors, *RTK* receptor tyrosine kinase, *TRIB3* tribbles 3, *VEGF* vascular endothelial growth factor

6 Cannabinoid-Based Combinational Therapies

The use of combinational anticancer therapies has a number of theoretical advantages over single-agent-based strategies as they allow the simultaneous targeting of tumour growth, progression and spreading at different levels. In line with this idea, recent observations suggest that the combined administration of cannabinoids with other anticancer drugs acts synergistically to reduce tumour growth. For example, the administration of THC and temozolomide (the benchmark agent for the management of glioblastoma) exerts a strong antitumour action in glioma xenografts, an effect that is also evident in temozolomide-resistant tumours (Torres et al. 2011). Of interest, no toxicity was observed in mice treated with combinations of THC and temozolomide (Torres et al. 2011). As most patients with glioblastoma undergo temozolomide treatment, these findings indicate that the combined administration of temozolomide and cannabinoids could be therapeutically exploited for the management of glioblastoma (Fig. 2).

Likewise, another study has recently shown that the combined administration of gemcitabine (the benchmark agent for the treatment of pancreatic cancer) and different cannabinoid agonists synergistically reduces the viability of pancreatic cancer cells (Donadelli et al. 2011). Other reports indicate that anandamide and HU-210 may also enhance the anticancer activity of paclitaxel (Miyato et al. 2009) and 5-fluorouracil (Gustafsson et al. 2009), respectively.

An additional approach has been to combine THC with CBD, a phytocannabinoid that reduces—although to a lower extent than THC—the growth of several types of tumour xenografts through a still poorly defined mechanism (Massi et al. 2006; McAllister et al. 2007; Shrivastava et al. 2011). Combined administration of THC and CBD enhances the anticancer activity of THC and reduces the doses of THC needed to induce its tumour growth-inhibiting activity (Marcu et al. 2010; Torres et al. 2011). Moreover, the combination of THC and CBD together with temozolomide produces a striking reduction in the growth of glioma xenografts even when low doses of THC are used (Torres et al. 2011). Of note, CBD has also been shown to alleviate some of the undesired effects of THC administration, such as convulsions, discoordination and psychotic events, and therefore improves the tolerability of cannabis-based medicines (Pertwee 2009). As mentioned above, *C. sativa* produces ~108 different cannabinoids and, apart from CBD, some of the other cannabinoids present in marijuana might attenuate the psychoactive side effects of THC or even produce other therapeutic benefits (Pertwee 2009). Thus, we think that clinical studies aimed at analysing the efficacy of cannabinoids as antitumour agents should be based on the use both of pure substances, such as THC and CBD, and of cannabis extracts containing controlled amounts of THC, CBD and other cannabinoids.

7 Clinical Antitumour Effects of Cannabinoids

Although the clinical approval of cannabinoids is largely restricted to palliative uses in various diseases, following promising preclinical data, the antitumour effects of cannabinoids are beginning to be clinically assessed. In a pilot Phase I clinical study, nine patients with actively-growing recurrent glioblastoma that had previously failed standard therapy underwent intracranial THC administration (Guzman et al. 2006). Under these conditions, cannabinoid delivery was safe and could be achieved without significant unwanted effects. In addition, although no statistically significant conclusions can be extracted from a cohort of nine patients, the results obtained in that study suggested that some patients responded—at least partially—to THC treatment in terms of decreased tumour growth rate, as evaluated by magnetic resonance imaging (Guzman et al. 2006). Importantly, analyses of samples obtained from two patients in this study before and after THC administration indicate that the molecular mechanism of cannabinoid antitumour action delineated in the previous sections, namely p8 and TRIB3 up-regulation (Carracedo et al. 2006b; Salazar et al. 2009), mTORC1 inhibition (Salazar et al. 2009), stimulation of autophagy and apoptosis (Carracedo et al. 2006b; Guzman et al. 2006; Salazar et al. 2009), inhibition of cell proliferation (Guzman et al. 2006), decreased VEGF signalling (Blazquez et al. 2004) and MMP-2 down-regulation (Blazquez et al. 2008), also operates in cancer patients. These findings were encouraging and reinforced the interest on the potential use of cannabinoids in cancer therapies. However, they also highlighted the need for further research aimed at optimizing the use of cannabinoids in terms of patient selection, combination with other anticancer agents and use of other routes of administration (see Box 3). Two clinical trials that are currently ongoing could shed some light on these issues. One of these studies is a Phase I/II trial aimed at evaluating the combined effect of Sativex (an oro-mucosal cannabis extract whose main active components are THC and CBD in a *ca.* 1:1 ratio) and temozolomide in patients with recurrent glioblastoma multiforme (http://clinicaltrials.gov/show/NCT01812603). The other study is a Phase II trial aimed at evaluating the effect of CBD as single treatment in patients with solid tumours (http://clinicaltrials.gov/ct2/show/NCT02255292?term=cbd+solid+tumour&rank=1). Hopefully, more clinical trials will add in the near future to these two to allow determining whether cannabinoids can be used, other than for their palliative effects, to treat cancer patients

Box 3. Different Pharmacological Approaches to Target Cancer Cells with Cannabinoids

Cannabinoid agonists or enhancers of endocannabinoid tone?

Administration of endocannabinoids or inhibitors of endocannabinoid-degrading enzymes has been shown to reduce the growth of different types of tumour xenografts (Bifulco et al. 2001; Ligresti et al. 2003) and, therefore,

(continued)

Box 3 (continued)
could be a reasonable strategy for targeting cannabinoid receptors for anti-cancer purposes. However, as discussed in Box 1, the role of the endocannabinoid system, including the endocannabinoid-degrading enzymes, in the control of tumour generation and progression is not well understood. Since enhancing endocannabinoid tone only has mild anti-tumour effects in mice and since no inhibitor of endocannabinoid degradation has been approved as yet for use in humans, clinical studies aimed at analysing the efficacy of cannabinoids as anti-tumour agents should be based on the use of plant-derived or synthetic agonists of cannabinoid receptors rather than on endocannabinoids or inhibitors of endocannabinoid degradation.

Cannabis extracts or pure cannabinoids?

The long-known therapeutic properties of *Cannabis sativa*—including amelioration of symptoms associated with cancer and its chemotherapy—have led to the authorization of the medical use of this plant and its extracts in several countries. As mentioned in the text, *C. sativa* produces ~108 different cannabinoids, including THC and CBD. Some of the other cannabinoids present in marijuana may contribute to the attenuation of THC psychoactive-side effects or even to the production of other therapeutic benefits (Pertwee 2009). However, pure drugs are more prone to standardization than complex molecular cocktails. Thus, it would be ideal that studies aimed at investigating the anticancer actions of cannabinoids in patients were performed comparatively with both pure substances and cannabis extracts containing controlled amounts of THC, CBD and other cannabinoids.

Which routes of cannabinoid administration?

The most widely used route of administration of recreational and self-medicated marijuana is smoking. Although THC and other phytocannabinoids are rapidly absorbed by inhalation, smoking is an unattractive clinical option. Preclinical work in animal models has typically administered cannabinoids intra peri-tumourally. Likewise, in the only clinical trial in which a cannabinoid has been assayed as an anti-tumour agent, THC was administered locally (intracranial delivery to Glioblastoma patients) (Guzman et al. 2006). Nevertheless, this route of administration has many obvious limitations. Currently available cannabis-based medicines are administered as capsules or using an oro-mucosal spray (Pertwee 2009). Preclinical animal models have yielded data indicating that systemic (oral or intraperitoneal) administration of cannabinoids effectively reduces tumour growth (author's unpublished observations), so it seems reasonable that future clinical studies directed at determining the efficacy of cannabinoids as anti-tumour agents use oral or oro-mucosal routes of administration.

8 Conclusions and Future Directions

It is widely believed that strategies aimed at reducing mortality from cancer should consist of targeted therapies capable of providing the most efficacious and selective treatment for each individual tumour and patient. Thus, the major focus of anticancer-drug development has progressively moved from non-specific chemotherapies to molecularly targeted inhibitors. However, despite the huge amount of preclinical literature on how these rationally designed compounds work, the advance of most of these drugs into the clinic is still limited.

How do cannabinoid-based medicines fit into this ongoing scenario? Let us consider gliomas, the type of cancer on which the most detailed cannabinoid research has been conducted to date. As discussed above, engagement of a molecular target (CB receptors) by a family of selective drugs (THC and other cannabinoid agonists) inhibits tumour growth in animal models through a well-established mechanism of action that seems to operate in patients. Moreover, cannabinoids potentiate the antitumour efficacy of temozolomide and ALK inhibitors in mice harbouring gliomas. These findings provide preclinical proof-of-concept that "cannabinoid sensitizers" could improve the clinical efficacy of classical cytotoxic drugs in glioblastoma (Fig. 2) and perhaps other highly malignant tumours such as pancreatic cancer, melanoma and hepatocellular carcinoma. However, further research is required to define the precise molecular crosstalk between cannabinoids and chemotherapeutic drugs and to optimize the pharmacology of preclinical cannabinoid-based combinational therapies. Of note, the role of the endocannabinoid system in cancer generation and progression needs to be explored in further detail as—depending on the experimental model—genetic inactivation of cannabinoid receptors may enhance or decrease tumourigenesis in animal models of cancer. Accordingly, whether pharmacological manipulation of endocannabinoid levels (e.g. by using inhibitors of the enzymes involved in the degradation of these local mediators) could be used alone or in combination with other anticancer agents as an anticancer strategy needs to be clarified.

Regarding patient stratification, we should unequivocally determine which particular individuals are potentially responsive to cannabinoid administration. For this purpose, high-throughput approaches should be implemented to find cannabinoid therapy-associated biomarkers in tumour biopsies or, ideally, in easily acquired fluids containing circulating cancer cells or enhanced levels of resistance factors that could have been released by cancer cells. These biomarkers would conceivably relate to cannabinoid pharmacodynamics—namely expression and activity of cannabinoid receptors and their downstream cell-death inducing effectors. This would be analogous to the biochemical evaluation of oestrogen and ERBB2 receptors, which predict the benefit from endocrine therapies and trastuzumab, respectively, in breast cancer. Predictive markers to define the sensitivity of a particular tumour to cannabinoid-based therapies could also include the status of growth factors, such as MDK in gliomas, as well as their receptors and signalling partners.

In conclusion, cannabinoids induce tumour cell death and inhibit tumour angiogenesis and invasion in animal models of cancer, and there are indications that they

do so as well in patients with glioblastoma. As cannabinoids show an acceptable safety profile, clinical trials testing them as single drugs or, ideally, in combination therapies in glioblastoma and other types of cancer are both warranted and urgently needed.

Acknowledgements Research in the groups of the authors is supported by grants from Spanish Ministry of Economy and Competitiveness (MINECO) (PI12/02248, FR2009-0052 and IT2009-0053 to GV; and PI11/00295 to CS), Comunidad de Madrid (S2011/BMD-2308 to MG), GW Pharmaceuticals (to GV, CS and MG); Fundación Mutua Madrileña (AP101042012 to GV) and Fundació Telemarató (20134031 to GV)

Potential Conflict of Interest

We declare that GW Pharmaceuticals funded part of the research of our laboratory. Likewise, part of the data obtained by the authors in relation with the antitumour action of cannabinoids is included in three patent applications presented by GW Pharmaceuticals.

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R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1