Milestones in Drug Therapy
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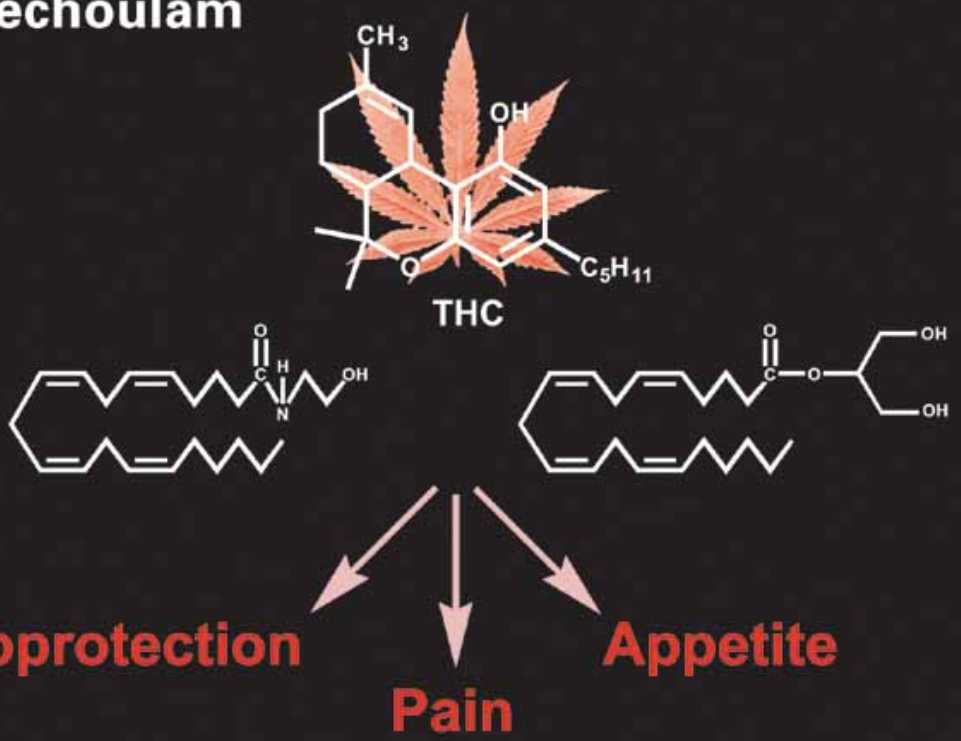
Cannabinoids as Therapeutics

Raphael Mechoulam
Editor



Birkhäuser

Milestones in Drug Therapy
MDT

Cannabinoids as Therapeutics

Edited by R. Mechoulam

Birkhäuser Verlag
Basel · Boston · Berlin

Editor
Raphael Mechoulam
Medical Faculty
Hebrew University
Ein Kerem, Jerusalem 91010
Israel

A CIP catalogue record for this book is available from the Library of Congress, Washington, D.C., USA

Bibliographic information published by Die Deutsche Bibliothek
Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the internet at http://dnb.ddb.de

ISBN 3-7643-7055-6 Birkhäuser Verlag, Basel - Boston - Berlin

Part of Springer Science+Business Media
Printed on acid-free paper produced from chlorine-free pulp. TFC ∞
Cover illustration: With the friendly permission of Lumír Hanuš

Printed in Germany
ISBN-10: 3-7643-7055-6
ISBN-13: 978-3-7643-7055-8

9 8 7 6 5 4 3 2 1 www. birkhauser.ch

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List of contributors

Itai A. Bab, Bone Laboratory, The Hebrew University of Jerusalem, P.O.B. 12272, Jerusalem 91120, Israel; babi@cc.huji.ac.il

Francis Barth, Sanofi-aventis, 371, rue du Professeur Joseph Blayac, 34184 Montpellier Cedex 04, France, e-mail: francis.barth@sanofi-aventis.com

Maurizio Bifulco, Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, and Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano (SA), Italy

Luciano de Petrocellis, Istituto di Cibernetica "Eduardo Caianiello", Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy

Vincenzo Di Marzo, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy; e-mail: vdimarzo@icmib.na.cnr.it

Javier Fernández-Ruiz, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain; e-mail: jjfr@med.ucm.es

Sara González, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain; e-mail: sgrc@med.ucm.es

Geoffrey W. Guy, GW Pharmaceuticals plc, Porton Down Science Park, Salisbury, Wiltshire, SP4 OjQ, UK

Lumír O. Hanuš, Department of Medicinal Chemistry and Natural Products, Pharmacy School, Medical Faculty, Hebrew University, Ein Kerem Campus, 91120 Jerusalem, Israel; e-mail: lumir@cc.huji.ac.il

Susan M. Huang, Department of Neuroscience Brown University, Providence, RI 02912, USA

Magdalena Kwiatkowska, Department of Psychology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada

Aron H. Lichtman, Department of Pharmacology and Toxicology, Virginia Commonwealth University, PO Box 980613, Richmond, VA 23298, USA; e-mail: alichtma@hsc.vcu.edu

Alessia Ligresti, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy

Cheryl L. Limebeer, Department of Psychology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada

Mauro Maccarrone, Department of Biomedical Sciences, University of Teramo,

Piazza A. Moro 45, 64100 Teramo, Italy; e-mail: mmaccarrone@unite.it
Raphael Mechoulam, Medical Faculty, Hebrew University, Ein Kerem Campus, 91120 Jerusalem, Israel; e-mail: mechou@cc.huji.ac.il
Richard E. Musty, Department of Psychology, University of Vermont, Burlington, VT 05405, USA; e-mail: musty@uvm.edu
Linda A. Parker, Department of Psychology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada; e-mail: lparker@wlu.ca
Daniela Parolaro, Center of Neuroscience, University of Insubria, Via A. da Giussano 10, 20152 Busto Arsizio (VA), Italy; e-mail: daniela.parolaro @uninsubria.it
Roger G. Pertwee, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland; e-mail: rgp@abdn.ac.uk
José Antonio Ramos, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain; e-mail: jara@med.ucm.es
Julián Romero, Laboratorio de Apoyo a la Investigación, Fundación Hospital Alcorcón, 28922 Alcorcón, Madrid, Spain; e-mail: jromero@fhalcorcon.es
Murielle Rinaldi-Carmona, Sanofi-aventis, 371, rue du Professeur Joseph Blayac, 34184 Montpellier Cedex 04, France, e-mail: murielle.rinaldi-carmona@sanofi-aventis.com
Tiziana Rubino, Center of Neuroscience, University of Insubria, Via A. da Giussano 10, 20152 Busto Arsizio (VA), Italy
Ethan Russo, GW Pharmaceuticals, 2235 Wylie Avenue, Missoula, MT 59809, USA; e-mail: erusso@montanadsl.net
Colin G. Stott, GW Pharmaceuticals plc, Porton Down Science Park, Salisbury, Wiltshire, SP4 OjQ, UK; e-mail: cgs@gwpharm.com
Stephen A. Varvel, Department of Pharmacology and Toxicology, Virginia Commonwealth University, PO Box 980613, Richmond, VA 23298, USA
J. Michael Walker, Department of Psychology, Indiana University, 1101 E 10th Street, Bloomington IN 47405-7007, USA; e-mail: walkerjm@indiana.edu

Preface

Twenty years ago the endocannabinoid system was unknown. We knew much about the use over millennia of *Cannabis* plant preparations both as a medicine and as "a drug that takes away the mind" (as so-well stated in ancient Assyrian clay tablets). During the early part of the last century considerable progress was made on the chemistry and pharmacology of *Cannabis*, but it was only after the identification in 1964 of Δ^9-tetrahydrocannabinol (Δ^9-THC) as the active constituent of the plant that this field caught the interest of many research groups and hundreds of papers on the chemistry, biochemistry, metabolism and clinical effects of this compound were published. However, its mechanism of action remained unknown for nearly two decades. In the mid-1980s the presence of a cannabinoid receptor in the brain was identified and shortly thereafter it was cloned. This was followed by the isolation of the major endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol, and the clarification of their biosyntheses and degradations. These advances led to an avalanche of publications in a wide variety of fields. We are now in the midst of major advances in biochemistry/physiology associated with the actions of the endocannabinoids.

This short volume tries to present an up-to-date picture in some of the major fields of endocannabinoid research. The first chapter in this book, on the use of *Cannabis* in India, can be viewed as an expression of thanks to the herbal practitioners, who for centuries passed on the medical traditions associated with the drug. The chemistry chapter is a short summary of active plant, synthetic and endogenous cannabinoids being investigated today, many of which are mentioned later in the book. Cannabidiol is an unusual cannabinoid – it does not bind to the known receptors and yet exerts a variety of effects. Hence a chapter is devoted to it. Most of the remaining chapters deal with the endocannabinoid system and the endocannabinoids in a variety of conditions and physiological systems. A chapter describes the research done on Sativex®, a standardized plant extract, shortly to be introduced in Canada as a drug for multiple sclerosis symptoms.

Numerous fields known to be affected by cannabinoids were not reviewed. The vast expanse of emotions is one of them. Most marijuana users smoke the drug in order to 'get high'. But we know very little about the mechanisms through which cannabis affects emotions. Under certain circumstances Δ^9-THC causes aggression, although usually it leads to sedation. Anxiety is another emotional aspect affected by cannabinoids. Although a short chapter is devoted to the calming of anxiety by cannabinoids it does not attempt to present a mechanistic picture. And we know next to nothing on the chemistry link-

ing endocannabinoids with stress, fear, love, satisfaction or despair. Are the endocannabinoids one of nature's tools to shape emotions? This is probably one of the fields which will be explored in the future. But books review the past. Possibly the next edition of this book, in 5 or 10 years time, will report on the progress made in associating endocannabinoids with emotions. Until then we shall have to remain content with more mundane topics such as neuroprotection, reproduction, appetite and effects on cancer.

The multitude of endocannabinoid effects seems like a fertile field for exploration by pharmaceutical firms. We soon expect to see the introduction of a synthetic cannabinoid antagonist in the treatment of obesity and, possibly later, drugs for neuroprotection, pain, multiple sclerosis, rheumatoid arthritis and cancer. Will post-traumatic stress disorder, schizophrenia and Tourette's syndrome come next?

Raphael Mechoulam Jerusalem, January 2005

Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabis in India: ancient lore and modern medicine

Ethan Russo

GW Pharmaceuticals, 2235 Wylie Avenue, Missoula, MT 59809, USA

Introduction: Ayurvedic medicine

India is a land steeped in faith and mysticism. *Ayurveda*, combining the Sanskrit words for life and knowledge, is a system of medicine intertwined inextricably with these traits. That a core of belief combined with empirical experimentation could produce a viable medical regimen still widely practiced after well over 3000 years is astounding to Western physicians. Cannabis was similarly bound to faith and mysticism in India in the past, in the Hindu and Islamic traditions, as well as in numerous other minority religions [1]. Merlin recently explained it well [2], "with the powerful tools of modern science and human imagination, our understanding of our deep-rooted desire to experience *ecstasy* in the original sense of the word (to break the mind free from the body and communicate with the 'gods' or the ancestors) will become clear with time". This chapter will seek to examine the medical claims for cannabis of the past, and place them in a contemporary light given current pharmacological knowledge.

Ayurveda is based on a conceptual medical system that seeks to balance three functional elements, called *doshas*, that the human body is composed of, and are commonly represented as *Vata* or *Vayu* (ether or air), *Pitta* (fire and water) and *Kapha* (phlegm or water and earth). Nadkarni [3] has rejected these simple relationships in favor of more abstract assignations [3]:

> "…the word *Vayu*, does not imply 'Wind' in Ayurvedic literature, but comprehends all the phenomena which come under the functions of the Central and Sympathetic Nervous Systems; that the word *Pitta* does not essentially mean 'Bile' but signifies the functions of Thermogenesis or heat production and metabolism, comprehending in its scope the process of digestion, coloration of blood and formation of various secretions and excretions and that the word *Kapha* does not mean 'Phlegm' but is used primarily to imply the functions of Thermo-taxis or heat regulation and secondarily formation of the various preservative fluids, e.g., Mucus, Synovia, etc., …"

Good health in Ayurveda is dependent upon attaining an equilibrium state of these factors. Disease is due to an imbalance or disharmony of the Tridosha system as the results of some cause, internal or external. A disease of prolonged extent will overflow its site of origin and spread through the body. Therapy is effected by a combination of religious, magical and prescriptive regimens, herbal therapy being an important element of the latter.

According to Kapoor [4], the *materia medica* of India comprises in excess of 2000 drugs, mostly of vegetal origin, with 700 medicinal plants known even during Buddhist times, *c*.250 BCE. Cannabis remains important among them.

Cannabis: its history in the medicine of India

Cannabis sativa seems to have diffused from a geographic point in Central Asia, according to classical plant explorers [5–8] and more modern authorities [9–11]. Sharma [12] felt its origin was in the Himalayan foothills, but offered little documentation. This botanical sleuthing has been supported by physical evidence of cannabis flowers and seeds associated with *haoma-soma* religious rites in ancient Bactrian sites in excavations by Sarianidi [13, 14] in Margiana (present day Turkmenistan), dating to the second millennium BCE. Philological support derives from the term *bhanga*, also seemingly originating among the Central Asian Arya peoples [15].

The *Zend-Avesta*, the holy book of Zoroastrianism, which survives in fragments, dating from around 600 BCE in Persia, alludes to the use of *Banga* in a medical context, identified as hemp [16]. Of this use, Bouquet stated [10]: "It is solely to its inebriating properties that hemp owes the signal honour of being sung in the *Vedas*, and it was probably the peoples of Northern Iran who discovered those properties, for they were already using the leaves (*Cheng*) and the resin (*Cers*) as inebriants before the Hindus." Mahdihassan [17] has attempted to draw a philological link between the *Ho-Ma* of the Chinese, the *Hao-Ma* of the *Avesta* and the *So-Ma* of Sanskrit, felt cognate to cannabis.

The earliest written reference to cannabis in India may occur in the *Atharvaveda*, dating to about 1500 BCE [18]: "We tell of the five kingdoms of herbs headed by Soma; may it, and *kusa* grass, and bhanga and barley, and the herb *saha*, release us from anxiety." Grierson [18] suggested this to be part of an offering, and ingestion or burning would both be typical of ancient practices for this purpose.

In the *Sushruta Samhita* (meaning the verses of Sushruta), perhaps dating from the third to the eighth centuries BCE, cannabis was recommended for phlegm, catarrh and diarrhea [18]. As noted, an anti-phlegmatic would be interpreted in Ayurvedic medicine as possessing a wide variety of effects. Similarly, Dwarakanath [19] has maintained that cannabis was employed in Indian folk medicine in aphrodisiacs and treatments for pain in the same era [19], while Sanyal observed [20] that "They also used the fumes of burning Indian Hemp (Canabis Indica) [*sic*] as an anaesthetic from ancient times…".

Watt [21] felt that by this early date the sexual dimorphism of cannabis was already evident to its cultivators, as well as the superiority of bhanga (mistakenly assigned as female) for cordage, and *bhang* (mistaken as male) for medical and mystical application. It was also likely about this time that the preparation of *ganja* (labeled *sinsemilla* in contemporary North America) was developed by isolating female cannabis plants to prevent fertilization, and increase resin production.

Aldrich [22] documented the development of tantric cannabis usage around the seventh century as a mingling of Shaivite Hinduism and Tibetan Buddhism. Apparently, the 11th century text, *Mahanirvana Tantra*, is currently still consulted with regard to sexual practices, withholding of male ejaculation and promotion of sexual pleasure in both genders.

An anonymous work, *Anandakanda*, added some 43 Sanskrit cannabis synonyms (Tab. 1) [23], many attesting the remarkable rejuvenating effects of cannabis. Dash [23] described the lengthy methods of cultivation, processing and mixing of cannabis with eight other medicinal plants, that when combined with personal isolation and celibacy for 3 years produce a result in which "it is claimed that the man lives for 300 years free from any disease and signs of old age". He dated this work to the 10th century, while Rao [24] placed it in the range of the 9th to the 12th centuries, and noted some 10 known manuscripts.

There is philological debate among Sanskrit scholars as to whether the identification of bhanga as cannabis can be authenticated before the year 1000 [25, 26]. Wujastyk [26] and Meulenbeld [25] dated the *Anandakanda*, or Root of Bliss, to *c*.1200, also noting its full accounting of cannabis' side effects. Their candidate for the first uncontested source for cannabis is the *Cikitsasarasangraha* of the Bengal author Vangasena, in the late 11th century, who included bhanga as an appetizer and digestive, noting it as "a drug like opium whose mode of action is to pervade the whole body before being absorbed and digested" [26]. It was also suggested in two recipes for a long and happy life. A contemporary work, the *Dhanvantariyanighantu*, observed a narcotic effect [26].

In the 12th–13th centuries from Gujarat, Nagarjuna's *Yogaratnamala* (The Garland of Jewels of Yoga), suggested cannabis smoke as a method by which to produce an impression of spirit possession in one's enemies [26].

The *Rajanighantu* of the 13th century added additional synonyms (Tab. 1), with attributed activities characterized as [18] (1) *katutva* (acridity), (2) *kashayatva* (astringency), (3) *ushnatva* (heat), (4) *tiktatva* (pungency), (5) *vatakaphapahatva* (removing wind and phlegm), (6) *samgrdhitva* (astringency), (7) *vakpradatva* (speech giving), (8) *balyatva* (strength-giving), (9) *medhakaritva* (inspiring of mental power) and (10) *sreshthadipanatva* (the property of a most excellent excitant).

According to interpretation of this source [27], "Its effects on man are described as excitant, heating, astringent; it destroys phlegm, expels flatulence, induces costiveness, sharpens the memory, excites appetite, etc."

Table 1. Indian names for cannabis in Sanskrit and Hindi

Indian name	Meaning
ajaya	the unconquered, invincible
ananda	the joyful, joyous, laughter moving, bliss
bahuvadini	causing excessive garrulousness
bhang, *bhanga*	hemp, mature cannabis leaves
bhangini	breaks three kinds of misery
bharita	the green one
capala	agile, capricious, mischievous, scatter-brained
capta	light-hearted
chapala	the light-hearted, causer of reeling gait, causer of vacillation
charas	cannabis resin (*hashish*), either hand-rubbed or sifted
cidalhada	gives happiness to mind
divyaka	gives pleasure, lustre, intoxication, beauty
dnayana vardhani	knowledge promoter
ganja	unfertilized female cannabis flowers
ganjakini	the noisy, vibrator
gatra-bhanga	body disintegrator
harshani	joy-giver
harshini	the exciter of sexual desire, the rejoicer, delight-giver, causer of elation
hursini	the exciter of sexual desire
Indrasana	Indra's food
jaya	victorious, the conquering
kalaghni	helps to overcome death
madhudrava	helps excrete nectar
madini	the intoxicator, sex intoxicator
manonmana	accomplishes the objects of the mind
matulani	wife of the datura
matkunari	an enemy of bugs
mohini	fascinating
pasupasavinaini	liberates creatures from earthly bonds
ranjika	causer of excitement
sakrasana	the worthy food of Indra
samvida manjari	flower causes garrulousness
sana	cannabis
sarvarogaghni	which cures all diseases
sawi	green leaved
Shivbooty	Shiva's plant
siddha	which has attained spiritual perfection
sidhamuli	on whose root is *siddha*
siddhapatri	vessel of highest attainment
siddhi	success giver
siddhidi	which endows *siddhi* on others
sidhdi	emancipation, beatitude, fruit of worship
suknidhan	fountain of pleasures
tandrakrit	causer of drowsiness
trailokya vijaya	victorious in the three worlds, conqueror of the three regions of the universe
trilok kamaya	desired in the three worlds
ununda	the laughter mover
urjaya	promoter of success
vijaya	victorious, promoter of success, all-conquering
vijpatta	the strong leaved
virapattra	leaf of heroes
vrijapata	strong nerved

About the same time, in the *Sharangadhara Samhita*, fresh extracts of bhang were employed medicinally [19], and it was linked to opium: "Drugs which act very quickly in the body first by spreading all over and undergoing change later are vyavayi; for example, bhanga, ahiphena" [28]. Additionally, cannabis was cited as an intoxicant and employed as the primary ingredient in a therapeutic mixture of herbs: "This recipe known as *jatiphaladi churna* if taken in doses of one karpa, with honey, relieves quickly grahani (sprue [chronic diarrhea]), kasa (cough), swasa (dyspnoea), aruchi (anorexia), kshaya (consumption) and pratishyaya [nasal congestion] due to vata kapha (rhinitis)" [28]. Inter-relationships of Tantra and Ayurveda in this work were explored by Sharma [29].

The 15th-century *Rajavallabha*, written by Sutradhar Mandan for Rana Kumhha of Mewar, attributed several additional qualities to cannabis [18]:

> "Indra's food (i.e., *ganja*) is acid, produces infatuation, and destroys leprosy. It creates vital energy, the mental powers and internal heat, corrects irregularities of the phlegmatic humour, and is an *elixir vitae.* It was originally produced, like nectar from the ocean by the churning with Mount Mandara, and inasmuch as it gives victory in the three worlds, it, the delight of the king of the gods, is called *vijaya*, the victorious. This desire-fulfilling drug was obtained by men on the earth, through desire for the welfare of all people. To those who regularly use it, it begets joy and destroys every anxiety."

Dymock added [27], "The Rahbulubha alludes to the use of hemp in gonorrhoea."

According to Chopra and Chopra [30], "In *Dhurtasamagama* (A.D. 1500), ganja is described as a soporific which 'corrects derangements of humours and produces a healthy appetite, sharpens the wit and act as an aphrodisiac'." In the *Ayurveda Saukhyam* of Todarananda [31] it was said of cannabis that "It causes unconsciousness, intoxication and talkativeness".

During the Renaissance European awareness of the psychoactivity of cannabis was kindled with the writings of Garcia da Orta, a Spanish Jew, who in the service of Portugal visited India in 1563. In addition to his descriptions of the plant as *bangue*, and a good illustration, he noted important medical properties [32], "The profit from its use is for the man to be beside himself, and to be raised above all cares and anxieties, and it makes some break into a foolish laugh." In another passage, stimulation of energy and appetite was noted: "Those of my servants who took it, unknown to me, said that it made them so as not to feel work, to be very happy, and to have a craving for food."

Soon thereafter, it was observed [30], "In *Bhavaprakash* (A.D. 1600), cannabis is mentioned as 'anti-phlegmatic, pungent, astringent and digestive'. On account of these marked narcotic properties it was probably also used as an anaesthetic, sometimes combined with alcohol, by the ancient Indian and Chinese surgeons."

The 18th century Persian medical text *Makhzan-al-Adwiya*, written by M. Husain Khan, was extremely influential in the *Unani Tibbi*, or Arabic-tradition medicine on the subcontinent. In it, cannabis was described in its various preparations as an intoxicant, stimulant and sedative, but also the following [33]:

> "The leaves make a good snuff for deterging the brain; the juice of the leaves applied to the head as a wash, removes dandriff [sic] and vermin; drops of the juice thrown into the ear allay pain and destroy worms or insects. It checks diarrhea, is useful in gonorrhea, restrains seminal secretions, and is diuretic. The bark has a similar effect.
>
> The powder is recommended as an external application to fresh wounds and sores, and for causing granulations; a poultice of the boiled root and leaves for discussing inflammations, and cure of erysipelas, and for allaying neuralgic pains."

Ali Gorji (personal communication, 2004) has recently consulted this work and added that it was helpful for stomach problems, nausea and uterine inflammation. Campbell [1], translated additional Persian names from this source: "Bhang is the Joy-giver, the Sky-flier, the Heavenly-guide, the Poor Man's Heaven, the Soother of Grief". Dymock and co-authors added a few more synonyms [34]: "the inebriating leaf", "fakir's grass", "the green tent" and "the throne giver". Chopra and Chopra [30] rendered another passage from the *Makhzan* as follows: "It is said that bhang is one of the best of God's gifts, it is a cordial, a bile absorber, and an appetizer, and its moderate use prolongs life. It quickens the fancy, deepens thought and sharpens judgment."

A nexus with Western medicine

The medical use of so-called Indian hemp was reintroduced to the West in the 19th century. In 1813, Ainslie [35] cited the use of *ganjah* and *bangie* as intoxicants, but also to treat diarrhea, and in a local application for hemorrhoids. In 1839, the seminal work of Sir William B. O'Shaughnessy on cannabis was written [36], then republished in England in 1843 [33]. His contribution was a model of modern investigation, involving a review of classical Sanskrit and Unani sources, a description of cannabis preparations including bhang (mature cannabis leaves), ganja (unfertilized female flowers), and *charas* (processed cannabis resin), an examination of contemporary Indian ethnobotanical uses and experiments of cannabis extracts in dogs, finally culminating with a series of human clinical trials with appropriate cautious dose titration. His treatise on the subject demonstrated the apparent clinical utility of cannabis in a wide range of disorders including cholera, rheumatic diseases, delirium tremens and infantile convulsions. For the first time miraculous recoveries were evidenced in a series of tetanus victims due to cannabis. Noting the anti-spasmodic and muscle-relaxant effects, it was tried in rabies, where [33] "the influence of a

narcotic, capable either of cheering or of inducing harmless insensibility, would be fraught with blessing to the wretched patient". Although no cure was forthcoming, the patient was visibly relieved of distress, and able to take some sustenance through his suffering. Its palliative benefit was not lost upon the physician, "the awful malady was stripped of its horrors; if not less fatal than before, it was reduced to less than the scale of suffering which precedes death from most ordinary diseases". Summing up his experience with cannabis, O'Shaughnessy concluded that "in hemp the profession has gained an anti-convulsive remedy of the greatest value".

A series of other practitioners both in India and in Great Britain soon noted success in extending the use of cannabis to treatment of migraine, and neuropathic and other pain conditions [37, 38]. Few clinical syndromes seemed unassailable: another Western physician in India observed the alleviation not only of an alcohol hangover with accompanying headache, but the patient's cholera as well [39]. Churchill employed cannabis to treat excessive uterine bleeding [40], and Christison applied it to childbirth [41] (reviewed in [42]).

In little more than a decade, a section on cannabis was deemed worthy of inclusion in Johnston's *The Chemistry of Common Life*, wherein the topic was treated at length [43]: "In India it is spoken of as the increaser of pleasure, the exciter of desire, the cementer of friendship, the laughter-mover, and the causer of the reeling gait, – all epithets indicative of its peculiar effects." About the same time, medical usage became common in North America [44].

In 1870, Dutt provided information on certain bhang preparations [45], "Numerous confections of *bhang* such a *Kamesvara modaka*, *Madana modaka*, *Balyasakrasana modaka*…are considered aphrodisiacs and are used in chronic bowel complaints, and nervous debility." A recipe for *Madana modaka* was then supplied, containing numerous herbs, but with "hemp leaves with flowers and seeds fried in clarified butter, equal in weight to all the other ingredients", which was "used in cough, chronic bowels complaints and impotence".

In 1877, Kerr submitted an extremely detailed report from Bengal encompassing history, religious context, cultivation and employment of cannabis in all its preparations [46]. This would form one source for the subsequent *Report of the Indian Hemp Drugs Commission* [47]. Documentation of ganja production, necessitating culling of male plants by the "ganja doctor" to prevent fertilization and increase resin production, was emphasized. Despite some apparent value judgments expressed, the author observed, "I am of opinion, however, that no moral gain whatever will be effected by the total suppression of ganja."

Watt noted that cannabis was [21] "valuable as a remedy for sick headache, and especially in preventing such attacks. It removes the nervous effects of a malady." Watt listed numerous contemporary European physicians on the subcontinent and their successes in treating a large variety of disorders with cannabis preparations. Dymock was one such [34]: "I have given the extract in doses of from ½ to 1 grain to a large number of European hospital patients suf-

fering from chronic rheumatism; it entirely relieved the pains and made them excessively talkative and jolly, complaining that they could not get enough to eat." Dymock also appreciated popular Indian descriptions of the time [34]: "When the *ganja* pipe begins to smoke all cares at once disappear" and "Smoke *ganja* and increase your knowledge".

Cannabis in its various forms remained the focus of intense scrutiny, and continued to harbor critics. Because of concerns of its moral dangers, the British and colonial authorities in India organized a commission to examine all aspects of the issue [47]. Its findings exceeded 3000 pages after exhaustive investigation and testimony, and may be summarized as follows [48]. (1) Moderate use of cannabis drugs had no appreciable physical effects on the body. As with all drugs, excessive use could weaken the body and render it more susceptible to diseases. Such circumstances were not peculiar to cannabis, however. (2) Moderate use of cannabis drugs had no adverse effect on the brain, except possibly for individuals predisposed to act abnormally. Excessive use, on the other hand, could lead to mental instability and ultimately to insanity in individuals predisposed by heredity to mental disorders. (3) Moderate use of cannabis drugs had no adverse influence on morality. Excessive usage, however, could result in moral degradation. Although in certain rare cases cannabis intoxication could result in violence, such cases were few and far between.

The commission advocated against governmental suppression of cannabis drugs. Many positive statements accompanied descriptions of their religious associations, and particularly their legion medical usage, both human and veterinary [1]:

> "It is interesting, however, to note that while the drugs appear now to be frequently used for precisely the same purposes and in the same manner as was recommended centuries ago, many uses of these drugs by native doctors are in accord with their application in modern European therapeutics. *Cannabis indica* must be looked upon as one of the most important drugs of Indian Materia Medica."

Particular attention remained focused on possible mental health sequelae of cannabis despite the lack of such findings from the Commission. In the conclusions of Mills [49]:

> "Indians used hemp narcotics for a variety of reasons and it is entirely possible that its use at certain times disagreed with certain individuals to the extent that they became muddled or even murderous. Yet the few of those that did become muddled or murderous and that were snared in the net of the colonial state came to be taken as representative of all those in India that used cannabis preparations. From this, colonial government developed an image of all Indian users of hemp narcotics as dangerous, lunatic and potentially violent."

Occasionally, colonial officials were enlightened enough to free themselves from ethnocentric chauvinism. One Captain R. Huddleston, a Deputy Commissioner in the Akola District, wrote in 1872 [50], "Therefore I should not condemn these compounds [cannabis preparations] as being directly connected with crime; that is to say, they are no more the cause of offence than is the bazar liquor with which the Banjara is so often primed when he does highway robbery, or the beer and gin guzzled by the British rough before he beats his wife and assaults a policeman." Modern epidemiological investigation refutes the etiological relationship of cannabis to violence and insanity [51], but the debate continues.

In 1897, cannabis retained a key indication [52], "The treatment of Tetanus by smoking gunjah…promises to supercede all other in India." Waring [52] went on to describe its effective application at the onset of spasms, and titration to patient requirements so long as was needed. In a previous source [53], smoking every few hours was recommended for the duration of need, which in four subjects ranged from 7 days to 1½ months. Lucas [54] introduced the concept of smoking cannabis for tetanus to the British medical press in 1880.

Meanwhile, cannabis spread to other British colonies with the Indian diaspora. Emigrants brought the herb along with them as a work accessory or medicine. In South Africa they adopted the local name *dagga* [55], whereas in Jamaica the Indian name, ganja, has been pre-eminent since the 19th century [56, 57], and its tonic effects are part of national medical lore today [57].

Politics and cannabis collide

At the dawn of the 20th century, cannabis suffered further downturns. In 1914 it was dropped from the pharmacopoeia of Ceylon (now Sri Lanka), over the vociferous objections of its adherents, such as Ratnam [58], whose points of debate included passionate defenses of its medical benefits and poignant political arguments comparing its benign nature to the relative dangers of other popular recreational agents, alcohol in particular. The status of cannabis was compounded by increasingly severe quality-control problems with material exported from India to the UK [59]. These two factors, political and pharmacological, were paramount in the decline of cannabis medicines in the West.

Cannabis use remained common in 20th-century India, however. It was noted [60]:

> "Labourers who have to do hard physical work use hemp drugs in small quantities to alleviate the sense of fatigue, depression and sometimes hunger. … This produces a sense of well-being, relieves fatigue, stimulates the appetite, and induces a feeling of mild stimulation which enables the worker to bear the strain and perhaps the monotony of this daily routine of life more cheerfully."

Similarly, by 1954, cannabis remained integral in Indian faith, as one Brahmin explained to a Western writer [61], "'It gives good *bhakti*', …the sort of devotional act which consists in emptying the mind of all worldly distractions and thinking only of God."

As late as 1957, two authorities in India noted [30], "Cannabis undoubtedly has remarkable therapeutic properties. …the drug has no constipating action, it does not depress the respiratory centre; and there is little or no liability to addiction formation." They went on to describe the usage in veterinary medicine for diarrhea in livestock, treating parasites, "footsore disease, increasing milk-flow in cows, and pacifying them, but also it is often administered to bullocks as a tonic, to relieve fatigue and to impart additional staying power." As a human household remedy, "A mild beverage made from bhang leaves is believed to sharpen the appetite and help the digestion." Religious mendicants were said to employ it for gastrointestinal and rheumatic afflictions during their peregrinations. Continued attestations were claimed for dysmenorrhea, gonorrhea, dysuria, asthma and spasmodic conditions. A fresh leaf poultice was said to reduce eye pain and conjunctivitis, swollen joints and local inflammations, while a piece of charas placed in dental caries was said to alleviate toothache. They noted, "Much of the sanctity attached to bhang is put down to its supposed properties 'clearing the head and stimulating the brain to think'." Finally, contributions to sexual performance were still claimed, as cannabis preparations "are frequently used by both young and middle-aged individuals for stimulating sexual desire and prolonging the sexual act".

Usage in Unani medicine at this time included treatment of insomnia, migraine, neuralgic pains, asthma, spasmodic conditions and previously noted gynecological conditions [30]. A continued contribution to Islamic mysticism was also noted as cannabis use "frees them from worldly bonds, and induces communion with the divine spirit".

In another book about medicinal plants of India [62], the author stated:

> "Charas…is a valuable narcotic, especially in cases where opium cannot be administered; it is of great value in malarial and periodical headaches, migraine, acute mania, whooping cough, cough of phthisis, asthma, anaemia of brain, nervous vomiting, tetanus, convulsion, insanity, delirium, dysuria, and nervous exhaustion; it is also used as an anaesthetic in dysmenorrhea, as an appetizer and aphrodisiac, as an anodyne in itching of eczema, neuralgia, severe pains of various kinds of corns, etc."

Indian charas of good quality is said to have a resin content of about 35–45% [63], which according to the calculations of Clarke [64], might yield a theoretical tetrahydrocannabinol (THC) content of up to 30%. Higher concentrations have been achieved with modern techniques.

Nadkarni [3] observed of cannabis, "All parts of the plant are intoxicating (narcotic), stomachic, antispasmodic, analgesic (anodyne), stimulating, aphrodisiac and sedative."

In 1977, Sharma observed [65] that "even today [cannabis] is used with restraint and judgment by students of Indian medicine. There are reports claiming the value of cannabis in the treatment of high blood pressure, migraine headaches, and even cancer."

In a modern review of Indian uses of cannabis, it was observed [66] that "Cannabis was used medicinally for almost all the ills flesh is heir to". Cannabis remained a key ingredient in two aphrodisiacal preparations, *Madana modaka* and *Kamesvara modaka* [67].

In a treatise entitled *Indigenous Drugs of India* [68] the authors noted the requirement of dose titration due to increasingly inconsistent cannabis preparations. This drawback was addressed in a prior study [69] in which the authors extracted local ganja to produce a 17% THC yield, which at intraperitoneal doses of 75 mg/kg in rats resulted in a potentiation of sub-analgesic doses of morphine.

In 1988 [70] cannabis was still mentioned as a remedy for malaria and blood poisoning, among many other indications. In neighboring Nepal, cannabis retains ethnobotanical applications among some 15 ethnic groups [71], for diarrhea, dysentery, local wound treatment and in veterinary medicine. In discussing the native use of cannabis and opium products by village doctors in India, who provided 80% of the population with their medical care in a report to the United Nations, the author felt that a legitimate role for them persisted [19]:

> "These drugs should be allowed to be used by Ayurvedic and Unani physicians until such time as the benefits of modern medicine are extended to rural areas. Banning their use by the large mass of Ayurvedic and Unani physicians for therapeutic purposes may create a vacuum which may not be easily filled for a long time to come."

Cannabis in contemporary Ayurvedic medicine

According to Chopra and Chopra [30], the modern Ayurvedic properties of cannabis are: *paphahari*, promoting loosening, separation and the elimination of phlegm; *grahini*, promoting retention and binding the bowels; *pachani*, promoting digestion; *ushna*, promoting heat; *pitala*, exciting the flow of bile; *mada-vardhani*, promoting talkativeness or releasing the volitional restraint of speech; *moda-vardhani*, promoting happiness; *vag-vardhani*, stimulating the digestive fire; *dipani*, stimulating appetite; *ruchya*, promoting taste; *nidraprada*, hypnotic. Kapoor [4] described its Ayurvedic attributes as follows [4]: its *rasa* (taste) is *tikta* (bitter); its *guna* (physical properties) are *laghu* (light, easy to digest), *teeshan* (acute, pungent) and *rooksha* (ununctuous); its *veerya*

(energy modality or potency) is *ushana* (heating, digestive); and its *vipaka* (transformation reactions after digestion) are *katu* (constipative, semen increasing). Among its properties and uses, it is conceived of as: *madakari* (causing intoxication), *nidrajanan* (sleep-inducing), *dipan* (affecting appetite), *grahi* (absorbable) and *pachan* (affecting digestion). Dwarakanath's [19] assignations were quite similar to these, but added Muslim descriptions such as constipative, stomachic, appetizer, causing elation, aphrodisiac, retentive, devitalizing, anodyne, hypnotic, anti-convulsant, causing delirium and intoxicating. The same author listed the names of 48 modern Ayurvedic and eight Unani Tibbi formulas containing cannabis for a wide range of indications.

A recent survey of bhang use in the holy city of Varanasi (formerly Benares) found it quite prevalent across socioeconomic strata, especially the working class, businessmen and among the more educated [72]. Most users in the third or fourth decades of life employed it for anxiety or mood disorders for the resulting pleasure, while older people cited benefits on gastrointestinal disorders with improvement in appetite and bowel habits, or for alleviating insomnia. Among the 100 subjects, 90% reported improvement in sleep without daytime fatigue. Improvement in "marital adjustment" was also claimed. All employed bhang orally, generally 1.5 g/day, for gastrointestinal indications, but 56% employed 4–10 g/day, without evidence of associated toxic adverse events.

In 1996, native cannabis was again extracted to a yield of 17% THC, which was then used to treat cancer pain in 42 human subjects [73]. In 11.9% there was no analgesia with doses of 25 mg, but 64.3% had up to 50% pain reduction, and 9.5% had greater than 75% pain relief with no use of adjunctive medicine.

Dash [23] identified cannabis as one of the primary herbs of rejuvenation and a synergist with other agents, promoting health, preventing disease and offering "side benefits". In order of therapeutic priority, its uses were listed as: sprue syndrome, sterility, impotency, diarrhea, indigestion, epilepsy, insanity and colic pain. In addition to the many indications above, the following were also noted: gastritis, anorexia, anal fistula, throat obstruction, jaundice, bronchitis, tuberculosis, torticollis, splenic disorder, delirium, obstinate urinary disorders, sinus problems, anemia, rhinitis, elephantiasis, edema, puerperal sepsis, gout and constipation.

The scientific basis of Indian cannabis claims

This chapter has enumerated the lore of Indian medicine with respect to therapeutic benefits of clinical cannabis, but what is its scientific rationale? The issues will be addressed systemically (Tab. 2).

The oldest cannabis claims are psychiatric from the *Atharvaveda*, citing its usage for anxiety. Current research is supportive, particularly for cannabidiol (CBD) as an anti-anxiety agent as well as an anti-psychotic (reviewed in [74]). Similar benefit may accrue in calming dementia, as THC proved beneficial in Alzheimer's disease patients [75]. Recently, cannabichromene (CBC)

Table 2. Indications for cannabis in India

Cannabis indication	Physiological basis	Reference
Psychiatric		
Anxiety	CBD reduces anxiety in humans	[74]
Extinction of aversive memories	EC control in hippocampus	[77]
Insomnia	Increased sleep in pain/multiple sclerosis patients	[79, 80]
Addiction treatment	Decreased usage of cocaine/alcohol	[84, 86]
Neurological		
Neuropathic pain	EC modulation of CNS pathways Clinical pain reduction	[87, 88] [79, 80]
Muscle relaxation	Spinal interneuron effects?	[79, 89]
Neuroprotection	THC/CBD antioxidant/NMDA antagonism	[91]
Migraine	Effects on periaqueductal grey, 5-HT, inflammation, etc.	[88, 92, 93]
Seizures	CBD anticonvulsant THC anticonvulsant, EC modulation of seizure threshold	[95] [96, 97]
Dermatological		
Anti-psoriatic?	TNF-α antagonism	[99]
Anti-pruritic	Peripheral anti-nociception	[100]
Rheumatic		
Benefit in rheumatoid arthritis	TNF-α antagonism	[99]
Endocrinological		
Appetite stimulation	Hypothalamic effect?	[101]
Oncological		
Anti-nausea	5-HT_3 antagonism or other?	[102, 103]
Tumor reduction	Promotes apoptosis Reduces angiogenesis Anti-prolactin effect Blocks pulmonary carcinogenesis	[104, 105] [104] [106] [107]
Pulmonary		
Asthma	Bronchodilation	[108, 110]
Gastroenterological		
Intestinal spasm	Smooth muscle relaxation	[88, 112]
Secretory diarrhea	EC modulation of secretion	[112]
Gastritis	Anti-inflammatory/gastric cytoprotection	[114, 115]
Jaundice	? immunomodulatory	[116]

(Continued on next page)

Table 2. (Continued)

Cannabis indication	Physiological basis	Reference
Gynecological		
Dysmenorrhea	Smooth muscle relaxation	Reviewed in [42]
Uterine bleeding	EC modulation in uterus	Reviewed in [42]
Lower-urinary-tract symptoms	Increased bladder capacity, decreased incontinence	[118]
Sexual		
Impotence	Pain reduction/spinal effects?	[119]
Premature ejaculation	EC modulation	[120]
Infectious		
Antibiotic	Effects of cannabinoids/terpenoids	[111, 121]
Anti-malarial	Caryophyllene, α-terpineol	[121, 123]
Insecticidal/pediculicidal	Octopamine/GABA	[126–128]

CBD, cannabidiol; CNS, central nervous system; EC, endocannabinoid; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine; 5-HT_3, serotonin type-3 receptor; NMDA, *N*-methyl-D-aspartate; TNF-α, tumor necrosis factor-α.

has also demonstrated anti-depressant effects in an animal model [76]. Additional support for benefits of cannabis on mood is evident from work demonstrating the regulation of extinction of aversive memories by the endocannabinoid system [77].

Insomnia treatment is another ancient claim that finds documentation in modern phase II–III clinical-trial results in multiple sclerosis patients and those with chronic neuropathic pain [78–81]. The 19th-century observation of benefit on addiction is echoed in modern studies of alcoholics [82] and cocaine users [83], with experimental support for decreased use rates in clinical experiments for each [84–86].

In the neurological realm, the ability of cannabis to treat pain, particularly of neuropathic origin, is the subject of a great deal of current research. Results to date are very encouraging, in terms of both basic science support (reviewed in [87, 88]) and the benefits in clinical trials [78–80].

Although tetanus is rarely observed in the modern age of immunization, the observed benefits on muscle relaxation underlie current application to treatment of spasms and spasticity in multiple sclerosis and spinal cord trauma [79, 89], where cannabis extracts have proven as effective as any currently available agent [90]. Although rabies remains invariably fatal, the neuroprotective effects of cannabis [91] may warrant new trials of cannabis extracts in its treatment, and that of slow virus (prion) diseases. Indian medical literature on migraine treatment is also supportive, as is a tremendous amount of pathophysiological data [88, 92, 93]. As for clinical trials, however, the words of Dr Mechoulam still ring true [94]: “no modern work exists”.

Another long-held claim pertains to cannabis in epilepsy. Previous experimental work showed some support for CBD [95], but this has been greatly bolstered by current experiments by Wallace et al. [96, 97], demonstrating the anti-convulsant properties of THC, and the modulation of seizure thresholds by anandamide.

Examining additional ectodermal tissue, both eczema and itch were cited in Indian literature as benefiting from cannabis treatment. Recent work demonstrating the value of tumor necrosis factor-α (TNF-α) antagonists in psoriasis [98] may justify the use of cannabis, particularly CBD-rich extracts, in the treatment of related diseases, as CBD shares this mechanism of action [99]. Similarly, the benefits of THC on peripheral pain and itch are becoming increasingly evident [88, 100].

Rheumatic diseases cited by O'Shaughnessy [36] and other authors remain an issue, but experiments underline the benefits of CBD in experimental rodent models of rheumatoid arthritis [99]. Phase II clinical trials are pending. Modern investigation demonstrates that cannabinoid treatments definitely have a clinical role to play in issues of appetite, with benefit seen in HIV/AIDS subjects [101], and in multiple sclerosis/neuropathic pain patients [79].

The role of cannabis in oncology may now extend far beyond its demonstrated ability to allay nausea in chemotherapy [102, 103], but include promotion of apoptosis, and suppression of angiogenesis in a wide variety of tissue types (reviewed in [104, 105]). Additionally, THC has anti-prolactin activity in breast carcinomas [106], and introduces a metabolic block in pulmonary carcinogenesis [107].

The role of cannabis in asthma has been much debated, but it is clear that THC is a bronchodilator [108], as is its terpenoid component, α-pinene [109], and that smooth muscle contraction in the lungs is mediated by endocannabinoids [110]. Given these facts, plus the prominent anti-inflammatory benefits of THC, CBD and terpenoids [111], it is apparent that additional investigation with vaporizer or other non-smoked inhalant technology with cannabis extracts is warranted.

The treatment of digestive issues with cannabis has figured prominently in India to the current day. Whether it be through reduction of intestinal spasms, constipation or inhibition of secretory diarrhea processes in cholera, cannabis components offer neuromodulatory amelioration (reviewed in [88, 112]). Given the combination of these factors mediated by THC, the TNF-α antagonism of CBD and the observed up-regulation of endogenous cannabinoids in human inflammatory bowel disease [113], there is every reason to believe that benefits will be forthcoming in clinical trials of cannabis extracts in Crohn's disease and ulcerative colitis. The gastritis claim finds support in studies documenting the benefit of cannabis in ulcer treatment [114], and the gastric cytoprotective effect of the cannabis essential-oil component, caryophyllene [115]. Even claims for treatment of jaundice may find support in recent claimed benefits seen in hepatitis C patients who use cannabis [116].

Hemorrhoids continue to plague mankind, and anecdotal evidence for the benefits of cannabis from rural Kentucky echo the Indian claims [117]. Myriad anti-inflammatory and anti-pruritic mechanisms may underlie the basis of such treatment. The benefits of cannabis in dysmenorrhea and excessive uterine bleeding are plausible given the expression of endocannabinoids in the uterus (reviewed in [42]). The benefits of cannabis in symptoms of the lower urinary tract have been strongly supported by increases in mean maximum cystometric capacity, decreased mean daytime frequency of urination, decreased frequency of nocturia and mean daily episodes of incontinence in multiple sclerosis patients treated with cannabis-based medicine extracts [118].

The persistence of claims of cannabis increasing sexual pleasure and performance is compelling, but not particularly amenable to simple experimental verification. Does cannabis treat impotence? There are frequent claims of such, including a successful pregnancy induced by one man who was previously impotent due to spinal damage, treated successfully with oromucosal cannabis-based medicine [119]. Additionally, recent data demonstrate that a cannabinoid agonist delayed ejaculatory responses in rats [120]. Thus, a convincing case may be made for human clinical trials [88].

Claims of the benefits of cannabis in infectious diseases have received little investigation since studies on bacteria in 1960 [121], wherein the authors demonstrated that an isolated resin from cannabis inhibited growth of *Mycobacterium tuberculosis* down to a dilution of 1:150000. Studies on human herpes simplex virus in 1980 revealed the inhibition of viral growth by THC even at low dosages [122]. A variety of cannabis components are anti-infective (reviewed in [111]), supporting such applications, as well as the use of cannabis in the treatment of malaria, where the essential oil components caryophyllene and α-terpineol demonstrate anti-protozoal activity [123]. Cannabis may yet prove useful in the treatment of dandruff, as suggested in Indian sources. Cannabichromene demonstrated anti-fungal activity [124], and ρ-cymene showed anti-candidal effects [125]. Cannabis effects on the causative yeast in dandruff, *Malassezia ovalis*, could be easily tested.

Clear benefits also seem likely in the treatment of lice, as this ancient indication has been supported by pediculicidal efficacy of cannabis terpenoid components [126], the activity of terpenoids on insect octopaminergic receptors [127], and their allosteric modulation of insect homo-oligomeric γ-aminobutyric acid (GABA) receptors [128]. A whole range of new applications of cannabis as an insecticide are possible [129]. Mechoulam decried the lack of investigation of cannabis effects on intestinal parasites [94], and this remains an area of deficiency in our cannabis knowledge.

Cannabis in India in context

As we have seen, the vast majority of claims for cannabis from India are fully corroborated by modern scientific and clinical investigation. In closing, a pas-

sage from Campbell [1] written for the *Report of the Indian Hemp Drugs Commission* more than a century ago offers a plaintive plea for this venerable herb:

> "By the help of bhang ascetics pass days without food or drink. The supporting power of bhang has brought many a Hindu family safe through the miseries of famine. To forbid or even seriously to restrict the use of so holy and gracious a herb as the hemp would cause widespread suffering and annoyance and to the large bands of worshipped ascetics deep-seated anger. It would rob the people of a solace in discomfort, of a cure in sickness, of a guardian whose gracious protection saves them from the attacks of evil influences, and whose mighty power makes the devotee of the Victorious, overcoming the demons of hunger and thirst, of panic fear, of the glamour of Maya or matter, and of madness, able in rest to brood on the Eternal, till the Eternal, possessing him body and soul, frees him from the having of self and receives him into the ocean of Being. These beliefs the Musalman devotee shares to the full. Like his Hindu brother the Musalman fakir reveres bhang as the lengthener of life, the freer from the bonds of self. Bhang brings union with the Divine Spirit. 'We drank bhang and the mystery I am He grew plain. So grand a result, so tiny a sin.'"

It is appropriate that modern-day cannabinoid researchers have acknowledged the integral role that Indian culture has played in our understanding of the biochemistry of cannabis. Thus, the first endocannabinoid, arachidonylethanolamide, was dubbed anandamide (*ananda* is Sanskrit for bliss; Tab. 1) [130]. In like manner, the most recently identified endocannabinoid, the cannabinoid antagonist *O*-arachidonylethanolamine, which is arachidonic acid and ethanolamine joined by an ester linkage, has been nicknamed virodhamine (*virodha* is Sanskrit for opposition) [131].

It is fascinating to note that our own endogenous cannabinoid physiology encompasses these positive and negative influences, in a manner analogous to THC and CBD effects from cannabis, the Indian phytopharmaceutical that leads us to this knowledge: nature and neurophysiology in symmetry and balance.

Acknowledgments
The author would like to thank Dominik Wujastyk, Jan Meulenbeld, Robert A. Nelson, Knut Movik, Ali Gorji and the dedicated staff of Interlibrary Loan at Mansfield Library, University of Montana, for their kind provision of resource materials for this chapter.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabinoid chemistry: an overview

Lumír O. Hanuš and Raphael Mechoulam

Department of Medicinal Chemistry and Natural Products, Medical Faculty, The Hebrew University of Jerusalem, Ein Kerem Campus, 91120 Jerusalem, Israel

Introduction

Cannabis sativa probably originates from neolithic China [1]. However the exact period of its domestication is unknown. The first known record of the use of cannabis as a medicine was published in China 5000 years ago in the reign of the Emperor Chen Nung. It was recommended for malaria, constipation, rheumatic pains, absent-mindedness and female disorders. Later its use spread into India and other Asian countries, the Middle East, Asia, South Africa and South America. It was highly valued in medieval Europe. In Western Europe, particularly in England, cannabis was extensively used as a medicine during the 19th century, while in France it was mostly known as a "recreational" drug [2].

Natural cannabinoids

The first successful attempt to identify a typical cannabis constituent was achieved by Wood et al. [3], who isolated cannabinol from the exuded resin of Indian hemp (*charas*), which was analysed as $C_{21}H_{26}O_2$. Another big step was made by Cahn, who advanced the elucidation of the structure of cannabinol [4], leaving as uncertain only the positions of a hydroxyl and a pentyl group. Several years later Todd's group in the UK [5, 6] and independently Adam's group in the USA [7] synthesized several cannabinol isomers and compared them with the natural one. One of the synthetic isomers was identical to the natural product. The correct structure of the first natural cannabinoid, cannabinol, was thus finally elucidated. These two groups assumed that the psychotropically active constituents were tetrahydrocannabinols (THCs), which however they could not isolate in pure form and therefore they could not elucidate their structures.

A second cannabis constituent, the psychotropically inactive cannabidiol, was also isolated, but its structure was only partially clarified [8]. Synthetic THC derivatives, which showed cannabis-like activity in animal tests, were prepared, but they obviously differed from the active natural product, on the basis of their UV spectrum [9–12].

In a systematic study of the antibacterial substances in hemp Krejčí and Šantavý found that an extract containing carboxylic acids was effective against *Staphylococcus aureus* and other Gram-positive micro-organisms. They isolated cannabidiolic acid and reported a nearly correct structure [13, 14] (Fig. 1).

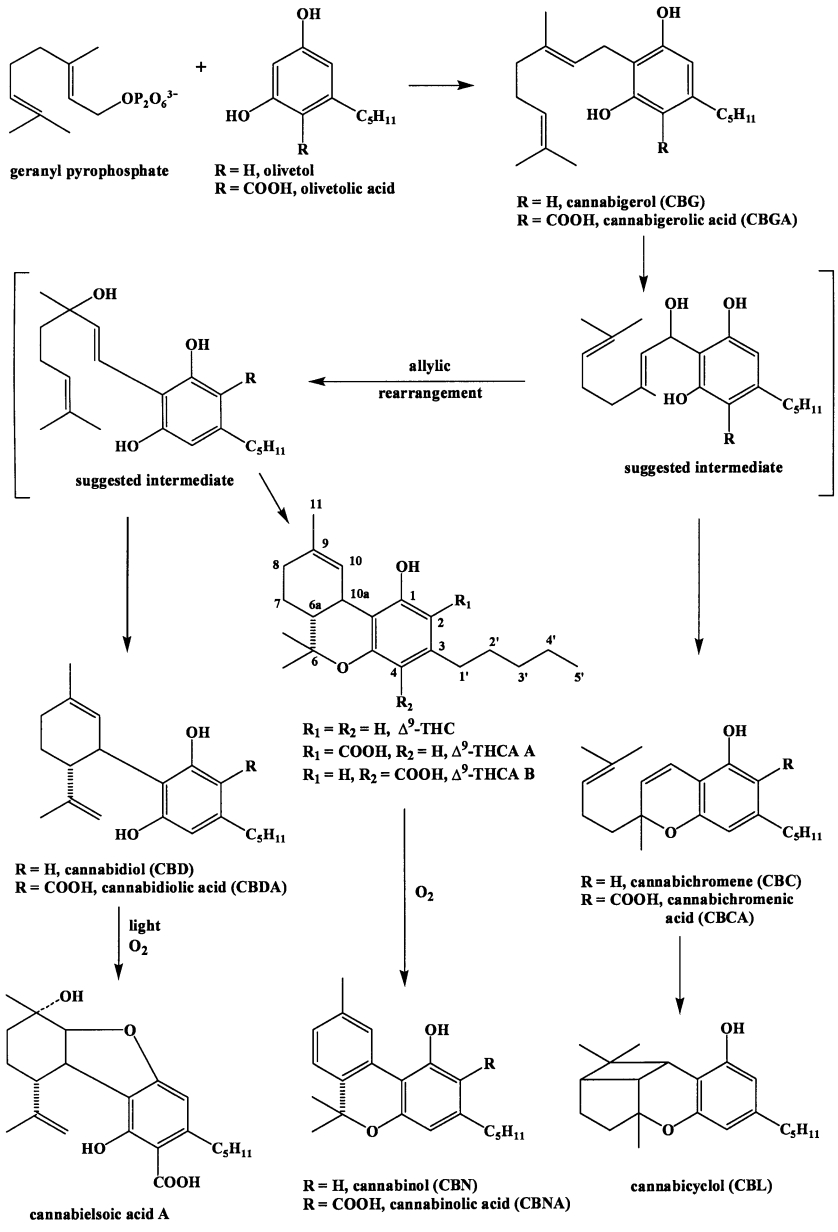


Figure 1. A tentative biogenesis of the plant cannabinoids

Advances in isolation methods made possible a clarification of the chemistry of cannabis. In 1963 our group reisolated cannabidiol and reported its correct structure and stereochemistry [15]. A year later we finally succeeded in isolating pure THC (Δ^9-THC); we elucidated its structure, obtained a crystalline derivative and achieved a partial synthesis from cannabidiol [16]. The absolute configuration of cannabidiol and of THC was established by correlation with known terpenoids [17]. Several years later a minor psychotomimetically active constituent, Δ^8-THC, was isolated from marijuana [18]. Whether this THC isomer is a natural compound, or an artifact formed during the drying of the plant, remains an open problem.

Several additional, non-psychotropic cannabinoids were also identified at that time. The best known are cannabigerol [19], cannabichromene [20, 21] and cannabicyclol [22]. For a better understanding of the biogenesis of a cannabinoids in the plant the isolation and identification of cannabinoid acids turned out to be essential. Alongside cannabidiolic acid, the cannabinolic and cannabigerolic acids were identified [23], followed by two Δ^9-THC acids, A and B [24, 25], as well as Δ^8-THC acid [26, 27] and cannabielsoic acid [28]. The decarboxylated product of cannabielsoic acid, cannabielsoin, is found in mammals as a metabolite of cannabidiol [29]. The syntheses of some of the cannabinoid acids have been reported [30].

A tentative pathway for the biogenesis of cannabinoids in the plant has been published [31–34]. However the only experimental support for Δ^9-THC acid formation from cannabigerolic acid (by direct oxidocyclization and not through cannabidiolic acid as was assumed before) has been reported by Shoyama's group [35]. They showed that the presence of a carboxyl group in the substrate is essential for enzymatic cyclization of the terpene moiety. This finding may explain the presence of THC and THC acids in certain cannabis strains (e.g. South African) that do not contain cannabidiol or its acid [36–38].

In a series of elegant publications Shoyama's group identified an enzyme forming cannabichromenic acid and showed that this acid is formed directly from cannabigerolic acid [39, 40].

It is possible that some of the natural neutral cannabinoids are artifacts formed through decarboxylation, photochemical cyclization (cannabicyclol), oxidation (cannabielsoic acid) or isomerization (Δ^8-THC and Δ^8-THC acid) of other constituents.

Endogenous cannabinoids

The discovery of a high-affinity, stereoselective and pharmacologically distinct cannabinoid receptor in a rat brain tissue [41] led to a search for natural endogenous ligands in the brain, which bind to this cannabinoid receptor. We assumed that the cannabinoid receptor in the brain is not present just to bind a plant constituent, but to be activated by specific endogenous ligands. Our approach involved first the synthesis of a potent labeled agonist (HU-243),

which made possible a sensitive bioassay. This compound is the most active cannabinoid known so far [65]. In a standard bioassay we expected that endogenous compounds with cannabinoid activity would displace tritiated HU-243 bound to the central cannabinoid receptor (CB_1).

Rat brains are too small and hence we started our isolations with porcine brains. After nearly 2 years of tedious work, which involved numerous chromatographic separations, we isolated from brain an endogenous compound that binds to the cannabinoid receptor with about the same potency as Δ^9-THC. This endogenous ligand was named anandamide [42], a name derived from the Sanskrit word for bliss, *ananda*. When administered intraperitoneally to mice it caused reduced activity in an immobility test and in open field tests, and produced hypothermia and analgesia, a tetrad of assays typical of the psychotropic cannabinoids [43]. Later we isolated two additional, apparently minor, endogenous cannabinoids, homo-γ-linoleoylethanolamide and 7,10,13,16-docosatetraenoylethanolamide [44].

The existence of a peripheral cannabinoid receptor (CB_2) led to the search for a ligand to this receptor. We isolated from canine gut another arachidonic acid derivative, 2-arachidonoyl glycerol (2-AG) [45]. At around the same time this compound was detected in brain [46] (see Fig. 2).

Hanuš et al. reported a third, ether-type endocannabinoid, 2-arachidonyl glyceryl ether (noladin ether), isolated from porcine brain [47]. It binds to the CB_1 cannabinoid receptor ($K_i = 21.2 \pm 0.5$ nM) and causes sedation, hypothermia, intestinal immobility and mild antinociception in mice. It binds very weakly to the CB_2 receptor. The presence of this endocannabinoid in brain has been questioned [48]. However as this type of natural glycerol derivative (an ether group on the 2-position) is unusual, we have repeated its isolation with an identical result (unpublished observations).

In the course of the development of a bioanalytical method to assay anandamide in brain and peripheral tissues, a compound with the same molecular weight as anandamide, but with a shorter retention time, was identified as *O*-arachidonoyl ethanolamine (arachidonic acid and ethanolamine joined by an ester linkage). This compound was named virodhamine [49].

On the basis of previous structure–activity relationship studies and on the existence in body tissues of biosynthetic precursors, Huang et al. assumed that *N*-arachidonoyl-dopamine (NADA) may exist as an endogenous "capsaicin-like" cannabinoid in mammalian nervous tissues and may possibly bind to the vanilloid receptor VR1 [50]. They found that NADA is indeed a natural endocannabinoid in nervous tissues, with high concentrations found in the striatum, hippocampus and cerebellum and lower concentrations in the dorsal root ganglion. NADA binds to the cannabinoid receptors with a 40-fold greater selectivity for the CB_1 ($K_i = 250 \pm 130$ nM) than the CB_2 receptor [50–52].

One of the typical endocannabinoid effects is pain suppression. Some endogenous fatty acid derivatives (palmitoylethanolamide, oleamide), which do not bind to CB_1 or CB_2, either enhance this effect (the so-called entourage

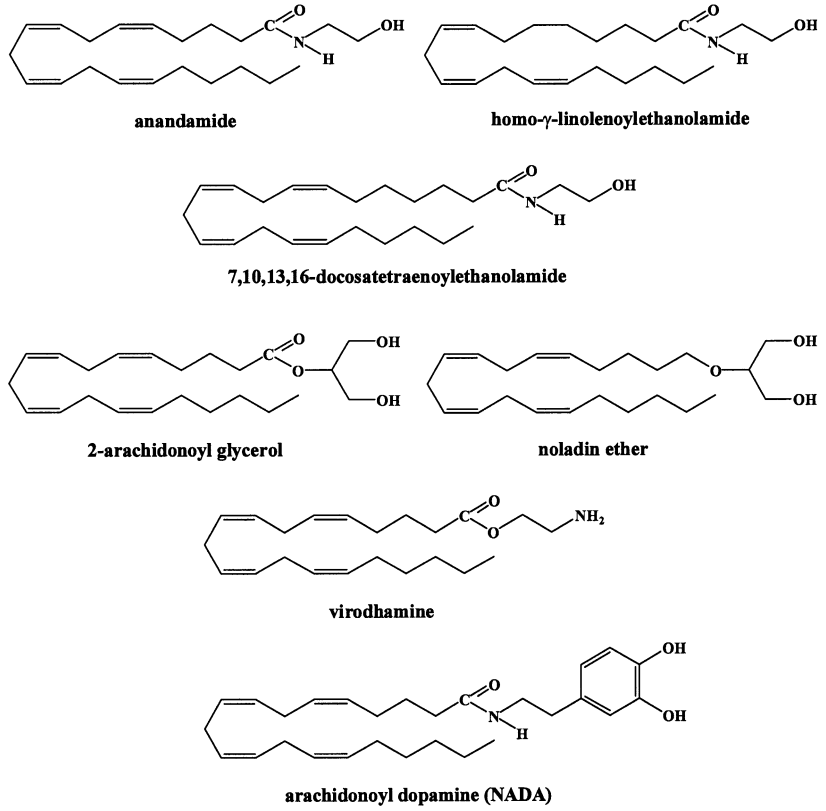


Figure 2. The main endocannabinoids

effect) or actually show activity by themselves, presumably by binding to as-yet unidentified cannabinoid receptors [53].

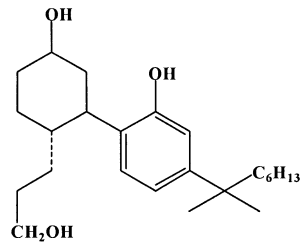
Shortly after the isolation of anandamide, its biosynthesis, metabolism and degradation in the body were studied [54, 55].

Synthetic cannabinoid receptors agonists/antagonists

In the late 1970s Pfizer initiated a cannabinoid project aimed at novel analgesic compounds. Numerous active bicyclic compounds were synthesized. The compound chosen for clinical evaluation was CP-55,940 [56, 57]. This compound is more potent than morphine and is at least 200-fold more potent than its enantiomer [55]. Structural and stereochemical evaluations led to highly active analogs [58]. The cannabinoid-type side effects observed with this group of "non-classical" cannabinoids led to the termination of the project [58]. However, these compounds helped advance the cannabinoid field as they

were the first cannabinoids that were widely used as labeled ligands. Indeed, in 1988 Allyn Howlett's group used tritium-labeled CP-55,940 for the identification of the first cannabinoid receptor [59]. [^{3}H]CP-55,940 is now an important tool in the study of cannabinoid receptors [60].

Structure 1



CP-55940

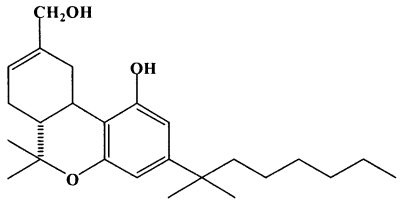
The need for stereospecific cannabinoid ligands led to further syntheses of enantiomers with essentially absolute stereochemical purity. This endevour culminated by the preparation of very potent cannabimimetic compounds [61]. Replacement of the n-pentyl side chain with a 1,1-dimethyl heptyl side chain in one of the major active primary metabolites of Δ^8-THC, 11-hydroxy-Δ^8-THC, led to the highly active ligand 11-hydroxy-Δ^8-THC-dimethylheptyl, or HU-210. The psychotropically inactive enantiomer, HU-211, is however analgesic, antiemetic and is at present being evaluated as an anti-trauma agent. Both compounds were synthesized with very high enantiomeric purity (99.8%) [62]. The high degree of enantioselectivity and potency of HU-210 was demonstrated in mice, dogs and pigeons [63, 64].

The synthetic HU-210 was used to prepare a novel probe for the cannabinoid receptor. Hydrogenation of this compound yielded two epimers of 5'-(1,1-dimethylheptyl)-7-hydroxyhexahydrocannabinol [65]. The equatorial epimer (designated HU-243) binds to the cannabinoid receptor with a K_D value of 45 pM, and is the most potent CB_1 agonist described so far. Tritiated HU-243 was used as a novel probe for the cannabinoid receptor.

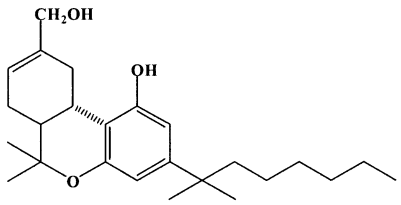
An effort to find new synthetic cannabinoids with increased therapeutic activity and few adverse side effects led to the preparation of ajulemic acid (HU-239), an analgetic and anti-inflammatory cannabinoid [66, 67]. This compound has anti-tumor effects in mice [68], binds to the peroxisome proliferator-activated receptor γ (PPARγ), a pharmacologically important member of the nuclear receptor superfamily [69], and induces apoptosis in human T lymphocytes [70]. However, it binds to CB_1 and has activity at the level of THC in the tetrad assay in mice [71].

A group at the Sterling pharmaceutical company prepared analogs of the anti-inflammatory drug pravadoline, an aminoalkylindole. To their surprise

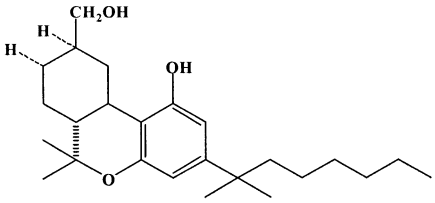
Structure 2



HU-210

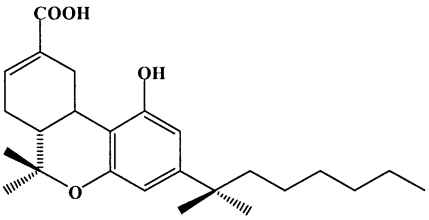


HU-211



HU-243

Structure 3

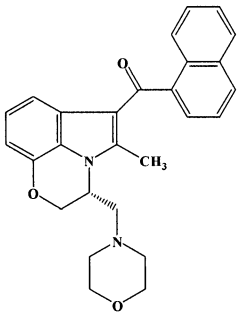


Ajulemic acid (CT_3, HU-239)

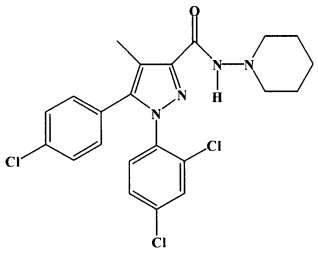
they discovered that these compounds acted not only as cyclooxygenase inhibitors, but also as cannabinoid agonists [72]. *In vitro* structure–activity relationship studies of these compounds led to numerous new compounds with cannabinoid receptor agonist activity [73, 74]. The best-known compound in this series is the conformationally restricted derivative WIN-55212-2 [75]. A binding assay in rat cerebellum membranes has been developed. It makes use of the stereospecific radioligand [^{3}H](*R*)-(+)-WIN-55212-2.

The first potent and selective antagonist of the central cannabinoid receptor (CB_1), SR-141716A, was reported in 1994 by a group at Sanofi [76]. This compound is not active on the peripheral cannabinoid receptor (CB_2) and has rapidly become a new tool in the study of cannabinoid receptor mechanisms and in research on new therapeutic agents. Another novel CB_1 antagonist, LY320135, which is not as selective as the previous one, was reported soon

Structure 4



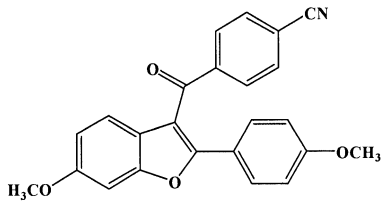
WIN-55212-2



SR-141716A
(rimonabant)

thereafter. This substituted benzofuran reverses anandamide-mediated adenylate cyclase inhibition and also blocks WIN-55212-2-mediated inhibition of N-type calcium channels [77].

Structure 5



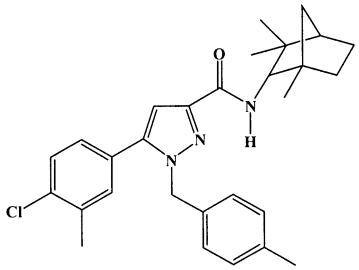
LY320135

The Sanofi group also described the first potent and selective antagonist of the peripheral cannabinoid receptor (CB_2), SR-144528 [78], and like the above-mentioned CB_1 antagonist, it soon became a major tool in cannabinoid research [79].

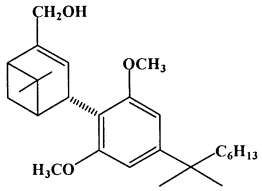
Our group reported the preparation of a CB_2-selective ligand, HU-308 [80], which is now being investigated as an anti-inflammatory drug by Pharmos, a pharmaceutical firm. It shows no central nervous system effects due to its essential lack of affinity for the CB_1 receptor. In HU-308 both phenolic groups are blocked as methyl ethers. This is in contrast to cannabinoid CB_1 agonists in which at least one of the phenolic groups has to be free.

Traumatic brain injury is a major cause of mortality and morbidity. There is no effective drug to treat brain-injured patients. We found that on closed head injury the amounts of 2-AG produced by the brain are increased 10-fold, and that this endocannabinoid apparently has a neuroprotective role, as administration of 2-AG to mice with head trauma reduces both the neurological damage and the edema [81]. Numerous other groups have recorded work on vari-

Structure 6



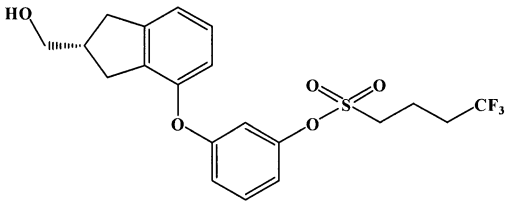
SR-144528



HU-308

ous aspects of cannabinoids as neuroprotective agents (see Chapter by Fernández-Ruiz et al. in this volume). On this basis a structurally novel, highly potent CB_1/CB_2 cannabinoid receptor agonist, BAY 38-7271, was prepared and shown to have pronounced neuroprotective efficacy in a rat model of traumatic brain injury [82–85].

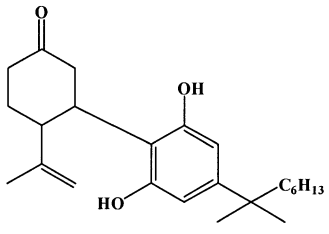
Structure 7



BAY 38-7271

Pharmos have developed a cannabinoid, PRS 211,096, that binds to the peripheral cannabinoid receptor and which is being assayed for treatment of multiple sclerosis [86].

Structure 8



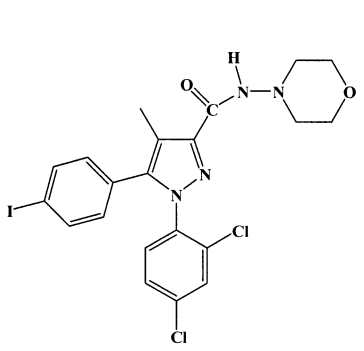
PRS 211,096

(*R*)-Methanandamide (AM-356) is a chiral analog of the endocannabinoid ligand anandamide, It is more stable than anandamide to hydrolysis by fatty acid amide hydrolase (FAAH), as the methyl group adjacent to the amide moiety apparently interferes with the enzyme. It has a K_i value of 20 ± 1.6 nM for the CB_1 receptor [87]. The K_i value for binding to the CB_2 receptor from mouse spleen is 815 nM [88]. Thus (*R*)-methanandamide has a high selectivity for the CB_1 receptor.

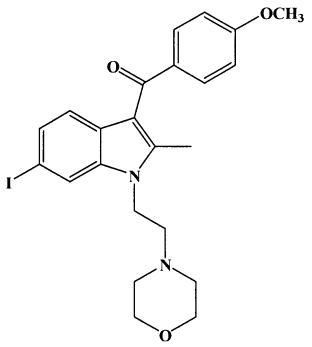
6-Iodo-pravadofine (AM-630), an aminoalkylindole, attenuates the ability of a number of cannabinoids to inhibit electrically evoked twitches of vas deferens isolated from mouse [89]. AM-630 behaves as a competitive antagonist of cannabinoid receptor agonists in the guinea-pig brain [90]. AM-630 also antagonizes the ability of the cannabinoid agonist WIN-55212-2 to stimulate guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding in mouse brain membrane preparations [91].

Gatley et al. [92] have developed a novel radioligand, [^{123}I]AM-281, structurally related to the CB_1-selective antagonist SR-141716A, that is suitable for *in vivo* studies of the central cannabinoid receptor and for imaging this receptor in the living human brain [92].

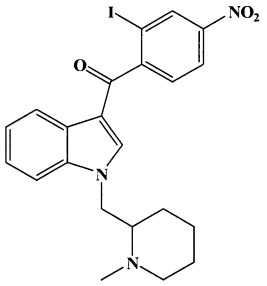
Structure 9



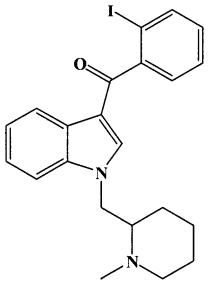
AM-281



AM-630



AM-1241

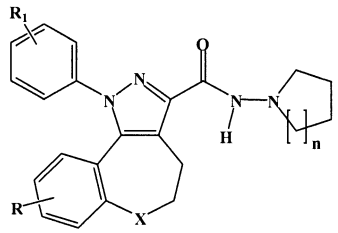


AM-2233

Scientists at the University of Connecticut have synthesized and studied a series of aminoalkylindoles as selective CB_2 agonists. The compounds are stated to be useful for the treatment of pain, glaucoma, multiple sclerosis and other diseases and disorders. Compound AM-1241 has a high affinity for the CB_2 receptor in a mouse spleen preparation ($K_i = 3.4 \pm 0.5$ nM), with good selectivity *versus* the CB_1 receptor in a rat brain preparation ($K_i = 280 \pm 41$ nM). This compound has recently been found to inhibit neuropathic pain in rodents [93].

AM-2233, a novel aminoalkylindole CB_1 agonist, was found to have a greater potency than WIN-55212-2 in assays *in vitro*, but has a similar potency to it in a mouse locomotor assay. It was suggested that its behavioral effects could have been mediated, in part, via an action on another receptor type in addition to the CB_1 receptor. AM-2233 represents the first agonist CB1 receptor ligand ($K_i = 0.4$ nM) with potential as an *in vivo* imaging agent for this receptor [94, 95]. Stoit et al. [96] have reported the syntheses and biological activities of potent pyrazole-based tricyclic CB_1 receptor antagonists. One can find additional information on cannabinoid receptor agonists and antagonists in Barth's review [97].

Structure 10



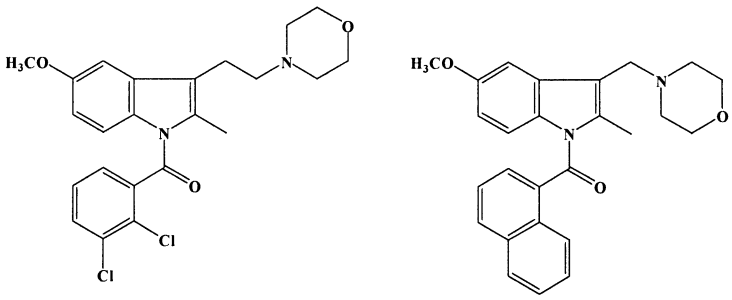
Pyrazole-based tricyclic CB_1 antagonists

Gallant et al. [98] have described two indole-derived compounds (see structures below), with binding potency for the human peripheral cannabinoid receptor (CB_2) in the nanomolar region, They are highly selective.

A new series of rigid 1-aryl-1,4-dihydroindeno[1, 2-c]pyrazole-3-carboxamides was recently designed [99]. Seven of the new compounds displayed very high *in vitro* CB_2-binding affinities. Four compounds showed very high selectivity for the CB_2 receptor.

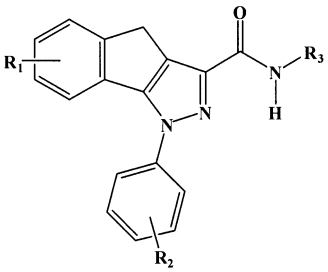
Cannabinoid structure–activity relationship data have indicated that the cannabinoid side chain and the phenolic hydroxyl are key elements in CB_1 receptor recognition. To test this hypothesis, the 1-deoxy analog, JWH-051, of the very potent cannabinoid 11-hydroxy-Δ^8-THC-dimethylheptyl (HU-210) was prepared and the affinity of this compound for the CB_1 receptor was determined [100]. Contrary to expectations, this 1-deoxy analog still had high affinity for the CB_1 receptor ($K_i = 1.2 \pm 0.1$ nM) and even greater affinity for the

Structure 11



Indole derivatives

Structure 12



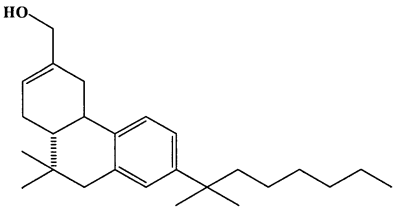
1,4-Dihydroindeno[1,2-c]pyrazole derivatives

CB_2 receptor (K_i = 0.032 ± 0.19 nM). On the basis of these data, it is apparent that a phenolic hydroxyl group is not essential for cannabinoid activity.

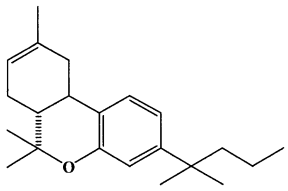
To obtain selective ligands for the CB_2 and to explore the structure–activity relationship of the 1-deoxy-cannabinoids, the same research group described the synthesis and pharmacology of 15 1-deoxy-Δ^8-THC analogues [101]. Five of these analogues had high affinity ($K_i \leq 20$ nM) for the CB_2 receptor. Four of them also had low affinity for the CB_1 receptor ($K_i \geq 295$ nM). 3-(1',1'-Dimethylbutyl)-1-deoxy-Δ^8-THC (JWH-133) had very high affinity for the CB_2 receptor (K_i = 3.4 ± 1.0 nM) and low affinity for the CB_1 receptor (K_i = 677 ± 132 nM).

In view of the importance of the CB_2 receptor, three series of CB_2-selective cannabinoid receptor ligands, 1-methoxy-, 1-deoxy-11-hydroxy- and 11-hydroxy-1-methoxy-Δ^8-THCs, were designed [102]. All of these compounds have greater affinity for the CB_2 receptor than for the CB_1 receptor; however, only 1-methoxy-3-(1',1'-dimethylhexyl)-Δ^8-THC (JWH-229) had essentially no affinity for the CB_1 receptor (K_i = 3134 ± 110 nM) with high affinity for CB_2 (K_i = 18 ± 2 nM).

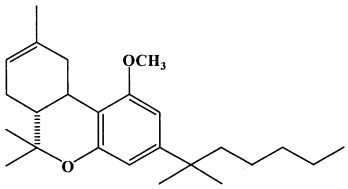
Structure 13



JWH-051



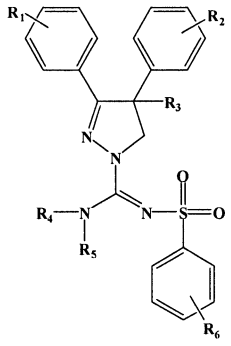
JWH-133



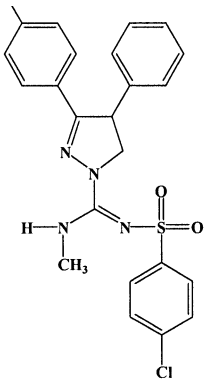
JWH-229

Recently the discovery of a further class of diarylpyrazolines with high potency and selectivity for the CB_1 receptor was described [103]. These compounds were found to be CB_1 antagonists. SLV319 was found to be a potent CB_1 antagonist ($K_i = 7.8$ nM) close to that of the Sanofi compound SR-141716A, with more than 1000-fold selectivity against CB_2.

Structure 14



Diarylpyrazolines

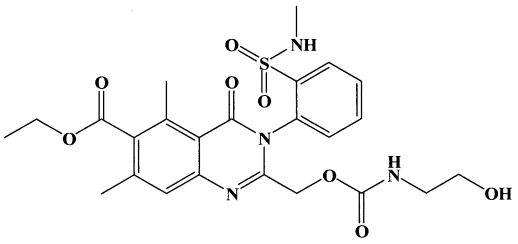


(–)-Enantiomer (SLV319)

Additional synthetic compounds that bind to the CB_1 and/or CB_2 receptors have been mentioned in patents. These were recently reviewed by Hertzog [104].

Novartis AG has recently filed a patent application on a series of quinazolines as cannabinoid agonists useful for the treatment of pain, osteoarthritis, rheumatoid arthritis and glaucoma, among other indications [105]. Compound **1** binds to both CB_1 (K_i = 34 nM) and CB_2 (K_i = 11 nM). The patent application refers to the compound as having CB_2 agonist activity. Additionally, this compound has been shown to be active in a rodent neuropathic pain model when administered at an oral dose of 0.5 mg/kg.

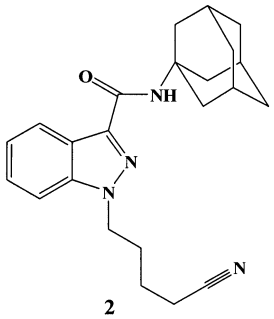
Structure 15



1

The University of Connecticut has disclosed a series of indazole derivatives that have been found to act as agonists of cannabinoid receptors [106]. The compounds exhibit a range of selectivities for CB_2 over CB_1. Compound **2**, for instance, exhibited K_i values of 2.28 and 0.309 nM for the CB_1 and CB_2 receptors, respectively. This compound produced dose-dependent anti-nociception to thermal stimulus in rats. The compound reduced locomotor activity in rats after intravenous administration, an effect attributed to activation of the CB_1 receptor.

Structure 16

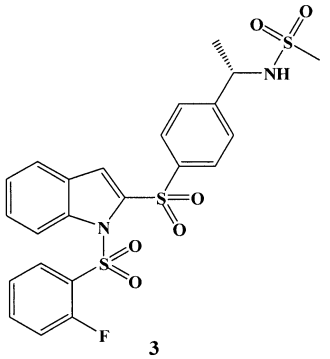


2

A series of aromatic CB_2 agonists has been disclosed by the Schering-Plough Research Institute [107, 108]. The compounds are reported to have anti-inflam-

matory and immunomodulatory activities, and to be active in cutaneous T cell lymphoma, diabetes mellitus and other indications. Compound **3** is stated to bind to CB_2 with a K_i value in the range 0.1–10 nM.

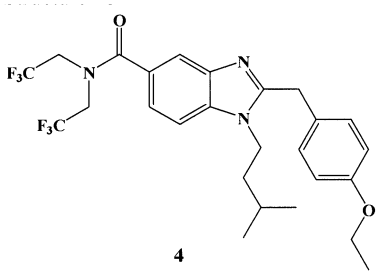
Structure 17



3

Researchers at AstraZeneca have disclosed a series of benzimidazoles and azabenzimidazoles to be CB_2 agonists [109]. The compounds are described as useful in the treatment of pain, cancer, multiple sclerosis, Parkinson's disease, Huntington's chorea, transplant rejection and Alzheimer's disease. Cannabinoid receptor selectivity data are provided for some of the new compounds. For instance, compound **4** binds to CB_2 (K_i = 3.1 nM) with much greater affinity than to CB_1 (K_i = 2.8 μM). No *in vivo* data are provided for the compounds.

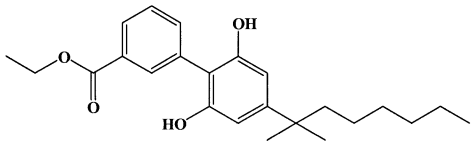
Structure 18



4

The University of Connecticut has disclosed a series of biphenyls as cannabinoid modulators [110]. These non-classical cannabinoids are described as useful for the treatment of peripheral pain, neuropathy, neurodegenerative diseases and other indications. Several of the compounds were found to bind selectively to the CB_2 receptor. For instance, compound **5** binds to CB_2 with a K_i value of 0.8 nM and to CB_1 with a K_i value of 241 nM.

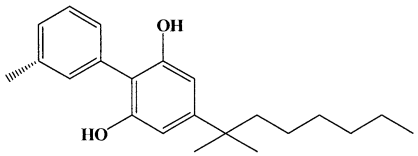
Structure 19



5

The Virginia Commonwealth University has filed a patent application on a series of resorcinol derivatives as selective CB_2 agonists useful for the treatment of pain, inflammation and autoimmune diseases [111]. Binding data for the compounds to CB_1 and CB_2 are provided, and the compounds were assayed for *in vivo* activity in mouse tail-flick, spontaneous activity and rectal temperature assays. Compound **6** had K_i values of 40 and 0.8 nM, respectively, for the CB_1 and CB_2 receptors. In addition, this compound was assessed by intravenous administration and exhibited ED_{50} values of 2.7, 2.4 and 3.6 mg/kg in the spontaneous activity, tail-flick and rectal temperature assays, respectively.

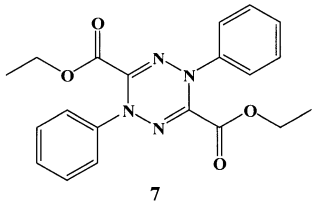
Structure 20



6

The University of Connecticut has disclosed a series of dihydrotetrazines and derivatives as CB_2 agonists [112]. Compound **7** is reported to be a potent CB_2 agonist (K_i = 19 nM) with 88-fold selectivity for the CB_2 over the CB_1 receptor. Such compounds are reported to be useful in the treatment of pain, glaucoma, multiple sclerosis, Parkinson's disease, Alzheimer's disease and other disorders.

Structure 21

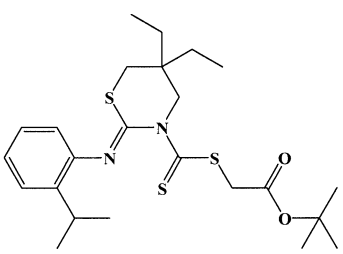


7

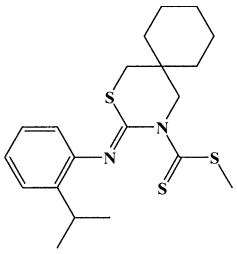
Shionogi has also disclosed two series of thiazine-containing CB_2 agonists, of which compounds **8** and **9** are examples [113, 114]. Selectivity data for several of the compounds with regard to CB_2/CB_1 affinities are described. For

example, compound **8** binds to CB_2 with a K_i value of 0.3 nM and a K_i value of >5000 nM for CB_1. Compound **9** displayed a K_i value of 1.2 nM at the CB_2 receptor and 80 nM at the CB_1 receptor. When dosed orally at 100 mg/kg in a mouse pruritis model, this compound reduced scratching by 98% relative to control animals.

Structure 22



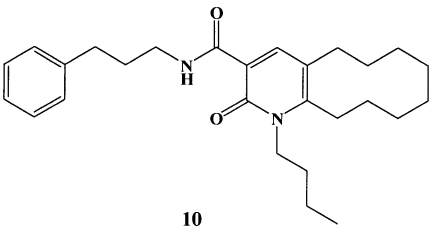
8



9

Shionogi has disclosed a series of amide-containing CB_2 modulators stated to be useful in the treatment of inflammation, nephritis, pain, allergies, rheumatoid arthritis, multiple sclerosis, brain tumors and glaucoma [115]. Compound **10** was found to bind to the CB_2 receptor with a K_i value of 4 nM, with very little affinity for CB_1 ($K_i < 5$ μM).

Structure 23

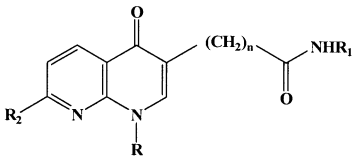


10

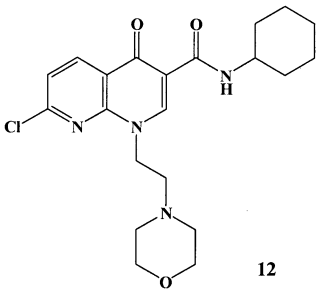
Recently 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives (**11**) were synthesized as new ligands of cannabinoid receptors [116]. Some of these compounds possess a greater affinity for the CB_2 receptor than for the CB_1 receptor. Compound 7-chloro-*N*-cyclohexyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (**12**) revealed a good CB_2 selectivity (CB_1, K_i = 1 μM; CB_2, K_i = 25 ± 1.8 nM).

Indole derivatives were prepared and tested for their CB_1 and CB_2 receptor affinities [117]. Three new highly selective CB_2 receptor agonists were identified, namely JWH-120 (CB_1, K_i = 1054 ± 31 nM; CB_2, K_i = 6.1 ± 0.7 nM), JWH-151 (CB_1, K_i >10000 nM; CB_2, K_i = 30 ± 1.1 nM) and JWH-267 (CB_1, K_i = 381 ± 16 nM; CB_2, K_i = 7.2 ± 0.14 nM).

Structure 24

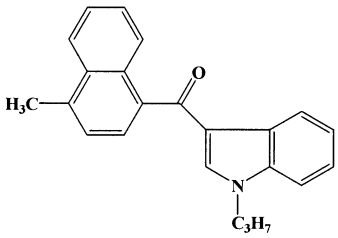


11

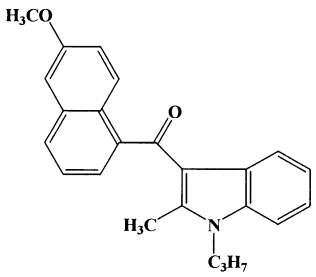


12

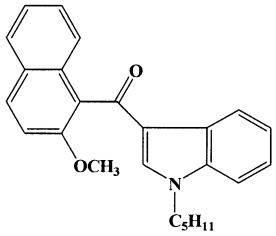
Structure 25



JWH-120



JWH-151



JWH-267

Conclusions

C. sativa L. has been used throughout history not only for its fiber, but also as a medicinal plant. It has been the object of scientific research over the past 150 years. After the isolation of the plant's constituents, biochemical work led to the identification of two receptors and of endogenous cannabinoids. Over the last decade numerous synthetic agonists and antagonists have been prepared. We may be approaching an important goal in cannabinoid research – the use of cannabinoids in medicine – which has been the dream of several generations of scientists.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabidiol as a potential medicine

Roger G. Pertwee

School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK

Introduction

Cannabidiol (CBD) is one of more than 60 oxygen-containing hydrocarbon constituents of cannabis that are collectively known as plant cannabinoids or phytocannabinoids [1, 2]. It was first isolated in 1940, by Roger Adams from Mexican marijuana and by Alexander Todd from Indian charas [3]. However, the correct structure of CBD was not determined until 1963 and its absolute stereochemistry until 1967 [4]. The CBD molecule is chiral and it is only the 3*R*,4*R*-(–)-enantiomer of this molecule that is found in cannabis. This enantiomer is referred to throughout this review as CBD. The chemical nomenclature of CBD differs from that of 6a*R*,10a*R*-(–)-Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of cannabis. Thus, as shown in Figure 1, whereas Δ^9-THC has a pyran ring which determines its numbering, CBD has no heterocyclic ring and its numbering is based on that of the terpene ring. Much of the Δ^9-THC and CBD that is extracted from harvested cannabis derives from the C-2 and C-4 carboxylic acids of Δ^9-THC or the C-3'/C-5' carboxylic acid of CBD (Fig. 1), all of which undergo decarboxylation when the plant material is stored or heated [1, 5]. The pharmacology of Δ^9-THC has been intensively investigated and it is now generally accept-

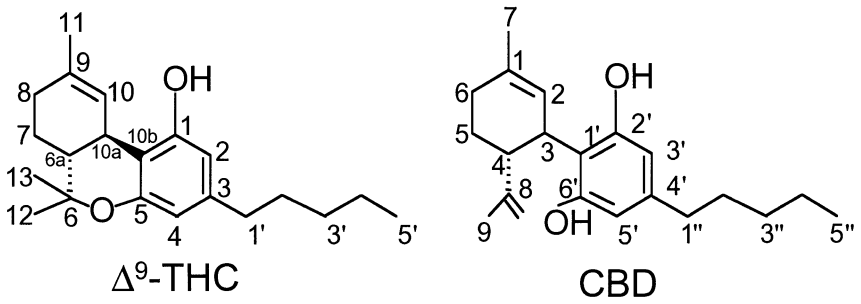

Figure 1. The structures of the phytocannabinoids (–)-Δ^9-tetrahydrocannabinol (Δ^9-THC) and (–)-cannabidiol (CBD)

ed that, in contrast to CBD, it produces many of its effects by acting on cannabinoid CB_1 receptors to modulate central and peripheral neurotransmission and on cannabinoid CB_2 receptors to modulate cytokine release from immune cells [6]. Additional pharmacological targets for Δ^9-THC have also been proposed [7]. Current knowledge about the pharmacological actions of CBD is much more limited. There is already no doubt, however, that this non-psychoactive phytocannabinoid is pharmacologically active and that its pharmacological actions differ markedly from those of Δ^9-THC [8, 9]. Moreover, as now discussed, it is likely that CBD will prove to have clinical applications (i) for the management of epilepsy and certain other central motor disorders, (ii) for the treatment of anxiety, psychotic illnesses and neurotoxicity associated for example with stroke, (iii) for the treatment of inflammation and (iv) for the attenuation of unwanted side effects produced by Δ^9-THC when this phytocannabinoid is used as a medicine. Other potential therapeutic targets for CBD include emesis, glaucoma, sleep and appetite disorders, and cancer.

Epilepsy

To date there have been two investigations into the effects of CBD on epileptic patients. One of these was performed with an epileptic patient who exhibited symmetrical spike and wave electroencephalographic (EEG) activity when in light sleep and was receiving medicine (unspecified) to prevent tonic-clonic seizures [10]. When this patient fell into a light sleep after receiving chloral hydrate, intravenous infusion of CBD at 2.4 mg/min for 17 min was associated with an increase in the occurrence of abnormal EEG. Whether CBD altered the incidence of tonic-clonic seizures in this patient was not determined. The other investigation, a double-blind clinical trial, was carried out with patients with secondary generalized epilepsy. These were patients who were experiencing at least one generalized convulsive crisis per week even though they were being given phenytoin, a barbiturate, primidone, clonazepam, carbamazepine, trimethadione and/or ethosuximide [11]. Of these patients, seven were given 200 or 300 mg of CBD daily by mouth for up to 4.5 months. There was also one patient who crossed over from the placebo group to CBD after 1 month. Seven other patients received placebo throughout the investigation. Within the CBD group, four patients improved markedly, three others showed some improvement and one patient did not improve. Only one patient in the placebo group improved with time. The most serious side effect, somnolence, was reported by four CBD patients and one placebo patient. As to EEGs, improvement was observed in two of the placebo patients, but only on one occasion, and in three of the CBD patients, with no change detected in any of the other patients. Because all patients received their usual anti-epileptic medicine(s) throughout this clinical trial, it is possible that instead of or as well as having a direct anti-convulsant effect, CBD may have been enhancing

the anti-convulsant effects of some these other drugs as, indeed, it has been found to do in some animal experiments (see below).

In line with its reported anti-epileptic effect in human subjects, CBD has been found to show anti-convulsant activity in several *in vivo* animal models of epilepsy. For example, as indicated in Table 1, it can prevent convulsions induced in mice or rats by electroshock, by sound or by convulsant agents such as pentylenetetrazol. In addition, it prevents clonic convulsions caused by chronic placement of cobalt wire in the dura and kindled convulsions produced by repetitive electrical stimulation of the subiculum or by repeated subcutaneous administration of pentylenetetrazol. There are also reports that CBD can enhance the ability of phenytoin to prevent audiogenic seizures in rats and of phenytoin and phenobarbitone to prevent convulsions induced by electroshock in mice [15, 30, 36, 37] and that it exhibits anti-convulsant activity in certain electrophysiological models of epilepsy (see [8]). Unlike Δ^9-THC, which has been found to produce a mixture of pro-convulsant and anti-convulsant effects in animal experiments, there is evidence that CBD has only anti-convulsant properties [32, 38, 39]. Indeed, there is one report that convulsions induced by Δ^9-THC in rabbits can be prevented by CBD when these two cannabinoids are co-administered, although not when CBD is injected before Δ^9-THC [16].

Little is yet known about the mechanism underlying the anti-convulsant effects of CBD. That this mechanism is specific in nature is suggested by the existence of a relationship between the structures of CBD analogues and the ability of these analogues to prevent convulsions in animals [8]. Such specificity is also supported by observations; firstly that CBD is not active in all animal models of epilepsy and secondly that CBD is effective as an anti-convulsant in

Table 1. Established rodent models of epilepsy in which CBD shows anti-convulsant activity

Measured response reduced or abolished by CBD	Reference
Convulsions induced in rats by corneal electroshock	[15, 19, 28]
Convulsions induced in mice by corneal electroshock	[13, 18, 20–25, 27, 30, 34, 35]
Convulsions induced in mice by ear electroshock	[29]
Convulsions induced in mice by pentylenetetrazol	[12, 18, 29, 30, 34]
Convulsions induced in mice or rabbits by convulsant agents other than pentylenetetrazol	[16, 18]
Convulsions induced by chronic placement of cobalt wire in the dura of rats	[14]
Kindled seizures induced in rats by repetitive subicular electroshock	[31]
Kindled seizures induced in mice by repeated injections of pentylenetetrazol	[26]
Audiogenic convulsions in rats	[15, 17]
Amplitude of electrically evoked cerebrocortical potentials in unanaesthetized rats	[32, 33]
Kindled afterdischarges induced in rats by repetitive subicular electroshock	[31]

All animals were unanaesthetized.

rats and mice at doses below those at which it produces a general impairment of motor function, for example in rotarod, bar-walk or open-field performance assays [8]. Also consistent with a specific mode of action is the finding that CBD shows quite high anti-convulsant potency, both in frogs, in which it has been shown to protect against electroshock-induced tonic convulsions with a potency at least 100 times greater than that of phenytoin [40] [this finding could not be replicated when the frog experiments were performed at a different time of the year (SA Turkanis, personal communication)], and in rats, in which it has been reported to exhibit signs of anti-epileptic activity at doses of 0.3 and 3 mg/kg administered intraperitoneally (i.p.) [19, 31]. In mice, however, CBD appears to have somewhat less anti-convulsant potency, reported ED_{50} values for the protection of this species from convulsions induced by electroshock being 38 mg/kg (administered intravenously, i.v.) and 80–120 mg/kg i.p. [21, 30, 34, 35]. Karler et al. [25] found the peak concentration of CBD in mouse brain to be 8 μg/g following its i.p. administration at a dose of 120 mg/kg. This approximates to 8 μg/ml and hence 25 μM, a concentration at which CBD would be expected to modulate central neurotransmission, for example by binding to cannabinoid CB_1 receptors and by inhibiting the transport of calcium, anandamide or certain neurotransmitters across neuronal membranes (Tab. 2). Interestingly, when mice were injected i.p. with an anti-convulsant dose of phenytoin (7 mg/kg), the brain concentration of this compound peaked at 6.6 μg/g [25], a concentration that approximates to 26 μM. Hence, it appears that although the doses at which CBD and phenytoin exhibit anti-convulsant activity in mice differ considerably, these disparate doses produce essentially the same concentration of CBD and phenytoin within the brain, suggesting that CBD may have much lower bioavailability in this species, at least when the intraperitoneal route is used.

There are reports that (+)- and (–)-CBD are equipotent against convulsions induced in rats by sound [52] or in mice by electroshock [27], making it unlikely that CBD prevents convulsions by acting on pharmacological targets such as CB_1 receptors that discriminate between these enantiomers [8, 42]. That CBD does not act through CB_1 receptors to prevent convulsions is also supported by a report that its ability to oppose electroshock-induced maximal convulsions in mice is not attenuated by the selective CB_1 receptor antagonist, SR-141716A, at a dose that does attenuate the anti-convulsant effect of Δ^9-THC or *R*-(+)-WIN-55212 [35].

Animal experiments have revealed several similarities between the anti-convulsant properties of CBD and phenytoin [8]. Therefore, as has been postulated for phenytoin, the anti-convulsant effect of CBD may depend at least in part on an ability to block the spread of seizure activity in the brain, possibly through suppression of post-tetanic potentiation. Indeed, there is already a report that CBD can abolish post-tetanic potentiation in bullfrog isolated ganglia, albeit at the rather high concentrations of 60–100 μM [53]. The pharmacology of CBD has less in common with ethosuximide than with phenytoin [8], suggesting that it may not share the ability of ethosuximide to

Table 2. Some actions of CBD expected to affect neurotransmission

Action	Tissue	Effective concentration	Reference
Antagonism of cannabinoid CB_1 receptor agonists	Mouse vas deferens	120 nM (K_B value)	[47]
Inhibition of Ca^{2+} uptake	Rat brainstem synaptosomes	100 nM	[45]
Inhibition of Ca^{2+} uptake	Mouse brain synaptosomes	1 μM	[45]
Inhibition of 5-HT uptake	Rat hypothalamic synaptosomes	1 μM	[41]
Inhibition of dopamine and noradrenaline uptake	Rat striatal or hypothalamic synaptosomes	1 μM	[41, 49]
Displacement of [^{3}H]SR-141716A from CB_1 receptors	CB_1-containing membranes	1.26 μM (K_i value)	[51]
Displacement of ^{3}H-CP-55,940 from CB_1 receptors	CB_1-containing membranes	2.28 μM (K_i value) 4.35 μM (K_i value)	[50, 51]
Enhancement of evoked neuronal release of noradrenaline and ATP	Mouse vas deferens	3.2 μM	[47]
Inhibition of dopamine and noradrenaline uptake	Mouse whole-brain synaptosomes	5 μM	[46]
Inhibition of 5-HT and GABA uptake	Mouse whole-brain synaptosomes	10 μM	[46]
Enhancement of basal release of dopamine and noradrenaline	Rat striatal and hypothalamic synaptosomes	10 μM	[49]
Antagonism of the cannabinoid CB_1 receptor agonist CP-55,940	Rat cerebellar membranes	10 μM	[48]
Inhibition of choline uptake	Rat hippocampal crude synaptosomal fraction	16 μM (EC_{50})	[28]
Inhibition of anandamide uptake	RBL-2H3 cells	22 μM (EC_{50})	[42]
Inhibition of anandamide metabolism	N18TG2 cell membranes	27.5 μM (EC_{50})	[42]
Attenuation of the affinity of dopamine D_2 receptor ligands for D_2 receptors	Mouse striatal membranes	30 μM	[43, 44]

GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine.

prevent petit mal epilepsy (absence seizures) in humans. However, because CBD differs from phenytoin in not eliciting any excitatory responses in behavioural and electrophysiological models of epilepsy [31, 39], it may also differ from phenytoin in not exacerbating absence seizures.

Clearly, there is now sufficient evidence to warrant further clinical investigations into the use of CBD for the management of epilepsy, particularly grand mal. Important objectives will be to identify all the types of epilepsy against which CBD is active and to determine whether this cannabinoid is more effective or has less serious, unwanted effects than established anti-epileptic drugs, whether tolerance develops to anti-convulsant effects of CBD in humans as it can in an animal model in which tolerance to phenytoin also develops [22, 25], and whether the synergism between CBD and phenytoin or phenobarbitone that has been observed in animal models of grand mal epilepsy also occurs in humans. At the non-clinical level, there is an urgent need for new research aimed at elucidating the mechanisms that underlie the anti-convulsant effects of CBD.

Other central motor disorders

In experiments directed at investigating the ability of CBD to improve chorea arising from Huntington's disease, positive results were obtained in one investigation in which four patients with this disease received CBD orally at 300 or 600 mg/day [54] but not in a subsequent clinical trial in which 15 Huntingtonian patients were given CBD orally for 6 weeks at about 700 mg/day [55]. The ability of CBD to reduce dystonia has also been investigated [56]. When administered to five patients at a dose of 100–600 mg/day *per os* (p.o.) for 6 weeks together with the standard medication, CBD reduced disease- or L-dopa-induced dystonia in all five patients. It also improved motor function in two of the patients with disease-induced dystonia when given once at 200 mg p.o. [57]. In two other patients, whereas CBD at 300–500 mg/day improved dystonia, it exacerbated hypokinesia and resting tremor [56]. CBD has also been reported to exhibit anti-dystonic activity in mutant hamsters [58]. However, its effect was marginal and produced only by the rather high dose of 150 mg/kg i.p. and not by 50 or 100 mg/kg i.p.

Anxiety

There is evidence that CBD has anxiolytic properties, at least in normal human subjects. Zuardi et al. [59] have reported that at a dose of 300 mg p.o. CBD relieves post-stress anxiety induced by a simulated public-speaking test and there are other reports that CBD has a sedative or somnolent effect in normal subjects at 200–600 mg p.o. [60, 61]. There is also evidence that the anxiolytic effect produced by CBD in normal human subjects is mediated by lim-

bic and paralimbic brain areas [62]. Although the question of whether CBD is effective against "pathological" anxiety states has still to be addressed, there is already evidence that CBD can oppose anxiety induced in humans by Δ^9-THC. Thus, Karniol et al. [63] found that groups of five human subjects who took 30 mg of Δ^9-THC p.o. together with 15, 30 or 60 mg of CBD experienced less Δ^9-THC-induced anxiety and panic and greater feelings of pleasure than when they took the same dose of Δ^9-THC by itself. Similarly, Zuardi et al. [64] found that whereas the incidence of feeling anxious, troubled, withdrawn, feeble, incompetent and discontented was greater in eight human subjects after Δ^9-THC at 0.5 mg/kg p.o. than after placebo treatment, CBD at 1 mg/kg p.o. attenuated these effects of Δ^9-THC when the two cannabinoids were co-administered and by itself increased the incidence of feeling quick witted and clear minded.

As discussed in greater detail elsewhere [8], CBD also shows signs of anxiolytic activity in animal models, experiments with rats or mice indicating that it can suppress the conditioned emotional response, increase conflict response rates and augment the proportion of time spent in the open arms of the elevated-plus maze. Interestingly, experiments with mice have also shown that the anxiogenic effect produced by Δ^9-THC in the elevated-plus maze can be opposed by a dose of CBD (0.01 mg/kg i.p.) that by itself is sub-anxiolytic in this bioassay [65]. CBD appears to have a bell-shaped dose-response curve for its anxiolytic effect, at least in animal assays [8]. For example, in rat experiments with the elevated-plus maze, it has been found to show greatest anxiolytic activity at 5 mg/kg i.p., less activity at 2.5 and 10 mg/kg and no activity at 20 mg/kg [66]. Why this should be remains to be established. There is also nothing yet unknown about the mechanism(s) by which CBD reduces anxiety other than that it appears to interact with its site(s) of action in a structure-dependent manner [8].

Psychotic illnesses

There is some very preliminary evidence that CBD may have anti-psychotic activity. Thus in experiments with nine normal human subjects, Leweke et al. [60] found co-administration of CBD (200 mg p.o.) to oppose the ability of the cannabinoid receptor agonist, nabilone (1 mg p.o.), to produce binocular depth inversion, a visual illusion that is thought to provide a model of psychosis. CBD did not affect this measured response when administered by itself, although it did decrease the vividness of mental imagery. Further evidence comes from some *in vivo* experiments with rats. These indicate that CBD shares the ability of established anti-psychotic drugs such as haloperidol to oppose certain effects of apomorphine, for example stereotyped sniffing and biting [67]. However, unlike at least some anti-psychotic drugs, CBD has been found not to induce catalepsy in rats or to elevate plasma prolactin in humans [61, 67].

Neurotoxicity

As discussed in greater detail elsewhere [8, 68–72], there is convincing evidence that CBD (and other cannabinoids that contain a phenol group) can protect neurons against oxidative stress and glutamate-induced excitotoxity by acting through a mechanism that is independent of CB_1 or CB_2 receptors. CBD has, for example, been found to protect against neurotoxicity induced by glutamate in primary cultures of rat cerebrocortical neurons (EC_{50} = 2–4 μM) [73, 74]. This was irrespective of whether the neurotoxicity was induced through *N*-methyl-D-aspartate (NMDA), 2-amino-3-(4-butyl-3-hydroxyisoxazol-5-yl)-propionic acid (AMPA) or kainate receptors. CBD was not antagonized by SR-141716A, an indication that its neuroprotective effect was not mediated by CB_1 receptors. In addition, it has been found that CBD concentrations of 1 μM or above oppose the release of calcium from intracellular stores stimulated by metabotropic or ionotropic glutamate receptor activation [75] and protect mouse hippocampal HT22 cells from oxidative death induced by hydrogen peroxide [71]. CBD also shows neuroprotective activity *in vivo*. Thus in rats with focal cerebral ischaemia induced by middle cerebral artery occlusion, it reduced behavioural signs of neurological impairment and decreased cerebral infarct volume when administered at ischaemia onset (5 mg/kg i.v.) and again 12 h after surgery (20 mg/kg i.p.) [74]. More recent experiments have shown that CBD can also protect from signs of brain damage caused by cerebral ischaemia in gerbils [76]. In these experiments the CBD dose-response curve was bell-shaped, the optimal dose being 5 mg/kg i.p. There is also evidence that *in vivo* treatment with CBD (2 mg/kg i.v.) can prevent retinal neurotoxicity induced in adult rats by intravitreal injection of NMDA [72]. It will now be important to establish whether CBD is neuroprotective in humans and, if it is, to establish how best to exploit this effect in the clinic.

Strong evidence has emerged, for example from experiments in which reactive oxygen species were generated in neuronal cultures [73], in mouse peritoneal granulocytes [77] or in a brain lipid oxidation assay [71], that, at concentrations in the low micromolar range, CBD possesses antioxidant (electron-donor) properties. Consequently, it is likely that the neuroprotective activity of CBD depends at least in part on an ability to act downstream of glutamate receptors to protect cellular structures from damage induced by reactive oxygen species generated in response to pathological events such as excessive glutamate release. Interestingly, Hampson et al. [73] have reported that CBD induces greater neuroprotection than α-tocopherol (vitamin E) or ascorbic acid, both of which are endogenous neuroprotective antioxidants. Other cannabinoids that possess neuroprotective properties include HU-211, which is not a CB_1 receptor ligand, and Δ^9-THC, which is. Whereas these phenolic cannabinoids both possess antioxidant activity, it is noteworthy that they probably owe their neuroprotective activity, at least in part, to an ability to block NMDA receptors (HU-211) or to inhibit glutamate release by activating presynaptic receptors (Δ^9-THC; see [6, 78]). Further support for the hypothesis that

CBD can prevent cell damage caused by reactive oxygen species comes firstly from evidence that in rats CBD (2 mg/kg i.v.) prevents NMDA-induced apoptotic death of retinal cells, at least in part, by opposing the accumulation of peroxynitrite [72] and secondly from the observation that at 100–700 nM, although not at concentrations above 1 or 2 μM, CBD protects serum-deprived human B lymphoblastoid cells or mouse NIH 3 T3 fibroblasts from oxidative cell death [79]. Certain other classical cannabinoids, including the non-psychotropic (+)-enantiomer of Δ^9-THC, were also found to exhibit protective activity in the latter investigation.

Inflammation

CBD has been reported to exhibit anti-inflammatory activity in several *in vivo* bioassays (Tab. 3) [77, 80–83], with results from some of these experiments indicating its dose-response curve to be bell-shaped. In addition, CBD has been shown to produce anti-nociception in the mouse phenylbenzoquinone abdominal stretch test [84], an effect that is consistent with its apparent anti-inflammatory activity. However, there are also reports that CBD does not exhibit anti-nociceptive activity in the mouse acetic acid abdominal stretch test or attenuate signs of hyperalgesia induced in rats by the injection of yeast into their hind paws [85]. In line with its inflammatory properties, there is evidence that CBD can inhibit lipoxygenase [81, 86] and reduce release of the proinflammatory cytokines interleukin-1 [87, 88] and tumour necrosis factor-α [77, 87, 88]. In addition, there is evidence that it can inhibit cyclooxygenase, albeit only at very high concentrations [86, 89–91]. However, CBD also possesses actions that are likely to be proinflammatory: it can activate phospholipase A_2 [90, 92–94] and inhibit release of the anti-inflammatory cytokine interleukin-10 [95].

Results from recent experiments with the mouse microglial cell line BV-2 indicate that CBD may also reduce inflammation in the central nervous system by affecting microglial cell migration [8, 96]. The data suggest that microglial cells co-express CB_2 receptors and receptors for abnormal CBD and that when these receptors are simultaneously activated they interact synergistically to trigger chemokinetic and chemotaxic migration of the microglial cells [96]. The data also suggest that 2-arachidonoyl glycerol can activate both these receptor types to stimulate migration of BV-2 cells and that this effect of 2-arachidonoyl glycerol is opposed by CBD, acting on the proposed abnormal CBD receptors. CBD was found to display the mixed agonist/antagonist properties that are typical of a partial agonist. Thus, at 0.3 μM, it opposed the stimulatory effect of 2-arachidonoyl glycerol on microglial cell migration but when administered by itself it produced a slight enhancement of basal migration (EC_{50} = 0.25 μM). There is evidence that microglial cells migrate towards neuroinflammatory lesion sites to release proinflammatory cytokines and cytotoxic agents and also that 2-arachidonoyl glycerol produc-

Table 3. Anti-inflammatory effects of CBD *in vivo*

Bioassay	Effect of CBD	Dose	Reference
Oedema induced in mice by sub-plantar injection of carrageenan	Inhibition	100 mg administered 19 h before carrageenan by abdominal transdermal patches using ethosomal carriers	[80]
Clinical signs of arthritis in mice (swelling, erythema, oedema, and/or rigidity of joints) and histologically assessed joint damage induced by type II collagen in complete Freund's adjuvant injected intradermally at the base of the tail	Inhibition	5, 10 or 20 mg/kg/day i.p. or 25 or 50 mg/kg/day p.o.*	[77]
Bovine type II collagen-induced IFN-γ release from lymph node cells taken from arthritic mice	Inhibition	5 mg/kg/day i.p.†	[77]
In vitro TNF release from synovial cells taken from knee joints of arthritic mice	Inhibition	5 mg/kg i.p.†	[77]
LPS-induced elevation of mouse serum TNF	Inhibition	10 mg/kg i.p. or s.c.	[77]
Ca^{2+} ionophore-induced stimulation of LTB_4 production in mouse plasma	Inhibition (*ex vivo*)	10 mg/kg p.o.	[81]
Ca^{2+} ionophore-induced stimulation of TXB_2 production in mouse plasma	Enhancement (*ex vivo*)	10 mg/kg p.o.	[81]
Plasma levels of PGE_2 in rats with carrageenan-inflamed paw tissue	Decrease	10–40 mg/kg p.o.	[82]
Basal PGE production by mouse peritoneal macrophages	Inhibition (*ex vivo*)	50 mg/kg p.o.	[83]

IFN-γ, interferon-γ; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; PGE, prostaglandin; s.c., subcutaneous; TNF, tumour necrosis factor; TXB_2, thromboxane B_2.

* Bell-shaped dose-response curve: CBD was more effective against clinical signs of arthritis (1) at 5 mg/kg/day i.p. than at 10 or 20 mg/kg/day i.p. and (2) at 25 mg/kg/day p.o. than at 50 mg/kg/day p.o. Optimal doses for reducing histologically assessed joint damage in arthritic mice were 5 mg/kg/day i.p. and 25 mg/kg/day p.o.

† Dose of CBD administered to the arthritic mice before they were killed.

tion by microglial cells can be increased by a pathological stimulus [96]. Consequently, it is possible that when given alone or in combination with a CB_2 receptor antagonist, CBD may have therapeutic potential for the management of neuroinflammation resulting from endocannabinoid-induced enhancement of microglial cell migration.

Other potential therapeutic targets

Emesis

Experiments in which rats were conditioned to display rejection reactions (gaping, chin rubbing and paw treading) in response to oral infusion of a flavour previously paired with the emetic agent lithium chloride have shown that the frequency of these rejection reactions can be reduced by both CBD and 4'-dimethylheptyl-CBD at 5 mg/kg i.p. [97]. Similar results have been obtained with Δ^9-THC, 11-hydroxy-Δ^8-THC-dimethylheptyl (HU-210) and the 5-HT_3 receptor antagonist, ondansetron [98–100]. CBD has also been found to modulate lithium-induced vomiting in the house musk shrew in a manner that was insensitive to antagonism by the CB_1-selective antagonist, SR-141716A [101]. Interestingly, although vomiting was suppressed by CBD at 5 and 10 mg/kg i.p., it was enhanced by higher doses (25 and 40 mg/kg i.p.). In contrast, Δ^9-THC exhibited only an anti-emetic effect which it seemed to produce by acting through CB_1 receptors. More recently, CBD has been found to share the ability of ondansetron and Δ^9-THC to suppress cisplatin-induced emesis in the house musk shrew [102]. Again, CBD differed from Δ^9-THC (and ondansetron) by producing a biphasic effect. It suppressed vomiting at 5 mg/kg i.p. and enhanced it at 40 mg/kg i.p. It is noteworthy that CBD (10 or 20 mg/kg i.p.) also differs from Δ^9-THC in not reducing 2-arachidonoyl glycerol-induced vomiting in shrews [103].

Glaucoma

CBD has been found to lower intraocular pressure when applied directly to the eyes of cats, acutely at 250 μg or continuously at 20 μg/h [104]. Δ^9-THC was also shown to lower cat intraocular pressure. However, whereas Δ^9-THC produced conjunctival hyperaemia, erythema and chemosis, CBD did not.

Sleep disorders

One notable side effect of CBD in epileptic patients is somnolence (see section on epilepsy). Consistent with this observation, rats injected with CBD at 20 or 40 mg/kg i.p. have been found to show signs of behavioural quiescence

followed by sleep, during which they exhibited cortical EEG patterns of the kind observed in physiological sleep [105]. Slow-wave sleep latency was decreased by the lower dose, whereas the higher dose increased the amount of slow-wave sleep. Rapid eye movement (REM) sleep was not affected by either dose. In a second investigation, CBD administered at doses of 25–100 mg/kg i.p. was found to increase sleep duration in rats [104]. In this investigation, however, CBD reduced the proportion of sleep time spent in REM sleep and delayed REM-sleep onset, indications that CBD may not be particularly effective clinically for the treatment of sleep disorders.

Appetite disorders

Experiments with rats have shown that, at 50 mg/kg i.p., CBD decreases the consumption of dry food, water and sucrose solutions [106] and that at 30 mg/kg i.p. it reduces consumption of sweetened milk candy [107]. The effect of CBD on appetite and food consumption in humans has yet to be investigated.

Cancer

As detailed elsewhere [8, 108–112], *in vitro* experiments have shown that at concentrations of 1 μM or more CBD can affect the growth and proliferation of cancer cells, the effect most usually observed being one of inhibition. There is also evidence that CBD has the ability to induce apoptosis in cultures of human HL-60 myeloblastic leukaemia cells and human U87 and U373 glioma cells [111, 112]. The data suggest that it produces this effect at 3.2 μM in γ-irradiated leukaemia cells, at 12.7 μM in non-irradiated leukaemia cells and at 25 μM but not 10 μM in the glioma cells [111, 112]. These and higher concentrations of CBD did not induce detectable apoptosis in γ-irradiated or non-irradiated monocytes obtained from normal individuals [111]. For the human glioma cell lines at least, the anti-tumour effects of CBD appear to be produced in a manner that is independent of CB_1 and vanilloid receptors, although possibly not of CB_2 receptors [112]. It has also been found that the growth of human glioma cells implanted subcutaneously into nude mice can be inhibited by CBD when this is administered repeatedly *in vivo* at a subcutaneous dose of 0.5 mg/mouse [112]. Future research directed at establishing whether CBD has potential as an anti-cancer drug should include the performance of additional CBD experiments with *in vivo* animal models of cancer and attempt to identify those types of tumour that are particularly susceptible to this compound. A recent finding by Kogan et al. [113] that a quinoid derivative of CBD, HU-331, shows marked anti-tumour activity *in vitro* and *in vivo* (in mice) also merits further investigation.

Alzheimer's disease

Iuvone et al. [114] have obtained evidence that one clinical application of CBD may be for the prevention of neuronal cell death that occurs in Alzheimer's disease. This evidence came from experiments performed with an *in vitro* model of this disease in which rat cultured pheocromocytoma PC12 cells were exposed to β-amyloid. It was found that CBD decreased β-amyloid-induced neurotoxicity in these non-neuronal cancer cells at 0.1–100 μM in a manner that appeared to depend, at least in part, on the ability of CBD to oppose β-amyloid-induced intracellular accumulation of Ca^{2+}, intracellular accumulation of reactive oxygen species, lipid peroxidation and apoptosis, as measured by caspase 3 accumulation and the occurrence of DNA fragmentation. This CBD seemed to do in a CB_1-receptor-independent manner. Whether CBD also shows protective activity in a neuronal model of Alzheimer's disease has yet to be established.

Concluding discussion

In conclusion, results largely from animal experiments indicate that CBD has a number of potential therapeutic applications. The evidence supporting its use for the management of grand mal epilepsy, anxiety, neurotoxicity and inflammation, both central and peripheral, is particularly convincing. However, it is possible that CBD will also come to have other clinical uses, for example the attenuation of unwanted effects of Δ^9-THC, when this psychoactive cannabinoid is used as a medicine (see [8]), or the treatment of cancer, acute schizophrenia, sleep or appetite disorders, disease- or drug-induced dystonia, glaucoma or nausea. As to future research, this should be directed at (1) establishing more conclusively whether CBD does indeed have therapeutic importance by performing clinical trials that measure its efficacy, provide information about the best dose regimens and delivery systems for particular applications and identify any unwanted effects of significance, including the development of tolerance to sought-after effects; (2) determining whether benefit-to-risk ratios could be improved by co-administering CBD with other drugs, for example with phenytoin for the management of grand mal epilepsy (see section on epilepsy) or with a cannabinoid CB_2 receptor antagonist to treat central neuroinflammation (see section on inflammation); (3) investigating the mode(s) of action of CBD more precisely and completely; (4) matching particular actions of CBD to particular therapeutic applications or side effects; (5) seeking out additional potential clinical uses for CBD for which there is currently little or no evidence.

There is also a need for CBD to be optimized as a medicine. In particular, it is important that the therapeutic applications of this phytocannabinoid are defined more precisely, for example by mounting clinical trials directed at establishing in greater detail (1) the types of epilepsy, neurotoxicity, dystonia

or cancer against which CBD is most effective or (2) the extent to which CBD can attenuate unwanted effects of Δ^9-THC or contribute additional beneficial effects without also producing unacceptable reductions in the clinically sought-after effects of the psychoactive cannabinoid. In addition, it will be important to determine the degree to which the apparent bell shape of the relationship between the dose of CBD and at least some of its sought-after effects (e.g. anxiolytic, neuroprotective and anti-inflammatory effects) limits the setting up of an acceptable dose regimen in the clinic. It will also be of interest to discover the cause(s) of these bell-shaped dose-response relationships which could, for example, arise because some actions produced only by high doses of CBD elicit responses (e.g. enhancement of tissue levels of anandamide through inhibition of its neuronal uptake and enzymic deamidation) that oppose effects produced by CBD at lower doses (e.g. antagonism of anandamide) (see [8]). Since CBD can modulate the activity of hepatic microsomal cytochrome P450 enzymes through both inhibition and induction (see [8]), there is also a need to be aware that CBD may undergo clinically significant pharmacokinetic interactions with some established medicines. Finally, it will be important to investigate the desirability/possibility of developing an analogue of CBD that, for example, has improved efficacy or potency for sought-after effects or that has a dose-response curve with a shape that is classically sigmoid rather than bell-shaped.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Endocannabinoids and regulation of fertility

Mauro Maccarrone

Department of Biomedical Sciences, University of Teramo, Piazza A. Moro 45, 64100 Teramo, Italy

Introduction

The adverse effects of cannabinoids, and in particular of Δ^9-tetrahydrocannabinol (Δ^9-THC), on reproductive functions have been known for a long time [1, 2], and include retarded embryo development, fetal loss and pregnancy failure (recently reviewed in [3, 4]). Δ^9-THC has been reported to account for the majority of the reproductive hazards of marijuana use, in particular in males it leads to impotency by suppressing spermatogenesis, by reducing the weight of reproductive organs and by decreasing the plasma concentration of circulating hormones like testosterone. In females, Δ^9-THC inhibits ovulation by prolonging the estrous cycle and decreasing the proestrous surge of luteinizing hormone. In addition, exposure to natural cannabis extracts during pregnancy has been correlated with embryotoxicity and specific teratological malformations in rats, hamsters and rabbits. Also the major endocannabinoid anandamide (*N*-arachidonoylethanolamine, AEA) has been shown to impair pregnancy and embryo development in mice [3], suggesting that endocannabinoids might regulate fertility in mammals. Consistently, down-regulation of AEA levels in mouse uterus has been associated with increased uterine receptivity, which instead decreased when AEA was up-regulated [5]. The levels of uterine AEA fluctuate with changes in the pregnancy status, which is important because successful implantation is the result of an intimate cross-talk between the active blastocyst and the receptive uterus [5]. AEA might be critical in regulating the so-called window of implantation through synchronization of trophoblast differentiation and uterine preparation to the receptive state. This hypothesis is consistent with the observation that low levels of cannabinoid agonists exhibit accelerated trophoblast differentiation and outgrowth, while higher doses inhibit trophoblast differentiation [6]. In the same context, the higher level of AEA in the nonreceptive uterus correlates well with the embryotoxic effect of the nonreceptive uterine environment, as well as with the *in vitro* observation that AEA inhibits embryo development and zona-hatching of blastocysts [5]. In the mouse, mRNAs of AEA-binding CB_1 and CB_2 receptors are expressed in the preimplantation embryos, and the levels of CB_1 receptors are much higher than those found in brain [3]. Activation of blastocyst CB_1 receptor is detrimental for mouse preimplantation and development [5, 6], but

it appears to accelerate trophoblast differentiation [3]. On the other hand, only CB_1 mRNA is present in the mouse uterus [3]. In the context of CB receptor modulation a recent study has shown a role for progesterone receptor in Δ^9-THC modulation of female sexual receptivity [7], further demonstrating that dysregulation of cannabinoid signalling disrupts uterine receptivity for embryo implantation [8]. Also, sea urchin (*Strongylocentrotus purpuratus*) sperm has been shown to have a CB receptor, and binding of AEA to this receptor reduces sperm-fertilizing capacity, by inhibiting the egg jelly-stimulated acrosome reaction [9, 10]. This observation has been recently extended to humans [11], and the implications for male fertility will be discussed later in this review. The biological activity of AEA via CB_1 and CB_2 receptors depends on its concentration in the extracellular space, which is controlled by its synthesis through a specific phospholipase D (PLD), by its cellular uptake through a specific AEA membrane transporter (AMT) and by its intracellular degradation by the enzyme fatty acid amide hydrolase (FAAH) [12]. Among these proteins, which together with AEA and congeners form the endocannabinoid system, FAAH has emerged as a pivotal check-point in several human diseases (for comprehensive reviews see [13–17]). Evidence in favour of its critical role in mammalian fertility will be discussed in the following sections.

Endocannabinoid degradation during pregnancy

FAAH activity has been demonstrated in mouse uterus [18], and the level of its mRNA has been shown to change during the peri-implantation period, in both mouse uterus and embryos [19]. FAAH localizes in the endometrial epithelium [20], where its activity and expression decrease during early pregnancy, due to a lower expression of the same gene rather than to FAAH isozymes with different kinetic properties [20].

Despite the growing evidence that AEA adversely affects uterine receptivity and embryo implantation (reviewed in [3, 4]) and that AEA degradation by FAAH may have physiological significance in these processes [18–20], the regulation of FAAH during early pregnancy is still obscure. Recently, we observed down-regulation of FAAH expression in pseudopregnant mice, and a fall of FAAH activity and expression from day 0 to day 5.5 of gestation [20]. We also reported that this fall was smaller in ovariectomized animals and larger in the same animals treated with estrogen, compared to controls [20]. These findings suggest that sex hormones might regulate FAAH activity by modulating gene expression at the translational level. The results of the treatment of virgin females with progesterone or estrogen, showing a similar down-regulation of FAAH compared to controls, strengthened this hypothesis. Therefore, it can be concluded that in mouse uterus sex hormones down-regulate FAAH activity by reducing gene expression at the level of protein synthesis. Interestingly, an AEA synthase activity was also measured in mouse uterus, and was found to respond to sex hormones in the same way as FAAH [20].

It can be proposed that down-regulation of FAAH during early pregnancy might allow higher local levels of AEA, which indeed have been shown to increase with pregnancy in the mouse uterus [5]. In turn, AEA might play a role in the endometrial changes associated with pregnancy, for instance through the inhibition of gap junctions and intercellular calcium signalling [21, 22]. On the other hand, nanomolar concentrations of AEA inhibit embryo development and blastocysts hatching *in vitro* [5, 20, 23]. This suggests that the local concentration of AEA around the implanting embryos must be low, implying that the blastocysts have the biochemical tools to dispose AEA and to prevent its detrimental effects. Indeed, AMT and FAAH activity have been demonstrated and characterized in these cells [20]. Moreover, AEA-induced inhibition of embryo development and blastocyst hatching is prevented by a CB_1 receptor antagonist, in line with the hypothesis that this activity of AEA is mediated by CB_1 receptors [3].

Collectively, these findings lead to a dual function of AEA in regulating fertility in mammals, a scenario that is schematically depicted in Figure 1. On one hand, a decreased FAAH activity in mouse uterus during early pregnancy might allow higher levels of AEA at the inter-implantation sites. Here, enhanced AEA can be instrumental in modifying endometrium by inhibiting gap junctions. On the other hand, a low level of AEA has to be granted at the implantation sites, in order to reduce the toxic effects of this lipid to the blastocysts. The reduction of AEA levels can be achieved by the active AMT and FAAH expressed by blastocysts, as well as by the uterine epithelial cells. It seems noteworthy that dual functions of AEA, depending upon its local concentration, have been already proposed to explain its anti-proliferative (high AEA) or pro-proliferative (low AEA) effects on trophoblast growth at the inter-implantation and implantation sites, respectively [18]. Consistently, AEA has been shown to regulate blastocyst function and implantation within a very narrow concentration range, by differentially modulating mitogen-activated protein kinase signalling and calcium channel activity via CB_1 receptors [24].

The embryo–uterine interactions are further complicated by the recent finding that mouse blastocysts rapidly (within 30 min of culture) release a soluble compound that increases by approximately 2.5-fold the activity of FAAH present in the mouse uterus without affecting gene expression at the translational level [25]. This "FAAH activator" is not present in uterine fluid, is released by neither dead blastocysts nor mouse embryonic fibroblasts, and is produced by trophoblast and inner cell-mass cells. Moreover, its activity is fully neutralized by lipase and is further potentiated by trypsin, whereas other proteases, phospholipases A_2, C or D, DNase I or RNase A are ineffective. Interestingly, the blastocyst-derived activator does not affect PLD, CB receptors or AMT in mouse uterus, pointing to a selective action towards FAAH. As yet the FAAH activator, the first ever reported to our knowledge, has not been identified with any factor known to be released by blastocysts, like platelet-activating factor, leukotriene B_4 or prostaglandins E_2 and $F_{2\alpha}$, and its molecular identity remains elusive [25]. However, the fact that a specific

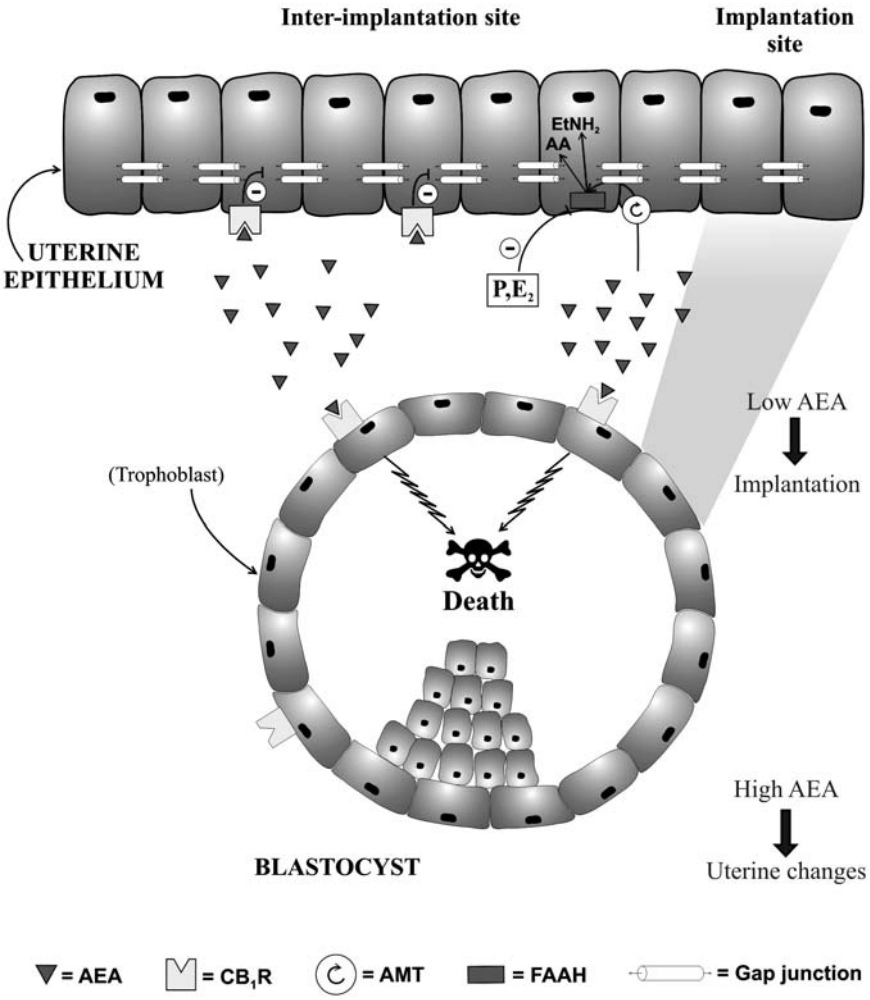

Figure 1. Local effects of anandamide on implantation and uterine changes. Binding of anandamide (AEA) to type 1 cannabinoid receptors (CB_1Rs) on the blastocyst leads to cell death, whereas its binding to CB_1 receptors on the uterine epithelium inhibits gap junctions and is instrumental in modifying the uterus during gestation. The AEA membrane transporter (AMT) and the fatty acid amide hydrolase (FAAH) present in the uterine epithelial cells cleave AEA into ethanolamine ($EtNH_2$) and arachidonic acid (AA), thus protecting the blastocyst against the noxious effects of AEA. Progesterone (P) and estrogen (E_2) down-regulate uterine FAAH. Also the blastocyst has active AMT and FAAH (omitted for the sake of clarity), which dispose of AEA and facilitate implantation.

FAAH activator is released by the implanting blastocysts further corroborates the hypothesis that these cells need to protect themselves against the noxious effects of uterine endocannabinoids. A lipid able to cross the cell membranes and to rapidly and specifically activate FAAH in uterine epithelial cells is suitable to confer this protection, as schematically shown in Figure 2. A defective

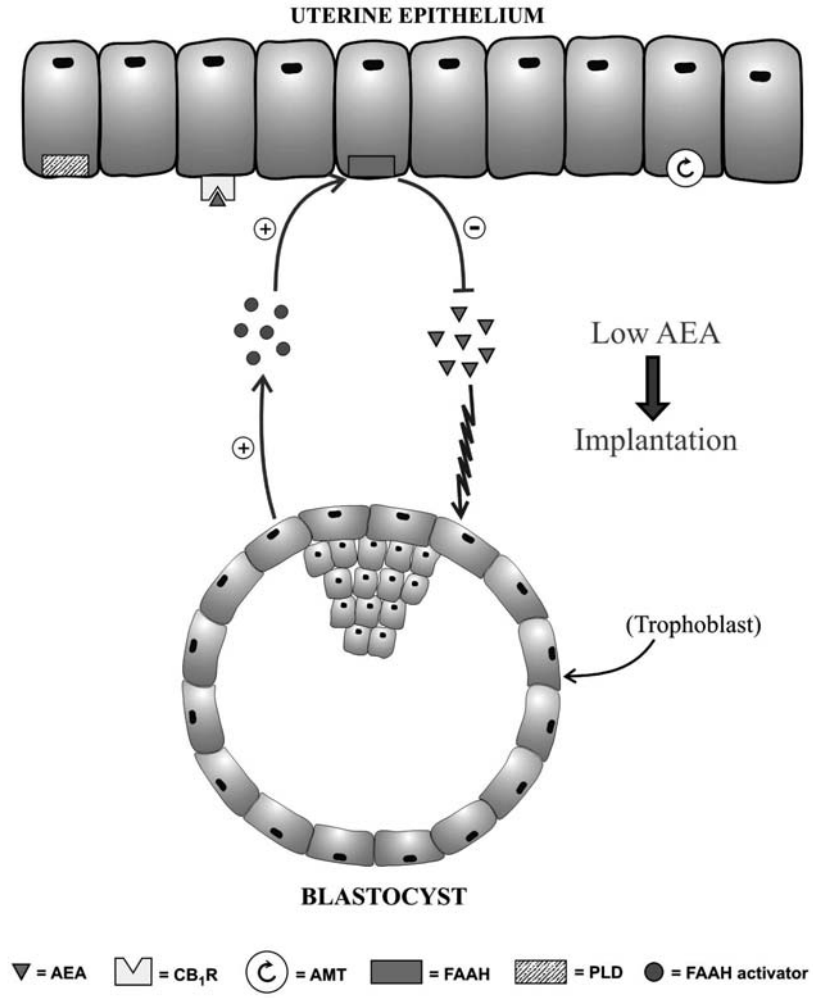

Figure 2. Interactions between blastocyst and uterine epithelium. At the site of implantation blastocysts release a lipid compound able to activate fatty acid amide hydrolase (FAAH) in uterine epithelial cells, termed the FAAH activator. This leads to the cleavage of anandamide (AEA), thus reducing its uterine levels and noxious effects towards the implanting blastocyst (see also Fig. 1). AEA-synthesizing phospholipase D (PLD), AEA membrane transporter (AMT) and AEA-binding type 1 cannabinoid receptors (CB_1Rs) of uterine epithelial cells are not modulated by the FAAH activator.

production of the activator by an unhealthy embryo might contribute to implantation failure. Alternatively, a non-receptive, unhealthy uterus might be unable to respond properly to a normal activator, leading to blastocyst death. Though the molecular details of the embryo–uterine cross-talks remain to be elucidated, present evidence strongly suggests that FAAH regulates these cross-talks by controlling the endogenous tone of AEA.

FAAH and human reproduction

Human reproductive fluids, such as seminal plasma, mid-cycle oviductal fluid, follicular fluid, amniotic fluid and milk have been reported to contain AEA, *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA) in the low nanomolar range, from 3 nM for AEA in the follicular fluid to 67 nM for OEA in human milk [26]. This suggests that endocannabinoids might regulate multiple physiological and pathological reproductive functions in humans, implying that exogenous cannabinoids delivered by marijuana smoke could threaten these processes. Consistently, Table 1 shows that blood AEA levels in women experiencing miscarriage are approximately 4-fold higher than the levels found in women with normal gestation [4]. Since the human reproductive tissues are only sparsely accessible to experimenters, we took advantage of the critical role of peripheral lymphocytes in embryo implantation and successful pregnancy [27] to investigate in these cells how the endocannabinoid system might affect human reproduction.

Table 1. The endocannabinoid system in human gestation. The endogenous levels of AEA were assayed in the blood of healthy women and of women who had miscarried. The binding to CB_1 receptors, and the activity of FAAH and AMT, were assayed in peripheral lymphocytes from the same subjects

Parameter	Women with normal gestation		Women who miscarried	
AEA content (pmol/ml)	0.9 ± 1.0	(100%)	4.0 ± 2.2	(444%)*
CB_1 binding (cpm/mg protein)	20380 ± 1930	(100%)	20400 ± 1795	(100%)
FAAH activity (pmol/min/mg protein)	133 ± 9	(100%)	48 ± 5	(36%)*
AMT activity (pmol/min/mg protein)	50 ± 4	(100%)	49 ± 4	(98%)

*$P < 0.01$ *versus* normal gestation ($P>0.05$ in all other cases).

The pathophysiological effects of AEA, and possibly of several congeners, are under control of FAAH. Indeed, FAAH-knockout mice have been shown to have 15-fold higher levels of AEA than wild-type littermates, suggesting that the hydrolase controls the *in vivo* levels of this endocannabinoid [28]. In particular, lymphocyte FAAH might be involved in controlling pregnancy failure by regulating the level of AEA at the feto–maternal interface, thus interfering with the lymphocyte-dependent cytokine networks around the mother and the fetus [27]. In this line, we have recently demonstrated that decreased activity and expression of FAAH in maternal lymphocytes is an early (<8 weeks of gestation) marker of spontaneous abortion in humans [29]. In a following study, we measured the activity of FAAH and AMT, and the binding to CB receptors in lymphocytes isolated from healthy women at 7–8 weeks of gestation, and we found that FAAH activity was lower in women

who miscarried compared to those who did not [30]. None of the other proteins of the endocannabinoid system were affected (Tab. 1). In addition, we found that the levels of AEA and FAAH in peripheral lymphocytes undergo specific variations during the various phases of the human ovulatory cycle. In particular, the highest levels of FAAH activity, paralleled by the lowest AEA concentrations, were found on day 21 of gestation, which is the period that temporally coincides with the putative window of implantation in humans [31]. Instead PLD, AMT and CB_1 receptors of lymphocytes did not change during the menstrual cycle (Tab. 2). This evidence strengthens the concept that high FAAH activity and low AEA levels may be among the factors that contribute to the success of implantation. Furthermore, they point towards a key role of FAAH, but not of the other proteins of the endocannabinoid system, in lymphocyte-mediated control of the hormone-cytokine networks at the feto–maternal interface. In this line, recent studies have shown that progesterone up-regulates FAAH, but not PLD, AMT or CB_1 receptors, in human lymphocytes [32]. Some molecular details of this activity have been unravelled, showing that progesterone, through formation of a complex with its intracellular receptor, enhances the level of the trancription factor Ikaros, which in turn enhances *FAAH* gene expression by binding to a specific sequence in the promoter region [32]. Also leptin, the product of the *obese* gene which controls fertility [33] and immune function [34], has been recently shown to enhance *FAAH* gene transcription through a STAT3-mediated activation of the FAAH promoter at a cAMP-response element (CRE)-like site [32]. These findings suggest that the hydrolase can be regarded as a molecular integrator of well-known fertility signals, and that it controls the activity of AEA in reproduction.

Table 2. The endocannabinoid system in human ovulatory cycle. The endogenous levels of AEA, the binding to CB_1 receptors, and the activity of PLD, FAAH and AMT, were assayed in peripheral lymphocytes of healthy women at different stages of the menstrual cycle

Parameter	Day 7		Day 14		Day 21	
AEA content (pmol/mg protein)	2.15 ± 0.20	(100%)	3.76 ± 0.35	(175%)*	1.29 ± 0.14	(60%)*
CB_1 binding (cpm/mg protein)	20000 ± 2030	(100%)	20000 ± 2050	(100%)	17400 ± 1795	(87%)
PLD activity (pmol/min/mg protein)	130 ± 15	(100%)	117 ± 12	(90%)	130 ± 15	(100%)
FAAH activity (pmol/min/mg protein)	115 ± 12	(100%)	46 ± 5	(40%)	253 ± 22	(220%)
AMT activity (pmol/min/mg protein)	50 ± 5	(100%)	43 ± 4	(86%)	45 ± 5	(90%)

*$P < 0.05$ *versus* day 7 ($P>0.05$ in all other cases).
$P < 0.01$ *versus* day 7 ($P>0.05$ in all other cases).

FAAH and the regulation of spermatogenesis

Despite the knowledge that chronic administration of Δ^9-THC to animals lowers testosterone secretion and reduces the production, motility and viability of sperm [35], a role for the endocannabinoid system in controlling male fertility remains to be elucidated. Evidence that AEA regulates human sperm functions has been recently presented [11], and *in vitro* studies have demonstrated that the AEA congener PEA may affect the time course of capacitation of human spermatozoa, by modulating the properties of their membranes [36]. In addition, rat testis is able to synthetize AEA [37], and this compound has been detected in human seminal plasma at approximately 10 nM [26]. More recently, the presence of CB_1 receptors in Leydig cells and their involvement in testosterone secretion have been demonstrated in mice [38]. Also the function of Sertoli cells has been shown to be altered by Δ^9-THC, though the molecular basis for this alteration has not been established [39]. As Sertoli cells of the mammalian seminiferous epithelium are involved in the regulation of germ cell development by providing nutrients and hormonal signals needed for spermatogenesis, we have recently investigated whether Sertoli cells are able to bind and degrade AEA, and whether this endocannabinoid might control survival and death of these cells. This is also in view of the well-documented pro-apoptotic activity of AEA [16]. In the same context, the effect of follicle-stimulating hormone (FSH) has been checked, because it dramatically impacts fetal and early neonatal Sertoli cell proliferation, and is critical in determining the spermatogenic capacity in the adult mammals [40]. To date this study on Sertoli cells represents the only characterization of the endocannabinoid system and its role in male reproductive function [41]. Therefore, the main outcomes will be briefly summarized here to put in a better perspective their physiological relevance and potential therapeutic implications.

We found that Sertoli cells have the biochemical machinery to bind and degrade AEA, and we have characterized this machinery in cells at a range of ages (4–24 days), largely used as a model for immature mice in endocrinological studies. Immature Sertoli cells express functional CB_2 receptors on their surface, and the level of these receptors is constant in ageing cells [41]. Instead, FAAH activity declines age-dependently, due to a lower gene expression, and also the uptake of AEA through AMT declines in ageing Sertoli cells. Incidentally, to the best of our knowledge this evidence represents the first demonstration of the modulation of the endocannabinoid system by ageing. In addition, we found that AEA uptake by Sertoli AMT, like that of other human peripheral cells, is significantly increased by NO donors [41], which might be relevant *in vivo* because NO plays several roles in regulating male fertility [42, 43]. In particular, NO regulates the contribution of Sertoli cells to fertility and inflammation-mediated infertility [42–44], and a faster removal of AEA from the extracellular space, which leads to termination of its biological activity, might be the rationale for these effects of NO.

An interesting observation is that AEA can force Sertoli cells to apoptosis, and that this process is more evident upon ageing [41]. The pro-apoptotic effect of AEA is not mediated by CB_1, CB_2 or so-called endothelial-type cannabinoid receptors, nor by vanilloid receptors. Instead, CB_2 receptors expressed by Sertoli cells have a protective role against the toxic effects of AEA, and so does FSH. In fact, this hormone dose-dependently inhibits apoptosis by inducing a remarkable (approximately 5-fold) increase in FAAH activity [41]. Therefore, it can be suggested that altered levels of FSH can affect testis development through the control of the pro-apoptotic potential of AEA. This observation, together with the well-established relationship of Sertoli cell number to the total spermatogenic output of the testis, can contribute to the negative effects exerted on testicular development by altered FSH concentrations, as well as by mutations of the FSH receptor gene [45]. Overall, the finding that Sertoli cells partake in the peripheral endocannabinoid system may open new perspectives to the understanding and treatment of male infertility. In particular, the observation that FAAH modulates the biological effects of AEA on Sertoli cells, and that this FAAH-mediated control is under hormonal regulation, extends to male reproduction the concept that AEA hydrolase is an important check-point, as described above for female fertility.

Conclusions and perspectives

Available evidence clearly shows that in mammals endocannabinoid signalling is intimately associated with embryo–uterine interactions during implantation. The exact physiological significance of this signalling pathway is not yet clear, and it is not known how widespread it might be among different species. At any rate, in humans low FAAH in lymphocytes correlates with spontaneous abortion, calling for attention on this enzyme as a key point in the control of the endocannabinoid system during pregnancy [29–32]. Moreover, available evidence seems to add endocannabinoids to the hormone-cytokine networks responsible for embryo–uterine interactions, and this might represent a useful framework for the interpretation of novel interactions between progesterone, leptin, cytokines, FSH and (endo)cannabinoids [7, 32, 41].

An interesting outcome of the reported findings is that quantitation of FAAH protein in maternal lymphocytes might become a diagnostic marker of spontaneous abortion, easy to measure in routine analyses. Peripheral lymphocytes are easily isolated from blood samples and immunochemical tests for FAAH could be run automatically, both important advantages for monitoring gestation in a low-risk population at large.

Since defective FAAH correlates with pregnancy failure, of interest is also the perspective that factors able to enhance FAAH activity might become useful therapeutic tools for the management of spontaneous abortion. Enhancers of promoter activity able to mimic the actions of Ikaros and STAT3, or lipid activators of FAAH like that released by mouse blastocysts, might open the

avenue to the development of new lead compounds of therapeutic value for the treatment of male and female infertility in humans.

Acknowledgements
I thank Professor A. Finazzi-Agrò (Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Rome, Italy) for continuing support, all colleagues who gave their valuable contribution to the fertility studies, and Mr G. Bonelli for the excellent production of the artwork. This investigation was supported by Ministero dell'Istruzione, dell'Università e della Ricerca (COFIN 2002 and 2003) and by Istituto Superiore di Sanità (IV AIDS Program), Rome.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabinoids in neurodegeneration and neuroprotection

Javier Fernández-Ruiz[1], Sara González[1], Julián Romero[2] and José Antonio Ramos[1]

[1] *Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain*
[2] *Laboratorio de Apoyo a la Investigación, Fundación Hospital Alcorcón, 28922-Alcorcón, Madrid, Spain*

Introduction

Among a variety of cellular and tissue functions, it has been suggested that the endocannabinoid system might also exert an important function in the cellular decision about death or survival (for review see [1–3]). This finding has derived from several experimental observations indicating that cannabinoids combine at the same time neuroprotective [4–6] and anti-proliferative [1, 3] properties. Thus, over the last decade, a considerable volume of work has accumulated evidence to assume that the endocannabinoid system plays a role in the protection against acute or chronic brain damage [4–6]. This fact is particularly relevant considering the postmitotic characteristics of neuronal cells, which makes repair processes after several types of brain injury extremely difficult. For instance, plant-derived, synthetic and/or endogenous cannabinoids provide neuroprotection in *in vitro* and *in vivo* models that replicate cytotoxic events, mainly energy failure and excitotoxicity, occurring during several types of accidental brain injury (i.e. ischemia and head trauma), that acutely trigger degeneration (see [4–6] for recent reviews). In addition, cannabinoids are also neuroprotective in several chronic neurodegenerative pathologies that also involve the occurrence of excitotoxicity, mitochondrial dysfunction, inflammation and/or oxidative stress, such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and multiple sclerosis (MS); see [4, 7] for review.

On the other hand, the activation of different elements of the endocannabinoid system, as part of an endogenous protectant response, has been documented in different experimental paradigms of neurodegeneration, although with variable results, depending on age, species, type and severity of injury, and mechanism(s) activated for cell death (reviewed in [5, 8, 9]). Thus, several studies have demonstrated that neuronal damage is accompanied by an increase in the production of endocannabinoids (see [5, 8] for recent reviews), although

other authors did not find this response [10]. For instance, Hansen and coworkers described an increase in the levels of anandamide (*N*-arachidonoylethanolamine, AEA) and its phospholipid precursor, but not of 2-arachidonoyl glycerol (2-AG), during acute degeneration in the neonatal rat brain [11, 12]. Similar results, increases in AEA with no changes in 2-AG, were found by Marsicano et al. [13] in a mouse model of kainate-induced excitotoxicity, and by Gubellini et al. [14] in a rat model of PD. However, Panikashvili et al. [15] showed that 2-AG is massively produced in the mouse brain after closed head injury. In addition, they found that this endocannabinoid has neuroprotective effects, as indicated by a reduction in edema and infarct volume and by improved clinical recovery after being administered to animals. This endogenous response has been also found in humans since elevated levels of AEA and other fatty acid amides have been also measured around the site of damage in a microdialysis study perfomed on a single stroke patient [16].

That the increases reported in endocannabinoid production during neurodegeneration [11–16] are part of an endogenous response may be also concluded from the observation that blockade of the endocannabinoid uptake with UCM707 increased protection against kainate-induced seizures in mice, where AEA levels were reported to be elevated [13]. However, this point is also controversial since, although van der Stelt et al. [10] found protection after exogenous administration of AEA in a neonatal model of secondary excitotoxicity, they did not record any increases in AEA or 2-AG levels and, concomitantly, they did not find any effect of another uptake inhibitor, VDM11, on lesion volume [10].

Cannabinoid receptor subtypes are also induced in nerve cells in response to injury and/or inflammation [12, 17–19]. Thus, Jin et al. [17] reported that CB_1 receptors are induced in neuronal cells after experimental stroke, whereas we described an increase of these receptors in response to excitotoxic stimuli in neonatal rats [12]. As regards CB_2 receptors, a receptor subtype that is mostly absent from the brain in healthy conditions (see below), recent reports have shown induction of this receptor subtype in several pathologies [18, 19]. This occurs in activated glial cells, mainly microglia surrounding senile plaques, in human AD brain samples [18], which might indicate that CB_2 receptors play a role in either reducing degenerative impact on neurons or, on the contrary, promoting cytotoxic events. Induction of CB_2 receptors at lesioned sites has been also documented in rat models of striatal degeneration replicating human HD pathology [19].

In contrast with the protective properties of cannabinoids in non-transformed nervous cells, these compounds are also able to elicit apoptosis in transformed nerve cells (C6 glioma, human astrocytoma U373MG and mouse neuroblastoma N18TG12 cells) *in vitro* [1, 20], and to promote the regression of glioblastoma *in vivo*, through a mechanism that involves the activation of mitogen-activated protein kinase and ceramide accumulation [21]. In addition, cannabinoids have been recently reported to inhibit angiogenesis, which represents a key process in tumorigenesis [22]. These anti-proliferative effects of

cannabinoids represent a novel potential utility of cannabinoid-based compounds in cancer treatment in the future [1, 3].

The present chapter will address the evidence concerning the first of these dual effects; that is, the capability exhibited by cannabinoids to influence, by either promoting or reducing, several biochemical mechanisms leading to the reduction of neuronal cell death by apoptosis or necrosis. We will divide the chapter into three parts. First, we will summarize the different cellular and molecular mechanisms that have been reported to underly the neuroprotective effects of plant-derived, synthetic or endogenous cannabinoids. Second, we will overview the information concerning these neuroprotective effects in acute neurodegeneration, in particular in the two major accidental causes of this pathology, cerebral ischemia and traumatic brain injury. Third, we will address the same properties in chronic neurodegeneration, with emphasis on the five disorders, PD, HD, AD, MS and ALS, where relevant information has been recently published.

Mechanisms involved in neuroprotection by cannabinoids

The molecular mechanisms underlying the neuroprotectant properties of cannabinoids are quite diverse and, frequently, complementary. They include some events not mediated by cannabinoid receptors [i.e. *N*-methyl-D-aspartate (NMDA) receptor antagonism, antioxidant properties] and others that are definitively mediated by either CB_1 or CB_2 receptors including their capability (1) to reduce processes such as glutamate release, calcium influx and/or inflammation, (2) to stimulate γ-aminobutyric acid (GABA) action and (3) to improve blood supply to the injured brain (for review, see [4–6]). Other additional processes also influenced by cannabinoids, such as improvement of glucose utilization, or alternatively the production of ketone bodies – which, produced by glial cells, may replace glucose as the major source of neuronal energy metabolism in ischemia (see [23, 24] for review) – or the lowering effect on body temperature (see [25] for review), might be also considered. However, they have been less explored in relation to their involvement in cannabinoid-induced neuroprotection, and therefore they will not be addressed here.

Anti-glutamatergic effects of cannabinoids

Excitotoxicity, reflected in excessive extracellular levels of glutamate and hyperactivation of glutamate receptors, mainly the NMDA receptor subtype, is a critical event in acute or chronic neurodegeneration (see [26] for review). It is bidirectionally related to an uncontrolled shift in sodium, potassium and, particularly, calcium concentrations that disrupt ionic homeostasis and leads to severe cell swelling and death [26]. Cannabinoid agonists are certainly

anti-glutamatergic substances since they are able to reduce excitotoxicity [4–6, 9, 27]. This has been demonstrated both *in vitro* (i.e. using cultures of hippocampal neurons [28] or spinal cord neurons [29]) and *in vivo* (i.e. rodent models of ischemic damage [30]).

These anti-glutamatergic effects of cannabinoid agonists are mainly exerted by inhibiting glutamate release, a fact that has been largely demonstrated using cultured neurons from numerous brain regions (see [4, 9] for recent reviews) and also *in vivo*, through the activation of CB_1 receptors located presynaptically in glutamatergic terminals (see Fig. 1, and [31] for review). This inhibitory effect of cannabinoid agonists on glutamate release is reversed by selective CB_1 receptor antagonists, such as SR-141716 [4, 9]. In addition, the antagonist by itself potentiated excitotoxicity in kainate-injected mice [13] and increased lesion volume in a rat model of HD generated by local injections of the complex II inhibitor malonate [32]. However, other authors, using neonatal models of excitotoxicity, reported no effects of SR-141716 by itself [10] or a neuroprotective effect that was counteracted by co-administration of cannabinoid agonists [33].

On the other hand, some specific cannabinoids, such as dexanabinol (HU-211) and AEA, are also able to directly act on NMDA glutamatergic receptors (see [4–6] for review). The case of HU-211 represents, together with cannabidiol (CBD) whose neuroprotective effects will be discussed below, an

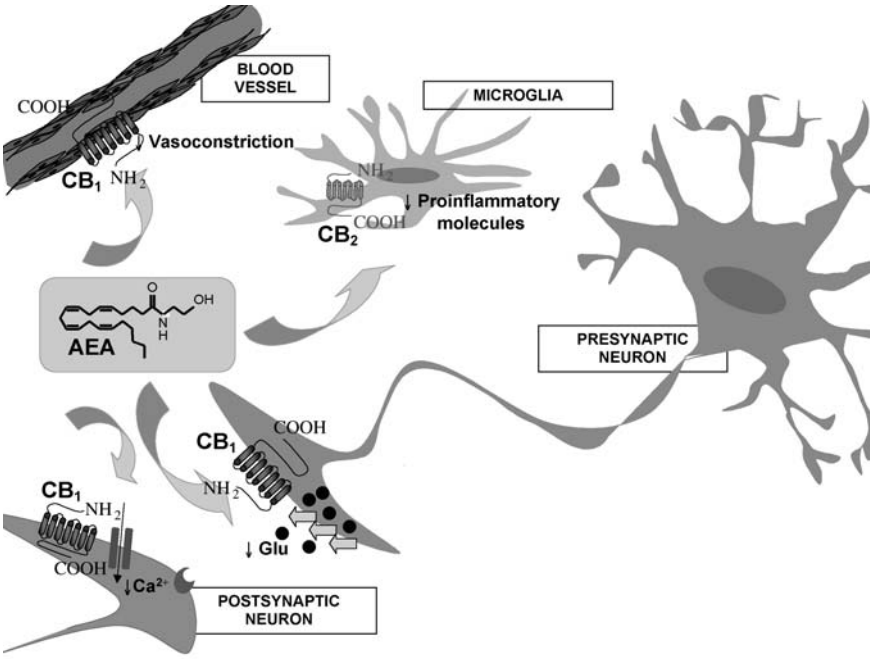

Figure 1. Cellular and molecular mechanisms involved in neuroprotective actions of cannabinoids.

interesting option because HU-211 has a cannabinoid structure, but does not bind cannabinoid receptors [4, 34]. Its neuroprotective activity originates from its capability to directly act on the glutamate system, by blocking the NMDA receptor at a site close to, but distinct from, that of non-competitive antagonists, such as MK-801 and phencyclidine, and from the recognition site for glutamate or glycine [4, 35]. Based on this antagonistic capability, HU-211 directly reduces NMDA receptor-mediated Ca^{2+} influx into neurons ([4, 36]; see more details below). However, it also provides neuroprotection because it is antioxidant [4, 37] and reduces the levels of tumor necrosis factor-α (TNF-α) [4, 34]. The result of these neuroprotective mechanisms activated by HU-211 is an improvement of motor and memory functions in association with reduced edema and blood–brain-barrier breakdown in rats subjected to closed head injury (see [4, 34] for review). AEA was also shown to directly interact with NMDA receptors in cortical, cerebellar and hippocampal slices, thereby producing a potentiation of NMDA-induced calcium responses [38]. However, this occurs only in the presence of SR-141716 [38]. This effect would be independent of its neuroprotective effects mediated by the activation of cannabinoid receptors (i.e. cannabinoid receptor-mediated reduction in Ca^{2+} influx, and anti-inflammatory and vascular effects; see details below).

Finally, it is interesting to also consider the recent evidence suggesting that one of the mechanisms of neuroprotection elicited by NMDA receptor blockade would imply the enhancement of GABA transmission [39]. Cannabinoids are able to increase inhibitory transmission mediated by GABA in some regions such as the basal ganglia [40, 41]. This would speak in favour of the critical importance of the imbalance between inhibitory and stimulatory innervations during processes of transneuronal delayed death. Cannabinoid agonists, by inhibiting glutamate release [4, 31] and/or increasing GABA presence at synapses – presumably by blocking GABA reuptake [40, 41] – might rectify this imbalance, thus delaying/arresting transneuronal death occurring in specific regions such as the substantia nigra *pars reticulata* [42].

Reduction of calcium influx by cannabinoids

As mentioned above, excitotoxicity causes hyperactivation of glutamate receptors that results in intracellular accumulation of cytotoxic levels of Ca^{2+}, which activate numerous destructive pathways involving calpains, caspases and other proteases, protein kinases, lypases, endonucleases, NO synthase, reactive oxygen species, etc. (for review, see [26]). In addition, voltage-sensitive ion channels are activated in response to the depolarization associated with NMDA-induced Ca^{2+} influx, and elevate intracellular levels of this and other ions. Cannabinoid agonists are able to close these voltage-sensitive ion channels, then reducing the overall Ca^{2+} current and the overactivation of destructive pathways which decrease the degree of neuronal death providing neuroprotection (see Fig. 1, and [4–6, 9, 25] for review). These effects would be

exerted preferentially through the activation of CB_1 receptors that, in this case, would be postsynaptically located (on neurons containing NMDA glutamate receptors) in contrast with those involved in the inhibition of glutamate release which would be presynaptically located (see Fig. 1). Several types of calcium channel (mainly N-, L- and P/Q-types) have been reported to be coupled to CB_1 receptors and are inhibited by the activation of these receptors [43–46]. In addition, AEA has been also reported to directly interact with T-type Ca^{2+} channels [47]. Cannabinoid agonists also affect K^+ currents by opening inwardly rectifying K^+ channels [44, 48, 49], an effect that might be also part of the neuroprotective action of cannabinoids.

This direct Ca^{2+}-lowering effect of cannabinoid agonists would add to the reduction of this ion produced indirectly as a consequence of the anti-glutamatergic effects of cannabinoids, which, through reducing glutamate release or blocking NMDA receptors (see above), would result in a reduction of glutamatergic receptor-mediated Ca^{2+} entry into the cells. As a consequence of this direct and/or indirect inhibition of Ca^{2+} influx produced by cannabinoid agonists, they would reduce the activation of Ca^{2+}-dependent cytotoxic cascades thus preventing neuronal damage [4–6]. In support of this hypothesis, several studies have demonstrated that the increase of Ca^{2+} influx produced by different neurotoxic stimuli, including NMDA and other excitotoxins, was reduced by different plant-derived, synthetic or endogenous cannabinoids [13, 28, 38, 50, 51], and that most of these effects were counteracted by SR-141716, thus suggesting involvement of CB_1 receptor activation [28, 38].

Antioxidant properties of cannabinoids

Brain injury in acute and chronic neurodegeneration triggers the accumulation of harmful products, such as reactive oxygen intermediates (see [52] for review), which, if not eliminated, damage DNA, proteins or membrane lipids, leading oxidative cell death. These oxidative species are produced, in response to excitotoxicity and/or mitochondrial dysfunction, from several sources, including arachidonic acid metabolism, mitochondrial defects, and the action of NO synthase and other enzymes (see [52, 53] for review). This process, so-called oxidative stress, appears when the normal balance between oxidative events and endogenous antioxidant mechanisms (i.e. antioxidative enzymes such as superoxide dismutase, catalase and peroxidase, glutation and small-molecule antioxidants such as vitamins A, C and E and ubiquinol) is disrupted, being responsible for secondary damage in conditions of acute neurodegeneration [54]. Certain classic cannabinoids, such as CBD, Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol, nabilone, levonantradol, dexanabinol and others, that contain phenolic groups in their chemical structure, are able to reduce oxidative stress [55]. These cannabinoids are potent antioxidant compounds against reactive oxygen species formed during the ischemic metabolism or in several chronic brain injuries where oxidative stress represents a crit-

ical event in the pathogenesis, such as PD [4–6]. However, it must be noted that these antioxidant properties of specific cannabinoids would be certainly CB_1 receptor-independent [37, 55–57]. This antioxidant capability has been proposed to explain the neuroprotective effects that Δ^9-THC and other related-cannabinoids showed in animal models of cerebral ischemia ([58, 59]; see [4–6] for review). Hampson et al. [56], using cultures of rat cortical neurons exposed to toxic levels of glutamate, also found that both Δ^9-THC and CBD provided neuroprotection, via a CB_1 receptor-independent mechanism, presumably based on the antioxidant properties of both compounds, which are relatively equivalent.

Cannabidiol is a plant-derived cannabinoid that presents an interesting pharmacological profile, comparable to that previously mentioned for HU-211 (see above). CBD is non-psychoactive, because does not bind significantly to CB_1 receptors. However, it exhibits an antioxidant potency comparable, and, even superior, to that of classic dietary antioxidants such as ascorbate and α-tocopherol [56]. CBD was equivalent to Δ^9-THC as an antioxidant compound, but it would be more advantageous than Δ^9-THC for a potential clinical use because it can be used at higher doses and for longer times than those possible with Δ^9-THC, due to its lack of psychoactivity. An additional advantage for CBD is that its use in prolonged treatment does not induce tolerance [60], a phenomenon often observed with Δ^9-THC [61]. On the other hand, it should be mentioned that recent evidence suggests that CBD might also act by blocking endocannabinoid uptake, thus increasing endocannabinoid levels [62], or by binding to hypothetical CBD receptors [63] still waiting to be isolated and/or cloned. All these properties, but particularly its antioxidant potential, enable CBD to be used as a neuroprotective compound, with minimal psychotropic side effects, against the brain damage produced by reactive oxygen species in brain ischemia [56] and also in several chronic neurodegenerative diseases (see [64] and details below). In this sense, we have recently found that in rat models of PD, either Δ^9-THC or CBD are able to delay/arrest the progression of neuronal death [64]. It is possible that they might also be effective in HD, as suggested by preliminary evidence [65], since it has been demonstrated that production of free radicals, originated as a consequence of a mitochondrial dysfunction, is one of the major cytotoxic events that takes place during the pathogenesis of this motor disorder (see [66] for review).

Anti-inflammatory properties of cannabinoids

Another mechanism potentially linked to various chronic and acute brain degenerative pathologies is the activation of inflammatory processes. Inflammation may induce or aggravate brain damage through increasing the release of neurotoxic mediators, such as TNF-α, interleukin (IL)-1β, IL-6, eicosanoids, NO and reactive oxygen species. Alternatively, it could enhance the neuronal vulnerability to these cytotoxic stimuli. These factors are predominantly produced by

glial cells (mainly reactive microglia) and impact on neurons to induce neurodegeneration (see [67] for review). For instance, IL-6 and TNF-α have been shown to promote demyelination, thrombosis, leukocyte infiltration and blood–brain-barrier disruption (see [68] for review). By contrast, glial cells (mainly astrocytes) are able to produce prosurvival factors which play a role in neuronal protection [69]. Both phenomena occur in ischemia [70], trauma [71], PD [72], HD [73], AD [74, 75] and other diseases [67]. In addition, inflammation can also elicit neurodegeneration through the activation of autoimmune responses against brain antigens, as happens in the case of MS and other demyelinating diseases (see [4, 76] for review). Cannabinoid agonists are able to reduce the inflammation that occurs in these diseases. This effect is possibly caused by local effects on glial cells, exerted by either reducing the release of cytotoxic factors or increasing the production of prosurvival molecules (see Fig. 1, and [4–6, 24, 25, 68] for review). This is consistent with the idea that the endocannabinoid signaling system would play crucial roles in glial cells both in healthy and pathological conditions (for review, see [24, 68]).

The anti-inflammatory potential of cannabinoid agonists in neurodegenerative diseases has been recently addressed in several studies that have revealed potent anti-inflammatory effects of selective agonists for CB_1 (arachidonoyl-2-chloroethylamide, ACEA) or CB_2 (JWH-133, JWH-015) receptors and also of non-selective cannabinoid agonists (see [4–6, 24, 68] for review). In part, this is the consequence of an effect of cannabinoids by protecting astrocytes and oligodendrocytes from death, which is also beneficial for neurons [77, 78]. On the other hand, cannabinoid agonists, possibly by activating CB_1 receptors [79], modulate proinflammatory cytokine production by glial cells, mainly IL-1, TNF-α, IL-6 and IL-12 which play a major role in the development of damage in neurodegenerative/neuroinflammatory conditions, such as those occurring in cerebral ischemia (see [4, 68] for review). Of particular interest is the inhibitory effect of cannabinoid agonists on the production of TNF-α since this is a major contributor to the pathophysiology of brain injury [80]. These inhibitory effects might be exerted by inhibiting the activation of the nuclear factor-κB (NF-κB), which is involved in the induction of cytokine gene expression. HU-211, which does not bind to cannabinoid receptors, was able to inhibit this transcription factor [34]. In addition, several cannabinoid agonists were able to reduce mRNA levels for certain cytokines in lipopolysaccharide-treated rat microglial cells but these effects were not cannabinoid receptor-mediated [81]. Another important inflammation-related mediator is NO, which is produced in response to immune-mediated cellular toxicity playing a role in neurodegeneration (for review, see [1, 68]). Strategies that reduce the expression of the inducible or neuronal forms of NO synthase may be neuroprotective (see [25] for review). In this sense, cannabinoid agonists have been reported to inhibit the release of NO in microglia [82], astrocytes [83], neurons [84] and macrophages [85].

Glial cells may also secrete various trophic factors, such as the transforming growth factor-β, the anti-inflammatory cytokine IL-10, and neurotrophins,

that could potentially rescue damaged neurons [79, 86], and whose production might be enhanced by cannabinoids. For instance, Molina-Holgado et al. [87] have recently reported that IL-1 receptor antagonist, an important anti-inflammatory cytokine that protects against experimentally induced ischemic, excitotoxic and traumatic brain insults, is produced in response to cannabinoid receptor activation in primary cultured glial cells. Interestingly, cannabinoid receptor activation failed to do this in knockout mice for this anti-inflammatory cytokine [87].

The anti-inflammatory properties of cannabinoid agonists also involve the activation of the CB_2 receptors which suggest an additional role of this receptor subtype in the inflammatory processes. This is obviously of great interest since this receptor subtype is not involved in psychotrophic effects of cannabinoids. This importance has been renewed in the light of recent evidence indicating that CB_2 receptors are also expressed in the brain, even in healthy conditions, located on cerebellar neurons [88], astrocytes [89], oligodendrocytes [78], reactive microglia [18, 19] and perivascular microglial cells [90]. Of special interest is the case of CB_2 receptors located in microglia since these cells are known to perform critical roles, as they are considered the resident macrophages in the central nervous system (CNS) [91]. Initially, it was suggested that microglial cells were involved in a protective role, eliminating died cells and allowing regeneration of viable axons after brief episodes of neuronal injury (i.e. physical trauma or ischemia/hypoxia). However, recent evidence suggests that a sustained activation of microglia contributes to the pathogenesis in chronic neurodegenerative diseases, as mentioned above (see [67, 68] for review). Recent reports [92, 93] suggest that CB_2 receptors play an important role in some key processes (i.e. microglial cell proliferation and migration at neuroinflammatory lesion sites), involved in the initial steps of microglial activation in response to infection, inflammation or tissue injury [94].

Therefore, it appears well-demonstrated that microglia, astrocytes and oligodendrocytes respond to cannabinoid agonists, so that the beneficial effects of these compounds in neuroinflammation/neurodegeneration might be related to some of the following events: (1) inhibition of proinflammatory mediator production, (2) enhancement of anti-inflammatory factor production, (3) inhibition of microglial recruitment and (4) enhancement of astrocyte or oligodendrocyte survival.

Vascular effects of cannabinoids

Brain damage, such as that caused by stroke or traumatic injuries, is also associated with the release of several endothelium-derived mediators, such as endothelin-1 (ET-1), NO and others, which affect the local vascular tone (for review, see [95]). The major of these mediators is ET-1, which, formed at endothelial cells, is able to produce vasoconstriction, thus limiting the blood supply to the injured area and aggravating brain damage [96]. Cannabinoids,

in particular 2-AG, are potent modulators of the vascular tone (see [97, 98] for review), which is suggestive that they might provide neuroprotection in part because of this property. In this sense, cannabinoids counteract the ET-1-induced vasoconstriction, thus helping to restore blood supply to the injured brain (see Fig. 1, and [99] for review). This effect was exerted by the activation of CB_1 receptors since it was prevented by SR-141716 [100], which indicates that this receptor subtype is located in brain microvasculature (see [101] for review). In addition, as mentioned above, cannabinoid agonists are able to reduce NO production, thus reducing the vascular effects of this additional endothelium-derived mediator [99]. Both effects might be part of the neuroprotective response provided by cannabinoid agonists, in particular in cases of acute neurodegeneration such as stroke and head trauma.

Cannabinoids in acute neurodegeneration

Traumatic brain injury is the leading cause of death in young people and represents, together with cerebral ischemia, two more frequent reasons for acute neurodegeneration resulting in permanent disability [102, 103]. Cell death during these acute insults is mainly necrotic and is characterized by a loss of plasma membrane integrity leading to subsequent inflammatory events. Apoptosis, characterized by activation of an endogenous mechanism of destructive enzymes called caspases, may also occur during acute degeneration but always as a secondary event. Unfortunately, neurodegeneration caused by either ischemia or trauma is currently without a satisfactory clinical treatment, despite several trials using compounds exhibiting anti-glutamatergic activity, calcium-blocking actions, antioxidant properties or anti-inflammatory effects [26, 104–108]. As cannabinoids combine all these properties, recent preclinical studies have tried to demonstrate that they may provide neuroprotection in acute degeneration produced by several types of accidental injuries, such as those producing glutamatergic excitotoxicity [13, 28, 56, 88], ischemic stroke [30, 109, 110], hypoxia [111], head trauma [15], oxidative stress [55, 56], ouabain-induced secondary excitotoxicity [10, 50] and others (see Tab. 1 for an overview, and [4–6] for recent reviews).

In vivo, treatment with cannabinoids reduced infarct size and associated edema, and produced a functional improvement (reduction of neurological deficits) in animal models reproducing acute degeneration [4–6], i.e. rodents with global (transient) or focal (permanent or transient) cerebral ischemia induced by occlusion of carotid and vertebral arteries or intracraneal vessels, respectively (see [9] for review). Neuroprotection by cannabinoids was also seen *in vitro* using cultured neurons subjected to hypoxia and/or glucose deprivation, or exposed to excitotoxic stimuli, where cannabinoids increased survival of neurons (see [6] for review). For instance, cannabinoid agonists protected cultured rat hippocampal neurons [28] and mouse spinal cord neurons [29] from excitotoxicity. Nagayama and coworkers [30] reported that

Table 1. Neuroprotective effects of cannabinoid-related compounds in acute or chronic neurodegenerative disorders

Disease	Therapeutic applications	References
Acute neurodegeneration		
	Reduction of infart size (and associated edema) and neurological deficits by cannabinoid agonists in rodents with global or focal cerebral ischemia, or subjected to closed head injury.	[4–6, 15, 30, 109]
	Increase of cell survival by several cannabinoid agonists in cultured neurons (from different regions) subjected to hypoxia and/or glucose deprivation, or exposed to excitotoxins.	[6, 28–30, 55, 56, 88, 111]
	Neuroprotection provided by direct or indirect cannabinoid agonists in rodents subjected to excitotoxic stimuli. Greater brain injury in CB_1 receptor-deficient mice subjected to ischemia.	[9, 10, 13, 50, 113]
Chronic neurodegeneration		
Huntington's disease (HD)	Reduction of striatal injury by Δ^9-THC in a non-apoptotic rat model (lesions with 3-nitropropionic acid).	[65]
	CB_2 receptor-mediated neuroprotection by cannabinoids in a rat model of striatal injury that progresses through apoptotic death (local applications of malonate); CBD exerted poor neuroprotective action in this model, whereas SR-141716 increased striatal damage.	[19, 32]
Parkinson's disease (PD)	Reduction of dopaminergic injury in the 6-hydroxydopamine rat model by Δ^9-THC and CBD.	[64]
	Increased cell survival *in vitro* exerted by HU-210 through enhancing glial influences to neurons.	[64]
Alzheimer's disease (AD)	Prevention of β-amyloid toxicity *in vitro* by AEA, noladin-ether or CBD.	[176, 177]
Multiple sclerosis (MS)	Reduction of motor deterioration in EAE rats by plant-derived cannabinoids or uptake inhibitors.	[186–188]
	Improvement of motor function, reduction of activated microglia, and promotion of remyelination by several cannabinoid agonists in a Theiler's murine encephalomyelitis model.	[189, 190]
	CB_1 receptor-deficient mice were more vulnerable to inflammatory and excitotoxic insults following immune attack in EAE.	[184]
Amyotrophic lateral sclerosis (ALS)	Delayed motor impairment and increased survival after Δ^9-THC administration in a genetic mouse model of ALS.	[192]

WIN-55,212-2 was protective *in vitro* and also in an *in vivo* model of ischemic damage. Anandamide and 2-AG have been also found to protect rat cortical neurons from *in vitro* ischemia [111]. In another studies using *in vivo* models,

2-AG was administered to mice subjected to closed head injury and significant reduction of brain edema and infart volume, better clinical recovery and reduced hippocampal cell death were documented [15]. Interestingly, the effects of 2-AG as a neuroprotective agent were enhanced by several 2-acylglycerols, which are present in the brain but that do not bind cannabinoid receptors. It was assumed that this effect, called the entourage effect, might be produced by partially blocking the mechanisms involved in 2-AG inactivation (uptake and hydrolysis) [25, 112].

Except in a few cases [30, 111], most of the neuroprotectant effects of several cannabinoid agonists were attenuated by SR-141716, thus supporting a mediation of CB_1 receptors, which can be also concluded from the studies of Parmentier-Batteur et al. [113]. These authors reported a greater brain injury (increased infart size and neurological deficits) in CB_1 receptor-deficient mice subjected to transient focal cerebral ischemia [113]. Similar results were recently reported by Marsicano et al. [13] in the same knockout mouse model but subjected to kainate injections. Conversely, in an *in vivo* neonatal model of NMDA-induced excitotoxicity, CB_1 receptor blockade reduced infart size and number of degenerating cortical neurons [33]. By contrast, other authors used a different *in vivo* neonatal model that, by blocking the Na^+/K^+-ATPase with ouabain, replicates the changes produced in ionic homeostasis during energy deprivation and/or mitochondrial dysfunction characteristic of acute (and also chronic) neurodegenerative diseases. They found a reduction of neuronal injury in neonatal rats by Δ^9-THC [50] or AEA [10], an effect that was prevented by SR-141716, mainly in the case of Δ^9-THC [50], thus indicating CB_1 receptor mediation. Lastly, it is important to note that, in all these examples, the neuroprotective capability of cannabinoid agonists is likely the consequence of their capability to reduce excitotoxicity, oxidative stress and/or inflammation, which are key events involved, to different extents, in the neurodegeneration occurring in these acute pathologies.

Despite the neuroprotectant properties that cannabinoids display in acute degeneration, the clinical development with cannabinoid-based compounds is still poor and only dexanabinol (HU-211) is presently being tested in a phase III clinical trial to reduce brain damage caused by head trauma or cerebrovascular injuries [34, 114] (see also www.pharmoscorp.com/product/dexanabinol.htm). This clinical trial has already demonstrated that HU-211 significantly improves the neurological outcome of head injured patients.

Cannabinoids in chronic neurodegeneration

Cannabinoids, based again on their anti-glutamatergic, antioxidant and/or anti-inflammatory properties, might be useful to delay/arrest the progression of neuronal degeneration also in chronic diseases, where processes such as excitotoxicity, mitocondrial dysfunction, energy failure, oxidative stress and inflammation are cooperative events in the pathogenesis (see [115–123] for

review). This would include diseases such as AD, ALS, HD, PD, MS and other pathologies (see Tab. 1 for an overview, and [4–7, 24, 25, 68, 76] for review). This adds to other benefits reported for cannabinoid-based compounds by alleviating specific clinical signs, such as the anti-hyperkinetic effect in HD [124, 125], the orexigenic action in AD [126] or the antispastic effects in MS [127–130] produced by direct or indirect agonists of cannabinoid receptors. By contrast, CB_1 receptor blockade has been reported to be effective to improve motor inhibition in PD [131, 132] and memory deficits in AD [133]. However, these effects on symptom relief will be addressed here only marginally.

HD

HD is an inherited neurodegenerative disorder characterized by motor abnormalities, cognitive dysfunction and psychiatric symptoms, which presents in mid life and is ultimately fatal (for review, see [115, 116]). The most striking neuropathological change in HD patients is the preferential and progressive degeneration of the striatum due to the selective death of striatal projection neurons (these neurons contain CB_1 receptors [134]), which is accompanied by a biphasic pattern of motor deterioration that evolves from an early hyperkinetic phase (choreic movements) to a late akinetic and more disabling phase [115, 116]). Although it has been demonstrated that HD is a disease of genetic origin (it is caused by an expansion of a polyglutamine tract in the N-terminal portion of the huntingtin protein [116]), mechanisms underlying striatal degeneration are still unknown. In addition, the therapeutic outcome for HD patients has been scarce and includes mainly (1) anti-dopaminergic drugs to reduce the excessive movement characteristic of first phases of the disease [135] and (2) anti-glutamatergic agents to reduce excitotoxicity [136]. However, both treatments have resulted to be poor in terms of improving quality of life for HD patients. In this context, cannabinoid agonists might provide therapeutic benefits in both aspects since they produce hypokinesia [7, 137] and also provide neuroprotection [4–6, 27, 137].

As mentioned above, recent studies have addressed the anti-hyperkinetic effects of direct or indirect cannabinoid agonists in animal models of HD [124, 125], based on the demonstration, in humans and laboratory animals, that the endocannabinoid transmission becomes hypofunctional in the basal ganglia in HD [138–145]. More recently, the neuroprotective potential of cannabinoids has been also tested in this disease [19, 32, 65], and, although the matter is still far from being clarified, some results have provided promising expectatives. The rationale for these studies is based on the idea that the losses and/or dysfunction of CB_1 receptors in the basal ganglia is a very early event that takes place before the appearance of major neuropathological signs and when cell death has not occurred or is minimal. This has been found in both humans and different models of transgenic mice that express mutated forms of huntingtin like the human pathology [140–142]. In addition, we have recently found that

rats with striatal atrophy caused by injection of mitochondrial toxins exhibited profound changes in G-protein activation by CB_1 receptor agonists, several days before overt striatal degeneration and appearance of severe motor symptoms, and in the absence of significant modifications of binding sites and mRNA levels for this receptor [65]. All these observations, collectively, support the notion that early functional changes in CB_1 receptors might be involved in the pathogenesis of HD but, more importantly, they might play an instrumental role in striatal neurodegeneration [65]. In other words, (1) these defects in CB_1 receptor signaling [65] could render neurons more vulnerable to the degenerative process associated with HD and (2) the stimulation of these receptors might reduce/delay the progression of striatal degeneration. This hypothesis has been also considered by van der Stelt et al. [9], who, considering the data obtained in HD and also in other pathologies, proposed that the malfunctioning of the endocannabinoid system (i.e. AEA or 2-AG synthesis is inhibited, CB_1 receptors are inactive or their expression is lost) might be a signal to trigger an unbalance in glutamate homeostasis and initiate excitotoxicity.

The neuroprotective potential of cannabinoids in HD would be based on one or more of the above-described mechanisms by which cannabinoids may reduce neuronal injury (i.e. acting as chemical antioxidants, inhibiting glutamate release, reducing Ca^{2+} influx and/or producing anti-inflammatory effects; for review, see [4, 5]). This is possible in HD because it is a neurodegenerative disorder where mitochondrial dysfunction, excitotoxicity, inflammation and oxidative stress have been proposed as cooperative events in the pathogenesis [116]. In a recent study [65] we have found a promising action of the non-selective plant-derived cannabinoid, Δ^9-THC, by protecting striatal neurons against the *in vivo* toxicity of 3-nitropropionic acid, a mitochondrial toxin that replicates the complex II deficiency characteristic of HD patients [146]. Striatal injury in this animal model progresses by mechanisms that mainly involve non-apoptotic death, since it is caspase 3-independent and produced via the Ca^{2+}-regulated protein calpain and activation of non-NMDA receptors [147, 148]. However, it remains to be demonstrated whether the neuroprotective effect of Δ^9-THC in this animal model of HD is caused by the activation of CB_1, CB_2 or the combined action of both receptors, as well as through other mechanisms available to Δ^9-THC. The involvement of CB_2 receptors, but not CB_1 receptors, has been demonstrated in other rat models of striatal injury generated by unilateral injections of malonate, another complex II inhibitor. Malonate produces cell death that progresses mainly through the activation of apoptotic machinery (it activates NMDA receptors and caspase 3 [149]). Thus, we found that activation of CB_2 (using HU-308) but not CB_1 (using ACEA) receptors provided neuroprotection, and that this effect was reversed by SR-144528, a selective CB_2 receptor antagonist [19]. This indicates a crucial role for this receptor subtype in neuroprotective effects of cannabinoids in this model. An important aspect of these observations is that CB_2 receptors are induced in response to malonate application in glial cells, possibly in reactive microglia [19]. As this receptor subtype is usually absent in the brain in

healthy conditions, its induction in this model of striatal injury might be interpreted as part of an endogenous response against the degeneration caused by the inhibition of the mitochondrial complex II. In addition, CBD was not effective in this HD model, thus indicating that neuroprotection exerted by cannabinoids is not due to their antioxidant properties [19].

Another aspect that remains to be elucidated concerns the mechanism(s) (i.e. reduction of excitotoxicity, antioxidant effects or anti-inflammatory action) that underlies the neuroprotective properties of cannabinoids in HD. In this sense, using an *in vitro* design to test 3-nitropropionic acid toxicity, we recently found a dose-dependent increase in the survival of cultured cerebellar granule cells when these cells were incubated in the presence of another non-selective cannabinoid agonist, HU-210 (I Lastres-Becker, F Molina-Holgado, JA Ramos and JJ Fernández-Ruiz, unpublished observations). Interestingly, this neuroprotective effect is slightly enhanced if the exposure to HU-210 is indirect, by incubating glial cells with the cannabinoid and the resulting conditioned medium being exposed to neurons (I Lastres-Becker, F Molina-Holgado, JA Ramos and JJ Fernández-Ruiz, unpublished observations). This would indicate that the neuroprotective effect of cannabinoids might be produced in part through modulating glial influence to neurons (i.e. by increasing prosurvival factors such as glial anti-inflammatory molecules, and/or by reducing cytotoxic ones such as NO, TNF-α or proinflammatory cytokines; see [24, 68] for review). Two specific observations support this hypothesis. First, it has been largely demonstrated that activation of glial cells (astrocytes, oligodendroglia or microglia) occurs in HD [73, 150] as in other neurodegenerative pathologies. Second, neuronal survival in these *in vitro* experiments was extremely enhanced if the conditioned media were generated after exposure to HU-210 of glial cells obtained from IL-1β-deficient mice (I Lastres-Becker, F Molina-Holgado, JA Ramos and JJ Fernández-Ruiz, unpublished observations).

PD

The major clinical neuropathology in PD includes bradykinesia (slowness of movement), rigidity and tremor caused by the progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta that leads to a severe dopaminergic denervation of the striatum (see a recent review in [117]). Although the etiology of PD is presently unknown, major pathogenic processes that trigger the progressive loss of nigral dopaminergic neurons are oxidative stress, mitochondrial dysfunction and inflammatory stimuli [72, 151, 152]. Dopaminergic-replacement therapy with L-dopa represents a useful remedy to release rigidity and bradikynesia in PD patients [153], at least in the early and middle phases of this disease. Later on, the chronic use of this therapy loses efficiency and elicits the appearance of an irreversible dyskinetic state characterized by involuntary movements. On the other hand, PD is in the

same situation that HD in terms of pharmacotherapy to delay/arrest the progression of neurodegeneration, substances that reduce excitotoxicity, calcium influx, oxidative stress and/or inflammation being the only options assayed to date (for review, see [154, 155]), although the results obtained so far are not as promising as expected [155]. Since cannabinoid agonists share many of the above properties (for review, see [4–6]), they have been proposed as potentially useful neuroprotective substances also in PD, although the issue has been explored only very recently. However, their hypokinetic profile is a disadvantage in this case, because, despite their neuroprotectant activity in long-term treatments, they acutely enhance rather than reduce motor symptoms in this disease, as revealed by a few clinical studies [7, 156, 157]. This is compatible with the observation of overactivity in the endocannabinoid transmission in the basal ganglia in PD in both patients [158] and animal models [14, 131, 158–160]. This explains the hypokinetic profile of this disease and the recent proposal of CB_1 receptor antagonists to alleviate bradykinesia and rigidity, in particular at later phases of PD when L-dopa therapy is less effective (for review, see [7, 161]), although the first experiences in laboratory animals have been controversial [131, 162]. There would be only one exception for cannabinoid agonists to be used for symptom relief in PD; that is, patients for whom tremor is the major symptom. CB_1 receptor agonists might be useful to alleviate this symptom, due to the well-know inhibitory effect of cannabinoid agonists on glutamate release from subthalamonigral neurons [163], whose hyperactivity is responsible for tremor [117]. However, the only small clinical study carried out to test the effects of cannabinoids on parkinsonian tremor led to negative results [164].

Coming back to the question of neuroprotection by cannabinoids in PD, recent preclinical studies carried out with Δ^9-THC have revealed that this compound could be also neuroprotective in PD [64]. The administration of Δ^9-THC reversed the impairment of dopaminergic transmission in the basal ganglia of rats with hemiparkinsonism caused by the unilateral application of 6-hydroxydopamine [64]. These effects did not occur in the contralateral structures, thus indicating that the effects of Δ^9-THC were produced by reducing dopaminergic cell death in the lesioned side rather than producing up-regulatory effects in surviving neurons [64]. The quantification of tyrosine hydroxylase mRNA levels in the substantia nigra of these animals corroborated this finding [64]. However, the fact that the same neuroprotective effects were elicited by CBD, another plant-derived cannabinoid with negligible affinity for the CB_1 receptor, suggests that these neuroprotective effects could be CB_1 receptor-independent, based on the antioxidant properties of both plant-derived cannabinoids [64]. This observation is similar to the results reported by Hampson et al. [56], who compared the neuroprotective effects of Δ^9-THC and CBD in rat cortical neuron cultures exposed to toxic levels of glutamate. This observation is particularly important in PD, a disease in which oxidative stress, together with excitotoxicity and mitochondrial failure, is a major hallmark in the pathogenesis of the disease [117].

However, we have also found evidence that glial-mediated effects are also involved in the neuroprotection provided by cannabinoids in PD. In this sense, although the cause of dopaminergic cell death in PD is still unknown, it has been postulated that alterations in glial cell function (i.e. microglial activation) may also play an important role in the initiation and/or early progression of the neurodegenerative process [165], especially in a region like the substantia nigra which is particularly enriched in microglia and other glial cells [166]. In fact, several glial-derived cytotoxic factors, such as TNF-α, IL-1β, NO and others, have been reported to be elevated in the substantia nigra and the caudate putamen of PD patients [167]. Based on this previous evidence, we recently performed an *in vitro* study to evaluate the effects of cannabinoid agonists on the neuronal toxicity of 6-hydroxydopamine. We found a marked increase in neuronal survival when cells were incubated with conditioned media generated by exposing glial cells to the non-selective cannabinoid HU-210, compared with the poor increase in neuronal survival produced by direct exposure of neuronal cells to HU-210 [64]. This supports the hypothesis that neuroprotection by cannabinoids in PD might be significantly dependent, not only on the antioxidant potential of certain cannabinoids, but also on the anti-inflammatory and glial cell-mediated effects reported for most of cannabinoids [24, 68]. Because of the role suggested for CB_2 receptors in glial-mediated effects of cannabinoids [68], it is possible that this receptor subtype may be involved in part of the effects observed in PD, as found in HD [19], although this question must be explored in further studies.

AD

AD is the leading cause of dementia in the elderly, affecting to more than 4 million people in the United States alone. The pathological hallmarks of AD are currently well known and include neuritic plaques (enriched in β-amyloid peptide, Aβ) and fibrillary tangles (enriched in hyperphosphorylated tau protein), neuronal loss, synaptic dysfunction and gliosis (see [94, 118, 168] for review). The cellular and molecular events involved in the pathogenesis of AD have been partially unveiled. Briefly, it is currently thought that aberrant processing of the β-amyloid precursor protein leads to the formation of Aβ deposits which, in conjuction with other factors, stresses nearby neurons, resulting in tau hyperphosphorylation and inducing the formation of neurofibrillary tangles [168]. Additionally, this process initiates an inflammatory response in which astrocytes and microglia play a critical role [118], as described for other neurodegenerative diseases (see above). The current therapies for AD are (1) acetylcholinesterase inhibitors that serve to improve memory deficits caused by depleted levels of acetylcholine resulting from neuronal loss [169] and (2) NMDA receptor blockers, such as the uncompetitive antagonist memantine that has provided efficacy against β-amyloid-induced neurodegeneration in rats and shown great promise in clinical trials [170].

Cannabinoids have been recently proposed as candidates for both symptom relief and slowing of degeneration (see [171] for review).

The evidence relating cannabinoids to AD is relatively recent and has been obtained from either biochemical or pharmacological studies. Thus, Westlake et al. [172] reported a decrease of CB_1 receptor gene expression in AD postmortem tissues, in particular in the basal ganglia, but which could not be attributable to the pathologic process. Thus, while CB_1 receptor protein levels remained unchanged, CB_1 mRNA exhibited a reduction that, as the authors argued in their study, was probably parallel to the neuronal loss that accompanies the progression of the disease. Studies conducted in aged rats have provided similar findings [173]. More recently, Benito et al. [18] reported that CB_1 receptor levels were unaltered in brain regions affected by Aβ deposits. In this immunohistochemical study, a slight decrease in the staining intensity of the samples was observed, but CB_1 receptor protein distribution was basically the same as in control cases.

In contrast with the lack of changes in CB_1 receptors, the analysis in postmortem tissues from AD patients revealed that CB_2 receptors are selectively overexpressed in the microglial cells that are associated with Aβ-enriched neuritic plaques [18]. This selectivity is especially striking, as parenquimal (silent) microglia seem not to express CB_2 receptors. Recent data [90] indicate that CB_2 receptors may be also expressed by a limited population of microglial cells in the healthy brain, i.e. perivascular microglial cells, which play a pivotal role in infectious processes affecting the CNS [174]. It may be hypothesized that the induction of CB_2 receptors in microglial cells surrounding neuritic plaques in AD may be part of an anti-inflammatory response of the CNS in order to protect neurons from degeneration. In addition, FAAH expression and enzymatic activity are increased in neuritic plaques from AD tissue samples; in particular, FAAH seems to be abundantly expressed by plaque-associated astroglia [18]. These results suggest that FAAH may participate in the important role that astrocytes play in the gliotic response to Aβ deposition [118].

Despite the observation of changes in specific elements of the endocannabinoid system, in particular CB_2 receptors, during the pathogenesis in AD, only few preclinical or clinical data exist regarding the potential therapeutic usefulness of cannabinoids in this disease. A part of these studies deals with the treatment of specific symptoms, as revealed by the clinical study of Volicer et al. [126], who demonstrated a beneficial effect of dronabinol (Δ^9-THC in oil solution for oral administration) by stimulating appetite and improving disturbed behavior in AD patients. In addition, the abundance of CB_1 receptors in the hippocampus and the parahippocampal and enthorinal cortices, as well as their involvement in the control of cholinergic activity, suggests an additional usefulness of cannabinoid-based compounds in memory deficits typical of these patients. In this sense, cannabinoid receptor agonists impair memory processing and cognition [175], whereas CB_1 receptor blockade with SR-141716 improves memory deficits in mice administered with Aβ, presumably by an increase in hippocampal acetylcholine levels [133].

Recent data on the putative neuroprotective and anti-inflammatory properties of cannabinoids have opened new perspectives that could be of interest in AD. For instance, the contribution of CB_1 receptors in an *in vitro* model of AD has been also studied by Milton [176]. This study shows that AEA and noladin-ether are able to prevent Aβ-induced neurotoxicity through a CB_1 receptor-mediated mechanism. Thus, after exposure to different fibrilogenic peptides, the two endocannabinoids, at nanomolar concentrations, were shown to prevent their toxic effect on a neuronal cell line [176]. Furthermore, this protective effect was reversed by the CB_1 receptor specific antagonist AM251, and seemed to be mediated by the mitogen activated protein kinase pathway, since a selective inhibitor for this signaling pathway also prevented the protective effects of the two endocannabinoids [176]. Similar results have been recently published by Iuvone et al. [177] using cultured PC12 cells. These authors found a marked reduction in cell survival following exposure of cells to Aβ, that was associated with increased reactive oxygen species production and lipid peroxidation, as well as caspase 3 activation, DNA fragmentation and increased intracellular calcium [177]. Interestingly, the treatment of the cells with CBD prior to Aβ exposure significantly elevated cell survival while it decreased oxidative stress, lipid peroxidation, caspase 3 levels, DNA fragmentation and intracellular calcium [177]. The authors concluded that CBD exerts a combination of neuroprotective, antioxidative and anti-apoptotic effects against Aβ toxicity, and that inhibition of caspase 3 appearance from its inactive precursor, pro-caspase 3, by CBD might be involved in the signalling pathway for this neuroprotection [177].

Therefore, taken together, the data obtained in the above studies suggest that cannabinoids could have an important role in the prevention of Aβ-induced neurotoxicity and counteract some of its devastating effects. Without excluding a role for CB_1 receptors or for other mechanisms available to certain cannabinoids, these data suggest that part of these beneficial effects of cannabinoids might be mediated by CB_2 receptors located on glial cells activated by the inflammatory process elicited by maturation of senile plaques. These effects would be similar to those described above concerning the anti-inflammatory role of cannabinoids exerted through the modulation of several cytotoxic mediators such as NO, TNF-α, cytokines and others (see [24, 68] for recent reviews).

MS

MS is the neurological disease that represents the most frequent cause of non-traumatic, chronic disability in young adults (for review, see [178]). It is an autoimmune disease that causes demyelination and axonal loss, in particular in the spinal cord, resulting in a variety of neurological signs, among which pain and motor impairment are the most characteristic [119]. It was initially thought that neurological signs in MS were exclusively caused by inflamma-

tory processes due to activated immune cells entering the CNS [119, 120]. This explains why current therapies addressed to delay disease progression include basically immunomodulatory agents (i.e. substances targeted against immune elements, such as interferon, glatiramer or mitoxantrone [179]). However, recent evidence also supports the ultimate occurrence of excitotoxicity and neurodegeneration (oligodendrocyte death and axonal loss) in this disease [121, 180–182]. It is for this reason that cannabinoids, in addition to their well-described relieving effects on specific symptoms in MS [127–130, 183], might be also used as neuroprotectant molecules to delay/arrest disease progression by protecting oligodendrocytes from death and by reducing axonal degeneration [76, 183, 184].

As regards the symptom-relieving effects of cannabinoids in MS, most of the studies have focused in the management of pain and motor-related symptoms such as spasticity, tremor and dystonia (for review see [76, 183]). They have tried to provide solid experimental support to previous anecdotal, uncontrolled or preclinical data that suggested a beneficial effect for marijuana when smoked by MS patients to alleviate specific symptoms such as spasticity, dystonia, tremor, ataxia, pain and others (for review see [76, 183]). This has been the basis for a clinical trial recently completed in the UK, which has proved that cannabinoids did not have a beneficial effect on spasticity in MS patients, but increased the patient's perception of improvement for different signs of this disease [185]. In animal studies, a series of studies by Baker and coworkers revealed a potent anti-spasticity effect of plant-derived, synthetic and endogenous cannabinoid agonists in a mouse model of MS, chronic relapsing experimental autoimmune encephalomyelitis (CREAE) [127–130]. They also demonstrated that these effects were mediated by cannabinoid CB_1 and, to a lesser extent, CB_2 receptors [127]. Using this mouse model, they have also described anti-spastic effects of compounds that are able to inhibit the process of termination of the biological action of endocannabinoids [128–130]. These data were concordant with the increase in endocannabinoid levels recorded in the brain and, in particular, in the spinal cord of these animals [128]. The chronic administration of plant-derived cannabinoids [186, 187] or specific endocannabinoid-uptake inhibitors [188] may also reduce or delay the incidence and severity of clinical signs in rats with experimental autoimmune encephalomyelitis (EAE), a monophasic model of MS where only inflammation takes place. However, the amelioration of experimental MS in this rat model is presumably due to the fact that cannabinoid agonists acted either as immunosuppressive agents, by preventing the accumulation of inflammatory cells in the CNS [186, 187], or by exerting a direct anti-inflammatory effect [188]. Beneficial effects of cannabinoids have been also reported by other authors [189, 190] in a MS mouse model generated by infection with the Theiler's murine encephalomyelitis virus. In these animals, cannabinoid agonists produced an improvement of motor function, and reduced activated microglia and promoted remyelination in the spinal cord [189], so cannabinoids might provide beneficial effects in MS that would go beyond symptom

relief. In this sense, a recent study by Pryce and coworkers [184] has demonstrated that CB_1 receptor-deficient mice tolerated inflammatory and excitotoxic insults poorly and developed substantial degeneration following immune attack in EAE.

Despite the progress in the pharmacological evaluation of cannabinoid-based medicines in MS in patients and animal models, there are no data on the possible changes in CB_1 and CB_2 receptors in the postmortem brain of patients with MS, while only a few studies have examined the status of the endocannabinoid transmission in animal models of this disease [128, 188, 191]. Thus, Baker and coworkers reported an increase of endocannabinoid levels in the brain and, in particular, in the spinal cord in the mouse model of MS [128], that was interpreted by these authors as indicative of an endocannabinoid influence on the control of some symptoms of MS in an environment of existing neurological damage (see [76] for review). In our laboratory, using EAE rats, we recently reported a decrease of CB_1 receptor binding and mRNA levels [191], although the decreases in CB_1 receptors were mainly circumscribed to the basal ganglia (lateral and medial caudate-putamen), and to a lesser extent to cortical regions. We have also recorded a reduction of endocannabinoid levels in these and in other brain structures [188]. However, as the pathology in MS models mainly occurs in the spinal cord, the relevance of the observations in the basal ganglia remains to be elucidated, although it is possible that they are a secondary adaptative event originated by primary changes at the spinal level. Thus they might be related to the motor deterioration which is one of the most prominent neurological signs in these rats [188, 191] and also in the human disease [76, 183]. Based on this fact, we hypothesized that the changes in CB_1 receptors and their ligands in the basal ganglia might be associated with disturbances in several neurotransmitters acting at this circuitry. If this were the case, the well-known effects of cannabinoid agonists on these neurotransmitters might underly the improving effects of these compounds in motor symptoms of MS (see [7, 27] for review). However, our hypothesis was wrong because we did not record any changes in dopamine, serotonin (5-hydroxytryptamine), GABA or glutamate in the basal ganglia of MS rats [188].

ALS

ALS is one of the most common neurodegenerative disorders, occurring both sporadically and as a familial disorder with demonstrated inherited cases in about a 10% of patients. Although it shows multiple clinical variants, it is characterized by degeneration of spinal motor neurons primarily and cortical neurons secondarily (see [122, 123] for recent reviews). Neuronal loss occurs from a combination of metal-elicited oxidative injury, excitotoxicity, aggregation and/or dysfunction of critical proteins, and genetic factors [122, 123]. Classic therapy in this disease includes riluzole, an inhibitor of glutamate release and sodium channel blocker, but it is unsatisfactory and does not arrest

the progression of this lethal disease whose duration averages approximately 2–3 years after diagnosis [122, 123]. Recent evidence has provided support to the possibility that cannabinoids may also function in ALS as neuroprotectant agents. This evidence has been obtained by Raman and coworkers [192] in a mouse genetic model of ALS ($HSOD^{G93A}$ transgenic mice) that overexpresses a mutated form of the enzyme copper/zinc superoxide dismutase 1 (SOD-1), which is linked to approximately 20% of familial cases of ALS [193, 194]. This enzyme plays a critical role as the endogenous scavenger of the superoxide anion, thus reducing the occurrence of oxidative stress. The mutation of SOD-1 increases the formation of superoxide anions and the oxidative tissue damage, and this is the key process that elicits all symptomatology characteristic of this ALS genetic mouse model. Raman and coworkers found that Δ^9-THC was effective in delaying motor impairment and prolonging survival if administered before or after the onset of signs in the ALS mouse model [192]. In addition, Δ^9-THC was also effective at reducing oxidative damage and excitotoxicity in spinal cord cultures [192]. No data exist on possible changes in specific elements of the endocannabinoid system in humans affected by this disease, but very recently Witting et al. [195] have published the first paper demonstrating endocannabinoid accumulation in the spinal cord of $HSOD^{G93A}$ transgenic mice, which was interpreted by these authors as part of an endogenous defense mechanism against the oxidative damage characteristic of this disease.

Concluding remarks and future perspectives

Among a variety of pharmacological effects, cannabinoids have been demonstrated as potentially useful and clinically promising neuroprotective molecules. In this chapter we have reviewed the cellular and molecular mechanisms that might be involved in these neuroprotective effects, paying emphasis in their potential (1) to reduce excitotoxicity exerted by either inhibiting glutamate release or, in some specific cases, blocking glutamatergic receptors, (2) to block NMDA receptor-induced calcium influx exerted directly, as a consequence of the antagonism of these receptors, or indirectly, through the inhibition of selective channels for this ion, (3) to decrease oxidative injury by acting as scavengers of reactive oxygen species, a property independent of cannabinoid receptor and restricted to specific classic cannabinoids, (4) to reduce inflammation by acting predominantly through the activation of CB_2 receptors on the glial processes that regulate neuronal survival and (5) finally, to restore blood supply to injured areas by reducing the vasocontriction produced by several endothelium-derived factors such as ET-1 or NO. Through one or more of these processes cannabinoids may provide neuroprotection in conditions of acute or accidental neurodegeneration, such as that occurring in traumatic injury or ischemic episodes. In fact, dexanabinol is already in a phase III clinical trial for therapeutic intervention in these pathologies.

Cannabinoids might be also used, in addition to several symptomatic utilities also described here, to delay/arrest the progression of neurodegeneration in chronic diseases affecting cognitive processes, such as AD, motor control or performance, such as PD, HD and ALS, or those initially produced by inflammatory processes, such as MS. Most of these diseases have scarcely been studied for applications of cannabinoids, or for changes in specific elements of the endocannabinoid system, but a rise in the number of studies is expected as soon as the promising results generated by these molecules progress from the present preclinical evidence to clinical applications.

Acknowledgements
The experimental work carried out by our group and that has been mentioned in this chapter has been supported during recent years by the Plan Regional de Investigación – CAM (grant nos 08.5/0029/1998 and 08.5/0063/2001) and MCYT (grant no. SAF2003–08269). The authors are indebted to all colleagues who contributed in this experimental work.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Role of the endocannabinoid system in learning and memory

Stephen A. Varvel and Aron H. Lichtman

Department of Pharmacology and Toxicology, Virginia Commonwealth University, PO Box 980613, Richmond, VA 23298, USA

Introduction

Following the discovery of an endocannabinoid system in the central nervous system, which consists of the endogenous ligands arachidonoylethanolamide (anandamide) and the monoacylglycerol 2-arachidonoyl glycerol (2-AG) that bind to the CB_1 receptor [1, 2], a great deal of effort has been focused on understanding the physiological function of this system. The identification of these and other putative endogenous cannabinoids, including noladin ether [3] and virodhamin [4], has sparked further in understanding the physiological functions of the endogenous cannabinoid system. A growing body of evidence suggests that this system serves several physiological functions including the modulation of pain [5–7], feeding [8], drug dependence [9–11], excitotoxicity [12], and cognition [13, 14]. In this review, we discuss recent *in vivo* and *in vitro* research investigating the role that the endocannabinoid system plays in learning and memory. Recent behavioral evidence indicates that the endocannabinoid system modulates key components of learning and memory, which include memory consolidation and extinction. On the molecular level, endocannabinoids have been demonstrated to modulate electrophysiological correlates of learning, suggesting that they play an important role in synaptic plasticity. Investigations into the role of the endocannabinoid system in learning processes should make important advances in the following three areas: (1) development of new cannabinoid-based pharmacotherapies with minimal undesirable side effects, (2) understanding the long-term consequences of marijuana use (which remains the most commonly used illicit drug [15]), and (3) shedding light on basic issues in neuroscience such as how are memories formed, stored, and forgotten?

Behavioral effects of cannabinoid agonists in learning paradigms

It has long been recognized that marijuana and its chief psychoactive component, Δ^9-tetrahydrocannabinol (Δ^9-THC), produce disturbances in various

aspects of learning and memory of humans (see [16, 17] for review) and in animal models of learning and memory (see Tab. 1 for an overview of rat, mouse, and nonhuman primate studies). Such deficits resulting from administration of Δ^9-THC and other cannabinoids have come under increasing scrutiny in recent years as new tools have become available, and as it has become clear that these deficits are the result of interactions with an endocannabinoid system that may play a crucial role in the physiological basis of learning and memory.

One strategy for investigating the role that the endocannabinoid system may play in learning and memory processes is through the use of animal learning models. While it is certain that exogenous administration of an agonist cannot closely mimic the actions of an endogenous system tightly integrated within neural circuits sensitive to specific spatio-temporal contexts, useful information about the endogenous system can be taken from these studies. For example, those particular memory tasks that are particularly sensitive to disruption by exogenous agonists may provide insight into processes that are modulated by endocannabinoids. Conversely, endocannabinoids are probably not crucial to aspects of memory that are insensitive to disruption by exogenous agonists. So which aspects of learning and memory appear most sensitive to agonists? The most consistent delineation made regarding the effects of CB_1 agonists is that they tend to disrupt aspects of short-term (i.e. working) memory, while leaving retrieval of well-learned information (i.e. long-term or reference memory) largely intact. Working memory is an evolving concept reflecting those processes necessary to learn and react to new information that changes over time – a mnemonic whiteboard of sorts. Clearly, the term working memory encompasses many distinct processes, including attentional mechanisms, as well as associational, consolidation, encoding and retrieval processes. Determining the impact of CB_1 agonists on these components is the focus of ongoing investigation. The section below reviews many of the key studies that address this issue by using a variety of animal models of cognition, including instrumental operant tasks, spatial maze paradigms, and conditioned avoidance tasks.

A major consideration in animal models of cognition is that learning and memory is not directly measured, but is inferred based on changes in performance. In particular, alterations in attentional, sensorimotor, and motivational processes can affect performance, independently of cognition. These potential confounds are of considerable concern in investigating the role of the endogenous cannabinoid system, as cannabinoid agonists and antagonists as well as $CB_1^{-/-}$ mice are known to affect many non-mnemonic functions that could impact specific animal models of learning, including locomotor activity, motivation, feeding behavior, and anxiety. In addition, there are many types of short-term and long-term memory that are reflected in a diversity of animal models, including recognition tasks, spatial tasks, food-motivated operant behavior, and fear-conditioning procedures. Moreover, within each particular form of memory, multiple processes are involved including acquisition, con-

Table 1. Effects of cannabinoid agonists in behavioral learning and memory paradigms

(a) Rats

Model	Drug	Observations	Reference
Operant: delayed match and delayed non-match to sample non-match to place	Δ^9-THC	Delay-dependent disruptions of performance (correlated with decreased hippocampal activity)	[18]
		Tolerance developed to Δ^9-THC-induced disruptions with repeated testing	[21]
	Δ^9-THC, anandamide, *R*-methanandamide	Δ^9-THC and *R*-methanandamide disrupted performance, blocked by SR-141716	[24]
	WIN-55,212-2	Delay-dependent disruptions	[19]
		Tolerance developed to daily dosing of 3.75 mg/kg	[170]
	Δ^9-THC, WIN-55,212-2	Decrease in performance associated with impaired hippocampal firing, blocked by SR-141716	[20]
	Δ^9-THC, anandamide (+PMSF)	Both produced working-memory deficits	[22]
		Both produced working-memory deficits, reversed by SR-141716	[23]
Operant: repeated acquisition	Δ^9-THC	Tolerance to performance deficits	[25]
		Deficits sensitive to estrogen	[26]
Radial arm maze	Δ^9-THC	Disruptions after acute and chronic dosing	[32]
		Increased errors	[37]
		Δ^9-THC-induced disruption blocked by SR-141716	[61]
		Microinjections in hippocampus, not other regions, produce deficits	[33, 90]
		Impaired retention, decreased activity	[171]
	CP-55,940	Impaired performance, blocked by SR-141716 and epstastigmine	[36]
	Δ^9-THC, WIN-55,212, CP-55940, anandamide	Δ^9-THC, WIN-55,212, and CP-55940 impaired performance	[33]
Water maze	HU-210	Impaired acquisition	[38]
	Δ^9-THC	Deficits in working memory	[172]
	Nabilone, Δ^8-THC	Δ^8-THC, not nabilone, disrupted place learning	[173]
	Δ^9-THC, WIN-55,212	WIN-55,212 disrupts learning, Δ^9-THC produces place-aversion	[174]

(Continued on next page)

Table 1. (Continued)

(a) Rats

Model	Drug	Observations	Reference
T-maze	Δ^9-THC	Working-memory deficits	[41]
		Working-memory deficits, inhibition of acetylcholine release. Blocked by SR-141716, sulpiride	[175]
		No tolerance after twice-daily injections for 14 days	[43]
Object recognition	Δ^9-THC	Disrupted at 10 mg/kg, lower doses potentiated by ethanol	[176]
	CP-55,940, methanandamide	Decreased learning/performance	[177]

(b) Mice

Model	Drug	Observations	Reference
Water maze	Δ^9-THC	Working-memory deficits, reversed by SR-141716	[39]
		Working-memory deficits reversed by bicuculline	[42]
		Deficits in acquisition and working memory, reversed by SR-141716	[178]
	Δ^9-THC, WIN-55,212, methanandamide	Working-memory deficits not present in CB_1-knockout mice	[40]
Passive avoidance	Anandamide	Post-training anandamide (short, not long, interval) impaired retention, blocked by D_1 and D_2 agonists	[44]
		Intra-hippocampal anandamide, post training, disrupted retention	[45]
		Post-training anandamide impaired retention in DBA/2 mice, improved it in C57BL/6 mice; both effects blocked by naltrexone	[48]
		Anandamide disrupted consolidation, effect modulated by stress, blocked by naltrexone	[46]
		Interaction between subthreshold doses of of anandamide + morphine on consolidation	[47]

(Continued on next page)

solidation, encoding, and retrieval processes. Thus, the endocannabinoid system may play selective roles in different components of memory, as well as in different types of memory.

Table 1. (Continued)

(c) Non-human primates

Model/species	Drug	Observations	Reference
DNMS, concurrent discrimination/Rhesus monkeys	Δ^9-THC	Δ^9-THC only disrupted DNMS task	[31]
Operant task battery: TRD DMTS, CPR, IRA, PR		Sensitivity of tasks (high–low): TRD>DMTS = IRA = CPR>PR	[28]
Repeated acquisition/Squirrel monkeys		Δ^9-THC disrupted task, blocked by SR-141716	[30]
Operant task battery (see above)/Rhesus monkeys	Marijuana smoke	Sensitivity of tasks (high–low): TRD = DMTS>IRA = CPR>PR	[27]
Repeated acquisition, conditional discrimination/ Rhesus monkeys	Δ^9-THC, WIN-55,212-2	Δ^9-THC and WIN-55,212 produced deficits in the repeated acquisition task, blocked by SR-141716	[29]

CPR, conditioned position responding; IRA, incremental repeated acquisition; PR, progressive ratio; TRD, temporal response diffentiation; DNMS, delay non-match to sample

Operant tasks

For decades behavioral researchers have made use of operant (instrumental) tasks to study the effects of drugs on mnemonic function. One implementation of this paradigm that relies heavily on working memory processes is the delayed-match (or non-match) to sample (DMTS or DNMS) instrumental task. These experiments generally consist of a subject being presented with a sample stimulus, an interval of time during which the stimulus is removed, and a subsequent test phase when the sample stimulus is presented simultaneously with a novel stimulus. The subject must indicate, usually by pressing a lever, which was the sample (match) or which was the novel (non-match) stimulus. Δ^9-THC and WIN-55,212-2, a potent synthetic cannabinoid analog, have both been shown to disrupt accuracy of such performance in a delay-dependent manner, consistent with a selective disruption of working memory, and are blocked by the CB_1 antagonist SR-141716 [18–20]. Importantly, these behavioral deficits were associated with a selective reduction in hippocampal cell ensemble firing during the sample phase, but not during the non-match phase of these experiments [18, 20]. Further work has also shown that tolerance develops to the disruptive effects of Δ^9-THC in this task [21], though it should be noted that the occurrence of rate suppression in this study obfuscated the assessment of choice accuracy.

Another series of experiments demonstrated that Δ^9-THC and anandamide [in the presence of PMSF (phenylmethylsulphonyl fluoride), a nonspecific amidase inhibitor], produced selective deficits in working memory performance in a two-component operant task [22]. One component (conditional dis-

crimination) required rats to press one of two levers in the presence of an auditory or visual stimulus in a test of reference memory, while the other component (delayed non-match to position) required rats to press the lever opposite the one that was appropriate for the first component – a test of working memory. Consistent with a CB_1 receptor mechanism of action, the selective deficits produced both by Δ^9-THC and anandamide in this task were reversed by SR-141716 [23]. Others have examined slightly different aspects of learning with other variations of operant tasks. For example, Δ^9-THC and methanandamide (an anandamide analog resistant to degradation) disrupted the repeated acquisition of a series of three responses in an SR-141716-reversible manner, though only at doses which also reduced response rates [24]. A refinement of this procedure added a performance component in which rats executed a previously learned series of responses that remained the same across sessions. Δ^9-THC reduced accuracy and rate of responding in both components, and as with the DNMS studies mentioned above, tolerance developed to these effects after daily administration [25]. Unfortunately, the sensitivity of the performance requirements of these tasks to Δ^9-THC-induced disruptions (as measured by response rates) precluded a straightforward interpretation regarding the selectivity of Δ^9-THC's effects. Interestingly, Δ^9-THC-induced deficits in this task were shown to be sensitive to estrogen, suggesting that sex differences may play an important modulatory role on cannabinoids effects on learning [26].

A series of experiments in rhesus monkeys tested the effects of Δ^9-THC or marijuana smoke on performance in a battery of instrumental tasks designed to assess different aspects of learning and memory [27, 28]. Both Δ^9-THC and marijuana smoke produced the most profound deficits in a temporal response differentiation task and a DMTS task. They also produced moderate disruptions of conditioned position responding and incremental repeated acquisition tasks, and were least disruptive of a progressive ratio task. Further studies with rhesus monkeys as well as squirrel monkeys have also demonstrated that Δ^9-THC as well as WIN-55,212-2 disrupted a repeated acquisition performance that was reversed by SR-141716 [29, 30]. Finally, another study showed that performance of a similar DNMS task was disrupted at doses of Δ^9-THC that did not interfere with a concurrent discrimination task [31], also consistent with a specific deficit in working memory processes.

Spatial tasks

Tests of spatial learning and navigation take advantage of strategies that animals use for foraging and avoidance of predators in their natural environment and represent an important approach to investigations of learning and memory. One such spatial memory task that has been used to study the effects of Δ^9-THC on spatial learning is the eight-arm radial maze, which requires rats to learn which arms contain food rewards, and to remember which arms have already been vis-

ited after an interposed delay. Nakamura et al. found that low dose of 1.25 mg/kg Δ^9-THC was shown to produce small deficits in retention (i.e. more errors) after a short delay that became more pronounced after daily administration [32]. These experiments suggested a cumulative detrimental effect of chronic Δ^9-THC on spatial memory, though the deficits disappeared after a period of drug washout. Lichtman et al. [33] showed that Δ^9-THC, WIN-55,212-2, and a potent bicyclic cannabinoid analog CP-55,940 all disrupted choice accuracy, while Δ^9-THC and CP-55,940 did so at doses lower than were required to increase task completion time (WIN-55,212-2 produced both effects at similar doses). While Δ^9-THC-induced memory impairment was blocked by SR-141716, the cholinesterase inhibitor physostigmine failed to diminish this effect [34]. However, the dose of physostigmine employed (0.06–0.24 mg/kg) produced excessive cholinergic activity (e.g. excessive salivation) and thus confounded the interpretation of this study. However, a subsequent study reported that a decreased doses range of physostigmine (0.01–0.05 mg/kg), as well as another cholinesterase inhibitor tetrahydroaminoacridine, improved Δ^9-THC-induced memory impairment in the radial-arm maze task [35]. Similarly, epstastigmine, a more selective cholinesterase inhibitor, reversed Δ^9-THC-induced deficits in the radial-arm maze [36]. Studies of serotonergic function found no relation between Δ^9-THC-induced deficits in the radial-arm maze and 5-hydroxytryptamine (5-HT) turnover [37].

Another spatial learning and memory task that has become increasingly popular is the Morris water maze. Unlike the radial-arm maze, this task does not entail food deprivation, but requires the subjects to navigate in a pool of water to locate a hidden platform by learning its relationship relative to salient visual cues. The highly potent cannabinoid agonist, (–)-11-hydoxy-Δ^8-THC-dimethylheptyl (HU-210), has been found to impair the ability of rats to acquire the hidden platform task, without disrupting their performance in a version of the maze in which the location of the platform was directly visible [38]. These deficits were accompanied by signs that are thought to reflect heightened anxiety, such as increased time spent around the outer edges of the pool (thigmotaxia) and increased vocalizations. Thus it was hypothesized that cannabinoids may produce an anxiety-like state that could contribute to their effects on learning [38].

Δ^9-THC also disrupted the performance of mice in a working-memory version of the water maze in which the location of the platform was changed before each session [39]. These effects were relatively selective as they occurred at significantly lower doses than those required to disrupt a reference memory version of the task (in which the platform remained in a constant position), or produce other effects characteristic of cannabinoid activity such as antinociception, hypothermia, catalepsy, and hypomotility. Subsequent water-maze experiments showed that the working-memory deficits produced by Δ^9-THC, as well as WIN-55,212 and methanandamide, at doses that did not impair a cued version of the task were fully reversed by SR-141716, and were not observed in $CB_1^{-/-}$ mice [40].

Another task that includes some spatial processing requirements is the delayed-alternation T-maze task. Δ^9-THC has been demonstrated to disrupt performance of rats in a delayed-alternation T-maze task, in which rats were required to remember which arm had been baited during a preceding sample trial [41]. These effects occurred at doses that did not interfere with a previously learned black/white discrimination, a reference-memory task. Similar effects of Δ^9-THC have been demonstrated in mice at a dose that did not affect choice latency [42]. Intriguingly, others replicated this effect and found that unlike in the operant tasks described above, tolerance did not develop to Δ^9-THC, even after 14 days of twice-daily injections [43].

Conditioned avoidance

Evidence in support of a further delineation of the specificity of cannabinoid effects on memory comes from several studies in mice that examined the effects of anandamide in an inhibitory avoidance procedure. In this paradigm, a chamber or runway is paired with an aversive consequence, most typically an electric shock. Following the conditioning procedure, the subject is returned to the apparatus and its memory is assessed by noting its latency to re-enter the area in which it had previously received the shock. Anandamide administered immediately after the training trial, but not 2 h after, impaired memory (decreased latencies) of DBA mice assessed 24 h later [44], suggesting a disruption of consolidation or encoding processes, since the mice were only susceptible for a short time following training. This effect appears to be delay-dependent, as intra-hippocampal administration of anandamide produced similar deficits in mice tested 24 h after training, while no deficits were observed when they were tested just 2 h after training [45]. Further work with this model demonstrated that the disruptive effects of anandamide on memory consolidation can be amplified by stress, potentiated by a low dose of morphine, and completely reversed by naloxone [46, 47]. Nonetheless, this effect appears to be dependent on the strain of mouse, as anandamide inexplicably appeared to improve memory performance in C57BL/6 mice [48].

In summary, cannabinoid agonists from several different chemical classes, as well as the endocannabinoid anandamide, tend to selectively disrupt tasks heavily dependent on working memory, as assessed in a variety of behavioral paradigms, at doses that do not affect reference memory tasks or produce many other commonly assessed cannabinoid effects. These effects have been shown to be mediated via the CB_1 receptor, as they are blocked by CB_1 antagonists and do not occur in $CB_1^{-/-}$ mice. Further, the working-memory deficits observed in the operant DNMS and spatial radial-arm maze tasks have been specifically linked to effects in the hippocampus, suggesting that endocannabinoids may play a particularly critical role in this brain area (see below). Many issues related to the chronic administration of cannabinoids on learning remain unresolved.

Most notably, tolerance has been shown to develop to the effects of Δ^9-THC in several operant tasks, but not in the delayed-alternation T-maze.

Endocannabinoid modulation of cognitive processes

Although it is well established that stimulation of CB_1 receptors by exogenously administered cannabinoids reliably produces potent and fairly specific memory deficits, the role that the endogenous cannabinoid system plays in learning and memory is less clear. Even prior to the discovery of anandamide, tonic activation of the endocannabinoid had been proposed to play a role in an active forgetting process, in which extraneous information is deleted from memory storage [78]. Using laboratory animal models, we hope to gain a better understanding of the role that the endocannabinoid system plays in learning and memory as well as in neurodegenerative disease states. Below, we describe evidence suggesting that blocking CB_1 receptors prolongs memory duration in a variety of animal models of cognition. In addition, there is some suggestion that this receptor may represent a potential therapeutic target to treat memory deficits associated with Alzheimer's disease. However, other research indicates that disruption of the endocannabinoid system can interfere with an important component of learning, the process of extinction.

Endocannabinoid modulation of memory duration

The disruption of CB_1 receptor signaling through the use of $CB_1^{-/-}$ mice and mice treated with CB_1 receptor antagonists are common approaches to investigate whether the endocannabinoid system is tonically active in whole animals. The role that the endocannabinoid system plays in learning and memory is typically inferred by the manner in which either approach alters performance in a memory test. The endocannabinoid system appears to play an inhibitory role on memory duration, as $CB_1^{-/-}$ mice and SR-141716-treated animals have exhibited improved performance in several memory tasks (see Tab. 2). Using a social recognition test, Terranova et al. [13] were the first to provide *in vivo* evidence that the endocannabinoid system tonically modulates memory. In this task a mature rodent spends more time investigating unfamiliar juvenile conspecifics than familiar ones. Subjects are presented with a juvenile conspecific on two separate 5-min trials separated by a delay of varying durations. A decrease in investigative time during the second trial compared with the first trial indicates that the subject 'remembered' the conspecific, while no difference in investigative time between the two presentations indicates that it no longer remembers the conspecific. SR-141716 given 5 min after the first trial dose-dependently enhanced memory 120 min later during the second trial [13]. The fact that SR-141716 was ineffective when administered at 15 or 90 min after trial 1 suggests the involvement of consolidation processes.

Table 2. Studies examining the impact of disrupting endocannabinoid signaling in various animal models of learning and memory

Model	$CB_1^{-/-}$ mice	CB_1 receptor antagonists	Reference
Social recognition	Not tested	Enhanced	[13]
Retroactive inhibition	Not tested	Enhanced	
Aged rats (24 months) and mice (10–12 months)	Not tested	Enhanced	
Object recognition	Enhanced	Not tested	[49]
Rat radial-arm maze, delayed non-match	Not tested	Enhanced	[51, 52]
Mouse conditioned freezing			
Acquisition (tone)	No effect	No effect	
Extinction (tone)	Impaired	Impaired	[14]
Extinction (context)	Impaired	Impaired	[63]
Mouse Morris water maze			
Acquisition	No effect	No effect	[40, 65]
Reversal learning	Impaired	Not tested	[40]
Extinction (space trials)	Impaired	Impaired	[65]
Operant tasks			
Pigeon fixed consecutive number responding	Not tested	No effect	[53]
Rat repeated-acquisition procedure	Not tested	No effect	[24]
Rat non-match to position	Not tested	No effect	[23]
Monkey repeated-acquisition procedure	Not tested	No effect	[29]
Rat delayed non-match to sample	Not tested	No effect	[20]
Mouse lever-pressing acquisition of lever pressing	Impaired	No tested	[54]
Scopolamine-induced memory impairment			
Rat social recognition	Not tested	No effect	[13]
Rat radial-arm maze	Not tested	No effect	[61]
Monkey repeated acquisition	Not tested	No effect	[30]
Rat passive avoidance retention			
i.c.v. β-amyloid peptide-(25–35) or -(1–42)	Not tested	Enhanced	[60]

i.c.v., intracerebrovascular.

Additionally, SR-141716 prevented the retroactive inhibition of memory elicited by a procedure in which the test subject was exposed to a different juvenile conspecific between the two trials. In a related task, $CB_1^{-/-}$ mice exhibited enhanced object-recognition memory compared to wild-type control mice [49]. Similarly, $CB_1^{-/-}$ mice have been shown to display enhanced long-term potentiation (LTP), an electrophysiological model of synaptic plasticity thought to underlie learning [50].

SR-141716 was also found to enhance memory in a modified eight-arm radial maze task [51]. When a long delay (6 h) was interposed between the first and second halves of the task, SR-141716-treated rats committed significantly fewer re-entry errors than committed by vehicle-treated rats. SR-141716 was

only effective when given immediately before phase 1, and failed to enhance memory when administered either immediately after phase 1 or 30 min before phase 2. In another study, the memory-enhancing effects of SR-141716 in a delay radial-arm maze task were found to be dose-related and occurred when administered immediately after the first session, suggesting that the drug enhanced consolidation processes [52]. However, studies using operant paradigms have shown no benefits of SR-141716 treatment on performance [20, 23, 24, 53]. In fact, $CB_1^{-/-}$ mice have been reported to display impaired acquisition in acquiring lever-pressing response using a simple fixed-ratio schedule [54]. However, other phenotypes of these mice, including, hypomotility [55], decreased feeding [8], and altered emotionality [56], may have indirectly disrupted acquisition through motivational or sensorimotor alterations. One salient difference between studies in which SR-141716 enhances memory and those that fail to find any memory improvement is the temporal components of the task. Memory paradigms that reveal enhancement require memory processes that are in the order of minutes or hours, while the studies in which SR-141716 was ineffective require the retention of information that is in the order of seconds.

Endocannabinoid involvement in neurodegenerative diseases

Alterations of the endocannabinoid system have been reported in at least two neurodegenerative disease states, suggesting the involvement of this system. Specifically, reductions of CB_1 receptors were reported in brains of patients diagnosed with either Alzheimer's disease [57] or Huntington's chorea [58]. A more recent report found that brains from Alzheimer's disease patients contained upregulated levels of CB_2 receptors, as well as increased expression of fatty acid amide hydrolase (FAAH), in microglia associated with β-amyloid plaques [59]. Preclinical studies investigating the role of the endocannabinoid system on memory generally utilize young, healthy animals, which arguably may not be optimal to investigate potential nootropic agents. Conversely, there is a great multitude of animal models of cognitive dementia and/or memory impairment that includes surgical procedures, traumatic brain injury, intracerebral administration of agents, transgenic mice, and drugs. Few studies have examined the functional role of the endocannabinoid system or cannabinoid-based therapies in these pathological states or animal models of dementia. Nonetheless, the fact that SR-141716 attenuated the deficits displayed by aged mice and rats in the social recognition task suggests that this agent may have some utility in treating memory deficits that are associated with aging [13]. Similarly, SR-141716 has been shown to prevent memory deficits in a passive avoidance task using a rat Alzheimer's model [60]. In this model, an intracerebroventricular injection of β-amyloid fragments disrupted memory when the rats were given the retention test 1 week, but not 1 day, following acquisition training. SR-141716 given 30 min prior to a 1-week retention test

prevented this memory deficit; however, it failed to enhance performance when given 30 min prior to acquisition. This pattern of findings suggests that acute administration of CB_1 receptor antagonists may be effective in preventing retrieval deficits associated with neurodegenerative states [60]. In contrast SR-141716 does not appear to be effective in the scopolamine model of memory impairment in which this nonselective muscarinic antagonist is known to induce a variety of memory deficits. SR-141716 failed to enhance scopolamine-induced deficits in a variety of animal models including the rat social recognition task [13] a rat radial-arm maze task [61], and a monkey repeated-acquisition task [30].

Endocannabinoid modulation of extinction

The studies outlined in Table 2, which found that blockade of CB_1 receptor signaling enhanced in memory tasks, suggest that SR-141716 may potentially serve as a memory-enhancing agent. However, disruption of CB_1 receptor signaling has also been shown to impair another important component of cognition, extinction. Extinction is defined as a process by which learned behaviors that are no longer reinforced become actively suppressed. SR-141716-treated mice and $CB_1^{-/-}$ mice exhibited impaired extinction of conditioned freezing to a tone that had been paired with foot shock [14]. Following the conditioning procedure, presentation of the tone during extinction (i.e. in the absence of shock) was found to increase endogenous levels of anandamide and 2-AG in the amygdala, a brain area associated with fear. Moreover, nonreinforced presentation of the tone following fear conditioning also led to differences in activation of extracellular signal-regulated kinases (ERKs) between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice [62]. Compared with wild-type mice, the $CB_1^{-/-}$ mice expressed different levels of phosphorylated ERKs, its downstream effector Akt, and the phosphatase calcineurin in different aspects of the amygdala and hippocampus. These findings are consistent with the notion that release of endocannabinoids plays a role in extinction learning. A subsequent study found that SR-141716 also impaired conditioned freezing to the test chamber in which the mice had received the shock [63]. Of consequence, conditioned freezing to a context is believed to involve hippocampal processes, while the hippocampus is not believed to play a role in conditioned freezing to a tone [64]. Interestingly, SR-141716 failed to disrupt the within-session (short-term) extinction of conditioned freezing, but did disrupt extinction when the mice were tested 24 h later, suggesting it was consolidation of the extinction learning that was impaired.

Similarly, the endocannabinoid system also appears to play a role in long-term extinction in the Morris water maze task [65]. In this model, SR-141716-treated mice, $CB_1^{-/-}$ mice, and appropriate C57Bl/6 control mice were trained in a fixed-platform procedure, the platform was removed after the mice learned the platform location, and then the mice were subjected to

either a spaced extinction procedure (i.e. a single 60-s extinction trial given every 2–4 weeks) or a massed extinction procedure (i.e. four daily 120-s trials given on 5 consecutive days). In the spaced extinction task, the control mice exhibited extinction across the probe trials, while both SR-141716-treated mice and $CB_1^{-/-}$ mice continued to return to where the platform had been located, indicating extinction deficits. Importantly, an additional group of wild-type mice that was given only a single probe trial 9 weeks following acquisition exhibited near-perfect performance, indicating that this task assessed extinction and not merely time-dependent forgetting. In contrast, disruption of CB_1 receptor signaling did not alter the rate of extinction when the massed extinction procedure was used. Collectively, these results suggest that the endocannabinoid system may play a specific role in long-term or spaced extinction procedures.

An implication of endocannabinoid modulation of extinction is that disruption of CB_1 receptor signaling may interfere with learning tasks that require the suppression of previously learned responses. In support of this notion, $CB_1^{-/-}$ mice as well as SR-141716-treated mice learn the location of the fixed-platform Morris water maze task at identical rates to wild-type mice [40, 65]. Following acquisition, however, $CB_1^{-/-}$ mice exhibited a significant impairment in a reversal task in which the location of the hidden platform was moved to the opposite side of the tank [40]. While the wild-type mice readily learned the new platform location, the $CB_1^{-/-}$ mice continued to swim to the original platform location, despite being repeatedly shown the new platform location. Endocannabinoid modulation of extinction also has implications related to drug abuse. Specifically, it has recently been demonstrated that Δ^9-THC and cannabidiol, a structurally related component of marijuana that does not bind to the CB_1 receptor, enhances extinction of cocaine-induced and amphetamine-induced conditioned place preference learning in rats [66]. Thus, augmenting the endocannabinoid system may be useful in treating a wide range of perseverant, maladaptive behaviors related to aversive situations, including post-traumatic stress disorders [14] and persistent drug-seeking behavior [66].

Potential confounds in manipulations altering CB_1 receptor signaling

Although findings in which SR-141716-treated animals or $CB_1^{-/-}$ mice exhibit altered performance in mnemonic tests are generally interpreted as evidence supporting endocannabinoid tone, other explanations can also account for these types of finding. In the former case, SR-141716 does not appear to act solely as a CB_1 receptor antagonist. For example, SR-141716 has been found to decrease [^{35}S]guanosine-5'-(γ-*O*-thio)triphosphate (GTPγS) binding in membranes isolated from human cannabinoid CB_1 receptor-transfected Chinese hamster ovary cells [67, 68], an effect opposite to that of cannabinoid agonists [69–71], suggesting inverse agonist activity. Since cannabinoid ago-

nists impair memory, a cannabinoid inverse agonist would be expected to enhance memory. On the other hand, SR-141716 was found to be 7000-fold more selective as a CB_1 receptor antagonist than as an inverse agonist [72], raising questions regarding the relevance of its inverse agonism in the whole animal. Another plausible explanation is that SR-141716 may act at non-CB_1 sites of action, though it does not bind to CB_2, histamine, dopamine, opioid, 5-HT, adenosine, and several other receptors and ion channels [73, 74]. SR-141716 has been reported to antagonize WIN-55,212-mediated inhibition of hippocampal excitatory transmission in $CB_1^{-/-}$ mice, suggesting activity at either an uncharacterized cannabinoid receptor or noncannabinoid site of action [75]. Curiously, AM-251, a CB_1 receptor antagonist that is structurally very similar to SR-141716, failed to block WIN-55,212-2-induced inhibition of excitatory transmission [76].

In the case of $CB_1^{-/-}$ mice, alternative interpretations related to the use of knockout models must be considered. For example, potential confounding factors include hitchhiking genes that are derived from the original cell line, epistasis in which the effect of gene disruption is modified by the genetic background in which it is placed, and pleiotropic effects in which other consequences of gene disruption indirectly affect the behavior of interest [77]. Nonetheless, a similar phenotype between the $CB_1^{-/-}$ mice and SR-141716-treated wild-type mice in any given behavioral test would support the notion that these processes are under tonic endocannabinoid tone.

Neuroanatomical locus of effects

The endocannabinoid system is heterogeneously distributed throughout the central nervous system, reflecting the diversity of physiological processes in which endocannabinoids have been implicated as playing a role. Several lines of evidence suggest that the hippocampus, an area long implicated with learning processes, plays a major role in the mediating both the effects of exogenous cannabinoids on memory and endocannabinoid modulation of memory. First, analysis of the distribution of CB_1 receptors shows that the hippocampus contains a high density of CB_1 receptors, as has been demonstrated with [^{3}H]CP-55,940 [78] and [^{3}H]SR-141716 [79] autoradiography, detection of CB_1 mRNA expression [80], as well as with CB_1 receptor antibodies [81, 82]. The hippocampus has also been shown to contain relatively large amounts of the endocannabinoids anandamide [83, 84] and 2-AG [84].

Immunocytochemical studies have revealed that FAAH, the enzyme responsible for anandamide catabolism [85, 86] and monoglyceride lipase, an enzyme that is believed to play a role in the hydrolysis of 2-AG [87, 88], are significantly present within the hippocampus. Collectively, the high abundance of CB_1 receptors, endogenous ligands, and enzymes associated with endocannabinoids within the hippocampus strongly suggest that this system plays a tonic role in physiological mechanisms of this brain area. Further support for

this hypothesis comes from the observation that anandamide levels were markedly decreased in the hippocampus of $CB_1^{-/-}$ mice, but not in other brain regions, suggesting that levels of anandamide in the hippocampus may be regulated in part by tonic activation of CB_1 receptors [89].

Additional evidence suggesting the hippocampus as an important locus for cannabinoid effects on learning and memory comes from experiments investigating the memory-impairing effects of CB_1 agonists. Not only do the effects of cannabinoid agonists resemble those of hippocampal lesions, but also the Δ^9-THC-induced deficits in the DMTS paradigm described above have been associated with specific decreases in firing of individual hippocampal neurons during the sample, but not the match, part of the experiment [18, 20] Site microinjection studies also confirm the importance of the hippocampus. For example, application of CP-55,940 directly into the dorsal hippocampus disrupted working memory performance in an eight-arm radial maze without producing other cannabinoid effects such as anti-nociception, hypomotility, catalepsy, and hypothermia, believed to be mediated in other brain areas [33]. This dissociation between choice accuracy in the radial maze and other pharmacological effects supports the notion that the hippocampus plays an integral role in the cognitive alterations produced by cannabinoids. Similarly, microinjections of Δ^9-THC into the dorsal and ventral hippocampus disrupted spatial memory in the eight-arm radial maze, while injections into 11 other brain regions, including different aspects of the cerebral cortex, amygdala, raphe, caudate putamen, and mammillary body, were without effect [90]. Interestingly, Δ^9-THC administration into the dorsal medial thalamus disrupted radial-arm maze performance, but it is likely that this was an indirect effect on menemonic function as an array of abnormal behavior (e.g. increased repetitive and pivoting [90]) was reported. Thus a strong case can be made for the hippocampus as a primary neuroanatomical locus for the exocannabinoids and endocannabinoid modulation of learning and memory.

Other brain regions linked to mnemonic and attentional tasks such as the prefrontal (frontal) cortex have also been shown to be sensitive to CB_1 agonists. For example, Δ^9-THC-induced working memory deficits have been associated with increased dopaminergic activity in the prefrontal cortex [41], and later studies have shown that Δ^9-THC produces increases of not only dopamine, but also of glutamate, while decreasing γ-aminobutyric acid (GABA) release [91]. Also, Δ^9-THC and WIN-55,212 have been shown to increase acetylcholine release in rat frontal cortex in an SR-141716-reversible manner when given systemically, but not locally [92]. CB_1 receptors are expressed in the frontal cortex [78, 84]. It seems likely that these activating effects of CB_1 agonists in the frontal cortex may be the result of a disinhibition mediated via depressed GABAergic activity in other parts of the brain. Another brain area which is just beginning to be fully appreciated for endocannabinoid modulation of learning processes is the striatum, a brain region whose role is in habit or procedural learning (e.g. [93]). As with the hippocampus, high levels of CB_1 receptors as well as anandamide and 2-AG are

expressed in the striatum [78, 84], and as discussed below the endocannabinoid system has been shown to play a critical role in synaptic plasticity in this structure.

Cellular effects/interactions with other transmitter systems

In addition to its activity at the CB_1 receptor, anandamide has been shown to have activity at the peripheral cannabinoid (i.e. CB_2) receptor, potassium channels [94], gap junctions [95], and the VR_1 vanilloid receptor [96, 97]. Nonetheless, endocannabinoid agonist activity at CB_1 receptors has been the focus of work to understand their roles in learning processes. Activation of CB_1 receptors leads to a cascade of events with consequences that affect the electrophysiological properties of cells. Well-characterized effects of CB_1 receptors include activation of adenylate cyclase activity [98–100], promotion of mitogen-activated protein kinases [101, 102], inhibition of N- and P/Q-type voltage-gated calcium channels [81, 103–105], and the opening of A-type and inwardly rectifying potassium channels through their coupling with $G_{i/o}$ proteins [105–107]. These effects on ion currents have a hyperpolarizing influence on cell membranes, inhibiting their excitability. As CB_1 receptors are found almost exclusively presynaptically, the main effect of this location is to inhibit transmitter release (see below).

Interactions with glutamatergic systems

Recent work has focused on the influence of CB_1 activity on glutamatergic activity. In rat hippocampal cultures, cannabinoid agonists have been shown to inhibit presynaptic glutamate release. This effect appears to be the result of activation of CB_1 receptors located on presynaptic nerve terminals, which subsequently inhibit N- and Q-type calcium channels via an inhibitory G protein [108–110]. Given glutamate's role as the primary excitatory input into the hippocampus and its importance in LTP, it is highly likely that CB_1-mediated inhibition of glutamate release is important to the mnemonic effects of cannabinoids. However, elucidating the nature of the relationship between endocannabinoids and glutamate in the whole animal represents a difficult challenge. The ability of glutamate agonists to reverse cannabinoid-induced memory impairments is limited by the behavioral toxicity associated with this class of compounds. Additional difficulties arise when the mnemonic effects of cannabinoids are compared with those of antagonists of glutamatergic receptors. There are several reports suggesting that working-memory (i.e. short time) systems are largely spared by doses of *N*-methyl-D-aspartate (NMDA) antagonists such as phencyclidine and dizocilpine (MK801) that disrupt the consolidation and retrieval of long-term reference memories [111–115], while cannabinoids tend to produce the opposite spectrum of effects.

Interactions with GABAergic systems

Cannabinoid receptors also clearly influence hippocampal GABAergic activity, though the nature of this interaction is far from clear, and may involve multiple pathways. Several laboratories have reported that CB_1 receptors in the hippocampus were located almost exclusively on nerve terminals of cholecystokinin (CCK)-containing GABAergic interneurons [116–119]. Furthermore, cannabinoid agonists have been shown to inhibit GABA release in several preparations [116, 119–121]. However, several other lines of evidence have suggested that cannabinoids may also play a facilitating role on GABAergic transmission by blocking its reuptake. Early studies showed that Δ^9-THC could inhibit the uptake of GABA as well as 5-HT, norepinephrine (NE), and dopamine (DA) in rat brain synaptosomes [122, 123]. In the striatal synaptosome preparations, WIN-55,212-2 inhibited GABA uptake [124]. However, in the hippocampus, Δ^9-THC-induced reductions in acetylcholine turnover were shown to be dependent on septal GABAergic interneurons, as this reduction in turnover was completely blocked by intra-septal administration of the GABA antagonist bicuculline [125]. In addition, WIN-55,212-2 has been shown to produce a tonic hyperpolarization of CA1 pyramidal cells in hippocampal slices, which was reversed by bicuculline [126]. Compelling evidence has also come to light suggesting that endocannabinoids act as a retrograde signal to inhibit GABA-mediated transmission that follows depolarization of hippocampal pyramidal neurons [121, 127]. In addition, a recent set of behavioral experiments has demonstrated the importance of GABAergic transmission *in vivo*, as shutting down the GABAergic interneuronal system with the GABA-A antagonist bicuculline completely reversed Δ^9-THC-induced deficits in both the Morris water maze working-memory task and an alternation T-maze task [42].

These results, taken together, suggest a complicated relationship between endocannabinoid and GABAergic influence in the hippocampus, in which CB_1 receptor stimulation may lead to a multitude of effects that depend on the specific pathway. Undoubtedly, the challenge is now to determine the functional significance of each of these pathways on cognition.

Interactions with cholinergic systems

It has long been recognized that an important element of the action of cannabinoids may be their ability to inhibit cholinergic transmission in the limbic system and cortex, and the memory deficits observed with cannabinoids resemble those seen following administration of cholinergic antagonists [39]. Early studies revealed that Δ^9-THC reduced uptake of choline in the hippocampus, thereby restricting acetylcholine synthesis [128, 129]. More recently, it has become clear that cannabinoids presynaptically inhibit the release of acetylcholine, possibly though CB_1 receptors located on the cholinergic nerve ter-

minals. Several cannabinoid agonists have been shown to inhibit electrically evoked acetylcholine release in hippocampal slices [130–132] and synaptosomes [133]. Similarly, microdialysis studies in awake rats also showed cannabinoid-induced decreases in acetylcholine release [134–136]. This effect on hippocampal acetylcholine release is clearly CB_1 receptor mediated, as all the afore-mentioned studies demonstrated that SR-141716 blocks the effect. Conversely, higher doses of SR-141716 increased the amount of released acetylcholine in the hippocampus, indicating either inverse agonist activity of SR-141716 or blockade of a tonic inhibitory influence by endocannabinoids. In support of the latter possibility, electrically evoked hippocampal (but not striatal) acetylcholine release was found to be 100% greater in $CB_1^{-/-}$ mice compared to wild-type controls [137]. Behavioral studies also support the hypothesis that acetylcholine plays a role in cannabinoid-induced memory impairment. Although an initial study found that the cholinesterase inhibitor physostigmine failed reverse Δ^9-THC-induced deficits in an eight-arm radial maze [61], subsequent studies demonstrated that low doses of physostigmine as well as other cholinesterase inhibitors blocked cannabinoid-induced memory impairment [35, 36].

Role of endocannabinoids in synaptic plasticity

Given that endocannabinergic mechanisms have been strongly implicated in behavioral paradigms of learning and memory, it is not surprising that a growing body of work has focused on understanding the role of the endocannabinoid system in the electrophysiological correlates of learning, synaptic plasticity. Synaptic plasticity is a network attribute of synapses, referring to their ability to change in structure and function in response to particular patterns of activation. These processes are believed to represent the neurobiological basis of learning in which the experiences of an organism can modify subsequent responses to stimuli.

Short-term plasticity

Great strides have been made in understanding the physiological role of the endocannabinoid system with the discovery that endocannabinoids may serve to mediate a short-term plasticity phenomenon referred to as depolarization-induced suppression of inhibition (DSI) and its corollary depolarization-induced suppression of excitation (DSE). DSI occurs on GABAergic synapses and has been studied in hippocampal CA1 pyramidal cells [138, 139] and in cerebellum [140, 141]. Depolarization of the postsynaptic cell results in the release of a retrograde messenger which diffuses back across the synapse and inhibits further GABA release, thus diminishing inhibitory tone for a brief period of a few seconds. Conversely, DSE involves the short-term inhibition of

glutamate release, and has also been demonstrated in several brain areas including the VTA [142].

Early reports suggested that the retrograde messenger may have been glutamate, acting on presynaptic metabotropic glutamate receptors [143, 144]. However, Wilson and Nicoll [121] as well as Ohno-Shosaku and colleagues [127] established that endocannabinoids play a critical role in DSI. Supporting such a role for endocannabinoids are the findings that DSI in the hippocampus is completely blocked by CB_1 antagonists [121, 127] and is absent in $CB_1^{-/-}$ mice [145]. Similarly, the DSI observed in cerebellar Purkinje cells has been shown to be mediated by CB_1 receptor activation located on presynaptic neurons [146–148]. Based on these findings it has been hypothesized that endocannabinoids are released from the postsynaptic neuron, and travel retrogradely to presynaptically located CB_1 receptors where they inhibit GABA release. Despite the potential importance of these phenomena, their physiological significance remains to be established. Hampson and colleagues attempted to induce DSI using pulse trains that mimic hippocampal cell-firing patterns that occur *in vivo* [149]. However, they found that these normal firing patterns of hippocampal neurons failed to elicit DSI.

Models of long-term synaptic plasticity: LTP

Initial studies examining the effects of cannabinoids on synaptic plasticity demonstrated that CB_1 agonists disrupt LTP. LTP refers to the phenomenon in which brief high-frequency stimulation applied to afferent pathways results in an increase in the excitatory synaptic potentials of postsynaptic neurons, which can last from hours to weeks. This phenomenon was first observed in the hippocampus [150] where it has been most extensively characterized, but has since been demonstrated in many other brain areas.

Nowicky et al. [151] first reported that Δ^9-THC significantly impaired the induction of LTP induced in CA1 region of hippocampal slices using two tetanizing trains (200 Hz, 0.5-s duration, 5-s intertrial interval (ITI)) delivered to the stratum radiatum. This effect of Δ^9-THC was also produced by HU-210 [152], and was shown to be mediated via the CB_1 receptor [153]. Terranova et al. [154] extended these findings to WIN-55212-2 and the endogenous cannabinoid anandamide, while Stella et al. [155] demonstrated that application of 2-AG can also inhibited the induction of hippocampal LTP, all of which were reversed by SR-141716. Furthermore, it has recently been shown that AM-404, an inhibitor of the putative anandamide transporter that also inhibits FAAH, can disrupt induction of LTP in an SR-141716-reversible manner [156]. Interestingly, $CB_1^{-/-}$ mice demonstrated enhanced hippocampal LTP induced by high-frequency stimulation to the Shaffer collaterals [50]. One likely mechanism given for this cannabinoid-induced disruption of LTP is that presynaptic CB_1 receptors inhibit release of the glutamate necessary to depolarize the postsynaptic cell and release NMDA receptors from the magnesium

blockade existent under normal conditions. The opening of these NMDA receptors and the subsequent influx of calcium triggers calcium-dependent second-messenger systems that initiate the induction of LTP [157]. While the studies described above lend support to the hypothesis that endocannabinoids may serve to diminish induction of LTP, these effects have been shown to depend on the induction method. In all of the cases mentioned above, LTP was induced by high-frequency titanic stimulation. However, LTP can also be observed following a theta burst protocol, which is thought to reflect a more physiologically relevant process. Lees and Dougalis [156] showed that while WIN-55,212 will block induction of LTP under both conditioning protocols in an AM-251-reversible manner, anandamide only prevents high-frequency stimulation LTP.

In contrast to the clear disruption of LTP observed when CB_1 agonists are exogenously administered to whole tissues, more subtle and potentially interesting effects have been reported when the effects of endocannabinoids have been looked at within their endogenous context. For example Carlson et al. [158] showed that endocannabinoids released via induction of DSI led to a facilitation of LTP in targeted cells, but not neighboring ones, by selectively disinhibiting postsynaptic cells (through decreased presynaptic GABA release) and lowering their thresholds for LTP. It was hypothesized that exogenous application of cannabinoids may disrupt learning by disrupting the spatial and temporal selectivity of coding mediated by endocannabinoids. Additionally, cannabinoids have been shown to promote signaling pathways such as ERK, which are known to be important for synaptic plasticity and learning [159].

Models of long term synaptic plasticity: long-term depression (LTD)

In contrast to high frequency stimulation that can lead to LTP, low-frequency stimulation leads to another form of long-term synaptic plasticity, known as LTD, in which synapse strength is weakened. LTD has been demonstrated in several brain areas, including the hippocampus, striatum, cerebellum, and various parts of the cortex (for reviews see [160, 161]). Over the past few years, endocannabinoids have been demonstrated to be crucial components of LTD in several brain areas including the striatum, amydala, frontal cortex, and nucleus accumbens.

LTD in the striatum, a process believed to be important in certain forms of learning, has been shown to be CB_1 receptor-dependent. LTD was absent in $CB_1^{-/-}$ mice and greatly reduced in slices preincubated with SR-141716, while it was potentiated by AM-404 (an inhibitor of both FAAH and the anandamide transporter) [162]. In addition, postsynaptic loading of anandamide resulted in reduced presynaptic excitatory transmission. These findings support the notion that endocannabinoids serve as retrograde messengers to reduce excitatory cortical inputs to striatal output neurons. A similar facilitative role of endocannabinoids on LTD has been proposed in the amygdala, where LTD induced

by WIN-55212 or amphetamine was blocked by AM-251, and the amphetamine-induced LTD was facilitated by AM-404 [163]. Additionally, LTD in the neocortex [164] as well as in the nucleus accumbens [165, 166] has been shown to be dependent on presynaptic activation of CB_1 receptors. Interestingly, chronic exposure to Δ^9-THC or WIN-552-2 has been shown to prevent the induction of LTD, suggesting a functional tolerance that may partially underlie putative, long-lasting cognitive deficits associated with chronic marijuana use [166].

This hypothesized role of endocannabinoids as initiators/mediators of LTD is also consistent with a series of experiments examining the effects of cannabinoid agonists and CB_1 antagonists on synaptic plasticity in the prefrontal cortex [167]. Tetanic stimulation induced plasticity in slightly more than half of the neurons examined, with approximately equal numbers of cells developing LTP and LTD. However, in the presence of WIN-55212-2 almost all of the plastic synapses showed LTD, while in the presence of SR-141716 almost all of the plastic synapses developed LTP. The consequences of such a shift on learning are unclear, though it may suggest that forms of learning heavily dependent on LTP could be attenuated by endocannabinoids, while forms of learning heavily dependent on LTD could actually be enhanced.

Other forms of synaptic plasticity

Endocannabinoids have also been shown to modify other forms of synaptic plasticity. For example, anandamide has been shown to inhibit an interesting though poorly understood phenomena referred to as long-term transformation (LTT), where GABAergic inhibition is "transformed" into excitation when presynaptic tetanic stimulation is paired with postsynaptic depolarization [168]. This LTT has been shown to play an important role in pairing-induced LTP, which is also inhibited by anandamide [168].

In addition to changes in presynaptic transmitter release and postsynaptic sensitivity, synaptic plasticity can be supported by the growth of new synapses, a process that has also been shown to be sensitive to CB_1 stimulation. Anandamide, Δ^9-THC, and WIN-55212-2 have all been shown to inhibit new synapse formation induced by forskolin, probably due to CB_1 receptor-mediated decreases in cAMP [169].

Conclusions on the effects of endocannabinoids on cognition

The weight of evidence clearly demonstrates that the endocannabinoid system is critically involved with physiological mechanisms underling learning and memory. It can be stated with a high degree of confidence that CB_1 receptors located in brain areas associated with learning and memory, including the hippocampus, frontal cortex, and striatum, are a crucial element of this influence.

CB_1 receptor activation by exogenous application of cannabinoid agonists consistently results in disturbances of learning and memory. The fact that such disturbances are generally observed at doses lower than those required to elicit other well-characterized effects (i.e. motor effects, analgesia, hypothermia) are consistent with the hypothesis that these agents have selective effects on memory. In general, exogenous administration of cannabinoids inhibits neurotransmitter release in the hippocampus. *In vivo* studies in which memory duration is enhanced in SR-141716-treated mice and $CB_1^{-/-}$ mice are consistent with the notion that endocannabinoids are tonically active to dampen memory. However, the specific actions of endocannabinoids, such as anandamide and 2-AG, in the tonic modulation of the neural pathways that underlie cognition remains an active area of research. The availability of selective CB_1 antagonists and transgenic mouse models promises to address this question and further our understanding of endocannabinoid systems. The possibility of developing drugs that target the endocannabinoid system to treat cognitive deficits associated with Alzheimer's disease, posttraumatic stress syndrome, and drug abuse is particularly intriguing.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabinoids and anxiety

Richard E. Musty

Department of Psychology, University of Vermont, Burlington, VT 05405, USA

Introduction

As stated in Ethan Russo's chapter the first known reference to the use of *Cannabis for the relief of anxiety was about 1500 bce* in India. In modern times the 1860 *Report of the Ohio State Medical Committee on Cannabis indica* [1] stated:

> "As a calmative and hypnotic, in all forms of nervous inquietude and cerebral excitement, it will be found an invaluable agent, as it produces none of those functional derangement or sequences that render many of the more customary remedies objectionable."

Anxiolytic effects of CB_1 receptor antagonists

Musty [2] found that cannabidiol (CBD) inhibited the development of stress-induced ulcers in rats as compared with diazepam, which produced an equivalent reduction in the number of stress-induced ulcers. Guimaraes et al. [3] tested rats in the elevated-plus maze. In the test, rats are placed in a plus-shaped maze which is elevated above the floor. Two of the maze arms are enclosed with walls and two are not. Time spent in the enclosed arms is taken as a measure of anxiety or fear. Both CBD and diazepam decreased the amount of time spent in the enclosed arms. Since these studies were conducted, Petitet et al. [4] and Thomas et al. [5] have reported CBD is an antagonist of the CB_1 receptor in the micromolar range, suggesting that CBD may have pharmacological effects an antagonist of the CB_1 receptor.

Musty et al. [6] found that CBD increased licking for water in the lick-suppression test, which reliably discriminates between anxiolytic drugs and those that are non-anxiolytic. Equivalent effects were found with the classic anxiolytic drug diazepam. In an effort to find more potent effects, they tested two analogs, 2-pinyl-5-dimethylheptyl resorcinol (PR-DMH) and Mono-methyl cannabidiol (ME-CBD-2). PR-DMH had anxiolytic activity, but was less potent than CBD, while ME-CBD-2 had no anxiolytic properties. CBD also decreased conditioned taste aversion in a dose-related fashion (40 mg/kg gave the peak effect).

Since the discovery of the synthetic, highly potent CB_1 receptor antagonist, SR-141716, by Rinaldi-Carmona et al. [7] several other studies seem to support the hypothesis that CB_1 receptor antagonists have anxiolytic properties. Onaivi et al. [8] used two tests of anxiety in mice, the elevated-plus maze (discussed above) and the two-compartment black and white box test. When administered SR-141716 in the elevated-plus maze, mice spent more time in the open arms, indicating a reduction of anxiety. In the second test, mice were allowed to choose to spend time in a two-compartment box, one of which was white and brightly lit, the other of which was black and dimly lit. Time spent in the dark compartment was taken as an index of anxiety. When administered SR-141716, mice spent more time in the white, brightly lit compartment, indicating a reduction in anxiety.

In a recent study Rodgers et al. [9], administered the CB_1 receptor antagonist SR-141716A and a reference benzodiazepine chlordiazepoxide (CDP). They argued that there were "qualitative" differences of cannabinoid ligands depending on the number of exposures (trials) in the elevated-plus maze. They tested the effects of SR-141716A (0.1–10.0 mg/kg) and the reference CDP (15 mg/kg) in mice that had never been exposed to the elevated-plus maze compared with mice that were given an exposure to the maze undrugged (day 1). Then, 24 h later (day 2), both groups of mice were given the same drug regimen as above. Those mice that had received CDP on day 1 did not exhibit any anxiolytic response. The authors suggest this indicated an immediate tolerance to CDP. On the other hand, mice given SR-1411716 on day 1 showed no anxiolytic response, but did so on day 2. This result is in contrast to the afore-mentioned study in which the effect of SR-141716 was observed on the first exposure to the elevated-plus maze. The authors state: "The apparent experientially induced 'sensitization' to the anxiolytic-like effects of SR-141716 in the plus-maze contrasts markedly with the widely reported loss of benzodiazepine efficacy in test-experienced animals." Further research is required to resolve these differences.

Cannabinoid receptor agonists

It seems that low doses of cannabinoid receptor agonists are anxiolytic in mice. In the light/dark box test cited above, 0.3 mg/kg of Δ^9-tetrahydrocannabinol (Δ^9-THC) reduced anxiety [10], but in lower doses of 0.03 and 0.1 mg/kg no effect was found. On the other hand, with doses of 5.0 [10] and 4.0 mg/kg, using a THC extract (Deyo R et al., personal communication), anxiogenic effects have been observed. Similarly, the potent CB_1 receptor agonist HU 210 has been shown to have anxiolytic effects at low doses (4 μg/kg) in the defensive withdrawal test in a novel environment (associated with fear/anxiety in rodents), but anxiogenic effects in a familiar environment [11]. These data seem to show that the context of administration is an important variable in the effect of CB_1 agonists.

CB_1 receptor-knockout studies

Martin et al. [12] tested CB_1-knockout mice and wild-type mice in the light/dark box test. They found that knockout mice spent more time in the dark section of the box and less time in the light part of the box compared with the wild-type mice, indicating that the knockout mice were more anxious, thus providing strong support that the CB_1 receptor system is involved in the control of emotional behaviors such as fear and anxiety.

Rodgers et al. [13] extended the findings of Martin et al. [12]. They tested CB_1-knockout mice and wild-types in the elevated-plus maze under two conditions, low light and high light, the latter having been shown to induce greater anxiety. They found that there were no differences or indications of anxiogenic activity in the low-light condition between knockout and wild-type mice. However, in the high-light condition knockout mice spent significantly more time the in the closed arms of the maze and less time in the open arms of the maze, as compared with the wild-type mice. These data provide more convincing evidence that the CB_1 receptor system is involved in the control of anxiety.

Anandamide hydrolysis and anxiety

Kathuria et al. [14] hypothesized that that anxiolytic effects of cannabinoids might be enhanced by endogenous cannabinoids by preventing their inactivation. Accordingly, this group synthesized several fatty acid amide hydrolase (FAAH) inhibitors. Two of these (named URB532 and URB597) selectively inhibited breakdown of anandamide *in vitro* while an inactive analog did not. These inhibitors were also tested in the elevated-plus maze. Both increased the time spent in the open arms of the maze, in a dose-dependent manner. These effects were blocked by several doses of SR-141716. The researchers also used the ultrasonic emission test. In this test rat pups are separated from their mother, which causes them to emit ultrasonic distress cries. Normally the mother uses these signals to locate and retrieve the pups. Anxiolytic drugs selectively reduce these cries dose-dependently. In this test both FAAH inhibitors reduced vocalizations in the pups which was reversed by co-administration of SR-141716. The authors conclude that raising anandamide levels seems to be important in the regulation of anxiety and suggest a potential new class of compounds which might be useful in the treatment of anxiety.

Discussion of animal studies

It seems that there is a paradox in the data discussed above. Regarding CB_1 antagonists, it seems that the preponderance of the data suggest that these compounds are anxiolytic. Agonists on the other hand seem to have biphasic

effects. Low doses seem to be anxiolytic, while high doses are anxiogenic. In addition, it seems that the context is important. Further research is needed to sort out the differences among various studies, but it is clear that both antagonists and agonists on the CB_1 receptor have anxiolytic properties. Standardization of behavioral testing procedures across laboratories would be helpful, the problem being that there are many variables which have not been explored with behavioral methods used to test for anxiolytic properties. Since it is widely known that activation and inactivation of CB_1 receptors has a multitude of modulator effects on neurotransmitter systems, it would be advantageous for researchers to examine what changes in neurotransmitter activity occur in conjunction with the pharmacological effects observed in the types of studies cited herein.

Human studies

Consroe et al. [15] found that anxiety was reduced in 85% of patients with multiple sclerosis in a self-report questionnaire. In another self-report study [16] patients with spinal cord injuries reported similar reductions in anxiety.

In a laboratory setting, when subjects were instructed to smoke marijuana until they reached their "usual" level of intoxication, regression analysis of a visual analog scale of the word "anxious" predicted decreased scores on this scale. These data support the hypothesis that THC has anxiolytic properties at low doses [17].

In normal volunteers, Zuardi et al. [18] tested the hypothesis that CBD would antagonize anxiety induced by THC. They used a dose of 0.5 mg/kg THC for a 68-kg subject, which is a rather large dose, exceeding the dose a person would take for the intoxicating effect of the drug. Subjects report a pleasant high at 0.25 mg/kg using the same route of administration without an increase in anxiety. In a second study Zuardi et al. [19] induced anxiety in normal subjects by having them prepare a 4-min speech about a topic from a course they had taken during the year. They were told the speech would be videotaped for later analysis by a psychologist. The subject began the speech while viewing his/her image on a video monitor. Anxiety measures were taken using the Visual Analogue Mood Scale (VAMS), which yields measures on four factors (anxiety, physical sedation, mental sedation and other feelings, e.g. interest) at five time points: baseline, immediately before instructions, immediately before the speech, in the middle of the speech and after the speech. Heart rate and blood pressure measures were also taken. The subjects were randomly assigned to one of four drug conditions: CBD (300 mg), isapirone (5 mg), diazepam (10 mg) or placebo. CBD, diazepam and isapirone decreased anxiety and systolic blood pressure. Neither CBD nor isapirone had effects on physical sedation, mental sedation, or other feelings, but diazepam induced feelings of physical sedation.

Crippa et al. [20] investigated CBD's effects on regional cerebral blood in normal postgraduate students. In addition, they administered the VAMS. Each subject was tested twice, 1 week apart. On week 1, half the subjects were given a single dose of 400 mg of CBD in corn oil (in a capsule) and the other subjects received a placebo capsule of corn oil only. On week 2 this procedure was reversed. During the regional cerebral blood scanning procedure subjects were resting for 30 min before the VAMS was administered. At the 30-min mark an intravenous cannula was inserted to administer the radioactive tracer material and the VAMS was given again. The cannula was removed and the scan was performed. The VAMS was given again at 60 and 75 min after drug ingestion. Anxiety decreased significantly by 60 and 75 min, when orally administered doses of CBD are known to be at peak blood levels. Tracer uptake in the CBD condition increased relative to placebo in the left parahippocampal gyrus and the left fusiform gyrus compared with placebo. Tracer uptake decreased in the CBD relative to placebo in the left amygdala-hippocampal complex and uncus, the hypothalamus and left superior portion of the posterior cingulate gyrus.

It seems clear that CBD decreased anxiety, which is often observed in people undergoing SPECT (single-photon emission computed tomography) or PET (positron emission tomography) scanning as measured by the VAMS. The brain area which showed increased activity in relation to placebo was the left parahippocampal gyrus. Deactivation of this area of the brain has been associated with panic attacks induced by lactate, anxiety induced by combat-related images and autobiographical memory scripts. It seems that anxiety is associated with reduced parahippocampal activity, consistent with the findings that CBD increases activity in this brain area. Because activity in the CBD condition decreased relative to the placebo, these data fit well since there are a lot of data linking amygdala activation in a large variety of anxiety states. Similarly, the hypothalamus is involved in various anxiety states: imaging studies in particular have shown increases in hypothalamic activity in anxiety induced in normal volunteers and panic patients, again consistent with the anxiolytic effect of CBD. In regard to the posterior cingulate gyrus, increased brain activity is associated with viewing anxiety-provoking videos, which provoked obsessions in obsessive patients. Patients with obsessive-compulsive disorder (OCD), if untreated, have increased metabolism in the brain area, which decreases with treatment and symptom remission, although there are some conflicting data (see [20] for references relating to all of the above discussion). While these data might be considered preliminary, they provide the first evidence of brain systems that are affected in humans. There seems to be quite strong convergence between animal research and human research, suggesting strongly that CBD is a true anxiolytic. Given the fact that this drug has no psychoactivity in terms of intoxication and is very safe, it seems important to pursue the potential of CBD, with further behavioral pharmacological studies, mechanistic studies employing neuropharmacological methods and in clinical studies.

Discussion

The data discussed in this review show there is converging evidence that the CB_1 receptor system is involved in the control of anxiety. Many studies have shown that both antagonists and agonists of the CB_1 receptor can produce anxiolytic effects both in animals and humans. Particularly strong evidence is the fact that CB_1-knockout mice are more anxious than wild-type mice The fact that anandamide hydrolysis inhibitors are anxiolytic and that they lead to an increase in anandamide levels in the brain is further support for the role of this system in the control of anxiety. Finally, the observations that CBD increases or decreases regional cerebral blood flow in areas of the brain predicted to be involved in various anxiety states provide strong supportive evidence that at least this cannabinoid is active in brain areas known to be involved in anxiety. At present, four cannabininoids are available for clinical trials: SR-141716 (Rimonbant), the GW Pharmaceuticals extract (Sativex®; a 1:1 ratio of Δ^9-THC/CBD), Δ^9-THC (Marinol) and Nabilone (Cesamet). It would seem reasonable to consider testing these compounds in specific anxiety states, which are refractory to traditional anxiolytics and related drugs.

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Edited by R. Mechoulam

Cannabinoid targets for pain therapeutics

Susan M. Huang[1] and J. Michael Walker[2]

[1] *Department of Neuroscience, Brown University, Providence, RI 02912, USA*
[2] *Department of Psychology, Indiana University, Bloomington, IN 47405, USA*

Introduction

Historical accounts and anecdotal reports show that among the most common medicinal uses of cannabis is pain relief [1]. Such accounts date back to the ancient Chinese physician Hoa-Gho thousands of years ago for surgical anesthesia, and to ancient Israel in 315–392 AD, likely for the control of pain in child birth [2]. The use of cannabis as medicine was so popular that there were at least 28 pharmaceutical preparations available in the United States prior to the passing of the Marijuana Tax Act in 1937 [3], which thwarted the legal use and development of cannabis-based medicine.

The isolation, structural elucidation and chemical synthesis of the active ingredient in marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), by Gaoni and Mechoulam [4] was a monumental step which revealed the molecular basis for the behavioral and physiological influences of cannabis reported throughout history. The subsequent recognition and cloning of specific cannabinoid CB_1 and CB_2 receptors (CB_1R and CB_2R) in the nervous system and the periphery [5–7] led to the realization of the likely importance of a cannabinoid neuromodulatory system in the body. More recently, the identification of several endogenous cannabinoids, beginning with anandamide [8], expanded the ever-more complex story of regulation of various physiological functions by the cannabinoid system.

Knowledge of the molecular components of the endocannabinoid system [9] allowed the development of pharmacological agents that target them [10]. Pharmacological agonists and antagonists have been synthesized for the cannabinoid CB_1R and CB_2R. The discovery of endogenous cannabinoids and studies of their biosynthetic and degradatory pathways also yielded molecular targets for perturbing the endocannabinoid system. Inhibitors of fatty acid amide hydrolase (FAAH), the enzyme that degrades several of the endogenous cannabinoids and inhibitors of the anandamide transporter, have been developed. These agents are useful research tools for studying the effects of cannabinoids on pain as well as potential therapeutic drugs. This review focuses on studies that evaluate the behavioral and physiological consequences of modulating the above-mentioned targets, as these are promising avenues for

the development of cannabinoid-based therapeutics for pain. Reviews covering basic research on the physiological role of endogenous cannabinoids in pain modulation can be found elsewhere [11].

Effects of direct-acting cannabinoid receptor agonists

Perhaps the earliest published preclinical experiment on the effects of cannabinoids on pain was that conducted by Dixon [12], who showed that dogs that inhaled cannabis smoke failed to react to pin pricks. After the isolation of Δ^9-THC, Bicher and Mechoulam [13] and Kosersky and colleagues [14] demonstrated that this chemical component of cannabis profoundly suppressed behavioral reactions to acute noxious stimuli and inflammatory pain. It was noted early on that the potency and efficacy of cannabinoids in acute pain paradigms rival that of morphine [15, 16]. However, cannabinoids also produce profound motor effects such as immobility and catalepsy [17], which raised a potential confounding factor for studies that assessed pain behavior, because escape or withdrawal responses to noxious stimuli are integral to the assessment. In part to address this potential confounding factor, experiments were initiated to determine whether cannabinoids suppress the spinal and thalamic circuits that give rise to pain sensations. These experiments demonstrated that cannabinoids selectively suppress noxious stimulus-evoked neuronal activity in spinal and thalamic nociceptive neurons [18–22]. This effect is observed with all modalities of noxious stimulation tested (mechanical, thermal, chemical), is mediated by cannabinoid receptors, and correlates with the pain-suppressive behavioral effects of cannabinoids [18–21]. Cannabinoids suppress C-fiber-evoked responses in spinal dorsal-horn neurons recorded in normal and inflamed rats [22–24]. Spinal expression of Fos protein, a marker of sustained neuronal activation [25], is also suppressed by cannabinoids in animal models of persistent pain [26–31].

Brain action of cannabinoid agonists

Intracerebroventricular administration of systemically inactive doses cannabinoid agonists suppresses pain with only miniscule amounts reaching the spinal cord at the time of peak analgesia [32]. When administered in this fashion, cannabinoids inhibit spinal nociceptive responses by actions on specific circuits in the brain that serve naturally to modulate pain sensitivity. These areas include the dorsolateral periaqueductal gray, dorsal raphe n., rostral ventral medulla, amygdala, lateral posterior and submedius regions of the thalamus, superior colliculus and noradrenergic nucleus A5 region [33–35]. It appears that the descending noradrenergic system is important in mediating the effects of cannabinoids in the brain [36, 37]. When spinal transection was performed in rats, there was a marked attenuation of the analgesic effects of systemically

administered cannabinoids [20, 38], suggesting substantial contribution sites in the brain. Hence it is possible that maximal analgesic effects could be difficult to attain without penetration into brain sites, either by selective routes of administration or with drugs that cannot penetrate the blood–brain barrier. However, the real measure of clinical success often lies in balancing the degree of pain relief with the extent of the unwanted side effects, which for cannabinoids are mainly due to actions in the brain leading to undesirable psychotropic effects.

Spinal action of cannabinoid agonists

Anti-nociceptive effects of cannabinoids are mediated in part at the spinal level, as inhibition of spinal reflexive responses to noxious stimuli was observed in spinally transected dogs [39]. Support for spinal mechanisms of cannabinoid analgesic action is also found in studies that demonstrated analgesia following intrathecal injections [40–42]. The behavioral data are consistent with the ability of spinally administered cannabinoids to suppress noxious heat-evoked and afterdischarge firing [19] and noxious stimulus-evoked Fos protein expression in spinal dorsal horn neurons [27]. Spinal administration of a CB_1R agonist also inhibits C-fiber- and A-δ-fiber evoked responses of spinal nociceptive neurons in a CB_1-dependent mechanism [24]. Systemic and intrathecally administered cannabinoids retain a weak but long-lasting anti-nociceptive effect in spinally transected rats [38, 40], providing compelling evidence for spinal mechanisms of cannabinoid anti-nociception.

Spinal administration of the ultra-potent cannabinoid HU-210 suppresses C-fiber-mediated neuronal hyperexcitability in carrageenan-inflamed and non-inflamed rats [23]; these effects were blocked by a CB_1R antagonist. Similar to the results with HU-210, spinal administration of anandamide also produced CB_1R-mediated effects in carrageenan-inflamed rats, but inconsistent effects were observed in non-inflamed rats [43]. Although not established following inflammation, upregulation of CB_1Rs is observed in the spinal cord following nerve injury, suggesting that regulation of spinal CB_1Rs may contribute to the therapeutic efficacy of cannabinoids in pathological pain states [44]. The observation that cannabinoids act spinally to inhibit pain implies that epidural cannabinoids may be effective in treating certain types of pain.

Peripheral action of cannabinoid agonists

Richardson and colleagues observed that peripheral administration of the cannabinoid agonist anandamide suppressed thermal hyperalgesia and edema in the carrageenan model of inflammation in a CB_1R-dependent manner [45]. The same dose administered to the non-inflamed contralateral paw was inactive, indicating that the compound did not produce its effects by absorption

into the systemic circulation. This finding indicates that CB_1R agonists acting in the periphery are sufficient to inhibit pain. Intraplantar administration of the mixed CB_1/CB_2 agonist WIN-55,212-2 also attenuated the development of carrageenan-evoked mechanical hyperalgesia, allodynia and spinal Fos protein expression in a CB_1R- and CB_2R-dependent manner [30].

Effects of cannabinoid CB_2-specific agonists

CB_2Rs are either absent or expressed in low levels by neural tissues [7, 46] but are present on immune cells and hence may have implications for pain. This distribution has led to the evaluation and validation of the CB_2R as a target for novel pharmacotherapies for pain, an attractive possibility because CB_2R agonists lack psychotropic side effects.

CB_2R agonists are anti-nociceptive in models of acute [47] and persistent [29, 48–51] pain. Hanuš and colleagues [49] demonstrated that the selective CB_2 agonist HU-308 produced marked decreases in pain behavior in rats receiving hindpaw injections of dilute formalin with no change in motor function, a side effect often seen with CB_1R agonists which may predict psychoactivity in humans. Another CB_2R agonist, AM-1241, has also been shown to induce CB_2R-mediated analgesia in acute pain paradigms while failing to elicit centrally mediated side effects such as hypothermia, catalepsy and hypoactivity [47]. In inflammatory pain models, AM-1241 also induces CB_2R-mediated suppression of carrageenan- and capsaicin-evoked thermal and mechanical hyperalgesia and allodynia [30, 50, 52].

Substances released by immune cells such as histamine, serotonin (5-hydroxytryptamine), eicosanoids, interleukin 1, tumor necrosis factor-α, and nerve growth factor sensitize nociceptors [53–55]. Therefore, it is plausible that activation of CB_2Rs on immune cells could suppress pain. Cannabinoid agonists have been shown to inhibit the release of inflammatory mediators from monocytic cells [56] and mast cells [57]. Direct effects on CB_2Rs localized to primary afferents have been postulated [58–62], though the basis for such effects is unclear in light of the paucity or lack of CB_2R expression in neural tissues. It would appear from these findings that CB_2R selective agonists are a promising target for drugs to treat pain and inflammation.

Effects of cannabinoid agonists in humans

The human trials of cannabis and Δ^9-THC are few in number and typically small in subject size. There are marked differences between studies in dose regimens and drug preparations, with some using smoked marijuana and others using Δ^9-THC by oral or intravenous routes. Some studies used healthy volunteers, whereas others used patients with clinical pain. Therefore, it is important to note that: (1) some negative results may have arisen from admin-

istration of doses that are ineffective; (2) the oral route of administration adds variability owing to the unpredictable absorption of Δ^9-THC; (3) smoked marijuana contains additional constituents that modify its actions; and (4) studies of experimental pain in healthy subjects provide information on the spectrum of the effects of cannabinoids in humans and initial indications with regard to the feasibility of their use in humans, while clinical trials on specific pathological pain syndromes are more relevant in assessing the effectiveness of cannabinoids on particular pain conditions.

Studies of cannabinoid agonists on experimental pain in humans

Several investigators have studied the effects of cannabinoids on pain perception in humans by administering controlled painful stimuli to healthy volunteers. One such study [63] found that an oral dose of 5 mg of Δ^9-THC given to healthy volunteers decreased their ability to distinguish between various intensities of painful heat stimuli with a time course that was distinguishable from the effects on memory and psycholinguistic measures. This effect is consistent with a pain-suppressive effect of the compound. Using sensory decision theory, they separated this effect from response bias, which refers to the tendency to respond either positively or negatively and is influenced by non-sensory factors such as the subject's culture, temperament and mood. Another study that used sensory decision theory reached the opposite conclusions [64], but in this study the large amount of Δ^9-THC that was consumed by the volunteers (an average of 19.4 marijuana cigarettes per day for high consumption and 13.1 for moderate users) almost certainly produced drug tolerance, which develops rapidly with cannabinoids, and may have confounded the results, making the data very difficult to interpret.

Raft and colleagues [65] demonstrated in healthy subjects that intravenously administered Δ^9-THC (0.022 and 0.044 mg/kg) increased pain threshold (the lowest intensity of stimulation that gives rise to pain) but not pain tolerance (the intensity at which pain becomes unbearable) to mechanical and electrical stimulation. Hill and colleagues [66] also measured pain thresholds and tolerance. In this study, healthy volunteers inhaled marijuana smoke. Marijuana smoking lowered the pain threshold as well as pain tolerance. A drawback of this study is the inability to state the dose with any accuracy, a possible basis for the fact that it is at variance with the results of Raft and colleagues [65].

A recent study employing topical administration of the cannabinoid agonist HU-210 has demonstrated its effectiveness in reducing the magnitude of pain produced by capsaicin as well as mechanical and thermal hyperalgesia and allodynia in human volunteers [67]. This was a particularly intriguing finding because topical application led to reduced pain sensation with no observable psychotropic effects.

Studies of cannabinoid agonists on clinical pain in humans

The studies discussed in this section are the most compelling, because the subject population was drawn from patients suffering from significant chronic clinical pain. Chronic pain differs from acute pain due to neural changes that occur with prolonged noxious stimulation. These changes lower the threshold for pain (allodynia) and heighten the painfulness of noxious stimulation (hyperalgesia). The mechanisms underlying different classes of pain (e.g. inflammatory pain *versus* neuropathic or nerve injury pain) differ. Consequently, different analgesics exhibit different degrees of efficacy in chronic pain of different etiologies. For example, morphine is an excellent analgesic for inflammatory pain, whereas it frequently lacks efficacy in neuropathic pain [68]. Therefore, studies of different types of clinical pain are necessary precursors to drawing sound conclusions about the efficacy of cannabinoids for pain pharmacotherapy.

Positive results of cannabinoids have been found in the studies of cancer pain conducted by Noyes and colleagues [69, 70]. The patients in the study ($n = 36$) reported continuous pain of moderate intensity. In a double-blind random pattern, patients received on successive days placebo, 10 and 20 mg of Δ^9-THC, and 60 and 120 mg of codeine. Pain ratings by the patients were used to estimate pain-relief and pain-reduction scores. The results indicated that 20 mg of Δ^9-THC was roughly equivalent to 120 mg of codeine. Five of the 36 patients experienced adverse reactions to Δ^9-THC, one following 10 mg of Δ^9-THC, four following 20 mg. The effectiveness of cannabinoids on cancer pain has also been observed in animal models [71].

Neuropathic pain is a potential target for cannabinoid pharmacotherapies, which has been validated in preclinical as well as clinical studies. A double-blind study evaluated the effect of intramuscular administration of various doses of the Δ^9-THC analog levonantradol in moderate to severe postoperative or trauma pain. Levonantradol provided pain relief at all four doses studied (1.5–3.0 mg) compared to placebo. More than half of the patients reported side effects, the most frequent being drowsiness with other symptoms such as dizziness, mild hallucinations and nervousness occurring less frequently. Recently, Δ^9-THC was evaluated in multiple sclerosis patients with central neuropathic pain in a double-blind, placebo-controlled crossover design [72]. Orally administered Δ^9-THC (10 mg daily for 3 weeks) lowered median spontaneous pain-intensity scores and increased the median pain-relief scores relative to placebo treatment. The modest but clear therapeutic effect was associated with improvements on the SF-36 quality-of-life scale with no change in the functional ability of the multiple sclerosis patients. During the first week of treatment, adverse side effects of Δ^9-THC treatment (dizziness, light-headedness) were more frequent with Δ^9-THC than placebo, but the adverse effects decreased over the therapeutic course, possibly due to tolerance [72]. In agreement with positive results from clinical data, a substantial number of preclinical studies have found cannabinoids to be effective in models of

neuropathic or nerve-injury pain [44, 51, 73–80], sometimes with increased potency compared to effects in naive animals.

Data from the clinical studies tend to agree with centuries of anecdotal data showing the effectiveness of systemically administered cannabinoids against clinical pain. However, the unfortunate fact is that for the compounds tested to date, maximal analgesia could not be obtained from systemic administration of direct-acting CB_1 cannabinoid agonists at doses that do not elicit psychotropic effects. While typical cannabinoids are relatively safe (no deaths linked to overdose) and do not appear to have serious toxicity problems, psychotropic effects have limited the dosing, preventing cannabinoid agonists from reaching their full potential for use in the clinic. More work on developing agonists that lack psychotropic side effects would be beneficial, and may be fruitful based upon recent studies described below.

Development of non-psychotropic cannabinoids

One possible strategy that has emerged from the study of the cannabinoid system at the cellular and molecular level is the exploitation of differential signal transduction mechanisms that can be coupled to the cannabinoid receptor. Several studies reported that a single cannabinoid agonist could elicit different degrees of signal amplification across various regions of the brain [81, 82], and the various types of G-protein subunits that exist throughout the brain were activated to varying degrees [83]. Moreover, different cannabinoid agonists were found to evoke different levels of activation of a single G protein subtype [84, 85], and the G protein subtype selectivity is conferred by distinct intracellular domains of the receptor [86]. Hence, it is conceivable that an agonist could be developed that would activate cannabinoid receptors and signal transduction pathways associated with pain suppression but not those associated with psychotropic effects and motor dysfunction. This possibility is further supported by the separation of neural circuits that mediate cannabinoid motor dysfunction (basal ganglia, cerebellum) from those that mediate analgesia (periaqueductal gray, rostral ventral medulla, spinal cord, peripheral nerve) [87–89], as it allows for the compartmentalization of distinct G protein subtypes and second messengers to particular regions of the brain, and hence physiological functions. If the circuits mediating cannabinoid agonist-induced pain-suppressive effects rely principally on second messengers different from those responsible for psychotropic effects, then it may be possible to develop drugs that preferentially activate these signalling mechanisms and achieve a pain-suppression-specific cannabinoid agonist. Further investigation into this avenue of drug development is necessary to determine its practical feasibility.

Examples of results from recent work aimed at developing cannabinoids lacking psychotropic side effects are the Δ^9-THC and cannabidiol acid derivatives ajulemic acid (CT-3) and HU-320. These compounds were reported to produce anti-inflammatory effects with a reduced side-effect profile [90–92],

perhaps because they possess either modest (ajulemic acid) or virtually no (HU-320) affinity for either CB_1 receptors or CB_2 receptors. While ajulemic acid produces its effects via CB_1 receptors (unpublished findings from the authors' laboratory), HU-320 produces its effects by an unknown mechanism that is unlikely to be the CB_1R. At sufficient doses, CT-3 produces catalepsy [92] similar to that observed with Δ^9-THC. However, extensive dose studies would be required to determine whether there is a greater dose separation between its anti-nociceptive/anti-inflammatory effects and its psychomotor side effects than that observed with typical cannabinoids. In a recent clinical trial of patients suffering from neuropathic pain, ajulemic acid possessed some efficacy [93]. While many questions about these and similar compounds are awaiting further research, this appears to be an important line of inquiry.

Development of inhibitors of FAAH

Shortly after the isolation of the first endocannabinoid anandamide [5], the enzyme responsible for anandamide hydrolysis, FAAH, was described [94] and cloned [95]. In addition to anandamide two other endocannabinoids, 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyldopamine (NADA), also appear to be susceptible to degradation by FAAH [96, 97]. Immunohistochemical studies show that FAAH is present in the ventral posterior lateral nucleus of the thalamus [98–100], the termination zone of the spinothalamic tract, which carries pain input from the periphery. FAAH is also found in Lissauer's tract, which comprises primary afferent fibers entering the spinal cord, and in small neurons in the superficial dorsal horn, which is the termination zone of nociceptive primary afferents. These observations demonstrate that a mechanism capable of inactivating anandamide, 2-AG and NADA is present in regions of the central nervous system related to nociceptive processing and thus suggest a role for these ligands in pain modulation.

FAAH-knockout mice exhibit enhanced analgesic effects of exogenously administered anandamide in a CB_1 receptor-dependent manner, suggesting that the lack of this degradatory enzyme prolonged the action of anandamide [101]. Moreover, these animals exhibit tonic CB_1R-mediated analgesia in both acute and chronic pain paradigms concurrent with a marked elevation of endogenous anandamide levels [101, 102]. The results indicate that enhanced activity of endogenous cannabinoid(s) from the lack of FAAH in the transgenic animals caused blunted sensitivity to pain.

The studies discussed above suggest that inhibitors of FAAH would enhance the action of endogenous cannabinoids, thereby inhibiting pain. In fact, pharmacological agents that inhibit FAAH, such as phenylmethylsulfonyl fluoride (PMSF), palmitylsulfonyl fluoride (AM-374), methyl arachidonyl fluorophosphonate (MAFP) and arachidonoyl serotonin (AA-5-HT) produce a number of effects [94, 103–105]. However, as FAAH inhibitors, these agents have potential for improvement either in potency (often requiring micromolar

concentrations in *in vitro* assays) or specificity (often also acting on the CB_1R or other hydrolases). More recently, efforts from Boger's and Cravatt's groups have resulted in the development of ultra-potent (effective at low nanomolar concentrations) inhibitors of FAAH [106–108]. Some of these compounds, such as URB532, URB597 and BMS-1, also affected other hydrolases, whereas OL-135 was highly selective for FAAH and lacked activity at cannabinoid receptors [109]. OL-135 suppressed pain in rodents in a CB_1-dependent manner concurrent with elevated levels of endocannabinoids [109]. These results are encouraging for the development of a clinically effective agent for pain based on selective inhibition of FAAH.

Development of inhibitors of cellular transport of endogenous cannabinoids

Another approach for manipulating the endocannabinoid system is with inhibitors of the putative transport mechanism for the endocannabinoid anandamide. Blocking cellular transport would be expected to cause increased anandamide levels to occur in the vicinity of cannabinoid receptors, a similar consequence as that caused by inhibition of FAAH. Several transport inhibitors have been synthesized, beginning with the compound AM-404 [110], followed by VDM11 [111], OMDM-1, OMDM-2 [112], UCM707 [113] and UCM719 [114]. These compounds provided good separation in potencies for anandamide uptake (low micromolar range) *versus* FAAH inhibition, but most also bind to CB_1Rs at doses similar to those required for inhibition of anandamide uptake. The best separation of these effects was found with UCM707 and OMDM-1, which offered a 5–6-fold separation in dose. Following similar rationales as those for FAAH inhibitors, it is likely that further development of inhibitors of the cellular transport mechanisms for endocannabinoids may be fruitful for clinical pain relief.

Synergism between cannabinoid and opioid agonists

The first indication of the existence of synergistic (greater than additive) pain-suppressive effects from co-administration of cannabinoid and opioid agonists came from a study by Ghosh and Bhattacharya [115] when they found that cannabis enhanced the analgesic effect of morphine in the rat. Further study into this phenomenon, with major contributions from Welch's group, have provided information on the particular cannabinoid agonists, the dosages, and the routes of administration required for the synergy to occur [41, 116–118]. An isobolographic analysis has been reported in rats, which plots the theoretical ED_{50} for the drug combinations codeine/Δ^9-THC and morphine/Δ^9-THC [119]. These synergistic effects of cannabinoid and opioid agonists appeared to be receptor-mediated. Moreover, not only do low or inactive

doses of cannabinoids enhance opioid analgesia, inactive doses of opioids can also enhance cannabinoid analgesia [120, 121].

The synergistic pain-suppressive effects of cannabinoid and opioid agonists observed in the animal studies may provide the basis for a promising approach in clinical pain management. The synergism would allow lower doses of opioids and cannabinoids to be administered for attainment of a given degree of pain suppression. As moderate to high doses of most cannabinoid or opioid agonists alone often cause unpleasant psychotropic or physiologic side effects in humans, the use of lower doses of these drugs may improve their clinical utility in patients. Additionally, the reduced exposure to these drugs may slow the development of tolerance. It is also possible that the inclusion of a cannabinoid agonist in the regime could prevent the development of opioid tolerance, as the results of an animal study by Cichewicz and Welch [122] seemed to indicate. As opioids are emetic substances and cannabinoids are anti-emetic, the combination may improve the side-effect profile of both drugs. Hence it appears that the use of carefully titrated doses of a combination of cannabinoid and opioid agonists may provide a better quality of pain management for many conditions.

Summary and conclusions

A large body of literature from numerous preclinical studies as well as some clinical studies has demonstrated the ability of cannabinoids to suppress pain. The realization of the clinical potential of cannabinoids still requires more work in the areas of target refinement, drug selectivity and drug delivery. Promising approaches include development of CB_1 or CB_2 agonists, inhibitors of endocannabinoid degradation or transport mechanisms, combination dosing of cannabinoids with other analgesics such as opioids, and new delivery systems such as transdermal patches. Highly potent and selective pharmacological agents that focus on normalization of pain threshold and sensitivity without causing psychotropic side effects in humans are much desired. Along with drug development, further characterization of the cannabinoid system is important in providing insights that are integral to identifying potential therapeutic approaches.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Potential use of cannabimimetics in the treatment of cancer

Luciano De Petrocellis[1], Maurizio Bifulco[2], Alessia Ligresti[3] and Vincenzo Di Marzo[3]

[1] *Istituto di Cibernetica "Eduardo Caianiello", Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy*
[2] *Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, and Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano (SA), Italy*
[3] *Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), Italy*

Introduction

The medicinal use of *Cannabis sativa* preparations has a millennial history [1] and is currently being critically re-evaluated [2]. The hemp plant *Cannabis sativa* produces about 66 compounds known as cannabinoids, and the exact chemical structure of the major psychotropic principal, (–)-Δ^9-tetrahydrocannabinol (Δ^9-THC) [3], was only identified in 1964, after decades of attempts and failures. Δ^9-THC is highly hydrophobic and was initially thought to work by interacting directly with biomembranes. A few pharmaceutical items, such as Marinol® and Dronabinol®, both based on Δ^9-THC, and Cesamet®, which instead is based on a synthetic Δ^9-THC analog, nabilone, have been prescribed in the USA as anti-emetics and appetite-stimulants to cancer or AIDS patients even before the molecular mode of action of Δ^9-THC was revealed [4]. It took the development of more-potent and enantiomerically pure Δ^9-THC analogs to understand that psychotropic cannabinoids act via specific sites of action to produce their typical effects. The long-standing issue of the mechanism of action of Δ^9-THC was solved with the discovery of cannabinoid receptors [5], and then of the *endocannabinoids*, endogenous agonists at cannabinoid receptors [6]. Two such receptor types have been cloned and characterized in mammalian tissues; they are coupled to $G_{i/o}$ proteins, through which they inhibit the adenylate cyclases, stimulate mitogen-activated protein kinases, and modulate the activity of Ca^{2+} and K^+ channels to transduce the binding of agonists into biological responses (see [7] for a review). CB_1 receptors are expressed in several brain regions, with very high concentrations in the basal ganglia, hippocampus, cerebellum and cortex, and mediate the typical psychotropic effects of *Cannabis*, marijuana and Δ^9-THC. Lower, albeit functionally active, amounts of CB_1 receptors are also found in peripheral neurons and various extra-neural sites

such as the testis, eye and vascular endothelium, as well as in many epithelial cells. CB_2 receptors are mostly confined to immune tissues and seem to underlie the immune-suppressant actions of Δ^9-THC [6]. Both CB_1 and CB_2 receptors are expressed from the early stages of fertilized oocyte development [8], and CB_1 expression in the developing brain is significantly different from that observed in the adult brain [9]. Several other plant cannabinoids, with little or no psychoactive action, have been identified and their possible therapeutic actions investigated. In particular, cannabidiol [10] appears as a promising therapeutic tool, even though its sites of action are not yet well understood.

The endogenous cannabinoid receptor ligands (endocannabinoids) identified so far are all derivatives (amides, esters, ethers) of long-chain polyunsaturated fatty acids, and exhibit varying selectivity for the two cannabinoid receptors [7] as well as for other molecular targets [5]. The two best-studied endocannabinoids are anandamide (*N*-arachidonoylethanolamine) and 2-arachidonoyl glycerol (2-AG) [11–13], and appear to be ubiquitous in mammalian tissues. Endocannabinoids, with their receptors [14, 15] and specific processes of ligand synthesis [16, 17], cellular uptake [16, 18] and degradation [19, 20], constitute the so-called endocannabinoid system.

The previous knowledge of Δ^9-THC pharmacology [21] and, most importantly, recent studies carried out by using multiple pharmacological, biochemical, analytical and genetic approaches [22], have revealed several possible functions of endocannabinoid signaling under both physiological and pathological conditions. Endocannabinoids have been proposed to act as retrograde messengers [23] being released from the post-synaptic cell following its depolarization, to then act back on CB_1 on pre-synaptic neurons to inhibit neurotrasmitter release. Due to their chemical nature as lipophilic compounds, and their peculiar biosynthetic mechanisms, endocannabinoids appear to act as local mediators in an autocrine and/or paracrine manner, and their modulatory activities on proteins and nuclear factors involved in cell proliferation, differentiation and apoptosis suggest that the endocannabinoid signaling system is involved, *inter alia*, in the control of cell survival, proliferation, transformation or death [24].

The anti-neoplastic activity of Δ^9-THC and its analogs was first observed in the early 1970s, when neither cannabinoid receptors nor endocannabinoids had yet been discovered. Although these observations were of potential interest, no in-depth investigations were performed on this topic until 7 years ago, when the effects of plant, synthetic and endogenous cannabinoids on cancer cell proliferation and apoptosis started to be revisited. By contrast, the beneficial effects of cannabinoids on some cancer-related disorders, such as emesis, nausea, depression, muscle tension, insomnia, chronic pain and appetite suppression, have been in part exploited pharmaceutically, since oral Δ^9-THC (dronabinol®, marinol®) can be prescribed legally in the USA for the treatment of nausea and emesis and as an appetite-stimulating drug for cancer patients undergoing chemotherapy and patients with AIDS, respectively. The results of a large body of recent studies now suggest that targeting the endocannabinoid

system might provide a significant contribution to both palliative and curative cancer therapies.

Cannabinoid receptor stimulation causes inhibition of cancer growth through multiple intracellular mechanisms and pathways

Based on *Cannabis* perturbation of the immune response, *in vivo* studies carried out in animals in the late 1990s investigated the possibility that marijuana smoking and long-term Δ^9-THC treatment may favor tumor growth. These studies, however, often produced opposing outcomes. For example, the enhancement of lung carcinoma was seen [25], and more recently it has been demonstrated that the treatment of glioma and lung carcinoma cell lines with nanomolar concentrations of Δ^9-THC, comparable with those detected in the serum of patients after Δ^9-THC administration, leads to accelerated cancer cell proliferation dependent on metalloprotease and epidermal growth factor receptor (EGFR) activity [26]. While in this study the involvement of cannabinoid receptors was not investigated, in another recent work low concentrations of Δ^9-THC also stimulated the proliferation of prostate carcinoma cells *in vitro*, in a CB_1-/CB_2-mediated manner and androgen receptor-dependent manner [27]. However, Munson and coworkers showed about 30 years ago that Δ^9-THC inhibits lung adenocarcinoma cell growth *in vitro* and after oral administration in mice [28], and a recent 2-year chronic administration study with high Δ^9-THC doses revealed a *reduction* of the spontaneous onset of hormone-dependent tumors in particular [29]. Experiments carried out *in vitro* seem to go more often in the direction of an anti-proliferative property of CB_1 and CB_2 receptor agonists (Fig. 1). For example, it was found that 4–5-day treatment of human breast cancer cell (HBCC) lines with sub-micromolar concentrations of endocannabinoids results in complete blockade of their proliferation [30]. CB_1 activation blocks the cell cycle at the G_0/G_1–S transition via the inhibition of adenylate cyclase and the cAMP/protein kinase A pathway. Protein kinase A phosphorylates and inhibits Raf1, and therefore anandamide, by preventing the inhibition of Raf1, induces the sustained activation of the Raf1/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling cascade [31]. These signaling events result in the inhibition of the expression of the long form of the receptor for endogenous prolactin [30], a hormone that HBCCs in culture use as an autocrine growth factor. In fact, agents activating the CB_1 receptor via the same mechanism also counteract the proliferation of human prostate cancer cells when induced by exogenous prolactin [32]. Indeed, both human breast and prostate cancer cells express high levels of CB_1 receptors that had never been detected previously in the corresponding healthy tissues. HBCCs also respond to nerve growth factor (NGF) by proliferating more rapidly, and 2-day treatment of HBCCs with CB_1 receptor agonists suppresses the levels of *trk* proteins, one of the two known types of NGF receptor, thus resulting in the inhibition of NGF-induced proliferation [32].

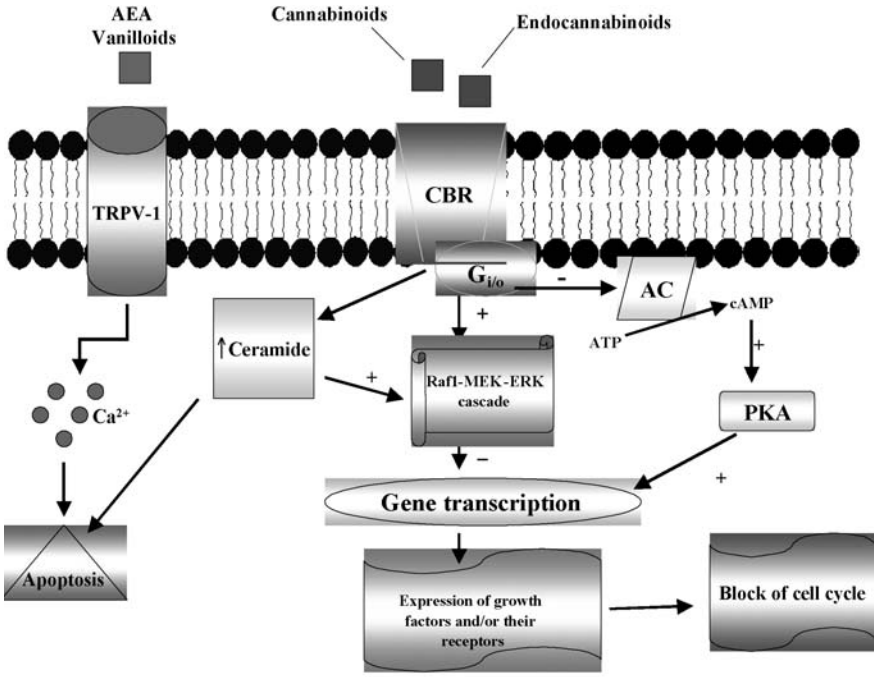

Figure 1. Possible ways for endocannabinoids to inhibit cancer growth. Stimulation of cannabinoid receptors by endocannabinoids can interfere with tumor growth in two possible ways: (1) by stopping the cancer cell cycle (anti-proliferative or anti-mitogenic effect) via inhibition of the activity of $p21^{ras}$ or of the expression of growth factors and/or of their receptors; (2) by promoting cancer cell apoptosis. The intracellular pathways mediating these two effects are schematically depicted in Figure 2. Furthermore, some endocannabinoids, like anandamide or *N*-arachidonoyldopamine [115], can also cause cancer cell apoptosis by both activating CB_1 receptors and gating vanilloid TRPV1 receptors. The latter effect causes a strong Ca^{2+} influx that can then lead to apoptosis and in some cases also to cell toxicity. AC, adenylate cyclase; AEA, *N*-arachidonoylethanolamine (anandamide); CBR, cannabinoid receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; PKA, protein kinase A; TRPV1, transient potential receptor vanilloid type 1 channel.

Substances that activate CB_1 receptors might also exert more general anti-tumor as well as anti-angiogenic effects by interfering with the expression of other growth and mitogenic factors (Fig. 2). CB_1 receptor activation also induces cell-cycle arrest at the G_0/G_1–S transition in thyroid epithelioma cells (KiMol Cells) obtained from the transformation of rat thyroid epithelial cells with the K-*ras* oncogene. Furthermore, repeated intra-tumor administration of a very low and non-psychotropic dose of a metabolically stable and more potent CB_1 receptor ligand, met-fluoro-anandamide, inhibits the growth of tumors induced in nude mice by injection of these cells [33]. This effect is accompanied by a strong reduction of the activity of the K-*ras* oncogene protein product, $p21^{ras}$. It was also shown that the expression of CB_1 receptors is regulated in healthy and transformed thyroid cells (as well as in tumors derived from these latter cells) in opposite ways following treatment with met-fluo-

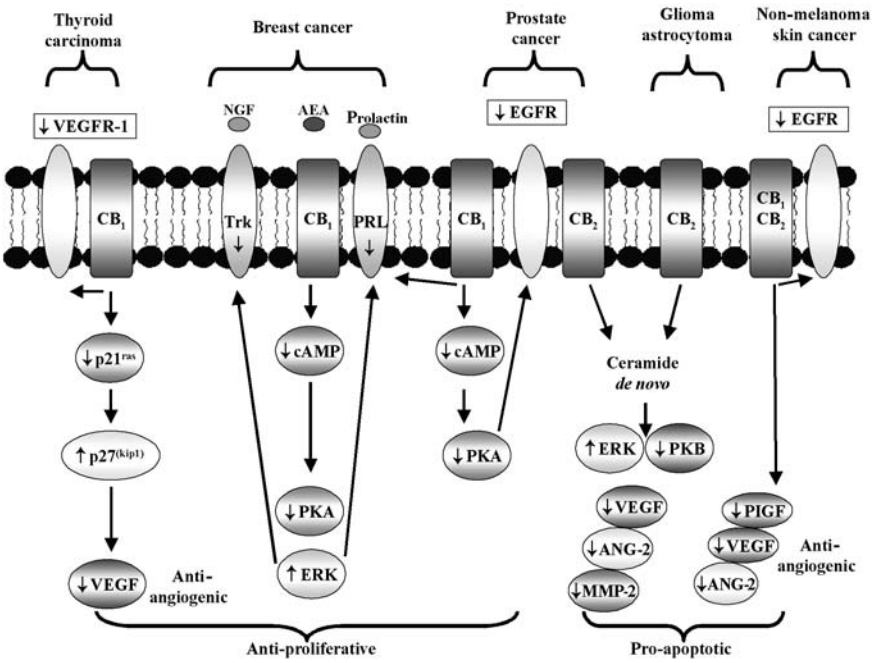

Figure 2. Mechanisms for cannabinoid receptor-mediated inhibition of cancer growth and spreading. The different intracellular signaling pathways implicated in cannabinoid receptor-mediated inhibition of cancer cell proliferation and metastasis, stimulation of cancer cell apoptosis and inhibition of endothelial cell proliferation and migration (and hence of angiogenesis) are shown. Under certain conditions, however, Δ^9-THC and anandamide have been found to stimulate either EGFR or androgen receptor expression, and to lead to enhanced proliferation *in vitro* of glioma and prostate carcinoma cells, respectively [26, 27]. AEA, *N*-arachidonoylethanolamine (anandamide); ANG-2, angioprotein-2; EGFR, epithelial growth factor receptor; ERK, extracellular signal-regulated kinase; MMP-2, matrix metalloproteinase-2; NGF, nerve growth factor; $p27^{(kip1)}$, cyclin-dependent kinase inhibitor; PIGF, placental growth factor; PKA, protein kinase A; PKB, protein kinase B; PRL, long form of the prolactin receptor; Trk, high-affinity neurotrophin receptor; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor-1. Adapted from [116].

ro-anandamide, by being suppressed or enhanced in healthy or cancer cells, respectively. Thus, the degree of CB_1 receptor expression determines the extent of the responsiveness of normal or transformed FRTL-5 cells to (endo)cannabinoids [33].

Inhibition of growth factor receptor signaling following CB_1 receptor activation has been shown also in pheochromocytoma [34], skin carcinoma [35] and prostate carcinoma cells [36] and is likely to be a general mechanism underlying the anti-proliferative actions by (endo)cannabinoids (Fig. 2). However, another molecule involved in cancer cell proliferation by governing cyclin-dependent kinase 2 activity, and whose over-expression can block the cell cycle in the G_1 phase during the G_1–S transition, is the p27 protein, which is also under the negative control of the *ras* oncogene. Accordingly, met-fluo-

ro-anandamide treatment of rat thyroid epitheliomas *in vitro* and *in vivo*, with subsequent inhibition of $p21^{ras}$, leads to sensibly increased levels of p27, an effect that may contribute to the anti-proliferative actions of (endo)cannabinoids in this model [37].

Finally, as shown by using various biochemical and pharmacological approaches, the stimulation of CB_2, or of both CB_1 and CB_2, receptors can also lead to significant counteraction of tumor growth via various mechanisms [38, 39]. In fact, inhibition of cell mitosis was shown not to be the only mechanism through which cannabinoids block solid tumor growth, particularly when CB_2 or other non-CB_1 receptors (see below) are involved. Δ^9-THC was found to induce the programmed death (apoptosis) of glioma and prostate cancer cells [40, 41]. The increased ceramide levels observed in glioma cells after cannabinoid action would drive the prolonged activation of the Raf1/MEK/ERK signaling cascade [42] and Akt inhibition [43], as well as induction of cyclooxygenase-2 (COX-2) expression [44]. While the relation between ERK activation and cell fate is complex and depends on many factors [45], the involvement of oxidative stress [46] and stress-activated protein kinases [47] cannot be ruled out during (endo)cannabinoid-induced apoptosis (Fig. 2). At any rate, these effects of CB_2 receptor stimulation result in glioma cell apoptosis *in vitro*, and in powerful inhibition of glioma growth *in vivo* [38, 42], although some of the tumors developed *in vivo* can be significantly less sensitive to Δ^9-THC than others [42].

Cannabinoid receptor stimulation leads to inhibition of tumor angiogenesis and metastasis

Expression of various oncogenes, particulary *ras*, can lead to a marked induction of a potent paracrine stimulator of angiogenesis, the vascular endothelial growth factor (VEGF) [48] (Fig. 2). The enhanced expression of VEGF is associated with a large number of human tumor types, including human thyroid tumors and cancer cells. The observation that met-fluoro-anandamide is able to block $p21^{ras}$ activity [33], and that endocannabinoids can inhibit the expression of several growth factors and/or their receptors (see above), suggested that CB_1 receptor stimulation could also interfere with VEGF and VEGF receptors. Indeed, in rat thyroid epitheliomas, met-fluoro-anandamide was found to inhibit the growth of already established tumors, in part by reducing the expression of VEGF and of the VEGF receptor Flt-1, which plays a crucial role in mediating VEGF-induced neo-angiogenesis and endothelial cell proliferation [37]. The expression of both VEGF and Flt-1 was suppressed not only in the tumor *in vivo* but also in tumor cells *in vitro*, indicating that the blockage of VEGF signaling may have a direct effect on tumor growth and metastasis, not only by blocking neo-angiogenesis but also by disrupting VEGF/VEGF receptor autocrine pathways [49].

It has been reported that CB_1 and CB_2 cannabinoid receptors are expressed in normal epidermis and in mouse skin tumors, and in this case both receptors

are functional in the regression of skin carcinomas, which may also rely on the inhibition of tumor angiogenesis. The blood vessels developed by cannabinoid-treated carcinomas were in fact small, and the expression of pro-angiogenic factors was depressed [35]. Once more, *ras* activation seems to be crucial in mouse skin carcinoma initiation and angiogenesis in which VEGF plays a pivotal role [50].

Also in a mouse model of glioma, local administration of a CB_2 receptor-selective agonist inhibits angiogenesis of malignant gliomas as determined by immunohistochemical and functional analyses [51]. *In vitro* and *in vivo* studies have shown a direct inhibition by cannabinoids of vascular hyperplasia characteristic of actively growing tumors into a pattern of small, differentiated and impermeable blood capillaries. This is once more associated with a decreased expression of VEGF and other vascular pro-angiogenic factors. Furthermore the activation of cannabinoid receptors inhibited endothelial cell migration and survival. Interestingly, the expression and activity of matrix metalloproteinase-2, a proteolytic enzyme that allows tissue remodelling during angiogenesis and metastasis, was also decreased by cannabinoids [51]. More recently, Δ^9-THC was also shown to reduce the expression in gliomas of the VEGF receptor, VEGFR-2, both *in vitro* and *in vivo*, and via blockade of ceramide biosynthesis [119].

Finally, it was observed that the CB_1-mediated anti-proliferative effect of met-fluoro-anandamide on thyroid cancer cells was much more efficacious on metastasis-derived cells than on the primary cancer line, possibly due to an upregulation of CB_1 receptors in the former cells (see below). Accordingly, in the Lewis lung carcinoma model of metastatic spreading, met-fluoro-anandamide was found to interfere efficaciously with the formation of lung metastatic nodules by acting at CB_1 receptors [37]. The mechanism through which stimulation of CB_1 receptors can lead to inhibition of the cellular processes involved in cancer cell metastatic spreading, including cell motility and adhesion, are currently under investigation in our laboratories. However, preliminary data on the inhibition of the migration of SW 480 colon carcinoma cells by anandamide and the selective CB_2 receptor agonist JWH133, via CB_1- and CB_2-receptor-mediated mechanisms, respectively, have been published [52]. Furthermore, experiments carried out by using human prostate cancer cells, also showed that 2-AG can inhibit invasion *in vitro* only in androgen-independent cells and via inhibition of cAMP- and protein kinase A-mediated signaling [120].

The endocannabinoid system attempts to provide protection from the growth and spread of cancer

The increasing expression of cannabinoid receptors in cancer cells and tissues observed with their increasing degree of malignancy and invasiveness, for example in astrocytomas and transformed thyroid cells [33, 38], might suggest

a possible role of the endocannabinoid system in the tonic suppression of the growth and spread of some tumors. In support of this hypothesis it was found that alterations also of anandamide or 2-AG levels occur in many tumors as compared to the corresponding healthy tissues ([53–55], see also [56]). In particular, an enhancement of endocannabinoid levels was observed in human breast cancers and prostate carcinomas ([55], see also [56]), in human pituitary tumors [53] and in human colorectal carcinomas [57]. In addition, human colorectal cancer Caco-2 cells loose their capability to respond to cannabinoid receptor agonists, and make less endocannabinoids, when they differentiate into non-malignant cells [57]. As cells from these tumors all respond to cannabinoids with inhibition of proliferation, or by entering apoptosis, it was suggested that endocannabinoids are endogenously over-produced in malignant tissues and cells, and cannabinoid receptors (and other endocannabinoid molecular targets) are subsequently over-stimulated, in the attempt to counteract cancer growth and spread. So far, evidence for this hypothesis has been obtained mostly *in vitro*. Anandamide and its congeners are produced by HBCCs, and a substance that elevates the levels of anandamide in these cells also inhibits cell proliferation [58], suggesting that synthetic compounds that selectively inhibit endocannabinoid degradation might also be used to inhibit cancer growth. In fact, two selective and specific inhibitors of endocannabinoid inactivation were recently found to inhibit Caco-2 cell proliferation in a CB_1-receptor-mediated manner and through elevation of cell endocannabinoid levels [57]. Palmitoylethanolamide, an anandamide congener that is synthesized in higher amounts than anandamide in all tumor cells analysed so far, inhibits the degradation of endocannabinoids in HBBCs, and may act as an endogenous enhancer of the tumor-suppressing activity of this endocannabinoid, whether this is exerted via CB_1 [59] or other ([60] and see below) receptors. Data from our laboratories indicate that this endogenous control of cancer growth by endocannabinoids can also occur *in vivo*, as two selective inhibitors of anandamide and 2-AG inactivation induce a strong CB_1-mediated inhibition of rat thyroid epithelioma growth in athymic mice by enhancing the tumor levels of these two compounds [117]. Furthermore, it was recently shown that inhibition of 2-AG degradation and biosynthesis can inhibit and enhance, respectively, the invasion of human prostate cancer cells *in vitro* [120].

Non-CB_1/-CB_2 receptors are also involved in (endo)cannabinoid anti-tumor actions

An ever-increasing number of reports (reviewed recently by Pertwee [61]) suggests that endocannabinoids might exert their biological effects also through non-CB_1/-CB_2 receptors. Regarding the inhibition of cancer growth, experiments carried out in our laboratories have shown that, rather than favoring cancer cell proliferation, the CB_1-selective antagonist SR-141716A causes a

slight, albeit significant, anti-proliferative effect both *in vitro* and *in vivo* [30, 32, 117]. This might suggest that non-CB_1-receptor-mediated anti-tumor effects of endocannabinoids might be unmasked when CB_1 receptors are blocked. In recent studies [62, 118] the apoptotic effect of anandamide on glioma cells was suggested to be mediated by another proposed target for this compound, the transient receptor potential (TRP) vanilloid type 1 (TRPV1) channel, also known as the VR1 receptor. This protein is a member of the large family of TRP non-selective cation channels, and is activated by heat, protons, plant toxins such as capsaicin and resiniferatoxin, and by some other endogenous arachidonate-derived metabolites (see [63, 64] for reviews). However, the mechanism through which anandamide induces apoptosis in TRPV1- and CB_1-expressing cells – that is, whether it involves both CB_1 and TRPV1 receptors, or only TRPV1 receptors with CB_1 receptors instead playing a protective role against TRPV1-induced apoptosis – is still controversial, and might depend on the experimental conditions used for the experiments [65–68]. In HBCCs, arvanil, a synthetic substance that activates both CB_1 and TRPV1, exhibits a more potent anti-proliferative activity *in vitro* than 'pure' agonists of either receptor class, and this action can be attenuated by both CB_1 and TRPV1 antagonists [65]. In glioma cells, anandamide causes apoptosis by acting via both CB_1 and TRPV1 receptors [66] or only via TRPV1, depending on the cell culture conditions [62]. In uterine cervix cancer cells *in vitro*, however, possibly due to aberrant over-expression of TRPV1, anandamide induces apoptosis only via activation of these receptors, and activation of CB_1 receptors again counteracts this effect [67]. Therefore, the actual role of TRPV1 channels in anandamide-induced inhibition of cancer cell growth (Fig. 1) is still not fully understood. Furthermore, it remains to be established whether the slight anti-cancer effect of SR-141716A is due to endocannabinoids being re-directed towards TRPV1, to counteract the protective action against apoptosis exerted by CB_1 under certain conditions, or to other, entirely unrelated and as-yet-identified, mechanisms of action of the CB_1 antagonist. Vanilloid compounds themselves have been shown to inhibit the proliferation and induce apoptosis of both cancer and non-transformed cells *in vitro* by acting via both TRPV1-and non-TRPV1-mediated mechanisms [68–70], and palmitoylethanolamide was found to enhance the antiproliferative effect on HBCCs not only of anandamide and other CB_1 agonists, but also of vanilloids [60]. Therefore, one could envisage the use of this compound, co-administered with either met-fluoro-anandamide or capsaicin derivatives, to lower the threshold of the anti-tumor effects of these compounds to doses that do not exhibit either undesired psychotropic activity or toxicity to healthy cells, respectively.

Plant cannabinoids such as cannabidiol, on the one hand, and Δ^9-THC and cannabinol, on the other, were shown to activate TRPV1 receptors [71] and the ANKTM1 channel (another member of the TRP family of proteins [72]), respectively. In fact, *Cannabis* components with little or no activity on cannabinoid CB_1 and CB_2 receptors have been shown in the past to exhibit anti-neoplastic activity *in vitro* [73]. Cannabidiol has anti-tumor effects on

human glioma cell lines [74–76], and inhibits the growth of human glioma cells subcutaneously implanted in nude mice [76] as well as of human acute myeloid leukaemia [77], in both cases through induction of apoptosis. Cannabigerol inhibits the growth of human oral epitheloid carcinoma cells [78]. The synthetic ajulemic acid (CT3), inhibits glioma cells *in vitro* and *in vivo* [79] and induces apoptosis in human T lymphocytes [80], although the lack of activity of this compound at CB_1 receptors is still a matter of controversy. The molecular mechanisms for the anti-cancer effects of most of these compounds are not yet understood, and are currently under investigation in our laboratories. TRPV1 receptors, however, do not seem to be involved in the inhibitory effect of cannabidiol against glioma [76].

Substances targeting the endocannabinoid system in cancer therapy – pros and cons

The anti-tumor potential of substances that modulate the activity of cannabinoid receptors or the levels of endocannabinoids, as well as of other possible targets for the anti-cancer action of these compounds, are still largely unexplored. It seems that cannabinoids selectively affect tumor cells but not their non-transformed counterparts and might even protect the latter from cell death (see above). For example, cannabinoids induce apoptosis of glioma cells in culture [38, 42, 74, 81], but, by contrast, they protect glial cells from apoptosis [82, 83], possibly due to a differential ability to synthesize ceramide [81]. Indeed, a general protective role of the endocannabinoid system is emerging from several recent studies (for a recent review see [84]). Cannabinoids appear to be well tolerated in animal studies and do not produce the generalized toxic effects in normal tissues that are a major limitation of most conventional agents used in chemotherapy, the median lethal dose of Δ^9-THC in animals being of several grams per kilogram of body weight [85]. However, together with obvious social, political and legal considerations, the therapeutic application of agonists selective for CB_1 receptors might be limited by the undesired psychotropic side effects expected from the stimulation of these receptors in the brain (even though some 'central' actions of CB_1 activation may be, instead, desirable, as discussed below). On the other hand, although devoid of psychotropic actions, the administration of compounds selective for the CB_2 receptor, as in the treatment of gliomas, skin carcinomas and lymphomas, might cause the immune-suppressive effects typical of Δ^9-THC, and this would then play against the organism's own defense against tumor growth. Yet selective CB_2 receptor agonists appear to be nowadays very efficacious against some types of pain [86], which could represent an additional benefit of anti-cancer drugs derived from these compounds. The limitations at least of CB_1-selective agonists might be overcome by the use of metabolically stable endocannabinoid analogs in combination with a non-psychotropic substance like palmitoylethanolamide, which might lower the threshold of concentra-

tions necessary to observe the tumor-suppressing effect [60]. Another approach for the development of new anti-cancer drugs would be the use of selective inhibitors of endocannabinoid degradation. These substances would be devoid of most psychotropic effects as they would preferentially act in those tissues where the levels of endocannabinoids are pathologically altered [87].

Another possible difficulty for the exploitation of (endo)cannabinoids against cancer growth is their very low solubility in water and their poor bioavailability when given orally. It seems that *Cannabis* is more efficient when smoked, but this administration route presents all the very well known and unwelcome consequences [88]. A very promising alternative has been recently proposed by GW Pharmaceuticals with Sativex®, a *Cannabis* extract containing Δ^9-THC and cannabidiol that is sprayed sublingually and is now undergoing clinical trials [89]. Alternatively, the use of water-soluble cannabinoids such as O-1057 might solve the solubility problems [90, 91], but to date the intra-tumor application of low doses of cannabinoids seems to represent the most viable option for those types of tumor that can be treated in this way, as it results, in animal models, in few if any undesired 'central' effects. The safety and efficacy of such type of administration of Δ^9-THC to treat glioma in humans is currently being assessed in a pre-clinical study in Spain [45].

Probably the greatest advantage offered by the use of cannabimimetics over other conventional anti-cancer agents might reside in their beneficial effects on some serious cancer-related disorders in humans, as follows. (1) Cannabinoids are anti-emetics in animal models of vomiting [92]. Marinol® and Cesamet® are approved to treat nausea and emesis associated with cancer chemoterapy [93, 94]. Modern anti-emetics are selective serotonin 5-HT_3-receptor antagonists, and, although cannabinoids can block 5-HT_3-receptors [95] they have a very distinct pharmacological profile. Hence, further studies should be performed to establish the mechanism of action and what types of cancer chemotherapies are suitable to cannabinoid anti-emetic treatment [96–99]. It is interesting to note that the potent TRPV1 agonist resiniferatoxin antagonizes cisplatin-induced emesis in dogs [100]. (2) Δ^9-THC and other cannabinoids reinforce appetite and increase food intake, seemingly via inhibition of anorexic signals [101–103]. Anorexia and cachexia are, in fact, primary problems in cancer patients. However, a recent phase III trial has questioned the efficacy of oral Δ^9-THC appetite-stimulating effects in advanced cancer [104]. (3) The neuromodulatory actions of endocannabinoids in the central, sensory and autonomic nervous systems result, mostly via CB_1 receptors, in the regulation of pain perception [105]. Cannabinoids produce spinal, supra-spinal and central analgesia by suppressing the activity of nociceptive circuits [106]. Peripheral CB_2 might mediate local analgesia [107] and might be important for cancer pain [108]. At the moment, cannabinoids seem to be no more potent than codeine (for a review see [109]), but clinical trials on their use for the treatment of cancer pain are in progress. Interestingly, the synergic actions of cannabinoid CB_1 receptor agonist with opioid receptor agonists produces, in animal models, analgesic actions stronger and longer-lasting (through the avoidance of the development of mor-

phine tolerance) than those obtained with each agonist alone [110, 111]. (4) Finally, studies in animals have shown that cannabinoids or agents that enhance the endogenous levels of endocannabinoids exert anti-anxiolytic effects [112, 113] and mediate the extinction of aversive memories [114], with potential beneficial psychological effects on cancer patients.

In conclusion, the use of cannabinoid receptor agonists and/or substances inhibiting endocannabinoid degradation might not only retard the growth of tumors via multiple mechanisms, but also alleviate simultaneously the weight loss, nausea and pain that so badly affect the life of women and men suffering from cancer. As multiple and different approaches are more and more likely to solve successfully the issue of cancer treatment, one can only hope that the further pre-clinical and clinical research that is certainly needed to substantiate this hypothesis will provide ever more encouraging results in this direction.

Acknowledgements
The authors thank the Associazione Italiana per la Ricerca sul Cancro and GW Pharmaceuticals, UK, for supporting their studies on this subject.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabinoids: effects on vomiting and nausea in animal models

Linda A. Parker, Cheryl L. Limebeer and Magdalena Kwiatkowska

Department of Psychology, Wilfrid Laurier University, Waterloo, Ontario, Canada N2L 3C5

Introduction

The development of chemotherapy treatment has prolonged the lives of many cancer patients. However, use of these powerful drugs presents a serious challenge to both clinicians and patients. Significant side effects of cancer chemotherapy include nausea and vomiting which may last for several days. These symptoms come to be dreaded by patients, often interfering with successful completion of treatment. The emetic reflex is conventionally considered to include vomiting, retching and the more subjective sensation of nausea. However, the organization of the reflex is very complex, because although nausea, retching and vomiting usually occur in a temporal sequence, they can be separated experimentally [1].

Vomiting is a widespread protective reflex that serves to expel accidentally ingested toxins from the upper gastrointestinal tract. The sensation of nausea serves as a warning (as does pain), and usually results in the cessation of ingestion and an associative aversion to the ingestant in the future. The act of vomiting is often followed by a feeling of well-being which may serve to reinforce that behavior [1]. In the case of chemotherapy patients, however, the vomiting reflex does not remove the perceived toxin; therefore, in contrast to the removal of an ingested toxin from the gut, vomiting is not self-limiting [1].

Chemotherapy patients experience three separate types of emetic episode: (1) acute nausea and/or vomiting occurs within minutes to hours of receiving a dose of a toxic chemotherapy drug, (2) delayed nausea and/or vomiting that has been arbitrarily defined as emesis begins or persists more than 24 h after chemotherapy, and (3) anticipatory nausea and/or vomiting (ANV) occurs when the patient is re-exposed to cues associated with the toxin. ANV occurs in nearly half of patients treated, frequently during later cycles of chemotherapy [2]. The more intense the initial acute emetic episode, the worse the resultant ANV.

A major advance in the control of emesis was the finding that blockade of one subtype of the 5-hydroxytryptamine (5-HT) receptor, the 5-HT_3 receptor, could suppress the acute emetic response (retching and vomiting) induced by cisplatin in the ferret and the shrew [3–7]. In clinical trials with humans, treatment with

5-HT_3 antagonists often combined with the corticosteroid, dexamethasone, during the first chemotherapy treatment has reduced the incidence of acute vomiting by 70–90% [1, 8–14]. If acute vomiting is prevented, the incidence of delayed and anticipatory vomiting is reduced [2, 8–11, 20]. However, the 5-HT_3 antagonists are less effective at suppressing acute nausea than they are at suppressing acute vomiting [1, 9, 10, 14, 20] and they are ineffective in reducing instances of delayed nausea/vomiting [13, 15–20] and ANV [1, 10, 11, 14, 20–22] when they do occur. Therefore, it is likely that another system may be involved in chemotherapy-induced nausea, delayed nausea/vomiting and ANV. Two such systems include the neurokinin 1 (NK_1) tachykinin receptors for substance P (e.g. [16, 17, 23]) and the endocannabinoid system [24–39]. The effect of cannabinoids on nausea and vomiting is the subject of this review.

Cannabinoids as anti-emetics

The marijuana plant has been used for several centuries for a number of therapeutic results, including nausea and vomiting [40]; however, it was only recently that Gaoni and Mechoulam [41] isolated the major psychotropic component, Δ^9-tetrahydrocannabinol (Δ^9-THC). Twenty-five years later, the specific brain receptors for this compound, cannabinoid$_1$ (CB_1) and cannabinoid$_2$ (CB_2), were identified (for review see [42]) and cloned [43]. Therefore, it was only natural to start the search for an endogenous ligand for the cannabinoid receptor, which was discovered 2 years later [44]. This ligand was the ethanolamide of arachidonic acid, and called anandamide. A second type of endocannabioid was discovered in 1995 [45], also a derivative of arachidonic acid, but its ester, 2-aracidonoyl glycerol (2-AG). Both anandamide and 2-AG are rapidly inactivated after their formation and release by the enzyme fatty acid amide hydrolase (FAAH) [46].

The anti-emetic effects of cannabinoids appear to be mediated by action at the CB_1 receptor. CB_1 receptors are found in the gastrointestinal tract and its enteric nervous system [47] as well as within the emetic system of the brain [34, 35] in the dorsal vagal complex, consisting of the area postrema (AP), nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMNX) in the brainstem of rats, ferrets and the least shrew [33, 34]. Recent reviews on the gastrointestinal effects of cannabinoids have concluded that cannabinoid agonists act mainly via peripheral CB_1 receptors to decrease intestinal motility [47], but act centrally to attenuate emesis [34, 35]. The dorsal vagal complex is involved in the nausea and/or vomiting reactions induced by either vagal gastrointestinal activation or several humoral cytotoxic agents. It is considered the starting point of a final common pathway for the induction of emesis in vomiting species. CB_1 receptors in the NTS are activated by Δ^9-THC and this activation is blocked by the selective CB_1 antagonists SR-141716 [28] and AM-251 [35]. Indeed, c-Fos expression induced by cisplatin in the DMNX, specific subnuclei of the NTS and AP is significantly

reduced by Δ^9-THC [34, 35]. Endogenous cannabinoid ligands, such as anandamide, as well as synthetic cannabinoids, such as WIN-55,212-2, also act on these receptors [33].

Recent findings indicate that the cannabinoid system interacts with the serotonergic system in the control of emesis. The dorsal vagal complex not only contains CB_1 receptors, but is also densely populated with 5-HT_3 receptors [48, 49], potentially a site of anti-emetic effects of 5-HT_3 antagonists. Anandamide has also been reported to interact with serotonin [50]. Cannabinoid receptors are co-expressed with serotonin 5-HT_3 receptors in some neurons in the central nervous system [51] and inhibitory functional interactions have been reported between cannabinoid CB_1 and 5-HT_3 receptors [52, 53]. Additionally, canabinoids reduced the ability of 5-HT_3 agonists to produce emesis [28] and this effect was prevented by pretreatment with the selective cannabinoid CB_1 receptor antagonist SR-141716. Cannabinoids may act at CB_1 presynaptic receptors to inhibit release of newly synthesized serotonin [28, 54, 55].

Anti-emetic effects of cannabinoids in human clinical trials

The potential for marijuana to suppress nausea and vomiting produced by chemotherapy is of considerable therapeutic interest. Indeed, three cannabis-based medicines are available: dronabinol, nabilone and levonantradol. Tramer et al. [56] present a thorough systematic review of 30 clinical trial comparisons of cannabis (oral nabilone, dronabinol (THC) and intramuscular levonantradol) with a placebo or other anti-emetics (predominantly dopamine antagonists). Tramer et al. [56] conclude that the cannabinoids were superior to the conventional dopaminergic antagonists in the treatment of nausea and vomiting.

There has been only one [57] comparison of cannabis with a 5-HT_3 antagonist using the short-acting emetic agent syrup of ipecac. Human participants compared the effectiveness of a single dose of ondansetron (8 mg) with one of two doses of smoked marijuana (8.4 and 16.9 mg Δ^9-THC) in attenuating nausea and vomiting produced by syrup of ipecac. Unlike cisplatin, ipecac produces short-lasting nausea and vomiting with a fast onset. They report that within the limited dose range tested, ondansetron was considerably more effective than smoked marijuana in attenuating vomiting and nausea and nausea produced by the ipecac. There have been no clinical trials comparing the relative efficacy of marijuana and 5-HT_3 antagonists in suppressing long-lasting nausea and vomiting produced by chemotherapy treatment.

Cannabinoids produce psychotropic side effects, which partially accounts for their lack of popularity in clinical use [58]. Patients who have not had any experience with cannabis often find the psychotropic effects unpleasant and disturbing. Most importantly, the development of 5-HT_3 antagonist anti-emetic drugs, with few side effects, has limited clinical use of cannabis-based medicines. The 5-HT_3 antagonist anti-emetic agents are highly effective at preventing chemotherapy-induced vomiting, but are much less effective at inhibit-

ing nausea, as well as delayed nausea and vomiting and ANV when they do occur. There is some evidence that cannabis-based medicines may be effective in treating these intractable symptoms. Abrahamov et al. [59] evaluated the anti-emetic effectiveness of Δ^8-THC, a close but less psychoactive relative of Δ^9-THC, in children receiving chemotherapy treatment. Two hours before the start of each cancer treatment and every 6 h thereafter for 24 h, the children were given Δ^8-THC as oil drops on the tongue or in a bite of food. After a total of 480 treatments, the only side effects reported were slight irritability in two of the youngest children (3.5 and 4 years old); both acute and delayed nausea and vomiting were controlled.

Furthermore, Tramer et al. [56] conclude that many patients have a strong preference for smoked marijuana over the synthetic cannabinoids delivered orally. This could be for various reasons: (1) possible advantages of self-titration with the smoked marijuana, (2) the difficulty of swallowing the pills while experiencing emesis, (3) faster speed of onset for the inhaled or injected Δ^9-THC than oral delivery, or (4) a combination of the action of other cannabinoids with Δ^9-THC that are found in marijuana. Although many marijuana users have claimed that smoked marijuana is a more effective anti-emetic than oral Δ^9-THC, no controlled studies have yet been published that evaluate this possibility. Most of the evidence is based upon anecdotal testimonials, such as that of the late Stephen Jay Gould [60]: "I was miserable and came to dread the frequent treatments with an almost perverse intensity. …Absolutely nothing in the available arsenal of medications worked at all. Marijuana, on the other hand, worked like a charm."

Smoking marijuana may represent a more efficient and rapid route of administration. However, it is also possible that as marijuana contains over 60 other compounds, some of these additional consitutents may contribute to the anti-emetic/anti-nausea effect. Another major cannabinoid found in marijuana is cannabidiol (CBD); however, unlike Δ^9-THC, CBD does not produce psychomimetic effects [61]. CBD, unlike Δ^9-THC, does not bind to the known cannabinoid receptors. It may act by blocking the reuptake of anandamide (an endogenous cannabinoid), or by inhibiting enzymatic hydrolysis of anandamide, or bind with some as-yet-unknown cannabinoid receptor [61–63]. In mice, CBD is a highly effective anti-inflammatory agent [63], as well as a neuroprotective antioxidant [64]. In shrews, CBD inhibits cisplatin-induced [32] and lithium-induced [31] emesis and in rats CBD inhibits nausea [38]. These effects are described more fully below.

Effects of cannabinoids on emesis in animals

In order to understand the pathways involved in the response to anti-cancer therapies to develop appropriate drug therapies, animal models have been developed. Since rats and mice do not vomit in response to a toxin challenge, it was necessary to develop other animal models of emesis. As indicated in

Table 1, there is considerable evidence that cannabinoids attenuate vomiting in emetic species. Cannabinoids have been shown to reduce vomiting in cats [30], pigeons [65, 66], ferrets [33–35], least shrews, *Cryptotis parva* [24–29] and the house musk shrew, *Suncus murinus* [31, 32].

Table 1. Effect of cannabinoid on emesis across species

Species	Emetogen	Cannabinoid	Effect on emesis
Cat	Cisplatin (7.5 mg/kg, iv)	Nabilone (0.025–0.1 mg/kg, iv)	↓ [30]
		N-Methyllevonantradol (0.003–0.02 mg/kg, iv)	↓ [30]
Dog	Cisplatin (3 mg/kg, iv)	Nabilone (0.1 mg/kg, iv)	– [67]
	Apomorphine (0.05–5 mg/kg, iv)	Δ^9-THC (0.003–0.3 mg/kg, iv)	– [68]
Pigeon	Cisplatin (10 mg/kg, iv)	Δ^9-THC (5.0 mg/kg) with $CuCl_2$	↓ [65]
		HU-211 (2.5 mg/kg) with $CuCl_2$	↓ [65]
	Cisplatin (7.5 mg/kg, iv)	HU-210 (0.012–0.05 mg/kg, sc)	↓ [66]
	Emetine (20 mg/kg, sc)	HU-210 (0.012–0.05 mg/kg, sc)	↓ [66]
Ferret	Morphine (1 mg/kg, sc)	WIN55,212–2 (0.03–0.13 mg/kg, sc)	↓ [33]
	Morphine-6-glucuronide (M6G; 0.05 mg/kg, sc)	Δ^9-THC (1 mg/kg, ip)	↓ [34]
		WIN-55,212-2 (1 mg/kg, ip)	↓ [34]
		Methanandamide (3 mg/kg, ip)	↓ [34]
	Cisplatin (10 mg/kg iv)	Δ^9-THC (0.1–1.0 mg/kg ip)	↓ [35]
C. parva (least shrew)	SR-141716A (20 mg/kg, ip)	CP-55,940 (1 mg/kg, ip)	↓ [24]
		WIN-55,212–2 (10 mg/kg, ip)	↓ [26]
		Δ^9-THC (20 mg/kg, ip)	↓ [24]
	Cisplatin (20 mg/kg, ip)	Δ^9-THC (1–10 mg/kg, ip)	↓ [25]
		WIN-55,212-2 (1–5 mg/kg, ip)	↓ [25]
		CP-55,940 (0.025–0.3 mg/kg)	↓ [29]
	2-AG (2.5–10 mg/kg, ip)	CP-55,940 (0.05–0.1 mg/kg, ip)	↓ [27]
		WIN-55,212–2 (1–5 mg/kg, ip)	↓ [27]
		Δ^9-THC (2.5–5 mg/kg, ip)	↓ [27]
		CBD (10–20 mg/kg, ip)	– [27]
		Anandamide (5 mg/kg, ip)	↓ [27]
		Methanandamide (10 mg/kg, ip)	↓ [27]
		SR-141716A (2.5–5 mg/kg, ip)	↓ [27]
	5-HTP (100 mg/kg, ip)	Δ^9-THC (5–20 mg/kg, ip)	↓ [28]
	5-HT (5 mg/kg, ip)	Δ^9-THC (20 mg/kg, ip)	↓ [28]
	2-methylserotonin (5-HT_3 agonist; 5 mg/kg, ip)	Δ^9-THC (20 mg/kg, ip)	↓ [28]
		Δ^9-THC (20 mg/kg, ip)	↓ [28]
S. murinus (house musk shrew)	Cisplatin (20 mg/kg, ip)	Δ^9-THC (2.5–10 mg/kg, ip)	↓ [32]
		CBD (5–10 mg/kg, ip)	↓ [32]
	$LiCl_2$ (390 mg/kg, ip)	Δ^9-THC (3–20 mg/kg, ip)	↓ [31]
		CBD (5–10 mg/kg, ip)	↓ [31]

?, reduced; –, no effect; iv, intravenous; sc, subcutaneous; ip, intraperitoneal.

Cats and dogs

Although the two studies that evaluated the potential of nabilone [(0.1 mg/kg, administered intravenously (iv)] to antagonize cisplatin-induced emesis [67] and apomorphine-induced emesis [68] in dogs failed to find an anti-emetic effect of the cannabinoid, studies with cats have shown more promising results. McCarthy and Borison [30] reported that the synthetic cannabinoids methyllevonantradol (0.003–0.02 mg/kg, iv) and nabilone (0.025–0.1 mg/kg, iv) protected cats against cisplatin-induced vomiting and reduced the number of vomiting episodes among cats not protected in a dose-dependent manner.

Pigeons

An early study [65] with pigeons demonstrated that the non-psychotropic synthetic cannabinoid, HU-211, was more effective than Δ^9-THC in suppressing cisplatin-induced vomiting. The anti-emetic effect of HU-211 was U-shaped over a narrow dose range, with maximal efficacy at 2.5 and 3 mg/kg, administered subcutaneously (sc). More recently, the potent psychoactive cannabinoid, HU-210 (0.0125–0.05 mg/kg, sc) has also been reported to suppress cisplatin-induced vomiting in the pigeon [66].

Ferrets

One of the most widely used animal models of emesis is the ferret. In this model, morphine-induced emesis [33] was suppressed by the synthetic cannabinoid agonist WIN-55,212-2 (0.03–0.13 mg/kg, sc) and this effect was reversed by the selective CB_1 receptor antagonist, AM-251. Van Sickle et al. [34] report that the emesis produced by morphine-6-glucuronide (M6G; sc) in ferrets was also inhibited by Δ^9-THC [1 mg/kg, administered intraperitoneally (ip)], WIN-55,212-2 (1 mg/kg, ip) and methanandamide (3 mg/kg, ip), and that this anti-emetic effect was also reversed by AM-251 (5 mg/kg, ip). Although AM-251 did not produce emetic episodes on its own, it did potentiate the emetogenic effects of M6G [34]. More recently, Δ^9-THC (0.05–1 mg/kg, ip) dose-dependently inhibited the emetic actions of cisplatin [35]. Furthermore, Δ^9-THC applied to the surface of the brain stem also inhibited emesis induced by intragastric hypertonic saline [35].

Shrews

Insectivores, such as the shrew, are the closest extant relatives to primates as well as the oldest group of eutherians. Insectivores have sensitive emetic reflexes. Shrews are smaller than carnivores (such as cats, dogs and ferrets)

that have typically been used to evaluate the anti-emetic properties of drugs and, therefore, are easier to maintain in a laboratory.

Considerable recent work by Darmani and colleagues [24–29] has evaluated the potential of different groups of cannabinoids to inhibit emesis induced by toxins in *C. parva* (the least shrew), which weighs 4–6 g. Cisplatin-induced emesis was inhibited by WIN-55,212-2 (1–5 mg/kg, ip) and Δ^9-THC (1–10 mg/kg, ip) in a dose-dependent manner with similar potency [25, 26]. However, the synthetic cannabinoid CP-55,940 (0.025–0.3 mg/kg, ip) more potently antagonized cisplatin-induced vomiting than WIN-55,212-2 or Δ^9-THC and also had a higher affinity for the CB_1 receptor [29]. The synthetic HU-210 has a higher affinity for the CB_1 receptor than CP-55,940, but has not been systematically evaluated for its anti-emetic capacity in the shrew model. Emesis induced by the precursor to serotonin, 5-hydroxytryptophan (5-HTP) (100 mg/kg, ip) was suppressed by Δ^9-THC [28] at doses of 5–20 mg/kg, ip; however, a dose of 20 mg/kg, ip, of Δ^9-THC was required to suppress the emesis produced by 5-HT (5 mg/kg, ip) and the selective 5-HT_3 agonist 2-methylserotonin (5 mg/kg, ip). Therefore, cannabinoids appear to suppress the emetic reaction to drugs that activate the serotonin system in the least shrew.

Since cannabinoid agonists prevent emesis, Darmani [24] predicted that blockade of the CB_1 receptor would induce vomiting. Indeed, at a dose of 10 mg/kg, ip, or 40 mg/kg, sc, SR-141716 induced vomiting in the least shrew; on the other hand, the CB_2 receptor antagonist, SR-144528, did not produce vomiting at any dose tested. WIN55,212-2 (minimal dose 10 mg/kg, ip) was more effective than Δ^9-THC (minimum dose 20 mg/kg, ip) in preventing SR-141716-induced vomiting [24, 29], which was also prevented by CP-55,940 (1 mg/kg, ip). The selective CB_1 receptor antagonist SR-141716 blocked the anti-emetic activity of cannabinoids at low doses [24–29] and produced vomiting on its own at higher doses in least shrews [24], suggesting that the endogenous cannabinoid system plays a role in the regulation of emesis.

Darmani [27] has also shown that the endocannabinoid 2-AG is a potent emetogenic agent, whereas anandamide may cause weak anti-emetic effects [27, 35]. The emetic effects of 2-AG were inhibited by CP-55,940 (0.05–0.1 mg/kg, ip), WIN-55,212-2 (1–5 mg/kg, ip), Δ^9-THC (2.5–5 mg/kg, ip), anandamide (5 mg/kg, ip), methanandamide (10 mg/kg, ip) and SR-141716 (2.5–5 mg/kg, ip), but not by CBD (10–20 mg/kg, ip). These results suggest that endogenous cannabinoids may also play a role in promoting emesis.

Over the past number of years, the *S. murinus* (house musk shrew) has been used as a model for emesis research [4, 6, 7]. These shrews weigh 30–60 g in contrast to the 4–6 g of the least shrew. Like rats, *Suncus* will avoid a flavor paired with lithium chloride [69]; however, unlike rats, these animals vomit in response to toxins, even though they possess similar neural circuitry in the emetic regions of the brain as rats [70]. In a series of experiments, Parker et al. [31] evaluated the emetogenic potential of lithium in *Suncus* and the ability of two principal cannabinoids found in marijuana, the psychoactive Δ^9-THC and the nonpsychoactive CBD, to reverse lithium-induced emesis. A prior study

[27] indicated that, unlike other cannabinoids tested including Δ^9-THC, CBD (10 and 20 mg/kg, ip) did not reverse retching and vomiting induced by the endogenous cannabinoid, 2-AG, in the much smaller least shrew. It has also been reported to be ineffective at inhibiting gastric motility in mice [71]. However, we had previously shown that CBD did prevent the establishment of lithium-induced conditioned gaping in rats [38], a putative rat model of nausea (reviewed below). We [31] found that Δ^9-THC produced a dose-dependent suppression of lithium-induced vomiting with higher doses producing greater suppression than lower doses. CBD, however, produced a biphasic effect with lower doses (5 and 10 mg/kg, ip), producing suppression and higher doses (20 and 40 mg/kg) producing enhancement of lithium-induced vomiting. The anti-emetic properties of CBD are of clinical relevance, because CBD does not produce the psychomimetic effects that are produced by Δ^9-THC. The suppression of lithium-induced vomiting by Δ^9-THC, but not by CBD, was reversed by pretreatment with SR-141716, confirming previous findings that CBD does not act at the CB_1 receptor [61].

Cannabinoid and serotonin systems interact centrally and both systems are involved in the control of emesis. There have been no studies with humans that have systematically evaluated the relative effectiveness of 5-HT_3 receptor antagonists and cannabinoid agonists to suppress acute and/or delayed nausea and vomiting produced by chemotherapeutic agents, such as cisplatin. Using the *Suncus* model of emesis, Kwiatkowska et al. [32] found that ip-injected ondansetron and Δ^9-THC both dose-dependently suppressed cisplatin-induced vomiting and retching; however, the minimally effective dose of ondansetron (0.2 mg/kg, ip) was considerably lower than the minimally effective dose of Δ^9-THC (2.5 mg/kg, ip). A combined pretreatment of doses of the two drugs that were ineffective alone (0.02 mg/kg ondansetron and 1.25 mg/kg Δ^9-THC) completely suppressed cisplatin-induced vomiting and retching. These results suggest that a combination of lower doses of ondansetron and Δ^9-THC may be an effective alternative treatment for the acute phase of chemotherapy-induced vomiting that may have fewer side effects than higher doses of either agent alone. The anti-emetic effects of ondansetron were not affected by pretreatment with SR-141716, suggesting that ondansetron does not act at the CB_1 receptor. Finally, as we saw with lithium-induced vomiting [31], low doses (5–10 mg/kg, ip) of the nonpsychoactive cannabinoid, CBD, effectively suppressed cisplatin-induced vomiting, but high doses of CBD (20–40 mg/kg, ip) potentiated vomiting.

The anti-emetic effect of low doses of CBD against lithium- and cisplatin-induced emesis in *Suncus* is inconsistent with the failure of a dose of 10 mg/kg to suppress emesis produced by 2-AG in the least shrew [27]. The difference may be related to the mechanism of action of the emetogens, the difference in body mass of the two species (the least shrew is 10 times smaller than the *Suncus*), or the biphasic effects of CBD on toxin-induced emesis. Our [32] results suggest that both primary cannabinoids found in cannabis, the

psychoactive Δ^9-THC and the nonpsychoactive CBD, effectively suppress lithium- and cisplatin-acute vomiting in *Suncus* at appropriate doses.

Conclusion

Until recently, there were few experimental studies that evaluated the anti-emetic properties of cannabinoids, probably for two reasons: (1) the mechanism of action of cannabinoids has only recently been discovered and (2) animal models of emesis relied on large animals, since rodents do not vomit. The recent work using the shrew provides the opportunity to evaluate the potential anti-emetic properties of drugs using smaller animal models.

Conditioned gaping in shrews: a model for ANV

ANV often develops over the course of repeated chemotherapy sessions [1, 2, 10, 11, 14, 20–22]. For instance, Nesse et al. [21] described the case of a patient who had severe nausea and vomiting with each treatment. After his third treatment, the patient became nauseated as soon as he walked into the clinic building and noticed a "chemical smell", that of isopropyl alcohol. He experienced the same nausea when returning for routine follow-up visits, even though he knew he would not receive treatment. The nausea gradually disappeared over repeated follow-up visits. Nesse et al. [21] reported that about 44% of the patients being treated for lymphoma demonstrated such anticipatory nausea. He suggested that "this syndrome of pretreatment nausea can be understood as a classically conditioned response" ([21], p. 33); certain odors have become aversive because of their association with chemotherapy-induced illness. Indeed, Bernstein [72] has clearly demonstrated that the flavor of foods also becomes aversive to patients receiving chemotherapy treatment.

Since it is best understood as a classically conditioned response [21, 73], control over ANV could be exerted at the time of conditioning or at the time of re-exposure to the conditioned stimulus (CS). If an anti-emetic drug is presented at the time of conditioning, then a reduction in ANV would be the result of an attenuated unconditioned response (UCR); that is, reduced nausea and vomiting produced by the toxin at the time of conditioning thereby attenuating the establishment of the conditioned response (CR). Indeed, when administered during the chemotherapy session, the 5-HT_3 antagonist granisetron has been reported to reduce the incidence of ANV in repeat cycle chemotherapy treatment [2]. On the other hand, if a drug is delivered prior to re-exposure to cues previously paired with the toxin-induced nausea and vomiting, then suppressed ANV would be the result of attenuation of the expression of the CR (conditioned nausea and/or vomiting); the 5-HT_3 antagonists are ineffective at this stage [1, 10, 11, 14, 20].

Anecdotal evidence suggests that Δ^9-THC alleviates ANV in chemotherapy patients [40, 59, 60]. Although there has been considerable experimental investigation of unconditioned vomiting in response to toxins, there have been relatively few reports of conditioned emetic reactions elicited by re-exposure to a toxin-paired cue (ANV). Conditioned gaping and retching has been observed to occur in coyotes, wolves and hawks upon re-exposure to cues previously paired with lithium-induced toxicosis [74, 75] and ferrets have been reported to display conditional emetic reactions during exposure to a chamber previously paired with lithium-induced toxicosis [76]. We [77] have presented a model of ANV based on the emetic reactions of *S. murinus*. Following two pairings of a novel distinctive contextual cue with the emetic effects of an injection of lithium chloride, the context acquired the potential to elicit gaping in the absence of the toxin. The expression of this conditioned reaction was completely suppressed by pretreatment with Δ^9-THC at a dose that did not suppress general activity. This provides the first experimental evidence in support of anecdotal reports that Δ^9-THC suppresses ANV.

Conditioned gaping in rats: a model of nausea

Nausea has been reported to be the most unpleasant and distressing aspect of chemotherapy, superceding even vomiting and retching [14]. Nausea is less effectively reduced by the 5-HT_3 receptor antagonists than is acute vomiting [1, 9, 10, 14, 20]. Even when the cisplatin-induced emetic response is blocked in the ferret by administration of a 5-HT_3 receptor antagonist, *c-fos* activation still occurs in the area postrema, suggesting that an action here may be responsible for some of the other effects of cytotoxic drugs, such as nausea or reduced food intake [12]. Grundy and colleagues [78–80] have demonstrated that in rats the gastric afferents respond in the same manner to physical and chemical (intragastric copper sulfate and cisplatin) stimulation that precedes vomiting in ferrets (presumably resulting in nausea that precedes vomiting). Furthermore, 5-HT_3 antagonists that block vomiting in ferrets also disrupt this preceding neural afferent reaction in rats. That is, in the rat the detection mechanism of nausea is present, but the vomiting response is absent [81]. Nauseogenic doses of cholecystokinin and lithium chloride induce specific patterns of brainstem and forebrain c-*fos* expression in ferrets that are similar to c-*fos* expression patterns in rats [82]. In a classic review paper, Borrison and Wang [83] suggest that the rats' inability to vomit can be explained as a species-adaptive neurological deficit and that, in response to emetic stimuli, the rat displays autonomic and behavioral signs corresponding to the presence of nausea, called the prodromata (salivation, papillary dilation, tachypnoea and tachycardia).

Over a number of years, our laboratory has provided considerable evidence that conditioned nausea in rats may be displayed as a conditioned rejection reaction [84–89] using the Taste Reactivity (TR) test [90]. When rats are intra-

orally infused with a bitter-tasting quinine solution, they display a distinctive pattern of rejection reactions (including gaping, chin rubbing and paw treading). Interestingly, following pairing with a toxin, sweet solutions come to elicit the rejection pattern of reactions. Therefore, conditioned rejection reactions provide an alternative measure of flavor aversion learning to that of a consumption test.

The consumption test is much less selective to emetic drugs than the TR test, because most psychoactive drugs (with rewarding or emetic properties) produce taste avoidance [84–87, 91]. In fact, rats will simultaneously avoid an amphetamine-paired flavor while demonstrating a preference for the amphetamine-paired place [92]. Considerable evidence indicates that not all taste avoidance is accompanied by conditioned aversion (rejection reactions) to the taste when assessed using the TR test. Stimuli that produce taste avoidance in the absence of nausea do not produce an aversion to the taste. Lower-intestinal discomfort [93], footshock [93], lesions of the lateral hypothalamus [94] as well as reinforcing drugs [84–87] produce taste avoidance but do not produce rejection reactions in the TR test. Conditioned rejection and conditioned avoidance are mediated by qualitatively, not quantitatively, different processes. Zalaquett and Parker [95] demonstrated that when the doses of lithium and amphetamine are adjusted such that rats develop stronger avoidance of the amphetamine-paired flavour, they still only reject the less-avoided lithium-paired flavour. That is, only drugs with emetic effects produce conditioned rejection reactions.

An early theory that attempted to explain paradoxical reports that reinforcing drugs produce taste avoidance suggested that any novel change in physiological state (be it hedonic or aversive) signals danger to the rat, a species that cannot vomit [96]. A flavor paired with this change in state comes to signal danger, resulting in subsequent avoidance of that taste. On the other hand, shrews do not avoid a flavor paired with the rewarding drugs, amphetamine or morphine; in fact, they develop a preference for a flavor paired with these drugs [97]. Presumably, the emetic shrew does not need to be as wary as the non-emetic rat about changes in state following ingestion, because it can vomit.

The most reliable conditioned rejection reaction in the rat is that of gaping [37, 87]. If conditioned gaping reflects nausea in rats, then anti-nausea drugs should interfere with this reaction. Limebeer and Parker [88] demonstrated that when administered prior to a saccharin/lithium pairing, the 5-HT_3 antagonist, ondansetron, prevented the establishment of conditioned gaping in rats, but not the establishment of conditioned taste avoidance. Presumably, ondansetron interfered with lithium-induced nausea preventing the conditioned gaping; on the other hand, it did not prevent the establishment of conditioned taste avoidance, suggesting that nausea was not necessary to suppress saccharin consumption. Ondansetron also interfered with the expression of previously established conditioned gaping, but not taste avoidance. Since ondansetron did not modify unconditioned gaping elicited by bitter quinine solution, the effect was

specific to nausea-induced gaping. In fact, ondansetron suppressed conditioned gaping elicited by exposure to a lithium-paired saccharin solution even during a drinking test, but it did not modify the amount consumed. Therefore, the effects of ondansetron on conditioned gaping, but not conditioned taste avoidance, cannot simply be attributed to differences in the delivery of the taste when using the two measures (e.g. [98]); conditioned gaping is suppressed by ondansetron whether the taste is presented by bottle or by intraoral infusion. Subsequently, Limebeer and Parker [89] demonstrated a very similar pattern following pretreatment with the 5-HT_{1A} autoreceptor antagonist, 8-hydroxy-2-di-N-propylaminotetraline (8-OH-DPAT), that also reduces serotonin availability and serves as an anti-emetic agent in animal models. Most recently, Limebeer et al. [99] report that lesions of the dorsal and median raphe that reduce forebrain serotonin availability interfere with the establishment and the expression of conditioned gaping consistent with reports that reduced serotonin availability interferes with nausea [99]. Since rats are incapable of vomiting, we have argued that the gape represents an incipient vomiting response. As is evident in Figure 1, the orofacial characteristics of the rat gape are very similar to those of the shrew just before it vomits [87].

Using the conditioned gaping measure of nausea in rats, we have demonstrated that a low dose (0.5 mg/kg, ip) of Δ^9-THC interferes with the establishment and the expression of cyclophosphamide-induced conditioned gaping [36]. Most recently, we found that CBD (5 mg/kg, ip), as well as its synthetic dimethylheptyl homolog (5 mg/kg, ip), suppressed the establishment and the expression of lithium-induced conditioned gaping, but not taste avoidance [38]. Finally, the potent agonist HU-210 (0.001–0.01 mg/kg) also suppressed lithium-induced conditioned gaping [37, 39] and this suppression was reversed by the CB_1 antagonist SR-141716, suggesting that the effect of HU-210 was mediated by its action at CB_1 receptors [39]. Although SR-141716 did not pro-

Figure 1. The rat gape is topographically similar to the shrew gape just before it vomits.

duce conditioned rejection on its own, it potentiated the ability of lithium to produce conditioned gaping. This same pattern has been reported in the emesis literature. Van Sickle et al. [34] reported that although the CB_1 antagonist AM-251 did not produce vomiting on its own, it potentiated the ability of an emetic stimulus to produce vomiting in the ferret.

Conclusions

Since the discovery of the mechanism of action of cannabinoids, considerable research using smaller animals (ferrets and shrews) confirms prior experimental reports [30, 65, 66] that cannabinoids serve as effective anti-emetics. In the ferret and shrew models, the site of action has been identified in the emetic area of the brainstem, the dorsal vagal complex [28, 29, 34, 35]. The shrew model, in particular, is cost-effective for the evaluation of the anti-emetic properties of agents. It is clear that many cannabinoids act on the CB_1 receptors to produce their anti-emetic properties; however, it is not known how the nonpsychoactive cannabinoid, CBD, which does not act at the CB_1 receptor, produces anti-emetic effects within a limited dose range in *S. murinus* [31, 32].

Research has also supported anecdotal reports that cannabis may attenuate ANV. Using *S. murinus*, Δ^9-THC effectively prevented conditioned gaping elicited by re-exposure to a lithium-paired chamber [77]. Further work on this model may reveal optimal agents for alleviating ANV, which is resistant to treatment with 5-HT_3 antagonists [1, 10, 11, 14, 20].

Finally, the conditioned gaping response appears to selectively reflect nausea in the rat [84–89], which is not capable of vomiting in response to a toxin. Rats display this response to flavors previously paired with emetic agents and this response is prevented by anti-emetic pretreatments [88, 89]. Cannabinoids suppress the establishment of this conditioned gaping when administered prior to a taste-toxin pairing [36–39]. Cannabinoids also suppress the expression of previously established conditioned gaping, again suggesting that they may serve to suppress ANV. Not only psychoactive cannabinoids, but also the non-psychoactive CBD, suppresses the establishment and the expression of lithium-induced conditioned gaping in rats [38].

The endogenous cannabinoid system clearly plays a role in the regulation of nausea and vomiting. The CB_1 antagonists SR-141716 and AM-251 respectively potentiated the strength of toxin-induced conditioned gaping in rats [37, 39] and toxin-induced vomiting in ferrets [34]. However, the finding that the endogenous cannabinoid 2-AG serves as an emetogenic agent in the least shrew [27] suggests that the endocannabinoid system may contribute to the control of nausea and vomiting in a complex manner.

Acknowledgements
The authors would like to thank Marion Corrick for care of the shrews. This research was supported by research grants to L.P. from the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

The skeleton: stone bones and stoned heads?

Itai A. Bab

Bone Laboratory, The Hebrew University of Jerusalem, Jerusalem 91120, Israel

Introduction

In vertebrates, bone mass is maintained constant between the end of linear skeletal growth, when the peak bone mass is established, and gonadal failure, when accelerated bone loss begins. The bone mass is preserved by a continuous destruction/formation process termed bone remodeling [1]. This destruction/formation cycle occurs at the same time in multiple foci that in humans encompass approximately 5% of trabecular, endosteal, and Haversian system surfaces. A cycle consists initially of a relatively rapid (i.e. a few weeks) resorption of pre-existing bone by a bone-specific, bone marrow hematopoietic cell type, the osteoclast, derived from the monocyte/macrophage lineage [2]. It is then followed by a slower (i.e. a few months) step of *de novo* bone formation by another bone-specific cell type, the osteoblast [3], which belongs to the stromal cell lineage of bone marrow [4]. Although different foci present different phases of the cycle, the overall net effect is that of a balance between bone destruction and formation. The physiologic importance of bone remodeling is best illustrated in osteoporosis, the most frequent degenerative disease in developed countries, which results from impaired remodeling balance that leads to bone loss and increased fracture risk mainly in females but also in males.

The synchronized occurrence of multiple remodeling sites has long been viewed as suggestive of a complex, local, autocrine/paracrine [5] as well as endocrine regulation. Indeed, experiments in knockout (KO) and transgenic mice have demonstrated paracrine regulation of osteoclast differentiation and activity by factors such as receptor activator of NFκB (RANK) ligand, osteoprotegerin, macrophage colony-stimulating factor (M-CSF) and interleukin 6, which are derived from neighboring stromal cells, including osteoblasts and osteoblast precursors [6–11]. The most convincing evidence for local osteoblast regulation is by bone morphogenetic proteins [12]. Systemically, ablation of gonadal hormones in females and males has been repeatedly demonstrated to favor bone loss in humans, rats, and mice [13, 14]. In addition, parathyroid hormone [15, 16], calcitonin [17], insulin-like growth factor I [18], and the osteogenic growth peptide [19] have been shown to regulate bone formation. More recently, it has been demonstrated that bone remodeling is also subject to a potent central control consisting of hypothalamic leptin and neuropeptide Y

signaling [20, 21] as well as downstream noradrenergic signaling by osteobalstic β2 adrenergic receptors [22]. It thus appears that the critical systems in the control of bone remodeling are gonadal and central nervous system-derived, and apply tonic inhibition of osteocalst and osteoblast function, respectively.

A couple of recent striking findings led us to study the involvement of the endocannabinoid system in the regulation of bone remodeling. One is that, as in the case of bone formation and bone mass, the central production of at least one major endocannabinoid, 2-arachidonoyl glycerol (2-AG), is subject to negative regulation by leptin [23]. The other observation is that traumatic head injury stimulates both bone formation [24, 25] and central 2-AG production [26].

Strategy

Our approach, designed to elucidate the regulatory role of the endocannabinoid system in bone, consisted of *in vitro* experiments in bone cells followed by *in vivo* skeletal phenotyping of cannabinoid receptor (CB)-deficient mice. After demonstrating a low bone mass (LBM) in these mice we assessed the prevention of bone loss in estrogen-deficient mice by CB agonists.

The initial experiments demonstrated CB mRNA in bone cells *in vitro*, confirmed by immunostaining *in vivo*. We then further used the *in vitro* system to demonstarte the regulation of osteoblast and osteoclast differentiation and activity by cannabinoid ligands. The relevance of the *in vitro* findings to the *in vivo* scenario was established by analyzing the cortical and trabecular bone of CB-KO and ovariectomized mice using micro-computed tomography and histomorphometry.

Expression of cannabinoid receptors in bone

Undifferentiated osteoblast progenitors, such as mouse bone marrow-derived stromal and MC3T3 E1 preosteoblastic cells [27, 28], exhibit very low levels of the neuronal CB_1 and peripheral CB_2 mRNA cannabinoid receptors [29], detectable only by ultrasensitive methods. When these cells are grown for 2–4 weeks in so-called osteogenic medium, which contains vitamin C, β-glycerophosphate, and dexamethasone [30], CB_1 mRNA remains at the same levels. However, CB_2 mRNA expression increases progressively in parallel to the expression of the osteoblastic marker genes which encode tissue non-specific alkaline phosphatase (*TNSALP*) [31], parathyroid hormone receptor 1 (*PTHRc1*) [32], and particularly the osteoblastic master regulatory gene, *RUNX2* [33]. CB_2, but not CB_1 mRNA transcripts are also present in high abundance in monocytic cells undergoing osteoclast differentiation induced by RANK ligand and M-CSF [34]. *In vivo*, CB_2 protein is present in trabecular osteoblasts and their decedents, the osteocytes [33], as well as in osteoclasts.

Cannabinoid ligands regulate bone cell differentiation and activity

CB_2 activation has different effects in early preosteoblasts and in more mature osteoblastic cells. In bone marrow derived partially differentiated osteoblasts, with limited CB_2 expression, the specific CB_2 agonist HU-308 [35] but not the specific CB_1 agonist noladin ether [36], triggers a G_i protein-mediated mitogenic effect. This response leads to a dose-response expansion of the preosteoblastic pool. In more mature osteoblastic cells, represented by the MC3T3 E1 cell line, HU-308 also stimulates osteoblast-differentiated functions such as alkaline phosphatase activity and matrix mineraliztion. Thus, CB_2 signaling has multiple regulatory osteogenic anabolic functions along the osteoblast differentiation pathway. CB_2 activation has an opposite effect on osteocalstogenesis, namely, it inhibits osteoclasts differentiation.

Cannabinoid receptor signalling regulates bone mass *in vivo*

Although only the CB_2 receptor is demonstrable in bone, both CB_1- and CB_2-deficient mice have LBM. However, the underlying mechanisms of these LBMs appear to be different inasmuch as a pronounced low trabecular bone density is already found in the CB_1-KO mice at a young age. At the same age the skeleton of CB_2-KO mice is almost normal, with severe bone loss reported only in nearly 1-year-old animals [38]. This age-related difference suggests that CB_1 is mainly involved in the establishment of peak bone mass, which develops during infancy, sexual maturation, and young adulthood [39, 40]. CB_2 appears to be an important regulator of bone remodeling and maintenance of constant bone mass in later life. Further support for this notion is derived from the different pathogenic processes that lead to the LBM in the CB_1- and CB_2-deficient mouse lines. Consistent with their early LBM, CB_1-KO mice have increased bone resorption associated with decreased bone formation [38]. Reminiscent of human postmenopausal osteoporosis [41], CB_2-KO mice have a high turnover LBM characterized by increases in both bone resorption and formation which are at a net negative balance [38].

Because CB_2 is only peripherally expressed, CB_2-specific ligands could provide an opportunity to augment bone mass while avoiding the cannabinoid psychotropic activity. Indeed, HU-308 attenuates bone loss induced by estrogen depletion in ovariectomized animals with significant respective bone anti-resorptive and anabolic activities in the trabecular and cortical skeletal compartments [38].

Summary

Although the CB_2 receptor was cloned more than a decade ago, its physiological role remained elusive. Using a combined approach encompassing mole-

cular and cellular biology, pharmacology, and genetic analyses in mice, we were able to show a role for CB_2 signaling in regulating bone mass. Furthermore, attenuation of the deleterious effects of ovariectomy on bone by a peripherally selective CB_2 cannabinoid receptor agonist have major implications for osteoporosis, offering new molecular targets for the diagnosis and treatment of this disease.

CB_1 is apparently not involved in age-related bone loss commonly diagnosed in humans, but may have an important role in early skeletal development.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabinoids and drugs of abuse

Daniela Parolaro and Tiziana Rubino

Center of Neuroscience, University of Insubria, Via A. da Giussano 10, 20152 Busto Arsizio (VA), Italy

Introduction

Derivatives of *Cannabis sativa*, such as marijuana and hashish, are the most widely consumed illicit drug: almost half of all 18-year olds in the USA and in most European countries admit to having tried it at least once, and 10% of that age group are regular users. There have been many subjective accounts of the cannabis 'high'. A typical 'high' is preceded initially by a transient stage of tingling sensations felt in the body and head accompanied by a feeling of dizziness or light-headedness. The 'high' is a complex experience, characterized by a quickening of mental association and a sharpened sense of humor, sometimes described as a state of "fatuous euphoria". As reported by Atha and Bianchard [1] in a survey of 1333 young British cannabis users the most common benefit reported were relaxation and relief from stress, insight/personal development and euphoria, but 21% of the users also described some adverse effects, including impaired memory, paranoia and amotivation/laziness. As with other intoxicant drugs, little is known about the brain mechanisms that underlie the cannabis high. The intoxicant effects are clearly mediated via CB_1 receptors. In a well-controlled study in 63 healthy cannabis users [2] who received either a CB_1 receptor antagonist (Rimonabant) or placebo and smoked either a Δ^9-tetrahydrocannabinol (Δ^9-THC)-containing or placebo marijuana cigarette, Rimonabant blocked the acute psychological effects of the active cigarettes. Moreover, self ratings of cannabis intoxication correlated most markedly with increased blood flow in the right frontal region as demonstrated using positron emission tomography (PET) to measure changes in cerebral blood flow.

The potential ability of cannabis derivatives to produce dependence in humans is still a controversial issue. Earlier clinical literature (for reviews see [3–5]) suggested that tolerance also occurs after repeated administration of Δ^9-THC in humans, although many of these studies were poorly controlled. But for many years cannabis was not considered to be a drug of addiction. Withdrawal of the drug did not lead to any obvious physical withdrawal syndrome either in people or in animals, and animals failed to self-administer the drug, a behavior usually associated with drugs of addiction.

Attitudes have changed markedly in recent years. According to the Diagnostic and Statistical manual of Mental Disorders (DSM IV) [5a] criteria

(American Psychiatric Association, 1994) for 'substance dependence' and 'substance abuse', surprisingly a high proportion of regular cannabis users appear to fall into these categories. Recent studies [6] indicated that almost one-third of regular cannabis users fell within the definition of 'substance abuse' or 'substance dependence'. Moreover, carefully controlled studies have also shown that a reliable and clinically significant withdrawal syndrome does occur in human cannabis users when the drug is withdrawn. The symptoms include craving for cannabis, decreased appetite, sleep difficulty and weight loss, and may sometimes be accompanied by anger, aggression, increased irritability, restlessness and strange dreams [7].

The existence of abuse liability of cannabinoids in animals is much more clearly observable. Processes involved in substance abuse are neurobiologically and behaviorally complex. Tolerance and withdrawal syndrome represent adaptive responses to the prolonged exposure of neurons to drugs, but the main factor common to all drugs of abuse is their ability to induce drug-seeking behavior, which is due to the positive reinforcing effects of the drugs. Several behavioral models have been used to evaluate tolerance and withdrawal, as well as the rewarding effects of cannabinoids, which will be briefly summarized here together their proposed molecular basis.

Tolerance

Chronic administration of natural or synthetic cannabinoid agonists in different animal species induces tolerance to most of their pharmacological effects (see [8, 9] for review). Although some papers have reported that pharmacokinetic events take place during the development of cannabinoid tolerance [10, 11], there is general agreement that this phenomenon is pharmacodynamic in nature. The best-known events that occur after development of cannabinoid tolerance are receptor downregulation and uncoupling from the G protein system, which ends in receptor desensitization (see [8] for review). Besides these alterations, other cellular adaptations are present in the brain of cannabinoid-tolerant rats, such as modulation of effector proteins. Specifically, it has been shown that increased activation of the cAMP pathway (i.e. cAMP accumulation and protein kinase A activity) [12–15], together with adaptations in the extracellular-signal-regulated kinase (ERK) cascade [16], were observed in some cerebral regions of chronic Δ^9-THC-treated animals. An elegant demonstration of the involvement of the Ras/ERK pathway in development of cannabinoid tolerance comes from studies in Ras-GRF1-knockout mice [16], a useful model where cannabinoid-induced ERK activation is lost. These animals did not develop tolerance to Δ^9-THC's analgesic and hypolocomotor effects, suggesting that the ERK cascade could play a pivotal role in the induction of synaptic plasticity due to chronic cannabinoid exposure. Finally, recent work reported that the pyrazolopyrimidine (PP1), the Src family tyrosine kinase inhibitor, reversed Δ^9-THC-induced tolerance, supporting a role for Src

tyrosine kinase in phosphorylation events in Δ^9-THC-tolerant mice [15]. Taken together, these recent data seem to indicate an outstanding role in cannabinoid tolerance for some protein kinases (protein kinase A, ERK, Src tyrosine kinase), suggesting that cannabinoid tolerance could be depicted as activity-dependent synaptic plasticity. Whether and how these kinases could contribute to CB_1 receptor downregulation or desensitization remains to be determined. In line with this view, large-scale analysis of gene-expression changes during acute and chronic exposure to Δ^9-THC in rat hippocampus [17] revealed that the altered genes were predominantly associated with membrane repair and synaptic structures, indicating that they are involved in transcription or proteosomal processes, possibly reflecting a change in neuronal capacity to deal with the ubiquitous consequences of chronic cannabinoid receptor activation over long time periods.

Finally, it cannot be ruled out that prolonged activation of cannabinoid receptors also leads to decreased endocannabinoid content and signalling in the striatum and to increased anandamide formation in the limbic forebrain [18], areas involved in the tonic control of movements and in reinforcement processes.

Physical dependence

Although the presence of spontaneous withdrawal after chronic cannabinoid treatment is also controversial in animals, there are no doubts that administration of the CB_1-selective antagonist SR-141716A precipitates a pronounced withdrawal syndrome in animals that have been chronically treated with cannabinoids (see [8, 9] for review). Biochemical indicies of adaptive changes have been demonstrated during cannabinoid withdrawal and they include compensatory changes in the cAMP pathway in the cerebellum [12–14, 19], which appears to be a key area in the modulation of somatic expression of cannabinoid abstinence syndrome. These findings directly demonstrated that, in analogy with other addictive drugs, the activation of the cAMP pathway is a crucial phenomenon at the onset of Δ^9-THC-withdrawing behaviors. Interestingly, a key structure in controlling this process could be the cerebellum, a region not previously associated with drug abuse, and whose participation in cognitive networks is actually a most exciting field of investigation. Moreover, activation of corticotropin-releasing factor [20] and a decrease in mesolimbic dopamine transmission [21, 22] have also been observed in withdrawn rats, strengthening the evidence that cannabinoids share with other drugs of abuse those neurochemical properties that are regarded as the biological substrate of drug addiction.

Behavioral sensitization

Behavioral sensitization represents another adaptive neurobiological alteration that occurs after repeated exposure to drugs and plays a role in drug

addiction, particularly in drug-seeking behavior that persists long after the discontinuation of drug use [23]. Rats repeatedly treated with Δ^9-THC for several days (3/5 days) and then challenged with Δ^9-THC 2/3 weeks after the last Δ^9-THC injection show a greater behavioral activation than rats repeatedly treated with vehicle [24, 25]. The molecular underpinnings of this phenomenon are still not well understood, but they involve altered CB_1 receptor functionality in the striatum and cerebellum of sensitized rats [26]. Moreover, in the cerebellum the cAMP pathway and the ERK cascade seem to lose their responsiveness to cannabinoids ([26] and T. Rubino et al., unpublished results). Preliminary data obtained in our laboratory indicate differential responsiveness of specific transcription factors in selected brain areas (striatum, prefrontal cortex and hippocampus) of pre-exposed rats, supporting the working hypothesis that relapse can be viewed as a certain kind of memory (addiction memory) since the brain obviously remembers the prior administration of the drug and induces craving.

Drug discrimination

Drug discrimination is a behavioral procedure based on the ability of a drug to induce a specific set of interoceptive stimulus conditions perceived by the animals that might be predictive of the subjective reports of perceptions/feelings induced by the same drug in humans. As a result, studies of the subjective effects of new drugs in both humans and animals have been relatively good predictors of either or not a drug will be abused. Since animals do not easily self-administer cannabinoids, the drug-discrimination procedure has long been the primary animal model available for evaluating the potential abuse liability of cannabinoids [27]. Cannabinoid drugs show a pharmacological specificity in this behavioral procedure. Thus, in animals trained to discriminate injections of Δ^9-THC from injections of saline, only drugs that possess the ability to activate CB_1 cannabinoid receptors fully generalize to the Δ^9-THC training stimulus (see [9] for review). Moreover, the discriminative stimulus effects of Δ^9-THC and other synthetic CB_1 agonists can be completely blocked by pre-treatment with the selective CB_1 receptor antagonist SR-141716A [28], further demonstrating that the cannabinoid discrimination is mediated by CB_1 receptors [29, 30]. In contrast, anandamide and stable analogs of this endocannabinoid do not fully substitute for Δ^9-THC in monkeys and rats [31–33], or has done so only at doses that severely decrease food-mantained responding [32]. The fast reuptake and rapid metabolism of anandamide by the fatty acid amide hydrolase enzyme is a likely explanation for why anandamide, which is a partial agonist of CB_1 receptors, just like Δ^9-THC, usually fails to produce Δ^9-THC-like discriminative-stimulus effects. Anandamide has been shown to have cannabinoid-like discriminative stimulus effects under some situations. Recently Jarbe et al. [33] demonstrated that methanadamide was successfully used as a training stimulus in rats,

and Δ^9-THC produced complete generalization. Anandamide was able to produce generalization to the methanandamide but not to the Δ^9-THC training stimulus that could be related to the different affinities of Δ^9-THC and methanadamide for CB_1 receptors, resulting in a discriminative stimulus for methanadamide with an intensity and a quality closer to the anandamide stimulus as compared to the Δ^9-THC stimulus. It could be also the case that anandamide and methanadamide but not Δ^9-THC possess affinity for a subpopulation of receptors other than CB_1. Unfortunately the ability of SR-141716A to block the generalization to anandamide was not tested. Among non-cannabinoid drugs, only the benzodiazepine diazepam has been found to produce partial generalization to cannabinoid training stimulus that was SR-141716A-insensitive, suggesting that this effect is mediated by an interaction through the GABAergic system [34].

Self-administration

Drug self-administration behavior has been one of the most direct and productive approaches for studying the rewarding properties of abused drugs. Using this methodology, it has been possible to study neuropharmacological mechanisms involved in such behaviors and preclinically evaluate therapeutic strategies for treatment of drug abuse. Since 1970, all attempts to obtain a robust procedure for Δ^9-THC self-administration have failed and this has been fundamental to claims of a differential status for cannabinoids with respect to major abused drugs. Within the last few years, however, reinforcing effects of some synthetic CB_1 cannabinoid agonists have been reported using intravenous self-administration procedures in rats and mice [35–37], although the experimental procedures employed in each of these studies limit the generality of the findings. Persistent intravenous self-administration of Δ^9-THC itself was first demonstrated in squirrel monkeys by Tanda et al. [38]. However, monkeys in this study had a history of cocaine self-administration, raising the possibility that persistent neurobiological adaptations might subsequently predispose animals to self-administer Δ^9-THC. This problem was successfully overcome by Justinova et al. [39], who demonstrated that Δ^9-THC-self-administration behavior was initiated and subsequently maintained at very high rates in monkeys with no history of exposure to other drugs, showing that this drug possesses reinforcing properties of its own that are not dependent on prior self-administration of other drugs. Thus self-administration of Δ^9-THC by squirrel monkeys provides a reliable animal model of human marijuana abuse, suitable for studying the relative abuse liability of other natural and synthetic cannabinoids and for developing new therapeutic strategies for the treatment or prevention of marijuana abuse in human.

Conditioned place preference

The conditioned place preference procedure is a classical procedure that provides an indication of drug-related reward/aversion effects in animals. Previous studies into the reinforcing properties of cannabinoids have produced conflicting evidence with respect to the generation of place preference. Some studies have shown that Δ^9-THC can produce place preference [40, 41], whereas others reported place aversion [12, 42–46] or no effect [41]. The discrepancies in results have been interpreted as being due to differences in apparatus, experimental design and the subjects used. Positive place preferences, when found, are usually highly dose-dependent, often occurring at only a single dose either in mice or in rats using Δ^9-THC as well as synthetic cannabinoid compounds [40, 41, 47, 48]. Indeed, place preference was obtained with a low dose of Δ^9-THC in mice (1 mg/kg) when they received a previous priming Δ^9-THC exposure in the home cage before the conditioning sessions [41]. Place aversion properties are often produced by Δ^9-THC and synthetic cannabinoids either in rats or in mice using similar dose ranges and standard place preference procedures [40, 43–46]. These apparently conflicting results could be explained by the possible dysphoric/anxiogenic consequences of the first cannabinoid exposure that could mask the development of positive place preference [41]. Discrepant results are also present for the CB_1 receptor antagonist SR-141716A: while some papers reported a positive place preference in rats [44, 49] some others failed to demonstrate either place preference or place aversion [45, 47]. These opposite results do not allow us to precisely indicate a role for endocannabinoid tone as a physiological system to suppress reward or to induce aversion.

Neurochemical correlates of cannabinoid rewarding properties

The mesolimbic dopaminergic system is part of a brain reward circuit that has been long thought to play a major role in mediating reinforcing/rewarding effects of drugs of abuse [50]. Many drugs abused by humans share the common property of selectively increasing dopamine release in the nucleus accumbens, the major terminal area of the mesolimbic dopamine system, but this has been a matter of debate with regard to Δ^9-THC and other cannabinoids. It is now well-accepted that cannabinoids are able to increase dopamine levels in the shell compared with the core of the nucleus accumbens, likely through an opioid receptor-mediated mechanism or a direct activation of dopaminergic neurotransmission in the nucleus accumbens (see [9] for review). Moreover, cannabinoids might exert part of their reinforcing effects through the endogenous opioid system [51, 52]. For example, Δ^9-THC-induced conditioned place preference is suppressed in μ-opioid receptor-knockout mice [48] and Δ^9-THC-induced self-administration can be blocked by μ-opioid receptor antagonists [36, 37]. The neurochemical mechanism of the interaction between

the endocannabinoid and opioid systems has not been elucidated, but might involve cannabinoid-induced synthesis and release of endogenous opioids or converging signal transduction pathways if the receptors are co-expressed [52].

Cannabinoid system and drug addiction

Animal models of drug reward provide evidence that endogenous cannabinoids play a role in determining the rewarding effects not only of cannabis but also of other psychoactive drugs, such as ethanol, cocaine, morphine, nicotine and amphetamine. Plenty of published works report the involvement of cannabinoid processes in positive reinforcement activated by both natural rewards and drugs of abuse. For example, in CB_1-knockout mice nicotine was not able to induce place preference as it does in wild-type mice [53], and administration of SR-141716 in the rat decreased nicotine self-administration [54]. These results suggest that activation of the endogenous cannabinoid system may participate in the motivational effect of nicotine; thus SR-141716 may be effective as an aid for smoking cessation.

Results on morphine-conditioned place preference in CB_1-knockout mice had controversial effect: in one study [55] morphine induced conditioned place preference in wild-type mice but failed to produce any response in knockout mice, indicating the inability of morphine to induce rewarding effects in the absence of CB_1 cannabinoid receptors. In a more recent work [56] CB_1 receptor-knockout mice developed a strong place preference to morphine, similar to that in wild-type Swiss-Webster mice, thus not supporting a contribution of the brain cannabinoid system to morphine reward. A possible explanation for this discrepancy could rely in the slightly more intensive conditioning paradigm and differences in the nature of conditioning chambers used for the experiment in the last paper. However, self-administration studies support the idea that the CB_1 cannabinoid receptor is essential for the modulation of morphine's rewarding effects. Cossu et al. [57] found that morphine did not induce intravenous self-administration in mutant CB_1 receptor-knockout mice, whereas it was significantly self-administered by the corresponding wild-type mice. Approaches involving the CB_1 antagonist SR-141716 gave more compelling results. Recently it was shown that SR-141716A pretreatment dose-dependently reduced operant heroin self-administration by male Wistar rats under a fixed-ratio schedule of reinforcement, and significantly lowered the breaking point of responding for heroin under a progressive-ratio schedule of reinforcement [58]. In the same line Solinas et al. [59] reported that SR-141716A markedly decreased heroin self-administration under the progressive-ratio schedule. In contrast, SR-141716A had no effect on heroin self-administration under the fixed-ratio schedule at heroin doses of 50 or 100 μg/kg per injection, but produced small decreases in self-administration at lower doses (25 and 12.5 μg/kg per injection). These data demonstrate that the cannabinoid CB_1 receptor antagonist SR-141716 produces a clear attenuation, but not a com-

plete blockade, of the reinforcing effects of heroin, suggesting a facilitatory modulation of opioid reinforcement by endogenous cannabinoid activity that is unmasked by CB_1 receptor blockade. All these lines of evidence provide support for the potential efficacy of cannabinoid CB_1 antagonists in the prevention and treatment of opioid addiction.

Evidence for endocannabinoid involvement in the rewarding effects of ethanol also exists (see [60] for review). Here we only cite the latest papers. Voluntary ethanol intake was significantly lower in $CB_1^{-/-}$ *versus* $CB_1^{+/+}$ young male mice [61–63]. Moreover, administration of the cannabinoid CB_1 receptor antagonist SR-141716 significantly reduced ethanol intake in CB_1 wild-type (+/+) mice [61] and rats [54]. The role of endocannabinoids and CB_1 receptor in alcohol-drinking behavior is now unequivocal; thus SR-141716 may be effective in reduction of alcohol consumption. Surprisingly the combination of the synthetic cannabinoid agonist CP-55,940 with MDMA (methylenedioxymethamphetamine; ecstasy) in rat reduced the number of drug-associated lever pressings compared to the single drugs [64] and pre-treatment with SR141716A significantly increased MDMA self administration. At first glance these data seem to suggest that the endocannabinoid system might have negative effects rather than the positive ones shown in the above cited studies. The nature of this interaction remains unclear due to the lack of studies on dopamine levels in mesolimbic structures that could add further insight on the neurochemical correlates of MDMA's reinforcing properties.

Finally, particularly relevant seems to be the role of endocannabinoid tone in relapse to drugs of abuse. This aspect of drug addiction assumes a striking interest in the human context. In fact, detoxification from drug addiction has been a medical problem for as long as drugs have been abused, due to relapse occurring even after prolonged drug-free periods. Several reinstatement models are currently available to investigate major factors contributing to relapse and have been used to study the involvement of the cannabinoid system. In recent work Fattore et al. [65] reported that the CB_1 receptor antagonist SR-141716A prevented heroin-induced reinstatement of heroin-seeking behavior but did not show any effect *per se*, suggesting that CB_1 receptor blockage alters the reinforcing consequences of heroin administration. Moreover, in animals with a history of heroin self-administration, cannabinoid primings elicit relapse to heroin-seeking behavior following an extended drug-free period. Similar results were also obtained by De Vries et al. [66]: the potent cannabinoid agonist HU-210 reinstated heroin seeking, whereas SR-141716A attenuated both heroin-primed and cue-induced heroin seeking following a 3-week extinction period. The same group [67] found very similar results also in animals withdrawn from cocaine self-administration: HU-210 provoked relapse to drug seeking after a prolonged withdrawal period, while blockade of CB_1 receptor attenuated the relapse induced by re-exposure to cocaine-associated cues or cocaine itself. The CB_1 cannabinoid antagonist was also used on alcohol-deprivation effects (i.e. the temporary increase in alcohol intake after a period of alcohol withdrawal) in Sardinian alcohol-preferring

(sP) rats [68]. As expected, alcohol-deprived rats virtually doubled voluntary alcohol intake during the first hour of re-access. Acute administration of SR-141716 completely abolished the alcohol-deprivation effect. These results suggest that the cannabinoid CB_1 receptor is part of the neural substrate mediating the alcohol-deprivation effect and that SR-141716 may possess anti-relapse properties.

Taken together, these results seem to indicate that SR-141716 could specifically counteract reward-related behaviors, whatever the specific factors involved in the action of each reinforcer, and that cannabinoid CB_1 receptors could be crucially involved in the neurobiological events evoked by appetitive reinforcers. However this does not necessarily mean that a permanent endogenous cannabinoid tone exists to ensure the organism a basal hedonic level. Thus it can be postulated that cannabinoid-related processes are elicited and maintained by pleasant reinforcers. This suggests that the activation of reward system could be under the permissive control of some complex CB_1-related cannabinoid processes which are required for the perception of the incentive value of positive reinforcements. Is the recent finding that Rimonabant reduces food intake in obesity and tobacco consumption in more than 500 adults underlying the relevance of therapeutic modulation of the endocannabinoid system?

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

Cannabinoids in appetite and obesity

Francis Barth and Murielle Rinaldi-Carmona

Sanofi-aventis, 371, rue du Professeur Joseph Blayac, 34184 Montpellier Cedex 04, France

Introduction

In contrast to most drugs, many of the pharmacological activities of cannabinoids were described in humans before being investigated by pharmacologists in laboratory animals. This peculiarity is of course due to the use of *Cannabis sativa* preparations for recreational purposes from ancient civilizations up to modern times.

The effects of cannabinoids on appetite makes no exceptions: the fact that cannabis can stimulate appetite has been observed since AD 300 [1]. Recent experiments using potent synthetic or natural endogenous cannabinoid agonists, as well as transgenic animals in which the cannabinoid system has been disrupted confirm the role of the cannabinoid system as a modulator of food intake.

Particularly interesting are the opposite effects of the newly developed cannabinoid antagonists. These compounds have been shown to decrease food intake and to regulate body-weight gain, and are expected to provide a new therapeutic approach to treat obesity, a condition that affects up to 27% of the US population and is now considered by the World Health Organization as a global epidemic that poses a serious threat to world health, particularly in adolescents [2].

However, the mechanism by which the cannabinoid system modulates food intake is far from fully understood, and its elucidation is the subject of much research at the moment.

Cannabinoid agonists and appetite

A large anecdotal and descriptive literature suggest that smoking cannabis stimulates hunger, and selectively increases the appetite for sweet and palatable food, which smokers sometimes refer to as ‘the munchies’. Starting in the 1970s, a series of well-controlled scientific studies was conducted to better characterize this effect [3].

An increased desire for sweet food (marshmallows) was indeed noted in subjects after smoking marijuana [4]. Other studies have shown that Δ^9-tetrahydrocannabinol (Δ^9-THC)-induced appetite stimulation was dependent on the route of administration [5], the dose used [6], the social environment [7] and the satiety status [8]. Higher appetite stimulation was observed with the suppository *versus* oral intake or inhalation, with low-Δ^9-THC *versus* high-Δ^9-THC cigarettes, in smoking in social groups *versus* isolated use and finally in fed *versus* fasted individuals.

Based on these findings, dronabinol (Marinol®), an oral formulation of Δ^9-THC, was approved by the US Food and Drug Administration (FDA) in 1992 for the treatment of AIDS-related anorexia. This wasting syndrome, which occurs in about 18% of AIDS patients, is of particular concern in that it may exacerbate the primary illness, decrease quality of life and also decrease survival.

Several clinical studies involving patients with anorexia related to AIDS, cancer or Alzheimer's disease demonstrated the efficacy of dronabinol [9, 10]. In a double-blind, placebo-controlled 6-week study involving 139 patients with AIDS-related anorexia, dronabinol (5 mg/day) was associated with increased appetite (38% above baseline *versus* 8% for placebo), improvement in mood (+10 *versus* –2%) and decreased nausea (–20 *versus* –7%). Body weight was stable in dronabinol patients, while placebo recipients had a mean loss of 0.4 kg [11]. These effects were later confirmed in a long-term, open-label follow-up study with 94 patients treated for up to 12 months [12]. Dronabinol therapy was generally well tolerated during long-term use, with most adverse events being associated with the known central effects of cannabinoids (euphoria, dizziness, confusion). No significant changes in hematology or blood-chemistry parameters due to the drug were noted.

The use of dronabinol or other cannabinoids, including smoked marijuana, to treat cancer or AIDS-related anorexia remains, however, controversial due to the psychotropic properties of these drugs, and also due to the reluctance to treat immuno-deficient patients with a cannabinoid drug, which may by itself negatively affect the immune system. This fear may however be unfounded, at least for dronabinol, if one considers a study that compared the use of dronabinol and megestrol acetate for the treatment of the HIV wasting syndrome in which no difference in the CD4 T-lymphocyte count due to the drugs during the 12-week study was noted [13]. Another study involving 67 patients with the HIV-1 infection concluded that smoked or oral Δ^9-THC did not produce any harmful effects on viral loads. The findings of this short-term (21-day) study should however be confirmed in additional long-term trials [14].

In laboratory animals, treatment with Δ^9-THC or synthetic cannabinoid agonists has shown inconsistent effects on food intake [1], with an increase [15–17], or decrease [18] of food intake, or without any effect [19]. The species, route of administration and dose used clearly influence the outcome of the experiments. In particular, the decreased locomotor performance induced by high doses of cannabinoids may often be responsible for the decreased food intake observed in the high-dose experiments.

Of particular interest are the experiments conducted with anandamide and 2-arachidonoyl glycerol (2-AG), the endogenous ligands for cannabinoid receptors. Subcutaneous injection of anandamide (0.5–10 mg/kg) induced significant overeating in pre-satiated male rats [20]. Subsequent studies have shown that very low doses of anandamide [0.001 mg/kg, administered intraperitoneally (i.p.)] also increased food intake in female mice while higher doses were not active in this paradigm [21]. Direct injection of anandamide (50 ng in 0.5 µl) into the ventromedial hypothalamus also initiated food intake in rats [22]. Similarly, injection of 2-AG into the nucleus accumbens shell, a limbic forebrain area strongly linked to eating motivation, potently and dose-dependently stimulated feeding in rats [23]. This effect was blocked by pre-treatment with the cannabinoid antagonist SR-141716 (Fig. 1), indicating the involvement of the cannabinoid CB_1 receptor.

These experiments clearly demonstrated that activation of central cannabinoid receptors by endocannabinoids stimulates eating behavior, and provided important evidence for the involvement of a central cannabinoid system in the normal control of feeding.

Interestingly, the selectivity for sweet or palatable food observed in humans was also observed in animal experiments. The potent synthetic cannabinoid agonist CP-55,940 (0.01–0.05 mg/kg i.p.) was shown to increase motivation for beer and for a sucrose solution in rats [24]. Δ^9-THC (0.5–2.5 mg/kg i.p.) also selectively increased the consumption of a high-fat or high-fat sweetened diet *versus* standard chow in free-feeding Lewis rats [16].

Recent studies also suggested that the endocannabinoid 2-AG present in milk may play a vital role in the initiation of milk suckling, and hence in growth and development during the early stages of mouse life [25, 26].

Cannabinoid antagonists and obesity

The first potent and selective CB_1 cannabinoid antagonist, SR-141716, was described in 1994 [27]. This compound was able to reverse the hyperphagia

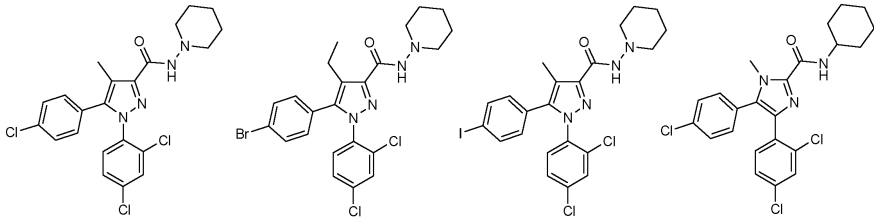

Figure 1. Selected cannabinoid CB1 antagonists acting on food intake.

induced by cannabinoid agonists such as Δ^9-THC [28], anandamide [20] and 2-AG [23].

More interestingly, SR-141716 also produced changes in ingestive behaviors when administered alone. This was first shown by Arnone et al. [29], who described the effects of SR-141716 [0.3–3 mg/kg, taken *per os* (p.o.)] on spontaneous sucrose feeding (sucrose pellets *versus* standard chow) and spontaneous or neuropeptide Y (NPY)-induced sucrose drinking in rats. SR-141716 markedly and dose-dependently reduced the consumption of sucrose, with only a marginal effect on regular chow and water consumption. Moreover, it also decreased ethanol consumption in C57BL/6 mice, a strain known for its genetic predisposition for ethanol consumption, without affecting water intake [29]. In another study, marmosets were given the choice between standard food or a sweet cane-sugar mixture; administration of SR-141716 (1–3 mg/kg p.o.) significantly and dose-dependently reduced the amount of sweet food ingested during the 6-h test period, with no effect on the standard food intake [30]. This preferential effect on palatable food intake compared with standard food suggested that an endogenous cannabinoid tone may modulate the appetitive value of food. Similar results were obtained by Colombo et al. [31], who described an overall decrease in food intake and body weight in rats treated with SR-141716 (2.5–10 mg/kg i.p.) for 14 days. Using a self-administration protocol it was also shown that SR-141716 reduces sucrose intake by acting on both the appetitive and the consummatory aspects of ingestive behavior in rats [32].

These results suggested a potential for cannabinoid antagonists in the treatment of eating disorders and, in particular, obesity. Therefore, the effect of chronic administration of SR-141716 in pharmacological models more relevant to obesity was investigated. One of these model is the diet-induced obese (DIO) mice, in which the animals are made obese simply through eating of a high-fat diet. Daily administration of SR-141716 (10 mg/kg/day p.o.) for 5 weeks produced a transient decrease in food intake with a sustained reduction in body weight. At the end of the treatment period, a significant 20% weight loss was obtained, together with a 50% reduction of adiposity. Moreover raised plasma leptin, insulin and free fatty acid levels were decreased to values found in lean (non-DIO) mice [33].

Similar results were obtained with AM-251, a close analogue of SR-141716 (Fig. 1). DIO mice were treated with AM-251(3–30 mg/kg/day p.o.) using a chronic, interrupted dosing schedule (2 weeks on treatment, 2 weeks off and a further 2 weeks on treatment). A significant reduction of body weight together with a reduction of adipose tissue mass was observed. While the anti-obesity effect was lost during the off-treatment period, it was recovered during the second treatment period, suggesting that chronic treatment of obese individuals with CB_1 antagonists is a viable pharmacological approach [34].

Other obesity models involve genetically obese animals; these include the *ob*/*ob* mice, which have an inherited lack of leptin, *db*/*db* mice, which have a defective leptin receptor, and Zucker (*fa*/*fa*) rats, which lack the leptin recep-

tor. In both *ob*/*ob* and *db*/*db* obese mice with unrestricted access to food, acute administration of SR-141716 (3 mg/kg i.p.) produced a significant reduction of food intake [35]. In Zucker (*fa*/*fa*) rats, SR-141716 induced a transient decrease in food intake and body-weight gain when administered orally for 4 weeks [36].

These encouraging results have stimulated the search for cannabinoid antagonists. Both publications and patent literature suggest that new cannabinoid antagonists with chemical structures distinct from that of SR-141716 may exhibit anti-obesity activity in animal models. The pyrazole derivative SR-147778 (Fig. 1) was able to reduce sucrose consumption in mice and rats, and food intake in fasted and non-deprived rats [37]. A new imidazole derivative acting as a potent cannabinoid CB_1 inverse agonist (Fig. 1, compound **1**) was recently shown to be effective in reducing body weight in DIO rats following 14 days treatment (3–10 mg/kg p.o.) [38] . Similar compounds are claimed in a Bayer patent application which describes significant reduction in food intake in normal rats and weight loss following chronic treatment in Zucker obese rats [39].

Following the results obtained in animal models of obesity, Sanofi-Synthelabo advanced SR-141716 (rimonabant) into clinical trials involving obese patients. Rimonabant was tested in obese male subjects with a body mass index (BMI = weight/height^2) of >27 kg/m^2 in a double-blind cross-over study (20 mg once a day *versus* placebo). The patients were treated for 7 days with a 28-days wash out. Rimonabant had no effect on taste and spit tests, yet induced a significant decrease in hunger (visual analogue scale), caloric intake and weight [40]. Moreover, in a subsequent study involving 287 obese patients (BMI values of between 29 and 41 kg/m^2) treated for 16 weeks with either placebo or rimonabant (5, 10 and 20 mg once daily), rimonabant was able to significantly reduce body weight as well as waist circumference. In the 20-mg group the mean decrease in body weight was 3.8 kg (*versus* 0.9 kg in the placebo group) and the mean decrease in waist circumference was 3.9 cm (*versus* 1.1 cm in the placebo group). The observed decrease in weight did not reach a plateau during the 4-month duration of the study and the tolerance of SR-141716 was excellent [41].

Rimonabant is currently being evaluated in multi-centre, randomized, double-blind phase III studies in order to assess its efficacy and long-term safety. The results of one of these studies, the RIO-Lipids trial, which enrolled 1036 overweight or obese patients (BMI values between 27 and 40 kg/m^2) with untreated dyslipidemia [high triglycerides and/or low high-density lipoprotein (HDL)-cholesterol] were presented at the 2004 American College of Cardiology annual meeting [42]. Patients were randomized to receive either a daily, fixed dose of rimonabant (5 or 20 mg) or placebo along with a mild hypocaloric diet for 1 year. Patients treated for 1 year with rimonabant 20 mg/day lost 8.6 kg (*versus* 2.3 kg in the placebo group). Over 72% of patients treated for one year with rimonabant (20 mg/day) lost over 5% of their body weight (*versus* 27.6% in the placebo group). Moreover, 44.3% of the

patients in the 20-mg rimonabant group lost more than 10% of their body weight (*versus* 10.3% in the placebo group). In addition to weight loss, the study was designed to assess a number of important associated cardiovascular risk factors. Study findings for rimonabant 20 mg include:

- a waist-circumference reduction of 9.1 cm in patients treated for 1 year (completers);
- an average increase in HDL-cholesterol (+23%) and a reduction in triglycerides (–15%) in completers;
- reduction of C-reactive protein (CRP), an important inflammatory marker predictive of cardiovascular risk (–27% *versus* –11% in the placebo group);
- and improved insulin sensitivity (oral glucose tolerance test) [42, 43].

These robust data were replicated in another phase III study (RIO-Europe) involving 1507 obese patients with or without comorbidities [44]. Rimonabant, which was well tolerated, could therefore become an important agent in the management of cardiovascular risk in obese patients.

Mechanism of action of cannabinoid ligands on food intake and energy balance

The mechanisms that control food-intake and energy homeostasis in mammals are particularly complex. Leptin and insulin, two hormones secreted by adipocytes and the pancreas cells respectively, are key elements of this process. Leptin and insulin are released from peripheral organs and interact with specific brain receptors in the hypothalamus in order to adapt food intake to body fat mass. However, it would appear that more than 16 other hypothalamic neuropeptides are involved in the precise control of energy homeostasis and act to either stimulate or inhibit food intake [45]. Moreover, monoamine neurotransmitters such as noradrenaline, serotonin and dopamine have also been shown to affect food intake, with noradrenaline and serotonin uptake being the pharmacological mechanism of sibutramine, one of the only two prescription drugs approved by the FDA for the treatment of obesity. How the endogenous and synthetic cannabinoids interact with all these systems remains largely unknown.

The involvement of cannabinoid CB_1 receptors in both the agonist-induced hyperphagia and antagonist-induced food-intake reduction has been demonstrated by several authors.

The involvement of cannabinoid CB_1 receptors in food consumption induced by anandamide and 2-AG was confirmed by its sensitivity to the specific CB_1 antagonist SR-141716, which reversed for example the hyperphagia induced by anandamide [20] and 2-AG [23]. On the other hand, the CB_2 antagonist SR-144528 [46] did not affect the Δ^9-THC-induced feeding in pre-satiated rats, excluding the participation of the CB_2 subtype in this effect [28]. The involvement of CB_1 receptors in the food-intake reduction induced by the

antagonist SR-141716 was confirmed by using CB_1 receptor-knockout mice. While SR-141716 significantly reduced food intake in wild-type mice, it was totally ineffective in mice lacking the CB_1 receptor [35, 47]. Moreover, it was shown that following temporary food restriction, the knockout mice eat less than their wild-type littermates. These results suggest the existence of an endogenous cannabinoid orexigenic tone which, when disrupted, may lead to decreased food consumption.

Interaction between cannabinoids and hormones controlling energy homeostasis

The link between cannabinoids and the anorexigenic hormone leptin was first suggested by Di Marzo et al. in 2001 [35]. Acute leptin treatment of normal mice and *ob*/*ob* mice (mutant mice lacking the leptin gene) not only decreased food intake but also resulted in decreased levels of anandamide and 2-AG in the hypothalamus. On the other hand, defective leptin signaling was associated with elevated hypothalamic but not cerebellar levels of endocannabinoids in obese *db*/*db* and *ob*/*ob* mice and Zucker rats [35]. These findings suggest a direct link between endogenous cannabinoids and the leptin-controlled energy homeostasis. Moreover, interactions between the cannabinoid system and other neuropeptides involved in the control of food intake have also been suggested in the literature. A cross-talk between the orexin-1 and the CB_1 receptors was recently described [48], and a synergistic interaction between CB_1 and melanocortin MC4 systems in feeding behavior has also been reported [49]. Moreover, the co-expression of hypothalamic CB_1 mRNA with corticotropin-releasing hormone (CRH), cocaine-amphetamine-regulated transcript (CART) and melanin-concentrating hormone (MCH) may also be indicative of possible interactions between the cannabinoid system and these peptides.

Cannabinoids and motivational processes

Ingestive behaviors, however, are not only linked to energy homeostasis control, as the brain-reward system, a complex neural network activated by pleasurable stimuli also mediates the incentive or hedonic value of food. The association of the cannabinoid system with the motivational processes is indicated by several lines of evidence. The preference for palatable sweet-food intake induced by administration of cannabinoid agonists in both humans and laboratory animals is most likely indicative of the involvement of an effect on the brain-reward systems. Moreover, the CB_1 antagonist SR-141716 not only selectively decreased the intake of sweet or palatable food, but also decreased both alcohol [29, 32] and nicotine self-administration in rats [50]. It also decreased heroin self-administration in rats [51] and was shown to reduce the reinforcing value of the median forebrain bundle (MFB) electrical stimulation

[52]. In addition, cannabinoids also interact with known opioid reward pathways, as indicated by the synergistic action of cannabinoid and opioid antagonists on food intake [53, 54]. These results suggest that the central cannabinoid system may act to amplify certain motivation indices and that blockade of its interaction with the reward system may contribute to the effects of CB_1 antagonists on food intake.

Peripheral mechanisms and effects on metabolic processes

Recently, a third, purely peripheral, mechanism of action was also proposed by several authors to explain some of the effects of SR-141716 on body weight. A direct effect of SR-141716 on the activation of metabolic processes was proposed by Ravinet-Trillou et al. [33]. This was supported by the fact that during the course of a 5-week study using DIO mice the drug produced only a transient effect on food intake, yet a sustained effect on body weight. Moreover, SR-141716-treated mice had an accelerated weight loss *versus* control during a 24-h fast and the weight loss was increased in SR-141716-treated mice compared with pair-fed animals [28]. Chronic treatment with SR-141716 also reduced the respiratory quotient of obese Zucker rats from 0.9 to 0.7, a change consistent with an increase in fat oxidation [55] and it was shown that metabolic factors contribute to the lean phenotype in adult mice with a disrupted CB_1 gene ($CB_1^{-/-}$ mice) [56]. A direct action of SR-141716 on adipocytes may be responsible for this peripheral effect, as CB_1 mRNA was detected in mouse and rat adipocytes [56, 57]. Interestingly, SR-141716 was shown to increase adiponectin mRNA expression in adipose tissue of obese *fa/fa* rats and in cultured mouse adipocytes. Adiponectin (Acrp 30) is a plasma protein exclusively expressed and secreted by adipose tissue which has been shown to increase fatty acid oxidation and improve insulin sensitivity and so reduce body weight. This effect of SR-141716 was abolished in $CB_1^{-/-}$ mice, indicating the involvement of CB_1 receptor [57]. All these results clearly underline that cannabinoid antagonists such as SR-141716 could exert a 'peripheral' metabolic action in addition to their known 'central' effect on food intake.

Other recent studies demonstrated that sensory deafferentation which destroyed the sensory terminals innervating the gut abolished both the hyperphagic effects of cannabinoid agonists and the hypophagic effect of SR-141716. These results suggest that CB_1 receptors, located in the gastrointestinal tract, may also participate in the modulation of feeding induced by cannabinoid agonists and antagonists [58].

Conclusion

Once considered as purely anecdotal, the effects of cannabinoids on appetite are now the subject of intense interest in the scientific community. The natural

cannabinoid agonist Δ^9-THC is indeed already used as a therapeutic agent to treat anorexia and cachexia associated with severe illness such as AIDS or cancer. New synthetic CB_1 agonists may be of therapeutic value for this therapeutic application if they are able to associate higher potency with a reduction of the side effects linked to the psychotropic effects of Δ^9-THC.

On the other hand, the opposite, anorectic effect of cannabinoid antagonists offers an important potential for the treatment of obesity, a condition which has become a major health problem in developed countries. In this respect, the results obtained in clinical studies with rimonabant (SR-141716), the first cannabinoid antagonist, are extremely encouraging. Moreover, based on preliminary pharmacological studies, CB_1 antagonists may also be useful for the treatment of alcoholism, and in smoking cessation. Clinical studies with rimonabant have already shown increased tobacco-smoking abstinence as well as prevention of the secondary weight gain often associated with smoking cessation [42, 59]. In any case, more research is needed to fully understand the complex role of the cannabinoid system in the regulation of feeding and body-weight control, a biological function which is particularly complex and involves interactions with many different biochemical messengers.

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Cannabinoids as Therapeutics
Edited by R. Mechoulam

The development of Sativex® – a natural cannabis-based medicine

Geoffrey W. Guy and Colin G. Stott

GW Pharmaceuticals plc, Porton Down Science Park, Salisbury, Wiltshire SP4 OJQ, UK

History of the development

Cannabis has been used medicinally for 4000 years [1–4] in a variety of cultures and was re-introduced into British medicine in 1842 by W. O'Shaughnessy [5]. It remained in the British pharmacopaeia until 1932, when cannabis, extract of cannabis and tincture of cannabis were among 400 medicines removed, though all three remained in the British Pharmaceutical Codex of 1949 [5].

However, following the 1961 UN Single Convention on Narcotic Drugs, cannabis and cannabis derivatives became scheduled products and were subject to special measures of control and parties could ban their use altogether. Following the 1971 UN Convention on Psychotropic Substances, the UK enacted the Misuse of Drugs Act 1971. Cannabinol and its derivatives, including Δ^9-tetrahydrocannabinol (Δ^9-THC), appeared in Schedule I to the Convention, and their regular medical use was prohibited. The introduction of the Misuse of Drugs Regulations in the UK in 1973 listed cannabis and cannabis products in Schedule 4 (now Schedule I in current legislation), thereby prohibiting medical use altogether [5].

Early research

Although the medicinal properties of cannabis had been well documented for a number of years, the constituent(s) responsible for therapeutic efficacy had, until recently, not been identified. The discovery, isolation (and subsequent synthesis) of the principal cannabinoid present in cannabis, Δ^9-THC, by Raphael Mechoulam and Yehiel Gaoni in 1964 [6] ensured that interest in cannabinoid chemistry remained and led to an expansion of cannabinoid research.

Despite the scheduling and prohibition of cannabis and the ban on medical use of cannabis-based products in the 1970s, research into the pharmacology and toxicology of Δ^9-THC continued through the 1970s and 1980s, mainly by the National Institute of Health (NIH) in the USA.

However, much of the work concentrated solely on Δ^9-THC (NTP program, NIH) [7]. In many cases, the investigation of the pharmacokinetics of cannabis components involved the delivery of smoked marijuana, and the measurement of Δ^9-THC levels and its primary metabolite, 11-hydroxy-tetrahydrocannabinol (11-OH-THC).

Recent research and development of a cannabis-based medicine

In January 1997, the White House Office of National Drug Control Policy (ONDCP) asked the Institute of Medicine (IOM) to conduct a review of the scientific evidence to assess the potential health benefits and risks of marijuana and its constituent cannabinoids. That review began in August 1997 and resulted in the report published in 1999 [8]. Reports were also published in August 1997 by the US NIH [9] and in December 1997 by the American Medical Association (AMA) [10].

In parallel with the timing of the IOM review, a number of expert bodies in the UK were asked to review the medical and scientific evidence for and against the use of cannabis as a medicine. The British Medical Association (BMA) published a report on the topic in 1997 [11]. The UK Department of Health commissioned three literature reviews on cannabis, at the request of the Advisory Council on the Misuse of Drugs (ACMD); and these were reviewed by the House of Lords Select Committee on Science and Technology in 1998. The authors of the report all gave evidence to the House of Lords inquiry [12–14].

Dr Geoffrey Guy was also invited to submit evidence to the House of Lords enquiry, and subsequently GW Pharmaceuticals Ltd was founded in the UK in early 1998. As GW's Executive Chairman, Dr Guy successfully floated the company (GW Pharmaceuticals plc) on the Alternative Investment Market (AIM) of the UK Stock Exchange in June 2001. The first UK Home Office licenses received by GW were to cultivate, possess and supply cannabis for research purposes were received in June 1998 and cultivation began in August 1998.

In November 1998, the House of Lords Select Committee on Science and Technology published its report *Cannabis: The Scientific and Medical Evidence* [15], which recommended that clinical trials of cannabis medicines should be carried out as a matter of urgency. The Committee warmly welcomed GW's research programme.

September 1999 saw the start of GW's first phase I clinical trials in healthy volunteers and in March 2000 GW received authorization from the Medicines Control Agency (MCA; now the Medicines and Healthcare Products Regulatory Agency, MHRA) to start phase II clinical trials in patients.

In March 2001, the same House of Lords Select Committee published a follow-up report, *Therapeutic Uses of Cannabis* [16], which confirmed the UK Government's intention to permit the prescription of cannabis-based medicines (CBMs) subject to the approval of the MHRA.

GW entered into its pivotal phase III clinical trials programme in March 2001. The initial phase III studies involved patients with multiple sclerosis (MS), neuropathic pain and cancer pain. The results of the first four phase III studies were reported in November 2002, and six of the trials have now been completed, yielding positive results, and a further three are due to report in 2005.

In March 2003 GW submitted an application to the MHRA for its first product, Sativex®.

In May 2003 GW entered into an exclusive UK marketing agreement for Sativex® with the German pharmaceutical company Bayer AG. This agreement was extended in November 2003, to add the Canadian market.

In May 2004 GW submitted a New Drug Submission for Sativex® to the Canadian regulatory authorities, Health Canada.

The endogenous cannabinoid system

The discovery and chemical synthesis of Δ^9-THC initiated the modern era of cannabis research because it enabled investigation of the effects and mode of action of individual cannabinoids in laboratory models [17]. The production of synthetic analogues of Δ^9-THC enabled structure – activity relationships of Δ^9-THC to be established. Further, pharmacological investigation of Δ^9-THC indicated that it might exert its effects by interacting with a specific receptor protein in the brain [18, 19]. The conclusion from this work was that the so-called cannabinoid receptor was a G-protein-coupled receptor. Once a CB receptor agonist, CP-55,940, was synthesized, radiolabelled binding studies were performed [20], and the distribution of CP-55,940-binding sites were found to be similar to those coded for by cDNA for another G-protein-coupled receptor, SKR6, a receptor without a known ligand (an orphan receptor). Further investigation using cannabinoid-binding assays revealed that SKR6 was indeed a cannabinoid receptor identified in rat brain [21]. Soon afterwards a human G-protein receptor was identified that had an amino acid sequence 98% identical to the SKR6 receptor in rat brain.

In 1993, a second G-protein-coupled cannabinoid receptor sequence (CX5) was identified among cDNAs from the human promyelocytic leukaemic cell line HL60 [22].

Munro et al. [22] suggested that the brain receptor be referred to as CB_1 and that the second receptor, which is expressed by cells of the immune system, be referred to as CB_2.

It has since become widely accepted that CB_1 receptors are widely distributed but are particularly abundant in some areas of the brain, including those concerned with movement and postural control, pain and sensory perception, memory, cognition and emotion, and autonomic and endocrine functions [23, 24]. They are also prevalent in the gut, testes and uterus. The role of the second type of receptor, CB_2 receptor, is still under investigation but it is believed to mediate the immunological effects of cannabinoids [23, 24].

In the meantime, Mechoulam and Devane isolated and elucidated the structure of a brain constituent that bound to the cannabinoid receptor [25]: arachidonylethanolamide (AEA, anandamide). During subsequent investigation of several lipid fractions collected from rat brain, it was discovered that the fractions also contained materials that bound to cannabinoid receptors [26]. Characterization of these fractions revealed that some contained polyunsaturated acid ethanolamides (similar to AEA), but others contained a distinct lipid component, 2-arachidonoyl glycerol (2-AG).

AEA is found to be a partial agonist at CB_1 receptors; whereas 2-AG binds to CB_1 and CB_2 with similar affinities, and is a full agonist at CB_1. 2-AG occurs in concentrations in the brain that are 170 times higher than those of AEA [26].

The role of these endogenous cannabinoids (so-called endocannabinoids) is currently unclear, and others have subsequently been identified: noladin ether [27], virodhamine [28], *N*-arachidonoyl-dopamine (NADA) [29] and arachidonoyl-serine (ARA-S) [30]. The identification of AEA and 2-AG has led to a resurgence of interest in the field of cannabinoid medicine, especially within the pharmaceutical industry, as they may represent potential molecular targets for the treatment of a number of disorders.

Cannabinoid receptor ligands

In the wake of widespread availability of synthetic CB receptor-specific ligands, research into the identification of potential sites of action of cannabinoids has increased around the world. However, until recently, the lack of significant available quantities of pure cannabinoids other than Δ^9-THC and cannabidiol (CBD) has been a constant source of frustration for researchers.

To date, of the synthetic research receptor ligands, only SR-141716A (CB_1 receptor antagonist) has shown sufficient potential to be developed into a pharmaceutical product (Rimonabant). A number of other synthetic cannabinoids have been developed into pharmaceuticals including Marinol®, Synhexyl, Nabilone and Levonantradol. However, regulatory approval of these products varies between territories and, as a result, they are not currently widely used or accepted.

Classification of cannabinoids

The existence of the various types of cannabinoid molecule available and their source has led to the proposal of four distinct classes of cannabinoids:

1. phytocannabinoids: those which occur naturally in the plant;
2. endocannabinoids: those that occur naturally in the body (AEA, 2-AG, etc.);
3. synthetic cannabinoids: cannabinomimetic compounds resulting from chemical synthesis (e.g. dronabinol, nabilone, HU-210, CP-55,940, SR-141716A);

4. fatty acid amide hydrolase (FAAH) inhibitors: compounds that affect AEA production, release, metabolism and re-uptake.

Production of cannabis-based medicines

Cannabis-based medicines may be produced according to the regulatory requirements in a variety of ways:

- isolation and purification of individual molecules from plant sources;
- chemical synthesis of required molecular components;
- extraction of required plant components;
- selective delivery of required components.

Rationale for the development of a cannabis-based medicine as a whole-plant extract

The cannabinoids that are currently of most interest and have received the most scientific interest to date are the principal components of cannabis, Δ^9-THC and CBD. Both have important pharmacology [31, 32]. Δ^9-THC has analgesic, anti-spasmodic, anti-tremor, anti-inflammatory, appetite-stimulant and anti-emetic properties; CBD has anti-inflammatory, anti-convulsant, anti-psychotic, anti-oxidant, neuroprotective and immunomodulatory effects. CBD is not intoxicating and indeed it has been postulated that the presence of CBD in cannabis may alleviate some of the potentially unwanted side effects of Δ^9-THC.

It is postulated that the beneficial therapeutic effects of cannabis result from the interaction of different cannabinoids [31]. This may explain why cannabis-based medicines made from whole-plant extracts may be more effective than single cannabinoid products, as the extracts consist of multiple cannabinoids in defined, specific ratios. Different ratios of cannabinoids may be effective in treating different diseases or conditions across a number of therapeutic areas.

Although research has focused primarily on the two principal cannabinoids, Δ^9-THC and CBD, it is possible that other components within the plant are also important, which is why GW Pharmaceuticals' medicines are made from whole-plant extracts. McPartland and Russo [31] cite a number of literature reports, which support this theory. Mechoulam et al. [33] suggested that other compounds present in herbal cannabis might influence Δ^9-THC activity. Carlini et al. [34] determined that cannabis extracts produced effects "two or four times greater than that expected from their THC content." Similarly, Fairbairn and Pickens [35] detected the presence of unidentified "powerful synergists" in cannabis extracts causing 330% greater activity in mice than Δ^9-THC alone.

Other compounds in cannabis may ameliorate the side effects of Δ^9-THC [31]. Whole cannabis causes fewer psychological side effects than synthetic

Δ^9-THC, seen as symptoms of dysphoria, depersonalization, anxiety, panic reactions and paranoia [36].

It is possible that the observed difference in side-effect profiles may also be due, in part, to differences in routes of administration: orally administered Δ^9-THC undergoes 'first-pass metabolism' in the small intestine and liver, to 11-OH-THC; and the metabolite has been reported to be psychoactive, albeit on the basis of limited evidence [37]. Inhaled Δ^9-THC undergoes little first-pass metabolism, so less 11-OH-THC is formed [38, 39]. The effect of the route of administration on tolerability has been known for years. Walton, in 1938, remarked that "smoking cannabis is a satisfactory expedient in combating fatigue, headache and exhaustion, whereas the oral ingestion of cannabis results chiefly in a narcotic effect which may cause serious alarm" [40].

The other classes of compounds present in cannabis also have their own pharmacology (e.g. terpenoids, flavonoids) [31, 32]. The potential for interaction and synergy between compounds within the plant may play a role in the therapeutic potential of cannabis as a medicine. This may explain why a cannabis-based medicine using extracts containing multiple cannabinoids, in defined ratios, and other non-cannabinoid fractions, may provide better therapeutic success and be better tolerated than the single synthetic cannabinoid medicines currently available.

CBD, as a non-psychoactive cannabinoid, is currently the cannabinoid of considerable interest. CBD, along with Δ^9-THC, has been demonstrated to have a wide range of pharmacological activity, with the potential to be developed for a number of therapeutic areas [41]. It is likely that other cannabinoids, present in small amounts in *Cannabis sativa* L., may also have interesting pharmacological properties, for example tetrahydrocannabivarin (THC-V), cannabichromene (CBC) and cannabigerol (CBG) [31, 32, 39].

Regulatory requirements

The pharmaceutical development of cannabis-based medicines is well documented [42, 43]. For cannabinoids to be made into pharmaceuticals, licensed by the regulatory bodies around the world, they must reach strict requirements laid down in terms of the product's quality, safety and efficacy and increasingly the healthcare industry requirement of cost-effectiveness. Such standards are achieved by adhering to the industry and regulatory standards of Good Laboratory Practice (GLP), Good Manufacturing Practice (GMP) and Good Clinical Practice (GCP), according to the guidance documents provided by the International Conference on Harmonisation [44]. All requirements are now implemented through European Union and national legislation. In the case of plant-based medicines they must also adhere to Good Agricultural Practice (GAP) standards.

As a result, quality control is required throughout the whole of the manufacturing chain, including the production of raw materials. For pharmaceuti-

cals produced from plants, the regulatory authorities have produced their own guidelines on the production of botanical drug products (BDPs) [45]. As botanical pharmaceuticals have more than a single chemical entity present, their control is paramount, and hence detailed characterization and specification is required.

Breeding of cannabis plants for generation of cannabis extracts

Cannabis is in most cases a dioecious plant; that is to say, the species produces separate male (staminate) and female (pistillate) plants [46].

Analysis of the various parts of the plant confirms that the major source of cannabinoids is the female flower. Cannabinoids are not detected in the roots. The richest sources of the principal cannabinoids Δ^9-THC and CBD are the leaves and flowers and hence these plant components are selected for the production of Δ^9-THC- and CBD-based medicines.

In the wild, *Cannabis* is a short-day-length plant. This means that the plant grows vegetatively through the long days of summer. Only when the day length falls, signalling the end of summer, does the female plant start to flower and hence the cannabinoids are produced. As an annual herb in the field, normally only one crop per year would be produced.

It is during the last few weeks of life that the female plant is most active in the production of cannabinoids and terpenes. The plant will produce variable inflorescences, these being complex clusters of flowers and bracts. Each flower consists of a furled specialized single leaf – the calyx – within which is housed the ovary. Each calyx is covered in minute sticky organelles – the stalked glandular trichomes. When viewed through a hand lens, each trichome resembles a golf ball (the resin head, also known as the glandular head) sitting on a tee (the trichome's stalk; Fig. 1)

The particular day length that induces flowering is termed the 'critical day length'. This will differ according to the geographical and genetic origin of the plant in question. Thus, flowering in response to exposure to a defined amount of light may be achieved through selective breeding.

Cannabinoid content varies in different varieties but the high cannabinoid content of modern varieties is purely due to plant breeding.

However, by growing under glass in controlled conditions, a succession of crops can be planned to meet production requirements. To be suitable for long-term commercial use, plants must have selected characteristics. Plants that are selectively bred for their characteristics are termed chemovars. In order to be commercially useful, they must possess the following characteristics:

- high rate of cannabinoid production;
- high yield of cannabinoid per unit area;
- high level of purity of the desired cannabinoid (purity as used here defines the consistency of cannabinoid content as a ratio);

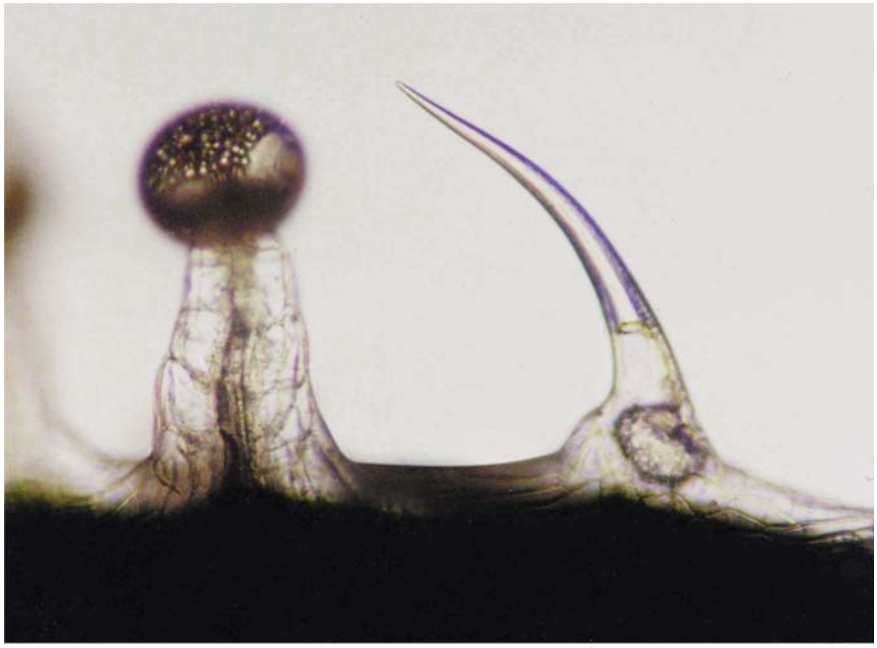

Figure 1. A glandular trichome from *C. sativa* L. (left) alongside a non-glandular trichome (right). The head on the glandular trichome is the main site of cannabinoid biosynthesis.

- high inflorescence-to-leaf ratio (the harvest index);
- natural resistance to pests and diseases;
- sturdy growth capable of bulk plant handling;
- ease of harvesting;
- minimal production of anthers on female plants.

The production of uniform high-quality botanical raw material (BRM) of defined composition is dependent upon the bulk production of cloned plants; that is to say, all plants are derived from cuttings taken from a few select mother plants. Being genetically identical, all the cloned plants have the potential to replicate exactly the characteristics of the mother plant.

BRM is obtained from distinct varieties of *C. sativa* plant hybrids to maximize the output of specific cannabinoids. The chemovars used are the result of an extensive breeding programme spanning more than 15 years.

GW's cannabis-based medicines are pharmaceutically formulated whole-plant extracts of chemovars of *C. sativa* produced by selective breeding to give a high content of defined cannabinoids, optimum habit and early flowering. A wide range of chemovars of *C. sativa* has been selectively bred by GW Pharmaceuticals. Each of these chemovars has a different cannabinoid profile, and the chemovars have been specifically bred to produce the required

level of specified cannabinoids. From this range, two separate chemovars, one that produces Δ^9-THC as the principal cannabinoid and one that produces CBD as the principal cannabinoid, have been selected for production of Sativex®.

Cultivation of chemovars for generation of cannabis extracts

Crops are produced from cuttings, which ensures that the genotype is fixed, giving a constant ratio of cannabinoid content. Cannabinoid content may be selectively bred to produce defined ratios of principal and other minor cannabinoids. By further careful, selective breeding, it is possible to cultivate chemovars which produce minor cannabinoids (CBC, CBG, THC-V, etc.) in greater amounts than have been observed to date in wild-type cannabis plants or in varieties produces by recreational growers. The pharmacology of the minor cannabinoids has yet to be clearly established, but may yet provide a whole new range of therapeutic options for both patient and clinician.

Mother plants

Potter [46] has described the use of "mother plants" to maintain the genotype for each subsequent generation of plants (rooted cuttings, termed "clones"). Once potted up and grown in continuous bright light [75 W/m^2 PAR (photosynthetically active radiation)] at 25 °C in optimized compost, a rooted cutting will reach a height of 2 m in 12 weeks. This plant is then capable of being heavily pruned; the removed branches being cut up to produce up to 80 cuttings per mother plant. If well kept, over the next 10–15 weeks the trimmed mother plant will regrow to produce at least two more flushes of cuttings. The vigour of the mother plant then wanes, and the plant is destroyed to make way for younger mothers.

Clones

Branches of the mother plant are removed where there are sufficient numbers of axial buds developing, these being the new growths that eventually develop into mature plants. Each branch is then cut into sections, each supporting only one axial bud. The cutting is then placed in rooting powder and immediately transferred into a very moist peat plug. In the correct environment, roots begin to appear after 7 days, and the cuttings allowed to acclimatize to their surroundings before they are potted up.

Rooted cuttings are transferred into large pots, filled with a proprietary growing media, which contains sufficient fertilizer to stimulate vegetative growth and flower production.

For the first 3 weeks after potting, plants are grown in continuous bright light. With no night-time breaks during this period the plant grows to around 50 cm and establishes a healthy root system.

After 3 weeks the lighting is switched to a 12-h light/12-h dark cycle. Having established themselves in a 24-h daylight environment in subtropical temperatures, the plants suddenly detect the change in light exposure, as if they had experienced the immediate arrival of the autumn equinox. For a short-day plant (i.e. late summer/autumn flowering) like cannabis, the response is dramatic. The GW chemovars flower within 5 days of the photoperiod switch. The inflorescences (flowers) increase in size over the next 6 weeks, becoming white with myriad receptive stigmas. The unfertilized stigmas then start to senesce to an orange/brown colour. After 8 weeks in flower, the bulk of stigmas have senesced and the rate of cannabinoid biosynthesis in the selected varieties slows rapidly. At this point, the crop is harvested.

Mother plants, seedlings and mature clones are produced under glass, which allows a very high degree of control of growing conditions to be exercised. The controls significantly exceed the controls possible for field-grown crops. In particular:

- proprietary compost is used, warranted free of artificial pesticides and herbicides by the supplier;
- the compost contains sufficient fertilizer to ensure optimum vegetative growth and eventual flowering;
- stringent hygiene conditions reduce ingressive pests and diseases – adventitious infestation is controlled biologically with predatory mites;
- fresh potable water, rather than stored or untreated water, is used for the irrigation of the plants; this reduces the potential for contamination with water-borne organisms;
- during growing, the plants are inspected regularly, and plants showing male characteristics are removed to avoid fertilization of plants;
- growing conditions are strictly controlled via computer technology to ensure that optimal cultivation conditions are maintained at all times in terms of light, temperature, humidity, airflow, etc.

Drying

At harvest, the entire plant is cut and dried in a temperature- and humidity-controlled environment until it meets the specification for loss on drying. Leaves and flowers are stripped from the larger stems to provide the BRM, which is stored in suitable containers protected from light under controlled conditions.

Drying the crop as quickly as possible reduces the cannabinoid losses, and this is achieved by keeping the plants in a stream of dehumidified air. Plants are crisp to the touch in less than 7 days.

As part of GAP and GMP, the BRM must conform to a specification. The specification for BRM includes tests for identification, extraneous matter and identification and assay for cannabinoids and cannabinoic acids, confirmatory thin-layer chromatography (TLC) and loss on drying. Additionally, BRM is tested for aflatoxins and microbial bioburden. The growing parameters employed have been selected to minimize the conditions that would be expected to result in microbial and fungal spoilage.

Extraction

Cannabinoids are present in the plant as the corresponding carboxylic acid and it is necessary to decarboxylate material before extraction. The conditions for efficient decarboxylation have been optimized to maximize decarboxylation and minimize oxidation. The process is time- and temperature-dependent and a criterion of not less than 95% efficiency was adopted for BRM used in subsequent manufacture of botanical drug substance (BDS; whole-plant extract).

Development work has shown that efficient extraction can be carried out using patented extraction technology. The conditions of the extraction have been carefully assessed during development and are essential to ensure the optimum conditions and hence the correct composition of the extract produced. The extraction produces a whole-plant extract, from which the BDS is prepared.

The whole-plant extract is subject to further processing (covered by intellectual proprietary rights) to remove unwanted materials from the extract. The exact content of the BDS is defined by a specific BDS specification. BDS is transferred to sealed, stainless steel containers and stored at -20 ± 5 °C to maintain stability.

A schematic diagram of the process flow from cultivation to final processing and quality-control release of the pharmaceutical product is detailed in Figure 2.

BDS content

Using any defined BRM, a corresponding BDS may be created using the above GW proprietary process. The contents of the BDS will depend on the genetically defined content of the BRM, and the technology used to extract the active constituents. Thus, BDSs may be produced which have defined levels of principal cannabinoids, other cannabinoids and other non-cannabinoid constituents. Thus a series of individual BDSs may be described.

Each BDS contains a cannabinoid fraction and a non-cannabinoid fraction. GW describes its BDSs individually as each BDS generated has a unique composition. The two BDSs used to generate Sativex® are Tetranabinex®, an extract of a chemically and genetically characterized cannabis plant, contain-

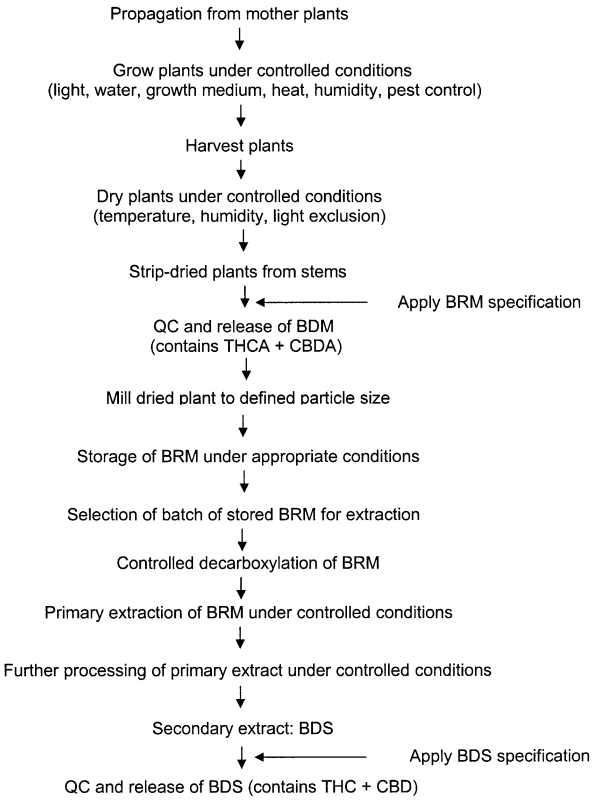

Figure 2. Schematic flow diagram of the production of GW Pharmaceuticals' BDSs. CBDA, cannabidiolic acid; QC, quality control; THCA, tetrahydrocannabinolic acid.

ing Δ^9-THC as the principal cannabinoid, and Nabidiolex®, an extract of a chemically and genetically characterized cannabis plant containing CBD as the principal cannabinoid. Other BDSs may be generated from extracts high in CBC, CBG, THC-V, cannabidivarin (CBD-V), etc.

Cannabinoid fraction

In addition to the principal cannabinoids present, each BDS contains other cannabinoids that may contribute to the activity of the whole extract.

Non-cannabinoid fraction

Each BDS also contains a non-cannabinoid fraction, which contains terpenes, sterols, fatty acids, anti-oxidants and flavonoids.

Characterization, control and specification of BDS

The ranges for the principal cannabinoids and other cannabinoids are defined in the BDS specification, as are the levels of non-cannabinoid compounds. The minor cannabinoids and non-cannabinoids are considered to be adjuvants to the principal cannabinoid rather than impurities. The non-cannabinoid fraction may be regarded as a diluent, rather than an impurity, making up the difference between assayed percentage of cannabinoids and 100% of the extract.

For regulatory approval, tight control of the content of the BRM, BDS and BDP is essential. Even though the pharmaceutical product is a botanical product, rather than a new chemical entity, characterization of more than 90% of the composition of the whole extract is required. GW has achieved this.

Stability

Stability studies are ongoing to assess the stability of Tetranabinex®, Nabidiolex® and the finished product Sativex® in order to establish a suitable shelf-life for the product. Such studies include temperature cycling and photostability, in compliance with international regulatory (International Conference on Harmonisation) conditions. Additionally, studies are being performed to investigate forced degradation.

Profile of a BDS

Typically, a GW Pharmaceuticals BDS contains the following.

- Principal cannabinoids
  Δ^9-THC (>90% of the cannabinoid fraction in THC BDS)
  CBD (>85% of the cannabinoid fraction in CBD BDS)
- Minor cannabinoids
  Cannabichromene (CBC)
  Cannabigerol (CBG)
  Cannabinol (CBN)
  Tetrahydrocannabivarin (THC-V)
  Cannabidivarin (CBD-V)
  Tetrahydrocannabinolic acid (THCA)
  Cannabidiolic acid (CBDA)
  Cannabicyclol (CBL)
  Cannabitriol (CBO)
  Cannabielsoin (CBE)
  Cannabichromivarin (CBC-V)
- Terpenes
  Monoterpenes: myrcene, limonene, linalool, α-pinene
  Sequiterpenoids: *trans*-caryophyllene, α-caryophyllene, caryophyllene

oxide, *cis*-nerolidol, *trans*-nerolidol
Diterpenoids: phytol
Triterpenoids: squalene

- Fatty acids
  Linolenic acid, palmitoleic acid, linoleic acid, palmitic acid, oleic acid, stearic acid, myristic acid, arachidic acid and behenic acid
- Sterols
  β-Sitosterol, Campesterol and Stigmasterol
- Carotenoids
  β-Carotene, lutein
- Chlorophylls and related compounds
  Phaeophytin
- Vitamins
  Vitamin E
- Phenolic compounds
  Flavonoids, coumarins, cinnamic acids and psoralens

Finished product – BDP: formulation and filling

The dosage form for Sativex® is a solution, consisting of a vehicle of ethanol, propylene glycol and peppermint, containing Tetranabinex® and Nabidiolex® extracts, that is sprayed into the oral cavity, on to the oromucosal surface.

Sativex® contains Tetranabinex® and Nabidiolex® extracts of *C. sativa* equivalent to 27 mg/ml Δ^9-THC and 25 mg/ml CBD per actuation. The container is an amber Type I glass vial, with a sealed pump, designed to deliver a uniform 100 μl volume. An actuator is used to produce the spray (Fig. 3).

Administration of Sativex®: achieving the therapeutic window

Appropriate delivery of the active components of a cannabis-based medicine is important in terms of patient acceptability, and achieving optimal and predictable effect. The rate of delivery of constituents to the site of action is as important as the amount delivered. Hence, the formulation selected to deliver cannabinoids is very important. The fact that cannabinoids are extremely lipophilic compounds limits the number of excipients that may be used to formulate cannabis-based medicines.

Sativex® is self-titrated by patients. Its frequency of use is determined by the type, severity and frequency of symptoms that patients endure. As patients vary enormously in terms of the symptoms they exhibit upon presentation to their physician, the administration of Sativex® is unique in each individual patient.

The ability of Sativex® to relieve a variety of single primary symptoms across different patient populations, coupled with its ability to relieve ‘clus-

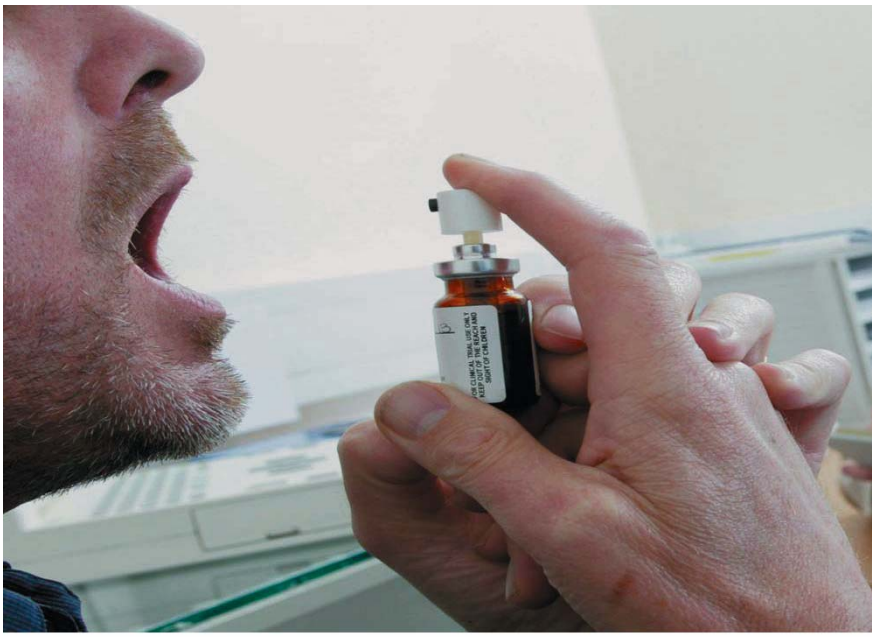

Figure 3. Administration of Sativex®.

ters' of symptoms in individual patients as reported in GW's clinical programme, demonstrates the real strength and potential of Sativex® as a medicine. These beneficial effects are not only due to the pharmacological actions of the medicine but also due to the flexibility of dosing that the medicine offers. It accommodates inter-individual variation, but also allows each patient to establish a dose regimen that provides patient benefits with minimal unwanted side effects. It allows patients the opportunity to develop their own dosing regimen, including dosing interval and acceptable dose range, and also enables them to assess the time course of symptom relief, using their own personal endpoints as markers of efficacy and tolerability. In this way, the patient is able to optimize the relief of their symptoms, while minimising and resolving the occurrence of any side effects that they may experience (i.e. patiens can target the therapeutic window).

By utilizing this approach, a number of significant clinical benefits of Sativex® have been reported in GW's clinical trial programme.

Clinical effects of Sativex®

The clinical effects of Sativex® have undergone investigation in an international clinical trials programme, with centres in UK, Romania, Belgium,

Ireland and Canada. More than 1400 subjects have participated in the clinical programme, which has initially targeted MS patients who have symptoms, and patients with neuropathic pain.

A summary of the programme is presented in Table 1. A total of 13 phase I studies have been undertaken to investigate the pharmacokinetics of Sativex®

Table 1. GW clinical programme, wave 1

Study number	Study population	Number enrolled	Study status
Acute studies			
Phase II studies			
GWN19901A	Various symptoms in MS and SCI[#]	34	C
GWN19902	Various symptoms in MS[#]	25	C
GWN19904	Various symptoms in MS, RA and SCI[#]	29	C
GWCRI016	Pain and stiffness caused by RA[*]	58	C
GWQSCBME01	Bladder dysfunction in MS[†]	21	C
Phase III studies			
GWMS0106	Spasticity in MS[*]	189	C
GWNP0101	Peripheral neuropathic pain characterized by allodynia[*]	125	C
GWMS0001	Multiple symptoms in MS[*]	160	C
GWPS0105	Chronic refractory pain in MS and other defects of neurological function[*]	70	C
GWMS0107	Neuropathic pain in MS[*]	66	C
GWBP0101	Pain in brachial plexus avulsion[#]	48	C
GWCA0101	Cancer pain[*]	176	O
GWMS0208	Bladder dysfunction in MS[*]	130[‡]	O
GWSC0101	Neuropathic pain in SCI[*]	120[‡]	O
Long-term extension studies			
Phase II			
GWN19901A	Various symptoms in MS and SCI[#]	29	O
GWN19902	Various symptoms in MS[#]	20	O
GWN19904	Various symptoms in MS, RA and SCI[#]	22	O
GWCRI016	Pain and stiffness caused by RA[*]	35	O
GWQSCBME01	Bladder dysfunction in MS[†]	16	C
Phase III			
GWEXT0101	Cancer pain[†]	40	O
GWMS0001 EXT	Multiple symptoms in MS[†]	137	O
GWEXT0102	Neuropathic pain and bladder dysfunction in MS[†]	494	O

C, complete; O, ongoing; MS, multiple sclerosis; SCI, spinal cord injury; RA, rheumatoid arthritis.
[#] Randomized, double-blind, placebo-controlled crossover study.
[*] Randomized, double-blind, placebo-controlled parallel group study.
[†] Open-label study.
[‡] Target recruitment figure.

and other formulations/products of GW's portfolio. To date, the results from three pharmacokinetic studies have been published [47–49].

Clinical programme results

Of the 11 efficacy studies completed to date (five phase II; six phase III), all 11 have yielded a range of positive results [50–60]. An additional three phase III trials commenced in 2002 and are due to complete in 2005.

In all studies all patients remained on the best current therapy available for their condition. However, they still had sufficient residual symptom-severity scores for them to seek further treatment (i.e. there was still a high clinical unmet need despite best available therapy). Sativex® was added to all their other medications, which were kept stable during the baseline/run-in periods and throughout the study period. The subsequent improvement in symptoms that was observed following treatment with Sativex® was *in addition* to any benefit they had previously derived from their existing therapy.

Phase II data

In phase II studies the following effects were seen:

- relief of neuropathic pain [50];
- improvement in spasticity [51, 52];
- improvement in muscle spasms [51, 53];
- improvement in bladder-related symptoms [52];
- improvement in sleep, mood and overall sense of well-being [50–52];
- improvement in morning pain in rheumatoid arthritis [54];
- opiate sparing effects

Phase III data

In randomized, double-blind, placebo controlled, phase III studies the following effects were seen:

- relief of central neuropathic pain (CNP) in MS [55] (see Fig. 4);
- relief of CNP in brachial plexus avulsion [56] (see Fig. 5);
- relief of chronic refractory pain of neurological origin [57];
- relief of spasticity in MS [58, 59] (see Figs 6 and 7);
- relief of peripheral neuropathic pain [60];
- relief of relief of sleep disturbance and improvement in sleep quality [55–58, 60] (see Fig. 8);
- improvement in patients quality of life [55, 60].

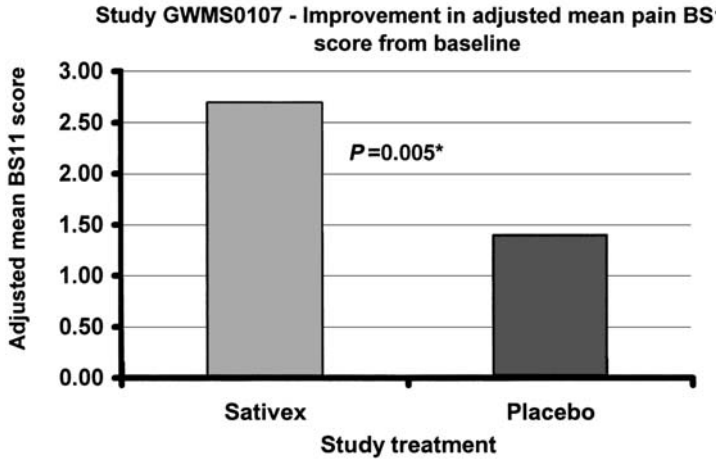

Figure 4. Relief of central neuropathic pain in MS [55]. BS11, Box Scale 11. Adapted from Rog and Young [55].

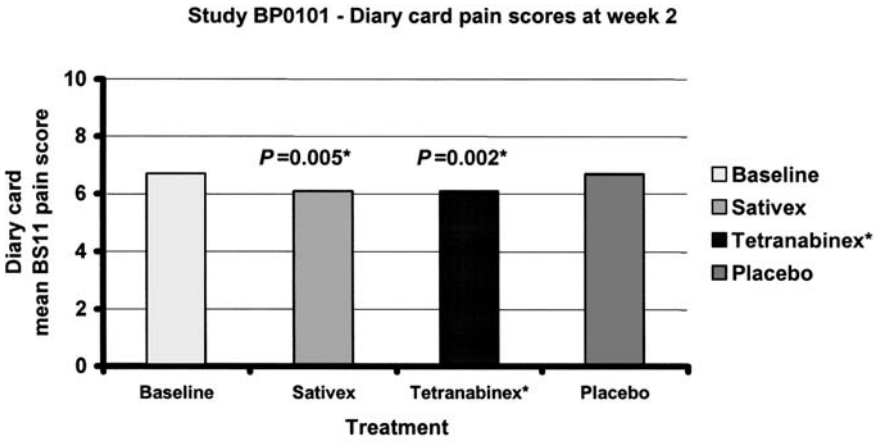

Figure 5. Relief of neuropathic pain in brachial plexus avulsion [56]. BS11, Box Scale 11. Adapted from Berman et al. [56].

Figures 4–8 present the primary efficacy data for Sativex®, from a number of the randomized, double-blind, placebo-controlled, phase III clinical studies conducted and presented to date.

Figure 9 presents the long-term data from patients who have reported pain as a symptom. The results encompass data from patients with a variety of pain syndromes who have completed the randomized studies and have elected to continue on the medicine long-term.

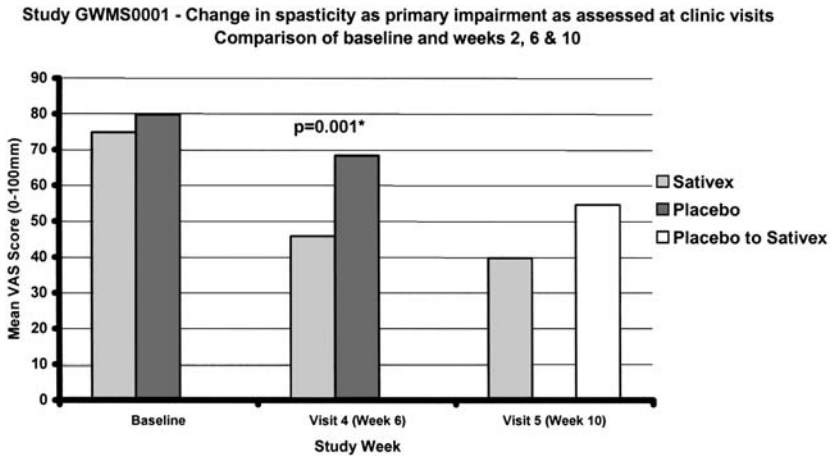

Figure 6. Relief of spasticity in MS (clinic assessments) [58]. Placebo was crossed over to Sativex® in weeks 7–10. Adapted from Wade et al. [58].

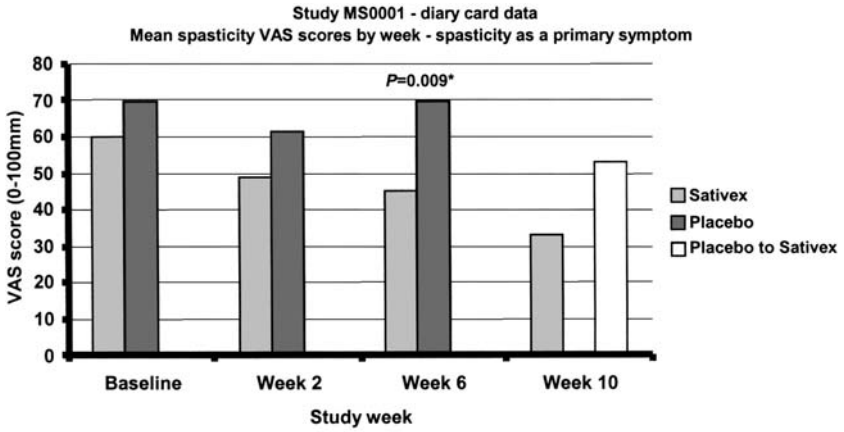

Figure 7. Relief of spasticity in MS (diary cards) [58]. Placebo crossed over to Sativex® in weeks 7–10. Adapted from Wade et al. [58].

Neuropathic pain in MS

Sativex® has been investigated for its effects on neuropathic pain from a variety of aetiologies. A study evaluating its effects in CNP in MS was undertaken in 2002 [55]. Following a baseline period during which their pain scores were assessed, 66 patients with CNP were randomized to receive either Sativex® or placebo for 4 weeks. The primary endpoint of the study was pain scores as measured on a patient diary card using an 11-point Numerical Rating

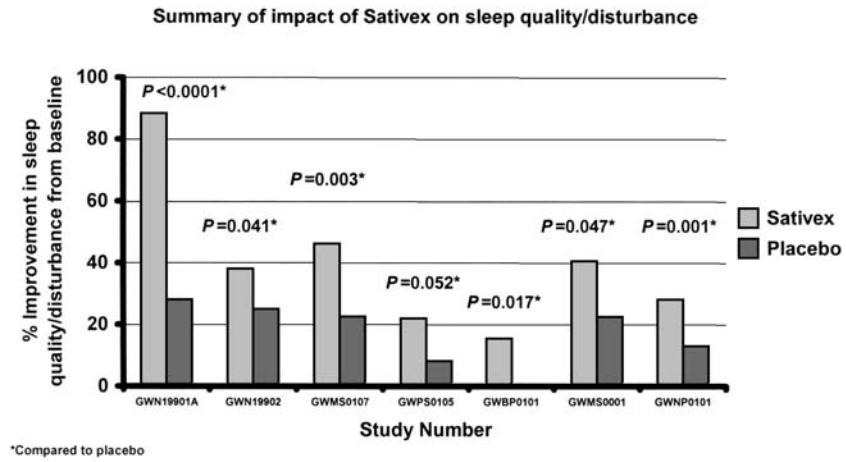

Figure 8. Relief of sleep disturbance [50, 51, 55–58, 60]

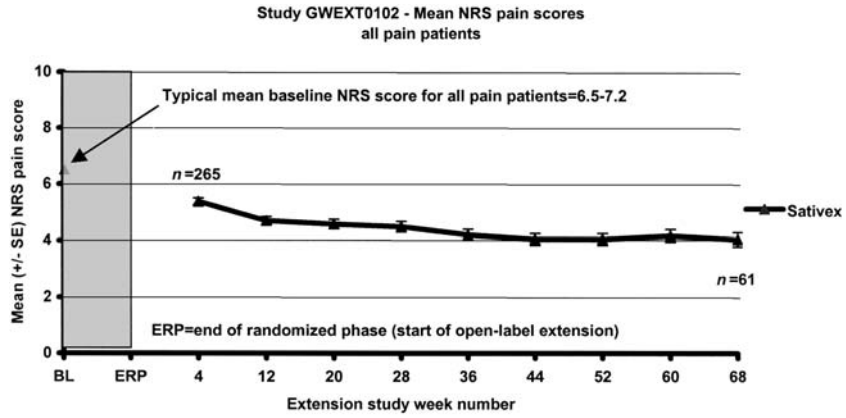

Figure 9. Sustained relief of neuropathic pain [64]

Scale (range 0–10). A summary of the results is given below, and the primary endpoint presented in Figure 4.

Sixty-four patients (96.9%) completed the trial. Fourteen patients were male, mean age 49.2 years (range 26.9–71.4, SD 8.3), mean expanded disability status scale (EDSS) 5.9 (range 2.0–8.5, SD 1.3) and mean duration of MS since diagnosis 11.5 years (range 1–36, SD 7.7).

The mean number of daily sprays taken in the final week of treatment was 9.6 of Sativex® (range 2–25, SD 6.1) and 19.1 of placebo (range 1–47, SD 12.9).

Thirty patients (88.2%) on Sativex® and 22 (68.8%) on placebo had at least one adverse event, none of which were serious. There was a statistically significant mean reduction in pain in favour of Sativex®, as measured using the

11-point numerical rating scale (NRS; 0 = none, 10 = worst), which was the primary outcome of the study [–1.25; 95% confidence interval (CI), –2.11, –0.39; $p = 0.005$].

There was a statistically significant improvement in mean sleep disturbance in favour of Sativex® (–1.39; 95% CI, –2.27, –0.50; $p = 0.003$). A significant mean reduction in pain with Sativex® compared with placebo was also demonstrated using the 10-item, 100-point neuropathic pain scale (–6.82; 95% CI, –13.28, –0.37; $p = 0.039$). On a seven-point Patient's Global Impression of Change (PGIC), those treated with cannabis-based medicine extracts were 3.9 times more likely (95% CI, 1.51, 10.06; $p = 0.005$) to feel "much" or "very much" improved than those receiving placebo, and no patient felt "much" or "very much" worse at the end of either treatment. No significant mean differences were found between treatment groups prior to treatment.

Neuropathic pain in brachial plexus avulsion

A further study evaluating the effects of Sativex® on CNP was undertaken in patients with brachial plexus avulsion [56]. Brachial plexus avulsion is a relatively uncommon condition but is characterized by severe, intractable neuropathic pain, which is difficult to treat. Due to the low numbers of patients available, even at the national treatment centre in the UK, the study was performed as a crossover study rather than to a parallel group design.

Following a baseline period during which their pain scores were assessed, 48 patients with brachial plexus avulsion were randomized to receive Sativex®, a formulated Δ^9-THC-rich extract (formulated Tetranabinex®), or placebo, each for a period of 2 weeks. The primary endpoint of the study was pain scores as measured on a patient diary card using an 11-point NRS (range 0–10). A summary of the results is given below, and the primary endpoint presented in Figure 5.

Forty-eight patients were enrolled. They all had at least one brachial plexus root avulsion for at least 18 months. They also had pain of at least 4 on an 11-point NRS at the time of enrolment. The study was a randomized, double-blind, crossover design consisting of three 2-week periods following a run-in period of 7–24 days. Patients continued on all previous stable medications including analgesics. During each 2-week period subjects received, in random order, either placebo, formulated Tetranabinex® or Sativex®. These were given as patient-activated oromucosal 100 µl sprays.

Efficacy endpoints were: 11 point NRSs for pain and sleep, short-form McGill (McGill Pain Questionnaire), General Health Questionnaire-12 (GHQ-12) and sleep quality and sleep disturbance were all recorded.

The mean number of daily sprays taken in the final week of treatment was 6.93 for Sativex®(range 1.1–22.2, SD 4.79), 7.26 for Tetranabinex® (range 1.2–21.6, SD 5.04) and 9.15 for placebo (range 2.0–35.6, SD 7.30). The results for the efficacy endpoints are shown in Table 2.

Table 2. Study GWBP0101 efficacy results

	Baseline	Placebo	Formulated Tetranabinex®	Sativex®
Pain NRS Score	6.7	6.7	6.1 ($P = 0.002$)	6.1 ($P = 0.005$)
McGill Pain Questionnaire (total intensity)	17.3	15.5	13.4 ($P = 0.04$)	13.8 ($P = 0.15$)
McGill (Part II) Pain-intensity VAS score	60.9	52.9	43.6 ($P = 0.04$)	45.1 ($P = 0.09$)
Sleep-quality NRS	4.8	5.2	6.0 ($P = 0.001$)	5.9 ($P = 0.02$)
GHQ-12	13.4	13.5	12.3 ($P = 0.18$)	10.9 ($P = 0.02$)

VAS, Visual Analogue Scale; GHQ-12, General Health Questionnaire-12.

These two studies [55, 56] and a third reported by Sharief [57] demonstrate that Sativex® has a significant analgesic effect in CNP. A further study yet to be fully reported also demonstrated a significant improvement in peripheral neuropathic pain characterized by allodynia [60]. These results are consistent with a recent report of dronabinol being effective in CNP in MS [61].

Symptoms of MS

In addition to reports of Sativex® being effective in the treatment of neuropathic pain, early studies indicated that it had a broad spectrum of activity across a variety of other symptoms in MS such as spasm, spasticity and bladder dysfunction [51–53]. In order to test the breadth of effect of the medicine, a study was undertaken evaluating a range of nominated primary symptoms in MS [58].

Patients chose one of five symptoms (pain, spasm spasticity, tremor or bladder dysfunction) as their nominated primary symptom. Despite their existing treatment prior to study entry, patients were required to have a symptom severity rated as >50 mm on a 100-mm VAS scale in order to be eligible. Other secondary impairments/symptoms (if present) were also monitored during the study.

A total of 160 patients entered a baseline period (14 days maximum); followed by a 6-week randomized, double-blind, placebo-controlled parallel-group comparison of Sativex® with placebo. Patients self-titrated to symptom resolution or maximum tolerated dose. Existing medication continued at a constant dose.

Primary efficacy comparisons were made between symptom scores recorded during baseline and scores recorded at the end of the 6-week parallel group period.

Patients then entered weeks 7–10 and all patients were re-titrated on to Sativex® and received open-label treatment for 4 weeks.

The results of the study are presented below and the outcome on the symptom of spasticity is presented in Figures 6 and 7.

Thirty-nine patients ($n = 19$ for Sativex®, $n = 18$ for the placebo) who nominated spasticity as their primary impairment showed a statistically significant improvement in their spasticity VAS scores as assessed at either their clinic visits or as recorded on their daily diary cards.

When the changes in each of the clinic visit spasticity VAS scores (in patients with spasticity as a primary impairment) were analysed, there was a highly statistically significant treatment difference of 22.79 mm in spasticity in favour of Sativex® ($P = 0.001$).

When the changes in each of the diary card spasticity VAS scores (in patients with spasticity as a primary impairment) were analysed, there was a highly statistically significant treatment difference of 18.41 mm in spasticity in favour of Sativex® ($P = 0.009$).

Effect on sleep

The most consistent endpoint in terms of response to Sativex® (measured in all GW studies except GWMS0106) has been the improvement in sleep quality/sleep disturbance reported by patients which chronic symptoms, irrespective of the aetiology. Patients with chronic refractory pain of neurological origin, CNP (from conditions such as MS and brachial plexus avulsion), peripheral neuropathic pain, and other symptoms of MS such as spasm, spasticity and bladder dysfunction have all reported statistically significant improvements in sleep (Fig. 8).

It is well accepted that sleep quality has a major impact on the quality of life of patients with chronic conditions. In the above clinical studies, Sativex® has not only produced statistically and clinically significant improvements in the patients primary symptoms, but also the ability to gain rest as a result of the relief of those symptoms. On average across the studies Sativex® has produced a 40% improvement in sleep quality/disturbance.

However, the effect of Sativex® on sleep is not due to a direct hypnotic effect of the medicine. The effect of Sativex® on the sleep process was investigated in a sleep laboratory study [62].

Nicholson et al. have reported the effects of Sativex® and formulated Tetranabinex® on nocturnal sleep and early-morning behaviour in young adults [62]. The effects of the medicines on nocturnal sleep, early-morning performance, memory and sleepiness were studied in eight healthy volunteers.

The study was double-blind and placebo-controlled with a four-way crossover design. The four treatments were placebo, Sativex® (six sprays, delivering a total dose of 15 mg of Δ^9-THC and 15 mg of CBD), formulated Tetranabinex® (six sprays, delivering a 15 mg dose of Δ^9-THC), and a "low-dose" Sativex® formulation (six sprays delivering a total dose of 5 mg of Δ^9-THC and 5 mg of CBD; i.e. identical to Sativex® formulation, but one-third

of the potency). Electroencephalogram (EEG) recordings made during the sleep period (11:00 PM to 7:00 AM). Performance, sleep latency and subjective assessments of sleepiness and mood were measured from 8:30 AM (10 h after drug administration).

There were no effects of 15 mg of Δ^9-THC (Tetranabinex®) on nocturnal sleep. Low-dose Sativex® (5 mg of Δ^9-THC and 5 mg of CBD) and Sativex® (15 mg of Δ^9-THC and 15 mg of CBD), produced a decrease in stage 3 sleep, but interestingly with Sativex® (15 mg) wakefulness was increased.

The next day, with Tetranabinex® (15 mg of Δ^9-THC), memory was impaired, sleep latency was reduced and the subjects reported increased sleepiness and changes in mood. However, interestingly, when 15 mg of CBD was added to the 15 mg of Δ^9-THC (i.e. following administration of 15 mg of Sativex®) there was no observed effect on daytime sleep latency and memory.

From this study, at the doses investigated, it appears that Δ^9-THC appears to have sedative properties, while CBD (present in Sativex®) appears to have alerting properties as it increased awake activity during sleep of patients taking Sativex® and counteracted the residual sedative activity of 15 mg of Δ^9-THC.

Thus Sativex® appears to promote sleep without changing the sleep architecture, but minimizes the residual effects that may be present if a Δ^9-THC-rich medicine (without the presence of CBD) is used.

What do patients want?

In a number of GW's clinical studies, patients have reported good overall improvement with Sativex®, as measured using the PGIC. Even small changes in symptom relief appear to be important to the patients, with a subset of the patients gaining large and sustained responses (e.g. ≥50% improvement from baseline).

This is reflected in reports from a number of patient groups. In the MS Society's (the UK's largest charity for people affected by MS) submission to the UK's National Institute for Clinical Excellence (NICE), the importance of small improvements in symptoms and sleep quality has been emphasized.

For example, the following quotes were included in their submission:

> "If cannabinoid-based medicines provide even minor symptom relief they could still have a major impact on people's quality of life and boost their self esteem."

> "An ideal treatment for spasticity would be short-acting so that it could reduce nocturnal spasms and aid sleep, but not compromise functioning during the daytime. Many of the existing treatments have long-term effects. Cannabinoid-based medicines have the advantage that they are short acting – they could therefore allow much better control of symptoms."

> *"Q*: What is it like to have MS?
> *Person with MS*: Get somebody to stay awake for 48 hours, make them drink loads of coffee so they just can't sleep, put weights on their ankles, a pack on their back, make them wear two lots of rubber gloves, the whole thing. Tell them it's for the rest of their life, because that's the most important thing."

> "…Others obtained pain relief or found that the drug (cannabis) simply helped them to sleep. Sleepless nights caused by spasms and nocturia can make the extreme fatigue in MS even worse. The importance of a good night's sleep cannot be overestimated. It has a major impact on Quality of Life."

Long-term data

The majority of patients (>70%) who participated in the GW randomized studies elected to receive the drug in long-term, open-label extension studies (>750 patients) [63, 64]. Efficacy with respect to a variety of symptoms has been maintained over an extended period of time (>1 year). To date, more than 200 patients have remained on treatment for more than 1 year, and a significant number have remained on treatment for more than 2 years (the maximum is 814 days as of November 2003), with no evidence of tolerance developing. Dosing has remained steady over the same period, and only minimal levels of intoxication have been reported using a 0–100 mm VAS scale (scores up to a maximum of approximately 20 upon initial exposure, diminishing over time). This, coupled with the low number of serious adverse reactions reported, demonstrates the tolerability of the product.

The effects observed in the randomized clinical studies have been sustained over the long-term (Fig. 9).

At baseline, patients in the randomized, placebo-controlled phase had the following NRS scores: brachial plexus injury, 6.8; neuropathic pain in MS, 6.5; peripheral neuropathic pain, 7.2; spinal cord injury (data not available, study ongoing).

Safety

GW has now generated more than 800 patient-years of exposure to Sativex® since the year 2000. By June 2004, more than 200 patients had been exposed to Sativex® for at least 1 year.

The most common adverse events reported during clinical studies were generally non-serious in nature and are mainly due to application site reactions (oral pain, dry mouth, oral mucosal disorder, tooth discolouration, mouth ulceration, oral discomfort, application-site pain, dysgeusia) or intoxication-like

reactions (fatigue, feeling drunk, lethargy, dizziness, somnolence, disturbance in attention, memory impairment, euphoric mood, disorientation).

Other common adverse events reported were nausea, vomiting, diarrhoea, constipation, dyspepsia, weakness and headache.

Intoxication

The long-standing concern regarding the development of cannabis-based medicines has been the psychoactivity of Δ^9-THC. Until now, this has been perceived as a major barrier to the safety and tolerability of such medicines. To date, patients have often reported that they are often unable to tolerate the synthetic cannabinoid medications currently available to them due to their side effects. The main concern for many patients regarding the use of cannabis-based medicines is the symptom of intoxication. Patients do not wish to get high and actively seek to avoid this as it interferes with their daily life, which in many cases has already been compromised by their symptoms and/or underlying condition. This is not a situation that is unique to cannabinoid medicines, as many other classes of licensed pharmaceuticals may produce intoxication-like effects (e.g. opioids, benzodiazepines, tricyclic antidepressants, etc.). Indeed, many patients suitable for treatment with cannabis-based medicines are already experiencing polypharmacy with such products.

The range of intoxication like reactions reported by patients taking Sativex® in clinical trials has consistently been reported [50–60, 63, 64]. Safety data have been collected in randomized, double-blind studies and in long-term open-label extension studies. Safety data from more than 500 patients in the long-term extension studies are now available, where patients were allowed to take up to 48 sprays per day (maximum Δ^9-THC dose = 130 mg/day). The most common intoxication like reaction reported is dizziness, reported initially in approx. 35% of patients. However, this includes patients who are new to the medication and are titrating their initial dose. In long-term use the incidence of such an event is approximately 25%. All other intoxication-like reactions are reported at incidences of less than 5% (with the exception of somnolence, 7%).

However, the most important issue regarding intoxication is not the incidence, but the severity of any intoxication-like reactions. This is where the composition of the medicine and its delivery become important. Sativex® not only produces a low incidence of intoxication, but when experienced by patients it is generally very low in severity. The ability of the patient to self-titrate with Sativex® makes it easier to target the therapeutic window, and makes the occurrence of any such side effects much more manageable, as the dose and dosage interval can be tailored to each patient's needs as required according to their daily circumstances.

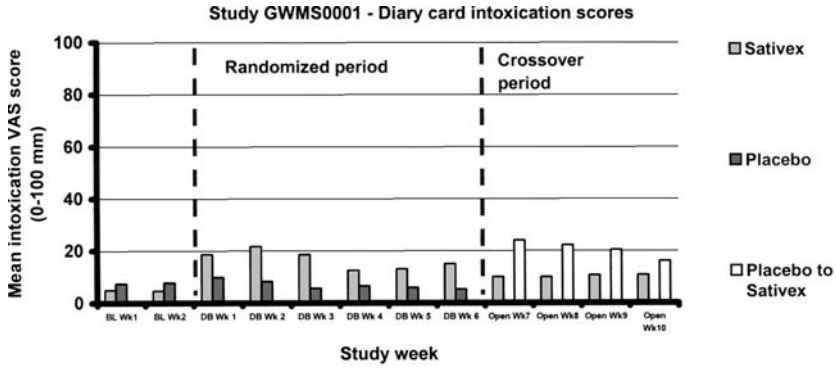

Figure 10. Intoxication produced by Sativex® [58]. BL, baseline; DB, randomized, double-blind period (weeks 1–6). Placebo crossed over to Sativex® in weeks 7–10. Adapted from Wade et al. [58].

As can be seen from Figure 10, the maximum severity of intoxication experienced by patients (measured using a VAS) was only approximately 20 out of 100 mm following initial exposure to Sativex®. This severity occurs early on in their initial titration period (within the first 2 weeks) and rapidly diminishes over time to scores less than 5 out of 100 mm. Figure 10 also shows that the picture is repeated in placebo patients who were then switched over to Sativex®. The long-term intoxication data presented in Table 3 also support this (see also Fig. 11).

So, although a relatively small amount of intoxication may occur initially in patients who use Sativex®, it subsides over time, and may be easily managed using patient self-titration, to minimize levels even further.

Table 3. Long-term intoxication produced by Sativex® [64]

Study week	No. of patients	Mean VAS score	SD	Median	Minimum	Maximum
4	330	4.84	11.69	1	0	75
12	268	3.08	8.33	0	0	62
20	211	2.04	4.75	0	0	35
28	205	2.46	6.26	0	0	42
36	184	2.83	6.77	0	0	45
44	150	3.69	10.54	0	0	77
52	121	2.26	7.29	0	0	50
60	90	1.37	6.02	0	0	53
68	62	1.92	8.94	0	0	69

VAS scale is 0–100 mm, where 0 means no intoxication and 100 is extreme intoxication.

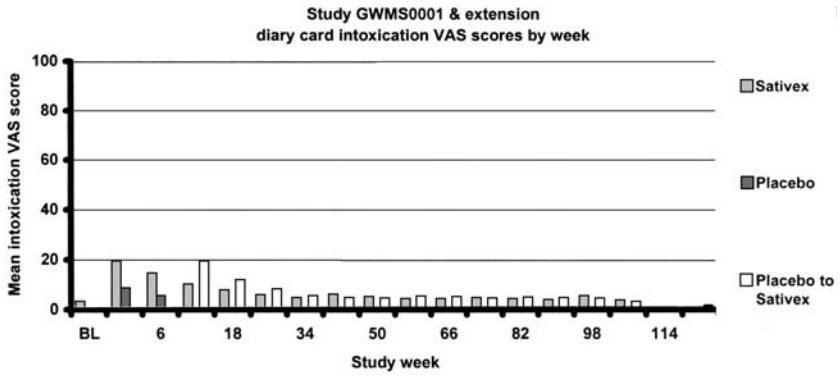

Figure 11. Long-term intoxication produced by Sativex® [63]. BL, baseline. Adapted from Wade et al. [63].

Dosing

The review of the efficacy and safety information above clearly demonstrates that there is a therapeutic window for Sativex® between the level at which patients can receive significant benefit without significant adverse effects, and the dose which may produce intoxicating effects. There is no evidence of tolerance, it can be seen that improvements in symptoms can be maintained while on a stable dose (Fig. 12).

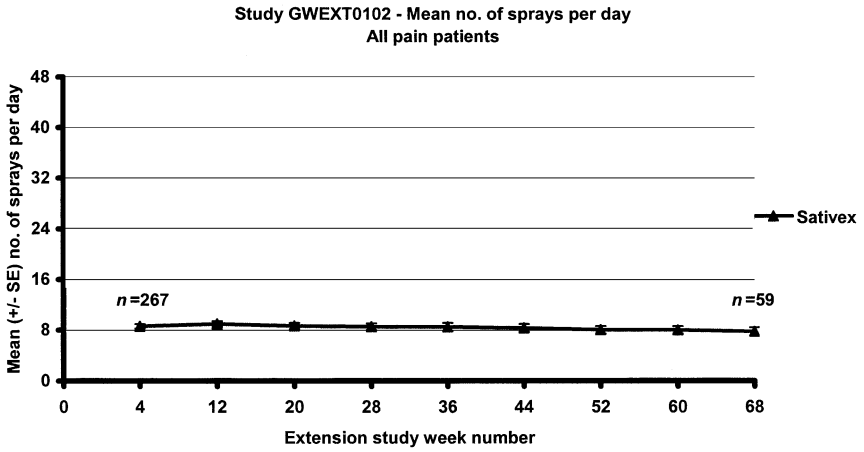

Figure 12. Long-term dosing of Sativex® in neuropathic pain [64]

Conclusion

There has been great debate with regard to merits of cannabis-based medicines with little scientific and clinical evidence to substantiate the anecdotal efficacy and safety. The discovery of the endogenous cannabinoid receptors and endocannabinoids such as AEA, 2-AG, noladin ether and NADA has spawned resurgence in the search for therapeutic agents to treat severe and chronic conditions.

To date, medicines made from single synthetic cannabinoid molecules have yet to be widely used, mainly due to their side-effect profiles. The development of a new product, Sativex®, made from whole-plant extracts of cannabis, may change the way cannabis is viewed, its therapeutic potential maximized and its universal approval as a medicine granted.

Sativex® is produced from botanical raw materials that have been specifically grown for their defined cannabinoid ratios. It is a blend of defined extracts, which ensure batch-to-batch reproducibility is attained. The other components of the extracts, in addition to the principal cannabinoids add to the benefits of the medicine.

Clinical studies with Sativex® have focused initially on symptom relief in chronic conditions, such as MS, neuropathic pain and rheumatoid arthritis, but it may have further potential as a disease-modifying agent in such conditions. Further clinical studies will be necessary to investigate this.

The clinical efficacy of Sativex® has been demonstrated in the largest programme of clinical studies of a cannabis-based medicine ever undertaken. Positive benefits have been observed in all 11 studies completed to date by GW. Dosing at levels of 8–15 sprays per day have produced significant improvements in central and peripheral neuropathic pain and improvement in a number of symptoms of MS (neuropathic pain, spasm, spasticity and bladder dysfunction) have also been reported. Further, the first study of cannabinoids in rheumatoid arthritis has demonstrated that Sativex® may have potential in relieving not only symptoms of rheumatoid arthritis, but it also may have a modulating effect on the disease process. A characteristic, which accompanies the symptom relief achieved with Sativex®, is an improvement in sleep quality.

Sativex® appears to improve symptom relief in the most difficult groups of patients – i.e. those who have significant residual symptoms even after best available therapy has been implemented. The benefits it confers are in addition to any relief patients may previously have attained with other medications. Patient groups continue to clamour for the approval of a cannabis-based medicine and have indicated that even a small reduction in symptoms is of major importance to patients, their quality of life and their overall sense of well being.

In addition to its considerable and sustained efficacy, Sativex®, in clinical studies, has a very acceptable safety and tolerability profile. It is generally well tolerated, and the flexibility offered to patients ensures they can quickly and

easily self-titrate to optimum benefit. Intoxication is not usually a limiting factor for the majority of patients, and any low levels of intoxication upon the patient's initial exposure to the medicine are further reduced as they become familiar with the medicine and the process of self-titration. Side effects experienced are usually mild or moderate in severity, and there have been few withdrawals from treatment in the clinical studies to date due to undesirable effects. Most adverse effects resolve without treatment, and some on a reduction of dosage of the medicine.

Long-term dosing with Sativex® maintains the clinical benefits initially observed in the acute setting, over prolonged periods. There is no evidence that tolerance to the beneficial effects develops. In some cases the benefits achieved with Sativex® have allowed patients to reduce the doses of, or even stop taking, other medications.

The approval of Sativex® as a pharmaceutical medicine by regulatory authorities around the world will represent a milestone in modern medicine and may catalyse a new era of BDPs.

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